WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

VOLUME 99

Some Aromatic Amines, Organic Dyes, and Related Exposures

LYON, FRANCE
2010

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Some Aromatic Amines, Organic Dyes, and Related Exposures

This publication represents the views and expert opinions
of an IARC Monographs Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon,

5–12 February 2008

2010

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, lifestyle factors and biological and physical agents, as well as those in specific occupations. The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed. The lists of IARC evaluations are regularly updated and are available on the Internet at http://monographs.iarc.fr/.

This programme has been supported since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the Health, Safety and Hygiene at Work Unit of the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities, and since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the U.S. National Cancer Institute, the U.S. National Institute of Environmental Health Sciences, the U.S. Department of Health and Human Services, or the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities.

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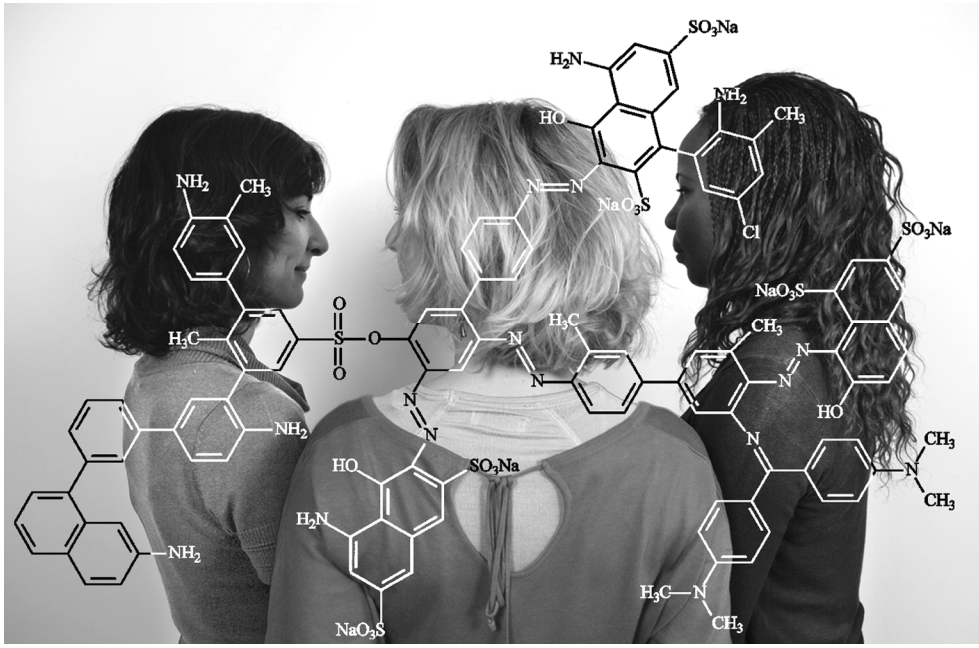

Cover: The composite structure brings together many of the chemical agents discussed and evaluated in this Volume; the background photograph illustrates occupational exposures of hairdressers and barbers, and personal use of hair colourants.

Cover photograph and design: Roland Dray (IARC). We gratefully acknowledge the gracious help of three of our colleagues in realizing the background photograph.

CONTENTS

NOTE TO THE READER

The term 'carcinogenic risk' in the *IARC Monographs* series is taken to mean that an agent is capable of causing cancer under some circumstances. The *Monographs* evaluate cancer hazards, despite the historical presence of the word 'risks' in the title.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a *Monograph* does not mean that it is not carcinogenic.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Section of IARC Monographs, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Section of IARC Monographs, so that corrections can be reported in future volumes.

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

VOLUME 99
SOME AROMATIC AMINES, ORGANIC DYES, AND RELATED EXPOSURES

Lyon, 5–12 February 2008

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[1] Working Group Members and Invited Specialists serve in their individual capacities as scientists and not as representatives of their government or any organization with which they are affiliated. Affiliations are provided for identification purposes only.

[2] Each participant was asked to disclose pertinent research, employment, and financial interests. Current financial interests and research and employment interests during the past 3 years or anticipated in the future are identified here. Minor pertinent interests are not listed and include stock valued at no more than US$10 000 overall, grants that provide no more than 5% of the research budget of the expert's organization and that do not support the expert's research or position, and consulting or speaking on matters not before a court or government agency that does not exceed 2% of total professional time or compensation. All grants that support the expert's research or position and all consulting or speaking on behalf of an interested party on matters before a court or government agency are listed as significant pertinent interests.

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[3] Dr Sorahan is expecting to be asked to carry out statistical analyses on a study of MOCA workers funded by the British Rubber Manufacturers' Association and the Polyurethane Manufacturers Association. There have been no other activities involving MOCA users or producers during the past 4 years, nor are any others anticipated.

[4] Dr Turesky holds stock in the Procter & Gamble Company, a manufacturer of hair dyes and other consumer products.

[5] Dr Nohynek is employed by L'Oréal, a manufacturer of hair dyes and other cosmetic products.

[6] In addition to her employment there, Dr Skare holds stock in the Procter & Gamble Company, a manufacturer of hair dyes and other consumer products.

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Martine Lézère's colleagues gratefully acknowledge her many years of service in the *IARC Monographs* programme, continuously since Volume 34 (October 1983).

PREAMBLE

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, the scientific principles and procedures used in developing a *Monograph*, the types of evidence considered and the scientific criteria that guide the evaluations. The Preamble should be consulted when reading a *Monograph* or list of evaluations.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after IARC was established in 1965, it received frequent requests for advice on the carcinogenic risk of chemicals, including requests for lists of known and suspected human carcinogens. It was clear that it would not be a simple task to summarize adequately the complexity of the information that was available, and IARC began to consider means of obtaining international expert opinion on this topic. In 1970, the IARC Advisory Committee on Environmental Carcinogenesis recommended '...that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented.' The IARC Governing Council adopted a resolution concerning the role of IARC in providing government authorities with expert, independent, scientific opinion on environmental carcinogenesis. As one means to that end, the Governing Council recommended that IARC should prepare monographs on the evaluation of carcinogenic risk of chemicals to man, which became the initial title of the series.

In the succeeding years, the scope of the programme broadened as *Monographs* were developed for groups of related chemicals, complex mixtures, occupational exposures, physical and biological agents and lifestyle factors. In 1988, the phrase 'of chemicals' was dropped from the title, which assumed its present form, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.

Through the *Monographs* programme, IARC seeks to identify the causes of human cancer. This is the first step in cancer prevention, which is needed as much today as when

IARC was established. The global burden of cancer is high and continues to increase: the annual number of new cases was estimated at 10.1 million in 2000 and is expected to reach 15 million by 2020 (Stewart & Kleihues, 2003). With current trends in demographics and exposure, the cancer burden has been shifting from high-resource countries to low- and medium-resource countries. As a result of *Monographs* evaluations, national health agencies have been able, on scientific grounds, to take measures to reduce human exposure to carcinogens in the workplace and in the environment.

The criteria established in 1971 to evaluate carcinogenic risks to humans were adopted by the Working Groups whose deliberations resulted in the first 16 volumes of the *Monographs* series. Those criteria were subsequently updated by further ad-hoc Advisory Groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987, 1988, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006).

The Preamble is primarily a statement of scientific principles, rather than a specification of working procedures. The procedures through which a Working Group implements these principles are not specified in detail. They usually involve operations that have been established as being effective during previous *Monograph* meetings but remain, predominantly, the prerogative of each individual Working Group.

2. Objective and scope

The objective of the programme is to prepare, with the help of international Working Groups of experts, and to publish in the form of *Monographs*, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* represent the first step in carcinogen risk assessment, which involves examination of all relevant information in order to assess the strength of the available evidence that an agent could alter the age-specific incidence of cancer in humans. The *Monographs* may also indicate where additional research efforts are needed, specifically when data immediately relevant to an evaluation are not available.

In this Preamble, the term 'agent' refers to any entity or circumstance that is subject to evaluation in a *Monograph*. As the scope of the programme has broadened, categories of agents now include specific chemicals, groups of related chemicals, complex mixtures, occupational or environmental exposures, cultural or behavioural practices, biological organisms and physical agents. This list of categories may expand as causation of, and susceptibility to, malignant disease become more fully understood.

A cancer 'hazard' is an agent that is capable of causing cancer under some circumstances, while a cancer 'risk' is an estimate of the carcinogenic effects expected from exposure to a cancer hazard. The *Monographs* are an exercise in evaluating cancer hazards, despite the historical presence of the word 'risks' in the title. The distinction between hazard and risk is important, and the *Monographs* identify cancer hazards even when risks are very low at current exposure levels, because new uses or unforeseen exposures could engender risks that are significantly higher.

In the *Monographs*, an agent is termed 'carcinogenic' if it is capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity. The induction of benign neoplasms may in some circumstances (see Part B, Section 3a) contribute to the judgement that the agent is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

The Preamble continues the previous usage of the phrase 'strength of evidence' as a matter of historical continuity, although it should be understood that *Monographs* evaluations consider studies that support a finding of a cancer hazard as well as studies that do not.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several different mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006; see also Part B, Sections 4 and 6). As mechanisms of carcinogenesis are elucidated, IARC convenes international scientific conferences to determine whether a broad-based consensus has emerged on how specific mechanistic data can be used in an evaluation of human carcinogenicity. The results of such conferences are reported in IARC Scientific Publications, which, as long as they still reflect the current state of scientific knowledge, may guide subsequent Working Groups.

Although the *Monographs* have emphasized hazard identification, important issues may also involve dose–response assessment. In many cases, the same epidemiological and experimental studies used to evaluate a cancer hazard can also be used to estimate a dose–response relationship. A *Monograph* may undertake to estimate dose–response relationships within the range of the available epidemiological data, or it may compare the dose–response information from experimental and epidemiological studies. In some cases, a subsequent publication may be prepared by a separate Working Group with expertise in quantitative dose–response assessment.

The *Monographs* are used by national and international authorities to make risk assessments, formulate decisions concerning preventive measures, provide effective cancer control programmes and decide among alternative options for public health decisions. The evaluations of IARC Working Groups are scientific, qualitative judgements on the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which public health decisions may be based. Public health options vary from one situation to another and from country to country and relate to many factors, including different socioeconomic and national priorities. Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments or other international organizations.

3. Selection of agents for review

Agents are selected for review on the basis of two main criteria: (a) there is evidence of human exposure and (b) there is some evidence or suspicion of carcinogenicity. Mixed exposures may occur in occupational and environmental settings and as a result of individual and cultural habits (such as tobacco smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. Ad-hoc Advisory Groups convened by IARC in 1984, 1989, 1991, 1993, 1998 and 2003 made recommendations as to which agents should be evaluated in the *Monographs* series. Recent recommendations are available on the *Monographs* programme website (http://monographs.iarc.fr). IARC may schedule other agents for review as it becomes aware of new scientific information or as national health agencies identify an urgent public health need related to cancer.

As significant new data become available on an agent for which a *Monograph* exists, a re-evaluation may be made at a subsequent meeting, and a new *Monograph* published. In some cases it may be appropriate to review only the data published since a prior evaluation. This can be useful for updating a database, reviewing new data to resolve a previously open question or identifying new tumour sites associated with a carcinogenic agent. Major changes in an evaluation (e.g. a new classification in Group 1 or a determination that a mechanism does not operate in humans, see Part B, Section 6) are more appropriately addressed by a full review.

4. Data for the *Monographs*

Each *Monograph* reviews all pertinent epidemiological studies and cancer bioassays in experimental animals. Those judged inadequate or irrelevant to the evaluation may be cited but not summarized. If a group of similar studies is not reviewed, the reasons are indicated.

Mechanistic and other relevant data are also reviewed. A *Monograph* does not necessarily cite all the mechanistic literature concerning the agent being evaluated (see Part B, Section 4). Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to epidemiological studies, cancer bioassays, and mechanistic and other relevant data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed. The same publication requirement applies to studies originating from IARC, including meta-analyses or pooled analyses commissioned by IARC in advance of a meeting (see Part B, Section 2c). Data from government agency reports that are publicly available are also considered. Exceptionally,

doctoral theses and other material that are in their final form and publicly available may be reviewed.

Exposure data and other information on an agent under consideration are also reviewed. In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, published and unpublished sources of information may be considered.

Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of each study description (see Part B). The reasons for not giving further consideration to an individual study also are indicated in the square brackets.

5. Meeting participants

Five categories of participant can be present at *Monograph* meetings.

(a) The Working Group is responsible for the critical reviews and evaluations that are developed during the meeting. The tasks of Working Group Members are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanisms of carcinogenesis; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans. Working Group Members generally have published significant research related to the carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Working Group Members are selected on the basis of (a) knowledge and experience and (b) absence of real or apparent conflicts of interests. Consideration is also given to demographic diversity and balance of scientific findings and views.

(b) Invited Specialists are experts who also have critical knowledge and experience but have a real or apparent conflict of interests. These experts are invited when necessary to assist in the Working Group by contributing their unique knowledge and experience during subgroup and plenary discussions. They may also contribute text on non-influential issues in the section on exposure, such as a general description of data on production and use (see Part B, Section 1). Invited Specialists do not serve as meeting chair or subgroup chair, draft text that pertains to the description or interpretation of cancer data, or participate in the evaluations.

(c) Representatives of national and international health agencies often attend meetings because their agencies sponsor the programme or are interested in the subject of a meeting. Representatives do not serve as meeting chair or subgroup chair, draft any part of a *Monograph,* or participate in the evaluations.

(d) Observers with relevant scientific credentials may be admitted to a meeting by IARC in limited numbers. Attention will be given to achieving a balance of Observers

from constituencies with differing perspectives. They are invited to observe the meeting and should not attempt to influence it. Observers do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations. At the meeting, the meeting chair and subgroup chairs may grant Observers an opportunity to speak, generally after they have observed a discussion. Observers agree to respect the Guidelines for Observers at *IARC Monographs* meetings (available at http://monographs.iarc.fr).

(e) The IARC Secretariat consists of scientists who are designated by IARC and who have relevant expertise. They serve as rapporteurs and participate in all discussions. When requested by the meeting chair or subgroup chair, they may also draft text or prepare tables and analyses.

Before an invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests to report financial interests, employment and consulting, and individual and institutional research support related to the subject of the meeting. IARC assesses these interests to determine whether there is a conflict that warrants some limitation on participation. The declarations are updated and reviewed again at the opening of the meeting. Interests related to the subject of the meeting are disclosed to the meeting participants and in the published volume (Cogliano *et al.*, 2004).

The names and principal affiliations of participants are available on the *Monographs* programme website (http://monographs.iarc.fr) approximately two months before each meeting. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC (Cogliano *et al.*, 2005).

All participants are listed, with their principal affiliations, at the beginning of each volume. Each participant who is a Member of a Working Group serves as an individual scientist and not as a representative of any organization, government or industry.

6. Working procedures

A separate Working Group is responsible for developing each volume of *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year in advance of the meeting of a Working Group, the agents to be reviewed are announced on the *Monographs* programme website (http://monographs.iarc.fr) and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are collected by IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as PubMed. Meeting participants who are asked to prepare preliminary working papers for specific sections are expected to supplement the IARC literature searches with their own searches.

For most chemicals and some complex mixtures, the major collection of data and the preparation of working papers for the sections on chemical and physical properties, on

analysis, on production and use, and on occurrence are carried out under a separate contract funded by the US National Cancer Institute. Industrial associations, labour unions and other knowledgeable organizations may be asked to provide input to the sections on production and use, although this involvement is not required as a general rule. Information on production and trade is obtained from governmental, trade and market research publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available for a variety of reasons (e.g. not collected or made public in all producing countries, production is small). Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants to prepare preliminary working papers. The working papers are compiled by IARC staff and sent, prior to the meeting, to Working Group Members and Invited Specialists for review.

The Working Group meets at IARC for seven to eight days to discuss and finalize the texts and to formulate the evaluations. The objectives of the meeting are peer review and consensus. During the first few days, four subgroups (covering exposure data, cancer in humans, cancer in experimental animals, and mechanistic and other relevant data) review the working papers, develop a joint subgroup draft and write summaries. Care is taken to ensure that each study summary is written or reviewed by someone not associated with the study being considered. During the last few days, the Working Group meets in plenary session to review the subgroup drafts and develop the evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections.

IARC Working Groups strive to achieve a consensus evaluation. Consensus reflects broad agreement among Working Group Members, but not necessarily unanimity. The chair may elect to poll Working Group Members to determine the diversity of scientific opinion on issues where consensus is not readily apparent.

After the meeting, the master copy is verified by consulting the original literature, edited and prepared for publication. The aim is to publish the volume within six months of the Working Group meeting. A summary of the outcome is available on the *Monographs* programme website soon after the meeting.

B. SCIENTIFIC REVIEW AND EVALUATION

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary

analyses are given in square brackets. When an important aspect of a study that directly impinges on its interpretation should be brought to the attention of the reader, a Working Group comment is given in square brackets.

The scope of the *IARC Monographs* programme has expanded beyond chemicals to include complex mixtures, occupational exposures, physical and biological agents, lifestyle factors and other potentially carcinogenic exposures. Over time, the structure of a *Monograph* has evolved to include the following sections:

1. Exposure data
2. Studies of cancer in humans
3. Studies of cancer in experimental animals
4. Mechanistic and other relevant data
5. Summary
6. Evaluation and rationale

In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and some key issues the Working Group encountered during the meeting.

This part of the Preamble discusses the types of evidence considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations.

1. Exposure data

Each *Monograph* includes general information on the agent: this information may vary substantially between agents and must be adapted accordingly. Also included is information on production and use (when appropriate), methods of analysis and detection, occurrence, and sources and routes of human occupational and environmental exposures. Depending on the agent, regulations and guidelines for use may be presented.

(*a*) *General information on the agent*

For chemical agents, sections on chemical and physical data are included: the Chemical Abstracts Service Registry Number, the latest primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. Information on chemical and physical properties that are relevant to identification, occurrence and biological activity is included. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

For biological agents, taxonomy, structure and biology are described, and the degree of variability is indicated. Mode of replication, life cycle, target cells, persistence, latency, host response and clinical disease other than cancer are also presented.

For physical agents that are forms of radiation, energy and range of the radiation are included. For foreign bodies, fibres and respirable particles, size range and relative dimensions are indicated.

For agents such as mixtures, drugs or lifestyle factors, a description of the agent, including its composition, is given.

Whenever appropriate, other information, such as historical perspectives or the description of an industry or habit, may be included.

(*b*) *Analysis and detection*

An overview of methods of analysis and detection of the agent is presented, including their sensitivity, specificity and reproducibility. Methods widely used for regulatory purposes are emphasized. Methods for monitoring human exposure are also given. No critical evaluation or recommendation of any method is meant or implied.

(*c*) *Production and use*

The dates of first synthesis and of first commercial production of a chemical, mixture or other agent are provided when available; for agents that do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided when available. In addition, methods of synthesis used in past and present commercial production and different methods of production, which may give rise to different impurities, are described.

The countries where companies report production of the agent, and the number of companies in each country, are identified. Available data on production, international trade and uses are obtained for representative regions. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice nor does it imply judgement as to their therapeutic efficacy.

(*d*) *Occurrence and exposure*

Information on the occurrence of an agent in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, plants, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. Such data may be available from national databases.

Data that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are reported. Information is presented on the range of human exposure, including occupational and environmental exposures. This includes relevant findings

from both developed and developing countries. Some of these data are not distributed widely and may be available from government reports and other sources. In the case of mixtures, industries, occupations or processes, information is given about all agents known to be present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with date and place. For biological agents, the epidemiology of infection is described.

(*e*) *Regulations and guidelines*

Statements concerning regulations and guidelines (e.g. occupational exposure limits, maximal levels permitted in foods and water, pesticide registrations) are included, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccination and therapy, are described.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part A, Section 4). Studies of biomarkers are included when they are relevant to an evaluation of carcinogenicity to humans.

(*a*) *Types of study considered*

Several types of epidemiological study contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies, correlation (or ecological) studies and intervention studies. Rarely, results from randomized trials may be available. Case reports and case series of cancer in humans may also be reviewed.

Cohort and case–control studies relate individual exposures under study to the occurrence of cancer in individuals and provide an estimate of effect (such as relative risk) as the main measure of association. Intervention studies may provide strong evidence for making causal inferences, as exemplified by cessation of smoking and the subsequent decrease in risk for lung cancer.

In correlation studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent under study. In correlation studies, individual exposure is not documented, which renders this kind of study more prone to confounding. In some circumstances, however, correlation studies may be more informative than analytical study designs (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

In some instances, case reports and case series have provided important information about the carcinogenicity of an agent. These types of study generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports and case series usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure.

The uncertainties that surround the interpretation of case reports, case series and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, these types of study may add materially to the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(*b*) *Quality of studies considered*

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. Bias is the effect of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between an agent and disease. Confounding is a form of bias that occurs when the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. The role of chance is related to biological variability and the influence of sample size on the precision of estimates of effect.

In evaluating the extent to which these factors have been minimized in an individual study, consideration is given to a number of aspects of design and analysis as described in the report of the study. For example, when suspicion of carcinogenicity arises largely from a single small study, careful consideration is given when interpreting subsequent studies that included these data in an enlarged population. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

Firstly, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Secondly, the authors should have taken into account — in the study design and analysis — other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis,

by statistical adjustment. In cohort studies, comparisons with local rates of disease may or may not be more appropriate than those with national rates. Internal comparisons of frequency of disease among individuals at different levels of exposure are also desirable in cohort studies, since they minimize the potential for confounding related to the difference in risk factors between an external reference group and the study population.

Thirdly, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(*c*) *Meta-analyses and pooled analyses*

Independent epidemiological studies of the same agent may lead to results that are difficult to interpret. Combined analyses of data from multiple studies are a means of resolving this ambiguity, and well-conducted analyses can be considered. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis) and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) (Greenland, 1998).

The advantages of combined analyses are increased precision due to increased sample size and the opportunity to explore potential confounders, interactions and modifying effects that may explain heterogeneity among studies in more detail. A disadvantage of combined analyses is the possible lack of compatibility of data from various studies due to differences in subject recruitment, procedures of data collection, methods of measurement and effects of unmeasured co-variates that may differ among studies. Despite these limitations, well-conducted combined analyses may provide a firmer basis than individual studies for drawing conclusions about the potential carcinogenicity of agents.

IARC may commission a meta-analysis or pooled analysis that is pertinent to a particular *Monograph* (see Part A, Section 4). Additionally, as a means of gaining insight from the results of multiple individual studies, ad-hoc calculations that combine data from different studies may be conducted by the Working Group during the course of a *Monograph* meeting. The results of such original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies or de-novo

analyses. Irrespective of the source of data for the meta-analyses and pooled analyses, it is important that the same criteria for data quality be applied as those that would be applied to individual studies and to ensure also that sources of heterogeneity between studies be taken into account.

(*d*) *Temporal effects*

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure, peak exposure (when appropriate) and time since cessation of exposure, are reviewed and summarized when available. Analyses of temporal relationships may be useful in making causal inferences. In addition, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although, at best, they allow only indirect inferences about mechanisms of carcinogenesis.

(*e*) *Use of biomarkers in epidemiological studies*

Biomarkers indicate molecular, cellular or other biological changes and are increasingly used in epidemiological studies for various purposes (IARC, 1991; Vainio *et al.*, 1992; Toniolo *et al.*, 1997; Vineis *et al.*, 1999; Buffler *et al.*, 2004). These may include evidence of exposure, of early effects, of cellular, tissue or organism responses, of individual susceptibility or host responses, and inference of a mechanism (see Part B, Section 4b). This is a rapidly evolving field that encompasses developments in genomics, epigenomics and other emerging technologies.

Molecular epidemiological data that identify associations between genetic polymorphisms and interindividual differences in susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. If the polymorphism has been demonstrated experimentally to modify the functional activity of the gene product in a manner that is consistent with increased susceptibility, these data may be useful in making causal inferences. Similarly, molecular epidemiological studies that measure cell functions, enzymes or metabolites that are thought to be the basis of susceptibility may provide evidence that reinforces biological plausibility. It should be noted, however, that when data on genetic susceptibility originate from multiple comparisons that arise from subgroup analyses, this can generate false-positive results and inconsistencies across studies, and such data therefore require careful evaluation. If the known phenotype of a genetic polymorphism can explain the carcinogenic mechanism of the agent being evaluated, data on this phenotype may be useful in making causal inferences.

(*f*) *Criteria for causality*

After the quality of individual epidemiological studies of cancer has been summarized and assessed, a judgement is made concerning the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group

considers several criteria for causality (Hill, 1965). A strong association (e.g. a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that estimates of effect of small magnitude do not imply lack of causality and may be important if the disease or exposure is common. Associations that are replicated in several studies of the same design or that use different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in exposure), and results of studies that are judged to be of high quality are given more weight than those of studies that are judged to be methodologically less sound.

If the risk increases with the exposure, this is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship. The demonstration of a decline in risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

A number of scenarios may increase confidence in a causal relationship. On the one hand, an agent may be specific in causing tumours at one site or of one morphological type. On the other, carcinogenicity may be evident through the causation of multiple tumour types. Temporality, precision of estimates of effect, biological plausibility and coherence of the overall database are considered. Data on biomarkers may be employed in an assessment of the biological plausibility of epidemiological observations.

Although rarely available, results from randomized trials that show different rates of cancer among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, a judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires firstly that the studies meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of effect of unity for any observed level of exposure, (b) when considered together, provide a pooled estimate of relative risk that is at or near to unity, and (c) have a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency that the relative risk of cancer increases with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the dose levels reported, and to the intervals between first exposure and disease onset observed in these studies. Experience with human cancer indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

3. Studies of cancer in experimental animals

All known human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (e.g. aflatoxins, diethylstilbestrol, solar radiation, vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) also present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, these agents are considered to pose a carcinogenic hazard to humans. Examples of additional scientific information are data that demonstrate that a given agent causes cancer in animals through a species-specific mechanism that does not operate in humans or data that demonstrate that the mechanism in experimental animals also operates in humans (see Part B, Section 6).

Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (see Part A, Section 4). In all experimental settings, the nature and extent of impurities or contaminants present in the agent being evaluated are given when available. Animal species, strain (including genetic background where applicable), sex, numbers per group, age at start of treatment, route of exposure, dose levels, duration of exposure, survival and information on tumours (incidence, latency, severity or multiplicity of neoplasms or preneoplastic lesions) are reported. Those studies in experimental animals that are judged to be irrelevant to the evaluation or judged to be inadequate (e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. OECD, 2002).

Other studies considered may include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies, co-carcinogenicity studies and studies in genetically modified animals); studies in which the end-point was not cancer but a defined precancerous lesion; experiments on the carcinogenicity of known metabolites and derivatives; and studies of cancer in non-laboratory animals (e.g. livestock and companion animals) exposed to the agent.

For studies of mixtures, consideration is given to the possibility that changes in the physicochemical properties of the individual substances may occur during collection, storage, extraction, concentration and delivery. Another consideration is that chemical and toxicological interactions of components in a mixture may alter dose–response relationships. The relevance to human exposure of the test mixture administered in the animal experiment is also assessed. This may involve consideration of the following aspects of the mixture tested: (i) physical and chemical characteristics, (ii) identified

constituents that may indicate the presence of a class of substances and (iii) the results of genetic toxicity and related tests.

The relevance of results obtained with an agent that is analogous (e.g. similar in structure or of a similar virus genus) to that being evaluated is also considered. Such results may provide biological and mechanistic information that is relevant to the understanding of the process of carcinogenesis in humans and may strengthen the biological plausibility that the agent being evaluated is carcinogenic to humans (see Part B, Section 2f).

(*a*) *Qualitative aspects*

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age and duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

Considerations of importance in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration of treatment and route of exposure were appropriate; (iv) whether the survival of treated animals was similar to that of controls; (v) whether there were adequate numbers of animals per group; (vi) whether both male and female animals were used; (vii) whether animals were allocated randomly to groups; (viii) whether the duration of observation was adequate; and (ix) whether the data were reported and analysed adequately.

When benign tumours (a) occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) appear to represent a stage in the progression to malignancy, they are usually combined in the assessment of tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent induces only benign neoplasms that appear to be end-points that do not readily undergo transition to malignancy, the agent should nevertheless be suspected of being carcinogenic and requires further investigation.

(*b*) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain, genetic background and age of the animal, and on the dose, route, timing and duration of the exposure. Evidence of an increased incidence of neoplasms with increasing levels of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Mechanisms such as induction of DNA damage or inhibition of repair, altered cell division and cell death rates and changes in intercellular communication are important determinants of dose–response relationships for some carcinogens. Since many chemicals require metabolic activation before being converted to their reactive intermediates, both metabolic and toxicokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce non-linearity in the dose–response relationship (Hoel *et al.*, 1983; Gart *et al.*, 1986), as could saturation of processes such as DNA repair. The dose–response relationship can also be affected by differences in survival among the treatment groups.

(*c*) *Statistical analyses*

Factors considered include the adequacy of the information given for each treatment group: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986; Portier & Bailer, 1989; Bieler & Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether or not there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel-Haenzel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the Poly-K test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other more complicated statistical procedures may be needed (Sherman *et al.*, 1994; Dunson *et al.*, 2003).

Formal statistical methods have been developed to incorporate historical control data into the analysis of data from a given experiment. These methods assign an appropriate weight to historical and concurrent controls on the basis of the extent of between-study and within-study variability: less weight is given to historical controls when they show a high degree of variability, and greater weight when they show little variability. It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical controls show high between-study variability and

are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals (Haseman *et al.*, 1984; Fung *et al.*, 1996; Greim *et al.*, 2003).

Although meta-analyses and combined analyses are conducted less frequently for animal experiments than for epidemiological studies due to differences in animal strains, they can be useful aids in interpreting animal data when the experimental protocols are sufficiently similar.

4. Mechanistic and other relevant data

Mechanistic and other relevant data may provide evidence of carcinogenicity and also help in assessing the relevance and importance of findings of cancer in animals and in humans. The nature of the mechanistic and other relevant data depends on the biological activity of the agent being considered. The Working Group considers representative studies to give a concise description of the relevant data and issues that they consider to be important; thus, not every available study is cited. Relevant topics may include toxicokinetics, mechanisms of carcinogenesis, susceptible individuals, populations and life-stages, other relevant data and other adverse effects. When data on biomarkers are informative about the mechanisms of carcinogenesis, they are included in this section.

These topics are not mutually exclusive; thus, the same studies may be discussed in more than one subsection. For example, a mutation in a gene that codes for an enzyme that metabolizes the agent under study could be discussed in the subsections on toxicokinetics, mechanisms and individual susceptibility if it also exists as an inherited polymorphism.

(*a*) *Toxicokinetic data*

Toxicokinetics refers to the absorption, distribution, metabolism and elimination of agents in humans, experimental animals and, where relevant, cellular systems. Examples of kinetic factors that may affect dose–response relationships include uptake, deposition, biopersistence and half-life in tissues, protein binding, metabolic activation and detoxification. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data from humans and animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be important for the extrapolation of hazards between species and in clarifying the role of in-vitro findings.

(*b*) *Data on mechanisms of carcinogenesis*

To provide focus, the Working Group attempts to identify the possible mechanisms by which the agent may increase the risk of cancer. For each possible mechanism, a representative selection of key data from humans and experimental systems is summarized. Attention is given to gaps in the data and to data that suggests that more than one mechanism may be operating. The relevance of the mechanism to humans is discussed, in particular, when mechanistic data are derived from experimental model systems. Changes in the affected organs, tissues or cells can be divided into three non-exclusive levels as described below.

(i) *Changes in physiology*

Physiological changes refer to exposure-related modifications to the physiology and/or response of cells, tissues and organs. Examples of potentially adverse physiological changes include mitogenesis, compensatory cell division, escape from apoptosis and/or senescence, presence of inflammation, hyperplasia, metaplasia and/or preneoplasia, angiogenesis, alterations in cellular adhesion, changes in steroidal hormones and changes in immune surveillance.

(ii) *Functional changes at the cellular level*

Functional changes refer to exposure-related alterations in the signalling pathways used by cells to manage critical processes that are related to increased risk for cancer. Examples of functional changes include modified activities of enzymes involved in the metabolism of xenobiotics, alterations in the expression of key genes that regulate DNA repair, alterations in cyclin-dependent kinases that govern cell cycle progression, changes in the patterns of post-translational modifications of proteins, changes in regulatory factors that alter apoptotic rates, changes in the secretion of factors related to the stimulation of DNA replication and transcription and changes in gap–junction-mediated intercellular communication.

(iii) *Changes at the molecular level*

Molecular changes refer to exposure-related changes in key cellular structures at the molecular level, including, in particular, genotoxicity. Examples of molecular changes include formation of DNA adducts and DNA strand breaks, mutations in genes, chromosomal aberrations, aneuploidy and changes in DNA methylation patterns. Greater emphasis is given to irreversible effects.

The use of mechanistic data in the identification of a carcinogenic hazard is specific to the mechanism being addressed and is not readily described for every possible level and mechanism discussed above.

Genotoxicity data are discussed here to illustrate the key issues involved in the evaluation of mechanistic data.

Tests for genetic and related effects are described in view of the relevance of gene mutation and chromosomal aberration/aneuploidy to carcinogenesis (Vainio

et al., 1992; McGregor *et al*., 1999). The adequacy of the reporting of sample characterization is considered and, when necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests. The available data are interpreted critically according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations and aneuploidy. The concentrations employed are given, and mention is made of whether the use of an exogenous metabolic system *in vitro* affected the test result. These data are listed in tabular form by phylogenetic classification.

Positive results in tests using prokaryotes, lower eukaryotes, insects, plants and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information on the types of genetic effect produced and on the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g. gene mutations), while others are associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour promotion, cell transformation and gap–junction intercellular communication may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. Critical appraisals of these tests have been published (Montesano *et al*., 1986; McGregor *et al*., 1999).

Genetic or other activity manifest in humans and experimental mammals is regarded to be of greater relevance than that in other organisms. The demonstration that an agent can induce gene and chromosomal mutations in mammals *in vivo* indicates that it may have carcinogenic activity. Negative results in tests for mutagenicity in selected tissues from animals treated *in vivo* provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence that rules out the carcinogenicity of agents that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative cell division, peroxisome proliferation) (Vainio *et al*., 1992). Factors that may give misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al*., 1986; McGregor *et al*., 1999).

When there is evidence that an agent acts by a specific mechanism that does not involve genotoxicity (e.g. hormonal dysregulation, immune suppression, and formation of calculi and other deposits that cause chronic irritation), that evidence is presented and reviewed critically in the context of rigorous criteria for the operation of that mechanism in carcinogenesis (e.g. Capen *et al.*, 1999).

For biological agents such as viruses, bacteria and parasites, other data relevant to carcinogenicity may include descriptions of the pathology of infection, integration and expression of viruses, and genetic alterations seen in human tumours. Other observations

that might comprise cellular and tissue responses to infection, immune response and the presence of tumour markers are also considered.

For physical agents that are forms of radiation, other data relevant to carcinogenicity may include descriptions of damaging effects at the physiological, cellular and molecular level, as for chemical agents, and descriptions of how these effects occur. 'Physical agents' may also be considered to comprise foreign bodies, such as surgical implants of various kinds, and poorly soluble fibres, dusts and particles of various sizes, the pathogenic effects of which are a result of their physical presence in tissues or body cavities. Other relevant data for such materials may include characterization of cellular, tissue and physiological reactions to these materials and descriptions of pathological conditions other than neoplasia with which they may be associated.

(*c*) *Other data relevant to mechanisms*

A description is provided of any structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent, the toxicological implications of the physical and chemical properties, and any other data relevant to the evaluation that are not included elsewhere.

High-output data, such as those derived from gene expression microarrays, and high-throughput data, such as those that result from testing hundreds of agents for a single end-point, pose a unique problem for the use of mechanistic data in the evaluation of a carcinogenic hazard. In the case of high-output data, there is the possibility to overinterpret changes in individual end-points (e.g. changes in expression in one gene) without considering the consistency of that finding in the broader context of the other end-points (e.g. other genes with linked transcriptional control). High-output data can be used in assessing mechanisms, but all end-points measured in a single experiment need to be considered in the proper context. For high-throughput data, where the number of observations far exceeds the number of end-points measured, their utility for identifying common mechanisms across multiple agents is enhanced. These data can be used to identify mechanisms that not only seem plausible, but also have a consistent pattern of carcinogenic response across entire classes of related compounds.

(*d*) *Susceptibility data*

Individuals, populations and life-stages may have greater or lesser susceptibility to an agent, based on toxicokinetics, mechanisms of carcinogenesis and other factors. Examples of host and genetic factors that affect individual susceptibility include sex, genetic polymorphisms of genes involved in the metabolism of the agent under evaluation, differences in metabolic capacity due to life-stage or the presence of disease, differences in DNA repair capacity, competition for or alteration of metabolic capacity by medications or other chemical exposures, pre-existing hormonal imbalance that is exacerbated by a chemical exposure, a suppressed immune system, periods of higher-than-usual tissue growth or regeneration and genetic polymorphisms that lead to

differences in behaviour (e.g. addiction). Such data can substantially increase the strength of the evidence from epidemiological data and enhance the linkage of in-vivo and in-vitro laboratory studies to humans.

(*e*) *Data on other adverse effects*

Data on acute, subchronic and chronic adverse effects relevant to the cancer evaluation are summarized. Adverse effects that confirm distribution and biological effects at the sites of tumour development, or alterations in physiology that could lead to tumour development, are emphasized. Effects on reproduction, embryonic and fetal survival and development are summarized briefly. The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is judged by the same criteria as those applied to epidemiological studies of cancer, but fewer details are given.

5. Summary

This section is a summary of data presented in the preceding sections. Summaries can be found on the *Monographs* programme website (http://monographs.iarc.fr).

(*a*) *Exposure data*

Data are summarized, as appropriate, on the basis of elements such as production, use, occurrence and exposure levels in the workplace and environment and measurements in human tissues and body fluids. Quantitative data and time trends are given to compare exposures in different occupations and environmental settings. Exposure to biological agents is described in terms of transmission, prevalence and persistence of infection.

(*b*) *Cancer in humans*

Results of epidemiological studies pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized. The target organ(s) or tissue(s) in which an increase in cancer was observed is identified. Dose–response and other quantitative data may be summarized when available.

(*c*) *Cancer in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species, study design and route of administration, it is stated whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or preneoplastic lesions were observed, and the tumour sites are indicated. If the agent produced tumours after prenatal exposure or in single-dose experiments, this is also mentioned. Negative findings, inverse relationships, dose–response and other quantitative data are also summarized.

(*d*) *Mechanistic and other relevant data*

Data relevant to the toxicokinetics (absorption, distribution, metabolism, elimination) and the possible mechanism(s) of carcinogenesis (e.g. genetic toxicity, epigenetic effects) are summarized. In addition, information on susceptible individuals, populations and life-stages is summarized. This section also reports on other toxic effects, including reproductive and developmental effects, as well as additional relevant data that are considered to be important.

6. Evaluation and rationale

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms. The strength of the mechanistic evidence is also characterized.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent to a higher or lower category than a strict interpretation of these criteria would indicate.

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency). A classification may change as new information becomes available.

An evaluation of the degree of evidence is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of the degree of evidence.

(*a*) *Carcinogenicity in humans*

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence. A statement that there is *sufficient evidence* is followed by a separate sentence that identifies the target organ(s) or tissue(s) where an increased risk of cancer was observed in humans. Identification of a specific target organ or tissue does not preclude the possibility that the agent may cause cancer at other sites.

***Limited evidence of carcinogenicity*:** A positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence of carcinogenicity*:** The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association between exposure and cancer, or no data on cancer in humans are available.

***Evidence suggesting lack of carcinogenicity*:** There are several adequate studies covering the full range of levels of exposure that humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and any studied cancer at any observed level of exposure. The results from these studies alone or combined should have narrow confidence intervals with an upper limit close to the null value (e.g. a relative risk of 1.0). Bias and confounding should be ruled out with reasonable confidence, and the studies should have an adequate length of follow-up. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the cancer sites, conditions and levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

When the available epidemiological studies pertain to a mixture, process, occupation or industry, the Working Group seeks to identify the specific agent considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

(*b*) *Carcinogenicity in experimental animals*

Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals.

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single

species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide *sufficient evidence*.

A single study in one species and sex might be considered to provide *sufficient evidence of carcinogenicity* when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

***Limited evidence of carcinogenicity*:** The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

***Inadequate evidence of carcinogenicity*:** The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

***Evidence suggesting lack of carcinogenicity*:** Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent is not carcinogenic. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the species, tumour sites, age at exposure, and conditions and levels of exposure studied.

(*c*) *Mechanistic and other relevant data*

Mechanistic and other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is highlighted. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and toxicokinetics, physicochemical parameters and analogous biological agents.

The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is evaluated, using terms such as 'weak', 'moderate' or 'strong'. The Working Group then assesses whether that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans derive from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

The conclusion that a mechanism operates in experimental animals is strengthened by findings of consistent results in different experimental systems, by the demonstration of

biological plausibility and by coherence of the overall database. Strong support can be obtained from studies that challenge the hypothesized mechanism experimentally, by demonstrating that the suppression of key mechanistic processes leads to the suppression of tumour development. The Working Group considers whether multiple mechanisms might contribute to tumour development, whether different mechanisms might operate in different dose ranges, whether separate mechanisms might operate in humans and experimental animals and whether a unique mechanism might operate in a susceptible group. The possible contribution of alternative mechanisms must be considered before concluding that tumours observed in experimental animals are not relevant to humans. An uneven level of experimental support for different mechanisms may reflect that disproportionate resources have been focused on investigating a favoured mechanism.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(*d*) *Overall evaluation*

Finally, the body of evidence is considered as a whole, in order to reach an overall evaluation of the carcinogenicity of the agent to humans.

An evaluation may be made for a group of agents that have been evaluated by the Working Group. In addition, when supporting data indicate that other related agents, for which there is no direct evidence of their capacity to induce cancer in humans or in animals, may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of agents if the strength of the evidence warrants it.

The agent is described according to the wording of one of the following categories, and the designated group is given. The categorization of an agent is a matter of scientific judgement that reflects the strength of the evidence derived from studies in humans and in experimental animals and from mechanistic and other relevant data.

Group 1: The agent is *carcinogenic to humans*.

This category is used when there is *sufficient evidence of carcinogenicity* in humans. Exceptionally, an agent may be placed in this category when evidence of carcinogenicity in humans is less than *sufficient* but there is *sufficient evidence of carcinogenicity* in experimental animals and strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity.

Group 2.

This category includes agents for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost *sufficient*, as well as those for which, at the other extreme, there are no human data but for which there is evidence of

carcinogenicity in experimental animals. Agents are assigned to either Group 2A (*probably carcinogenic to humans*) or Group 2B (*possibly carcinogenic to humans*) on the basis of epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used simply as descriptors of different levels of evidence of human carcinogenicity, with *probably carcinogenic* signifying a higher level of evidence than *possibly carcinogenic*.

Group 2A: The agent is *probably carcinogenic to humans*.

This category is used when there is *limited evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals. In some cases, an agent may be classified in this category when there is *inadequate evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent may be classified in this category solely on the basis of *limited evidence of carcinogenicity* in humans. An agent may be assigned to this category if it clearly belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A.

Group 2B: The agent is *possibly carcinogenic to humans*.

This category is used for agents for which there is *limited evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals. It may also be used when there is *inadequate evidence of carcinogenicity* in humans but there is *sufficient evidence of carcinogenicity* in experimental animals. In some instances, an agent for which there is *inadequate evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals together with supporting evidence from mechanistic and other relevant data may be placed in this group. An agent may be classified in this category solely on the basis of strong evidence from mechanistic and other relevant data.

Group 3: The agent is *not classifiable as to its carcinogenicity to humans*.

This category is used most commonly for agents for which the evidence of carcinogenicity is *inadequate* in humans and *inadequate* or *limited* in experimental animals.

Exceptionally, agents for which the evidence of carcinogenicity is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents that do not fall into any other group are also placed in this category.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that further research is needed, especially when exposures are widespread or the cancer data are consistent with differing interpretations.

Group 4: The agent is *probably not carcinogenic to humans*.

This category is used for agents for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents for which there is *inadequate evidence of carcinogenicity* in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of mechanistic and other relevant data, may be classified in this group.

(*e*) *Rationale*

The reasoning that the Working Group used to reach its evaluation is presented and discussed. This section integrates the major findings from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic and other relevant data. It includes concise statements of the principal line(s) of argument that emerged, the conclusions of the Working Group on the strength of the evidence for each group of studies, citations to indicate which studies were pivotal to these conclusions, and an explanation of the reasoning of the Working Group in weighing data and making evaluations. When there are significant differences of scientific interpretation among Working Group Members, a brief summary of the alternative interpretations is provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

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GENERAL REMARKS

This ninety-ninth volume of the *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of some aromatic amines, organic dyes, and related occupational or consumer exposures. Each had been evaluated previously in Volumes 1, 4, 29, 57, or 77, or in Supplement 7. Newer epidemiological and experimental studies have been published and are reviewed in this volume. As most of these compounds have been associated with bladder tumours in humans or experimental animals, they are being considered at one time with a view towards determining whether the overall evaluations can be improved through consideration of common mechanisms of carcinogenesis.

Several studies where hair dyes were applied to the skin of experimental animals would not be considered adequate tests of carcinogenicity by current standards. These studies did not involve administration at a maximum tolerated concentration, and signs of toxicity, reduced body-weight gain, or reduced survival were not often reported. Rather, the hair dyes were generally applied at concentrations representative of consumer use. In one set of experiments, the concentration applied to the animals was diluted to one-tenth of the strength of the commercial product as intended for use by people. These low concentrations, coupled with the small numbers of animals tested in some studies, reduce the informative value of these experiments and suggest a pressing need for publication of proper carcinogenicity studies that meet current standards.

A summary of the findings of this volume appears in *The Lancet Oncology* (Baan *et al.*, 2008).

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GENERAL DISCUSSION OF COMMON MECHANISMS FOR AROMATIC AMINES

Ever since certain aromatic amines have been shown to be carcinogenic in humans the question has been raised how the chemical structure determines the biological effects, because a better understanding of this relationship could help assess the hazard and the risk associated with exposure to these chemicals. The common denominator is an amino-group bound to an aromatic system. The chemical reactivity of this amino group depends on the mesomeric interaction with the aromatic system, which is determined by further substituents and steric factors (Beland *et al.*, 1997; Marques *et al.*, 1997).

Recent progress in cancer research has revealed the complexity of the interaction between exogenous exposures and the physiology of an organism. The existing knowledge favours the idea that common principles combine the many aromatic amines and it might be concluded that most, if not all aromatic amines have a carcinogenic potential. The following considerations should help to answer the questions:

1. Do aromatic amines have generally a carcinogenic potential?
2. Is there a common mode of action that allows us to draw this conclusion?

1. Metabolic Activation

Metabolic activation was the leading concept to find out how aromatic amines cause biological effects. Both acute and chronic toxicity are held to depend on the metabolic activation of the amino group. The key reaction responsible for all the biological activities is the *N*-oxidation to aryl-*N*-hydroxylamines. Either the free amine or the acetamide can be *N*-oxidized. Thus an equilibrium exists between the two, which is determined by the competing activity of *N*-acetyltransferases and *N*-deacetylases. Frederick *et al.* (1985) described the equilibrium between benzidine, *N*-acetylbenzidine and *N,N′*-diacetylbenzidine in liver slices. The distribution in this eqilibrium is important, since acetylation of the amine to the acetamide is an inactivating reaction, as is the *C*-oxidation of the aromatic system. Dogs develop more readily bladder tumours with benzidine than several other species, because dogs as "non-acetylators" lack one of the inactivating metabolic steps (Lakshmi *et al.*, 1995).

Both the *N*-hydroxylamine and the *N*-hydroxyacetamide may be further activated by activating the leaving group through conjugation of the hydroxy group with sulfate or acetate, the sulfate being usually a better mutagen than the acetate. Eventually, the biological activity depends on the bioavailability of a nitrenium ion, an ultimate reactive metabolite that reacts with DNA, RNA, and proteins. The ultimate metabolites of most arylamines react *in vitro* and *in vivo* with C-8 of guanine and the respective adduct has been made responsible for point mutations.

The metabolic activation of benzidine is an interesting example that shows the complexity that follows from competing metabolic pathways and how these depend on the experimental system investigated. Assuming that *N*-hydroxy- *N*,*N*′-diacetylbenzidine (*N*-OH-DABZ) is a proximate carcinogen, it was used as the starting material in in-vitro experiments in which the esterification by cytosolic sulfotransferase to a reactive protein-binding metabolite could be demonstrated, and it was suggested that this pathway is involved in benzidine-induced carcinogenesis (Morton *et al.*, 1980).

Later on, the question was where the activating metabolism takes place. Acid-labile glucuronides are formed in the liver, which are transported to the bladder. In the acid urine they are hydrolysed either to *N*-acetylbenzidine, which could be activated by peroxidases, or to *N*′-hydroxy-*N*-acetylbenzidine, which could be further activated for instance by *O*-acetylation. The formation of the resulting guanine-C-8-adduct was explained by the reaction of the nitrenium ion with the intermediary formation of benzidine-diimine (Babu *et al.*, 1992; Zenser *et al.*, 1998). Already this summary outline of activating metabolism shows how many competing steps may be influenced by the circumstantial situation, the individual susceptibility, nutritional habits, voiding volume, dwelling time in the bladder, etc. It is clear that toxicokinetics will be influenced by species-, tissue- and cell-specific conditions.

The concept of metabolic activation was soon generally accepted as an essential prerequisite to explain the biological activity of an amine and it was expected that once the relationships between chemical structure of the amine and these conditions were understood, it would be possible to account for quantitative differences in the level of reactive metabolites and explain the diverse biological effects.

Other pathways have been proposed, such as the formation of reactive oxygen species which are made responsible for oxidative DNA damage and mutations induced by, e.g., 2-naphthylamine (Ohnishi *et al.*, 2002), 4-aminobiphenyl (ABP) and benzidine (Makena & Chung, 2007). But this is also a general property of aromatic amine metabolism. Human lung chromosomes contain high levels of arylamine peroxidase activity which readily activates ABP, benzidine, 4,4′-methylenebis(2-chloroaniline) (MOCA), 2-amino-fluorene (AF) and 2-naphthylamine as measured by DNA-adduct formation (Culp *et al.*, 1997). Prostaglandin H synthase activates *N*-acetylbenzidine leading to the typical guanine-C-8-acetylbenzidine-adduct (Lakshmi *et al.*, 1998). Peroxidase-mediated activation of aromatic amines can also be demonstrated by activating polymorphonuclear leukocytes with tumour promoters. Binding of metabolites to leukocyte DNA has been

found with benzidine, 2-aminofluorene and methylaminoazobenzene (Tsuruta *et al.*, 1985).

By far the most studies of aromatic amine metabolism were performed with 2-acetylaminofluorene (AAF) and to a lesser degree with 4-aminobiphenyl (ABP) and 2-aminonaphthalene (AN) and benzidine (BZ), which were among the first chemicals classified as human carcinogens. The ultimate goal of these studies – mostly performed in cell and tissue culture and less *in vivo* – was to find the critical metabolic pathway and the critical biological lesion, primarily in DNA. Many positive correlations were found and many species- and tissue-specific effects of individual arylamines could be explained by quantitative differences in toxicokinetics rather than by specific properties of the individual amine.

The role of metabolic activation for the individual susceptibility has particularly attracted interest with aromatic amines (Carreón *et al.*, 2006). One of the first examples for an enzymatic polymorphism was the observation that workers occupationally exposed to benzidine who were slow acetylators were at greater risk to develop bladder tumours than rapid acetylators (Golka *et al.*, 2002; Gu *et al.*, 2005. Sinués *et al.*, 1992). This was not confirmed in later studies, which incorporated phenotypic and genotypic analysis (Hayes *et al.*, 1993: Ma *et al.*, 2004). Similarly, there was no overall increase in bladder-cancer risk in the *GSTM1*-null genotype of benzidine-exposed workers (Shinka *et al.*, 1998), which is in contrast to its association with elevated bladder-cancer risk in the general population (Rothman *et al.*, 1996).

On the other hand, an elevated bladder-cancer risk for formerly benzidine-exposed workers in the Chinese dyestuff industry was associated with a homozygous mutant genotype of UDP-glucuronosyltransferase 2B7. This polymorphism is different from that in Caucasian populations (Lin *et al.*, 2005).

In summary, many enzymatic polymorphisms of enzymes involved in the metabolism of aromatic amines are now known and the various equilibria between activating and inactivating steps inevitably must be influenced by the individual set-up. One of the consequences is that epidemiological effects are likely to show up only in particularly exposed and rather homogeneous populations.

2. Mechanisms of Carcinogenesis

The concept of metabolic activation to an ultimate reactive metabolite was clearly supported by the finding that c-H-*ras*, the first oncogene that has been found in normal liver as well as in mouse-liver tumours, could be activated by a point mutation caused by exactly the guanine-C-8-AF adduct described above (Wiseman *et al.* 1986). This activation was considered an early effect in the development of liver tumours in the mouse (Anderson *et al.* 1992). In the meantime many proto-oncogenes have been identified that are activated and tumour-suppressor genes that are inactivated by genotoxic

effects. Most activated oncogenes found in human tumours are identical with those found in experimental animals.

Are there DNA-specific effects? Numerous carcinogens produce mutations in typical codons, such as 12, 13 and 61 of the H-*ras* gene, and the corresponding mutations were identified in different target tissues, i.e. in human lung and colon, but the pattern of mutations is too different to establish a clear cause-effect relationship. It is for instance not known why mutations of the H-*ras* gene are seen in mouse-liver tumours, but not in rat-liver tumours (Bitsch *et al.*, 1993). They seem to represent tumour-initiating lesions in mouse skin and liver and in rat mammary tissue (Stanley, 1995). Similar but distinguishable mutation profiles were seen with ABP, 2-aminoanthracene and PhIP in the *lac*Z-reversion assay (Garganta *et al.*, 1999). The selectivity of mutations in the *ras* oncogene in AAF-induced mouse lung and liver tumours was proposed to be tissue-specific as compared with those in spontaneously occurring mouse lung and liver tumours (Wang *et al.*, 1993).

Although both AAF and ABP produce the same type of DNA-adduct, i.e. dG-C8-AF and dG-C8-ABP, the pattern of mutations is different. AAF induces frame-shift and base-substitution mutations (G-T-transversions), ABP only base-substitution mutations (G-A-transversions). The mutagenic efficiency per adduct is greater with AAF than with ABP (Beland *et al.*, 1990). The dG-8-ABP adducts have been identified in human-bladder tumours (Zayas *et al.*, 2007), in bladder epithelial cells (Skipper & Tannenbaum, 1994), and in exfoliated bladder epithelial cells (Talaska *et al.*, 1993). The adduct levels correlate positively with cigarette smoking, type of tobacco and slow-acetylator phenotype. Do these results reflect bladder-specific or amine-specific effects?

From the very beginning interest focused on bladder tumours, because they were the first to be associated in humans with occupational exposure to aromatic amines. But as we know now, adduct formation and genotoxic effects are not target tissue-specific. The dG-C8-ABP adduct has been demonstrated in many human tissues, for instance in mammary tissue (Faraglia *et al.*, 2003).

The formation of dG-C8-ABP adducts correlates well with the formation of protein adducts such as that with haemoglobin (Talaska *et al.*, 1993; Kadlubar *et al.*, 1991). This protein adduct can be measured in blood samples and be used as a biomarker of exposure and as a biomarker of effect. It indicates that the *N*-hydroxylamine (or the nitroso-derivative) is distributed throughout the organism and in agreement with the general experience is available in most if not all tissues. Nukui *et al.* (2007) demonstrated the transplacental exposure during the pregnancy of smoking mothers.

The molecular basis by which 4-ABP mediates carcinogenic activity was believed to be its ability to produce mutations in the human genome. However, additional mechanisms have come up recently. Bladder cancer is now proposed to be the result of gross chromosome aberrations rather than point mutations (Saletta *et al.* 2007). Cells carrying chromosome instability and microsatellite instability have a selective advantage. Exposure to specific carcinogens can select for tumour cells with distinct forms of genetic instability (Bardelli *et al.*, 2002).

Despite many open questions the key steps outlined above are basically the same (Goodrow, 1996), and the similarities exceed the unexplained differences. The same metabolic scheme, the same kind of genotoxic lesions and in many cases the same or analogous tissue-specificity – like the generation of bladder tumours – support the view that the mode of action is the same for this whole group of chemicals.

Moreover, Benigni and Pino (1998) studied tumour profiles (target tissues) of 536 rodent carcinogens in the four experimental systems usually employed (rat/mouse, male/female). Aromatic amines and nitroarenes were among the classes most represented in the database. The authors come to the conclusion that no obvious association exists between chemical/mode of action class and tumour profile. It rather appears that each class produces tumours at a wide range of sites. It is suggested that the events surrounding the ultimate mechanism of reaction with DNA determine the differences in tumour profile.

Benigni and Passerini (2002) evaluated several QSAR-models and concluded that the gradation of potency of aromatic amines depends first on their hydrophobicity, and second on electronic properties (reactivity, propensity to be metabolically transformed) and steric characteristics. Although this regards some basic properties, it appears not to be possible to predict carcinogenicity and potency of an aromatic amine. However, the models help to verify the proposed mode of action and in fact support it.

In summary, a common mode of action is at the basis of the carcinogenic properties of aromatic amines and a carcinogenic potential seems to be associated with this whole group of chemicals. When and where a tumour will develop depends on the interaction of the chemical, with its specific properties, in a highly adaptable organism.

3. The Role of Monocyclic Aromatic Amines

It was believed for a long time that only the polycyclic aromatic amines, but not the monocyclic amines have carcinogenic potential. This conviction was abandoned when occupational exposure to 4-chloro-*ortho*-toluidine was shown to produce bladder tumours in workers. *ortho*-Toluidine had also to be classified as a carcinogen and the experimental results with aniline eventually put an end to this hypothesis. With each of a vast variety of monocyclic aromatic amines, *N*-hydroxylamines are metabolically formed under suitable conditions, and reactions with DNA and mutagenic activity can be demonstrated (Marques *et al.*, 1997). No criterium can be defined at present that would allow to seperate genotoxic from non-genotoxic, or carcinogenic from non-carcinogenic monocyclic arylamines. This is primarily due to results indicating that the role of genotoxicity was overestimated. It dominates potential and potency far less than hitherto believed.

This became particularly apparent with the recent developments concerning aniline and structurally related amines. The discussion focused for a long time on the question: is

aniline a genotoxic carcinogen and if not, should it be classified at all as a carcinogen. Tests for mutagenicity gave contradictory results and because of the low genotoxic potency these data were considered not to be sufficient to explain the spleen tumours observed in rats (Wilmer *et al.*, 1984; Bomhard and Herbold, 2005). It was concluded that these tumours must be caused by a non-genotoxic mechanism, with the possibility to establish a NOAEL (Bus & Popp, 1987 called it a threshold). It was hypothesized that with increasing doses more damaged erythrocytes are eliminated in the spleen, which causes vascular congestion, pericapsular inflammation, fibrosis and eventually sarcoma and angiosarcoma of the spleen. This would represent a typical high-dose phenomenon. In addition it was argued that spleen tumours in male rats are not relevant for the human situation.

The process starts with the *N*-oxidation of aniline to *N*-phenylhydroxylamine in the liver. In the erythrocytes, phenylhydroxylamine is then co-oxidized to nitrosobenzene, and Fe2+-haemoglobin is oxidized to Fe3+-methaemoglobin. Methaemoglobin has a reduced capacity to bind oxygen and causes a hypoxic situation. Both reactions are reversible, nitrosobenzene is reduced back to phenylhydroxylamine and Fe3+ to Fe2+. This regenerating process depends largely on the availability of reduced glutathione, which keeps methaemoglobin at a tolerable level. At the work-place only methaemoglobin levels of more than 5% are considered adverse. Khan *et al.*, (1997) expected that detrimental effects occur only when the degradation of erythrocytes in the spleen is overloaded. One of the hypotheses is that erythrocyte membranes become less plastic and like senescent erythrocytes are sequestered and degraded by the spleen. Iron is released in this process (Ciccoli *et al.*, 1999) which could activate oxygen, which in turn modifies cellular DNA. This would be an indirect genotoxic mechanism. At the same time lipids and proteins are oxidized and heme is excessively degraded. All these reactions contribute to cytotoxicity. Although iron is also released within the erythrocytes during methaemoglobin formation, the intravasal degradation of these cells is not thought to play a significant role (Pauluhn, 2004).

The example aniline shows how intimately genotoxic and non-genotoxic effects are connected and that genotoxicity alone will not answer the question.

Is it possible now to close the discussion and decide whether or not aniline has a carcinogenic potential, or more precisely, can a threshold be defined below which it does not contribute to carcinogenic risk? First of all, when metabolic activation and bioavailability of reactive metabolites are used as an end-point, an NEL was not reached at low doses in a 4-week study in male rats (Zwirner-Baier *et al.* 2003). It was, therefore, concluded that any exposure to aniline contributes to a background of methaemoglobin formation. A variety of endogenous and exogenous chemicals make up this background; other aromatic amines are particularly involved.

In addition to methaemoglobin formation, erythrocytes are damaged by reactive metabolites that react with proteins and membranes. Nitrosobenzene, for instance, reacts with the SH-groups of cysteine in the β-chain of haemoglobin. A stable sulfinamide-

adduct is formed, which has been used as a biomarker of effect (Albrecht and Neumann, 1985, Neumann, 2000, Sarkar *et al.* (2006).

The use of Hb-adducts as biomarkers clearly demonstrates that the general population is exposed to many monocyclic and polycyclic aromatic amines (Bryant *et al.*, 1987, Neumann *et al.*, 1995). The biomonitoring results make also clear that acute toxicity follows the same direction in humans as in experimental animals. Consequently, any quantitative considerations have to take into account additive or synergistic effects for most steps within this common mode of action.

4. The Role of Aromatic Nitrocompounds

At this point it is necessary to direct the attention to the fact that aromatic nitro-compounds have to be included into the group of chemicals whose correct collective name is *N*-substituted aryl compounds. The same *N*-hydroxylamine is formed by reducing the nitrogroup as by oxidizing the amine. These reactions take place at different locations. Nitrogroups are reduced to nitrosobenzene primarily in the reductive environment of the intestine, whereas amines are oxidized predominantly in liver. The ultimate metabolites may therefore be distributed differently. It is interesting to look at corresponding pairs of amino- and nitrocompounds, such as aniline and nitrobenzene (Neumann *et al*., 1995; Neumann, 2005). The reactive metabolites – phenylhydroxylamine and nitrosobenzene – are identical, the location of tumours in the rat, however, is different. Aniline causes sarcomas predominantly in the spleen, nitrobenzene produces liver adenoma and carcinoma in the rat. Both agents acutely produce methaemoglobin and chronic anaemia, and liver and kidney damage in rat and mouse. The biological tolerance values for aniline and nitrobenzene have therefore been set the same in Germany (DFG 2007; list of MAK and BAT values). The relationship between nitroarenes and amines becomes particularly important if it is realized that aromatic nitro-compounds are ubiquitously present in the environment as combustion products. Wherever organic material is combusted not only polycyclic aromatic hydrocarbons but – in the presence of nitrogen – also polycyclic aromatic nitro-compounds are formed (Neumann, 2001). Already in 1978, Johnson & Cornish (1978) studied in rats the conversion of 1- and 2-nitronaphthalene to 1- and 2-aminonaphthalene.

5. Genotoxicity Is Not the Only Mechanism

Soon it became clear that a single mutation was not sufficient to generate a tumour, but two or three such critical lesions in combination should be able to control the multistep process of tumour formation (Brandau & Böhle, 2001). In the case of large-bowel tumours up to eight irreversible alterations were postulated. The underlying

paradigm was that a genotoxic chemical, like an aromatic amine, is able to transform a normal cell into a tumour cell, which gains increasing growth advantage and ultimately grows to a tumour. Numerous types of genotoxic lesions may contribute, chromosome instability included. All the knowledge about the spectrum of DNA lesions formed upon administration of a single carcinogen as an initiator has not yet led to the identification of those critical lesions that predispose a cell to the development of neoplasia (Dragan and Pitot, 1992).

Despite many positive correlations between genotoxic lesions and species- and tissue-specific effects, genotoxic effects are necessary but not sufficient to explain the process of tumour formation. Early on, observations were reported indicating that pre-neoplastic lesions in rat liver were only seen when carcinogen treatment was coupled with a proliferative stimulus, partial hepatectomy being one of the possible triggers (Columbano *et al.*, 1981; Neumann, 1986, Nguyen-Ba & Vasseur, 1999).

A well known example for non-correlation of genotoxicity and tumour formation came from a dose–response study, the so-called megamouse experiment. Chronic administration of AAF to BALB/c mice produced liver as well as bladder tumours. The level of the typical guanine-C-8-AF-adduct as the relevant lesion increased linearly with dose in both tissues, more so in bladders than in livers. Tumor incidence however, increased linearly only in livers starting at the level of spontaneous liver tumours. Despite the higher adduct levels in bladder, the tumour incidence increased in this tissue steeply and nonlinearly only at some higher doses. This increase was associated with an increase in cell proliferation. This means that independent of the significantly higher DNA-damage in the bladder, tumours developed only when cell proliferation was stimulated by the carcinogenic agent.

In a corresponding experiment with 4-aminobiphenyl, bladder tumours were also obtained only with increased cell proliferation. Adduct levels were 2 to 3 times higher in bladder than in liver. In this case the yield of liver tumours was rather low, which was explained by an increased formation and the transport of *N*-hydroxy-4-aminobiphenyl-*N*-glucuronide from the liver, which led to lower exposures in the liver and higher exposures to the reactive metabolite in the bladder, where the glucuronide is hydrolized. This shows how pharmacokinetics can modify the genotoxic effect and how toxicity may determine tissue specificity (Poirier *et al.*, 1995).

In another example the carcinogenic effects of three polycyclic aromatic amines were compared: trans-4-acetylaminostilbene (AAS), 2-acetylaminophenanthrene (AAP), and 2-acetylaminofluorene (AAF). All three agents produce initiated, i.e. promotable cells in rat liver, but only one of them (AAF) produces liver tumours and, therefore, is a complete carcinogen for this tissue. A fundamental difference between the three agents is that only the complete carcinogen is hepatotoxic. In this case the adverse effect could be attributed at the molecular level as a non-genotoxic effect. AAF metabolites specifically uncouple the mitochondrial respiratory chain by detracting electrons, which opens the mitochondrial transition pore and interferes with the regulation of apoptosis (Bitsch *et al.*,

2000). Inhibition of apoptosis may help damaged cells to escape cell death and acquire a tumorigenic phenotype (Nguyen-Ba & Vasseur, 1999).

All three examples show that two different properties were required to make the aromatic amine a complete carcinogen: it must be mutagenic and cytotoxic. Other end-points, like progressive loss of histone H4 lysine 20 trimethylation, and increased histone H3 serine 10 phosphorylation, which were detected in rat liver, but not in kidney and spleen, indicate clearly the importance of epigenetic changes in carcinogenesis (Pogribny *et al.*, 2007).

Among the first authors who proposed a role for toxicity were Radomski *et al.* (1971). 1-Naphthylamine (1-NA) in contrast to the 2-isomer was considered to be non-carcinogenic, and the rat resistant to the formation of bladder tumours. In a study that compared the two isomers, the isomeric *N*-hydroxylamines (*N*-OH-NA) and their nitroso-derivatives (NO-N) were tested directly by i.p. injection in rats, and both oxidation products produced tumours (fibromas, fibrosarcoma and lymphosarcomas), but they also turned out to be hepatotoxic, such that the survival time was significantly reduced.. Both, 1-NOH-NA and 1-NO-N were more carcinogenic than the 2-isomers, and both gave the same type of tumours. When administered to newborne mice, it was the other way around: 2-NOH-NA was more carcinogenic than 1-NOH-NA, and 2-NOH-N more efficient than 2-NO-N. The original testing for carcinogenicity of the amines was evidently insufficient and both isomers have carcinogenic potential under suitable conditions. It also shows that the rat is not completely resistant to oral doses of 2-NA (Hicks *et al.* 1982). Toxicity has strongly influenced the outcome of the test results. The promoting effects of AAF have often been used in models of carcinogenicity testing and undefined toxicity was made responsible for this effect (Sparfel *et al.*, 2002).

6. Conclusions

The study of carcinogenic *N*-substituted aryl compounds, a large group of chemicals not only present at many workplaces but also in the general environment, teaches us an important lesson. If suitable conditions are chosen it is possible to demonstrate, with practically all of them, the formation of ultimate metabolites, their reaction with DNA, RNA and proteins, mutagenic activity, the formation of methaemoglobin and other acute toxic effects. Only in a few cases has it been possible so far to prove a causal relationship in humans, sufficient to classify the agent in IARC's Group 1.

What kind of information would be necessary to label an agent as hazardous to humans? It appears impossible to exclude the suspicion of a carcinogenic potential for this type of chemical. Together with the fact that many of these *N*-substituted chemicals are present in the environment and due to their common mode of action, additive or synergistic effects have to be expected. Tumour-promoting effects have been seen with

mixtures in which the level of most of the individual chemicals was below that expected to have such an effect (Crisp database, Benjamin, 2010).

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GENERAL INTRODUCTION TO THE CHEMISTRY OF DYES

1. Principles of Colour Chemistry

1.1 Basis for colour

Unlike most organic compounds, dyes possess colour because they 1) absorb light in the visible spectrum (400–700 nm), 2) have at least one chromophore (colour-bearing group), 3) have a conjugated system, i.e. a structure with alternating double and single bonds, and 4) exhibit resonance of electrons, which is a stabilizing force in organic compounds (Abrahart, 1977). When any one of these features is lacking from the molecular structure the colour is lost. In addition to chromophores, most dyes also contain groups known as *auxochromes* (colour helpers), examples of which are carboxylic acid, sulfonic acid, amino, and hydroxyl groups. While these are not responsible for colour, their presence can shift the colour of a colourant and they are most often used to influence dye solubility. Figure 1 shows the relationships between wavelength of visible and colour absorbed/observed. Other factors contributing to colour are illustrated in Figures 2–4.

Fig. 1. Wavelength of light absorption versus colour in organic dyes

Wavelength Absorbed (nm)	Colour Absorbed	Colour Observed
400–435	Violet	Yellow-Green
435–480	Blue	Yellow
480–490	Green-Blue	Orange
490–500	Blue-Green	Red
500–560	Green	Purple
560–580	Yellow-Green	Violet
580–595	Yellow	Blue
595–605	Orange	Green-Blue
605–700	Red	Blue-Green

Fig. 2. Examples of chromophoric groups present in organic dyes

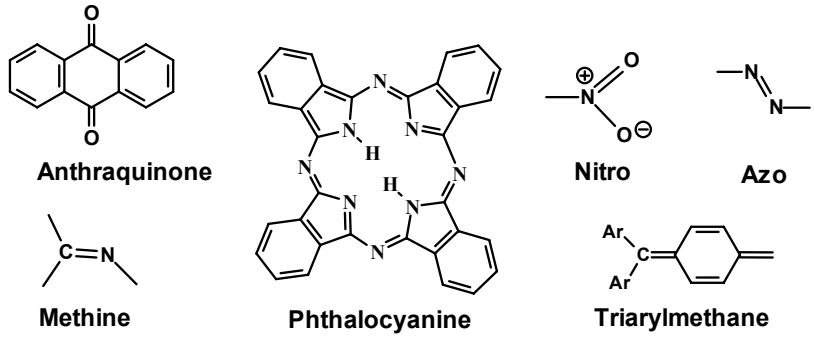


Fig. 3. Conjugated systems in Vitamin A (top) and β-carotene (bottom)

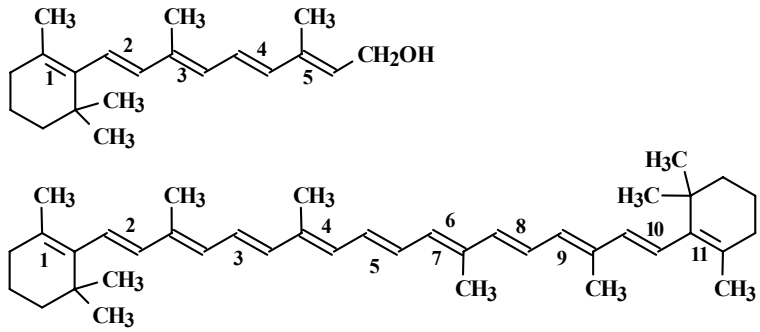
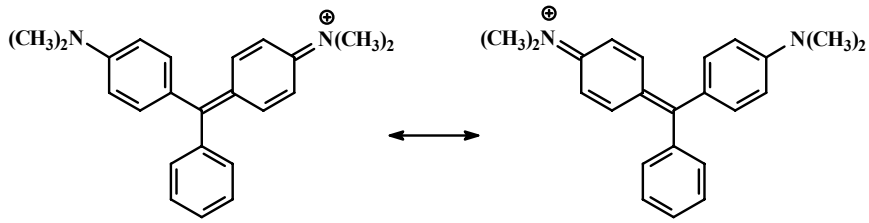


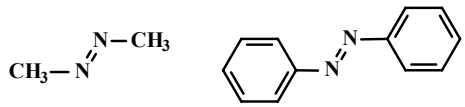
Fig. 4. A pair of resonance structures for Malachite Green (C.I. Basic Green 4)



Regarding the requirement of a chromophore generating colour in organic compounds, it is important to note that the chromophore must be part of a conjugated system. This is illustrated through the examples in Figure 5 where it can be seen that placement of an azo group between methyl groups produces a colourless compound, while a yellow-orange colour is obtained when the azo group is placed between aromatic rings. Similarly, the structures in Figure 3 demonstrate the importance of having an extended conjugated system. It is evident that doubling the length of the conjugated

system in Vitamin A to give β-carotene causes a significant bathochromic shift, i.e. to a darker colour.

Fig. 5. Importance of having a chromophore within a conjugated system

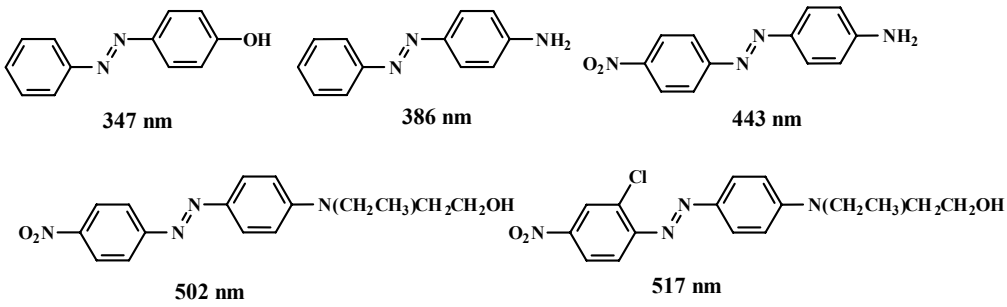


Colourless **Orange**

In addition to influencing solubility, auxochromes are essential ring substituents in providing target colours. This is illustrated in Figure 6, where the following effects of substituents are shown:

- Adding groups of increasing electron-donating ability to the azobenzene structure has a bathochromic effect (*cf*. OH *vs* NH_2).
- Electron-donating (NH_2) and electron-accepting (NO_2) groups placed in conjugation provide a bathochromic effect. In this regard, nitro groups are especially beneficial, contributing to their prevalence in disperse dye structures.
- Increasing the number of electron-attracting groups conjugated with the electron-donor has a bathochromic effect.
- The electron-donating effects of an amino group are enhanced by adding alkyl groups to the N-atom.

Fig. 6. Effects of substituent groups within an azo-dye system



347 nm **386 nm** **443 nm**

502 nm **517 nm**

1.2 Dyes versus Pigments

With regard to their solubility, organic colourants fall into two classes, *viz*. dyes and pigments (Allen 1971). The key distinction is that dyes are soluble in water and/or an organic solvent, while pigments are *insoluble* in both types of liquid media. Dyes are used to colour substrates to which they have affinity. Pigments can be used to colour any

polymeric substrate but by a mechanism quite different from that of dyes, in that surface-only colouration is involved unless the pigment is mixed with the polymer before fibre or moulded article formation.

2. Considerations in Dye Design

2.1 Dye-substrate affinity

Dyes containing one or more azo groups (i.e. azo dyes) comprise by far the largest family of organic dyes. Prominent types are 1) acid dyes for polyamide and protein substrates such as nylon, wool, and silk; 2) disperse dyes for hydrophobic substrates such as polyester and acetate, and 3) direct and reactive dyes for cellulosic substrates such as cotton, rayon, linen, and paper. Generally, the synthesis of azo dyes involves two steps. Step 1 is the conversion of an aromatic amine to a diazo compound (i.e. Ar-NH2 → Ar-N2+), a process known as diazotization, and step 2 is the reaction of the diazo compound with a phenol, naphthol, aromatic amine, or a compound that has an active methylene group, to produce the corresponding azo dye, a process known as diazo coupling (e.g. Ar-N2+ + Ar'-OH→ Ar-N=N-Ar'-OH). This process is suitable for forming both azo dyes and pigments. Typical structures of colourants that fall into the two groups are shown in Figure 7.

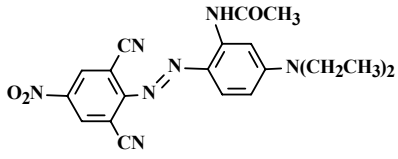
Since the effectiveness of a dyeing or printing process often hinges on the affinity between the dye and substrate, dyes are designed with a specific substrate in mind. In this regard, dyes must be designed that have a) greater affinity for the substrate than the medium (usually water) from which it is applied and b) a high degree of permanence under end-use conditions (e.g. stability to fading upon exposures to water (wet fast) and/or sunlight (light fast)). The following is a summary of the types of considerations associated with the development of dyes for polymeric (especially fibre-based) substrates (Aspland, 1997). The availability of colourants for a specific substrate type is the result of a deliberate molecular design process that takes the target substrate and end-use application into consideration.

2.1.1 *Dyes for polyesters*

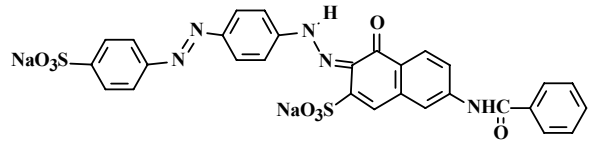
Dyes developed for polyesters are known as disperse dyes. In this case, the mechanism of coloration involves "dissolving" the dye in the polymer matrix to form a solid–solid solution. Taking advantage of the well known principle that "like dissolves like", disperse dyes are designed that are hydrophobic in nature. Such colourants are very sparingly soluble in water and derive their name from the fact that they are dispersed rather than fully dissolved in water to carry out the dyeing process. An example is C.I. Disperse Blue 165 (Figure 7). Disperse dyes have no affinity for hydrophilic polymers

such as cellulose, which makes them unsuitable for colouring cotton, cellophane, and paper, but quite suitable for poly(ethylene terephthalate) and cellulose acetate.

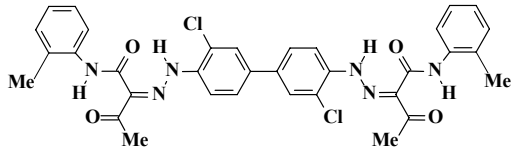
Fig. 7. Structures of some commercial azo dyes and pigments



Disperse Blue 165



Direct Red 81



Pigment Yellow 14

2.1.2 *Dyes for polyamides and proteins*

Dyes for these substrates normally form ionic bonds (Fig. 8) within the polymer matrix. In this case dyes bearing a negative (anionic) charge are used because polyamides such as nylon and proteins such as wool, silk, and leather carry a positive (cationic) charge – especially during the dyeing process. Anionic dyes for polyamide and protein substrates are known as *acid dyes*, an example of which is C.I. Acid Black 1 (Fig. 9). They derive their name from the fact that they are typically applied to suitable substrates from a medium containing acid. These dyes have little to no affinity for polyester, cellulosic, or cationic polymers, since such substrates cannot form an ionic bond with them.

Fig. 8. Schematic representation of dye–polymer binding via ionic bonding on nylon

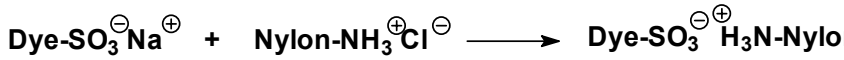
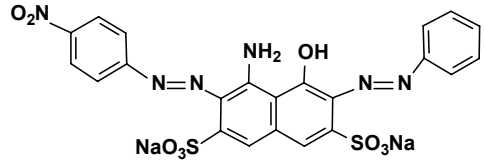
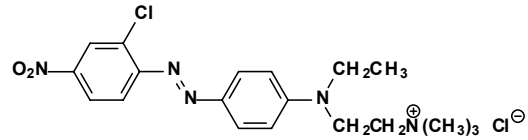


Fig. 9. Structure of a commercial acid and cationic (basic) dye



Acid Black 1



Basic Red 18

2.1.3 *Dyes for cationic polymers*

Dyes for these substrates also form ionic bonds within the polymer matrix. In this case, dyes bearing a positive (cationic) charge are used because polymers such as poly(acrylonitrile) carry a negative (anionic) charge in their backbone, making the ionic character of the interacting substances the reverse of that described above for acid dyes. Cationic dyes for acrylic substrates were initially known as *basic dyes*, an example of which is C.I. Basic Red 18 (Figure 9). Today, they derive their name from the fact that they possess a cationic group. These dyes have no affinity for polyester, cellulosic, or polyamide polymers, since such substrates cannot form an ionic bond with them. However, cationic dyes can be used to dye protein fibres and, in fact, the first synthetic dye Mauveine was a basic dye that was used for dyeing silk. This takes advantage of the presence of carboxylate ($–CO_2^-$) groups in silk and wool.

2.1.4 *Dyes for cellulosic polymers*

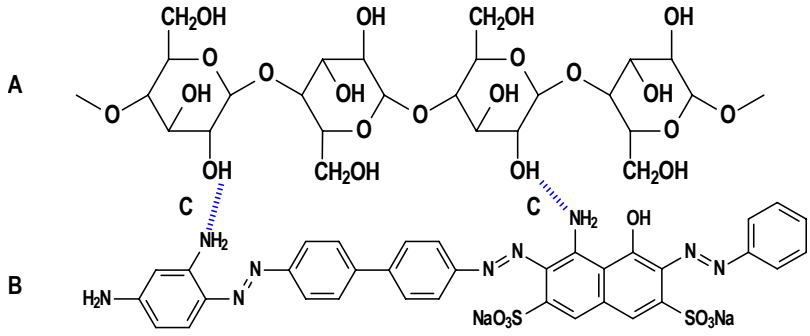
Cellulosic substrates include cotton, rayon, cellophane, linen, and paper, all of which are very hydrophilic and, therefore, require hydrophilic (water soluble) dyes for their coloration from a dyebath. In addition, dyes must be designed that maintain affinity when the substrate is exposed to water. This allows the colour to remain on the substrate, when, for instance, a cotton fabric is laundered or a cup of coffee is inadvertently spilled on a sheet of paper containing printed information. The ease with which cellulosic substrates such as cotton swell and lose colourants during laundering has led to the design and development of more dye colourant families for cellulosic fibres than any other substrate.

Dyes designed for cellulosic polymers are direct, azoic, vat, sulfur, and reactive dyes. *Direct dyes* are so named because they were the first colourants that had affinity for cotton in the absence of a binding agent known as a mordant. Because these dyes are water-soluble, many have low wet fastness. Figure 10 illustrates two key properties of benzidine-based direct dyes, *viz*. 1) they tend to be linear molecules, and 2) they are able to gain close proximity to the cellulose chain, to maximize the effects of intermolecular interactions such as H-bonding.

To enhance wet fastness on cellulosic fibres, methods were developed to apply water-insoluble dyes to cotton. Such dyes include those that are either water insoluble in their

natural form or synthesized inside the polymer matrix as a water-insoluble dye. The idea is that placing a water-insoluble dye within the polymer matrix would prevent colour removal upon exposure of the substrate to water. In turn, this led to the development of *vat dyes* and *sulfur dyes* (Figure 11a,b). Vat dyes owe their name to the vatting process associated with their application, while sulfur dyes are so named because of the essential use of sulfur in their synthesis. The structures of vat dyes are rather well known but sulfur dye structures are less well defined, because their polymeric nature makes them unsuitable for standard methods for structure characterization. In their application, vat and sulfur dyes are converted to a water-soluble form that has affinity for cellulose and are subsequently converted back to their water-insoluble form, giving good permanence under wet conditions. Included in the family of vat dyes is the well known natural dye *indigo*. While indigo is still the most important colourant for denim fabric, its a-typically small size causes this vat dye to be very susceptible to removal in a laundering process, giving jeans a faded appearance even after one wash. This underscores the importance of designing colourants for cellulosic substrates that possess the features required for them to remain within the polymer matrix when water swells the substrate.

Fig. 10. Representation of H-bonding (C) between a cellulose (A) and a direct dye (B)



Azoic colourants (cf. Figure 11c) are also known as naphthol dyes because naphthol compounds are used in their synthesis. These dyes do not exist per se, but they are generated inside the polymer matrix by applying the two necessary components to the substrate separately. Following their application to the substrate, the two components find each other and combine to form a water-insoluble colourant.

The final class of suitable colourants for cellulosic fibres is known as *reactive dyes* (*cf*. Figure 11d). They derive their name from the fact that they undergo a chemical reaction with cellulose to form a covalent bond (Figure 12). Reactive dyes opened the door to bright wet-fast shades on cellulosic fibres that were not previously attainable.

Fig. 11. Characteristic structures of sulfur (a), vat (b), azoic (c) and reactive (d) dyes

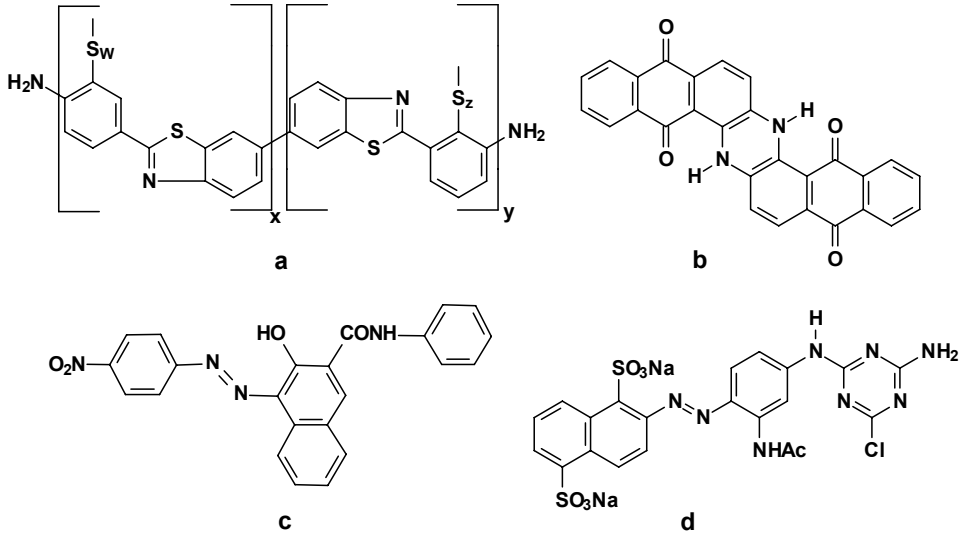
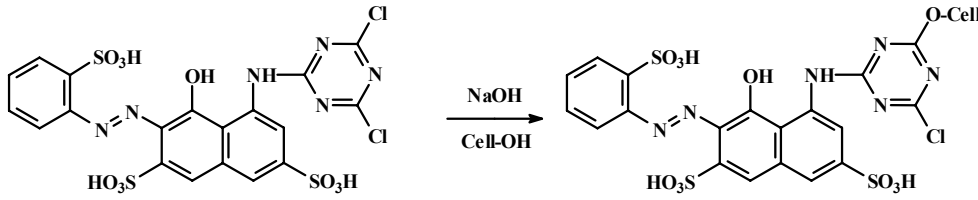


Fig. 12. Dye-fiber fixation via covalent bonding, where Cell-OH represents cellulose

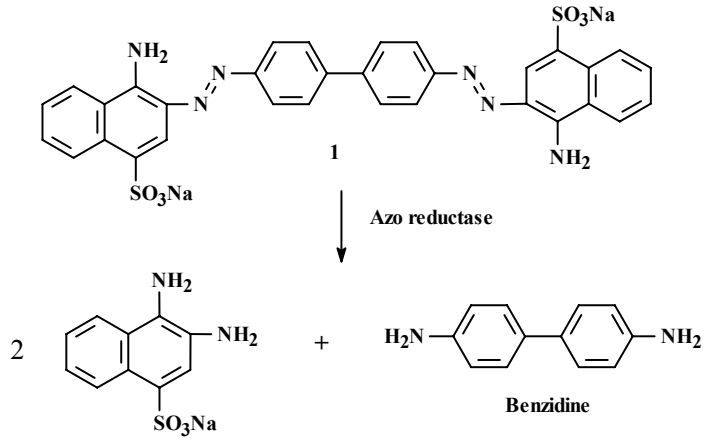


2.2 Toxicological considerations

While dye–substrate affinity is critical, synthetic dyes cannot be commercialized unless they pose little health risk under end-use conditions. Consequently, environmental safety is an essential consideration in molecular design. In this regard, the raw materials employed in the manufacture of synthetic dyes should not involve compounds known to pose health risks. This would include a large group of aromatic amines (Anon, 1996) that are either cancer-suspect agents or established mutagens in the standard *Salmonella* mutagenicity assay (Maron and Ames, 1983). It is clear, therefore, that dye design must take into consideration the likely genotoxicity of the potential metabolites generated in mammalian systems (Prival *et al.*, 1984). In the case of azo dyes, the enzyme-mediated formation of genotoxic aromatic amines as metabolites must be considered, since it is possible that the intact dye is safe but not all of its metabolites. For instance, the use of

dye **1** in Figure 13 could lead to the formation of the bladder carcinogen benzidine if this dye were taken up in the body.

Fig. 13. Reductive-cleavage of Direct Red 28 (1) using an azo-reductase enzyme



2.2.1 *Structure–Property Relationships*

Following the recognition that certain aromatic amines used in azo-dye synthesis caused bladder cancer, a wide variety of chemicals were evaluated in animal studies, and the results showed that aromatic amines and azo compounds of the type shown in Figure 14 were carcinogenic (Weisburger, 1978). In this regard, it is generally believed that the ultimate carcinogen arises from the metabolic conversion of these compounds to electrophilic species (*cf*. Figure 15) that interact with electron-rich sites in DNA to cause DNA adducts, mutations and subsequent adverse effects on the cell. It is also clear that ring substituents that enhance the hydrophobic character increase carcinogenic potential: this is the case when adding a methyl group to 2-naphthylamine (**2**; R = H) or *meta*-phenylenediamine (*cf*. analogue **4**).

As a follow-up to the study pertaining to carcinogenicity data assessment (Longstaff, 1983), correlations between dye structure and carcinogenicity data were established. In a comparison of hydrophobic azo dyes containing amino groups in the *para*- or *ortho*-position, it was found that the *para*-isomers were carcinogenic while the *ortho*-isomers were not. To account for these results, the chemistry in Figure 16 was proposed (Gregory, 1986). It is believed that the isomeric arylazo amines produce nitrenium ions (**9**) that either interact with DNA (*para*-isomer) or undergo intramolecular cyclization (*ortho*-isomer) to produce adducts (**10**) or benzotriazoles (**11**), respectively.

Fig. 14. Examples of carcinogenic aromatic amines and azo compounds

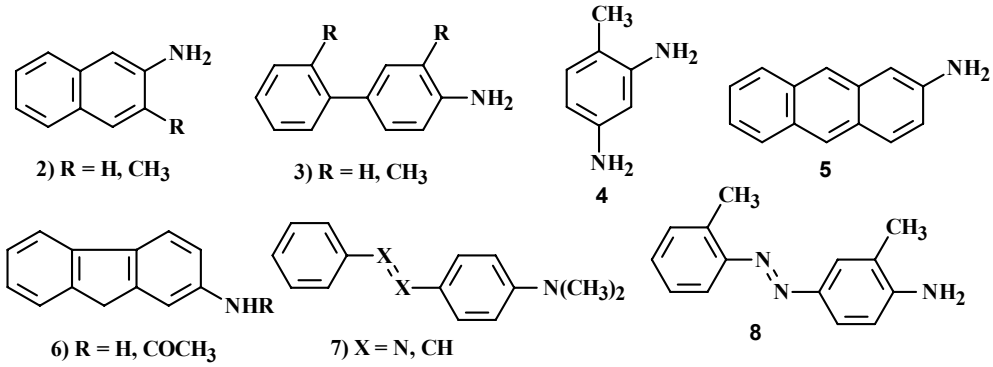


Fig. 15. Nitrenium ion formation from the metabolism of aromatic amines

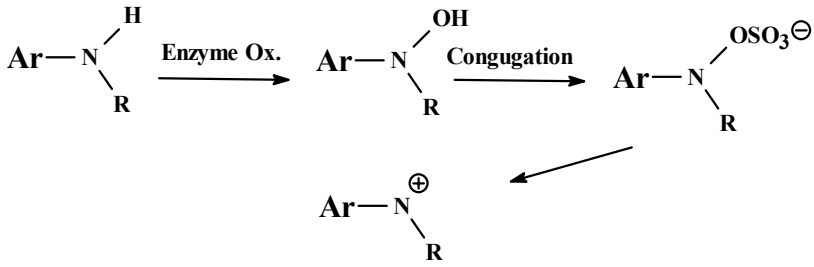
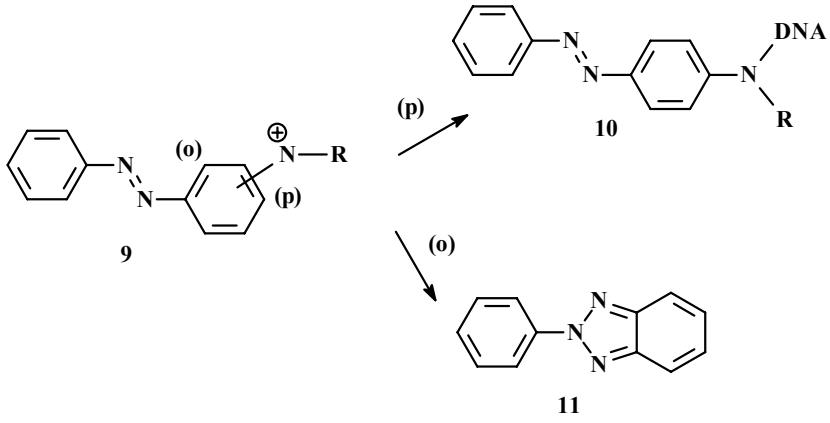


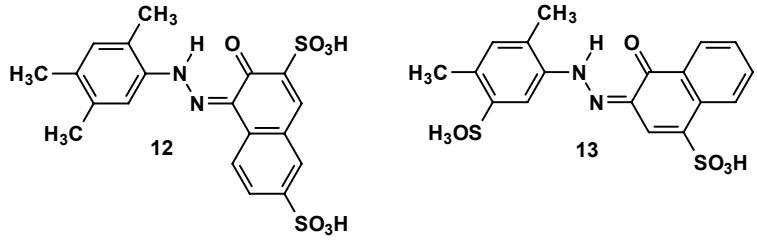
Fig. 16. Reaction pathways for nitrenium-ion (9) metabolites of azobenzene isomers



A correlation of carcinogenicity data with the structures of hydrophilic azo dyes was also presented (Gregory, 1986). Water-soluble dyes of type **12** were carcinogenic, while type **13** dyes (Fig. 17) were not. In this case, the nature of the reductive-cleavage products

was the determining factor in the observed carcinogenicity data. Whereas dye **12** produces a lipophilic amine (2,4,5-trimethylaniline), dye **13** produces water-soluble sulfonated amines only, making aromatic amine genotoxicity an important consideration in azo-dye design.

Fig. 17. Carcinogenic (12) and non-carcinogenic (13) water-soluble monoazo dyes



3. Hair Dyes

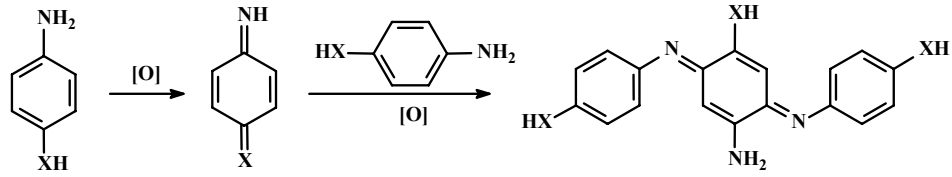
A great majority of the dyes used in hair colouring are known as oxidation hair dyes (Corbett, 1985, 2000). A much smaller number of the commercial hair dyes are synthetic dyes that have affinity for protein substrates such as wool. Oxidation dyes, the more permanent of the two groups, are produced directly on the hair by oxidizing aromatic diamines such as *para*-phenylenediamine or 2,5-diaminotoluene with an oxidizing agent. Suitable diamines have been referred to as "primary intermediates" and the oxidizing agents (e.g. hydrogen peroxide) as "developers." Other useful primary intermediates are aminodiphenylamines, aminomethylphenols, and *para*-aminophenol.

When used alone, the primary intermediates provide a very limited shade-range following their oxidation on hair. To enhance the range of available hair colours, the primary intermediates are oxidized in the presence of suitable "couplers." While most couplers do not produce colours when exposed to developers alone, they give a wide range of shades on hair when applied in combination with primary intermediates. Appropriate couplers include 3-aminophenol, resorcinol, and α-naphthol.

The chemistry associated with the oxidation of primary intermediates is now reasonably well known. For *para*-phenylenediamine and *para*-aminophenol (*cf.* **14**), oxidation-induced self-coupling proceeds via the process outlined in Fig. 18, where it can be seen that permanent hair-dye formation involves oxidation followed by coupling to give type-**15** structures. Fig. 19 provides chemistry representative of combinations arising from joining an α-naphthol-based coupler (**16**) and a sulfonated *N*-phenyl-*para*-phenylenediamine primary intermediate (**17**) to produce experimental dyes **18** and **19**. This chemistry also illustrates the fact that oxidation dyes are often mixtures rather than single products.

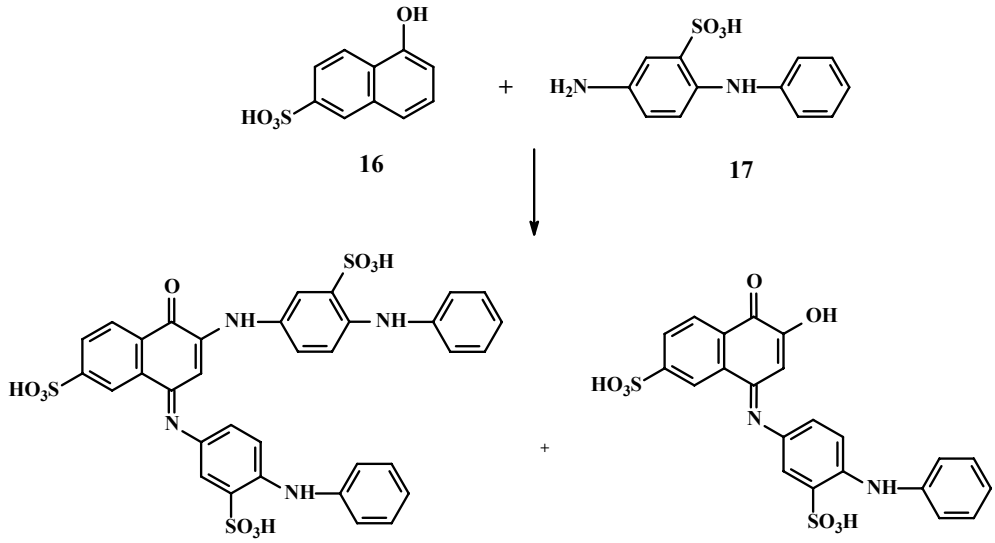
An analytical method has been developed for characterizing the reactants and reaction products of oxidative hair-dye formulations. The results indicated that a significant amount, i.e. ≈20% or more of the initial concentrations of precursor(s) and coupler(s), is always present in the formulation that is not diffused into hairs (see Section 1 of the Monograph on hair dyes in this volume).

Fig. 18. Oxidation hair-dye formation from primary intermediates (X = O, NH)



14 **15**

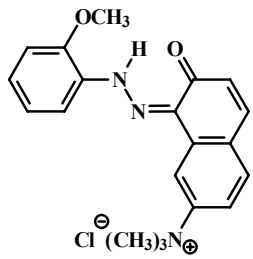
Fig. 19. Oxidation hair-dye formation from a primary intermediate and coupler



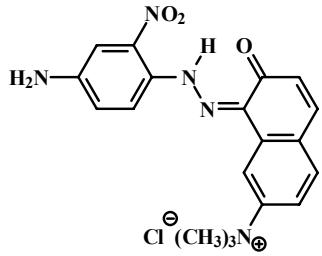
16 **17** **18** **19**

C.I. Basic dyes such as Yellow 57, Red 76, Blue 99, Brown 16, and Brown 17 have been used in colour refreshener shampoos and conditioners. Similarly, C.I. Acid dyes such as Yellow 3, Orange 7, Red 33, Violet 43, and Blue 9 have been used in shampoos, in this case to deliver highlighting effects (Corbett, 2000). Example structures of non-permanent hair dyes are provided in Fig. 20, where it can be seen that these dyes are drawn from those known to have affinity for protein-based textile fibres.

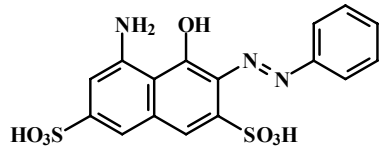
Fig. 20. Examples of non-permanent hair dyes



Basic Red 76



Basic Brown 17



Acid Red 33

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THE MONOGRAPHS

4-AMINOBIPHENYL

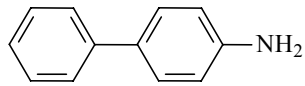
1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 92–67–1
CAS Name: [1,1'-Biphenyl]-4-amine
Synonyms: 4-Amino-1,1'-biphenyl; 4-aminobiphenyl; *p*-aminobiphenyl; 4-aminodiphenyl; *para*-aminodiphenyl; biphenyl-4-ylamine; (1,1'-biphenyl-4-yl)amine; 4-biphenylamine; *para*-biphenylamine; 4-biphenylylamine; 4-phenylaniline; *para*-phenylaniline; 4-phenylbenzenamine; *para*-xenylamine; xenylamine

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



$C_{12}H_{11}N$ Rel. mol. mass: 169.22

1.1.3 *Chemical and physical properties of the pure substance*

Description: Leaflets from alcohol or water (O'Neil, 2006); colourless to tan crystalline solid that turns purple on exposure to air (Pohanish, 2008)
Boiling-point: 302°C (Lide, 2008)
Melting-point: 53°C (O'Neil, 2006)
Solubility: Slightly soluble in cold water; readily soluble in hot water, acetone, chloroform, diethyl ether, and ethanol (O'Neil, 2006; Lide, 2008)
Octanol/water partition coefficient: log P, 2.80 (Verschueren, 2001)

1.1.4 *Technical products and impurities*

No information was available to the Working Group.

1.1.5 *Analysis*

The first analytical studies on 4-aminobiphenyl were reported during the 1960s. Method developments have enabled the detection of 4-aminobiphenyl at extremely low concentrations, down to the picogram level. GC/MS on derivatized samples and LC/MS on non-derivatized samples are most often used. Table 1.1 presents a selection of recent studies of the analysis of 4-aminobiphenyl in various matrices.

1.2 Production and use

1.2.1 *Production*

Because of its carcinogenic effects, 4-aminobiphenyl has not been produced commercially in the USA since the mid-1950s (Koss *et al.*, 1969). It was present in the drug and cosmetic colour additive D&C Yellow No. 1; however, use of this colour additive was discontinued in the late 1970s. 4-Aminobiphenyl also has been reported as a contaminant in diphenylamine (NTP, 2005; HSDB, 2009).

Available information indicates that 4-aminobiphenyl was produced and/or supplied in research quantities in the following countries: China, Germany, Hong Kong Special Administrative Region, India, Switzerland, and the USA (Chemical Sources International, 2008).

1.2.2 *Use*

4-Aminobiphenyl has been used formerly as a rubber antioxidant. It is reportedly still used in the detection of sulfates and as a model carcinogen in mutagenicity studies and cancer research (O'Neil, 2006).

1.3 Occurrence and exposure

1.3.1 *Natural occurrence*

4-Aminobiphenyl is not known to occur in nature.

1.3.2 *Occupational exposure*

Historically, occupational exposure to 4-aminobiphenyl mainly occurred during its production and its use as a rubber antioxidant and dye intermediate. No exposure measurements are available for these occupational exposure situations.

Occupational exposure can also occur in workers exposed to products contaminated with 4-aminobiphenyl, or in workers exposed to benzidine and benzidine-based dyes, from which 4-aminobiphenyl can be metabolically released (Lakshmi *et al.*, 2003; Beyerbach *et al.*, 2006). Other circumstances with potential exposure to 4-aminobiphenyl

Table 1.1. Selected methods of analysis of 4-aminobiphenyl in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Finger paints	Paint containing amine is applied to an inert surface and dried. Painted sample and modifier (methanol) are placed in SFE cartridge for extraction.	SFE/GC	< 0.5µg/g	Garrigós *et al.* (1998, 2000)
Toy products	Supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), or Soxhlet extraction with methanol	HPLC/UV	< 0.2µg/g	Garrigós *et al.* (2002)
Hair dyes	Isolation from dyes by solvent extraction with hexane, followed by silica gel chromatography, either with or without chemical treatment of the extract with zinc/HCl, and final purification with a mixed cation exchange reversed-phase resin	HPLC-ESI-MS	0.3ppb	Turesky *et al.* (2003)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/dichloromethane (50:50).	GC/MS	5ng/mL	Doherty (2005)
Dyes, cosmetics, inks and finger paints	Extract with methanol. Separate and detect on a phenyl ether linked stationary phase	HPLC/MS	40–500 pg	Hauri *et al.* (2005)
Urine	Acid hydrolysis of arylamine conjugates in urine, extraction with n-hexane, derivatization with pentafluoropropionic anhydride, and analysis	GC/MS	1ng/L	Riedel *et al.* (2006)

Table 1.1 (contd)

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Blood	Hydrolyse 4-aminobiphenyl-haemoglobin adduct using 1N sodium hydroxide. Purify and concentrate using solid phase extraction and elute on C-18 using chloroform. Evaporate and derivatize with pentafluoropropionic anhydride at room temperature. Dissolve in ethyl acetate for analysis	GC/MS	0.5 pg/g	Sarkar *et al.* (2006)
Air	Pre-concentration by percolating air through an acidic solution, ion-pair extraction with bis-2-ethylhexylphosphate (BEHPA), derivatization with isobutyl chloroformate (IBCF), and analysis	GC/MS	0.01 ng/m^3	Akyüz (2007)
Hair dye	Ion-pair extraction from aqueous samples with bis-2-ethylhexyl-prosphate released after solving the samples in acid solution, followed by sonication, derivatization of compounds with isobutyl chloroformate and analysis	GC/MS	0.02–0.2 ng/g	Akyüz & Ata (2008)

ESI, electrospray ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SFE, supercritical fluid extraction; UV, ultraviolet

include exposure to environmental tobacco smoke (Hammond *et al.* 1995), and exposure of laboratory workers working with 4-aminobiphenyl as a model in cancer research or for the detection of sulfate ions.

In workers exposed to benzidine or to benzidine-based dyes and a non-exposed control group in a dye factory in India, urine samples were analysed for 4-aminobiphenyl and acetylated 4-aminobiphenyl (Ac4ABP). 4-Aminobiphenyl was found in 30 of 33 urine samples from exposed workers (mean, 109 pmol/mL) and one of the 13 control workers. The benzidine-exposed workers had significantly higher 4-aminobiphenyl levels (57 pmol/mL) than did the benzidine-based dye-exposed workers (29.3 pmol/mL). In the sample that belonged to the person with the highest 4-aminobiphenyl concentration, acetylated 4-aminobiphenyl was found at a concentration of 79.5 pmol/mL, while it was not detected in any of the other urine samples (Beyerbach *et al.*, 2006).

1.3.3 *Environmental occurrence and exposure of the general population*

The main sources of exposure to 4-aminobiphenyl for the general population are cigarette smoking and environmental tobacco smoke. Other potential sources include emissions from cooking oils, hair dyes, and food and other dyes contaminated with it.

Living near benzidine-contaminated sites could entail exposure to 4-aminobiphenyl, as benzidine in the environment can be degraded to 4-aminobiphenyl by bacterial action (Bafana *et al.*, 2007).

(a) Occurrence in tobacco smoke and fumes from cooking oils

4-Aminobiphenyl is formed during tobacco combustion. Mainstream cigarette smoke was reported to contain 4-aminobiphenyl at levels of 2.4–4.6 ng per cigarette without a filter and 0.2–23 ng per cigarette with a filter; sidestream smoke contains up to 140 ng per cigarette (Patrianakos & Hoffmann 1979; Hoffmann *et al.*, 1997). A non-smoker exposed to environmental tobacco smoke during one month can ingest as much 4-aminobiphenyl as by smoking 17 cigarettes (Hammond *et al.,*1995)

4-Aminobiphenyl has also been detected in emissions from cooking oils. In a study from Taiwan, China, concentrations of 4-aminobiphenyl were 35.7 μg/m^3 in emissions from sunflower oil, 26.4 μ/m^3 from vegetable oil and 23.3 μg/m^3 from refined-lard oil (Chiang *et al.*, 1999).

(b) Occurrence as a contaminant

4-Aminobiphenyl can occur as contaminant in 2-aminobiphenyl, which is used in the manufacture of dyes. 4-Aminobiphenyl has also been detected in aniline (Reilly, 1967), in the drug and cosmetic colour-additive D&C Yellow No. 1 (HSDB, 2009) and in the food dye FD&C Yellow No. 6 (Richfield-Fratz *et al.*, 1985). 4-Aminobiphenyl also has been reported as a contaminant in diphenylamine, a fungicide used on apples (Olek, 1988).

4-Aminobiphenyl has been detected in hair dyes. In a study from the USA, Turesky *et al.* (2003) found 4-aminobiphenyl in eight of 11 different oxidative and direct hair

dyes, with levels of 4-ABP ranging from non-detectable (< 0.29 ppb) to 12.8 ppb. 4-Aminobiphenyl was found in blonde, red, and black, but not in brown dyes. The same study reported that research-grade 1,4-phenylenediamine (PPD), a key constituent for colour development of many permanent hair dyes, can be contaminated with 4-aminobiphenyl in concentrations up to 500 ppb. In a study from Turkey (Akyüz & Ata, 2008), 4-aminobiphenyl was found in concentrations up to 8.12 µg/g in 28 of the 54 hair-dye samples, up to 2.23 µg/g in 11 of 25 henna samples, and up to 2.87 µg/g in four of 10 commercial natural henna samples tested.

1,4-Phenylenediamine is manufactured by use of three methods: (1) reduction of *para*-nitroaniline, (2) diazotization of aniline, or (3) direct nitration of benzene without chlorinating. The major manufacturer of 1,4-phenylenediamine in the USA produces this chemical for use in hair dyes via the third process; it is > 99% pure.

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Council Directive 89/677/EEC

According to Council Directive 89/677/EEC, 4-aminobiphenyl may not be sold to the general public. The packaging shall be legible and indelibly marked as follows: "Restricted to professional users" (European Economic Community, 1989).

(b) Directive 98/24/EC

According to EU legislation, the manufacture of 4-aminobiphenyl has been prohibited since 1998 (European Commission, 1998). The Council Directive 98/24/EC in Annex III prohibits the production, manufacture or use at work of 4-aminobiphenyl and its salts and activities involving 4-aminobiphenyl and its salts. The prohibition does not apply if 4-aminobiphenyl and its salts are present in another chemical agent, or as a constituent of waste, provided that its concentration therein is less than 0.1% w/w.

(c) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azo-colourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes which, by reductive cleavage of one or more azo groups may release 4-aminobiphenyl in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(d) Directive 2004/37/EC

4-Aminobiphenyl is regulated by the Directive 2004/37/EC (European Commission, 2004a), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(e) Cosmetics Directive

The Commission Directive 2004/93/EC of 21 September 2004 amends Council Directive 76/768/EEC for the purpose of adapting Annexes II and III thereto to technical progress (European Commission, 2004b). In this Directive, biphenyl-4-ylamine (i.e. 4-aminobiphenyl) and its salts are listed in Annex II as substances that must not form part of the composition of cosmetic products.

1.4.2 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of 4-aminobiphenyl in Group 1 (IARC, 1987).

1.4.3 *Germany*

4-Aminobiphenyl is classified as a Category-1 carcinogen by the MAK Commission. The MAK Commission listed 4-aminobiphenyl as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set. A biological tolerance value (BAT) was set at 10.0 ng/L in blood, released from the 4-aminobiphenyl-haemoglobin-adduct (MAK, 2007).

1.4.4 *USA*

(a) ACGIH

4-Aminobiphenyl has been assigned an A1 notation, *Confirmed Human Carcinogen* (ACGIH, 2001). Accordingly, a numerical TLV (threshold limit value) is not recommended for occupational exposure. A ‘Skin notation’ is recommended (*potential significant contribution to the overall exposure by the cutaneous route*) as an additional precaution for undue exposure. As for all substances designated as A1 carcinogens without a TLV, workers should be properly equipped to eliminate all exposure to 4-aminodiphenyl to the fullest extent possible.

(b) NIOSH

The National Institute for Occupational Safety and Health lists 4-aminobiphenyl as one of thirteen OSHA-regulated carcinogens. Exposures of workers are to be controlled through the required use of engineering controls, work practices, and personal protective equipment, including respirators (NIOSH, 2005).

(c) FDA

FDA has set limits for 4-aminobiphenyl for the following certified colours (FDA, 1985, 1986, 1988): FD&C Yellow No. 5: ≤ 5 ppb; FD&C Yellow No. 6: ≤ 15 ppb; D&C Red No. 33: ≤ 275 ppb (FDA, 2009).

(d) NTP

4-Aminobiphenyl is listed in the NTP Report on Carcinogens as *known to be a human carcinogen* (NTP, 2005).

1.4.5 *Other*

(a) GESTIS

Table 1.2 presents some international limit values for 4-aminobiphenyl (GESTIS, 2007).

Table 1.2. International limit values (2007) for 4-aminobiphenyl

Country	Limit value – Eight hours		Limit value – Short-term	
	ppm	mg/m^3	ppm	mg/m^3
France	0.001	0.007		
Hungary				10
Italy				0.001

(b) Recent bans

The use and import of 4-aminobiphenyl were banned recently in the Republic of Korea in 2003, and in Switzerland in 2005 (UN/UNEP/FAO, 2007).

2. Studies of Cancer in Humans

2.1 Descriptive studies

In a case series, Melick *et al.* (1955) reported 19 cases of bladder cancer in 171 (11.1%) male workers engaged in the production of 4-aminobiphenyl. The exposure took place in a chemical plant in the USA between 1935 and 1955. In a later follow-up study, it was reported that among 315 male workers exposed to 4-aminobiphenyl, 53 had developed bladder tumours. The interval until development of bladder cancer varied from 15 to 35 years after beginning of the exposure (Melick *et al.*, 1971).

2.2 Cohort studies

Following the cessation of industrial production of 4-aminobiphenyl in 1955 in the USA, a large surveillance programme was started on workers reportedly exposed to this chemical. In the first study, a total of 601 specimens of urinary sediment from 285 men exposed to 4-aminobiphenyl were examined for cytological features. Among these men, 31 were found to have significantly abnormal epithelial cells in urinary sediments, of whom 10 were diagnosed with histologically confirmed bladder carcinoma (Melamed *et al.*, 1960). A later follow-up study was undertaken of the 22 patients whose urinary sediment in 1960 contained suspicious or frankly malignant cells, but did not have evidence of clinical bladder cancer. The study provided complete data on 18 patients, 11 of whom developed histologically confirmed carcinoma of the bladder (Koss *et al.*, 1965). In a third follow-up of this screening study, now extended to 503 male workers exposed to 4-aminobiphenyl, 35 workers developed histologically confirmed bladder cancer (Koss *et al.*, 1969). In a further extension, a group of 541 workers exposed to 4-aminobiphenyl were examined for abnormalities in cytologic specimens over a maximum period of 14 years. Among 86 men who had suspicious or positive cytology, 43 developed histologically confirmed carcinoma of the bladder (Melamed 1972).

Cancer mortality was studied among 884 male workers at the Nitro chemical plant in West Virginia (USA), which produced a variety of chemicals (Zack & Gaffey 1983). A ten-fold increase in mortality from bladder cancer was reported, with nine cases observed and 0.91 expected over the period 1955–1977. All nine cases started work in the plant before 1949; 4-aminobiphenyl was used in the plant from 1941 until 1952.

An updated and enlarged cohort study was conducted among workers at the same rubber chemicals plant in Nitro, West Virginia (USA) (Collins *et al.*, 1999). The aim of the study was to investigate any bladder cancer risk from exposure to 2-mercapto-benzothiazole (MBT). As 4-aminobiphenyl was shipped to the Nitro plant during 1935–1955 and used to produce a rubber antioxidant, MBT-exposed workers also had potential exposure to 4-aminobiphenyl. Exposure during the 20 years of manufacturing was limited to a few areas, e.g. where samples were taken for analysis, where distillation residues

were removed and where leaks occurred. The greatest exposure was found where workers were involved in repair of equipment and in cleaning-up of accidental leaks. As no information was available to determine which workers were involved in the clean-up of 4-aminobiphenyl, workers were considered to be exposed if they had a job in the department where 4-aminobiphenyl was used or were considered to have potential exposure to 4-aminobiphenyl if they were employed between 1935 and 1955, the years during which this chemical was used in the plant. The study examined the mortality of 1059 full-time white male hourly production workers employed at the rubber chemicals plant between 1955 and 1977; 600 of these workers were exposed to MBT. Follow-up was from 1955 to 1996. SMRs were computed by use of mortality rates for the white male population in the four counties in West Virginia within a 20-mile radius of the plant. For all study subjects, the SMRs for lung cancer, prostate cancer and other cancer sites were at expected levels. However, rates for bladder cancer were highly elevated in the total cohort (16 deaths, SMR, 6.3; 95% CI: 3.6–10.3), and in the subcohort of workers with exposure to 4-aminobiphenyl (eight deaths, SMR 27.1, 95% CI: 11.7–53.8). There was no excess of leukaemia, based on four deaths (SMR, 1.0; 95% CI: 0.3–2.6).

Cigarette smoking is an established cause of bladder cancer, and aminobiphenyls have been implicated in bladder-cancer etiology in smokers (IARC, 2004). Haemoglobin adducts of 4-aminobiphenyl are considered to be valid biomarkers of the internal dose of aminobiphenyl to the bladder (Probst-Hensch *et al.*, 2000). However, these biomarker studies have not been able to disentangle the role of 4-aminobiphenyl compared with other bladder carcinogens in tobacco smoke. Therefore, studies of tobacco smokers and bladder cancer are not reviewed here; the reader is referred to the Monograph on tobacco smoke (IARC, 2004).

3. Studies of Cancer in Experimental Animals

3.1 Oral administration

3.1.1 *Mouse*

A group of Ab × IF F_1 male and female hybrid mice, 10 weeks of age, were given commercial 4-aminobiphenyl (source and purity not stated) by gavage (0.2 mL of a 0.25% solution in arachis oil) twice weekly for 9 months. The estimated total intake was 38 mg per animal. Two of 12 mice surviving to 90 weeks developed bladder carcinoma. [The Working Group assumed there were none in 38 control animals.] Hepatomas were found both in treated animals and controls in similar frequencies (Clayson *et al.*, l965).

A group of C57 × IF F_1 male and female hybrid mice, 12 weeks of age, received 4-aminobiphenyl (Koch-Light Laboratories Ltd; purity not stated) by gavage (0.2 mL of a 0.25% solution in arachis oil) three times per week for 50 weeks. The estimated total intake was 75 mg per animal. The mice were killed approximately 20 weeks after the end

of the treatment. Thirteen of 28 (46%) female and four of 21 (19%) male mice had malignant liver tumours. One male mouse had a bladder carcinoma. In 50 control mice, one benign hepatoma was observed (Clayson *et al.*, 1967).

Groups of 840 male and 840 female BALB/cStCrlfC3Hf/Nctr mice from the NCTR breeding colony, four weeks of age, were given 4-aminobiphenyl-HCl (purity, > 99.5%) in the drinking-water at doses of 0, 7, 14, 28, 55, 110, and 220 ppm (males) and 0, 7, 19, 38, 75, 150, and 300 ppm (females). The 220-ppm and 300-ppm doses for one week corresponded to the LD_{50} (in mg/kg bw given by gavage) for male and female mice, respectively. Necropsies were performed at 13, 26, 39, 52, and 96 weeks, on approximately 45 organs and tissues of each animal. Induction of angiosarcomas (all sites), hepatocellular tumours and bladder carcinomas were dose-related (see Table 3.1) (Schieferstein *et al.*, 1985).

Table 3.1 Incidence of bladder carcinoma, hepatocellular carcinoma and angiosarcoma in BALB/c mice following chronic dosing with 4-aminobiphenyl in drinking-water

Sex	Dose (ppm)	Angiosarcoma (all sites)	Bladder carcinoma	Hepatocellular carcinoma
Female	0	1/119 (1%)	0/118 –	0/117 –
	7	4/120 (3%)	0/118 –	0/120 –
	19	4/120 (3%)	0/119 –	2/120 (2%)
	38	2/120 (2%)	1/118 (1%)	4/119 (3%)
	75	14/120 (12%)	0/118 –	10/119 (8%)
	150	26/118 (22%)	5/117 (4%)	14/118 (12%)
	300	11/117* (11%)	1/117 (1%)	7/117* (6%)
Male	0	1/118 (1%)	0/116 –	2/118 (2%)
	7	1/117 (1%)	1/117 (1%)	1/117 (1%)
	14	1/118 (1%)	1/118 (1%)	0/118 –
	28	2/119 (2%)	0/118 –	0/117 –
	55	4/115 (3%)	6/115 (5%)	0/114 –
	110	5/119 4%)	15/118 (13%)	3/118 (3%)
	220	14/118* (12%)	23/118* (19%)	2/117 (2%)

Adapted from Schieferstein *et al.* (1985)
*Positive trend, $P \leq 5.10^{-5}$

3.1.2 *Rabbit*

Seven rabbits (strain unspecified) were given 4-aminobiphenyl (source and purity not stated) orally (dose and dose regimen not given) to the limit of tolerance, and the treatment was continued until the onset of the final illness. Three animals were sacrificed in the first two years and two each between three and four years and between five and six years after the start of treatment. Bladder carcinomas were observed in three rabbits, the

earliest after four years of treatment. No tumours were observed in twelve control rabbits [no statistical analysis was provided] (Bonser, 1962).

3.1.3 *Dog*

Two male Beagle dogs, seven months of age, were given 4-aminobiphenyl (source and purity not stated; b.p. 302°C) in a gelatin capsule daily, six times per week until termination of the study. The dose level was lowered during the course of the experiment and dosing was also interrupted temporarily due to severe toxicity. The experiment was terminated after two years and nine months (total dose per dog: 2.9 and 3.3 g/kg bw, respectively); both dogs had bladder carcinoma [while no concurrent controls were included, historical data from this laboratory show that thirty Beagle dogs ranging from three to nine years of age did not develop bladder tumours] (Walpole *et al.*, 1954).

Four young adult female mongrel dogs were given a small quantity of 'purified' 4-aminobiphenyl (source not given) admixed into the food on five days a week for one year; after that, the animals received three times weekly by mouth a capsule containing 0.3 g of this compound. Bladder carcinomas were observed in all four dogs after 21–34 months. The total dose until first appearance of tumours was 87.5–144.0 g per dog, which corresponded to 8.2–14.1 g/kg bw (Deichmann *et al.*, 1958) [no controls were included].

Six female pure-bred beagle dogs, 6–12 months of age, were given 4-aminobiphenyl (source and purity not indicated) by mouth in a capsule at 1.0 mg/kg bw, five times per week for two years and 10 months (four dogs) or three years and one month (two dogs). The total dose range was 5.35–7.34 g per dog. Three bladder papillomas and three bladder carcinomas (transitional cell type) were observed in the six dogs (Deichmann *et al.*, 1965).

Two male and four female pure-bred beagle dogs were each given a single oral dose of 50 mg/kg bw 4-aminobiphenyl (purity, 98–99%) by capsule; the male dogs died within 18 hours, the females suffered severe acute intoxication, but survived and remained in good health for five years, as did two other female dogs that received a single oral dose of 15 mg/kg bw. There were no tumours in the urinary bladder of any of these female dogs (Deichmann & MacDonald, 1968). [The Working Group noted the inadequate dosing in this study.]

In an initiation-promotion study, four female pure-bred beagle dogs were given a single initiating dose of 4-aminobiphenyl (50 mg/kg bw; source and purity not specified) in corn oil by capsule. After recovery from severe acute methaemoglobinaemia, the dogs received a daily supplement of 6 g D,L-tryptophan as a promoter admixed into 300 g dog chow during the rest of the experiment. One dog killed due to injury after six months showed preneoplastic changes in the bladder of the type produced by tryptophan alone. After 4.5 years, the bladder of the three other dogs also showed typical changes seen after tryptophan treatment: macrophagic and lymphocytic infiltration, epithelial hyperplasia, irregularity of the basal layer and erosion of the luminal surface. One dog had developed a

small papillary bladder tumour. Since a single dose of 4-aminobiphenyl or chronic treatment with tryptophan had not produced tumours or preneoplastic changes in the bladder in previous studies (see Deichmann & MacDonald, 1968; Radomski *et al.*, 1971), this result was considered by the authors as suggestive of a promoting or co-carcinogenic effect of tryptophan (Radomski *et al.*, 1977). [The Working Group noted that the lack of a tryptophan-only treated group; the inadequate design of the Deichmann & MacDonald (1968) study made the authors' conclusion hard to believe.]

Twenty-four female pure-bred beagle dogs, four months of age, were given 4-aminobiphenyl (source, purity and dose (*sic*) not stated) in a corn oil suspension contained in a capsule on five days a week for three years. Twenty control animals were included. Two dogs remained without detectable tumours, two showed Grade-1 tumours very late in the study, and 20 animals developed tumours that ultimately progressed into Grade-2 and -3 bladder tumours (mainly transitional cell carcinomas) (Block *et al.*, 1978). [The Working Group noted the absence of information on the twenty control animals.]

3.2 Subcutaneous administration

3.2.1 *Mouse*

Fifty-two newborn Swiss mice were given a subcutaneous injection of 200 μg of 4-aminobiphenyl (Koch-Light; distilled before use) in 0.02 mL of 3% aqueous gelatin on each of the first three days of life. Forty-three mice survived until 48–52 weeks. Among the survivors, 19 of 20 (95%) male and 4 of 23 (17%) female mice had 'hepatomas'. Only three (3%) mice developed hepatomas in a control group of 42 males and 48 females (Gorrod *et al.*, 1968).

In a multistep in-vitro/in-vivo system, SV40-immortalized human urothelial cells were treated for 24h with 4-aminobiphenyl and maintained in culture for six weeks. The cells were then inoculated subcutaneously into female athymic nude (*nu/nu*) mice (age 4–6 weeks). After six months, 28 of 45 (62%) treated mice had developed carcinomas, and none of nine controls inoculated with cells treated with DMSO (Bookland *et al.*, 1992a).

3.2.2 *Rat*

Groups of 10 male and 12 female albino rats, three months of age and weighing 100 g, from a closed but randomly mated colony, were each subdivided in two groups and given 4-aminobiphenyl (purified, b.p. 302°C) in arachis oil by subcutaneous injection daily during 250–376 days, to a total dose of 3.6–5.8 g/kg bw. In the control group that received arachis oil only, one of 12 (8%) male and four of 11 (36%) female rats developed injection-site sarcomas. One female (9%) rat developed an intestinal tumour. In the treated groups, seven of 23 (30%) animals had intestinal tumours, one (4%) had a liver sarcoma, three of 12 females (25%) had mammary tumours and two (17%) had carcinoma of the uterus (Walpole *et al.*, 1952).

3.3 Intraperitoneal injection

3.3.1 *Mouse*

In a comparative carcinogenicity study of heterocyclic amines, newborn male B6C3F$_1$/nctr mice [initial number not clear] were given 4-aminobiphenyl (purity > 98%) by intraperitoneal injection. The amounts administered were 1.25 and 0.625 μmol dissolved in 35 μl dimethyl sulfoxide, injected in portions of 5, 10 and 20 μl on days 1, 8 and 15 after birth, respectively. Surviving pups were weaned on day 21 and designated for necropsy at 8 or 12 months of age. Mortality was 43% for the low-dose and 58% for the high-dose group. At 12 months, the 19 and 15 surviving mice in the low- and high-dose groups, respectively, all had liver adenomas (*vs* 11% in the 44 DMSO controls; $P < 0.001$, Fisher exact test). Among these, five mice in each group had liver carcinomas (26 and 33%, respectively) (Dooley *et al.*, 1992).

Twenty-four newborn B6C3F$_1$ mice were treated with 0.3 μmol 4-aminobiphenyl (re-purified to > 99%, verified by HPLC) dissolved in DMSO, by a series of intraperitoneal injections: 1/7th of the dose on postnatal day 1, 2/7th on day 8, and 4/7th on day 15. Eighteen control mice received DMSO only. Adenomas were found in 22.2% of the controls and in 79.2% of the treated mice. Hepatocellular carcinomas were observed in two treated animals (Parsons *et al.*, 2005).

Newborn male CD1 mice were injected intraperitoneally with 4-aminobiphenyl (purity > 99%) at a total dose of 625 nmol in 35 μl DMSO, given in portions of 5, 10 and 20 μl on days 1, 8, and 15 after birth, respectively. At weaning, the animals were divided into two groups, which were fed *ad libitum* until they were 14 weeks of age. Thereafter, one group received 90% of the calories of the *ad libitum* feeding regimen during one week, followed by 75% of the calories during one week, and then 60% of the calories in the diet until sacrifice at 12 months. Of 22 mice fed *ad libitum*, 12 (55%) had liver adenomas, six (27%) had hepatocellular carcinomas, and two mice (9%) had bronchio-alveolar adenoma. No liver tumours were seen in the calorie-restricted group ($n = 19$), but two mice (11%) developed bronchio-alveolar adenomas (Von Tungeln *et al.*, 1996). [The Working Group noted the lack of untreated controls.]

In a study to investigate the role of CYP1A2 in carcinogenesis induced by 4-aminobiphenyl, groups of mice deficient in cytochrome P450 1A2 (CYP1A2-*null*), derived from a mixed background of 129/Sv and C57BL/6 strains, received intraperitoneal injections of 10 and 20 μl of solutions of 4-aminobiphenyl (Aldrich; re-purified and re-crystallized) in DMSO on days 8 and 15 of age, respectively. One dose group was given a 20-mM solution and the other a 40-mM solution of 4-aminobiphenyl. The cumulative amounts of the test compound in the two dose groups were 600 and 1200 nmol, respectively. The mice were killed at 16 months of age and the livers analysed by histology. Mice proficient in CYP1A2 (CYP1A2+/+) were similarly treated. In the CYP1A2+/+ mice, both the high-dose and low-dose treatments caused a significant increase ($P < 0.01$) in liver adenomas (69–70%) and hepatocellular carcinomas (15–20%) in males compared with a control group consisting of a combination of CYP1A2+/+, +/–

and –/– mice treated with DMSO only. In females, the percentages were 3–17% and 0–4%, respectively. No differences in tumour incidence were seen with the different genotypes (CYP1A2-proficient or deficient) or with different doses of 4-aminobiphenyl (Kimura *et al.*, 1999). [The Working Group noted the inadequacy of the DMSO controls.]

3.4 Intravesicular implantation

Thirty-five albino mice [sex, age and strain not specified] received an intravesicular implant of 4-aminobiphenyl (British Drug Houses; dose unclear) in paraffin wax. After 40 weeks, three of 35 mice (9%) had developed an invasive bladder carcinoma. This tumour yield was not significantly different from that in the control mice implanted with paraffin wax alone (2/56; 4%) (Bonser *et al.*, 1956).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, excretion

4.1.1 *Humans*

Grimmer *et al.* (2000) analysed the amounts of the aromatic amines 1- and 2-naphthylamine and 2- and 4-aminobiphenyl in the urine of 48 German smokers and non-smokers. Both groups excrete these four aromatic amines, with smokers excreting approximately twice as much as non-smokers (736 ng/24h *vs* 327 ng/24h). Similar amounts of urinary 2-naphthylamine and 4-aminobiphenyl were found in the two groups. The origin of the aromatic amines found in the urine of non-smokers is at present unknown. Based on the cotinine levels in the urine of non-smokers, environmental tobacco smoke can be excluded as a major source of aromatic amines. In addition, neither diesel exhaust-related nitroarenes nor the corresponding amino-derivatives to which they may be metabolically converted were detected. The aromatic amines in urine arising from sources other than tobacco smoke or diesel exhaust may play a role in the etiology of bladder cancer in non-smokers (Grimmer *et al.*, 2000).

A method for measuring *ortho*-toluidine, 2-naphthylamine and 4-aminobiphenyl in the urine of smokers and non-smokers used acid hydrolysis of the arylamine conjugates in urine, extraction with n-hexane, derivatization with pentafluoropropionic anhydride, and subsequent analysis with gas chromatography combined with mass spectrometry using negative-ion chemical ionization. The limits of detection were 4 ng/L for *ortho*-toluidine and 1 ng/L for 2-naphthylamine and 4-aminobiphenyl. Smokers ($n = 10$) excreted significantly higher amounts of *ortho*-toluidine (204 *vs* 104 ng/24 hours), 2-amino-naphthalene (20.8 *vs* 10.7 ng/24 hours), and 4-aminobiphenyl (15.3 *vs* 9.6 ng/24 hours) than non-smokers ($n = 10$). All non-smokers had quantifiable amounts of *ortho*-toluidine,

2-naphthylamine, and 4-aminobiphenyl in their urine, confirming that there are other environmental sources of exposure to these compounds (Riedel *et al.*, 2006).

4.1.2 *Experimental systems*

4-aminobiphenyl (4-ABP) is activated by cytochrome P450 (CYP), to produce the genotoxic *N*-hydroxy-4-ABP metabolite, which reacts with DNA.

Anderson *et al.* (1997) examined pancreatic tissues from 29 organ donors (13 smokers, 16 non-smokers) for their ability to metabolize aromatic amines and other carcinogens. Microsomes showed no activity in CYP1A2-dependent *N*-oxidation of 4-aminobiphenyl. Antibodies were used to examine microsomal levels of CYP1A2, 2A6, 2C8/9/18/19, 2E1, 2D6, and 3A3/4/5/7 and epoxide hydrolase. Immunoblots detected only epoxide hydrolase at low levels; CYP levels were < 1% of those in the liver. In pancreatic cytosols and microsomes, 4-nitrobiphenyl reductase activity was present at levels comparable to those in human liver. The *O*-acetyltransferase activity (AcCoA-dependent DNA-binding of radiolabelled *N*-hydroxy-4-aminobiphenyl) of pancreatic cytosols was high, about two thirds the levels measured in human colon. Cytosols showed high activity for *N*-acetylation of *para*-aminobenzoic acid, but not of sulfamethazine, indicating that acetyltransferase-1 is predominantly expressed in this tissue. Cytosolic sulfo-transferase was detected at low levels. ^{32}P-postlabelling showed putative arylamine-DNA adducts in most samples. In eight of 29 DNA samples, a major adduct was observed, which was chromatographically identical to the predominant ABP-DNA adduct, *N*-(deoxyguanosin-8-yl)-aminobiphenyl.

Chou *et al.* (1995) investigated the role of human sulfotransferase(s) in the bio-activation of the *N*-hydroxy metabolite of 4-aminobiphenyl (*N*-OH-ABP) *in vitro* with human tissue cytosols. Using an enzymatic assay consisting of a PAPS-regenerating system, radio-labelled *N*-OH-ABP, calf-thymus DNA and tissue cytosols, the sulfotransferase-mediated metabolic activation of *N*-OH-ABP was determined as the PAPS-dependent covalent binding of the *N*-OH substrate to DNA. The sulfotransferase(s) in human liver, and to a lesser extent colon, can readily metabolize *N*-OH-ABP. No metabolic activation was detected with cytosols prepared from human pancreas or from the carcinogen target tissue, the urinary bladder epithelium. The *N*-OH-ABP sulfotransferase activities of liver and colon cytosols from different individuals were highly correlated with their thermostable phenol sulfotransferase (TS-PST) activity (liver, $r = 0.99$, $P < 0.01$; colon, $r = 0.88$, $P < 0.01$). *N*-OH-ABP sulfotransferase activity was highly sensitive to inhibition by the selective TS-PST inhibitor 2,6-dichloro-4-nitrophenol ($IC_{50} = 0.7$ microM), and by *para*-nitrophenol. These data show that human liver TS-PST can metabolically activate the proximate human carcinogen *N*-OH-ABP to a reactive sulfuric acid ester intermediate that binds to DNA. In addition, in view of the putative role of *N*-OH-ABP as a major transport form of the carcinogen to the urinary bladder and of the absence of sulfotransferase activity in this tissue, sulfotransferase activation in the liver may actually decrease the bioavailability of *N*-OH-ABP to extrahepatic tissues and

thus serve as an overall detoxification mechanism for the urinary bladder (Chou *et al.*, 1995).

Adris and Chung (2006) investigated *Pseudomonas aeruginosa*, an opportunistic pathogen of the human urinary tract, and other selected human endogenous bacteria for metabolic activation of the bladder pro-carcinogens 2-aminofluorene, 4-aminobiphenyl, and benzidine. When incubated with each of those agents, the cell-free extracts of *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, and the intestinal anaerobes *Bacteroides fragilis*, *Clostridium perfringens*, and *Eubacterium aerofaciens* produced increased numbers of histidine revertants with the tester strain *Salmonella typhimurium* TA98 in the *Salmonella* mutagenicity assay. In addition, the cell-free extracts of *Pseudomonas aeruginosa*, *Bacteroides fragilis*, and *Eubacterium aerofaciens* each showed the presence of a CYP absorption peak in the carbon monoxide difference spectrum. This was not observed with the other bacteria. These findings indicate that human endogenous bacteria, which are opportunistic pathogens of the urinary bladder, can metabolically activate these bladder pro-carcinogens into mutagens. The metabolic activation by *Pseudomonas aeruginosa*, *Bacteroides fragilis*, and *Eubacterium aerofaciens* is mediated by a CYP enzyme. For those organisms that induced metabolic activation but did not show a CYP absorption peak with the cell-free extracts, other oxidative enzymes may be involved.

4.1.3 *Other in-vitro studies*

In-vitro studies have been conducted with human and animal microsomal preparations of liver and urinary bladder, and with purified CYPs and recombinant CYPs (Beland & Kadlubar, 1985). CYP1A2 displays the highest catalytic acitivity of the CYPs for *N*-oxidation of 4-ABP, and it is the principal CYP involved in *N*-hydroxylation of 4-ABP in human and rat liver microsomes (Butler *et al.*, 1989). The k_{cat} of *N*-oxidation of 4-ABP is about fivefold greater with pooled human liver microsomes than with human urinary bladder microsomes fortified with NADPH, but the K_m values are comparable between the two microsomal preparations (Nakajima *et al.*, 2006). Several human endogenous bacteria, which are pathogens of the urinary bladder, can metabolically activate the 4-ABP and other bladder procarcinogens. For some of these microorganisms, metabolic activation is mediated by a CYP enzyme (Adris & Chung, 2006).

A large inter-individual variation in *N*-oxidation of 4-ABP in human liver microsomes is attributed to the variable CYP1A2 protein content (Butler *et al.*, 1989; Turesky *et al.*, 1998). Subjects who display relatively high CYP1A2 activity and who are rapid *N*-oxidizers of 4-ABP may be at elevated risk for cancer arising from exposure to aromatic amines or heterocyclic aromatic amines (Butler *et al.*, 1992). However, recent data from studies on laboratory animals have questioned the importance of CYP1A2 in the toxicity and carcinogenicity of 4-ABP. In *CYP1A2*-knockout animals, formation of methaemoglobin, which occurs by co-oxidation of *N*-hydroxy-4-ABP with oxy-

haemoglobin (Kiese, 1966), was lower in *CYP1A2*-knockout mice than in *CYP1A2*-wild-type mice (Shertzer *et al.*, 2002), and 4-ABP-DNA adducts were formed in livers of *CYP1A2*-knockout mice at even higher levels than in livers of *CYP1A2* wild-type mice (Tsuneoka *et al.*, 2003). In the neonatal mouse cancer model, *CYP1A2-null* mice had the same level of hepatocellular carcinoma (HCC) as the *CYP1A2*-wild-type mice following exposure to 4-ABP (Kimura *et al.*, 1999). From these findings, it appears that CYP1A2 is not the primary enzyme responsible for the *N*-hydroxylation 4-ABP in the mouse; thus other enzymes must be involved in the bioactivation and toxicities of 4-ABP. Indeed, liver microsomes from *CYP1A2-null* mice displayed an activity as least half as great as the liver microsomes from *CYP1A2*-wild-type mice in *N*-oxidation of 4-ABP (Kimura *et al.*, 1999). The enzymes responsible for this bio-activation remain to be determined. The high doses of 4-ABP employed in these animal studies could have triggered the activities of other enzymes to catalyse the *N*-oxidation of 4-ABP, a reaction process that normally does not occur after low-dose treatments. The *N*-oxidation of 4-ABP is known to be carried out principally by CYP1A2 in rat and human liver microsomes (Kimura *et al.*, 1999); these studies reveal species differences in 4-ABP metabolism by CYPs among mice, rats, and humans.

Extrahepatic CYPs, peroxidases, and prostaglandin-H synthase have been reported to catalyse the bioactivation of 4-ABP. Recombinant CYPs 1A1, 1B1 and 2A13 have been shown to bioactivate 4-ABP (5 μM), when induction of *umu* gene expression in *S. typhimurium* NM2009 was used as an endpoint (Shimada *et al.*, 1996; Nakajima *et al.*, 2006). These CYP enzymes could contribute to 4-ABP damage in extrahepatic tissues. Enzymes in bovine bladder epithelium and dog-bladder epithelium bioactivate arylamines, including 4-ABP (10–30 μg/plate) into bacterial mutagens in *S. typhimurium* TA98 (frame-shift specific) (Hix *et al.*, 1983), and they catalyse 4-ABP binding to DNA (Wise *et al.*, 1984). Intact cells or cell homogenates (S-9 fraction) from bovine bladder were found to be more effective than were liver preparations in the activation of 4-ABP (1–20 μg/plate) into a bacterial mutagen, in *S. typhimurium* TA98 (Hix *et al.*, 1983) and TA100 (point-mutation specific) strains (Oglesby *et al.*, 1983). Intact bovine urothelial cells also weakly bioactivated 4-ABP (5–20 μg/mL) into a mutagen in Chinese hamster V79 cells (Oglesby *et al.*, 1983). Prostaglandin H synthase, with arachidonic acid serving as a co-factor, has been shown to catalyse the binding of 4-ABP to DNA (Flammang *et al.*, 1989). This enzyme is present in urinary bladder epithelium, prostatic epithelium, colonic mucosa, and peripheral lung tissue of many species. Peroxidases, which are expressed at appreciable levels in the lung (Culp *et al.*, 1997) and mammary gland (Josephy 1996), also catalyse the binding of 4-ABP to DNA (Gorlewska-Roberts *et al.*, 2004). These results demonstrate the capacity of enzymes in extrahepatic tissues, including the bladder urothelium, to metabolically activate aromatic amines, and they suggest a role for the target organs in carcinogen bioactivation.

N-hydroxy-4-ABP can undergo further metabolism by phase-II enzymes to produce highly unstable esters. The esters undergo heterolytic cleavage to produce the reactive nitrenium ion, which readily forms adducts with DNA. NAT1 and NAT2 isoforms of

human and rodent *N*-acetyltransferases catalyse the formation of *N*-acetoxy-4-ABP (Minchin *et al.*, 1992), while sulfotransferase (SULT1A1) (Chou *et al.*, 1995) produces the *N*-sulfate ester of 4-ABP. These products may be the penultimate carcinogenic metabolites of 4-ABP formed *in vivo*. There are several metabolic reactions, including *N*-acetylation, that can compete with the CYP-mediated *N*-oxidation of 4-ABP. The resulting acetamide of 4-ABP is a poor substrate for CYP1A2, and *N*-acetylation is considered to be primarily a detoxification reaction (Cohen *et al.*, 2006). The rate of *N*-acetylation of 4-ABP with recombinant human NAT1 was threefold greater than with recombinant human NAT2, whereas the *O*-acetylation of *N*-hydroxy-4-ABP was about twofold higher with NAT2 (Minchin *et al.*, 1992). Thus, human NAT isoforms are involved in the detoxification of 4-ABP as well as in the bioactivation of *N*-hydroxy-4-ABP.

4.2 Genetic and related effects

4.2.1 *DNA adducts*

DNA adducts of 4-ABP have been reported *in vitro*, in bacterial and mammalian cells, in experimental animals exposed to 4-ABP or its metabolites, and in human tissues. Three major adducts are formed when *N*-hydroxy-4-ABP is reacted with calf-thymus DNA at pH 5.0: *N*-(deoxyguanosin-8-yl)-4-ABP (dG-C8–4-ABP) is the principal adduct and formed in 80% yield, followed by *N*-(deoxyadenosin-8-yl)-4-ABP (15% yield) and *N*-(deoxyguanosin-N^2-yl)-4-ABP (5% yield) (Beland *et al.*, 1983; Beland & Kadlubar, 1985). The N^2-deoxyguanosine adduct is unusual in that it contains a hydrazo linkage. More recently, 3-(deoxyguanosin-N^2-yl)-4-ABP and *N*-(deoxyguanosin-N^2-yl)-4-azobiphenyl have been identified as minor DNA adducts of 4-ABP (Hatcher & Swaminathan, 2002; Swaminathan & Hatcher, 2002).

(a) Humans

The adduct dG-C8–4-ABP was first detected by ^{32}P-postlabelling in biopsy samples of the human urinary bladder (Talaska *et al.*, 1991). Thereafter, the adduct was detected by gas-chromatography/negative-ion chemical ionization mass spectrometry (GC-NICI-MS) in mucosa specimens of human lung and urinary bladder; it was found at levels ranging from < 0.32–49.5 adducts per 10^8 nucleotides in lung, and from < 0.32–3.94 adducts per 10^8 nucleotides in the bladder samples (Lin *et al.*, 1994). A subsequent study reported the detection of the dG-C8–4-ABP adduct by immuno-histochemistry, ^{32}P-postlabelling, or GC-NICI-MS in bladder and lung tissue from smokers and ex-smokers (Culp *et al.*, 1997). The adduct levels measured by immuno-histochemistry ranged from three adducts per 10^8 nucleotides (the limit of detection) up to 505 adducts per 10^8 nucleotides. Comparable data were obtained through GC-NICI-MS measurements. The ^{32}P-postlabelling analyses underestimated the adduct level by up to 30-fold, suggesting that human lung DNA may be resistant to enzymatic hydrolysis,

possibly as a consequence of the high levels of DNA damage among current smokers. The 4-ABP adduct levels did not correlate with the numbers of cigarettes smoked per day or the duration of smoking, so the 4-ABP adducts in lung were proposed to originate from environmental exposure to 4-nitrobiphenyl (Culp *et al.*, 1997). The frequent detection of dG-C8–4-ABP adducts in non-smokers indicates that there may be other environmental sources of exposure as well: one such source may be hair dyes (Turesky *et al.*, 2003).

Wang *et al.* (1998) used immunohistochemistry to detect 4-ABP-DNA adducts in livers of subjects from Taiwan, China, with hepatocellular carcinoma (HCC). The mean relative staining intensity for 4-ABP-DNA was slightly higher in tumour tissues than in non-tumour tissues obtained from the same HCC patients. The mean intensities were significantly higher than the mean intensities from control tissues taken from non-HCC patients. However, no difference in mean relative staining intensity was found between smokers and nonsmokers in tissues obtained from non-HCC patients, or between tumour or non-tumour tissues taken from HCC cases. After stratification of the relative staining intensity data for 4-ABP-DNA adducts, there was a monotonically increasing risk for HCC with higher 4-ABP adduct levels; the linear relationship between adduct levels in liver tissue and HCC risk remained significant after adjustment for covariates, including hepatitis B surface-antigen status.

The DNA from the induced sputum of smokers, representing DNA of the lower respiratory tract, was shown to possess significantly higher levels of 4-ABP-DNA adducts than in the sputum of non-smokers, when assessed by immunohistochemical staining. The levels of adducts were related to indices of current smoking (cigarettes/day: $r = 0.3$, $P = 0.04$ and tar/day: $r = 0.4$, $P = 0.02$) (Besaratinia *et al.*, 2000). Faraglia *et al.* (2003) also detected 4-ABP-DNA adducts in female breast-tissue biopsy samples, when visualized by immunohistochemistry; the woman's smoking status was correlated with the levels of 4-ABP-DNA in tumour-adjacent normal tissues, but not in tumour tissue. 4-ABP-DNA adducts were also detected in laryngeal biopsies by immunohistochemical methods. Staining-intensity data showed a log-normal distribution, and values determined in tumour-adjacent tissue from individuals who smoked were significantly higher (median: 173.5, geometric mean: 159.9) than those values measured in tumour-adjacent tissue from non-smokers (median: 75.5, geometric mean: 7.40) (Faraglia *et al.*, 2003). Epithelial cell DNA isolated from human breast milk was shown by ^{32}P-postlabelling to contain *N*-(deoxyguanosin-8-yl)-4-ABP, and the adduct level was significantly associated with the use of hair-colouring products (OR = 11.2, 95% CI = 1.1–109.2), but not with tobacco usage (Ambrosone *et al.*, 2007).

Recent studies have employed liquid chromatography/electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) methods to quantify dG-C8–4-ABP in human tissues. Zayas *et al.* (2007) detected dG-C8–4-ABP in urinary bladder epithelium in 12 of 27 subjects in DNA of tumour or non-tumour surrounding tissue. The level of adducts ranged from five to 80 adducts per 10^9 bases. The level of DNA adducts did not correlate with the level of the 4-ABP-haemoglobin-sulfinamide adduct; the latter is derived from 4-nitrosobiphenyl, an oxidation product of *N*-hydroxy-4-ABP, and a biomarker of exposure

to an internal dose of 4-ABP (Skipper & Tannenbaum, 1994; Zayas *et al.*, 2007). The lack of correlation of 4-ABP-DNA and -protein biomarkers may be attributed to an unknown time of formation of the observed adducts and to a variable persistence of dG-C8–4-ABP in the bladder. Because the activation or detoxification processes of 4-ABP metabolism as well as DNA-repair mechanisms may be tissue-specific, a correlation between haemoglobin (Hb) adducts and biopsied DNA adducts may not exist. The metabolism of 4-ABP is complex and/or may take place in one or more compartments that are distant from the urinary bladder. Thus, prediction of the relationship between exposure and adduct levels is not straightforward. Another pilot study reported the presence of dG-C8–4-ABP in six of 12 human pancreas samples (Ricicki *et al.*, 2005). The levels ranged anywhere from 1 to $60/10^8$ nucleotides, but there were no correlations among between the level of adducts, smoking preference, age, or gender. The frequency of hair-dye usage was not reported in the study.

4-ABP forms a major adduct at the 93β cysteine amino acid of human Hb (Green *et al.*, 1984; Ringe *et al.*, 1988). Adduct formation occurs following the co-oxidation reaction of *N*-hydroxy-4-ABP with oxy-Hb (Kiese 1966). The resulting 4-nitrosobiphenyl product reacts with the sulfhydryl group of cysteine to form a sulfinamide adduct (Green *et al.*, 1984; Ringe *et al.*, 1988; Bryant *et al.*, 1988). Several human biomonitoring studies have shown a relationship between the frequency of tobacco smoking, the extent of 4-ABP-Hb-adduct formation, and bladder-cancer risk (Bryant *et al.*, 1988; Skipper & Tannenbaum, 1994; Jiang *et al.*, 2007). Thus far, investigations have not examined the relationship between the frequency of hair dye-usage and 4-ABP-Hb adduct levels; however, statistically significant increases in levels of 3,5-dimethylaniline-Hb adducts have been found in women who regularly used permanent hair dyes (Gan *et al.*, 2004).

(b) Experimental systems

The methods of 4-ABP-DNA adduct detection include: HPLC with radiometric detection of tritium-labelled adducts; ^{32}P-postlabelling; immunhistochemistry; GC-NICI-MS of alkali-treated DNA, which cleaves the bond between the guanyl C8 atom and the 4-amino group of 4-ABP in dG-C8–4-ABP; and LC-ESI-MS/MS (Beland & Kadlubar, 1985; Lin *et al.*, 1994; Culp *et al.*, 1997; Doerge *et al.*, 1999).

N-Hydroxy-4-ABP reacts with DNA at neutral pH (pH 7.0) to form covalent adducts, but the reactivity is enhanced tenfold under acidic conditions (pH 5.0), which catalyses the generation of the highly reactive nitrenium ion (Beland *et al.*, 1983). Such mildly acidic pH conditions can occur in the urinary bladder and can cause the hydrolysis of the metabolite N^4-(β-glucosiduronyl)-*N*-hydroxy-4-ABP to produce *N*-hydroxy-4-ABP and its nitrenium ion, leading to DNA adducts in the bladder epithelium (Beland *et al.*, 1983; Kadlubar *et al.*, 1991).

Beagle dogs were treated with tritium-labelled 4-ABP at a single dose of 60 µmol/kg bw and sacrificed after one, two, or seven days. DNA adducts in urothelial cells were monitored by HPLC with radiometric detection (Beland & Kadlubar, 1985). The level of adducts in the animals was essentially constant at each time point, with

N-(deoxyguanosin-8-yl)-4-ABP accounting for 76% of the total binding. The next most abundant product was the N^2-deoxyguanosine substituted adduct (15%), followed by the *N*-(deoxyadenosin-8-yl)-4-ABP adduct (9%). These same adducts and 3-(deoxyguanosin-N^2-yl)-4-aminobiphenyl and *N*-(deoxyguanosin-N^2-yl)-4-azobiphenyl were detected by ^{32}P-postlabelling of DNA in human uroepithelial cells exposed to *N*-hydroxy-4-ABP (Hatcher & Swaminathan, 2002; Swaminathan & Hatcher, 2002).

DNA adducts of 4-ABP were quantified, by ^{32}P-postlabelling and immunohistochemistry, in liver and bladder of male and female BALB/c mice, following treatment with a range of concentrations of 4-ABP (0, 7, 14, 28, 55, 110 or 220 ppm; male) and (0, 7, 19, 38, 75, 150 or 300 ppm; female) in the drinking-water for 28 days (Poirier *et al.*, 1995). The principal adduct in both tissues, for both genders, was dG-C8–4-ABP. A comparison between DNA-adduct formation and tumourigenesis indicated a linear correlation between adduct levels and incidence of liver tumours in female mice. The relationship between adducts and tumourigenesis was distinctly nonlinear in the bladders of male mice, and tumour incidence rose rapidly above the 50-ppm dose of 4-ABP: toxicity and cell proliferation may have increased the bladder-tumour incidence.

4.2.2 *Mutations and other related effects*

(a) Mutagenesis in bacteria

4-Aminobiphenyl and other primary arylamines are frameshift mutagens in *S. typhimurium* strain TA1538 (Beland *et al.*, 1983). When revertants were expressed as a function of DNA binding, *N*-hydroxy-*N*′-acetylbenzidine was found to be the most mutagenic arylamine, followed by *N*-hydroxy-2-aminofluorene, and then by *N*-hydroxy-2-naphthylamine (*N*-OH-2-NA) and *N*-hydroxy-4-aminobiphenyl; the latter two showed approximately the same number of revertants per adduct. In a similar study conducted with *S. typhimurium* strain strain TA 1535, which detects base-substitution revertants, only *N*-OH-2-NA induced mutations (Beland *et al.*, 1983).

More recent studies with the TA100 strain (base substitution-specific) containing plasmid pKM101 (which enhances the mutagenic potential of some genotoxicants through error-prone DNA-repair polymerases) have shown that 4-ABP (3–300 µg/plate) induces revertants in the TA100 strain, in the presence of a rat liver S-9 mix from animals pretreated with polychlorinated biphenyls (PCBs), at levels five- to tenfold higher than in TA98 (frameshift-specific) (Chung *et al.*, 2000). The mutagenic potency of 4-ABP (1–1000 µg/plate) was also greater in strain YG1029 (base substitution-specific strain), which has multiple copies of the bacterial *O*-acetyltransferase (Dang & McQueen, 1999), indicating formation of the reactive *N*-acetoxy-4-ABP intermediate.

The induction of the *umu* response of 4-ABP (0.1–20 µM) in *S. typhimurium* strain NM6001, containing human NAT1, was about fourfold greater than in strain NM6002 expressing human NAT2, when recombinant human CYP1A2 was used for bioactivation of 4-ABP (Oda, 2004). Consistent with these data, NAT1 was superior to NAT2 in inducing the binding of *N*-hydroxy-4-ABP to DNA (Minchin *et al.*, 1992). 4-ABP

(5 μg/plate), in the presence of rat liver S-9 (PCB pretreatment), also induced mutations in *S. typhimurium* strain TA102, which is sensitive to agents producing reactive oxygen species (Makena & Chung, 2007). The generation of reactive oxygen species may occur through redox cycling of *N*-hydroxy-4-ABP and its nitroso metabolite (Kim *et al.*, 2004) via a hydronitroxide radical as an intermediate (Makena & Chung, 2007).

Verghis *et al.* (1997) studied mutagenesis of 4-ABP in *E.coli* in the *lacZ* gene using M13 cloning vectors able to detect many base-pair substitution mutations and most insertions and deletions. To minimize the effect of host DNA-repair processes, mutagenicity experiments were performed with single-stranded DNA randomly modified with 4-ABP instead of double-stranded DNA. Sequence analysis of 4-ABP-induced mutations in the *lacZ* gene revealed exclusively base-pair substitutions, with over 80% of the mutations occurring at G sites. Among the sequence changes at G sites, G:C → to T:A transversions predominated, followed by G:C → C:G transversions, and G:C → A:T transitions. An oligonucleotide containing the major DNA adduct, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8–4-ABP), was situated within the *Pst*I site of a single-stranded M13 genome to examine the mechanism of genotoxicity of this adduct. After *in vivo* replication of the adduct-containing 4-ABP-modified and control (unadducted) genomes, the mutational frequency and mutational specificity of the dG-C8–4-ABP lesion were determined. The targeted mutational efficiency was ~0.01%, and the primary mutation observed was the G:C → C:G transversion (69%), followed by G:C → to T:A transversions (23%). In the mutagenesis studies with the randomly 4-ABP-modified vector, G:C → T:A transversions were the most numerous, possibly reflecting a sequence-context effect for the dG-C8–4-ABP lesion. Thus, although dG-C8–4-ABP is weakly mutagenic at the *Pst*I site, it can contribute to the mutational spectrum of 4-ABP lesions (Verghis *et al.*, 1997).

(b) Mutagenesis in mammalian cells

(i) *Mutations in* HGPRT*, chromosomal instability*

4-Aminobiphenyl and three of its proximate *N*-hydroxylated carcinogenic metabolites (0.5–400 μM) were mutagenic in an SV40-immortalized human uroepithelial cell line, as assayed by use of induction of mutations in the hypoxanthine-guanine phosphoribosyl-transferase (*HGPRT* or *HPRT*) locus (Bookland *et al.*, 1992b). 2-ABP did not induce mutations in this cell line at these concentrations.

Human bladder-cancer (RT112) cells treated with 4-ABP (125 μg/ml), with rat liver S-9 (after pretreatment with PCB) used for bio-activation, developed chromosomal instability in 50–60% of the cells. 4-ABP could affect tumour occurrence by inducing chromosomal instability, which represents the predominant form of genetic instability in human solid tumours (including those in bladder and colon) (Saletta *et al.*, 2007).

N-Hydroxy-acetylaminobiphenyl (*N*-OH-AABP) at a range of doses (0.5, 1.0, and 10.0 μM) induced mutations by up to five- and 16-fold over background levels,

respectively, at the *HPRT* and thymidine kinase (*TK*) loci of human lymphoblastoid TK6 cells (Luo *et al.*, 2005; Ricicki *et al.*, 2006).

(ii) *H*-ras *mutations caused by 4-ABP*

DNA adducts and mutations were induced in the livers of neonatal wild-type B6C3F$_1$ and CD-1 mice, and in Big Blue B6C3F$_1$ transgenic neonatal and adult mice treated with a regimen of 4-ABP known to induce tumours in neonatal mice (total doses: 3, 9 or 31 mg/kg 4-ABP at days 8 and 15 postnatal; 31 mg/kg bw for adults) (Manjanatha *et al.*, 1996). dG-C8–4-ABP was the major DNA adduct identified in the livers of Big Blue B6C3F$_1$ mice. Adduct levels for adult females were about twofold higher than for neonatal females, while adduct levels in adult males were less than half those in neonatal males. In the neonatal wild-type B6C3F$_1$ mice, 4-ABP preferentially induced C:G → A:T mutations (reflecting G:C → T:A transversions in the non-coding strand) in H-*ras* codon 61, followed by G:C → C:G mutations. However, in CD-1 mice, 4-ABP primarily induced A:T → T:A transversions in H-*ras* codon 61, a molecular feature consistent with the formation of the *N*-(dA-C8-yl)-4-ABP adduct (Manjanatha *et al.*, 1996). In the C57B1/10J mouse strain, mutations in codon 61 of the H-*ras* gene were not significantly implicated in chemical induction of liver tumours by 4-ABP (ABP was administered in diet at 200 and 600 ppm for nine months) (Lord *et al.*, 1992), suggesting that the incidence of *Ras* mutations in chemically-induced mouse liver tumours is strain-dependent.

(iii) *Transgene mutations caused by 4-ABP*

4-ABP was evaluated in the Muta^{TM}Mouse transgenic mouse mutation assay. A single oral dose of 75 mg/kg bw induced, respectively, 6.9-, 1.8- and 2.2-fold increases in the mutation frequency (MF) in the bladder, liver and bone marrow. Ten daily oral doses of 10 mg/kg body weight of 4-ABP increased the MF in the bladder, liver, and bone marrow by 13.7-, 4.8- and 2.4-fold the control value, respectively (Fletcher *et al.*, 1998).

4-ABP treatment significantly increased the mutations in the liver *cII* transgene in both genders of neonatal Big Blue B6C3F$_1$ transgenic mice but not in the adult mice, following treatment with a regimen of 4-ABP known to induce tumours in neonatal mice (total doses of 3, 9 or 31 mg/kg 4-ABP at days 8 and 15 postnatal; 31 mg/kg bw for adults). Sequence analysis of *cII* mutant DNA revealed that 4-ABP induced a unique spectrum of mutations in the neonatal mice, characterized by a high frequency of G:C → T:A transversions, while the mutational spectrum in 4-ABP-treated adults was similar to that in control mice. These authors suggested that neonates are more sensitive than adults to 4-ABP, because the relatively high levels of cell division in the developing animal facilitate the conversion of DNA damage into mutations (Chen *et al.*, 2005). The mutational spectrum data are consistent with the data seen for the tetracycline resistance gene of the plasmid pBR322, where 4-ABP modification resulted in G:C → to T:A and G:C →C:G transversions; frameshift mutations occurred with a lower frequency (Melchior *et al.*, 1994).

N-Hydroxy-4-acetylaminobiphenyl (*N*-hydroxy-AABP) (10–320 μM) caused a dose-dependent increase in mutation frequency – up to 12.8-fold over the background level – in the *cII* transgene in embryonic fibroblasts of the Big Blue mouse (Besaratinia *et al.*, 2002). A high frequency of G:C → T:A transversions occurred in the *cII* transgene of treated cells. The authors concluded that the pattern of mutations induced in the *cII* gene by this metabolite of 4-ABP is at odds with the mutational spectrum of the *TP53* gene in human bladder cancer, where G:C → A:T transitions are the dominant type of mutation (Olivier *et al.*, 2002).

(iv) TP53 *mutations induced by 4-ABP*

About 50% of bladder cancers contain a mutation in the tumour-suppressor gene *TP53* (Olivier *et al.*, 2002). The spectrum of mutations in the *TP53* gene in smokers and non-smokers with bladder cancer shows base substitutions occurring at G:C and A:T base pairs. Codon 285 of the *TP53* gene, a mutational hotspot at a non-CpG site in bladder cancer, was found to be the preferential binding site for *N*-hydroxy-4-ABP (30 μM) *in vitro* (Feng *et al.*, 2002b). Moreover, C5-cytosine methylation greatly enhanced *N*-hydroxy-4-ABP binding at CpG sites; two mutational hotspots at CpG sites, codons 175 and 248, became preferential binding sites for *N*-hydroxy-4-ABP only after being methylated. The distribution of 4-ABP–DNA adducts was mapped in the *TP53* gene at the nucleotide-sequence level in human bladder cells (HTB-1) treated with *N*-hydroxy-4-ABP or *N*-hydroxy-AABP (30 μM) and mutational hotspots in bladder cancer at codons 175, 248, 280, and 285 were preferential sites for 4-ABP adduct formation (Feng *et al.*, 2002a). The authors suggested that 4-ABP contributes to the mutational spectrum in the *TP53* gene in human bladder cancer. These data provide some molecular evidence that links 4-ABP to bladder cancer, but the roles of methylation status and transcriptional activity in the mutational spectrum in the *TP53* gene induced by 4-ABP have yet to be determined.

4.2.3 *Other biological effects of 4-ABP in mammalian cells*

In mammalian cells, 4-ABP and three of its proximate *N*-hydroxylated carcinogenic metabolites (0.5–400 μM) were genotoxic in an SV40-immortalized human uroepithelial cell line, in which mutation induction was measured at the hypoxanthine-guanine phosphoribosyl-transferase (*HGPRT*) locus (Bookland *et al.*, 1992b). The isomeric 2-ABP did not induce mutations at these concentrations. At very high concentrations, 2-ABP and 4-ABP (500 μM), among other arylamines, were reported to induce DNA damage in human lymphocytes, as revealed by use of the comet assay (Chen *et al.*, 2003).

With *N*-hydroxy-AABP at a range of doses (0.5, 1.0 and 10.0 μM), the levels of dG-C8–4-ABP adducts in the cells ranged from 18 to 500 adducts per 10^9 nucleotides at 27 hours, and resulted, respectively, in 95%, 85%, and 60% cell-survival rates. By means of statistical regression, 2250 genes were identified that showed statistically significant changes in expression after treatment with *N*-hydroxy-AABP; they included induced

stress-response genes such as members of the class of heat-shock proteins [Hsp40 homologue (DNAJ), Hsp70, Hsp105, and Hsp125], and metal regulatory transcription factor 1 (MTF1). Another gene whose expression was upregulated was the *XPA* gene, which encodes a DNA-damage recognition protein involved in excision repair. Several of the genes were also induced in TK6 cells treated with benzo[*a*]pyrene diol epoxide (Luo *et al.*, 2005; Ricicki *et al.*, 2006). The subsets of commonly regulated genes were indicative of a general cellular response to toxicity to these two classes of carcinogenic agents.

DNA adducts were formed in transitional cell carcinoma (TCC) cell lines from the huan urinary bladder expressing wild-type or mutant *TP53*, following exposure to *N*-hydroxy-ABP, *N*-hydroxy-AABP or *N*-acetoxy-4-acetylaminobiphenyl (*N*-OAc-AABP) (Swaminathan *et al.*, 2002). The major adduct in the cell lines was identified as dG-C8–4-ABP with all three chemicals. The number of adducts ranged from 0.1 to 20 per 10^6 nucleotides, with *N*-OAc-AABP yielding the highest adduct levels after 2 hours of treatment. Only *N*-OAc-AABP (5 μM) induced an apoptotic response, which was independent of the *TP53* status. However, the *TP53* status did affect repair rates, and the level of the dG-C8–4-ABP adduct was approximately 2-fold higher in TCC cells with mutant *TP53* than with wild-type *TP53*, at 24 hours post-treatment. The authors concluded that *TP53* could be modulating the repair of 4-ABP-DNA adducts in the human uroepithelial cells, and that unrepaired DNA damage that accumulates in TP53-deficient cells could cause an accumulation of mutation, increase genomic instability, and accelerate neoplastic progression.

4.3 Mechanistic considerations

Since biomonitoring was introduced to assess exposure to aromatic amines, 4-aminobiphenyl (4-ABP) was found in human samples. Originally the analysis of haemoglobin adducts pointed to cigarette smoke as a source. Recently, 4-ABP as well as *ortho*-toluidine and 2-naphthylamine were detected in the urine of smokers and non-smokers. The concentrations were significantly higher in smokers than in nonsmokers in all three cases, but it was concluded that 4-ABP is a general environmental contaminant (Riedel *et al.*, 2006). Looking for the sources, 4-nitrobiphenyl must be considered to contribute to the exposure. This applies not only to the assessment of haemoglobin adducts, but also to guanine-C8-ABP-adducts in DNA. The biomarkers correlate with three risk factors for bladder cancer: cigarette smoking, tobacco brand and slow-acetylator phenotype. DNA adducts have not only been demonstrated in human bladder tumours (Zayas *et al.*, 2007), bladder epithelium (Skipper & Tannenbaum, 1994) and in exfoliated urothelial cells (Talaska *et al.*, 1993), but also in mammary (Faraglia *et al.*, 2003) and other tissues (Cohen *et al.*, 2006; Saletta *et al.*, 2007).

The common activation product of aminobiphenyls is the *N*-hydroxy derivative. Studies in dogs indicate that *N*-oxidation to *N*-hydroxyaminobiphenyl takes place in the liver, from where the metabolite is transferred to the bladder. The hydroxylamine may be

further activated by conjugation to form sulfate or acetate. The conjugates are unstable and give rise to the nitrenium ion that adds to DNA and gives promutagenic DNA adducts. Modifying factors of DNA-adduct formation in the bladder are the pH of the urine and the frequency of urination (Kadlubar *et al.*, 1991). Although CYP1A2 is considered to be the enzyme primarily responsible for the oxidation of arylamines in the liver, experiments with *CYP1A2*-knockout mice show that this is not the case with 4-ABP (Kimura *et al.*, 1999; Shertzer *et al.*, 2002). The lack of CYP1A2 did not influence the decrease of the thiol SH-pool in the liver, did not interfere with the consumption of glutathione in red cells, and increased the formation of methaemoglobin (Shertzer *et al.*, 2002). This bears on the interpretation of individual susceptibility of workers exposed to 4-ABP. The activity of CYP1A2 varies by a factor of 60 in humans, and workers with high levels of active enzyme have been assumed to be more susceptible to developing bladder tumours than those with low levels of this enzyme. Another possible route of activation was suggested by the results of studies in *S. typhimurium* strain TA102. In this case 4-ABP, activated by liver S9, induced mutations through generation of reactive oxygen species (ROS) (Makena & Chung, 2007).

The formation of haemoglobin adducts and DNA adducts in tissues other than the bladder supports the view that the activated metabolite is not only available in the target tissue for tumour formation but throughout the organism, and factors other than bladder-specific activation must be responsible for the development of the bladder tumours.

In addition to the dG-C8-ABP adduct, the dG-*N*2-ABP adduct and the dA-C8-ABP adduct were observed (Beland & Kadlubar, 1985; Swaminathan & Hatcher, 2002). Although 4-ABP like 2-aminofluorene (2-AF) produces the typical guanine-C8-adduct, the mutation pattern of the two chemicals is different. dG-C8-AF produces both frameshift and base-substitution (G to T) mutations, and dG-C8-ABP only base-substitution (G to A) mutations, 2-AF being more mutagenic than ABP. These differences are considered to arise from the different conformational changes induced in DNA by aromatic amines, including conversion from the *anti*- to the *syn*-conformation (Beland *et al*., 1983; Beland & Kadlubar, 1985). So far a key to explain tissue specificity and individual susceptibility has not been found.

Gene-expression profiles were measured in the TK6 lymphoblastoid cell line after exposure to *N*-hydroxy-4-aminobiphenyl. The amount of the major dG-C8-ABP adduct was correlated with cell toxicity, mutation at the *TK* (thymidine kinase) and *HPRT* loci and changes in gene expression. The expression of 2250 genes was significantly altered. Five genes related to functions of cell survival and cell growth were downregulated; all the others were upregulated (Ricicki *et al.*, 2006). The results indicate a possible approach to relate gene-expression patterns with phenotypic markers, but also show the complexity of the cellular response to a chemical insult.

More recent results emphasize that the induction of mutations in the human genome may not be the only relevant genotoxic effect. Saletta *et al.* (2007) propose that gross chromosomal alterations rather than point mutations are responsible for the formation of bladder tumours. Chromosome aberrations and chromosomal instability are held to play a

central role. The induction of chromosomal instability can be carcinogen-specific. 4-ABP induces chromosomal instability (CIN) in genetically stable RT112 bladder cells, but no microsatellite instability (MIN). Conversely, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produces MIN but not CIN in these cells. CIN is generally defined as the ability to gain and lose chromosomes, a property frequently observed in tumours, which makes them resistant to the toxic effects of carcinogens and gives them a selective advantage. However, the actual role of chromosomal instability in ABP-induced bladder cancer remains to be established (Saletta *et al.*, 2007).

The expanding knowledge about metabolic activation of 4-ABP and the causal relationship between DNA-adduct formation and mutagenesis stimulated the expectation that differences in tissues and species, as well as individual susceptibility can be explained on the basis of the pharmacokinetic behaviour. However, Beland and Kadlubar and others stated as early as 1985: "It is clear that adduct formation *per se* is not sufficient for tumour initiation and that other processes, such as error-prone repair and stimulation of cell replication, play an essential role." Even today, the tissue-specific effects of 4-ABP can neither be answered by specific mutations or mutation rate nor by selective advantage. After so many years of research about one of the first classified human carcinogens it still seems unlikely that a critical pathway can be found that leads from exposure to cell transformation and tumour development. However, the data collected thus far support a common mode of action for many aromatic amines, in which genotoxic effects and toxicity both have a role. This mode of action is defined as interaction with cellular regulation pathways. In their review, Cohen *et al.* (2006) call it "synergy of DNA reactivity and cell proliferation." The emphasis so far has been on DNA-reactivity. The role of toxicity and the underlying biochemistry and molecular biology has been neglected and is essentially unknown. Epidemiology and animal experiments support the classification of 4-ABP as carcinogenic to humans. The toxicological profile shows many properties typical for the group of carcinogenic aromatic amines and their common mode of action. Since 4-ABP and 4-nitrobiphenyl are present in the general environment, any further assessment requires more knowledge about the background exposure and the incremental contribution of specific sources.

5. Summary of Data Reported

5.1 Exposure data

4-Aminobiphenyl was formerly used as a rubber oxidant, as a dye intermediate and for the detection of sulfates. Currently it is only used for research purposes. Its production has been prohibited in the European Union since 1998 and ceased in the USA in the 1950s. 4-Aminobiphenyl is still produced in some countries and supplied to countries where it is no longer produced. 4-Aminobiphenyl may occur as a contaminant in 2-aminodiphenyl, in some cosmetic colour additives, in hair dyes and in the fungicide

diphenylamine. Occupational exposure may occur during production and use of the compound. The main sources of exposure for the general population are tobacco smoking and environmental tobacco smoke, although the use of hair dye contaminated with 4-aminobiphenyl is also a potential source of exposure.

5.2 Human carcinogenicity data

Excesses of bladder-cancer risk have been found in a case series and a cohort study in workers producing and using many chemicals including 4-aminobiphenyl. The most marked excess of bladder cancer occurred in workers exposed to 4-aminobiphenyl. Chance, bias and confounding from other chemical exposures or smoking offer no credible explanation for these increased risks.

5.3 Animal carcinogenicity data

4-Aminobiphenyl was tested for carcinogenicity by oral administration in mice, rabbits and dogs, and by subcutaneous administration in rats. Following its oral administration, it induced bladder papillomas and carcinomas in rabbits and dogs, and neoplasms at various sites in mice, including dose-related increases in the incidences of angiosarcomas, hepatocellular tumours and bladder carcinomas. When administered subcutaneously to rats, it induced tumours of the intestine. When administered by intraperitoneal injection to newborn mice or *CYP1A2-null* mice, it caused an increase in the incidence of liver tumours.

5.4 Other relevant data

The bioactivation of 4-aminobiphenyl (4-ABP) *in vitro*, in experimental animals and in humans is well documented. The genotoxic metabolite that binds to DNA is *N*-hydroxy-4-ABP; its oxidised derivative 4-nitrosobiphenyl binds to haemoglobin. Human CYP1A2 displays the highest catalytic activity of the P450 enzymes for *N*-oxidation of 4-ABP; however, extrahepatic CYPs, peroxidases and prostaglandin H synthase (PHS) can catalyse bioactivation within target sites. *N*-acetylation is an important detoxification pathway of 4-ABP. Some epidemiological investigations on aromatic amine exposure have reported that individuals who are slow NAT2 *N*-acetylators are at elevated risk for bladder cancer, and 4-ABP has been implicated as one of the causal agents. More recent studies have established that NAT2 is the enzyme responsible for the observed differences. Elevated levels of 4-ABP haemoglobin- or DNA-adducts have been detected in these populations and support a role for 4-ABP in urinary bladder cancer and possibly other cancers.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of 4-aminobiphenyl. 4-Aminobiphenyl causes bladder cancer in humans.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of 4-aminobiphenyl.

6.3 Overall evaluation

4-Aminobiphenyl is *carcinogenic to humans (Group 1)*.

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European Commission (2004a) Directive 2004/37/EC of the European Parliament and of the council of 29 April 2004 on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Sixth individual Directive within the meaning of Article 16(1) of Council Directive 89/391/EEC) (codified version)

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AURAMINE AND AURAMINE PRODUCTION

1. Exposure Data

1.1 Chemical and Physical Data

1.1.1 *Nomenclature*

Auramine

Chem. Abstr. Serv. Reg. No.: 492–80–8
CAS Name: 4,4'-Carbonimidoylbis[*N*,*N*-dimethylbenzenamine]
Synonyms: C.I. 41000B; C.I. Solvent Yellow 34; 4,4'-dimethylaminobenzophenonimide; 4,4'-(imidocarbonyl)bis(*N*,*N*-dimethylaniline); glauramine; Solvent Yellow 34; yellow pyoctanine

Auramine hydrochloride

Chem. Abstr. Serv. Reg. No.: 2465–27–2
CAS Name: 4,4'-Carbonimidoylbis[*N*,*N*-dimethylbenzenamine], hydrochloride (1:1)
Synonyms: Auramine chloride; 4,4'-carbonimidoylbis[*N*,*N*-dimethylbenzenamine], monohydrochloride; C.I. 41000; C.I. Basic Yellow 2; C.I. Basic Yellow 2, monohydrochloride

Michler's base

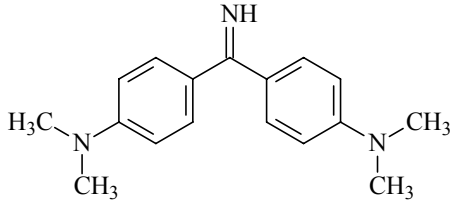
Chem. Abstr. Serv. Reg. No.: 101-6-1
CAS Name: 4,4'-Methylenebis(*N*,*N*-dimethyl)benzenamine
Synonyms: 4,4'-methylenebis(*N*,*N*-dimethyl) aniline; tetramethyldiaminodiphenylmethane; 4,4'-bis(dimethylamino) diphenylmethane; tetra base; methane base; Michler's hydride; Michler's methane
Use: 4,4'-Methylenebis(*N*,*N*-dimethyl)benzenamine (Michler's base) is an intermediate in the synthesis of several organic dyes, e.g., auramine.

Michler's ketone

Chem. Abstr. Serv. Reg. No.: 90-94-8
CAS Name: Bis[4-(dimethylamino)phenyl]methanone.
Synonyms: 4,4'-bis(dimethylamino)benzophenone; *p,p'*-bis(dimethylamino)benzophenone; bis[*p*-(*N,N'*-dimethylamino)phenyl]ketone; tetramethyldiaminobenzophenone
Use: Bis[4-(dimethylamino)phenyl]methanone (Michler's ketone) is a chemical intermediate used in the synthesis of a number of dyes and pigments, particularly auramine derivatives. It is a hydrolysis product of auramine.

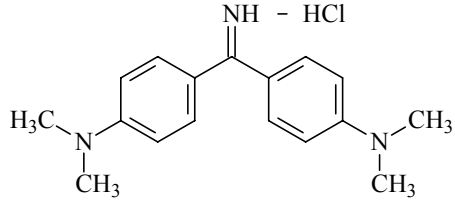
1.1.2 *Structural formula, molecular formula, and relative molecular mass*

Auramine



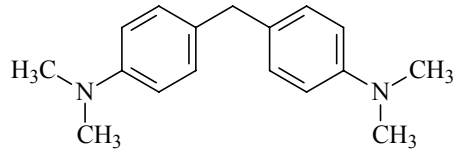
$C_{17}H_{21}N_3$ Rel. mol. mass: 267.37

Auramine hydrochloride



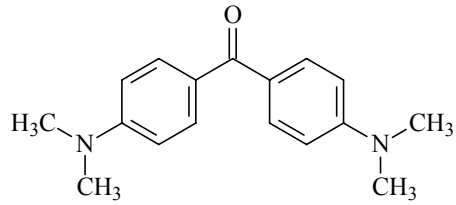
$C_{17}H_{21}N_3.HCl$ Rel. mol. mass: 303.83

Michler's base



$C_{17}H_{22}N_2$ Rel. mol. mass: 254.41

Michler's ketone



$C_{17}H_{20}N_2O$ Rel. mol. mass: 268.36

1.1.3 *Chemical and physical properties of the pure substance [hydrochloride salt]*

Description: Yellow needles from water (Lide, 2008)
Melting-point: 267 °C (Lide, 2008)
Solubility: Slightly soluble in water (Lide, 2008); 10 mg/ml in water; 20 mg/ml in ethanol; 60 mg/ml in ethylene glycol methyl ether (Green, 1990)

1.1.4 *Technical products and trade names*

Trade names for auramine include: Auramine Base; Auramine N Base; Auramine O Base; Auramine OAF; Auramine OO; Auramine SS; Baso Yellow 124; Brilliant Oil Yellow; Orient Oil Yellow 101; and Waxoline Yellow O.

Trade names for auramine hydrochloride include: ADC Auramine O; Aizen Auramine; Aizen Auramine Conc. SFA; Aizen Auramine OH; Aizen Auramine OW 100; Arazine Yellow; Auramine 0–100; Auramine A1; Auramine Extra; Auramine Extra 0–100; Auramine Extra 0–125; Auramine Extra Conc. A; Auramine FA; Auramine FWA; Auramine II; Auramine Lake Yellow O; Auramine N; Auramine O; Auramine ON; Auramine OO; Auramine OOO; Auramine OS; Auramine Pure; Auramine SP; Auramine Yellow; Basic Flavine Yellow O; Basic Light Yellow; Basic Light Yellow O; Basic Yellow 2; Basic Yellow O; Basonyl Yellow120; Calcozine Yellow OX; Flexo Yellow 110; and Mitsui Auramine O.

1.1.5 *Analysis*

Analytical studies on auramine began in the 1970s and continued during the 1980s, by use of high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) to assess auramine levels in shrimp tissue and biological dyes, respectively. Analyses conducted since 1990 use the more sensitive HPLC and mass-spectrometric methods. Table 1.1 presents selected methods of detection and quantification of auramine in various matrices.

Table 1.1. Selected methods of analysis of auramine in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Shrimp tissue	Extract with hexane; remove hexane; extract residue with ethanol; remove ethanol; heat residue with ethanol/ammonia water; shake liquid with acetic acid and with Amberlite LA-2; shake with Amberlite in ethyl acetate; wash ethyl acetate with sodium chloride solution and with 5% ammonia water	HPLC	Not given	Tonogai *et al.* (1983)
Biological dyes	Dissolve dye; elute with n-butanol-acetic acid-water (4:1:5) or n-butanol-ethanol-water (9:1:1)	TLC	Not given	Allison & Garratt (1989)
Ball-point pen inks	A 1-mm section is cut from an ink line drawn on paper; extract with methanol, and analyse	FD-MS	Not given	Sakayanagi *et al.* (1999)
Soybean	Mill auramine-soybean mixture; extract with ethanol using ultrasound; filter; wash filtercake with ethanol; combine ethanol fractions; dilute further with ethanol, and analyse	HPLC	0.25 µg/mL	Luo *et al.* (2005)

FD, field desorption; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

1.2 Production and use

1.2.1 *Production*

The diphenylmethane dyes are usually grouped with the triarylmethane dyes. The dyes of the diphenylmethane subclass are ketoimine derivatives, and include auramine O (hydrochloride salt). These dyes are still used extensively for the colouration of paper and in the preparation of pigment lakes (Thetford, 2000).

Auramine and its salts can be manufactured by first heating 4,4'-bis(dimethylaminodiphenyl)methane (Michler's base; CAS No. 101–61–1) with a mixture of urea, sulfamic acid, and sulfur in ammonia at 175°C. The auramine sulfate formed in the reaction may be used directly in the dyeing process or can be converted into auramine base or auramine hydrochloride (auramine O). Highly concentrated solutions for use in the paper industry can be prepared by dissolving auramine base in formamide containing sodium bisulfate. The nitrate and nitrite salts exhibit excellent solubility in alcohols, which facilitates their use in lacquers and flexographic printing colours (Kirsch *et al.*, 1978; Thetford, 2000).

Production of auramine took place first in Europe (Switzerland, Germany, United Kingdom, France), and later also in the USA. Production in these locations has generally been discontinued. Auramine manufacturing is currently mainly located in India and China.

In 1993, approximately 9000 tonnes of basic diphenylmethane and triphenylmethane dyes were sold. Crystal violet, methyl violet, malachite green, auramine, and Rhodamine B are suitable for many purposes and are among the economically most important dyes. Auramine O and its ethyl homologue, ethylauramine, are brilliant yellow dyes with high colour strength. Worldwide annual sales of these dyes are approximately 1000 tonnes (Gessner & Mayer, 2000).

Information was collected from 1996 to 1998 in Europe for the IUCLID database for substances with a production or import volume between 10 and 1000 tonnes/year (Low Production Volume Chemicals (LPVCs)). Auramine hydrochloride was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including those imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the aggregate production volumes that were reported for auramine hydrochloride.

Available information indicates that auramine was produced and/or supplied in research quantities in the following countries: Hong Kong Special Administrative Region, India, the People's Republic of China, and the USA (Chemical Sources International, 2008).

Available information indicates that auramine hydrochloride was produced and/or supplied in research quantities in the following countries: Canada, Germany, Hong Kong Special Administrative Region, India, Japan, the Netherlands, Switzerland, the United Kingdom, and the USA (Chemical Sources International, 2008).

Table 1.2. Auramine hydrochloride production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	NR
1994	10–500
1998	10–500
2002	NR
2006	NR

NR, not reported

1.2.2 *Use*

Auramine dyes are used for dyeing of leather, jute, tanned cotton, and paints, and as dye components in inking ribbons, ballpoint pastes, oils and waxes, and carbon paper. The most important areas of application are in dyeing paper and in flexographic printing. For the latter, a large number of salts are produced, which have a high solubility in ethanol and ethanol-water mixtures (e.g. nitrates, nitrites, bromides, iodides, salts of alkyl-, aralkyl-, or arylsulfonic acids, thiocyanates, and phosphates). For dyeing paper, solutions of the hydrochloride in organic solvents (e.g., thiodiglycol) are employed, and tartaric and citric acids have been used as stabilizers. The chlorides can also be converted into more easily soluble acetates or propionates to obtain highly concentrated solutions. The hydrogen sulfates of auramine are also readily soluble. Coloured salts based on auramine with tannic acid, phosphomolybdic acid, and preferably phosphotungsto-molybdic acid are used as yellow toner pigments for the development of latent electrostatic images (Fierz-David & Blagey, 1949; Gessner & Mayer, 2000; Thetford, 2000; Varella *et al.*, 2005).

Auramine was used as a fluorescent staining agent to stain acid-fast bacteria in sputum or infected tissue, and also in combination with the dye rhodamine in the Truant auramine-rhodamine stain for *Mycobacterium tuberculosis* (Silver *et al.*, 1966).

Auramine was historically used as a component in brilliantine, a grooming product intended to soften men's hair (Gubéran *et al.*, 1985), particularly in the 1930s, which could have resulted in occupational exposure as well as customer exposure to auramine.

Auramine has also been used in some countries as a food colourant (D'Aquino & Santini, 1977). Auramine has been detected in a small percentage of food samples from India (Tripathi *et al.*, 2007), including fresh peas (Rao & Bhat, 2003). In China, auramine has been detected in bean products (Lin, 2007).

Auramine has been used to colour smoke, in military applications (Department of the Army, 1990) and in firework displays (Shimizu, 1981).

1.3 Occurrence and exposure

1.3.1 *Natural and environmental occurrence*

Auramine is not known to occur as a natural product.

No data have been reported on levels of auramine in environmental matrices such as water and soil.

1.3.2 *Occupational exposure*

The US National Occupational exposure survey (1981–1983) estimated that about 19 000 workers were exposed to auramine. The industries with the largest numbers of exposed workers included the paper and allied products industry, and the health services industry (laboratory workers) (NIOSH, 1990).

The only well-described groups of workers exposed to auramine include British (Case & Pearson, 1954) and German auramine production workers (Kirsch *et al.*, 1978; Thiess *et al.*, 1982). Case reports of bladder cancer among Swiss auramine-production workers have also been published (Müller, 1933). Among a cohort of 4772 laboratory workers, 8% were reported to have been exposed to auramine and its salts (Kauppinen *et al.*, 2003). Exposure measurements in the workplace or biological samples of workers employed in the production of auramine are not available.

The manufacture of auramine involves potential exposure to its process chemicals (e.g., dimethylaniline, formaldehyde, sulfur, ammonium chloride, ammonia, Michler's base), as well as to other chemicals used and produced at the same location (e.g., benzidine, 1-naphthylamine, 2-naphthylamine, magenta, aniline) (Case & Pearson, 1954).

1.4 Regulations and guidelines

1.4.1 *Auramine*

(a) Europe

(i) *Directive 2004/37/EC*

The manufacture of auramine and auramine hydrochloride is regulated by Directive 2004/37/EC (European Commission, 2004a), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 or 2. This Directive specifies rules

regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(ii) *Directive 2004/93/EC*

The Commission Directive 2004/93/EC of 21 September 2004 amends the Council Directive 76/768/EEC for the purpose of adapting Annexes II and III thereto to technical progress (European Commission, 2004b). In this Directive, 4,4′-Carbonimidoylbis[*N,N*-dimethylaniline] (auramine) and its salts are listed in Annex II as substances that must not form part of the composition of cosmetic products.

(b) Germany

Deviating from the EU classification, auramine and auramine hydrochloride are classified as Category-2 carcinogens by the MAK Commission. The MAK Commission listed auramine and auramine hydrochloride as substances where percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set (MAK, 2007).

(c) Japan

The Japan Society for Occupational Health (2007) follows the classification by IARC of auramine in Group 2B.

(d) Other

(i) *GESTIS*

Table 1.3 presents some international limit values for auramine (GESTIS, 2007).

Table 1.3. International limit values (2007) for auramine

Country	Limit value – Eight hours (mg/m^3)	Limit value – Short-term (mg/m^3)	Comments
Austria	0.08 inhalable aerosol	0.32 inhalable aerosol	Technical guidance concentration (based on technical feasibility)
Switzerland	0.08		

1.4.2 *Michler's Base* [CAS No. 101–61–1]

(a) Europe

(i) *Directive 2004/37/EC*

N,N,N′,N′-Tetramethyl-4,4′-methylenedianiline [Michler's base] is regulated by Directive 2004/37/EC (European Commission, 2004a), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. This Directive specifies rules regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(ii) *Directive 2005/90/EC*

In Directive 2005/90/EC, the list of substances classified as carcinogenic, mutagenic or toxic to reproduction (c/m/r) of Directive 76/769/EEC was amended to include *N,N,N′,N′*-tetramethyl-4,4′-methylenedianiline [Michler's base] (European Commission, 2005a).

(iii) *Directive 2005/80/EC*

The Commission Directive 2005/80/EC of 21 November 2005 amends Council Directive 76/768/EEC, concerning cosmetic products, for the purposes of adapting Annexes II and III thereto to technical progress (European Commission, 2005b). In this Directive, *N,N,N′,N′*-tetramethyl-4,4′-methylenedianiline [Michler's base] is listed in Annex II as a substance that must not form part of the composition of cosmetic products.

(b) Germany

4,4'-Methylenebis(*N,N*-dimethylaniline) [Michler's base] is classified as a Category-2 carcinogen by the MAK Commission. The MAK Commission listed 4,4'-methylenebis(*N,N*-dimethylaniline) as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set (MAK, 2007).

(c) Japan

The Japan Society for Occupational Health (2007) follows the classification by IARC of 4,4'-methylenebis(2-methylaniline) [Michler's base] in Group 2B.

(d) USA

4,4′-Methylenebis(*N,N*-dimethylbenzenamine) [Michler's base] is listed in the NTP *Report on Carcinogens* as *reasonably anticipated to be a human carcinogen* (NTP, 2005).

(e) Other

(i) GESTIS

Table 1.4 presents some international limit values for Michler's base (GESTIS, 2007).

Table 1.4. International limit values (2007) for Michler's base

Country	Limit value – Eight hours (mg/m^3)	Limit value – Short-term (mg/m^3)	Comments
Austria	0.1 inhalable aerosol	0.4 inhalable aerosol	Technical guidance concentration (based on technical feasibility)
Switzerland	0.1 inhalable aerosol		

2. Studies of Cancer in Humans

2.1 Case report

Müller (1933) described two cases of bladder cancer in men occupied in auramine manufacture.

2.2 Cohort studies

Case & Pearson (1954) showed a relatively high incidence of bladder tumours in 238 workers engaged in the manufacture of auramine, with a latent period ranging between 9 and 28 years. Care had been taken to eliminate workers who were recorded as also having been in contact with benzidine, 1-naphthylamine, or 2-naphthylamine. Overall, there were six death certificates mentioning bladder tumours, where only 0.45 would have been expected from the overall male population of England and Wales (SMR 13.3, 95% CI: 4.9–29.0).

A mortality study was conducted among 191 workers who had been employed for > 1 month in auramine production at the Badische Anilin- und Sodafabrik (BASF) in Ludwigshafen (Federal Republic of Germany, FRG) during the period 1932–1976. Subjects were followed-up until death or until 15–08–1976, whichever came first. A total of 20 workers were lost to follow-up. The mortality in the group was compared with those in the populations of Ludwigshafen (1970–1973), in the district of Rheinhessen-Pfalz (1970–1975), and in the FRG (1971–1974). A comparison with another group of workers in the plant could not be made. Among a total of 45 deaths, ten were the result of malignant tumours (proportional mortality ratio 1.35, compared with FRG). The tumours occurred in the bladder ($n = 2$), lung ($n = 2$), prostate ($n = 3$), and stomach ($n = 3$). All ten

cases had been employed before 1950, and six of them before 1940. Co-exposure to 1- and 2-naphthylamine could not be excluded (Kirsch *et al.*, 1978).

A cohort of 703 male and 677 female hairdressers born in or after 1880 who started to run salons in Geneva between 1900 and 1964 was followed-up to the end of 1982. Cause-specific mortality was analysed for the period 1942–1982 using sex-, age- and year-specific death rates for Switzerland as the reference; a significant excess mortality from bladder cancer (observed deaths, 10; expected, 3.9) was found among males. Cancer incidence recorded for the years 1970–1980 showed a significant increase among males for all neoplasms (obs, 65; exp, 51.4), for cancer of the buccal cavity and pharynx (obs, 6; exp, 2.5), for cancer of the prostate (obs, 12; exp, 6.1), and for bladder cancer (obs, 11; exp, 5.3). Among female hairdressers, bladder cancer was observed in two cases, where 1.5 would be expected. It was suggested that the excess in male hairdressers might be related to some colouring agent(s) in brilliantines, which were widely used in men's hairdressing salons in Geneva until about 1950. Auramine was one of the commonly used dyes in brilliantines during the 1930s. However, the impurities of dyes raise the concern that the extra deaths from bladder cancer could have been caused by other agents, e.g., 2-naphthylamine, a known human bladder carcinogen (Gubéran *et al.*, 1985).

3. Studies of Cancer in Experimental Animals

3.1 Auramine

3.1.1 *Oral administration*

(a) Mouse

Thirty mice (15 males, 15 females; strain and age unspecified) were given a diet containing 0.1% of commercial auramine (BDH; purity unspecified) in arachis oil for 52 weeks (estimated total intake, 728 mg per animal), and kept for their life-span. Nineteen mice died before termination of the experiment (90 weeks). In the treated group, seven mice (23%) developed hepatomas and 11 (37%) developed lymphomas, compared with none and five (8%), respectively, in 60 control animals treated with arachis oil only. One subcutaneous sarcoma was also reported in the treated group (Bonser *et al.*, 1956).

A group of thirty stock mice (15 males, 15 females) was given 0.1% auramine, and a group of 27 CBA mice (12 males, 15 females) was given 0.2% auramine (BDH; purity not specified) dissolved in acetone in the diet for 52 weeks (approximate total estimated intake, 1820 mg and 3640 mg per mouse, respectively). In stock mice, four of the seven (57%) males and three of the 10 (30%) females that survived to tumour-bearing age showed hepatomas. No hepatomas were seen in 16 stock control mice. In CBA mice, seven of 12 males (58%) and 11 of 15 females (73%) that survived to tumour-bearing age showed hepatomas. Seven of 90 CBA control mice developed hepatomas (Walpole, 1963).

(b) Rat

Twelve male Wilmslow-Wistar rats were given a diet containing 0.1% of commercial auramine (ICI Ltd; purity not specified) for 87 weeks (estimated total intake, 10 g per rat), followed by normal diet until death. Eleven animals (92%) developed hepatomas between the 91st and the 122nd week after the start of treatment. Twelve control rats were tumour-free at death between 90 and 120 weeks (Williams & Bonser, 1962; Walpole, 1963).

Female Sprague-Dawley rats (age 50–55 days) received a single dose of 150 mg auramine O (source not clear) in sesame oil by oral gavage and were autopsied after six months. No tumours were seen in 19 rats observed. Another group received an oral dose of 80 mg per animal every three days between 40 and 70 days of age (total dose, 800 mg/rat), followed by autopsy after nine months. No tumours were seen in 15 animals observed (Griswold *et al.*, 1966, 1968).

Groups of 40 Sprague-Dawley rats (20 females, 20 males) received technical grade auramine (BASF; purity, 87%) at 0, 50, 100 and 200 ppm in the diet for 24 months. In these four dose groups, tumours (benign and malignant combined) were seen in 6, 13, 8 and 10 male rats, respectively, and in 19, 18, 15 and 19 female rats. The corresponding total numbers of tumours were 12 (one malignant), 15 (none malignant), nine (none malignant), and 10 (two malignant) in male rats, respectively, and 34 (four malignant), 41 (6), 36 (4), and 53 (6) in females, respectively. The tumour induction was not statistically significant (Kirsch *et al.*, 1978).

(c) Other animal species

(i) *Rabbit*

In a preliminary comparative experiment, nine rabbits (strain unspecified) were given auramine (source and purity not stated) orally (dose and dose regimen not given) to the limit of tolerance, and the treatment was continued until the onset of the final illness. Six animals were sacrificed in the first two years, and three between three and four years after the start of treatment. Metaplasia of the urinary tract epithelium, suggestive of pre-cancerous change, was seen in two of five (40%) rabbits examined. No tumours were seen in control and treated animals (Bonser, 1962). [The Working group noted the lack of experimental details.]

(ii) *Dog*

No abnormalities were detected in dogs (strain not specified) given auramine (source and purity not stated) orally (dose not given) daily for about seven years (total ingested amount, 66 g per animal) (Walpole, 1963). [The Working Group noted the lack of description of experimental design and lack of use of controls.]

3.1.2 *Subcutaneous administration*

(a) Rat

Twenty-four male Wilmslow Wistar rats, 8–10 weeks of age, were given subcutaneous injections (0.1 mL per 100 g bw) of a 2.5% suspension of commercial auramine (purity not specified) in arachis oil on five days per week for 21 weeks (estimated total dose, 110–120 mg per animal). In 20 surviving animals, 11 fibrosarcomas (tumour yield: 55%) and three hepatomas (tumour yield: 15%) were observed. Three intestinal carcinomas were also reported (Williams & Bonser, 1962). [The Working Group noted that no control data were given.]

3.2 Michler's base

3.2.1 *Oral administration*

(a) Mouse

A bioassay for the possible carcinogenicity of technical-grade Michler's base was conducted with B6C3F1 mice. Michler's base was administered in the feed, at either of two concentrations, to groups of 50 male and 50 female animals. The high and low dietary concentrations of Michler's base were, respectively, 2500 and 1250 ppm for the two sexes. Twenty animals of each sex were placed on test as controls. The compound was administered for 78 weeks, followed by an observation period of 13 weeks. There were no significantly positive associations between the concentrations of Michler's base administered and mortality among mice of either sex. Adequate numbers of animals survived sufficiently long to be at risk from late-developing tumours. The mean body weights of dosed mice were significantly lower than those of the controls. There were elevated incidences of hepatocellular adenomas in dosed mice when compared with controls (i.e., 2/20 (10%), 3/50 (6%), and 16/48 (33%) in control, low-dose, and high-dose males, respectively; and 1/19 (5%), 18/49 (37%), and 22/48 (46%) in control, low-dose, and high-dose females, respectively). The incidences of hepatocellular carcinomas in dosed mice did not differ greatly from those in controls. Among both sexes of mice, there was a significant positive association between the concentrations of the chemical administered and the incidences of a combination of hepatocellular adenomas and hepatocellular carcinomas. For male mice, the Fisher exact-test comparisons were not significant; however, for females, both the comparisons of high-dose with control and low-dose with control were significant. Under the conditions of this bioassay, Michler's base was carcinogenic in female B6C3F1 mice, inducing liver neoplasms (National Cancer Institute, 1979a).

(b) Rat

A bioassay for the possible carcinogenicity of technical-grade Michler's base was conducted with Fisher 344 rats. Michler's base was administered in the feed, at either of

two concentrations, to groups of 50 male and 50 female animals. The high and low dietary concentrations of Michler's base were, respectively, 750 and 375 ppm for the two sexes. Twenty animals of each sex were placed on test as controls. The compound was administered for 59 weeks, followed by an observation period of 45 weeks. There were no significantly positive associations between the concentrations of Michler's base administered and mortality among rats of either sex. Adequate numbers of animals survived sufficiently long to be at risk from late-developing tumours. There was slight dose-related mean body-weight depression among female rats, the mean body weight of high-dose male rats was slightly less than that for controls. For both male and female rats, there was a significant positive association between the concentrations of Michler's base administered and the incidences of follicular-cell carcinomas of the thyroid (i.e., 1/18 (6%), 4/50 (8%), and 21/46 (46%) in the control, low-dose, and high-dose males, respectively; and 0/20, 3/46 (7%), and 23/45 (51%) in the control, low-dose, and high-dose females, respectively). The high-dose to control Fisher exact-test comparisons were also significant for each sex. Under the conditions of this bioassay, Michler's base was carcinogenic in Fisher 344 rats, inducing thyroid follicular-cell carcinomas in both males and females (National Cancer Institute, 1979a).

3.3 Michler's ketone

3.3.1 *Oral administration*

(a) Mouse

A bioassay for the possible carcinogenicity of technical-grade Michler's ketone was conducted with B6C3F1 mice. Michler's ketone was administered in the feed, at either of two concentrations, to groups of 50 male and 50 female animals. The high and low dietary concentrations of Michler's ketone were 2500 and 1250 ppm, respectively, for mice of both sexes. Twenty animals of each sex were placed on test as controls. The compound was administered for 78 weeks. The period of compound administration was followed by an observation period of 13 weeks. There were significant positive associations between the concentrations of Michler's ketone administered and mortality in mice of both sexes. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumours. There was distinct dose-related mean body-weight depression in mice of both sexes. There were significant positive associations between the concentrations of Michler's ketone administered and the incidences of hepatocellular carcinomas in female mice and haemangiosarcomas in male mice. Incidences in hepatocellular carcinomas in females were: 0/19 (P for trend < 0.001), 16/49 (33%), and 38/50 (56%), for increasing doses, respectively. Incidences in haemangiosarcomas in males were: 0/19 (P for trend < 0.001), 5/50 (10%), and 20/50 (40%), for increasing doses, respectively. In all of these cases the high-dose to control Fisher exact-test comparison of incidences was also significant ($P < 0.001$) (National Cancer Institute, 1979b).

(b) Rat

A bioassay for the possible carcinogenicity of technical-grade Michler's ketone was conducted with Fischer 344 rats. Michler's ketone was administered in the feed, at either of two concentrations, to groups of 50 male and 50 female animals. The high and low dietary concentrations of Michler's ketone were, respectively, 500 and 250 ppm for male rats, and 1000 and 500 ppm for female rats. Twenty animals of each sex were placed on test as controls. The compound was administered for 78 weeks. The period of compound administration was followed by an observation period of 28 weeks for male and high-dose female rats, and 29 weeks for low-dose female rats. There were significant positive associations between the concentrations of Michler's ketone administered and mortality in rats of both sexes. Adequate numbers of animals in all groups survived sufficiently long to be at risk of late-developing tumours. There was distinct dose-related mean body-weight depression in female rats, and the mean body weight among dosed male rats was slightly lower than that in controls. There were significant positive associations between the concentrations of Michler's ketone administered and the incidences of hepatocellular carcinomas in both sexes of rats. Incidences in males were: 0/20 (p for trend < 0.001), 9/50 (18%), and 40/50 (80%) for increasing doses, respectively. Incidences in females were: 0/20 (p for trend < 0.001), 41/47 (87%), and 44/49 (90%), for increasing doses, respectively. In all of these cases the high-dose to control Fisher exact-test comparison of incidences was also significant ($P < 0.001$) (National Cancer Institute, 1979b).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

No data were available to the Working Group.

4.2 Genetic and related effects (see Table 4.1 for details)

[Some general comments on the data in Table 4.1: a) results were obtained with commercial ("technical grade") auramine, the grade of purity presumably varying from 65% to 85%; b) purified auramine was tested only in the study by Parodi *et al.* (1982), together with the technical-grade product.]

Commercial preparations of auramine gave a positive outcome in the prophage-induction test in the presence of metabolic activation (Ho & Ho, 1981). They were mutagenic to *Salmonella typhimurium* strains TA98, TA1535, TA1538, and YG10, but only in the presence of metabolic activation. The effect was observed in four of nine studies with TA98 (Parodi *et al.*, 1981; Nagao & Takahashi, 1981; Zeiger *et al.*, 1992; Varella *et al.*, 2005), in one of four studies with TA1535 (Richold & Jones, 1981), in four of six studies with TA1538 (Rowland & Severn, 1981; Richold & Jones, 1981; Simmon

Table 4.1. Genetic and related effects of auramine

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Prophage, induct/sos/strand breaks/x-links	NT	+	300 µg/ml	Ho & Ho (1981)
S. typhimurium, forward mutation	NT	+	100 µg/plate	Skopek *et al.* (1981)
S. typhimurium TA100, TA98, TA97, reverse mutation	–	–	100 µg/plate	Brams *et al.* (1987)
S. typhimurium TA100, reverse mutation	NT	–	2000 µg/plate	Parodi *et al.* (1981)
S. typhimurium TA98, reverse mutation	NT	(+)	2000 µg/plate	Parodi *et al.* (1981)
S. typhimurium TA100, TA1535, TA1537, TA98, reverse mutation	–	–	NR	Kier *et al.* (1986)
S. typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	125 µg/plate	Simmon (1979a)
S. typhimurium TA100, TA98, reverse mutation	–	NT	NR	Ichinotsubo *et al.* (1981)
S. typhimurium TA100, TA1537, TA98, reverse mutation	–	–	500 µg/plate	Richold & Jones (1981)
S. typhimurium TA100, TA98, reverse mutation	–	–	NR	Venitt & Crofton-Sleigh (1981)
S. typhimurium TA100, reverse mutation	–	–	100/1000 µg/plate[b]	Zeiger *et al.* (1992)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
S.typhimurium TA100, reverse mutation	–	–	1200 µg/plate	Varella *et al.* (2004)
S.typhimurium TA100, reverse mutation	–	–	500 µg/plate	Hakura *et al.* (2005)
S.typhimurium TA1535,TA1538, reverse mutation	–	NT	125 µg/plate	Rosenkranz *et al.* (1976)
S. typhimurium TA1535, TA1538, reverse mutation	–	–	125 µg/plate	Rosenkranz & Poirier (1979)
S. typhimurium TA1535, reverse mutation	–	+	250 µg/plate	Richold & Jones (1981)
S. typhimurium TA1538, reverse mutation	?	+	50 µg/plate	Rowland & Severn (1981)
S. typhimurium TA1538, reverse mutation	–	+	50 µg/plate	Richold & Jones (1981)
S. typhimurium TA1538, reverse mutation	–	+	250 µg/plate	Simmon & Shepherd (1981)
S. typhimurium TA1538, reverse mutation	NT	+	33–667 µg/plate	Zeiger *et al.* (1992)
S. typhimurium TA98, reverse mutation	–	+	250 µg/plate	Nagao & Takahashi (1981)
S. typhimurium TA98, reverse mutation	–	+	100/33 µg/plate[b]	Zeiger *et al.* (1992)
S. typhimurium TA98, reverse mutation	–	+	300 µg/plate	Varella *et al.* (2004)
S. typhimurium YG10, reverse mutation	–	+	150 µg/plate	Varella *et al.* (2004)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
S. typhimurium (other), reverse mutation	–	–	50 µg/plate	Gatehouse (1981)
E. coli (other), reverse mutation	–	–	40 µg/plate	Gatehouse (1981)
Bacillus subtilis, multigene test	?	?	NR	Macgregor & Sacks (1976)
E. coli WP2 *uvrA*, reverse mutation	–	–	NR	Venitt & Crofton-Sleigh (1981)
Saccharomyces, differential tox	+	+	100 µg/ml	Sharp & Parry (1981b)
S. cerevisiae, intrachromosomal recombination	–	NT	1200 µg/ml	Schiestl *et al.* (1989)
S. cerevisiae, deletion assay	+	NT	1200 µg/ml	Schiestl *et al.* (1989)
S. cerevisiae, gene conversion	+	NT	75 µg/ml	Sharp & Parry (1981a)
S. cerevisiae, gene conversion	+	NT	78 µg/ml	Zimmermann & Scheel (1981)
S. cerevisiae, homozygosis	+	+	500 µg/ml	Simmon (1979b)
S. cerevisiae, reverse mutation	–	?	889 µg/ml	Mehta & von Borstel (1981)
D. melanogaster, intrachromosomal recombination	–		134 µg/ml	Consuegra *et al.* (1996)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Wheat, aneuploidy	+	NT	NR	Rédei & Sandhu (1988)
S. cerevisiae, aneuploidy	+	NT	200 µg/ml	Parry & Sharp (1981)
DNA strand breaks/x-links, rat primary hepatocytes	+	NT	0.8 µg/ml	Sina *et al.* (1983)
DNA strand breaks/x-links, rat primary hepatocytes	+	NT	2.67 µg/ml	Martelli *et al.* (1998)
Gene mutation, Chinese hamster lung V79 cells, *Hprt* locus	–	+	401/1069 µg/ml	Fassina *et al.* (1990)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus	NT	(–)	45 µg/ml	Amacher *et al.* (1980)
SCE, Chinese hamster cells *in vitro*	–	+	100 µg/ml	Perry & Thomson (1981)
Micronucleus test, rat hepatocytes *in vitro*	?	NT	8.54 µg/ml	Martelli *et al.* (1998)
Micronucleus test, Syrian hamster embryo cells *in vitro*	+	NT	NR	Fritzenschaf *et al.* (1993)
Cell tranformation, SHE, clonal assay	NT	+	2 µg/ml	Pienta & Kawalek (1981)
Cell tranformation, SHE, clonal assay	–	NT	1 µg/ml	Pienta *et al.* (1977)
DNA strand breaks/x-links, human cell line HuF22 *in vitro*	+	NT	80 µg/ml	Parodi *et al.* (1982)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks/x-links, human primary hepatocytes	+	NT	4.81 µg/ml	Martelli *et al.* (1998)
Micronucleus test, human hepatocytes *in vitro*	?	NT	8.54 µg/ml	Martelli *et al.* (1998)
Host-mediated assay, microbial cells	–		660 mg/kg	Simmon *et al.* (1979)
DNA strand breaks/x-links, rat liver cells, *in vivo*	+		29.4 mg/kg	Parodi *et al.* (1981)
DNA strand breaks/x-links, rat liver cells *in vivo*	+		9 mg/kg	Brambilla *et al.* (1985)
DNA strand breaks/x-links, rat liver cells *in vivo*	+		125 mg/kg	Martelli *et al.* (1998)
DNA strand breaks/x-links, rat urinary bladder cells *in vivo*	+		125 mg/kg	Martelli *et al.* (1998)
DNA strand breaks/x-links, mouse liver cells *in vivo*	+		80 mg/kg	Sasaki *et al.* (1997)
DNA strand breaks/x-links, mouse kidney cells *in vivo*	(+)		80 mg/kg	Sasaki *et al.* (1997)
DNA strand breaks/x-links, mouse lung cells *in vivo*	(+)		80 mg/kg	Sasaki *et al.* (1997)
DNA strand breaks/x-links, mouse spleen cells *in vivo*	–		80 mg/kg	Sasaki *et al.* (1997)
DNA strand breaks/x-links, mouse bone-marrow cells *in vivo*	–		80 mg/kg	Sasaki *et al.* (1997)
DNA strand breaks/x-links, mouse bone-marrow cells *in vivo*	+		30 mg/kg	Parodi *et al.* (1982)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks/x-links, rat liver cells *in vivo*	+		7.5 mg/kg	Parodi *et al.* (1982)
DNA strand breaks/X-links, rat liver cells *in vivo*	–		30 mg/kg	Parodi *et al.* (1982)[c]
DNA strand breaks/x-links, rat liver cells *in vivo*	+		300 mg/kg	Kitchin and Brown (1994)
DNA strand breaks/x-links, rat kidney cells *in vivo*	+		15 mg/kg	Parodi *et al.* (1982)
SCE, mouse bone-marrow cells *in vivo*	–		15 mg/kg	Parodi *et al.* (1983)
SCE, mouse bone-marrow cells *in vivo*	–		15 mg/kg	Parodi *et al.* (1982)[c]
SCE, mouse bone-marrow cells *in vivo*	–		15 mg/kg	Parodi *et al.* (1982)
Micronucleus test, mice *in vivo*	–		82 mg/kg	Salamone *et al.* (1981)

In-vitro tests, µg/ml; in-vivo tests, mg/kg bw
[a] +, positive; (+) weakly positive; –, negative; ?, inconclusive; NT, not tested
[b] In the absence/in the presence of an exogenous metabolic system
[c] These data were obtained with purified auramine
HID, highest ineffective dose; LED, lowest effective dose; NR, not reported

& Shepherd, 1981; Zeiger *et al.*, 1992), and in one study with YG10 (Varella *et al.*, 2004). Commercial auramine gave a negative outcome in both the absence and the presence of metabolic activation in eight studies, and in one study in the absence of metabolic activation (Ichinotsubo *et al.*, 1981), with TA100 (Brams *et al.*, 1987; Kier *et al.*, 1986; Simmon, 1979a; Richold & Jones, 1981; Venitt & Crofton-Sleigh, 1981; Zeiger *et al.*, 1992; Varella *et al.*, 2004; Hakura *et al.*, 2005), in three studies with TA1537 (Simmon, 1979a; Kier *et al.*, 1986; Richold & Jones, 1981). It also gave a negative outcome in both the absence and the presence of metabolic activation (Rosenkranz & Poirier, 1979) and in the absence of metabolic activation (Rosenkranz *et al.*, 1976) with TA1535 and TA1538. Auramine induced forward mutation in *S. typhimurium* in the presence of metabolic activation (Skopek *et al.*, 1981). In both the absence and the presence of metabolic activation it was non-mutagenic in two studies with *E. coli* (Venitt & Crofton-Sleigh, 1981; Gatehouse, 1981), and gave equivocal results in the *Bacillus subtilis* multigene test (Macgregor & Sacks, 1976).

In studies with *Saccharomyces cereviasiae*, auramine (technical grade) induced deletions (Schiestl *et al.*, 1989), gene conversion (Sharp & Parry, 1981a; Zimmermann & Scheel, 1981), homozygosis (Simmon, 1979b) and aneuploidy (Parry & Sharp, 1981) in the absence of metabolic activation, but it did not produce intrachromosomal recombination (Schiestl *et al.*, 1989) and reverse mutation (Mehta & von Borstel, 1981). Auramine (technical grade) gave a positive result in the differential toxicity assay, both in the absence and the presence of metabolic activation (Sharp & Parry, 1981b). It induced aneuploidy in wheat (Rédei & Sandhu, 1988). Auramine did not induce intra-chromosomal mitotic recombination in *D. melanogaster* (Consuegra *et al.*, 1996).

In cultured, non-human mammalian cells, both positive and negative results were obtained. Commercial auramine induced DNA strand breaks in primary cultures of rat hepatocytes (Sina *et al.*, 1983; Martelli *et al.*, 1998). In the presence of metabolic activation, mutations were induced at the *Hprt* (Fassina *et al.*, 1990) but not at the *Tk* (Amacher *et al.*, 1980) locus, sister chromatid exchange was induced in Chinese hamster cells (Perry & Thomson, 1981), and morphological transformation in Syrian hamster embryo cells (Pienta & Kawalek, 1981). Cell transformation was also observed in the absence of metabolic activation (Pienta *et al.*, 1977). Micronucleus formation was observed in Syrian hamster embryo cells in the absence of metabolic activation (Fritzenschaf *et al.*, 1993), but not in primary rat hepatocytes (Martelli *et al.*, 1998). Auramine induced DNA strand-breaks in the human cell line HuF22 in the absence of metabolic activation (Parodi *et al.*, 1982), and it caused DNA fragmentation but not micronucleus formation in primary human hepatocytes (Martelli *et al.*, 1998).

In vivo, auramine gave negative results in a host-mediated assay with microbial cells (Simmon *et al.*, 1979). It induced DNA fragmentation in the liver (Parodi *et al.*, 1981; Brambilla *et al.*, 1985; Martelli *et al.*, 1998; Parodi *et al.*, 1982; Kitchin & Brown, 1994), in the kidney (Parodi *et al.*, 1982) and in the urinary bladder of rats (Martelli *et al.*, 1998). In mice, DNA fragmentation was induced in the liver, kidney and lung, but not in the spleen (Sasaki *et al.*, 1997); in the bone marrow, contrasting results were obtained in two

studies (Sasaki *et al.*, 1997; Parodi *et al.*, 1982). In mice, auramine did not induce sister chromatid exchange in bone-marrow cells (Parodi *et al.*, 1982, 1983) and it gave a negative outcome in the micronucleus test (Salamone *et al.*, 1981).

Taken as a whole, the results listed in Table 4.1, obtained with commercial auramine, show that this compound is potentially mutagenic and genotoxic, but only in the presence of an appropriate system of metabolic activation. Importantly, purified auramine was inactive in tests for induction of DNA fragmentation in rat liver and induction of sister chromatid exchange in bone-marrow cells of mice.

5. Summary of Data Reported

5.1 Exposure data

Auramine is manufactured in two steps by reaction of *N*,*N*-dimethylaniline with formaldehyde to form Michler's base, followed by reaction of this intermediate with ammonium chloride and sulfur in the presence of ammonia. This process can lead to auramine that contains Michler's ketone, probably from the hydrolysis of the target dye. Auramine is used as a colourant for paper and inks, to a lesser degree for textiles and leather, in laboratories as a biological stain, and to colour smoke. Auramine is not known to occur in nature. Occupational exposure to auramine can occur during its production or during its use in paper and allied products industries. Auramine has been detected in food samples from India, including fresh peas, and in bean products in China. The production of auramine is prohibited in Europe and the USA; its manufacture continues mainly in India and China.

5.2 Human carcinogenicity data

A single landmark study in the United Kingdom has shown a marked excess of bladder cancer in workers engaged in the manufacture of auramine. Workers exposed to benzidine and β-naphthylamine were excluded. Studies are not available to evaluate the role of pure auramine in carcinogenicity.

5.3 Animal carcinogenicity data

Auramine (technical-grade) was tested for carcinogenicity by oral administration in mice, rats, rabbits and dogs, and by subcutaneous injection in rats. Following its oral administration, it induced hepatomas and lymphomas in mice and hepatomas in rats. The studies in rabbits and dogs were inadequate for evaluation. After subcutaneous injection in one study in rats, it induced local sarcomas. In a well-designed study, dietary administration of Michler's ketone caused an increased incidence of hepatocellular

carcinomas in male and female rats and female mice, and of hemangiosarcomas in male mice. In a well-designed study, dietary administration of Michler's base caused increased incidence of hepatocellular carcinoma and adenomas in mice and follicular-cell carcinomas of the thyroid in rats.

5.4 Other relevant data

There are no data on the toxicokinetics of auramine.

In genotoxicity tests, purified auramine was used in one study only, along with the technical-grade product. The purified sample [composition not analysed] was inactive in inducing in-vivo DNA fragmentation in rat liver and sister chromatid exchange in mouse bone-marrow cells. All other data on mutagenicity and genotoxicity of auramine were obtained with commercial preparations of the compound with varying degrees of purity, which presumably was never higher than 85%. This may explain the variability of the results reported.

Taken as a whole, the results show that commercial auramine is potentially mutagenic and genotoxic both in bacteria and cultured cells in the presence of metabolic activation. In one study, purified auramine was inactive in a test to assess DNA fragmentation.

In in-vivo experiments in rats and mice, the technical-grade auramine induced liver-DNA fragmentation in four of five experiments; it was also positive in this assay in urinary bladder cells of the rat, weakly positive in kidney and lung cells of the mouse, but negative in mouse spleen and bone-marrow cells.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of auramine production. Auramine production causes bladder cancer in humans.

There is *inadequate evidence* in humans for the carcinogenicity of auramine.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of auramine, technical grade.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Michler's ketone.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Michler's base.

6.3 Overall evaluation

Auramine production is *carcinogenic to humans (Group 1)*.
Auramine is *possibly carcinogenic to humans (Group 2B)*.
Michler's ketone is *possibly carcinogenic to humans (Group 2B)*.

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BENZIDINE

1. Exposure Data

This section includes data on benzidine-based dyes, benzidine congeners and benzidine-congener-based dyes.

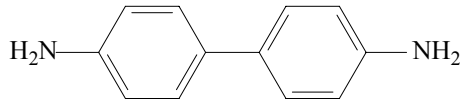
1.1 Benzidine and benzidine-based dyes – Chemical and physical data

1.1.1 *Benzidine*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 92–87–5
CAS Name: [1,1'-Biphenyl]-4,4'-diamine
Synonyms: 4'-Amino-[1,1'-biphenyl]-4-ylamine; 4-(4-aminophenyl)-aniline; benzidine; 4,4'-bianiline; *p,p′*-bianiline; 4,4'-biphenyldiamine; 4,4'-diamino-1,1'-biphenyl; C.I. 37225; C.I. Azoic Diazo Component 112; 4,4'-diaminobiphenyl; *p,p′*-diaminobiphenyl; 4,4'-diaminodiphenyl; *p*-diaminodiphenyl; 4,4'-diphenylenediamine

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{12}N_2$ Rel. mol. mass: 184.24

(c) Chemical and physical properties of the pure substance

Description: White or slightly-reddish, crystalline powder (O'Neil, 2006)
Boiling-point: 401 °C (Lide, 2008)
Melting-point: 120 °C (Lide, 2008)
Solubility: Slightly soluble in water, diethyl ether, and dimethyl sulfoxide; soluble in ethanol (Lide, 2008)

(d) Trade name

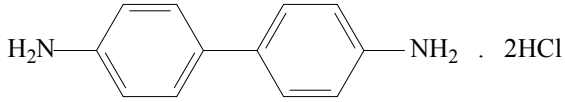
Trade name: Fast Corinth Base B.

1.1.2 *Benzidine dihydrochloride*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 531–85–1
CAS Name: [1,1'-Biphenyl]-4,4'-diamine, hydrochloride (1:2)
Synonym: Benzidine hydrochloride; [1,1'-biphenyl]-4,4'-diamine, dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{12}N_2.2HCl$ Rel. mol. mass: 257.16

(c) Chemical and physical properties of the pure substance

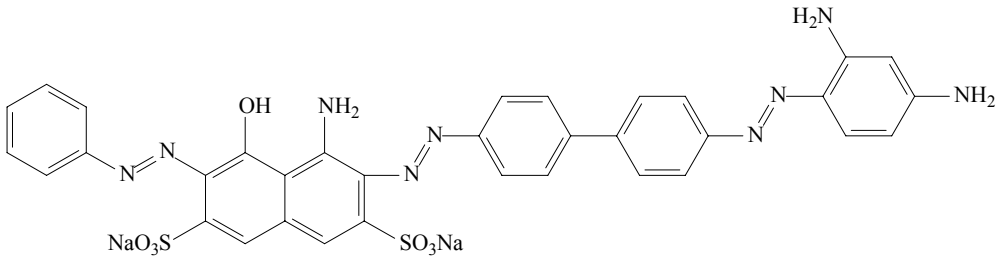
Description: Crystals (O'Neil, 2006)
Solubility: Soluble in water and ethanol (O'Neil, 2006)

1.1.3 *C.I. Direct Black 38*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 1937–37–7
CAS Name: 4-Amino-3-[2-[4'-[2-(2,4-diaminophenyl)diazenyl][1,1'-biphenyl]-4-yl]diazenyl]-5-hydroxy-6-(2-phenyldiazenyl)-2,7-naphthalenedisulfonic acid, sodium salt (1:2)
Synonyms: 4-Amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-2,7-naphthalenedisulfonic acid, disodium salt; C.I. 30235; C.I. Direct Black 38; C.I. Direct Black 38, disodium salt; Direct Black 38; disodium 4-amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-2,7-naphthalenedisulfonate; disodium 4-amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)naphthalene-2,7-disulfonate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{34}H_{25}N_9O_7S_2.2Na$ Rel. mol. mass: 781.73

(c) Chemical and physical properties of the pure substance

Description: Grey-black powder (IARC, 1982)
Solubility: Soluble in water; moderately soluble in ethanol and ethylene glycol monoethyl ether; insoluble in other organic solvents (IARC, 1982)

(d) Trade names

Trade names: ATul Direct Black E; Ahco Direct Black GX; Airedale Black ED; Aizen Direct Deep Black EH; Aizen Direct Deep Black GH; Aizen Direct Deep Black RH; Amanil Black GL; Amanil Black WD; Apomine Black GK; Apomine Black GX; Atlantic Black BD; Atlantic Black C; Atlantic Black E; Atlantic Black EA; Atlantic Black GAC; Atlantic Black GG; Atlantic Black GXCW; Atlantic Black GXOO; Atlantic Black SD; Azine Deep Black EW; Azocard Black EW; Azomine Black EWO; Belamine Black GX; Bencidal Black E; Benzamil Black E; Benzanil Black E; Benzo Deep Black E; Benzo Leather Black E; Benzoform Black BCN-CF; Black 2EMBL; Black 4EMBL; Brasilamina Black GN; Brilliant Chrome Leather Black H; Calcomine Black; Calcomine Black EXL; Carbide Black E; Chloramine Black C; Chloramine Black EC; Chloramine Black ERT; Chloramine Black EX; Chloramine Black EXR; Chloramine Black XO; Chloramine Carbon Black S; Chloramine Carbon Black SJ; Chloramine Carbon Black SN; Chlorazol Black E; Chlorazol Black EA; Chlorazol Black EN; Chlorazol Burl Black E; Chlorazol Leather Black ENP; Chlorazol Silk Black G; Chlorazol black; Chrome leather Black E; Chrome leather Black EC; Chrome leather Black EM; Chrome leather Black G; Chrome leather Brilliant Black ER; Coir Deep Black C; Columbia Black EP; Columbus Black EP; Coranil Direct Black F; Diacotton Deep Black; Diacotton Deep Black RX; Diamine Deep Black EC; Diamine Direct Black E; Diaphtamine Black V; Diazine Black E; Diazine Direct Black E; Diazine Direct Black G; Diazol Black 2V; Diphenyl deep Black G; Direct Black A; Direct Black BRN; Direct Black CX; Direct Black CXR; Direct Black E; Direct Black EW; Direct Black EX; Direct Black FR; Direct Black GAC; Direct Black GW; Direct Black GX; Direct Black GXR; Direct Black JET;

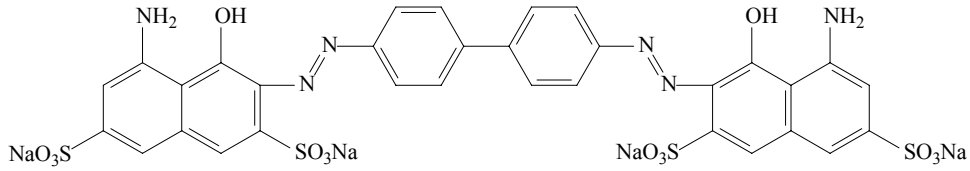
Direct Black Meta; Direct Black Methyl; Direct Black N; Direct Black RX; Direct Black SD; Direct Black WS; Direct Black Z; Direct Deep Black E; Direct Deep Black E Extra; Direct Deep Black EA-CF; Direct Deep Black EAC; Direct Deep Black EW; Direct Deep Black EX; Enianil Black CN; Erie Black B; Erie Black BF; Erie Black GAC; Erie Black GXOO; Erie Black JET; Erie Black NUG; Erie Black RXOO; Erie Brilliant Black S; Erie Fibre Black VP; Fenamin Black E; Fibre Black VF; Fixanol Black E; Formaline Black C; Formic Black C; Formic Black CW; Formic Black EA; Formic Black MTG; Formic Black TG; Hispamin Black EF; Interchem Direct Black Z; Kayaku Direct Deep Black EX; Kayaku Direct Deep Black GX; Kayaku Direct Deep Black S; Kayaku Direct Leather Black EX; Kayaku Direct Special Black AAX; Lurazol Black BA; META Black; Mitsui Direct Black EX; Mitsui Direct Black GX; Nippon Deep Black; Nippon Deep Black GX; Paper Black BA; Paper Black T.

1.1.4 *C.I. Direct Blue 6*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 2602–46–2
CAS Name: 3,3′-[[1,1'-Biphenyl]-4,4'-diylbis(2,1-diazenediyl)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], sodium salt (1:4)
Synonyms: 3,3′-[[1,1'-Biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; 3,3′-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; 2,2'-(4,4'-biphenylylenebisazo)bis[8-amino-1-naphthol-3,6-disulfonic acid], tetrasodium salt; C.I. 22610; C.I. Direct Blue 6; C.I. Direct Blue 6, tetrasodium salt; tetrasodium 3,3′-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonate]

(b) Structural formula, molecular formula, and relative molecular mass



$C_{32}H_{20}N_6O_{14}S_4.4Na$ Rel. mol. mass: 932.76

(c) Chemical and physical properties of the pure substance

Description: Blue-violet solid (IARC, 1982)

Solubility: Soluble in water; slightly soluble in ethanol and ethylene glycol monoethyl ether; insoluble in other organic solvents (IARC, 1982)

(d) Trade names

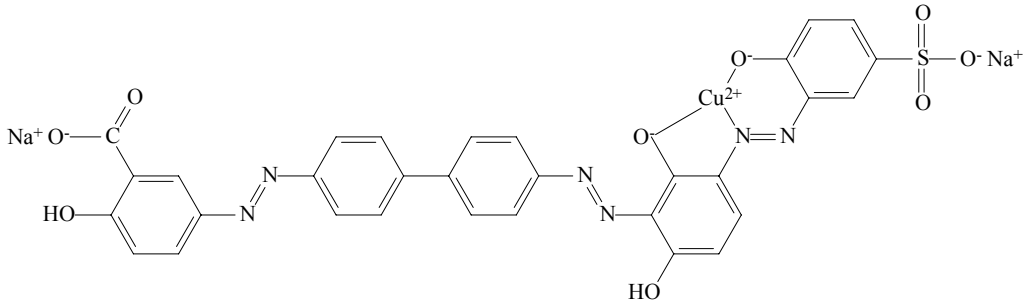
Trade names: Airedale Blue 2BD; Aizen Direct Blue 2BH; Amanil Blue 2BX; Atlantic Blue 2B; Atul Direct Blue 2B; Azocard Blue 2B; Azomine Blue 2B; Belamine Blue 2B; Bencidal Blue 2B; Benzanil Blue 2B; Benzo Blue 2B; Benzo Blue BBA-CF; Benzo Blue BBN-CF; Benzo Blue GS; Blue 2B; Blue 2B salt; Brasilamina Blue 2B; Calcomine Blue 2B; Chloramine Blue 2B; Chlorazol Blue B; Chlorazol Blue BP; Chrome Leather Blue 2B; Cresotine Blue 2B; Diacotton Blue BB; Diamine Blue; Diamine Blue 2B; Diamine Blue BB; Diaphtamine Blue BB; Diazine Blue 2B; Diazol Blue 2B; Diphenyl Blue 2B; Diphenyl Blue KF; Diphenyl Blue M2B; Direct Blue 2B; Direct Blue 2BA; Direct Blue 6; Direct Blue A; Direct Blue BB; Direct Blue GS; Direct Blue K; Direct Blue M2B; Direct Sky Blue K; Enianil Blue 2BN; Fenamin Blue 2B; Fixanol Blue 2B; Hispamin Blue 2B; Indigo Blue 2B; Kayaku Direct; Kayaku Direct Blue BB; Mitsui Direct Blue 2BN; Modr Prima 6; Naphtamine Blue 2B; Niagara Blue 2B; Nippon Blue BB; Paramine Blue 2B; Phenamine Blue BB; Pheno Blue 2B; Pontamine Blue BB; Tertrodirect Blue 2B; Vondacel Blue 2B.

1.1.5 *C.I. Direct Brown 95*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 16071–86–6
CAS Name: [2-hydroxy-5-[2-[4'-[2-[2-(hydroxy-**κ**O)-6-hydroxy-3-[2-[2-(hydroxy-**κ**O)-5-sulfophenyl]diazenyl-**κ**N1]phenyl]diazenyl][1,1'-biphenyl]-4-yl]diazenyl]benzoato(4-)]-cuprate(2-), sodium (1:2)
Synonyms: C.I. 30145; C.I. Direct Brown 95; 5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoic acid, copper complex; [dihydrogen 5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulphophenyl)azo]phenyl]azo]-4-biphenylyl]azo]salicylato(2-)]-copper, disodium salt; [5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoato(4-)]-cuprate(2-), disodium; [5-[[4'-[[2-(hydroxy-**κ**O)-6-hydroxy-3-[[2-(hydroxy-**κ**O)-5-sulfophenyl]azo-**κ**N1]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoato(4-)]-cuprate(2-), disodium

(b) Structural formula, molecular formula, and relative molecular mass



$C_{31}H_{18}CuN_6O_9S.2Na$ Rel. mol. mass: 760.10

(c) Chemical and physical properties of the pure substance

Description: Reddish-brown powder
Solubility: Soluble in water; slightly soluble in ethanol; insoluble in acetone (IARC, 1982)

(d) Trade names

Trade names: Aizen Primula Brown BRLH; Aizen Primula Brown PLH; Amanil Fast Brown BRL; Amanil Supra Brown LBL; Atlantic Fast Brown BRL; Atlantic Resin Fast Brown BRL; Belamine Fast Brown BRLL; Benzamil Supra Brown BRLL; Benzanil Supra Brown BRLL; Benzanil Supra Brown BRLN; Brown 4EMBL; Calcodur Brown BRL; Chloramine Fast Brown BRL; Chloramine Fast Brown BRLL; Chloramine Fast Cutch Brown PL; Chlorantine Fast Brown BRLL; Chrome Leather Brown BRLL; Chrome Leather Brown BRSL; Cuprofix Brown GL; Derma Fast Brown W-GL; Dermafix Brown PL; Dialuminous Brown BRS; Diaphtamine Light Brown BRLL; Diaphthamine Light Brown BRLL; Diazine Fast Brown RSL; Diazol Light Brown BRN; Dicorel Brown LMR; Diphenyl Fast Brown BRL; Direct Brown BRL; Direct Fast Brown BRL; Direct Fast Brown LMR; Direct Light Brown BRS; Direct Supra Light Brown ML; Durazol Brown BR; DuroFast Brown BRL; Eliamina Light Brown BRL; Enianil Light Brown BRL; Fastolite Brown BRL; Fastusol Brown LBRSA; Fastusol Brown LBRSN; Fenaluz Brown BRL;Helion Brown BRSL; Hispaluz Brown BRL; Ismafast Brown BRSL; KCA Light Fast Brown; KCA Light Fast Brown BR; Kayarus Supra Brown BRS; Paranol Fast Brown BRL; Peeramine Fast Brown BRL; Pontamine Fast Brown BRL; Pontamine Fast Brown NP; Pyrazol Fast Brown BRL; Pyrazoline Brown BRL; Saturn Brown LBR; Sirius Supra Brown BRL; Sirius Supra Brown BRS; Solantine Brown BRL; Solar Brown PL; Solex Brown R; Solius Light Brown BRLL; Solius Light Brown BRS; Sumilight Supra Brown BRS; Suprazo Brown BRL; Suprexcel Brown BRL; Tertrodirect Fast Brown BR; Tetramine Fast Brown BRDN Extra; Tetramine Fast Brown BRP;

Tetramine Fast Brown BRS; Triantine Brown BRS; Triantine Fast Brown OG; Triantine Fast Brown OR; Triantine Light Brown BRS; Triantine Light Brown OG.

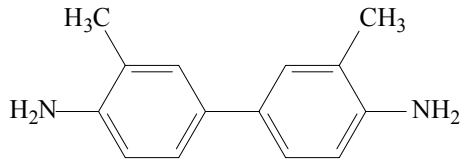
1.2 3,3′-Dimethylbenzidine and 3,3′-dimethylbenzidine-based dyes – Chemical and physical data

1.2.1 *3,3′-Dimethylbenzidine*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 119–93–7
CAS Name: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine
Synonyms: 4′-Amino-3,3′-dimethyl[1,1′-biphenyl]-4-ylamine; 4,4′-bi-*ortho*-toluidine; C.I. 37230; 4,4′-diamino-3,3′-dimethyl-1,1′-biphenyl; 4,4′-diamino-3,3′-dimethylbiphenyl; 4,4′-diamino-3,3′-dimethyldiphenyl; diaminoditolyl; diaminotolyl; 3,3′-dimethyl-(1,1′-biphenyl)-4,4′-diamine; 3,3′-dimethylbiphenyl-4,4′-diamine; 3,3′-dimethyl-4,4′-biphenyldiamine; 3,3′-dimethyl-4,4′-diphenyldiamine; 3,3′-dimethyldiphenyl-4,4′-diamine; 4,4′-di-*ortho*-toluidine; 3,3′-tolidine; *ortho*, *ortho*′-tolidine; 2-tolidine; *ortho*-tolidine

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2$ Rel. mol. mass: 212.29

(c) Chemical and physical properties of the pure substance (O'Neil 2006)

Description: White to reddish crystals or crystalline powder
Melting-point: 129–131°C
Solubility: Slightly soluble in water; soluble in ethanol, diethyl ether, and dilute acids

(d) Technical products and impurities

Trade names: Fast Dark Blue Base R; and C.I. Azoic Diazo Component 113.

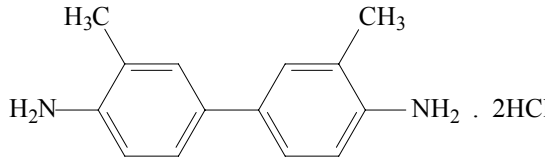
1.2.2 *3,3′-Dimethylbenzidine dihydrochloride*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 612–82–8

CAS Name: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine, hydrochloride (1:2)
Synonyms: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine, dihydrochloride; *ortho*-tolidine dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



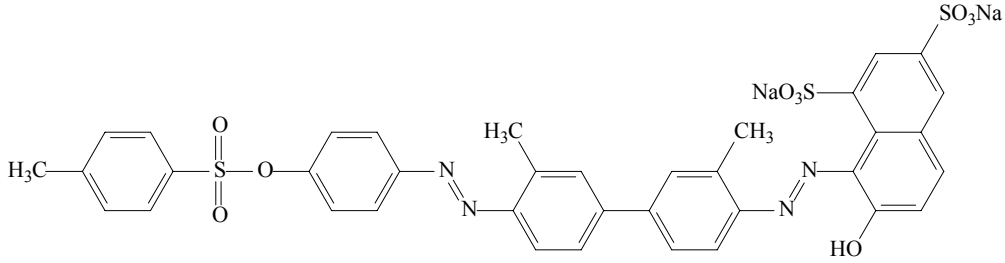
$C_{14}H_{16}N_2.2HCl$ Rel. mol. mass: 285.21

1.2.3 *C.I. Acid Red 114*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 6459–94–5
CAS Name: 8-[2-[3,3′-Dimethyl-4'-[2-[4-[[(4-methylphenyl)sulfonyl]oxy]phenyl]diazenyl] [1,1'-biphenyl]-4-yl]diazenyl]-7-hydroxy-1,3-naphthalenedisulfonic acid, sodium salt (1:2)
Synonyms: C.I. 23635; C.I. Acid Red 114; C.I. Acid Red 114, disodium salt; 8-[[3,3′-Dimethyl-4'-[[4-[[(4-methylphenyl)sulfonyl]oxy]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-7-hydroxy-1,3-naphthalenedisulfonic acid, disodium salt; disodium 8-((3,3′-dimethyl-4'-(4-(4-methylphenylsulphonyloxy)phenylazo)(1,1'-biphenyl)-4-yl)azo)-7-hydroxynaphthalene-1,3-disulphonate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{37}H_{28}N_4O_{10}S_3.2Na$ Rel. mol. mass: 830.82

(c) Chemical and physical properties of the pure substance (O'Neil 2006)

Description: Red powder (NTP, 1991a)
Solubility: Soluble in water (NTP, 1991a)

(d) Trade names

Trade names: Acid Leather Red BG; Acid Milling Red BS; Acid Milling Red RS; Acid Red F-RS; Acid Red P-RS; Acid Red RS; Amacid Milling Red PRS; Anadurm Red M-R; Apollo Nylon Fast Red R; Atul Acid Milling Red RS; Benzyl Fast Red BG; Benzyl Red BR; Best Acid Milling Red RS; Colomill Red RS; Concorde Acid Red M-RS; Concorde Leather Red RSN; Covalene Red RS; Covalene Scarlet RS; Covanyl Scarlet RS; Daedo Acid Red RS; Dinacid Milling Red RG; Dycosweak Acid RS; Elcacid Milling Fast Red RS; Eniacid Fast Red R; Erionyl Red RS; Erionyl Red RS 125; Everacid Milling Red RS; Everlan Red RS; Fabracid Red M-RS; Fenafor Red PB; Folan Red B; Indacid Milling Red RS; Intrazone Red BR; Kayanol Milling Red RS; Kayanol Milling Red RS 125; Kenamide Red K 2R; Kenanthrol Red R; Leather Fast Red B; Lerui Acid Red F-RS; Levanol Red GG; Midlon Red PRS; Milling Fast Red B; Milling Fast Red R; Milling Red B; Milling Red BB; Milling Red SWB; Monacid Red RS; Polar Red RS; Sandolan Red N-RS; Sella Fast Red RS; Sulphonol Fast Red R; Sulphonol Red R; Suminol Milling Red RS; Supranol Fast Red 3G; Supranol Fast Red GG; Supranol Red PBX-CF; Supranol Red R; Telon Fast Red GG; Tertracid Milling Red B; Tetracid Milling Red B; Tetracid Milkling Red G; Vondamol Fast Red RS.

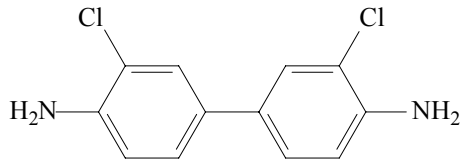
1.3 3,3′-Dichlorobenzidine – Chemical and physical data

1.3.1 *3,3′-Dichlorobenzidine*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 91–94–1
CAS Name: 3,3′-Dichloro-[1,1'-biphenyl]-4,4'-diamine
Synonyms: 4'-Amino-3,3′-dichloro[1,1'-biphenyl]-4-ylamine; C.I. 23060; 4,4'-diamino-3,3′-dichlorobiphenyl; 4,4'-diamino-3,3′-dichlorodiphenyl; *ortho*, *ortho*'-dichlorobenzidine; 3,3′-dichloro-*para*, *para*'-bianiline; 3,3′-dichlorobiphenyl-4,4'-diamine; 3,3′-dichloro-4,4'-diamino(1,1-biphenyl); 3,3′-dichloro-4,4'-diaminobiphenyl

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{10}Cl_2N_2$ Rel. mol. mass: 253.13

(c) Chemical and physical properties of the pure substance

Description: Needles from alcohol (O'Neil, 2006; Lide, 2008)
Melting-point: 132.5°C (Lide, 2008)
Solubility: Insoluble in water; soluble in acetic acid, benzene, and ethanol (Lide, 2008)

(d) Trade names

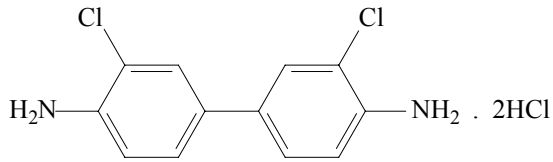
Trade names for 3,3′-dichlorobenzidine include: Curithane C 126.

1.3.2 *3,3′-Dichlorobenzidine dihydrochloride*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 612–83–9
CAS Name: 3,3′-Dichloro-(1,1'-Biphenyl)-4,4'-diamine, dihydrochloride
Synonyms: 3,3′-Dichlorobenzidine dihydrochloride; 3,3′-dichlorobenzidine hydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{10}Cl_2N_2.2HCl$ Rel. mol. mass: 326.05

(c) Chemical and physical properties of the pure substance

Description: Leaflets from water (O'Neil, 2006)
Solubility: Insoluble in water; very soluble in ethanol (Lide, 2008)

1.4 3,3-Dimethoxybenzidine and dimethoxybenzidine-based dyes – Chemical and Physical Data

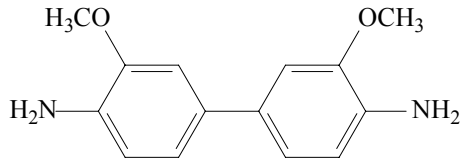
1.4.1 *3,3′-Dimethoxybenzidine*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 119–90–4
CAS Name: 3,3′-Dimethoxy-[1,1'-biphenyl]-4,4'-diamine

Synonyms: 4,4′-Bi-*ortho*-anisidine; C.I. 24110; C.I. Disperse Black 6; 4,4'-diamino-3,3′-dimethoxy-1,1'-biphenyl; 4,4'-diamino-3,3′-dimethoxy-1,1'-diphenyl; dianisidine; 3,3′-dianisidine; *ortho*-dianisidine; 3,3′-dimethoxybiphenyl-4,4'-diamine; 3,3′-dimethoxy-4,4'-diaminobiphenyl; 3,3′-dimethoxy-4,4'-diaminodiphenyl

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2O_2$ Rel. mol. mass: 244.29

(c) Chemical and physical properties of the pure substance

Description: Leaflets or needles from water (Lide, 2008)
Melting-point: 137°C (Lide, 2008)
Solubility: Insoluble in water; soluble in acetone, benzene, chloroform, diethyl ether, and ethanol (Lide, 2008)

(d) Trade names

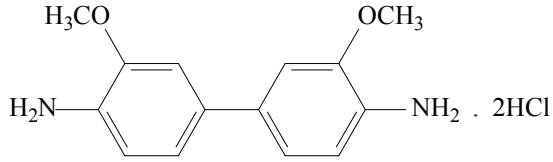
Trade names for 3,3′-dimethoxybenzidine include: Acetamine Diazo Black RD; Acetamine Diazo Navy RD; Amacel Developed Navy SD; Azoene Fast Blue Base; Azofix Blue B Salt; Azogene Fast Blue B; Azogene Fast Blue B Salt; Blue BN Base; Blue Base NB; Blue Base IRGA B; Brentamine Fast Blue B Base; C.I. Azoic Diazo Component 48; Cellitazol B; Cibacete Diazo Navy Blue 2B; Diacel Navy DC; Diacelliton Fast Grey G; Diato Blue Base B; Diazo Fast Blue B; Fast Blue B Base; Fast Blue Base B; Fast Blue DSC Base; Hiltonil Fast Blue B Base; Kayaku Blue B Base; Lake Blue B Base; Meisei Teryl Diazo Blue HR; Mitsui Blue B Base; Naphthanil Blue B Base; Neutrosel Navy BN; Setacyl Diazo Navy R; Spectrolene Blue B.

1.4.2 3,3′-Dimethoxybenzidine dihydrochloride

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 20325–40–0
CAS Name: 3,3′-Dimethoxy-[1,1'-biphenyl]-4,4'-diamine, hydrochloride (1:2)
Synonyms: C.I. Disperse Black 6, dihydrochloride; *ortho*-dianisidine dihydrochloride; 3,3′-dimethoxy-[1,1'-biphenyl]-4,4'-diamine dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2O_2.2HCl$ Rel. mol. mass: 317.21

(c) Chemical and physical properties of the pure substance

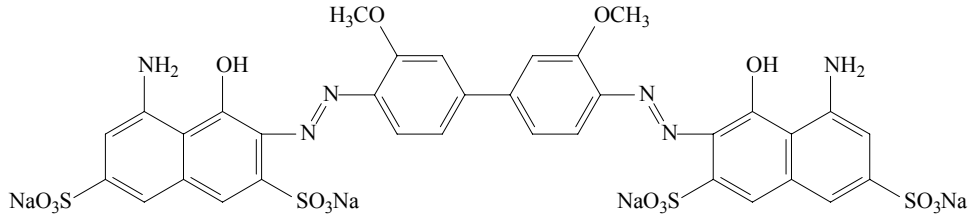
Description: Off-white powder (NTP, 1990)
Melting-point: 274°C (NTP, 1990)
Solubility: Readily soluble in hot water and sparingly soluble in cold water and alcohol (Schwenecke & Mayer, 2005)

1.4.3 *C.I. Direct Blue 15*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 2429–74–5
CAS Name: 3,3′-[(3,3′-Dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(2,1-diazenediyl)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], sodium salt (1:4)
Synonyms: C.I. 24400; C.I. Direct Blue 15; C.I. Direct Blue 15, tetrasodium salt; 3,3′-[(3,3′-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; tetrasodium 3,3′-[(3,3′-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonate];

(b) Structural formula, molecular formula, and relative molecular mass



$C_{34}H_{24}N_6O_{16}S_4.4Na$ Rel. mol. mass: 992.81

(c) Chemical and physical properties of the pure substance

Description: Dark blue powder (NTP, 1992)
Solubility: Soluble in water (NTP, 1992)

(d) Trade names

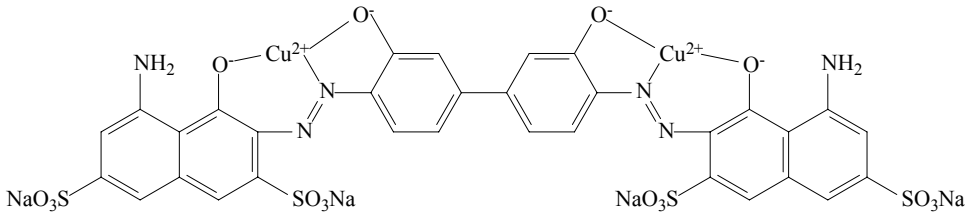
Trade names for C.I. Direct Blue 15 include: Airedale Blue D; Aizen Direct Sky Blue 5B; Aizen Direct Sky Blue 5BH; Amanil Sky Blue; Atlantic Sky Blue A; Atul Direct Sky Blue; Azine Sky Blue 5B; Belamine Sky Blue A; Benzanil Sky Blue; Benzo Sky Blue A-CF; Benzo Sky Blue S; Cartalsol Blue 2GF; Cartasol Blue 2GF; Chloramine Sky Blue 4B; Chloramine Sky Blue A; Chrome Leather Pure Blue; Cresotine Pure Blue; Diacotton Sky Blue 5B; Diamine Blue; Diamine Blue 6B; Diamine Sky Blue; Diamine Sky Blue CI; Diaphtamine Pure Blue; Diazol Pure Blue 4B; Diphenyl Brilliant Blue; Diphenyl Sky Blue 6B; Direct Blue 10G; Direct Blue FFN; Direct Blue FFN-B 15; Direct Blue HH; Direct Lake Blue 5B; Direct Pure Blue; Direct Pure Blue M; Direct Pure Blue N; Direct Sky Blue; Direct Sky Blue 5B; Direct Sky Blue A; Enianil Pure Blue AN; Fenamin Sky Blue; Hispamin Sky Blue 3B; Kayafect Blue Y; Kayaku Direct SKH Blue 5B; Kayaku Direct Sky Blue 5B; Mitsui Direct Sky Blue 5B; Naphtamine Blue 10G; Niagara Blue 4B; Niagara Sky Blue; Nippon Direct Sky Blue; Nippon Sky Blue; Nitsui Direct Sky Blue 5B; Nitto Direct Sky Blue 5B; Oxamine Sky Blue 5B; Paper Blue S; Phenamine Sky Blue A; Pontacyl Sky Blue 4BX; Pontamine Sky Blue 5 BX; Pontamine Sky Blue 5BX; Shikiso Direct Sky Blue 5B; Sky Blue 4B; Sky Blue 5B; Tertrodirect Blue F; Vondacel Blue HH.

1.4.4 *C.I. Direct Blue 218*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 28407–37–6
CAS Name: [μ-[[3,3′-[[3,3′-di(hydroxy-κO)[1,1'-biphenyl]-4,4'-diyl]bis(1,2-diazenediyl-κN1)]bis[5-amino-4-(hydroxy-κO)-2,7-naphthalenedisulfonato]](8-)]]dicuprate(4-), sodium (1:4)
Synonyms: C.I. 24401; C.I. Direct Blue 218; 2,2’-(3,3′-dihydroxy-4,4′-biphenylylenebisazo)bis[8-amino-1-naphthol-3,6-disulfonic acid, dicopper derivative, tetrasodium salt; 3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid, copper complex; [μ-[[3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato]](8-)]]dicuprate(4-), tetrasodium; Direct Blue 218; [tetrahydrogen-3,3′-[(3,3′-dihydroxy-4,4'-biphenylylene)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato](4-)]dicopper, tetrasodium salt; tetrasodium [μ-[[3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonato]](8-)]]dicuprate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{32}H_{16}Cu_2N_6O_{16}S_4.4Na$ Rel. mol. Mass: 1087.82

(c) Chemical and physical properties of the pure substance

Description: Dark blue solid (NTP, 1994)
Solubility: Limited solubility in water (NTP, 1994)

(d) Trade names

Trade names for C.I. Direct Blue 218 include: Amanil Supra Blue 9GL; Carta Blue VP; Fastusol Blue 9GLP; Intralite Blue 8GLL; Pontamine Bond Blue B; Pontamine Fast Blue 7GLN; and Solantine Blue 10GL.

1.5 Analysis

Analytical studies on benzidine began in the 1950s. Recent studies include the use of gas chromatography/mass spectrometry (GC/MS) to detect very low (ppm-ppb) levels in water and paint samples. While GC analysis invariably requires derivatization of the amine before the analysis, analysis by liquid chromatography (LC) in combination with mass spectrometry does not. Also, the use of modern LC-MS/MS methods permits the analysis of complex mixtures. Table 1.1 presents a selection of recent studies on the analysis of benzidine and benzidine-based dyes in various matrices.

Table 1.1. Selected methods of analysis of benzidine and benzidine congeners in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Benzidine				
Water & soil	pH adjustment, extraction (dichloromethane), evaporation, residue dissolved in mobile phase	CZE	1 ppm	Bromley & Brownrigg (1994) in Choudhary (1996)
Finger paints	Paint containing amine is applied to an inert surface and dried. Painted sample and modifier (methanol) are placed in SFE cartridge for extraction and GC analysis	SFE/GC	< 0.5 μg/g	Garrigós *et al*. (2000, 2002)
Food polyurethane packaging	Dissolve in ethanol at 500μg/mL; dilute to 5μg/mL; refrigerate up to 5 weeks; protect from light by covering containers with aluminum foil	LC-ESI-MS/MS	0.9 μg/L	Mortensen *et al*. (2005)
Food colourants	Dissolve 100 mg in 5ml of pH9 borate buffer	μLC/ECD	36 pmol/L	Shelke *et al*. (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	LC/ECD	4.5 nmol/L	Mazzo *et al*. (2006)
Water	Extract from water at pH 8.5 with dichloromethane; evaporate solvent; silylate	GC/MS	4 ng/L	Shin & Ahn (2006)
3,3'-Dimethylbenzidine				
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	<20 μg/g	Garrigós *et al*. (2002)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/ dichloromethane (50:50)	GC/MS	5 ng/mL	Doherty (2005)

Table 1.1 (contd)

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
3,3'-Dimethylbenzidine (contd)				
Food polyurethane packaging	Dissolve in ethanol at 500µg/mL; dilute to 5µg/mL; refrigerate up to 5 weeks; protect from light by covering containers with aluminum foil	LC-ESI-MS	0.7 µg/L	Mortensen *et al*. (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	HPLC/ECD	7.69 nmol/L	Mazzo *et al*. (2006)
3,3'-Dicholorobenzidine				
Urine	Urine specimens (100mL) were extracted at pH 6-7 with chloroform; extract were evaporated to dryness after adding *p*-chlorobiphenyl as an internal standard. The residue was dissolved in 100 µL of benzene containing 1% (v/v) 1-aminobutane	GC/MS	10-20 pg	Hurst *et al*. (1981)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	20.1 µg/L	Sutthivaiyakit *et al.* (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	LC/ECD	5.15 nmol/L	Mazzo *et al*. (2006)
Water	Extract from water at pH 8.5 with dichloromethane; evaporate solvent; silylate	GC/MS	20 nl/L	Shin & Ahn (2006)

Table 1.1 (contd)

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
3,3'-Dimethoxybenzidine				
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	<20 μg/g	Garrigós *et al*. (2002)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/ dichloromethane (50:50)	GC/MS	5 ng/mL	Doherty (2005)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	47.8 μg/mL	Sutthivaiyakit *et al*. (2005)

CZE, capillary zone electrophoresis; ECD, electro-chemical detection; ESI, electrospray ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; SFE, supercritical fluid extraction; UV, ultraviolet

1.6 Production

1.6.1 *Benzidine and benzidine-based dyes*

(a) Benzidine

Benzidine and its substitution products (*ortho*-tolidine [3,3′-dimethylbenzidine], 3,3′-dichlorobenzidine, and *ortho*-dianisidine [3,3′-dimethoxylbenzidine]) represent the group called the diphenyl bases. They are used mainly as intermediates in the production of azo dyes and azo pigments. Symmetrically or asymmetrically coupled products can be produced by simultaneous or successive diazotization (coupling), respectively. The diphenyl bases have been of interest as cross-linking agents, e.g., in polyurethane plastics, in which they can noticeably increase temperature stability. The diphenyl radical has a chain-stiffening effect in polyamides. The ability of the diphenyl bases to react with numerous cations, anions, and organic substances, such as oxidizing agents and blood, is used for analytical and diagnostic purposes (Schwenecke & Mayer, 2005).

Benzidine and the other diphenyl bases are produced in three separate processing stages: 1) reduction of nitro groups to form hydrazo compounds; 2) benzidine rearrangement; 3) isolation of the bases. Benzidine has been produced from nitrobenzene on an industrial scale since about 1880. Commercial production methods include alkaline iron reduction, amalgam reduction, and electrochemical reduction. The resultant hydrazobenzene is rearranged with hydrochloric acid or sulfuric acid during cooling. The base is then isolated in the form of benzidine hydrochloride or benzidine sulfate. The conversion of these salts to the free base is avoided as much as possible because of the chronic toxicity of benzidine (Schwenecke & Mayer, 2005).

The most important reaction commercially is the diazotization of the two amino groups. Reaction with nitrous acid converts benzidine into the tetrazonium compound, in which the first diazonium group is coupled very vigorously whereas the second reacts more slowly. As a result it is possible to produce asymmetrical diazo dyes. Gradual diazotization is also possible (Schwenecke & Mayer, 2005).

The manufacturing of benzidine is prohibited in several countries, e.g., Japan, Republic of Korea, Canada and Switzerland (UN/UNEP/FAO, 2009). According to EU legislation, the manufacture of benzidine has been prohibited in Europe since 1998 (European Commission, 1998).

Benzidine is no longer manufactured for commercial purposes in the USA. All large-scale production was discontinued in 1976, and only small quantities remain available for use in diagnostic testing. Estimated US benzidine production in 1983 was 500 pounds (227 kg) (possibly excluding some captive production), compared with 10 million pounds (4500 tonnes) in 1972 (ATSDR, 2001).

Available information indicates that benzidine was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, the People's Republic of China, Switzerland, and the USA

(Chem Sources-International, 2010). Available information indicates that benzidine hydrochloride was produced and/or supplied in research quantities in the following countries: Belgium, Canada, Germany, Hong Kong Special Administrative Region, India, Switzerland, and the USA (Chem Sources-International, 2010).

(b) Benzidine-based dyes

Benzidine-based dyes were produced in commercial quantities in the United States starting no later than 1914. Total production in the USA reached 14 million kg (31 million pounds) in 1948, which dropped to about 2.9 million kg (6.4 million pounds) in 1976 and about 780 000 kg (1.7 million pounds) in 1978 (IARC, 1982). In 1978, Direct Black 38 accounted for about 48% of the production, followed by Direct Blue 2 (12.8%) and Direct Green 6 (6.4%). In 1974, nine manufacturers produced benzidine-based dyes; by 1979, only one manufacturer remained, producing 17 benzidine-based dyes (NTP, 2005a).

Information was collected in Europe from 1996 to 1998 for the IUCLID database for substances with a production or import volume between 10 and 1000 tonnes/year (Low Production Volume Chemicals (LPVCs)). Direct Black 38 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Available information indicates that Direct Black 38 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, the United Kingdom, and the USA (Chem Sources-International, 2010).

Direct Blue 6 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, and the USA (Chem Sources-International, 2010).

Direct Brown 95 was produced and/or supplied in research quantities in the following countries: India, Japan, and the USA (Chem Sources-International, 2010).

1.6.2 *Dimethylbenzidine and dimethylbenzidine-based dyes*

(a) 3,3′-Dimethylbenzidine

ortho-Nitrotoluene undergoes alkaline reduction with zinc dust, electrolytic reduction, or catalytic reduction to form 2,2′-dimethylhydrazobenzene. This is rearranged in dilute hydrochloric acid or 20% sulfuric acid at 5–50°C. The free base (3,3′-dimethylbenzidine) or the dihydrochloride can be isolated (Schwenecke & Mayer, 2005).

In 1978, the major company producing 3,3′-dimethylbenzidine in the USA ceased production; its annual production had averaged approximately 200 000 pounds (NTP, 2005b). The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the

aggregate production volumes that were reported for 3,3′-dimethylbenzidine dihydrochloride. 3,3′-Dimethylbenzidine was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Available information indicates that 3,3′-dimethylbenzidine was produced or supplied in the following countries: Canada, Germany, Hong Kong Special Administrative Region, India, Japan, the Netherlands, the People's Republic of China, South Africa, Switzerland, and the USA (Chem Sources-International, 2010).

Table 1.2. 3,3′-Dimethylbenzidine dihydrochloride production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	NR
1998	NR
2002	NR
2006	NR

USEPA (2003, 2007)
NR, not reported

(b) Dimethylbenzidine-based dyes

Acid Red 114 can be prepared by coupling *ortho*-tolidine [3,3′-dimethylbenzidine] to phenol, which is then coupled to G-acid (2-naphthol-6,8-disulfonic acid), followed by reaction of the phenolic hydroxyl group with *para*-toluenesulfonyl chloride (Chudgar & Oakes, 2003). Acid Red 114 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Table 1.3 presents the aggregate production volumes that were reported for Acid Red 114 by the USEPA.

Available information indicates that Acid Red 114 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, and the USA (Chem Sources-International, 2010).

Table 1.3. Acid Red 114 production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	NR
1998	10–500
2002	NR
2006	NR

USEPA (2003, 2007)
NR, not reported

1.6.3 *Dichlorobenzidine*

3,3′-Dichlorobenzidine is commercially produced by reduction of *ortho*-nitrochlorobenzene to form a hydrazo compound, which is rearranged in the presence of mineral acids to form 3,3′-dichlorobenzidine (Schwenecke & Mayer, 2005). The commercial product is usually provided in the form of the dihydrochloride salt because of its greater stability. 3,3′-Dichlorobenzidine dihydrochloride was included on the list of HPVCs with a range of 10 000 to 50 000 tonnes (Allanou *et al.*, 1999; European Commission, 2000).

Table 1.4 presents the aggregate production volumes that were reported for 3,3′-dichlorobenzidine by the USEPA.

Table 1.4. 3,3′-dichlorobenzidine production volumes

Year	Volume (in thousands of pounds)
1986	NR
1990	>500–1000
1994	10–500
1998	>1000–10 000
2002	10–500
2006	NR

USEPA (2003, 2007)
NR, not reported

Table 1.5 presents the aggregate production volumes that were reported by the USEPA for 3,3′-dichlorobenzidine dihydrochloride.

The US International Trade Commission reported a production volume of 3,3′-dichlorobenzidine-based dyes of over 18 million pounds in the USA in 1983; 3,3′-dichlorobenzidine is no longer used to manufacture dyes in the USA (ATSDR, 1998).

Available information indicates that 3,3′-dichlorobenzidine was produced and/or supplied in the following countries: Hong Kong Special Administrative Region, India, the People's Republic of China, the United Kingdom and the USA (Chem Sources-International, 2010), whereas 3,3′-dichlorobenzidine dihydrochloride was produced and/or supplied in the following countries: Belgium, Hong Kong Special Administrative Region, India, Japan, the People's Republic of China, Switzerland, the United Kingdom and the USA (Chem Sources-International, 2010).

Table 1.5. 3,3′-dichlorobenzidine dihydrochloride production volumes

Year	Volume (in millions of pounds)
1986	>1–10
1990	>1–10
1994	>10–50
1998	>10–50
2002	>10–50
2006	10–<50

USEPA (2003, 2007)

1.6.4 *Dimethoxybenzidine and dimethoxybenzidine-based dyes*

(a) Dimethoxybenzidine

3,3′-Dimethoxybenzidine has been produced commercially since the 1920s. 3,3′-Dimethoxybenzidine and 3,3′-dimethoxybenzidine dihydrochloride were included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Data on production of 3,3′-dimethoxybenzidine in the USA were last reported in 1967, when five companies produced approximately 368 000 pounds (IARC, 1974). Table 1.6 presents the aggregate production volumes that were reported for 3,3′-dimethoxybenzidine dihydrochloride by the USEPA.

Available information indicates that 3,3′-dimethoxybenzidine was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, Switzerland, the United Kingdom and the USA (Chem Sources-International, 2010), whereas 3,3′-dimethoxybenzidine dihydrochloride was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, the People's Republic of China and the USA (Chem Sources-International, 2010).

Table 1.6. 3,3′-dimethoxybenzidine dihydrochloride production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	10–500
1998	>500–1 000
2002	10–500
2006	<500

USEPA (2003, 2007)

(b) Dimethoxybenzidine-based dyes

Direct Blue 15 is prepared by coupling *ortho*-dianisidine (3,3′-dimethoxybenzidine) to two moles of H-acid (4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid) under alkaline conditions. Direct Blue 218 is produced from Direct Blue 15 by metalizing and elimination of methyl groups from the methoxide to form the copper complex (Chudgar & Oakes, 2003). Direct Blue 15 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Table 1.7 presents the aggregate production volumes that were reported by the USEPA for Direct Blue 15 and Direct Blue 218.

Available information indicates that Direct Blue 15 was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan and the USA (Chem Sources-International, 2010).

Table 1.7. Production volumes for Direct Blue 15 and Direct Blue 218

Year	Volume (in thousands of pounds)	
	Direct Blue 15	Direct Blue 218
1986	10–500	10–500
1990	>1000–10 000	10–500
1994	>500–1000	10–500
1998	>500–1000	10–500
2002	>500–1000	10–500
2006	NR	<500

USEPA (2003, 2007)
NR, not reported

1.7 Use

1.7.1 *Benzidine and benzidine-based dyes*

(a) Benzidine

Benzidine has been used since the 1850s as the reagent base for the production of a large number of dyes, particularly azo dyes for wool, cotton, and leather. However, because benzidine was found in the 1970s to be carcinogenic to humans, there has been a considerable decline in the use of the benzidine dyes. Benzidine is used for the quantitative determination of sulfuric acid and for the detection and determination of numerous anions and metal ions. The reaction of benzidine with pyridine in the presence of elemental chlorine is suitable for detecting traces of free chlorine or pyridine in drinking-water. The green to blue colouration that occurs when benzidine reacts with hydrogen peroxide in the presence of peroxidases can be used to detect blood. Benzidine still plays a role in many chemical syntheses (Schwenecke & Mayer, 2005).

In the past, benzidine also has been used as a rubber compounding agent, in the manufacture of plastic films, for detection of hydrogen peroxide in milk, and for quantitative determination of nicotine. Most of these uses have been discontinued, although some dyes that may contain benzidine as an impurity are still used as stains for microscopy and similar laboratory applications (ATSDR, 2001).

(b) Benzidine-based dyes

Benzidine-based dyes were used primarily to colour textiles, leather, and paper products and also in the petroleum, rubber, plastics, wood, soap, fur, and hair-dye

industries (NTP, 2005b). Approximately 40% was used to colour paper, 25% to colour textiles, 15% to colour leather, and 20% for diverse applications. By the mid-1970s, most manufacturers started phasing-out the use of benzidine-based dyes and replacing them with other types of dyes (NIOSH, 1980). Access to these dyes for home use is no longer permitted in the US; however, some dyes (particularly direct browns, greens, and blacks) were available as consumer products in the 1970s (ATSDR, 2001).

1.7.2 *Dimethylbenzidine and dimethylbenzidine-based dyes*

(a) 3,3′-Dimethylbenzidine

3,3′-Dimethylbenzidine is a starting material in the production of a large number of azo dyes and pigments. 3,3′-Dimethylbenzidine is used in the determination of oxygen and chlorine in water, and for the colorimetric determination of cations of gold, cerium, and manganese. An important derivative of 3,3′-dimethylbenzidine is its diacetoacetyl compound, 4,4’-bisacetoacetylamino-3,3′-dimethyldiphenyl. It is a coupling agent that is frequently used; in combination with chloroanilines it gives yellow shades. 3,3′-Dimethylbenzidine diisocyanate is used as a cross-linking agent for the synthesis of polymers (Schwenecke & Mayer, 2005).

(b) Dimethylbenzidine-based dyes

Dimethylbenzidine-based dyes and pigments have been used in printing textiles, as biological stains, and in colour photography (NTP, 2005b).

1.7.3 *Dichlorobenzidine*

3,3′-Dichlorobenzidine was introduced in the early 1930s and is an important diphenyl base. It is used as the starting material for pigments with yellow and red shades. These are used for coloring printing inks, paints, plastics, and rubbers. The important diarylide yellow pigments, which are incorrectly known as benzidine yellows, are formed by the combination of 3,3′-dichlorobenzidine with acetic acid arylides. 3,3′-Dichlorobenzidine is also used in the production of polyurethane rubbers (Schwenecke & Mayer, 2005).

Diarylide pigments are important economically, particularly in the production of printing ink. 3,3′-Dichlorobenzidine is by far the most important bisdiazo component. The term “benzidine pigments” is still sometimes used for this group, but this is incorrect because benzidine has never been used to produce diarylide pigments. Diarylide pigments are produced by the bisdiazotization of 3,3′-dichlorobenzidine, followed by coupling with two equivalents of an acetoacetic arylide (Herbst & Hunger, 2004).

1.7.4 *Dimethoxybenzidine and dimethoxybenzidine-based dyes*

(a) Dimethoxybenzidine

3,3′-Dimethoxybenzidine is used almost exclusively as a chemical intermediate for producing dyes and pigments. The Society of Dyers and Colourists reported its use in the production of 89 dyes in 1971. 3,3′-Dimethoxybenzidine is also used as a chemical intermediate to produce *ortho*-dianisidine diisocyanate for use in adhesives and as a component of polyurethanes (IARC, 1974).

(b) Dimethoxybenzidine-based dyes

3,3′-Dimethoxybenzidine-based dyes and pigments have been used as colourants for paper, plastics, rubber, textiles, and leather (IARC, 1974).

1.8 Occurrence

1.8.1 *Natural occurrence*

Benzidine and its congeners are not known to occur naturally.

1.8.2 *Occupational exposure*

Occupational exposure to benzidine, benzidine congeners and their related dyes can occur during the production and use of these substances. Other workers potentially exposed to benzidine include laboratory personnel using benzidine-containing laboratory chemicals. Steinberg (1977) reported the results of a 1974 survey of US forensic laboratories, which showed that 54 of 276 laboratories were familiar with the benzidine test for blood.

Benzidine-based dyes and benzidine congener-based dyes can also be metabolized to benzidine and the respective congener, which may result in additional exposure to the aromatic amine. Exposure studies in benzidine-based dye workers therefore measured benzidine rather than the benzidine-based dyes.

Studies reporting airborne and urine levels and dermal wipes of benzidine in the benzidine and benzidine-based dye industry are listed in Tables 1.8–1.10.

(a) Airborne benzidine

Benzidine concentration in workplace air has been reported for different work settings; the results are summarized in Table 1.8.

In a Moscow aniline-dye factory, benzidine was produced from 1930 to 1988, with a six-year lapse from 1941 to 1947, after which the plant was reconstructed (Bulbulyan *et al.*, 1995). Factory area air-samples were obtained between 1930 and 1971. Benzidine-in-air concentrations of up to 6 mg/m^3 were reported. Levels were lower after the reconstruction

Table 1.8. Benzidine concentration in air in different occupational settings

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Meigs *et al*., (1951, 1954)	USA, 1948–1952	Benzidine manufacturing – press room	26	mean (max)	0.018 (0.087)
		– other areas	5		<0.001
Zavon *et al*., (1973)	USA, >1958	Benzidine manufacturing – reducers	NR	NR	<0.007
		– conversion tubs	NR	NR	<0.007
		– clarification tub	NR	NR	0.005
		– filter press	NR	NR	0.072–0.415
		– salting-out tub	NR	NR	0.152
		– centrifuge	NR	NR	<0.005
		– location for shoveling benzidine into drums	NR	NR	17.6
Krajewska *et al*., (1980)	Poland, 1976	Benzidine manufacturing – old production process	253	GM +/- SD	0.017 +/- 0.63
		– new, automated production process	275	GM +/- SD	0.0008[a] +/- 0.37 (91% < LOD of 0.0027)
				max	0.031
Bi *et al*., (1992)	China, Tianjin, 1962	Direct dye & benzidine production – transformation	17	mean (max)	0.05 (0.9)
		– deposition	13	mean (max)	0.13 (0.25)
		– filtration	13	mean (max)	0.24 (0.38)
		– oil pump	10	mean (max)	0.16 (0.35)
	China, Jilin, 1965	– packaging	4	mean (max)	0.39 (1.18)
	China, Jilin, 1970	– underground tub	2	mean (max)	0.27 (0.33)

Table 1.8 (contd)

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Bulbulyan *et al*., (1995)	Russian Federation, 1930–1941	Aniline dye production	NR	range	0.06–1.8
	Russian Federation, 1947–1948		NR	range	0–6 (n undetected unknown)
	Russian Federation, 1956		16	range	0–2.2 (4 undetected)
	Russian Federation, 1957 summer		4	range	0–1.2 (1 undetected)
	Russian Federation, 1957 winter		44	range	0–0.18 (33 undetected)
	Russian Federation, 1971		39	range	0 (all undetected)

Table 1.8 (contd)

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Kim *et al*., (2007)	Republic of Korea, 1998	Benzidine-based dye manufacture – drying	5		ND
		– packaging	5		ND
		– material treatment	1		ND
		– filtering	5	range	ND–0.65
		– drying	2		ND
		– transport	2		ND
		– maintenance	1		ND
		Benzidine and benzidine-based dyes use	3		ND
		– material treatment			
		– coupling	3		Trace
		– coupling/dissolution	5	range	ND–trace
		– dissolution	3	range	ND–trace
		– filtering	4	range	ND–trace
		– drying	2		ND
		– grinding/packaging	7	mean (range)	0.0417 (ND–0.24)
		– mixing	1		0.1131
		– maintenance	3	range	trace–0.0149

[a] estimated value from probability distribution model
GM, geometric mean; LOD, limit of detection; ND, not detected; NR, not reported; SD, standard deviation

of the plant and airborne benzidine levels were all below the limit of detection in 1971.

In a chemical plant in the USA, benzidine production started in 1929 (Zavon *et al.*, 1973). After an employee had noted haematuria in 1958, concentrations of benzidine in the air at different locations of the plant were assessed. These measurements showed that major exposure occurred at an activated charcoal discard press during hand cleaning as well as during shovelling of dry benzidine into barrels (air concentration, 17.6 mg/m^3).

In another US chemical plant in Connecticut, USA, benzidine was produced between the mid-1940s and mid-1965, while dichlorobenzidine production continued until 1989 (Ouellet-Hellstrom & Rench, 1996). Due to bladder-cancer concerns a permanent biological monitoring programme was instituted in 1949, which continued until 1965. Air concentrations measured in 1948 and 1949 were reported (Meigs *et al.*, 1951, 1954) with a maximum of 0.087 mg/m^3.

In a Polish benzidine-manufacturing plant (Krajewska *et al.*, 1980), air samples showed lower airborne benzidine concentrations after the production process had been changed.

Direct dye-production facilities in three cities in China (Tianjin, Shanghai, Jilin) used imported powdered benzidine until about 1956, when production of benzidine began in Tianjin and Jilin (Bi *et al.*, 1992). Benzidine production ceased in 1977. In addition to the two benzidine-production facilities, in 1971 there were eight benzidine-using facilities in Tianjin and eight in Shanghai. Benzidine was measured in ambient air in the factories in Tianjin and Jilin during 1962–1970, with a maximum of 1.18 mg/m^3, during packaging.

In the Republic of Korea (Kim *et al.*, 2007) benzidine exposure levels in 1998 were available from one benzidine-production facility and two facilities that used benzidine. In many samples benzidine was not detectable, and the highest concentration of 0.65 mg/m^3 was measured during filtering in the benzidine-based dye manufacturing plant.

(b) Biomonitoring of urinary concentrations

Measurements of benzidine and benzidine derivatives in the urine of workers in various factories are summarized in Table 1.9.

In a benzidine production plant in the USA (Zavon *et al.*, 1973) and in a chemical plant in Connecticut, USA (Meigs *et al.*, 1954), urine concentrations were measured pre- and post-shift. In both studies, leves were higher after the workshift than before.

In direct dye-production facilities in three cities of China (Bi *et al.*, 1992), urine levels were determined for selected workers in Tianjin, in 1962. Levels of renal benzidine excretion ranged between non-detectable and 0.77 mg/24 hours.

Table 1.9. Urinary levels of benzidine or benzidine derivatives in exposed workers

Reference	Country, year of study	Task	Number of workers (samples)		Level benzidine
Meigs *et al*., 1954	USA, 1950	Benzidine manufacturing – press room, 6.30 am	12 (36)	mean	0.406 +- 0.080 mg/L
		– press room, 4 pm	12 (81)	mean	1.125 +- 0.213 mg/L
Zavon *et al*., 1973	USA, >1958	Benzidine manufacturing – Monday morning	14	[see graph]	<0.02 mg/L
		– before shift	33	[see graph]	<0.07 mg/L
		– after shift	24	[see graph]	<0.159 mg/L
Lowry *et al*., 1980	USA	dye manufacturing I (Bzd)	7		0
		dye manufacturing I (MoAcBzd)	7	range	0–7 ppb (5 undetected)
		dye manufacturing II (Bzd)	4	mean +- SD	48 +- 46 ppb
		dye manufacturing II (MoAcBzd)	4	mean +- SD	233 +- 257 ppb
		textile dyeing I (Bzd)	4	–	0
		textile dyeing I (MoAcBzd)	4	range	0–4 ppb (3 undetected)
		textile dyeing II (Bzd)	8	range	0–39 ppb (6 undetected)
		textile dyeing II (MoAcBzd)	8	mean +- SD	16.7 +- 18.5 ppb (5 undetected)
		leather dyeing (Bzd)	12	–	0
		leather dyeing (MoAcBzd)	12	–	0
		paper dyeing, only using direct black 38 (Bzd)	47	range	0–1 ppb (45 undetected)
		paper dyeing, only using direct black 38 (MoAcBzd)	47	mean +- SD	3.4 +- 2.1 ppb (38 undetected)
Meal *et al*., 1981	UK	Textile dye houses	20 (114)	range	1.0–25.4 nmol/mmol creatinine (86 undetected)
		Tannery, duestuff quality control laboratories	9 (95)		ND

Table 1.9 (contd)

Reference	Country, year of study	Task	Number of workers (samples)		Level benzidine
Dewan *et al*., 1988	India, NR	Direct Black 38 manufacture	18	range	0.0024–0.3625 mg/L
Bi *et al*., 1992	China, 1962	Direct dye and benzidine production – pressure filter	5	range	0.04–0.77 mg/24h
		– transformation	3	range	0.29–0.44 mg/24h
		– reducer	2	range	ND
Rothman *et al*., 1997	India, 1993	Production of benzidine dihydrochloride and benzidine based dyes	33		
		– free benzidine		mean	1.6 ng/μmol creatinine
		– *N*-acetyl benzidine		mean	19.6 ng/μmol creatinine
		– *N,N'*-diacetylbenzidine		mean	1.0 ng/μmol creatinine
Krajewska *et al*., 1980	Poland, 1976	Benzidine manufacturing, new production process	73	range	0.0004–0.0123 mg/L (64% undetected)

ND, not detected; NR, not reported; ppb, parts per billion; SD, standard deviation

As part of a US National Institute for Occupational Safety and Health (NIOSH) industry-wide study, urine samples were collected from workers exposed to azo dyes during the dye manufacture (two sites) and use (four sites) (Lowry *et al.*, 1980). Levels of benzidine and monoacetylbenzidine were reported for different departments (see Table 1.9). Diacetylbenzidine and 4-aminobiphenyl were not detected in the urine of the workers.

In a Polish benzidine-manufacturing plant, urinary levels were below the detection limit in 64% of the samples. The detected concentrations ranged between 0.004 and 0.0123 mg/L (Krajewska *et al.*, 1980).

In a study in the United Kingdom (Meal *et al.*, 1981), 200 samples from 29 workers exposed to benzidine-derived dyes in three textile-dye houses, two tanneries, and two dyestuff quality-control laboratories were analysed. Of the 29 workers, five (from one woollen-textile industry) had detectable levels of free benzidine in their urine after acid hydrolysis, ranging between 1.0–25.4 nmol/mmol creatinine.

Indian workers in a small-scale unit manufacturing Direct Black 38 provided urine samples. Acetylated benzidine metabolites were detected in all and benzidine in all but two samples (Dewan *et al.*, 1988).

In Indian factories that manufactured benzidine dihydrochloride or benzidine-based dyes, levels of free urinary benzidine and acetyl-benzidine were measured in 33 workers. One subject had non-detectable levels of benzidine, *N*-acetylbenzidine and *N,N′*-diacetylbenzidine in his post-shift urine sample. Mean levels of free benzidine and benzidine metabolites in the remaining 32 workers were reported (Rothman *et al.*, 1997).

(c) Dermal exposure

Three studies reported dermal exposure in workers by measuring levels of benzidine in dermal wipes. They are summarized in Table 1.10.

1.8.3 *Environmental occurrence and exposure of the general population*

Benzidine-based dyes can contain varying amounts of benzidine due to contamination. Twenty-six US-produced dyes based on benzidine were found to contain < 1–20 mg/kg benzidine and one contained 270 mg/kg (IARC, 1982). Eight of 33 benzidine-based dye samples obtained from Belgium, Egypt, India, the Netherlands, Poland, Romania, and the Republic of Korea were found to contain 38–1254 mg/kg of benzidine; the others had 24 mg/kg or less (IARC, 1982).

The general population can be exposed to benzidine when in contact with consumer goods that contain benzidine or benzidine based-dyes, such as leather products (Ahlström *et al.*, 2005), clothes and toys (Garrigós *et al.*, 2002). Some food colours such as tartrazine

Table 1.10. Benzidine concentrations on dermal wipes taken in various industries

Reference	Country, year of study	Task	Number of workers		Level benzidine
Krajewska *et al*., 1980	Poland, 1976	Benzidine manufacturing, old production process – torso	27	mean (range)	4.2 (0.8–28) µg/dcm^2 (7 undetected)
		– right palm	28	mean (range)	444 (4–1800) µg/dcm^2 (2 undetected)
		Benzidine manufacturing, new production process – torso	214	mean (range)	7 (1–300) µg/dcm^2 (75% undetected)
		– right palm	224	mean (range)	13 (1.5–680) µg/dcm^2 (45% undetected)
Bi *et al*., 1992	China, 1962	Direct dye and benzidine production – transformation, handpalm	9	mean (range)	5.7 (0.5–22.2) µg/cm^2
		– transformation, hand back	9	mean (range)	3.3 (0.7–8.6)
		– transformation, front arm	9	mean (range)	5.3 (0.9–19.4)
		– transformation, breast	9	mean (range)	2.6 (0.3–12.3)
		– pressure filter, handpalm	11	mean (range)	11.7 (1.4–51.2)
		– pressure filter, hand back	11	mean (range)	5.8 (0.9–27.6)
		– pressure filter, front arm	11	mean (range)	7.9 (1.1–32.2)
		– pressure filter, breast	11	mean (range)	1.6 (0.7–3.1)
Bulbulyan *et al*., 1995	Russia, 1937–1938	Aniline dye production	NR	range	56.22 mg (sample size unknown)
	Russia, 1947–1948		NR	range	13.16–39.80 mg (sample size unknown)
	Russia, 1957		NR	range	0.22–6.08 mg (sample size unknown)
	Russia, 1971		NR	range	0.013–0.025 mg (sample size unknown)

NR, not reported

and sunset yellow FCF have been reported to contain trace amounts of benzidine (< 5 to 270 ng/g) (Lancaster & Lawrence, 1999).
The general population can also be exposed to benzidine when living near former manufacturing or disposal sites where benzidine and benzidine-based dyes were manufactured or disposed of. Benzidine and benzidine-based dyes have been detected in effluent from plants manufacturing and using dyes. In the effluent of a Brazilian textile-dye processing plant, benzidine was detected at concentrations of 47 µg/L (Alves de Lima *et al.*, 2007). In 1990, benzidine was detected at 240 µg/L (on site) and 19 µg/L (off site) in groundwater at a hazardous-waste site that was the former location of a large dye manufacturer (ATSDR, 2001). Microbial degradation of these benzidine-based dyes may release free benzidine into the environment (ATSDR, 2001).

1.9 Regulations and guidelines

Table 1.11 gives an overview of the regulations and guidelines detailed below.

1.9.1 *Europe*

(a) Council Directives 89/677/EEC and 97/56/EC

According to Council Directive 89/677/EEC, benzidine and its salts are restricted from sale to the general public (EEC, 1989). In Council Directive 97/56/EC, 3,3′dimethylbenzidine, 3,3′-dichlorobenzidine and 3,3′-dimethoxybenzidine are restricted from sale to the general public (European Commission, 1997).

(b) Council Directive 98/24/EC

According to EU regulations, the manufacture of benzidine and its salts has been prohibited since 1998. The Council Directive 98/24/EC in Annex III prohibits the production, manufacture or use at work of benzidine and its salts and activities involving benzidine and its salts. The prohibition does not apply if benzidine and its salts are present in another chemical agent, or as constituents of waste, provided that its individual concentration therein is less than 0.1% w/w (European Commission, 1998).

(c) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes which, by reductive cleavage of one or more azo groups, may release one or more of the aromatic amines (benzidine, dimethylbenzidine, dichlorobenzidine, dimethoxylbenzidine) in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance

Table 1.11. Regulations and guidelines for benzidine, 3,3′-dimethylbenzidine, 3,3′-dichlorobenzidine and 3,3′-dimethoxybenzidine (see references in text)

Country	Directive or regulatory body	Comment	Benzidine	3,3′-dimethyl-benzidine	3,3′-dichloro-benzidine	3,3′-dimethoxy-benzidine
Europe	89/677/EEC	Packaging, labelling Amendment to 76/769/EEC	x			
	97/56/EC	Packaging, labelling Amendment to 76/769/EEC		x	x	x
	98/24/EC	Ban of production and use	x			
	2002/61/EC	Marketing and use of azocolourants; amendment to CD 76/769/EEC	x	x	x	x
	2004/37/EC	Exposed workers	x	x	x	x
	76/768/EEC	Cosmetics directive	x			
	2004/93/EC	Cosmetics directive; amendment to CD 76/768/EEC		x	x	x
	2005/90/EC	List of CMR	x			
Germany	MAK (2007)		skin No BLV	2 No limit	skin No BLV	2 No limit
Japan	JSOH (2007)		1	2B	2B	2B
USA	ACGIH (2001)		A1; skin No TLV	A3; skin No TLV	A3; skin No TLV	
	NIOSH (2005)		x		x	x
	NTP (2005a,b,c,d)		K	RAHC	RAHC	RAHC

x, indicates that the regulation applies to this agent
A1, confirmed human carcinogen; A3, confirmed animal carcinogen with unknown relevance to humans; BLV, biological limit value; K, known to be a human carcinogen; RAHC, reasonably anticipated to be a human carcinogen; skin, potential significant contribution to the overall exposure by the cutaneous route; TLV, tolerable limit value;

with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(d) Directive 2004/37/EC

Benzidine and its salts are regulated by the Directive 2004/37/EC (European Commission, 2004a). The directive applies to activities in which workers are exposed to carcinogens or mutagens of category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(e) Cosmetics Directive (2004/93/EC)

The Commission Directive 2004/93/EC of 21 September 2004 amends Council Directive 76/768/EEC for the purpose of adapting Annexes II and III thereto to technical progress (European Commission, 2004b). In this directive, the following substances are listed in *Annex II* as substances that must not form part of the composition of cosmetic products: benzidine dihydrochloride; benzidine-based azo dyes; dimethylbenzidine (4,4′-bi-*ortho*-toluidine); dimethylbenzidine dihydrochloride; dimethylbenzidine-based dyes (*ortho*-tolidine-based); dichlorobenzidine; dichlorobenzidine dihydrochloride; dimethoxylbenzidine and its salts; and dimethoxylbenzidine-based azo dyes.

(f) Directive 2005/90/EC

In the Directive 2005/90/EC, the list of substances classified as carcinogenic, mutagenic or toxic to reproduction (c/m/r) of Directive 76/769/EEC was amended to include benzidine (European Commission, 2005).

1.9.2 *Germany*

Benzidine and its salts are classified as Category-1 carcinogens by the MAK Commission. The MAK Commission listed benzidine and its salts as substances where percutaneous absorption may significantly contribute to systemic exposure.

3,3′-Dichlorobenzidine is classified as a Category-2 carcinogen by the MAK Commission. The MAK Commission listed 3,3′-dichlorobenzidine as a substance where percutaneous absorption may significantly contribute to systemic exposure.

3,3′-Dimethylbenzidine and 3,3′-dimethoxybenzidine are classified as Category-2 carcinogens by the MAK Commission. No MAK values were set for these substances (MAK, 2007).

1.9.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of benzidine in Group 1; of 3,3′-dimethylbenzidine, 3,3′-dichlorobenzidine, 3,3′-dimethoxybenzidine, C.I. Acid Red 114, and C.I. Direct Blue 15 in Group 2B; and of C.I. Direct Black 38, C.I. Direct Blue 6, and C.I. Direct Brown 95 in Group 2A.

1.9.4 *USA*

(a) ACGIH

Benzidine has been assigned an A1 notation, *Confirmed Human Carcinogen*. No numerical TLV (threshold limit value) is recommended for occupational exposure for agents assigned an A1 notation. A skin notation is recommended based on the skin being a significant route of entry into the body, leading to systemic toxicity. As for any substance with no recommended TLV and an A1 carcinogenicity notification, worker exposure should be carefully controlled to the fullest extent possible (ACGIH, 2001).

3,3′-Dimethylbenzidine (*ortho*-tolidine) and 3,3′-dichlorobenzidine and its dihydrochloride salt, have been assigned an A3 notation, *Confirmed Animal Carcinogen with Unknown Relevance to Humans*. No numerical TLVs are recommended for occupational exposure to these substances. A skin notation is recommended based on the skin being a significant route of entry into the body, leading to systemic toxicity (ACGIH, 2001).

(b) NIOSH

The National Institute for Occupational Safety and Health (NIOSH, 2005) lists benzidine and 3,3′-dichlorobenzidine among 13 OSHA-regulated carcinogens. Exposures of workers to these chemicals should be controlled through the required use of engineering controls, work practices, and personal protective equipment, including respirators. OSHA and NIOSH concluded that benzidine and benzidine-based dyes were potential occupational carcinogens and recommended that worker exposure be reduced to the lowest feasible level. OSHA and NIOSH further concluded that *ortho*-tolidine (3,3′-dimethylbenzidine) and *ortho*-dianisidine (3,3′-dimethoxybenzidine) and dyes based on these compounds may present a cancer risk to workers and should be handled with caution.

(c) NTP

Benzidine and dyes that are metabolized to benzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005a) as *known human carcinogens*.

3,3′-Dimethylbenzidine and dyes that are metabolized to 3,3′-dimethylbenzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005b) as *reasonably anticipated to be human carcinogens*.

3,3′-Dichlorobenzidine and 3,3′-dichlorobenzidine dihydrochloride are listed in the NTP *Report on Carcinogens* (NTP, 2005c) as *reasonably anticipated to be human carcinogens*.

3,3′-Dimethoxybenzidine and dyes that are metabolized to 3,3′-dimethoxybenzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005d) as *reasonably anticipated to be human carcinogens*.

1.9.5 *Other*

(a) GESTIS

Table 1.12 presents some international limit values for benzidine and its congeners (GESTIS, 2007).

Table 1.12. International limit values (2007) for benzidine and its congeners

Country	Limit value – Eight hours		Limit value – Short term		Comments
	ppm	mg/m³	ppm	mg/m³	
Benzidine					
France	0.001	0.008			
Hungary				0.008	
Italy			0.001		
3,3′-Dimethylbenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
USA-NIOSH				0.02	Ceiling limit value (60 minutes)
3,3′-Dichlorobenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
Hungary				0.03	
Switzerland	0.003	0.03			
3,3′-Dimethoxybenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
Switzerland	0.003	0.03			

From: GESTIS (2007)
TRK, technical guiding concentration

2. Studies of Cancer in Humans

2.1 Case reports

Numerous case reports from different countries were reviewed and described in the IARC Monographs Volume 1, Volume 29 and Supplement 7 (IARC, 1972, 1982, 1987). Relevant studies are discussed below.

Vigliani and Barsotti (1962) reported 47 tumours of the urinary bladder (31 carcinomas, 16 papillomas) that occurred between 1931 and 1960 in six Italian dyestuff factories among workers involved in benzidine production and utilization [number of workers at risk not available]. Twenty of the 47 cases occurred between 1931 and 1948 among 83 Italian dyestuff workers. Airborne concentrations of benzidine measured in one of the plants ranged from 0 to 2.0 μg/m^3 (13 samples, mean = 0.3 μg/m^3), and urinary concentrations ranged from 6 to 25 μg/L [the number of specimens analysed was not provided].

Zavon *et al.* (1973) followed for 13 years a group of 25 men occupationally exposed to benzidine during its manufacture in a plant in Cincinnati (USA). All of the workers were exposed to benzidine, three of the workers were also exposed to 2-naphthylamine for about one year, and three to α-toluidine. Airborne benzidine concentrations at various locations within the plant varied from < 0.005 to a maximum of 17.6 mg/m^3 at a location where the workers shovelled benzidine into drums; the approximate mean urinary concentration reached 0.04 mg/L by the end of the workshift. Thirteen men (52%) developed transitional cell bladder carcinoma after a mean exposure of 13.6 years and an average latency (time from first exposure) of 16.6 years. The mean duration of exposure for those who did not develop tumours was 8.9 years. Four renal tumours were observed in three men. [The Working Group of Volume 29 considered that the high incidence of bladder cancer in this cohort was remarkable evidence of the carcinogenic potency of benzidine].

Since the publication of Supplement 7 (IARC, 1987), four case reports have documented the presence of bladder cancer cases among workers exposed to benzidine (Matsushima, 1989; Mason *et al.* 1992; Szeszenia-Dabrowska *et al.* 1997; Miyakawa *et al.* 2001). Based on their observations, Miyakawa *et al.* (2001) suggested that the latency period of occupational bladder cancer after exposure to benzidine could be longer than 40 years.

2.2 Cohort studies (see Table 2.1)

Case *et al.* (1954) studied workers from 21 dyestuff factories in England and Wales. Bladder cancer occurred approximately 15–20 years after exposure to different aromatic amines, including benzidine only, aniline, 1-naphthylamine only, 2-naphthylamine only, magenta, auramine and mixed exposures. They found a total of 127 deaths for which the

Table 2.1. Summary of cohort studies of workers exposed to benzidine

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Case *et al.* (1954), England and Wales	Cohort of 4622 dyestuff workers exposed to BZ and other aromatic amines; mortality follow-up 1921–49	Inspection of 21 participating facilities and work history data of cases; workers classified by exposure to different aromatic amines	Bladder	Overall BZ only	127 10	**SMR** 31.1 [25.9–36.9] 13.9 [6.7–25.5]		Reference, England and Wales; 34 cases reported among workers exposed only to BZ
Mancuso & el-Attar (1967), Ohio	Cohort of 639 white men exposed to BZ and/or BNA employed in 1938–39; incidence follow-up through 1965	Based on company records	Bladder and kidney	*Exposure group* BZ only BZ and BNA	 7 18	**Cumulative Incidence per 100 000** 237 1590		
Sun & Deng (1980), China	Cohort of 1601 men exposed to BZ in the chemical dye industry	Duration of employment	Bladder	*Exposure (years)* <5 5–9 10+ *p* for trend	 1 7 13	**Morbidity %** 0.1 1.4 4.7 <0.01		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Morinaga *et al.* (1982), Osaka, Japan	Cohort of 3322 men employed in BZ and BNA manufacture during 1950–78; vital status follow-up 100%	Occupational history from plant records				**SMR**		Among 244 exposed workers with previous genito-urinary cancer 11 developed second primary cancer; SMRs presented for second primary cancer; local reference
			Large intestine	Overall	2	6.9 [0.8–24.9]		
			Liver, gallbladder and bile ducts	Overall	3	8.6 [1.8–25.0]		
			Respiratory system	Overall	4	3.1 [0.9–8.1]		
Rubino *et al.* (1982); Decarli *et al.* (1985); Piolatto *et al.* (1991), Turin, Italy	Cohort of 664 male workers employed >1 year during 1922–70 in a dyestuff manufacturing plant and exposed to arylamines; mortality follow-up 1946–89, 94% complete	Occupational history from plant records included categories of exposure to selected chemicals; overall classification as exposed to aromatic amines	Bladder	Overall	49	**SMR** 30.4 [23.0–40.2]		National reference
				Time since last exposure (years)				
				During exposure	15	100.8 [60.8–167.2]		
				<10	15	39.8 [24.0–66.0]		
				10–19	12	19.5 [11.1–34.3]		
				20+	7	14.8 [7.1–31.0]		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Meigs *et al.* (1986), Connecticut, USA	Cohort of 984 workers (830 men, 154 women) of a BZ manufacturing plant employed ≥1 day during 1945–65; incidence follow-up 1945–78	Occupational history from employment, production and sales records; expert reconstruction of employment histories and estimation of time exposed to BZ	Bladder			**SIR**		State reference; cancer incidence decline[d] >1950, coinciding with measures to reduce exposure
				Overall	8	3.4 [1.5–6.8]		
				BZ exposure in men				
				≤1 day	1	1.8 (0.1–10.1)		
				>1 day–6 months	0	0 (0–4.7)		
				>6 months–<2 years	1	1.9 (0.1–10.7)		
				≥2 years	6	13.0 (4.8–28.4)		
				Employment (years)				
				<1	0	0 (0–3.2)		
				1–5	2	3.4 (0.4–12.4)		
				5+	6	10.0 (0.6–21.7)		
Wu (1988), Shanghai, China	Cohort of 2525 workers (1860 men, 665 women) of BZ manufacturing plants employed >1 year during 1972–81	N/A	Bladder			**SIR**		Local reference
				Overall	30	26.1 (18.8–35.4)		
						RR		
				Interaction analysis				
				BZ -, smoking -		1.0		
				BZ -, smoking +		6.2 (p=0.05)		
				BZ +, smoking -		63.4 (p <0.05)		
				BZ +, smoking +		152.3 (p <0.01)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Delzell (1989); Sathiakumar and Delzell (2000), New Jersey, USA	Cohort of 3266 workers at a dye and resin manufacturing plant (2859 men, 407 women) employed >6 months during 1952–1995; mortality follow-up 1952–1995; vital status 99%; cause of death 97%	Occupational history from plant records; subjects classified by dates of employment and years worked in 8 major work areas; North Dyes area used BZ from 1959–1970				**SMR**		State reference
			Bladder	Overall	8	1.4 (0.6–2.7)		
				BZ use	4	5.2 (1.4–13.2)		
			Lymphopoietic	Overall	12	0.5 (0.2–0.8)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
You *et al.* (1990), Shanghai, China	Cohort of 736 BZ dyes production workers (550 men, 186 women) employed >6 months; mortality and incidence follow-up from first entry to 1982; vital status 100%; 100% histologically confirmed	Occupational history from plant records; jobs classified into pre-synthesis (chemical changes from BZ to dyes) and post-synthesis (processes leading to finished dyes) groups	Bladder			**SMR**		Local reference
				Men	5	14.7 (p <0.01)		
				Women	0	0		
				Jobs among men				
				Pre-synthesis	5	31.3 (p <0.01)		
				Material treatment	2	66.7 (p <0.01)		
				Synthetic reaction	3	30.0 (p <0.01)		
				Maintenance and others	0	0		
				Post-synthesis	0	0		
						SIR		
				Men	14	19.2 (p <0.01)		
				Women	1	50.0 (p <0.05)		
				Jobs among men				
				Pre-synthesis	14	35.0 (p <0.01)		
				Material treatment	6	75.0 (p <0.01)		
				Synthetic reaction	7	26.9 (p <0.05)		
				Maintenance and others	1	20.0 (p <0.05)		
				Post-synthesis	0	0		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Szeszenia-Dabrowska *et al.* (1991), Poland	Cohort of 6978 male rubber goods production workers employed >3 months during 1945–73; mortality follow-up 1945–85; vital status 90%	Occupational history from plant records; type and concentration of chemical exposure indirectly estimated				**SMR**		National reference; multiple exposures, including BNA
			Bladder	Overall	10	1.2 [0.6–2.2]		
				Subcohort employed during 1945–53	6	2.8 [1.2–6.1]		
			Lympho-haematopoietic (200–208)	Overall	7	0.5 [0.2–1.03]		
Shinka *et al.* (1991); Shinka *et al.* (1995), Wakayama City, Japan	Cohort of 363 workers of 9 dye manufact-uring plants; incidence follow-up to 1964–94; vital status 100%	Occupational history from plant records; workers classified by potential BZ or BNA exposure				**OR**		
			Urothelial	*All factories*				
				BZ exposure	49	8.3 (1.6–42.6)		
				BNA exposure	3	1		
				BZ and BNA	6	4.3 (0.9–19.7)		
				Factory A only				
				BZ exposure	4	12.7 (2.0–81.2)		
				BNA exposure	2	1		
				BZ and BNA	6	6.2 (1.1–35.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Bi *et al.* (1992); Hayes *et al.* (1993); Carreón *et al.* (2006), Tianjin, Shanghai, Jilin, Henan and Chonquin, China	Nested case-control study in cohort of 2515 workers (1850 men, 665 women) employed >1 year during 1945–77 in BZ production and use facilities; 68 cases (diagnosed 1965–1991), 107 controls frequency-matched by 10 year age	Based on knowledge of operations and limited IH data, BZ exposure level assigned to each job and multiplied by duration of exposure	Bladder			**OR**	Lifetime cigarette smoking	
				BZ cumulative level years				
				Low (<30)	32	1.0		
				Medium (30–59)	15	2.7 (1.1–6.3)		
				High (≥60)	17	4.4 (1.8–10.8)		
Bulbulyan *et al.* (1995), Moscow, Russia	Cohort of 4581 aniline dye production workers (2409 men, 2172 women) employed on Jan.1, 1975 and exposed >1 month to BZ or BNA, or employed for >2 years; 514 men, 287 women exposed to BZ or BNA; mortality and incidence follow up 1975–89; ca 90% histologically confirmed cases	Limited industrial hygiene air and environmental measurements; jobs classified into groups based on BZ or BNA exposure	Bladder			**SIR**		Local reference, no lymphohaematopoietic cancer SIR provided for group exposed to BZ or BNA
				Ever exposed to BZ or BNA				
				Men	19	10.8 [6.9–17.0]		
				Women	5	21.0 [8.7–50.4]		
				Ever exposed to BZ				
				Employment (years)				
				<10	6	11.2 (4.1–24.3)		
				10–19	7	17.2 (6.9–35.4)		
				20–29	2	5.7 (0.6–20.6)		
				30–39	2	4.7 (0.1–26.1)		
				40+	1	13.6 (0.2–75.9)		
				p for trend		0.22		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Szymczak *et al.* (1995); Sitarek *et al.* (1995), Poland	Cohort of 10 529 dye production workers (8523 men, 2006 women) employed >3 months during 1945–74; mortality follow-up 1945–91	Workers classified into 4 exposure groups: I- BZ only, II-BZ & other occupational hazards, III -involved in dye production with no BZ exposure, IV- not involved in dye production	Bladder	*Men*		**SMR**	Age, gender, calendar time	National reference
				Exposed to BZ only	9	14.7 [7.6–28.2]		
				Exposed to BZ & other occupational hazards	15	16.3 [9.9–27.1]		
			Lympho-haematopoietic (200–208)	Exposed to BZ only	2	1.7 [0.4–6.7]		
Naito *et al.* (1995), urban area, Japan	Cohort of 442 workers of a BZ production and dye manufacturing plant (437 men, 5 women) during 1935–88; mortality and incidence follow-up 1935–92; vital status 100%	Duration of employment at BZ manufacture or use facility as surrogate of duration of exposure	Urinary tract (188, 189)	BZ manufacture	14	**SMR** 45.1 (24.7–75.7)		National reference; incidence rates reported by duration of exposure; PPE reportedly used among all workers
				BZ use	6	15.8 (5.8–34.3)		
			Bladder	BZ manufacture	10	63.6 (30.5–117.0)		
				BZ use	5	27.0 (8.8–63.0)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Rosenman & Reilly (2004), Michigan, USA	Cohort of 488 white men employed in a chemical manufacturing facility during 1960–77; mortality follow-up 1979–2001; incidence follow-up 1981–2002 (Michigan Tumour Registry)	Time and length of employment estimated from social security records; workers classified as exposed to BZ or not if employed before or after 1973	Bladder	Overall	3	**SMR** 8.3 (1.7–24.4)		National reference for SMR and SEER for SIR
				Year started work				
				<1973	3	9.6 (2.0–28.1)		
				≥1973	0	0		
			Lympho-haematopoietic	Overall	6	2.8 (1.04–6.2)		
				Year started work				
				<1973	3	1.8 (0.4–5.3)		
				≥1973	3	6.6 (1.4–19.4)		
			Leukemia	Overall	4	5.1 (1.4–12.9)		
			Bladder	Overall	22	**SIR** 6.9 (4.3–10.4)		

ANA, 1-naphthylamine; BNA, 2-naphthylamine; BZ, benzidine; IH, industrial hygiene; ND, not determined; OR, odds ratio; PPE, personal protective equipment; SEER, Surveillance, Epidemiology and End Results Program; SIR, standardized incidence ratio; SMR, standardized mortality ratio

death certificate indicated bladder cancer (4.09 expected). For workers exposed exclusively to benzidine, 10 confirmed bladder-cancer deaths were found (0.72 expected). The authors also reported a total of 34 incident bladder-cancer cases among workers exposed to benzidine only.

In a study of 639 white men exposed to benzidine and/or 2-naphthylamine in a plant in Ohio (USA), Mancuso & el-Attar (1967) reported 14 bladder-cancer deaths and a total of 18 genitourinary cancer deaths. The mortality rate for bladder cancer in the cohort was 78/100 000 versus 4.4/100 000 expected on the basis of Ohio male mortality rates. The cumulative risk for incident bladder cancer in workers exposed to benzidine was reported to be 237 per 100 000. The authors also noted six cases of pancreatic cancer, with a cumulative mortality rate of 39 per 100 000 (*vs* 7.5/100 000 expected).

Among 1601 workers in the chemical-dye industry in China who were exposed to benzidine, methylnaphthylamine and dianisidine, An & Deng (1980) reported 21 cases of bladder cancer. All cases had a history of exposure to benzidine, and no cancer was found among workers exposed to methylnaphthylamine or dianisidine. An exposure-response relationship was suggested, as the percentage of cases among exposed workers increased with length of exposure (test for trend $P < 0.01$).

Morinaga *et al.* (1982) ascertained the incidence of second primary cancers in 3322 workers employed from 1950 to 1978 in industries in Japan that manufactured benzidine and 2-naphthylamine. Of the 244 workers who had developed cancer of the genitourinary organs, 11 men subsequently developed histologically confirmed cancers of the liver, gallbladder, bile duct, large intestine, and lung. An unexposed group of 177 male bladder-cancer patients, assembled from the Osaka Cancer Registry during 1965–1975, showed eight cases of a second primary cancer, five being stomach cancer. No stomach cancer was observed in the study cohort. A statistically significant excess risk for liver, gallbladder, and bile-duct cancer ($P < 0.05$) was found. The number of observed deaths from respiratory cancer was greater than expected, but not statistically significant.

In 1982, Rubino *et al.* reported the results of a retrospective cohort study in dye-manufacturing workers of Turin, Italy. A very high bladder-cancer risk was observed among workers exposed to benzidine (five deaths observed, SMR = 83.3). An extended follow-up conducted by Decarli *et al.* (1985) reported 41 deaths (SMR 46.1; 95% CI, 33.9–62.6) for the total cohort. Piolatto *et al.* (1991) added eight years of follow-up. The cohort included 664 male workers employed more than one year from 1922 to 1970. Occupational history was obtained from plant records and included categories of exposure to selected chemicals including benzidine. The overall bladder-cancer mortality risk was very high (49 deaths, SMR 30.4; 95% CI, 23.0–40.2). The risk for bladder cancer was also found to vary inversely both with age at first exposure and time since last exposure. The authors reported elevated SMRs for upper digestive and respiratory tract cancers.

Meigs *et al.* (1986) reported a statistically significant excess of bladder tumours in a cohort of 984 workers at a benzidine-manufacturing facility in Connecticut. Benzidine-exposure status was determined from information contained in employment, production

and sales records. Eight cases of bladder cancer were observed (SIR 3.4; 95% CI, 1.5–6.8). Risk was greatest among those in the highest exposure category (SIR 13.0; 95% CI, 4.8–28.4). Risk also showed an increasing trend with length of employment: < 1 year, SIR = 0 (1.15 expected; 95% CI, 0–3.2); 1–5 years, SIR = 3.4 (95% CI, 0.4–12.4); and > 5 years, SIR 10.0 (95% CI, 0.6–21.7). The authors report a decline in the overall bladder-cancer incidence among those employed after 1950, coincident with the implementation of major preventive exposure measures, but indicate that this finding is limited by the small number of cases.

Wu (1988) reported the results of studies conducted by The Cooperative Group in China. One of these studies was a retrospective cohort study of 2525 workers exposed to benzidine for at least one year from 1972 through 1981 [the author reports that the production and use of benzidine in China was stopped in 1977]. Twelve deaths and 30 incident bladder-cancer cases were observed. An excess incidence of bladder cancer compared with the Shanghai general population was observed (SIR 26.1; 95% CI, 18.8–35.4). A synergistic effect of smoking on benzidine-associated bladder-cancer risk was also observed. When compared with non-smoking and non-exposed workers, the relative risk for bladder cancer of smoking non-exposed workers was 6.2, compared with a risk of 63.4 for non-smoking exposed workers, and a risk of 152.3 for smoking exposed workers. In addition to the principal findings related to bladder cancer, a slight increase in the incidence of lung and stomach cancers was noted in workers exposed to benzidine. [The Working Group noted that quantitative data were not provided.]

A cohort of workers employed at a New Jersey (USA) dye and resin manufacturing plant was examined from 1952 to 1985 as part of a larger retrospective study of 2642 workers (Delzell *et al.* 1989). Occupational history was obtained from plant records, and department titles were classified into 10 work areas. The azo-dye area involved exposures to dye-related compounds including benzidine. Eighty-nine of the workers had former employment at the Cincinnati Chemical Works (CWW), which had produced or used benzidine and 2-naphthylamine. The 2553 workers who had never worked at the CWW, and therefore had little potential for benzidine exposure, had fewer than expected deaths from all causes combined and from diseases of all major organ systems. The former CWW workers had an excess of cancer, which was due to excess mortality from bladder (SMR 12, $P = 0.004$), kidney (SMR 9.5, $P = 0.04$), and central nervous system (SMR 9.1, $P = 0.04$) cancers. Sathiakumar and Delzell (2000) added 10 years of follow-up by extending the cohort through 1995. The expanded cohort included 3266 workers (2859 men, 407 women) employed more than 6 months. The bladder-cancer mortality excess observed in the earlier study among former CWW workers remained in the expanded cohort. The overall SMR for bladder cancer among all white men employed in the North Dyes area (where benzidine was used from 1959 to 1970) was 5.2 (95% CI, 1.4–13.2). The authors attributed the bladder-cancer excess to exposure to aromatic amines at the CWW, since plant employees that had not worked at the CWW had approximately equal observed and expected deaths from bladder cancer.

You *et al.* (1990) conducted a retrospective cohort study in seven factories producing benzidine-based dyes in Shanghai, China. The cohort included 736 production workers (550 men, 186 women) employed for more than six months. Occupational history was obtained from plant records and included accumulated working time in benzidine production. Men were classified based on their jobs into two groups: pre-synthesis (thought to have been exposed to benzidine) and post-synthesis (exposed mainly to finished benzidine-based dyes). Five deaths and 14 cases of bladder cancer in men were observed, all in the pre-synthesis group. Increased mortality and incidence were observed in the whole cohort and in the pre-synthesis group, particularly among those workers involved in material treatment (SIR 75.0, $P < 0.01$) and synthetic reaction (SIR 26.9, $P < 0.05$).

Szeszenia-Dabrowska *et al.* (1991) studied a cohort of 6978 men employed in rubber goods production, predominantly rubber footwear, in Poland. The cohort included workers employed for more than three months from 1945 to 1973. Occupational history was obtained from plant records. The authors indicated that aromatic amines, including benzidine, are among the chemicals used as additives in rubber factories. The type and level of chemical exposure were indirectly estimated. Ten deaths from bladder cancer were observed in the whole cohort (8.4 expected), and six were among workers employed in production. Among the subcohort employed during 1945 and 1953, and presumably exposed to aromatic amines including benzidine, the SMR for bladder cancer was 2.8 (95% CI, 1.2–6.1). Seven deaths from lymphopoietic cancers were observed (14.2 were expected).

Shinka *et al.* (1991) observed that 105 of 874 Japanese workers of nine dye-manufacturing plants who were engaged in the manufacture and handling of benzidine developed urothelial (primarily bladder) cancer. In a more recent study, Shinka *et al.* (1995) extended the follow-up of 363 exposed workers. Occupational history was obtained from plant records and workers were classified into exposure categories with regard to benzidine or 2-naphthylamine. Workers were also classified according to their work in benzidine manufacture, benzidine use, or both. The risk factors significantly related to tumour occurrence in all nine plants were benzidine as a dye intermediate (OR 8.3; 95% CI, 1.6–42.6) and manufacturing work (OR 4.6; 95% CI, 1.9–11.0).

In a cohort of 1972 benzidine-exposed workers in China between 1972 and 1977, Bi *et al.* (1992) examined bladder-cancer mortality and incidence through 1981. Limited industrial hygiene data on benzidine were available, and were used in combination with knowledge of operations to classify the job held for the longest time as being associated with a high, medium or low exposure to benzidine. The authors reported an overall SMR of 17.5 (eight deaths; 95% CI, 7.5–34.5) and an overall SIR of 25 (30 cases, 95% CI, 16.9–35.7), with risks ranging from 4.8 (95% CI, 1.0–14.1) to 158.4 (95% CI, 67.6–309.0) for low to high exposures. Further, bladder cancer was positively associated with exposure duration. Benzidine-exposed workers who also smoked cigarettes had a 31-fold increased risk for bladder cancer, compared with an 11-fold increased risk observed in

non-smoking workers. Hayes *et al.* (1993) conducted a nested-case control study in 38 bladder cancer cases and 43 controls from this cohort. Using the exposure categories developed by Bi *et al.*, and assigning them a score of 1 for low, 3 for medium and 9 for high, the authors estimated cumulative benzidine exposure as the product of each score times duration of exposure. Cumulative benzidine level-years were categorized into low (< 30), medium (30–59) and high (≥ 60) exposure. Compared with low exposure, the risks for medium and high exposure were OR 2.6 (95% CI, 0.8–8.9) and OR 6.7 (95% CI, 1.7–33.6), respectively. Carreón *et al.* (2006) expanded the previous study to include 68 cases and 107 controls frequency-matched by 10-year age groups. Using the same cumulative benzidine-exposure categories, the risks for medium and high exposure, compared with low, were OR 2.7 (95% CI, 1.1–6.3) and OR 4.4 (95% CI, 1.8–10.8). These risks were adjusted for lifetime cigarette smoking. Additionally, Carreón *et al.* (2006) reported that *NAT2* genotype slow acetylators had a reduced risk for bladder cancer when compared with rapid acetylators (OR, 0.3; 95% CI, 0.1–1.0). The study did not have sufficient power to evaluate the interaction between *NAT2* polymorphisms and benzidine exposure. To overcome this limitation, the authors compared the result presented above with the result of a meta-analysis of eight case–control studies of NAT2 acetylation and bladder cancer in Asian populations not exposed occupationally to aromatic amines. The authors concluded that there was evidence of a gene-environment interaction, as the upper limit of the estimate obtained in their study adjoined the lower limit obtained in the meta-analysis (pooled OR, 1.4; 95% CI, 1.0–2.0).

Bulbulyan *et al.* (1995) evaluated a cohort of 4581 aniline dye-production workers for cancer incidence and mortality. The study included limited industrial hygiene air and environmental measurements of benzidine and 2-naphthylamine dating back to the 1930s; jobs were classified into groups based on exposure to benzidine and 2-naphthylamine. In a group of 514 men and 287 women who had been ever exposed to benzidine or 2-naphthylamine, there were 115 observed cases of all cancers *vs* 62.57 expected cases. Among men ever exposed to benzidine or 2-naphthylamine, increased incidence was observed for cancers of the oesophagus (SIR 3.5; 95% CI, 1.4–8.4), lung (SIR 2.3; 95% CI, 1.5–3.4), and bladder (SIR 10.8; 95% CI, 6.9–17.0). Women had a statistically significant excess of bladder cancer (21.0; 95% CI, 8.7–50.4). Excess cancer rates, including for bladder cancer, were also found in men and women exposed only to "other" chemicals. The risk for bladder cancer did not increase with duration of employment for workers ever-exposed to benzidine, but it did for workers ever-exposed to 2-naphthylamine.

Naito *et al.* (1995) conducted a retrospective cohort mortality study of 442 workers (437 men, five women) exposed to one or more substances (mainly benzidine, 2-naphthylamine, 1-naphthylamine, and *ortho*-dianisidine) at a benzidine-production and dye-manufacturing plant in Japan. No industrial hygiene data for the plant were available; therefore, duration of employment at the facility was used as a surrogate of duration of exposure. The authors reported that all the workers wore work clothes, gloves, high

rubber boots, and a gas mask in the factory, and that it had wide windows in all directions. A significantly increased risk for bladder cancer was found among workers engaged in the manufacture (SMR 63.6; 95% CI, 30.5–117.0) and use (SMR 27.0; 95% CI, 8.8–63.0) of benzidine. Increased risks for cancer mortality for other organs were observed, but these were not statistically significant. [No information was provided for lymphohaematopoietic cancers.] Incidence rate-ratios of urothelial cancer increased with duration of exposure for both benzidine manufacture and use.

Szymczak *et al.* (1995) and Sitarek *et al.* (1995) carried out a mortality study among 10 529 workers in a dye-manufacturing plant in Poland, involving exposure to benzidine. Workers were classified into four groups based on their potential to benzidine exposure. A statistically significant increase in mortality from bladder cancer was observed among men exposed to benzidine only (SMR 14.7; 95% CI, 7.6–28.2) and benzidine and other occupational hazards (SMR 16.3; 95% CI, 9.9–27.1). Increased mortality risks were also observed among men exposed to benzidine and other occupational hazards for pancreatic cancer (SMR = 3.3; 95% CI, 1.2–8.7). No excess mortality for lymphohaematopoietic cancers was observed among men exposed to benzidine alone or in combination with other occupational hazards.

Rosenman and Reilly (2004) analysed a cohort of 488 white men employed in a chemical manufacturing facility in Michigan, USA. The facility had produced benzidine from 1960 through 1972 and 3,3′-dichlorobenzidine from 1961 to 2001. Workers were identified from social security records. Since no plant records were available, social security data were used to estimate time of first work and years worked. Analyses were conducted for the entire cohort and separately for people who began to work in 1973 or later, after benzidine production had been discontinued. For the whole cohort, an excess of bladder-cancer mortality was observed (SMR 8.3; 96% CI, 1.7–24.4). All cases occurred in those with five or more years of duration of work. There were six deaths from lymphohaematopoietic cancer (SMR 2.8; 95% CI, 1.04–6.2) including one from non-Hodgkin lymphoma, one from multiple myeloma, two from chronic lymphocytic leukaemia, one from acute leukaemia, and one from chronic myelogenous leukaemia (SMR for leukaemia 5.1; 95% CI, 1.4–12.9). The SIR for bladder cancer was 6.9 (95% CI, 4.3–10.4); no additional cases of lymphohematopoietic cancer were identified from the cancer registry. All bladder-cancer deaths and 21 of 22 cases of bladder cancer occurred among those employed before 1973. A statistically significant increase in mortality from lymphohematopoietic cancer was observed among workers who began work in 1973 or later.

2.3 **Case-control studies** (see Table 2.2)

Since the publication of Supplement 7, a few case–control studies of benzidine exposure and the risk for bladder and other cancers have been published. These studies have a high potential for misclassification of exposure, as information is collected on past

Table 2.2. Summary of case-control studies of workers exposed to benzidine

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors & comments
Schumacher and Slattery (1989), Utah, USA	Bladder	417 (332 men, 85 women) from state cancer registry, aged 21-84; response rate 76%	877 (685 men, 192 women) population-based controls, frequency-matched for age and sex in a 2:1 ratio; response rate 79%	Interviewer-administered standardized questionnaire; occupational-exposure linkage system used to identify workers exposed to BZ	*BZ exposure men* Never Ever <10 years 10+ years *BZ exposure women* Never Ever	 1.0 1.2 (0.7–2.1) 1.0 (0.5–2.1) 1.6 (0.6–4.1) 1.0 1.0 (0.4–2.2)	Adjusted for age, smoking, religion, education smoking
You *et al.* (1990), Shanghai, China	Bladder	317 men from Shanghai industry, aged 23–78 years; 41 had occupational exposure to BZ; response rate 100%	317 hospital-based non-cancer controls, matched by hospital, gender, age within 5 years, from same industrial and residential districts as cases	Employment record, workers employed >6 months in the dyestuffs, rubber, cable, ink, dress pressing and cigarette industries were considered exposed	Occupational exposure to BZ	5.7 (p <0.001) ND	(Smoking)
Ugnat *et al.* (2004), British Columbia, Alberta, Saskatchewan, Manitoba, Canada, 1994–97	Bladder	549 men from provincial cancer registries aged 20–75 years; 14 reported BZ exposure; response rate 60%; 100% histologically confirmed	1099 population-based controls (15 reported BZ exposure) frequency-matched by sex, 5-year age group; response rate 59%	Mailed standardized questionnaire, telephone interview to those who failed to return it	Ever exposed to BZ *BZ exposure (years)* Never 1–9 10–19 20+ p for trend	2.2 (1.0–4.9) 1.0 2.7 (0.7–10.5) 0 2.7 (0.7–9.1) 0.26	Adjusted for age, province, education, smoking, exposure years, coffee and tea intake

Table 2.2 (contd)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors & comments
Mao *et al.* (2000), British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Prince Edward Island, Nova Scotia and Newfoundland, Canada, 1994–97	Non-Hodgkin lymphoma	1469 (764 males, 705 females) from provincial cancer registries, aged 20–74 years; 24 (21 men, 3 women) reported BZ exposure; response rate 75%; 100% histologically confirmed	5073 population-based controls (55 reported BZ exposure) frequency-matched by 5-year age group, sex and province; response rate 67%	Mailed standardized questionnaire, telephone interview to those who failed to return it	*Ever exposed to BZ* Men Women *BZ exposure in men (years)* Never 1–3 ≥4 *p* for trend	 1.9 (1.1–3.4) 0.6 (0.2–2.2) 1.0 1.1 (0.3–4.4) 2.2 (1.1–4.0) 0.02	Adjusted for age, province, BMI, milk intake
Hu *et al.* (2002), British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Prince Edward Island, Nova Scotia and Newfoundland, Canada, 1994–97	Renal cell carcinoma	1279 (691 men, 588 women) from provincial cancer registries, aged 20–70+ years; 32 (28 men, 4 women) reported BZ exposure; response rate 79%; 100% histologically confirmed	5370 population-based controls (66 reported BZ exposure) frequency-matched by 5-year age group, sex and province; response rate 71%	Mailed standardized questionnaire, telephone interview to those who failed to return it	*Ever exposed to BZ* Men Women *BZ exposure in men (years)* Never 1–10 11+ *p* for trend	 2.1 (1.3–3.6) 1.0 (0.3–3.1) 1.0 1.8 (0.8–3.9) 2.5 (1.2–5.0) 0.004	Adjusted for age, province, education, BMI, smoking, alcohol, meat intake

BMI, body mass index; BZ, benzidine; SD, standard deviation

events that may be inaccurately recorded or not available, and information obtained from study participants may be biased. In addition, the level of exposure in these studies is likely to be, on average, much lower than that in the cohort studies, which tend to focus on workers with the highest exposures to benzidine.

In a population-based case–control study in Utah (USA), Schumacher *et al.* (1989) examined the associations between bladder cancer and occupational exposures, including benzidine. The authors included 417 bladder-cancer cases diagnosed between 1977 and 1983, identified from a rapid ascertainment system and the state cancer-registry. Population-based controls were randomly selected and frequency-matched to cases by age and sex in a 2:1 ratio. The number of controls was 877 (685 men, 192 women). Trained interviewers conducted home interviews to obtain complete occupational histories and information on bladder-cancer risk factors. An occupation-exposure linkage system was used to identify workers exposed to suspect bladder carcinogens, including benzidine and 2-naphthylamine. The system graded exposures as "light," "moderate," "heavy" and "unknown". Those with heavy exposures were classified as exposed, and the rest were considered unexposed. The numbers of workers exposed to benzidine and 2-naphthylamine were identical, since the same occupations were linked to both chemicals. Among men, an increased risk for bladder cancer was observed among those exposed to benzidine, even though the association did not reach statistical significance (OR, 1.2; 95% CI, 0.7–2.1). A non-statistically significant risk increase was observed among men with more than 10 years of exposure (OR, 1.6; 95% CI, 0.6–4.1). No increased risk for bladder cancer was observed among women ever-exposed to benzidine. Smoking did not confound these associations.

You *et al.* (1990) selected 317 men with bladder cancer from Shanghai, China, 41 of whom were considered to have been occupationally exposed to benzidine if employed for more than six months in the dyestuffs, rubber, cable, ink, dress pressing or cigarette industries. The study included 317 hospital-based non-cancer controls, matched by hospital, gender, and age within five years. Controls were from the same industrial and residential districts as the cases. Occupational exposure to benzidine was associated with bladder cancer incidence (OR, 5.7; $P < 0.001$).

A series of case–control studies for different cancers and occupations have been conducted in Canada. All of the studies involved the selection of 19 types of cancer (20 755 cases) from eight provincial cancer registries. The cancer registries ascertained cases on the basis of pathology reports. Cancer-free population-based controls (5039) were selected from a random sample of individuals within the provinces. Sampling strategies for controls varied among provinces. Controls were frequency-matched to all cancer cases by age and sex distribution. A standardized questionnaire including occupation and cancer risk factors was mailed to study participants. The questionnaire also recorded information on exposure to 17 different chemicals, including benzidine, for at least a year, and the duration of that exposure. Telephone interviews were conducted with those subjects who failed to return the questionnaire. [These studies have large

sample sizes, are population-based, and control for the known risk factors in each analysis, but have the known limitations of case–control studies, including the low reliability of self-reported exposures and exposure misclassification].

In the most recent analysis of the Canadian series, Ugnat *et al.* (2004) studied 549 men with histologically-confirmed bladder cancer and 1099 male controls. They observed an increased risk for bladder cancer among people who reported exposure to benzidine (adjusted OR, 2.2; 95% CI, 1.0–4.9). The exposure-response relationship was not statistically significant (p for trend = 0.26).

Mao *et al.* (2000) studied 1469 newly diagnosed cases of non-Hodgkin lymphoma and 5073 population-based controls. The authors found increased risks for non-Hodgkin lymphoma among men exposed to benzidine (adjusted OR, 1.9; 95% CI, 1.1–3.4). Among men, an exposure-response effect was observed with increased length of exposure to benzidine (p for trend = 0.02).

Hu *et al.* (2002) analysed the risk for renal cell cancer among 1279 cases and 5370 population-based controls. An increased risk for renal cell carcinoma was observed among men (adjusted OR, 2.1; 95% CI, 1.3–3.6) but not among women (adjusted OR, 1.0; 95% CI, 0.3–3.1). An increased risk with duration of exposure to benzidine was observed among men (p for trend = 0.004).

3. Studies of Cancer in Experimental Animals

Animal bioassays conducted with benzidine were reviewed in IARC Monograph Volumes 1 and 29, and in Supplement 7. The studies mentioned in Volume 1 were all reconsidered in Volume 29. This Section provides a summary of these studies and a more detailed review of more recent ones.

3.1 Benzidine

3.1.1 *Oral administration*

(a) Mouse

Four groups of 50 male B6C3F_1 mice, six weeks of age, were fed 150 ppm benzidine dihydrochloride (certified American Chemical Society (ACS) grade) in the diet for 45 weeks. Groups of 50 mice were killed at 45, 60, 75 and 90 weeks of age, respectively, to evaluate the occurrence of liver-cell tumours. At 45 weeks, 8/50 (16%) mice had tumours, 4% of which were hepatocellular carcinomas. At 60, 75 and 90 weeks the proportions of mice with tumours were 20/50 (40%), 31/50 (62%) and 35/50 (70%). Of these tumours, 10%, 28% and 48%, respectively, were hepatocellular carcinomas. In

historical controls, the incidence of hepatocellular tumours was 1/98 (1%) (Vesselinovitch *et al.,* 1975). [No statistical analysis was applied.]

Three groups of 50 male B6C3F$_1$ mice, six weeks of age, were fed 150 ppm benzidine dihydrochloride (certified ACS grade) in the diet until 45, 60, or 90 weeks of age. All animals were killed at 90 weeks of age. The incidences of mice bearing liver tumours, mostly hepatocellular carcinomas, are given in Table 3.1. A negative relationship was observed between the incidence of liver tumours and the duration of treatment, which may have been related to toxicity (Vesselinovitch *et al.,* 1975). [No statistical analysis was applied.]

Table 3.1. Incidences of liver-cell tumours in male B6C3F$_1$ mice fed benzidine dihydrochloride

Duration of treatment (weeks)	Estimated consumption of benzidine (mg/mouse)	Effective no. animals	Liver-cell tumours	%
39	117	50	35	70
54	162	50	25	50
84	188	50	22	44

From Vesselinovitch *et al.* (1975)

To evaluate the effect of mode of administration in benzidine-induced carcinogenesis, groups of 50 male and 50 female B6C3F1 mice were given doses of 50 or 100 ppm benzidine dihydrochloride (certified ACS grade) in the feed, or twice weekly by stomach tube, at 0.5 or 1.0 mg/treatment. No effects on survival were noted, and all animals were killed at 90 weeks of age. Continuous feeding of benzidine in the diet produced liver-cell tumours in 3/50 (6%) males and in 13/50 (26%) females at the lower dose, and in 11/50 (22%) males and 32/50 (64%) females at the higher dose, which indicates a greater susceptibility of female animals. One liver tumour was seen in 98 male control mice, none in 100 control females. Twice-weekly administration of benzidine by stomach tube seemed to have a weaker hepatocarcinogenic effect than continuous feeding at comparable amounts, especially in female mice: 4/75 (5%) had tumours after intermittent feeding *vs* 13/50 (26%) upon continuous feeding of 50 ppm [no statistical analysis was applied]. Benzidine also caused Harderian gland tumours and lung adenomas in both treatments, and had a marginal effect on the development of lymphoreticular tumours (Vesselinovitch *et al.*, 1975).

Groups of 43–100 B6C3F$_1$ male and female mice [age not specified] were fed a diet containing 150 ppm benzidine dihydrochloride [purity unspecified] (1) from the 12th day of gestation (prenatal) to delivery; (2) to mothers with litters from delivery to weaning; (3) to offspring from weaning to 90 weeks of age; (4) during the pre-natal and pre-weaning period; or (5) prenatally, during pre-weaning and in adulthood. Groups of

untreated controls were also available. Administration pre-natally or during pre-weaning induced a marked increase in the incidence of hepatocellular tumours in male mice (31 and 95%, respectively) but not in females (3 and 5%, respectively). In mice treated from weaning to 90 weeks of age, the tumour incidences were 59% in males and 96% in females. In the group treated both pre-natally and during pre-weaning, the incidences were 100% in males and 25% in females. When mice were treated pre-natally, during pre-weaning and then up to 90 weeks of age, the incidences of hepatocellular tumours were 100% in males and 94% in females [no statistical analysis applied]. The incidences of hepatocellular tumours in untreated controls were 1% in males and 0% in females (Vesselinovitch *et al.,* 1979).

Groups of F_1 (C57BL/6Jf C3Hf/Nctr females × BALB/cStCrLfC3Hf/Nctr males) and mono-hybrid (F_1 females and F_1 males) weanling mice were fed diets containing 0, 30, 60, 120, 200 or 400 ppm of benzidine hydrochloride [purity unspecified]. The 400-ppm dose was chosen on the basis of preliminary tests, the highest dose being probably the maximum tolerated dose. Groups of mice were killed after 40, 60 or 80 weeks of treatment. The incidences of hepatocellular adenomas and carcinomas in the control and treated groups, summarized in Table 3.2, were increased in treated mice (Nelson *et al.,* 1982).

In studies designed to assess the susceptibility of mice to liver tumours at different stages of development, groups of $B6C3F_1$ mice were fed diets containing 150 ppm of benzidine dihydrochloride [purity not specified]. Pregnant female mice were fed from the 12th day of gestation to delivery (group 1), to mothers with litters from delivery to weaning (group 2) and to offspring from weaning through 90 weeks (group 3). Ninety-eight to 100 $B6C3F_1$ male and female mice were killed at 52, 90 and 142 weeks and served as controls. The incidences of hepatocellular adenomas and carcinomas are summarized in Table 3.3. The incidences of carcinomas were increased in the pre-weaning and adult mice (Vesselinovitch, 1983). [No statistical analysis was applied.]

Groups of 72–120 F_1 (BALB/cStCr1fC3Hf/Nctr males × C57BL/6Jf C3Hf/Nctr females) and mono-hybrid cross (MC) (F_1 males and F_1 females) mice, 4–5 weeks of age, weighing 8–15 g were given drinking-water containing 0, 30, 40, 60, 80, 120 or 160 ppm (for males) and 0, 20, 30, 40, 60, 80 or 120 ppm (for females) of benzidine dihydrochloride [purity unspecified] and killed after 33 months of exposure. Dose levels were selected using data from a prior study (Frith & Dooley, 1976). The incidence of hepatocellular carcinomas, along with data on body weights, water consumption, the dose received by animals, the overall mortality, adjusted liver-tumour mortality and the time to liver tumour were reported (Littlefield *et al.*, 1984). Effects on body weight and survival were noted in both strains. The incidences of malignant liver tumours are summarized in Table 3.4. For all four strain/sex combinations, there was a significant dose-related trend for fatal liver tumours, incidental liver tumours, and the pooled estimate using Peto's test [details on statistics not provided]. At the lowest doses, the incidence in Harderian gland

Table 3.2. Incidences of hepatocellular adenomas and carcinomas in mice fed benzidine dihydrochloride

Sacrifice period (weeks)	Dose (ppm)	F1				Mono-hybrid			
		Males		Females		Males		Females	
		%	r/n[a]	%	r/n	%	r/n	%	r/n
40	0	0.0	0/49	0.0	0/48	0.0	0/50	0.0	0/48
	30	0.0	0/98	2.0	2/98	1.0	1/101	0.0	0/97
	60	0.0	0/72	1.4	1/72	0.0	0/71	0.0	0/72
	120	0.0	0/51	0.0	0/49	2.1	1/48	5.9	3/51
	200	6.0	3/50	10.0	5/50	0.0	0/52	12.0	6/50
	400	3.6	1/28	44.8	13/29	3.7	1/27	38.5	10/26
60	0	2.1	1/48	2.1	1/48	0.0	0/48	2.1	1/48
	30	0.0	0/73	4.1	3/74	4.3	3/69	9.7	7/72
	60	8.2	4/49	7.7	4/52	6.5	3/46	22.2	12/54
	120	18.8	9/48	41.4	24/58	16.0	8/50	46.4	26/56
	200	19.1	9/47	88.5	54/61	18.6	8/43	78.3	47/60
	400	52.2	12/23	100.0	41/41	26.9	7/26	86.8	33/38
80	0	0.0	0/46	0.0	0/47	4.4	2/45	0.0	0/48
	30	11.4	5/44	20.9	9/43	4.9	2/41	27.9	12/43
	60	12.8	6/47	53.5	23/43	16.3	7/43	47.6	20/42
	120	28.9	13/45	91.9	34/37	31.8	14/44	96.9	31/32
	200	38.1	8/21	100.0	9/9	36.8	7/19	87.5	7/8
	400	80.0	16/20	0.0	0/1	64.7	11/17	83.3	5/6

From Nelson *et al.* (1982)
[a] r/n = number of animals with tumour / number of animals examined

tumours was also increased compared with that in the controls (4 and 5% in the F_1 females and males, and 5 and 8% in the MC males and females, respectively), and then remained higher at the other dose levels in the males. The incidences in the high doses for F_1 and MC males were 25 and 20%, respectively (dose effect, $P = 0.02$ with a linear and quadratic term). The females of both strains reached a high incidence of 24 to 29% in the mid-doses, which then decreased to about 11 or 12% at the high dose ($P = 0.002$ with a linear and quadratic effect). A dose effect was also observed for angioma of the uterus ($P = 0.07$ with a linear effect). The incidences in the control animals were 3% and 2%, for the F_1 and MC strains of mice, respectively; then it increased with dose to 14% for F_1 mice and 7% for MC mice (Littlefield *et al.*, 1983, 1984).

Table 3.3. Incidences of hepatocellular adenomas and carcinomas in mice fed benzidine dihydrochloride

Group	Treatment Period	Effective Number	Adenoma		Carcinoma		Total	
			No.	%	No.	%	No.	%
Males								
1	Prenatal	36	5	14	3	8	8	22
2	Pre-weaning	52	9	17	26	50	35	67
3	Adult	26	5	19	17	65	22	85
Control	52 weeks	100	0	0	0	0	0	0
	90 weeks	98	1	2	0	0	1	2
	142 weeks	100	4	8	3	6	7	14
Females								
1	Prenatal	56	1	2	1	2	2	4
2	Pre-weaning	43	4	9	5	12	9	21
3	Adult	25	0	0	16	64	16	64
Control	52 weeks	99	0	0	0	0	0	0
	90 weeks	96	0	0	0	0	0	0
	142 weeks	100	1	1	0	0	1	1

From Vesselinovitch (1983)

(b) Rat

Two groups, each of 10 female Wistar rats [age not specified] were fed a diet containing 0.017% benzidine [purity not specified] with casein or a diet containing benzidine with casein hydrolysate-tryptophan. All rats given benzidine plus casein were dead by 224 days after the start of treatment; 2/10 (20%) had liver tumours (1 hepatoma seen at 125 days and 1 bile-duct carcinoma at 178 days). Animals fed benzidine-tryptophan survived longer (424 days): 3/7 (43%) animals examined had hepatocellular tumours (1 carcinoma at 202 days, 1 cholangioma at 236 days and a bile-duct carcinoma at 424 days). None of the animals in the two experimental groups developed bladder tumours (Boyland *et al.*, 1954). [The Working Group noted the small number of animals and the lack of controls.]

Four groups of 10–20 female Sprague-Dawley rats, 40 days of age, were given benzidine [purity was tested, but not described in detail] at doses of 12, 25, 35 or 50 mg/rat in sesame oil by stomach tube for 30 days. A control group was fed the sesame-oil vehicle. At the end of the nine-month period of observation, when the experiment was terminated, 10/10 (100%), 8/10 (80%), 0/20 and 4/20 (20%) animals were still alive in the four treatment groups, respectively. In the vehicle control group, 127/140 (91%) were still alive at nine months. Thus, mortality was high in animals fed the two highest doses of

Table 3.4. Incidence of malignant liver tumours in mice exposed to benzidine dihydrochloride in drinking-water

Dose(ppm)	Male		Female	
	F1	Monohybrid cross	F1	Monohybrid cross
0	14/125 (11%)	17/123 (14%)	3/124 (2%)	10/125 (8%)
20	—	—	51/120 (43%)	54/119 (45%)
30	24/119 (20%)	20/118 (17%)	52/95 (55%)	43/95 (45%)
40	30/96 (31%)	20/95 (21%)	45/72 (63%)	31/71 (44%)
60	23/71 (32%)	23/71 (32%)	55/71 (77%)	37/72 (51%)
80	35/71 (49%)	24/71 (34%)	60/69 (87%)	51/69 (74%)
120	51/71 (72%)	37/71 (52%)	64/72 (89%)	56/72 (78%)
160	49/71 (69%)	32/71 (45%)	—	—

From Littlefield *et al.* (1984)

benzidine, and only five rats (at the highest dose) were autopsied; four of these showed multiple mammary carcinomas. In the groups receiving 12 and 25 mg benzidine/rat, 5/10 (50%) and 7/9 (78%) animals autopsied also showed multiple mammary carcinomas (one rat in the group fed 12 mg had a fibroadenoma). Five of 132 sesame-oil vehicle controls examined had mammary tumours. In the benzidine-treated groups, the first palpable mammary lesions appeared about 60 days after the first treatment. At this point the mean number of mammary masses per rat showed a dose–response relationship [no statistical analysis applied]. No effect was reported in organs other than the mammary gland (Griswold *et al.*, 1968).

(c) Hamster

Groups of 30 male and 30 female random-bred Syrian golden hamsters, nine weeks of age, were fed diets containing 0.1% (w/w) benzidine or benzidine dihydrochloride (certified grade) for life. A control group of the same size was also available. No bladder pathology was seen in either the treated or the control group. In the benzidine-treated group, an increased incidence of liver tumours was observed: 19/22 (86%) males and 6/26 (23%) females developed multiple cholangiomatous tumours, most of which had signs of malignancy; 12 males and three females also developed benign and malignant hepatocellular tumours. In the group fed benzidine dihydrochloride, the liver was also the only target organ: 10/20 (50%) male and 12/27 (44%) female hamsters developed cholangiomas, mostly benign; seven males and four females also developed hepatomas. No liver tumours were seen in females or males of the untreated control group (Saffiotti *et al.,* 1967).

(d) Other animal species

(i) *Rabbit*

An invasive bladder carcinoma was induced in one out of nine rabbits [sex, age and strain not specified] given oral tolerance limit doses of benzidine [purity not specified] (Bonser, 1962). [The Working Group noted the lack of description of experiment or results.]

(ii) *Dog*

Seven mongrel dogs (one male and six females, full grown; ~11.3 kg) were given a total dose of 325 g benzidine [purity not specified] by oral capsules over 5 years (200 mg per day for 15 months and then 300 mg per day for 45 months, on six days a week). One of the female dogs developed bladder carcinomas (Spitz *et al.*, 1950). [The Working Group noted the lack of description of experiment or results.]

(iii) *Frog*

A group of five frogs (*Rana temporaria*), 1–1.5 years of age, received a total oral dose of 60 mg benzidine [purity not specified] and were observed for 20 weeks, after which the experiment was terminated. One liver tumour was observed (Khudoley, 1977). [The Working Group noted the lack of description of experiment or results.]

3.1.2 *Subcutaneous and/or intramuscular administration*

(a) Mouse

Three groups of 12–24 male, albino Delph mice, 10 weeks of age, were given subcutaneous injections of 300 mg benzidine base [purity unspecified] in olive oil, or received olive oil alone, three times a week for 45 weeks. One group served as untreated controls. The survival rates were good in all groups up to 45 weeks when the experiment was terminated. No changes in the bladder were observed in the benzidine-treated animals. In 2/19 (10%) control mice receiving olive oil alone hyperplasia of the bladder was noted. Five of nine (55%) mice given benzidine base had hepatomas, compared with 3/19 (16%) in the olive oil group and 5/17 (29%) in the untreated controls (Baker, 1950). [The Working Group noted the short duration of the experiment.]

A group of 54 male and 13 female C3HA mice [age at start not specified], weighing 18–20 g, were injected subcutaneously with 6 mg/mouse of benzidine [source and purity not specified] dissolved in 0.2 mL of sunflower oil once per week over eight months (total dose, 210 mg/mouse). At the appearance of the first tumour (16 months), 46 mice [sex not specified] were still alive. Liver tumours (hepatocellular carcinomas, adenomas and cholangiomas) developed in 13 mice [sex not specified]; and lung adenocarcinomas were found in two mice. A further group of 114 males were exposed by the same treatment schedule for 13 months (total dose, 336 mg/mouse). At 16 months, 24 mice were still

alive, and 18 developed liver tumours. Hepatomas developed in 1% of historical controls (Prokof'eva, 1971). [The Working Group noted the low survival rates.]

(b) Rat

Groups of Sherman rats, two months of age, average weight 150 g, were given 15 mg of benzidine [technical and purified grades] or benzidine sulfate [technical grade] by subcutaneous injection, once weekly for life. A suitable control group was given the olive oil vehicle. The experimental design and data on survival and tumour incidence are summarized in Table 3.5 (Spitz *et al.*, 1950). [The Working Group noted the poor survival in both treated and control groups.]

Table 3.5. Tumour incidences in rats given subcutaneous injections of benzidine and salts

Compound	Average weekly dose (mg)	Total dose (g)	No. of rats at start	No. of rats surviving more than 300 days	Rats with tumours					
					Liver (neoplasms)		External auditory canal carcinomas		Colon adeno-carcinoma	
					No.	%	No.	%	No	%
Olive oil	910	92.82	50	28	–	–	–	–	–	–
Technical benzidine	15	1.28	233	36	8	3.4	54	23	–	–
Pure benzidine	15	0.96	152	24	6	3.9	32	21	7	1.8
Benzidine sulphate	15	0.94	153	5	1	0.65	16	10.5	–	–

From Spitz *et al*. (1950)

A group of 25 male and 25 female rats [strain and age at start not specified], weighing 100–120 g, were injected subcutaneously with an initial dose of 15 mg benzidine [purity not specified] in 0.5 mL of sunflower-seed oil, once a week for 14 weeks. Due to severe toxicity, a smaller dose of 10 mg per week was then given for the next six weeks to each rat, and finally once every 15 days for six weeks. By six months of treatment, each animal had received a total dose of 300 mg benzidine. Another group of 50 rats [sex unspecified] served as controls: 25 received subcutaneous injections with the solvent for six months while the remaining 25 rats were kept untreated. Of the 15 treated males surviving, 12 developed tumours: two hepatomas, four malignant tumours of the Zymbal gland, six sarcomas at the injection site and two other sarcomas; two of the five surviving treated females developed tumours: one malignant tumour of the Zymbal gland and one myeloid

leukaemia. None of the 25 controls injected with the solvent [sex not specified] developed tumours at the injection site (Pliss, 1964).

A group of 28 rats [strain and sex not specified], 6–8 weeks of age, were given benzidine [purity, source and vehicle not specified] by weekly subcutaneous injections of 5 mg/rat for 32–60 weeks (total dose, 170 mg/rat). When the first tumour appeared at 210 days, 28 rats were still alive. Intestinal tumours developed in four rats between 252 and 318 days (Pliss *et al.*, 1973) [The Working Group noted the lack of available controls and the lack of experimental detail.]

Groups of 16 female and 14 male white non-bred rats [strain and age not specified] were injected subcutaneously with benzidine [source and purity not specified] (5 mg/rat) dissolved in 0.5 mL of sunflower oil, weekly for about 52 weeks (total dose, 160–260 mg/rat). At 219 days, when the first tumour (a skin epithelioma) was detected, 24 rats [sex not specified] were still alive; all animals were killed at 357 days. Tumours were found in 23 rats (95.8%), with an average latent period of 275 days. Nine of 24 rats (39.1%) had multiple primary tumours. Zymbal gland tumours developed in 18 rats (78.3%); five had local fibrosarcomas and one a local rhabdomyosarcoma (Pliss and Iogannsen, 1974). [The Working Group noted that no untreated or solvent controls were available.]

Groups of 18 male and 16 female albino non-inbred rats [age at start not specified] weighing 120–140 g were injected subcutaneously once a week for about 33 weeks with benzidine (5 mg/rat; source and purity not specified) suspended in 0.5 mL of oil [not specified] to give a total dose of 170 mg/rat. At 210 days, when the first tumour appeared, 16 males and 12 females were still alive. A total of 26 tumours developed in 14 males: six local sarcomas, nine tumours of the Zymbal gland, nine liver tumours (cystocholangiomas and hepatocellular carcinomas) and two intestinal tumours (polyposis and adenocarcinoma). A total of 20 tumours developed in 11 females: five local sarcomas, six tumours of the Zymbal gland, four mammary adenocarcinomas, one mammary adenoma, two liver tumours (cystocholangioma and hepatocellular carcinoma) and two intestinal tumours (Pliss & Vol'fson, 1974). [The Working Group noted that no untreated or solvent controls were available.]

(c) Frog

A group of 37 grass frogs (*Rana temporaria*) of both sexes, 1–1.5 years of age, received weekly subcutaneous injections of 0.2–0.5 mL of a 0.5% solution of benzidine [purity not specified] in mineral oil for up to 38 weeks (total dose, 45–114 mg/animal). A group of 120 untreated frogs were observed for 56 weeks (three of these developed skin cystadenopapillomas), and a further group of 67 frogs were given subcutaneous injections of 0.2–0.5 mL mineral oil, weekly for 42 weeks as controls. When the first tumour appeared at 16 weeks, 14 animals in the treated group were still alive, of which six (43%) had tumours of the liver and haematopoietic system [not further specified], with an

average latent period of 24.8 weeks. No tumours were observed in the control group (Khudoley, 1977).

3.1.3 *Intraperitoneal administration*

(a) Rat

Three groups of 30 female CD rats, 30 days of age, were given intraperitoneal injections twice weekly for 4 weeks, of 0, 10 or 30 μmol/kg bw benzidine [purity not specified] as a suspension in trioctanoin. Control rats received trioctanoin only. All survivors were killed 46 weeks after the first injection. No tumours were seen in the kidney or bladder in treated or control groups. In the benzidine-treated groups, a dose-related increase in the incidence of mammary tumours, benign and malignant, was noted: 3/30 (10%) in controls, 7/30 (23%) in the low-dose group and 12/29 (41%) ($P < 0.01$, χ^2 test) in the high-dose group. Zymbal gland tumours (adenomas or carcinomas) were observed in 1/30 (3%) controls, 1/30 (3%) low-dose animals and 7/29 (24%) ($P < 0.05$) high-dose animals. No tumours of the liver were found; however, altered cellular foci in the liver were observed in 9/30 (19%) controls, 14/30 (46%) low-dose animals and 20/29 (76%) ($P < 0.01$) high-dose rats (Morton *et al.,* 1981).

3.1.4 *Inhalation exposure*

(a) Rat

A group of 48 white out-bred rats of both sexes [age at start non specified], weighing 100–120 g, were exposed to an aerosol containing 10–20 mg/m^3 [1.3–2.7 ppm] benzidine [source and purity not specified] in inhalation chambers during four hours/day, for five days a week over 20 months (total dose, 27 mg/rat). Control rats [number not specified] were kept in inhalation chambers and exposed to air during the same period. Animals were kept until moribund. The first myelogenous leukaemia was found in a treated rat 13 months after the start of the experiment, at which time 28 rats were still alive. By the end of the study (28 months), five myeloid leukaemias, two breast fibroadenomas, one squamous-cell cancer of the Zymbal gland, one hepatoma and one breast adenocarcinoma were found in eight animals. Mammary adenomas were found in two of 21 control rats (Zabezhinskiĭ, 1970). [The Working Group noted the lack of information on the size of the aerosol particles and on the survival of controls.]

3.1.5 *Other experimental systems*

(a) Mouse

Following surgical implantation of a 45-mg glass bead in the urinary bladder of female mice (strain 150 ICR) at five weeks of age, the animals were divided into three groups: one group (30 mice) served as controls and was fed a commercial basal diet; the

second group (60 mice) received a diet containing 0.2% benzidine [purity not specified]; the third group (60 mice) was fed a diet containing a mixture of 0.2% benzidine and 2% DL-tryptophan. The experimental groups received their diets starting at six weeks of age for 20 weeks and were then fed the control diet for 40–43 weeks. The experiment was terminated 63 weeks after the start of treatment. Of the group that received benzidine alone, only 19% of the animals were still alive at the end of the experiment, while 65.5% of controls and 49.2% of the group treated with benzidine plus tryptophan were still alive at that time. Hepatomas were observed in 34 of 41 (82.9%) mice treated with benzidine and in 24 of 51 (47.1%) mice treated with the benzidine-tryptophan mixture, indicating an inhibitory effect of tryptophan; no hepatomas were seen in the controls. No bladder tumour was found in any of the animals; however, the authors reported hyperplasia in all bladders observed (Miyakawa and Yoshida, 1980).

(b) Rat

Five groups of 30–40 male Fischer rats (age at start not specified), weighing approximately 200 g, were implanted with a heterotopic bladder, which was then instilled once a week for 20 weeks with 0.5 ml phosphate-buffered saline:dimethyl sulfoxide solution (PBS/DMSO, 4:1) or this solution containing 1 μmol benzidine or the derivatives, *N*′-hydroxy-*N*-acetylbenzidine, the *N*′-glucuronide of *N*′-hydroxy-*N*-acetylbenzidine, or the *N*-glucuronide of *N*-hydroxy-2-aminofluorene [chemicals were synthesized and analysed by authors]. These bladders were then instilled once a week for an additional 30 weeks with PBS without DMSO. The experiment was terminated at the end of 50 weeks. Transitional cell carcinomas were observed in 1 of 39 (3%) of the control group, 1 of 29 (3%) in the benzidine group, 18 of 30 (60%) in the *N*′-hydroxy-*N*-acetylbenzidine group, 28 of 28 (100%) in the *N*′-hydroxy-*N*-acetylbenzidine-*N*′-glucuronide group, and in 24 of 29 (83%) of the *N*-hydroxy-2-aminofluorene-*N*′-glucuronide rats (Wang *et al.*, 1990).

(c) Fish

Benzidine [purity not specified] was mixed into a diet and given to a group of 100 fish (guppies) of both sexes, 10–12 months of age, at a dose of 300 mg/kg dry diet for 56 weeks, at which point the experiment was terminated. The six fish that survived the treatment period had no detectable tumours; however, signs of hepatotoxicity (focal necrosis, fatty dystrophy and diffuse hyperplasia of hepatocytes) were noted. None of the 120 control guppies fed the standard diet developed tumours or preneoplastic changes (Pliss & Khudoley, 1975). [The Working Group noted the high mortality in the treated group.]

3.2 3,3′-Dichlorobenzidine and its dihydrochloride

Studies in experimental animals of carcinogenicity of 3,3′-dichlorobenzidine and its dihydrochloride by oral exposure were previously reviewed by IARC (1982, 1987). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.2.1 *Oral administration*

(a) Mouse

A group of 26 male ICR/JCL mice [age at start not specified] were fed a diet containing 0.1% 3,3′-dichlorobenzidine [purity unspecified, not clear if it was the free amine or the dihydrochloride salt] for up to 12 months. All eight animals killed after six months of treatment (100%) had hepatomas, as did all 18 animals (100%) killed after 12 months of treatment. Of 39 control mice maintained on a normal diet and killed at six, 12 and 18 months, 0/5 (0%), 2/21 (9%) and 5/13 (38%) had hepatomas respectively (Osanai, 1976). [The Working Group noted the absence of information on survival of treated and control animals.]

A group of 22 female and 51 male D mice ($cC_{57}W$ x $C_{57}Bl$ hybrids, age not specified) weighing 12–20 g, received food containing 0.1 ml of a 1.1% suspension of 3,3′-dichlorobenzidine (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in sunflower oil, which was administered on six days a week for 12 months (total dose, 127–135 mg/mouse). The animals were observed for life. There was no control group but the authors used historical control data. The numbers of mice that survived were: 37 at six months, 34 at 12 months, and 18 mice at the time of appearance of the first tumour (18.5 months). Four of 18 animals (22%) had tumours: 2/18 (11%) had hepatomas, 2/18 (11%) had liver hemangiomas, 1/18 (5%) had a carcinoma of the sebaceous gland, and 1/18 (5%) had a lung adenoma. No liver hepatomas or hemangiomas had been seen in the historical controls (Pliss, 1959). [The Working Group noted the absence of adequate controls].

(b) Rat

A group of 15 female and 35 male outbred Rappolovo rats weighing 110–130 g [age at start not specified] received food containing 0.5–1.0 ml of a 4.4% suspension of 3,3′-dichlorobenzidine (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in sunflower oil at a dose of 10–20 mg/day, which was administered on six days a week for 12 months (total dose, 4.5 g/rat). The animals were observed for life. The numbers of animals that survived were: 34 at six months and 27 at 12 months. At the time of the appearance of the first tumour (11 months), 29 rats were alive. Twenty-three rats (23/29, 79%) developed tumours, including seven (24%) Zymbal gland tumours, three (10%) skin tumours, seven (24%) mammary gland tumours, two (7%) adenocarcinomas

of the ileum, three (10%) bladder tumours, three (10%) tumours of the haematopoietic system, two (7%) connective tissue tumours, two (7%) salivary gland tumours, one (3%) liver tumour and one (3%) thyroid tumour. Among a group of 130 controls injected with octadecylamine and methylstearylamine, no tumours were found within 23 months (Pliss, 1959). [The Working Group noted the absence of adequate controls.]

Groups of 50 male and 50 female ChR-CD rats, 38 days of age, were given a diet containing 1000 ppm 3,3′-dichlorobenzidine [purity unspecified] for 16 months. An equal number of animals were maintained on a control diet for a period of 24 months. Six rats per group and per sex were killed at 12 months for an interim evaluation. Of the remaining treated rats, six survived up to 16 months, at which time they were killed. A statistically significant ($P < 0.05$) increase in the incidence of tumours was observed in treated compared with control animals for the following target sites in males: granulocytic leukaemias, 9/44 (20%) treated, 2/44 (4%) control; mammary adenocarcinomas, 7/44 (16%) treated, 0/44 control; Zymbal gland carcinomas, 8/44 (18%) treated, 0/44 control. In females, mammary adenocarcinomas were seen in 26/44 (59%) treated, 3/44 (7%) control (Stula *et al.*, 1975).

A group of 20 female Sprague-Dawley rats, 40 days of age, were given 10 doses of 3,3′-dichlorobenzidine dihydrochloride [purity and impurities unspecified] in sesame oil every three days by gastric intubation (total dose, 300 mg/rat, which was the maximum tolerated dose). The observation period was nine months, when the 14 surviving animals were killed. No mammary tumours were observed in 15 treated rats autopsied, while 5/132 (4%) animals treated with sesame oil only had mammary tumours (Griswold *et al.,* 1968).

(c) Hamster

Groups of 30 male and 30 female random bred Syrian golden hamsters, nine weeks of age, were given 0.1% of a technical grade 3,3′-dichlorobenzidine (mixture of 40% as the dihydrochloride and 60% as free base) in powdered diet throughout their life-span (total intake, 3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. No information on survival of the treated or control animals was given. Exposure to this dose level of 3,3′-dimethylbenzidine did not induce any significant carcinogenic effect at any site or bladder pathology (Saffiotti *et al.*, 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

(d) Dog

Six female beagle dogs, one year of age, were each given 100 mg 3,3′-dichlorobenzidine (reported to be 100% pure) in a gelatin capsule three times per week for six weeks, then five times per week continuously for periods up to 7.1 years. Six untreated female beagle dogs served as controls for several studies and were killed after

8.3 to 9.0 years on test. The intake of 3,3′-dichlorobenzidine was between 9.1 and 12.8 mg/kg bw per dose. One dog sacrificed after 3.5 years on test had no tumours. Another sacrificed after 6.6 years on test (total intake, 164 g) had an undifferentiated carcinoma of the urinary bladder. Of the remaining dogs killed at 7.1 years (total intake, 176 g/dog), 4/4 (100%) had papillary transitional-cell carcinomas of the urinary bladder and 3/4 (75%) had hepatocellular carcinomas. None of the six control dogs had these tumours. However, 4/6 (67%) control animals killed at 8–9 years of age had major tumours of the mammary gland (adenocarcinomas and carcinosarcoma) (Stula *et al.*, 1978).

3.2.2 *Subcutaneous and/or intramuscular administration*

(a) Mouse

A group of 15 female and 8 male D mice ($cC_{57}W$ x $C_{57}Bl$ hybrids, age not specified), weighing 12–20 g, received twice weekly 0.1-ml subcutaneous injections of an 11% or a 5.5% suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a total dose of 265 mg/mouse over 11 months. The numbers of mice that survived were: nine at six months, eight at 12 months, and eight at the time of appearance of the first tumour (12.5 months). The animals were observed for life. Five animals [sex not identified] (62%) had tumours at different sites: 2/8 (25%) had local sarcomas, 3/8 (37%) had liver tumours, 1/8 (12%) had a tumour of the haematopoietic system, 1/8 (12%) had a lung adenoma. Another group of 31 female and 36 male D mice received weekly 0.1-ml subcutaneous injections of an 11% or a 5.5% suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a dose of 5 to 2.5 mg/mouse. The total dose was 130 mg/mouse over 11 months. The numbers of mice that survived were: 32 at six months, 23 at 12 months, and 20 at the time of appearance of the first tumour (13.5 months). The animals were observed for life. Eight animals [sex not identified] (40%) had tumours at different sites: 1/20 (5%) had local sarcomas, 5/20 (25%) had liver tumours, 1/20 (5%) had a tumour of the haematopoietic system, 2/20 (10%) had a lung adenoma, and 1/20 (5%) had a squamous-cell keratinizing tumour of the lower jaw. No tumour occurred within 23 months in 130 controls injected with octadecylamine or methylstearylamine (Pliss, 1959). [The Working Group noted the absence of adequate controls and poor description of the pathology findings.]

(b) Rat

A group of 61 sexually mature white rats weighing 110–130 g (strain, age, and distribution by sex not specified) initially received twice weekly subcutaneous injections of a suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a dose of 60 mg/rat. Because of initial high mortality, the dose was reduced beginning from the 6th month to weekly injections of

20 mg/rat. The total dose of 3,3′-dichlorobenzidine was estimated to range from 1.6 to 3 g/rat. The animals were observed for life. A group of 130 rats (strain, age, and distribution by sex not given) served as controls and received subcutaneous injections of octadecylamine or methylstearylamine over a 10-month period and were then observed for life. Thirty-five animals survived to the time of appearance of the first tumour (time not specified). Eighteen animals (51%) had tumours: 9/35 (26%) had Zymbal gland tumours, 7/35 (20%) had mammary tumours, 5/35 (14%) had local sarcomas, 1/35 (3%) had liver tumours, 2/35 (6%) had a tumour of the haematopoietic system, and 1/35 (3%) had a thyroid tumour. No tumour occurred within 23 months in 130 controls injected with octadecylamine or methylstearylamine (Pliss, 1958). [The Working Group noted the inadequate reporting of the experiment.]

A group of rats weighing 100–130 g (strain, age, number and distribution by sex not specified) received subcutaneous injections of 15–60 mg/rat of 3,3′-dichlorobenzidine [purity and impurities unspecified] in sunflower seed oil or glycerol and water at unspecified intervals for 10 to 13 months. No information on survival was provided. Tumours were reported to occur in 74% of animals. No information on number of tumour-bearing animals or time to first tumour was reported. Skin, sebaceous and mammary gland tumours were observed most frequently, and there were also intestinal, urinary bladder and bone tumours. Among 50 control rats injected with the vehicle alone or left untreated, a single sarcoma was reported (Pliss, 1963). [The Working Group noted the inadequate reporting of the experiment.]

3.2.3 *Transplacental exposure*

(a) Mouse

A group of BALB/c mice [number and age not specified] were treated with 5 subcutaneous injections of 3,3′-dichlorobenzidine in sunflower oil (2 mg/injection; total dose, 10 mg/mouse) during the last week of pregnancy. The progeny (13 males and 11 females) were kept with the treated animals throughout lactation and weaning at 3–4 weeks, and were then observed until natural death. Of the offspring that lived 12–20 months, 13/24 (54%) had tumours, compared with 6/30 (20%) of the control progeny. A significant increase in the incidence of lymphoid leukaemias (7/24, 29%) in treated and 0/30 in control animals [sex unspecified]) was observed in the offspring. Lung tumours (5/24 (21%) in treated and 3/30 (10%) in control animals) and mammary tumours (4/11 (36%) in treated and 3/19 (16%) in control animals) were also reported (Golub *et al.,* 1974).

3.2.4 *Administration with known carcinogens*

(a) Rat

Nine groups of 22 and one group of 96 male Wistar rats (age not specified) received the following compounds alone or in sequence for a period of four weeks per compound: *ortho*-*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (0.01% in drinking water), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (0.15% in the diet), *N*-fluorenyl-acetamide (0.025% in the diet) and 3,3′-dichlorobenzidine (0.3% in the diet). An untreated control group consisted of 12 rats. The animals were killed when 40 weeks old. 3,3′-Dichlorobenzidine given in sequence with one or more of the other compounds induced histological changes of the liver (cystic change of bile ducts and oval-cell proliferation) in 44–50% of animals ($P < 0.05$ when compared with groups that did not receive 3,3′-dichlorobenzidine). No change was seen in the liver when 3,3′-dichlorobenzidine was given alone. The incidence of urinary bladder tumours was not significantly increased when 3,3′-dichlorobenzidine was added to the sequence of the chemicals studied (Tatematsu *et al.,* 1977).

3.3 3,3′-Dimethoxybenzidine

Studies in experimental animals of carcinogenicity of 3,3′-dimethoxybenzidine or its dihydrochloride salt by oral exposure were previously reviewed by IARC (1982). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.3.1 *Oral administration*

(a) Mouse

Groups of 166 male and 165 female BALB/c mice, four weeks of age, were given drinking water containing 0, 20, 40, 80, 160, 315, or 630 ppm of 3,3′-dimethoxybenzidine dihydrochloride [purity unspecified] for up to 112 weeks. Interim sacrifices and histopathological assessments were conducted on all dose groups after 13, 26, 39, 52, 78, or 112 weeks. Water consumption was monitored and was depressed in all the groups, especially the high-dose group. Although body-weight gain was suppressed at the highest dose level during the first year, administration of 3,3′-dimethoxybenzidine dihydrochloride did not affect the mortality of either males or females. No increased incidences of neoplasms were observed in any of the tissues examined, which included spleen, Harderian gland, liver, and lung (Schieferstein *et al.*, 1990).

(b) Rat

A group of 42 male and female rats (strain, distribution and age not specified) were given 30 mg of 3,3′dimethoxybenzidine [purity unspecified] by gavage in sunflower-seed oil three times a week for three weeks. The dose was then reduced to 15 mg because of

poor survival and continued for an additional 13 months. Eighteen animals survived to 14 months. Two of the 18 animals (11%) that survived to 18 months had neoplasms of the Zymbal gland, one (5.5%) had a fibroadenoma of the mammary gland, and one (5.5%) had an ovarian neoplasm. None of the 50 rats in the control groups (25 injected with sunflower-seed oil subcutaneously and 25 untreated) developed tumours at these sites (Pliss, 1963, 1965). [The Working Group noted the inadequate reporting of the experiment.]

Groups of 3 or 14 (10-mg dose only) male and 3 or 15 (10-mg dose only) female Fischer rats (age not given) were administered 3,3′-dimethoxybenzidine [purity unspecified] by gavage at dose levels of 0, 0.1, 0.3, 1, 3, 10, or 30 mg per animal per day on five days per week. A proprietary mixture composed of sodium chloride, sodium carboxymethylcellulose, polysorbate 80, and benzyl alcohol in water was used as a vehicle in this study. The animals were treated for 52 weeks and then observed for an additional six months. Groups of male and female rats received 0.5 ml of the vehicle five days per week and served as the vehicle controls. In addition, there was a separate group of 90 males and 90 female rats that served as untreated controls. Neoplasms occurred as early as day 293, but most were detected at necropsy 18 months after the initial administration of 3,3′- dimethoxybenzidine. A variety of neoplasms were reported, and pooled results for all dosed male and female groups (59 animals) included neoplastic lesions of the urinary bladder (two papillomas), mammary gland (three carcinomas, two fibroadenomas), skin (five carcinomas), intestinal tract (three carcinomas), and Zymbal gland (eight carcinomas). Incidences of neoplasms were significantly increased over those of the 360 pooled vehicle and untreated control rats (Hadidian *et al.*, 1968).

Groups of 60, 45, 75, and 60 male and female F344/N rats, seven weeks of age, were given drinking water containing 3,3′-dimethoxybenzidine dihydrochloride at 0, 80, 170, or 330 ppm respectively (corresponding to 0, 6, 12, or 21 mg/kg per day for males and 0, 7, 14, or 23 mg/kg per day for females) for up to 21 months. Although initially planned as a two-year study, this experiment was terminated early because of reduced survival in all dose groups associated with the appearance of treatment-related neoplasms. Survival decreased markedly with increasing dose. Among males, the number of rats surviving at study termination was 44 in the control group and eight in the low-dose group. None of the male rats in the medium- and-high-dose groups survived the study duration. Among females, 45, 15, and 6 rats survived in the control, low-dose, and medium-dose groups, respectively, and none of the rats in the high-dose group survived. The tumour incidences in male and female rats are shown in Tables 3.6 and 3.7. Histopathological examination of the tissues revealed tumours at various sites, including benign and malignant tumours of the skin, Zymbal gland, preputial gland, clitoral gland, mammary gland, uterus, oral cavity, intestine, liver, and mesothelium. The observed increase in the incidence of astrocytomas of the brain may also have been related to exposure to 3,3′-dimethoxybenzidine dihydrochloride (NTP, 1990).

(c) Hamster

Groups of 30 male and 30 female, random bred Syrian golden hamsters, nine weeks of age, were given 0.1% 3,3′-dimethoxybenzidine (purity not given) in powdered diet throughout their lifespan (3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. The only malignant neoplasm observed was a transitional cell carcinoma of the urinary bladder in one animal after 144 weeks of exposure to dimethoxybenzidine. This neoplasm is rare in hamsters and therefore was attributed to dimethoxybenzidine exposure (Saffiotti *et al.* 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

Table 3.6. Tumour incidences in male rats given 3,3′-dimethoxybenzidine dihydrochloride in drinking-water for up to 21 months

Tumour type	Concentration (ppm) in drinking-water			
	0	80	170	330
	Tumour incidence/number examined			
Skin: basal cell or sebaceous gland adenoma or carcinoma	2/60**[a] (3%)	33/45**[b] (73%)	56/75**[b] (75%)	41/60**[b] (68%)
Skin: squamous cell papilloma	0/60**[a]	13/45**[b] (29%)	28/75**[b] (37%)	22/60**[b] (37%)
Zymbal gland: adenoma or carcinoma	0/59**[a]	10/45**[b] (22%)	25/75**[b] (33%)	30/60**[b] (50%)
Preputial gland: adenoma or carcinoma	16/60*[a] (27%)	12/43 (28%)	33/73*[b] (45%)	29/59*[b] (49%)
Oral cavity: papilloma or carcinoma	1/60*[a] (2%)	8/45*[b] (18%)	10/75*[b] (13%)	11/60*[b] (18%)
Small intestine: adenocarcinoma	0/60	4/45*[a] (9%)	7/75*[a] (9%)	5/60*[a] (8%)
Large intestine: adenomatous polyp or adenocarcinoma	0/60**[a]	1/45 (2%)	8/75*[b] (11%)	8/60*[b] (13%)
Liver: Neoplastic nodule or hepatocellular carcinoma	1/60**[a] (2%)	4/45 (9%)	7/74*[b] (9%)	8/60**[b] (13%)
Mesothelium: mesothelioma	2/60*[a] (3%)	1/45 (2%)	7/75 (9%)	6/60 (10%)
Brain: astrocytoma	0/60	2/44 (5%)	3/75 (4%)	1/60 (2%)

[a] Statistical significance by Cochran-Armitage trend test based on effective rates: *P <0.05, **P ≤0.001
[b] Statistical significance by Fisher exact test based on effective rates: *P <0.05, **P ≤0.001
From NTP (1990)

Table 3.7. Tumour incidence in female rats given 3,3′-dimethoxybenzidine dihydrochloride in drinking-water for up to 21 months

Tumour type	Concentration (ppm) in drinking-water			
	0	80	170	330
	Tumour incidence/number examined			
Skin: basal cell adenoma or carcinoma	0/60	4/45*[a] (9%)	3/75 (4%)	2/60 (3%)
Skin: Squamous cell papilloma	0/60	0/45	3/75 (4%)	0/60
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60*[b]	1/44 (2%)	0/75	3/60 (5%)
Zymbal gland: adenoma or carcinoma	1/60*[b] (2%)	12/45**[a] (27%)	21/75**[a] (28%)	16/60**[a] (27%)
Mammary gland: adenocarcinomas	1/60**[b] (2%)	2/45 (4%)	14/75**[a] (19%)	20/60**[a] (33%)
Oral cavity: papilloma or adenoma	2/60 (3%)	2/45 (4%)	6/75 (8%)	5/60 (8%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60*[b]	1/45 (2%)	1/75 (1%)	3/60* [b] (5%)
Clitoral gland: adenoma or carcinoma	7/58**[b] (12%)	27/44**[a] (61%)	48/74**[a] (65%)	41/45**[a] (91%)
Uterus: adenoma or carcinoma	0/60	4/45*[a] (9%)	2/75 (3%)	2/60 (3%)
Brain: astrocytoma	0/60	1/45 (2%)	1/75 (1%)	0/60

[a] Statistical significance by Fisher exact test based on effective rates: *P <0.05, **P ≤0.001
[b] Statistical significance by Cochran-Armitage trend test based on effective rates: *P <0.05
**P ≤0.001
From NTP (1990)

3.4 3,3′-Dimethylbenzidine

Studies in experimental animals of the carcinogenicity of 3,3′-dimethylbenzidine or its dihydrochloride salt by exposure via oral and subcutaneous administration were previously reviewed by IARC (1982). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.4.1 *Oral administration*

(a) Mouse

Groups of 120 male and 120 female BALB/c mice, four weeks of age, were given drinking-water containing 0, 5, 9, 18, 35, 70, or 140 ppm of 3,3′-dimethylbenzidine dihydrochloride [purity unspecified] for up to 112 weeks. Interim sacrifices and histopathological assessments were conducted on all dose groups after 13, 26, 39, 52, 78, and 112 weeks. Water consumption was monitored, and average weekly 3,3′-dimethylbenzidine dihydrochloride doses (mg/kg) were determined to range from 5 to 126 mg/kg per week. 3,3′-Dimethylbenzidine dihydrochloride in oral doses exceeding 100 mg/kg per week was well tolerated, as evidenced by the absence of treatment-related changes in water consumption, body-weight gain, or mortality. Incidences of alveolar-cell adenomas and adenocarcinomas ($P < 0.0002$, carcinomas; $P < 0.0001$, adenomas and carcinomas combined) of the lung were increased in a dose-related fashion among males that were either found dead or sacrificed in moribund condition. Similar increases were not observed in females or in animals randomly selected for interim sacrifice (Table 3.8). The incidences of tumours of the skin, spleen, liver, and Harderian gland were unaffected by the administration of 3,3′-dimethylbenzidine dihydrochloride (Schieferstein *et al.,* 1989).

(b) Rat

A group of twenty female Sprague-Dawley rats, 40 days of age, were given a suspension of 3,3′-dimethylbenzidine (purity not given) in sesame oil by gavage at a tota1 dose of 500 mg per rat, fractionated in 10 doses at 3-day intervals and then held for an additional eight months. A group of 140 female Sprague-Dawley rats, 40 days of age, served as the controls and received the sesame oil only. Survival was 80% in treated animals versus 90% in the controls. Sixteen rats treated with 3,3′-dimethylbenzidine were alive at the end of the nine-month observation period. Neoplastic responses included significantly increased incidences of mammary tumours. Three of the 16 treated animals (19%) showed a total of four mammary carcinomas. Among 132 surviving control rats, five (4%) had a total of three mammary carcinomas, one fibroadenoma and five hyperplasias (Griswold *et al.*, 1968).

Groups of 70, 45, 75, and 70 male and female F344/N rats, 6 weeks of age, were given drinking-water containing 3,3′-dimethylbenzidine dihydrochloride [purity unspecified] at 0, 30, 70, or 150 ppm, respectively, for up to 14 months. Although initially

Table 3.8. Lung alveolar-cell adenomas and adenocarcinomas in BALB/c mice exposed to 3,3′-dimethylbenzidine dihydrochloride in drinking-water for up to 104 weeks

Drinking-water concentration (ppm)	Specified sacrifice times (wk)						Animals found dead or moribund
	13	26	39	52	78	112	
	Incidence of lung alveolar-cell adenomas and adenocarcinomas/ number of animals examined (incidence of adenocarcinomas)						
Males							
0	0/24	0/24	0/8	1/15	11/23	3/10 (1)	5/16 (2)
5	0/24	1/24	0/8	3/16	4/20	5/10 (3)	7/16 (2)
9	0/24	1/24	1/8	1/14	8/18	0/4	5/25 (2)
18	0/24	0/24	0/8	5/14	8/23 (2)	6/10	5/18 (2)
35	0/24	0/24	2/8	2/15	5/18	3/8 (1)	7/24 (6)
70	0/24	0/24	0/8	4/16 (1)	7/21	4/7 (1)	11/20 (5)
140	0/24	0/24	0/8	2/16	8/20	4/7 (1)	13/20 (10)
Females							
0	0/24	0/24	0/8	0/16	4/21	1/7	7/19 (5)
5	0/24	0/24	0/8	1/15	1/23	2/8	4/17 (3)
9	0/24	0/24	1/8	2/16	8/20 (1)	4/9	3/19 (3)
18	1/24	0/24	0/8	1/13	5/21 (1)	4/5 (2)	4/20 (2)
35	0/24	0/24	0/8	3/16	4/20	5/11 (3)	5/17 (2)
70	0/24	1/24	1/8	0/16	2/21	5/10	4/15 (2)
140	0/24	0/24	0/8	4/16 (1)	5/18 (2)	3/11 (1)	4/18 (2)

From Schieferstein *et al.*, (1989)

planned as a two-year study, this experiment was terminated early because of reduced survival in all dose groups associated with the appearance of treatment-related neoplasms. A scheduled interim sacrifice and histopathological assessment of 10 controls and 10 high-dose animals of each sex was conducted during the ninth month of the study. Although the incidences of tumours observed in 3,3′-dimethylbenzidine dihydrochloride-dosed rats were not significantly elevated at this interim sacrifice, the appearance of malignant tumours of the liver (male only), lung, mammary gland (female only), skin, preputial gland (male only), oral cavity (female only), small intestine (male only), clitoral gland, and Zymbal gland after only nine months suggested a treatment-associated early onset of some tumours. Tumour incidences, summarized in Table 3.9, were unequivocally increased in a dose-related manner after 14 months of 3,3′-dimethylbenzidine dihydrochloride administration. Administration of 3,3′-dimethylbenzidine dihydrochloride significantly increased the incidences of a wide array of malignant and benign tumours in both sexes of F344/N rats. (NTP, 1991b).

Table 3.9. Tumour incidences in F344/N rats administered 3,3'-dimethylbenzidine hydrochloride in drinking-water for 14 months

Tumour type	Daily dose (ppm)			
	0	30	70	150
	Tumour incidences/number examined[a]			
Males				
Skin: Basal cell adenoma or carcinoma	0/60	11/45** (24%)	54/75** (72%)	30/60** (50%)
Sebaceous gland adenoma	0/60	0/45	7/75*	5/60*
Squamous cell papilloma or carcinoma	0/60	2/45 (4%)	17/75** (23%)	27/60** (45%)
Keratoacanthoma	1/60 (2%)	1/45 (2%)	8/75* (11%)	5/60* (8%)
Zymbal gland: Adenoma or carcinoma	1/60 (2%)	3/45 (7%)	32/75** (43%)	36/60** (60%)
Preputial gland: Adenoma or carcinoma	2/60 (3%)	4/45 (9%)	6/75 (8%)	9/60* (15%)
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	35/75** (47%)	33/60** (55%)
Oral cavity: Squamous cell papilloma or carcinoma	0/60	0/45	4/75 (5%)	5/60* (8%)
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	4/75 (5%)	8/60* (13%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	6/75* (8%)	15/60** (25%)
Lung: Neoplasms	1/60 (2%)	0/45	8/75* (11%)	6/60* (10%)
Females				
Skin: Basal-cell adenoma or carcinoma	0/60	3/45 (7%)	10/75** (13%)	9/60** (15%)
Squamous cell papilloma or carcinoma	0/60	3/45 (7%)	9/75* (12%)	12/60** (20%)
Zymbal gland: Adenoma or carcinoma	0/60	6/45* (13%)	32/75** (43%)	42/60** (70%)
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	7/74* (9%)	4/60* (7%)
Oral cavity: Squamous cell papilloma or carcinoma	0/60	3/45 (7%)	9/75* (12%)	13/60** (22%)
Clitoral gland: Adenoma or carcinoma	0/60	14/45** (31%)	42/75** (56%)	32/59** (54%)

Table 3.9 (contd)

Tumour type	Daily dose (ppm)			
	0	30	70	150
	Tumour incidences/number examined[a]			
Females (contd)				
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45 (2%)	3/75 (5%)	5/60* (8%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45 (2%)	7/75* (9%)	4/60* (7%)
Lung: Neoplasms	1/60 (2%)	1/45 (2%)	3/74 (4%)	4/60 (7%)
Mammary gland: Adenocarcinoma	0/60	1/45 (2%)	3/75 (4%)	6/60* (10%)

[a] Statistical significance by Fisher exact test: *P <0.05; **P <0.001
From NTP (1991)

(c) Hamster

Groups of 30 male and 30 female random bred Syrian golden hamsters, nine weeks of age, were given 0.1% 3,3′-dimethylbenzidine (purity not given) in powdered diet throughout their life-span (3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. Exposure to this dose level of 3,3′-dimethylbenzidine did not induce any significant carcinogenic effect or bladder pathology (Saffiotti *et al.*, 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

3.4.2 *Subcutaneous administration*

(a) Rat

A group of 105 male and female (distribution not given) Sherman rats, two months of age, were given a mixture of technical grade *ortho*-tolidine (3,3′-dimethylbenzidine) in olive oil by subcutaneous injection for their lifetime. The weekly dose level was 60 mg per rat (maximum cumulated dose, 5.5 g). Of the treated animals, 48 (46%) survived more than 300 days. Five rats (4.8%) developed cancer of the external auditory canal (Zymbal gland), with all tumours appearing after the 354th day. Twenty-eight of the 50 control rats treated with olive oil only (56%) survived more than 300 days. No details on any tumours observed in this control group were reported. While an untreated control group was not run concurrently in this experiment, the authors reported 56 tumours

occurring among 578 untreated rats (490 rats not otherwise incorporated in the study and 88 animals in "diet and vehicle control groups") of the same colony. None of these tumours were located in the external auditory canal (Spitz *et al.*, 1950). [The Working Group noted that this study is limited by poor survival of the animals, which can be attributed to the reported lack of climate control in the animal rooms and widespread disease in the treated and control animals].

Groups of 27 male and 26 female random-bred white rats [strain and age not specified] received weekly subcutaneous injections of a 4% suspension of purified *ortho*-tolidine (3,3′-dimethylbenzidine) in 0.5 ml sunflower oil for 13 months. Doses were 20 mg per rat per week, for a total dose of 1160 mg/rat. Use of control animals was not reported. Twenty-five male and 25 female rats survived for at least eight months (time of occurrence of the first tumour), and 11 males and 5 females lived up to 18 months. Of the animals that survived for at least eight months, 17/25 (68%) males and 13/25 (52%) females developed a total of 41 tumours. Zymbal gland tumours accounted for 14/27 (52%) tumours observed in males and 6/14 (43%) in females. Other sites where tumours were observed included mammary gland (five, female only), skin (three, male and female), preputial gland (three, male and female), and forestomach (one male). An additional group of rats (24 of each sex) received a weekly subcutaneous implant of a pellet containing 20 mg of purified *ortho*-tolidine and 10 mg of glycerol for 14 months. A third group (20 of each sex) received a weekly subcutaneous implant of a pellet containing 20 mg of *ortho*-tolidine that had been subjected to ultraviolet irradiation before the preparation of the pellet. The difference in response to these exposures between the two groups was minimal. Of a total of 68 animals that were alive at the time of appearance of the first tumour (11–12 months), 48 developed a total of 60 tumours. Among these were 27 Zymbal gland carcinomas, and tumours at other sites (Pliss & Zabezhinsky, 1970). [The Working Group noted this study was limited by the lack of a control group. However, in a preliminary report on these studies it was stated that rats from the same colony did not spontaneously develop tumours of the Zymbal gland (Pliss, 1965)].

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Absorption*

(a) Humans

Benzidine is light and fluffy, but solid and vapour forms can be rapidly absorbed through the skin (Barsotti and Vigliani, 1952; Budavari *et al.*, 1989; Ferber *et al.*, 1976; Meigs *et al.*, 1951, 1954; Zavon *et al.*, 1973). Exposure to benzidine can occur from

breathing contaminated air, wearing contaminated clothing, or by ingestion of contaminated food or water (Meigs *et al.*, 1951). Breathing or ingesting benzidine-based dyes also expose humans to benzidine, because the intestine contains bacteria that can break down these dyes into benzidine (Chung, 1983; Chung *et al.*, 1992).

Absorption follows after exposure by inhalation, or by the oral and dermal routes. Inhalation and skin contact are probably the predominant exposure routes for humans (ATSDR, 2001). It has been reported that benzidine and *3,3′*-dimethylbenzidine can readily penetrate intact skin (Meigs *et al.*, 1951). Industrial workers who handle benzidine and perspire freely were reported to have higher urinary concentrations of benzidine (Meigs *et al.*, 1954).

(b) Animals

Radioactivity was observed in tissues, urine, and faeces following application of 1 mg/kg bw of radioactive benzidine or benzidine derivatives for 1, 8, and 24 hours onto the shaved skin of F344 rats in a well controlled study, in which the animals were prevented from grooming themselves and licking at the site of benzidine application (Shah & Guthrie, 1983). About 25% of the initial dose of benzidine and benzidine derivatives penetrated into rat skin within 8 hours. At 24 hours after dosing, 49% of the radioactivity was recovered from the skin, which indicated that approximately half of the applied benzidine had penetrated into the skin and about 50% of the applied dose had been absorbed.

Aldrich *et al.* (1986) applied radiolabelled Direct Black 38 to the shaved dorsal skin of male F344 rats and New Zealand rabbits that were prevented from licking the site of application. Radioactivity was measured in urine and faeces 24–144 hours after application of the dye. At 144 hours, approximately 3% of the applied radioactivity was detected in the urine and 5% in the faeces of the rabbits. Excretion of radioactive substances was eventually negligible in rats (0.05% in urine and 0.16% in faeces). So skin penetration by the benzidine-based dye was unlikely; the absorbed and excreted radioactive material in the rabbits was presumed to represent benzidine that had been liberated by azo reduction of the dye (ATSDR, 2001). Bos *et al.* (1986) demonstrated transport of benzidine but not benzidine-based dyes across the mucosa of an isolated segment of rat intestine in a perfusion chamber, suggesting that benzidine but not benzidine-based dyes could be absorbed in the intestine.

Qiao *et al.* (1996) and Williams *et al.* (1996) developed a unique approach to study the absorption of chemicals from complex mixtures by use of "mechanistically defined chemical mixtures (MDCM)" applied to pig skin, which is similar in structure and function to human skin. Baynes *et al.* (1996) employed this system to investigate the absorption of mixtures of chemicals consisting of a marker chemical (benzidine), a solvent (acetone or DMSO), a surfactant (0% or 10% sodium lauryl sulfate), a vasodilator (0 μg or 180 μg methyl nicotinate), and a reducing agent (0% or 2% $SnCl_2$). It was found that acetone and DMSO enhanced dermal penetration of benzidine in most of the

mixtures. Compared with other mixtures evaluated, $SnCl_2$ inhibited benzidine absorption irrespective of the solvent present. $SnCl_2$ also inhibited benzidine penetration in DMSO mixtures containing sodium lauryl sulfate only, but not in acetone mixtures. It was proposed that interactions between benzidine and $SnCl_2$ may be the cause of the inhibition of benzidine absorption. The chemical-biological interaction between methyl nicotinate, sodium lauryl sulfate, and the skin may enhance benzidine absorption.

4.1.2 *Distribution*

(a) Humans

There is no information available on the distribution of benzidine in humans.

(b) Animals

In general, there appears to be a rapid plasma clearance of absorbed benzidine, followed by a more gradual metabolism and clearance of its metabolites (Shah & Guthrie, 1983; Lakshmi *et al.*, 1990).

When rabbits were given oral doses of 60–120 mg/kg of benzidine for periods ranging from 42 days to 128 days, the highest concentrations were found in the heart and lungs. Benzidine metabolites were not determined (Oida 1958a,b).

Soloimskaia (1968) reported that benzidine was rapidly absorbed after injection into rats with maximum concentrations of free and bound benzidines found at 2 and 3 hours, respectively. The highest concentrations were found in the blood, followed by liver, kidney, spleen, heart, and lung.

The body distribution of benzidine in various tissues and in urine of rats at 4 and 12 hours after intraperitoneal (i.p.) injection of 100 mg/kg benzidine was as follows: high concentrations were found in the stomach, stomach contents, and small intestine at 4 hours, and in the small intestine and its contents at 12 hours. Tissue concentrations of conjugated metabolites were high at 12 hous, and benzidine concentrations in the liver were high and constant over the 12-hour period (Baker and Deighton, 1953).

When radiolabelled benzidine was applied to the skin of rats, radioactivity was distributed approximately as follows (percentage of applied radioactivity at 1, 8, and 24 hours after application): blood (0.2, 0.3, and 0.7%), liver (1.5, 1.0, and 0.7%), lung (0.09, 0.2, and 0.2%), intestines (1.0, 14.0, and 1.3%), and stomach (0.5, 0.4, and 0.08%). Twenty-four h after dosing approximately half of the applied radioactivity had remained at the site of application (Shah & Guthrie, 1983). In a similar experiment in rats, in which radiolabelled benzidine was injected intravenously, tissues retaining the most radioactivities after three days were muscle, liver, and the stomach. Only a small amount was found in the bladder (Lynn *et al.*, 1984).

Chipman & Mohn (1989) demonstrated that biliary benzidine and benzidine metabolites could be reabsorbed from the intestine and transported again to the liver in both rats and mice. This entero-hepatic re-circulation could contribute to the persistence,

the further metabolism, and presumably the hepatoxicity and carcinogenicity of benzidine and its metabolites.

Sanderson & Clark (1993) showed that intraperitoneal administration of benzidine to pregnant mice resulted in the induction of micronuclei in the maternal bone marrow and in the liver of the fetuses; however, when pregnant mice were orally exposed to benzidine, there was no increase in micronucleated cells either in the maternal livers or the livers of the fetuses (Harper *et al.*, 1989). There was no information on whether benzidine could be stored in maternal tissues and be mobilized during pregnancy or lactation, nor was it known whether benzidine could be excreted in breast milk (ATSDR, 2001).

Kellner *et al.* (1973) studied the distribution of benzidine by injecting 0.2 mg/kg of uniformly labelled [^{14}C]-benzidine into animals of various species. In rats, substantial radio-activity was found after 4 hours in the lung, small and large intestines, the bladder, and the kidney, with smaller amounts in all other tissues and fluids examined. The findings in dogs were generally similar except for the 10- to 15-fold higher levels of radioactivity in bladder tissue, and the much lower activity (about 10% that of rats) in the lung. This was consistent with the high carcinogenicity of benzidine in the bladder of dogs. Approximately 90% of the radioactivity was cleared from the blood during the first 24 hours after dosing, the remainder being cleared more slowly. Half-lives of radioactivity from day one to day six or seven were 68 hours in the rats and 88 hours in the dogs. After seven days, the radioactivity was much reduced in all organs examined from rats, dogs, and monkeys. Highest residual activity was found in the liver for all three species. Expressed as concentration of benzidine in wet tissue, the mean liver concentrations were 0.042 μg/g for rats, 0.087–0.19 μg/g for three dogs, and 0.01 and 0.027 μg/g for the two monkeys (ATSDR, 2001).

In dogs, plasma clearance of benzidine is fairly rapid. Approximatelty 10% remained in the plasma after 5 hours, while metabolism and metabolite clearance occurred more gradually. In a study of four dogs monitored over a 5-hour period following intravenous administration of 1 mg/kg radiolabelled benzidine, Lakshmi *et al.* (1990) found that the initial plasma half-life of benzidine was approximately 30 minutes, while it was about 3 hours for total radiolabel (benzidine and metabolites). Five hours after infusion, 75% of recovered radioactivity was found in the bile (12–25%), urine (23–52%), and carcass muscle (15–30%). Significant amounts of radioactivity were also detected in fat (3–8%), the liver (4–8%), and plasma (2–7%). Small quantities were found in the stomach, intestines, spleen, kidneys, heart, and lungs. The bladder transitional epithelium showed a higher concentration of bound radioactivity than did bladder muscle. In liver, kidney, bladder muscle, and bladder epithelium, the majority of radioactivity was bound to protein, while smaller amounts were bound to DNA.

The differential serum protein-binding of benzidine- and benzidine congener-based dyes and derivatives was studied by use of crossed immuno-electrophoresis (X-IEP) techniques. The binding of these chemicals to certain serum proteins could be observed in

electrophoretic and immunoprecipitation patterns in X-IEP. Benzidine- and dimethylbenzidine-based dyes bound to albumin α_1-lipoprotein, β-lipoprotein, and hemopexin, whereas benzidine and dimethylbenzidine did not produce any electrophoretic shifts. However, autoradiographic analyses with benzidine and 3, 3′-dimethylbenzidine did show binding of benzidine to both α_1- and β-lipoprotein precipitation peaks. Although the physiological or pathological consequences of dye-binding to these serum proteins are not well understood, dyes derived from benzidine and its congeners may be carried by the proteins to different parts of the cell. For example, α_1-lipoprotein delivered the dyes to macrophage lysosomes where they inhibited several lysosomal enzymes, causing prolonged impairment of macrophage function with teratogenic, anti-immune, and potentially carcinogenic consequences (Crowle & May, 1982; Emmett *et al.*, 1985).

4.1.3 *Metabolism*

(a) Humans

Cerniglia *et al.* (1982) studied the metabolism of the azo-dye Direct Black 38 in intestinal bacteria and found that the azo linkage in Direct Black 38 was reduced by azoreductase in these bacteria, resulting in release the carcinogen benzidine. Chung *et al.* (1978) reported that many intestinal bacteria isolated from faeces of patients with polyposis could reduce azo dyes. The bacteria isolated from intestine and/or the skin was also reported to have azoreductase activity (Chung *et al.*, 1992; Chung & Stevens, 1993; Chen, 2006; Platzek *et al.*, 1999; Xu *et al.*, 2007). The azoreductase in different preparations was affected by various dietary factors such as cellulose, proteins, fibres, antibiotics, or supplementation with live cultures of lactobacilli (Chung *et al.*, 1992). Many benzidine congener-based dyes including 3, 3′-dimethylbenzidine, 3, 3′-dimethoxybenzidine, and 3, 3′-dichlorobenzidine were also reported to be reduced by azoreductase in intestinal bacteria and/or other environmental microorganisms to release the benzidine congeners and many other metabolites such as monoacetyl- and diacetylbenzidine and their congeners (Bowman & Nony, 1981; Bowman *et al.*, 1982, 1983; Manning *et al.*, 1985; Cerniglia *et al.*, 1986). These metabolites were also detected in workers exposed to these azo dyes, benzidine or benzidine-based pigments (Vigliani & Barsotti, 1961; Haley, 1975, 1982). Therefore, azo-reduction was considered the first step of azo dye- induced carcinogenesis (Chung, 1983; Chung & Cerniglia, 1992) and control of azoreduction becomes important in azo dye-induced cancer (Chen *et al.*, 2006). It should be pointed out that metabolic conversion of benzidine-, 3, 3′-dimethybenzidine- and 3, 3′-dimethoxybenzidine-based dyes to their respective carcinogenic amine precursors in vivo is a general phenomenon: exposure to benzidine-based dyes has caused bladder cancer in humans. However, studies in which azo pigments based on 3, 3′-dichlorobenzidine such as Pigment Yellow 12 had been orally administered to rats, hamsters, rabbits, and monkeys did not generally show significant amounts of 3, 3′-

dichlorobenzidine in the urine. The aromatic amine components from azo pigments based on *3, 3′*-dichloro-benzidine appear not to be readily bio-available. Therefore, it seems unlikely that occupational exposure to insoluble azo pigments (3, 3′-dichloro-benzidine-based azo dyes such as Pigment Yellow 12) would be associated with a substantial risk for bladder cancer in humans (Golka *et al.*, 2004).

Since benzidine is the major mutagenic and carcinogenic moiety of carcinogenic azo dyes (Chung & Cerniglia, 1992) the mechanisms of activation of benzidine have been extensively studied (Morton *et al.*, 1979).

Rothman *et al.* (1996a) conducted a cross-sectional study of workers exposed to benzidine and benzidine-based dyes, and unexposed controls. Benzidine, *N*-acetyl-benzidine, and *N,N′*-diacetylbenzidine were not detected in the urine of control subjects. Urinary levels of these compounds were low in workers producing benzidine-based dyes and about 17-fold higher in workers manufacturing benzidine. Upon analysis by ^{32}P-postlabelling, four DNA adducts were found to be significantly elevated in urothelial cells of exposed workers compared with controls, the predominant adduct being *N*-(deoxyguanosin-8-yl)-*N′*-acetylbenzidine. This is the only adduct that was significantly associated with the total amount of urinary metabolites of benzidine. These results suggest that *N*-monoacetylation is involved in activation of benzidine, while *N*-diacetylation is likely part of a detoxification pathway. In this study, there was no significant association between *NAT2* genotype and adduct levels.

Zenser *et al.* (1996) assessed *N*-acetylation of benzidine to *N*-acetylbenzidine by use of human recombinant NATs. Km and V_{max} values were higher for NAT1 than for NAT2. The clearance ratios (NAT1/NAT2) for benzidine and *N*-acetylbenzidine were 54 and 535, respectively, suggesting that NAT1 is a more efficient enzyme for *N*-acetyl-benzidine than NAT2. The much higher Km values of NAT1 and NAT2 for *N*-acetyl-benzidine compared with benzidine appear to favour the metabolism of benzidine over that of *N*-acetylbenzidine, for low exposures.

In human liver slices incubated with [^{3}H]-labelled benzidine, the relative amounts of benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine were 19 ± 5, 34 ± 4, and 1.6 ± 0.5%, respectively. Similar results were observed if slices were incubated with [^{3}H]-acetylbenzidine instead of [^{3}H]-benzidine (Zenser *et al.*, 1996). Thus, in these studies, conditions in liver slices favour the formation of *N*-acetylbenzidine rather than *N,N′*-diacetylbenzidine. With paraoxon, a deacetylase inhibitor, the formation of *N,N′*-diacetyl-benzidine increased 32-fold. *para*-Aminobenzoic acid, a NAT1-selective substrate, increased the amount of benzidine and decreased the amount of *N*-acetylbenzidine produced, resulting in a decreased ratio of acetylated products. This is consistent with benzidine being an NAT1 substrate. Individuals with rapid *NAT2* genotypes did not form significantly more *N*-acetylbenzidine than did slow acetylators. There was no apparent correlation of *N,N′*-diacetylbenzidine formation with *NAT2* genotype. HPLC analysis of the liver-slice extracts detected *N*-glucuronides of both benzidine and *N*-acetyl-benzidine.

These *N*-glucuronides represent 7 and 16%, respectively, of the total radioactivity recovered by HPLC (Zenser *et al.*, 1996).

Ciotti *et al.* (1999) assessed the capacity of five different human recombinant UDP-glucuronosyltransferases (UGTs) expressed in COS-1 cells. [^{14}C]-Labelled UDP-glucuronic acid was used as a co-substrate. Benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine, the *N*-OH derivatives of acetyl- and diacetylbenzidine, the 3-OH derivatives of diacetylbenzidine and benzidine were used as substrates. *N,N′*-diacetyl-benzidine was not a substrate for glucuronidation. UGT1A9 showed the highest relative rate of metabolism, with a preference for the *N*-OH derivatives of acetyl- and diacetyl-benzidine. The overall results suggest the following relative ranking of transferase metabolism: UGT1A9 > UGT1A4 >> UGT2B7 > UGT1A6 ≈ UGT1A1.

Since the *N*-glucuronides of benzidine and *N*-acetyl-benzidine are acid-labile (Babu *et al.*, 1992; 1993), this property was used to indirectly assess these glucuronides in urine from workers in India manufacturing benzidine or benzidine-based dyes, in comparison with those from workers at a construction company. The pH of post-workshift urine was inversely correlated with the proportions of benzidine and *N*-acetylbenzidine present as free (non-glucuronidated) compounds. When controlling for internal dose, individuals with urine at pH < 6 had a tenfold higher level of the deoxy-guanosine adduct of acetylbenzidine in their exfoliated bladder cells, compared with subjects with urine at pH ≥ 7 (Rothman *et al.*, 1997). These results suggest that a low pH of the urine may be a risk factor in bladder cancer.

In both bladder cells and white blood cells, dGp-acetylbenzidine is the major adduct (Zhou *et al.* 1997). The sum of urinary benzidine metabolites (benzidine, *N*-acetyl-benzidine, and *N,N′*-diacetylbenzidine) is an index of internal dose that correlates with the level of the dGp-acetylbenzidine adduct in both peripheral white blood cells and in exfoliated bladder cells. Moreover, adduct levels in human peripheral white blood cells correlate with those in exfoliated bladder cells. Similar mechanisms of adduct formation may exist in both cell types, with white blood cells serving as a surrogate biomarker. Thus, dGp-acetylbenzidine is an important adduct, and human peripheral white blood cells are a relevant cell type for studying this adduct formation.

Lakshmi *et al.* (2000a; 2000b) assessed the metabolic pathways leading to dGp-acetylbenzidine formation in human peripheral white blood cells. Transformation of [^{3}H]-labelled acetylbenzidine was assessed by use of myeloperoxidase (MPO) or hypochlorous acid (HOCl). MPO-mediated metabolism required H_2O_2. While transformation by HOCl was completely inhibited by 10 mM taurine, the metabolism of acetylbenzidine by MPO was only reduced 56%. Transformation by either MPO or HOCl was inhibited by 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 1 mM glutathione, and 1 mM ascorbic acid. Two previously identified oxidation products of acetylbenzidine, *N′*-hydroxy-*N*-acetylbenzidine and 4′-nitro-4-acetylaminobiphenyl, were not detected. With DNA or dGp present, a new product was observed that corresponded to synthetic dGp-acetylbenzidine. The HOCl-derived adduct was identified by electrospray-ionization mass

spectrometry (ESI/MS) and NMR as dGp-acetylbenzidine. Upon analysis by ^{32}P-postlabelling, dGp-acetylbenzidine increased more than 300-fold if either DNA or dGp was present. Indomethacin (0.1mM) did not alter adduct formation. These results are consistent with human neutrophils forming dGp-acetylbenzidine by a peroxidative mechanism involving MPO.

Studies with many aromatic amines and human liver microsomes (Beland & Kadlubar, 1990) and with acetylbenzidine and rat-liver microsomes have demonstrated CYP-mediated *N*-oxidation (Lakshmi *et al.*, 1997). With rat-liver microsomes, *N′*-hydroxy-*N*-acetylbenzidine is formed. However, *N*-oxidation of acetylbenzidine with human liver microsomes has not been demonstrated.

(b) Experimental systems

N-Acetylation plays an important role in biotransformation of aromatic amines and was assessed for benzidine. In rat-liver slices incubated with 0.05 mM [^{3}H]-labelled benzidine, the acetylated products *N*-acetylbenzidine and *N,N′*-diacetylbenzidine represented 8.8 ± 3.6 and 73 ± 2.5%, respectively, of the total radioactivity recovered after HPLC (Lakshmi *et al.*, 1995a). No unmetabolized benzidine was observed.

Excretion of aromatic amines is facilitated by UDP-glucuronosyltransferases (UGTs). When *N*-glucuronidation of benzidine and *N*-acetylbenzidine was assessed, microsomes from dog and rat produced an identical new HPLC peak, which was dependent upon the presence of UDP-glucuronic acid (Babu *et al.*, 1992, 1993). Whether incubated in the presence or absence of detergents, microsome-catalysed glucuronidation of *N*-acetylbenzidine and *N,N′*-diacetylbenzidine decreased as follows: human > dog > rat. No glucuronidation of *N,N′*-diacetylbenzidine was observed, which is consistent with the lack of glucuronidation of arylamides (Babu *et al.*, 1993). To determine the specificity of the UGT reaction with benzidine and *N*-acetylbenzidine, a wide range of inhibitors (known substrates) was tested. Results were consistent with multiple transferases metabolizing benzidine and *N*-acetylbenzidine.

To correlate results with microsomal glucuronidation to those in intact tissues, dog-liver slices were incubated with 0.05 mM [^{3}H]-labelled benzidine. An HPLC peak corresponding to the glucuronide conjugate of benzidine represented as much as 30% of the total radioactivity recovered (Babu *et al.*, 1992). Neither benzidine nor acetylbenzidine glucuronide was detected under these incubation conditions with rat liver, which rapidly *N*-acetylates benzidine to acetyl- and diacetylbenzidine (Babu *et al.*, 1993; Lakshmi *et al.*, 1995b). This is consistent with *N*-acetylation and *N*-glucuronidation being competing pathways and likely playing a role in benzidine-induced liver cancer in rats and bladder cancer in dogs and humans (Case *et al.*, 1954; Haley, 1975).

The pH of the urine is affected by diet and was considered to be a potential modifier of benzidine-induced bladder carcinogenesis. After 4 or 5 min at pH 5.3 and 37ºC, half of the *N*-glucuronides of benzidine and acetylbenzidine are hydrolysed to their parent amine (Babu *et al.*, 1992; 1993). At pH 7.4, the half-lives of these glucuronides are 104 and 140

min, respectively. The *O*-glucuronides of the hydroxamic acids, *N*-hydroxy-*N*-acetylbenzidine and *N*-hydroxy-*N,N′*-diacetylbenzidine, were not acid-labile. The *N*-glucuronide of *N′*-hydroxy-*N*-acetylbenzidine was acid-labile, with a half-life at pH 5.5 of 3.5 hours, compared with 7.5 min for the *N*-glucuronide of acetylbenzidine. Thus the *N*-glucuronide of *N*-acetylbenzidine is much more likely to be involved in acidic urine-catalysed hydrolysis than is its *N*-hydroxy *N*-glucuronide. The glucuronides of 4-aminobiphenyl and *N*-OH-4-aminobiphenyl were both acid-labile with half-lives of 10 and 32 min, respectively, at pH 5.5 (Babu *et al.*, 1996). In contrast, the *O*-glucuronide of *N*-OH-*N*-acetyl-4-aminobiphenyl was not acid-labile, with half-lives at pH 5.5 and 7.4 of 55 and 68 min, respectively. Thus other *N*-glucuronides of aromatic amines are also acid-labile and may have a shorter half-life than their corresponding *N*-OH *N*-glucuronides. *O*-Glucuronides are not acid-labile (Babu *et al.*, 1996).

To evaluate NADPH-dependent oxidation of benzidine, Lakshmi *et al.* (1996) used liver microsomes from control and β-naphthoflavone-treated rats. Beta-naphthoflavone treatment increased the metabolism of benzidine compared with the control, as judged from the HPLC metabolite-profile and protein/DNA binding. The CYP inhibitors ellipticine and α-naphthoflavone, selective for CYP1A1/1A2, elicited 50% inhibition at approximately 0.2 and 0.5 μM, respectively. Mass spectrometry identified the only metabolite formed as 3-hydroxybenzidine. *N*-hydroxybenzidine formation by CYP has never been reported.

CYP-induced metabolism and activation of acetyl- and diacetylbenzidine was assessed by incubating liver microsomes from control and β-naphthoflavone-treated rats with either substrate (Lakshmi *et al.*, 1997). With β-naphthoflavone-induced microsomes, *N*-hydroxy-*N*-acetylbenzidine formation was eight-fold higher than in the control: a significant formation of ring-oxidation products was demonstrated, and *N′*-hydroxy-*N*-acetylbenzidine formation was at the limit of detection. With control microsomes, more *N′*-hydroxy-*N*-acetylbenzidine was produced than *N*-hydroxy-*N*-acetylbenzidine. While oxidation of diacetylbenzidine was not observed with control microsomes, significant *N*-hydroxy-*N,N′*-diacetylbenzidine formation and ring-oxidation was seen with β-naphthoflavone treatment. Metabolism by β-naphthoflavone-induced microsomes was completely blocked by selective CYP1A1/1A2 inhibitors, α-naphthoflavone and ellipticine. *N′*-Hydroxy-*N*-acetylbenzidine formation by control microsomes was not prevented by these inhibitors. A non-specific CYP inhibitor, SKF-525A, exhibited partial dose-response inhibition of *N′*-hydroxy-*N*-acetylbenzidine. The adduct *N′*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine was detected by ^{32}P-postlabelling in incubations containing DNA and acetylbenzidine, but not diacetylbenzidine. More adduct was detected with control than with β-naphthoflavone-treated microsomes. Thus, while *N*-hydroxybenzidine formation by CYP was not observed, acetyl- and diacetylbenzidine are substrates for these enzymes and form *N*-hydroxy metabolites. These results are consistent with CYP-mediated activation of acetylbenzidine to *N′*-hydroxy-*N*-acetylbenzidine with subsequent binding to

DNA and adduct formation. This is likely a mechanism responsible for the formation of the dGp-acetylbenzidine adduct in humans (Lakshmi *et al.*, 1997).

A study investigating the metabolism of benzidine in slices of the inner renal medulla of rabbits showed that benzidine induced a dose-dependent reversible inhibition of prostaglandin E2 (PGE2) synthesis. Binding of [^{14}C]-labelled benzidine metabolites to medullary tissue was observed. This binding was increased by arachidonic acid, and arachidonic acid-mediated binding was prevented by inhibitors of prostaglandin H synthase. Inhibitors of mixed function oxidase activity (metyrapone and SKF-525A) did not inhibit binding of benzidine metabolite(s). These findings are consistent with previous studies and demonstrate the microsomal co-oxidative metabolism of benzidine by prostaglandin H synthase in the inner medulla (Rapp *et al.*, 1980).

Since the bladder is a possible site for activation of arylamine bladder carcinogens, the dog bladder was studied and found to contain little CYP but substantial prostaglandin H synthase (PHS) activity (Wise *et al.*, 1984). Lakshmi *et al.* (1998) assessed the possible formation of the dGp-acetylbenzidine adduct by peroxidatic activation of acetylbenzidine. Adduct formation was measured by ^{32}P-postlabelling. Ram seminal vesicle microsomes were used as a source of PHS. The peroxidatic activity of PHS induced formation of the adduct, whether DNA or dGp was present. Adduct formation was dependent upon the presence of peroxidase and a specific substrate, i.e. arachidonic acid or H_2O_2. Adduct formation was inhibited by indomethacin (0.1 mM), ascorbic acid (1 mM), and glutathione (10 mM), but not by DMPO (100 mM), a radical scavenger. Since the PHS activity in cultured urothelial cells from humans and dogs is enhanced by bradykinin, calcium ionophore, arachidonic acid, and phorbol ester (Danon *et al.*, 1986; Zenser *et al.*, 1988; 1990), a corresponding increase in benzidine activation could occur.

Zenser *et al.* (1999) examined the mechanism by which PHS from ram seminal vesicle microsomes catalyses the oxidation of the reducing co-factor acetylbenzidine. During the conversion of this compound to its final end product 4′-nitro-4-acetylaminobiphenyl, a new metabolite was detected when 1 mM ascorbic acid was present. Similar results were observed whether arachidonic acid or H_2O_2 was used as substrate. This metabolite co-eluted with synthetic *N*′-hydroxy-*N*-acetylbenzidine but not *N*-hydroxy-*N*-acetylbenzidine. The new metabolite was identified as *N*′-hydroxy-*N*-acetylbenzidine by ESI/MS/MS. It represented as much as 10% of the total radioactivity recovered after HPLC. When *N*′-hydroxy-*N*-acetylbenzidine was substituted for *N*-acetylbenzidine, 4′-nitro-4-acetylaminobiphenyl was formed. Inhibitor studies demonstrated that the metabolism was due to PHS, not CYP. Oxygen-uptake studies did not demonstrate a requirement for molecular oxygen. When [^{18}O]H_2O_2 was used as substrate, [^{18}O] enrichment was observed. These results demonstrate a peroxidative mechanism of oxidation of *N*-acetylbenzidine and *N*′-hydroxy-*N*-acetylbenzidine by PHS and suggest stepwise oxidation of acetylbenzidine to *N*′-hydroxy, 4′-nitroso, and 4′-nitro products (Zenser *et al.,* 1999). In contrast, horseradish peroxidase and myeloperoxidase (MPO) appear to activate acetylbenzidine by a radical mechanism not involving *N*′-hydroxy-*N*-

acetylbenzidine (Lakshmi *et al.*, 1998; 2000a). Thus, dGp-acetylbenzidine is formed by activation of acetylbenzidine with CYP, PHS or MPO, each representing a different mechanism of activation.

To gain more insight into peroxidative activation of acetylbenzidine, glutathione was used to trap the activated intermediate (Lakshmi *et al.*, 2000b; Zenser *et al.*, 2001). Myeloperoxidase, like horseradish peroxidase, metabolizes acetylbenzidine by a mechanism that does not produce 4′-nitro-4-acetylaminobiphenyl. While the thiol conjugate of horseradish peroxidase-activated acetylbenzidine was expected to be similar to that formed with benzidine, *i.e.* 3-(glutathione-*S*-yl)-benzidine (Wise *et al.*, 1985; Lakshmi *et al.* 1994), this was not the case (Lakshmi *et al.*, 2000b). The product was identified by mass spectrometry and NMR as *N*′-(glutathion-*S*-yl)-acetylbenzidine-*S*-oxide. The lack of effect of mannitol and superoxide dismutase suggests that neither the hydroxyl radical nor superoxide is involved in this reaction. Studies also indicated that molecular oxygen is not a source of the sulfinamide oxygen. Methaemoglobin (acting as a peroxidase) catalysed the formation of the same conjugate (Zenser *et al.*, 2001). The proposed mechanism for sulfinamide formation, involving two consecutive one-electron oxidations with subsequent arrangement to a sulfur-stabilized nitrenium ion, suggests that the oxygen may be derived from water. A less active ring-activated intermediate, such as a diimine monocation may be formed, which is a resonance structure of the acetyl-benzidine nitrenium ion. This intermediate may play a role in the activation of acetylbenzidine by horseradish peroxidase (Lakshmi *et al.*, 1998) and MPO (Lakshmi *et al.*, 2000a), leading to formation of the dGp-acetylbenzidine adduct.

Reduction of benzidine-based dyes is a potential source of human exposure to benzidine. Since aromatic amines can be activated to bind haemoglobin, these adducts offer a method for assessing exposure. When female Wistar rats were given an oral dose of 0.5 mmol/kg benzidine and haemoglobin was isolated after 24 hours (Birner *et al.*, 1990; Zwirner-Baier & Neumann, 1998), the haemoglobin binding index (HBI) was 2.4 (benzidine), 18.9 (acetylbenzidine), and 3.0 (4-aminobiphenyl, 4-ABP). Since benzidine is rapidly *N*-acetylated in rats, acetylbenzidine adducts are expected. Diacetylbenzidine is not expected to form haemoglobin adducts, and no adducts were detected after administration of this compound. The presence of 4-ABP adducts was unexpected, and demonstrates an unknown pathway of the metabolism of benzidine. This method was then used to monitor the bioavailability of benzidine and its metabolites following oral administration of Direct Red 28, a benzidine-derived azo-dye, at 1 mmol/kg. The HBI indices for benzidine, acetylbenzidine and 4-ABP were 0.3, 1.8, and 2.2. This demonstrates exposure to these three compounds derived from a benzidine-based dye (Birner *et al.*, 1990). Reactive nitric oxygen species transform benzidine to 4-ABP and 4'-OH-4-amino-biphenyl and this reaction, involving components of the inflammatory response, may be a source of 4-ABP formation from benzidine *in vivo* (Lakshmi *et al.*, 2003).

4.1.4 *Excretion*

(a) Humans

A single oral dose of 100 mg of benzidine in humans resulted in urinary excretion of free benzidine and its mono- and di-acetylated derivatives. However, only less than 1 mg of the initial dose was recovered (Engelbertz & Babel, 1953).

Benzidine and its metabolites were measured in the urine of exposed industrial workers in March and August. The mean urinary concentrations of the compounds after exposure in the spring were as follows: benzidine, 0.28 mg/L; *N*-acetylbenzidine, 0.27 mg/L; *N,N'*-diacetylbenzidine, 0.52 mg/L; conjugated 3-hydroxybenzidine, 3.9 mg/L. During the month of August, a mean overall concentration of 21.8 mg/L was measured for benzidine and its metabolites, the peak excretion being 31 mg/L. Dermal contact with dust containing benzidine was the primary source at the benzidine plants. Daily showers and clean working clothes reduced the quantity of benzidine and its metabolites excreted in the urine of exposed workers (Meigs *et al.*, 1951).

Excretion of 0–363 µg/L benzidine, 6–1117 µg/L acetylbenzidine, and 4–160 µg/L diacetylbenzidine was measured in the urine of workers potentially exposed to several benzidine-based dyes. Exposure was assumed to have been largely by inhalation, but dermal exposure may have been significant as well (NIOSH, 1980; Dewan *et al.*, 1988).

Many workers in dye, printing, warehouse, and colour room shops who were exposed to benzidine-based dyes including Direct Black 4, Direct Blue 2, Direct Brown 2, Direct Green 1, Direct Orange 1, Direct Orange 8, Direct Red 28, Direct Blue 6, Direct 38, and Direct Brown 95 excreted benzidine in their urine (Walker, 1970; Genin, 1977; Lynn *et al.*, 1980; Robens *et al.*, 1980; Haley, 1982).

(b) Animals

(i) *Rat*

Excretion of benzidine after an intravenous dose of 0.2 mg/kg in rats, dogs, and monkeys was 97%, 96%, and 88%, respectively, one week after dosing. Dogs and monkeys excreted benzidine via the urinary route, whereas rats used the biliary route (Kellner *et al.*, 1973). Following intravenous exposure of rats to 0.2 or 2.5 mg/kg radiolabelled benzidine, most of the radioactivity (63–80%) was excreted in the faeces during the first 3 to 7 days, and much less via the urine (17–29%) (Kellner *et al.*, 1973; Lynn *et al.*, 1984). Experiments with bile duct-cannulated rats indicated that virtually all fecal metabolites originated from biliary excretion. Urinary metabolites included 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide (25%), *N,N'*-diacetylbenzidine (12%), and *N*-hydroxy-*N,N'*-diacetylbenzidine glucuronide (4%). Metabolites and relative amounts were similar in bile, except that about half of the 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide was replaced by the 3-glutathion-*S*-yl-*N,N'*-diacetylbenzidine conjugate (Lynn *et al.*, 1984).

After dermal application of radiolabelled benzidine to rats, radioactivity was detected in both urine and faeces as early as one hour after treatment. Excretion was significantly greater (6–8-fold) in urine than in faeces during the first eight hours, but it was comparable for both routes after 24 hours (23% in urine, 19% in faeces) (Shah & Guthrie, 1983).

In rats, the major route of excretion after single oral doses of 0.5, 5 or 50 mg/kg radiolabelled benzidine appeared to be via the faeces. At the lowest dose, 74% of the radioactivity was excreted in the faeces during the first days after exposure and only 17% in the urine. With increasing doses the percentage of radioactive compounds excreted in the faeces decreased, while that in the urine increased. At the low- and mid-level doses (0.5 and 5.0 mg/kg), the major radioactive compounds were identified as 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide (39% and 37%), *N,N'*-diacetylbenzidine (13% and 17%), *N*-hydroxy-*N,N'*-diacetylbenzidine glucuronide (4% and 5%), *N*-acetylbenzidine (3% and 4%), and free benzidine (2% and 2%, respectively). At the high dose (50 mg/kg), the percentage of *N*-hydroxy-*N,N'*-diacetylglucuronide increased substantially to 24%, largely at the expense of *N,N'*-diacetylbenzidine (reduced to 4%). No radioactivity was detected in expired air (Lynn *et al.*, 1984).

The analyses of urine and faeces after intravenous injection of radiolabelled benzidine confirmed that the main excretion route of radioactivity was via the faeces (Kellner *et al.*, 1973; Lynn *et al.*, 1984). However, other studies reported that benzidine, its metabolites, and their conjugates were excreted approximately equally in urine and bile/faeces (Shah and Guthrie, 1983; Lakshmi *et al.*, 1990).

(ii) *Mouse*

After injection of mice with 100 mg/kg benzidine, the following compounds were found excreted in the urine: benzidine (10%), *N*-acetylbenzidine (3.4%), *N,N'*-diacetylbenzidine (2.6%), 3-hydroxy ethereal sulfate (29%), 3-hydroxybenzidine glucuronide (12%), *N*-hydrogen sulfate or glucuronide conjugates (18%), and monoacetylated 3-hydroxy ethereal sulfate or glucuronide-benzidine conjugate (25%) (Sciarini & Meigs, 1961).

(iii) *Dog*

After intraperitoneal injection of benzidine, dogs excreted this compound in the urine, but the fecal excretion was 11 times greater than via the urine (Sciarini & Meigs, 1958). The urinary excretion in dogs after intravenous injection of benzidine was reported to range from 1–2.5 times that found in the bile or faeces (Kellner *et al.*, 1973; Lakshmi *et al.*, 1990). About 30% of the radioactivity excreted in the urine or bile was free benzidine. 3-Hydroxybenzidine was a major metabolite (6%) found in the bile, but not in the urine. *N*-Acetylated metabolites were not found. This is in agreement with the fact that dogs are deficient in *N*-acetyltransferase activity (Lakshmi *et al.*, 1990). The urinary concentration

of free benzidine ranged from 2 to 9%, and the concentration of 3-hydroxybenzidine or its sulfur conjugate ranged from 25 to 50%.

(iv) *Monkey*

When benzidine was intravenously injected in three monkeys (0.2 mg/kg bw), the cumulative excretion during the first seven days varied from 30% to 70% in the urine and from 5 to 36% in the faeces. There were some indications of the presence of *N*-acetylbenzidine, but this was not chemically confirmed (Kellner *et al.*, 1973).

Oral exposure of monkeys to 10 or 100 mg benzidine resulted in urinary excretion of free benzidine and *N*-acetylbenzidine. The combined 72-hour excretion of these two compounds represented only a small fraction (1.5%) of the administered dose (Rinde & Troll, 1975). This contrasts with the 6% reported in the rat as excreted fraction for these two compounds (Lynn *et al.*, 1984).

4.2 Genetic and related effects

4.2.1 *Humans*

Mirkova and Lalchev (1990) studied the cytogenetic effects of occupational exposure to benzidine and benzidine-based dyes (Direct Black 38 and Direct Blue 6) in workers at a manufacturing plant in Bulgaria, who had a recognized high risk for occupational cancer. Twenty-three workers (13 men, 10 women), 47 ± 8.3 years of age and exposed for a mean of 15 years, were compared with 30 controls presumed to have had no exposure. A statistically significant (10-fold) increase in the number of circulating peripheral lymphocytes displaying chromosomal aberrations was observed in exposed workers when compared with controls. The highest frequencies of aberrant lymphocytes were associated with the highest airborne dust concentrations of benzidine (0.42–0.86 mg/m^3) or benzidine-based dyes (7.8–32.3 mg/m^3), and with the highest mean levels of benzidine found in the urine (1.8–2.3 μg/L). The frequency of polyploid lymphocytes was also elevated in workers when compared with controls. No significant association with smoking was observed. A major strength of this study is the monitoring and biomonitoring of benzidine. These data provide clear evidence of benzidine's genotoxicity in humans under occupational exposure conditions, and are in agreement with oral genotoxicity results from animals and in-vitro test systems (see below).

4.2.2 *Experimental systems*

(a) In-vivo studies

There are conflicting reports on the ability of benzidine to induce micronucleated polychromatic erythrocytes in the rat. It was inactive at doses of up to 250 mg/kg bw (Trzos *et al.,* 1978), but was positive (with no dose–response) when tested at comparable

doses (100, 200, 300 mg/kg bw) in another study (Cihák, 1979). It was also reported to be active in inducing micronucleus formation when given at the high dose of 409 mg/kg bw, either dermally or subcutaneously (Urwin *et al.,* 1976).

Several studies have addressed the in-vivo genotoxicity of benzidine in animals following oral or parenteral exposure. Although early studies were conflicting or equivocal, benzidine was clearly demonstrated to induce bone-marrow micronuclei in two strains of male mice (C57BL6 and CBA), 24 and 48 hours after a single administration of 300 mg/kg bw benzidine by oral gavage (Mirkova & Ashby, 1988). The number of micronucleated cells per 1000 normal cells for the test groups (5.75–8.75) was three times that observed in control groups (2.0–2.9). These findings were extended in a subsequent study in which male C57BL6 mice, treated by oral gavage with either a single dose (900 mg/kg bw) or with three consecutive daily doses (150 or 300 mg/kg bw), showed a positive dose–response for bone-marrow micronucleus induction (Mirkova, 1990) [The Working Group noted the high doses used in some of these studies]. Negative results, however, were reported for a different strain of mice (ICR), 6–8 weeks of age, treated with single oral gavage doses of 100 or 200 mg benzidine/kg bw. Harper *et al.* (1989) observed no significant increases in micronucleated cells in the bone marrow of treated male, female, or pregnant female mice (gestation days 16–17), nor in the livers from the fetuses of treated pregnant female mice. When given by single oral gavage to male rats, 200 mg benzidine/kg bw induced unscheduled DNA synthesis in liver cells, which is a repair response to DNA damage (Ashby & Mohammed, 1988). In a study with Swiss-albino mice, 9–13 weeks of age, intraperitoneal administration of benzidine to pregnant dams increased the frequency of micronucleated polychromatic erythrocytes in the liver of fetuses, which suggests that benzidine (or metabolites) can cross the placenta (Sanderson & Clark, 1993).

(b) In-vitro studies

Benzidine has consistently been found to be mutagenic to *Salmonella typhimurium* strain TA1538 when tested in the presence of an exogenous metabolic activation system from Sprague-Dawley rats (see, *e.g.*, Ames *et al.,* 1973; Anderson & Styles, 1978) or humans (see, *e.g.*, Neis *et al.*, 1985). The urine of rats fed benzidine was mutagenic to *S. typhimurium* TA1538, TA98 or TA100 when tested in the presence of a rat-liver metabolic activation system or to *S. typhimurium* TA1538 in the presence of a rat-liver cytosolic fraction; addition of glucuronidase increased the mutagenic activity in TA1538 (Bos *et al.,* 1980).

N-Hydroxy-*N,N'*-diacetylbenzidine was mutagenic to *S. typhimurium* TA1538 in the presence of a partially purified *N,O*-acyltransferase preparation (Morton *et al.*, 1979). Benzidine was negative in the *Escherichia coli* pol A test (Fluck *et al.,* 1976) and in the prophage-induction test (Speck *et al.,* 1978), when tested either in the presence or absence of a rat-liver metabolic activation system. Mutagenic activity on the X-chromosome recessives (visibles and lethals) and RNA genes of *Drosophila melanogaster* has been

reported (Fahmy & Fahmy, 1977). Benzidine (6×10^{-4} M for 30 min) inhibited DNA synthesis in HeLa cells *in vitro* in the absence of activation (Painter, 1978), and *in vivo* in renal and hepatic cells when given intraperitoneally or intragastrically to 14–18-day-old suckling mice in doses of 15–30% of the LD50 (Amlacher & Ziebarth, 1979).

Unscheduled DNA synthesis was induced by benzidine (active dose range, 10^{-7}–10^{-3} M) in HeLa cells in the presence of a phenobarbital-induced rat-liver activation system (Martin *et al.,* 1978) and in rat hepatocytes (Williams, 1978; Brouns *et al.,* 1979). Benzidine, when tested in the presence of a rat-liver metabolic activation system, induced DNA strand-breaks in Chinese hamster V79 cells (Swenberg *et al.,* 1976). When measured by the alkaline elution assay, there was a dose-related increase in DNA strand-breaks in the livers of rats exposed to benzidine *in vivo* (Petzold & Swenberg, 1978). Benzidine (2.5 μg/mL) transformed BHK21 $Cl^{-}13$ cells in the presence of an Aroclor 1254-induced rat-liver metabolic system (Ashby *et al.*, 1978), and was shown to transform Syrian hamster embryo cells (Pienta, 1980).

When tested in many in-vitro assays published since 1982 (IARC, 1982), benzidine has generally shown positive results for reverse mutation in *Salmonella typhimurium* in the presence of exogenous metabolic activation (*e.g.*, liver S-9) (Chung *et al.*, 2000; Dorado & Pueyo, 1988; Duverger-van Bogaert *et al.*, 1995; Gregory *et al.*, 1981; Zeiger *et al.*, 1992); negative for SOS DNA repair in *Escherichia coli* (von der Hude *et al.*, 1988); positive for mutation in yeast (Buchholz *et al.*, 1992; Mitchell & Gilbert 1991); positive (Oberly *et al.*, 1990) or negative (Phillips *et al.*, 1990) for gene mutation in Chinese hamster ovary cells; positive (Fassina *et al.*, 1990; Suter *et al.*, 1992) or negative (Oglesby *et al.*, 1983) for gene mutation in Chinese hamster V79 cells; and positive (*Tk* locus) or negative (*Hgprt* locus) for gene mutation in mouse-lymphoma cells (Henderson *et al.*, 1990; Myhr & Caspary, 1988). Benzidine has also given a positive response when tested for chromosome breakage (Swenberg *et al.*, 1976) and sister chromatid exchange (Grady *et al.*, 1986; Lindahl-Kiessling *et al.*, 1989) in cultured human and animal cells; it was generally positive in cultured hepatocytes for unscheduled DNA synthesis (Kornbrust & Barfknecht 1984a, 1984b; Steinmetz *et al.*, 1988; Williams, 1978); positive for animal cell transformation (Ashby *et al.*, 1978; Pienta, 1980); and negative for DNA-adduct formation in cultured mammalian cells, but positive with calf-thymus DNA, in the absence of exogenous activation (Phillips *et al.*, 1990).

4.2.3 *Effects on cell function*

The results of a study on the expression of mutant p53 protein in workers exposed to benzidine and in bladder-cancer patients (Shen *et al.*, 2005) indicated that the expression level of mutant p53 increased with the exposure-intensity index in exposed workers; the expression was significantly higher in bladder-cancer patients than in the group of workers with the highest exposure-intensity index. Moreover, there was a strong correlation between the Papanicolau grade of exfoliated urothelial cells and the expression

level or the quantity of mutant p53 protein for the higher benzidine-exposure category (Shen *et al.*, 2005).

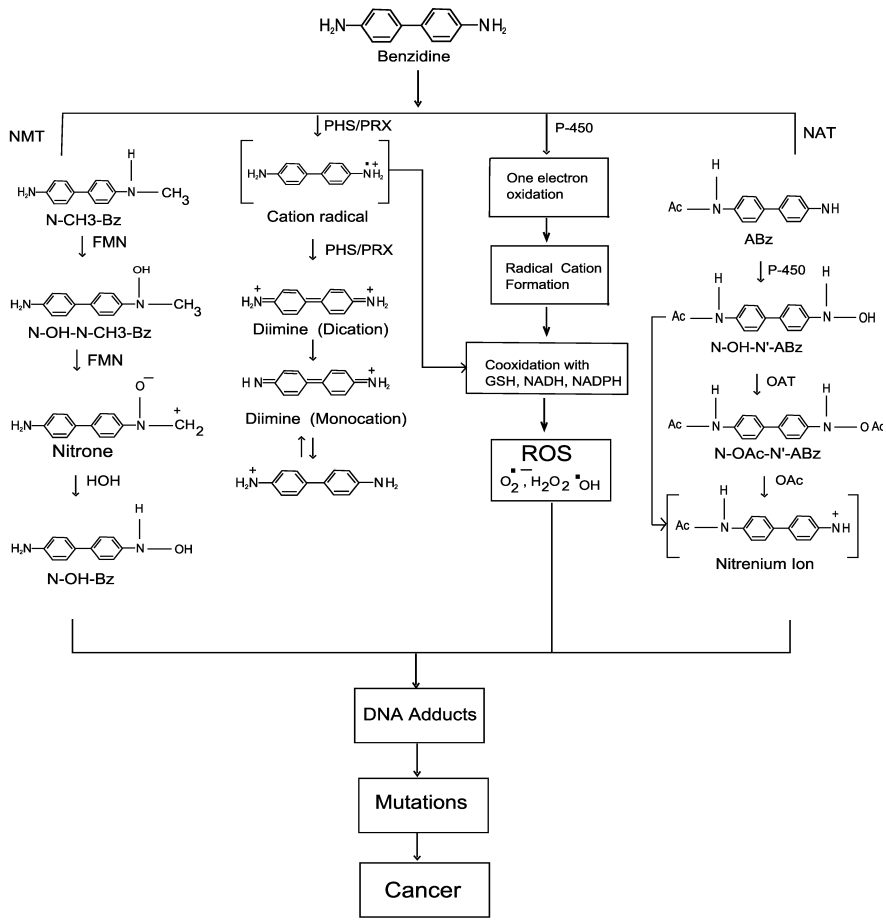
To investigate the expression of mutant p53 protein in relation to benzidine exposure, Xiang *et al.* (2007) analysed mutant p53 protein by use of an immuno-polymerase chain reaction (immuno-PCR) method in the serum of 331 healthy benzidine-exposed workers, while classifying exfoliated urothelial cells in the urine of these workers with Papanicolau's grading (PG). When the workers were divided according to benzidine-exposure level, the amounts of mutant p53 protein in the mid- and high-exposure groups were significantly higher than in the low-exposure group, and also significantly higher in the PG-II and PG-III groups than in the PG-I group (Xiang *et al.*, 2007).

Wu and Heng (2006) found DNA lesions in exon 7 of the *Tp53* gene in rats treated intraperitoneally with benzidine, its major targets being bladder, liver and lung. This suggests that the toxicity of benzidine is probably related to damage in the *Tp53* gene (Wu & Heng, 2006).

4.3 Mechanistic considerations

Benzidine metabolism has been extensively studied (Whysner *et al.*, 1996, Zenser *et al.*, 2002). Pathways involved in benzidine-initiated bladder cancer include the following: benzidine is *N*-acetylated to *N*-acetylbenzidine, which can be *N*-glucuronidated or *N*-hydroxylated in the liver (Zenser *et al.* 2002). *N*-Glucuronides of acetylbenzidine or *N′*-OH-*N*-acetylbenzidine can be transported by the blood and filtered by the kidneys, leading to their accumulation in urine within the lumen of the bladder. The *N*-Glucuronides are acid-labile and are converted back to *N*-acetylbenzidine or *N′*-OH-*N*-acetylbenzidine in acidic urine. Note that while the *N*-glucuronide of acetylbenzidine has an estimated half-life of 7.5 minutes, that for the hydroxylated acetyl-derivative is 3.5 hours. (Babu *et al.* 1995). Thus, *N*-acetylbenzidine is more likely to be hydrolysed than *N′*-OH-*N*-acetylbenzidine during a short transit time of urine in bladder. Within bladder cells, *N′*-OH-*N*-acetylbenzidine could react directly with DNA or, following conversion to the *N*-acetoxy derivative by *N,O*-acetyltransferase, form the dGp-acetylbenzidine adduct. *N*-acetyl-benzidine will require further activation before it can bind to DNA and form this adduct. This activation could involve *N*-oxidation by CYP and/or prostaglandin H synthase (Lakshmi *et al*. 1998). The dGp-acetylbenzidine adduct initiates carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumour formation. *N*-Acetylation is both an activation (forming *N*-acetylbenzidine) and inactivation (forming *N,N′*-diacetyl-benzidine) reaction. dGp-acetylbenzidine can be formed from acetylbenzidine by several different enzymatic pathways. Thus, benzidine-induced initiation of bladder cancer is complex, involving multiple organs (i.e. liver, kidney, and bladder) and metabolic pathways (i.e. *N*-acetylation, *N*-glucuronidation and *N*-oxidation by CYP and/or peroxidation) (see Figure 4.1).

Figure 4.1. Metabolic pathways involved in the bladder cancer initiation by benzidine



Adapted from Makena and Chung, 2007

ABz, N-acetylbenzidine; Ac, Acetyl; Bz, Benzidine; P-450, cytochrome P-450; FMN, flavin monooxygenase; GSH, glutathione; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NAT, N-acetyltransferase; N-CH3-Bz, N-methylbenzidine; N-OAc-N'-ABz, N-acetoxy-N'-acetylbenzidine; N-OH-Bz, N-hydroxybenzidine; N-OH-N'-ABz, N-hydroxy-N'-acetylbenzidine; N-OH-N-CH3Bz, N-hydroxy-N-methylbenzidine; NMT, N-methyltransferase; OAc, acetoxy; OAT, O-acetyltransferase; PHS, prostaglandin H synthetase; PRX, peroxydase; ROS, reactive oxygen species

The results obtained in recent years are compatible with what could be expected from the present understanding of the mode of action of aromatic amines. However, there are still some unanswered questions. Why does benzidine not produce bladder tumours in the rat in contrast to several other species? Although the data are in favour of a genotoxic mechanism, not all species-specific differences can be explained by metabolic activation (Whysner *et al.*, 1996). Interestingly, little is known about the acute toxicity of benzidine, except that it does not stimulate but rather inhibits cell proliferation.

One of the most advanced approaches uses quantitative single-cell proteomics to select biomarkers of effect and to develop profiles of the sequence of events in a complex network of signalling pathways in bladder cancer (Hemstreet & Wang, 2004). A comprehensive view was obtained of the alterations induced by reactive metabolites of 4-aminobiphenyl by gene-expression profiles in human lymphoblastoid TK6 cells. The activity of 2250 genes was altered by treating these cells with *N*-hydroxy-acetylaminobiphenyl. Gene-expression patterns have been linked in this way to phenotypic markers, such as DNA-adduct levels, toxicity and mutagenicity. So far, the results tell us something about the complexity of the responses of a cell exposed to a non-physiological agent, which should caution against searching for monocausal explanations and pathways (Ricicki *et al.*, 2006, Srinivas *et al.*, 2001). The gene-expression profile in livers from mice fed *N*-2-acetylaminofluorene in combination with partial hepatectomy showed that from 2304 cDNA clones 69 were upregulated in comparison with the expression seen after partial hepatectomy alone. The increased gene expression may be associated with the activation of oval cells (Arai *et al.*, 2004).

4.4 Susceptibility

Taking into account that extensive epidemiological studies have indicated a relationship between bladder cancer in populations exposed to arylamines and the slow phenotype for their acetylation, the knowledge of the human acetylator phenotype may be a useful indicator of possible risk for bladder cancer due to exposure to these chemicals. Within the same human liver preparations, benzidine and sulfamethazine acetylation were directly and significantly correlated ($r = 0.672$; $P < 0.05$) (Peters *et al.*, 1990).

In rat-liver slices incubated with [3H]-labelled benzidine, *N,N′*-diacetylbenzidine represented 73 ± 2.5% of the total radioactivity recovered by HPLC, *N*-acetylbenzidine represented 8.8 ± 3.6%, while no unmetabolized benzidine was observed. In human liver slices, benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine represented 19 ± 5%, 34 ± 4% and 1.6 ± 0.5%, respectively. Thus in human liver slices the formation of *N*-acetylbenzidine rather than *N,N′*-diacetylbenzidine is favoured. Individuals with rapid *N*-acetyltransferase 2 (*NAT2*) genotypes formed 1.4-fold more *N*-acetylbenzidine than did slow acetylators, but this increase was not significant. These data suggest that in humans the enzyme deacetylase influences hepatic metabolism of benzidine and its subsequent

carcinogenic effects more than *N*-acetyltransferase, and helps to explain the species- and organ-specificity of benzidine-induced carcinogenesis (Lakshmi *et al.*, 1995a).

According to a cross-sectional study among 33 workers exposed to benzidine and 15 unexposed controls (Rothman *et al.*, 1996a), four benzidine-related DNA adducts were significantly elevated in the exfoliated urothelial cells of exposed workers compared with controls. The predominant adduct co-chromatographed with *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine and it was the only adduct significantly associated with total urinary benzidine metabolites ($r = 0.68$; $P < 0.0001$). This supports the concept that monofunctional acetylation is an activation rather than a detoxification step for benzidine. Almost all benzidine-related metabolites measured in the urine of exposed workers were acetylated among slow acetylators as well as rapid acetylators (95 ± 1.9% *vs* 97 ± 1.6%), and NAT2 activity did not affect the levels of any DNA adduct measured; it is thus unlikely that inter-individual variations in NAT2 function are relevant for benzidine-associated bladder carcinogenesis.

The glutathione *S*-transferase M1-null (*GSTM1-null*) genotype had no impact on DNA adducts in urothelial cells and urinary mutagenicity levels in workers currently exposed to benzidine, and GSTM1 did not conjugate benzidine or its metabolites. These results led to the conclusion that the *GSTM1-null* genotype does not have an impact on bladder cancer caused by benzidine. This is in contrast to studies in the general population suggesting that subjects with the *GSTM1-null* genotype are at a higher risk for bladder cancer (Rothman *et al.*, 1996b).

Studies designed to assess the metabolism of benzidine and *N*-acetylbenzidine by the *N*-acetyltransferases NAT1 and NAT2, conducted with human recombinant NAT1 and NAT2 and human liver slices, indicated that benzidine and *N*-acetylbenzidine are substrates of NAT1. *N*-acetylation of benzidine and *N*-acetylbenzidine did not correlate with the *NAT2* genotype. A higher average acetylation ratio was observed in human liver slices possessing the *NAT1*10* compared with the *NAT1*4* allele, suggesting that *NAT1* may exhibit a polymorphic expression in human liver (Zenser *et al.*, 1996).

The results of studies performed to assess the role of *GSTP1* polymorphism in the development of benzidine-related bladder cancer (Ma *et al.*, 2003) indicated that carriers of the *GSTP1 AG* or *GC* genotypes are found more frequently, but not to a significant extent (OR = 1.95; 95% CI 0.70–5.46), among benzidine-exposed bladder-cancer patients than in benzidine-exposed workers without known disease. Significant differences were found between all benzidine workers without known disease and all workers with known disease with respect to the degree of changes in exfoliated urothelial cells. These findings show the existence of an association between the *GSTP1 AG* or *GC* genotype and higher cytological gradings of exfoliated urothelial cells from formerly benzidine-exposed workers.

Inflammation and infection may play an important role in the activation of benzidine. As a matter of fact, reactive nitrogen/oxygen species (RNOS), which are components of the inflammatory response, were found to react with benzidine forming azo-benzidine.

Glutathione prevented the RNOS-mediated transformation of benzidine (Lakshmi *et al.*, 2003).

A study to evaluate the influence of urinary pH on the levels of free benzidine and *N*-acetylbenzidine and on DNA adducts in urothelial cells (Rothman *et al.*, 1997) demonstrated that individuals with urine at pH < 6 had tenfold higher DNA-adduct levels than did individuals with urine at pH ≥ 7. The pH of the urine was inversely correlated with the proportion of benzidine ($r = -0.78$; $P < 0.0001$) and *N*-acetylbenzidine ($r = -0.67$; $P < 0.0001$) present as free components.

N′-(3′-monophospho-deoxyguanosin-8-yl)-*N*-acetylbenzidine was the major adduct detected in bladder cells from workers exposed to benzidine, and an inverse relationship was observed for the pH of the urine and levels of this adduct, as well as for urinary pH and levels of free (unconjugated) benzidine and *N*-acetylbenzidine (Zenser *et al.*, 1998).

5. Summary of Data Reported

5.1 Exposure data

Benzidine has been used for over a century, mainly for the production of azo dyes and as a rubber-compounding agent. 3,3′-Dimethylbenzidine (*ortho*-tolidine) is produced mainly as an intermediate for dyes and pigments but also for manufacturing polyurethane-base elastomers. 3,3′-Dichlorobenzidine is used primarily in the production of yellow, and some red and orange pigments for the printing ink, textile, paper, paint, rubber, plastic, and related industries. It also has application as a compounding ingredient for rubber and plastics. 3,3′-Dichlorobenzidine is also used with 4,4’-methylenebis(2-chloroaniline) (MOCA) as a curing agent for polyurethane elastomers. 3,3′-Dimethoxybenzidine (*ortho*-dianisidine) is used almost exclusively for the production of azo dyes and azo pigments.

Benzidine and its congeners are not known to occur naturally. Occupational exposure occurs during their production and use. Only studies for benzidine itself are available. Airborne concentrations in the workplace reached maximum values of 6 mg/m^3, measured in Russia in 1947–1948. In more recent studies, from China (1962–1970) and the Republic of Korea (1998), the maximum values were 1.18 and 0.65 mg/m^3, respectively.

Since benzidine-based dyes are known to be metabolized to benzidine, exposure studies in workers have measured the benzidine concentration in urine. The highest reported value was 56 mg/L (Russia 1937–1938). In a dye-manufacturing industry in India, values up to 0.36 mg/L were measured.

The general population can be exposed when living near factories or disposal sites, through plant effluents or groundwater contamination. An additional source of exposure is the use of consumer products containing benzidine- and congener-based dyes, which can be contaminated with the respective amine, and also via uptake of the dyes from those

products and ensuing metabolization. The manufacturing of benzidine is now prohibited in the EU and several other countries, e.g. Japan, the Republic of Korea, Canada and Switzerland.

5.2 Human carcinogenicity data

Many case reports and cohort studies have shown that occupational exposure to benzidine increases the risk for cancer of the urinary bladder among workers in various countries. The studies show consistent positive associations with some indication of dose–response relationships.

In addition, studies that have documented a decreasing bladder-cancer risk in occupational cohorts after removing exposures to benzidine support a causal interpretation of the observed association between benzidine exposure and bladder cancer.

5.3 Animal carcinogenicity data

Benzidine or its dihydrochloride salt was tested in mice, rats, hamsters and dogs by oral administration, in mice and rats by subcutaneous administration and in rats by inhalation and intraperitoneal injection. Following oral administration to newborn and adult mice of different strains and of both sexes, it significantly increased the incidence of benign and malignant liver tumours. In female rats, it markedly increased the incidence of mammary tumours; in male and female hamsters, it increased the incidence of liver tumours; and in dogs it produced bladder tumours. The subcutaneous administration of benzidine or its sulfate to mice produced significant increases in the incidence of benign and malignant liver tumours. In rats, benzidine produced a high incidence of Zymbal-gland tumours; colonic tumours were also reported. The intraperitoneal administration of benzidine to rats resulted in a marked increase in the incidence of mammary and Zymbal-gland tumours. Studies in fish, rabbits and frogs could not be evaluated. The results of the inhalation study in rats could not be interpreted.

3,3′-Dichlorobenzidine or its dihydrochloride salt was tested in mice, rats, hamsters and dogs by oral administration, in mice by transplacental exposure and in mice and rats by subcutaneous administration. When administered in the diet, 3,3′-dichlorobenzidine induced hepatomas in male mice, granulocytic leukaemia and Zymbal-gland carcinomas in male rats, mammary adenocarcinomas in rats of both sexes, and transitional cell carcinomas of the urinary bladder and hepatocellular carcinomas in female dogs. When administered by transplacental exposure, 3,3′-dichlorobenzidine increased the incidence of lymphoid leukaemia in mice. A feeding study in hamsters and the studies with subcutaneous administration in mice and rats could not be evaluated.

3,3′-Dimethoxybenzidine was tested in mice, rats and hamsters by oral administration. When given by stomach intubation to both male and female rats, 3,3′-dimethoxybenzidine caused tumours at various sites, including the Zymbal gland, the

intestine (carcinoma), skin (carcinoma), and urinary bladder (papilloma). When the dihydrochloride salt of 3,3′-dimethoxybenzidine was administered in the drinking-water to male and female rats, increased incidences of Zymbal-gland tumours (adenoma and carcinoma), liver neoplasms and tumours (neoplastic nodules and hepatocellular carcinoma), large intestine tumours (adenomatous polyps and adenocarcinoma), skin tumours (basal-cell adenoma and carcinoma), and oral cavity tumours (squamous-cell papilloma and carcinoma) were observed. Male rats also had increased incidences of tumours of the preputial gland, the small intestine (adenocarcinoma), and mesothelioma, and female rats had increased incidences of tumours of the clitoral gland (adenoma and carcinoma), mammary gland (adenocarcinoma), and uterus or cervix (adenoma and carcinoma). A feeding study in hamsters could not be evaluated.

3,3′-Dimethylbenzidine was tested in mice, rats and hamsters by oral administration and in rats by subcutaneous administration. Oral exposure of mice of both sexes to 3,3′-dimethylbenzidine in the drinking-water as the dihydrochloride salt caused increased incidences of lung tumours (alveolar-cell adenomas and adenocarcinomas). Oral exposure of rats of both sexes to 3,3′-dimethylbenzidine in the drinking-water as the dihydrochloride salt increased the incidence of Zymbal-gland tumours (adenomas and carcinomas), liver tumours (neoplastic nodules or hepatocellular carcinomas), large intestine tumours (adenomatous polyps or adenocarcinomas), skin tumours (basal cell adenomas and carcinomas), and oral cavity tumours (squamous cell papillomas and carcinomas) in both males and females; preputial gland tumours (carcinomas), small intestine tumours (adenocarcinomas) and lung tumours in males; and clitoral gland tumours (adenomas and carcinomas) and mammary gland tumours (adenocarcinomas) in females. In rats, subcutaneous injection of 3,3′-dimethylbenzidine caused significant increases in Zymbal-gland tumours in both sexes and skin, preputial gland and forestomach tumours in males and mammary gland tumours in females. A feeding study in hamsters could not be evaluated.

5.4 Other relevant data

Pathways involved in benzidine-initiated bladder cancer include the following steps in human metabolism: benzidine is *N*-acetylated to acetylbenzidine, which can be *N*-glucuronidated or *N*-oxidized in the liver. *N*-Glucuronides of acetylbenzidine or *N*-hydroxyacetylbenzidine can be transported by the blood and filtered by the kidneys, which results in accumulation in urine within the lumen of the bladder. *N*-Glucuronides are acid-labile and could be converted to acetylbenzidine or *N*′-hydroxy-*N*-acetyl-benzidine in acidic urine. Note that while the *N*-glucuronide of acetylbenzidine has an estimated half-life of 7.5 minutes, that for *N*′-hydroxy-*N*-acetyl-benzidine is 3.5 hours at pH 5.5. Thus, acetyl-benzidine is more likely to be hydrolysed than *N*′-hydroxy-*N*-acetyl-benzidine during a short transit time of urine in the bladder. Within bladder cells, *N*′-hydroxy-*N*-acetylbenzidine could react directly with DNA, or following conversion to the

N-acetoxy derivative by *O*-acetyltransferases, form the adduct *N*-(deoxyguanosin-8-yl)-*N*′-acetylbenzidine (dGp-acetylbenzidine). Acetyl-benzidine requires further activation before it can bind DNA and form this adduct. This activation could involve *N*-oxidation by cytochrome P450 (CYP) enzymes and/or prostaglandin H synthase. The adduct dGp-acetylbenzidine initiates carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumour formation. Levels of this DNA adduct in human peripheral white blood cells correlate with those in exfoliated bladder cells. Benzidine can be metabolized to 4-aminobiphenyl and form haemoglobin adducts in rats. Thus, initiation of bladder cancer by benzidine is complex, involving multiple organs (i.e. liver, kidney, and bladder) and metabolic pathways (i.e. *N*-acetylation, *N*-glucuronidation and *N*-oxidation by CYP enzymes or peroxidation). Uroepithelial cells contain substantial prostaglandin H synthase activity along with bladder infiltration with polymorphonuclear leukocytes. Myeloperoxidase activity, an index of infiltration with neutrophils, has also been observed. Both peroxidases could contribute to the activation of acetylbenzidine in bladder epithelium.

Conjugates of benzidine and free benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine have been measured in urine of workers exposed to benzidine-based azo dyes, and more specifically to Direct Black 38. One study reported formation of haemoglobin adducts derived from benzidine, acetylbenzidine, 4-aminobiphenyl and aniline in workers exposed to Direct Black 38. Likewise, studies in rhesus monkeys, Syrian golden hamsters, dogs and rats exposed to various dyes based on benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, e.g., Direct Black 38, Direct Blue 6 and Direct Brown 95, consistently show the presence of the free amines or acetylated amines in the urine. In addition, several studies demonstrated anaerobic bacteria in the intestine of mice, rats and humans to be capable of cleaving the azo-linkage in the dyes, thereby liberating the amine.

Benzidine has been found mutagenic to *Salmonella* when tested in the presence of an exogenous metabolic system from rats as well as from humans. Also *N*-acetylbenzidine and *N*-hydroxy-*N*,*N*′-diacetylbenzidine, which are urinary metabolites of benzidine in the rat, were positive in *Salmonella* in the presence of an activation system. The urine of rats that received benzidine in the food was mutagenic to *Salmonella* in the presence of metabolic activation.

Benzidine consistently showed negative results in *E. coli* tests. Mutagenic activity in the X-linked recessive lethal assay and induction of mutations in RNA genes of *Drosophila* has been reported.

Benzidine was tested in many in-vitro assays in cultured mammalian cells; positive and negative results were observed in gene-mutation tests. Benzidine was active in tests for DNA fragmentation and induction of sister chromatid exchange in cultured human and animal cells; benzidine was generally active in inducing unscheduled DNA synthesis in cultured hepatocytes of rats.

In-vivo tests in animals showed conflicting results with respect to the ability of benzidine to induce micronuclei in polychromatic erythrocytes, but several studies demonstrated micronucleus induction in mice treated orally with a wide range of doses (150 to 900 mg/kg bw).

When benzidine was administered to pregnant female mice, no significant increase in the micronucleus frequency was observed in the liver of the fetuses. Positive results, however, were reported in a different study, in which the frequency of micronucleated polychromatic erythrocytes in the liver was found increased.

There are data on genotoxic effects of benzidine in workers of a manufacturing plant in Bulgaria, who were exposed to benzidine or benzidine-based dyes. A statistically significant (ten-fold) increase in the number of circulating peripheral lymphocytes with chromosomal aberrations was observed in exposed workers. The highest frequencies of aberrant lymphocytes were associated with the highest levels of exposure and correlated with the concentrations of benzidine found in urine. Also, mutant p53 protein was increased in workers exposed to benzidine.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of benzidine. Benzidine causes bladder cancer in humans.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of benzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3,3′-dichlorobenzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3.3′-dimethoxybenzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3,3′-dimethylbenzidine.

6.3 Overall evaluation

Benzidine is *carcinogenic to humans (Group 1)*.

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DYES METABOLIZED TO BENZIDINE

1. Exposure Data

See Monograph on Benzidine in this volume.

2. Studies of Cancer in Humans

The Working Group reviewed available epidemiological studies that evaluated the association between exposure to benzidine derivatives or azo dyes metabolized to benzidine and cancer. Three benzidine derivatives used in the manufacture of azo dyes were reviewed: 3,3′-dimethylbenzidine (*ortho*-tolidine), 3,3′-dimethoxybenzidine (*ortho*-dianisidine), and 3,3′-dichlorobenzidine. These benzidine congeners have been evaluated in previous Monographs and classified in Group 2B (*possibly carcinogenic to humans*) (IARC, 1972, 1974, 1982, 1987). The Working Group also reviewed available human studies on the carcinogenicity of three azo dyes metabolized to benzidine: Direct Brown 95, Direct Blue 6, and Direct Black 38, all classified in Group 2A as benzidine-based dyes (IARC, 1982, 1987). Most studies that analysed the association between benzidine derivatives and cancer were conducted in workers employed in the manufacture of azo dyes. In these studies, confounding by concomitant exposure to the Group-1 carcinogens benzidine and 2-naphthylamine is likely, and precludes the evaluation of the effect of carcinogenicity by the individual congeners (see Monographs on benzidine and 2-naphthylamine in this volume). The Working Group was aware of studies among workers likely to be exposed to azo dyes metabolized to benzidine. One of these occupational groups, hairdressers and barbers, was evaluated in another Monograph in this volume by this Working Group (see Monograph on occupational exposures of hairdressers and barbers). Other occupations, such as shoe and leather workers, textile workers, and painters, have been evaluated in previous volumes (IARC 1987, 1990, Vol 98). For this evaluation, studies on these occupations were reviewed, but only when they specifically listed exposure to benzidine derivatives or azo dyes metabolized to benzidine. In some instances noted below, exposure to a specific azo dye was not mentioned.

2.1 Benzidine derivatives

2.1.1 *Cohort studies* (Table 2.1)

IARC Monograph Volume 29 (IARC, 1982) reported the results of three studies of workers exposed to 3,3′-dichlorobenzidine (Gerarde and Gerarde, 1974; Gadian, 1975; MacIntyre, 1975). At the time, the Working Group noted that these studies examined relatively small cohorts of workers, and that the time since first exposure to 3,3′-dichlorobenzidine was 20 or fewer years for over two thirds of the workers. Also, in the study by Gerarde and Gerarde (1974), follow-up of exposed workers was less than 85% complete. [The Working Group agreed that the significance of these findings is uncertain.]

As part of a notification programme in a dye-intermediary production plant, Schulte *et al.* (1985) identified a cohort of 1385 workers employed from 1940 to 1972. The cohort was potentially exposed to 3,3′-dimethylbenzidine, 2-naphthylamine, benzidine and 1-naphthylamine, with 2-naphthylamine as the major exposure. A questionnaire was used to obtain information on occupational history, particularly relating to working in the 2-naphthylamine-grinding room, use of protective measures, and history of other jobs with potential exposure to bladder carcinogens. Additionally, information was obtained on alcohol, tobacco, coffee and artificial sweetener use, and personal health history. Follow-up was conducted from date of first employment until 1982. Incidence rates in the cohort were compared to estimated incidence rates for bladder cancer in the United States. For the entire cohort, 13 cases of bladder cancer were observed (overall RR, 3.9; 95% CI, 2.2–6.8). Stratified analyses by race and length of employment also showed statistically significant associations. The most remarkable was among black workers with invasive cancer and more than 10 years of employment (RR, 111.1; 95% CI, 35.0–352.5). The authors reported that, in general, black workers had jobs involving greater exposure to 2-naphthylamine.

In a nested case–control study, Schulte *et al.* (1986) evaluated the effect of potential risk factors on the risk for bladder cancer. The analysis included the aforementioned 13 bladder-cancer cases and the remaining 1372 workers in the cohort as controls. Workers were considered exposed if they had more than one year of employment or any employment in two departments with potential 2-naphthylamine exposure. The crude odds ratio between exposure and bladder cancer was 7.0 (95% CI, 3.9–12.4). The odds ratio for the association between bladder cancer and duration of employment, controlling for smoking and source of drinking water, was 4.3 (95% CI, 1.8–10.3).

Sinks *et al.* (1992) evaluated cancer risk at a paperboard-printing manufacturing plant in Georgia, USA. The cohort consisted of 2050 workers employed for more than one day, with duration of employment obtained through company records. Company material safety-data sheets were reviewed and potential carcinogens were identified. From supplier information, the authors determined that pigments were manufactured from 3,3′-dichlorobenzidine and *ortho*-toluidine, but these substances were not identified in laboratory tests. One bladder-cancer death (SMR 2.6; 95% CI, 0.1–14.5) and one renal cell cancer death (SMR, 1.4; 95% CI, 0.0–7.8) were observed. For the incidence analysis, six cases of

Table 2.1. Summary of cohort studies of populations exposed to [benzidine derivatives and] azo dyes metabolized to benzidine

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Benzidine derivatives								
Schulte *et al.* (1985); Schulte *et al.* (1986) Georgia, USA	Nested case–control study from cohort of 1385 workers employed in a chemical plant during 1940–72	Workers potentially exposed to 3,3′-dimethylbenzidine, BZ and BNA. Questionnaire used to obtain occupational history and other risk factors; workers considered exposed if employed >1 year or any time in any 2 departments with potential BNA exposure	Bladder			**OR**		
				Exposed	13	7.0 (3.9–12.4)		
				Duration of employment	13	4.3 (1.8–10.3)	Smoking, source of drinking water	

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Sinks *et al.* (1992) Georgia, USA	Cohort of 2050 workers (1828 men, 222 women) employed >1 day at a paperboard printing manufacturing plant during 1957–88; mortality follow up 1957–88; incidence follow-up to 1990; vital status 90%; cause of death 82%; nested case–control study of 6 renal cell cancer cases and 48 controls	Length of employment and department from plant records; materials used from review of MSDS and supplier information				**SMR**	Age, sex	Mortality, national reference; incidence, local reference; which workers were exposed to DCB was not determined
			Renal cell	Overall	1	1.4 (0.0–7.7)		
			Bladder	Overall	1	2.6 (0.1–14.7)		
						SIR		
			Renal cell	Overall	6	3.7 (1.4–8.1)		
			Bladder	Overall		1.1 (0.2–3.1)		
						OR		
				Department (>5 years)				
			Renal cell	Finishing	3	16.6 (1.7–453.1)		
				Maintenance	1	5.3 (0.1–223.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Naito *et al.* (1995) Urban area, Japan	Cohort of 442 workers of a BZ production and dye manufacturing plant (437 men, 5 women) during 1935–88; mortality and incidence follow-up 1935–92; vital status 100%	Workers exposed to one or more substance, including 3,3′-dimethoxybenzidine, BZ and BNA. Duration of employment at BZ manufacture or use facility as surrogate of duration of exposure				**SMR**		National reference; incidence rates reported by duration of exposure; PPE reportedly used among all workers
			Urinary tract (188, 189)	Dye manufacture	6	15.8 (5.8–34.3)		
			Bladder	Dye manufacture	5	27.0 (8.8–63.0)		
Ouellet-Hellstrom & Rench (1996) Connecticut, USA	Cohort of 704 workers (585 men, 119 women) first employed at a chemical plant between 1965 (when BZ production was discontinued) and 1989; incidence follow-up 1965–94; vital status 96%	Personnel records for occupational history; exposure scoring for arylamines (3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, DCB, *o*-toluidine, *o*-chloroaniline) for each job title based on expert judgement		**Men**		**SIR**		State reference; workers with testicular cancer had no exposure to arylamines
			Bladder	Overall	7	8.3 (3.3–17.1)		
				Annual cumulative exposure score (CES)				
				No exposure	0	0		
				<2.5	2	5.5 (0.7–19.8)		
				2.5+	5	16.4 (5.3–38.2)		
				CES among smokers				
				<2.5		11.6 (1.4–41.8)		
				2.5+		23.6 (7.7–55.2)		
			Testis	Overall	2	11.4 (1.4–41.1)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Rosenman & Reilly (2004) Michigan, USA	Cohort of 488 white men employed in a chemical manufacturing facility during 1960–77; mortality follow-up 1979–2001; incidence follow-up 1981–2002 (Michigan Tumor Registry)	Workers classified as exposed to BZ and DCB if employed before 1973, those employed during or after 1973 were exposed to DCB only. Time and length of employment estimated from social security records.	Bladder	Overall	3	**SMR** 8.3 (1.7–24.4)		National reference for SMR and SEER for SIR
				Year started work				
				<1973	3	9.6 (2.0–28.1)		
				≥1973	0	0		
			Lympho-haematopoietic cancer	Overall	6	2.8 (1.4–6.2)		
				Year started work				
				<1973	3	1.8 (0.4–5.3)		
				≥1973	3	6.6 (1.4–19.4)		
			Leukemia	Overall	4	5.1 (1.4–12.9)		
						SIR		
			Bladder	Overall	22	6.9 (4.3–10.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Azo dyes metabolized to benzidine								
Stern *et al.* (1987) Minnesota and Wisconsin, USA	Cohort of 9365 tannery production workers (7085 men, 2280 women), employed during 1940–79 at tannery A and 1940–80 at tannery B; mortality follow-up 1940–82; vital status 95%; cause of death 97%	Occupational history from plant records; duration of employment as surrogate for cumulative exposure; BZ measured in 2 samples of bulk dyes: 2.0 & 55 ppm	Bladder			**SMR**		State reference; no increased SMR for other cancers
				Latency (15+ years)	4	0.5 (0.1–1.3)		
				Department				
				Retan, color, fat-liquor	2	1.0 (0.2–3.2)		
				Finishing	3	0.9 (0.2–2.5)		
			Leukemia (ICD-7, 204)	Latency (15+ years)	10	1.0 (0.5–1.9)		
				Department				
				Retan, color, fat-liquor	3	1.0 (0.2–2.9)		
				Finishing	7	1.3 (0.5–2.6)		
			Lymphomas (ICD-7, 200-203, 205)	Latency (15+ years)	12	0.9 (0.5–1.5)		
				Department				
				Retan, color, fat-liquor	1	ND		
				Finishing	7	0.9 (0.4–1.9)		
Costantini *et al.* (1989) Florence and Pisa, Italy	Cohort of 2926 male workers newly employed in tanneries during 1950–81; at least one period of employment of >6 months; mortality follow-up 1950–83; vital status 99%	Employment in the tannery industry from municipal records	Bladder			**SMR**		National reference
				Overall	5	1.5 (0.5–3.5)		
				Latency (years)				
				<15		0		
				15–19		1.6		
				20–24		2.8		
				25–29		3.9		
				p for trend		>0.05		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
You *et al.* (1990) Shanghai, China	Cohort of 1210 workers (1060 men and 150 women) employed >1 year in weighing and formulating or dyeing at textile printing and dyeing plants during 1949–83; vital status 98%	Workers divided in 2 groups based on BZ-based dyes usage: high exposure (>500 kg/month) and low exposure (<500 kg/month)	Bladder	Overall	1	ND		Local reference
Montanaro *et al.* (1997) Genoa, Italy	Cohort of 1244 workers at a tannery (870 men, 374 women) employed >6 months during 1955–88; mortality follow-up to 1994; vital status 96%; cause of death 98%	Length of employment from plant records; information on department only for 25% workers				**SMR**		National and regional reference
			Bladder	Overall	10	2.4 (1.2–4.5)		
				Duration of exposure (years)				
				<5	3	4.4 (0.9–12.8)		
				5–14	0	0 (0–2.8)		
				15+	7	3.0 (1.2–6.1)		
			Lymphoma (200-202)	Overall	1	0.4 (0.01–2.0)		
			Leukemia (204-208)	Overall	0	0 (0.0–0.9)		

BNA, 2-naphthylamine; BZ, benzidine; DCB, 3,3'-dichlorobenzidine; MSDS, material safety data sheets; ND, not determined; SIR, standardized incidence ratio; SMR, standardized mortality ratio

renal-cell cancer were seen (SIR, 3.7; 95% CI, 1.4–8.1), but no increase in bladder-cancer risk was observed (3 cases; SIR, 1.1; 95% CI, 0.2–3.1). In a nested case–control study, the risk for renal-cell cancer by duration of employment and department or work process was evaluated. Employment in the finishing department for five or more years was associated with an increased risk for this cancer type (three cases; OR, 16.6; 95% CI, 1.7–453.1). The authors could not determine if workers from the finishing department were exposed to inks.

Naito *et al.* (1995) conducted a retrospective cohort-mortality study of 442 workers (437 men, 5 women) exposed to one or more substances (mainly benzidine, 2-naphthylamine, 1-naphthylamine, and 3,3′-dimethoxybenzidine) at a benzidine production and dye-manufacturing plant in Japan. No industrial hygiene data for the plant were available; therefore, duration of employment at the facility was used as a surrogate for duration of exposure. Nineteen workers were potentially exposed to 3,3′-dimethoxybenzidine during dye manufacture, and of these only three were exposed solely to 3,3′-dimethoxybenzidine. The authors reported that all workers in the factory wore work clothes, gloves, high rubber boots, and a gas mask. An increased risk for bladder cancer was found among workers engaged in dye manufacture (SMR, 27.0; 95% CI, 8.8–63.0). Increased risks for cancer mortality for other organs were observed, but none were statistically significant [results for lymphohaematopoietic cancers were not reported]. Incidence rate ratios of urothelial cancer increased with duration of exposure (*P* for trend, 0.04). [Due to the small number of workers exposed to 3,3′-dimethoxybenzidine alone or in combination with other arylamines, it is not possible to attribute the increased risk for bladder cancer to exposure to this substance. Furthermore, reduced exposures are likely due to the reported use of personal protective equipment].

Ouellet-Hellstrom and Rench (1996) studied workers at the same chemical plant previously studied by Meigs *et al.* (1986) in Connecticut, USA. The cohort consisted of 704 workers (585 men and 119 women) first employed at a plant during the period 1965–89, after benzidine production was discontinued, and were therefore never exposed to benzidine. Workers were presumably exposed to 3,3′-dichlorobenzidine (predominantly used at the plant), 3,3′-dimethoxybenzidine and 3,3′-dimethylbenzidine. The authors used personnel records at the plant and a questionnaire to obtain occupational history and information on other risk factors such as smoking. Each job title was assigned an exposure score to arylamines based on expert judgment. An annual cumulative exposure score to arylamines was calculated for each worker. Expected numbers of cancers were estimated using cancer-incidence rates for the state of Connecticut. Men had elevated SIRs for cancers of the buccal cavity, bladder, kidney, brain and testis. Only for bladder cancer (SIR, 8.3; 95% CI, 3.3–17.1) and testicular cancer (SIR, 11.4; 95% CI, 1.4–41.1) did this increase reach statistical significance. No excess of lymphohaematopoietic cancers was observed (SIR, 1.1; 95% CI, 0.1–4.1). The two workers with testicular cancer had no exposure to arylamines, and one had worked at the plant for only 15 days. Women had a statistically insignificant increase in breast cancer (SIR, 1.9; 95% CI, 0.4–5.6). The SIR for bladder cancer increased with increasing exposure. This excess bladder cancer

occurred among chemical operators (who worked with arylamines over long periods) and mechanics (who had short periods of exposure that was likely intense). The bladder cancer SIRs by cumulative exposure among smokers were higher than for the total cohort. [This study provides strong evidence of the association between exposure to benzidine-based dyes—or benzidine derivatives metabolised to benzidine—and bladder cancer].

Rosenman and Reilly (2004) analysed a cohort of 488 white men employed in a chemical manufacturing facility in Michigan, USA. The facility produced benzidine from 1960 through 1972 and 3,3′-dichlorobenzidine from 1961 to 2001. Workers were identified from social security records. Since no plant records were available, social security data were used to estimate time of first work and years worked. Analyses were conducted for the entire cohort and separately for people who began to work in 1973 or later, after benzidine production was discontinued. For the whole cohort, an excess of bladder-cancer mortality was observed (SMR, 8.3; 96% CI, 1.7–24.4). All cases occurred in those with five or more years of duration of work. There were six deaths from lymphohaematopoietic cancer (SMR, 2.8; 95% CI, 1.04–6.2), including one from non-Hodgkin lymphoma, one from multiple myeloma, two from chronic lymphocytic leukaemia, one from acute leukaemia, and one from chronic myelogenous leukaemia (SMR for leukaemia, 5.1; 95% CI, 1.4–12.9). Only one bladder cancer case (of 22 observed) occurred among workers starting employment after 1972, when the plant was only producing 3,3′-dichlorobenzidine. A statistically significant increase in mortality from lymphohaematopoietic cancer was observed among workers who began work in 1973 or later. [Not enough latency among those exposed only to 3,3′-dichlorobenzidine may have accumulated].

2.2 **Dyes metabolized to benzidine** (Table 2.2)

2.2.1 *Case reports and proportional mortality studies*

Genin (1977) analysed the urine of 22 workers involved in the drying and grinding of azo dyes metabolized to benzidine (e.g. Direct Black 38) and dyes metabolized to 3,3′-dimethoxybenzidine (e.g. Direct Blue 15) (IARC, 1982, 1993). Benzidine was found in the urine of eight workers, and 3,3′-dimethoxybenzidine in the urine of three. A retrospective search of plant records showed five cases of bladder cancer in dryers and grinders.

In a proportional mortality study of 1429 bleachers and dyers presumed to be exposed to dyes metabolized to benzidine in the United Kingdom, Newhouse (1978) showed no excess deaths from cancer of the bladder (14 deaths observed, 13.1 expected) [The Working Group of Volume 29 noted that the study was limited in that no certificates of deaths occurring in the first 20 years after start of exposure were available, and only approximately one third of the workers included in the analysis had actually been exposed to dyes.]

Table 2.2. Summary of case-control studies of populations exposed to azo dyes metabolized to benzidine

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors	Comments
Yoshida *et al.* (1971), Kyoto, Japan 1954–1971	Bladder	200 men from three Kyoto hospitals	148 men from two of the three hospitals with urologic diseases other than bladder cancer	Interview with patients or their family or extracts from hospital charts to assess exposure to dyes. BZ-based dyes reportedly used	Overall	6.8 (p=0.002)		3 cases reported the habit of licking brushes dipped in dyes
Myslak *et al.* (1991) East Ruhr area, Germany 1984–87	Bladder (malignant and benign)	403 (290 carcinomas, 113 papillomas) (21 painters) from 3 hospitals; aged 69.6±9.3 (mean±SD); response rate 82%; 100% histologically confirmed	426 (8 painters) with benign prostate disease from same hospitals; response rate 84%	Mailed standardized questionnaire for occupational history	Painters overall	2.8 (1.2–6.3)		Benzidine-based dyes heavily manufactured in Germany before 1950, painters prepared paints themselves

BZ, benzidine; SD, standard deviation

2.2.2 *Cohort studies*

Stern *et al.* (1987) conducted a mortality study in a cohort of 9365 tannery production workers (7085 men, 2280 women), employed at two chrome-leather tanneries in Minnesota and Wisconsin. The processes in both tanneries have remained more or less the same since the end of the 19th century. The authors reported detectable concentrations of benzidine in bulk dyes in the dye room [but exposure was likely to be due to benzidine-based dyes]. Chemical sampling was conducted at both plants. Detectable concentrations (2 and 55 ppm) of benzidine in two samples of bulk dyes were found in the retan/colour/fat-liquor department. Occupational history was obtained from plant records; duration of employment was used as a surrogate for cumulative exposure, and cancer mortality analyses for select causes also examined workers ever employed at specific departments. Expected mortality rates were calculated using US and State death rates (no major differences were observed in risk estimates using both rates; only the latter are presented here). The authors did not observe an excess in the risk for bladder-cancer mortality (tannery A: one observed death; tannery B: four observed deaths; SMR, 1.0; 95% CI, 0.3–2.5). Risk was not increased for other primary sites of cancer mortality. Analysis by duration of exposure did not show increased mortality risk for those workers with more than 15 years of latency (SMR, 0.5; 95% CI, 0.1–1.3). The authors reported that of the five deaths from bladder cancer, four occurred among workers of tannery B, and two of these deaths occurred in the retan/colour/fat-liquor department (1.0 expected), but each of those had worked for less than two months at the tannery.

A cohort mortality-study of 2926 male workers newly employed in tanneries between 1950 and 1981 and with at least one period of employment of more than six months was conducted by Costantini *et al.* (1989) in Florence and Pisa, Italy. Known exposures in the plants include dyes metabolized to benzidine, dyes metabolized to 3,3′-dimethylbenzidine and dyes metabolized to 3,3′-dimethoxybenzidine. Employment in the tannery industry was obtained from municipal records, including beginning and end of work. Non-statistically significant increases in mortality were observed for cancers of the kidney, pancreas, lung, bladder (5 deaths; SMR, 1.5; 95% CI 0.5–3.5) and lymphohaematopoietic system. Analyses by latency did not show positive trends, except for bladder cancer, where the SMR increased with latency, although the trend was not statistically significant. All bladder-cancer deaths occurred in workers who entered the cohort between 1950 and 1964.

You *et al.* (1990) conducted a retrospective cohort study among workers in 17 knitting factories, 10 stocking factories, nine silk printing and dyeing factories and seven printing and dyeing factories in Shanghai, China. The cohort of 1210 workers (1060 men and 150 women) had worked for more than a year in weighing and formulating or dyeing, where they had been exposed to the dust of dyes metabolized to benzidine. Fifteen types of benzidine-derived dyes have been used in these factories, and the dyes used in the

largest quantities included Direct Black 38. Cancer mortality and incidence were analysed among exposed workers by dividing them into two groups based on their usage of benzidine-based dyes: high exposure (> 500 kg/month) and low exposure (< 500 kg/month). Only one case of bladder cancer was observed in the exposed group [no risk estimate was provided].

Montanaro *et al.* (1997) studied mortality in a cohort of 1244 workers employed at a chrome tannery in Genoa, Italy, between 1955 and 1988, where workers were exposed to azo dyes metabolized to benzidine and other chemicals used in the tanning process. Length of employment was obtained from plant records; information on department was available only for 25% of the workers. Mortality for all cancers was 12% higher than expected. An excess of bladder-cancer mortality was observed (10 deaths; SMR, 2.4; 95% CI, 1.2–4.5) as well as an excess of colorectal cancer deaths (SMR, 1.8; 95% CI, 1.1–2.9). No excess deaths due to lymphoma or leukaemia were reported (SMR, 0.4; 95% CI, 0.01–2.0 and SMR, 0; 95% CI, 0.0–0.9, respectively).

2.2.3 *Case–control studies*

A hospital-based case–control study of 200 male bladder-cancer cases and 148 male controls of the same age range with urinary disorders in Kyoto, Japan, showed that 17 (8.5%) of the cases and 2 (1.4%) of the controls had worked in the silk-dyeing industry. The odds ratio for employment in the silk-dyeing industry was 6.8 ($P = 0.002$). At least 7 of the 17 patients with bladder cancer who had worked in the dyeing industry were kimono painters, some of whom may have ingested dyes by holding brushes or spatulas in their mouths while working. Reportedly, benzidine-based dyes were used by these kimono painters (Yoshida, 1971). [The Working Group noted that no data on potential confounding factors were provided.]

In a study in a major industrial area of Germany, Myslak *et al.* (1991) selected 403 malignant and benign bladder-cancer cases from three hospitals, and 426 controls with benign prostate disease from the same hospitals. All of the cases were histologically confirmed. Study participants were mailed a questionnaire to obtain information on complete occupational history and smoking habits. The questionnaires were coded for occupational categories, and study participants were classified as painters if they had been employed in this occupation for at least six months and did not have another occupation known to be associated with bladder cancer. Painters were of interest because benzidine-based dyes were manufactured on a large scale in Germany before 1950, and during that time painters usually prepared the paints themselves. Among the cases, 21 were painters and among the controls eight were painters. The overall relative risk for bladder cancer among painters was 2.8 (95% CI, 1.2–6.3).

3. Studies of Cancer in Experimental Animals

3.1 Direct Black 38

3.1.1 *Oral administration*

(a) Mouse

A group of 60 ICR mice [sex unspecified], four weeks of age, weighing 25–30 g, received 3000 mg/L Direct Black 38 [purity unspecified] in their drinking-water for 55–60 weeks, at which time the 59 surviving animals were killed. Hepatocellular carcinomas were found in 46/59 (78%) mice, and mammary carcinomas in 20/59 (34%); nine animals developed both types of tumours. A further 40 mice were given the same concentration of Direct Black 38 in drinking-water, and two mice were killed every two weeks starting from week 16 of treatment. The first liver tumour occurred in a mouse killed 20 weeks after the start of treatment. No liver or mammary tumour was reported in a group of 20 untreated controls (Asada *et al.,* 1981).

(b) Rat

Groups of 10 male and 10 female Fischer 344 rats, six weeks of age, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Black 38 and 1.3% corn oil for 13 weeks. The compound was determined by HPLC to be 87.1 ± 3.4% pure, with the following components: water, 7.13 ± 0.54%; NaCl, 7.9%; benzidine, < 0.004%; and traces of at least eight other impurities. The infrared spectrum was as expected. Surviving rats were killed at 13 weeks. All animals that were given 3000 mg/kg Direct Black 38 died before termination of the experiment: male rats survived for less than five weeks and female rats less than 12 weeks. Of the nine surviving males that received 1500 mg/kg, four (44%) had hepatocellular carcinomas and five (55%) had neoplastic nodules. No male receiving another dose exhibited a tumour, although 7/10 (70%) male animals given 375 mg/kg, 9/10 (90%) males given 750 mg/kg, and 5/9 (55%) males given 1500 mg/kg had foci of cellular alteration or basophilic foci in the liver. Of the females, 5/10 (50%) given 1500 mg/kg exhibited neoplastic nodules in the liver at the termination of the experiment, and all females administered 750 or 1500 mg/kg had foci of cellular alteration in the liver (NTP, 1978; Robens *et al.,* 1980). [The Working Group noted the short duration of the experiment, the small number of animals tested, and the impurity of the compound used.]

Groups of 12–15 Wistar rats were administered 100 mg/L or 500 mg/L commercial Direct Black 38 [purity unspecified; direct Deep Black EX; benzidine-free, as shown by HPLC] in their drinking-water. When the eight rats of the 12 that received 100 mg/L were killed at 60 weeks, no tumour was observed. Of 15 rats administered 500 mg/L Direct Black 38, 13 survived until 60 weeks; two (15%) papillomas and three (23%) carcinomas of the urinary bladder, three (23%) carcinomas of the liver and two (15%) adenocarcinomas of the colon were seen in six animals. No tumour was observed in a control

group of nine rats (Okajima *et al.,* 1975). [The Working Group noted the small number of animals.]

A group of 20 male and 25 female rats (strain and age unspecified) were given 400 mg/L Direct Black 38 [source and purity unspecified] in their drinking-water (0.04%) for 14 months, at which time four males and two females were still alive. One of the females had 'breast cancer' [pathological designation not specified]; no other neoplasm was noted (Niitsu, 1973). [The Working Group noted the poor survival of the animals and the short duration of the experiment; in addition, the number of control animals was not specified.]

3.1.2 *Bladder implantation*

Two groups of 50 female dopamine-deficient mice (weight, 20 g) received either a paraffin wax pellet (20 mg) containing 10% Direct Black 38 [purity unspecified] or a wax pellet alone implanted in the bladder. After 40 weeks, when the surviving animals were killed, three bladder carcinomas were observed among the 21 mice still alive. In the control group, one bladder carcinoma was observed in 36 surviving mice (Niitsu, 1973). [The Working Group noted the short duration of the experiment.]

3.2 Direct Blue 6

3.2.1 *Oral administration*

Groups of 10 male and 10 female Fischer 344 rats, six weeks old, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Blue 6 and 1.3% corn oil for 13 weeks. The compound was determined by HPLC to be 59.9 ± 1.9% pure, with the following components: water, 9.18 ± 0.51%; NaCl, 20.8%; benzidine, < 0.004%; and traces of at least eight other impurities. Survivors were killed at 13 weeks. All animals that were given 3000 mg/kg Direct Blue 6 and one male rat that received 1500 mg/kg diet died before termination of the study; all males given the highest dose died before five weeks on the study, and all females at that dose were dead by 10 weeks. Liver-cell tumours were seen in eight of 10 (80%) males given 1500 mg/kg; two (20%) were hepatocellular carcinomas and six (60%) were neoplastic nodules. Of animals given 3000 mg/kg, 1/9 (11%) males and 7/9 (78%) females were found to have liver-cell tumours at autopsy before the termination of the experiment: four of the tumours in females were hepatocellular carcinomas and three were neoplastic nodules. No neoplastic lesion was seen in animals of either sex given lower doses. The first tumours appeared after four weeks of feeding in the males and after five weeks of feeding in the females. Almost all animals fed 750 or 1500 mg/kg exhibited foci of cellular alterations in the liver and some basophilic foci were seen in the livers of animals receiving 3000 mg/kg. In the same bioassay, no increased incidence of tumours, compared with that in controls, was found in groups of 10 male and 10 female B6C3F1 mice fed diets containing 750, 1500, 3000, 6000 or 12,500 mg/kg [ppm] of Direct Blue 6 and killed 13 weeks later (NTP, 1978;

Robens *et al.*, 1980). [The Working Group noted the short duration of the experiment, the limited number of animals tested, and the impurity of the compound used.]

Twenty female Wistar rats [age unspecified] were given 400 mg/L Direct Blue 6 [purity unspecified] in their drinking-water (0.04%) for 14 months. At 12 months, 12 animals were still alive, and one (8%) had a squamous-cell carcinoma of the outer ear. No other neoplasm was found (Niitsu, 1973). [The Working Group noted the small number of animals and the lack of a control group.]

3.2.2 *Subcutaneous and/or intramuscular administration*

(a) Rat

A group of 20 male and female rats (strain unspecified), 3–4 months of age and weighing 100–159 g, were treated with doses of 10 mg Direct Blue (purity, 97%) by weekly or bi-weekly subcutaneous injections up to a total dose of 200 mg. Animals survived less than 270 days. No tumours were seen. No control group was included (Fujita *et al.,* 1957). [The Working Group noted the poor survival and lack of proper controls.]

3.2.3 *Bladder implantation*

A group of 50 female dd mice (20 g) had either a paraffin wax pellet (20 mg) containing 10% Direct Blue 6 [purity unspecified] or a wax pellet without dye implanted in the bladder. After 40 weeks, when the surviving animals were killed, bladder carcinomas were found in 3 of 21 (14%) [$P = 0.13$; Fisher exact test] treated mice and in 1 of 36 (3%) controls still alive at that time (Niitsu, 1973).

3.3 Direct Brown 95

3.3.1 *Oral administration*

(a) Rat

Groups of 10 male and 10 female Fischer 344 rats, 6 weeks old, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Brown 95 and 1.3% corn oil. The compound was determined by HPLC to be 72.2 ± 7.0% pure, with the following components: water, 4.99 ± 0.22%; NaCl, 14.9%; benzidine, < 0.004%; and traces of at least eight other impurities. Surviving rats were killed at 13 weeks. All male and female animals that received 1500 or 3000 ppm Direct Brown 95 died before termination of the study: male rats survived for less than five weeks, females given the high dose survived less than six weeks on the study, and females fed 1500 ppm up to 12 weeks; two males receiving 750 ppm Direct Brown 95 also died before the end of the study. Among male rats, basophilic foci or foci of cellular alteration were seen in 2/9 (22%) animals given 3000 ppm, in 7/8 (87%) given 1500 ppm and in 8/10 (80%) given 750 ppm. Among

females, 4/8 (50%) given the 1500-ppm dose exhibited neoplastic nodules, and one of these showed a hepatocellular carcinoma; basophilic foci or foci of cellular alteration in the liver were seen in 3/8 (37%) females given 3000 ppm, 6/8 (75%) given 1500 ppm and 3/10 (30%) given 750 ppm. No other relevant findings in relation to neoplastic development were seen in these animals.

(b) Mouse

In the same bioassay, groups of 10 male B6C3F1 mice, 6–7 weeks of age, were fed a diet containing 750, 1500, 3000, 6000 or 12,500 ppm [ppm] Direct Brown 95 and 1.3% corn oil. Groups of 10 female B6C3F1 mice, 6–7 weeks of age, were fed similar diets containing 350, 750, 1500, 3000 or 6000 ppm of the dye. Control diets contained corn oil in amounts equal to that in the diets of groups given the highest doses. The compound was administered for 13 weeks, when all animals were killed. The only suggestive neoplastic lesion observed was foci of basophilic cellular alteration in the liver of one male mouse administered 12,500 ppm Direct Brown 95 (NTP, 1978; Robens *et al.*, 1980). [The Working Group noted the short duration of the experiment, the limited number of animals tested, and the impurity of the compound used. Other samples of the compound may have different impurities.]

3.4 CI Acid Red 114

3.4.1 *Oral administration*

Groups of 45–75 male and female F344/N rats, 5 weeks of age, received doses of 0, 70, 150, or 300 ppm (male) or 0, 150, 300, or 600 ppm (female) of CI Acid Red 114 (purity, 82–85%; 15 organic impurities: two represented ~3%, benzidine < 1 ppm) in the drinking-water. Seventy animals were in the control and high-dose groups, 45 in the low-dose groups, and 75 in the mid-dose groups. Ten animals were evaluated from the control and high-dose groups at nine months, and ten animals from all dose groups were evaluated at 15 months. The average amount of compound consumed per day was 4, 8, or 20 mg/kg for males and 9, 21, or 69 mg/kg for females. Survival at 105 weeks for male rats receiving 0, 70, 150, or 300 ppm was 24/50, 15/35, 26/65, and 1/50; for females receiving 0, 150, or 300 ppm, survival was 36/50, 13/35, and 6/64 (see Table 3.1). All female rats receiving 600 ppm had died by week 89. The decreased survival in treated groups was due primarily to the development of chemical-related neoplasms. Of the surviving animals, the final mean body weights for males receiving 70 or 150 ppm were 94% and 90% of control and for females receiving 150 or 300 ppm, 99% and 84% of control. These weight differences began in the second year of the studies and were attributed in part to the development of neoplasms in the dosed groups.

Table 3.1. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Acid Red 114 in the drinking-water for 104 weeks

Survival and tumour types[a]	Dose (mg/L [ppm])				*p* Value[b]
Males	0	70	150	300	
Females	0	150	300	600	
Males					
Survival[c]	24/50 (48%)	15/35 (43%)	26/65 (40%)	1/50 (2%)	
Skin					
Basal-cell adenoma or carcinoma	1/50 (2%)	5/35 (14%)	28/65 (43%)	32/50 (64%)	<0.001
Sebaceous-cell adenoma or carcinoma	1/50 (2%)	1/35 (3%)	5/65 (8%)	6/50 (12%)	=0.007
Squamous-cell papilloma or carcinoma	1/50 (2%)	2/35 (6%)	11/65 (17%)	9/50 (18%)	=0.001
Keratoacanthoma	1/50 (2%)	1/35 (3%)	4/65 (6%)	7/50 (14%)	<0.001
Zymbal gland adenoma or carcinoma	0/50 –	0/35 –	8/65 (12%)	7/50 (14%)	=0.005
Liver neoplasms	2/50 (4%)	2/35 (6%)	15/65 (23%)	20/50 (40%)	<0.001
Females					
Survival[c]	36/50 (72%)	13/35 (37%)	6/64 (9%)	0/50 –	
Basal-cell adenoma or carcinoma of the skin	0/50 –	4/35 (11%)	7/65 (11%)	5/50 (10%)	=0.012
Zymbal gland adenoma or carcinoma	0/50 –	3/35 (8%)	18/65 (28%)	19/50 (38%)	<0.001
Clitoral gland adenoma or carcinoma	11/48 (23%)	17/32 (53%)	28/62 (45%)	23/50 (46%)	<0.001
Liver neoplasms	0/50 –	0/35 –	19/64 (30%)	8/50 (16%)	<0.001
Lung adenoma or carcinoma	1/50 (2%)	2/35 (6%)	9/65 (14%)	4/50 (8%)	=0.007
Oral cavity squamous-cell papilloma or carcinoma	0/50 –	3/35 (8%)	9/65 (14%)	6/50 (12%)	=0.017
Small intestine polyps or adenocarcinoma	0/50 –	0/35 –	1/63 (16%)	2/50 (4%)	NS
Large intestine polyps or adenocarcinoma	0/50 –	1/35 (3%)	0/64 –	3/50 (6%)	NS

From US National Toxicology Program (1991b)
[a] Terms used by authors
[b] Logistic regression test for trend
[c] At 22 months; reduced survival in exposed groups due to tumour development
NS, not significant

At nine and 15 months, a few neoplasms were seen in the liver, lung, clitoral gland, skin, Zymbal gland, oral cavity epithelium, and small and large intestine, and the number of neoplasms at these sites increased as the studies progressed (see Table 3.1). At two years, there was a clear carcinogenic response in the skin, Zymbal gland, and liver of male and female rats, and in the clitoral gland, oral cavity epithelium, small and large intestine, and lung in female rats. Treatment-related increases were also seen in the incidence in neoplasms of the oral cavity epithelium, adrenal gland, and lung of male rats,

and in mononuclear cell leukaemia and in neoplasms of the mammary gland and adrenal gland in female rats. The incidence of these neoplasms was generally lower, but was significant and considered to be marginally related to chemical treatment. The same neoplastic effects had been previously observed in some or all of the NTP studies with dimethoxybenzidine, dimethylbenzidine, or C.I. Direct Blue 15 (NTP, 1991a,b).

3.5 CI Direct Blue 15

3.5.1 *Oral administration*

At study initiation, 70 F344/N rats of each sex, 40–47 days of age, were given 0 or 2500 ppm CI Direct Blue 15 [purity, ~50%; ~35 impurities, including 3,3′-dimethoxybenzidine (836–1310 ppm) and benzidine (< 1 ppm)], 45 rats of each sex were given 630 ppm and 75 rats of each sex were given 1250 ppm in the drinking-water. Interim evaluations were made at nine and 15 months. The average amounts of compound consumed per day by the six dose groups after week 52 of the studies were estimated to be 45, 90, and 215 mg/kg for male rats and 50, 100, and 200 mg/kg for female rats. The studies were terminated at 22 months due to extensive mortality associated with chemical-related neoplasia. Survival of control, 630-, 1250-, and 2500-ppm males at 22 months was 37/50, 8/35, 11/65, and 2/50 (see Table 3.2); survival of females was 40/50, 13/35, 22/65, and 4/50. At 22 months, the mean final body weights of the 630-, 1250-, and 2500-ppm groups were 95%, 91%, and 81% of those of the control for male rats and 91% of those of the control for all female dose groups. At the nine-month interim evaluations, one adenoma of the Zymbal gland was seen in a high-dose male rat, and three carcinomas of the clitoral gland were seen in the high-dose females. At the 15-month interim evaluations, Zymbal gland neoplasms were seen in low- and high-dose males and in all treated female dose groups. Mid- and high-dose males and females also had preputial or clitoral gland neoplasms, and a few neoplasms were present in the skin, small and large intestine, liver, and oral cavity of treated animals at 15 months. At the end of the study (see Table 3.2), neoplasms related to chemical administration were found in the Zymbal gland, skin, oral cavity, and the preputial or clitoral gland in both male and female rats. Neoplasms related to chemical administration were also seen at other sites including the small and large intestine, liver, uterus, and brain. The incidence of mononuclear cell leukaemia was also increased in treated rats (NTP, 1992a).

3.6 C.I. Direct Blue 218

3.6.1 *Oral administration*

(a) Mouse

Groups of 50 male and 50 female B6C3F1 mice, 7 weeks of age, were administered C.I. Direct Blue 218 [purity approximately 60%; no attempt was made to identify major

Table 3.2. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Direct Blue 15 in the drinking-water for 96 weeks

Survival and tumour types[a]	Dose (mg/L[ppm])				*P* Value[b]
	0	630	1250	2500	
Males					
Survival[c]	37/50	8/35	11/65	2/50	
Skin					
Basal-cell adenoma or carcinoma	2/50 (4%)	9/35 (26%)	27/65 (42%)	28/50 (56%)	<0.001
Sebaceous gland adenoma	0/50 –	1/35 (3%)	7/65 (11%)	3/50 (6%)	=0.002
Squamous-cell papilloma or carcinoma	2/50 (4%)	4/35 (11%)	11/65 (17%)	19/50 (38%)	<0.001
Zymbal gland: adenoma or carcinoma	1/50 (2%)	5/35 (14%)	10/65 (15%)	20/50 (40%)	<0.001
Preputial gland: adenoma or carcinoma	8/49 (16%)	5/35 (14%)	23/64 (36%)	9/48 (19%)	<0.001[d]
Hepatocellular neoplasms nodule or carcinoma	0/50 –	6/35 (17%)	9/65 (14%)	11/50 (22%)	<0.001
Oral cavity: squamous-cell papilloma or carcinoma	1/50 (2%)	10/35 (29%)	24/65 (37%)	17/50 (34%)	<0.001
Small intestine: adenocarcinoma	0/50 –	0/35 –	0/65 –	2/50 (4%)	=0.078
Large intestine: polyps or adenocarcinoma	0/50 –	1/35 (3%)	6/65 (9%)	8/50 (16%)	<0.001
Mononuclear-cell leukaemia	17/50 (34%)	19/35 (54%)	28/65 (43%)	20/50 (40%)	<0.001[d]
Females					
Survival	40/50	13/35	22/65	4/50	
Squamous-cell papilloma or carcinoma of the skin	0/50 –	2/35 (6%)	6/65 (9%)	5/50 (10%)	=0.001
Zymbal gland: adenoma or carcinoma	0/50 –	4/35 (11%)	11/65 (17%)	17/50 (34%)	<0.001
Clitoral gland: adenoma or carcinoma	7/50 (14%)	11/31 (35%)	24/64 (38%)	27/50 (54%)	< 0.001
Hepatocellular neoplastic nodule or carcinoma	0/50 –	0/35 –	2/65 (3%)	5/50 (10%)	<0.001
Oral cavity: squamous-cell papilloma or carcinoma	2/50 (4%)	4/35 (11%)	19/65 (29%)	15/50 (30%)	<0.001
Small intestine: adenocarcinoma	0/50 –	0/35 –	1/65 (2%)	3/50 (6%)	=0.032
Uterine adenoma or adenocarcinoma	1/50 (2%)	0/35 –	1/65 (2%)	4/50 (8%)	=0.004
Mononuclear-cell leukaemia	7/50 (14%)	13/35 (37%)	27/65 (42%)	15/50 (30%)	<0.001[d]

From US National Toxicology Program (1992a)
[a] Terms used by authors
[b] Logistic regression trend test
[c] At 22 months; reduced survival in exposed groups due to neoplasia
[d] Life-table test

impurities. However, benzidine was not detected at levels greater than 1 ppm and dimethoxybenzidine was detected at 7 ppm] in the diet at 0, 1000, 3000 or 10,000 ppm for 103 weeks. Mean body weights of males and females receiving 10,000 ppm were 19% lower than controls for males and 27% lower than controls for females. Mortality was similar to that of the controls. In male mice, the incidence of hepatocellular adenomas or carcinomas (combined) was increased: 21/50 (42%), 20/50 (40%), 23/50 (46%) and 45/50 (90%) ($P < 0.001$, Fisher exact test and Logistic regression trend test) in control, low-, mid- and high-dose groups, respectively. In female mice, the incidence of hepatocellular adenomas or carcinomas (combined) was increased: 10/49 (20%), 15/50 (30%), 21/49 (43%) and 45/49 (92%) ($P < 0.001$, Fisher exact test; $P < 0.001$ trend test) in control, low-, mid- and high-dose groups, respectively (NTP, 1994).

(b) Rat

Groups of 50 male and 50 female Fischer 344 rats, 6–7 weeks of age, were administered C.I. Direct Blue 218 [purity, see study above] in the diet at 0, 1000, 3000 or 10,000 ppm for 103 weeks. Mean body weights of males and females receiving 10,000 ppm were 11% lower than controls for males and 9% lower than controls for females. Mortality among females receiving 10,000 ppm was slightly but not significantly lower than that of the controls. In males, the incidence of squamous cell papillomas or carcinomas (combined) of the pharynx was increased: 0/50, 0/50, 0/50 and 6/50 (12%) ($P = 0.013$, Fisher exact test and $P < 0.001$, Logistic regression trend test) in the control, low-, mid- and high-dose groups, respectively. In females, the incidence of tumours in the treated groups was not significantly different from that in the control groups (NTP, 1994).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

Lowry *et al.* (1980) measured benzidine and monoacetylbenzidine in the urine of workers exposed to benzidine-based azo dyes during their manufacture, or during textile or paper dyeing. A colorimetric screening method, based on reaction of extracted aromatic amines with 2,4,6-trinitrobenzene-sulfonic acid, and a specific electron-capture gas chromatographic (EC-GC) detection method were used. Alkali-labile conjugates of benzidine and 2,4-diaminoazobenzene were found together with free 2,4-diamino-azobenzene and traces of benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxy-benzidine.

Dewan *et al.* (1988) used high performance liquid chromatography (HPLC) to analyse the presence of benzidine and mono- and diacetyl-benzidine in post-shift urine samples of 18 workers in a small-scale unit manufacturing Direct Black 38. Acetylated

metabolites were found in all of the urine samples (range, 6–131.8 μg/L for monoacetyl-benzidine), and benzidine was found in all except two (range, 2.4–48.9 μg/L). Two workers who lived on the factory premises excreted very high levels of benzidine (362.5 and 260.3 μg/L) and its metabolites (1117.2 and 660.2 μg/L, for monoacetylbenzidine) in their urine. None of the urine samples showed the presence of 4-aminobiphenyl. Environmental sampling showed high dust levels during powdering of the finished product. Exposure to benzidine by inhalation at this stage was considered unlikely, because no benzidine was detectable in the finished dye.

Beyerbach *et al.* (2006) used gas chromatography/mass spectrophotometry (GC/MS) to identify and quantify, in groups of Indian workers producing benzidine-2HCl or Direct Black 38 from benzidine-2HCl, aniline and other compounds, the following haemoglobin adducts: benzidine-Hb, N-acetylbenzidine-Hb, 4-aminobiphenyl-Hb, aniline-Hb. The latter two were quantitatively the major adducts. The amounts of adducts were highly correlated in all the exposed workers ($n = 33$). The benzidine-exposed group had 10- to 17-fold higher adduct levels than the dye workers. Since 4-aminobiphenyl can be metabolically released from benzidine and the azo dye, and aniline can be released from the azo dye, the presence of 4-aminobiphenyl-Hb and aniline-Hb may be the consequence of exposure to the parent compounds, or of exposure to benzidine and the azo dye after metabolic release of the arylamine moiety.

4.1.2 *Experimental systems*

(a) In-vivo studies

Rhesus monkeys excreted an average of 1.25% benzidine plus monoacetyl-benzidine of the benzidine moiety in Direct Black 38, Direct Blue 6, and Direct Brown 95, respectively, in the urine after receiving two different doses by gavage, whereas gavage with pure benzidine yielded 1.45%. These analyses were done by use of selective extraction followed by thin-layer chromatography (TLC). The authors thus postulated a nearly complete metabolic conversion of these three dyes to benzidine (Rinde & Troll, 1975).

Following oral administration of a single dose of 10 mg/kg bw Direct Black 38 to Syrian golden hamsters, 10.7 μg benzidine, 535 μg monoacetylbenzidine, 27.6 μg diacetylbenzidine, 11.5 μg 4-aminobiphenyl and, as alkali-hydrolysable conjugates, 328.5 μg benzidine and 6.3 μg 4-aminobiphenyl were identified in the urine by parallel electron-capture gas chromatography and HPLC. Peak excretion occurred between 0–8 and 8–16 hours. These results indicate that a total of 10% of the dye is metabolized to benzidine and its metabolic follow-up products (Nony *et al.*, 1980b).

Several bisazobiphenyl dyes derived from benzidine, 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine were studied in the dog and rat. The dyes were given orally and the urine was analysed for benzidine, 3,3′-dimethyl- or 3,3′-dimethoxybenzidine by use of a specific gas chromatographic assay. The identity of the peaks was confirmed by GC/MS. Dogs treated acutely (100 mg/kg bw) with benzidine-derived dyes excreted substantial

quantities of benzidine (166–1675 μg) in urine (0–48 hours). No benzidine was detected in urine before treatment. Benzidine present in dog urine following dye administration exceeded by at least ninefold the benzidine present as impurity in the administered dyes, and was comparable to that excreted in urine when pure benzidine was fed (100 mg/kg bw). Rats chronically dosed (100 mg/kg bw/day) with benzidine-based dyes excreted *N*-acetylbenzidine (3–54 μg/day) and traces of benzidine in urine. Bisazobiphenyl dyes derived from 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine were metabolized to 3,3′-dimethyl- and 3.3′-dimethoxybenzidine, respectively, in both the dog and rat. The *N*-acetyl derivatives of 3,3′-dimethyl- and 3.3′-dimethoxybenzidine were identified in urine from rats treated with dyes derived from 3.3′-dimethyl- and 3.3′-dimethoxybenzidine, respectively. The results indicate that the metabolic conversion of bisazobiphenyl dyes, derived from benzidine, 3,3′-dimethyl- and 3,3′-dimethoxybenzidine, to carcinogenic aromatic amines is a general phenomenon, and therefore with few exceptions should be anticipated for each member of this class of chemicals (Lynn *et al.*, 1980).

The mutagenic activation *in vivo* of three azo dyes was studied. Wistar rats received solutions of benzidine, Direct Black 38 and Direct Brown 95 orally or by intraperitoneal injection. Urine was collected for 24 hours. For Direct Black 38, significantly higher mutagenicity values were found in the urine after oral administration than after intraperitoneal treatment. Such differences were not observed for benzidine and Direct Brown 95. The results suggest that for some compounds like Direct Black 38, extrahepatic enzymes, most likely present in the intestinal flora, play a substantial role in the azo cleavage (Bos *et al.*, 1984). After oral administration of these dyes to germ-free Wistar rats, no mutagenicity was observed in the urine. A germ-free rat that received benzidine produced urine with mutagenicity comparable to that of a normal rat. These results show that after oral administration, reduction by the intestinal microflora is the first step in the bio-toxification of benzidine-based dyes (Bos *et al.*,1986).

(b) In-vitro studies

Preparations of rat and mouse intestine *in vitro* have been shown to convert Direct Black 38 to benzidine (Niitsu, 1973). After increasing the microbial activity in rats by feeding a meat-based diet, the azo-reductase level was also enhanced (Goldin *et al.* 1978).

Anaerobic bacterial suspensions isolated from human faeces and from the intestinal contents of rhesus monkeys and CD rats were incubated for 48 hours with Direct Black 38 (a benzidine-based azo dye), Direct Red 2 (3,3′-dimethylbenzidine-based), and Direct Blue 15 (3,3′-dimethoxybenzidine-based). The respective free amines, benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, formed by azo reduction of the dyes by intestinal bacteria were isolated and identified by GC/MS. Within six hours of incubation, 90–100% of each dye was reduced by all three bacterial suspensions. The results suggest that anaerobic intestinal bacteria may play a significant role in the metabolism of dyes derived from benzidine (Cerniglia *et al.*, 1982a).

The metabolism of Direct Black 38 (a benzidine-based azo dye), Direct Red 2 (3,3′-dimethylbenzidine-based) and Direct Blue 15 (3,3′-dimethoxybenzidine-based) has been

studied both in pure cultures of anaerobic bacteria and in bacterial suspensions derived from the intestinal contents of rats. All of the pure cultures and the rat intestinal bacteria were able to reduce the azo linkages of Direct Black 38, Direct Red 2 and Direct Blue 15 with the subsequent formation of benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, respectively. The metabolites were isolated and identified by GC/MS, and had chromatographic and mass-spectral properties similar to those of authentic standards. In-vitro anaerobic incubations of rat intestinal microorganisms are able to reduce and cleave the azo bonds of dyes derived from benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine to form potentially carcinogenic aromatic amines (Cerniglia *et al.*, 1982b).

Metabolism of the benzidine-based dye Direct Black 38 was examined by use of a semi-continuous culture system that simulates the lumen of the human large intestine. The system was inoculated with freshly voided human faeces, and an active flora was maintained as evidenced by volatile fatty acid and gas production. Within seven days after exposure to the dye, the following metabolites were isolated and identified by GC/MS: benzidine, 4-aminobiphenyl, monoacetylbenzidine, and acetylamino-biphenyl. Benzidine reached its peak level after 24 hours, accounting for 39.1% of the added dye. Its level began to decline, and by day seven the predominant metabolite was acetylaminobiphenyl, which accounted for 51.1% of the parent compound. Formation of the deaminated and *N*-acetylated analogues of benzidine, which are more mutagenic and lipophilic, has not been attributed so far to the intestinal microbiota (Manning *et al.*, 1985).

The role of the rat intestinal flora in the azo reduction of some benzidine-based dyes was studied by measurement of the formation of benzidine after anaerobic incubation of 1 mM Direct Black 38, Direct Blue 6 and Direct Brown 95 in the presence of caecal bacteria *in vitro*. After about six hours, the concentration of benzidine had reached a maximum at 160 μM for Direct Black 38, at 40 μM for Direct Blue 6, and at 30 μM for Direct Brown 95 (Bos *et al.*, 1986).

The azo reduction and acetylation *in vitro* of three azo dyes were studied. In the presence of rat-liver S9, benzidine was released from Direct Black 38 and Direct Brown 95, whereas hardly any benzidine was produced during incubation of Direct Blue 6. Incubation of benzidine with isolated rat hepatocytes resulted in the formation of diacetylbenzidine. No diacetylbenzidine was formed during incubation of benzidine with rat-liver S9, unless the cofactor for the acetylation reaction, acetyl coenzyme A, was added to the incubation medium. Isolated rat hepatocytes were capable of producing diacetylbenzidine from the three test dyes without supplementation with acetyl coenzyme A (Bos *et al.*, 1984).

Ingested azo dyes can be metabolized to aromatic amines by intestinal microorganisms, but hepatic enzymes can also catalyse the reductive cleavage of the azo linkage to produce the parent amines. The intestinal microbial azoreductase may be more important than the liver enzymes in azo reduction. Anaerobic bacteria were isolated from caecal or faecal contents from experimental animals and humans. The significance of the capacity of intestinal bacteria to reduce azo dyes and the conditions of azo reduction were

investigated. The azoreductase(s) that catalyse these reactions have been found to be oxygen-sensitive and to require flavins for optimal activity. The azoreductase activity in a variety of intestinal preparations was influenced by dietary factors such as cellulose, proteins, fibres or antibiotics (Chung *et al.* 1992).

In a plate assay for the detection of anaerobic bacteria that produce azoreductases, ten strains of anaerobic bacteria capable of reducing azo dyes were tested. The strains were isolated from human faeces and identified as *Eubacterium hadrum* (2 strains), *Eubacterium* spp. (2 species), *Clostridium clostridiiforme*, a *Butyrivibrio* sp., a *Bacteroides* sp., *Clostridium paraputrificum*, *Clostridium nexile*, and a *Clostridium* sp. The average rate of reduction of Direct Blue 15 (a dimethoxybenzidine-based dye) in these strains ranged from 16–135 nmol of dye per min per mg of protein. The enzymes were inactivated by oxygen. In seven isolates, a flavin compound (riboflavin, flavin adenine dinucleotide, or flavin mononucleotide) was required for azoreductase activity. In the other three isolates and in *Clostridium perfringens*, no added flavin was required for activity. Each bacterium expressed only one azoreductase isozyme. At least three types of azoreductase were produced by the different isolates. All of the azoreductases were produced constitutively and released extracellularly (Rafii *et al.*, 1990; Rafii & Cerniglia 1995).

4.2 Genetic and related effects

4.2.1 *3,3′-Dimethylbenzidine dihydrochloride*

3,3′-Dimethylbenzidine dihydrochloride was mutagenic in *Salmonella typhimurium* strain TA98 with exogenous metabolic activation; it was not mutagenic in strains TA100, TA1535, or TA97 with or without activation. 3,3′-Dimethylbenzidine dihydrochloride induced sister-chromatid exchange and chromosomal aberrations in Chinese hamster ovary (CHO) cells in the absence of exogenous metabolic activation; these effects were not evident in tests with S9 activation. Sex-linked recessive lethal mutations were induced in germ cells of adult male *Drosophila melanogaster* given 3,3′-dimethylbenzidine dihydrochloride in the feed or by injection. No reciprocal translocations occurred in *D. melanogaster* germ cells following exposure to 3,3′-dimethylbenzidine dihydrochloride (NTP, 1991a).

4.2.2 *3,3′-Dimethoxybenzidine dihydrochloride*

3,3′-Dimethoxybenzidine was mutagenic in *S. typhimurium* strain TA100 with exogenous metabolic activation and in strain TA98 without activation; a weakly positive response was observed in strain TA1535 with metabolic activation. 3,3′-Dimethoxybenzidine induced sister chromatid exchange and chromosomal aberrations in CHO cells with and without exogenous metabolic activation. 3,3′-Dimethoxybenzidine did not

induce sex-linked recessive lethal mutations in adult male *D. melanogaster* exposed via feeding or by injection (NTP, 1990).

4.2.3 *Direct Black 38*

Direct Black 38 was mutagenic in *Salmonella typhimurium* strains TA98 and TA100 when tested in the presence of a mouse-liver metabolic activation system; no mutagenicity was observed in the absence of activation (Lazear & Louie 1978). Monoacetylbenzidine, a major metabolite of benzidine (see above), and urine from hamsters given Direct Black 38 (100 mg/kg bw) were mutagenic in *S. typhimurium* strain TA1538, but only when tested in the presence of metabolic activation (Lazear & Louie, 1978; Nony & Bowman 1980a). Urine from rats given 500 mg/kg bw Direct Black 38 was also mutagenic in *S. typhimurium* strains TA98 and TA100 in the presence of metabolic activation (Tanaka, 1980).

4.2.4 *CI Acid Red 114*

In a standard pre-incubation protocol, CI Acid Red 114 was mutagenic in *Salmonella typhimurium* strain TA98 in the presence of induced hamster liver S9, and an equivocal response was noted in strain TA100 with hamster liver S9. However, no significant mutagenic activity was noted in strains TA1535 or TA1537, with or without S9 activation. In a modified *S. typhimurium* gene-mutation test that employed reductive metabolism followed by oxidative metabolism with S9 liver enzymes, CI Acid Red 114 was strongly mutagenic in strain TA1538. This dye did not induce sister chromatid exchange or chromosomal aberrations in CHO cells with or without S9 activation; reductive metabolism was not used in these cytogenetic tests. No increase in sex-linked recessive lethal mutations was observed in germ cells of male *Drosophila melanogaster* administered CI Acid Red 114 by feeding or injection (NTP, 1991b).

4.2.5 *CI Direct Blue 15*

CI Direct Blue 15 was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98 when tested in a standard pre-incubation protocol with or without exogenous metabolic activation; however, when a specialized reductive metabolism protocol was used, C.I. Direct Blue demonstrated mutagenic activity in *Salmonella* strain TA1538. C.I. Direct Blue 15 did not induce sister chromatid exchange or chromosomal aberrations in CHO cells with or without S9 activation; reductive metabolism was not used in these cytogenetic tests (NTP, 1992a).

4.2.6 *CI Direct Blue 218*

CI Direct Blue 218 was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 tested with and without exogenous metabolic activation

(S9). It was also tested in a modified *Salmonella* test-protocol that employed reductive metabolism supplied by flavin mononucleotide or rat caecal bacteria, followed by oxidative metabolism; results of this test with strain TA1538 were also negative. C.I. Direct Blue 218 induced a small but significant increase in sister chromatid exchange in CHO cells at the highest dose tested, without S9. No increase in chromosomal aberrations was observed in these cells, with or without S9. C.I. Direct Blue 218 did not induce sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* (NTP, 1994).

4.2.7 *CI Pigment Red 23*

CI Pigment Red 23, an azo dye, was mutagenic in *Salmonella typhimurium* strains TA100, TA1537, and TA98, with and without exogenous metabolic activation (S9), but it was not mutagenic in strain TA1535. CI Pigment Red 23 induced sister chromatid exchange in CHO cells in the absence of S9, but not with S9 activation. The pigment did not induce chromosomal aberrations in these cells in the presence or absence of S9 (NTP, 1992b).

4.3 Mechanistic considerations

This section discusses the general problem of how to approach the hazard evaluation of precursors of established carcinogens, e.g., azo-colourants. These are synthesized by coupling aryl amines with single or multiple diazo moieties. Benzidine and the benzidine derivatives 3,3′-dimethyl-, 3,3′-dimethoxy-, and 3,3′-dichlorobenzidine are compounds that can be double-diazotized. The azo group can be cleaved reductively by intestinal bacteria or azo reductases in the liver and in other tissues, and the amines released. Aminonaphthalene and monocyclic aromatic amines are also encountered. In many cases the released amines or their metabolites have been detected in experimental animals as well as in humans. Most of these basic components are carcinogenic in experimental animals, benzidine itself being an IARC Group-1 carcinogen. Hundreds of such azo colourants exist, and they obviously cannot be all tested individually for carcinogenic potential.

As an example, Direct Red 28 was orally administered to rats and the haemoglobin adducts were used as biomarkers to demonstrate that benzidine is released from the dye. The same haemoglobin adducts were found with benzidine itself. Upon hydrolysis of the haemoglobin adduct benzidine, monoacetylbenzidine and 4-aminobiphenyl were identified in the same relative amounts (Birner *et al.*, 1990). This shows that the dye is metabolized to benzidine, and one of its two amino groups is *N*-oxidized to the nitroso-derivative that binds to haemoglobin in the erythrocytes. A radical-based mechanism may also play a role. It shows also that the reactive metabolite is widely distributed and can be expected to produce DNA lesions in most tissues. It should be noted that 4-amino-biphenyl is a metabolite of the dye and of benzidine, which links this exposure to that of

nitrobiphenyl, a combustion product and common environmental pollutant (Neumann, 2001).

Similarly, benzidine and 4-aminobiphenyl are released from Direct Black 38 and Direct Brown 1, commonly used in leather and textile industries. The particular role of skin bacteria for reductive cleavage and the subsequent absorption of the amines from textiles is emphasized (Gnanamani *et al.*, 2004).

It is concluded that all azo colourants whose metabolism can liberate a carcinogenic aromatic amine are potentially carcinogenic. It has therefore been recommended that the colourants be dealt with as if they were classified in the same categories as the corresponding carcinogenic or suspected carcinogenic amine (Deutsche Forschungsgemeinschaft, 2007). There are, however, colourants that have been claimed to be insoluble and that may not contribute to the amine exposure. This can be tested by use of biomarkers. In case of Pigment Yellow 17, a diarylide azo pigment with a 3,3′-dichlorobenzidine component, the expected haemoglobin adducts were identified after intratracheal instillation and oral administration to rats, but the level was very low. In a 4-week feeding study, Zwirner-Baier & Neumann (1994) calculated that 0.6% of the dose was cleaved in the intestine and the 3,3′-dichlorobenzidine absorbed. Others have suggested that the amine component of 3,3′-dichlorobenzidine-based dyes is in general practically not bioavailable (Golka *et al.*, 2004).]

When the contribution of a benzidine-based dye to cancer risk is claimed to be low or negligible, the bio-availability of the carcinogenic component should be excluded, e.g. by use of biomarkers of exposure or biomarkers of effect. However, if this is not the case, it does not seem justified to classify benzidine-based dyes differently from benzidine.

5. Summary of Data Reported

5.1 Exposure data

See the Monograph on Benzidine in this volume.

5.2 Human carcinogenicity data

The epidemiological evidence has been reviewed for workers exposed to benzidine derivatives (3,3′-dimethyl-, 3,3′-dimethoxy-, and 3,3′-dichlorobenzidine) and benzidine-based dyes. Studies of occupations with potential exposure to dyes metabolized to benzidine were reviewed only in those instances in which the authors specifically noted that such an exposure was occurring, including shoe and leather workers, and textile workers.

Consistently elevated risks for bladder cancer were observed among workers exposed to benzidine derivatives. There is also some evidence of increased risks with intensity of exposure and duration of exposure.

The Working Group identified four cohort studies and two case–control studies in populations occupationally exposed to dyes metabolized to benzidine. The risk for bladder cancer for workers exposed to dyes metabolized to benzidine was not consistent across studies, with some reports showing excess risk and others not showing an effect. A limitation of most studies is that while they indicate the potential for exposure to these compounds, no quantitative exposure data are presented.

The difficulty of evaluating the cancer risk from exposure to these compounds arises from concomitant exposures to benzidine and 2-naphthylamine, which are bladder carcinogens in humans. Potential cross-contamination with residual benzidine may also occur. Consequently, it is not possible to establish if the risks observed in certain studies are due to potential confounders or to benzidine precursors of dyes metabolized to benzidine.

5.3 Animal carcinogenicity data

Direct Black 38 was tested for carcinogenicity in mice by oral administration, producing hepatocellular carcinomas and mammary carcinomas. In rats, Direct Black 38 produced hepatocellular carcinomas within 13 weeks after administration in the diet, and carcinomas in the urinary bladder, liver and colon after administration in drinking-water.

In a single well-conducted study, Direct Blue 6 produced hepatocellular carcinomas in male and female rats within 13 weeks after its oral administration.

Direct Brown 95 was tested for carcinogenicity in a single well-conducted study under Good Laboratory Practice. After oral administration, it produced neoplastic nodules in the livers of 4/8 female rats, one of which showed a hepatocellular carcinoma. This study was terminated after 13 weeks. The finding of preneoplastic lesions after such a short exposure period suggests a carcinogenic effect similar to that of Direct Black 38 and Direct Blue 6.

C.I. Acid Red 114 was tested for carcinogenicity in rats by administration in the drinking-water. It produced a clear carcinogenic response in the skin, Zymbal gland and liver of male and female rats, and in the clitoral gland, oral cavity epithelium, small and large intestine, and lung in female rats after two years. Treatment-related increases were also seen in the incidences of neoplasms of the oral cavity epithelium, adrenal gland, and lung of male rats, and of mononuclear cell leukaemia and neoplasms of the mammary gland and adrenal gland in female rats.

C.I. Direct Blue 15 was tested for carcinogenicity in rats by administration in the drinking-water. It produced neoplasms in the Zymbal gland, skin, oral cavity, and the preputial or clitoral gland in both male and female rats. The 24-month study was terminated at 22 months because of rapidly declining animal survival, which was due primarily to neoplasia. Neoplasms related to chemical administration were also seen at other sites including the small and large intestine, liver, uterus, and brain.

C.I. Direct Blue 218 was tested for carcinogenicity in mice and rats by administration in the diet. It produced hepatocellular adenomas and carcinomas in male and female mice,

and squamous cell papillomas or carcinomas of the pharynx in male rats after two years of treatment.

5.4 Other relevant data

See the Monograph on Benzidine in this volume.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of dyes metabolized to benzidine.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Black 38.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Blue 6.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Brown 95.

6.3 Overall evaluation

Dyes metabolized to benzidine are *carcinogenic to humans (Group 1)*

In reaching this evaluation, the Working Group considered the following:

(1) Benzidine is a Group-1 *human carcinogen,* (2) metabolism of benzidine-based dyes results in the release of free benzidine in humans and in all experimental animal species studied, and (3) exposure to benzidine from exposure to benzidine-based dyes is equivalent to exposure to large doses of benzidine.

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MAGENTA AND MAGENTA PRODUCTION

Historically, the name Magenta has been used to refer to the mixture of the four major constituents comprising Basic Fuchsin, namely Basic Red 9 (Magenta 0), Magenta I (Rosaniline), Magenta II, and Magenta III (New fuchsin). Although samples of Basic Fuchsin can vary considerably in the proportions of these four constituents, today each of these compounds except Magenta II is available commercially under its own name. Magenta I and Basic Red 9 are the most widely available.

1. Exposure Data

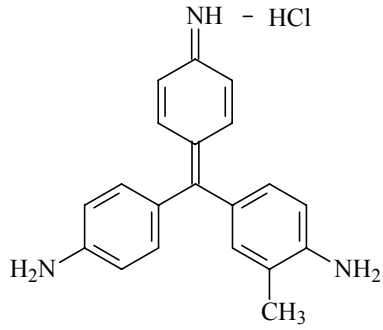
1.1 Chemical and physical data

1.1.1 *Magenta I*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 632–99–5
CAS Name: 4-[(4-Aminophenyl)(4-imino-2,5-cyclohexadien-1-ylidene)methyl]-2-methylbenzenamine, hydrochloride (1:1)
Synonyms: 4-[(4-Aminophenyl)(4-imino-2,5-cyclohexadien-1-ylidene)methyl]-2-methylbenzenamine, monohydrochloride; Basic Fuchsin hydrochloride; C.I. 42510; C.I. Basic Red; C.I. Basic Violet 14; C.I. Basic Violet 14, monohydrochloride; 2-methyl-4,4'-[(4-imino-2,5-cyclohexadien-1-ylidene)methylene]dianiline hydrochloride; rosaniline chloride; rosaniline hydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{20}H_{19}N_3.HCl$ Rel. mol. mass: 337.85

(c) Chemical and physical properties of the pure substance

Description: Metallic green, lustrous crystals (O'Neil, 2006; Lide, 2008)
Melting-point: Decomposes above 200 °C (O'Neil, 2006; Lide, 2008)
Solubility: Slightly soluble in water (4 mg/mL); soluble in ethanol (30 mg/mL) and ethylene glycol methyl ether (30 mg/mL); insoluble in diethyl ether (Green, 1990; O'Neil, 2006; Lide, 2008)

(d) Trade names

Trade names for Magenta I include: Aizen Magenta; Aniline Red; Astra Fuchsine B; Basic Fuchsine; Basic Fuchsin; Basic Magenta; Basic Magenta E-200; C-WR Violet 8; Calcozine Fuchsine HO; Calcozine Magenta RIN; Calcozine Magenta RTN; Calcozine Magenta XX; Cerise B; Diabasic Magenta; Diamond Fuchsin; Diamond Fuchsine; Fuchsin; Fuchsin Basic; Fuchsine; Fuchsine A; Fuchsine CS; Fuchsine G; Fuchsine HO; Fuchsine N; Fuchsine RTN; Fuchsine SBP; Fuchsine Y; Magenta; Magenta DP; Magenta E; Magenta G; Magenta PN; Magenta S; Magenta Powder N; Magenta Superfine; Magenta Super Fine; Methyl Fuchsin; Mitsui Magenta; Orient Basic Magenta; RGB 20; RGN 10.

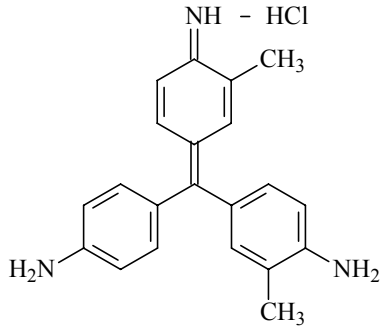
1.1.2 *Magenta II*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 26261–57–4
CAS Name: 4-[(4-Aminophenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl]-2-methylbenzenamine, hydrochloride (1:1)
Synonyms: 4-[(4-Aminophenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl]-2-methylbenzenamine, monohydrochloride; α4-(*p*-aminophenyl)-

α4-(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)-2,4-xylidine, monohydrochloride; dimethyl fuchsin

(b) Structural formula, molecular formula, and relative molecular mass



$C_{21}H_2N_3.HCl$ Rel. mol. mass: 351.87

(c) Chemical and physical properties of the pure substance

No information was available to the Working Group.

(d) Technical products and impurities

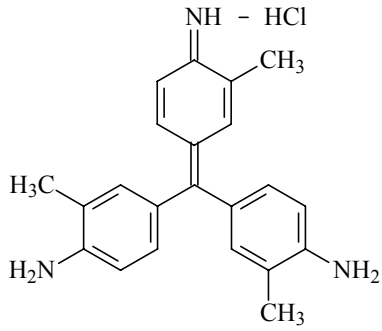
Magenta II is not available as a separate commercial product.

1.1.3 *Magenta III*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 3248–91–7
CAS Name: 4,4'-[(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methylene]bis[2-methylbenzenamine], hydrochloride (1:1)
Synonyms: 4-[(4-Amino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene) methyl]-2-methylbenzenamine, monohydrochloride; 4-[(4-amino-m-tolyl)(4-imino-3-methylcyclohexa-2,5-dien-1-ylidene)methyl]-o-toluidine, monohydrochloride; Basic Violet 2; C.I. 42520; C.I. Basic Violet 2; C.I. Basic Violet 2, monohydrochloride; 4,4'-[(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methylene]bis[2-methylbenzenamine], monohydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{22}H_{23}N_3.HCl$ Rel. mol. mass: 365.90

(c) Chemical and physical properties of the pure substance

Description: Green crystalline powder (Green, 1990)
Solubility: Soluble in water (20 mg/mL), ethanol (20 mg/mL), and ethylene glycol methyl ether (20 mg/mL) (Green, 1990)

(d) Trade names

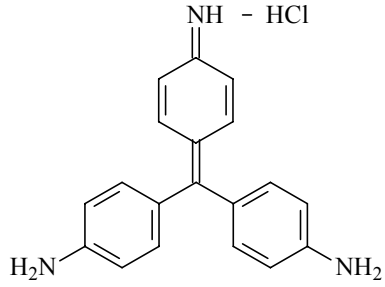
Trade names for Magenta III include: Astra New Fuchsine G; Astrazon Fuchsine GN; Calcozine New Fuchsine; Fuchsin NB; Leather Ruby HF; Magenta ABN; Neofuchsine; New Fuchsin; New Fuchsine; New Fuchsine G Crystal; New Magenta; Remacryl Magenta B.

1.1.4 *Basic Red 9 (Magenta 0)*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 569–61–9
CAS Name: 4,4'-[(4-imino-2,5-cyclohexadien-1-ylidene)methylene]bis[benzenamine], hydrochloride (1:1)
Synonyms: Basic Parafuchsine; Basic Red 9; C.I. 42500; C.I. Basic Red 9; C.I. Basic Red 9, monohydrochloride; *para*-Fuchsin; *para*-Fuchsine; 4,4'-[(4-imino-2,5-cyclohexadien-1-ylidene)methylene]bis[benzenamine], monohydrochloride; 4,4'-(4-iminocyclohexa-2,5-dienylidenemethylene)dianiline hydrochloride; Magenta 0; Parafuchsin; Parafuchsine; Parafuchsin hydrochloride; Para Magenta; Pararosaniline; Pararosaniline chloride; Pararosaniline hydrochloride; *para*-Rosaniline hydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{19}H_{17}N_3.HCl$ Rel. mol. mass: 323.82

(c) Chemical and physical properties of the pure substance

Description: Pale violet powder (Lide, 2008)
Melting-point: 269 °C (decomposes) (Lide, 2008)
Solubility: Slightly soluble in water (3 mg/mL); soluble in ethanol (25 mg/mL) and ethylene glycol methyl ether (70 mg/mL) (Green, 1990)

(d) Trade names

Trade names for C.I. Basic Red 9 (Magenta 0) include: Calcozine Magenta N; Fuchsine DR 001; Fuchsine SP; Fuchsine SPC; and Orient Para Magenta Base.

1.1.5 *Analysis*

The first reports on analysis of magenta were published during the 1960s. The more recent studies have involved the use of LC/MS, in which laser-desorption electrospray ionization was employed to analyse paper and textiles for the presence of magenta. Assessments using high-resolution TLC, including an electromolecular propulsion method, have been reported (Table 1.1).

An LC-MS method involving electrospray ionization has been developed to distinguish Magenta 0 from its homologues, which have identical UV-visible absorption spectra (Huang *et al.*, 2005).

1.2 Production and use

1.2.1 *Production*

Magenta was among the first synthetic dyes to be produced, beginning in the 1850s (Bannister & Elliot, 1983). Magenta was produced commercially in England (Bannister & Olin, 1965) and in the USA (US Tariff Commission, 1922). In the United Kingdom, the process for manufacturing magenta has involved condensation of *ortho*-toluidine (see

IARC, 1982a, 1987) and formaldehyde (see IARC, 1982b, 1987) in the presence of nitrotoluene, resulting mainly in the production of Magenta III (Howe, 1977). Magenta I is prepared by the reaction of a mixture of aniline (see IARC, 1982a, 1987), *ortho*- and *para*-toluidine and their hydrochlorides with nitrobenzene or a mixture of nitrobenzene and *ortho*-nitrotoluene in the presence of ferrous chloride, ferrous oxide and zinc chloride (US National Library of Medicine, 1992). C.I. Basic Red 9 is prepared by the reaction of aniline with formaldehyde in the presence of hydrogen chloride, forming 4,4'-methylenedianiline (see IARC, 1986), which is then heated with aniline and aniline hydrochloride in the presence of nitrobenzene and ferric chloride (US National Library of Medicine, 1992).

Table 1.1. Selected methods of analysis of magenta in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Solvent mixtures	Apply to stationary phase, develop plate	EMP-TLC	Not given	Haber (1998)
Natural and synthetic samples	Dissolve dye (10^{-3} M) in ethanol–water (4:1, v/v).	IPTLC	500ng	Misra & Gupta (2002)
Dyestuffs and textile fibres	Place dye sample or cut dyed cotton fibre in glass vials, add 2-propanol/water (4:1), heat, remove fibres, and evaporate. Dissolve residue in methanol for analysis.	LC-MS with ESI	5ppm	Huang *et al.*, (2005)

EMP, electromolecular propulsion; ESI, electrospray ionization; IPTLC, ion-pair thin-layer chromatography; LC-MS, liquid chromatography-mass spectrometry; TLC, thin-layer chromatography

Magenta III is prepared by condensation of two moles of *ortho*-toluidine with formaldehyde in nitrobenzene in the presence of iron salts to give the corresponding substituted diphenylmethane base. This base is not isolated, but undergoes an oxidative condensation with another mole of *ortho*-toluidine to produce the dye (Thetford, 2000).

No recent data were available on the production of magenta or C.I. Basic Red 9. Production data for Magenta I in the USA were last reported for 1964, when the combined production of five producers was reported as 53 tonnes (US Tariff Commission, 1965). Production of C.I. Basic Red 9 in the USA was estimated as greater than 0.9 tonnes in 1972 and 0.5 tonnes in 1975 (US National Library of Medicine, 1992).

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for

chemicals manufactured (including imported) in amounts of 10 000 pounds [4500 kg] or greater (in 1986) or 25 000 pounds [11 000 kg] or greater (in 2003) at a single site. The aggregate production volume reported for Magenta I in 1990 was 10 000-500 000 pounds [4500–227 000 kg].

Available information indicates that Magenta I was produced and/or supplied in research quantities in the following countries: Canada, Germany, Hong Kong Special Administrative Region, India, the Netherlands, Switzerland, the United Kingdom, and the USA (Chemical Sources International, 2008).

Magenta III was produced and/or supplied in research quantities in the following countries: Canada, Germany, India, Japan, Switzerland, and the USA (Chemical Sources International, 2008).

Magenta 0 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, the Netherlands, Switzerland, and the USA (Chemical Sources International, 2008).

1.2.2 *Use*

The general population can be exposed to magenta through a variety of uses. Under the name of Basic Violet 14, magenta is used in hair dyes and is also used in cosmetic products not intended to come in contact with mucous membranes (EU Directive 76/768/EEC). Magenta stains animal fibres directly and vegetable fibres after mordanting. Under the name of Basic Red 9, magenta is also used as a colourant in artists' paints. Magenta III is used as a thin-layer chromatography developing agent for perfluorinated organics (Williams *et al.*, 1992).

Magenta is antiseptic against gram-positive bacteria and can be used in dermatology for the treatment of pyoderma, dermatitis, intertrigo, eczema, and burns in solutions of 2% to 5% (Balabanova *et al.*, 2003). Known as Castellani's paint or magenta paint, it has been used topically since it was introduced in the 1920s (Castellani, 1968) to treat skin conditions such as fungal skin lesions (Whitwell, 1968) or infective dermatitis (Shelley, 2004). Carbol-Fuchsin solution, containing less than 1% of CI Basic Red 9, is used to treat postoperative phenol nail procedures and as a dermal first-aid antiseptic drying agent (Biogenex, 2003).

Magenta is reported to be used as a food-irradiation dosimeter in an aqueous solution of 3.13×10^{-5} mol/L (Khan & Naz, 2007) and as a meat-marking colour in New Zealand (Dacre, 1971).

1.3 Occurrence

1.3.1 *Natural occurrence*

Magenta is not known to occur as a natural product.

1.3.2 *Occupational exposure*

The only well-described groups of workers exposed to magenta include those in a dyestuffs-manufacturing plant in Ludwigshafen, Germany (Rehn, 1895), the manufacture of magenta in the British chemical industry (1910–1952) (Case & Pearson, 1954) and the manufacture of "new fuchsin" in an Italian dyestuffs factory (Rubino *et al.*, 1982; Piolatto *et al.*, 1991). No environmental or biological measurements have been reported for these plants or any other plants that have produced or are currently producing magenta.

Production of magenta may expose workers to process chemicals (e.g., aniline, *ortho*- and *para*-toluidine, and – historically – arsenic acid). Exposure to other chemicals used and produced at the same location may also occur (e.g., benzidine, 1-naphthylamine, 2-naphthylamine, auramine, aniline) (Case & Pearson, 1954).

Occupational exposure can also occur during the use of magenta as a dye intermediate and when dyeing textile fibres, fabrics and paper products (IARC, 1993). The levels of exposure to magenta have not been reported for these occupational groups.

Exposure to magenta can also occur in laboratories, where it is widely used as a biological stain (basic fuchsin dye) to stain bacteria and as a component of Schiff's reagent to detect aldehydes. Under laboratory conditions, magenta has also been produced directly on nylon fabric by a fungal strain closely related to *Phoma herbarum* (Chiba *et al.*, 2006).

The US National Exposure Survey (1981–1983) estimates the number of employees potentially exposed to Basic Red 9 as approximately 900, the largest group being laboratory technicians and medical scientists. A total of around 12 700 workers were potentially exposed to Magenta I in six industries (NIOSH, 1990).

1.3.3 *Exposure of the general population*

Magenta may be present in the waste effluents from plants where it is produced or used. Concentrations of magenta in water or soil have not been reported. Wastewater containing dyes from the textile industry is difficult to treat using conventional methods, because the dyes are stable to light and oxidizing agents, and are resistant to aerobic digestion (Rai *et al.*, 2007). Various methods for the degradation of magenta in wastewater have been developed (Li *et al.*, 1999; Yiyun & Jiepin, 2005).

1.4 Regulations and guidelines

1.4.1 *Magenta*

(a) Germany

The MAK Commission listed Magenta (basic fuchsin hydrochloride) as a substance being examined for the establishment of MAK values based on its carcinogenic effects (MAK, 2007).

(b) Japan

The Japan Society for Occupational Health (2007) follows the classification by IARC of Magenta (containing C.I. Basic Red 9) in Group 2B.

1.4.2 *Basic Red 9 (Magenta 0)*

(a) Europe

(i) *Commission Directive 2000/32/EC*

Commission Directive 2000/32/EC of 19 May 2000 adapts to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (European Commission, 2000). 4,4'-(4-iminocyclohexa-2,5-dienylidenemethylene)dianiline hydrochloride (C.I. Basic Red 9) is listed in Annex I to Directive 67/548/EEC, which contains a list of dangerous substances, together with details on the classification and labelling of each substance.

(ii) *Directive 2004/37/EC*

C.I. Basic Red 9 is regulated by Directive 2004/37/EC (European Commission, 2004), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 or 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(b) Japan

The Japan Society for Occupational Health (2007) follows the classification by IARC of C.I. Basic Red 9 in Group 2B.

(c) USA

C.I. Basic Red 9 monohydrochloride is listed in the NTP *Report on Carcinogens* as *reasonably anticipated to be a human carcinogen* (NTP, 2005).

2. Studies of Cancer in Humans

2.1 Case report

Rehn (1895) was the first to report the appearance of bladder tumours in three of 45 workers involved in the manufacture of fuchsin in one factory in Germany. At the time, this process involved heating a mixture of chemicals, including toluidine, aniline

and nitrobenzene to obtain crude fuchsin, which was then purified and crystallized. The three workers had been employed for 15–29 years. One worker developed a "fibroma papillare", another an oedematous papilloma, and the third a carcinoma of the bladder.

2.2 Cohort studies

Case & Pearson (1954) surveyed workers employed for at least six months between 1910 and 1952 in the manufacture of magenta in the British chemical industry. Workers who had been exposed to benzidine or 1- or 2-naphthylamine were excluded. Bladder-cancer occurrence was determined from factory and hospital records. Deaths were identified from alphabetical lists of death certificates, and the numbers were compared with mortality rates for England and Wales for the period 1921–1952. Among 85 magenta production workers known to have been in contact with magenta, but not exposed to auramine, 1- or 2-naphthylamine or benzidine, there were five cases of bladder cancer, with exposure duration ranging from 1–19 years. Three of these cases were mentioned on death certificates, whereas only 0.13 would have been expected for the whole male population in England and Wales (SMR 23.1; $P < 0.005$). One case of bladder cancer was observed among nine subjects who had been exposed to both magenta and auramine, but no death was seen from this cause (0.02 expected). [The Working Group noted that during the manufacture of magenta exposure to other aromatic amines cannot be ruled out.]

Rubino *et al.* (1982) studied case-specific mortality of 53 male workers who had been employed for ≥ 1 month in the manufacture of 'new fuchsin' (Magenta III, Basic Violet 2, CI No. 42520) and Safranine T (Basic Red 2, CI No. 50240) during the period 1922–1970 in a factory in the province of Torino, Italy. Workers engaged in the manufacture and use of 1- or 2-naphthylamine or benzidine were excluded from the study. Subjects and their work histories were identified from factory personnel records, and the workers were followed for mortality from 1946 to 1976, as identified from factory records and from the municipal registries of their current residence. Among the 53 workers exposed, five deaths from bladder cancer were observed, while 0.08 were expected on the basis of mortality rates for Italy in 1951–76 (SMR 62.5; $P < 0.001$). The cases had been occupationally exposed to Magenta III and Safranine T for 12–40 years. The exposures also involved *ortho*-toluidine and 4,4'-methylenebis(2-methylaniline). The same cohort of workers was followed to the end of 1981 (Decarli *et al.*, 1985) and further until 1989 (Piolatto *et al.*, 1991). No additional deaths from bladder cancer were reported.

2.3 Case–control studies

A total of 512 male cases of bladder cancer and 596 hospital-based controls were studied for occupation and bladder-cancer risk during 1978–1983 in the province of Torino, Italy (Vineis & Magnani, 1985). Complete occupational histories and related information were obtained via hospital interviews. Exposures to specific chemicals,

including magenta, were estimated from ILO occupation and industry titles, using information on the industrial uses of these chemicals as described in published sources. On the basis of industrial branches in which magenta exposure could have occurred, 41 cases were classified as having been exposed before the age of 60, to give a relative risk of 1.8 (95% confidence interval, 1.1–2.9). On the basis of job titles in which exposure to magenta could have occurred, two cases were classified as having been exposed before the age of 60, with an associated relative risk of 3.0 (95% confidence interval, 0.4–20.0).

3. Studies of Cancer in Experimental Animals

3.1 Magenta

3.1.1 *Oral administration*

(a) Mouse

A group of sixty stock mice (30 males, 30 females; strain and age unspecified) received 12 mg magenta (BDH; purity not specified) in arachis oil by gastric instillation once per week for 52 weeks (total dose, 624 mg), and were kept for their life-span. Eighteen mice were used as controls. Four (20%) lymphomas and one (5%) hepatoma were found in 20 surviving treated animals, and five (25%) lymphomas were found in 18 controls (Bonser *et al.* 1956).

(b) Rat

A group of eighty Sprague Dawley rats (40 males, 40 females), 12 weeks of age, were treated by intragastric instillation with magenta (purity unspecified) dissolved in saline, twice weekly for life. Initial dose was 400 mg/kg bw per week, but due to severe toxicity the treatment had to be interrupted and continued at half the original dose level. A control group of similar size received the solvent only. Tumour incidences reported were 5% for the magenta-treated rats and 40% for the controls (males and females combined) (Ketkar & Mohr 1982).

(c) Hamster

Syrian golden hamsters (40 males, 40 females), 12 weeks of age, were treated by intragastric instillation with magenta dissolved in saline, twice weekly for life. Dose levels were 400 and 600 mg/kg bw. The 600-mg/kg bw dose was not tolerated by the animals. A control group was given the solvent only. Tumour incidences reported at the low dose were 5% (one nasal cavity fibroma and a bronchiogenic adenoma in two male hamsters; one adrenal cortex adenoma and one submandibular gland adenoma in two females), and 10% for the solvent controls (except for one adrenal cortex adenoma, all

nine other tumours in eight control animals – three males, five females – were seen at sites different from those in the treated hamsters) (Green *et al.*, 1979).

3.2 CI Basic Red 9 (*para*-magenta)

3.2.1 *Oral administration*

(a) Mouse

Groups of 50 male and 50 female B6C3F$_1$ mice, 6–10 weeks of age, were given a diet containing 0, 500 or 1000 mg/kg (ppm) CI Basic Red 9 for 103 weeks and were killed at 110–115 weeks of age. Two lots of the test chemical were used, with purities of 93 and 99% (water was the major impurity). Mean body weights of treated mice were lower than those of controls throughout the study. At the end of the experiment, 42/50 (84%) control; 32/50 (64%) low-dose; 35/50 (70%) high-dose males and 31/50 (62%) control, 12/50 (24%) low-dose, and 6/50 (12%) high-dose females were alive ($P < 0.001$). In male mice, CI Basic Red 9 caused a dose-related increase in the incidence of hepatocellular carcinomas (10/50 (20%) control; 20/50 (40%) low-dose; 27/50 (54%) high-dose; $P < 0.001$, incidental tumour trend test). The incidence of hepatocellular adenomas was 22/50 (44%) control, 21/50 (42%) low-dose, and 17/50 (34%) high-dose. The combined incidence of liver tumours was 29/50 (58%) control, 37/50 (74%) low-dose, and 41/50 (82%) high-dose ($P = 0.005$, incidental tumour trend test). In female mice, the compound caused a dose-related increase in the incidence of hepatocellular carcinomas (3/49 (6.1%) control; 19/50 (38%) low-dose; 37/49 (75%) high-dose; $P < 0.001$, Cochran-Armitage trend test). The incidence of hepatocellular adenomas was 2/49 (4%) control, 18/50 (36%) low-dose and 4/49 (8%) high-dose ($P < 0.001$, Fischer exact test). The combined incidence of liver tumours in females was 5/49 (10%) control, 35/50 (70%) low-dose, and 41/49 (84%) high-dose ($P < 0.001$, Cochran-Armitage trend test). An increase in the incidence of benign and malignant adrenal phaeochromocytomas (combined) was found in females (1/48 (2%) control; 8/47 (17%) low-dose; 8/45 (18%) high-dose; $P = 0.015$, Cochran-Armitage trend test) (NTP, 1986).

(b) Rat

Groups of 40 male and 40 female Sprague Dawley rats, 12 weeks of age, were treated intragastrically twice a week with 0 or 600 mg/kg bw CI Basic Red 9 [purity unspecified] in 0.9% saline. The dose of 600 mg/kg was found to be toxic and, after 12 weeks, treatment was discontinued for one week; after a further six weeks, half of the original dose (300 mg/kg bw) was used for the remaining treatment, for life. Average survival times were 104 weeks for control males, 70 weeks for treated males, 92 weeks for control females and 69 weeks for treated females. No treatment-related increase in the incidence of tumours was observed in rats of either sex (Ketkar & Mohr, 1982). [The Working Group noted the poor survival in the treated groups and the inadequate reporting of the study.]

Groups of 50 male and 50 female Fischer 344/N rats, 6–7 weeks of age, were given a diet containing 0, 1000 or 2000 mg/kg (ppm) (males) and 0, 500 or 1000 ppm (females) CI Basic Red 9 for 103 weeks and were killed at 110–113 weeks of age. Two lots of the test chemical were used, with purities of 93 and 99% (water was the major impurity). Increased mortality was seen in high-dose males and females, and at the end of the experiment, 36/50 (72%) control, 29/50 (58%) low-dose and 0/50 high-dose males and 37/50 (74%) control, 35/50 (70%) low-dose, and 14/50 (28%) high-dose females were still alive. CI Basic Red 9 caused significant increases in the incidences of benign and malignant tumours at various sites in both males and females (Table 3.1) (NTP, 1986).

Table 3.1. Trends in tumour incidences at specific sites in Fischer 344/N rats fed diets containing CI Basic Red 9

Tumour site and type	Control	Low-dose	High-dose	*P* (trend)[a]
Male				
Dose (mg/kg diet)	0	1000	2000	
Skin				
Squamous-cell carcinoma	0/50 -	1/50 (2%)	10/50 (20%)	< 0.001
Trichoepithelioma	0/50 -	0/50 -	7/50 (14%)	= 0.001
Sebaceous adenoma	0/50 -	0/50 -	5/50 (10%)	= 0.006
Subcutis				
Fibroma	2/50 (4%)	20/50 (40%)	16/50 (32%)	< 0.001
Zymbal gland				
Carcinoma	1/50 (2%)	1/50 (2%)	13/50 (26%)	< 0.001
Thyroid gland				
Follicular adenoma	0/49 -	0/46	9/44 (20%)	< 0.001
Follicular carcinoma	0/49 -	5/46 (11%)	18/44 (41%)	< 0.001
Combined	0/49 -	5/46 (11%)	25/44 (57%)	< 0.001
Liver				
Hepatocellular neoplastic nodule	5/50 (10%)	14/50 (28%)	6/50 (12%)	= 0.447
Hepatocellular carcinoma	0/50 -	2/50 (4%)	8/50 (16%)	= 0.001
Combined	5/50 (10%)	15/50 (30%)	14/50 (28%)	= 0.021
Females				
Dose (mg/kg diet)	0	500	1000	
Subcutis				
Fibroma	0/50 -	15/50 (30%)	10/50 (20%)	= 0.005
Zymbal gland				
Carcinoma	0/50 -	2/50 (4%)	7/50 (14%)	= 0.003
Thyroid				
Follicular adenoma	0/47 -	0/48 -	4/50 (8%)	= 0.017
Follicular carcinoma	0/47 -	2/48 (4%)	2/50 (4%)	> 0.05
Combined	0/47 -	2/48 (4%)	6/50 (12%)	= 0.009

From NTP (1986)
[a] Cochran-Armitage trend test

(c) Hamster

Syrian golden hamsters (40 males, 40 females), 12 weeks of age, were treated by intragastric instillation with CI Basic Red 9 dissolved in saline, twice weekly for life. Dose levels were 300 and 600 mg/kg bw. The high dose was not tolerated by the animals. Tumour incidences reported at the low dose were 6% (one adrenal cortex adenoma, one tracheal papillary polyp and two intestinal adenocarcinomas, all in one male hamster; one tracheal papillary polyp, one subcutaneous fibrosarcoma and two intestinal adenocarcinomas in four females), and 10% for the solvent controls (except for one adrenal cortex adenoma, all nine other tumours in eight control animals – three males, five females – were seen at sites different from those in the treated hamsters) (Green *et al.*, 1979).

3.2.2 *Subcutaneous administration*

(a) Rat

Twenty BD III rats received 10 mg *para*-magenta as a 1% aqueous solution by subcutaneous injection once per week for 65 weeks (total dose, 650 mg). The first local sarcoma appeared at 10 months (total dose, 370 mg); six more were observed in subsequent months in 12 surviving animals. The spontaneous incidence of sarcomas in these rats was < 0.5% (Druckrey *et al.* 1956). [The Working Group noted the lack of use of controls and the lack of adequate description of the experiment.]

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

No data were available to the Working Group.

4.2 Genetic and related effects

4.2.1 *Magenta* (see Tables 4.1 and 4.2)

[The data reported in the Table are the results of experiments with commercial preparations of magenta; in most cases, the exact composition of the products tested and the degree of purity is not known. In the experiments with Magenta I the test compound seems to be better identified, but also in this case its degree of purity is not reported.]

Magenta came out negative in the prophage-induction test, both in the absence and the presence of metabolic activation (Speck *et al.*, 1978). It was mutagenic in *Salmonella typhimurium* strains TA98, TA100, and TA1535, but only in the presence of metabolic activation. The effect was observed with TA98 (Mortelmans *et al.*, 1986), TA100

Table 4.1. Genetic and related effects of Magenta and Magenta 1

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Magenta				
Prophage induction/SOS/strand breaks/x-links	–	–	500 µg/ml	Speck *et al.* (1978)
S. typhimurium TA100, reverse mutation	–	+	50 µg/plate	Mortelmans *et al.* (1986)
S. typhimurium TA100, reverse mutation	–	+	167 µg/plate	Dunkel *et al.* (1984)
S. typhimurium TA100, reverse mutation	NT	+	5 µg/plate	Yamaguchi (1988)
S. typhimurium TA100, TA98, reverse mutation	–	–	1000/5000 µg/plate	Japan Chemical Industry Ecology (JETOC) (1996)
S. typhimurium TA1535, TA1538, reverse mutation	–	–	125 µg/plate	Rosenkranz & Poirier (1979)
S. typhimurium TA1535, reverse mutation	–	+	17 µg/plate	Mortelmans *et al.* (1986)
S. typhimurium TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	167 µg/plate	Dunkel *et al.* (1984)
S. typhimurium TA1535, TA1537, TA1538, reverse mutation	–	–	200/5000 µg/plate	Japan Chemical Industry Ecology (JETOC) (1996)
S. typhimurium TA1537, reverse mutation	–	–	17 µg/plate	Mortelmans *et al.* (1986)
S. typhimurium TA98, reverse mutation	–	+	166 µg/plate	Mortelmans *et al.* (1986)

Table 4.1 (contd)

Test system	Result[a]		Dose (HID/LED)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
E. coli WP2 *uvrA*, reverse mutation	–	–	166 µg/plate	Dunkel *et al.* (1984)
E. coli WP2 *uvrA*, reverse mutation	–	–	1000/5000 µg/plate	Japan Chemical Industry Ecology (JETOC) (1996)
S. cerevisiae, homozygosis	–	–	300 µg/ml	Simmon (1979b)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	1 µg/ml	Williams *et al.* (1982)
Cell transformation, SHE, clonal assay	–	NT	1 µg/ml	Pienta *et al.* (1977)
Magenta 1				
S. typhimurium TA100, reverse mutation	–	+	17 µg/ml	Mortelmans *et al.* (1986)
S. typhimurium TA1535, TA1537, reverse mutation	–	–	16.5 µg/plate	Mortelmans *et al.* (1986)
S. typhimurium TA98, reverse mutation	–	+	166 µg/plate	Mortelmans *et al.* (1986)

[a] +, positive; –, negative
HID, highest ineffective dose; LED, lowest effective dose; NT, not tested

Table 4.2. Genetic and related effects of CI Basic Red 9 (para-rosaniline)

Test system	Result[a]		Dose (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Prophage induction/SOS repair test, DNA strand breaks, cross-links or related damage	–	NT	500 µg/ml	Speck *et al.* (1978)
Escherichia coli pol A^+/pol A^-W3110-P3478 differential toxicity (liquid suspension)	+	NT	20 µg/ml	Rosenkranz & Poirier (1979)
Escherichia coli WP2/WP67/CM871, differential toxicity	+	–	155 µg/ml	De Flora *et al.* (1984a)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, TA1586, reverse mutation	–	–	250 µg/plate	Simmon (1979a)
Salmonella typhimurium TA100, T1535, TA1537, reverse mutation	–	–	1000 µg/plate	Bonin *et al.* (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1070 µg/plate	De Flora (1981)
Salmonella typhimurium TA100, reverse mutation	–	+	167 µg/plate	Dunkel *et al.* (1984)
Salmonella typhimurium TA100, reverse mutation	–	+	17 µg/plate	Mortelmans *et al.* (1986)
Salmonella typhimurium TA102, reverse mutation	–	–	4 µg/ml	De Flora *et al.* (1984b)
Salmonella typhimurium TA1535, TA1538, reverse mutation	–	–	125 µg/plate	Rosenkranz & Poirier (1979)
Salmonella typhimurium TA1535, TA1537, TA1538, reverse mutation	–	–	167 µg/plate	Dunkel *et al.* (1984)

Table 4.2 (contd)

Test system	Result[a]		Dose (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1535, reverse mutation	–	(+)	500 µg/plate	Mortelmans *et al.* (1986)
Salmonella typhimurium TA1537, reverse mutation	–	–	167 µg/plate	Mortelmans *et al.* (1986)
Salmonella typhimurium TA1538, reverse mutation	–	+	1000/320 µg/plate	Bonin *et al.* (1981)
Salmonella typhimurium TA98, reverse mutation	–	(+)	167 µg/plate	Dunkel *et al.* (1984)
Salmonella typhimurium TA98, reverse mutation	NT	+	0.2 µg/ml	Arni *et al.* (1985)
Salmonella typhimurium TA98, reverse mutation	–	+	50 µg/plate	Mortelmans *et al.* (1986)
Salmonella typhimurium TA97, reverse mutation	–	(+)	1600 µg/plate	De Flora *et al.*(1984b)
Escherichia coli exclusive of strain K12, forward mutation	+	(+)	5000 µg/plate	Hayes *et al.* (1984)
Escherichia coli WP2 *uvrA*, reverse mutation	–	–	167 µg/plate	Dunkel *et al.* (1984)
Saccharomyces cerevisiae, homozygosis by mitotic recombination	–	–	300 µg/plate	Simmon (1979b)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	1 µg/ml	Williams *et al.* (1982)
Unscheduled DNA synthesis, rat primary hepatocytes	+	NT	2.2 µg/ml	NTP (1986)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	3.24 µg/ml	Kornbrust & Barfknecht (1984)

Table 4.2 (contd)

Test system	Result[a]		Dose (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Unscheduled DNA synthesis, Syrian hamster primary hepatocytes	+	NT	3.24 µg/ml	Kornbrust & Barfknecht (1984)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus	+	+	1 µg/ml	Mitchell *et al.* (1988)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus	?	–	7.5/100 µg/ml	Myhr & Caspary (1988)
Gene mutation, Chinese hamster CHL/IU cell line with *gpt* shuttle vector	–	NT	20 µg/ml	Yamada *et al.* (2000)
Sister chromatid exchange, Chinese hamster cells *in vitro*	–	–	15 µg/ml	Anderson *et al.* (1990)
Cromosomal aberrations, Chinese hamster cells *in vitro*	–	–	50 µg/ml	Anderson *et al.* (1990)
Cromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	0.29 µg/ml	Hagiwara *et al.* (2006)
Cromosomal aberrations, Chinese hamster cells CHL/IU *in vitro*	–	NT	20 µg/ml	Yamada *et al.* (2000)
Cell transformation, BALB/c3T3 mouse cells	+	NT	0.04 µg/ml	Dunkel et al. (1981)
Cell transformation, Syrian hamster embryo cells, clonal assay	–	NT	1 µg/ml	Pienta *et al.* (1977)

Table 4.2 (contd)

Test system	Result[a]		Dose (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cell transformation, Syrian hamster embryo cells, clonal assay	–	+	2 μg/ml	Pienta & Kawalek (1981)
Cell transformation, RLV/Fischer rat embryo cells	+	NT	1.4 μg/ml	Dunkel et al. (1981)
Urine from mouse and rat, microbial mutagenicity	+	+	120 mg/kg x 2 po	Lawlor *et al.* (1987)
Host-mediated assay, *Salmonella typhimurium* in mice	–		1600 mg/kg x 1 po	Simmon *et al.* (1979)
Host-mediated assay, *Salmonella typhimurium* in mice	–		1600 mg/kg x 1 im	Simmon *et al.* (1979)
Host-mediated assay, *Saccharomyces cerevisiae* in mice	–		1600 mg/kg x 1 po	Simmon *et al.* (1979)

[a] +, positive; (+), weakly positive; –, negative; ?, inconclusive (variable response in several experiments within an adequate study)
HID, highest ineffective dose; LED, lowest effective dose; NT, not tested

(Dunkel *et al.*, 1984; Mortelmans *et al.*, 1986; Yamaguchi, 1988), and TA1535 (Mortelmans *et al.*, 1986). Magenta was negative with TA1537 (Dunkel *et al.*, 1984; Mortelmans *et al.*, 1986; JETOC, 1996), TA98 and TA100 (JETOC, 1996) and TA1538 (Rosenkranz and Poirier, 1979; Dunkel *et al.*, 1984; JETOC, 1996). In both the absence and the presence of metabolic activation, it did not induce reverse mutations in *Escherichia coli* (Dunkel *et al.*, 1984; JETOC, 1996) or homozygosis with *Saccharomyces cerevisiae* (Simmon, 1979b). In cultured, non-human mammalian cells, magenta did not induce unscheduled DNA synthesis in primary rat hepatocytes (Williams *et al.*, 1982) and, tested only in the absence of metabolic activation, failed to induce morphological transformation in Syrian hamster embryo cells (Pienta *et al.*, 1977). Mutagenic activity of Magenta I in bacteria was similar to that of magenta; it showed a positive result, but only in the presence of metabolic activation, in *Salmonella typhimurium* TA98 and TA100, and gave a negative response in TA1535 and TA1537 (Mortelmans *et al.*, 1986).

These data indicate that in the presence of an exogenous system of activation, both magenta and Magenta I are mutagenic in bacteria.

4.2.2 *CI Basic Red 9 (*para-*rosaniline)* (see Table 4.2)

[In the large majority of the studies reported in the Table, information on the degree of purity of CI Direct Red 9, a common constituent of magenta, is lacking; commercial products were used.]

In the absence of metabolic activation, CI Basic Red 9 came out negative in the prophage-induction test (Speck *et al.*, 1978), but induced repairable DNA damage in two *E. coli* differential toxicity assays (Rosenkranz & Poirier, 1979; De Flora *et al.*, 1984a). It was more often non-mutagenic in *Salmonella typhimurium* (Simmon 1979a; Bonin *et al.*, 1981; De Flora, 1981). A positive response was obtained only in the presence of metabolic activation with TA98 (Dunkel *et al.*, 1984; Arni *et al.*, 1985; Mortelmans *et al.*, 1986), with TA100 (Dunkel *et al.*, 1984; Mortelmans *et al.*, 1986), with TA1535 (Mortelmans *et al.*, 1986), with TA1538 (Bonin *et al.*, 1981), and with TA97 (De Flora *et al.*, 1984b). The response was negative with TA1537 (Mortelmans *et al.*, 1986), with TA102 (De Flora *et al.*, 1984b) and TA1586 (Simmon, 1979a). CI Basic Red 9 induced forward mutation in *Escherichia coli* in both the absence and the presence of metabolic activation, but not reverse mutation (Hayes *et al.*, 1984). In a study with *Saccharomyces cereviasiae*, CI Basic Red 9 did not induce homozygosis by mitotic recombination (Simmon, 1979b).

In cultured, non-human mammalian cells, both positive and negative results were obtained. CI Basic Red 9 induced unscheduled DNA synthesis in Syrian hamster hepatocytes (Kornbrust & Barfknecht, 1984) and in one study with rat primary hepatocytes (NTP, 1986) and was negative in two other studies in rats (Williams *et al.*, 1982; Kornbrust & Barfknecht, 1984). Mutation at the thymidine kinase (*Tk*) locus in mouse lymphoma cells was induced in both the absence and the presence of metabolic

activation in only one of two studies (Mitchell *et al.*, 1988), and a negative result was obtained in a different gene-mutation assay (Myhr & Caspary, 1988; Yamada *et al.*, 2000). Similarly, chromosomal aberrations were induced in one study (Hagiwara *et al.*, 2006), but not in two others (Anderson *et al.*, 1990; Yamada *et al.*, 2000), and sister chromatid exchange was not observed (Anderson *et al.*, 1990). Variable responses were obtained in four assays for cell transformation: two positive (Dunkel *et al.*, 1981) and two negative (Pienta *et al.*, 1977; Pienta & Kawalek, 1981) results in the absence of metabolic activation, and one positive result in the presence of an exogenous metabolic system (Pienta & Kawalek, 1981). Oral administration of CI Basic Red 9 to mice or rats resulted in urine that was mutagenic to bacteria (Lawlor *et al.*, 1987), but in a host-mediated assay the dye did not induce mutation in *Salmonella typhimurium* recovered from the peritoneal cavity of mice (Simmon *et al.*, 1979).

The inconsistency in the genotoxicity data could be attributed to the different grades of purity of the material employed.

5. Summary of Data Reported

5.1 Exposure data

Historically, the name magenta has been used to refer to a mixture of the four major constituents comprising Basic Fuchsin, namely Basic Red 9, Magenta I, Magenta II and Magenta III. Today all except Magenta II are available commercially under their own names. Magenta I and Basic Red 9 are the most widely available.

Magenta has not been reported to occur as such in nature. Under the name of Basic Violet 14 (Magenta I), magenta is used in hair dyes and in cosmetic products not intended to come in contact with mucous membranes. Basic Red 9 is used as colourant in artist's paints.

Magenta has antiseptic properties; it has been used in dermatology since the 1920s under the name of Castellani's paint or magenta paint. Carbol-Fuchsin solution, which contains Basic Red 9, is used topically as a first-aid antiseptic drying agent. It is also widely used in laboratories as a biological stain.

Magenta is used as a food-irradiation dosimeter and was used as a meat-marking colour in New Zealand.

Occupational exposure to magenta can occur during its production, during its use as a dye intermediate, when dyeing textile fibres, fabrics and paper products, and in laboratories.

Production of magenta may involve exposure to process chemicals such as aniline, *ortho*- and *para*-toluidine, and – historically – arsenic acid. Exposure to other chemicals used and produced at the same location may also occur (e.g., benzidine, 1-naphthylamine, 2-naphthylamine, auramine, aniline).

Magenta may be present in the waste effluents from plants where it is produced or used.

5.2 Human carcinogenicity data

One case report from Germany and two small cohort studies from the United Kingdom and Italy have reported an increased risk for bladder cancer among workers engaged in the manufacture of magenta. These cohort studies excluded workers exposed to benzidine and β-naphthylamine. One case–control study from Italy has shown an increased risk for bladder cancer associated with occupational exposure to magenta. Taken together, these studies indicate that excess bladder cancer risks are caused by the production of magenta, but co-exposures preclude a similar evaluation for magenta itself.

5.3 Animal carcinogenicity data

No adequate study was available to evaluate the carcinogenicity of magenta in experimental animals.

CI Basic Red 9 (Magenta 0) was tested for carcinogenicity in one study in mice and in one study in rats by oral administration in the diet, and in one study in rats by subcutaneous administration. After oral administration, the compound induced hepatocellular carcinomas in male and female mice and in male rats; adrenal gland phaeochromocytomas in female mice; benign and malignant skin tumours in male rats; and subcutaneous fibromas, thyroid gland follicular-cell tumours, and Zymbal gland carcinomas in male and female rats. Subcutaneous administration to rats resulted in a high incidence of local sarcomas. A study in hamsters was found inadequate for evaluation.

5.4 Other relevant data

There are no data on the toxicokinetics of magenta or CI Basic Red 9.

Few data are available on the mutagenicity and genotoxicity of magenta and Magenta I. Both were mutagenic in *S. typhimurium* TA100 and TA 98 (magenta in two out of four experiments) in the presence of an exogenous metabolic activation system, but they were inactive in all other strains tested. Magenta did not induce homozygosis in *S. cerevisiae*, unscheduled DNA synthesis (UDS) in primary hepatocytes or cell transformation of SHE cells.

The data for CI Basic Red 9 are also inconsistent. In the mutagenicity tests in *Salmonella*, it was always negative in the absence of an exogenous system of activation, while in the presence of bioactivation, the results were clearly positive only in strain TA100 (two out of three experiments). Similar conflicting results were obtained in different tests in *E. coli*.

In mammalian cells *in vitro*, CI Basic Red 9 was positive for UDS induction in one of three experiments in rat hepatocytes and in one study with hamster hepatocytes. In a

recent test for chromosomal aberrations in Syrian hamster embryo cells, the compound was positive, in the absence of activation, at a very low dose (0.29 microg/mL), while it was negative for the same test in two different lines of Chinese hamster cells. Similar conflicting results were obtained in other tests *in vitro*.

Mutagenicity was detected, both in the absence and in the presence of metabolic activation, in the urine of mice and rats fed with a diet containing CI Basic Red 9. However, the host-mediated assay with *Salmonella* and *S. cerevisiae* in mice, in the absence of a system of activation, was consistently negative.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of magenta production. Magenta production causes bladder cancer in humans.

There is *inadequate evidence* in humans for the carcinogenicity of magenta.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of CI Basic Red 9 (para-magenta).

There is *inadequate evidence* in experimental animals for the carcinogenicity of magenta.

6.3 Overall evaluation

Magenta production is *carcinogenic to humans (Group 1)*.

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4,4'-METHYLENEBIS(2-CHLOROANILINE)

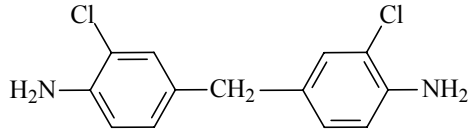
1. Exposure Data

1.1 Chemical and Physical Data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 101-14-4
CAS Name: 4,4'-Methylenebis(2-chlorobenzenamine)
Synonyms: Bis(4-amino-3-chlorophenyl)methane; bis(3-chloro-4-aminophenyl)methane; 4,4'-diamino-3,3′-dichlorodiphenylmethane; di(4-amino-3-chlorophenyl)methane; di(3-chloro-4-aminophenyl)methane; 3,3′-dichloro-4,4'-diaminodiphenylmethane; 2,2'-dichloro-4,4'-methylenedianiline; 4,4'-methylenebis(2-chloroaniline); 4,4'-methylenebis(*ortho*-chloroaniline); methylenebis(chloraniline); methylenebis(*ortho*-chloroaniline); methylenebis(3-chloro-4-aminobenzene); MOCA; MBOCA

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



$C_{13}H_{12}Cl_2N_2$ Rel. mol. mass: 267.15

1.1.3 *Chemical and physical properties of the pure substance (OSHA, 2009)*

Description: Colourless to yellow or light brown crystalline solid with a faint amine-like odor
Melting-point: 110 °C

Solubility: Slightly soluble in water; soluble in diluted acids, diethyl ether, benzene, ethanol, and soluble to varying degrees in most organic solvents

1.1.4 *Technical products and impurities*

Pure 4,4'-methylenebis(2-chloroaniline) (MOCA) is a colourless crystalline solid. Historically, the technical grade of MOCA that is available in the United States came mainly from Japan in the form of tan/yellow fused prills or pastilles. The diamine purity is 99.8%, typically with 0.2% free *ortho*-chloroaniline (monomer) (ATSDR, 1994).

Trade names for 4,4'-methylenebis(2-chloroaniline) include: Bisamine A; Bisamine S; CPP 100; Cuamine M; Cuamine MT; Curene 442; Diamet Kh; Ihara Cuamine MT; Isocross SM; Millionate M; Pandex E; Pandex M 3202; Quodorole; SL 4037.

1.1.5 *Analysis*

Analyses of 4,4'-methylenebis(2-chloroaniline) were first reported in the 1970s. Two interesting recent studies have involved the use of gas chromatography/mass spectrometry to analyse water samples for the presence of 20 carcinogenic amines, and high-performance liquid chromatography in conjunction with UV detection to determine amine levels in extracts from toys, at ppm–ppb (10^{-6}–10^{-9}) concentrations (Garrigós *et al*., 2002; Doherty, 2005). Table 1.1 presents selected recent studies of the analysis of 4,4'-methylenebis(2-chloroaniline) in various matrices.

1.2 Production and use

1.2.1 *Production*

MOCA is produced commercially by reacting formaldehyde with *ortho*-chloroaniline. By-products such as trimers and tetramers—diamines with three- and four-ring structures joined by methylene groups—constitute up to 8–10% of commercial MOCA. 4,4'-Methylenebis(2-chloroaniline) comprises up to 90–92% of the commercial MOCA produced for coatings and cast polyurethanes. There is no commercial use for pure 4,4'-methylenebis(2-chloroaniline) other than for laboratory work (ATSDR, 1994).

Reports in early 1983 indicated that US manufacturers no longer produced MOCA and that any MOCA used in the USA is imported (NTP, 2005). Since the production of MOCA in the US ceased in 1982, the amount of MOCA imported into the US has increased from 1.5 million pounds in 1983 to approximately 2.0 million pounds in 1991. Most of the MOCA used in the US is manufactured in Taiwan, China, where the annual production is about 5000 to 10 000 tonnes (Chen *et al.*, 2005).

Although production of MOCA ceased in the United Kingdom in 1987, the amount imported has increased from 90–120 tonnes in 1995 to more than 200 tonnes in 2006 (Cocker *et al.*, 2009).

Table 1.1. Selected methods of analysis of 4,4'-Methylene-bis(2-chloroaniline) (MOCA) in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Urine	Ion-paired solid-phase extraction on a disposable octadecylsilica column with an acidic methanol solution containing 1-heptane-sulfonic acid	HPLC-ECD	1 µg/L	Okayama *et al.* (1988)
Toy products	Supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), or Soxhlet extraction with methanol	HPLC-UV	< 0.2 µg/g	Garrigós *et al.* (2002)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/dichloromethane (50:50).	GC-MS	5 ng/mL	Doherty (2005)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *t*-butylmethyl ether; concentrate, and dilute with methanol	LC-MS/MS	14.1 µg/L	Sutthivaiyakit *et al.* (2005)

ECD, electro-chemical detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; UV, ultraviolet

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the aggregate production volumes that were reported for MOCA.

Available information indicates that 4,4'-methylenebis(2-chloroaniline) was produced and/or supplied in the following countries: Germany; Hong Kong Special Administrative Region; Japan; the People's Republic of China; South Africa; Switzerland; Taiwan, China; and the USA (Chemical Sources International, 2008).

Table 1.2. 4,4'-methylenebis(2-chloroaniline) (MOCA) production volumes

Year	Volume (in millions of pounds)
1986	>1–10
1990	>1–10
1994	>1–10
1998	>1–10
2002	>1–10
2006	0.5–<1

1.2.2 *Use*

4,4'-Methylenebis(2-chloroaniline) (MOCA) is an aromatic amine used for curing epoxy resins. It is mixed with diisocyanate-based pre-polymer resins to produce tough, resistant polyurethane products (Cocker *et al.*, 2009). The polyurethane prepolymers are used in the manufacture of castable urethane rubber products such as shock-absorption pads and conveyor belting (IARC, 1993). In the laboratory, MOCA is used as a model compound for studying carcinogens (NTP, 2005; O'Neil, 2006).

1.3 Occurrence and exposure

1.3.1 *Natural occurrence*

MOCA is not known to occur as a natural product.

In MOCA-contaminated soil, the compound is rapidly adsorbed to the soil matrix and probably exists largely in a covalently bound state (Voorman & Penner, 1986).

1.3.2 *Occupational exposure*

Occupational exposure to MOCA can occur during its production and during its use in the polyurethane industry. Workers can be exposed to MOCA in the form of a liquid emulsion, solid pellets with dust, or solid pellets without dust. In most cases, dermal absorption after contact with contaminated surfaces is the most important occupational exposure route, with inhalation and ingestion representing minor routes of exposure (IARC, 1993).

According to Rappaport and Morales (1979), in 1972 some 10 000 persons in industrialized countries were exposed occupationally to MOCA in the context of manufacturing and processing (Will *et al.*, 1981).

The Health and Safety Executive (HSE) in the United Kingdom estimates that in 2005–2006, 300 workers in the United Kingdom were directly exposed to MOCA during polyurethane-elastomer production, and over 1000 workers such as office staff were indirectly exposed (HSE, 2007a).

Estimates of the number of workers in the USA potentially exposed to MOCA in 1977 ranged from 2100 to 33 000. In 1979 an estimated 1400 workers in the USA were directly exposed and 7400 indirectly exposed while working in polyurethane manufacturing processes involving MOCA (Ward *et al.*, 1987). In 1982, the EPA estimated that 1400–2720 workers were directly exposed and 7600–15200 were indirectly exposed.

(a) Exposure measurements

Concentrations of MOCA in air, blood and urine and in surface-wipe samples have been reported for workers employed in the production and use of MOCA from a range of countries, including Australia, France, Germany, Japan, Taiwan (China), the United Kingdom, and the USA. Different analytical methods have been applied, which can complicate comparisons of reported MOCA levels (see Section 1.1.5).

Monitoring of airborne MOCA alone is considered ineffective in the assessment of worker exposure (Robert *et al.*, 1999), and post-shift urine measurement is the most-employed method to assess exposure. Concentrations of MOCA in urine reflect recent exposure, since the biological half-life of this compound is approximately 23 hours (Osorio *et al.*, 1990).

Some studies have determined urinary concentrations of acetyl-MOCA in addition to MOCA, showing that *N*-acetyl-MOCA is a minor urinary metabolite compared with the elimination of the parent amine (Cocker *et al.*, 1988; Shih *et al.*, 2007).

An alternative to measuring MOCA in urine is to determine the concentrations of haemoglobin adducts of MOCA in blood. These adducts are stable for the lifespan of haemoglobin, which in humans is about 120 days (Vaughan & Kenyon, 1996).

(b) MOCA-production workers

Air concentrations of MOCA have been reported from two MOCA-production plants. In a study from the USA (Linch *et al.*, 1971), the airborne concentration of MOCA was below the detection limit (< 0.01 mg/m^3). In a study from Taiwan, China (Chen *et al.*, 2005), the highest concentrations in air were measured during the purification of MOCA (0.41 mg/m^3).

Urinary concentrations of MOCA in production workers have been reported from France, Taiwan (China), and the USA; they are summarized in Table 1.3.

In a full-scale commercial MOCA-manufacturing plant in the USA, urinary concentrations as high as 3000 μg/L were reported for the year 1969 (Linch *et al.*, 1971). The use of gloves and protective equipment such as suits and respirators was shown to lower exposure considerably.

During a biological monitoring programme in a French factory that periodically produces MOCA, urinary concentrations were reported to range from < 0.5μg/L up to 1600 μg/L (Ducos *et al.*, 1985).

In workers from a production plant in the USA, urinary concentrations of MOCA were measured several months after production had ceased. The maximum level measured was 50 000 μg/L (Ward *et al.*, 1990). [The Working Group noted that the high exposure levels reported may be due to continued exposure to MOCA after its production had ceased, through workplace and environmental contamination].

In 10 workers from a MOCA-manufacturing plant in Taiwan, China (Liu *et al.*, 2005), urinary concentrations ranged between 268 and 15701 μg/g creatinine (Table 1.3).

In another study from Taiwan, China (Shih *et al.*, 2007), 54 urine samples were collected from workers in three MOCA-manufacturing factories. MOCA and acetyl-MOCA (*N*-acetyl-4,4'-methylenebis(2-chloroaniline)) were measured, with median values of 38.6 and 1.8 ng/mL, respectively. MOCA concentrations correlated significantly with the corresponding acetyl-MOCA concentrations in urine.

(c) Polyurethane production workers

Concentrations of MOCA have been measured in the urine of polyurethane-production workers from Australia, Canada, France, Germany, Japan, the United Kingdom and the USA; results are summarized in Table 1.4.

Five hours after an accidental spill of hot MOCA onto the face of a worker cleaning out a MOCA-delivery line in 1976, the urinary level of the exposed worker was 1400 μg/g creatinine (Hosein & Van Roosmalen, 1978).

MOCA concentrations were measured in 49 urine specimens from MOCA-exposed personnel of a plastics manufacturing and processing plant in Germany (Will *et al.*, 1981). The concentrations ranged between 15–100 μg/L.

During a biological monitoring programme conducted in 1978–1981 in a factory in the United Kingdom that manufactured polyurethane elastomers by use of MOCA pellets, urinary MOCA concentrations were measured before and after introduction of safety

Table 1.3. Urinary levels of MOCA in MOCA production workers

Reference	Country, year of study	Task	No. of samples	Measurement	Concentration of MOCA	
					µg/L	µg/g creatinine
Linch *et al.* (1971)	USA, 1962	MOCA production	1	Max	25 000	
	USA, 1969–70	Operators	4	Range	40–3800	
		Operators and moulders without gloves	14	Mean (SD)	278 (252)	
		Operators and moulders with gloves	14	Mean (SD)	80 (92)	
		Operators with gloves, suit, respirator	3	Mean (SD)	3.8 (1.9)	
Ducos *et al.* (1985)	France, 1982	Before improvements	12	Mean	600	450
	France, 1983	After improvements	11	Mean	62	63
Ward *et al.* (1990)	USA, 1981	Production workers	385	Max	50 000[a]	
Liu *et al.* (2005)	Taiwan, China, 2002	MOCA purification process	10	Mean (range)		5544 (267.9–15701)
Shih *et al.* (2007)	Taiwan, China, NR	3 factories, MOCA	54	Range	1.7–1663	2.5–2267
		3 factories, acetyl-MOCA	54	Range	0.08–93.1	0.16–102.8

[a] several months after production ceased
NR, not reported

measures (ventilation, protective clothing, dry-cleaning scheme for overalls) (Thomas & Wilson, 1984). Concentrations dropped from an average of 50 nmol/mmol creatinine to less than 5 nmol/mmol creatinine.

More than 340 analyses were performed on urine samples from 150 workers from 19 polyurethane factories in France (Ducos *et al.*, 1985). In 17 factories where MOCA was used as solid pellets or in solutions for manufacturing a variety of products by coating, moulding or foaming urethane resins, mean concentrations of excreted MOCA in the urine of exposed workers varied from undetectable to 660 μg/L, with a maximum of 1600 μg/L (1540 μg/g creatinine). Process improvements resulted in a significant reduction in urinary MOCA concentrations, to averages of 20–62 μg/L.

The Michigan Department of Public Health tested urine samples of nine manufacturing workers, and reported MOCA concentrations ranging from 13 to 458 μg/L (mean, 145 μg/L) (Keeslar, 1986).

In a NIOSH study of mixers and moulders in a polyurethane elastomer factory, MOCA urinary concentrations were increased during the week and dropped over the weekend (NIOSH 1986).

Between 1980 and 1983, 3323 urinary samples from 54 companies in the USA were analysed: MOCA concentrations exceeded 50 μg/L in 16.9% of the samples and exceeded 100 μg/L in 9.2% (Ward *et al.*, 1987). In 1985, the urinary concentration exceeded 50 μg/L in 12% of all samples tested. In 1990, 8% of the samples showed concentrations that were still above that level (Lowry & Clapp, 1992).

Urinary MOCA concentrations were measured on a regular basis in seven factories in Australia that used MOCA from 1984 onwards (Wan *et al.*, 1989). The measurements were done in five factories before and after a training programme promoting the safe use of MOCA; concentrations decreased from 29.6 μg/L to 10.4 μg/L.

In a study from Japan, MOCA urinary concentrations were measured in five workers over one week (Ichikawa *et al.*, 1990). The concentrations at the beginning and the end of the workshift were 3.1–81.5 and 2.4–96.6 μg/g creatinine, respectively. The highest concentrations were measured for workers pouring the MOCA mix.

Following the accidental spill of MOCA onto the face of a polyurethane worker, urinary MOCA concentrations reached 1700 ppb [μg/L] 4 hours after the accident (Osorio *et al.*, 1990).

In 1986 a study was conducted at a company in the USA that used large amounts of MOCA in manufacturing polyurethane products (Clapp *et al.*, 1991). Sixty-six percent of the urine samples had detectable levels of MOCA, and the highest concentration measured was 159 μg/L.

Urine samples were obtained from five workers involved in the production of polyurethane elastomers in Australia (Vaughan & Kenyon, 1996). The urine contained MOCA at 4.5–2390 nmol/L. Blood samples contained MOCA at 0.13–17.37 nmol/L.

In another study from Australia (Murray & Edwards, 1999), 12 workers in the manufacture of polyurethane showed a median MOCA urinary concentration of

6.5 μmol/mol creatinine (range, 0.4–48.6 μmol/mol creatinine). MOCA was not detected in the urine of control workers.

In a study from France (Robert *et al.*, 1999), postshift urinary MOCA concentrations were determined in 40 workers from four factories producing polyurethane resin. Workers exposed directly to crystallized MOCA on a daily basis had the highest concentration of MOCA in their urine, with a median value of 84 μg/L (49 μg/g creatinine). Concentrations by job category and trends between 1982 and 1996 were also reported (see Table 1.4).

In a small company manufacturing pliable polyurethane, urinary MOCA concentrations were determined (Fairfax & Porter, 2006). None of the 13 employees had detectable amounts of MOCA except the one who performed the urethane casting (15 μg/L total MOCA in urine). Personal air samples collected from the location where the urethane caster worked contained no detectable amounts of MOCA.

A survey of occupational exposure to MOCA in the polyurethane-elastomer industry in Great Britain conducted in 2005–2006 (HSE, 2007a) included 20 polyurethane elastomer manufacturers and two suppliers of MOCA. Urinary concentrations ranged between 1.3–25.0 μmol/mol creatinine.

(d) Occupational surface contamination

Studies from the United Kingdom and USA reported work-surface contamination in the polyurethane industry by determining MOCA concentrations in surface-wipe samples (Table 1.5).

Data from occupational health and safety inspections from 41 polyurethane-production facilities in the USA were assembled (PEDCo Environmental, 1984) and reported surface contamination at facilities using solid and liquid MOCA.

In 1986 a study was conducted at a company in the USA that manufactured polyurethane products and was a large user of MOCA (Clapp *et al.*, 1991). Wipe sampling indicated moderate contamination of the workplace by MOCA dust, with averages up to 19 μg/100 cm^2. The average MOCA concentration found on skin pads worn on workers' hands was generally less than 10 μg/set, with a high of 25 μg/set.

In a small company manufacturing pliable polyurethane (Fairfax & Porter, 2006), the presence of MOCA on work surfaces was reported. Nine locations had non-detectable amounts of MOCA, including a desktop 15 feet from the urethane-casting area; the door handles of the mould oven, respirator locker, gloves locker and restroom; the top of an oven; the handle of the water dispenser in the breakroom; and the handle of a coffee mug. The top of a metal scale table had the highest amount, at 209.7 μg/m^2.

In an occupational exposure survey conducted in 2005–2006 in the polyurethane-elastomer industry in Great Britain, contamination of various surfaces with MOCA was reported (HSE, 2007a). The amounts detected were similar for most surfaces. Contamination around the hopper was generally above that at the other sites, which was thought to be due to excess spillage of MOCA during hopper filling and failure to clean it up immediately.

Table 1.4. Urinary levels of MOCA in polyurethane production workers

Reference	Country, year of study	Task	Number of samples	Measurement	Concentration of MOCA	
					µg/L	µg/g creatinine[1]
Hosein & Van Roosmalen, 1978	Canada, 1976	5 hours after accidental spill (case report)	1			1400
		23 hours after the spill	1			30
Will *et al.*, 1981	Germany	Plastics manufacturing and processing plant	49	range	< 15–100 (LOD = 15)	
PEDCo Environmental, 1984	USA	Without gloves	14	mean	278	
		With gloves	14	mean	80	
		With gloves, respirators and suits	3	mean	3.8	
Thomas & Wilson, 1984	United Kingdom, 1978	Process workers using pelletized MOCA before improvements	~12–15	mean		50 µmol/mol
	United Kingdom, 1982	After improvements	~12–15	mean		< 5 µmol/mol
Ducos *et al.*, 1985	France, 1982	Blending solid MOCA with polyol before improvements	4	range	75–940	31–510
	France, 1983	Blending solid MOCA with polyol after improvements	3	range	ND–9	ND–11
		17 other factories	NR	max	1600	1400
Keeslar, 1986	USA	Manufacturing workers	9	range	13–458	

Table 1.4 (contd)

Reference	Country, year of study	Task	Number of samples	Measurement	Concentration of MOCA	
					µg/L	µg/g creatinine[1]
NIOSH, 1986	USA	Mixers, week	NR	mean	30	
		Mixer, weekend	NR	mean	8.9	
Ward *et al.*, 1987	USA, 1980–83	54 companies	3323	–	16.9% > 50	
				–	9.2% > 100	
Wan *et al.*, 1989	Australia, 1984–87	Workers from 5 factories before training programme		GM (max day)	29.6 (327.2)	
	Australia, 1987	After training programme		GM (max day)	10.4 (21.3)	
Ichikawa *et al.*, 1990	Japan, NR	Production workers (no respirators or gloves)	4	range	[4–120]	2.4–64.0
		Poured mix (no respirators or gloves)	1	–	[200]	96.6
Osorio *et al.*, 1990	USA	Accidental spill of molten MOCA, 4 hours after	1	–	1700 ppb [µg/L]	
Clapp *et al.*, 1991	USA	1 plant	77	–	6.5% > 50	
		Mixers	10	mean (max)	61.9 (158.9)	
		Moulders	35	mean (max)	14.8 (40.0)	
Lowry & Clapp, 1992	USA, 1985	33 companies	1228	–	12% > 50	
	USA, 1990	38 companies	1441	–	8% > 50	

Table 1.4 (contd)

Reference	Country, year of study	Task	Number of samples	Measurement	Concentration of MOCA	
					µg/L	µg/g creatinine[1]
Vaughan & Kenyon, 1996	Australia	5 manufacturers of polyurethane elastomers		range	4.5–2390 nmol/L	
Murray & Edwards, 1999	Australia, 1998	Polyurethane manufacture workers	12	range		0.4–48.6 µmol/mol
Robert *et al.*, 1999	France	Mixer (n = 6)	17	range	0.5–375	0.5–149
		Moulder (n = 10)	26	range	0.5–58	0.5–22
		Maintenance (n = 6)	17	range	5–570	0.5–456
		Others (n = 13)	38	range	0.5–35	0.5–28
Fairfax & Porter, 2006	USA	Urethane casting	1	–	15	
		Other tasks	NR		ND	
HSE, 2007a	United Kingdom, 2005–06	Casting	3	range		3.3–17.0 µmol/mol
		Moulding	12	range		2.2–25.0 µmol/mol
		All exposed	40	range		1.3–25.0 µmol/mol

ND, not detected; NR, not reported; GM, geometric mean
[1] concentration in µmol/mol creatinine when indicated

Table 1.5. MOCA levels in workplace surface wipe samples

Reference	Country, year	Task	Number of samples	Amount of MOCA (μg/100 cm^2)
				Median
PEDCo Environmental, 1984	USA	Solid MOCA: Storage and manual transfer of solid MOCA to melting operations	37	847
		Solid MOCA: Melting	38	11
		Solid MOCA: Transfer of molten MOCA to mixing operations and mixing	9	1650
		Solid MOCA: Transfer of mixture to moulds and pouring of mould	19	5
		Solid MOCA: Storage and manual transfer to melting operations, after controls	19	25
		Solid MOCA: Melting, after controls	2	369
		Solid MOCA: Transfer of molten MOCA to mixing operations and mixing, after controls	12	8
		Solid MOCA: Transfer of mixture to moulds and pouring of mould, after controls	8	8
		Liquid MOCA: Storage and transfer to mixing, uncontrolled	25	30
		Liquid MOCA: Mixing, uncontrolled	4	50 000
		Liquid MOCA: Transfer to moulding and moulding, uncontrolled	6	4.3
		Liquid MOCA: Storage and transfer to mixing, controlled	19	9
		Liquid MOCA: Mixing, controlled	6	4.4
		Liquid MOCA: Transfer to moulding and moulding, controlled	1	0.25

Table 1.5 (contd)

Reference	Country, year	Task	Number of samples	Amount of MOCA (μg/100 cm^2)
				Mean
Clapp *et al.*, 1991	USA, 1986	MOCA room near melting pot	3	19.1
		Top of standing cabinet (moulding dept)	2	4.7
		MOCA room dispensing counter	3	1.4
		Trimmer work table	1	0.1
		Moulder work table	14	0.5
				Mean
Fairfax & Porter, 2006	USA	Top of transformer adjacent to electric oven (where MOCA is heated)	1[a]	0.533
		Top of metal scale table	1	2.097
		Seal of urethane caster respirator	1	0.015
		Top of scale table	1	0.051
				GM
HSE, 2007a	United Kingdom	Fume cupboard	27	3800
		Storage	34	2700
		Weighing/pouring	21	2660
		Mixing	9	1910
		Oven	21	1910
		Hopper	4	96 000
		Casting	6	790
		Other	34	2340

[a]Only those samples with detectable levels are presented (LOD, 0.2 μg per sample)
GM, geometric mean.

1.3.3 *Exposure to the general population*

The general population can be exposed to MOCA in an area that has been contaminated with MOCA or upon consumption of certain types of plants (e.g. root crops) grown in MOCA-contaminated soil. Also, immediate family members of workers exposed to MOCA can be affected. Concentrations of MOCA in urine of up to 15 μg/L have been reported (Keeslar, 1986).

1.3.4 *Accidental release of MOCA in the environment*

Extensive environmental contamination with MOCA on several hundred hectares of land surrounding a MOCA plant occurred in 1979 in Adrian, MI, USA (Keeslar, 1986). Levels up to several milligrams per kilogram were found in gardens and community recreation areas. Of 12 selected children, aged 2 to 16 years, half were found to have detectable concentrations of MOCA ranging from 0.3 to 1.0 ppb [μg/L] in the urine. These children were all under the age of six years. Contact with contaminated soil during playing and going barefoot was considered the most likely route of exposure. The general adult population living within the MOCA-contaminated area had no detectable levels of MOCA in the urine samples tested.

The concentrations in sediment samples collected from the lagoon used by the MOCA plant mentioned above ranged from 1600 to 3800 ppm [mg/kg dry weight]. Effluent water from the lagoon had a concentration of 250 ppb [μg/L], deep-well water from under the plant had a concentration of 1.5 ppb [μg/L], and surface runoff water contained 1 ppb [μg/L]. Activated sludge from the sewage-treatment plant contained an estimated 18 ppm [mg/kg]. MOCA was not detected in sewage treatment-plant influent or effluent water (detection limit 0.5 μg/L) or in the water of a river located near the plant (detection limit 0.1 μg/L) (Parris *et al.*, 1980).

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Directive 97/56/EC

According to Directive 97/56/EC on the restrictions on the marketing and use of certain dangerous substances and preparations, the packaging of 4,4′-methylenebis(2-chloroaniline) and preparations containing this compound must be marked legibly and indelibly as follows: “Restricted to professional users” (European Commission, 1997).

(b) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes which, by reductive cleavage of one or more azo groups, may release 4,4′-

methylenebis(2-chloroaniline) (MOCA) in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(c) Directive 2004/37/EC

4,4′-Methylenebis(2-chloroaniline) (MOCA) is regulated by the Directive 2004/37/EC (European Commission, 2004), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

1.4.2 *Japan*

The Japan Society for Occupational Health (JSOH; 2007) has reported an occupational exposure limit (OEL) value of 0.005 mg/m^3 with a skin notation for 3,3′-dichloro-4,4′-diaminodiphenylmethane [MOCA]. An Occupational Exposure Limit based on Biological Monitoring (OEL-B) was mentioned of 50 µg/g-creatinine (total MOCA) in urine sampled at the end of the shift at the end of the work week. The JSOH follows the classification by IARC of 3,3′-dichloro-4,4’-diaminodiphenylmethane in Group 2A.

1.4.3 *Germany*

4,4′-Methylenebis(2-chloroaniline) (MOCA) is classified as a Category-2 carcinogen by the MAK Commission. The MAK Commission listed 4,4′-methylenebis(2-chloroaniline) as a substance where percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set (MAK, 2007).

1.4.4 *USA*

(a) ACGIH

4,4′-Methylenebis(2-chloroaniline) (MOCA) has been assigned an A2 notation, *suspected human carcinogen* (ACGIH, 2001). A TLV-TWA (threshold limit value–time-weighted average) of 0.01 ppm (0.11 mg/m^3) is recommended. A skin notation is assigned (*potentially significant contribution to the overall exposure by the cutaneous route*) in recognition of the consensus that skin absorption from direct contact is the major source of occupational exposure. Implementation of a urine-monitoring programme to ensure the effectiveness of dermal exposure control is encouraged.

(b) NIOSH

4,4'-Methylenebis(2-chloroaniline) is listed with a TWA of 0.02 ppm (0.22 mg/m^3) [skin] as a recommended exposure limit (REL) not in effect (NIOSH, 1989).

(c) NTP

4,4′-Methylenebis(2-chloroaniline) is listed in the NTP *Report on Carcinogens* as *reasonably anticipated to be a human carcinogen* (NTP, 2005).

1.4.5 *United Kingdom*

The Health and Safety Commission (HSE, 2007b) has set an 8-hour time-weighted average Workplace Exposure Limit (WEL) of 0.005 mg/m^3 for 2,2'-dichloro-4,4'-methylenedianiline (MOCA), with a skin notation. HSE also lists MOCA as capable of causing cancer and/or heritable genetic damage, and has defined a Biological Monitoring Guidance Value (BMGV) of 15 μmol total MOCA/mol creatinine in urine sampled after the work-shift.

1.4.6 *Other*

(a) GESTIS

Table 1.6 presents some international limit values for MOCA (GESTIS, 2007).

Table 1.6. International limit values (2007) for MOCA

Country	Limit value – Eight hours		Limit value – Short-term		Comments
	ppm	mg/m^3	ppm	mg/m^3	
Austria		0.02		0.08	TRK value (based on technical feasibility)
Canada, Québec	0.02	0.22			
Denmark	0.01	0.11	0.02	0.22	
France	0.02	0.22			
The Netherlands		0.02			
Spain	0.01 (skin)	0.1 (skin)			
Switzerland		0.02			
United Kingdom		0.005			

TRK, technical guiding concentration

2. Studies of Cancer in Humans

2.1 Screening studies

Ward *et al.* (1988; 1990) reported three cases with noninvasive papillary tumours of the bladder identified in a screening study of 385 workers who had been exposed to MOCA in a chemical plant in Michigan, USA, from 1968 to 1979. Later on, Hogan (1993) assessed detailed occupational exposures for the three cases by reviewing the chemical plant's product catalogues, material safety-data sheets, and written documentation about chemicals used to produce specific products, and by examining job histories, the time frames of exposure, and job classifications. The study found that the three cases had been exposed to many other chemicals in addition to MOCA during their time at the chemical plant and throughout their work history. Some of these chemicals are known or suspected bladder carcinogens (*ortho*-toluidine, 4-chloro-*ortho*-toluidine).

In 1989, Fox Chase Cancer Center worked with DuPont Chamber Works in New Jersey, USA to develop a bladder-cancer screening programme for the high-risk population of workers who had been exposed to MOCA, β-naphthylamine, benzidine, or *ortho*-toluidine at this plant (Mason and Vogler 1990; Mason *et al.* 1992). During the first seven quarterly periods of screening, two new cases and one recurrent case of transitional cell carcinoma of the bladder were detected, but they had been exposed to occupational bladder carcinogens other than MOCA.

Chen *et al.* (2005) screened for bladder cancer at four MOCA-manufacturing factories in Taiwan, China that employed 70 workers who were directly involved in MOCA-manufacturing processes, including the reaction, neutralization, washing, purification, and packing, or who were indirectly involved in the research and development laboratory. Ninety-two workers were not involved in the MOCA manufacturing or packing, nor were they working in the same building as the employees involved in these activities. The prevalence of atypical urinary cells and the nuclear matrix protein 22 tumour marker was not significantly different between the two groups of workers. However, the prevalence of positive occult blood was borderline-significantly ($P = 0.055$) greater in male exposed workers (18%) than in male non-exposed workers (7%). Among the 70 workers who had exposure to MOCA, there was one person with suspected malignant cells on urine cytology, one person with atypical cytology combined with gross haematuria, and one simply with atypical cytology. One worker was absent from the screening programme because he was admitted to the hospital, where he was diagnosed with bladder cancer. Liu *et al.* (2005) presented detailed information for the confirmed bladder-cancer case in his occupational history and environmental monitoring data. The confirmed bladder-cancer case had worked in the purification process area for 14 years (1987–2001), where the concentration of MOCA in the air was the highest (0.23–0.41 mg/m^3) and exceeded permissible OSHA exposure levels (0.22mg/m^3), without wearing any personal protective equipment at work. Furthermore, the worker was a non-smoker without a history of exposure to any other bladder carcinogen, except for

occasional pesticides application during agricultural work before he began working at this factory.

3. Studies of Cancer in Experimental Animals

Animal bioassays conducted with 4,4′-methylenebis(chloroaniline) (MOCA) were reviewed in IARC Monograph Volume 57 (IARC, 1993).

3.1 Oral administration

3.1.1 *Mouse*

Groups of 25 male and 25 female HaM/ICR mice, 6–8 weeks of age, were fed diets containing 0, 1000 or 2000 ppm MOCA as the hydrochloride (purity 97%) for 18 months. The doses were chosen on the basis of preliminary tests, the highest dose being the maximum tolerated dose. The effective numbers of animals at the end of the study were 18, 13, and 20 males, and 20, 21, and 14 females in the control, low-dose and high-dose groups, respectively. Haemangiomas or haemangiosarcomas (mainly subcutaneous) combined occurred in 0/18, 3/13 (23%), and 8/20 (40%) in the control, low-dose, and high-dose groups of the male mice, and in 1/20 (5%), 0/21, and 6/14 (43%) of the female mice. 'Hepatomas' occurred in 0/20, 9/21 (43%), 7/14 (50%) in the control, low-dose, and high-dose groups of female mice ($P < 0.01$, Fisher exact test) and in 3/18 (17%), 3/13 (23%), 4/20 (20%) male mice. The incidence of lymphosarcomas and reticulum-cell sarcomas was decreased in treated females. The authors stated that the incidence of vascular tumours in the high-dose groups was comparable with that in historical controls of the same strain [and probably not treatment-related] (Russfield *et al.*, 1975).

3.1.2 *Rat*

Groups of 25 male and 25 female Wistar rats, 100 days of age, were fed 0 or 1000 ppm MOCA (purity unspecified) in a protein-deficient diet [not otherwise specified] for 500 days (total dose, 27 g/kg bw), followed by an observation period on protein-deficient diet. Animals were killed when moribund; mean survival of treated males and females was 565 days and 535 days, respectively, and mean survival of male and female controls on the protein-deficient diet was 730 days. Of the 25 treated males, 23 died with tumours; 'hepatomas' occurred in 22/25 (88%) ($P < 0.001$, Fisher exact test), and lung tumours (mainly carcinomas) in 8/25 (32%) ($P = 0.002$, Fisher exact test). Among the treated females, 20 rats died with tumours; 'hepatomas' occurred in 18/25 (72%) ($P < 0.001$ Fisher exact test), and lung tumours were observed in 5/25 (20%) ($P = 0.025$, Fisher exact test). No 'hepatoma' or lung tumour was observed among 50 control animals (Grundmann & Steinhoff, 1970).

Groups of 25 male Charles River CD-1 rats, 6–8 weeks of age, were given diets containing 0, 500 or 1000 ppm MOCA as the hydrochloride (purity 97%) for 18 months. The doses were chosen on the basis of preliminary tests, the highest dose being the maximum tolerated dose. All surviving animals were killed 24 months after the start of the study; about 55% of the control and treated animals were still alive at 20–22 months. The effective numbers were 22 control, 22 low-dose and 19 high-dose animals. 'Hepatomas' occurred in 0/22 control, 1/22 (5%) low-dose and 4/19 (21%) high-dose rats ($P < 0.05$, Cochran-Armitage trend test) (Russfield *et al.*, 1975).

Groups of 50 male and 50 female Charles River CD rats, 38 days of age, were given 0 (control) or 1000 ppm MOCA (purity approximately 95%) in a standard diet (23% protein) for two years. The average duration of the experiment was 560 days for treated males, 548 days for treated females, 564 days for male controls and 628 days for female controls. Six animals from each group were sacrificed after one year for interim evaluation. Lung adenocarcinomas occurred in 21/44 (48%) ($P < 0.05$, χ^2-test) treated males and 27/44 (61%) ($P < 0.05$, χ^2-test) treated females. Squamous-cell carcinoma of the lung was observed in one treated male and one treated female. No lung tumour was observed among control animals. Lung adenomatosis, considered to be a preneoplastic lesion, developed in 14/44 (32%) treated males and 11/44 (25%) treated females, and in 1/44 (2%) males and 1/44 (2%) females in the controls ($P < 0.05$). Pleural mesotheliomas occurred in 4/44 (9%) treated males and 2/44 (5%) treated females; no such tumour was observed among controls. Hepatocellular adenomas and hepatocellular carcinomas occurred in 3/44 (7%) and 3/44 (7%) treated males and in 2/44 (5%) and 3/44 (7%) treated females, respectively, but not in controls. Ingestion of MOCA resulted in a lower incidence of pituitary tumours in treated females than in controls (1/44 (2%) *vs* 12/44 (27%)) (Stula *et al.*, 1975).

In the same study, another 25 male and 25 female Charles River CD rats, 36 days of age, were given 0 (control) or 1000 ppm MOCA (purity approximately 95%) in a low-protein diet (7%) for 16 months. Six animals from each group were sacrificed after one year for interim evaluation. The average duration of the experiment was 400 days for treated males, 423 days for treated females, 384 days for control males and 466 days for control females. Lung adenocarcinomas occurred in 5/21 treated males ($P < 0.05$, χ^2-test) and 6/21 females ($P < 0.05$, χ^2-test); no such tumour developed in 21 untreated male or female controls. Lung adenomatosis was observed in 8/21 (38%) treated males and 14/21 (67%) treated females and in 1/21 (5%) male controls and 1/21 (5%) female controls ($P < 0.05$, χ^2-test). Hepatocellular adenomas occurred in 5/21 (24%) treated males ($P < 0.05$, χ^2-test) and 2/21 (10%) treated females; hepatocellular carcinomas were observed in 11/21 (52%) treated males ($P < 0.05$, χ^2-test) and 1/21 (5%) treated females; no hepatocellular tumour was observed among 21 untreated males or females. Fibroadenomas of the mammary gland occurred in 1/21 (5%) treated and 7/21 (33%) control female rats ($P < 0.05$). Mammary gland adenocarcinomas developed in 6/21 (29%) treated and in 0/21 untreated females ($P < 0.05$, χ^2-test) (Stula *et al.*, 1975).

Groups of 100, 100, 75 and 50 male Charles River CD rats, 35 days of age, weighing 90–167 gr, were fed either a "protein-adequate" (27%) diet containing 0, 250, 500 or 1000 mg/kg (ppm) MOCA (industrial grade [purity unspecified]) or a "protein-deficient" (8%) diet containing 0, 125, 250 and 500 ppm MOCA for 18 months, after which they were maintained on their respective diet without MOCA for 6 months, followed by a 32-week observation period. The mean survival time (in weeks) for the protein-adequate diet was: control, 89; low-dose, 87; mid-dose, 80 ($P < 0.01$) (two-sided test, but method not specified); high-dose, 65 ($P < 0.001$); for the protein-deficient diet, these values were: control, 87; low-dose, 81; mid-dose, 79; high-dose, 77 ($P < 0.05$). The numbers of rats on the protein-adequate diet still alive at week 104 were: control, 20/100; low-dose, 14/100; mid-dose, 10/75; and high-dose, 0/50 (at 84 weeks, there were six surviving rats in this group). The numbers of animals on the protein-deficient diet still alive at week 104 were: control, 34/100; low-dose, 22/100; mid-dose, 14/75; and high-dose, 5/50. MOCA induced several tumour types in both groups; the incidences of the predominant tumours are shown in Table 3.1. Dose-related increases in the incidences of lung tumours, mammary adenocarcinomas, Zymbal gland carcinomas and hepatocellular carcinomas were observed in both experiments. The highest tumour incidence was observed in the lung. An increased incidence of haemangiosarcomas was observed only in the group on the protein-deficient diet. In groups given 500 ppm MOCA, tumour incidence was generally lower in those fed the protein-deficient diet, but hepatocellular carcinomas and Zymbal gland carcinomas occurred at a higher incidence in this group (18 and 12%) than in the protein-adequate group (4 and 7%) (Kommineni *et al.*, 1979).

3.1.3 *Dog*

A group of six pure-bred female beagle dogs, approximately one year of age, were given a daily oral dose of 100 mg MOCA (~90%, ~10% polyamines with a three-ring structure and ~0.9% *ortho*-chloroaniline) in a gelatin capsule on three days per week for six weeks, then on five days per week for up to nine years. A further group of six female dogs served as untreated controls. One treated dog died early, at 3.4 years of age, because of intercurrent infection; the other animals were killed between 8.3 and nine years. Transitional-cell carcinomas of the urinary bladder occurred in 4/5 (80%) treated dogs, and a composite tumour (transitional-cell carcinoma/adenocarcinoma) of the urethra developed in the 5th treated dog. No such tumours were observed among the six untreated control dogs ($P < 0.025$, Fisher exact test) (Stula *et al.*, 1978).

3.2 Subcutaneous administration

3.2.1 *Rat*

In a study reported as a short communication, groups of 17 male and 17 female Wistar rats (age unspecified) were injected subcutaneously with 500 or 1000 mg/kg bw

Table 3.1. Percentages of male rats with tumours at specific sites after feeding of MOCA in diets with different protein contents

Dietary protein	MOCA (ppm)	No. of rats autopsied	Lung adeno-carcinomas	All lung tumours	Mammary adeno-carcinomas	Zymbal gland carcinomas	Hepatocellular carcinomas	Haemangio-sarcomas	Pituitary adenomas[a]
Adequate	0	100	0	1	1	1	0	2	42
(27%)	250	100	14***	23***	5	8*	3	4	36
	500	75	27***	37***	11**	7	4	4	25*
	1 000	50	62***	70***	28***	22***	36***	0	4***
Deficient	0	100	0	0	0	0	0	1	23
(8%)	125	100	3	6**	1	0	0	2	16
	250	75	9**	15***	4	5*	0	5	12*
	500	50	16***	26***	6*	12***	18***	8*	20

From Kommineni *et al.* (1979).

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

[a] Includes pituitary adenocarcinomas (0–2 per group).

MOCA (94% pure) as a suspension in saline either once a week or at longer time intervals for 620 days (88 weeks) (total dose, 25 g/kg bw). The rats were fed a laboratory diet with normal protein content. The mean observation period was 778 days (111 weeks). A total of 22 animals developed 29 malignant tumours. Hepatocellular carcinomas occurred in 9/34 (26%) ($P < 0.0042$, Fisher exact test), and malignant lung tumours (six adenocarcinomas, one carcinoma) were observed in 7/34 (20%) animals ($P < 0.016$, Fisher exact test). A malignant subcutaneous tumour (unspecified) was found in one rat (sex unspecified). Among 25 male and 25 female untreated controls (mean observation period, 1040 days (148 weeks)), a total of 13 malignant tumours, including one lung tumour, developed; no hepatocellular carcinoma was observed in the control group (Steinhoff & Grundmann, 1971). [The Working Group noted the inadequate reporting of the experiment.]

3.3 Initiation-promotion studies

3.3.1 *Mouse*

Groups of 80 male and female SENCAR mice, 7–9 weeks of age, were given a single dermal application to the dorsal area of 0, 0.1, 1, 10, 100 or 200 mg MOCA (purity not specified), and after a one-week interval 2 µg of 12-*O*-tetradecanobylphorbol-13-acetate (TPA) was applied twice a week for 26 weeks as a promoter. MOCA did not induce significant numbers of mouse skin papillomas (Table 3.2) (Nesnow *et al.*, 1985).

Groups of 20 female hairless albino HRA/Skh mice, about six weeks of age, were given a single dermal application of 12.5, 25, 50 or 100 mg MOCA (purity ≥ 90–100%). After one week, 5 µg of 12-*O*-tetradecanonylphorbol-13-acetate (TPA) was applied twice a week for 21 weeks as a promoter. In this experiment MOCA was tested as an initiator. Mice were observed for one year; the minimum diameter of the papillomas scored was approximately 1 mm. The group treated with 25 mg MOCA had a slightly higher tumour incidence (35%) than the controls (27%) at 25 weeks, and at 52 weeks (20% *vs* 16%). However, comparison of the control group response with any of the MOCA-treated groups did not show any statistically significant difference (Rozinova *et al.*, 1998).

Table 3.2. Mouse skin-tumour initiation by MOCA

Dose µg[a]	Number of mice surviving		Mice bearing papillomas	
	Male	Female	Male	Female
0	40	36	10	11
100	35	38	6	3
1000	39	39	23	15
10 000	38	39	5	5
100 000	38	40	16	15
200 000	37	37	14	5

From Nesnow *et al.*, 1985.

[a] dermal application of MOCA, dermally promoted with 2 µg TPA

In the same study, another group of 20 female hairless albino HRA/Skh mice, about six weeks of age, were given single dermal application of 2.56 µg 7,12-dimethylbenz[*a*]anthracene (DMBA). After one week, dermal applications of 2.5 or 5 mg of MOCA (purity ≥ 90–100%) were started and continued twice a week for twenty weeks. In this experiment MOCA was tested as a promoter. Mice were observed for one year; the minimum diameter of the papillomas scored was approximately 1 mm. The negative control group received only a single application of DMBA. In this group, the first tumour occurrence was observed at week 19; the maximum tumour yield was 0.55 ± 0.21 tumours/mouse. In the 2.5-mg and 5-mg MOCA-promoted groups, the first tumour occurrence was observed at weeks 9 and 11, and the maximum tumour yields were 0.65 ± 0.17 and 0.30 ± 0.14 tumours/mouse, respectively (Rozinova *et al.*, 1998).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, excretion

4.1.1 *Humans*

Vaughan and Kenyon (1996) measured the concentrations of MOCA in urine, plasma and blood, and the amounts of haemoglobin (Hb) adducts in exposed workers. Samples from five workers involved in the production of polyurethane elastomers were examined. Gas chromatography/negative-ion chemical ionization mass spectrometry (GC-NICI-MS) was used for the determination of pentafluoropropionyl derivatives of MOCA. Both 3, 3′-dichlorobenzidine and [$^2H_6$]-MOCA were used as internal standards. Urinary concentrations of MOCA ranged over 500-fold, from 4.5 nmol/l (worker A) to 2390 nmol/l (worker E). Adducted MOCA was released by alkaline hydrolysis, and adduct levels were similar with or without dialysis of Hb. The Hb adducts ranged from 0.73 pmol/g Hb for worker A, who carried out general duties in the factory, to 43.3 pmol/g Hb for worker E, who was involved in mixing MOCA with a prepolymer. The former value is similar to that reported for 4-aminobiphenyl-Hb adducts in non-smokers (0.19 ± 0.08 pmol/g Hb) and smokers (0.91 ± 0.28 pmol/g Hb) (Skipper & Tannenbaum, 1990). The higher values for MOCA adducts are similar to concentrations of Hb adducts of 4,4'-methylenedianiline (MDA), a structurally similar compound. Concentrations of 10.88 pmol MDA/g Hb and higher values of 25.33 pmol *N*-acetyl-MDA/g Hb were reported for a worker (Bailey *et al.*, 1990), with a ratio of *N*-acetyl-MDA to MDA of 2.3. Although the actual value for pmol *N*-acetyl-MOCA/g Hb was not given, the ratio of *N*-acetyl-MOCA to MOCA was reported to range from 0.016 to 0.05 (Vaughan & Kenyon, 1996). This suggests that *N*-acetylation may not be an important pathway for MOCA metabolism. Blood MOCA concentrations were determined after alkaline hydrolysis. Values for worker A (0.13 nmol/l) and worker E (17.37 nmol/l) represented the lowest and highest levels in blood. Plasma values after alkaline hydrolysis were similar to those

in blood from corresponding workers, with those of workers A and E being 0.05 and 21.95 nmol/l, respectively. When the latter sample was subjected to ultrafiltration, 85% was found to be associated with the high-molecular-weight fraction, representing Hb and possibly other protein adducts. Hb adducts were thought to occur by activation of MOCA in a manner similar to DNA-adduct formation. While the half-life for excretion of MOCA in urine has been estimated to be 23–24 hours, Hb has a life-span of about 120 days, and Hb adducts would reflect exposure over this extended time course (Osorio *et al.*, 1990).

While *N*-acetylation has been shown to be an important detoxifying pathway in the human biotransformation of many aromatic amines, this may not be the case for MOCA (see previous study). In workers exposed to benzidine, more than 95% of measured urinary benzidine metabolites were present in *N*-acetylated form (Rothman *et al.*, 1996). Urinary levels of MOCA and its metabolites have been monitored in exposed workers, with only low concentrations of *N*-acetyl-MOCA and *N*,*N*′-diacetyl-MOCA observed (Ducos *et al.*, 1985; Cocker *et al.*, 1988). To evaluate whether *N*-acetylated products might be heat-labile conjugates, urine was heated for 1.5 hours at 80°C (Cocker *et al.*, 1988). This improved the detection of *N*-acetyl-MOCA. However, this did not produce ratios of *N*-acetylated to parent amine of greater than 0.09. More than half of the urine samples in this study had undetectable levels of *N*-acetyl-MOCA and thus were not used in the analysis. For heat-treated urine, ratios for *N*-acetylated MOCA to MOCA ranged from 1 to 24 (Cocker *et al.*, 1988). Human liver homogenates catalysed *N*-acetylation of MOCA with rates for rapid and slow acetylators similar to that observed with benzidine (Glowinski *et al.*, 1978). These results suggest that MOCA is either not *N*-acetylated *in vivo* or that it is acetylated and then rapidly deacetylated. The latter was suggested when human liver slices incubated with [^{3}H]-benzidine doubled their production of *N*-acetylated products with paraoxon (Lakshmi *et al.*, 1995). In urothelial cells of workers exposed to benzidine, the most predominant DNA adduct formed was *N*-acetylated, i.e. *N*′-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (Rothman *et al.*, 1996). In contrast, in urothelial cells of workers exposed to MOCA, the major adduct was shown to be a non-*N*-acetylated monocyclic adduct, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (Kaderlik *et al.*, 1993). *N*-Acetyl-MOCA is less mutagenic than MOCA as measured by the Ames test (Hesbert *et al.*, 1985), and no difference has been observed in the induction of DNA repair in hepatocytes from either rapid or slow acetylator rabbits (McQueen *et al.*, 1983). Thus, the metabolism and activation of MOCA seems significantly different from that of benzidine or even 4,4′-methylenedianiline (MDA).

4.1.2 *Experimental animals*

N-Acetylation plays an important role in metabolism of aromatic amines in rats. For example, [^{3}H]-labelled benzidine (180 mCi/mmol) is rapidly metabolized to its mono- and diacetylated products by male Fischer 344 rats. After 30 min, the recirculating perfusate from the isolated perfused rat liver showed a ratio of *N*-acetylated metabolites to benzidine of 14 (Lynn *et al.*, 1983; 1984). In contrast, *N*-acetylated heterocyclic amines

are not observed following administration of the parent compound to male Fischer 344 rats (Armbrecht *et al.*, 2007). A study of female LAC:Porton rats given an intraperitoneal injection of [methylene-^{14}C]-MOCA (8.3 mCi/mmol, radiochemical purity unspecified) reported at least nine metabolites and showed evidence of an *O*-glucuronide, an *O*-sulfate, and amino-chlorophenol. However, no *N*-acetylated metabolites were reported (Farmer *et al.*, 1981). Another extensive study with male CD rats (30 days old; immature) assessed the metabolism of orally dosed [methylene-^{14}C]-MOCA (4 to 7 mCi/mmol; ~93% radiochemical purity). Evidence of an *O*-glucuronide and an *O*-sulfate in urine was found, along with the major metabolite in bile, i.e. the mono-*N*-glucuronide of MOCA. However, no *N*-acetylated products were reported (Morton *et al.*, 1988).

Rat microsomal CYPs catalysed formation of three hydroxylated MOCA metabolites, *N*-OH, 5-OH, and methylene-OH. *N*-OH-MOCA is thought to participate in MOCA binding to DNA. The major MOCA-DNA adduct in rats was shown to be a non-*N*-acetylated monocyclic adduct, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (Silk *et al.*, 1989). This adduct was also formed by reaction of *N*-OH-4-amino-3-chlorobenzyl alcohol with DNA.

While MOCA is a substrate for rat *N*-acetyltransferases, *N*-acetylated products of MOCA have not been reported in urine or bile of rats that were given this amine.

Sabbioni and Neumann (1990) developed a quantitative method to determine MOCA-protein adducts. Female Wistar rats received oral doses of 3.82, 14.2 and 16.2 μmol/kg bw [^{14}C]-ring-labelled MOCA or 0.25 and 0.50 mmol/kg bw unlabelled MOCA. MOCA bound in decreasing amounts to macromolecules in the lung, liver and kidney. Fractions of 0.19% and 0.026% of the dose were bound to the blood proteins haemoglobin and albumin, respectively. MOCA was released by hydrolysis from haemoglobin, and determined by HPLC with electrochemical detection or by GC-MS. However, in contrast to many other aromatic amines, only 54% of the haemoglobin-bound [^{14}C]-labelled metabolites are hydrolysable, while none of the radioactivity could be released from albumin. This implies the existence of additional reactive species, presumably originating from metabolic cleavage at the methylene-bridge in MOCA, which yields stable protein adducts.

Macromolecular binding in rats after oral and dermal application of [^{14}C]-labelled MOCA has also been described (Cheever *et al.*, 1990).

Chen *et al.* (1991) studied the capacity of *N*-oxidized metabolites of MOCA to form haemoglobin (Hb) adducts in animals with or without induction of CYP enzymes with phenobarbital or β-naphthoflavone. Intravenous administration of as little as 0.04 μmol/kg *N*-hydroxy-MOCA to rats resulted in measurable formation of MOCA-Hb adducts (0.9 ng/50 mg Hb). Intraperitoneal administration of 0.5–50 mg/kg MOCA to rats, and subcutaneous administration of 5–500 mg/kg MOCA to rats and 4–100 mg/kg to guinea-pigs resulted in dose-related formation of Hb adducts. MOCA-Hb adducts remained elevated in blood for longer than 10 weeks following a single subcutaneous dose in guinea-pigs. Pretreatment of rats with phenobarbital would induce microsomal benzphetamine *N*-demethylase (BND) activity in rats, and result in a small increase in in-

vitro *N*- and *ortho*-hydroxylation of MOCA, but did not increase in-vivo Hb adduct levels. Pretreatment of rats with β-naphthoflavone induced microsomal aryl hydrocarbon hydroxylase as well as ethoxyresorufin-*O*-deethylase and increased the formation of MOCA-Hb adducts when the animals were dosed with MOCA at 100 and 500 mg/kg, but not 20 mg/kg subcutaneously (Chen *et al.* 1991).

Cheever *et al.* (1991) studied the multiple oral administration of MOCA in adult male rats. As many as 28 consecutive daily doses of [^{14}C]MOCA at 28.1 μmol/kg bw (5 μCi/day) were given. Rats were killed at weekly intervals for seven weeks. MOCA adduct formation for globin and serum albumin was evaluated by determination of [^{14}C]MOCA covalent binding. The covalent binding associated with globin showed a linear increase over the 28-day exposure period with 342 fmol/mg globin 24 hours after the final dose. The covalent binding with albumin was 443 fmol/mg albumin. After cessation of dosing, the albumin and globin adduct levels decreased rapidly with a half-life of the respective proteins.

Bailey *et al.* (1993) studied the exposure of rats to [^{14}C]ring-labelled MOCA. At 24 hours after a single intraperitoneal dose (3.74 μmole/kg bw), 0.08% of the administered dose was adducted to haemoglobin (Hb) and alkaline hydrolysis liberated 38% of the bound radioactivity as the parent MOCA. The formation of adducts correlated linearly with the dose of MOCA (3.74–44.94 μmole/kg bw).

The biological availability of the *N*-hydroxylated derivatives of *ortho*-substituted diamines and of known carcinogenic diamines was investigated in female Wistar rats by determining haemoglobin (Hb) adducts. Hb from rats dosed with 0.5 mmol/kg diamine and from untreated control rats were isolated and hydrolysed. The released diamine and monoacetyldiamine were quantified by HPLC with electrochemical detection or GC/MS. MDA, 4,4′-oxydianiline (ODA), 4,4′-ethylenediamine, and 4,4′-thiodianaline (TDA) bound to haemoglobin as diamine and as monoacetyl-diamine. 4,4′-Methylenebis(2,6-dimethylaniline), 4,4′-methylenebis(2,6-diethylaniline), MOCA, and 4,4′-sulfonyl-dianiline (daspone) bound only as diamine to Hb. 4,4′-Methylenebis (2,6-dichloroaniline) did not bind to Hb. Thus, the presence of two substituents in the *ortho* position and the presence of electron-withdrawing groups in the *para* position relative to the amino group drastically reduced the formation of Hb adducts. The extent of haemoglobin binding of the bicyclic diamines (daspone, 3,3′-dichlorobenzidine, MDA, MOCA, TDA, ODA, and benzidine) increases with their carcinogenic potency (Sabbioni & Schütze, 1998).

4.1.3 *In-vitro studies*

Because occupational exposure to MOCA occurs mainly through skin penetration, Hewitt *et al.* (1995) evaluated decontamination procedures *in vitro* using fresh full-thickness human breast skin. Flow-through diffusion cells were used to determine the effect of four washing solutions (100% ethanol, 100% water, 1 and 10% (v/v) aqueous soap). All solutions were equally effective at removing MOCA (ring-[^{14}C]-MOCA, specific activity 59 mCi/mmol; radiochemical purity > 96%; 10–18 μg/cm^2) from the

surface of human skin, with 22–47% of the applied dose removed at 72 hours. The penetration of MOCA into and through human skin at 72 hours was significantly reduced (two- to threefold) by washing the skin surface at 3 or 30 min. Washing at one hour after application significantly reduced total uptake, but this was less effective than at earlier time points. These results suggest that MOCA is rapidly absorbed from the skin surface into the skin. To reduce systemic exposure, the authors suggested that skin should be washed within the first 30 minutes after contamination. The choice of washing solution is less critical than is the time of washing. Similar results were observed for MDA. These results are consistent with previous studies showing that the uptake of MOCA from the skin surface into the skin itself is rapid, reaching a maximum only one hour after application (Hewitt *et al.*, 1993). This suggests the presence of a cutaneous reservoir within the skin.

Wiese *et al.* (2001) showed that recombinant human COX-1 and COX-2, isoforms of prostaglandin H synthase, activate MOCA, given as [4,4'-methylene-^{14}C]MOCA (8.66 mCi/mmol), to bind to DNA. Besides MOCA, several different aromatic and heterocyclic amines were studied. Prostaglandin H synthase is composed of two separate isoenzymes, cyclooxygenase and hydroperoxidase. The latter utilizes these amines as reducing substrates. Binding was found to be arachidonic acid-dependent. The highest DNA binding was observed with MOCA. hCOX-2 activated MOCA nearly twice as effectively as did hCOX-1. In contrast, activation of the other amines by both enzymes was similar. While benzidine-DNA binding was reduced by BSA, this was not observed with MOCA. The peroxidative activity of human lung microsomes was shown to activate MOCA (^{14}C-MOCA, specific activity 46.7 mCi/mmol) to bind DNA (Culp *et al.*, 1997). With specific antisera it was demonstrated that myeloperoxidase was responsible for DNA binding. Binding was dependent on H_2O_2 and was inhibited by azide, but not by indomethacin or eicosatetraynoic acid (Culp *et al.*, 1997). This is consistent with myeloperoxidase-mediated rather than prostaglandin H synthase-mediated binding. The source of myeloperoxidase was thought to be the polymorphonuclear neutrophils. These cells are involved in the inflammatory response, which can contribute to carcinogenesis (Parsonnet, 1999). Results demonstrate that different enzymes mediate peroxidative activation of MOCA to bind to DNA.

While human CYP1A2 *N*-hydroxylates many arylamines, MOCA is an exception, preferring CYP2A6 or CYP3A4 (Butler *et al.*, 1989; Yun *et al.*, 1992).

[^{3}H]*N*-OH-MOCA (20 mCi/mmol) was found to be metabolically activated by 3′-phosphoadenosine-5′-phosphosulfate (PAPS)-dependent human liver sulfotransferases to bind to DNA (Chou *et al.*, 1995). In 12 human hepatic cytosols, binding was significantly correlated with levels of thermostable phenol-sulfotransferase activity, but not with thermolabile phenol- or dehydro-epiandrosterone-sulfotransferase activities. Binding was prevented by 2,6-dichloro-4-nitrophenol, a potent selective inhibitor for human thermostable phenol sulfotransferase. *N*-OH-MOCA was a good substrate for sulfotransferase in human liver cytosols, but not in cytosols from human urinary bladder epithelium. Thermostable phenol-sulfotransferase is polymorphically distributed and

could contribute to individual differences in carcinogen susceptibility. *N*-Hydroxyarylamines are thought to initiate DNA-adduct formation (Chou *et al.*, 1995).

Walraven *et al.* (2006) used recombinant rat *N*-acetyltransferases to assess *N*-acetylation of MOCA by the enzymes Nat1, Nat2, and Nat3 via HPLC analysis of non-radioactive metabolites. MOCA was *N*-acetylated by all three transferases. Nat3 (the recently discovered third rodent *N*-acetyltransferase) metabolized MOCA along with six other arylamines. However, Nat3-mediated metabolism of MOCA was at the limit of detection. Nat1 and Nat2 each metabolized 12 of the 13 arylamines tested. MOCA was only one of three amines found to be selectively *N*-acetylated by recombinant rat Nat1. In contrast, 4,4'-methylenedianiline was one of nine substrates selectively metabolized by rat Nat2. Previous studies have demonstrated *N*-acetylation of MOCA by human and rabbit liver homogenates (Glowinski *et al.*, 1978). *N*-Acetylation of MOCA by human rapid and slow acetylators was similar to that observed with benzidine.

4.2 Genetic and related effects

4.2.1 *Humans*

Exfoliated urothelial cells were recovered from urine samples from a worker accidentally sprayed with molten MOCA. Samples were collected at different times—up to 430 hours—after exposure (Osorio *et al.*, 1990). The occurrence of MOCA-DNA adducts was investogated in these exfoliated urothelial cells by ^{32}P-post-labelling analysis. The major DNA adduct was *N*-(deoxyadenosin-8-yl)-4-aminochlorobenzyl alcohol. This MOCA-DNA adduct was detected in samples obtained between 4 and 98 hours after initial exposure but not in samples collected at later times. The level of DNA adducts 4 hours after exposure was determined to be 516 adducts/10^8 nucleotides. A five-fold decrease in adduct level was observed 14 hours later, followed by a gradual decrease over subsequent days (Kaderlik *et al.* 1993).

An increased frequency of sister chromatid exchange, consistent with their apparent exposure to MOCA, was seen in peripheral lymphocytes from a small number of workers exposed to MOCA during the manufacture of polyurethane (Edwards & Priestly, 1992).

In a study from Australia, micronucleus induction was measured in peripheral lymphocytes and exfoliated urothelial cells of workers exposed to MOCA. Twelve male workers (age 24–42 years) were recruited for this study from four work locations where exposure was noted. Exfoliated urothelial cells from pre-work urine samples on a midweek work-day were assessed for micronucleus (MN) frequency. Post-work urine samples were analysed for total MOCA. Blood samples were collected on the same day and were cultured for 96 hours. Cytochalasin-B-blocked cells were scored for MN. Eighteen male control subjects (age 23–59 years) provided corresponding urine and blood samples. Average urinary MOCA concentrations were 6.5 µmol/mol creatinine (range 0.4–48.6 µmol/mol creatinine) in post-work samples of MOCA-exposed workers. MOCA was not detected in the urine of control workers. Mean MN frequencies were higher in

urothelial cells and lymphocytes of MOCA-exposed workers (14.27 ± 0.56 and 13.25 ± 0.48 MN/1000 cells) than in control subjects (6.90 ± 0.18 and 9.24 ± 0.9 MN/1000 cells). The mean number of micronucleated cells was also higher in both tissues of exposed workers (9.69 ± 0.32 and 8.54 ± 0.14 MN cells/1000 cells) than in control subjects (5.18 ± 0.11 and 5.93 ± 0.13 MN cells/1000 cells) (Murray & Edwards, 1999).

More recently, the same authors studied cytogenetic endpoints, including the formation of micronuclei (MN) in exfoliated cells and lymphocytes, to estimate the risk for genotoxic events in workers exposed to MOCA and bitumen fumes (which contain skin and lung carcinogens). Twelve men employed in polyurethane manufacture, who had been exposed to MOCA, 12 bitumen road-layers (exposed to bitumen fumes containing PAHs) and 18 hospital-store personnel (controls) were recruited for the study. All provided blood and urine samples on the same day. Blood cultures were prepared by use of a cytochalasin B-block method. Exfoliated urothelial cells were collected from urine and stained for microscopy. The number of MN was higher in MOCA-exposed workers (14.27 ± 0.56 MN/1000 cells; 9.69 ± 0.32 micronucleated cells/1000 cells) than in bitumen-exposed workers (11.99 ± 0.65 MN/1000 cells; 8.66 ± 0.46 micronucleated cells/1000 cells). The MN frequency in control subjects was 6.88 ± 0.18 MN/1000 cells (5.17 ± 0.11 micronucleated cells/1000 cells). Conversely, in lymphocytes, MN frequencies were higher in bitumen-exposed workers (16.24 ± 0.63 MN/1000, 10.65 ± 0.24 MN cells/1000) than in MOCA-exposed workers (13.25 ± 0.48 MN/1000, 8.54 ± 0.14 MN cells/1000) or in control subjects (9.24 ± 0.29 MN/1000 or 5.93 ± 0.13 MN cells/1000). The MN frequency values for the various groups were all significantly different from each other ($P < 0.01$). The results suggest that these genotoxins could cause MN formation to a different extent in different tissues (Murray & Edwards, 2005).

4.2.2 *Experimental systems*

(a) Animals

In an early study, Kugler-Steigmeier *et al.* (1989) showed that MOCA produced DNA adducts in the liver of Sprague-Dawley rats at levels typically found for moderately strong genotoxic carcinogens.

In rats given a single dose of 95 μmol/kg bw [methylene-^{14}C]MOCA by gavage, DNA adducts were found after 24 hours at 7 pmol/mg DNA in liver, 2 pmol/mg in lung and 0.5 pmol/mg in kidney. These adducts, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol and *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene were eliminated from rat liver with nonlinear kinetics. The selective reaction of *N*-hydroxy-MOCA with adenine in DNA, and the formation of a single arylamine ring adduct suggest a substitution mechanism involving an intermediate with a strong S_N1 character, aided by the negative inductive effect of the *ortho*-chlorine. Due to tautomer formation, the initial adduct may be inherently unstable and undergo cleavage at the 1′-carbon-methylene bond to yield the observed adducts (Segerbäck & Kadlubar, 1992).

Segerbäck *et al.* (1993) applied ^{32}P-postlabelling analysis to determine DNA adducts of MOCA in target and non-target tissues in dogs. Beagle dogs were treated with single and multiple doses of MOCA, and DNA-adduct levels were determined in liver and bladder epithelium. After a single dose, the level of adducts in the liver was 1.5-fold higher than that in the bladder epithelium. The amounts of adducts in these two organs increased three- to five-fold after 10 doses, and adducts in the liver were then 2.8-fold higher than in the bladder epithelium. The amounts found in these two organs after single exposures were compared, per unit exposure dose, with those reported for other carcinogenic aromatic amines. The comparison showed that MOCA was as effective in DNA-adduct formation as were most other potent urinary bladder carcinogens. These results suggest that MOCA may have high carcinogenic potential in humans.

DeBord *et al.* (1996) studied adduct formation by ^{32}P-postlabelling in rats treated with MOCA and in human uro-epithelial cells treated with *N*-OH-MOCA. In both cases, the major adduct corresponded to *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol.

(b) In-vitro studies

Shivapurkar *et al.* (1987) found that MOCA could bind with DNA of explant cultures of human and dog bladders. In both humans and dogs, there appeared to be a population of cells with high DNA binding and another with low DNA binding. The binding of MOCA to human bladder DNA appeared to be higher than to dog bladder DNA.

MOCA formed adducts with DNA in cultured canine and human bladder cells, and in liver, lung and kidney of rats treated topically, intraperitoneally or orally. One of three HPLC peaks of an enzymatic digest of DNA derived from rats treated *in vivo* was identified tentatively as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (Silk *et al.*, 1989).

Kuslikis *et al.*, (1991) found that *N*-Hydroxy-MOCA was mutagenic to *Salmonella typhimurium* TA98 and TA100 in the absence of an exogenous metabolic activation system. Other MOCA metabolites, *ortho*-hydroxy-MOCA, 4-amino-3,3′-dichloro-4′-nitrosodiphenyl-methane (mononitroso derivative) and di-(3-chloro-4-nitrosophenyl) methane (dinitroso derivative), were not mutagenic to *S. typhimurium* TA98 or TA100. The mutagenic activity of the mononitroso derivative towards strain TA100, however, appeared to be masked by its toxicity.

Reaction of *N*-hydroxy[methylene-^{14}C]-MOCA with DNA *in vitro* resulted in the formation of two major adducts, which were identified by mass spectroscopy as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol and *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene (Kuslikis *et al.*, 1991).

The SV40-immortalized human uroepithelial cell line SV-HUC.PC was used to study the malignant transformation capacity of *N*-OH-MOCA. SV-HUC.PC cells were exposed *in vitro* to various concentrations of *N*-OH-MOCA. The carcinogen-treated cells were propagated in culture for about six weeks and subsequently injected subcutaneously into athymic nude mice. Two of the 14 different groups of SV-HUC.PC cell cultures treated with different concentrations of N-OH-MOCA formed carcinomas in these mice.

^{32}P-postlabelling analyses of DNA isolated from SV-HUC.PC cells after exposure to *N*-OH-MOCA revealed one major and one minor adduct. The major adduct has been identified as the *N*-(deoxyadenosin-3′,5′-bisphosph-8-yl)-4-amino-3-chlorobenzyl alcohol (pdAp-ACBA) and the minor adduct as *N*-(deoxyadenosin-3′,5′-bisphospho-8-yl)-4-amino-3-chlorotoluene (pdAp-ACT). Furthermore, the SV-HUC.PC cytosols catalysed the binding of *N*-OH-MOCA to DNA in the presence of acetyl-CoA, to yield similar adducts (Swaminathan *et al.*, 1996).

Reid *et al.* (1998) explored the genotoxic potential of MOCA by monitoring the induction of mutations at the *HPRT* locus of AHH-human lymphoblastoid cells. Exposure of AHH-1 cells to *N*-OH-MOCA was found to induce a six-fold increase in mutant frequency and resulted in base-pair substitution primarily at A:T base pairs. The induction of mutations at A:T sites by *N*-OH-MOCA provides a marker of genotoxic damage for an exposed population (Reid *et al.*, 1998).

Birner *et al.* (1990) have shown that *ortho*-substitution with methyl groups of benzidine to produce 3,3′-5,5′-tetramethylbenzidine results in non-mutagenic derivatives (Chung *et al.*, 2000) that also do not bind to haemoglobin. It is thought that in 4,4′-methylenediamine (MDA) or MOCA, bulky substituents in the *ortho* position eliminate the mutagenicity and carcinogenicity of MDA and MOCA, presumably by inhibiting the formation of the *N*-OH-arylamines, which are the putative genotoxic intermediates.

The major metabolite of MOCA in the urine of dogs is identified as 3,3′-dichloro-4,4′-diaminodiphenylmethane-5-sulfate. Upon hydrolysis of this metabolite with arylsulfatase, time- and enzyme concentration-dependent protein binding and time-dependent DNA binding of this material were observed. However, the metabolites were not mutagenic towards *Salmonella* tester strain TA98 at up to 20 μg/plate, and cytotoxic to the tester strain at 50 μg/plate (Manis & Braselton, 1984).

(c) Summary of short-term genotoxicity tests (IARC, 1993)

MOCA was shown to cause prophage induction in *Escherichia coli* (Thomson, 1981) and differential toxicity in *Bacillus subtilis rec*-deficient strains (Kada, 1981). It was mutagenic to *Salmonella typhimurium* (Bridges *et al.*, 1981), *Escherichia coli* (Matsushima *et al.*, 1981) and at the *Tk* locus in mouse lymphoma L5178Y cells (Mitchell *et al.*, 1988; Myhr & Caspary, 1988), but not to *Saccharomyces cerevisiae* (Mehta & von Borstel, 1981). MOCA caused aneuploidy in *S. cerevisiae* (Parry & Sharp, 1981) but gave equivocal results with regard to gene conversion and did not induce mitotic crossing-over in the same organism (Jagannath *et al.*, 1981). It induced mutation in *Drosophila melanogaster* (Kugler-Steigmeier *et al.*, 1989) and unscheduled DNA synthesis in primary cultures of hepatocytes from mice (McQueen *et al.*, 1981), rats (McQueen *et al.*; 1981; Williams *et al.*; 1982; Mori *et al.*, 1988), and Syrian hamsters (McQueen *et al.*, 1981). Sister chromatid exchange but not chromosomal aberration was induced in Chinese hamster ovary cells (Galloway *et al.*, 1985). MOCA induced cell transformation in mammalian cells (Daniel & Dehnel, 1981; Dunkel *et al.*, 1981; Styles,

1981) and inhibited gap-junctional intercellular communication in cultured rat-liver cells (Kuslikis *et al.*, 1991). MOCA induced sister chromatid exchange in lymphocytes of rats treated *in vivo* (Edwards & Priestly, 1992).

4.3 Mechanistic considerations

The toxicological profile of MOCA is quite similar to that of monocyclic aromatic amines: MOCA causes an increased incidence of bladder tumours in exposed workers, bladder tumours in dogs, lung and liver tumours in rodents, and hemangiosarcoma and hepatoma, among others, in rats and mice. The genotoxic properties of MOCA have been broadly documented previously (IARC, 1993).

MOCA is *N*-oxidized, and the resulting metabolites bind to DNA, RNA and proteins. Interestingly, two different kinds of activation product are formed, and the question of how they contribute to the biological effects has not been answered. A hydrolysable haemoglobin adduct has been found in humans and in experimental animals. MOCA is practically the only cleavage product released from the adduct after oral administration to rats. This indicates that the non-acetylated MOCA and not *N′*-acetyl-MOCA is *N*-oxidized (Sabbioni & Neumann, 1990). MOCA is different from other bifunctional amines, such as benzidine and 3,3′-dimethoxybenzidine, for which predominantly *N′*-acetylated adducts are formed (Neumann, 1988).

The expected 2-chloro-4-methylaniline (2-chloro-*meta*-toluidine) was not detected upon hydrolysis of the haemoglobin adducts found in rats. It should have been formed when it is a major metabolite that is N-oxidized to the respective hydroxylamine and nitroso-derivative. Obviously other reactive forms yield the nonhydrolyzable adducts of haemoglobin and albumin. Structurally, 2-chloro-*meta*-toluidine is related to 4-chloro-*ortho*-toluidine, in which the two substituents are exchanged. In both cases a bulky substituent is in the *ortho*-position to the amine and the *para*-position is blocked. Although experimental data do not seem to exist, similar biological properties would be expected. Overall, MOCA shows many properties typical for aromatic amines and monocyclic amines in particular, which supports the idea of a common mode of action for this class of chemicals.

5. Summary of Data Reported

5.1 Exposure data

4,4′-Methylenebis(2-chloroaniline) (MOCA) is widely used as a curing agent in the polyurethane industry. Occupational exposure to MOCA has been reported in industries that manufacture and use MOCA. Dermal absorption after contact with contaminated surfaces appears to be the most important route of exposure, with inhalation and ingestion representing minor routes. Post-shift urine measurements represent the most appropriate

method to measure workers' exposure. Urinary levels of MOCA have been reported for MOCA production workers and polyurethane production workers in Australia, France, Germany, Japan, Taiwan (China), the United Kingdom, and the USA.

MOCA does not occur in nature. The general population can be exposed to MOCA if they live in an area contaminated with this compound.

5.2 Human carcinogenicity data

No adequate epidemiological studies were available to the Working Group to evaluate an association between exposure to 4,4′-methylenebis(2-chloroaniline) and bladder cancer risk.

5.3 Animal carcinogenicity data

MOCA was tested for carcinogenicity by oral administration in the diet in mice in one study, in rats of each sex in two studies, in male and female rats in a further two studies using normal and low-protein diets, and in one study using capsules in female dogs. It was also tested by subcutaneous administration to rats in one study.

Oral administration of MOCA increased the incidence of liver tumours in female mice. In a series of experiments in which rats were fed either standard or low-protein diets, it induced liver-cell tumours and malignant lung tumours in males and females in one study, a few liver-cell tumours in male rats in another, lung adenocarcinomas and hepatocellular tumours in males and females in a third, and malignant lung tumours, mammary gland adenocarcinomas, Zymbal gland carcinomas and hepatocellular carcinomas in a fourth. Oral administration of MOCA to female beagle dogs produced transitional-cell carcinomas of the urinary bladder and urethra. Subcutaneous administration to rats produced hepatocellular carcinomas and malignant lung tumours.

5.4 Other relevant data

The toxicological profile of MOCA is typical for biologically active monocyclic aromatic amines. It is metabolically activated by *N*-oxidation to metabolites that react with DNA and proteins. The genotoxicity of MOCA is well documented. The acute toxicity is reflected by methaemoglobin formation, spleen toxicity, fibrosis in spleen and often in liver and kidney of rodents. The profile is comparable with those of *ortho*-toluidine and 4-chloro-*ortho*-toluidine, thus indicating a common mode of action.

MOCA is mutagenic to *Salmonella typhimurium*, *E. coli*, *Drosophila melanogaster*, and human lymphoblastoid cells. It causes prophage induction in *E. coli*, differential toxicity to *Bacillus subtilis* rec-deficient strains, and mutations at the *Tk* locus in mouse lymphoma L5178Y cells. It causes aneuploidy in *S. cerevisiae* and unscheduled DNA synthesis in the primary cultures of hepatocytes from mice, rats, and Syrian hamsters.

MOCA induces sister chromatid exchange in lymphocytes of rats. It forms DNA adducts in cultured canine and human bladder cells, and in the lung, liver, and kidney of rats treated with MOCA. It inhibits intercellular communication in cultured rat liver cells. The MOCA metabolite, *N*-OH MOCA, forms DNA adducts in rat liver, causes malignant transformation in SV40-immortalized human SV.HUC.PC uroepithelial cells, which subsequently produce carcinomas in athymic nude mice. MOCA also binds to protein RNA, and DNA of rats.

MOCA has been shown to interact with DNA to form adducts in urothelial cells, and with haemoglobin to form adducts in the blood of exposed workers. MOCA has been shown to cause the formation of sister chromatid exchange and micronuclei in urothelial cells and lymphocytes of exposed humans. Chromosomal aberrations have been shown to be a good predictor of carcinogenicity.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of 4,4′-methylenebis(2-chloroaniline).

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of 4,4′-methylenebis(2-chloroaniline).

6.3 Overall evaluation

4,4′-methylenebis(2-chloroaniline) is *carcinogenic to humans (Group 1)*.

6.4 Rationale

In reaching this evaluation the Working Group considered the following:

- MOCA shows many properties typical for monocyclic aromatic amines, *e.g., ortho*-toluidine, which supports the notion of a common mode of action for this class of chemicals.
- MOCA is genotoxic in numerous assays for genotoxicity.
- In rats and dogs, species in which MOCA has shown to produce tumours, this compound is metabolized to *N*-hydroxy-MOCA, which forms DNA adducts.
- One of the two major MOCA-DNA adducts found in the target tissues for carcinogenicity in animals (rat liver and lung; dog urinary bladder) was also

found in urothelial cells from a worker with known occupational exposure to MOCA.

- An increased frequency of sister chromatid exchange and micronuclei was seen in urothelial cells and lymphocytes of workers exposed to MOCA.

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2-NAPHTHYLAMINE

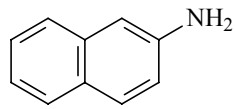
1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 91–59–8
CAS Name: 2-Naphthalenamine
Synonyms: 2-Aminonaphthalene; β-aminonaphthalene; *ortho*-aminonaphthalene; C.I. 37270; β-naphthalenamine; naphthalen-2-ylamine; 2-naphthylamine; β-naphthylamine

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



$C_{10}H_9N$ Rel. mol. mass: 143.19

1.1.3 *Chemical and physical properties of the pure substance* (O'Neil, 2006)

Description: White to reddish crystals
Boiling-point: 306 °C
Melting-point: 111–113 °C
Solubility: Soluble in water, diethyl ether, and ethanol (Lide, 2008)
Octanol/water partition coefficient: log P, 2.25 (Verschueren, 2001)

1.1.4 *Technical products and impurities*

The commercial product contains several polycyclic hydrocarbons including dibenzo(*a,h*)phenazine, which is formed from 2-naphthylamine in the presence of air. 2-Amino-1,4-naphthoquinone-*N*^{4},2-naphthylimine has also been reported as a contaminant of 2-naphthylamine (Radomski *et al.*, 1971).

Trade names for 2-naphthylamine include Fast Scarlet Base B.

1.1.5 *Analysis*

Analyses of 2-naphthylamine were first reported in the 1960s. Historically, water, urine, and textile samples have been analysed for 2-naphthylamine content with gas chromatography (GC) and liquid chromatography-mass spectrometry (LC-MS). These methods permit detection at concentrations down to the ng/L level. Recent studies involve the use of GC/MS to determine the concentrations of 2-naphthylamine (in derivative form) in cigarette smoke and in the urine of smokers. Table 1.1 presents selected recent studies of the analysis of 2-naphthylamine in various matrices.

1.2 Production and use

1.2.1 *Production*

2-Naphthylamine was commercially produced in the US from the early 1920s to the early 1970s. In 1955 (the latest year for which production data were found), 581 000 kg (1.3 million pounds) were produced (IARC, 1974). Since its commercial manufacture and use in the US were banned in the early 1970s, 2-naphthylamine has been available only in small quantities for laboratory research.

According to EU legislation, the production, manufacture and use of 2-naphthylamine has been prohibited since 1998 (European Commission, 1998).

Available information indicates that 2-naphthylamine was produced and/or supplied in research quantities in the following countries: Belgium, Germany, Hong Kong Special Administrative Region, the People's Republic of China, Switzerland, and the USA (Chemical Sources International, 2010)

1.2.2 *Use*

2-Naphthylamine is now used only in laboratory research. It formerly was used commercially as an intermediate in the manufacture of dyes, as an antioxidant in the rubber industry, and to produce 2-chloronaphthylamine (IARC, 1974; HSDB, 2003).

1.3 Occurrence

1.3.1 *Natural and environmental occurrence*

2-Naphthylamine is formed during the pyrolysis of nitrogen-containing organic matter and can occur as such in nature (Masuda *et al*.,1967; Talukder & Kates, 2000).

Table 1.1. Selected methods of analysis of 2-naphthylamine in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Textiles	Solutions are prepared in dry tetrahydrofuran; add penta-fluoropropionic anhydride and heat; cool; dilute with methanol	GC/MS	[2 ng/mL]	Narvekar & Srivastava (2002)
Urine	Extract twice with *n*-hexane; centrifuge; add dry pyridine; derivatize using pentafluoropropionic anhydride; extract with phosphate buffer; centrifuge; evaporate organic solvent under N_2 stream	GC/MS	75 ng/L	Weiss & Angerer (2002)
Water	Pass water samples at neutral pH through C18 cartridges; add 10 mM tributylamine–acetic acid and formic acid (pH 3); conduct ion-pair solid phase extraction	HPLC-ESI-MS	2 ng/L	Loos *et al.* (2003)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/dichloromethane (50:50)	GC/MS	5 ng/mL	Doherty (2005)
Dyes, cosmetics, inks and finger paints	Extract with methanol. Separate and detect on a phenyl ether-linked stationary phase	HPLC/MS	NR	Hauri *et al.* (2005)
Urine	Acid hydrolysis of arylamine conjugates in urine, extraction with n-hexane, derivatization with pentafluoropropionic anhydride, and analysis	GC/MS	1 ng/L	Riedel *et al.* (2006)
Cigarette smoke	Cigarettes are conditioned at 22 °C and 60% humidity, machine smoked; mainstream smoke constituents are collected in glass filters; constituents are extracted with hydrochloric acid and hexane, derivatized with pentafluoropropionic acid anhydride and trimethylamine, and analysed.	GC/MS	1 ng/cig. [0.5–0.8 g tobacco/cig.]	Hyodo *et al.* (2007)
Water	River water samples are filtered and then spiked with stock solutions of analyte.	HPLC/ECD	8.8 nmol/L	Zima *et al.* (2007)

ECD, electrochemical detection; GC, gas chromatography; HPLC-ESI, high performance liquid chromatography–electrospray ionization; MS, mass spectrometry; NR, not reported

2-Naphthylamine may occur in the waste streams from plants where it is produced and used; it has been reported to be present in the effluent from certain dyestuff factories in Japan (Takemura *et al.*, 1965).

1.3.2 *Occupational exposure*

Occupational exposure to 2-naphthylamine (2-NA) mainly occurs during its production and when used in the manufacture of azo dyes. Occupational exposure to 2-NA can also occur in laboratories where it is used for research purposes, and when workers are exposed to pyrolysis fumes containing 2-NA (e.g., foundry fumes, environmental tobacco smoke, heated cooking oils), or to 2-nitronaphthalene (e.g., foundry workers), a nitro-PAH that can be metabolized to 2-NA. Exposure can also occur in workers exposed to products containing 2-NA as a contaminant, such as certain rubber chemicals.

Subjects reported to have been exposed to 2-NA during its production include workers from Italy (Rubino *et al.*, 1982), Japan (Tsuchiya *et al.*, 1975; Morinaga *et al.*, 1982), the Russian Federation (Bulbulyan *et al.*, 1995), the United Kingdom (Case & Pearson, 1954) and the USA (Schulte *et al.*, 1986; Cassidy *et al.*, 2003).

Exposure measurement data were reported for a dye factory that manufactured 2-NA and benzidine in Moscow (Bulbulyan *et al.*, 1995). 2-NA levels in factory-air samples taken between 1939 and 1948 ranged from < 0.001 mg/L to > 0.003 mg/L. Factory-wall wipes contained 60.0–115 mg/m^2 wall surface. Dermal wipes taken after a shower at work showed amounts of 2-NA between 0.018–37.5 mg [data on the skin surface-area were not available].

In a study among a group of workers in Germany primarily exposed to aniline and 4-chloroaniline, urinary 2-NA concentrations ranged between 0–9.8 µg/L (mean 3.9±2.2) in 22 smokers and between 0.0–11.6 µg/L (mean 2.1±2.8) in 21 nonsmokers; levels measured in non-smoking non-exposed workers were 0.0–1.6 µg/L (mean 0.5±0.7). No difference was observed between slow and fast acetylators among smokers or non-smokers (Riffelmann *et al.*, 1995).

In a study in two Danish iron foundries, 2-NA was proposed as a possible marker of 2-nitronaphthalene in the urine of PAH-exposed workers (Hansen *et al.*, 1994). The concentrations of 2-NA in urine were significantly higher in PAH-exposed workers compared with controls (matched for smoking habits), with hand moulders, finishing workers and truck drivers having the highest levels. This may be explained by the presence of 2-nitronaphthalene (which can be metabolized to 2-NA), of aromatic amines (e.g., in moulding sand) or of nitrogen oxides (e.g., in diesel exhaust). A study of 19 human volunteers showed that up to 0.03% of a single 10-mg dose of *N*-phenyl-2-naphthylamine is converted internally to 2-naphthylamine (IARC, 1978). More recently, it has been estimated that a maximum of 1% of total *N*-phenyl-2-naphthylamine uptake can be metabolized into 2-NA (Weiss *et al.*, 2007).

1.3.3 *Occurrence as an impurity in other compounds*

Several dye intermediates have been shown to contain small amounts of 2-naphthylamine. 2-NA concentrations of up to 0.5% have been reported in commercially produced 1-naphthylamine (substance profile). A 4% contamination level has been reported in 1-NA produced in Japan, which after 1970 was reduced to less than 1% (Tsuchiya *et al.*, 1975). 2-NA has also been detected in 6-amino-2-naphthalene sulfonic acid (Bronner's acid) (Cassidy *et al.*, 2003), a pigment intermediate, as well as in auramine.

2-NA has been detected in hair dyes. A study from Turkey showed 2-NA in 20 out of 54 hair dyes and in 15 out of 25 hennas, in concentrations up to 5.47 and 4.15 μg/g respectively (Akyüz & Ata, 2008). [The Working Group noted that the hair dyes analysed in this study are not necessarily representative of hair dyes used in other countries].

2-NA has been detected in rubber antioxidants such as Nonox S and Agerite Resin at levels of 0.25% [2500 ppm] (Veys, 2004). *N*-phenyl-2-naphthylamine, also a rubber antioxidant, can be contaminated with 2-NA, and can also be metabolized (dephenylated) into 2-NA to some extent.

In a polyethylene pipe manufacturing plant in the USA, contamination of a resin additive with 2-NA was detected in 1996, which may have resulted in worker exposure to 2-NA from 1970 to 1996 (Felknor *et al.*, 2003).

1.3.4 *Exposure of the general population*

The general population can be exposed to 2-NA through environmental exposure, via tobacco smoke, via other fumes containing 2-NA, or when in contact with dyes and hair dyes contaminated with 2-NA. Exposure to the nitro-PAH 2-nitronaphthalene, which is formed by incomplete combustion of organic material and generally occurs in the environment as a mixture of other nitro-PAH and non-nitro-PAH compounds, can also represent an indirect source of exposure to 2-NA.

Mainstream cigarette-smoke from eight different US conventional market cigarettes contained 2-naphthylamine at concentrations of 1.47 to 14.06 ng per cigarette (Stabbert *et al.*, 2003). Amounts in mainstream cigarette-smoke range from 1–22 ng per cigarette (IARC, 2004), those in sidestream cigarette-smoke range from 113.5–171.6 ng per cigarette (IARC, 2004).

In a study from Germany (Riffelmann *et al*., 1995), urinary concentrations were higher in smokers (3100 ng/L±2100; $n = 8$) compared with non-smokers (500 ng/L±700; $n = 8$). [The Working Group noted that the very high levels reported are likely due to the unspecific detection by the GC-EC method used (Riedel *et al*., 2006)]. In a later German study (Grimmer *et al.*, 2000), freebase 2-NA was found at comparable levels in the urine of non-smokers (120.8 ng/24 hours), of smokers (84.5 ng/24 hours) and of individuals exposed to environmental tobacco smoke (94.9 ng/24 hours). In a study by Riedel *et al.* (2006), smokers ($n = 10$) excreted significantly higher amounts of 2-NA compared with nonsmokers ($n = 10$) (20.8 *vs* 10.7 ng/24 hours).

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Council Directive 89/677/EEC

According to Council Directive 89/677/EEC, 2-naphthylamine and its salts may not be used in concentrations equal to or greater than 0.1% by weight in substances and preparations placed on the market (European Economic Community, 1989).

(b) Council Directive 98/24/EC

According to EU regulations, the manufacture and use of 2-naphthylamine has been prohibited since 1998 (European Commission, 1998). The Council Directive 98/24/EC in Annex III prohibits the production, manufacture or use at work of 2-naphthylamine and its salts and activities involving 2-naphthylamine and its salts. The prohibition does not apply if 2-naphthylamine is present in another chemical agent, or as a constituent of waste, provided that its individual concentration therein is less than 0.1% w/w.

(c) Council Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes which, by reductive cleavage of one or more azo groups, may release 2-naphthylamine in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(d) Directive 2004/37/EC

2-Naphthylamine is regulated by the Directive 2004/37/EC (European Commission, 2004a), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(e) Directive 2004/93/EC

The Commission Directive 2004/93/EC of 21 September 2004 amends the Council Directive 76/768/EEC for the purpose of adapting Annexes II and III thereto to technical progress (European Commission, 2004b). In this Directive, 2-naphthylamine and its salts are listed in *Annex II* as substances that must not form part of the composition of cosmetic products.

(f) Directive 2005/90/EC

In the Directive 2005/90/EC, the list in Directive 76/769/EEC of substances classified as carcinogenic, mutagenic or toxic to reproduction was amended to include 2-naphthylamine (European Commission, 2005).

1.4.2 *Germany*

2-Naphthylamine is classified as a Category 1 carcinogen by the MAK Commission. The MAK Commission listed 2-naphthylamine as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set (MAK, 2007).

1.4.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of 2-naphthylamine in Group 1.

1.4.4 *USA*

(a) ACGIH

2-Naphthylamine has been assigned an A1 notation, *Confirmed Human Carcinogen* (ACGIH, 2001). Accordingly, a numerical TLV (threshold limit-value) is not recommended for occupational exposure. As for all substances designated as A1 carcinogens without a TLV, workers should be properly equipped to eliminate all exposure to 2-naphthylamine to the fullest extent possible.

(b) NIOSH

The National Institute for Occupational Safety and Health (NIOSH, 2005) lists 2-naphthylamine as one of 13 OSHA-regulated carcinogens. Exposures of workers are to be controlled through the required use of engineering controls, work practices, and personal protective equipment, including respirators.

(c) NTP

2-Naphthylamine is listed in the NTP Report on Carcinogens as *known to be a human carcinogen* (NTP, 2005).

1.4.5 *Other*

(a) GESTIS

Table 1.2 presents some international limit values for 2-naphthylamine (GESTIS, 2007).

Table 1.2. International limit values (2007) for 2-naphthylamine

Country	Limit value – Eight hours		Limit value – Short-term	
	ppm	mg/m^3	ppm	mg/m^3
France	0.001	0.005		
Hungary				0.005
Italy				0.001

(b) Recent bans

The use and import of 2-naphthylamine was banned recently in the Republic of Korea in 2003, and in Switzerland in 2005 (UN/UNEP/FAO, 2007).

2. Studies of Cancer in Humans

Cancer risk related to exposure to 2-naphthylamine has been assessed in several cohort studies and case reports. In spite of the fact that, in general, production ceased several decades ago, follow-ups were continuously updated until very recent dates.

2.1 Case reports, case series

Several reports from the 1960s and 1970s described cases of bladder cancer among workers exposed to 2-naphthylamine in France (Billiard-Duchesne, 1960), Italy (Vigliani & Barsotti, 1962) and Japan (Tsuji, 1962, Tsuchiya *et al.* 1975)

Goldwater *et al.* (1965) found that the cumulative incidence of bladder cancer among British coal-tar dye workers exposed to 2-naphthylamine, and not to other aromatic amines, was 25% (12 cases among 48 exposed workers).

In another study from Japan, 112 cases of recognized occupational bladder cancer (10.3%) were found among 1085 workers employed in the synthesis and handling of 2-naphthylamine and/or benzidine (Nakamura *et al.*, 1980; Shinka *et al.,* 1991).

Hashmi *et al.,* (1995) found that among 250 consecutive bladder-cancer patients admitted to the Dow Medical College in Karachi, Pakistan, three had been exposed to 2-naphthylamine.

In a group of 438 persons engaged in the production of aromatic amines, including 2-naphthylamine, in Japan, 88 (20%) developed uroepithelial cancer (Hamasaki *et al.*, 1996).

[The studies listed above represent a mere sample of many case series spanning four decades.]

In addition to the case series presented above, three screening programmes detected cases of bladder cancer among workers exposed to 2-naphthylamine. These were the DuPont Chambers Works programme (Mason *et al.*, 1986, 1992; Mason & Vogler, 1990), the Drake programme (Marsh and Cassidy 2003) and the screening programme of a dyestuff plant in Tokyo, Japan (Miyakawa *et al.*, 2001). [The Working Group raised concerns about the validity of the results of these screening programmes as far as it concerns etiological research with a substantial potential for detection bias and because no measure of effect is available relating β-naphthylamine exposure to cancer.]

2.2 Cohort studies

One of the first studies to review cases of bladder cancer among workers exposed to 2-naphthylamine was that of Case *et al.* (1954), who reviewed 341 cases of bladder cancer of a total of 455 occurring in employees of participating firms in the British chemical industry who had been exposed to 2-naphthylamine only. There were 26 cases of bladder cancer mentioned on the death certificate, where only 0.3 would have been expected from the overall male population of England and Wales ($P < 0.001$). The mean induction period was 16 years.

Mancuso & el Attar (1967) followed a cohort of 639 male workers employed in a company manufacturing 2-naphthylamine and benzidine in Ohio (USA) for 27 years (1938–1965), with respect to cause-specific mortality. Based on 14 cases, the observed mortality rate for bladder cancer was elevated when compared with that expected for the population of Ohio (78/100 000 *vs* 4.4/100 000). Among white men, all cases occurred among the "exposed" group. There was an underreporting of cases of bladder cancer on the death certificates. The "attack rate", taking together those who had died and those who were living with bladder neoplasms, for those exposed to 2-naphthylamine was 952/100 000. Based on six cases, the mortality rate for pancreatic cancer was also elevated (39/100 000 *vs* 7.5/100 000).

Veys (1969, 2004) followed a cohort of male workers employed in the British rubber industry with respect to bladder-cancer morbidity and mortality. Among these workers, 2090 were considered likely to be exposed to 2-naphthylamine, as they were employed between 1945 and 1949, while 3038 workers who started their employment after 1950 were considered to be not exposed to 2-naphthylamine. For morbidity, 58 bladder cancers were identified in the first group, while 33.9 were expected according to national standardized incidence rates (SIR, 1.7; 95% CI, 1.3–2.2). In the second group, 39 cases were traced while 38.3 were expected (SIR, 1.0; 95% CI 0.7–1.4). Bladder-cancer mortality data from the same cohort did not show an increased risk (SMR, 1.0; 95% CI, 0.60–1.6); in only 16 of the 46 deceased was bladder cancer certified as the underlying cause of death.

Morinaga *et al.* (1982) ascertained the incidence of second primary cancers among 3322 workers exposed to 2-naphthylamine and benzidine during 1950–1978 in Japan. Among these workers, 244 had previously had genito-urinary cancer. Within this subgroup, 11 male workers developed subsequent cancers of the liver, gallbladder, bile duct, large intestine and lung. Compared with a control group of 177 male bladder-cancer patients, the excess occurrence of liver, gallbladder and bile-duct cancer was statistically significant ($P < 0.05$). One case of maxillary sinus cancer occurred in a worker exclusively exposed to 2-naphthylamine and one case of prostate cancer occurred in a worker exposed to both 2-naphthylamine and benzidine. The nine remaining cases appeared in workers exposed to benzidine.

Piolatto *et al.* (1991) carried out a cohort study among 906 dyestuff workers in Turin, Northern Italy, who were employed for ≥ 1 year between 1922 and 1970 and followed-up through 1989. Causes of death were ascertained through death certificates. Overall, 49 bladder-cancer cases were observed, while 1.6 were expected (SMR, 30.4; 95% CI, 23.0–40.2) Results were stratified by specific employment in the plant. Among those involved in manufacturing of naphthylamine or benzidine, the SMR for bladder cancer was 142.11 (27 deaths; 95% CI, 97.5–207.2) (Decarli *et al.*, 1985). Those who used naphthylamines or benzidine showed an SMR of 16.7 (three deaths; 95% CI, 5.4–51.7). An earlier study of the same cohort provided results specific to exposure during 2-naphthylamine manufacture; the SMR for bladder cancer was 150 (95% CI, 67.4–333.9) based on six cases (Rubino *et al.*, 1982).

A cohort study in the USA included 1384 workers employed between 1940 and 1972 in a chemical plant in Georgia manufacturing and using 2-naphthylamine and other aromatic amines. The cohort was followed-up until 1992. The initial cohort comprised approximately 70 female workers, but these were excluded from the mortality study because they were generally not employed in jobs involving exposure to 2-naphthylamine. The vital status at the end of follow-up was determined by use of the National Death Index and US postal service data. Incident cases of bladder cancer were identified by community neurologists and a bladder-cancer screening programme. The mortality experience of the cohort was compared with that of the US and Georgia populations. The SMR corresponding to bladder cancer as underlying cause of death was 2.4 (three deaths; 95% CI, 0.5–7.0) and that corresponding to bladder cancer as listed anywhere on the death certificate was 5.6 (eight deaths; 95% CI, 2.4–11.1). There was also an excess of deaths for esophageal cancer (seven deaths; SMR, 2.0; 95% CI, 0.8–4.1), lung cancer (41 deaths; SMR, 1.67; 95% CI, 1.20–2.3), and prostate cancer (11 deaths; SMR, 2.1; 95% CI, 1.1–3.8) (Schulte *et al.*, 1985, 1986; Stern *et al.*, 1985; Axtell *et al.*, 1998).

Delzell *et al.* (1989) followed a cohort of 2642 men employed at a dye- and resin-manufacturing plant in New Jersey (USA) from the opening of the plant in 1952 through 1988. Data on work history were obtained through several independent sets of records. A subcohort of 89 men who had previously worked in a chemical plant that produced benzidine and 2-naphthylamine showed an excess of total cancer deaths based on

17 cases (SMR, 1.99; 95% CI, 1.16–3.18). This excess was due essentially to bladder cancer (three cases observed, 0.25 expected; SMR, 12; 95% CI, 3.9–37.2), kidney cancer (two cases observed, 0.21 expected; SMR, 9.52; 95% CI, 2.4–38.1) and tumours of the central nervous system (two cases observed, 0.22 expected; SMR, 9.1; 95% CI, 2.3–36.3).

Morinaga *et al.* (1990) followed from 1970 to 1986 a cohort of 604 workers from two factories in Osaka (Japan) that produced 2-naphthylamine and benzidine in the period 1945–1971. The causes of death of these workers were compared with those expected based on the mortality of the Osaka population. Those who had been exposed exclusively to 2-naphthylamine showed a significant increase in urinary cancer (two cases observed; SMR, 11.76; 95% CI, 2.9–47.0). Those exposed to both 2-naphthylamine and benzidine had a higher risk with two cases observed (SMR, 25.00; 95% CI, 6.3–100.0). The increase in risk for other cancers (liver and lung) was not statistically significant.

Szeszenia-Dabrowska *et al.* (1991) conducted a cohort study among 6978 male workers employed in the rubber industry in Poland for at least three months during 1945 to 1973 and followed-up through 1985. These workers were predominantly engaged in rubber footwear manufacture. A total of 299 deaths from cancer were registered in the cohort (SMR, 112.7; 95% CI, 100.6–126.2). Bladder cancer was significantly increased for workers employed between 1945 and 1953, when 2-naphthylamine was used (six deaths; SMR, 2.76; 95% CI, 1.24–6.14).

Bulbulyan *et al.* (1995) evaluated cancer incidence and mortality in a cohort of 4581 aniline-dye production workers (2409 men and 2172 women) in Moscow, exposed to 2-naphthylamine or benzidine during ≥ 1 month, or employed during > 2 years, with a follow-up time of 15 years, from January 1975 to December 1989. Cases were ascertained through oncology registries that were available in the 33 Moscow regions. Job history for up to four jobs per person was ascertained through employment records. Incidence and mortality for several cancer sites were determined with regard to exposure to 2-naphthylamine and benzidine. Standardized incidence ratios were calculated by comparison with the Moscow general population. For workers ever exposed to 2-naphthylamine, the incidence of bladder cancer was significantly increased for those employed > 3 years (eight cases; SIR, 19.5; 95% CI, 8.4–38.5). There was a positive exposure-response relationship with years worked ($P = 0.07$); those who were first employed before the age of 20 showed a larger increase in risk (four cases; SIR, 49.4; 95% CI, 13.3–126.3) than those hired at later ages (p for trend = 0.04).

Naito *et al.* (1995) followed a cohort of 442 dyestuff workers in an urban area in Japan from date of entry (1935–1988) through 1992, on average for 39 years. The SMR for bladder cancer for those exposed to 2-naphthylamine was 48.4 (three deaths; 95% CI, 10.0–141.5). The SMR risk for all sites was 1.9 (nine deaths; 95% CI, 0.9–3.7) for 2-naphthylamine manufacture and 0.4 (2 deaths; 95% CI, 0.1–1.4) for 2-naphthylamine use. In addition to mortality, the authors reported on the incidence by periodic urologic screenings. The adjusted incidence rate of urothelial carcinoma increased with duration of exposure during 2-naphthylamine manufacture.

The Drake Health Registry Study provides for bladder-cancer screening of all former employees of the Drake Chemical Co. (Lock Haven, Pennsylvania, USA). The company and its predecessors produced 2-naphthylamine as a main product from 1940 until 1962 and possibly as a residual contaminant through 1981, when the plant was closed. Based on the Drake Health Registry, a cohort study was conducted among 400 workers (374 men, 26 women) with sufficient data for analysis. Causes of death were determined through the National Death Index and death certificates. The mortality experience of the cohort from 1960 to 1998 was examined, and expected numbers of deaths were computed with the US population and the local area population as a standard. For all cancers, based on 28 deaths, the SMR was 3.1 (95% CI, 2.1–4.5). For bladder cancer, the SMR was 16.8 (95% CI, 4.6–43.1) based on four deaths. For cancer of the respiratory system, based on 12 deaths, the SMR was 3.9 (95% CI, 2.0–6.8) (Cassidy *et al.*, 2003). Two decades earlier in an ecological study, Budnick *et al.* (1984) had analysed age-, sex-, and race-adjusted cancer-mortality rates for the county where the plant was located, and reported a statistically significant increase in bladder cancer among white men.

3. Studies of Cancer in Experimental Animals

3.1 Oral administration

3.1.1 *Mouse*

A group of 13 male and 12 female IF mice, weighing 25 g, were given 5 mg of 2-naphthylamine (BDH; purified by distillation and crystallization) in arachis oil via stomach tube twice weekly at a dose of 400 mg/kg bw per week for life (90 weeks). A group of six males and five females served as arachis-oil controls. Liver cholangiomas were observed in 5/13 (38%) male and 5/12 female (41%) mice. No tumours were observed in control animals (Bonser *et al.*, 1952).

Groups of nine male and 14 female CBA mice, weighing 30 to 35 g (age not specified) were given five mg 2-naphthylamine (BHD; purified by distillation and crystallization) in arachis oil by gavage, twice weekly for a weekly dose of 240 mg/kg bw for life (90 weeks). A group of seven male and seven female mice served as arachis-oil controls. Hepatomas were observed in 6/9 males (67%) and 7/14 females (50%). No tumours were observed in the control animals. The authors reported the incidence of hepatomas in the control breeding mice of the laboratory to be 8% (Bonser *et al.*, 1952).

Groups of 14–15 male and 12–15 female CBA mice (weight and age not given) were fed four different synthetic diets containing 2-naphthylamine (concentration not specified) for a dose of 160 mg/kg bw per week for 90 weeks. No information on use of controls was provided. Hepatomas occurred at similar incidences in all four diet groups, with a total of 24/57 (42%) hepatomas observed in males and 25/54 (46%) in females. The

authors reported that malignant hepatomas were found in 16 mice (sex not specified) (Bonser *et al.*, 1952). [The Working Group noted the lack of controls.]

Groups of 20 female BALB/c mice, eight weeks of age, were fed a diet containing 2000 ppm 2-naphthylamine [purity not specified] for 40 weeks, followed by basal diet for 15 weeks. At 55 weeks, hyperplasia of the bladder epithelium was seen in 6 of 16 (37%) surviving mice. In the livers of these mice, hyperplastic nodules were seen in 14 animals (87%), adenomas in 10 (62%) and hepatomas in three (19%) (Yoshida *et al.*, 1979).

A group of 16 female and 16 male A/J mice, 6–8 weeks of age, received 2-naphthylamine in tricaprylin by gavage three times per week for eight weeks, resulting in a total dose per animal of 600 mg/kg bw. At 24 weeks, 8 of 14 (57%) male and 4 of 13 (31%) female survivors had lung tumours. This was not significantly different from the lung tumour rate in the vehicle controls. Tumour multiplicity was significantly increased in male mice (0.93 ± 1.00 *vs* 0.27 ± 0.59; $P < 0.05$) (Stoner *et al.*, 1986).

3.1.2 *Rat*

A group of 18 male, 29 female and three unknown albino rats were fed 0.01% 2-naphthylamine [purity unspecified] in the diet for life; this resulted in four bladder papillomas among 50 rats that survived for up to 102 weeks. No carcinomas were seen in the treated group. There were no tumours observed in the 43 controls fed an untreated diet (Bonser *et al.*, 1952).

Oral administration by gavage of 0.5–150 mg 2-naphthylamine [purity unspecified] per week to rats for 52 weeks, followed by an observation period until up to 80 weeks did not result in a significant increase in tumours compared with controls (Hadidian *et al.*, 1968).

A group of 25 female Wistar rats, 6–8 weeks of age, received 2-naphthylamine [purity unspecified] suspended in arachis oil by stomach tube at a dose rate of 300 mg/kg bw per week for one year, and were then kept until symptoms of bladder disease were seen. A group of 55 males and females served as controls. The first tumour appeared at 57 weeks. Histology indicated that five of 17 (29%) animals showed the presence of bladder tumours. Three animals were reported to be "still alive" (Hicks & Chowaniec, 1977).

A group of 20 female Wistar rats, 10 weeks of age, received 2-naphthylamine (purity not specified) at a dose of 300 mg/kg bw in arachis oil by gastric intubation once a week for 57 weeks. Animals were killed when moribund, or after 100 weeks. A group of 20 animals served as arachis-oil controls. Nineteen animals survived 57 weeks or longer. The bladders of 18 rats were examined histologically: eight of 18 (44%) had urothelial hyperplasia, and four of these eight had also carcinomas (Hicks *et al.*, 1982).

3.1.3 *Hamster*

A group of 30 male and 30 female Syrian golden hamsters, nine weeks of age, received 1% or 0.1% (w/w) 2-naphthylamine (purity unspecified) in the diet for life. The estimated intake was 600 mg 2-naphthylamine/week. Bladder carcinomas were observed in 10/23 (43%) effective males and in 8/16 (50%) effective females treated with the 1% concentration, with the first tumours appearing after 45 weeks. One male and one female hamster also developed hepatomas. Dietary concentrations of 0.1% did not produce carcinomas in 60 animals after 97 weeks. Repeated administration of the chemical by gavage (10 mg, twice weekly for 40 weeks) did not produce any tumours. In two control groups of 60 and 40 animals, fed the diet only, no bladder carcinomas were observed (Saffiotti *et al.*, 1967).

3.1.4 *Rabbit*

Oral doses (200 mg, twice weekly) of 2-naphthylamine (purity unspecified) given to six rabbits (age and sex unspecified) for more than five years induced a transitional-cell bladder papilloma at 4.75 years in one animal, and epithelial hyperplasia with downgrowth in the bladder in another after 5.25 years (Bonser *et al.*, 1952).

3.1.5 *Dog*

A group of 16 medium-sized to large female mongrel dogs, weighing 8–20 kg, received daily subcutaneous injections of commercial 2-naphthylamine (4 mg/day for dogs ≤ 12 kg: 5 mg/day for larger dogs) for 14 weeks. The dose was doubled during the next 12 weeks and tripled during the subsequent 63 weeks. During the last 54 weeks of this period, the dogs also received a daily dose of 2-naphthylamine (by capsule) in the food. The number of dogs on this dual regimen was gradually increased, as was the oral dose (starting with 100 mg/day). By the time the subcutaneous injections were terminated, all dogs received a daily oral dose of 300 mg of 2-naphthylamine. During the first five months of this experimental period, eight dogs received sodium bicarbonate (100 mg/kg bw) to alkalinize the urine. Four control dogs received no treatment. Over a total treatment period of 20–26 months, 13 of the 16 (81%) dogs had developed papillomas and carcinomas in the bladder (Hueper *et al.*, 1938). [This evidence was based partly on autopsy and partly on cystoscopy and biopsy.]

A group of four medium-sized mongrel Airedale dogs (one female and three males) received 2-naphthylamine (BDH; purified by distillation and crystallization) at 150 mg per day in milk for three weeks. The dose was then reduced to 100 mg/day (for an unspecified period). The dose was gradually increased to 700 mg per day after the end of the first year. One male dog died after one year of treatment. Multiple papillomas in the bladder were seen in the three other dogs after 3.7–5 years of treatment. In one animal, one anaplastic carcinoma was found. There were no metastases, and the renal pelves, ureters and urethra were tumour-free, as were all other organs (Bonser, 1943).

A group of four female mongrel dogs received 200 mg 2-naphthylamine (purity unspecified) orally in a gelatin capsule on six days per week for six months. The daily dose was then increased to 600 mg and continued for up to two years. The maximum cumulative dose of 2-naphthylamine given to a dog was 310 g. One dog died after 14.5 months of treatment without neoplastic changes in the bladder. Another dog received 2-naphthylamine for one year and was killed nine months later with no neoplastic changes in the bladder epithelium. Multiple transitional-cell tumours, some of which were histologically malignant, were seen in the bladder of the remaining two dogs. One of these died after two years of treatment; the other was killed one year later. The latter dog had developed an invasive bladder carcinoma (Bonser *et al.*, 1956a).

A group of four female mongrel dogs, each weighing 12–14 kg, were fed 400 mg 2-naphthylamine [purity unspecified] daily for two years. Bladder tumours were noted and confirmed by microscopic examination of biopsy sections in all four animals within 9–18 months after the start of the experiment. The dogs received intravenous and intracavitary therapy with 5-fluorouracil, whereupon transient tumour regression was observed. All dogs were then kept on standard diet and followed for 23 to 55 additional months until death or sacrifice. Metastases of the bladder carcinomas were seen in the lungs of two dogs and in the kidney in one (Harrison *et al.*, 1969).

A group of 34 beagle dogs, 8–10 months of age, received 2-naphthylamine [purity unspecified] mixed with lactose, orally in gelatin capsules on 6 days per week in doses of 6.25, 12.5, 25, and 50 mg/kg bw (dose groups A, B, C, D) per day for 2–26 months. Two male and two female control dogs received capsules with lactose only. Within 30 months, bladder tumours were induced across all dose groups in 24/34 (70%) dogs: invasive transitional-cell carcinomas were seen in two dogs each in dose groups A, B, and D, and in five dogs in dose group C; invasive squamous-cell carcinomas were observed in one, two, three and two dogs in dose groups A, B, C and D, respectively; papillary carcinomas were noted in one, three and four dogs in dose groups B, C and D, respectively. Overall, carcinomas were present in 9/11 (81%) dogs that had received a total of 100–200 g 2-naphthylamine and in 6/23 (26%) dogs receiving a total amount of < 100 g. No tumours were seen in the controls. The total amount of 2-naphthylamine required for tumour induction was less for dose group A after 24–30 months than for dose groups C and D after 9–18 months (Conzelman & Moulton 1972).

In a study designed to investigate the morphology of 2-naphthylamine-induced bladder tumours, eight female dogs, weighing 18–30 kg, were treated with 2-naphthylamine (5–30 mg/kg bw, 4–6 days per week, for 7.5 months and 30 mg/kg bw, 4–6 days per week, for 8.5 months). After 55 months since the beginning of the experiment, bladder carcinomas developed in seven (87%) animals (Romanenko & Martynenko, 1972).

A group of 15 female dogs, weighing 16–22 kg, received an oral capsule containing 500–600 mg 2-naphthylamine [purity not specified] daily for 20–26 months. All dogs had haematuria, and all developed bladder carcinoma (Rigotti *et al.*, 1977).

Eight female pure-bred beagle dogs were given a daily dose of 2-naphthylamine (5 mg/kg bw; source and purity not specified; route of administration not given) for 30 days. Four dogs then started on a daily supplement of 6 g D,L-tryptophan admixed into 300 g dog chow during the rest of the experiment. The other four dogs were kept on standard diet. Four additional dogs received only the dietary supplement but no 2-naphthylamine. After three years, all 12 dogs were killed and the bladders were examined grossly and by histology. The bladders of dogs that received only 2-naphthylamine showed no abnormal effects. The dogs that were given tryptophan with 2-naphthylamine showed gross and histological changes, such as papillary tumours and epithelial hyperplasia. The bladder mucosa of dogs given tryptophan alone showed similar hyperplastic changes (Radomski *et al.*, 1977).

Five male purebred beagle dogs received capsules with 2-naphthylamine in corn oil orally in doses of 25 mg/kg bw, daily on five days per week for 26 weeks. In a second experiment, groups of four dogs (two females, two males) were similarly treated for one, six or 36 weeks, then promptly killed. In a third experiment, eight dogs (four females, four males) were treated with the same regimen for 26 weeks, then four dogs were killed and the others kept without further treatment for three years. Loss of bladder luminal membrane, hyperplasia of the bladder epithelium, and lymphocyte infiltration of the submucosa were seen after one and six weeks of treatment in some dogs, and to a more severe degree after 36 weeks of treatment in all dogs. Two of the four dogs that were kept for three years after a 26-week treatment period had papillary carcinomas of the bladder (Rdomski *et al*., 1978).

A group of two female and three male beagle dogs, approximately nine months of age, received 400 mg pure 2-naphthylamine daily on five days per week for 34 months. The chemicals were given as tablets enclosed in a gelatin capsule. Control dogs (four males and four females) received capsules with lactose. All treated dogs developed transitional cell carcinomas of the bladder within 34 months. No neoplasia of the bladder was found in the controls (Purchase *et al.*, 1981).

3.1.6 *Monkey*

Twenty-four young male and female rhesus monkeys (*Macaca mulatta*; weighing 2–3 kg) received 2-naphthylamine mixed with lactose, orally in gelatin capsules on six days per week in doses of 6.25, 12.5, 25, 50, 100, 200, and 400 mg/kg bw per day for 33–60 months. Some animals received a fixed dose during the entire experiment; others received different doses over the course of five years. Papillary carcinoma, transitional-cell carcinoma or carcinoma in situ were observed in the bladder of nine treated monkeys, the earliest of these tumours appearing after 33 months. The majority of tumours occurred in animals given high doses of 2-naphthylamine. No other types of tumour were observed, and no tumours occurred in three control monkeys (Conzelman *et al.* 1969).

3.2 Subcutaneous or intra-peritoneal injection

3.2.1 *Mouse*

Swiss albino mice, approximately 12 weeks of age, received 0.1 ml of a freshly made 3% solution of 2-naphthylamine (BDH; purified by distillation and crystallization) in arachis oil by subcutaneous injection, twice weekly for 50 weeks. None of 13 mice surviving 33 weeks of treatment had subcutaneous sarcomas; two of four mice (50%; all females) surviving > 77 weeks had hepatomas. In a similar experiment with 2-naphthylamine (RCH; purified by gradient sublimation), local subcutaneous sarcomas were seen in two of 12 mice (17%; at 37 and 41 weeks, respectively) and one hepatoma at 52 weeks. When a 3% solution of 2-naphthylamine (BDH; purified by distillation and crystallization) was allowed to stand for four weeks and injected subcutaneously into Swiss albino mice (0.1 ml per mouse; twice weekly for 50 weeks), 10 of 16 (62%) mice that survived ≥ 20 weeks had subcutaneous injection-site sarcomas, and four of five (80%) mice that survived ≥ 80 weeks had hepatomas. No sarcomas or hepatomas developed in 17 male and female controls treated with arachis oil only (Bonser *et al.*, 1956a).

CBA mice, 12 weeks of age, received 0.1 mL of a freshly made 3% solution of 2-naphthylamine-HCl (BDH; purified by distillation and crystallization) by subcutaneous injection, twice weekly for six months and then once weekly for a further four months. No sarcomas were seen in 11 mice surviving ≥ 56 weeks of treatment, and hepatomas were observed in four of 11 (36%) mice dying after 82 weeks. In a similar experiment with 2-naphthylamine-HCl purified by gradient sublimation, no subcutaneous sarcomas were seen in ten mice surviving ≥ 58 weeks of treatment, and hepatomas were observed in four of 11 (36%) mice dying after 82 weeks. One (7%) hepatoma developed in 15 male and female control animals (Bonser *et al.*, 1956a).

Groups of 45 male and 45 female mice (from a local dealer, strain and age not specified) were given 2-naphthylamine (BDH, purified by distillation and crystallization via subcutaneous injection (0.1 mL of a freshly prepared or four-week-old 30-mg/ml suspension in arachis oil) twice weekly for 52 weeks (total dose, 312 mg per mouse). A total of 12 mice had subcutaneous sarcomas, seven mice had hepatomas, and two mice had intestinal tumours (one benign, one malignant) (Bonser *et al.*, 1956b). [The Working Group noted the lack of concurrent controls.]

Groups of 10 male and 10 female inbred A/St mice, 6–8 weeks of age, received intraperitoneal injections of 2-naphthylamine in tricaprylin. The injections were given three times per week for eight weeks. Doses per injection were 62.5, 125 or 250 mg/kg bw. At 24 weeks after the first injection the surviving mice were killed and the lungs were excised. The average number of lung tumours per mouse (1.38 ± 0.30) was statistically different ($P < 0.01$) from that in the corresponding solvent control (0.19 ± 0.10) in the mice treated with the highest dose of 2-naphthylamine (total dose 6 g/kg bw). The tumour yield in mice treated with 2-naphthylamine was not significantly different from that in the controls (Theiss *et al.*, 1981).

Three groups of 16 females and 16 males A/J mice, 6–8 weeks of age, received 2-naphthylamine (purity not specified) in tricaprylin by intraperitoneal injection three times per week for eight weeks, resulting, respectively, in total doses per animal of 120, 300 and 600 mg/kg bw. At 24 weeks, the incidence of lung tumours varied from 27% to 47%, which was not significantly different from the lung tumour rate in the vehicle controls (Stoner *et al.*, 1986).

A group of 91 newborn BALB/c mice received a single 0.02-mL subcutaneous injection containing 50 μg 2-naphthylamine (purity unspecified) in 1% aqueous gelatine on the first day of life. Among 71 surviving mice that were killed between the 36th and 43rd weeks, 15 (21%) had lung tumours [not significant], and one had a hepatoma. In a concurrent control group, 2/21 (10%) mice developed lung tumours (Roe *et al.*, 1963).

Twelve groups of 50–60 newborn BALB/c mice received subcutaneous injections of 100 μg 2-naphthylamine in 20 μL arachis oil once within 24 hours after birth, or once daily on the first five days of life. After 40 weeks, the numbers of pulmonary adenomas in the surviving mice in the two groups were not statistically different from those in the controls. In a second experiment, newborn BALB/c mice (number not specified) received subcutaneous injections of 100 μg 2-naphthylamine in 20 μl 3% aqueous gelatin once daily on the first five days of life. Surviving mice were killed at 50 weeks of age. Lung adenomas were seen in 9/41 (22%) surviving mice and in 4/48 (8%) controls, which was not significant. No other tumours were found. The failure of 2-naphthylamine to induce tumours in newborn mice was ascribed to immaturity of their metabolic system (Walters *et al.*, 1967).

A group of 68 newborn Swiss mice (sex unspecified) ($n = 68$) were given a single subcutaneous injection of 30 μL commercial, recrystallized 2-naphthylamine as 3% suspension in gelatin [purity unspecified] within 24 hours after birth. Ten months later, bronchogenic adenomas were found in six of 63 (10%) autopsied mice [not significant], and no tumours in 57 controls (Radomski *et al.*, 1971).

A group of 28 males and 23 females newborn Swiss mice were given subcutaneous injections of 30 μL commercial, recrystallized 2-naphthylamine as 3.3-mg/mL suspension in 3% gelatin (100 μg per dose) on the first, third and fifth day of life. Twelve months later, 2/26 (8%) autopsied male mice had hepatomas, and one of the 21 (5%) autopsied females had a lymphosarcoma. Among the controls (20 males, 30 females) one male mouse had a lymphosarcoma (Radomski *et al.*, 1971).

3.2.2 *Rat*

A group of 16 male Chester Beatty random inbred albino rats, weighing 200 g, received intraperitoneal injections of 50 mg/kg bw 2-naphthylamine freshly suspended in arachis oil twice weekly for three months. Thereafter, the rats were maintained until tumours were palpable or until death (survival period, 123–622 days). Of 14 rats examined, three had tumours: two (14%) had sarcomas, one (7%) had a salivary gland tumour (Boyland *et al.*, 1963). [The Working Group noted the absence of control rats.]

3.3 Topical application

3.3.1 *Mouse*

A group of twenty-five IF mice (sex not specified), weighing on average 25 g, received a dorsal dose of a saturated solution of 2-naphthylamine in benzene (applied by skin-painting; no details given) once a week for 99 weeks. No skin tumours or liver tumours were found (Bonser *et al.*, 1952). [The Working group noted the absence of control mice.]

3.4 Intravesicular implantation or instillation

3.4.1 *Mouse*

A group of 89 albino mice received an intravesicular implant of 2-naphthylamine (dose unclear) in paraffin wax. Forty-one mice received the compound after purification as in Bonser (1943), fourty-eight mice were given the compound after purification by gradient sublimation (Henson *et al*., 1954). After 40 weeks, eight mice (9%) had developed bladder carcinomas, four of which were invasive. This tumour yield was not significantly different from that in the control mice implanted with paraffin wax alone, but, according to the authors, the tumours were better established histologically (Bonser *et al.*, 1956c). In contrast, among 74 stock mice that survived 40 weeks after having received an intravesicular implant of a stearic-acid pellet containing 2-naphthylamine (dose unclear), no bladder adenomas, papillomas or carcinomas were found (Boyland *et al.*, 1964).

3.4.2 *Dog*

Four beagle dogs (age and sex unspecified) were given 10 mg commercial 2-naphthylamine (recrystallized before use) dissolved in 5 ml DMSO once every two weeks for 30 months. The solution was instilled by catheter, directly into the bladder lumen. No tumours were seen when the dogs were sacrificed at 45 months (Radomski *et al.*, 1971).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

4.1.1 *Humans*

Because of the known carcinogenicity of 2-naphthylamine in humans, few metabolic studies have been carried out. However, *N*-(2-naphthyl)-hydroxylamine and bis-(2-amino-

1-naphthyl) phosphate have been identified in the urine of some hospital patients given small doses of 2-naphthylamine (Troll & Nelson, 1961; Troll *et al.*, 1963).

Grimmer *et al.* (2000) analysed the amounts of the aromatic amines 1- and 2-naphthylamine and 2- and 4-aminobiphenyl in the urine of 48 German smokers and non-smokers. The results indicate that both groups excrete these four aromatic amines, with smokers excreting approximately twice as much (736 ng/24 hours *vs* 303 ng/24 hours). Similar amounts of urinary 2-naphthylamine and 4-aminobiphenyl were found in the two groups. The excreted aromatic amines decompose in the urine within a few hours, which explains why aromatic amines are difficult to detect in this matrix (in this study *para*-toluidine was added to the urine as a stabilizer). The origin of the aromatic amines found in the urine of non-smokers is unknown at present. Based on the cotinine levels in the urine of non-smokers, environmental tobacco smoke can be excluded as a major source of aromatic amines. In addition, neither diesel exhaust-related nitroarenes, nor the corresponding amino-derivatives to which they may be metabolically converted, were detected. The aromatic amines in urine arising from sources other than tobacco smoke or diesel exhaust may play a role in the etiology of bladder cancer in non-smokers.

4.1.2 *Animals*

Boyland (1958) and Boyland & Manson (1966) identified 24 metabolites of 2-naphthylamine in the urine of rats, rabbits, dogs or monkeys. Metabolism occurs along four main pathways: (i) *N*-hydroxylation followed by conversion to 2-amino-1-naphthyl-mercapturic acid, 2-nitrosonaphthalene and rearrangement to 2-amino-1-naphthol; (ii) oxidation at positions C5 and C6 to an arene oxide, which rearranges to 5-hydroxy-2-naphthylamine, reacts with water to form a 5,6-dihydroxydihydro derivative and forms a 5-hydroxy-6-mercapturic acid; (iii) conjugation of the amino group with acetic, sulphuric or glucuronic acid; (iv) secondary conjugation of the hydroxyl group with phosphate sulphuric or glucuronic acid. The proportion of these metabolites excreted in the urine of various experimental animals is different (Bonser *et al.*, 1951; Boyland & Manson, 1966; Conzelman *et al.*, 1969), although in most species 2-amino-1-naphthyl sulfate is the predominant metabolite. In early studies it was proposed that the *ortho*-hydroxylation metabolite 2-amino-1-naphthyl glucuronide was hydrolysed by β-glucuronidase present in urine, yielding 2-amino-1-naphthol (Allen *et al.*, 1957), which may act as the proximate carcinogen. The latter compound may be oxidized to form 2-amino-1-naphthoquinone, which can form DNA adducts (Beland & Kadlubar, 1985; Yamazoe *et al.*, 1985).

In dogs a small percentage of 2-naphthylamine was metabolized to bis-(2-amino-1-naphthyl) hydrogen phosphate (Boyland *et al.*, 1961; Troll *et al.*, 1963). *N*-Oxidation of 2-naphthylamine leads to more reactive metabolites (Deichmann & Radomski, 1969). Radomski & Brill (1971) showed that after a single oral dose of 70 mg/kg bw of both the carcinogenic 2-isomer and the weakly active or inactive 1-isomer of naphthylamine, the proportion of the dose converted into the corresponding *N*-hydroxylamine and nitroso

compound and excreted in the urine was about the same. When a dose of 5 mg/kg bw was given, the proportion of the 2-isomer converted to these metabolites in the urine remained about the same (0.2%), whereas the 1-isomer gave rise to barely detectable traces of these compounds. The unstable *N*-hydroxy metabolites are excreted in the urine as glucuronic acid conjugates and hydrolysed by acid or β-glucuronidase. These conjugates have been considered as the carcinogenic urinary metabolites of aromatic amines in the dog (Radomski *et al.,* 1973). Methaemoglobinaemia, which is a measure of *N*-hydroxy compounds in the blood, was higher in the dog with the 2-isomer at the 70-mg/kg bw dose (Radomski & Brill, 1971).

4.1.3 *In-vitro systems*

Dermal penetration of 2-naphthylamine and *ortho*-toluidine through human skin was studied by use of diffusion cells. Both compounds penetrate rapidly (lag time approximately 1.2 and 0.8 hours, respectively) and in high percentages (54 and 50%, respectively, of the applied dose within 24 hours) (Lüersen *et al.*, 2006).

Duverger-van Bogaert *et al.* (1991) evaluated the ability of the cytosol from human red blood cells to activate aromatic amines using the Ames test with *S. typhimurium* strain TA98 under liquid preincubation conditions. While negative results were obtained with 1-naphthylamine, a slight response was observed for 2-naphthylamine.

The cytosol from nine fresh autopsy specimens of human bladder tissue was analysed for the presence of *N*-acetyltransferase (NAT) activity towards *para*-aminobenzoic acid, 4-aminobiphenyl, 2-aminofluorene, and 2-naphthylamine. Apparent Km values indicated little difference in NAT affinity (100–300 microM) for any of the substrates between the nine individual bladders. However, the apparent V_{max} values showed that the bladders could be divided in rapid or slow acetylator phenotypes, based on their NAT activity towards 4-aminobiphenyl, 2-aminofluorene, and 2-naphthylamine. Four of the bladder cytosols had mean activities significantly higher (approximately 10-fold, $P < 0.01$) than the mean NAT activities of the other five bladder cytosols towards each arylamine carcinogen. However, no significant difference was detected in their NAT activities when *para*-aminobenzoic acid ws used as a substrate. The human bladder cytosols were also tested for their capacity to activate *N*-hydroxy-3,2'-dimethyl-4-aminobiphenyl to a DNA-binding electrophile through direct *O*-acetyltransferase (OAT)-mediated catalysis. The *N*-hydroxyarylamine OAT activity also discriminated between two levels of activation, being significantly higher (about twofold, $P = 0.0002$) in the rapidly *N*-acetylating bladder cytosols, which correlated ($r = 0.94$) with the measured levels of NAT activity in each cytosol. These results suggest that NAT activity and OAT activity of the human bladder vary concordantly with *N*-acetylator phenotype. The polymorphic expression of these acetylation activities may be important risk factors in human susceptibility to bladder cancer from arylamine carcinogens (Kirlin *et al.*, 1989).

The metabolic activation of 4-aminobiphenyl, 2-naphthylamine, and several heterocyclic amines has been shown to be catalysed by rat cytochrome P450ISF-G

[CYP1a2] and by its human ortholog, cytochrome P450PA [CYP1A2] (Butler *et al*., 1989a). In humans, hepatic microsomal caffeine 3-demethylation is the initial major step in caffeine biotransformation, which is selectively catalysed by cytochrome P450PA. Caffeine 3-demethylation was highly correlated with 4-aminobiphenyl *N*-oxidation ($r = 0.99$; $P < 0.0005$) in hepatic microsomal preparations obtained from 22 human organ donors, and both activities were similarly decreased by the selective inhibitor 7,8-benzoflavone. A rabbit polyclonal antibody raised against human cytochrome P450PA strongly inhibited these activities as well as the *N*-oxidation of the carcinogen 2-naphthylamine and other amines. Human liver cytochrome P450PA also catalysed caffeine 3-demethylation, 4-aminobiphenyl *N*-oxidation, and phenacetin *O*-deethylation. Thus, estimation of caffeine 3-demethylation activity in humans may be useful in the characterization of arylamine *N*-oxidation phenotypes and to assess whether or not the hepatic levels of cytochrome P450PA, as affected by environmental or genetic factors, contribute to inter-individual differences in susceptibility to arylamine-induced cancers (Butler *et al.*, 1989b).

Moore *et al.* (1984) investigated the metabolism of benzidine and 2-naphthylamine in organ cultures of human and rat bladder. There was little oxidative metabolism of either carcinogen in either species. In particular, *N*-hydroxy-2-naphthylamine, a proximate carcinogen of 2-naphthylamine could not be detected. In contrast, large amounts of the acetylated metabolites, *viz*. *N*-acetylbenzidine, *N,N*-diacetylbenzidine and *N*-acetyl-2-naphthylamine were formed both in rat and human bladder cultures.

Microsomal enzyme preparations from dog liver, kidney, and bladder were used to assess the prostaglandin H synthase-catalysed activation of carcinogenic aromatic amines to bind covalently to proteins and nucleic acids. Bladder transitional epithelial microsomes activated *ortho*-dianisidine, 4-aminobiphenyl, and 2-naphthylamine to bind to protein and tRNA, and benzidine and *ortho*-dianisidine to bind to DNA. Co-substrate and inhibitor specificities were consistent with activation by prostaglandin H synthase. Binding of benzidine to protein was not observed with either hepatic or renal cortical microsomes upon addition of arachidonic acid or reduced nicotinamide adenine dinucleotide phosphate. Prostaglandin H synthase and mixed-function oxidase-catalysed binding of 2-naphthylamine to protein and to tRNA were compared by use of liver and bladder microsomes. Only mixed-function oxidase-catalysed binding was observed in liver, and only prostaglandin H synthase-catalysed binding was seen in bladder. The rate of binding catalysed by bladder microsomes was considerably greater than that catalysed by hepatic microsomes. In addition, the bladder content of prostaglandin H synthase activity was approximately 10 times that in the inner medulla of the kidney, a tissue reported to have a relatively high content of this enzyme in other species. These results are consistent with involvement of bladder transitional epithelial prostaglandin H synthase in the development of primary aromatic amine-induced bladder cancer (Wise *et al.*, 1984)

Hammons *et al.* (1985) studied the in-vitro hepatic metabolism of 2-aminofluorene (2-AF), 2-naphthylamine and 1-naphthylamine by use of high-pressure liquid chromatography. Hepatic microsomes from rats, dogs, and humans were shown to

catalyse the *N*-oxidation of 2-AF and of 2-naphthylamine, but not of 1-naphthylamine; and the rates of *N*-oxidation of 2-AF were 2- to 3-fold greater than the *N*-oxidation rate of 2-naphthylamine. In each species, rates of 1-hydroxylation of 2-naphthylamine and 2-hydroxylation of 1-naphthylamine were comparable and were 2- to 5-fold greater than 6-hydroxylation of 2-naphthylamine or 5- and 7-hydroxylation of 2-AF. Purified rat hepatic monooxygenases, cytochromes P450UT-A, P450UT-H, P450PB-B, P450PB-D, P450BNF-B, and P450ISF/BNF-G but not P450PB-C or P450PB/PCN-E, catalysed several ring oxidations as well as the *N*-oxidation of 2-AF. Cytochromes P450PB-B, P450BNF-B, and P450ISF/BNF-G were most active; however, only cytochrome P450ISF/BNF-G, the isosafrole-induced isozyme, catalysed the *N*-oxidation of 2-naphthylamine. The purified porcine hepatic flavin-containing monooxygenase is known to carry out the *N*-oxidation of 2-AF, but only ring-oxidation of 1-naphthylamine and 2-naphthylamine was detected. No *N*-oxidation of 1-naphthylamine was found with any of the purified enzymes, which is in line with the fact that no carcinogenicity is observed with this compound. Furthermore, carcinogenic arylamines appear to be metabolized similarly in humans and experimental animals. Enzyme mechanisms accounting for the observed product distributions were evaluated by Hückel molecular-orbital calculations on neutral, free-radical, and cation intermediates. The authors proposed a reaction pathway that involves two consecutive one-electron oxidations to form a paired substrate cation-enzyme hydroxyl-anion intermediate that collapses to ring- and *N*-hydroxy products (Hammons *et al.*, 1985; see also Sasaki *et al*., 2002).

Boyd and Eling (1987) examined the oxidation of the bladder carcinogen 2-naphthylamine by prostaglandin H synthase (PHS) *in vitro*. Oxygen-uptake studies of 2-naphthylamine oxidation in the presence of glutathione, as well as extensive product analysis yielded data that were consistent with a one-electron mechanism of 2-naphthylamine oxidation by PHS. The formation of 2-nitroso-naphthalene was not observed under any condition. Metabolism studies with a purified PHS preparation confirmed that 2-naphthylamine oxidation is dependent on the peroxidase activity of the enzyme complex, and that a variety of organic hydroperoxides may support the reaction. Horseradish peroxidase oxidized 2-naphthylamine to the same products but, depending on the pH, in very different proportions from those obtained with PHS. Oxidation of 2-naphthylamine by a one-electron chemical oxidant resulted in a product profile similar to that obtained in the enzymatic systems. These results are consistent with a one-electron mechanism of 2-naphthylamine oxidation by PHS. The metabolism data also provide evidence for the formation of two types of potentially reactive electrophile: 2-imino-1-naphthoquinone and a free-radical species.

Poupko *et al.* (1983) studied microsome-mediated *N*-hydroxylation of 4-aminobiphenyl in mucosal tissue of bovine and canine bladder relative to the activity in liver. Bovine bladder microsomes mediated the *N*-hydroxylation of this amine at an exceptionally high rate, whereas no detectable activity was found with bovine liver microsomes. Dog-bladder microsomes were 40–100 times less active than bovine bladder microsomes and contained approximately one third the amount of cytochrome P450

(CYP). Dog liver microsomes were as active as dog bladder microsomes per nanomole CYP, and an order of magnitude more active when normalized to microsomal protein. Rat liver microsomes contained the highest level of CYP of all the preparations studied, and *N*-hydroxylase activity was approximately twice that in dog liver. Metabolic conversion of 4-ABP, 2-naphthylamine, and 1-naphthylamine into mutagens with S9 from bovine bladder mucosa was investigated in *Salmonella typhimurium* and found to parallel the carcinogenic potency of these compounds. These results demonstrate considerable tissue-, species-, and compound-specificity for the metabolic activation of aromatic amines, and provide further evidence in support of activation of the amines in the bladder as a mechanism of aromatic amine-induced bladder cancer (Poupko *et al.*, 1983).

4.2 Genetic and related effects

4.2.1 *Experimental systems*

(a) DNA adducts of 2-naphthylamine

Three DNA adducts are formed by the reaction of *N*-hydroxy-2-naphthylamine with DNA *in vitro* at pH 5.0 (Beland *et al.*, 1983; Beland & Kadlubar, 1985). The major adduct has been characterized as an imidazole ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-naphthylamine (50% of the total adducts); there were smaller amounts of 1-(deoxyguanosin-N^2-yl)-2-naphthylamine (30% of total adducts) and 1-(deoxyadenosin-N^6-yl)-2-naphthylamine (15% of total adducts). These same three DNA adducts were formed in target (urothelium) and non-target (liver) tissues of dogs two days after the oral administration of 2-naphthylamine (Beland & Kadlubar, 1985). A four-fold higher binding level of 2-naphthylamine was found in urothelial DNA compared with that in the liver. The major adduct in both tissues was the ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-naphthylamine; there were smaller amounts of 1-(deoxyadenosin-N^2-yl)-2-naphthylamine and 1-(deoxyguanosin-N^2-yl)-2-naphthylamine. The N^2-deoxyguanosine adduct persisted in the dog liver, and both this adduct and the ring-opened C8-deoxyguanosine adduct persisted in the bladder. The differential loss of adducts indicates that active repair processes are ongoing in both tissues, and the relative persistence of the ring-opened C8-deoxyguanosine adduct in the target but not the non-target tissue suggests that this adduct is a critical lesion for the initiation of urinary bladder tumours.

Peroxidative enzymes, such as prostaglandin H synthase (PHS), catalyse both the *N*-oxidation and ring-oxidation of 2-naphthylamine, a major ring-oxidation product being 2-amino-1-naphthol (Yamazoe *et al.*, 1985). When PHS was used to catalyse the binding of 2-naphthylamine to DNA, the same three adducts arising from *N*-hydroxy-2-naphthylamine were detected. In addition there were three other adducts, which appeared to be formed from 2-imino-1-naphthoquinone, the oxidative product of 2-amino-1-naphthol. The major product was characterized as N^4-(deoxyguanosin-N^2-yl)-2-amino-1,4-naphthoquinone-imine; two minor products were tentatively identified as

N^4-(deoxyadenosin-N^6-yl)-2-amino-1,4-naphthoquinone-imine and a deoxyguanosin-N^2-yl adduct of a naphthoquinone-imine dimer (Beland & Kadlubar, 1985; Yamazoe *et al.*, 1985). These DNA adducts, formed via peroxidation, accounted for approximately 60% of the total DNA binding that was observed by incubation of 2-naphthylamine with PHS *in vitro*. *In vivo*, in dogs, the DNA adducts derived from 2-imino-1-naphthoquinone accounted for approximately 20% of the 2-naphthylamine bound to urothelial DNA, but they were not detected in liver DNA (Yamazoe *et al.*, 1985). The remaining adduction products were derived from *N*-hydroxy-2-naphthylamine. Thus, PHS expressed in the bladder could play a significant role in bioactivation of arylamines directly in the bladder and could contribute to carcinogenesis of 2-naphthylamine and other arylamines that serve as substrates of PHS.

(b) Genotoxicity of 2-naphthylamine

Most of the genetic effects of 2-naphthylamine have been reviewed (Mayer 1982) and are summarized in Supplement 7 of the IARC Monographs (IARC, 1987).
2-Naphthyl-amine exhibits weak mutagenicity in the microbial assays and in some mammalian systems *in vitro*. Results for the mutagenicity of 2-naphthylamine in yeast assays have been inconsistent. In the assays *in vivo*, 2-naphthylamine was positive in the sex-linked recessive lethal assay with *Drosophila melanogaster*, but it failed to induce sister chromatid exchange in the mouse. Several micronucleus tests and sperm-abnormality assays with mice yielded inconclusive results. In the bone-marrow micronucleus assay, very high doses of 2-naphthylamine (200–800 mg/kg bw) were required to elicit effects in C57BL6 male mice (Mirkova & Ashby, 1988). 2-Naphthylamine was positive in the mammalian spot test in T stock mice given nine doses of 2-naphthylamine (50 to 100 mg/kg bw), but there was no clear dose-response effect (Chauhan *et al.*, 1983). Few other data exist about the mutagenic effects of 2-naphthylamine in whole organisms, although it is known to induce DNA fragmentation in rodent liver after treatment *in vivo* (Parodi *et al.*, 1981).

(i) *Bacterial mutagenesis*

The metabolic conversion of 2-naphthylamine (5–50 µg/plate) to a bacterial mutagen in *S. typhimurium* TA100 was catalysed much more efficiently by the hamster than by any of the other species tested (human, pig, rat, mouse). Mouse preparations displayed the weakest activity (Phillipson & Ioannides, 1983). In two other studies, the activities of liver-S9 preparations from rats pre-treated with PCBs or 3-methylcholanthrene (3-MC) were comparable to the activities of liver-S9 obtained from guinea-pigs pre-treated with the same CYP450 inducers in bio-activation of 2-naphthylamine (2.5–10 µg/plate) to a bacterial mutagen in strain TA100 (Baker *et al.*, 1980). Liver-S9 preparations from rats pre-treated with PCB or 3-MC also displayed comparable activity in bio-activation of 2-naphthylamine (10–50 µg/plate) in strain TA1535 (Bock-Hennig *et al.*, 1982). Bovine urinary bladder cells, but not hepatocytes, were able to activate 2-naphthylamine to bacterial mutagens in *S. typhimurium* TA98; the minimal amount of 2-naphthylamine

required to increase the level of revertants over background was 20 μg/plate (Hix *et al.*, 1983). Bovine bladder cells were also able to bioactivate 2-naphthylamine (20–80 μg/plate) to a mutagen in *S. typhimurium* strain TA100, with the activity being about six- to eight-fold higher than in TA98; however, bladder cells did not activate 2-naphthylamine (20 μg/ml) to a mutagen in hamster V79 cells when resistence to 6-thioguanine was used as a genetic marker (Oglesby *et al.*, 1983). The finding of bacterial mutagenicity of 2-naphthylamine (4–5000 μg/ml) was corroborated by bioactivation with rat-liver S9 (PCB-pretreatment) in the Ames II assay (Flückiger-Isler *et al.*, 2004).

Recombinant human CYPs 1A1, 1A2 and 1B1 were unable to activate 2-naphthylamine (5 μM) to a DNA-damaging agent, when the induction of *umu* was used as an endpoint, in *S. typhimurium* strain NM2009, which expresses multiple copies of bacterial *O*-acetyltransferase (Shimada *et al.*, 1996). 2-Naphthylamine (up to 100 μM) also failed to induce the *umu* response in *S. typhimurium* tester strains expressing human CYP1A1, 1A2, 1B1, 2C9, 2D6, 2E1 or 3A4 with bacterial *O*-acetyltransferase (Oda *et al.*, 2001). However, 2-naphthylamine (≥ 1 μM) in the presence of recombinant human CYP1A2 did induce the *umu* response in *S. typhimurium* tester strains NM6001 and NM6002, which expressed recombinant human acetyltransferase NAT1 and NAT2 isoforms, respectively, but not in strain NM6000, which is *O*-acetyltransferase-deficient (Oda 2004). The level of *umu* induction by 2-naphthylamine was about twofold higher in strain NM6001 than in NM6002 (Oda 2004). Purified rat CYP4B1 was the most efficient haemoprotein among 10 different CYP450s in bioactivating 2-naphthylamine (10 μM) and inducing the *umu* response in *S. typhimurium* NM2009 (Imaoka *et al.*, 1997).

In the *E. coli* K-12 *uvrB/recA* DNA-repair host-mediated assay in male NMRI mice, 2-naphthylamine (i.p. 200 mg/kg bw) elicited differential killing in *E. coli* retrieved from the kidney and testicles, but no activity was observed in blood, liver or lung. Surprisingly, 1-naphthylamine (i.p. 33 and 100 mg/kg bw) showed a quite different pattern, inducing differential killing of *E. coli* retrieved from blood, liver, lung and kidneys (Hellmér & Bolcsfoldi, 1992).

The results of studies on bacterial mutagenesis of 2-naphthylamine with recombinant CYP450 enzymes are inconsistent. This may be due to varying assay conditions and the presence of different phase-II enzymes that modulate the metabolism of 2-naphthylamine.

(ii) *Mammalian mutagenesis*

2-Naphthylamine (50 μg/mL and 100 μg/mL) in the presence of liver microsomes from PCB-pretreated rats was shown to increase the level of mutations by up to tenfold over background levels at five independent genetic loci in Chinese hamster ovary (CHO) cells (Gupta & Singh, 1982). Mutation induction was also observed at these loci in the absence of a metabolic activation system, indicating that the CHO cells are capable of bioactivating 2-naphthylamine (Gupta & Singh, 1982). However, 2-naphthylamine (20 μg/ml) was not mutagenic in hamster V79 cells, based on selection of 6-thioguanine-resistant mutants in the presence of bovine urinary bladder cells for bioactivation (Oglesby *et al.*, 1983).

(iii) *DNA damage induced by human carcinogens in cell-free assays*

2-Naphthylamine (0.2–1 mM) induced DNA damage—measured by ^{32}P-postlabelling—when calf-thymus DNA was co-incubated with 2-naphthylamine and liver microsomes from rats pretreated with phenobarbital or β-naphthoflavone as CYP inducers. However, the bioactivated 2-naphthylamine did not induce DNA fragmentation (Adams *et al.*, 1996).

5. Summary of Data Reported

5.1 Exposure data

2-Naphthylamine was formerly used as a dye intermediate and in the rubber industry; it is no longer used commercially and its manufacture has been banned in the European Union since 1998. 2-Naphthylamine can be formed by pyrolysis of nitrogen-containing organic matter. Recent analytical methods permit detection at the parts-per-billion level.

Occupational exposure occurred during production, during its use in the manufacturing of dyes, and when present as a contaminant in antioxidants used in rubber production. In workers exposed primarily to aniline and 4-chloroaniline, urinary levels of 2-naphthylamine of both smokers and non-smokers were higher than in non-smoking non-exposed workers. The rubber antioxidant *N*-phenyl-2-naphthylamine can be contaminated with 2-naphthylamine and may also be metabolised to 2-naphthylamine.

The major sources of exposure for the general population are environmental exposure to 2-naphthylamine and 2-nitronaphthalene, tobacco smoke and fumes from cooking oil.

5.2 Human carcinogenicity data

Numerous case series and 11 cohort studies (four in the USA, two in the United Kingdom, two in Japan, and one each in Italy, Poland and the Russian Federation) are available concerning bladder-cancer risks in workers engaged in the manufacture and use of 2-naphthylamine. All of these case series and cohort studies indicate that markedly elevated bladder-cancer risks are associated with the manufacture and use of 2-naphthylamine. In some of these studies, it was not possible to quantify the relative contributions of benzidine and 2-naphthylamine exposures in the overall excess risks. However, a United Kingdom study on bladder-cancer risks in the British rubber industry showed that the elimination of the antioxidants contaminated with 2-naphthylamine (without other changes in industrial practices) was enough to eliminate the excess risk for bladder cancer in the industry.

5.3 Animal carcinogenicity data

2-Naphthylamine was tested for carcinogenicity by oral administration in mice, rats, hamsters, rabbits, dogs and monkeys. Following oral administration, it induced bladder neoplasms in monkeys, dogs and hamsters, and liver tumours in mice. A low incidence of bladder carcinomas was observed in rats after oral administration. In lung-adenoma bioassays in mice by oral and intraperitoneal injection, 2-naphthylamine produced an increase multiplicity in lung tumours.

5.4 Other relevant data

2-Naphthylamine undergoes bioactivation by human and animal CYP450s to produce *N*-hydroxy-2-naphthylamine, which reacts with DNA to form several adducts. A second bioactivation pathway occurs with peroxidases to form 2-amino-1-naphthol. This metabolite can undergo oxidation to produce 2-imino-1-naphthoquinone, which reacts with DNA to form a unique adduct in the urothelium of experimental animals. Liver and bladder tissues from various species activate 2-naphthylamine to a genotoxicant *in vitro* in bacteria and mammalian cells. Both CYPs and peroxidases may play a significant role in inducing genetic damage of 2-naphthylamine in the urothelium.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of 2-naphthylamine. 2-Naphthylamine causes bladder cancer in humans.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of 2-naphthylamine.

6.3 Overall evaluation

2-Naphthylamine is *carcinogenic to humans (Group 1)*.

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ortho-TOLUIDINE

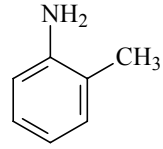
1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 95–53–4
CAS Name: 2-Methylbenzenamine
Synonyms: 1-Amino-2-methylbenzene; 2-amino-1-methylbenzene; 2-aminotoluene; *ortho*-aminotoluene; 2-methyl-1-aminobenzene; 2-methylaniline; *ortho*-methylaniline; *ortho*-methylbenzenamine; 2-methylphenylamine; 2-tolylamine; *ortho*-tolylamine

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



C_7H_9N Rel. mol. mass: 107.15

1.1.3 *Chemical and physical properties of the pure substance*

Description: Light yellow liquid becoming reddish brown on exposure to air and light (O'Neil, 2006)
Boiling-point: 200–201 °C (O'Neil, 2006)
Melting-point: −14.41 °C (Lide, 2008)
Solubility: Slightly soluble in water; soluble in diethyl ether, ethanol and dilute acids (O'Neil, 2006)
Octanol/water partition coefficient: log P, 1.29–1.32 (Verschueren, 2001)

1.1.4 *Technical products and impurities*

No information was available to the Working Group.

1.1.5 *Analysis*

ortho-Toluidine is the most widely studied of the aromatic amines reviewed in this Volume. Waste-water, air and urine samples have been analysed. The use of LC/MS and LC with electrochemical detection provides detection limits down to the nanomol level. *ortho*-Toluidine is one of the amines detected as a reduction product of azo dyes used as toy colourants. Table 1.1 presents selected methods of detection and quantification of *ortho*-toluidine in various matrices.

Table 1.1. Selected methods of analysis of *ortho*-toluidine in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	0.2 µg/g	Garrigós *et al.* (2002)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	37.9 µg/mL	Sutthivaiyakit *et al.* (2005)
Water	Dissolve in methanol (0.001 mol/L); dilute; add to deionized water	HPLC/ECD	1.94 nmol/L	Mazzo *et al.* (2006)

ECD, electrochemical detection; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; UV, ultraviolet

1.2 Production and use

1.2.1 *Production*

The synthesis of toluidines begins with a mixed-acid (nitric/sulfuric acid) mononitration of toluene, which produces all three isomers (*ortho*, *meta*, *para*), usually in the ratio 15:1:9, respectively. Since the isomeric toluidines themselves cannot be effectively separated by distillation, separation of the isomers is achieved at the nitrotoluene stage. The literature describes a multitude of methods for reducing nitrotoluenes to toluidines. These range from dissolving metal reductions to catalytic

hydrogenation. Currently, high-volume manufacture of toluidines utilizes the same type of continuous vapour-phase hydrogenation process used for aniline manufacture. The hydrogenation catalysts include various supported metals, Raney nickel, copper, molybdenum, tungsten, vanadium, and noble metals (Bowers, 2000).

Commercial production was first reported in the USA in 1922 for *ortho*-toluidine and in 1956 for *ortho*-toluidine hydrochloride (IARC, 1982, 2000). In the late 1970s, production volumes were estimated to be 1.1 to 11 million pounds/year, but this increased to 14.5 to 28.2 million pounds/year by the early 1990s (IARC, 2000). *ortho*-Toluidine hydrochloride has not been commercially produced in the USA since 1975 (HSDB, 2009).

Information was collected in 1994 in Europe for the IUCLID database for substances with a production or import volume greater than 1000 tonnes/year (High Production Volume Chemicals (HPVCs)). *ortho*-Toluidine was included on the list of HPVCs with a range of 100 000 to 500 000 tonnes (Allanou *et al.*, 1999; European Commission, 2000).

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the aggregate production volumes that were reported for *ortho*-toluidine.

Available information indicates that *ortho*-toluidine was produced and/or supplied in the following countries: Canada, France, Germany, Hong Kong Special Administrative Region, India, Italy, Japan, Mexico, the Netherlands, the People's Republic of China, South Africa, Switzerland, the United Kingdom, and the USA (Chemical Sources International, 2010).

Table 1.2. *ortho*-Toluidine production volumes

Year	Volume (in millions of pounds)
1986	>10–50
1990	>10–50
1994	>10–50
1998	>50–100
2002	>10–50
2006	10–<50

USEPA (2003, 2007)

1.2.2 *Use*

ortho-Toluidine, the isomer produced in greatest volume, has found many commercial applications. By far the single largest use for *ortho*-toluidine is in the preparation of 6-ethyl-*ortho*-toluidine, an intermediate in the manufacture of two very large-volume herbicides, metolachlor and acetochlor. Another important use for *ortho*-toluidine is in rubber chemicals, where it is used in the manufacture of a rubber antioxidant, and of di-*ortho*-tolylguanidine, a nonstaining rubber accelerator. Acetoacet-*ortho*-toluidine, 3-hydroxy-2-naphthoyl-*ortho*-toluidine, 2-toluidine-5-sulfonic acid, and *ortho*-aminoazotoluene are four of the more important dye and pigment intermediates manufactured from *ortho*-toluidine. In addition, *ortho*-toluidine is used to manufacture epoxy resin hardeners such as methylene-bis-2-methylcyclohexylamine, fungicide intermediates such as 2-amino-4-methylbenzothiazole, and *ortho*-fluorobenzoyl chloride. Certain pharmaceutical intermediates are also prepared starting with *ortho*-toluidine (Bowers, 2000).

Other minor uses of *ortho*-toluidine and its hydrochloride salt are as intermediates in organic synthesis and as ingredients in a clinical laboratory reagent for glucose analysis (HSDB, 2009).

1.3 Occurrence and exposure

1.3.1 *Natural occurrence*

ortho-Toluidine is not known to exist as a natural substance.

1.3.2 *Occupational exposure*

Occupational exposure to *ortho*-toluidine can occur during its production, or during the production of dyes, pigments and rubber chemicals manufactured from this compound. Laboratory and medical personnel may be exposed when using *ortho*-toluidine for staining tissues.

(a) Production of ortho-toluidine

In the former USSR (Khlebnikova *et al.*, 1970), air samples at a plant manufacturing *ortho*-toluidine from *ortho*-nitrotoluene (215 samples) generally exceeded the maximum permissible concentrations of 3 mg/m^3 (IARC 1982) by 2–7 times. The highest airborne exposure concentrations were observed during distillation and extraction processes (25–28.6 mg/m^3). Concurrently, in 80–90% of the air samples *ortho*-nitrotoluene levels exceeded the maximum permissible concentration of 1 mg/m^3: values up to approximately 5 mg/m^3 were reported. Dermal deposition of *ortho*-toluidine at 0.01–0.03 mg/dm^2 of skin was measured by collecting 1% acetic acid washes from the wrists, chest and back of individuals at the end of work-shifts (n = 168). After post-shift showers,

dermal levels of *ortho*-toluidine had decreased by a factor of 10. Patches of cloth placed on the workers' overalls collected about 0.10 mg/dm^2 ($n = 46$).

(b) Use of ortho-*toluidine in dye production*

Measurements in the 1940s in a dye-production plant in the United States indicated that the concentration of *ortho*-toluidine was below 0.5 ppm (2.19 mg/m^3) in the workroom air (breathing zone and area samples) and ranged from < 0.3 ppm to 1.7 ppm in the urine of workers engaged in the production of thioindigo. In addition to inhalatory exposure, exposure from ingestion and skin contact may have occurred (Ott & Langner, 1983).

Exposure to *ortho*-toluidine was reported to occur in an Italian plant producing fuchsin (magenta) and safranine T-based dyes (Rubino *et al.*, 1982), in a German plant producing 4-chloro-*ortho*-toluidine (Stasik, 1988), and in a plant producing azo-dyes in New Jersey, USA (Delzell *et al.*, 1989). No data on exposure levels were provided.

(c) Production and use of rubber antioxidant

ortho-Toluidine, aniline, hydroquinone and toluene were used to synthesize a rubber antioxidant in a chemical plant in the United States. Despite low air concentrations (< 1 ppm; 4.38 mg/m^3), elevated pre-shift urinary *ortho*-toluidine levels of 18 ± 27 µg/L ($n = 46$) and post-shift levels of 104 ± 111 µg/L were detected. The average concentration of *ortho*-toluidine in the pre-shift samples was 17 times higher than that in urine samples of unexposed workers (Ward *et al.*, 1991; Teass *et al.*, 1993).

Sorahan *et al.* (2000) reported worker exposure to *ortho*-toluidine in a plant that produced rubber chemicals in the United Kingdom, but no data on exposure concentrations were provided.

Korinth *et al.* (2006) reported on four workers involved in vulcanising hydraulic rubber articles, who were exposed to *ortho*-toluidine contained in di-*ortho*-tolylguanidine (used as an accelerator for the vulcanization of rubber products) by dermal absorption. The concentrations of *ortho*-toluidine in the air at the workplace ranged between 26.6–93.9 µg/m^3 and *ortho*-toluidine concentrations in urine were between 54.7–242.9 µg/L.

(d) Demolition workers of an SO_2 plant polluted with ortho-*toluidine*

Labat *et al.* (2006) measured urinary *ortho*-toluidine levels in workers employed in the demolition of a liquid-SO_2 plant polluted with *ortho*-toluidine. The plant had stopped production 20 years earlier. For unexposed workers, urinary concentrations of *ortho*-toluidine ranged between 0.17 and 2.46 µg/g creatinine. Post-shift urinary concentrations for exposed workers ranged between 26.1 and 462 µg/g creatinine. After protective measures were taken, this decreased to 2.4–20.1 µg/g creatinine.

(e) Laboratory workers

Although medical and laboratory personnel represent a significant population of workers potentially exposed to *ortho*-toluidine, air concentrations have been determined to be below 22 µg/l (Environmental Protection Agency, 1984). Kauppinen *et al.* (2003) reported that in 1988 in Finland, five of 26 pathological laboratories used *ortho*-toluidine for staining tissues with a median use of 10 g/year, three of 30 clinical laboratories used it with a median use of 180 g/year, and six of 20 other laboratories with a median use of 10 g/year.

1.3.3 *Environmental occurrence and exposure of the general population*

The general population is known to be exposed ubiquitously to *ortho*-toluidine, although its origin is not known. The levels found are not explained by known exposures.

(a) Air

According to the Toxics Release Inventory (Environmental Protection Agency, 1997), air emissions of *ortho*-toluidine from 23 industrial facilities were approximately 5260 kg in 1995 in the United States.

(b) Water

Surface-water discharges of *ortho*-toluidine from 23 industrial facilities in the United States, as reported in the Toxics Release Inventory, decreased from 242 kg in 1994 to 116 kg in 1995 (Environmental Protection Agency, 1996, 1997).

ortho-Toluidine has been reported to be present in surface-water samples taken from three rivers in Germany, at levels of 0.3–1 µg/L (Neurath *et al.*, 1977). *ortho*-Toluidine has been identified in one secondary effluent from seven industrial and publicly owned treatment works (Ellis *et al.*, 1982) and in wastewaters from synthetic fuel production (Leenheer *et al.*, 1982; Stuermer *et al.*, 1982). It has also been detected in effluents from refineries and chemical production facilities, in process water, river water, groundwater and seawater in the United States (Shackelford & Keith, 1976; Environmental Protection Agency, 1984; NTP, 2005).

(c) Soil

Soil discharges of *ortho*-toluidine from 23 industrial facilities in 1995 in the United States amounted to 5.5 kg, as reported in the Toxics Release Inventory (Environmental Protection Agency, 1997). An estimated 10 000 kg of *ortho*-toluidine were released via underground injection.

(d) Food and beverages

Neurath *et al.* (1977) found unspecified isomers of toluidine in commercially available samples of kale and celery (1.1 mg/kg) and carrots (7.2 mg/kg). *ortho*-Toluidine has been identified in the volatile aroma components of black tea (Vitzthum *et al.*, 1975).

DeBruin *et al.* (1999) measured *ortho*-toluidine at part per billion levels in breast-milk samples from both smokers and nonsmokers (< 0.01 to 0.26 ppb). The concentrations observed did not correlate with smoking status, suggesting that tobacco smoking is not the main source of exposure.

(e) Tobacco smoke

Mainstream smoke from eight US commercial cigarette brands has been found to contain 8.6–144.3 ng *ortho*-toluidine per cigarette (Stabbert *et al.*, 2003). The concentrations of *ortho*-toluidine tend to be much higher in sidestream smoke than in mainstream smoke (IARC, 2004).

Riedel *et al.* (2006) reported urinary excretion of *ortho*-toluidine at 70.1–139.6 ng/24 hours (mean ± SD, 105.2 ± 25.6) for nine nonsmokers and 107.9–258.7 (mean ± SD, 204.2 ± 59.1) for 10 smokers. Higher amounts were reported in an earlier study (El-Bayoumy *et al.*, 1986), with a mean *ortho*-toluidine excretion of 4.1 µg/24 hours in 12 nonsmokers and 6.3 µg/24 hours in 16 smokers.

In a study by Riffelmann and colleagues (1995), urinary concentrations of *ortho*-toluidine in eight smokers averaged 1.7 ± 1.6 µg/L, while it was not detected in eight non-smokers. In another study, current smoking had no influence on background values and on the increase of *ortho*-toluidine adducts to haemoglobin (Gaber *et al.*, 2007).

(f) Hair dyes

In a study from Turkey (Akyüz & Ata, 2008), *ortho*-toluidine was found in 34 of the 54 hair dyes tested, at quantities up to 1547 µg/g.

(g) Prilocaine

Prilocaine, a widely used anaesthetic, is metabolized to *ortho*-toluidine. A component of EMLA cream, it is also used as a pain reliever in neonates (Taddio *et al.*, 1998), during circumcision (Taddio *et al.*, 1997) and during venipuncture in children (Tak & van Bon, 2006).

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Directive 97/56/EC

According to Directive 97/56/EC on the restrictions on the marketing and use of certain dangerous substances and preparations, the packaging of *ortho*-toluidine and its

preparations must be marked legibly and indelibly as follows: "Restricted to professional users" (European Commission, 1997).

(b) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes that, by reductive cleavage of one or more azo groups, may release *ortho*-toluidine in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the test method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(c) Directive 2004/37/EC

ortho-Toluidine is regulated by the Directive 2004/37/EC (European Commission, 2004), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

1.4.2 *Germany*

Deviating from the EU classification, *ortho*-toluidine is classified as a Category 1 carcinogen by the German MAK Commission (MAK, 2007). The MAK Commission listed *ortho*-toluidine as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set. Furthermore, it was classified as a germ-cell mutagen, Class 3A: substances that have been shown to induce genetic damage in germ cells of humans or animals, or that produce mutagenic effects in somatic cells of mammals *in vivo* and have been shown to reach the germ cells in an active form.

1.4.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of *ortho*-toluidine. An occupational exposure limit (OEL) of 1 ppm (4.4 mg/m^3) was reported; skin absorption was noted.

1.4.4 *United Kingdom*

The Health and Safety Commission (HSE, 2007) has set an 8-hour time-weighted average Workplace Exposure Limit (WEL) of 0.2 ppm (0.89 mg/m^3) for *ortho*-toluidine, with a skin notation. HSE also lists *ortho*-toluidine as capable of causing cancer.

1.4.5 *USA*

(a) ACGIH

ortho-Toluidine has been assigned an A3 notation, *Confirmed Animal Carcinogen with Unknown Relevance to Humans* (ACGIH, 2001). A TLV-TWA (threshold limit value–time-weighted average) of 2 ppm (8.8 mg/m^3) is recommended. Reported significant percutaneous absorption and its systemic health effects warrant assigning the skin notation (*potential significant contribution to the overall exposure by the cutaneous route*). A methaemoglobin level in blood of 1.5% of total haemoglobin measured during or at the end of the shift is recommended as a Biological Exposure Index (BEI) for biological monitoring of exposure to *ortho*-toluidine.

(b) NTP

ortho-Toluidine and *ortho*-toluidine hydrochloride are listed in the NTP *Report on Carcinogens* as *reasonably anticipated to be human carcinogens* (NTP, 2005).

1.4.6 *Other*

(a) GESTIS

Table 1.3 presents some international limit values for *ortho*-toluidine (GESTIS, 2007).

Table 1.3. International limit values (2007) for *ortho*-toluidine

Country	Limit value: Eight hours		Limit value: Short-term		Comments
	ppm	mg/m³	ppm	mg/m³	
Austria	0.1	0.5	0.4	2	TRK value (based on technical feasibility)
Belgium	2	8.9			
Canada, Québec	2	8.8			
Denmark	2	9	4	18	
France	2	9			
Hungary				0.5	
Poland		3		9	
Spain	0.2 (skin)	0.89 (skin)			
Switzerland	0.1	0.5			
USA, OSHA	5	22			
United Kingdom	0.2	0.89			

OSHA, Occupational Safety and Health Administration; TRK, technical guiding concentration

2. Studies of Cancer in Humans

2.1 Case reports

Uebelin and Pletscher (1954) studied the occurrence of bladder tumours in workers employed at a factory in Switzerland producing dyestuff intermediates. A total of 97 cases were found in 300 workers exposed to various combinations of 2-naphthylamine, benzidine and other aromatic amines for unspecified periods during 1924–53. A further 650 workers had been exposed only to aniline or other aromatic amines, but not to 2-naphthylamine or benzidine; three of these men developed bladder tumours, but their exact exposures were uncertain. The authors distinguished a subgroup of 35 men who prepared 4-chloro-*ortho*-toluidine from *ortho*-toluidine; no bladder tumours were found among these men. [The Working Group noted that insufficient details were provided concerning person–years at risk or follow-up to evaluate the significance of these observations.]

Gropp (1958) described 98 cases of bladder tumours that occurred from 1903 to 1955 among workers at a German factory. Oettel (1959, 1967) and Oettel *et al.* (1968) also reported these cases and some additional ones. Most of Gropp's cases had had exposure to 2-naphthylamine alone (23 men) or together with other amines, including benzidine, aniline and toluidines (45 men). However, 11 cases were reported as having had exposure only to aniline/toluidine; the population at risk is not known. [The Working Group noted that insufficient details were provided concerning person–years at risk or follow-up to evaluate the significance of these observations.]

Khlebnikova *et al.* (1970) reported bladder tumours in workers engaged in the production of *ortho*-toluidine and/or *para*-toluidine in the USSR during the 1960s. The concentration range of *ortho*-toluidine measured in the air of the working environment was 0.5–28.6 mg/m^3. Two cases of bladder tumours (papillomas) were found when 75 of 81 current toluidine-exposed workers were examined cystoscopically. The 81 workers comprised 35 operators, 18 fitters, and 10 cleaners. A total of 41 workers had worked in *ortho*-toluidine production for more than five years; most of these workers had also been in contact with *para*-toluidine. Six other cases of bladder tumours (including four carcinomas) had been found earlier, upon cystoscopic examination of 16 former workers who had worked with the toluidines for periods ranging from 12 to 17 years. [The Working Group noted that the information contained in the paper with regard to person–years of exposure and to the other substances to which these workers may have had prior or concomitant exposure is insufficient to evaluate the significance of these observations].

Lipkin (1972), in a further report from the USSR, referred to 27 cases of bladder cancer occurring in individuals exposed occupationally to *ortho*- and *para*-toluidine, and to 21 cases in workers exposed to both these chemicals and to 1-naphthylamine. Genin *et al.* (1978) referred to 36 people with bladder cancer who had been occupationally exposed to *ortho*- and *para*-toluidine and to "some other aromatic amino and nitro compounds" in a chemical plant. The cancers were diagnosed in men and women aged

between 25 and 61. [The Working Group noted that no information was given on the sizes of the populations at risk. It is possible, but not explicitly stated, that these cases included some or all of those reported by Khlebnikova *et al.* (1970).]

Conso and Pontal (1982) reported three cases of bladder cancer occurring among 50 workers employed in a factory where *para*-toluenediamine was synthesized from *ortho*-toluidine and *ortho*-aminoazotoluene. [The Working Group noted that although a formal estimate of expected numbers of bladder cancer cases was not provided, three bladder cancers among 50 persons is likely to represent a substantial excess. The relationship of these bladder cancers to *ortho*-toluidine exposure cannot be determined because of co-exposure to *ortho*-aminoazotoluene, which has been classified as possibly carcinogenic to humans (IARC, 1975, 1987).]

2.2 Cohort studies (see Table 2.1)

Case and Pearson (1954) and Case *et al.* (1954) reported a historical cohort study of bladder tumours among workers in the British chemical industry engaged in the manufacture of dyestuff intermediates. Groups were defined in terms of exposure to aniline, benzidine, 1- and 2-naphthylamine, auramine and magenta. Many aniline-exposed workers were also exposed to toluidines produced in the same plants. The 21 firms in the survey included the major British producers of aniline, and mortality was observed from 1921 to 1952. There were 812 men classified as having had exposure to aniline but not to the other suspected carcinogens. The death certificate for one of these workers mentioned a bladder tumour; 0.52 such deaths were expected on the basis of national rates [The Working Group noted that exposure of these workers to *ortho*-toluidine is not specified in this paper but can be inferred from knowledge of aniline-production processes].

Rubino *et al.* (1982) reported mortality findings for 906 men first employed in a dyestuff factory in northern Italy between 1922 and 1970 and followed from 1946 to 1976. Mortality was compared with that of the Italian male population. Follow-up was 96% complete and 260 deaths were identified. Thirty-six deaths from urinary bladder cancer were observed (SMR, 29.3; 95% CI, 20.5–40.5). Thirty-one of these deaths occurred among 610 men with exposure to one or more of benzidine, 1-naphthylamine, and 2-naphthylamine (SMR, 35.6; 95% CI, 24.2–50.6). Some of these workers were also exposed to *ortho*-toluidine, among other chemicals, and a significant excess of bladder cancer mortality was observed in the 53 men engaged solely in the manufacture of magenta (IARC, 1993) and safranine T, also involving exposure to one or more of the following chemicals: toluene, *ortho*-nitrotoluene and 4,4′-methylene bis(2-methylaniline), 2,5-diaminotoluene, *ortho*-aminoazotoluene and aniline (5 deaths; SMR, 62.5; 95% CI, 20.3–145.9). No quantitative exposure measurements or data on smoking were available. [The Working Group noted that the clear occupational excess of bladder cancer could not be specifically attributed with certainty to exposure to *ortho*-toluidine or to any one of the other compounds involved.]

Table 2.1. Summary of principal cohort studies of workers exposed to *ortho*-toluidine

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
Industry	Dyestuff production	Dye production	4-Chloro-*ortho*-toluidine production and processing	Rubber chemicals	Rubber chemicals
Size of the total cohort	906 men	342 men	116 men	1749 (1643 men)	2160 men
Cohort definition	First employed 1922–70	Some employment during 1940–58	Employed before 1970 >1929–82 (mortality) <1967–86 (incidence)	Employed 1946–88	Employed ≥ six months; some employment between 1955–84
Period of follow-up	1946–76	1940–75		1946–94 (mortality) 1973–88 (incidence	1955–96 (mortality) 1971–92 (incidence)
Deaths					
All causes					
N	260	118	19	[190]	1131
SMR (95% CI)	1.5 [1.4–1.7]	1.0 [0.8–1.2]	1.1 (0.7–1.7)	[0.9] [0.8–1.0]	1.0 (1.0–1.1)

Table 2.1 (contd)

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
All cancers					
N	96	27	5	[49]	305
SMR (95% CI)	2.7 [2.1–3.2]	1.3 [0.8–1.8]	1.5 (0.5–3.4)	[1.0] [0.7–1.3]	1.0 (0.9–1.1)
Bladder cancer mortality					
N	36	0	0	2	17
SMR (95% CI)	29.3 [20.5–40.5]	1.5 urinary cancers expected	0.5 urogenital cancers expected	[2.1] [0.2–7.4]	1.4 (0.8–2.3)
Bladder cancer incidence					
N			8	13	19
SIR (95% CI)			72.7 (31.4–143.3)	3.6 [1.9–6.2]	1.1 (0.6–1.7)
Subgroup exposed to ortho-toluidine					
Size	53	117	Same as full cohort	708	53
Bladder cancer mortality					
N	5	0	0	1	3
SMR (95% CI)	62.5 [20.3–145.9]	[expected number N/A]	0.5 urogenital cancers expected	3.8 [0.1–21.1]	(15.9) [3.3–46.4]

Table 2.1 (contd)

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
Bladder cancer incidence *N* SIR (95% CI)			8 72.7 (31.4–143.3)	7 6.5 [2.6–13.3]	
Other exposures among subgroup exposed to *ortho*-toluidine[a]	4,4′-Methylene bis (2-methylaniline) (2B) Magenta (2B) Safranine T *ortho*-Nitrotoluene (3) 2,5-Diaminotoluene (3) Aniline (3) *ortho*-Aminoazotoluene (2B) Toluene (3)	Multiple exposures including 4-chloro-*ortho*-toluidine (2A) and 4-chloroacetyl-*ortho*-toluidine	*N*-Acetyl-*ortho*-toluidine 6-Chloro-*ortho*-toluidine 4-Chloro-*ortho*-toluidine (2A)	Aniline (3) Hydroquinone (3) Toluene (3) Carbon disulfide Sulfur Benzothiazole 4-Aminobiphenyl (contaminant) (1) 2-Mercaptobenzothiazole (Ward *et al.*, 1996) Nitrobenzene (2B) (Viet *et al.*, 2009)	Aniline (3) 2-Mercaptobenzothiazole Phenyl-β-naphthylamine (3)

[a] Previous IARC overall evaluations of carcinogenicity are given in parentheses.
N, number; SMR, standardized mortality ratio; SIR, standardized incidence ratio

Ott and Langner (1983) studied the mortality of 342 male employees assigned to three aromatic amine-based dye-production areas from 1914 to 1958 at a chemical plant in the United States. All study subjects were currently working as of 1st January 1940 or hired after that date. Expected numbers of deaths for the period 1940–1975 were based on mortality rates for US white males. There were no deaths from bladder cancer, with 1.5 deaths expected from malignant neoplasms of the urinary organs. In one area of the plant, 117 men produced bromindigo and thioindigo with potential exposure to 4-chloro-*ortho*-toluidine and other raw materials and intermediates, including *ortho*-toluidine. No bladder cancer deaths occurred in this subcohort. [The expected figure was not reported.] There was one death from lymphoma [SMR, 0.83] and two deaths from leukaemia [SMR, 2.22]. [The Working Group noted that the interpretation of this study was limited by the small size of the population exposed to 4-chloro-*ortho*-toluidine, and by the ascertainment of deaths only.]

Stasik (1988) reported findings from a mortality study of 335 male workers employed for at least twelve months in the period 1929–1982 in the production and processing of 4-chloro-*ortho*-toluidine at a dyestuffs-manufacturing plant in Frankfurt, Hessen, Germany. Three other monocyclic amines had been used at the plant: *N*-acetyl-*ortho*-toluidine, 6-chloro-*ortho*-toluidine and *ortho*-toluidine. Exposure to 4-chloro-*ortho*-toluidine was reported to be predominant. No deaths from bladder cancer were identified in the period 1929–1982 [expected figure unspecified but estimated on the basis of mortality rates for the Federal Republic of Germany to be about 0.2.] [The Working Group noted that the study had inadequate tracing of deaths and description of methods.] Two incident cases of urothelial carcinoma were subsequently identified in this workforce in workers employed in the 4-chloro-*ortho*-toluidine production plant before improvements in industrial hygiene were made in 1970. As a result of this discovery, a cancer incidence study was established for the 116 subjects employed at this plant before 1970. There was no cancer registry for the region in which the plant was located; expected numbers were therefore based on rates for Saarland, a neighbouring province of the Federal Republic of Germany. A marked excess of bladder cancer cases based on eight cases was reported (SIR, 72.7; 95% CI, 31.4–143.3). No quantitative measure of exposure to 4-chloro-*ortho*-toluidine was available, and exposure to other amines was also present. Median exposure time of the cases before 1970 was 14 years. Three of the eight cases were nonsmokers, one was a former smoker, two were smokers and the smoking habits of the remaining two were unknown. [The Working Group noted that the definition of the subcohort for the cancer incidence survey was made *a posteriori,* the obervational period was unspecified, and case ascertainment was inadequately described. Consequently, some bias in the estimate of excess risk may be present. The excess of bladder cancer could not be attributed with any certainty to *ortho*-toluidine or to any one of the other compounds present.]

Ward *et al.* (1991) reported findings for bladder cancer incidence in workers exposed to *ortho*-toluidine and aniline at a US chemical-manufacturing plant. The study was initiated at the request of union representatives who had noted several bladder cancers

among workers in the department that manufactured chemicals for the rubber industry. Among the major reactants used in these processes were two primary aromatic amines, *ortho*-toluidine and aniline; other reactants and intermediates included 2-mercaptobenzothiazole, hydroquinone, toluene, carbon disulfide, benzothiazole and a proprietary chemical, later identified as nitrobenzene (Ward *et al.*, 1991; Ward *et al.*, 1996; Viet *et al.*, 2009). The study cohort comprised 1749 workers (1643 males, 106 females) employed at the plant in the period 1946–1988. A total of 708 of these workers were considered to have been definitely exposed to *ortho*-toluidine and aniline; 288 workers assigned to maintenance, janitorial, yard work and shipping departments were considered to have been possibly exposed, and the remaining 753 workers were considered to be probably unexposed. Vital status was identified for 1973 through 1988. Bladder-cancer cases were identified from company or union records and confirmed through medical records, or through matching with records at the local cancer registry. Expected numbers of incident bladder cancers were based on local incidence rates. Overall, 13 cases of bladder cancer were observed for the period 1973–1988 (SIR, 3.6; 95% CI, 1.9–6.2), seven of which occurred in the definitely exposed group (SIR, 6.5; 95% CI, 2.6–13.3), four in the possibly exposed group (SIR, 3.7; 95% CI, 1.0–9.4) and the remaining two cases in non-exposed workers (SIR, 1.4; 95% CI 0.2–5.0). Bladder-cancer incidence was particularly elevated in employees who had worked in the exposed department for more than 10 years; six of the seven exposed cases occurred in this subcohort (SIR, 27.2; 95% CI, 10.0–59.2). Data on smoking were available for only 143 study subjects but suggested that confounding from smoking could explain no more than a small fraction of the observed bladder-cancer excess. Other chemical exposures at the plant should be considered. Aniline was present but is not known to induce bladder cancer in humans or animals (IARC, 1987). 4-Aminobiphenyl was identified as a potential low-level contaminant (< 1 ppm) in some bulk samples of process chemicals at the plant in 1990 (Ward & Dankovic, 1991). 4-Aminobiphenyl is an IARC Group-1 carcinogen and is known to be a highly potent human bladder carcinogen (IARC, 1972, 1987). 2-Mercaptobenzothiazole has not been reviewed by IARC, but has shown some evidence of carcinogenicity in rats and equivocal evidence of carcinogenicity in mice (NTP, 1988). An exposure-assessment study conducted at the plant in 1990 (Ward *et al.*, 1996) showed substantially higher urinary concentrations and levels of haemoglobin adducts of *ortho*-toluidine and aniline among exposed workers compared with in-plant controls. Levels of 4-aminobiphenyl adducts were much lower than those of *ortho*-toluidine and aniline, and were similar for exposed and unexposed groups. [The Working Group noted that haemoglobin adducts reflect only recent exposures (Hemminki, 1992), and it is therefore possible that higher levels of 4-aminobiphenyl contamination existed in the past.] A subsequent mortality analysis of the same cohort for the period 1946–1994 (Prince *et al.*, 2000) found only two deaths from bladder cancer in the total cohort (SMR, 2.1; 95% CI, 0.2–7.4). One of these deaths occurred in the definitely exposed group (SMR, 3.8; 95% CI, 0.1–21.1). There were two deaths from lymphohaematopoietic cancers in the definitely exposed group (SMR, 1.2; 95% CI, 0.1–4.2). A further 19 cases

of bladder cancer occurring in this cohort, 18 of which were diagnosed in the later period of 1989–2003, have been identified (Markowitz & Levin, 2004; Markowitz, 2005). Ten of these cases were stated to be members of the definitely exposed subgroup. [The Working Group noted that the authors did not have access to the study master files and were not able to provide expectations for their findings. Nevertheless, this article indicates a continuing excess of occupational bladder cancer at the plant under study.]

Sorahan *et al.* (2000) updated a study of workers exposed to several aromatic amines in a factory manufacturing chemicals for the rubber industry in the United Kingdom (Sorahan & Pope, 1993). All subjects had had at least six months' employment in the factory and some employment in the period 1955–1984. Mortality was examined for the period 1955–1996 and cancer incidence for the period 1971–1992. The updated study included 2160 male production workers, 605 of whom had been exposed to one or more of the four chemicals under investigation (aniline, 2-mercaptobenzothiazole, phenyl-β-naphthylamine, *ortho*-toluidine), including 53 workers who were exposed to *ortho*-toluidine. In the latter subcohort, three bladder cancer deaths were observed (SMR, 15.9; 95% CI, 3.3–46.4). A total of 30 bladder cancers were identified in the overall cohort on the basis of death certificate or cancer registration data. Internal analysis (Poisson regression) revealed a significant association between the risk for bladder cancer and duration of exposure to *ortho*-toluidine (1–4 years, $n = 2$; RR, 6.7; 95% CI, 1.6–28.4; ≥ 5 years, $n = 1$; RR, 7.7; 95% CI, 1.0–56.9).

[The Working Group noted that individual smoking habits were not available for all subjects in any of the published cohort studies. However, the excesses of bladder cancer reported in the four positive studies were much too large to have been due to smoking alone.]

3. Studies of Cancer in Experimental Animals

Studies in experimental animals of carcinogenicity of *ortho*-toluidine and its hydrochloride salt were previously reviewed by IARC (1978, 1982, 1987, 2000). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.1 Oral administration

3.1.1 *Mouse*

Groups of 25 male and 25 female Swiss CD-1 mice, 6–8 weeks of age, were treated with *ortho*-toluidine hydrochloride (purity, 97–99%) in the diet at levels of 16 000 or 32 000 mg/kg diet (ppm) for three months and then, due to toxicity, at levels of 8000 or 16 000 ppm for a further 15 months. Subsequently, the animals were kept without treatment for an additional three months and then killed. The doses were chosen on the

basis of preliminary tests, the higher being the maximum tolerated dose. A simultaneous control group of 25 untreated mice of each sex was used, plus additional controls used for the other compounds tested in the study, and tumour incidences of matched and pooled controls were compared statistically (both separately and together) with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. In male mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined, observed in abdominal viscera) was 0/14, 5/99 (5%), 5/14 (36%) ($P < 0.025$, Fisher exact test) and 9/11 (82%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively. In female mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined, observed in abdominal viscera) was 0/15, 9/102 (9%), 5/18 (28%) ($P < 0.05$, Fisher exact test) and 9/21 (43%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively [the separate incidences for haemangiomas and haemangiosarcomas were not reported] (Weisburger *et al.*, 1978).

Groups of 50 male and 50 female $B6C3F_1$ mice, six weeks of age, were given *ortho*-toluidine hydrochloride (purity > 99%) in the diet at 1000 or 3000 ppm for 102–103 weeks. Concurrent control groups consisted of 20 male and 20 female untreated mice. The mean body weights of both treated males and females were lower than those of the corresponding controls, and were dose-related. Mortality was not significantly related to treatment in either sex. In male mice, the incidence of haemangiomas or haemangiosarcomas (combined, all sites, mainly observed in the abdominal viscera) was increased: 1/19 (5%), 2/50 (4%) and 12/50 (24%) ($P < 0.002$, Cochran–Armitage trend test) in control, low-dose and high-dose groups, respectively. In female mice, the incidence of hepatocellular adenomas or carcinomas (combined) was also increased: 0/20, 4/49 (8%) and 13/50 (26%) ($P < 0.007$, Fisher exact test; $P = 0.001$ trend test) in control, low-dose and high-dose groups, respectively (National Cancer Institute, 1979).

3.1.2 *Rat*

Groups of 25 male Sprague-Dawley CD rats, 6–8 weeks of age, were treated with *ortho*-toluidine hydrochloride (purity 97–99%) in the diet at dose levels of 8000 or 16 000 ppm for three months and then, due to toxicity, at levels of 4000 or 8000 ppm for a further 15 months. Animals were kept without treatment for an additional six months and then killed. The doses were chosen on the basis of preliminary tests, the higher being the maximum tolerated dose. A concurrent control group of 25 untreated male rats was used, plus additional controls used for the other compounds tested in the study, and tumour incidences of matched and pooled controls were compared with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. The incidence of subcutaneous fibromas and fibrosarcomas (combined) was 0/16, 18/111 (15%), 18/23 (78%) ($P < 0.025$, Fisher exact test) and 21/24 (88%) ($P < 0.025$, Fisher exact test, compared with all controls) in simultaneous controls, pooled controls, low-dose and high-dose groups, respectively. A non-statistically significant

increase in the incidence of transitional-cell carcinomas of the urinary bladder was also observed: 0/16, 5/111 (5%), 3/23 (13%) and 4/24 (17%) in simultaneous controls, pooled controls, low-dose and high-dose groups, respectively (Weisburger *et al.*, 1978).

Groups of 30 male Fischer 344 rats, eight weeks of age, were treated with *ortho*-toluidine hydrochloride [recrystallized product, purity not specified] in the diet at a concentration of 4000 ppm (0.028 mol/kg of diet) for 72 weeks. A control group of 30 untreated male rats was included in the study. The experiment was terminated at 93 weeks. The mean daily dose of *ortho*-toluidine hydrochloride was 0.062 g (0.00043 mol) per rat, and the total dose was 31.3 g (0.22 mol) *ortho*-toluidine hydrochloride per rat. Mean body weights were higher in the treated group than in the control group. Survival of the treated animals was comparable to controls through month 18, then steadily decreased until termination at 22 months. The incidence of fibromas of the skin was 1/27 (4%), 25/30 (83%; $P < 0.001$, Fisher exact test); that of fibromas of the spleen was 0/27, 10/30 (33%; $P < 0.001$, Fisher exact test); that of mammary fibroadenomas 0/27, 11/30 (37%; $P < 0.001$, Fisher exact test); and that of peritoneal sarcomas was 0/27, 9/30 (30%; $P < 0.01$, Fisher exact test) for control and treated groups, respectively (Hecht *et al.*, 1982).

Groups of 50 male and 50 female Fischer 344 rats, six weeks of age, were given *ortho*-toluidine hydrochloride (purity > 99%) in the diet at concentrations of 3000 or 6000 ppm for 101–104 weeks. Matched control groups consisted of 20 male and 20 female untreated rats. Mean body weights of treated male and female rats were lower than those of the corresponding controls, and were dose-related. Mortality was significantly affected by the treatment ($P < 0.001$, Tarone test for positive trend). In males, the incidence of sarcomas, fibrosarcomas, angiosarcomas or osteosarcomas (combined) of multiple organs (mainly subcutis and spleen or bone) was 0/20, 15/50 (30%; $P = 0.003$, Fisher exact test) and 37/49 (76%; $P < 0.001$, Fisher exact test); that of subcutaneous integumentary fibromas was 0/20, 28/50 (56%; $P < 0.001$, Fisher exact test) and 27/49 (55%; $P < 0.001$, Fisher exact test); and that of mesotheliomas of multiple organs or tunica vaginalis was 0/20, 17/50 (34%; $P < 0.001$, Fisher exact test) and 9/49 (18%; $P = 0.036$, Fisher exact test) in controls, low-dose and high-dose groups, respectively. In females, the incidence of transitional cell carcinomas of the urinary bladder was 0/20, 9/45 (20%; $P = 0.028$, Fisher exact test) and 22/47 (47%; $P < 0.001$, Fisher exact test); that of sarcomas, fibrosarcomas, osteosarcomas or angiosarcomas (combined) of multiple organs (mainly subcutis and spleen or bone) was 0/20, 3/50 (6%) and 21/49 (43%; $P = 0.001$, Fisher exact test); and that of mammary adenomas and fibroadenomas (combined) was 7/20 (35%), 20/50 (40%) and 35/49 (71%) ($P = 0.006$), in controls, low-dose and high-dose groups, respectively (National Cancer Institute, 1979).

Three groups of 20 male Fischer 344/N rats, 45 days of age, were given *ortho*-toluidine hydrochloride (purity > 99%) at a concentration of 5000 ppm in the diet for up to 26 weeks. Group 1 was treated with *ortho*-toluidine hydrochloride for 13 weeks and then killed. Group 2 was treated for 13 weeks and then kept untreated for an additional 13 weeks, when animals were killed. Group 3 was treated with *ortho*-toluidine

hydrochloride for 26 weeks and then killed. The average of group mean compound consumption was 301 mg/kg/day for Group 1, 304 mg/kg/day for Group 2 and 285 mg/kg/day for Group 3. Groups of 10 untreated male Fischer 344/N rats served as controls for each of the treated groups. Hyperplasia in the transitional epithelium of the urinary bladder was observed in 10/20 (50%) ($P < 0.01$, Fisher exact test) rats exposed to *ortho*-toluidine hydrochloride for 13 weeks and in 17/20 (85%) ($P < 0.01$, Fisher exact test) rats exposed for 26 weeks. No hyperplasia was observed in the concurrent control animals (0/10). It was also reported that mesotheliomas in the epididymis were observed in 2/20 (10%) male rats exposed to *ortho*-toluidine hydrochloride for 13 weeks and held for an additional 13 weeks. No mesotheliomas were seen in concurrent controls (0/10) (NTP, 1996).

3.2 Subcutaneous injection

3.2.1 *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, eight weeks of age, were given subcutaneous injections of 1.9 mmol/kg bw (2 mg/kg bw) *ortho*-toluidine (free base) [recrystallized product, purity not specified] in peanut oil once per week for 52 weeks. Control groups of 15 male and 15 female hamsters were given 52 subcutaneous injections of peanut oil. Animals were observed until moribund. The experiment was terminated after 87 weeks. Mean body weights in the treated groups were similar to those of the control groups. Mean survival times were shorter in the treated groups, being 61.3 and 57.8 weeks in male and female treated hamsters, respectively, compared with 75.5 and 68.7 weeks in male and female controls, respectively. The incidence of tumours in the treated groups was not significantly different from that in the control groups [details on the incidence of specific tumours were not reported] (Hecht *et al.*, 1983). [The Working Group noted the small number of animals, low dose and short duration of treatment.]

3.3 Carcinogenicity of metabolites

3.3.1 *Rat*

Groups of 30 male Fischer 344 rats, eight weeks of age, were treated with *ortho*-nitrosotoluene [recrystallized product, purity not specified] in the diet at a concentration of 3380 ppm (0.028 mol/kg of diet) for 72 weeks. A control group of 30 untreated male rats was used. The experiment was terminated at 93 weeks. The mean daily dose of *ortho*-nitrosotoluene was 0.051 g (0.00042 mol) per rat and the total dose was 25.7 g (0.21 mol) *ortho*-nitrosotoluene per rat. Mean body weights were higher in the treated group than in the control group. The incidence of fibromas of the skin was 1/27 (4%), 19/29 (66%) ($P < 0.001$, Fisher exact test); that of fibromas of the spleen was 0/27, 14/29 (48%) ($P < 0.001$, Fisher exact test); that of hepatocellular carcinomas was 0/27, 18/29 (62%) ($P < 0.001$, Fisher exact test); and that of urinary bladder tumours was 0/27, 15/29 (52%)

($P < 0.01$, Fisher exact test) in control and treated groups, respectively (Hecht *et al.*, 1982).

3.3.2 *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, eight weeks of age, were given subcutaneous injections of 1.9 mmol/kg bw (2 mg/kg bw) *ortho*-nitrosotoluene [recrystallized product, purity not specified] in peanut oil once per week for 52 weeks. Control groups of 15 male and 15 female hamsters were given 52 subcutaneous injections of peanut oil. Animals were observed until moribund. The experiment was terminated after 87 weeks. Mean body weights in the treated groups were similar to those of the control groups. Mean survival times were shorter in the treated groups, being 45.4 and 51.1 weeks in male and female treated hamsters, respectively, compared with 75.5 and 68.7 weeks in male and female controls, respectively. The incidence of tumours in the treated groups was not significantly different from that in the control groups [details on the incidence of specific tumours were not reported] (Hecht *et al.*, 1983). [The Working Group noted the small number of animals, the low dose and the short duration of treatment.]

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

4.1.1 *Humans*

ortho-Toluidine occurs in the environment and is a constituent of tobacco smoke (Patrianakos & Hoffmann, 1979). Riedel *et al.* (2006) measured this amine in urine from smokers and nonsmokers using gas-chromatography/mass-spectrometry/negative-ion chemical ionization (GC-MS-NICI) with a deuterated *ortho*-toluidine standard to assess recovery. Excretion of *ortho*-toluidine was significantly higher in 10 smokers (204 ng/24 h, 117 ng/L) than in 10 nonsmokers (104 ng/24 h, 55 ng/L). A large variation was observed among nonsmokers, which was attributed to one subject with *ortho*-toluidine concentrations of 318 ng/L or 731 ng/24 h, the highest of all subjects. After excluding this subject, the difference for *ortho*-toluidine excretion between smokers and nonsmokers remained significant. Urinary arylamine excretion in smokers was associated with the extent of smoking, as assessed by daily cigarette consumption ($P < 0.177$), urinary excretion of nicotine equivalents (nicotine plus its five major metabolites; $P < 0.021$), cotinine in saliva ($P < 0.118$), and carbon monoxide in exhaled breath ($P < 0.373$). All nonsmokers had quantifiable amounts of *ortho*-toluidine in their urine, confirming that other environmental sources of exposure also occur. This study evaluated its results in comparison with those of three other studies on *ortho*-toluidine excretion in smokers and

nonsmokers (el-Bayoumy *et al.*, 1986; Riffelmann *et al.*, 1995; Ward *et al.*, 1996). While only one of those studies reported significant differences between smokers and nonsmokers (Riffelmann *et al.*, 1995), higher absolute values were reported for non-occupationally exposed nonsmokers in all three studies. Possible reasons for this were suggested to be alkaline rather than acid hydrolysis, leading to cleavage of *N*-acetylated metabolites; the method of detection, i.e. electrochemical or electron capture *vs* GC-MS-NICI; and non-occupational exposure to *ortho*-toluidine.

ortho-Toluidine is a major metabolite of prilocaine (*N*-[α-propyl-aminopropionyl]-*ortho*-toluidine), a widely used local anaesthetic. Gaber *et al.* (2007) assessed the impact of prilocaine-treatment on the formation of haemoglobin (Hb) adducts from *ortho*-toluidine by obtaining blood samples from 20 patients undergoing head and neck surgery and six healthy volunteers, before and 24 h after receiving local anaesthesia with prilocaine (Xylonest®, 100 mg). Hb adducts of *ortho*-toluidine and 4-aminobiphenyl were determined by GC/MS. *ortho*-Toluidine-Hb adducts were significantly increased at 24 h after prilocaine treatment by 22 ± 13 ng/g Hb ($P < 0.0001$), which corresponds to about 0.034 ± 0.021% of the prilocaine dose. This corresponds to a 6–360-fold increase of *ortho*-toluidine adduct levels in patients. Because of an extremely high background level, the increase was only 1.6-fold in one patient (40.9 ng/g before *vs* 64.4 ng/g Hb at 24 h after prilocaine injection). Self-reported smoking status and 4-aminobiphenyl-Hb adducts were used to control for smoking-related effects. Current smoking had no influence on background values or on the increase of *ortho*-toluidine adducts by prilocaine. The latter did not alter 4-aminobiphenyl-Hb adduct levels, which were significantly higher in eight smokers, 0.15 ± 0.1 ng/g Hb, than in 16 nonsmokers, 0.04 ± 0.04 ng/g Hb ($P < 0.01$). Data for *ortho*-toluidine are similar to those given in a previous report (Ward *et al.*, 1996), which found similar values of Hb adducts in smoking and nonsmoking unexposed workers; among exposed workers, smokers and non-smokers showed similar results. Since smokers have significantly higher CYP1A2 activity compared with nonsmokers (Sesardic *et al.*, 1988), the absence of any effect of smoking status on the increase of *ortho*-toluidine adducts after exposure to prilocaine does not support a role for this enzyme in activation of *ortho*-toluidine. The results are also consistent with those of studies in rats demonstrating no significant increase in *ortho*-toluidine-Hb adducts after induction of CYP1A2 by β-naphthoflavone (DeBord *et al.*, 1992). Human CYP2A6 and 2E1 have also been shown to play a role in the activation of another monocyclic amine, 2,6-dimethylaniline (Gan *et al.*, 2001).

4.1.2 *Experimental systems*

The metabolism of prilocaine in the mouse has been investigated (Akerman *et al.*, 1966). In female Sprague-Dawley rats, the tissue distribution of ^{14}C-prilocaine, dosed intramuscularly at 10 mg/kg bw, was in the following decreasing order: lung, kidney, spleen, brain, heart, liver and blood. Lung distribution was maximal after 10 min and remained at the highest level of ^{14}C-prilocaine during the entire 120 min of the study. The

rapid in-vivo decomposition of ^{14}C-prilocaine, when given intraperitoneally at 10 mg/kg bw to DSS mice, was unaffected by the CYP inhibitor SKF 525A (25 mg/kg bw, i.p.). However, SKF 525A greatly reduced the metabolism of lidocaine (a substituted xylidene, *ortho*-diethylamino-2,6-dimethylacetanilide). In experiments with rat and mouse liver slices *in vitro*, *ortho*-toluidine was tentatively identified as a metabolite of lidocaine.

The ability of *ortho*-toluidine to affect xenobiotic biotransformation in male Sprague-Dawley rats was investigated (Leslie *et al.*, 1988). After intraperitoneal injections of 10 mg/kg bw *ortho*-toluidine, daily for 7 consecutive days, there was an increase in hepatic CYP content and activities of ethoxyresorufin-*O*-deethylase, ethoxycoumarin-*O*-deethylase, and aldrin epoxidase. At 100 mg/kg bw, *ortho*-toluidine increased metabolic activity at several hydroxylation sites of androstenedione and caused a small decrease in testosterone synthesis. Administration of *ortho*-toluidine at either 10 or 100 mg/kg bw, was not associated with a change in aniline *para*-hydroxylase, epoxide hydrolase, or aminopyrine *N*-demethylase activities. Similar results were observed with male Wistar rats given intraperitoneal injections of 75 mg/kg bw *ortho*-toluidine for three consecutive days (Gnojkowski *et al.*, 1984). The hepatic activities of microsomal aryl hydrocarbon hydroxylase (predominantly catalysed by CYP1A iso-enzyme activity) and NADPH-cytochrome *c* reductase and the content of cytochrome *b5* were enhanced. No effect was observed on epoxide hydrolase, aminopyrine demethylase or glutathione *S*-transferase activities or CYP content. Another study used caffeine metabolism to investigate whether the three isomers of toluidine induce CYP1A2 activity (Jodynis-Liebert & Matuszewska, 1999). Male Wistar rats were fasted overnight and given an oral dose of 1, 10, or 60 mg/kg bw of each isomer. After 24 hours, rats were orally dosed with 10 mg/kg bw of caffeine and killed three hours later. All toluidines were inducers of CYP1A2. However, *ortho*-toluidine was the most effective with large increases observed at 1 mg/kg bw, and its effect was dose-dependent. This study is quite different from the two studies discussed above with regard to route of administration and total time of exposure to *ortho*-toluidine.

In-vivo biotransformation of *ortho*-toluidine has been assessed in male F344 rats (Son *et al.*, 1980). Following a 50- or 400- mg/kg bw subcuteneous dose of *ortho*-[methyl-^{14}C]toluidine, > 75% of the radioactivity was recovered in urine and < 3.5% in faeces after 48 hours. Major routes of metabolism were *N*-acetylation and hydroxylation at the 4-position. Minor pathways included hydroxylation at the 6-position, oxidation of the methyl group, and oxidation of the amino group. Sulfate conjugates predominated over glucuronides. Studies should be designed to determine specific P450s, *N*-acetyl-transferases, or other enzymes involved in the metabolism of *ortho*-toluidine.

4.2 Genetic and related effects

The genetic toxicology of *ortho*-toluidine has been extensively studied in two international collaborative trials for evaluation of short-term tests for carcinogens (Ashby, 1981; 1985). A review (Danford, 1991) summarized the conclusions of these trials. The genetic toxicology of *ortho*-toluidine has also been reviewed more briefly, in the context

of carcinogenesis, by Sellers and Markowitz (1992). There seems to be substantial variation in results between different laboratories and minor variations in protocols (see Table 4.1 for details and references; this Table is reproduced from Monograph Volume 77; IARC, 2000).

Most of the data from bacterial or bacteriophage assay-systems show negative or at most weakly positive results. *ortho*-Toluidine gave positive results for induction of bacteriophage lambda, but only when tested in the presence of exogenous metabolic activation (Thomson, 1981). It failed to induce SOS activity in *Salmonella typhimurium* TA1535/PSK1002 (Nakamura *et al.*, 1987). At very high concentrations (20 mg per plate), it was differentially toxic towards *Escherichia coli* strains differing in capacity for recombinational repair, in the absence of S9 mix (Rosenkranz & Poirier, 1979). However, this result was not reproduced in two further studies carried out in other laboratories, with lower concentrations (Green, 1981; Tweats, 1981). *ortho*-Toluidine gave positive results for forward mutation in recombination-deficient strains of *Bacillus subtilis* (Kada, 1981).

A large series of studies have been reported using *S. typhimurium* strains TA100, TA102, TA1535, TA1537, TA1538, TA98 and TA97. Almost all of the results were negative, although there are sporadic reports of positive responses, only in the presence of S9 mix, with strains TA100, TA98, TA1535 and TA1538 (Table 4.2).

Miller *et al.* (1986) tested the mutagenic potential of some chemical components of dental materials including *ortho*-toluidine, and found that this compound was not mutagenic in the Ames *Salmonella*/microsome mutagenicity spot test and the plate-incorporation test with tester strains TA97, TA98, TA100, and TA104, with or without S9 mix. Analogues of *ortho*-toluidine, such as *para*-toluidine and *N,N*-dimethyl-*para*-toluidine were also not mutagenic.

Gupta *et al.* (1987) tested the mutagenicity of *ortho*-toluidine and its potential metabolites in the *Salmonella*/mammalian microsome mutagenicity assay. The compound was not mutagenic in the presence or absence of rat liver S9 in *Salmonella* strains TA98 and TA100. However, its *N*-oxidized metabolites, *N*-hydroxy-*ortho*-toluidine and *ortho*-nitrotoluene showed a marked mutagenic response in TA100, but only in the presence of S9 mix at dose levels of 0.5–2.0 μM per plate; a linear dose–response curve was observed. Other potential metabolites such as *N*-acetyl-*ortho*-toluidine, *N*-acetyl-*N*-hydroxy-*ortho*-toluidine, *N*-acetoxy-*N*-acetyl-*ortho*-toluidine, 2-OH-6-methyl-acetanilide, *ortho*-azoxytoluene, and *ortho*-azotoluene were found to be inactive in TA100 and TA98, with or without activation.

In *Saccharomyces cerevisiae*, *ortho*-toluidine induced "petite" mutants in strain D5 (Ferguson, 1985) but it was negative for mitotic crossing-over and gene conversion in strain D7 (Metha and von Borstel, 1985; Brooks *et al.*, 1985). The *S. cerevisiae* deletion (DEL) assay can detect a wide variety of nonmutagenic carcinogens, including carcinogens that are not detectable with the Ames test (Schiestl *et al.* 1989). Carls and Schiestl (1994) employed the *S. cerevisiae* DEL assay to test the genotoxicity of *ortho*-toluidine, and found that it increased the frequency of DEL recombination

Table 4.1. Genetic and related effects of *ortho*-toluidine (reproduced from *IARC Monographs* Volume 77)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Prophage induction, SOS repair, DNA strand breaks or cross-links	NT	+	2500	Thomson (1981)
Prophage induction, SOS repair, DNA strand breaks or cross-links (*Salmonella typhimurium* TA1535/pSK1002)	–	–	1670	Nakamura *et al*. (1987)
Escherichia coli pol A/W3110-P3478, differential toxicity (liquid suspension tests)	–	–	250 µg/plate	Rosenkranz *et al.* (1981)
Escherichia coli rec strains, differential toxicity	+	NT	20 µL/disc	Rosenkranz & Poirier (1979)
Escherichia coli rec strains, differential toxicity	?	–	2500	Green (1981)
Escherichia coli rec strains, differential toxicity	–	–	1000	Tweats (1981)
Bacillus subtilis rec strains, forward mutation	+	+	20 µL/disc	Kada (1981)
Salmonella typhimurium, TM677, forward mutation	NT	–	500 µg/plate	Skopek *et al.* (1981)
Salmonella typhimurium, forward mutation	–	–	500 µg/plate	Liber (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation	–	–	5000 µg/plate	McCann *et al.* (1975)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Simmon (1979)
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	1000 µg/plate	Tanaka (1980)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	10 µL/plate	Baker & Bonin (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA92, TA98, reverse mutation	–	–	2000 µg/plate	Brooks & Dean (1981)
Salmonella typhimurium TA100, TA98, TA1537, reverse mutation	–	–	5000 µg/plate	MacDonald (1981)
Salmonella typhimurium TA100, TA1537, TA98, reverse mutation	–	–	1000 µg/plate	Nagao & Takahashi (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	10 000 µg/plate	Richold & Jones (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2000 µg/plate	Rowland & Severs (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, G46, C3076, reverse mutation	–	–	1000 µg/mL agar	Thompson *et al.* (1983)
Salmonella typhimurium TA100, TA98, TA97, TA102, reverse mutation	–	–	10 000 µg/plate	Baker & Bonin (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Falck *et al.* (1985)
Salmonella typhimurium TA100, TA102, TA98, TA97, reverse mutation	–	–	2000 µg/plate	Matsushima *et al.* (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	5000 µg/plate	Rexroat & Probst (1985)
Salmonella typhimurium TA100, reverse mutation	–	+[c]	2000 µg/plate	Zeiger & Haworth (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium BA13 (L-arabinose resistance), forward mutation	NT	+	480 µg/plate	Dorado & Pueyo (1988)
Salmonella typhimurium TA1535, TA1538, reverse mutation	–	–	250 µg/plate	Rosenkranz & Poirier (1979)
Salmonella typhimurium TA1535, TA1537, TA98, reverse mutation	–	–	300 µg/mL	Gatehouse (1981)
Salmonella typhimurium TA1535, reverse mutation	–	–	1000 µg/plate	Zeiger & Haworth (1985)
Salmonella typhimurium TA1538, reverse mutation	–	–	50 µg/plate	Ferretti *et al.* (1977)
Salmonella typhimurium TA1538, reverse mutation	–	–	50 µg/plate	Garner & Nutman (1977)
Salmonella typhimurium TA1538, reverse mutation	–	+[d]	10 µg/mL	Gatehouse (1981)
Salmonella typhimurium TA98, reverse mutation	–	NT	100 µg/plate	Nagao *et al.* (1977)
Salmonella typhimurium TA98, reverse mutation	NT	–	25 µg/plate	Nagao *et al.* (1978)
Salmonella typhimurium TA98, reverse mutation	NT	+	1.3 µg/plate	Kawalek *et al.* (1983)
Salmonella typhimurium TA98, TA97, reverse mutation	–	–	500 µg/plate	Zeiger & Haworth (1985)
Escherichia coli WP2 *uvrA*, reverse mutation	–	–	1000 µg/plate	Gatehouse (1981)
	–	–	1000 µg/mL agar	Thompson *et al.* (1983)
Escherichia coli WP2 *uvrA*, reverse mutation	–	–	1000 µg/plate	Falck *et al.* (1985)
Aspergillus nidulans, forward mutation	–	NT	504	Carere *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Aspergillus nidulans, genetic crossing-over	–	NT	2520	Carere *et al.* (1985)
Saccharomyces cerevisae, DNA repair-deficient strains, differential toxicity	+	+	300	Sharp & Parry (1981a)
Saccharomyces cerevisiae, gene conversion	–	–	333 µg/plate	Jagannath *et al.* (1981)
Saccharomyces cerevisiae, strain XV185-14C, gene conversion	–	+	2222	Mehta & von Borstel (1981)
Saccharomyces cerevisiae, gene conversion	+	NT	50	Sharp & Parry (1981b)
Saccharomyces cerevisiae, gene conversion	NT	–	2 µL/mL	Zimmermann & Scheel (1981)
Saccharomyces cerevisiae, gene conversion, reverse mutation	–	–	500	Arni *et al.* (1985)
Saccharomyces cerevisiae, gene conversion	–	–	2000	Brooks *et al.* (1985)
Saccharomyces cerevisiae, gene conversion, forward/reverse mutation	–	–	1000	Inge-Vechtomov *et al.* (1985)
Saccharomyces cerevisiae, gene conversion, reverse mutation	–	–	500	Parry & Eckardt (1985a)
Saccharomyces cerevisiae, deletion assay	+	+	1000	Carls & Schiestl (1994)
Saccharomyces cerevisiae, interchromosomal recombination	–	–	5000	Carls & Schiestl (1994)
Saccharomyces cerevisiae, forward ‘petite’ mutation	+	NT	2500	Ferguson (1985)
Saccharomyces cerevisiae, strain XV185-14C, reverse mutation	–	–	2222	Mehta & von Borstel (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Saccharomyces cerevisiae, reverse mutation	–	–	21.2	Harrington & Nestmann (1985)
Saccharomyces cerevisiae, gene conversion	–	+	1512	Mehta & von Borstel (1985)
Saccharomyces cerevisiae, aneuploidy	+	+	50	Parry & Sharp (1981)
Saccharomyces cerevisiae, aneuploidy	+	+	NR	Parry & Eckardt (1985b)
Saccharomyces cerevisiae, aneuploidy	–	NT	1.5 µL/mL	Zimmermann *et al.* (1985)
Drosophila melanogaster, genetic crossing-over, somatic mutation or recombination	+		0.94 mM in feed[e]	Würgler *et al.* (1985)
Drosophila melanogaster, somatic mutation (and recombination)	(+)		10 700	Fujikawa *et al.* (1985)
Drosophila melanogaster, somatic mutation (and recombination)	+		1 mM in feed	Vogel (1985)
Drosophila melanogaster, somatic mutation (and recombination)	+		1 mM in feed	Batiste-Alentorn *et al*. (1991)
Drosophila melanogaster, somatic mutation	–		2 mM in feed	Batiste-Alentorn *et al.* (1994)
Drosophila melanogaster, somatic mutation (and recombination), wing-spot test	+		5 mM in feed	Batiste-Alentorn *et al*. (1995)
DNA strand breaks, cross-links or related damage, rat hepatocytes *in vitro*	+	NT	319	Bradley (1985)
DNA strand breaks, cross-links or related damage, Chinese hamster ovary cells *in vitro*	+	+	4280	Douglas *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks, cross-links or related damage, Chinese hamster ovary cells *in vitro*	–	(+)	2140	Lakhanisky & Hendrickx (1985)
DNA strand breaks (Comet assay), MCL-5 cells	+	NT	454	Martin *et al.* (1999)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	500 nmol/mL	Thompson *et al.* (1983)
Unscheduled DNA synthesis, Sprague-Dawley rat primary hepatocytes	–	NT	10.7	Kornbrust & Barfknecht (1984)
Unscheduled DNA synthesis, rat primary hepatocytes	+	NT	107	Glauert *et al.* (1985)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	53.5	Probst & Hill (1985)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	10	Williams *et al.* (1985)
Unscheduled DNA synthesis, Sprague-Dawley rat primary hepatocytes	–	NT	10.7	Barfknecht *et al.* (1987)
Unscheduled DNA synthesis, golden Syrian hamster primary hepatocytes *in vitro*	–	NT	10.7	Kornbrust & Barfknecht (1984)
Unscheduled DNA synthesis, golden Syrian hamster primary hepatocytes *in vitro*	–	NT	10.7	Barfknecht *et al.* (1987)
Gene mutation, *Hprt* locus, ouabain resistance, Chinese hamster ovary cells *in vitro*	–	–	500	Zdzienicka & Simons (1985)
Gene mutation, Chinese hamster lung V79 cells *Hprt* locus *in vitro*	–	–	2000	Fox & Delow (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, Chinese hamster lung V79 cells, *Hprt* locus *in vitro*	(+)	–	500	Kuroda *et al.* (1985)
Gene mutation, Chinese hamster lung V79 cells *Hprt* locus *in vitro*	–	–	10 µL/mL	Lee & Webber (1985)
Gene mutation, Chinese hamster lung V79 cells, oubain resistance *in vitro*	–	–	535	Kuroki & Munakata (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	1100[f]	Amacher & Turner (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus and *Hprt* locus *in vitro*	–	–	1.3 µL/mL	Knaap & Langebroek (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	10 µL/mL	Lee & Webber (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	+	NT	0.3 µL/mL	Myhr *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	500[g]	Oberly *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	(+)	–	1004	Styles *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, ouabain or thioguanine resistance *in vitro*	–	+	200	Garner & Campbell (1985)
Gene mutation, mouse lymphoma L5178Y cells, ouabain or trifluorothymidine resistance, *in vitro*	–	–	1004	Styles *et al.* (1985)
Gene mutation, Balb/c 3T3 cells, ouabain resistance *in vitro*	NT	(+)	250	Matthews *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	–	300	Perry & Thomson (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	1070	Douglas *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	+	50	Gulati *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	–	500	Lane *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	–	–	2140	Natarajan *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	+	268	van Went (1985)
Sister chromatid exchange, RL_4 rat liver cells *in vitro*	+	NT	21.8	Priston & Dean (1985)
Micronucleus formation, Chinese hamster ovary cells *in vitro*	–	–	1070	Douglas *et al.* (1985)
Micronucleus formation, Syrian hamster embryo cells *in vitro*	+	NT	NR	Fritzenschaf *et al.* (1993)
Chromosomal aberrations, Chinese hamster CH1-L liver fibroblasts *in vitro*	+	NT	12	Danford (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	+	+	250	Gulati *et al.* (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	+	1000	Ishidate & Sofuni (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	–	2140	Natarajan *et al.* (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	(+)	300	Palitti *et al.* (1985)
Chromosomal aberrations, RL_4 rat liver cells *in vitro*	+	NT	700	Priston & Dean (1985)
Aneuploidy, Chinese hamster CH1-L liver fibroblasts *in vitro*	+	NT	60	Danford (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cell transformation, C3H/10T1/2 mouse cells	–	(+)	600	Lawrence & McGregor (1985)
Cell transformation, BALB/c3T3 mouse cells	–	+[h]	150	Matthews *et al.* (1985)
Cell transformation, C3H/10T1/2 mouse cells	+	NT	500	Nesnow *et al.* (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	1	Barrett & Lamb (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	100	Sanner & Rivedal (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	750	Kerckaert *et al.* (1998)
Cell transformation, baby hamster kidney BHK-21 cells	+	+	NG	Daniel & Dehnel (1981)
Cell transformation, baby hamster kidney BHK-21 cells	NT	+	250	Styles (1981)
Cell transformation, Chinese hamster ovary cells	–	–	0.5 µL/mL	Zdzienicka *et al.* (1985)
Cell transformation, RLV/Fischer rat embryo cells	(+)	NT	10	Suk & Humphreys (1985)
Cell transformation, SA7/Syrian hamster embryo cells	(+)	NT	965	Hatch & Anderson (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells rodent *in vitro*	+	NT	5	Elmore *et al.* (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells rodent *in vitro*	+	NT	5	Scott *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*[i]	–	NT	535	Umeda *et al.* (1985)
Gene mutation, human TK6 cells *in vitro*	+	+	150	Crespi *et al.* (1985)
Gene mutation, human AHH-1 cells *in vitro*	NT	+	300	Crespi *et al.* (1985)
Sister chromatid exchange, human lymphocytes *in vitro*	?	–	100	Obe *et al.* (1985)
Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	1000	Lindahl-Kiessling *et al.* (1989)
Micronucleus formation, human lymphocytes *in vitro*	+	–	2000	Vian *et al.* (1993)
Unscheduled DNA synthesis, HeLa S3 cells *in vitro*	–	+	0.05 µL/mL	Barrett (1985)
Body fluids from Sprague-Dawley rats (urine), microbial mutagenicity (*S. typhimurium* TA98)	–	+[c]	300 mg/kg bw, po × 1	Tanaka (1980)
Body fluids from WAG/Rij rats (plasma), sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+		400 mg/kg bw, ip × 1	Darroudi & Natarajan (1985)
DNA strand breaks, cross-links or related damage, animal cells *in vivo*	+		100	Cesarone *et al.* (1982)
Sister chromatid exchange, $B6C3F_1$ mouse bone marrow cells *in vivo*	(+)		200	Neal & Probst (1983)
Sister chromatid exchange, animal cells *in vivo*	+		600	McFee *et al.* (1989)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Micronucleus formation, B6C3F$_1$ mice *in vivo*	–		0.338 mL/kg ip × 2	Salamone *et al.* (1981)
Micronucleus formation, CD-1 mice *in vivo*	–		0.16 mL/kg ip × 2	Tsuchimoto & Matter (1981)
Chromosomal aberrations, B6C3F$_1$ mice bone marrow cells *in vivo*	–		300 ip × 1	McFee *et al*. (1989)
Micronucleus formation, B6C3F$_1$ mice bone marrow cells *in vivo*	–		300 ip × 1	McFee *et al*. (1989)
Micronucleus test, *Pleurodeles waltl in vivo*	+		20 µg/mL	Fernandez *et al.* (1989)
Binding (covalent) to RNA or protein, Crl:CD rat liver *in vivo*	+		500 po × 1	Brock *et al*. (1990)
Sperm morphology, (CBA × BALB/c)F$_1$ mice *in vivo*	?		0.25 ip × 5	Topham (1981)
Sperm morphology, (CBA × BALB/c)F$_1$ mice *in vivo*	–		0.4 ip × 5	Topham (1980)

[a] +, positive; (+), weakly positive; –, negative; NT, not tested; ?, inconclusive; NR, not reported
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro test, µg/mL; in-vivo test, mg/kg bw/day; ip, intraperitoneal; po, oral
[c] Active only with 30% hamster liver S9; not with rat liver S9
[d] S9 from phenobarbital-treated rats
[e] Acute feeding
[f] Cytotoxic dose; higher toxicity observed with S9
[g] Toxicity higher in the presence of S9
[h] Activation by co-cultivation with X-irradiated primary rat hepatocytes
[i] Growth of V79 (T2-14) 6-thioguanine-resistant cells

Table 4.2. Genetic and related effects of metabolites of *ortho*-toluidine (reproduced from *IARC Monographs* Volume 77)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
***N*-Hydroxy-*ortho*-toluidine**				
Salmonella typhimurium TA100, reverse mutation	–	+	0.16 µg/plate	Gupta *et al*. (1987)
Salmonella typhimurium TA98, reverse mutation	–	–	0.62 µg/plate	Gupta *et al*. (1987)
***N*-Acetyl-*ortho*-toluidine**				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	3.75 µg/plate	Gupta *et al*. (1987)
***N*-Acetyl-*N*-hydroxy-*ortho*-toluidine**				
Salmonella typhimurium TA100, reverse mutation	–	–	4.1 µg/plate	Gupta *et al*. (1987)
Salmonella typhimurium TA98, reverse mutation	–	+	2.1 µg/plate	Gupta *et al*. (1987)
***N*-Acetoxy-*N*-acetyl-*ortho*-toluidine**				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	5.2 µg/plate	Gupta *et al*. (1987)

[a] +, positive; –, negative
[b] LED, lowest effective dose; HID, highest ineffective dose

2.6-fold in the absence of S9 and more than five-fold in the presence of S9. In the absence of S9 an increase in DEL recombination-frequency was first seen at a concentration of 3 mg/ml, whereas in the presence of S9 an increase appeared at 1 mg/ml. The frequency of intrachromosomal recombination was not increased in this test.

In *S. cerevisiae*, treatment with *ortho*-toluidine resulted in differential toxicity in repair-proficient and -deficient strains (Sharp and Parry, 1981a). However, inconsistent data were seen in all other assays with this species of yeast. Of eight assays for gene conversion carried out in different laboratories, one positive result was reported only when exogenous metabolic activation was present (Mehta and von Borstel, 1981) and another only when it was absent (Sharp and Parry, 1981b). Although *ortho*-toluidine caused a recombinogenic event leading to deletion (in either the presence or absence of exogenous metabolic activation), it failed to cause intra-chromosomal recombination in the same yeast strain (Carls and Schiestl, 1994). It was a mitochondrial "petite" mutagen (Ferguson, 1985), but failed to give a positive response for forward mutation in a nuclear gene (Inge-Vechtomov *et al.*, 1985). It gave a positive result in one of seven assays for reverse mutation. However, it caused aneuploidy in two of three assays (Parry and Sharp, 1981; Parry and Eckardt, 1985b). Assays for forward mutation or genetic crossing-over in *Aspergillus nidulans* gave completely negative results, as did a forward mutation assay in *Schizosaccharomyces pombe*.

Uniformly negative results were found for reverse mutation in *E. coli* strains WP2 or WP2 *uvrA*. Where positive responses have been seen in microbial assays, they have generally required variations to the standard test procedures, including the use of the fluctuation protocol, or incorporating the addition of norharman or lithocholic acid. High concentrations of S9 mix, or special types of S9 mix may also be important.

In the *Escherichia coli* K-12 *uvrB/recA* DNA repair host-mediated assay, *ortho*-toluidine was shown to be not mutagenic, with or without metabolic activation (Hellmér and Bolcsfoldi, 1992).

ortho-Toluidine caused DNA strand breakage in various animal cell lines *in vitro*, in the absence of exogenous metabolic activation. The alkaline single-cell gel electrophoresis (comet) assay revealed DNA breakage after *ortho*-toluidine treatment in a metabolically competent human mammary cell line, MCL-5, as well as in primary cultures of cells isolated from human breast milk. The response was substantially increased when the cells were incubated in the presence of the DNA-repair inhibitors hydroxyurea and cytosine arabinoside. Only one of eight studies showed that treatment with *ortho*-toluidine could lead to unscheduled DNA synthesis (Glauert *et al.*, 1985). A single study suggested a weak positive effect in gene mutation at the *Hprt* locus in V79 Chinese hamster cells, although two other similar studies gave negative results. *ortho*-Toluidine failed to cause mutation to ouabain resistance in V79 Chinese hamster cells (Zdzienicka and Simons, 1985). Two of six studies suggested a positive response at the *Tk* locus but not usually at other loci in mouse lymphoma L5178Y cells. However, there are isolated reports of *ortho*-toluidine increasing gene mutations at loci other than *Tk* in

L5178Y cells or in other animal cells *in vitro*, but only in the presence of exogenous metabolic activation.

There have been occasional reports of *ortho*-toluidine causing chromosomal aberrations (Danford, 1985; Gulati *et al.*, 1985; Ishidate and Sofuni, 1985; Palitti *et al.*, 1985; Priston and Dean, 1985) or micronuclei (Fritzenschaf *et al.*, 1993) in various cultured cell lines. Manifestation of these effects required incubation times longer than three hours. In some of these studies, S9 mix was required, while in others it reduced the effect. Most studies of effects on sister chromatid exchange, in either animal or human cells, have revealed positive results, even in the absence of exogenous metabolic activation. *ortho*-Toluidine caused aneuploidy in mammalian cells *in vitro* (Danford, 1985), and increased cell transformation in all but one of 11 studies. The latter effects did not generally appear to require exogenous metabolic activation, although it should be noted that the cell types have some endogenous metabolic capability. In two of three studies, *ortho*-toluidine inhibited intracellular communication (Elmore *et al.*, 1985; Scott *et al.*, 1985).

Several in-vivo studies have been conducted. *ortho*-Toluidine gave a positive result in a host-mediated assay for bacterial mutagenesis. It increased somatic mutation (Fujikawa *et al.*, 1985; Vogel, 1985) but not genetic crossing-over in *Drosophila melanogaster*, and enhanced sister chromatid exchange in rodent models (McFee *et al.*, 1989). Only one of four studies in mice and one study in a newt model (Fernandez *et al.*, 1989), suggested that it enhanced micronucleus frequency. Studies on sperm morphology have given equivocal data (Topham, 1980; 1981).

Although *ortho*-toluidine shows negative results in most genotoxicity tests, there are several reports that show positive results. The bacterial mutagenicity tests, predominantly negative, were in general agreement with other studies with monocyclic aromatic amines. Only 37 of 87 of the short-term tests used in a large collaborative study showed a positive result (Ashby *et al.*, 1985). *ortho*-Toluidine was negative in the Ames mutagenicity test (Brennan and Schiestl, 1999) but it gave positive results in assays detecting unscheduled DNA synthesis or strand breaks (Danford, 1991).

The *ortho*-toluidine metabolite 4-amino-3-methylphenol increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf-thymus DNA in the presence of Cu(II). This metabolite auto-oxidizes to form the aminomethylphenoxyl radical, which reacts with O_2 to form the superoxide ($O_2^{\cdot-}$) radical and H_2O_2. The reactive species generated by H_2O_2 in the presence of Cu (I) contributes to the formation of DNA damage. Metal-mediated DNA damage by *ortho*-toluidine metabolites through H_2O_2 may play a role in the carcinogenicity of *ortho*-toluidine (Ohkuma *et al.*, 1999).

DNA-adduct formation was measured in in-vitro and in-vivo experiments with *ortho*-toluidine and other amines. Calf-thymus DNA was modified *in vitro* by reaction with activated *ortho*-toluidine. Female Wistar rats ($n = 2$) were given a single dose of the arylamine by oral gavage and were killed after 24 hours. Hepatic DNA and DNA modified *in vitro* were hydrolysed enzymatically to individual 2′-deoxyribonucleosides. Adducts were determined by use of HPLC/MS/MS by comparison with synthesized

standards. *ortho*-Toluidine formed adducts to 2′-deoxyguanosine and 2′-deoxyadenosine after in-vitro reaction with DNA. It also formed hydrolysable haemoglobin adducts in treated rats (Jones and Sabbioni 2003).

Bolognesi *et al.* (1980) investigated the DNA damage induced by administration to mice of 2,4-dinitroaniline, *ortho*-toluidine, and *para*-toluidine. DNA damage was measured by use of the alkaline filter-elution technique. Target organs for the ultimate carcinogens in mice appear to be the liver and kidney. The DNA damage was evident four hours after administration of a single dose of *ortho*-toluidine and *para*-toluidine. The value obtained after treatment with 2.4-dinitroaniline fell within the range of controls.

Brock *et al.* (1990) studied the hepatic macromolecular binding and tissue distribution of *ortho*-toluidine and *para*-toluidine in rats. The degree of binding to hepatic macromolecules appeared to be at a maximum for both compounds at 24–48 hours following dosing. At 24 hours, the level of DNA binding of *ortho*-toluidine was about 1.2-fold lower than that of *para*-toluidine. The binding to RNA and protein was also lower for *ortho*-toluidine than for *para*-toluidine, although the difference was not as large as that observed for DNA binding. There were subtle differences in tissue distribution for each isomer.

The formation of *ortho*-toluidine-Hb adducts was first detected by Birner and Neumann (1988) in a study with rats. The HbI (haemoglobin-binding index) was in the same range as that observed for aniline and *ortho*-chlorotoluidine.

Haemoglobin (Hb) and albumin (Alb) adducts of *ortho*-toluidine were quantified in blood samples collected from rats after a single injection (Cheever *et al.*, 1992). Mild alkaline hydrolysis of Hb adducted with [^{14}C]-labelled *ortho*-toluidine followed by extraction with ethyl acetate resulted in recovery of 63% of the bound radioactivity. A single radio-labelled peak identified as *ortho*-toluidine by GC-MS was found after HPLC analysis. In subsequent experiments Hb and Alb adduct levels were determined by HPLC analysis of this cleavage product by means of fluorescence detection. The detection limit for *ortho*-toluidine was 450 pg/injection or 5 pmol/mg Hb. Mean adduct levels for Hb increased rapidly over the first four hours, with the highest level (ng/mg Hb ± SD) 3.7 ± 0.5 detected 24 hours after administration of *ortho*-toluidine at a dose of 50 mg/kg bw. The adduct levels for pooled Alb samples increased from 0.7 ng/mg Alb at two hours to 2.5 ng/mg Alb at four hours, but were not detectable at 24 hours after dosing. Hb adducts showed a linear relationship with *ortho*-toluidine doses of 10, 20, 40, 50, and 100 mg/kg bw. The Hb-adduct half-life was 11 days after a single 100 mg/kg bw dose. Hb-adduct levels were still quantifiable (1.3 ± 0.2 ng/mg Hb) by HPLC/fluorescence at 28 days after a 100-mg/kg bw dose of *ortho*-toluidine (Cheever *et al.*, 1992).

The binding characteristics of *ortho*-toluidine to rat haemoglobin (Hb) and albumin (Alb) were studied by DeBord *et al.* (1992). Sprague-Dawley rats were given [^{14}C]-labelled *ortho*-toluidine intraperitoneally at 10, 20, 40, 50, or 100 mg/kg bw, and were killed at 2, 4, 8, 18, 24, 48, or 72 hours, or 7, 14, 28 days. Haemoglobin and albumin were isolated from blood, and *ortho*-toluidine was determined by liquid scintillation counting. For albumin, a maximum binding occurred at 50 mg/kg bw at the 4-h time point (15.6 ng

ortho-toluidine/mg Alb); the maximum binding to Hb was observed at 24 h at the 100-mg/kg bw dose (23.0 ± 5.1 ng *ortho*-toluidine/mg Hb). *ortho*-Toluidine-Alb binding was not linear, but *ortho*-toluidine-Hb appeared to increase linearly in a dose-dependent manner. The biological half-lives of *ortho*-toluidine bound to Alb or Hb were observed to be 2.6 and 12.3 days, respectively, after rats were given a single dose of [^{14}C]-labelled *ortho*-toluidine. An approximately two-fold increase in radioactivity bound to Hb was observed after i.p. administration of 100 mg/kg bw [^{14}C]-labelled *ortho*-toluidine compared with oral intubation.

Suzuki *et al.* (2005) evaluated the liver and peripheral blood micronucleus assays with nine chemicals including *ortho*-toluidine in young male Fischer F344 or SD rats. *ortho*-Toluidine significantly increased the numbers of micronucleated hepatocytes (MNHEPs), micronucleated reticulocytes (MNRETs) and the number of reticulocytes (RETs) in the peripheral blood of the test animals. Results of this study were in agreement with the report that *ortho*-toluidine induced hepatocellular carcinoma and hemangiosarcoma in mice, and cancer in multiple organs in rats (IARC, 2000). However, Nakai *et al.* (1994) reported that *ortho*-toluidine was negative in a mouse bone-marrow micronucleus assay.

McFee *et al.* (1989) tested the in-vivo genotoxicity of *ortho*-toluidine in B6C3F1 mice, and found that bone-marrow cells from mice given intraperitoneal injections to up to the maximum tolerated dose of *ortho*-toluidine hydrochloride did not show an increased frequency of chromosomal aberrations or micronuclei, but the frequency of sister chromatid exchange was increased in two successive tests.

ortho-Toluidine induced an increase of intra-chromosomal recombination in *S. cerevisiae* strains RS112 and EG133 of greater than three- and six-fold at doses of 5 and 6 mg/ml, respectively, although statistical significance ($P < 0.05$) was reached only at the highest dose. The frequency of recombination was reduced by the presence of the antioxidant *N*-acetyl-cysteine. The cytotoxicity of *ortho*-toluidine was also reduced in the presence of this free-radical scavenger. Superoxide dismutase-deficient strains of *S. cerevisiae,* however, were hypersensitive to the cytotoxicity induced by *ortho*-toluidine (Brennan and Schiestl, 1999). These results indicate that the genotoxicity of *ortho*-toluidine was at least in part due to the formation of free radicals.

In *Drosophila melanogaster*, both positive and negative responses were obtained in the somatic cell w/w^{co} test with repair-proficient strains (Vogel, 1985; Würgler and Vogel, 1986). Inconclusive results were also obtained for the reversion at w/w^{-} in the excision-repair deficient *mei*-9 strain (Fujikawa *et al.*, 1985; Batiste-Alentorn *et al.*, 1991). Mutagenicity of several aromatic amines was tested by means of the w/w^{+} somatic assay of *D. melanogaster* with the wild-type strain Leiden Standard (LS) and an insecticide-resistant stock Hikone-R (HK-R). *ortho*-Toluidine was found to be positive in this test (Rodriguez-Arnaiz & Aranda, 1994). Batiste-Alentorn *et al.* (1995) further conducted the *D. melanogaster* wing-spot somatic mutation and recombination assay on 10 selected carcinogens including *ortho*-toluidine. Third-instar larvae, 72 hours old and trans-heterozygous for two recessive wing-cell markers, i.e. *multiple wing hairs (mwh*) and

flare^3 (*flr*^{3}), were given three concentrations of each carcinogen in the feed during the rest of their development until pupation, and genotoxic effects were measured as significant increases in the appearance of visible mutant-hair clones on the adult wing-blade. It was found that six of the carcinogens tested, including *ortho*-toluidine, produced significant increases in wing-spot frequency.

Ward *et al.* (1996) monitored aromatic amine exposures in workers at a chemical plant with a known bladder-cancer excess. Data were obtained for a total of 73 workers, including 46 of 64 exposed workers who were employed in the rubber-chemicals department and had the potential for exposure to aniline and *ortho*-toluidine, and 27 of 52 unexposed workers employed in other departments where aniline and *ortho*-toluidine were not used or produced. They found that post-shift urinary *ortho*-toluidine concentrations averaged (± standard deviation) 2.8 µg/L (± 1.4 µg/L) in unexposed subjects and 98.7 µg/L (± 119.4 µg/L) in exposed subjects ($P = 0.0001$). Average aniline-Hb adducts and *ortho*-toluidine-Hb adducts were also significantly higher ($P = 0.0001$) among exposed workers than among unexposed control subjects (3163 pg/g ± 1302 pg/g for unexposed *vs* 17 441 ± 8867 pg/g for exposed in the case of aniline-Hb adducts; 3515 ± 6036 pg/g for unexposed *vs* 40, 830 ± 32 518 pg/g for exposed in the case of *ortho*-toluidine-Hb adducts). Average levels of adducts to *4*-aminobiphenyl, a potential contaminant of process chemicals, were not significantly different ($P = 0.48$). The adduct data suggest that among these workers, *ortho*-toluidine exposure substantially exceeded aniline exposure and that *4*-aminobiphenyl exposure, if it occurred at all, was not widespread. The authors concluded that occupational exposure to *ortho*-toluidine was the most likely causal agent of the bladder-cancer excess observed among workers in the rubber-chemicals department of the plant under study (Ward *et al.*, 1996).

Marques *et al.* (1996) investigated the ability of *N*-(acyloxy)arylamine derived from 2-, 3- and 4-methylaniline (*ortho*-toluidine is 2-methylaniline), 2,3- and 2,4-dimethyl-aniline to bind to DNA by reacting with deoxyguanosine (dG), and dG nucleotides. The predominant products from reactions with dG and the nucleotides were characterized as *N*-(deoxyguanosine-8-yl)-arylamines. Analyses of the [^{1}H]- and [^{13}C]-NMR spectra suggested that the adducts containing a methyl substituent *ortho* to the arylamine nitrogen (i.e. toluidine) had a higher percentage of *syn* conformers. With other aromatic amines, the occurrence of *syn* conformers has been associated with higher propensity for base mispairing and higher tumorigenic responses (Cho *et al*., 1994; Eckel and Krugh 1994). It was observed that some aromatic amines containing methyl substituents in the *ortho* position tend to be more mutagenic and tumorigenic than analogues with no substituents in the *ortho* position (El-Bayoumy *et al.*, 1981; Nussbaum *et al.*, 1983). Theoretical simulation studies indicated substantial percentages of low-energy *syn* conformers, increasing with the substitution pattern in the order *para* < *meta* < *ortho* < *ortho-para* < *ortho-meta*. The results demonstrate that although single-ring arylamines are considered weak carcinogens, their electrophilic *N*-acetoxy derivatives, which are plausible metabolic intermediates, react with DNA to yield covalent adducts structurally identical to those derivatives from carcinogenic polyarylamines such as 2-aminofluorene and

4-aminobiphenyl. Furthermore, the conformational perturbation induced in DNA by the formation of monoarylamine-DNA adducts, especially those with *ortho* substituents, may contribute to the biological activities of these compounds (Marques *et al.*, 1996). [The Working Group noted as a *caveat* that these conformational studies were done with the nucleosides but not with oligonucleotides.]

Marques *et al.* (1997) further studied the effects of the substitution site on the oxidation potentials of these alkylanilines, the mutagenicities of the corresponding *N*-hydroxyalkylanilines, and the conformations of the alkylaniline-DNA adducts. It was found that the adducts from *ortho*-substituted alkylanilines may be intrinsically more mutagenic than their *meta*- and *para*-substituted analogues. There were higher percentages of low-energy *syn* conformers in the adducts that contained alkyl groups *ortho* to the arylamine nitrogen as opposed to adducts not bearing *ortho* substituents. It was suggested that the conformational properties of the DNA adducts, in particular their ability to adopt the sy*n* conformation, may be determinant factors for the genotoxic responses elicited by certain alkylanilines (i.e. *ortho*-toluidine [2-methylaniline] and 2,6-dimethylaniline). Beland *et al.* (1997) confirmed, on the basis of spectroscopic and theoretical data, that DNA adducts of these single-ring amines containing alkyl groups *ortho* to the amine function (e.g. *ortho*-toluidine) had a greater percentage of *syn* conformers around the glycosyl bond than those not bearing such groups.

4.3 Mechanistic considerations

The classification of *ortho*-toluidine as carcinogenic to humans has been controversial for some time. Recently, the classification has been upgraded to Category 1 (carcinogenic to humans) of the MAK List in Germany (DFG, 2007). Epidemiological observations were impaired by the fact that workers, for instance in the rubber industry, were usually exposed to other aromatic amines as well, mostly aniline or 4-aminobiphenyl. Haemoglobin adducts have been used to monitor rubber-industry workers from the rubber-chemical department. Their average blood levels of *ortho*-toluidine-Hb adducts were significantly higher than those in non-exposed controls, whereas 4-aminobiphenyl-Hb adduct levels were not higher than in controls (Ward *et al.*, 1996). Interestingly, the adduct levels were increased even under conditions where airborne exposures were below the OSHA time-weighted average permissible exposure limits. There has been discussion about the possibility that workers with bladder tumours could have been exposed to 4-aminobiphenyl in the 1950s and early 1960s, when diphenylamine was produced that often contained 4-aminobiphenyl as a contaminant (Freudenthal & Anderson, 1997). It remains remarkable that even today haemoglobin adducts of *ortho*-toluidine are increased at certain workplaces under improved hygiene conditions. This may partly be due to the fact that these amines are readily absorbed through the skin, particularly if the skin barrier is damaged. Frequent use of skin-barrier creams increases the absorption and internal exposure (Korinth *et al.*, 2007). Drugs may also contribute to the exposure; prilocaine, a

local anaesthetic, is hydrolysed *in vivo* and leads to a massive increase of the amount of *ortho*-toluidine-Hb adducts (Gaber *et al.*, 2007).

In line with the hypothesis that alkylation in the *ortho*-position relative to the amino group enhances the activity, *ortho*-toluidine is more potent than aniline and *para*-toluidine. It produces predominantly sarcoma in rats. The acute toxic effects of *ortho*-toluidine are comparable to those of other monocyclic aromatic amines, i.e. methaemoglobin formation, haemosiderosis, fibrosis, while both liver and kidneys are affected. In contrast to the carcinogenic potency, however, *ortho*-toluidine is less toxic than aniline, and as toxic as *para*-toluidine. The haemoglobin binding-indices for these three compounds – 4, 22 and 2, respectively – reflect this well (Neumann, 2005). It is concluded that both genotoxic and acute toxic effects are necessary to explain the experimental tumour formation induced by *ortho*-toluidine.

5. Summary of Data Reported

5.1 Exposure data

The aromatic amine *ortho*-toluidine is used in the production of dyes, pigments and rubber chemicals, and in laboratories to stain tissues. The main route of occupational exposure is by dermal contact. *ortho*-Toluidine is detected ubiquitously in the general population, but its origin is not known. It is not known to exist as a natural substance. Detectable levels of *ortho*-toluidine have been found in surface water, in effluents and soil near industrial facilities, in breast milk and in other food items. *ortho*-Toluidine is also present in tobacco smoke, although this does not appear to be a main source of exposure. *ortho*-Toluidine was detected in patients following treatment with the anaesthetic prilocaine, a metabolic precursor of *ortho*-toluidine. Analytical techniques have been developed to detect *ortho*-toluidine in very small quantities in toys.

5.2 Human carcinogenicity data

The five principal relevant cohort studies of chemical production workers available for evaluation were carried out in Germany, Italy, the United Kingdom, and the United States (two studies). Four of these studies reported highly elevated bladder-cancer risks in *ortho*-toluidine-exposed workers; the earlier study from the USA had limited power to detect any excess risk. Smoking differences could be excluded with confidence as the sole explanation of the elevated risks. In the Italian study, confounding by concomitant exposure to various recognized occupational bladder carcinogens was considered to be possible. This is not the case for the positive German, United Kingdom and US studies, because any known bladder carcinogens were present only at trace levels. In addition,

other exposures were very different from study to study. The epidemiological data provide strong evidence that *ortho*-toluidine causes bladder cancer.

5.3 Animal carcinogenicity data

ortho-Toluidine was tested for carcinogenicity as its hydrochloride salt in two experiments in mice and in four experiments in rats, and as the free base in one limited experiment in hamsters. When administered in the diet to mice, *ortho*-toluidine hydrochloride increased the incidences of hepatocellular carcinomas or adenomas and hemangiosarcomas at multiple sites. When administered in the diet to rats, *ortho*-toluidine hydrochloride increased the incidences of sarcomas of multiple organs, subcutaneous fibromas and mesotheliomas, transitional cell carcinomas of the urinary bladder, and mammary gland fibroadenomas and adenomas. When *ortho*-toluidine was administered as the free base by subcutaneous injection to hamsters, the number of tumours produced was not significantly different from that in controls. *ortho*-Nitrosotoluene, a metabolite of *ortho*-toluidine, was tested in one study in rats and one study in hamsters. When administered in the diet to rats, *ortho*-nitrosotoluene increased the incidence of fibromas of the skin and spleen, hepatocellular carcinomas and urinary bladder tumours. When administered by subcutaneous administration to hamsters, *ortho*-nitrosotoluene did not produce an increase in tumours.

5.4 Other relevant data

In contrast to most other aromatic amines, *ortho*-toluidine is metabolised in rats and humans by CYPs other than 1A2. Candidates for the responsible CYPs are CYP2A6 and 2E1. The major routes of metabolism (assessed in the rat) were *N*-acetylation and hydroxylation at the 4-position. The oxidation of the amino group was a minor pathway. Adducts to haemoglobin, albumin and DNA are formed in rodents after *ortho*-toluidine administration *in vivo*. An additional pathway to DNA-damage induction by *ortho*-toluidine is inferred by the observation that the *ortho*-toluidine metabolite 4-amino-3-methylphenol increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA in the presence of Cu(II). The levels of *ortho*-toluidine-Hb adducts were tenfold higher among exposed workers than among unexposed control subjects. Prilocaine, a widely used local anaesthetic, is metabolized to *ortho*-toluidine, leading to *ortho*-toluidine-Hb adducts after prilocaine treatment. In line with what is generally observed with monocyclic aromatic amines, *ortho*-toluidine was inactive in most bacterial genotoxicity tests, although there are a few reports showing positive results, most of them either at very high doses or after introduction of variations in the standard test procedures. *ortho*-Toluidine produced increases in intra-chromosomal recombination in *S. cerevisiae*. In cultured mammalian cells, *ortho*-toluidine showed predominantly negative results with some exceptions: in liver and peripheral blood of rats *ortho*-toluidine significantly increased the number of micronucleated hepatocytes and micronucleated reticulocytes.

DNA damage measured by the alkaline filter-elution technique was induced by administration of *ortho*-toluidine to mice. In line with the hypothesis that alkylation in the *ortho*-position to the amino group enhances carcinogenicity, *ortho*-toluidine is a more potent animal carcinogen than are aniline and *p*-toluidine. Both genotoxicity and acute toxic effects, necessary to explain the experimental tumour formation by *ortho*-toluidine, have clearly been shown.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of *ortho*-toluidine. *ortho*-Toluidine causes cancer of the urinary bladder.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of *ortho*-toluidine.

6.3 Overall evaluation

ortho-Toluidine is *carcinogenic to humans (Group 1).*

The Working Group was aware of the existence of numerous dyes and colourants that contain *ortho*-toluidine as a structural element, but a full evaluation of this group of dyes was beyond the scope of this Monograph. The local anaesthetic prilocaine, which is metabolized to *ortho*-toluidine, has been shown to cause methaemoglobinaemia and haemoglobin-adduct formation in treated patients.

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4-CHLORO-*ortho*-TOLUIDINE

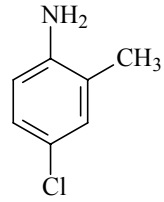
1. Exposure Data

1.1 Chemical and Physical Data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 95-69-2
CAS Name: 4-Chloro-2-methylbenzenamine
Synonyms: 2-Amino-5-chlorotoluene; 3-chloro-6-aminotoluene; 4-chloro-2-methylaniline; 4-chloro-6-methylaniline; (4-chloro-2-methylphenyl)amine; 4-chloro-2-toluidine; 4-chloro-*ortho*-toluidine; 5-chloro-2-aminotoluene; *para*-chloro-*ortho*-toluidine; 2-methyl-4-chloroaniline; 2-methyl-4-chlorobenzeneamine

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



C_7H_8ClN Rel. mol. mass: 141.60

1.1.3 *Chemical and physical properties of the pure substance* (from Lide, 2008)

Description: Leaves (from alcohol); dark violet solid (Sigma-Aldrich, 2010)
Boiling-point: 244 °C
Melting-point: 30.3 °C
Solubility: Soluble in ethanol; slightly soluble in carbon tetrachloride

1.1.4 *Trade names*

Trade names for 4-chloro-*ortho*-toluidine include: Amarthol Fast Red TR Base; Azoene Fast Red TR Base; Azoic Diazo Componenet 11, Base; Brentamine Fast Red TR Base; Daito Red Base TR; Deval Red K; Deval Red TR; Diazo Fast Red TRA; Fast Red 5CT Base; Fast Red Base TR; Fast Red TR Base; Fast Red TR-T Base; Fast Red TRO Base; Kako Red TR Base; Kambamine Red TR; Mitsui Red TR Base; Red Base Ciba IX; Red Base IRGA IX; Red Base NTR; Red TR Base; Sanyo Fast Red TR Base; Tulabase Fast Red TR.

1.1.5 *Analysis*

4-Chloro-*ortho*-toluidine can be readily detected at the ppb (10^{-9}) level by means of GC/MS methods, with or without prior derivatization. Two recent studies have involved methods for detecting this amine in complex mixtures and as reduction products of azo dyes used as toy colourants. Table 1.1 presents a selection of recent studies on the analysis of 4-chloro-*ortho*-toluidine in various matrices.

1.2 Production and use

1.2.1 *Production*

4-Chloro-*ortho*-toluidine is produced by direct chlorination of acetyl-protected *ortho*-toluidine. After removal of the protecting group with base, 4-chloro-*ortho*-toluidine is separated by distillation from the 6-chloro-*ortho*-toluidine isomer (Bowers, 2000).

Commercial production of 4-chloro-*ortho*-toluidine began in Germany in 1924 and was first reported in the United States in 1939 (IARC, 1990, 2000). An IARC Working Group reported that commercial production of 4-chloro-*ortho*-toluidine in the USA stopped in 1979, and all importation and distribution of the compound were discontinued in 1986 (IARC, 1990).

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the aggregate production volumes that were reported for 4-chloro-*ortho*-toluidine.

Available information indicates that 4-chloro-*ortho*-toluidine was produced and/or supplied in research quantities in the following countries: Belgium, China, Hong Kong Special Administrative Region, Japan, Switzerland, the United Kingdom, and the USA (Chemical Sources International, 2010).

Table 1.1. Selected methods of analysis of 4-chloro-*ortho*-toluidine in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Water	Evaporate solution; derivatize using heptafluorobutyric acid and heat; add phosphate buffer; extract with methanol	GC/MS	0.4pg	Longo & Cavallaro (1996)
Toy products	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	< 0.2µg/g	Garrigós *et al*. (2002)
Textiles	Solutions are prepared in dry tetrahydrofuran; add pentafluoropropionic anhydride and heat; cool; dilute with methaol	GC/MS	[2ng/mL]	Narvekar & Srivastava (2002)
Water	Dissolve amine in methanol; dilute aliquots with deionized water	SPE/HPLC	~1ng/mL	Chang *et al*. (2003)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/ dichloromethane (50:50).	GC/MS	5ng/mL	Doherty (2005)

GC, gas chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; SPE, solid phase microextraction; UV, ultraviolet

1.2.2 *Use*

4-Chloro-*ortho*-toluidine and its hydrochloride salt have been used commercially to produce azo dyes for cotton, silk, acetate, and nylon, and as intermediates in the production of Pigment Red 7 and Pigment Yellow 49. As an azoic diazo component, 4-chloro-*ortho*-toluidine is used in the synthesis of some azoic dyes, which are prepared by a two-step process involving diazotization of a primary amine component and coupling of the diazotized amine with a naphthol-derived coupling component (IARC, 1990, 2000; National Cancer Institute, 1979). Since the 1960s, 4-chloro-*ortho*-toluidine has also been used in the manufacture chlordimeform, a pesticide and acaricide (IARC, 1990). As the hydrochloride salt, 4-chloro-*ortho*-toluidine occurs as a contaminant of chlordimeform. It is also a metabolite of this insecticide, which has recently been banned in several countries (IARC, 1983, 1990, 2000; Bowers, 2000). Chlordimeform is no longer used in the USA since 1989 (EPA, 2007).

Table 1.2. 4-Chloro-*ortho*-toluidine production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	NR
1998	NR
2002	<10
2006	NR

USEPA, 2003, 2007
NR, not reported

1.3 Occurrence and human exposure

1.3.1 *Natural occurrence*

4-Chloro-*ortho*-toluidine and its hydrochloride salt are not known to occur as natural products.

1.3.2 *Occupational exposure*

Occupations with the greatest potential for exposure to 4-chloro-*ortho*-toluidine include those involved in the production and use of this chemical as an intermediate for the manufacture of dyestuffs, pigments, and in the production of chlordimeform. Since

4-chloro-*ortho*-toluidine is a major metabolite of this insecticide, exposure can also occur in field workers applying chlordimeform.

*(a) Exposure during the manufacture of 4-chloro-*ortho*-toluidine, and 4-chloro-*ortho*-toluidine-based dyes*

Workers in production and processing of 4-chloro-*ortho*-toluidine in Germany were reported to have been exposed to this substance between 1929 and 1982 (Stasik, 1988). The same author reported that 4-chloro-*ortho*-toluidine was detected at concentrations of 1700 and 2100 µg/L, respectively, in the urine of two workers, at 12 and 48 hrs after exposure to [unknown concentrations of] chlordimeform (Stasik, 1991).

In a dye factory in Switzerland, 4-chloro-*ortho*-toluidine was manufactured from *ortho*-toluidine between 1924 and 1953 (Uebelin and Pletscher, 1954); in an organic-dye factory in the USA, 4-chloro-*ortho*-toluidine was used in the thioindigo production-area (Ott & Langner, 1983). No exposure measurements were reported for either study.

(b) Exposure during the manufacture and use of chlordimeform

4-Chloro-*ortho*-toluidine has been detected in the urine of workers exposed to chlordimeform in the production, packaging and agricultural use of this insecticide.

After substantial improvement of working conditions in a chlordimeform-manufacturing plant in 1980, the concentrations of 4-chloro-*ortho*-toluidine and chlordimeform were measured in the urine of production workers and reported to be minimal (Popp *et al.*, 1992). Quantitative information on concentrations before and after the improvements was not given.

Among employees of a chemical plant, nine of 22 workers who packaged chlordimeform became suddenly ill; chlordimeform and 4-chloro-*ortho*-toluidine were present in their urine three days after exposure (Folland *et al.*, 1978).

Kurtz *et al.* (1987) monitored agricultural workers exposed to chlordimeform used as a pesticide on cotton in California, USA, during the 1982 application season. Chlordimeform and its metabolites were detected in the urine.

Geyer and Fattal (1987) found 4-chloro-*ortho*-toluidine at a concentration of 240 µg/L in the urine of an agricultural worker exposed to chlordimeform in a cotton-growing area.

Among 1000 urine samples from 130 cotton growers, two thirds had no detectable levels of 4-chloro-*ortho*-toluidine. For six mixer/loader/applicators, the concentration was higher than 1 ppm (Coye *et al.*, 1986).

(c) Other uses

Workers can be exposed to 4-chloro-*ortho*-toluidine during its laboratory use as an immunochemical stain. Under the name Fast Red TR, 4-chloro-*ortho*-toluidine is also reported to be used in a colorimetric method to assess the authenticity of drugs (Green *et al.*, 2000). In a study from Finland (Kauppinen *et al.*, 2003), one of five molecular-

biology laboratories used 4-chloro-*ortho*-toluidine, albeit in very small amounts (< 1 g per year). According to the 1981–83 National Occupational Exposure Survey (NOES, 1983), 250 chemists employed in health services in the United States were potentially exposed to 4-chloro-*ortho*-toluidine.

1.3.3 *Environmental occurrence and exposure in the general population*

The general population can be exposed to 4-chloro-*ortho*-toluidine via the environment and in food products as a decomposition product of chlordimeform. Riffelmann *et al.* (1995) reported concentrations of 4-chloro-*ortho*-toluidine in the urine of occupationally non-exposed smokers ($n = 8$) and nonsmokers ($n = 8$) as 3.0 μg/L (0.0–8.0 μg/L) and 2.2 μg/L (0.0–6.3 μg/L).

(a) Water

4-Chloro-*ortho*-toluidine can occur in water as a result of the hydrolysis of chlordimeform via hydrolysis of the intermediate *N*-formyl-4-chloro-*ortho*-toluidine (WHO, 1998).

(b) Soil

The microbial degradation of chlordimeform in soils by several bacterial and fungal species has led to formation of 4-chloro-*ortho*-toluidine (Johnson & Knowles, 1970).

(c) Plants and foods

4-Chloro-*ortho*-toluidine has been identified in field samples of plant materials treated with chlordimeform, e.g. in young bean leaves at concentrations of < 0.1–0.2 ppm [mg/kg], in grape stems at 0.02–0.3 ppm, in a mixture of grape stems and berries at 0.02–0.5 ppm and in prunes and apples at < 0.04 ppm (Kossmann *et al.*, 1971). In an experimental field application (one, two or three treatments with chlordimeform, harvest 42 days after last treatment), 4-chloro-*ortho*-toluidine was detected in rice grains at 25, 53 and 61 ppb [μg/kg], respectively, and in straw parts at 1600, 7200 and 6900 ppb, respectively (Iizuka & Masuda, 1979). 4-Chloro-*ortho*-toluidine was detected as a metabolic product in cotton plants following treatment with chlordimeform (Bull, 1973) and similarly in cucumbers and apples (Grübner, 1977). 4-Chloro-*ortho*-toluidine could also potentially occur in honey, as chlordimeform is used in some countries against the mite *Varroa jacobsoni* Oud., which seriously affects honey beehives (Jiménez *et al.*, 2002). 4-Chloro-*ortho*-toluidine was not detected in cargo rice and husks sprayed or treated on the soil with chlordimeform (limit of detection, 0.028 ppm) (Fan & Ge, 1982).

(d) Finger paint

Garrigós *et al.* (2000) detected 4-chloro-*ortho*-toluidine in five commercially available fingerpaints at concentrations between 0.3–0.6 ng/g dry paint [total number of

samples not indicated; values read from graph]. In an earlier study, 4-chloro-*ortho*-toluidine was detected in one of three tested fingerpaints (0.3 ng/g dry paint) (Garrigós *et al.*, 1998).

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azo-colourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes that, by reductive cleavage of one or more azo groups, may release 4-chloro-*ortho*-toluidine in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(b) Directive 2004/37/EC

4-Chloro-*ortho*-toluidine is regulated by Directive 2004/37/EC (European Commission, 2004), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(c) Directive 2005/90/EC

In the Directive 2005/90/EC, the list of substances classified as carcinogenic, mutagenic or toxic to reproduction of Directive 76/769/EEC was amended to include 4-chloro-*ortho*-toluidine (European Commission, 2005).

1.4.2 *Germany*

Deviating from the EU classification, 4-chloro-*ortho*-toluidine is classified as a Category-1 carcinogen by the MAK Commission (MAK, 2007). The MAK Commission listed 4-chloro-*ortho*-toluidine as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set. Furthermore, it was classified as germ-cell mutagen, Class 3A (Substances that have been shown to induce genetic damage in germ cells of humans or animals, or that produce mutagenic effects in somatic cells of mammals *in vivo* and have been shown to reach the germ cells in an active form).

1.4.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of 4-chloro-*ortho*-toluidine in Group 2A.

1.4.4 *USA*

4-Chloro-*ortho*-toluidine and 4-chloro-*ortho*-toluidine hydrochloride are listed in the NTP Report on Carcinogens as *reasonably anticipated to be human carcinogens* (NTP, 2005).

2. Studies of Cancer in Humans

2.1 Cohort studies

Uebelin & Pletscher (1954) studied the occurrence of bladder tumours in workers at a factory in Switzerland producing dyestuff intermediates. The authors distinguished a group of 35 men who prepared 4-chloro-*ortho*-toluidine from *ortho*-toluidine during the years 1924–1953; no bladder tumours were found among these men [The Working Group noted that insufficient details concerning person-years at risk and period of follow-up were provided to evaluate the significance of this observation].

Ott & Langner (1983) studied the mortality of 342 male employees assigned to three aromatic amine-based dye production areas from 1914 to 1958 at a chemical plant in the United States. All study subjects had some period of employment at the plant after 1st January, 1940. Expected numbers of deaths for the period 1940–1975 were based on mortality rates for US white males. There were no deaths from cancer of the bladder and other urinary organs [SMR, 0.0; 95% CI, 0–2.46]. In one area of the plant, 117 men produced bromindigo and thioindigo with potential exposure to 4-chloro-*ortho*-toluidine and other raw materials and intermediates, including *ortho*-toluidine. No bladder cancer deaths occurred in this subcohort. [The expected figure was unspecified but estimated to be about 0.5.] [The Working Group noted that the interpretation of this study was limited by the small size of the population exposed to 4-chloro-*ortho*-toluidine, and the ascertainment of deaths only.] There was one death from lymphoma [SMR 0.83] and two deaths from leukaemia [SMR 2.22].

Stasik *et al.* (1985) reported findings from a mortality study of 335 male workers employed for at least twelve months in the period 1929–1982 in the production and processing of 4-chloro-*ortho*-toluidine at a dyestuffs-manufacturing plant in Saarland, Germany. Three other monocyclic amines had been used at the plant, *viz N*-acetyl-*ortho*-toluidine, 6-chloro-*ortho*-toluidine and *ortho*-toluidine. Exposure to 4-chloro-*ortho*-toluidine was reported to be predominant. No deaths from bladder cancer were identified in the period 1929–1982 [expected figure unspecified but estimated on the basis of

mortality rates for the Federal Republic of Germany to be about 0.2.] [The Working Group noted that the study had inadequate tracing of deaths and description of methods.] Two incident cases of urothelial carcinoma were subsequently identified in two workers employed in the 4-chloro-*ortho*-toluidine production-plant before improvements in industrial hygiene were made in 1970. As a result of this discovery, a cancer-incidence study was established (Stasik, 1988) for the 116 subjects employed at this plant before 1970. There was no cancer registry for the region in which the plant was located; expected numbers were based, therefore, on rates for Hessen, a neighbouring province of the Federal Republic of Germany. A marked excess of bladder-cancer incidence based on eight cases was reported (SIR, 72.7; 95% CI, 31.4–143.3). No quantitative measure of exposure to 4-chloro-*ortho*-toluidine was available, and exposure to other amines was also present. Cigarette smoking was not considered to be an important confounding variable as three of the eight cases were nonsmokers. [The Working Group noted that the definition of the subcohort for the cancer incidence survey was made *a posteriori,* the observational period was unspecified, and case ascertainment was inadequately described. Consequently, some bias in the estimate of excess risk may be present. The excess of bladder cancer could not be attributed with any certainty to 4-chloro-*ortho*-toluidine or to any one of the other compounds present.]

Popp *et al.* (1992) reported the results of a bladder-cancer incidence study among 49 male workers exposed on an irregular basis to 4-chloro-*ortho*-toluidine in the synthesis of chlordimeform from 1965 to 1986 in a German chemical plant. The period of follow-up was stated to be 1950–1990. Expected numbers were available from incidence rates based on cancer registry data from Saarland, Germany. There was a marked excess of bladder tumours based on seven observed cases (SIR, 53.8; 95% CI, 21.7 –110.9), all of which were found in workers exposed to 4-chloro-*ortho*-toluidine before 1976, when working conditions were improved. Some of these workers were also exposed to the aromatic amine 4-chloroaniline (classified as Group 2B, possibly carcinogenic to humans (IARC, 1993)), which was used for appreciably shorter periods and in smaller quantities than 4-chloro-*ortho*-toluidine. No bladder tumours were noted among a further group of 121 individuals exposed only to the final product, chlordimeform. [The Working Group noted that the study methods were not fully described, including allocation of person-years and case ascertainment. Concomitant exposure to chlordimeform and 4-chloroaniline could not be excluded as confounders. The Working Group considered that the excess of bladder cancer reported was too large to have been due to smoking alone.]

3. Studies of Cancer in Experimental Animals

Studies in experimental animals to assess the carcinogenicity of 4-chloro-*ortho*-toluidine, when given orally as the hydrochloride salt, were previously reviewed by IARC (1978, 1983, 1987, 1990, 2000). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.1 Oral administration

3.1.1 *Mouse*

Groups of 25 male and 25 female Swiss CD-1 mice, 6–8 weeks of age, were fed 4-chloro-*ortho*-toluidine hydrochloride (97–99% pure) in the diet at dose levels of 0, 750 or 1500 mg/kg diet (ppm) (males) or 0, 2000 or 4000 mg/kg diet (ppm) (females) for 18 months. Animals were then kept without treatment for three further months and then killed. The doses were chosen on the basis of preliminary tests, the higher being the maximum tolerated dose. A parallel control group of 25 untreated mice of each sex was used, plus additional controls used for the other compounds tested in the study. Tumour incidences of matched and pooled controls were compared statistically (both separately and together) with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. In male mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined, observed mainly in the spleen and in the subcutaneous or subperitoneal adipose tissue) was 0/14, 5/99 (5%), 12/20 (60%) ($P < 0.025$, Fisher exact test) and 13/20 (65%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively. In female mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined) was 0/15, 9/102 (9%), 18/19 (95%) ($P < 0.025$, Fisher exact test) and 12/16 (75%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively [the separate incidences for haemangiomas and haemangiosarcomas were not reported] (Weisburger *et al.*, 1978).

Groups of 50 male and 50 female B6C3F$_1$ mice, six weeks of age, were given 4-chloro-*ortho*-toluidine hydrochloride (purity > 99%) in the diet at concentrations of 3750 or 15 000 ppm (males) and 1250 or 5000 ppm (females) for 99 weeks, except high-dose females, which had all died by 92 weeks. Concurrent controls consisted of 20 male and 20 female untreated mice. Mean body weights of all treated groups were lower than those of the corresponding controls and were dose-related. Mortality was dose-related for both males and females ($P < 0.001$, Tarone test for dose-related trend). The incidence of haemangiosarcomas (originating in the adipose tissue adjacent to the genital organs) was 0/20, 3/50 (6%) and 37/50 (74%) ($P < 0.025$, Fisher exact test) in control, low- and high-dose group males, respectively, and 0/18, 40/49 (82%) ($P < 0.025$, Fisher exact test) and 39/50 (78%) ($P < 0.025$, Fisher exact test) in control, low- and high-dose group females, respectively (National Cancer Institute, 1979).

3.1.2 *Rat*

Groups of 25 male Sprague-Dawley CD rats, 6–8 weeks of age, were treated with 4-chloro-*ortho*-toluidine hydrochloride (97–99% pure) in the diet at dose levels of 2000 or 4000 ppm for three months and then, due to toxicity, lowered to levels of 500 or 1000 ppm for a further 15 months. Animals were kept without treatment for an additional six months and then killed. The doses were chosen on the basis of preliminary tests, the higher dose being the maximum tolerated dose. A concurrent control group of 25 untreated male rats was used, plus additional controls used for the other compounds tested in the study. Tumour incidences of matched and pooled controls were compared with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. There was no treatment-related increase in the incidence of tumours at any site (Weisburger *et al.*, 1978). [The Working Group noted the non-standard protocol and the lack of information on survival.]

Groups of 50 male and 50 female Fischer 344 rats, six weeks of age, were given 4-chloro-*ortho*-toluidine hydrochloride (purity > 99%) in the diet at concentrations of 1250 or 5000 ppm for 107 weeks. Concurrent controls consisted of 20 male and 20 female untreated rats. Mean body weights of the high-dose males and females were lower than those of the corresponding controls. Mortality was not significantly affected by treatment in rats of either sex. At the end of the study, survival in treated groups of males was > 75% compared with > 55% in control males, and > 80% in treated females compared with > 75% in control females. No tumours occurred at incidences that could be related to the treatment (National Cancer Institute, 1979).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

4.1.1 *Humans*

No data were available to the Working Group

4.1.2 *Experimental systems*

4-Chloro-*ortho*-[^{14}C]toluidine is a major metabolite of the insecticide chlordimeform, *N'*-(4-chloro-*ortho*-tolyl)-*N,N*-dimethyl-formamidine. Knowles & Gupta (1970) assessed the metabolism of both compounds in Sprague-Dawley rats. Chlordimeform ([^{14}C]-Galecron^{R}, tolyl methyl-[^{14}C], CIBA Agrochemical Co.) was orally administered to rats. Urinary excretion of [^{14}C]-Galecron after 72 hours represented 88% of a 3-μCi (specific activity, 1.43 mCi/mmol) dose, with 7.5% excreted in faeces. The chloroform-soluble radioactive material in urine (recovery not provided) was separated by means of silica-gel TLC and tentatively identified by co-chromatography with authentic standards.

Besides Galecron, the urine contained demethyl-Galecron, *N*-formyl-4-chloro-*ortho*-toluidine, and 4-chloro-*ortho*-toluidine. Three other metabolites were also observed, but not identified. The highest levels of radioactive equivalents were observed in liver > muscle > fat. In further experiments, [^{14}C]-4-Chloro-*ortho*-toluidine (specific activity, 1.43 mCi/mmol) was also orally administered to Sprague-Dawley rats. Urinary excretion accounted for 71% of the 3-μCi dose and faecal excretion was 25% after 72 hours. Approximately 50% of the radioactivity in urine was recovered in the ethyl-acetate extract and analysed by TLC as described for Galecron above. Besides 4-chloro-*ortho*-toluidine, urinary metabolites included 5-chloroanthranilic acid and 4-chloro-2-methylacetanilide. The latter is an *N*-acetylated metabolite, and its excretion never exceeded that of 5-chloroanthranilic acid. Five unknown metabolites were also indicated by TLC, but not further identified. The highest levels of 4-chloro-*ortho*-[^{14}C]toluidine equivalents were found in the fat > liver > kidney. The aqueous fraction from the urinary extracts was not further analysed. The major pathways proposed for chlordimeform metabolism in the rat are described as follows: *N*-demethylation forms demethyl-chlordimeform; both the latter and chlordimeform are cleaved at the carbon-nitrogen bond to form *N*-formyl-4-chloro-*ortho*-toluidine, which can undergo deformylation to 4-chloro-*ortho*-toluidine; oxidation of the methyl group on 4-chloro-*ortho*-toluidine produces 5-chloroanthranilic acid, while oxidation of *N*-formyl-4-chloro-*ortho*-toluidine produces *N*-formyl-5-chloroanthranilic acid; the latter can undergo deformylation to 5-chloroanthranilic acid.

4-Chloro-*ortho*-toluidine was one of thirteen different monocyclic aromatic amines given orally to female Wistar rats and female B6C3F1 mice (0.5 mmol [71 mg]/kg bw) (Birner & Neumann, 1988). The haemoglobin binding index (HBI) for 4-chloro-*ortho*-toluidine was 11-fold higher (HBI 28) in rats than in mice (HBI 2.5). Twenty-four hours after an oral dose of chlordimeform, 0.5 mmol/kg bw, to female Wistar rats, the HBI for 4-chloro-*ortho*-toluidine was 2.5 (Sabbioni and Neumann, 1990). The latter value is less than one tenth of that reported for 4-chloro-*ortho*-toluidine above in rats. This is because chlordimeform must be metabolized to 4-chloro-*ortho*-toluidine before it can be activated to bind Hb. In contrast to other aromatic amines, *N*-acetylation is not important in the metabolism and activation of 4-chloro-*ortho*-toluidine.

In Osborne-Mendel rats that were given 4-chloro-*ortho*-[*methyl*-^{14}C]toluidine intraperitoneally at a dose of 14 mg/kg bw, the levels of adducts bound to protein (480 pCi), RNA (560 pCi), and DNA (170 pCi) were significantly higher in the liver than in 10 other tissues examined (Hill *et al.*, 1979). In in-vitro studies with liver microsomes, irreversible binding of radioactivity was detected which was dependent upon NADPH and linear with respect to incubation time and protein concentration. Binding to macromolecules *in vitro* was increased in rats pretreated with phenobarbital intraperitoneally at a dose of 100 mg/kg bw for two days, implying that cytochrome P450 is involved in the reaction. The major and minor microsomal metabolites were identified by mass spectrometry as 5-chloro-2-hydroxyaminotoluene and 4,4′-dichloro-2,2′-dimethylazobenzene, respectively. The former product suggested the presence of an *N*-OH intermediate, which is further oxidized to an *N*-nitroso derivative as indicated by

the latter product. Consistent with the studies described above (Birner & Neumann, 1988; Sabbioni & Neumann, 1990), these authors propose that the *N*-OH derivatives of these monocyclic aromatic amines are co-oxidized within the red blood cell to nitroso metabolites that bind to SH groups in haemoglobin.

Following oral administration of 25 mg/kg bw 4-chloro-*ortho*-toluidine to male Sprague-Dawley-derived [Tif:RAIf (SPF)] rats and male mice [Tif:MAGf (SPF)], both from CIBA-GEIGY, covalent binding to both DNA and protein occurred (Bentley *et al.*, 1986a). Three times more radioactivity was shown to be covalently bound to rat liver protein (199 ± 18 pmol/mg) than to mouse-liver protein (62 ± 126 pmol/mg). The results with protein binding are consistent with the study mentioned above, which showed that the Hb-binding index for 4-chloro-*ortho*-toluidine was 11-fold higher in rats than in mice (Birner & Neumann, 1988). However, twice as much liver-DNA binding was detected in the mouse study than in the rat study. Two DNA adducts were observed by two-dimensional TLC on silica-gel plates for both mouse and rat. One adduct was present at six- to 30-fold higher levels in mice than in rats. Furthermore, more radioactivity was observed to bind to calf-thymus DNA when 4-chloro-*ortho*-toluidine was activated with mouse- *vs* rat-liver subcellular fractions. The higher levels of DNA-adduct formation of 4-chloro-*ortho*-toluidine in mice may mechanistically explain the greater susceptibility of the mouse compared with the rat to 4-chloro-*ortho*-toluidine-induced haemangiosarcoma.

The ability of chlordimeform and 4-chloro-*ortho*-toluidine to affect hepatic xenobiotic biotransformation in male Sprague-Dawley rats was assessed (Leslie *et al.*, 1988). Chlordimeform did not affect total CYP content, aniline *p*-hydroxylase or glutathione *S*-transferase activities, but induced ethoxyresorufin-*O*-deethylase, ethoxy-coumarin-*O*-deethylase and epoxide-hydrolase, while decreasing the activities of aldrin epoxidase and aminopyrine *N*-demethylase. These effects were observed after seven consecutive daily intraperitoneal injections of 50 or 100 mg/kg bw, but not with 1 or 10 mg/kg bw chlordimeform, except for a reduction in aminopyrine *N*-demethylase, which was also seen at 10 mg/kg bw chlordimeform. A similar experiment with 7 × 100 mg/kg bw 4-chloro-*ortho*-toluidine increased the CYP content and the activities of ethoxyresorufin-*O*-deethylase, ethoxycoumarin-*O*-deethylase, glutathione *S*-transferase and epoxide hydrolase. At 10 mg/kg bw, 4-chloro-*ortho*-toluidine also increased the activities of ethoxyresorufin-*O*-deethylase and ethoxycoumarin-*O*-deethylase. Both compounds caused an enhanced activity on certain hydroxylation sites of androstenedione and a small decrease in testosterone synthesis. Increased biotransformation by CYPs is likely to involve CYP1A1 and 1A2 (Leslie *et al.*, 1988).

The metabolism of chlordimeform ([^{14}C]-Galecron^{R}, tolyl methyl-[^{14}C], CIBA Agrochemical Co.) in animals and plants has been reviewed. The cumulative percentages of the dose excreted in 24-hour urine were 75% in mongrel dogs, 65% in a lactating goat, and 80% for a male goat. The percentage of the dose excreted in faeces after 72 hours was 0.6 and 1.8% in dog and goat, respectively. After 24 hours, 25% of radioactive material in rat urine partitioned into chloroform, but < 10% was extractable in organic solvents from dog and goat urine. The amounts of chlordimeform, referred to as chlorphenamidine,

expressed as a percentage of organic-soluble radioactivity at 24 and 72 hours were 9.9% and 2.1% for the rat, 1.3% and 0.2% for the dog, and 0.1% and < 0.1% for the goat. The organic-soluble metabolites in all three species included demethyl-Galecron, *N*-formyl-4-chloro-*ortho*-toluidine, 4-chloro-*ortho*-toluidine, *N*-formyl-5-chloroanthranilic acid, and 5-chloroanthranilic acid. The conversion of chlordimeform to *N*-formyl-5-chloro-anthranilic acid, and 5-chloroanthranilic acid was goat > dog > rat. The water-soluble radioactivity was further analysed in dog and goat urine. Treatment with β-glucuronidase or β-glucuronidase-arylsulfatase yielded aglycones tentatively identified by means of TLC as glucuronide or sulfate conjugates. The biliary excretion was also examined in a mongrel dog treated orally with chlordimeform at 0.3 mg/kg bw. After 72 hours, 5% of the dose was recovered in bile, with peak concentrations of radioactivity occurring after eight hours. Metabolites in bile, present in free and conjugated form, included all those mentioned above and several unknown compounds. *N*-formyl-4-chloro-*ortho*-toluidine was the major metabolite in bile. Rat-liver microsomal metabolism of [^{14}C]-Galecron produced demethyl-Galecron, which was sensitive to SKF 525A, but not to diisopropyl-fluorophosphate. Microsomes from the abdomens of houseflies, *Musca domestica L*., also produced demethyl-Galecron. In addition, [^{14}C]-*N*-formyl-4-chloro-*ortho*-toluidine was converted to 4-chloro-*ortho*-toluidine by a rat-liver soluble fraction, which was inhibited by diisopropylfluorophosphate, but not SKF 525A (Knowles, 1970; Knowles & Gupta, 1970).

4.2 Genetic and related effects (see Table 4.1 for details and references)

Data on the genetic and related effects of 4-chloro-*ortho*-toluidine that were available up to 1990 and 1993 have been reviewed by IARC (1990) and Jackson *et al.* (1993), respectively.

4-Chloro-*ortho*-toluidine has been tested in several laboratories with a series of *Salmonella* strains, and the results have been generally negative. There were single positive results with strains TA100 and TA98 (with metabolic activation) and with TA1535 (without metabolic activation). Mutation tests (with and without activation) and DNA repair assays (without activation only) in *Escherichia coli* were negative, while a positive response was observed for differential toxicity in *S. typhimurium*, indicating the induction of DNA damage.

Positive results have been obtained in several tests in mammalian systems, including induction of DNA strand-breaks *in vitro*, unscheduled DNA synthesis in rat primary hepatocytes, sister chromatid exchange and chromosomal aberrations (only in the presence of an external metabolizing system) in Chinese hamster cells *in vitro*, and transformation of BALB/c 3T3 mouse cells. The mouse-spot test was also positive. 4-Chloro-*ortho*-toluidine bound to hepatic DNA and RNA in mice and rats *in vivo*, with a greater extent of binding in mice than in rats. In contrast, it gave negative results in both the sister chromatid exchange and chromosomal aberration assays in human lymphocytes *in vitro* and in the mouse heritable translocation assay *in vivo*.

4.2.1 *Macromolecular binding*

Bentley *et al.* (1986a) examined the covalent binding of 4-chloro-*ortho*-toluidine to macromolecules in rats and mice, and found that the extent of covalent binding of 4-chloro-*ortho*-toluidine to hepatic DNA in mice was about twice as high as that in rats. This was apparent at all time points, i.e. 6, 12, 28, and 68 hours after a single oral dose of 25 mg/kg bw of [^{14}C]-ring-labelled 4-chloro-*ortho*-toluidine. The extent of covalent binding to DNA decreased with time after the application, indicating that the DNA damage was being repaired, the extent and rate of repair being very similar in both species. Binding, assessed 20 hours after application, was proportional to the total dose given, following repeated daily application of 4-chloro-*ortho*-toluidine for up to five consecutive days in both rats and mice. Two major hydrophobic DNA-adducts were formed in both species, one of these adducts being 6–30-fold more prominent in the mouse than in the rat. The nature of the DNA adducts has not been clarified. Also, more radioactivity was bound to calf-thymus DNA *in vitro* when [^{14}C]-4-chloro-*ortho*-toluidine was activated by mouse-liver subcellular fractions than when rat-liver fractions were used. These findings suggest that mice were more efficient than rats at forming reactive metabolites from 4-chloro-*ortho*-toluidine. However, there was more covalent binding of this compound with hepatic proteins in rats than in mice. Twenty hours after a single application of [^{14}C]-4-chloro-*ortho*-toluidine, the amount bound to hepatic proteins of rats was 199 ± 18 pmol equivalents per mg, which was more than 3 times higher than in mice (62 ± 16 pmol equivalents per mg). The species differences in the metabolism of *4*-chloro-*ortho*-toluidine could account for the fact that mice were more susceptible to the carcinogenic effects of 4-chloro-*ortho*-toluidine.

A similar study conducted by the same group (Bentley *et al.,* 1986b) reported that at all time points after a single administration of [^{14}C]4-chloro-*ortho*-toluidine the extent of binding decreased in the order: protein > RNA > DNA in both species. In-vitro experiments showed that mouse-liver subcellular fractions catalysed the binding of *4*-chloro-*ortho*-toluidine to calf-thymus DNA more readily than did rat-liver fractions. Conversely, binding to protein and RNA was more marked in the rat than in the mouse. It seemed that different patterns of reactive metabolites are formed from *4*-chloro-*ortho*-toluidine in the two species, such that mice produced more DNA-reactive metabolites, while rats produced more metabolites with a high affinity for proteins and RNA.

The high incidence of haemangiosarcomas associated with chronic feeding of 4-chloro-*ortho*-toluidine suggests that blood-vessel endothelial cells are most susceptible to the effects of this compound. Binding of 4-chloro-*ortho*-toluidine to DNA isolated from non-parenchymal cells in mouse liver was investigated, but no preferential DNA-binding in these cells was found (Bentley *et al.*, 1986b). Metabolic activation by microsomal fractions and NADPH were essential for activation, but inducers of several CYP-dependent mono-oxygenases had no demonstrable effect on the rate of DNA binding. Cytosolic enzymes did not alter metabolic activation since the rate of binding to DNA was similar, whether microsomes or S9-supernatant were used for activation. When

Table 4.1. Genetic and related effects of 4-chloro-*ortho*-toluidine

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium, repair-deficient strains, TA1538, TA1978, differential toxicity	+	NT	250 mg/disc	Rashid *et al*. (1984)
Escherichia coli rec strains, differential toxicity	–	NT	1000 mg/disc	Rashid *et al*. (1984)
Salmonella typhimurium TA100, reverse mutation[c]	–	+	17.8 µg/plate	Zimmer *et al*. (1980)
Salmonella typhimurium TA100, reverse mutation[c]	–	–	333 µg/plate	Haworth *et al.* (1983)
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation[c]	–	–	1000 µg/plate	Haworth *et al*. (1983)
Salmonella typhimurium TA100, TA1537, TA1538, TA98, reverse mutation[c]	–	NT	325 µg/plate	Rashid *et al*. (1984)
Salmonella typhimurium TA100, reverse mutation	–	+	100 µg/plate	Göggelmann *et al*. (1996)
Salmonella typhimurium TA1535, reverse mutation[c]	+	NT	200 µg/plate	Rashid *et al*. (1984)
Salmonella typhimurium TA1535, TA1537, reverse mutation	–	–	1500 µg/plate	Göggelmann *et al*. (1996)
Salmonella typhimurium TA1537, TA98, reverse mutation[c]	–	–	NR	Zimmer *et al*. (1980)
Salmonella typhimurium TA98, reverse mutation	–	+	375 µg/plate	Göggelmann *et al*. (1996)
Escherichia coli WP2 *uvrA*, WP2, other strains, reverse mutation[c]	–	–	2000 µg/plate	Rashid *et al*. (1984)

Table 4.1 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks, cross-links or related damage, Chinese hamster V79 lung cells *in vitro*[c]	(+)	NT	534	Zimmer *et al*. (1980)
Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	+	NT	14.2	Williams *et al*. (1989)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*[c]	+	+	50	Galloway *et al*. (1987)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*[c]	+	+	400	Galloway *et al*. (1987)
Cell transformation, BALB/c 3T3 mouse cells[c]	+	NT	75	Matthews *et al.* (1993)
Sister chromatid exchange, human lymphocytes *in vitro*	–	–	283	Göggelmann *et al*. (1996)
Chromosomal aberrations, human lymphocytes *in vitro*	–	–	283	Göggelmann *et al*. (1996)
Mouse spot test, C57BL6J×T mice[c]	+		100 po × 3[d]	Lang (1984)
Mouse heritable translocation test, NMRI/SPF mice[c]	–		200 po; 7 d/w, 7 w	Lang & Adler (1982)
Binding (covalent) to DNA, rat and mouse liver *in vivo*[c]	+		25 po × 1	Bentley *et al.* (1986a)
Binding (covalent) to RNA or protein, rat and mouse liver *in vivo*[c]	+		25 po × 1	Bentley *et al.* (1986a,b)

[a] +, positive; (+), weak positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw/day
[c] Test performed with the hydrochloride salt of 4-chloro-*ortho*-toluidine
[d] Treatments on days 8, 9 and 10 of embryonic development

added, S9-supernatant cofactors for sulfate, acetate, or glutathione conjugation did not affect the DNA binding. This suggested that activation involving *N*-hydroxylation followed by sulfate conjugation did not happen in this system. Different reactive metabolites may be responsible for binding to DNA and proteins.

Capillary endothelial cells are most probably the target for 4-chloro-*ortho*-toluidine-induced carcinogenesis, but endothelial cells of the aorta (Juchau *et al.*, 1976) and the liver (Baron *et al.*, 1981) were reported to have very low CYP mono-oxygenase activity. This may also be the case for capillary endothelial cells, but endothelial cells are generally active producers of prostaglandins (Moncada *et al.*, 1977). The co-oxidation of several aromatic amines with arachidonic acid was catalysed by prostaglandin synthase (Zenser *et al.*, 1980). Reactive intermediates that bind to calf-thymus DNA may be formed from 4-chloro-*ortho*-toluidine by horse-radish peroxidase in the presence of H_2O_2. Therefore, the peroxidative activation of 4-chloro-*ortho*-toludine may be carried out by prostaglandin synthase in the capillary endothelial cells (Bentley *et al.*, 1986b).

Sabbioni & Neumann (1990) investigated haemoglobin adducts of 4-chloro-*ortho*-toluidine released from pesticides. Female Wistar rats were dosed orally with the pesticide chlordimeform—a precursor of 4-chloro-*ortho*-toluidine—up to 1 mmol/kg bw. Blood was obtained after 24 hours, and haemoglobin isolated and hydrolysed with 1N NaOH. The amines were extracted and quantified by gas chromatography with nitrogen-specific or mass-selective detection. The haemoglobin binding index (binding in nmol/mol Hb, per dose in nmol/kg) of 4-chloro-*ortho*-toluidine was 2.4. This indicated that 4-chloro-*ortho*-toluidine was produced from chlordimeform and bound to haemoglobin in rats.

4.2.2 *Cytogenetic effects*

Galloway *et al.* (1985) developed a screening protocol for detecting chemically-induced cytogenetic changes *in vitro*. This protocol was used to test several chemicals including 4-chloro-*ortho*-toluidine for their ability to induce chromosomal aberrations and sister chromatid exchange (SCE) in Chinese hamster ovary cells, with and without a rat-liver metabolic activation system (S9 mix). The SCE test of 4-chloro-*ortho*-toluidine was positive under conditions of cell-cycle delay; the response was stronger without S9 mix (Galloway *et al.*, 1987). Chromosomal aberrations were not seen without S9 when cells when fixed at 19.5 hours, but this may be due to insufficient allowance for cell-cycle delay, since aberrations were observed on SCE slides in the first metaphase (MI) cells at later fixation times (26–33 hours). With S9, there was a high frequency of aberrations in cells fixed at 19.5 hours (37% aberrant cells; 1.4 aberrations per cell) at the high dose of 400 µg/ml. A high frequency of endoreduplicated mitoses (over 20% of the mitotic cells) was noticed in all of the treated cultures (Galloway *et al.*, 1987).

4.2.3 *Mutagenicity*

Zimmer *et al.* (1980) tested the mutagenicity and DNA-damage induction by several substituted anilines including 4-chloro-*ortho*-toluidine, and found that this amine caused mutations in a dose-dependent manner with the *Salmonella*/microsome mutagenicity assay with tester strain TA100 (but not TA98), with metabolic activation by S9 mix. When the DNA damage in Chinese hamster lung fibroblast (V79) cells was measured by alkaline elution as described by Swenberg *et al.* (1976), 4-chloro-*ortho*-toluidine caused a slight increase in the elution of DNA from the filters following a 2-hour exposure and a significant increase when the incubation time was 4 hours.

Lang and Adler (1982) studied the mutagenic potential of 4-chloro-*ortho*-toluidine in the mouse heritable translocation assay. The maximal tolerated dose of the compound was given daily to male mice, by gavage, for seven consecutive weeks. After mating with untreated females, about 1000 F1 male offspring per group including a vehicle-control group and a positive control (tretamine, TEM) group were tested for their reproductive performance by use of a sequential decision procedure on litter sizes to select males with translocation heterozygosity. Partially sterile, sterile, and non-classifiable F1 males were examined cytogenetically by scoring chromosomes for translocation multivalents or analysing mitotic divisions for marker chromosomes. 4-Chloro-*o*-toluidine tested at doses that showed toxic effects did not induce translocation heterozygosity.

The mutagenic potential of 4-chloro-*ortho*-toluidine was further tested with the mammalian spot test. Female C57BL/6J mice were mated to T-stock males that had been treated by gavage with a maximal tolerated dose of 4-chloro-*ortho*-toluidine. Mutation induction was monitored in the off-spring by checking the fur of the young mice for coloured spots that resulted from expression of a recessive gene involved in coat-colour determination. It was found that 4-chloro-*ortho*-toluidine was mutagenic in the spot test, which is in line with in-vitro experiments and with its carcinogenic potential in the mouse. However, its precursor chlordimeform and another metabolite of chlordimeform, *N*-formyl-4-chloro-*ortho*-toluidine, were negative in this assay (Lang, 1984).

4-Chloro-*ortho*-toluidine is a metabolite of chlordimeform, a pesticide. The bacterial mutagenicity of chlordimeform, its metabolite 4-chloro-*ortho*-toluidine, and another metabolite, 4-chloro-*N*-formyl-*ortho*-toluidine, was evaluated (Rashid *et al.*, 1984). It was found that 4-chloro-*ortho*-toluidine did not cause mutation in strains TA98, TA1537, TA1538 and TA100, with or without S9 activation. However, 4-chloro-*ortho*-toluidine caused a dose-dependent response in tester strain TA1535. At a concentration of 325 µg/plate the number of revertant colonies was almost threefold increased over the control. At > 400 µg/plate, the number of revertant colonies declined. The same authors reported that 4-chloro-*ortho*-toluidine did not cause mutation in tryptophan-dependent *E. coli* WP2, with or without activation by rat-liver microsomal enzymes. However, 4-chloro-*ortho*-toluidine was active in inducing DNA damage in the *S. typhimurium* TA1538/TA1978 and *E. coli* multirepair-deficient systems (Rashid *et al*., 1984).

Hamzah & Eltorkey (1995) studied the mutagenicity of aromatic amines including 4-chloro-*ortho*-toluidine, *ortho*-toluidine, 4,4'-methylene dianiline (MDA), 4,4'-methylene-bis(2-chloroaniline) (MOCA) and its three possible substitutes, ethacure 300, cyanacure, and polacure 740M in *Salmonella typhimurium* tester strains TA98 and TA100. *ortho*-Toluidine, 4-chloro-*ortho*-toluidine, MDA, MOCA and its substitutes ethacure 300 and cyanacure showed mutagenic activity, while polacure 740M showed no mutagenic activity. All of the mutagens caused an increase in ethoxyresorufin *O*-deethylase (EROD) activity, while polacure 740M showed no appreciable increase in EROD activity. Thus, there was excellent correlation between mutagenicity and EROD induction. [The ability of a chemical to induce EROD activity has been suggested to bear some relationship to its carcinogenic potential.]

Göggelmann *et al.* (1996) studied the genotoxicity of 4-chloro-*ortho*-toluidine in *S. typhimurium*, human lymphocytes, and V79 Chinese hamster cells. In the absence of a metabolizing system (S9 mix), 4-chloro-*ortho*-toluidine did not induce mutations in *S. typhimurium* or chromosome aberrations and sister chromatid exchange in human lymphocytes. It also did not induce spindle disturbances in V79 Chinese hamster cells. In the presence of S9 mix, 4-chloro-*ortho*-toluidine induced revertants in *S. typhimurium* strains TA98 and TA100, but it was inactive in producing structural or numerical chromosomal changes in mammalian cells.

Sekihashi *et al.* (2002) conducted a comparative investigation of some carcinogenic chemicals including 4-chloro-*ortho*-toluidine in multiple organs from mice and rats, using the single-cell gel electrophoresis (Comet) assay. The species difference in genotoxic sensitivity was analysed at an equitoxic level but not at an equidose. Groups of four mice or rats were treated intraperitoneally or orally with a test chemical at the highest dose that did not cause death or distinct toxic effects. Mice were treated with 600 mg/kg bw, rats with 500 mg/kg bw. The stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow were sampled at 3, 8, and 24 hours after treatment. Significantly increased DNA migration was observed in the liver (both species), kidney (rats only) and bladder, lung and brain (mouse only).

4-Chloro-*ortho*-toluidine induced chromosomal aberrations and sister chromatid exchange in Chinese hamster ovary cells (Galloway *et al.*, 1987) and gave conflicting results for mutagenicity in *S. typhimurium* strain TA1535 (negative, Haworth *et al.*, 1983; positive, Rashid *et al.*, 1984) (see Jackson *et al.*, 1993).

4.3 Mechanistic considerations

As a general rule, *ortho*-alkylsubstitution increases the biological activity of aniline, as does blocking the *para*-position relative the amino-group. *ortho*-Toluidine has a higher biological activity than aniline, and 4-chloroaniline is also more active than aniline. It could therefore be expected that 4-chloro-*ortho*-toluidine is exceptionally potent among the monocyclic aromatic amines. This is not really the case as far as carcinogenicity in rats is concerned. Two cancer bioassays were considered negative (IARC, 2000). In mice,

however, the typical hemangiosarcoma and hemangioma in spleen developed at doses of 2000 and 4000 mg/kg diet (ppm), i.e. considerably lower doses than with *ortho*-toluidine. All three of these amines are acutely toxic and produce methaemoglobin and Heinz bodies as well as anaemia upon chronic administration. Toxic effects are seen in the spleen, liver and kidney, the LD50s in rats being in a range comparable with *ortho*-toluidine and 4-chloro-*ortho*-toluidine (600–1300 mg/kg bw, oral dose), but lower than 4-chloro-aniline (300–400 mg/kg bw). Excessive binding to haemoglobin reflects the greater toxicity of 4-chloro-aniline. The haemoglobin binding index is over 300, as compared to 4 with *ortho*-toluidine and 28 with 4-chloro-*ortho*-toluidine. However, it is not clear how acute toxicity participates in the carcinogenic process and how to explain the difference between the formation of rodent sarcoma and the human bladder tumours.

Evaluating the available information, the Working Group determined that 4-chloro-*ortho*-toluidine has no properties that would elevate it from the group of monocyclic aromatic amines. The toxicological profile rather supports the idea of a common mode of action for the aromatic amines in general and the monocyclic amines in particular.

5. Summary of Data Reported

5.1 Exposure data

4-Chloro-*ortho*-toluidine and its hydrochloride derivative are not known to occur naturally. 4-Chloro-*ortho*-toluidine is used primarily in the manufacture of organic colourants. Traditionally, it has been used in the manufacture of the acaricide and insecticide chlordimeform.

Occupational exposures to 4-chloro-*ortho*-toluidine occur predominantly among workers involved in its production or use in the manufacture of organic colorants (dyes and pigments) and of chlordimeform. 4-Chloro-*ortho*-toluidine was detected at levels of 1700–2100 μg/L in the urine of workers involved in the production of this amine. Other occupational exposures to 4-chloro-*ortho*-toluidine occur among workers in research laboratories who use this amine as a biological stain or in colorimetry.

The general population can be exposed to 4-chloro-*ortho*-toluidine as a contaminant in certain consumer products. 4-Chloro-*ortho*-toluidine has been detected at ppm levels in field samples of plant-based foods (e.g. bean leaves, grape stems, and berries) and cotton plants treated with chlordimeform. Similar levels have been found in finger paints.

5.2 Human carcinogenicity data

Three small cohort studies of workers exposed to 4-chloro-*ortho*-toluidine were available: one among dye production workers in the USA, and two studies of 4-chloro-*ortho*-toluidine production workers in Germany. The US study did not show an excess of bladder cancer, but the study was small and had limited power to detect any excess. The

two German studies showed high relative risks of bladder cancer. Co-exposure to *ortho*-toluidine could not be excluded as the cause of the excess risk in the 4-chloro-*ortho*-toluidine production workers.

5.3 Animal carcinogenicity data

4-Chloro-*ortho*-toluidine was tested for carcinogenicity by oral administration of its hydrochloride salt in two experiments in mice and in two experiments in rats. A significant increase of hemangiosarcomas or hemangiomas was observed in both sexes of two strains of mice that received chronic dietary administration of 4-chloro-*ortho*-toluidine hydrochloride. 4-Chloro-*ortho*-toluidine hydrochloride, however, was not found to be a carcinogen when administered chronically in the diet to two strains of rats.

5.4 Other relevant data

The toxicokinetics of 4-chloro-*ortho*-toluidine were studied in experimental animals by administering the compound itself or the insecticide chlordimeform, which is metabolized to 4-chloro-*ortho*-toluidine.

When 4-chloro-*ortho*-toluidine was given orally to rats and mice the haemoglobin binding index was 11-fold higher in the rat than in the mouse. After intraperitoneal administration of 4-chloro-*ortho*-toluidine to rats, the level of adducts bound to protein and DNA was higher in the liver than in ten other tissues.

In studies with liver microsomes isolated from rats treated with radiolabelled 4-chloro-*ortho*-toluidine, irreversible binding of radioactive material to macromolecules was detected, which was increased in rats pretreated with phenobarbital. The major and minor microsomal metabolites were identified as 5-chloro-2-hydroxy-aminotoluene and 4,4'-dichloro-2,2'-dimethylazobenzene, respectively. The former product suggests the presence of an *N*-OH intermediate, which is further oxidized to an *N*-nitroso derivative. *N*-Oxidized metabolites of 4-chloro-*ortho*-toluidine bind to protein, RNA and DNA. Bacterial mutagenicity tests with 4-chloro-*ortho*-toluidine gave predominantly negative results, but tests with cultured mammalian cells gave positive results. *In vivo*, 4-chloro-*ortho*-toluidine binds to hepatic DNA in mice and rats; the damage seems to be repaired rapidly and the structure of the adducts has not been elucidated. The only positive in-vivo mutagenicity test reported is a mouse spot test.

There are no data on toxicokinetics or genetic effects of 4-chloro-*ortho*-toluidine in humans.

6. Evaluation

6.1 Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of 4-chloro-*ortho*-toluidine.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of 4-chloro-*ortho*-toluidine.

6.3 Overall evaluation

4-Chloro-*ortho*-toluidine is *probably carcinogenic to humans (Group 2A)*.

7. References

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OCCUPATIONAL EXPOSURES OF HAIRDRESSERS AND BARBERS AND PERSONAL USE OF HAIR COLOURANTS

1. Exposure Data

1.1 Introduction

Throughout history and across cultures, women as well as men have felt the need to change the natural colour of their skin, lips and hair, or to restore the colour of greying hair. For thousands of years, cosmetic dyes have been a part of all human cultures. The use of hair dyes can be traced back at least 4000 years; evidence from royal Egyptian tombs suggests the use of henna for dyeing of hair and fingernails. Henna contains the dye Lawsone (2-hydroxy-1,4-naphthoquinone), which, in its pure form, is also used as a synthetic direct (semi-permanent) hair dye. In the days of the Roman Empire, grey hair was darkened by combing it with lead combs dipped in vinegar. Interestingly, it has recently been shown that this application produces darkening of grey hair by deposition of lead sulfide nanoparticles (diameter of about 5 nm) on the surface of the hair (Walter *et al.*, 2006; Nohynek *et al.*, 2004a). Traditional, lead acetate-based products for darkening grey hair are still found on the international market, although their importance is minor.

Today, millions of consumers use a large variety of cosmetic dyes and pigments to change the appearance of their skin, lips, nails or hair. Hair dyeing has become a common practice in modern industrialised societies; the hair-dye industry has estimated (unpublished data) that between 50 and 80% of women have used hair dyes in the USA, Japan and the European Union. During the last century, synthetic dyes have taken a pivotal role in hair colouration. Their chemistry, use and safety have been reviewed (Corbett 1999, 2000; Corbett *et al.*, 1999; Nohynek *et al.*, 2004a; Zviak & Millequant 2005a,b).

1.2 Composition of modern hair dyes

Modern hair dyes may be classified into the following categories: oxidative (permanent) dyes, direct (temporary or semi-permanent) dyes, and natural dyes.

In the frequently used code system introduced by the European Cosmetic Association (COLIPA), hair-dye ingredients are classified into classes A, B or C: class A includes

ingredients of oxidative hair dyes, class B those of semi-permanent hair dyes, and Class C those of temporary hair dyes (see examples in Tables 1.4, 1.5, 1.6).

1.2.1 *Oxidative (permanent) hair dyes*

(a) Composition

Oxidative (permanent) hair dyes are the most important group and have a market share in the EU or the USA of approximately 80% (Corbett *et al.*, 1999).

Oxidative (permanent) hair dyes consist of two components that are mixed before use and generate the dye within the hair by chemical reactions. Their chemistry and use has recently been reviewed (Corbett *et al.*, 1999; Zviak and Millequant, 2005b). Modern oxidative dyes contain several ingredients with different functions (examples in 507Table 1.1) as follows:

Primary intermediates: arylamines (*para*-phenylenediamine (PPD), *para*-toluenediamine (PTD), substituted *para*-diamines), *para*-aminophenols (*para*-aminophenol, 4-amino-*meta*-cresol), 4,5-diaminopyrazole and pyrimidines. Oxidation of these substances and their chemical coupling with modifier (coupler) molecules result in coloured reaction products.

Couplers or modifiers: these include *meta*-substituted aromatic derivatives (*m*-phenylene-diamines, *meta*-aminophenols, resorcinol), pyridines and naphthols. Couplers determine the final shade by reaction with the oxidized form of primary intermediates, followed by further oxidative coupling reactions.

Oxidants: hydrogen peroxide, urea peroxide, sodium percarbonate, perborate.

Alkalinising agents: ammonia, monoethanolamine or aminomethylpropanol.

(b) Relative concentration of the components

The actual colouring mixture is prepared extemporaneously, before application to the hair, by mixing, generally weight by weight, a solution containing the precursors and the other components of the formula with a solution containing hydrogen peroxide called developer. Each of these two solutions amounts in general to 50 g per use, but it is not uncommon to use 40 g of a colourant formula mixed with 60 g of developer. With non-lightening oxidative colouring the amount of developer may be twice the amount of colourant formula. The final mixture applied to the hair amounts to about 100 g but can vary according to the amount of hair to be treated.

Given that it is diluted by the developer, if the original concentration of a precursor (base or coupler) is X, its concentration in the final mixture coming into contact with the hair, is, at most X/2.

In practice, the initial concentration X used lies between 0.006% and 7%. This range of concentrations corresponds to a spectrum of shades from very pale blond to black. This

Table 1.1. Examples of dye-substance classes used in modern oxidative hair dyes (from Corbett, 1999 and 2000; Hair-dye industry data, 2007)

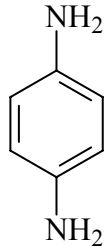
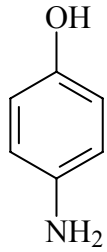
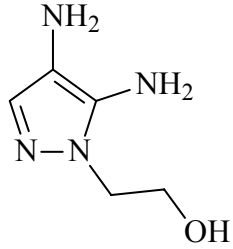
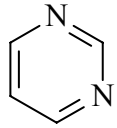
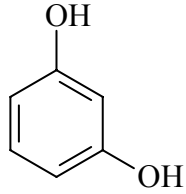
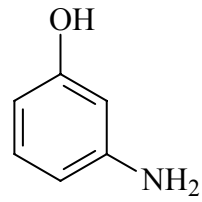
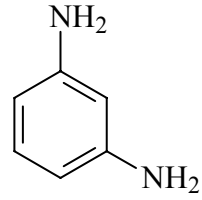
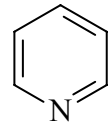
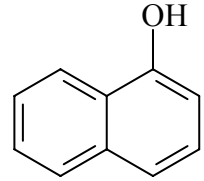
SUBSTANCE CLASS	STRUCTURE	FUNCTION
Para-Phenylenediamine		Primary Intermediate
Para-Aminophenol		Primary Intermediate
4,5-Diaminopyrazole		Primary Intermediate
Pyrimidine		Primary Intermediate
Resorcinol		Oxidative Coupler

Table 1.1 (contd)

SUBSTANCE CLASS	STRUCTURE	FUNCTION
Meta-Aminophenol		Oxidative Coupler
Meta-Phenylenediamine		Oxidative Coupler
Pyridine		Oxidative Coupler
1-Naphthol		Oxidative Coupler

is how the 20–70 shades of an oxidation-dye formulation are built up. It is worth noting that in the market:

- About 50% of shades contain no more than 0.5% precursors or colourants as a whole;
- About 25% of shades contain between 0.5% and 0.75% overall precursors or colourants; and
- About 25% of shades contain more than 0.75% of total precursors or colourants, with even fewer shades with a concentration reaching or exceeding 3.5% before mixing the developer.

The concentrations mentioned above represent the total content of precursors or dyestuffs (sometimes as many as 10) that is necessary to achieve the desired shade. More precisely, oxidation-dye products consist of a mixture of two or three bases, four or five

couplers and sometimes one or two direct colourants (see below) (Zviak & Millequant, 2005b).

The shade/colour achieved on the hair depends on the ingredients and their concentrations. Given the number of ingredients and the different resulting colour tones, a clear correlation of hair-dye shade with the concentration of the primary intermediate and coupler cannot be made. The dye shade, however, permits an estimation of the ingredient concentrations: dark hair dyes tend to contain the highest concentration of ingredients, whereas light (blond) shades tend to contain lower concentrations.

1.2.2 *Direct hair dyes*

Direct hair dyes include semi-permanent (resisting several shampooing processes) or temporary (resisting one or few shampooing processes) hair dyes. Direct hair dyes represent the second category of economically important hair colorants.

Semi-permanent colouring agents contain low-molecular-weight dye molecules, such as nitro-phenylenediamines, nitro-aminophenols, and some azo or anthraquinone dyes. These dyes may be used on their own in semi-permanent hair dyes or in combination with oxidative hair dyes in permanent hair-dye products to improve the tone of the final colour on the hair.

Temporary dyes represent the third class of hair colours. Temporary colouring agents are relatively large molecules and include azo-, triphenylmethane-, indophenol- or indamine-type dyes (Zviak & Millequant, 2005a) that are less resistant to washing and may be rinsed off by a single or a few shampooing processes. Typical formulations of semi-permanent hair dye are presented in Table 1.2.

1.2.3 *Natural hair dyes*

The majority of natural dyes use henna (produced by extraction of the leaves of a North African shrub (*Lawsonia inermis*) or the pure dye ingredient of henna, i.e. Lawsone (2-hydroxy-1,4-naphthoquinone). Henna has a long history of widespread use as a natural hair and body dye; however, a warning was raised against Lawsone by the Scientific Committee on Consumer Products (SCCP) of the European Commission (SCCNFP 2004). In addition, the reported increase in the use of Henna mixed with synthetic dye molecules, such as *para*-phenylenediamine (Black Henna) or other aromatic amines as a direct hair dye or body paint is of concern (Onder, 2003; Arranz Sánchez *et al.*, 2005).

Other natural dyes include extracts of Chamomile, indigo and various woods, barks or flowers (Zviak and Millequant, 2005a).

Natural dyes extracted from plants are of relatively small but growing economic importance.

Table 1.2. Ingredients of typical semi-permanent hair-colouring products

Light blond	*Reddish brown*
Water	Water
Ethoxydiglycol	Butoxyethanol
Polyethyleneglycol-50 tallow amide	Coconut acid diethanolamide
Hydroxyethylcellulose	Hydroxyethylcell ulose
Lauric acid diethanolamide	Lauric acid
Aminomethyl propanol	*N*-Methylaminoethanol
Erythorbic acid	HC Blue No. 2
Fragrance	2-Nitro-5-glyceryl methylaniline
Oleic acid	Fragrance
Triethanolammonium dodecylbenzenesulfonate	Butylparaben
CI Disperse Black 9	Ethylparaben
CI Disperse Blue 3	Methylparaben
CI Disperse Violet 1	Propylparaben
FD&C Yellow No. 6	3-Methylamino-4-nitrophenoxyethanol
HC Blue No. 2	3-Nitro-*para*-hydroxyethylaminophenol
HC Orange No. 1	HC Yellow No. 6
HC Red No. 3	CI Disperse Violet 1
HC Yellow No. 2	2-Amino-3-nitrophenol
HC Yellow No. 4	4-Amino-3-nitrophenol
	CI Disperse Blue 1
Red	*Dark brown*
Water	Water
Ethoxydiglycol	Butoxyethanol
Polyethyleneglycol-50 tallow amide	Polyglyceryl-2 oleyl ether
Hydroxyethylcellulose	Coconut acid diethanolamide
Lauric acid diethanolamide	Hydroxyethylcellulose
Aminomethyl propanol	HC Blue No. 2
Erythorbic acid	Lauric acid
Fragrance	*N*-Methylaminoethanol
Oleic acid	2-Nitro-5-glyceryl methylaniline
Triethanolammonium dodecylbenzenesulfonate	Fragrance
CI Disperse Black 9	HC Violet No. 2
HC Orange No. 1	CI Disperse Blue 1
HC Red No. 1	Butylparaben
HC Red No. 3	Ethylparaben
HC Yellow No. 2	Methylparaben
	Propylparaben
	HC Yellow No. 7
	3-Methylamino-4-nitrophenoxyethanol
	CI Disperse Violet 1
	3-Nitro-*para*-hydroxyethylaminophenol

From Cosmetic, Toiletry, and Fragrance Association (1992)
It should be noted that CI Disperse Blue 3, HC Yellow No. 6, 2-amino-3-nitrophenol, and CI Disperse Blue 1 are now banned by the European Union (*cf* Table 1.9).

1.2.4 *Trends in composition over time*

The invention, history and use of oxidative hair dyes and their ingredients has been reviewed (Corbett, 1999). The components of oxidative hair dyes have considerably changed since their introduction at the end of the 19th century. In the early phase, aromatic amines (primary intermediates, *para*-diamines, *para*-aminophenols) were used on their own in combination with hydrogen peroxide. During the period up the 1920s it was found that primary intermediates (*para*-diamines, *para*-aminophenols) may be combined with coupler molecules (resorcinol, *meta*-diamines, *meta*-aminophenols) to form coloured end products on oxidation. Thus in the early 1930s, the principal primary intermediates and couplers used today were already on the market. From the 1930s up to the 1970s there was little innovation in the components of oxidative and direct hair dyes; in the 1960s most manufacturers used a basic palette of 10–15 typical dye components; substances used in the period between the early 1930s to the 1970s are shown in Table 1.3.

Table 1.3. Some dye components used in typical oxidative and direct hair dyes in the period 1930s–1970s. US data (Corbett, 1999b)

SUBSTANCE	FUNCTION
para-Phenylenediamine	Primary intermediate
2,5-Diaminotoluene	Primary intermediate
N-Phenyl-*para*-phenylenediamine	Primary intermediate
4-Chlororesorcinol	Coupler
2,4-Diaminotoluene	Coupler
2,4-Diaminoanisole	Coupler
ortho-Aminophenol	Primary intermediate
para-Aminophenol	Primary intermediate
2-Amino-4-chlorophenol	Primary intermediate
Hydroquinone	Coupler
meta-Aminophenol	Coupler
Resorcinol	Coupler
Picramic acid	Direct dye
2-Nitro-*para*-phenylenendiamine	Direct dye
4-Amino-2-nitrophenol	Direct dye

In the 1970s and 1980s, major changes took place in the composition of direct and oxidative hair dyes. First, numerous innovative dyes were discovered, and several new direct and oxidative substances were introduced on the market. Second, in 1975, the positive results of mutagenicity tests in *Salmonella typhimurium* (Ames test) initiated a long-lasting controversy about the genotoxic and/or carcinogenic potential of some hair dyes, when Ames *et al.* suggested that nearly 90% of oxidative hair-dye ingredients were mutagenic in *Salmonella typhimurium* and might therefore pose a carcinogenic risk to consumers (Ames *et al.*, 1975).

On the basis of a worldwide survey coordinated by the EU, US and Japanese Cosmetic Associations during late 2007/early 2008, there would currently be 50

ingredients of oxidative, 43 ingredients of semi-permanent, and 88 ingredients of temporary hair dyes on the international market (EU, North- and Latin-America, Asia).

1.3 Production volumes

The major ingredients in oxidative hair dyes and their approximate annual worldwide tonnage of use (2005) are shown in Table 1.4. These data suggest that the bulk of substances used in oxidative hair dyes (total worldwide use at 50 to 250 tonnes) consist of traditional ingredients, such as resorcinol, *para*-phenylenediamine, 2,5-diaminotoluene and 4-amino-2-hydroxytoluene, *para*- and *meta*-aminophenol, 2-methyl-5-hydroxyethyl-aminophenol and 4-amino-*meta*-cresol. A few other ingredients are used at an annual tonnage of 10 to 50 tonnes, whereas most oxidative hair-dye ingredients are used at relatively minor amounts at 5 tonnes or less.

The estimated worldwide annual (2005) use of semi-permanent hair dyes is shown in Table 1.5. These data reveal a tonnage significantly lower than that of oxidative hair dyes and reflect their lesser economic importance. With the exception of two nitro-phenol-type semi-permanent dye ingredients (2-amino-6-chloro-4-nitrophenol and 4-hydroxypropyl-amino-3-nitrophenol), which are used at 5 to 15 tonnes per year, most substances are below 5 tonnes, whereas three substances are below 0.5 tonnes per year.

The estimated worldwide annual (2005) use of temporary hair dyes is shown in Table 1.6. The total annual use of most of these dyes is < 5 tonnes, except for Acid Violet 43 and Acid Red 33, which are used at 5 to 10 tonnes per year.

1.4 Application and formation of hair dyes

1.4.1 *Application of hair dyes*

Oxidative and direct hair dyes are applied to the hair in aqueous solutions or as a gel at a maximal concentration of 2.0 to 3.0% of the primary dye ingredient (dark-shade hair dyes) or < 0.05% (light-shade hair dyes) (see paragraph 1.2.1). After a contact time varying from a few to approximately 30 min permitting the hair-dyeing process to take place, the dye is rinsed off, and the hair is shampooed, rinsed, cut and dried. Detailed modern hair-dyeing techniques were recently reviewed (Zviak & Millequant, 2005a and 2005b).

1.4.2 *Reaction products of hair dyes*

Taking into account the standard volume of 80 mL of commercial hair-dye formulations, human external exposure during hair colouring would be in the range of 40 to 2400 mg. The frequency of hair-dye use varies between once per month (temporary hair dyes) to once per 6–8 weeks (oxidative hair dyes).

Table 1.4. Approximate total (North America, Latin America, EU, rest of the world) annual (2005) worldwide use (metric tonnes) of oxidative hair-dye ingredients. Data were collected by the international hair-dye industry and cover approximately 90% of the world market

INGREDIENT	COLIPA Code	2005 Total Use (tonnes)
2,5-Diaminotoluene	A005	150–200
para-Phenylenediamine	A007	150–200
Resorcinol	A011	200–250
4-Chlororesorcinol	A012	5–10
meta-Aminophenol	A015	50–100
para-Aminophenol	A016	50–100
1-Naphthol	A017	5–10
1,5-Naphthalenediol	A018	1–5
2,7-Naphthalenediol	A018	0.1–0.5
para-Methylaminophenol	A022	10–15
Hydroxybenzomorpholine	A025	0.5–1.0
4-Amino-2-hydroxytoluene	A027	150–200
2-Methyl-5-hydroxyethylaminophenol	A031	50–100
Phenylmethylpyrazolone	A039	10–15
2,4-Diaminophenoxyethanol	A042	20–25
3-Amino-2,4-dichlorophenol	A043	0.1–0.5
2-Methylresorcinol	A044	10–50
N,N-Bis-(2-hydroxyethyl)-*para*-phenylenediamine	A050	5–10
2,4,5,6-Tetraminopyridine	A053	1–5
4-Amino-*meta*-cresol	A074	50–100
6-Amino-*meta*-cresol	A075	1–5
1,3-Bis(2,4-diaminophenoxy)-propane	A079	1–5
Hydroxyethyl-*para*-phenylenediamine	A080	5–10
2-Amino-4-hydroxyethylanisole	A084	10–15
5-Amino-6-*ortho*-cresol	A094	1–5
Hydroxyethyl-3,4-methylenedioxylaniline	A098	1–5
2,6-Dihydroxy-3,4-duimethylpyridine	A099	0.1–0.5
2,6-Dimethoxy-3,5-pyridinediamine	A101	0.1–0.5
Hydroxypropyl-bis-(*N*-hydroxyethyl-*para*-phenylenediamine)	A121	1–5
6-Hydroxyindole	A128	1–5
3-Amino-3-hydroxypyridine	A132	10–15
2,6-Diaminopyridine	A136	0.1–0.5
2,6-Dihydroxyethylaminotoluene	A138	0.1–0.5
2,5,6-Triamino-4-pyrimidinol	A143	1–5
Dihydroxyindoline	A147	1–5
1-Acetoxy-2-methylnaphthalene	A153	1–5
1-Hydroxyethyl-4,5-diaminopyrazole	A154	10–50
2,2'-Methylenebis-4-aminophenol	A155	1–5

Table 1.5. Approximate annual (2005) worldwide use (metric tonnes) of semi-permanent hair-dye ingredients. Data were collected by the major international hair-dye industry and cover approximately 90% of the world market

INGREDIENT	COLIPA Code	2005 Total Use (tonnes)
Acid Yellow 1	B001	1–5
Disperse Red 17	B005	1–5
Basic Brown 17	B007	1–5
4-Nitro-*ortho*-phenylenediamine	B024	1–5
Picramic acid	B028	1–5
HC Red No. 13	B031	1–5
2-Nitro-5-glyceryl methylaniline	B060	1–5
HC Red No. 10 and 11	B071	1–5
2-Hydroxyethyl picramic acid	B072	1–5
4-Amino-2-nitrophenyl-amine-2'-carboxylic acid	B087	0.1–0.5
2-Chloro-6-ethylamino-4-nitrophenol	B089	1–5
2-Amino-6-chloro-4-nitrophenol	B099	10–15
4-Hydroxypropylamino-3-Nitrophenol	B100	5–10
2,6-Diamino-3-((pyridine-3-yl)azo)pyridine	B111	1–5
Basic Violet 2	B115	0.1–0.5
Basic Red 51	B116	0.1–0.5
Basic Yellow 87	B117	1–5
Basic Orange 31	B118	1–5

Table 1.6. Approximate annual (2005) worldwide use (metric tonnes) of major temporary hair-dye ingredients. Data were collected by the international hair-dye industry and cover approximately 90% of the world hair-dye market

INGREDIENT	COLIPA Code	2005 Total Use (tonnes)
Basic Red 76	C008	1–5
Basic Brown 16	C009	1–5
Basic Yellow 57	C010	1–5
Acid Orange 7 sodium salt	C015	1–5
Acid Red 33	CO22	5–10
Acid Yellow 23 trisodium salt	C029	1–5
Acid Yellow 3 mono- and disodium salt	C054	1–5
Basic Blue 99	C059	1–5
Acid Violet 43	CO63	5–10
Curry Red disodium salt	C174	1–5
Acid Red 18 trisodium salt	C175	1–5
Acid Red 52 sodium salt	C177	0.1–0.5
HC Blue 15 phosphate	C182	1–5
Tetrabromophenol blue	C183	1–5

An analytical methodology based essentially on HPLC was developed, which allowed the study of the kinetics of oxidative hair-dye coupling chemistry under conditions reflecting consumer usage, i.e. colour formation over 30 min (SCCNFP 2004, opinion 0941/05; Rastogi *et al.*, 2006). The methodology was applied to 11 different combinations of hair-dye precursors and couplers, and demonstrated that the amount of colour formed increases with time, while the amounts of free precursors and couplers decrease. Only the expected reaction products – based on the chemistry of the oxidative coupling of precursors and couplers – were formed, and no significant additional reactions or unexpected products were detected. Self-coupling products (such as Bandrowski´s base) or transient intermediates were not detected in the hair-dye formulations.

During the dyeing process, the consumer is exposed to the precursor(s), the coupler(s) and the expected reaction product(s). The kinetics of colour formation also revealed that the exposure of the consumer to the reaction product (hair dye) is much less than the exposure to the precursor and coupler over the whole application time. The total concentrations of unreacted precursors and couplers in various experiments were 12–84% of the applied dose. The typical concentration of reaction products in the formulations after 30 min varied from 0.02% to 0.65%. A worst-case scenario for the exposure from hair dyes in the dyeing process was derived from the data: the maximum content of a hair dye formed in the formulation 30 min after application was 0.65%. In a 70-ml (= 70-g) formulation this equals to 455 mg of hair dye formed.

1.5 Personal use of hair colourants

The main route of exposure to hair-dye components during personal use of hair colourants is dermal. Several studies have measured dermal and systemic exposure to hair-dye components, mainly by use of *para*-phenylenediamine (PPD) or [^{14}C]-labelled PPD.

In a study from the USA, seven hair dyes (oxidative and direct) were [^{14}C]-labelled and applied onto volunteers (Wolfram & Maibach, 1985). The extent of scalp penetration was slightly higher for direct dyes but in neither case did it exceed 1% of the applied dose.

In a study on percutaneous absorption of PPD during an actual hair-dyeing procedure, urinary levels of PPD metabolites were monitored during 24 or 48 hours after the dye had been applied (Goetz *et al.*, 1988). The fraction of the applied dose found in the urine 24 hours after application ranged from 0.04% to 0.25%. This study also showed a five- to ten-fold decrease in PPD penetration when the scalp was protected with clay before applying the dye.

In a study from Taiwan, China (Wang & Tsai, 2003), five volunteers dyed their own hair with various dye products containing different concentrations of PPD (2.2–3.2% or 1.1–1.6 g). The PPD excretion in the urine after 48 hours was 0.02–0.45% of the total dose.

Eight volunteers (Hueber-Becker *et al.*, 2004) received an oxidative hair-dye application containing [^{14}C]-PPD. The dye remained on the hair and scalp for 30 min. In the urine samples collected afterwards, 0.50±0.24% of the total applied radioactivity was recovered. The mean systemic dose was calculated to be approximately 0.09±0.04mg [^{14}C]-PPD-equivalents/kg body weight. In an *in-vitro* human skin study, a total of 2.4±1.6% of the applied radioactivity was absorbed (found in epidermis, dermis and receptor fluid), corresponding to an absorption rate of 10.6±6.7 $\mu g_{eq}/cm^2$. In the same eight volunteers, specific PPD metabolites were measured: five different metabolites were found, mainly *N*-mono-acetylated and *N,N′*-diacetylated PPD (Nohynek *et al.*, 2004b).

1.5.1 *Estimation of the internal dose to the user of hair-dye ingredients*

According to its "Notes of Guidance", the Scientific Committee for Consumer Products (SCCP) assesses hair dyes based on data of percutaneous absorption, mainly from in-vitro studies with human or pig skin. In a typical study, 20 mg of a representative hair-dye formulation per cm^2 is applied onto the skin. Following a 30-min contact time, the amount of hair dye in the epidermis (*stratum corneum* excluded), dermis and the receptor fluid after 24 hours is determined and summed-up to provide a worst-case value for the systemic (internal) dose. Taking the commercially important hair dye *para*-phenylenediamine (PPD) as an example, and using a worst-case scenario for a 60-kg adult person, the systemic exposure dose was estimated to be 0.052 mg/kg bw, calculated as follows:

A maximum concentration of 4.0% of PPD is mixed before use with H_2O_2. Thus the usage volume of 100 ml contains at maximum 2.0% PPD. Assuming the highest penetration (0.00447 mg/cm^2) and a typical human body weight (60 kg) and exposed scalp area (700 cm^2), this would give a systemic exposure dose of 0.00447 mg/cm^2 x 700 cm^2/60 kg = 0.052 mg/kg body weight. (source: SCCP's "Notes of Guidance" for the testing of cosmetic ingredients and their safety evaluation, 6th revision, adopted by the SCCP during the 10th plenary meeting of 9 December 2006).

1.6 Occupational exposure as a hairdresser and barber

Occupational exposure studies in hairdressers have mainly focused on airborne exposure. Only few studies have measured dermal and systemic exposure of hairdressers to certain chemicals.

1.6.1 *Airborne exposure in hairdressing salons*

In addition to hair dyes, hairdressers can be exposed to a wide variety of chemicals. Studies to measure the airborne occupational exposure of hairdressers are summarized in Table 1.7. In these studies, ethanol is generally used as a marker for solvent exposure, and *para*-phenylenediamine (PPD) is often used as a marker for dye exposure.

The exposure to organic solvents is generally highest in the hairdressers' breathing zone during the mixing and application of chemicals to the hair. The exposure time is usually short, varying from tens of seconds (hair sprays) to tens of minutes (permanent solutions and dyes) (Leino, 2001), but these tasks may be repeated many times a day. Several studies showed that local exhaust ventilation can significantly reduce airborne exposure (Hollund & Moen, 1998; Leino, 2001), but also indicated that it is seldom used.

The exposure of hairdressers to oxidative hair dyes was measured under controlled conditions. Three separate phases of hair dyeing were monitored: (1) dye preparation/hair dyeing, (2) rinsing/shampooing/conditioning, and (3) cutting/drying/styling, on eighteen hair dressers working for six hours with a dark-shade oxidative hair dye containing 2% of [^{14}C]-*para*-phenylenediamine (PPD). The detected PPD-equivalents in personal air samples (charcoal cartridges) were higher during the hair-dyeing phase than during the other phases, and ranged from < 0.25 μg (detection limit) to 0.88 μg with a mean exposure time of ~30 min (Hueber-Becker *et al.*, 2007).

Table 1.7. Airborne occupational exposure levels in hairdressing salons

Work task Exposure	Concentration in air	Remarks	Reference
Permanenting			
Ammonium thioglycolate	0.5–10 μg/m^3		Leino (2001)
Glyceryl monothioglycolate	0–1.8 μg/m^3		Leino (2001)
Ammonia	1.4–3.5 mg/m^3		Leino (2001)
	0.5–4.4 mg/m^3		Hollund & Moen (1998)
	105 mg/m^3	2 min	Van der Wal *et al.* (1997)
	5–25 mg/m^3		Rajan (1992)
	0.7–7.2 mg/m^3		Hakala *et al.* (1979)
Hydrogen sulfide	0.14–0.7 mg/m^3		Hakala *et al.* (1979)
Organic solvents	45 mg/m^3	Peak	Leino (2001)
Ethanol	2–36 mg/m^3		Almaguer *et al.* (1992)
	2–30 mg/m^3		Rajan (1992)
	0–3 mg/m^3	Breathing zone	Gunter *et al.* (1976)
Isopropanol	0–9 mg/m^3		Rajan (1992)
	0.4–14.8 mg/m^3		Hollund & Moen (1998)
Toluene	0.04–0.11 mg/m^3		Hollund & Moen (1998)
Hydrogen peroxide	0.014–0.14 mg/m^3	Spot measurement	Van der Wal *et al.* (1997)

Table 1.7 (contd)

Work task Exposure	Concentration in air	Remarks	Reference
Dyeing			
p-Phenylenediamine (PPD)	<1.0 µg/m^3	Detection limit	Gagliardi *et al.* (1992)
	<1.0–0.1 µg/m^3	Detection limit	Hollund & Moen (1998)
Diaminotoluene	<1.0–0.1 µg/m^3	Detection limit	Hollund & Moen (1998)
Ammonia	1.4–3.5 mg/m^3		Leino (2001)
	0.4–4.5 mg/m^3		Hollund & Moen (1998)
Hydrogen peroxide	0.007 mg/m^3	Spot measurement	Van der Wal *et al.* (1997)
Organic solvents	25 mg/m^3	Peak	Leino (2001)
Bleaching			
Ammonium persulfate	0–4.7 µg/m^3		Leino (2001)
	30 µg/m^3	Peak	Leino (2001)
Ammonia	1.4–3.5 mg/m^3		Leino (2001)
	0.3–10 mg/m^3		Hollund & Moen (1998)
Hydrogen peroxide	0.014 mg/m^3	Spot measurement	Van der Wal *et al.* (1997)
Hair lacquering			
Organic solvents	45 mg/m^3	Peak	Leino (2001)
Ethanol	150 µl/l		Van der Wal *et al.* (1997)
	0.007–3 mg/m^3		Gunter *et al.* (1976)
Isobutane	373–1935 mg/m^3		Gunter *et al.* (1976)
Butane	30 µl/l	1 min	Van der Wal *et al.* (1997)
Polyvinylpyrrolidone	7–70 µg/m^3		Gunter *et al.* (1976)
Particulates	100 mg/m^3	5 s	Van der Wal *et al.* (1997)
Ambient air in salons			
CO2	400–4500 µl/l		Van der Wal *et al.* (1997)
Volatile organic compounds	0.084–0.465 mg/m^3		Leino (2001)
	0.14–0.66 mg/m^3	8-h TWA C6–C16	Van der Wal *et al.* (1997)

Table 1.7 (contd)

Work task Exposure	Concentration in air	Remarks	Reference
Ethanol	0.1–56.6 mg/m^3	8-h TWA pers. samples	van Muiswinkel *et al.* (1997)
	0.1–43 mg/m^3	8-h TWA ambient air	van Muiswinkel *et al.* (1997)
	4.4–57 mg/m^3	8-h TWA breathing zone	Van der Wal *et al.* (1997)
	2.3–26 mg/m^3	8-h TWA stationary	Van der Wal *et al.* (1997)
	2–59 mg/m^3		Hakala *et al.* (1979)
Ammonia	0.1–1.2 mg/m^3		Hollund & Moen (1998)
	2.6–4.9 mg/m^3	8-h TWA breathing zone	Van der Wal *et al.* (1997)
	0.02–0.44 mg/m^3	8-h TWA winter	Van der Wal *et al.* (1997)
Hydrogen peroxide	0.01–0.069 mg/m^3	8-h TWA	Van der Wal *et al.* (1997)
Total dust	0.066–0.133 mg/m^3		Leino (2001)
	0.28–2.7 mg/m^3	8-h TWA breathing zone	Van der Wal *et al.* (1997)
	0.11–1.01 mg/m^3	8-h TWA stationary	Van der Wal *et al.* (1997)
Particulates	8500–17000 /l	>0.5 µm	Leino (2001)
	160–400 /l	<0.5 µm	Leino (2001)
	0.03–0.39 mg/m^3	8-h TWA stationary	Van der Wal *et al.* (1997)
	0.3–0.6 mg/m^3	8-h personal samples	Palmer *et al.* (1979)

Adapted from Leino (2001)
CO_2, carbon dioxide; TWA, time-weighted average

1.6.2 *Dermal and systemic exposure*

In a study from Sweden (Lind *et al.*, 2005) the dermal exposure of 33 hairdressers was assessed with a hand-rinse method (Lind *et al.*, 2004). Samples were taken in the hairdressing salons during normal working hours: before the hair-dyeing procedure, after the application of the hair dye, and after cutting of the newly dyed hair. The samples were analysed for five different compounds used in common commercial hair-dye products in Sweden: 1,4-phenylenediamine (PPD), toluene-2,5-diaminesulphate (TDS), 3-aminophenol (MAP), resorcinol (RES), and 2-methyl-resorcinol (MRE). The results are shown in Table 1.8. The maximum levels detected after application of hair dyes were: 939 nmol per

Table 1.8. Amounts of hair-dye compounds in hand rinse from professional hair dressers

Compound	Dominant hand		Serving hand	
	Positive samples (*n*)	Amount (nmol per hand) mean (range)	Positive samples (*n*)	Amount (nmol per hand) mean (range)
a. Taken before mixing hair-dye cream with hydrogen peroxide *Total number of samples = 33*				
PPD	3	294 (197–406)	3	263 (201–311)
MAP	19	116 (25–478)	20	100 (21–450)
TDS	7	149 (26–386)	6	192 (55–323)
RES	6	138 (24–433)	5	133 (24–397)
MRE	3	13 (7–20)	1	6
b. Taken after application of hair dye *Total number of samples = 33*				
PPD	4	454 (22–939)	4	426 (36–839)
MAP	22	94 (31–244)	22	73 (23–154)
TDS	12	118 (19–379)	11	142 (13–741)
RES	21	185 (30–513)	21	136 (24–773)
MRE	5	57 (10–187)	3	44 (19–82)
c. Taken after cutting newly dyed hair *Total number of samples = 29*				
PPD	5	178 (33–360)	5	153 (36–324)
MAP	15	49 (26–102)	14	52 (17–116)
TDS	14	71 (11–162)	13	120 (19–365)
RES	20	99 (19–364)	20	158 (22–736)
MRE	3	36 (10–82)	2	62 (14–109)

Adapted from Lind *et al*. (2005)
MAP, 3-aminophenol; MRE, 2-methyl-resorcinol; PPD, 1,4-phenylenediamine; RES, resorcinol; TDS, toluene-2,5-diaminesulphate

hand for PPD, 244 for MAP, 741 for TDS, 773 for RES and 187 for MRE. Positive findings were also reported for samples taken before mixing hair-dye cream with oxidizing cream. This exposure may derive from previous hair-dyeing activities on the same day or from background exposure from contaminated surfaces. Hand exposure was not significantly lower in hairdressers working with gloves compared with hairdressers not using gloves while dyeing hair. It was noted, however, that gloves were often re-used and could be a source of contamination.

In a later study, the penetration of PPD, TDS, and RES through protective gloves during hairdressing was investigated: when properly used, all the tested gloves gave

considerable protection against permeation of the hair-dye components studied (Lind *et al.*, 2007).

A study among 18 hairdressers in controlled conditions using radioactive PPD (2%) in hair dyes measured systemic exposure to PPD (Hueber-Becker *et al.*, 2007). No radioactivity above the limit of detection (< 10 ng PPD_{eq}/ml) was found in blood samples. Several urine samples contained no measurable or quantifiable radioactivity. Using the detected urinary levels and the urine volume, the mean urinary excretion across all hairdressers during the working-day was calculated to be < 25 ± 5.2 μg [^{14}C]-PPD_{eq}.

1.7 Regulations and guidelines

The legislation of cosmetics and the regulation of ingredients in hair-dye formulations in the EU and the USA differ. During the 1980s, several putative carcinogenic hair-dye substances were banned in the EU, but not in the USA. Furthermore, in April 2003 the EU Scientific Committee on Consumer Products (SCCP) started a strategy to ensure the safety of hair-dye products. The SCCP foresees banning of all permanent and non-permanent hair dyes for which industry has not submitted a safety file for the substances involved, and of those on which the SCCP has issued a negative opinion. Table 1.9 gives the list of ingredients that are currently not permitted in hair-dye products in the European Union. A substantial number of those banned substances were of limited commercial interest.

The Japanese regulation of cosmetics is the most restrictive. All cosmetic products are considered equivalent to drugs and are thus subject to premarket approval by the Ministry of Health and Welfare, and may contain only those ingredients included in the Comprehensive Licensing Standards of Cosmetics by Category (CLS); these ingredients must be conform certain defined specifications. Other Asian countries (e.g. Republic of Korea and China) have developed regulatory requirements for hair dyes and their ingredients similar to those in Japan (Corbett *et al.* 1999; Nohynek *et al.* 2004a).

In response to occupational safety concerns, i.e. the risk for developing contact dermatitis, international hair-dye label warnings recommend that hairdressers wear protective gloves during the hair dyeing and rinsing processes (Wilkinson and Shaw, 2005).

Table 1.9. List of 135 hair-dye substances banned by the European Union (updated September 2007)

Ref. No. Annex II to CD	Chemical Name / INCI - Name	CAS No.
363	*ortho*-Phenylenediamine	95-54-5
364	2,4-Diaminotoluene	95-80-7
376	1-Methoxy-2,4-diaminobenzene	615-05-4
377	1-Methoxy-2,5-diaminobenzene	5307-02-8
380	Basic Violet 3	548-62-9
383	2-Amino-4-nitrophenol	99-57-0
384	2-Amino-5-nitrophenol	121-88-0
386	CI 42640	1694-09-3
387	Acid Yellow 36	587-98-4
388	Basic Violet 1	8004-87-3
398	CI 45170, CI 45170:1	81-88-9; 509-34-2
406	4-Ethoxy-2,4-diaminobenzene	5862-77-1
407	1-beta-Hydroxyethyl-2,4-diaminobenzene	14572-93-1
408	Catechol	120-80-9
409	Pyrogallic acid	87-66-1
412	4-Amino-2-nitrophenol	119-34-6
413	1-Methyl-2,6-diaminobenzene	823-40-5
700	Disperse Blue 1	2475-45-8
1188	Basic Green 4	569-64-2
1204	*meta*-Phenylenediamine	108-45-2
1212	6-Methoxy-2,3-pyridinediamine HCl	94166-62-8
1213	2,3-Naphthalenediol	92-44-4
1214	2,4-Diaminodiphenylamine	136-17-4
1215	2,6-Bis(2-hydroxyethoxy)-3,5-pyridinediamine HCl	117907-42-3
1216	2-Methoxymethyl-*p*-aminophenol HCl	29785-47-5 (HCl)
1217	4,5-Diamino-1-methylpyrazole HCl; sulfate	21616-59-1 HCl
1218	4,5-Diamino-1-((4-chlorophenyl)methyl)-1H-pyrazole sulfate	163183-00-4
1219	4-Chloro-2-aminophenol	95-85-2
1220	4-Hydroxyindole	2380-94-1
1221	4-Methoxytoluene-2,5-diamine HCl	56496-88-9
1222	5-Amino-4-fluoro-2-methylphenol sulfate	163183-01-5
1223	*N*,*N*-Diethyl-*meta*-aminophenol	91-68-9
1224	*N*,*N*-Dimethyl-2,6-pyridinediamine HCl	-
1225	*N*-cyclopentyl-*meta*-aminophenol	104903-49-3
1226	*N*-Methoxyethyl-*para*-phenylenediamine HCl	72584-59-9
1227	2,4-Diamino-5-methylphenetol HCl	113715-25-6
1228	1,7-Naphthalenediol	575-38-2
1229	3,4-Diaminobenzoic acid	619-05-6
1230	2-Aminomethyl-*para*-aminophenol HCl	135043-65-1; 79352-72-0
1231	Solvent Red 1	1229-55-6
1232	Acid Orange 24	1320-07-6
1233	Acid Red 73	5413-75-2

Table 1.9 (contd)

Ref. No. Annex II to CD	Chemical Name / INCI - Name	CAS No.
1234	PEG-3,2′,2′-di-*para*-Phenylenediamine	144644-13-3
1235	6-Nitro-*ortho*-toluidine	570-24-1
1236	HC Yellow No 11	73388-54-2
1237	HC Orange No 3	81612-54-6
1238	HC Green No 1	52136-25-1
1239	HC Red No 8 and its salts	97404-14-3; 13556-29-1
1240	Tetrahydro-6-nitroquinoxaline and its salts	158006-54-3; 41959-35-7
1241	Disperse Red 15, except as impurity in Disperse Violet 1	116-85-8
1244	1-Methyl-2,4,5-trihydroxybenzene and its salts	1124-09-0
1245	2,6-Dihydroxy-4-methylpyridine and its salts	4664-16-8
1246	5-Hydroxy-1,4-benzodioxane and its salts	10288-36-5
1247	3,4-Methylenedioxyphenol and its salts	533-31-3
1248	3,4-Methylenedioxyaniline and its salts	14268-66-7
1249	Hydroxypyridinone and its salts	822-89-9
1250	3-Nitro-4-aminophenoxyethanol and its salts	50982-74-6
1251	2-Methoxy-4-nitrophenol (4-Nitroguaiacol) and its salts	3251-56-7
1252	C.I. Acid Black 131 and its salts	12219-01-1
1253	1,3,5-Trihydroxybenzene (Phloroglucinol) and its salts	108-73-6
1254	1,2,4-Benzenetriacetate and its salts	613-03-6
1255	Ethanol, 2,2'-iminobis-, reaction products with epichlorohydrin and 2-nitro-1,4-benzenediamine (HC Blue No. 5) and its salts	68478-64-8; 158571-58-5
1256	N-Methyl-1,4-diaminoanthraquinone, reaction products with epichlorohydrin and monoethanolamine (HC Blue No. 4) and its salts	158571-57-4
1257	4-Aminobenzenesulfonic acid and its salts	121-57-3
1258	3,3'-(Sulfonylbis(2-nitro-4,1-phenylene)imino)bis(6(phenylamino)) benzenesulfonic acid and its salts	
1259	3(or 5)-((4-(Benzylmethylamino)phenyl)azo)-1,2-(or1,4)dimethyl-1H-1,2,4-triazolium and its salts	
1260	2,2'-((3-Chloro-4-((2,6-dichloro-4-nitrophenyl)azo)phenyl)imino)-bisethanol (Disperse Brown 1) and its salts	23355-64-8
1261	Benzothiazolium, 2-[[4-[ethyl(2hydroxyethyl)amino]phenyl]azo]-6-methoxy-3-methyl- and its salts	
1262	2-[(4-Chloro-2-nitrophenyl)azo]-*N*-(2-methoxyphenyl)-3-oxobutanamide (Pigment Yellow 73) and its salts	13515-40-7
1263	2,2'-[(3,3'-Dichloro[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[3-oxo-*N*-phenylbutanamide] (Pigment Yellow 12) and its salts	6358-85-6
1264	2,2'-(1,2-Ethenediyl)bis[5-((4ethoxyphenyl)azo]benzenesulfonic acid) and its salts	

Table 1.9 (contd)

Ref. No. Annex II to CD	Chemical Name / INCI - Name	CAS No.
1265	2,3-Dihydro-2,2-dimethyl-6-[(4-(phenylazo)-1naphthalenyl)azo]-1H-pyrimidine (Solvent Black 3) and its salts	4197-25-5
1266	3(or 5)-[[4-[(7-amino-1-hydroxy-3-sulphonato-2-naphthyl)azo]1-naphthyl]azo]salicylic acid and its salts	
1267	2-Naphthalenesulfonic acid, 7-(benzoylamino)-4-hydroxy-3[[4-[(4-sulfophenyl)azo]phenyl]azo]- and its salts	
1268	(μ-((7,7'-Iminobis(4-hydroxy-3-((2-hydroxy-5-(*N*-methylsulphamoyl)phenyl)azo)naphthalene-2-sulphonato))(6-)))dicuprate(2-) and its salts	
1269	3-[(4-(Acetylamino)phenyl)azo]-4-hydroxy-7-[[[[5-hydroxy-6-(phenylazo)-7-sulfo-2-naphthalenyl]amino]carbonyl]amino]-2-naphthalenesulfonic acid and its salts	
1270	2-Naphthalenesulfonic acid, 7,7'-(carbonyldiimino)bis(4hydroxy-3-[[2-sulfo-4-[(4-sulfophenyl)azo]phenyl]azo]-, and its salts	25188-41-4
1271	Ethanaminium, *N*-(4-[bis[4-(diethylamino)phenyl]methylene]-2,5-cyclohexadien-1-ylidene)-*N*-ethyl- and its salts	
1272	3H-Indolium, 2-[[(4-methoxyphenyl)methylhydrazono]methyl]-1,3,3-trimethyl- and its salts	
1273	3H-Indolium, 2-(2-((2,4-dimethoxyphenyl)amino)ethenyl)-1,3,3-trimethyl- and its salts	
1274	Nigrosine spirit soluble (Solvent Black 5)	11099-03-9
1275	Phenoxazin-5-ium, 3,7-bis(diethylamino)-, and its salts	47367-75-9
1276	Benzo[a]phenoxazin-7-ium, 9-(dimethylamino)-, and its salts	
1277	6-Amino-2-(2,4-dimethylphenyl)-1H-benz[de]isoquinoline1,3(2H)-dione (Solvent Yellow 44) and its salts	2478-20-8
1278	1-Amino-4-[[4[(dimethylamino)methyl]phenyl]amino]-anthraquinone and its salts	12217-43-5
1279	Laccaic Acid (CI Natural Red 25) and its salts	60687-93-6
1280	Benzenesulfonic acid, 5-[(2,4-dinitrophenyl)amino]-2-(phenylamino)-, and its salts	15347-52-1
1281	4-[(4-Nitrophenyl)azo]aniline (Disperse Orange 3) and its salts	730-40-5
1282	4-Nitro-*meta*-phenylenediamine and its salts	5131-58-8
1283	1-Amino-4-(methylamino)-9,10-anthracenedione (Disperse Violet 4) and its salts	1220-94-6
1284	*N*-Methyl-3-nitro-*para*-phenylenediamine and its salts	2973-21-9
1285	*N*1-(2-Hydroxyethyl)-4-nitro-*ortho*-phenylenediamine (HC Yellow No. 5) and its salts	56932-44-6
1286	*N*1-(Tris(hydroxymethyl))methyl-4-nitro-1,2-phenylenediamine (HC Yellow No. 3) and its salts	56932-45-7
1287	2-Nitro-*N*-hydroxyethyl-*para*-anisidine and its salts	57524-53-5
1288	*N*,*N'*-Dimethyl-*N*-Hydroxyethyl-3-nitro-*para*-phenylenediamine and its salts	10228-03-2

Table 1.9 (contd)

Ref. No. Annex II to CD	Chemical Name / INCI - Name	CAS No.
1289	3-(*N*-Methyl-*N*-(4-methylamino-3-nitrophenyl)amino)propane1,2-diol and its salts	93633-79-5
1290	4-Ethylamino-3-nitrobenzoic acid (*N*-Ethyl-3-Nitro-PABA) and its salts	2788-74-1
1291	(8-[(4-Amino-2-nitrophenyl)azo]-7-hydroxy-2-naphthyl)trimethylammonium and its salts, except Basic Red 118 (CAS 71134-97-9) as impurity in Basic Brown 17	
1292	5-((4-(Dimethylamino)phenyl)azo)-1,4-dimethyl-1H-1,2,4-triazolium and its salts	
1293	*meta*-Phenylenediamine, 4-(phenylazo)-, and its salts	495-54-5
1294	1,3-Benzenediamine, 4-methyl-6-(phenylazo)- and its salts	
1295	2,7-Naphthalenedisulfonic acid, 5-(acetylamino)-4-hydroxy-3((2-methylphenyl)azo)- and its salts	
1296	4,4'-[(4-Methyl-1,3-phenylene)bis(azo)]bis[6-methyl-1,3-benzenediamine] (Basic Brown 4) and its salts	4482-25-1
1297	Benzenaminium, 3-[[4-[[diamino(phenylazo)phenyl]azo]-2-methylphenyl]azo]-*N,N,N*-trimethyl- and its salts	
1298	Benzenaminium, 3-[[4-[[diamino(phenylazo)phenyl]azo]-1-naphthalenyl]azo]-*N,N,N*-trimethyl- and its salts	
1299	Ethanaminium, N-[4-[(4(diethylamino)phenyl)phenylmethylene]-2,5-cyclohexadien-1-ylidene]-*N*-ethyl- and its salts	
1300	9,10-Anthracenedione, 1-[(2-hydroxyethyl)amino]-4(methylamino)- and its derivatives and salts	86722-66-9
1301	1,4-Diamino-2-methoxy-9,10-anthracenedione (Disperse Red 11) and its salts	2872-48-2
1302	1,4-Dihydroxy-5,8-bis[(2-hydroxyethyl)amino]anthraquinone (Disperse Blue 7) and its salts	3179-90-6
1303	1-[(3-Aminopropyl)amino]-4-(methylamino)anthraquinone and its salts	
1304	*N*-[6-[(2-Chloro-4-hydroxyphenyl)imino]-4-methoxy-3-oxo-1,4-cyclohexadien-1-yl]acetamide (HC Yellow No. 8) and its salts	66612-11-1
1305	[6-[[3-Chloro-4-(methylamino)phenyl]imino]-4-methyl-3-oxocyclohexa-1,4-dien-1-yl]urea (HC Red No. 9) and its salts	56330-88-2
1306	Phenothiazin-5-ium, 3,7-bis(dimethylamino)- and its salts	
1307	4,6-Bis(2-Hydroxyethoxy)-*meta*-Phenylenediamine and its salts	
1308	5-Amino-2,6-Dimethoxy-3-Hydroxypyridine and its salts	104333-03-1
1309	4,4'-Diaminodiphenylamine and its salts	537-65-5
1310	4-Diethylamino-*ortho*-toluidine and its salts	148-71-0
1311	*N,N*-Diethyl-*para*-phenylenediamine and its salts	93-05-0
1312	*N,N*-Dimethyl-*para*-phenylenediamine and its salts	99-98-9
1313	Toluene-3,4-Diamine and its salts	496-72-0
1314	2,4-Diamino-5-methylphenoxyethanol and its salts	141614-05-3
1315	6-Amino-*ortho*-cresol and its salts	17672-22-9

Table 1.9 (contd)

Ref. No. Annex II to CD	Chemical Name / INCI - Name	CAS No.
1316	Hydroxyethylaminomethyl-*para*-aminophenol and its salts	110952-46-0
1317	2-Amino-3-nitrophenol and its salts	603-85-0
1318	2-Chloro-5-nitro-*N*-hydroxyethyl-*para*-phenylenediamine and its salts	50610-28-1
1319	2-Nitro-*para*-phenylenediamine and its salts	5307-14-2
1320	Hydroxyethyl-2,6-dinitro-*para*-anisidine and its salts	122252-11-3
1321	6-Nitro-2,5-pyridinediamine and its salts	69825-83-8
1322	Phenazinium, 3,7-diamino-2,8-dimethyl-5-phenyl- and its salts	
1323	3-Hydroxy-4-[(2-hydroxynaphthyl)azo]-7-nitronaphthalene-1-sulphonic acid and its salts	16279-54-2
1324	3-[(2-nitro-4-(trifluoromethyl)phenyl)amino]propane-1,2-diol (HC Yellow No. 6) and its salts	104333-00-8
1325	2-[(4-chloro-2-nitrophenyl)amino]ethanol (HC Yellow No. 12) and its salts	59320-13-7
1326	3-[[4-[(2-Hydroxyethyl)methylamino]-2-nitrophenyl]amino]-1,2-propanediol and its salts	173994-75-7
1327	3-[[4-[Ethyl(2-hydroxyethyl)amino]-2-nitrophenyl]amino]-1,2-propanediol and its salts	114087-41-1
1328	Ethanaminium, *N*-[4-[[4-(diethylamino)phenyl][4-(ethylamino)-1-naphthalenyl]methylene]-2,5-cyclohexadien-1-ylidene]-*N*-ethyl- and its salts	

2. Studies of Cancer in Humans

2.1 Occupational exposures of hairdressers and barbers

2.1.1 *Cohort studies* (Table 2.1) [Only studies not included in the previous IARC Monograph (Volume 57) are listed in the Table].

Alderson (1980) followed a sample of 1831 male hairdressers identified at the 1961 census of England and Wales until 1978. Mortality from all cancers was similar to that expected (134 obs., 126.1 exp.), and no specific cancer showed a significant excess: oesophagus, 5 obs., 3.4 exp.; lung, 52 and 50.8; bladder, 7 and 5.6; leukaemia, 3 and 2.7.

Kono *et al.* (1983) followed the mortality of a cohort of 7736 registered female beauticians from 1948 to 1960 in Fukuoka Prefecture, Japan, for an average of 22.5 years. Among the site-specific cancers examined, only stomach cancer occurred in significant excess (61 observed, 45.59 expected; 95% CI, 1.02–1.72). They found no case of bladder

Table 2.1. Cohort studies of occupational exposure to hair dyes

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Lynge (1990-1991)	4874 male and 9497 female hairdressers at the 1970 census aged 25–64 years from Denmark; follow-up from 1970–1980				**SIR**	NR	
			Bladder				
			Men	41	205 (151–278)		
			Women	7	176 (71–363)		
Skov & Lynge (1994)	1177 male and 4160 female hairdressers at the 1970 census aged 25–64 years from Denmark; follow-up from 1970–1987	Census data	*Bladder*				Includes data from Lynge (1990-1991) and possibly Skov & Lynge (1991)
			Men	67	158 (124–201)		
			Women	12	123 (64–215)		
			Lung				
			Men	127	120 (101–143)		
			Women	31	097 (68–138)		
			NHL				
			Men	12	118 (61–206)		
			Women	16	192 (110–312)		
			HD				
			Men	6	200 (73–435)		
			Women	3	90 (19–264)		
			Leukemia				
			Men	13	102 (54–174)		
			Women	8	94 (40–185)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Boffetta *et al.* (1994)	29 279 female hairdresser at the 1970 census aged 25–64 years from Denmark, Sweden, Norway and Finland; follow-up from 1971–1985; follow-up started in 1987 for Denmark	Census data	*Ovarian* Female *NHL* Female	 127 36	 1.18 (0.98–1.40) 1.20 (0.84–1.66)	NR	
Pukkala *et al.* (1992)	3637 female and 168 male hairdressers born in or before 1946; followed up between 1970–1987; members of the Finnish Hairdressers' Association	Data from National Cancer Registry	**SIR** *Overall* Female *Breast* Female *Urinary bladder* Female *Multiple Myeloma* Female *Leukemia* Female	 247 70 1 1 4	 1.27 (1.11–1.42) 1.24 (0.97–1.57) 0.4 (0.01–2.35) 0.42 (0.01–2.35) 0.96 (0.26–2.47)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Morton (1995)	2052 women with usual occupation as beauticians among residents in the Portland-Vancouver area of Oregon; age 16–68 years; diagnosed between 1963–1977	Hospital records and death certificates	Breast	28	**SIR** 125.2		
Calle *et al*. (1998)	Main lifetime occupation as beauticians, among 563 395 female participants of Cancer Prevention Study II; 46 433 person-years accrued in 1982–1991	Questionnaire	Breast	16	1.02 (0.62–1.69)	Age, race, family history of breast cancer, body mass index, education, smoking, alcohol, exercise, breast cysts, age at menarche, age at menopause, oral contraceptive use, estrogen replacement therapy, number of livebirths, age at first livebirth	Housewives used as reference

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Andersen *et al.* (1999)	10 298 male and 26 545 female hairdressers at the 1970 census aged 25–64 years from Denmark, Sweden, Norway and Finland; follow-up from 1971–1991	Census data			**SIR**		
			Breast				
			Male	4	204 (56–523)		
			Female	643	105 (97–113)		
			Bladder				
			Male	147	147 (125–173)		
			Female	37	89 (63–123)		
			Lung				
			Male	249	121 (107–137)		
			Female	122	122 (102–146)		
			Ovary				
			Female	164	118 (101–138)		
			HD				
			Male	7	97 (39–199)		
			Female	8	88 (40–166)		
			NHD				
			Male	33	101 (69–142)		
			Female	48	106 (78–141)		
			MM				
			Male	18	100 (59–158)		
			Female	19	80 (48–125)		
			Acute Leukemia				
			Male	11	91 (46–163)		
			Female	18	94 (56–148)		
			Other Leukemia				
			Male	25	123 (80–182)		
			Female	18	91 (54–143)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Pollán & Gustavsson, (1999)	1 101 669 women with a gainful occupation at the 1970 census, age 25–64 years. from Sweden; follow-up from 1971–1989	Record linkage between the Swedish cancer registry and a population registry comprising all individuals included in the 1970 census	*Breast* Female	258	**SIR** 110 (98–124)	Age, geographical category, period, town size	Comparisons within occupation and with other occupations reported as well. Total number employed as hairdresser or beauticians not given
Vasama Neuvonen *et al.* (1999)	892 591 economically active women in Finland, with occupation as reported in the 1970 census, born between 1906 and 1945; follow-up from 1971–95	Finnish Cancer Registry and exposures based on Finnish job-exposure matrix (FINJEM) developed at the Finnish Institute of Occupational Health	*Ovary* Hairdressers and barbers Beauticians	57 3	**SIR** 1.3 (1.0–1.7) 1.0 (0.2–2.9)	Birth cohort, follow-up period, social status	Total number employed as hairdresser or beauticians not given

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Mutanen & Hemminki, (2001)	Swedish children born from 1935 to 1996 with a father (2.7 million) or a mother (1.0 million) with active occupation in the 1960 census; follow up from 1958–96 for the age group 0–14 years	Census data			Not reported	Not reported	Four cases of kidney cancer among children of male hairdressers: SIR=10.6 (2.9–27.2) and 2 cases among children of female hairdressers: SIR=1.0 (0.1–3.7)
Shields *et al.* (2002)	1 670 517 women with a gainful occupation at the 1960 or 1970 censuses, years from Sweden. follow-up from 1971–89	Swedish Cancer Environment Register (CER III) and census data	*Ovary* 1970 data 1960 data Both censuses	 14 36 51	 0.56 (0.3–0.9) 0.87 (0.6–1.2) 1.21 (0.9–1.6)	5-year age grouping	Total number employed as barbers, beauticians etc not given

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Czene *et al.* (2003)	38 866 women and 6866 men from Sweden who declared to be employed as "hairdressers, barbers, beauticians and others" in at least one of the four censuses of 1960, 1970, 1980 and 1990; follow-up from 1960–1998	Census data	**Males**				
			Any census				
			Bladder	87	1.22 (0.98–1.51)		
			Lung	141	1.38 (1.16–1.63)		
			NHL	29	0.91 (0.61–1.31)		
			HD	8	1.17 (0.50–2.32)		
			MM	18	1.17 (0.69–1.85)		
			Leukemia	29	0.97 (0.65–1.39)		
			1960 census				
			Bladder	82	1.25 (1.01–1.55)		
			Lung	133	1.41 (1.18–1.68)		
			NHL	24	0.86 (0.55–1.28)		
			HD	7	1.34 (0.53–2.78)		
			MM	17	1.19 (0.69–1.92)		
			Leukemia	25	0.94 (0.61–1.38)		
			Females				
			Any census				
			Breast	913	1.02 (0.95–1.09)		
			Bladder	51	1.09 (0.81–1.43)		
			Lung	160	1.35 (1.15–1.58)		
			Ovary	192	1.11 (0.96–1.28)		
			NHL	64	0.94 (0.72–1.20)		
			HD	11	0.58 (0.29–1.03)		
			MM	31	1.30 (0.88–1.84)		
			Leukemia	57	1.01 (0.77–1.31)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Czene *et al*. (2003) (contd)			*1960 census*				
			Breast	565	1.01 (0.93–1.09)		
			Bladder	33	0.95 (0.65–1.34)		
			Lung	109	1.22 (1.00–1.47)		
			Ovarian	111	0.97 (0.80–1.16)		
			NHL	41	0.96 (0.69–1.31)		
			HD	6	0.72 (0.26–1.59)		
			MM	19	1.14 (0.69–1.79)		
			Leukemia	41	1.16 (0.83–1.57)		
Ji *et al*. (2005)	4639 male hairdressers at the census of 1960; follow-up from 1960–2000	Census data	**Bladder**		**SIR**	Age, period, socioeconomic status	Same cohort used in all three publications
			SEI-adjusted				
			1960 census	88	1.26 (1.01–1.54)		
			1960–1970 census	62	1.14 (0.88–1.45)		
			1960–1970–1980 census	33	1.35 (0.91–1.84)		
			Smoking corrected				Correction for smoking was done by dividing the SIR by 35% of the excess of lung cancer
			1960 census	88	1.10 (0.88–1.34)		
			1960–1970 census	62	1.00 (0.76–1.26)		
			1960–1970–1980 census	33	1.17 (0.81–1.60)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Ji & Hemminki, (2005a)	4639 male hairdressers at the census of 1960 and 16 360 female hairdressers at the census of 1970. Sweden; follow-up from 1960–2000 for males; 1970–2000 for females.	Census, Swedish Family-Cancer Database and Swedish Cancer Registry data	*Lung* Males Females	 144 92	**SIR** 1.42 (1.20–1.66) 1.19 (0.96–1.44)	Age, period, socioeconomic status	**UADT**: *Males*: 49 cases, SIR=1.39 (1.03–1.81) SIR significant also for hairdressers at 1960 & 1970 censuses, and 1960, 1970 & 1980 censuses, and for tongue and larynx. *Females*: 34 cases, SIR=1.45 (1.01–1.98); SIR significant for pharynx.

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Ji & Hemminki, (2005b)	Same as in Ji & Hemminki (2005a); Sweden; follow-up from 1960–2002 for males; 1970–2002 for females	Census, Swedish Family-Cancer Database and Swedish and Swedish Cancer Registry data	*Males*		**SIR**	Age, period, socioeconomic status	
			Leukemia	27	0.85 (0.56–1.21)		
			CLL	9	0.71 (0.32–1.25)		
			AML	6	0.94 (0.34–1.84)		
			CML	3	1.03 (0.19–2.52)		
			PV	4	1.03 (0.27–2.29)		
			Females				
			Leukemia	39	1.02 (0.73–1.37)		
			CLL	8	0.73 (0.31–1.33)		
			AML	14	1.41 (0.77–2.24)		
			CML	3	0.86 (0.16–2.10)		
			PV	7	1.26 (0.50–2.66)		

AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; HD, Hodgkin's disease; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NR, not reported; PV, polycythemia vera; RR, relative risk; SEI, socio-economic index; SIR, Standardized Incidence Ratio

cancer (1.01 expected), five cases of breast cancer (8.5 expected) and nine cases of lung cancer (7.4 expected).

Teta *et al.* (1984) examined cancer incidence during 1935–1978 in 11 845 female and 1805 male cosmetologists in Connecticut (USA) who had held licences for five years or more and had begun hairdressing school before 1 January 1966. A significant excess of lung cancer (standardized incidence ratio [SIR], 1.41) and excesses of brain (SIR, 1.68) and ovarian cancer (SIR, 1.34) of borderline significance were observed among women; the SIR for bladder cancer was 1.36 (95% CI, 0.74–2.27), on the basis of 14 cases. No significant cancer risk was evident for female cosmetologists licensed since 1935, even for those with 35 years or more of follow-up, although the SIRs for brain cancer, lymphoma and leukaemia were elevated. Female cosmetologists who had entered the profession between 1925 and 1934, however, experienced a significant overall increase in cancer incidence (SIR, 1.29) and significant excesses of respiratory cancer and cancers of the breast, *corpus uteri* and ovary. Among the men in the cohort, there was no excess of cancers at all sites (77 observed, 73.4 expected), but cancers of the brain occurred more frequently than expected (4 observed, 1.9 expected). [The Working Group noted that no other numbers were given for cancers at specific sites in men.]

Gubéran *et al.* (1985) studied cancer mortality during the period 1942–1982 and incidence in the years 1970–80 in a cohort of 703 male and 677 female hairdressers in Geneva, Switzerland. Increased mortality from bladder cancer was observed among men (10 observed, 3.9 expected; $P < 0.01$) and women (2 observed, 1.0 expected). The corresponding values for incident cases were 11 and 5.3 for men ($P < 0.01$) and 2 and 1.5 for women. Significant ($P < 0.05$) excesses of incident cases of cancer of the buccal cavity and pharynx (6 observed, 2.5 expected) and of prostate cancer (12 observed, 6.1 expected) were seen in men in the period 1970–80. No case of cancer of the buccal cavity and pharynx was seen in women (0.8 expected); for neither of these sites, however, was there an excess in the longer period covered by the mortality analysis (1942–1982). A nested case–control study of 18 cases of bladder cancer among men in this cohort (10 deceased, six incident cases that occurred during 1970–1980 and two incident cases that occurred in 1981) showed a non-significantly longer duration of exposure (measured from the start of apprenticeship) among those who dressed men's hair but not among those who dressed women's hair. Enquiries indicated that the great majority of male hairdressers in this study never dyed men's hair. In the period 1900–1950, application of brilliantines to men's scalps after haircuts was widespread in Geneva. The authors stated that those preparations may have contained colouring agents that are bladder carcinogens, such as *para*-dimethylaminoazobenzene, chrysoidine and auramine, which have been found in brilliantines in other countries. They also mentioned that 2–naphthylamine has been found as an impurity in Yellow AB and Yellow OB, which have been used in cosmetics.

In a brief note, Shibata *et al.* (1990) reported three deaths from leukaemia (3.84 expected) and two from lymphoma (3.01 expected) in a cohort of 8316 male and female barbers surveyed in the period 1976–1987 in the Aichi Prefecture, Japan.

In a cohort study of 248 046 US male veterans who served during 1917–1940 and were interviewed in 1954 or 1957 on smoking habits and occupations, Hrubec *et al.* (1992) analysed the mortality pattern of 740 barbers through 1980. Smoking-adjusted relative risks were 1.2 for all cancers (110 deaths; 95% CI, 1.06–1.45), 1.6 for respiratory cancers (95% CI, 1.22–2.20), 1.5 for prostate cancer (95% CI, 1.03–2.15) and 2.5 for multiple myeloma (95% CI, 1.08–5.63). No excess was found for bladder cancer (3 deaths; OR, 0.7).

Morton (1995) estimated breast-cancer incidence by compiling all cases first diagnosed during the period 1963–1977 among residents of the Portland-Vancouver Standard Metropolitan Statistical Area by searching records of all 24 hospitals (16 of which had tumour registries) in the four counties. An additional 1.8% of total cases were identified through death certificates. The total number of breast-cancer cases identified was 7368. For resident women aged 16–68 the usual occupation was retrieved from census data, and mean age-standardized incidence and mortality rates per 100 000 women were computed for each occupational category. The mean annual incidence rate among beautician was 125.2/100 000, not significantly different from that for all women. The mortality rate was 55.2/100 000, significantly higher than the rate for all women.

In a cohort of 563 395 female participants in Cancer Prevention Study II, a prospective mortality study enrolling volunteers from all US states interviewed in 1982 and followed up until 1991, 1780 fatal breast cancers were identified. Among women whose main lifetime occupation was beautician, the rate ratio, adjusted for age and several known or potential risk factors, was 1.02 (95% CI: 0.62–1.89), based on 16 cases (Calle *et al*., 1998).

(a) Scandinavian cohorts

Several cohort studies were conducted in the Scandinavian countries, often based on overlapping populations. Consequently, their results are not always independent from each other. They have been grouped by country to better understand these interdependencies.

(i) *Denmark*

Bladder-cancer incidence was investigated in a cohort of 4874 men and 9497 women aged 20–64 years from Denmark, who declared "hairdresser" as occupation in the 1970 census. Follow-up was performed up to 1980 through record linkage with national mortality and migration databases. The observed number of cases of bladder cancer was obtained through record linkage with the national Danish Cancer Registry, which also provided incidence rates to compute the expected number of cases. In men, there were 41 observed cases, as compared with 19.97 expected ones (SIR, 2.05; 95% CI, 1.51–2.78). In women, the observed and expected numbers of cases were 7 and 3.97, respectively, thus giving a SIR of 1.76 (95% CI, 0.71–3.63) (Lynge, 1990-1991).

As a follow-up of the previous study the cohort was then updated for the period 1981–1987 and extended to several other cancer sites. During this period, 26 further cases

of bladder cancer occurred among men (SIR, 1.17, 95% CI, 0.77–1.72) and 5 among women (SIR, 0.88; 95% CI, 0.28–2.04). Thus, for the overall period 1970–1987, the SIRs were 1.58 (95% CI, 1.24–2.01) in men and 1.23 (95% CI, 0.64–2.15) in women. In males during 1970–97 there were one case of cancer of the oral cavity (SIR = 0.37, 95% CI: 0.01–2.07), one of pharynx cancer (SIR, 0.29; 95% CI, 0.01–1.64), 10 of larynx cancer (SIR, 1.06; 95% CI, 0.51–1.95), 127 of lung cancer (SIR,,1.20; 95% CI, 1.01–1.43), 12 of NHL (SIR, 1.18; 95% CI, 0.61–2.06), 6 of Hodgkin disease (SIR, 2.00; 95% CI, 0.73–4.35), 13 of leukaemia (SIR, 1.02; 95% CI, 0.54–1.74) and 520 of all cancers (SIR, 1.12; 95% CI, 1.02–1.22). In females there were no cases of cancer of the oral cavity (SIR, 0; 95% CI, 0.00–2.29), three of pharynx cancer (SIR, 2.01; 95% CI, 0.42–5.89), one of larynx cancer (SIR, 0.58; 95% CI, 0.01–2.33), 31 of lung cancer (SIR, 0.97; 95% CI, 0.68–1.38), 16 of NHL (SIR, 1.92; 95% CI, 1.10–3.12), three of Hodgkin disease (SIR, 0.90; 95% CI, 0.19–2.64), eight of leukaemia (SIR, 0.94; 95% CI, 0.40–1.85) and 507 of all cancers (SIR, 1.05; 95% CI, 0.96–1.15) (Skov & Lynge, 1994).

(ii) *Finland*

In a study not entirely independent of that of Skov *et al.* (1990), a cohort of 3637 female and 168 male hairdressers, born in or before 1946, who were members of the Finnish Hairdressers' Association between 1970 and 1982, were followed-up for cancer incidence through the National Cancer Registry between 1970 and 1987 (Pukkala *et al.*, 1992). Expected numbers of cases were calculated by multiplying the number of person-years in each age group by the corresponding overall cancer incidence in Finland during the period of observation. Among women, there were 247 cases of cancer, versus 195.0 expected. Non-significant excesses were seen for breast cancer (70 cases, 56.3 expected), cervical cancer (11 cases, 7.1 expected), lung cancer (13 cases, 7.6 expected) and ovarian cancer (21 cases, 12.8 expected). Risks were not elevated for cancers at other sites, including the bladder (1 and 2.5), leukaemia (4 and 4.2) and multiple myeloma (1 and 2.4). The risk for all cancers was higher during the period 1970–1975 ($P < 0.05$) than during 1976–1981 ($P > 0.05$) or 1982–1987 ($P > 0.05$). Among men, 25 cases of cancer were observed (17.9 expected; 95% CI, 0.90–2.06); nonsignificantly elevated risks were found for cancers of the lung and pancreas, on the basis of seven and three cases, respectively.

The incidence of ovarian cancer was studied in a cohort that included all 892 591 economically active women in Finland, with occupations as reported in the 1970 census, who were born between 1906 and 1945. The follow-up period was 1971–1995. The National Cancer Registry was used for the identification of ovarian cancer cases ($n = 5072$). The expected numbers of cases were standardized by age, time period and social status, using rates of the entire cohort as the standard. The SIR for hairdressers and barbers was 1.3 (95% CI, 1.0–1.7), based on 57 cases, and that for beauticians was 1.0 (95% CI, 0.2–2.9), based on three cases (Vasama-Neuvonen *et al.*, 1999).

(iii) *Sweden*

Pollán and Gustavsson (1999) studied the incidence of breast cancer in a cohort of 1 101 669 Swedish women, alive and 25–64 years of age on 1 January 1971, who were gainfully employed at the time of the 1970 census and had also been present in the country during the 1960 census. The follow-up period was 1971–1989. Vital status was ascertained from a population registry, while emigration was not considered. Linkage to the Swedish Cancer Environment registry was used to identify breast cancer cases ($n = 29\,284$). Information on occupation was extracted from the 1970 census. For each occupation, expected numbers of cases were computed by use of age- and period-adjusted rates from the whole cohort. Among women employed as hairdressers or beauticians (total number not given), there were 284 observed and 258 expected cases of breast cancer, yielding a SIR of 1.10 (95% CI, 0.98–1.24). When further adjustment was made for area of residence and town size, defined from data extracted from the 1970 census, the relative risk (RR) was 1.09 (95% CI, 0.97–1.23) when the reference was set to all women in the cohort, and 1.21 (95% CI, 1.08–1.37) when the reference was set to women in other occupations of the same group (services and military work). Among women who reported hairdresser or beautician as their occupation in the 1970 census only, there were 85 cases of breast cancer, and the RR compared with women in other occupations of the same group was 1.09 (0.88–1.35). Among women reporting this occupation in both censuses (1960 and 1970), the number of breast cancers was 199, and the RR was 1.27 (95% CI: 1.11–1.47).

Mutanen & Hemminki (2001) studied the incidence of childhood cancer in a cohort that comprised all Swedish children born between 1935 and 1996 with a father (2.7 million) or a mother (1.0 million) with active occupation in the 1960 census, registered in the Swedish Family Cancer Database. The follow-up was performed between 1958 and 1996 for the age group 0–14 years. Cancer data were obtained from the Swedish Cancer Registry. Expected numbers of cases were calculated from reference incidence rates specific for 5-year age, area and socioeconomic status. The total active population was used as the reference population. Among the children of all economically active fathers there were 3376 childhood-cancer cases, eight of which were among hairdressers. Among children with economically active mothers there were 1408 childhood-cancer cases, 37 of which among hairdressers. Expected numbers for the whole group were not presented. There were four cases of kidney cancer among children of male hairdressers, compared with 0.4 expected (SIR, 10.6; 95% CI, 2.9–27.2). Among children of female hairdressers, there were 2 and 1.9 observed and expected cases, respectively, for a SIR of 1.0 (0.1–3.7).

All 1 670 517 women in Sweden who participated in both the 1960 and the 1970 censuses, who were gainfully employed in 1960 or 1970, were followed-up until 1989 with regards to ovarian cancer incidence, through linkage to the Swedish Register of Causes of Death. The Swedish Cancer Register was used to identify 9591 cases of ovarian cancer occurring during follow-up. The total numbers of women in the cohort employed as "barbers, beauticians, etc.," or in haircutting and beauty salons are not given.

The relative risk associated with various occupations was calculated by Poisson regression, stratified by time categories based on the census when employment was reported (1960 only, 1970 only, or both). Adjustment was performed for age. Among women who reported being employed as "barbers, beauticians etc." in 1970 only, there were 14 cases of ovarian cancer (RR, 0.56; 95% CI, 0.3–0.9), there were 36 cases among women reporting it in 1960 only (RR, 0.87; 95% CI, 0.6–1.2) and 51 cases among women reporting it in both censuses (RR, 1.21, 95% CI, 0.9–1.6). Among women who reported being employed in haircutting and beauty salons in 1970 only, 1960 only, and in both censuses, there were 12 (RR, 0.50; 95% CI, 0.3–0.9), 37 (RR, 0.88; 95% CI, 0.6–1.2), and 52 (RR, 1.26; 95% CI, 0.96–1.7) cases of ovarian cancer, respectively (Shields *et al.*, 2002).

Czene *et al.* (2003) studied the incidence of various cancers in a cohort comprising 38 866 women and 6866 men in Sweden who declared being employed as "hairdressers, barbers, beauticians and others" in at least one of the four censuses of 1960, 1970, 1980 and 1990. Of these, 67% of male and 41% of female hairdressers kept their occupation for at least two censuses. The follow-up period was 1960–1998. Tumour data were retrieved from the Swedish Cancer Registry. Expected numbers of cases were computed with reference to those who were economically active at least during one of the censuses, for those who were hairdressers in at least one census, and to those economically active in 1960 for those who were hairdressers in 1960. The rates used were sex-, period (5 years)- and age (5 years)-specific. For male hairdressers at any census, direct significant associations were found for cancers of the upper aerodigestive tract (51 cases; SIR, 1.51; 95% CI, 1.13–1.99), colorectal adenocarcinoma (135 cases; SIR, 1.24; 95% CI, 1.04–1.47) and lung cancer (141 cases; SIR, 1.38; 95% CI, 1.13–1.99), and a borderline association for bladder cancer (87 cases; SIR, 1.22; 95% CI, 0.98–1.51). No significant associations were found for other cancers of the digestive tract, urinary tract, skin, nervous system, thyroid, endocrine glands, connective tissue or for haematopoietic neoplasms. Results for male hairdressers at the 1960 census were similar. For female hairdressers at any census, direct significant associations were found for cancer of the pancreas (68 cases; SIR, 1.33; 95% CI, 1.03–1.68), lung cancer (160 cases; SIR, 1.35; 95% CI, 1.15–1.58), cancer of the cervix (213 cases; SIR, 1.28; 95% CI, 1.13–1.48), and cancer of the skin *in situ* (110 cases; SIR, 1.30; 95% CI, 1.07–1.55). No significant associations were found for other cancers, including in the breast, ovaria, and lung and for haematopoietic neoplasms, where the estimated SIRs were below 1.10 (except for multiple lyeloma (MM)). Results for female hairdressers at the 1960 census were largely similar, although the associations with pancreas cancer (38 cases; SIR, 1.01; 95% CI, 0.71–1.38) and cervical cancer (97 cases; SIR, 0.99; 95% CI, 0.80–1.21) were no longer evident.

Using the Swedish Family Cancer Database, a cohort was formed of all 1 644 958 Swedish men economically active at the 1960 census and all 1 154 091 women economically active at the 1970 census. In the cohort there were 4639 male and 16 360 female hairdressers. Follow-up ended in 2000. Linkage to the Swedish Cancer Registry

identified 24 041 bladder, 35 776 lung and 11 627 upper aerodigestive tract (UADT) cancers in men, and 3405, 8352 and 1767 in women, respectively. Expected numbers of cases were computed using sex-, age (5 years)-, period (10 years)- and socioeconomic status (6 groups)- specific rates from the whole cohort. Among male hairdressers there were 144 cases of lung cancer (SIR, 1.42; 95% CI, 1.20–1.54) and 92 (SIR, 1.19; 95% CI, 0.96–1.44) among female hairdressers (Ji & Hemminki, 2005a). A significant increase in risk for bladder cancer was found in men (SIR, 1.26; 95% CI, 1.01–1.54; 88 cases). The authors tried to adjust for the effect of smoking by dividing the SIR by 35% of the excess of lung cancer. The smoking-adjusted SIR was 1.10 (95% CI, 0.88–1.34). Among hairdressers in two consecutive censuses (1960 and 1970) there were 62 bladder-cancer cases, and the SIR was 1.14 (95% CI, 0.88–1.45) before and 1.00 (0.76–1.26) after adjustment for smoking. The corresponding values for those who were hairdressers at the censuses of 1960, 1970 and 1980 were 1.35 (95% CI, 0.91–1.84) and 1.17 (95% CI, 0.81–1.60), based on 33 cases. For women, data for bladder cancer were not presented, because no significant association was found (Ji *et al.*, 2005). Among male hairdressers, there were 49 UADT cancers (SIR, 1.39; 95% CI, 1.03–1.81). When subsites were considered, the SIR was significantly increased for cancers of the tongue (9 cases; SIR, 2.41; 95% CI, 1.09–4.25) and larynx (21 cases; SIR, 1.78; 95% CI, 1.10–2.62). The excess in UADT cancers was still significant when analysis was restricted to men who were employed as hairdressers in both the 1960 and the 1970 censuses (34 cases; SIR, 1.45; 95% CI, 1.01–1.98), or in all three censuses of 1960, 1970 and 1980 (16 cases; SIR, 1.96; 95% CI, 1.12–3.04). For female hairdressers the SIR of UADT cancers was 1.57 (95% CI, 1.02–2.23), based on 26 cases. When subsites were considered, the observed numbers were small, and a significant increase was found for cancer of the pharynx only (nine cases; SIR, 2.49; 95% CI, 1.13–4.39) (Ji & Hemminki, 2005a).

A subsequent cohort study was based on the same data, and had the same design as that described above (Ji & Hemminki, 2005b), and although the number of hairdressers was not given, it appears to be the same as mentioned before (4639 male and 16 360 female hairdressers). In this case, the follow-up was extended to 2002, and the neoplasms investigated were leukaemias. In the whole cohort of economically active men in 1960 there were 11 002 leukaemia cases, and in economically active women in 1970 there were 4040. No excess was found for all leukaemias for male (27 cases; SIR, 0.85; 95% CI, 0.56–1.21) or female (39 cases; SIR, 1.02; 95% CI, 0.73–1.37) hairdressers. When major leukaemia subtypes were considered separately, no excess was seen for CLL, AML, CML or PV in either sex. When analysis was restricted to women who were hairdressers in both censuses of 1960 and 1970, the SIR for PV was significantly elevated (7 cases; SIR, 3.54; 95% CI, 1.40–6.64) (Ji & Hemminki, 2005b).

(iv) *More than one Scandinavian country*

Following a report by Lynge and Thygesen (1988) on Danish hairdressers, Skov *et al.* (1990) carried out an analysis of the incidence of bladder cancer and lung cancer in men

and women employed as hairdressers and beauticians in 1960 in Norway and Sweden and as hairdressers and barbers in 1970 in Denmark and Finland.

Lynge and Thygesen (1988) found an increased risk for bladder cancer in hairdressers in Denmark: the RR was 2.05 for men, on the basis of 41 cases (95% CI, 1.51–2.78), and 1.76 for women, on the basis of seven cases (95% CI, 0.71–3.63). No corresponding increase in lung cancer was observed. In Finland, Norway and Denmark, the expected numbers of cancer cases were calculated by multiplying the person-years at risk for each of the five-year birth cohorts of hairdressers by the sex-specific incidence rate for the equivalent five-year birth cohort of all people who were economically active at the time of the census. In Sweden, the expected number of cancer cases was calculated by multiplying the number of hairdressers in a given region of Sweden in each five-year birth cohort at the time of the census by the sex-specific estimated cancer probability for the equivalent five-year birth cohort of all people in the region. National figures were obtained by aggregating the observed and the expected numbers across the 27 Swedish regions. The pattern of excess bladder-cancer incidence without a corresponding increase in lung-cancer incidence was not found in any of the other Nordic countries (Skov *et al.*, 1990).

In Sweden (Malker *et al.*, 1987; Skov *et al.*, 1990), the incidence of lung cancer was increased in male (98 cases; RR, 1.5; 95% CI, 1.2–1.8) and female (31 cases; 1.6; 1.1–2.2) hairdressers, and bladder-cancer incidence was increased in men (54 cases; 1.5; 1.1–1.9) but not in women (six cases; 0.4; 0.2–1.0). The authors noted that a national survey of smoking in Sweden carried out in 1963 had found that 74% of male barbers and beauticians aged 50–69 were regular smokers, compared with 46% of all men aged 50–69 years. In Norway, the incidences of bladder cancer and lung cancer were increased in hairdressers (RR, 1.4–1.6), but the increase was significant only for lung cancer in men. In the data from Finland, no case of bladder cancer was recorded among male hairdressers in the period 1971–80 (expected, 0.3), but three cases occurred in women (1.8 expected). The incidence of lung cancer was not increased: three observed, 2.0 expected in men and two observed, 4.4 expected in women (Skov *et al.*, 1990). The incidence of non-Hodgkin lymphoma was examined in Denmark (1970–80) by occupational category by Skov and Lynge (1991) using a similar method. No significant excess was observed in female hairdressers (RR, 1.98; 95% CI, 0.24–7.15; two cases); no case was recorded among male hairdressers. When all groups of hairdressers were included (self-employed/barber, work in beauty shops and hairdresser), the RRs were 1.3 (0.48–2.83; six cases) for men and 2.0 (0.81–4.14; seven cases) for women.

Boffetta *et al.* (1994) studied the incidence of ovarian cancer and NHL in a cohort of 29 279 women from Denmark, Sweden, Norway and Finland, aged 20–64 years in 1970, who declared being employed as a hairdresser in the 1970 census questionnaire. They were followed-up between 1970 and 1985 (1987 for Denmark). Date of death or emigration was obtained through linkage to appropriate population registries; linkage to the four national cancer registries identified cases of ovarian cancer and NHL in the cohort. The observed incidence was compared with the expected one using national

5-year age-specific rates in the overall female population at the censuses. SIRs were calculated as the ratio of observed and expected cases, and 95% CIs by use of Byar approximation. Overall, 127 ovarian cancer cases were observed in the cohort, compared with 107.8 expected (SIR, 1.18; 95% CI, 0.98–1.40). The SIRs were 1.4 (95% CI, 1.0–1.9), 1.1 (95% CI, 0.0–1.6) and 1.1 (95% CI, 0.8–1.4) for the periods 1971–1975, 1976–1980 and 1981–1985, respectively. The overall SIR for NHL was 1.20 (95% CI, 0.84–1.66), from 36 observed cases.

Using the same methods as Boffetta *et al.*, Andersen *et al.* (1999) conducted a systematic analysis of cancer risk in 10 298 male and 26 545 female hairdressers from Denmark, Finland, Norway and Sweden. In men, there was a significant increase for all sites combined (SIR, 1.12; 95% CI, 1.06–1.18; 1302 cases). Significant increases were found for lung cancer (SIR, 1.21; 95% CI, 1.07–1.37; 249 cases), bladder (SIR, 1.47; 95% CI, 1.25–1.73; 147 cases) and skin cancers other than melanoma (SIR, 1.22; 95% CI, 1.01–1.48; 105 cases). No association was found for NHL (SIR = 1.01), multiple myeloma (SIR = 1.00) or other haemolymphopoietic malignancies, while the SIR for lip cancer was significantly decreased (SIR, 0.34; 95% CI, 0.11–0.76; 5 cases). For women, the SIR for all sites was 1.05 (95% CI, 1.00–1.09) based on 2178 cases. Significant increases were found for cancers of the pharynx (SIR, 2.16; 95% CI, 1.18–3.62; 14 cases), lung (SIR, 1.22; 95% CI, 1.02–1.46; 122 cases), *cervix uteri* (SIR, 1.21; 95% CI, 1.03–1.43) and ovary (SIR, 1.18; 95% CI, 1.01–1.38). No increase in risk was found for bladder (SIR, 0.89; 95% CI, 0.63–1.23; 37 cases), breast (SIR, 1.05; 0.97–1.13; 643 cases) or haemolymphopoietic cancers (SIR 1.06 for NHL, 0.80 for multiple myeloma).

2.1.2 *Case–control studies* (Tables 2.2–2.6)

(a) Bladder cancer (Table 2.2) [Only studies not included in the previous IARC Monograph (Volume 57) are listed in this Table]

In a case–control study, Glashan and Cartwright (1981) interviewed all patients with bladder cancer presenting in three Yorkshire (United Kingdom) centres over a 3-year period (744 men and 247 women). Controls were 993 men and 345 women without malignant disease, age- and sex-matched to cases. Mantel-Haenszel ORs adjusted for age, sex and year of diagnosis were computed; the OR for "hairdressers" was 0.9 (95% CI, 0.3–3.2).

Schoenberg *et al.* (1984) conducted a population-based case–control study in 1978–79 in New Jersey, USA, on 706 white male patients aged 21–84 years with newly diagnosed, histologically confirmed cancer of the bladder or papilloma not specified as benign, and 1392 controls selected with random-digit dialling (<65 yrs) or from Medicare lists (age 65–84). Participation rates were 89.7% for cases, 86.6% for controls. Twenty cases and 38 controls were excluded from the analysis because of incomplete or unreliable interview. ORs were estimated by multiple logistic regression models adjusted for age, smoking and 19 employment categories. Twelve cases and 17 controls were ever employed as barbers or hairdressers, and the corresponding OR was 1.27 (0.59–2.73).

Table 2.2. Case–control studies of bladder cancer and occupational exposure of hair dyes

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Glashan & Cartwright (1981)	744 male and 247 female bladder cancer cases admitted to 3 centres in Yorkshire, UK	993 men and 345 women; matched on age and sex	Interview	Employed as a hairdresser	NR	0.9 (0.3–3.2)	Sex, age, year of diagnosis	
Schoenberg *et al.* (1984), New Jersey, USA, 1978–79	686 white male cases of histologically confirmed carcinoma of the urinary bladder; aged 21–84	1354 population controls; aged 21–84; matched on age; selected through random digit dialing or HCFA/Medicare lists	Interview	Ever employed as a barber/ hairdresser	12	1.27 (0.59–2.73)	Age, smoking, occupation	
Kunze *et al.* (1992), Northern Germany, 1977–84	531 male and 144 female cases of histologically confirmed benign or malignant epithelial tumors of the urinary bladder, ureters, renal pelves, and urethra; selected from four urologic wards in three cities	675 hospital controls with benign urological diseases; matched on age (±5 years), sex	Interview	Ever employed as a hairdresser	10	1.7 (0.6–4.5)	Age, smoking	Same study population as reported in Claude *et al.* (1988)

Table 2.2 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Cordier *et al.* (1993), France, 1984–1987	658 male and 107 female cases of histologically confirmed bladder cancer; aged <80 years; Five French Regions.	658 male and 107 female hospital controls randomly selected in the same hospital among patients admitted for causes other than cancer, respiratory disease or symptoms suggestive of bladder cancer; controls were matched with cases for sex, age (±5 years), ethnic origin and place of residence	Interviews	Having worked as a hairdresser for ≥6 months (males only)	5	2.21 (0.41–11.94)	Sex, age, ethnicity, residence, smoking.	Data for female hairdressers not reported
Bolm-Audorff *et al.* (1993) Northern Germany 1989–1992	300 histologically confirmed cases of lower urinary tract cancer	300 hospital-based controls admitted for non-malignant diseases of the urinary tract matched on sex, age, residence	Interviews	*Employed as hairdresser* Men Women Total	 5 2 7	 4.05 (0.63–26.09) Not calculated 6.48 (1.15–36.51)	Smoking	

Table 2.2 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Teschke *et al.* (1997), British Columbia, Canada, 1990–1992	88 male and 17 female cases of histologically confirmed bladder cancer registered by the British Columbia Cancer Agency	112 male and 27 female population controls selected from provincial voters lists; frequency matched on age (± 2 years) and sex	Interview	Ever employed as hairdresser or barber	3	3.2 (0.2–176)	Sex, age, smoking	For nasal cancer: 1 case, OR=2.5 (0–225)
				Most recent 20 years removed	2	2.6 (0.1–159)		
Sorahan *et al.* (1998), West Midland Region, UK 1991–1993	Primary analysis: 624 male and 179 female cases of urothelial cancer; Secondary analysis: 1106 male and 321 female cases of urothelial cancer born in 1915–70	*Primary analysis:* 2135 population controls; *Secondary analysis:* 2199 controls; controls were matched on sex, year of birth and GP	Interview	Ever employed as a hairdresser	11	Primary analysis 1.70 (0.74–3.89), Secondary analysis 1.63 (0.86–3.12)	Sex, year of birth, GP, smoking.	Two types of analyses performed: a matched/paired analysis and group analysis comparing all cancer cases and controls
				Ever employed as a hairdresser	22			

Table 2.2 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Kogevinas *et al.* (2003), Germany, France, Italy, Spain, Denmark, Greece,1976–1996	Pooled analysis of 3346 male incident cases of bladder cancer, from 11 case–control studies; aged 30–79 years	6840 male controls identified through hospitals and the general population; controls individually or frequency matched to cases on age (within 5 years) and geographic area	Job exposure matrix was used to calculate the prevalence of exposure and average levels of exposure were evaluated for each occupation in different time periods	Ever employed as a hairdresser	37	1.09 (0.70–1.70)	Age, smoking study centre	Same study population as reported in 't Mannetje *et al.* (1999); Reference: for males not employed in 8 a priori defined high risk occupation (including hairdressers), for females never employed as hairdresser.
Gago-Dominguez *et al.* (2001a), Los Angeles, USA, 1987–1996	1514 incident cases of bladder cancer; aged 25–64	1514 neighborhood controls; matched by sex, date of birth (within 5 years), ethnicity and neighborhood of residence at the time of cancer diagnosis	In-person interview	*Employed as hairdresser or barber* Never Ever *Duration of employment* <10 years ≥10 years	 1494 20 6 14	 1.0 1.5 (0.7–3.2) 0.5 (0.2–1.6) 5.1 (1.3–19.2)	Age, sex, smoking, ethnicity, residence.	

Table 2.2 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Zheng *et al.* (2002), Iowa, USA, 1986–1989	1135 male and 317 female cases of histologically confirmed cases of bladder cancer; identified by the State Health Registry of Iowa; between 1986 to 1989	1601 male and 833 female population controls; frequency-matched by gender and 5-year age group to all cases in a larger study, which also included cancers of the brain, kidney, pancreas, colon, and rectum.; randomly selected from computerized state driver's license records or HCFA lists	Self-administered mailed questionnaire	Employed in a barber shop for ≥5 years	5	1.8 (0.4–8.0)	Age, smoking, family history of bladder cancer	Data for occupational exposure for women not given.
Gaertner *et al.* (2004), Canada, 1994–1997	535 male and 352 female cases, with incident, histologically confirmed bladder cancer; aged 20–74 were identified through the provincial cancer registries in seven Canadian provinces	1430 male and 1417 female population controls; frequency matched on age and gender; identified through the National Enhanced Cancer Surveillance System (NECSS)		**Employed as hairdresser** *Male* *Female* >1–5 years >5–15 years >15 years	 8 6 1 4 3	 3.42 (1.09–10.8) 0.75 (0.28–2.01) NR (no exposed controls) 4.7 (0.79–27.9) 1.98 (0.4–9.7)	Age, province, race, smoking, consumption of fruit, fried food and coffee, employment in potentially hazardous occupations	

Table 2.2 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Dryson *et al.* (2008), New Zealand, 2003–2004	165 male and 48 female incident cases of bladder cancer; aged 25–70; reported to the New Zealand Cancer Registry	221 male and 250 female population controls; randomly selected from the New Zealand Electoral Roll; frequency matched by age	Interviews	*Employed as hairdresser for >1 year* Total Men Women	 6 2 4	 9.15 (1.60–52.22) NR 9.95 (1.37–72.21)	Age, sex, smoking	Risk ratio not reported for men because no controls were identified
Golka *et al.* (2008), North Rhine-Westphalia, Germany, 1988–95	156 male cases with histologically confirmed urinary bladder cancer	336 prostate cancer cases	Mail questionnaire	Employed as hairdresser for >1 year	4	4.90 (0.85–28.39)	Age, smoking	There were less than 10 cases and/or 10 controls available for analysis

CI, confidence interval; NR, not reported; OR, odds ratio

Kunze *et al.* (1992) conducted a hospital-based case–control study in northern Germany (1977–1984), including 531 male patients with an epithelial neoplasm of the lower urinary tract and an equal number of controls admitted to urological wards with benign urological diseases (64% with prostatitis or prostatic hyperthropy) individually matched by age to cases. Two percent of the cases refused to participate. The smoking-adjusted conditional OR for having ever worked as a hairdresser was 1.7 (95% CI, 0.6–4.5), based on 10 exposed cases and six exposed controls.

Cordier *et al.* (1993) conducted a hospital-based case–control study in seven French hospitals between 1984 and 1987. Cases were 765 patients (658 men and 107 women) below age 80 years, with a histologically confirmed bladder cancer diagnosed after 1 January 1982 (527 (69%) were interviewed less than one year after diagnosis) and no previous history of cancer. Controls were patients with no history of cancer admitted to the same hospitals for causes other than cancer, respiratory disease or symptoms suggestive of bladder cancer, individually matched to cases by sex, age, ethnic origin and place of residence. Participation rates were not given. ORs were estimated by unconditional logistic regression including the matching variables and smoking. For subjects who had worked at least six months as hairdressers (five cases and two controls) the OR was 2.21 (95% CI, 0.41–11.94).

Bolm-Audorff *et al.* (1993) conducted a hospital based case–control study on 239 male and 61 female histologically confirmed cases of lower urinary tract cancers (90% of the bladder) identified in four clinics in two areas of Germany between 1989 and 1992, and an equal number of controls admitted to the same clinics for non-malignant diseases of the urinary tract, individually matched to cases by sex, age and residence. Response rate was 93% for cases and 98% for controls. Among men, five cases and three controls were employed as hairdressers, and the smoking-adjusted OR was 4.05 (95% CI, 0.63–26.09). Among women, two cases and no controls were exposed. The OR for both sexes combined was 6.48 (95% CI, 1.15–36.51).

Teschke *et al.* (1997) conducted a population-based case–control study in British Columbia, Canada, between 1990 and 1992, including 105 cases of bladder cancer (88 men and 17 women) and 48 cases of cancer of the nasal cavity or sinus (33 men and 15 women), all histologically confirmed. Controls were 128 men and 31 women randomly selected in age and sex strata from the provincial voters list. Since recruitment of bladder-cancer cases was restricted to those born after 1915, only 112 male and 27 female controls were used for this cancer. Participation rates were 89% for nasal cancer, 88% for bladder cancer, and 81% for controls, and next-of-kin interviews were conducted for 15%, 18% and 14%, respectively. ORs were adjusted for sex, age and smoking. Three bladder-cancer cases and one control had ever worked as a hairdresser or barber; the OR was 3.2 (95% CI, 0.2–176). When the more recent 20 years were removed, two cases and one control were exposed (OR 2.6; 95% CI, 0.1–159). One nasal cancer case and one control were exposed (OR 2.5; 95% CI, 0–225).

Sorahan *et al.* (1998), in a population-based case–control study from the West Midland Region, United Kingdom, interviewed 1427 cases (1106 males and 321 women)

born in 1915–70 and diagnosed with a primary urothelial tumour between 1991 and 1993. A first set of controls was selected by asking general practitioners (GP) of the cases to each select three patients matched on sex and year of birth. Of the 908 GPs contacted, 55% accepted to participate, representing a total of 615 cases. Out of 1845 potential controls, identified 1147 (62%) filled in the questionnaire. A further set of 1768 potential controls matched to cases on age, sex and GP were selected through the Family Health Service Authorities, 1052 (60%) of whom returned the questionnaire. [Note: sex distribution of controls not given]. The primary analysis was performed on the 803 cases (624 men and 179 women) for which at least one matched control was available, and 2135 controls correctly matched to cases. A further analyses was conducted on all 1427 cases and 2199 controls. The conditional OR adjusted for smoking and for ever having been employed as a hairdresser in the primary analysis was 1.70 (95% CI, 0.74–3.89), based on 11 cases and 18 controls. When all cases and controls were considered, there were 22 exposed cases and 19 exposed controls, and the OR adjusted for age, year of birth and smoking was 1.63 (95% CI, 0.86–3.12).

Data from 11 case–control studies on bladder cancer conducted between 1976 and 1996 were combined, and results were published separately for women (’t Mannetje *et al.*, 1999) and men (Kogevinas *et al.*, 2003). Subjects outside the age range 30–79 years and prevalent cases were excluded. Among women, 700 cases of bladder cancer and 2425 controls (761 hospital and 1664 population controls) were included. Eleven cases and 56 controls had worked as hairdresser, barber, or beautician, and the OR adjusted for age, smoking, study centre and centre-age interaction was 0.8 (95% CI, 04.–1.7), compared with those never employed as a hairdresser. Among men, 3346 cases and 6840 controls were used for the analyses, of whom 37 and 62, respectively, had ever been employed as a hairdresser. Compared with men never employed in eight previously defined high-risk occupations (including hairdressers), the OR adjusted for age, smoking and study centre of those ever employed as a hairdresser was 1.09 (95% CI, 0.70–1.70) (’t Mannetje *et al.*, 1999; Kogevinas *et al.*, 2003).

Gago-Dominguez *et al.* (2001a) conducted a population-based case–control study in Los Angeles, California, USA, which involved 1514 (72%) non-Asian patients aged 25–64 years with incident histologically confirmed bladder cancer diagnosed between 1987 and 1996, identified through the SEER cancer registry of Los Angeles County. For each case a neighbourhood control was matched on sex, date of birth, ethnicity and neighbourhood of residence (69% were the first eligible control) [Sex distribution of cases and controls was not given]. Twenty cases and 13 controls were employed as hairdressers or barbers for at least one year, and the smoking-adjusted OR was 1.5 (95% CI, 0.7–3.2). The OR was 0.5 (95% CI, 0.2–1.6) for those employed for less than 10 years, based on six cases and 10 controls, and 5.1 (95% CI, 1.3–19.2) for those employed for 10 or more years, based on 14 cases and three controls.

Zheng and colleagues (2002) conducted a population-based case–control study on 1452 histologically confirmed incident bladder-cancer cases (1135 men and 317 women) aged 40–85 years identified by the State Health Registry of Iowa, US, between 1986 and

1989, and 2434 (1601 men and 833 women) population controls, frequency-matched by sex and age to cases of cancers at several organ sites. Participation rate was 85% for cases and 80–82% for controls. For 156 cases, proxy interviews were conducted. Among men, five cases and three controls were exposed for at least five years in barber shops, giving an OR (adjusted for age, smoking, family history of bladder cancer) of 1.8 (95% CI, 0.4–8.0). All exposed subjects had worked for 10 or more years in barber shops. No data were presented for women.

Gaertner *et al.* (2004) conducted a population-based case–control study in seven Canadian provinces, including 535 male and 352 female patients aged 20–74 years with incident, histologically confirmed bladder cancer between 1994 and 1997, identified through cancer registries. Controls were 1430 men and 1417 women randomly selected either by random-digit dialling (two provinces) or from the provincial health insurance database (five provinces) in 1996. Participation rates were 58% for male cases, 61% for female cases, 59% for male controls and 65% for female controls. ORs were computed by means of unconditional logistic regression models and adjusted for age, province, race, smoking, consumption of fruit, fried food and coffee, and employment in potentially hazardous occupations (as printers, rubber workers, metal workers, truck drivers, painters, dry cleaners, mechanics and machinists). Occupations held for at least one year full-time equivalent were considered relevant. Among men, eight cases and six controls had worked as hairdressers (OR, 3.42; 95% CI, 1.09–10.8): one case and no controls for less than five years, four cases and two controls for 5–15 years (OR 4.7; 95% CI, 0.79–27.9) and three cases and four controls for over 15 years (OR 1.98; 95% CI, 0.4–9.7; *P* for trend, 0.24). Among women there were six cases and 34 controls exposed (OR 0.75; 95% CI, 0.28–2.01).

Dryson *et al.* (2008) interviewed 213 out of all incident cases of bladder cancer aged 25–70, reported to the New Zealand Cancer Registry in 2003–2004 (64%; 165 men and 48 women), together with 471 population controls (48% of eligible controls; 221 men and 250 women) in a population-based case–control study. ORs were adjusted for age, sex, ethnicity and smoking. Six cases and three controls had worked as a hairdresser for more than one year; the OR was 9.15 (95% CI, 1.60–52.22). Among men, two cases and no controls were exposed, and among women four cases and three controls (OR 9.95; 95% CI, 1.37–72.21). Similarly, for the industrial classification "hairdressing and beauty salon" the OR was 5.35 (95% CI, 1.37–20.9), based on seven cases and five controls, three and none respectively among males, and four and five among women (OR, 4.79; 95% CI, 0.90–25.32). No consistent pattern by duration of employment was seen, but numbers were small. For hairdressers, the OR was 2.87 (95% CI, 0.59–13.89) in ever-smokers and 9.66 (95% CI, 0.62–151.42) in non-smokers.

A mailed questionnaire was sent to 332 male subjects with histologically ascertained bladder cancer, and as controls 566 with prostate cancer, who requested an after-care treatment in Bochum, Germany between 1992 and 1995. Of these, 63% of bladder and 72% of prostate cancer cases responded. After further exclusion of 53 bladder and 69 prostate cancer cases because they either were not resident in North-Rhine Westphalia,

had both bladder and prostate cancer, were first diagnosed before 1988 or did not specify first diagnosis, had asked for aftercare for another disease, or did not complete the questionnaire, 156 bladder and 336 prostate cancer cases were available for analysis. Four bladder and two prostate cancer cases had worked as a hairdresser for more than one year, and the corresponding age- and smoking-adjusted OR was 4.90 (95% CI, 0.85–28.39) (Golka *et al.*, 2008).

(b) Childhood cancers (Table 2.3)

The association between parental occupation and the risk for neuroblastoma was evaluated in a population-based case–control study conducted in 139 hospitals in the United States and Canada from 1992 to 1996. Of 741 eligible cases, information was obtained for 538 (73%) case mothers and 472 (64%) case fathers (directly for 405 and from the mother for 67). Controls were selected by random-digit dialling (RDD), and individually matched to cases on date of birth. The response rate for the RDD screening phase was 74%. Of 708 eligible controls, information was obtained for 504 (71%) mothers and 446 (64%) fathers (directly for 304 and from the mother for 142). Conditional logistic regression was used to obtain the OR adjusted for age, mother's age, race and education, and household income in birth year. Exposed subjects were defined as those who had worked as a barber or hairdresser for at least six months. Two case fathers and one control father had worked as a barber or hairdresser; the OR was 3.3 (95% CI, 0.2–45.7). There were 24 exposed case mothers and 10 control mothers, giving an OR of 2.8 (95% CI, 1.2–6.3) (Olshan *et al.*, 1999).

(c) Breast cancer (Table 2.4)

Band *et al.* (2000) conducted a population-based case–control study on occupational risk factors and breast-cancer risk by menopausal status in British Columbia, Canada. Cases were women below age 75 years with Canadian citizenship, residing in British Columbia, English-speaking and without previous history of breast cancer, diagnosed with histologically-confirmed breast cancer between June 1, 1988 and June 30, 1989, identified through the British Columbia Cancer Registry. Controls were women randomly selected from the 1989 British Columbia Provincial Voters List, matched to the cases by 5-year age group, with no diagnosis of breast cancer before. For breast-cancer cases, permission from the physician was requested before contacting the patient. A questionnaire eliciting information on lifetime job descriptions, occupation and industry titles, duration and period of work was mailed to cases and controls. The questionnaire collected information also on socio-demographic, anthropometric, menstrual and reproductive factors, smoking and drinking habits, exogenous estrogen use and family history of breast cancer. If the questionnaire was incomplete, subjects were contacted by telephone to collect missing data. Of 1489 eligible breast-cancer cases identified, 1018 (68.4%) returned a completed questionnaire (for 58 cases the physician did not grant

Table 2.3. Case-control studies of childhood cancer and occupational exposure to hair dyes

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Olshan *et al*., (1999), United States and Canada, 1992–1996	504 newly diagnosed cases of neuroblastoma; aged <19 years; 538 case mothers and 405 case fathers were identified	504 control mothers and 446 control fathers of children selected by RDD, individually matched on date of birth	Interview	*Employed ≥6 months as barber or hairdresser* Men Women	 2 24	 3.3 (0.2–45.7) 2.8 (1.2–6.3)	Race, age, education, household income in birth year	

CI, confidence interval; OR, odds ratio; RDD, random-digit dialling

Table 2.4. Case-control studies of breast cancer and occupational exposure to hair dyes

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Band *et al*. (2000) British Columbia, Canada, 1988–1989	995 female breast cancer cases; aged <75 years; identified through the British Columbia Cancer Registry	1020 population controls; no diagnosis of breast cancer before 1989; randomly selected from the 1989 British Columbia Provincial Voters List	Mailed questionnaire	*Employed as barber/hairdresser* Ever Usual occupation	 22 12	 1.92 (1.04–3.56) 1.02 (0.53–2.11)	Age, weight, smoking, alcohol, family history of breast cancer, history of breast biopsies, menopausal status.	Reported 90% CI
	Pre-menopausal 297 cases	332 controls		*Employed as barber/hairdresser* Ever usual occupation	 14 7	 5.45 (1.85–16.09) 2.71 (0.84–8.74)	Age, smoking, history of breast biopsies, and family history of breast cancer	
	Post-menopausal 690 cases	675 controls		*Employed as barber/hairdresser* Ever usual occupation	 8 5	 0.84 (0.35–2.01) NR	Age, weight, alcohol, family history of breast cancer, history of breast biopsies	
Habel *et al*. (1995) King County, Washington, USA, 1988–1990	537 white female cases of breast cancer; aged 50–64; identified through the SEER program	492 population controls (RDD); stratified by age	Interview	Worked as cosmetologist	7	1.5 (0.5–4.8)	Age, education, parity, BMI, alcohol	

BMI, body mass index; CI, confidence interval; OR, odds ratio; RDD, random-digit dialling; SEER, Surveillance Epidemiology and End Results

permission, 376 cases refused to participate, and 37 had either died or were lost to follow-up). Of 1502 women selected as controls, 359 refused to participate, 10 were deceased, 108 were lost to follow-up and 1025 (68.2%) returned the questionnaire. After further exclusion of cases and controls with no matches or with missing information, 995 cases (297 pre-menopausal and 690 post-menopausal) and 1020 controls (332 pre-menopausal and 675 post-menopausal) were available for the analysis. Conditional logistic regression models were used to estimate ORs and 90% CIs for occupational variables. At first, potential confounders to be included in the model were selected from several factors by keeping the ones significantly associated with breast-cancer risk in a forward selection procedure. Then the ORs for each occupation and industry type for which at least three cases were exposed were estimated in turn, for all women and separately for pre- and post-menopausal women. Usual occupation/industry (job held for the longest period) and ever occupation/industry (job ever held) were investigated. Overall, 22 breast-cancer cases reported to have ever worked as a barber or hairdresser, for an OR of 1.92 (90% CI, 1.04–3.56) and 12 cases reported barber/hairdresser as their usual occupation, for an OR of 1.02 (90% CI, 0.53–2.11). Among pre-menopausal women, 14 cases had ever been a barber or hairdresser (OR, 5.45; 90% CI, 1.85–16.09) and 7 cases reported barber or hairdresser as their usual occupation (OR, 2.71; 90% CI, 0.84–8.74). In post-menopause, there were eight cases who had ever been a barber/hairdresser (OR, 0.84; 90% CI, 0.35–2.01) and five cases whose usual occupation was barber/hairdresser (OR not given).

Habel *et al.* (1995) conducted a population-based case–control study of breast cancer in King County, Washington, USA. Cases were 537 white women aged 50–64 years, resident in King County, who were diagnosed with histologically confirmed first primary invasive or *in situ* carcinoma of the breast between January 1988 and June 1990. Controls were 492 women selected by random-digit dialling, stratified by age, without history of breast cancer. Participation rate was 81% for cases and 73% for controls. ORs were computed by means of logistic regression models and adjusted for age, education, parity, body-mass index, and alcohol. The three longest-held occupations were recorded for each woman. Seven cases and five controls reported having worked as a cosmetologist, and the OR was 1.5 (95% CI, 0.5–4.8). For all exposed women exposure began at least 10 years before diagnosis, while four cases and five controls had had the occupation for at least 5 years (OR, 0.9; 95% CI, 0.2–3.4).

(d) Cancers of the haemolymphopoietic system (Table 2.5)

Herrinton *et al.* (1994) conducted a population-based case–control study on 681 patients (321 women and 360 men) with incident multiple myeloma diagnosed from 1977 to 1981, identified through cancer registries in four geographical areas in the USA (Seattle, Utah, Detroit, Atlanta), and 1679 population controls (746 women and 933 men) frequency-matched to cases on sex, age and race. Participation rate was 89% for cases and 83% for controls. Proxy interviews were conducted for 36% of cases and 1% of controls. ORs were adjusted for age, sex, race, study centre and education. Among women, 12 cases and 22 controls had been employed as a hairdresser for six months or more, and

Table 2.5. Case–control studies of haemolymphopoietic malignancies and occupational exposure to hair dyes

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Herrinton *et al*. (1994), Seattle, Utah, Detroit, Atlanta, USA, 1977–1981	360 male and 321 female incident cases of multiple myeloma identified through cancer registries participating in the SEER program in four geographic areas; aged <82 years	933 male and 746 female population controls were matched on age, sex, and race; identified using area sampling methods and random-digit dialing	Interview	**Employed as hairdresser for >6 months** *Men* Never Ever *Women* Never Ever *Duration (Women)* <2 years 2–5 years >5 years	 359 1 309 12 3 5 4	 1.0 1.5 (0.12–17) 1.0 1.3 (0.60–2.7) 1.2 (0.25–5.3) 6.6 (1.2–36) 0.66 (0.21–2.0)	Age, sex, race, study centre, education	Results were similar when only self-responders were included
Mele *et al.* (1994), Rome, Bologna, Pavia, Italy, 1986–1990	619 cases (273 women, 346 men) of newly diagnosed acute myeloid leukemia, acute lympocytic leukemia, chronic myeloid leukemia and RAEB; aged ≥15 years; identified through hematology departments at selected hospitals in Rome, bologna and Pavia	1 161 outpatients from the same hematology departments as the cases; randomly selected; aged ≥15 years	Interview	*Women employed as hairdresser* AML ALL RAEB CML	 [2] [2] [3] [8]	 0.8 (0.1–6.8) 0.9 (0.1–8.0) 3.9 (0.3–45.3) 5.8 (1.3–26.1)	Sex, age, education, residence, selected occupations	Data for males not given.

Table 2.5 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Miligi *et al.* (1999), Italy	1217 female cases of newly diagnosed NHL, HD, leukemia and MM; aged 20–74 years who resided in the areas under study; cases were identified through periodic surveys of the hospital and pathology departments, the archives of Cancer Registries and cancer institutes	861 population controls; random sample of the general population, ages 20–74 residing in each of the areas under study; stratified by sex and 5-year groups	Interview	*Employed as "hairdresser, barber, beautician and related workers" for at least 5 years, excluding the 5 years before diagnosis* NHL Leukemias MM HD	 9 5 3 5	 1.9 (0.7–5.8) 2.2 (0.7–7.1) 11.1 (1.8–67.0) 2.1 (0.7–6.5)	Age	
Costantini *et al.* (2001), Italy, 1991–1993	1520 newly diagnosed male cases of: NHL, 811 HD, 193 Leukaemia, 383 MM, 133; among residents; aged 20–74 years; cases were identified through periodic surveys of the hospital departments.	918 population controls; random sample residents in each of the areas under study; stratified by sex and 5-year age groups.	Interviews	*Employed as "hairdresser, barber, beautician and related workers" for at least 5 years, excluding the 5 years before diagnosis* NHL Leukemias MM	 5 5 5	 0.6 (0.2–1.6) 1.0 (0.3–3.2) 2.2 (0.7–6.9)	Age	

Table 2.5 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
't Mannetje *et al.* (2008), New Zealand, 2003–2004	157 male and 134 female incident cases of NHL; aged 25 to 70 years; notified to the New Zealand Cancer Registry	221 male and 250 female cases; randomly selected from the New Zealand Electoral Roll for 2003, frequency matched by age according to the age distribution of New Zealand cancer registrations for NHL, bladder cancer and leukaemia in 1999	Interview	*Employed as hairdresser, beauty therapist or related activity for ≥1 year* Total Men Women	 4 0 4	 1.09 (0.27–4.35) - 0.94 (0.22–3.98)	Gender, age group, smoking status, Maori ethnicity, occupational status	
Coté *et al*. (1993), USA 1987–1989	2153 dead cases of NHL and AIDS	8612 persons who died of AIDS without NHL frequency matched on sex, age and race	Death certificate	Worked as beautician or cosmetologist	9	0.65 (0.3–1.13)	Sex, age, race	

AIDS, acquired immunodeficiency syndrome; ALL acute lymphocytic leukaemia; AML, acute myeloid leukaemia; CI, confidence interval; CML, chronic myeloid leukaemia; HD, Hodgkin disease; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; OR, odds ratio; RAEB, refractory anaemia with excess blasts

the OR was 1.3 (95% CI, 0.60–2.7). Of these, four cases and 15 controls were employed for longer than five years (OR, 0.66; 95% CI, 0.21–2.0). Only one male case and two male controls had worked as a hairdresser, yielding an OR of 1.5 (95% CI, 0.12–17). Results were similar when the analysis was restricted to self-responders (OR 1.1 for women and 2.2 for men ever employed as a hairdresser).

Mele *et al.* (1994) carried out a hospital based case–control study on leukaemia and pre-leukaemia in three hospitals located in Rome, Bologna and Pavia, Italy, between 1986 and 1990. Cases were 15 years or older, and included 252 patients with acute myeloid leukaemia (AML) (129 men and 123 women), 100 with acute lymphoblastic leukaemia (ALL) (48 men and 52 women), 111 with refractory anaemia with excess of blasts (72 men and 111 women) and 156 with chronic myelogenous leukaemia (CML) (97 men and 59 women). Controls were 1161 patients (399 men and 762 women) identified in outpatient departments on their first visit. ORs were adjusted for sex, age, education, residence and selected occupations. Among female controls, 1.6% had worked as a hairdresser. The percentage among female cases were: 0.8% (OR, 0.8; 95% CI, 0.1–6.8) for AML, 1.9% (OR, 0.9; 95% CI, 0.1–8.0) for ALL, 2.6% (OR, 3.9; 95% CI, 0.3–45.3) for refractory anaemia with excess of blasts, and 4.1% (OR, 5.8; 95% CI, 1.3–26.1).

A population-based case–control study on haemolymphopoietic neoplasms was conducted in 12 areas of Italy. Included were all newly diagnosed cases that occurred between 1991 and 1993 among residents, aged 20–74 years, identified through periodic survey of the relevant hospital departments in the study areas and in regional cancer institutes or university-affiliated haematology departments. Controls were random samples of the general population resident in the study areas, stratified by sex and age. Of 3357 eligible cases and 2391 eligible controls, an interview was obtained for 2737 (82%; 1520 men, 1217 women) cases and 1779 (74%; 918 men and 861 women) controls. Proxy interviews were conducted for 19% of cases and 5% of controls. The diagnosis of cases was NHL for 811 men and 639 women, Hodgkin disease (HD) for 193 men and 172 women, leukaemia for 383 men and 269 women, and multiple myeloma (MM) for 133 men and 137 women. Mantel-Haenszel ORs adjusted for age were computed. Subjects were considered exposed if they had worked as "hairdressers, barbers, beauticians and related workers" for at least five years, excluding the five years before diagnosis. For women, there were nine exposed cases with NHL/CCL (OR, 1.9; 95% CI, 0.7–7.1), five exposed leukaemia cases (OR, 2.2; 95% CI, 0.7–7.1), three exposed MM cases (OR, 11.1; 95% CI, 1.8–67.0) and five exposed HD cases (OR, 2.1; 95% CI, 0.7–6.5) (Miligi *et al.*, 1999; Costantini *et al.*, 2001).

Coté *et al.* (1993) conducted a study to investigate whether beauticians and cosmetologists with AIDS had an increased risk for NHL compared with other persons with AIDS. Cases were 2153 persons who died between 1987 and 1989 in 23 US states and had both AIDS and NHL listed as cause of death. Controls were 8612 subjects selected among persons who died of AIDS without NHL listed as a cause of death, frequency-matched to cases by sex, age and race. For nine (0.42%) cases and 56 (0.65%)

controls lifetime occupation on the death certificate was beautician or cosmetologist, and the OR was 0.65 (95% CI, 0.3–1.13).

(e) Other cancers

(i) *Cancer of the upper aerodigestive tract* (Table 2.6)

Boffetta *et al.* (2003) conducted a multicentre population-based case–control study of cancer in the larynx and hypopharynx in the early 1980s in six high-incidence areas: Calvados in France, the province of Varese and the city of Turin in Italy, the canton of Geneva in Switzerland, and the provinces of Zaragoza and Navarra in Spain. All of these the areas were covered by cancer registration. Cases were 1010 men with histologically-verified incident epidermoid carcinomas of the larynx and hypopharynx (ICD-9 146.4, 146.5, 148, 149.8 and 161). Participation rate was around 75% in Spain and Italy, > 90% in Geneva and lower in Calvados, where recruitment was limited to the main cancer hospital. Controls were 2176 men who were a representative sample from the general population, selected by use of different sources in the different areas, frequency-matched to the age and sex distribution of controls. Participation rate for controls was > 75% in Spain, Varese and Calvados, 64% in Geneva and 56% in Turin. The occupational section of the questionnaire elicited the list of jobs held for at least one year since 1945. Unconditional logistic regression models were used to estimate ORs, which were adjusted for age, study area, and tobacco and alcohol use. Thirteen cases and 18 controls had worked as a barber or hairdresser, and the estimated OR was 2.33 (95% CI, 1.00–5.40). The ORs were 2.7, 2.2 and 2.2 for duration of employment of 1–10, 11–20 or 21 or more years, respectively (*P* for trend, 0.09).

Swanson & Burns (1997) conducted a population-based case–control study in the three-county Detroit area, USA between 1984 and 1991, including 163 (84 men and 79 women) incident salivary gland cancer cases aged 40–84 years, and 3751 controls (1807 men and 1944 women) who were population referents selected by random-digit dialling. Response rates were 96% and 97%, respectively, and proxy interviews were conducted for 23% of the cases and 8% of the controls. ORs were estimated with multiple logistic regression models, adjusted by age, race and smoking status. Exposed subjects were those ever employed as a hairdresser or in a beauty shop, and were compared with those only employed in occupations with little or no exposure to carcinogens. For men, too few cases were exposed to calculate ORs, while for women, seven cases and 56 controls had ever worked as a hairdresser (OR, 2.7; 95% CI, 1.1–6.5) and eight women and 61 controls had worked in a beauty shop (OR, 3.4; 95% CI, 1.4–7.9).

(ii) *Lung*

Two studies reported results on the association between occupational exposure to hair dyes and lung cancer (Schoenberg *et al.*, 1987; Jahn *et al.*, 1999). Both had small numbers of exposed cases and exposed controls and were considered uninformative.

Table 2.6. Case-control studies of cancer of the upper aerodigestive tract and occupational exposure to hair dyes

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Swanson & Burns (1997), Detroit, USA, 1984–1991	84 male and 79 female cases of salivary gland cancer, identified through the SEER program	1807 male and 1944 female population controls selected via RDD	Telephone interview	Ever employed as hairdresser	7	2.7 (1.1–6.5)	Age, race, smoking.	For males too few exposed subjects to compute ORs. Reference category: subjects only employed in occupations with little or no exposure to carcinogens.
				Ever worked in a beauty shop	8	3.4 (1.4–7.9)		
Boffetta *et al.* (2003), France, Italy, Switzerland and Spain, 1980–1983	1010 males with histologically verified incident epidermoid carcinomas of the larynx and hypopharynx	2176 male population controls selected from census lists, electoral rolls, or population registries	Interviews	Employed as barber/hairdresser for 1+ years since 1945	13	2.33 (1.00–5.40)	Age, study area, alcohol, tobacco	95% CI not reported
				Duration of employment				
				1–10 years	5	2.7		
				11–20 years	2	2.2		
				≥21 years	6	2.2		
						p trend=0.09		

CI, confidence interval; OR, odds ratio; RDD, random-digit dialling; SEER, Surveillance Epidemiology and End Results

2.2 Personal use of hair colourants

2.2.1 *Cohort studies* (see Table 2.7 for studies of urinary bladder, breast and haematopoietic cancers)

Hennekens *et al.* (1979) carried out a cross-sectional postal questionnaire survey in 1976 on 172 413 married female nurses, aged 30–55, in 11 US states, whose names appeared in the 1972 register of the American Nurses' Association. Of the 120 557 responders, 38 459 reported some use of permanent hair dyes; of these, 773 had been diagnosed as having a cancer. The risk ratio for the association of cancers at all sites with hair-dye use (at any time) was 1.10 ($P = 0.02$). When 16 cancer sites were examined separately, significant associations with permanent hair-dye use were found for cancer of the *cervix uteri* (RR, 1.44; $P < 0.001$) and for cancer of the vagina and vulva (RR, 2.58; $P = 0.02$). These associations weakened but remained significant after adjustment for smoking habits. There was no consistent trend of cancer risk with increasing interval from first use of hair dyes, although women who had used permanent dyes during 21 years or more before the onset of cancer had a significant increase in risk for cancers at all sites combined (RR, 1.38 adjusted for smoking; $P = 0.02$), largely because of an excess of breast cancers (RR, 1.48), which, however, was balanced by a decrease of similar magnitude 16–20 years before the onset of cancer. Among the other cancer sites evaluated, no significant associations were found that were related to personal use of hair dyes and lymphoma (RR, 0.59; $P = 0.102$) or urinary cancer (RR, 0.62; $P = 0.296$). Analyses of cases of cancer that had occurred only after 1972 (the year the study population was defined from the nurses' register) and were reported by surviving cases in 1976 yielded essentially the same results, thus indicating that self-selection for the study, early retirement and loss from the professional register were not sources of bias in this study.

Green *et al.* (1987) examined hair-dye use in relation to breast cancer in a follow-up study of a subgroup of the population described above, comprising 118 404 nurses who had no cancer in 1976 and were followed up to 1982. No relationship was detected: the rate ratio for ever-use was 1.1 (95% CI, 0.9–1.2) on the basis of 353 cases, compared with 505 for never-use. The risk for breast cancer did not increase with frequency or duration of use.

Another prospective cohort study from the Nurse's Health Study (Grodstein *et al.*, 1994) examined the relationship between permanent hair-dye use and risks for incident lymphoma, leukaemia, and multiple myeloma. A total of 99 067 women aged 30–55 years were followed through 1990. After eight years of follow-up, 244 incident haematopoietic cancers were identified, including 24 cases of Hodgkin lymphoma, 144 of non-Hodgkin lymphoma, 44 of leukaemia and 32 of multiple myeloma. No positive associations were observed between ever-use of permanent hair dyes and risk for all haematopoietic cancers (OR, 0.9; 95% CI, 0.7–1.2) or specific types, including Hodgkin lymphoma (OR, 0.9: 95% CI, 0.4–2.1), non-Hodgkin lymphoma (OR, 1.1; 95% CI, 0.8–1.6), multiple myeloma (OR, 0.4; 95% CI, 0.2–0.9), chronic lymphocytic leukaemia

Table 2.7. Cohort studies of personal use of hair dye

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)	Adjustment factors	Comments
Hennekens *et al*. (1979) USA 1972–76	120 557 active female nurses, aged 30–55 yrs; living in 11 U.S. states and registered in the American Nurses' Association	Self-administered questionnaire	*Breast cancer*			Age at first hair dye use, total number of years used, smoking	30% non-respondents
			Never used	861	1.0		
			1–5 years	102	1.10		
			6–10 years	79	1.02		
			11–15 years	49	1.05		
			16–20 years	16	0.64		
			>21 years	24	1.48		
			Bladder cancer				
			Never used	32	1.0		
			1–5 years	2	0.8		
			6–10 years	1	0.43		
			11–15 years	1	0.67		
			16–20 years	1	1.43		
			>21 years	0	0		
			Lymphoma				
			Never used	22	1.0		
			1–5 years	1	0.18		
			6–10 years	2	0.38		
			11–15 years	6	2.07		
			16–20 years	0	0		
			>21 years	1	1.67		

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)	Adjustment factors	Comments
Green *et al*. (1987) USA 1976–82	118 404 active female nurses, aged 30–55 yrs; living in 11 U.S. states and registered in the American Nurses' Association; followed prospectively for 6 years	Self-administered questionnaire	*Breast cancer* Never used Ever used 1–5 years 6–10 years 11–15 years 16–20 years >21 years	 505 353 98 66 66 49 40	 1.0 1.1 (0.9–1.2) 1.3 (1.0–1.6) 0.9 (0.7–1.2) 1.0 (0.8–1.3) 1.2 (0.9–1.6) 1.2 (0.9–1.6)	Age, smoking, age at first birth, maternal history of breast cancer, menopausal status, benign breast disease	Nurses enrolled in this study are from the same cohort as Hennekens *et al*. (1979)
Thun *et al*. (1994) USA 1982–89	573 369 women aged 30 and older enrolled from the ACS Cancer Prevention Study II (CPS-II); follow-up through 1989	Self-administered questionnaire	*Breast cancer* Never used Ever used 1–9 years 10–19 years ≥20 years *Bladder cancer* Never used Ever used 1–9 years 10–19 years ≥20 years	 	 1.0 1.0 (0.8–1.1) 0.9 (0.8–1.1) 0.9 (0.8–1.1) 0.9 (0.7–1.2) 1.0 0.6 (0.3–1.0) 0.8 (0.4–1.7) 0.5 (0.2–1.5) 0.3 (0.1–1.1)	Age, hair dye colour	Assessed risk based on very few exposed cases

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)	Adjustment factors	Comments
Thun *et al*. (1994) (contd)			*Lymphoma*				
			Never used	263	1.0		
			Ever used	87	1.0 (0.7–1.2)		
			1–9 years	28	0.8 (0.5–1.3)		
			10–19 years	35	1.1 (0.8–1.6)		
			≥20 years	24	1.0 (0.7–1.5)		
			Urinary system				
			Never used		1.0		
			Ever used		0.7 (0.5–0.9)		
			1–9 years		0.9 (0.6–1.3)		
			10–19 years		0.6 (0.4–0.9)		
			≥20 years		0.5 (0.3–0.9)		
			Multiple myeloma				
			Never used	144	1.0		
			Ever used	51	1.1 (0.8–1.5)		
			1–9 years	18	0.9 (0.5–1.5)		
			10–19 years	15	1.0 (0.6–1.9)		
			≥20 years	18	1.4 (0.9–2.3)		
			All hematopoietic cancers				
			Never used	714	1.0		
			Ever used	227	0.9 (0.8–1.1)		
			1–9 years	81	0.9 (0.7–1.2)		
			10–19 years	88	1.0 (0.8–1.3)		
			>20 years	58	0.9 (0.7–1.2)		

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)	Adjustment factors	Comments
Grodstein *et al*. (1994) USA 1976–90	99 067 active female nurses, aged 30–55 yrs; living in 11 U.S. states and registered in the American Nurses' Association	Self-administered questionnaire	*All cancers*				Same cohort as Hennekens *et al*. (1979)
			Never used	140	1.0		
			Ever used	104	0.9 (0.7–1.2)		
			Hodgkin lymphoma				
			Never used	14	1.0		
			Ever used	10	0.9 (0.4–2.1)		
			Non-Hodgkin lymphoma				
			Never used	74	1.0		
			Ever used	70	1.1 (0.8–1.6)		
			CLL				
			Never used	15	1.0		
			Ever used	8	0.6 (0.3–1.5)		
			Multiple myeloma				
			Never used	24	1.0		
			Ever used	8	0.4 (0.2–0.9)		

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Altekruse *et al*. (1999) USA 1982–94	547 586 women enrolled in the ACS Cancer Prevention Study II (CPS II)	Self-administered questionnaire	*All cancers*			Age, race, cigarettes smoked per day, age at quitting smoking, education and blue collar occupation, BMI, reproductive factors, dietary factors, alcohol consumption, exercise, aspirin use, X-ray exposure, treatment with radium, coal tar/pitch/asphalt, diesel engine exhaust, dyes, gasoline exhaust, pesticides/herbicides, textiles fibres/dust, wood dust, coal or stone dust, X-rays/radioactive isotopes	
			Never used	13 420	1.0		
			Ever used	5179	0.9 (0.9–1.0)		
			Breast				
			Never used	1894	1.0		
			Ever used	782	0.9 (0.9–1.0)		
			Bladder				
			Never used	154	1.0		
			Ever used	48	1.0 (0.7–1.4)		
			All hematopoietic cancers				
			Never used	1417	1.0		
			Ever used	574	1.1 (1.0–1.2)		
			All leukemias				
			Never used	511	1.0		
			Ever used	207	1.1 (0.9–1.3)		
			Non-Hodgkin lymphoma				
			Never used	536	1.0		
			Ever used	227	1.1 (1.0–1.3)		
			Multiple myeloma				
			Never used	329	1.0		
			Ever used	131	1.0 (0.8–1.3)		

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Henley & Thun (2001)	547 571 women enrolled in the ACS Cancer Prevention Study II (CPS II)	Self-administered questionnaire	**Bladder cancer** *Total cohort* Never used Ever used *Never smokers* Never used Ever used	 244 92 128 28	 1.0 1.1 (0.8–1.4) 1.0 0.9 (0.6–1.4)		

ACS, American Cancer Society; BMI, body mass index; CLL, chronic lymphocytic leukemia

(RR, 0.6; 95% CI, 0.3–1.5), and other leukemias (RR, 0.8; 95% CI, 0.3–1.9). In the absence of information on non-permanent hair-dye use and without repeated assessment of exposure in the follow-up period may introduce misclassification of exposure, but the exposure misclassification is more likely to be non-differential and drives the association towards the null, because the exposure assessment was done before disease onset.

Thun *et al.* (1994) examined prospectively the relationship between hair-dye use and the development of fatal cancer in 573 369 women. Women who had ever used permanent hair dyes showed a reduced risk for all fatal cancer combined (RR, 0.9; 95% CI, 0.9–1.0) and of urinary system cancers (RR, 0.7; 95% CI, 0.5–0.9), and no increase in risk for any type of haematopoietic cancer. Women who had used black hair dyes for 20 years or more had an increased risk for fatal non-Hodgkin lymphoma (RR, 4.4; 95% CI, 1.3–15.2) and multiple myeloma (RR, 4.4; 95% CI, 1.1–18.3); however, these results are based on a very few exposed cases. No relationship was found between the use of permanent hair dyes and fatal cancers of the mouth, breast, lung, bladder or cervix.

Altekruse *et al.* (1999) examined cancer deaths linked to use of permanent hair dye in a cohort of 547 586 women in the USA. Exposure was assessed at the start of the follow-up period. A small increase in risk for death from haematopoietic cancers (RR, 1.1; 95% CI, 1.0–1.2) and leukemias was observed in association with more than 20 years of use (RR, 1.3; 95% CI, 1.0–1.7). However, patterns by duration of use of dyes and type of colour were inconsistent.

Henley & Thun (2001) analysed the mortality associated with hair-dye use based on a sample of 547 571 women from the ACS Cancer Prevention Study (CPS II). After 16 years of follow-up, the death rate from bladder cancer was similar among women who reported ever using permanent hair dye to that of never-users (RR, 1.1; 95% CI, 0.8–1.4) and restricted to lifelong nonsmokers (RR, 0.9; 95% CI, 0.6–1.4). Among women who had used permanent hair dyes, no consistent increase was seen in either the death rate from bladder cancer or the rate ratio associated with hair-dye use.

2.2.2 *Case–control studies* (see Tables 2.8–2.11)

The Working Group systematically reviewed studies dealing with exposures of cases of cancer of the urinary bladder and breast (sites that have been studied extensively), lymphatic and haematopoietic neoplasms and childhood cancer. No systematic review was made of studies of other cancer sites.

(a) Cancers of the urinary bladder and renal pelvis (Table 2.8)

Lockwood (1961) performed a case–control study of bladder tumours in Copenhagen, Denmark. All patients diagnosed with bladder tumours from 1942 until 1 March 1956 and able to be interviewed in 1956–57 were eligible for inclusion. Of the 428 patients, 369 (282 men) were interviewed, together with 369 population controls (282 men) selected from the electoral rolls and matched for sex, age, marital status, occupation and residence, and interviewed in 1956–59. Later in the study, a question on use of brilliantine

Table 2.8. Case–control studies of urinary bladder and renal pelvis cancer and personal hair dye use

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Lockwood (1961) Copenhagen, Denmark 1942–56	369 patients with bladder tumors reported to the Danish Cancer Registry between 1942 and 1956 who were residents of Kobenhavn and the Borough of Frederiksberg; follow-up until June 1959	369 population-based controls were selected from election rolls and matched on age, sex, marital status, occupation and residence	Interview	**Daily brilliantine use** *Men* Yes *Women* Yes	 51 2	 1.7 (1.1–2.6) 1.1 (0.2–6.6)	Matched on sex, age, marital status, occupation, residence	
Dunham *et al*. (1968) New Orleans, USA 1958–64	487 patients with a bladder cancer identified between 1958 and 1964; cases selected through physician and hospital records and through the Tumor Registry. Office of Vital Statistics	527 control patients diagnosed with conditions unrelated to genitor-urinary tract or to neoplastic disease; controls selected from the same hospitals as the patients	Interview	*Preparations for hair and scalp* Not used Used	 87 42	 1.0 [0.9]	Not reported	

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Howe *et al.* (1980) Canada 1974–76	480 male and 152 female patients diagnosed with bladder cancer in 3 provinces between 1974 and 1976	480 male and 152 female neighbourhood controls matched on age (± 5 years) and sex	Interview	*Use of hair dyes* *Males* No Yes Females No Yes	 472 8 136 16	 1.0 Not calculated 1.0 0.7 (0.3–1.4)	History of bladder and kidney conditions,analgesic use, cigarette smoking, other tobacco products, exposure to a priori suspect industries, exposure to dust and fumes in occupations other than those expected a priori, exposure to specific chemicals, age, coffee and other beverage use, nitrate and nitrite sources in the diet, fiddlehead greens, diabetes, education, use of non-public water supply	Hair dye use not included in the analysis of male patients due to no exposed male controls

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Hartge *et al.* (1982) USA 1977–78	2982 incident cases of histologically confirmed bladder cancer among residents living in 10 geographic areas within the USA; aged 21–84; cases identified through cancer registries	5782 randomly selected population controls stratified by age, sex and geographical area; random digit dialing (RDD) used to select controls; aged 21–64	Interview	**History of hair dye use**			Age, sex, race and cigarette smoking	No trend with frequency or duration in either sex
				Males				
				Never dyed hair	2065	1.0		
				Ever dyed hair	172	1.1 (0.9–1.4)		
				Unknown	12			
				Females				
				Never dyed hair	288	1.0		
				Ever dyed hair	443	0.9 (0.8–1.1)		
				Unknown	2			
				Duration of hair dye use				
				Males				
				Never dyed hair	2065	1.0		
				<5 years	115	1.4		
				5–10 years	27	0.8		
				10–19 years	23	1.1		
				≥20 years	6	0.7		
				Unknown	13	NR		
				Females				
				Never dyed hair	288	1.0		
				<5 years	109	1.1		
				5–10 years	68	0.9		
				10–19 years	149	1.1		
				≥20 years	104	0.8		
				Unknown	15	NR		

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Ohno *et al.* (1985) Nagoya, Japan 1976–78	Male and female cases of urinary cancer selected through the Nagoya Bladder Tumor Registry; aged >20 years; residents of metropolitan Nagoya	Controls randomly selected from the general population through electoral registries; aged 20 and older; frequency matched on age, sex and residence	Interview	**Frequency of hair dye use** *Non smokers* Not tinted <1 time/month ≥1 time/month *Smokers* Not tinted <1 time/month ≥1 time/month	 21 12 12 2 8 10	 1.0 0.84 (0.37–1.92) 0.97 (0.42–2.23) 1.0 10.0 (1.56–63.97) 25.0 (3.48–179.9)		Analysis for men not conducted. Only crude RR presented.
Claude *et al.* (1986) Northern Germany 1977–82	431 cases admitted to 3 hospitals in northern Germany; cases with an initial diagnosis of bladder cancer were included in the study	431 hospital controls matched on age (± 5 years) and sex	Interview	Personal hair dye use		Not reported	Not reported	No association was observed with urinary tract tumours for use of hair dyes.

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Nomura *et al*. (1989) Hawaii, USA 1977–86	261 patients diagnosed with urinary tract cancer from 7 hospitals on the island of Oahu; aged 30–93	522 population controls 2:1 matched on age (± 5 years), sex, race and residence; controls selected through the Hawaii State Department of Health's surveillance program	Interview	**Hair dye use** *Male* Nonuser User 1–5 years 6+ years *p* trend *Female* Nonuser User 1–5 years 6+ years *p* trend	 180 15 12 3 25 41 15 26 	 1.0 1.3 (0.6–2.8) 2.1 (0.9–4.8) 0.7 (0.2–2.4) 1.00 1.0 1.5 (0.8–2.9) 2.4 (1.0–6.0) 1.2 (0.6–2.4) 0.64	Pack-years of cigarette smoking	No trend with frequency or duration for either sex

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Gago-Dominguez *et al*. (2001a) California, USA 1987–1996	897 incident cases histologically confirmed non-Asian cases of bladder cancer; aged 25–64 years; cases identified through the Los Angeles County Cancer Surveillance Program (SEER Los Angeles County) between 1987–1996	1:1 matched 897 neighborhood controls for each interviewed case; controls matched on date of birth (within 5 years), sex, ethnicity, place of residence at the time of diagnosis	Interview	**Hair dye use** *Male* Regular use No Yes Duration of use (years) Non-users <15 15–<30 30+ *p* for trend *Female* Regular use No Yes Duration of use (years) Non-users <15 15–<30 30+ *p* for trend	 655 39 670 9 2 2 79 124 105 22 38 22 	 1.0 0.8 (0.5–1.3) 1.0 0.7 (0.2–1.9) 1.1 (0.2–7.1) - 0.99 1.0 1.3 (0.8–2.2) 1.0 1.1 (0.5–2.5) 1.7 (0.8–3.6) 3.7 (1.2–11.2) 0.01	Age, sex, ethnicity, neighborhood, smoking status, number of cigarettes smoked per day, number of years smoking	For exclusive use of permanent hair dyes in women: OR=1.8 (1.01–3.3) with dose response relationship.

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Gago-Dominguez *et al*. (2001b) California, USA 1987–1996	203 cases of bladder cancer from Los Angeles County; NAT2 analysis on 124 cases and 122 controls	203 matched controls		**Hair dye use** *NAT2 Slow* Non-users Exclusive use* Number of times per year <12 12+ *p* for trend Number of years of use <15 15+ *p* for trend *NAT2 Fast* Non-users Exclusive use* Number of times per year <12 12+ *p* for trend Number of years of use <15 15+ *p* for trend	 31 27 15 12 6 21 20 20 10 10 4 16 	 1.0 2.7 (1.01–7.2) 2.1 (0.7–6.5) 4.3 (0.9–20.0) 0.04 1.1 (0.3–4.7) 4.2 (1.3–14.1) 0.02 1.0 1.1 (0.4–2.7) 0.8 (0.3–2.4) 1.3 (0.4–4.5) 0.79 0.3 (0.07–1.3) 1.7 (0.6–5.0) 0.42	Age, ethnicity, current smoking status, number of cigarettes smoked per day and number of years smoking	*, exclusive users of permanent hair dyes

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Gago-Dominguez *et al*. (2003) California 1992–1996	363 non-Asian women; aged 25–64 with histologically confirmed cases of bladder cancer; cases identified through the Los Angeles County Cancer Surveillance Program (SEER Los Angeles County) between 1992–1996	1:1 matched neighborhood controls; controls matched on sex, date of birth (within 5 years), ethnicity and neighborhood	Interview	**Hair dye use** *NAT2 Rapid* Non-users Exclusive use* Number of times per year <12 12+ *p* for trend Number of years of use <15 15+ *p* for trend *NAT2 Slow* Non-users Exclusive use* Number of times per year <12 12+ *p* for trend Number of years of use <15 15+ *p* for trend	 28 32 15 17 7 25 36 33 18 15 7 26 	 1.0 1.3 (0.6–2.8) 1.0 (0.4–2.6) 1.6 (0.6–4.6) 0.39 0.5 (0.2–1.7) 2.0 (0.8–5.0) 0.20 1.0 2.9 (1.2–7.5) 2.2 (0.8–6.5) 5.3 (1.2–23.2) 0.02 1.1 (0.3–4.5) 4.9 (1.6–15.4) 0.008	Age, ethnicity, smoking variables	*, exclusive use of permanent hair dyes. Sub-analysis by *NAT1* genotype among non-smokers, exclusive use of permanent hair dyes: NAT1*10, 1.0 (0.2–4.3); non-NAT1*10, 6.8 (1.7–27.4)

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Gago-Dominguez *et al*. (2003) California 1992–1996 (contd)				*CYP1A2 Rapid*			Age and ethnicity, current smoking status (Yes/No), number of cigarettes smoked per day, number of years of smoking	
				Non-users	36	1.0		
				Exclusive use*	26	1.2 (0.6–2.7)		
				Number of times per year				
				<12	10	1.2 (0.4–3.5)		
				12+	16	1.2 (0.5–3.4)		
				p for trend		0.63		
				Number of years of use				
				<15	6	0.4 (0.1–1.3)		
				15+	20	2.3 (0.9–6.2)		
				p for trend		0.19		
				CYP1A2 Slow				
				Non-users	27	1.0		
				Exclusive use*	37	2.5 (1.04–6.1)		
				Number of times per year				
				<12	21	1.6 (0.6–4.2)		
				12+	16	7.6 (1.5–39.4)		
				p for trend		0.01		
				Number of years of use				
				<15	7	0.8 (0.2–2.8)		
				15+	30	4.4 (1.5–13.0)		
				p for trend		0.01		

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Andrew *et al*. (2004) New Hampshire, USA 1994–1998	459 cases of bladder cancer diagnosed among residents of New Hampshire; ages 25–74; from 1994–1998	Control subjects from a study on non-melanoma skin cancer; controls frequency matched on age and gender; all controls less than 65 years were selected by using population lists obtained from the New Hampshire Department of Transportation; controls 65 year of age and older were chosen from data files provided by the Centers for Medicare and Medicaid Services (CMS) of New Hampshire	Interview	**Hair dye use** *Men* Any dye use No Yes *Women* Any dye use No Yes **Duration of use** *All Dyes* Nonuser 1–11 years >11 years *p* trend *Permanent dyes* Nonuser 1–11 years >11 years *p* trend	 332 19 29 69 29 43 26 0.17 57 16 16 0.72	 1.0 0.5 (0.3–0.8) 1.0 1.1 (0.6–1.9) 1.0 1.4 (0.8–2.5) 1.0 (0.5–1.9) 1.0 1.7 (0.8–3.7) 1.5 (0.7–3.2)	Age, smoking, education	

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Kelsey *et al*. (2005) New Hampshire, USA 1994–1998	330 cases of bladder cancer identified through the New Hampshire State Cancer Registry between 1994–1998; aged 25–74;	Controls were selected from the population	Pathological and cytological samples; personal interview	**Hair dye use** *TP53 mutation* Never Ever *TP53 immunohisto-chemistry (IHC) inactivation* Never Ever *TP53 mutation/IHC inactivation* Never Ever	 282 73 282 73 282 73	 1.0 1.4 (0.4–4.4) 1.0 3.2 (1.4–7.2) 1.0 4.1 (1.0–17.0)	Age, sex, stage	TP53 mutation negative served as the "control" group in the analysis

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Lin *et al*. (2006) Texas, USA 1999–2001	712 incident cases of bladder cancer identified through the University of Texas MD. Anderson Cancer Center and Baylor College of Medicine	Controls were recruited in collaboration with the Kelsey-Seybold clinics; controls were frequency matched to cases by age (±5 years), gender, and ethnicity; controls have no prior history of cancer (except non-melanoma skin cancer)	Interview	**Regular hair dye use** *Overall* Non users Permanent *Men* Nonusers Regular users *Women* Nonusers Regular users **Duration of use (years)** *Women* <15 15–29 ≥30 *Lifetime use (total number of times)* <100 100–200 ≥200 *Frequency of use (number of times per year)* <6 6–11 ≥12	 523 100 489 59 34 127 33 18 26 40 20 17 26 6 18	 1.0 0.81 (0.50–1.30) 1.0 0.71 (0.47–1.07) 1.0 1.0 (0.54–1.85) 0.75 (0.26–2.12) 1.31 (0.43–3.98) 0.86 (0.34–2.17) 0.78 (0.29–2.14) 1.56 (0.54–4.48) 0.66 (0.23–1.88) 0.26 (0.03–2.18) 0.76 (0.07–8.59) 8.88 (0.65–121.42)	Age, gender, ethnicity, smoking status	Duration, lifetime and frequency of use not analysed separately in men

Table 2.8 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	Relative risk (95% CI)	Adjustment factors	Comments
Kogevinas *et al*. (2006) Spain 1998–2001	128 newly diagnosed and histologically confirmed cases of bladder cancer from 18 hospitals in 5 areas of Spain between 1998–2001	131 patients hospitalized with diagnoses believed to be unrelated to the exposures of interest; 1:1 matched on age (±5 yrs), gender, ethnicity and study hospital; controls included subjects who were mainly hospitalized for trauma or minor surgery	Computer-aided personal interviews	*Hair dye use* Never used hair dyes Used hair dyes at least 10 times Only permanent hair dye use at least 10 times Only dark colour use Self applying without gloves *Duration of use* Never users <10 years 11–24 years 25–32 years >32 years *NAT2 genotype* slow fast	 42 78 60 23 4 42 15 20 15 20 32 24	 1.0 0.8 (0.5–1.4) 0.8 (0.5–1.5) 1.1 (0.5–2.5) 1.2 (0.2–6.1) 1.0 0.4 (0.2–0.9) 1.0 (0.4–2.4) 0.5 (0.2–1.2) 1.2 (0.5–2.7) 0.6 (0.3–1.4) 0.9 (0.3–2.6)	Age, region, smoking, socio-economic status	Sub-analysis by *NAT1* genotype: NAT1*10, 2.9 (0.7–11.6); non-NAT1*10, 0.6 (0.2–1.6)

CI, confidence interval; OR, odds ratio; RR, relative risk

was added, and this question was answered by 51% of the male and female patients and by 93% of male and 80% of female controls. The crude OR for brilliantine use, relative to those reporting no use, was [1.7] for men (51 exposed patients; 95% CI, 1.1–2.6) and [1.1] for women (two exposed patients; 95% CI, 0.2–6.6).

Jain *et al.* (1977) reported (in a letter) data on hair-dye use among 107 patients with bladder cancer and an equal number of sex- and age-matched controls in Canada. All male controls had benign prostatic hypertrophy, and all female controls had stress incontinence. The OR for bladder cancer in association with any exposure to hair dyes (based on 19 pairs discordant for use of hair dye) was 1.1 (95% CI, 0.41–3.03). [The Working Group noted that the choice of controls was unusually limited and may have introduced a detection bias.]

Neutel *et al.* (1978) reported (in a letter) data on hair-dye use in a subset of 50 case–control pairs (matched by sex and 10-year age group) re-interviewed after a previous, larger case–control study of bladder cancer in Canada. Use of hair dyes was reported by 18 cases and 19 controls. Frequent use of hair dyes and hairdressing as an occupation, however, were said to show protective effects (the former being significant, $P < 0.01$) against bladder cancer, although the numbers on which these statements were based are not given in the report.

In a study that examined bladder cancer and occupational exposure of hairdressers and barbers, Howe *et al.* (1980) found that eight male cases (including two of the barbers) and no male control had a history of personal use of hair dyes ($P = 0.004$, one-tailed test); only one of them had used hair dyes for more than six years before diagnosis of bladder cancer. There was no evidence in women of an increased risk for bladder cancer associated with personal use of hair dyes (OR, 0.7; 95% CI, 0.3–1.4 for ever-use *vs* never-use).

Hartge *et al.* (1982) examined hair-dye use among participants in the US National Bladder Cancer Study in a case–control study of bladder cancer involving 2982 incident cases and 5782 controls, of whom 615 cases and 1164 controls had ever dyed their hair. The overall ORs for hair-dye users were 1.1 (95% CI, 0.9–1.4) among men and 0.9 (0.8–1.1) among women. ORs by frequency or duration of use ranged for both sexes from 1.0 to 1.7, with no clear relationship with increasing duration or frequency among people of either sex. Use of black hair dye was associated with elevated ORs in both men and women; the OR was of borderline significance for the two sexes combined (OR, 1.4; 95% CI, 1.0–1.9; 68 exposed cases).

Ohno *et al.* (1985) conducted a case–control study of 65 female bladder-cancer patients in Nagoya, Japan, in the period 1976–78. Hair-dye use was associated with an increased RR among those who smoked but not among non-smokers (RR, 1.31; 95% CI, 0.64–2.71). There was a positive relationship between smoking and hair-dye use more than once a month (RR, 25; 95% CI, 3.48–179.86); after adjustment for smoking, no significant effect of hair dyes remained (RR, 1.7; 95% CI; 0.82–3.52; 22 exposed cases).

A matched case–control study was carried out by Claude *et al.* (1986) of 340 men and 91 women with bladder cancer in Lower Saxony, Germany, in the period 1977–82. It was stated that no association with hair-dye use was found, but details were not provided.

Nomura *et al.* (1989) carried out a case–control study among 137 Caucasian and 124 Japanese cases of cancer of the lower urinary tract in Hawaii (USA) and two population-based controls for each case, in the period 1977–86. A weak, non significant association with hair-dye use was found for both men and women, but there was no positive trend with increasing duration of use.

Gago-Dominguez *et al.* (2001a) studied 897 incident cases of bladder cancer and 897 matched controls in a population-based case–control study conducted in Los Angeles between 1987 and 1996. In a cigarette-smoking-adjusted model based on 82 exposed female cases and 56 exposed female controls, exclusive use of permanent hair dyes was related to an increased risk for bladder cancer (OR, 1.9; 95% CI, 1.01–3.3). The longest female users of permanent hair dyes (i.e. > 30 years) had a 4-fold increase in the risk for bladder cancer. Men exposed to hair dyes were not at an increased risk for bladder cancer, although the estimates were based on small numbers. Based on a subset of the population, Gago-Dominguez *et al.* (2001b) found a higher bladder-cancer risk among NAT-2 slow acetylators than among fast NAT-2 acetylators (OR, 2.7; 95% CI, 1.01–7.2 and OR, 1.1; 95% CI, 0.4–2.7, respectively). In an extension of the study, Gago-Dominguez *et al.* (2003) confirmed this association and also found indications of an interaction between CYP1A2 slow metabolizers and permanent hair-dye use in relation to bladder-cancer risk.

In a population-based case control study conducted in New Hampshire in 1994–1998 that included 459 incident bladder cancer cases and 665 controls, Andrew *et al.* (2004) found no overall indication of an increased bladder-cancer risk among hair-dye users, after adjustment for age, smoking and education level. However, women exposed to permanent hair dyes who had started their use before age 37 were at a 2.3-fold increased risk for bladder cancer (95% CI, 1.1–4.6), based on 22 exposed cases and 32 exposed controls. Women with greater frequency of use and prolonged time since first use of permanent hair dyes were also at an increased risk for bladder cancer. Subjects exposed for less than 11 years to permanent dyes had a 1.7-fold excess in risk (95% CI, 0.8–3.7), and among those exposed for more than 11 years the risk was 1.5 (95% CI, 0.7–3.2, *P*-value for linear trend, 0.72). In an extension of the New Hampshire study, Kelsey *et al.* (2005) found that among bladder-cancer cases, hair-dye use was significantly associated to TP53 immunohisto-chemistry inactivation (OR, 3.2; 95% CI, 1.4–7.2), but not to *TP53* mutation (OR, 1.4; 95% CI, 0.4–4.4), based on 282 cases who were never-users of hair dyes and 73 cases who had ever used hair dyes.

In a case–control study conducted in Texas among 712 incident bladder-cancer cases and 712 hospital-based controls, Lin *et al.* (2006) found no indication of an increased risk for bladder cancer among users of permanent hair dyes after adjustment for age, sex, race and smoking, based on 100 exposed cases and 115 exposed controls (OR, 0.81; 95% CI, 0.50–1.30). No increased risks were found for other types of hair dyes or when

duration variables were assessed. Restriction of the analyses to women only also showed no significant increase in risks.

In a case–control study conducted in Spain between 1998 and 2001 among 152 female bladder-cancer cases and 166 female controls, Kogevinas *et al.* (2006) found no overall or specific indication of an elevated risk for bladder cancer among hair-dye users (OR, 0.8; 95% CI, 0.5–1.4), based on 78 exposed cases after adjustment for age, region and smoking. No increase in risk was observed for only permanent users for at least 10 times (OR, 0.8; 95% CI, 0.5–1.5), among users of only dark colour (OR, 1.1; 95% CI, 0.5–2.5), or for self-applying of the dyes with no gloves (95% CI, 0.2–6.1). Users for more than 32 years showed a non-significantly increased risk of 1.2 (95% CI, 0.5–2.7). ORs were below 1.0 for high cumulative exposure. There was no indication of an increased risk for bladder cancer associated with the *NAT2* genotype.

Takkouche *et al.* (2005) published a meta-analysis evaluating the association between use of hair dyes and risk for cancer. This meta-analysis of 10 studies (9 case–control and 1 cohort) reached an overall risk estimate for bladder cancer of 1.01 (95% CI, 0.89–1.14). The pooled OR for permanent dye use was 1.13 (95% CI, 0.93–1.38) and 1.0 (95% CI, 0.82–1.22) for intensive exposure using fixed effects models. For random effects models the estimates were 1.13 (95% CI, 0.93–1.38) and 1.33 (95% CI, 0.69–2.56), respectively.

(b) Breast cancer (Table 2.9)

Shafer & Shafer (1976) reported on 100 consecutive breast-cancer patients in a clinical practice in New York, USA, 87% of whom had been long-term users of hair-colouring agents, and a group of age-comparable controls, 26% of whom were regular users of permanent hair dyes over prolonged periods. [The Working Group noted the dissimilarity of the exposure definitions for the two groups and the lack of information on the number of controls or the manner of eliciting details on the use of hair dyes.]

Kinlen *et al.* (1977) reported a study of 191 breast-cancer patients interviewed in the hospital in 1975 and 1976 in Oxford, United Kingdom, and 561 controls without cancer, matched to the patients by age (within three years), marital status and social class. Seventy-three cases and 213 controls had used permanent or semi-permanent hair dyes, giving an OR of 1.01. There was no evidence of an increasing risk for breast cancer with increasing duration of use of hair dyes or with use beginning more than four or more than nine years before diagnosis. Stratification by age at first pregnancy showed a deficit of cases in which hair-dye use was reported among women whose first pregnancy occurred at ages 15–19 (33.3% of cases used hair dyes, compared with 64.7% of controls) and an excess of cases with use of hair dyes among women whose first pregnancy had occurred at 30 years of age or older (38.3% of cases and 25.5% of controls). There were two hairdressers among cases (1.0%) and 10 among controls (1.8%).

Shore *et al.* (1979) compared the hair-dye use of 129 breast-cancer patients and 193 control subjects aged 25 and over, identified from the records of a multiphasic screening clinic in New York City, USA. Adjusted ORs for use of permanent hair dyes

Table 2.9. Case–control studies of breast cancer and personal hair dye use

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Kinlen *et al.* (1977) Oxford, UK 1975–76	191 women with breast cancer; interviewed in hospital between 1975–1976	561 matched controls; 506 inpatients/outpatients from the same hospitals and did not have malignant disease as the cases and 55 women selected from an age-sex register in a general practice and interviewed at home; matched on age (within three years), marital status, and social class	Interview	**Anytime before diagnosis** *Length of use* <1 year 1–4 years 5–9 years 10–14 years 15–19 years ≥20 years **More than 4 years before diagnosis** *Length of use* <1 year 1–4 years 5–9 years 10–19 years ≥20 years	 10 31 17 11 2 2 10 23 12 7 0	 0.67 1.34 0.88 1.25 0.66 0.74 0.87 1.17 1.04 0.86 -	Age, social status, parity, age at menopause, smoking	No trend with duration of use

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Kinlen *et al*. (1977) (contd)				**More than 9 years before diagnosis**				
				Length of use				
				<1 year	5	0.56		
				1–4 years	15	1.37		
				5–9 years	2	0.53		
				10–19 years	5	1.12		
				≥20 years	0	-		
Shore *et al*. (1979) New York, USA 1964–76	129 breast cancer cases identified through a breast cancer clinic registry.	193 controls comprised a sequential sample of women who attended the clinic in 1968–1969; aged ≥25 years old	Interview	**Use of oxidative hair dye**				
				Number of years used before diagnosis				Cases and controls selected from a previous study of breast cancer (Thiessen 1974)
				0 years	43	1.08		
				5 years	35	1.31		
				10 years	23	1.58		
				15 years	15	1.44		

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Stavraky *et al.* (1979) Ontario, Canada 1976–79	All patients with breast cancer admitted to one of two hospital clinics located in Toronto and London, Ontario Canada. 50 cases of breast cancer were identified in London, Ontario and 35 cases were identified in Toronto; aged 30-69 years	A total of 170 controls were indentified; 70 controls from Toronto and 100 controls from London, Ontario	Interview	**Use of hair dyes** *London, Ontario* Any hair dye use Unexposed Exposed Permanent hair dye use Unexposed Exposed Semi-permanent hair dye use Unexposed Exposed Color rinse used Unexposed Exposed Streaking product used Unexposed Exposed	 14 36 22 28 46 4 33 17 45 5	 1.0 1.5 (0.7–3.1) 1.0 1.3 (0.6–2.5) 1.0 1.7 (0.4–6.5) 1.0 1.2 (0.6–2.5) 1.0 1.0 (0.3–3.2)	Education, income, place of residence, family history of cancer, age at menarche and first pregnancy, use of other household products	

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Stavraky *et al.* (1979) (contd)				*Toronto, Ontario*				
				Any hair dye use				
				Unexposed	16	1.0		
				Exposed	21	0.7 (0.3–1.7)		
				Permanent hair dye use				
				Unexposed	21	1.0		
				Exposed	16	1.1 (0.5–2.4)		
				Semi-permanent hair dye use				
				Unexposed	33	1.0		
				Exposed	2	0.3 (0.1–1.7)		
				Color rinse used				
				Unexposed	31	1.0		
				Exposed	4	0.8 (0.1–2.7)		
				Streaking product used				
				Unexposed	33	1.0		
				Exposed	2	0.5 (0.1–2.7)		

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Nasca *et al*. (1980) New York, USA 1975–76	118 breast cancer cases identified through diagnostic index files of hospitals from 3 upstate New York hospitals between 1975–1976; aged 20–84, with a diagnosis of primary tumor of the breast	233 population controls select through random digit dialing; 2:1 matched on age and county	Interview	**Use of any hair dye** *Total times used* Never used <50 50–99 100–149 150–199 ≥200	 60 26 12 4 7 7	 1.0 1.11 (0.67–1.84) 1.19 (0.57–2.41) 0.87 (0.24–2.68) 1.53 (0.56–3.98) 1.70 (0.61–4.57)	Previous benign breast disease (BBD), parity, age at first pregnancy, menopause induced by operation, age at menarche, education	
Wynder & Goodman (1983) New York, USA 1979–81	401 breast cancer patients admitted to Memorial Sloan-Kettering Cancer Center between 1979–1981; white women, aged 20–80 years	625 controls admitted to the Cancer Center within 2 months of the cases; hospitalized without a primary diagnosis of breast cancer or a history of the disease; 1;1 or 2:1 matched on age of diagnosis	Interview	**Any hair dye use** *Overall* Never Ever *Smoker* Never used Ever used *Ex-smoker* Never used Ever used *Never smoker* Never used Ever used	 132 267 32 82 33 60 67 125	 1.0 1.02 (0.78–1.32) 1.0 0.87 (0.52–1.45) 1.0 0.76 (0.43–1.36) 1.0 1.32 (0.90–1.93)	Age at diagnosis, smoking, religion, age at first menarche, oral contraceptive use, age at first pregnancy, age at menopause, age, history of BBD, family history of breast cancer	No dose–response relationship

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Koenig *et al.* (1991) New York, USA 1977–1981	398 cases of breast cancer were identified through breast cancer screening at Guttman Breast Diagnostic Institute between 1977–1981	790 randomly selected controls screened during the same study period but without breast cancer	Telephone interview	**Hair dye use** No hair dye use Ever use *Permanent dye* 1–9 10–49 50–149 150–906 *Semi-permanent dye* 1–9 10–49 50–776 *Temporary dye* 1–49 50–1 824	 97 294 50 44 65 41 24 8 17 23 14	 1.0 0.8 (0.6–1.1) 0.9 (0.6–1.3) 0.8 (0.6–1.2) 0.9 (0.6–1.3) 0.8 (0.5–1.2) 1.3 (0.8–2.3) 0.4 (0.2–1.0) 0.8 (0.4–1.4) 1.2 (0.7–2.0) 0.7 (0.4–1.4)	Age, family history of breast cancer, age at first full-term birth, height, Latin American birth place, history of receiving Medicaid, race, average darkness of dye color, religion	No trend with number of uses; models not adjusted for smoking
Boice *et al.* (1995), USA, 1926–1982	528 breast cancer cases; identified through the American Registry of Radiologic Technologists; certified between 1926 and 1982;	2628 controls; 5:1 matched on sex, date of birth (+ 5 years), calendar year of certification, length of time between certification and diagnosis	Interview	*Hair dye use* Never Yes	 368 155	 1.0 1.08 (0.87–1.33)	age, age at menarche, menopause, first birth, family history of breast cancer	

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Cook *et al*. (1999) Western Washington, USA 1983–1990	844 cases identified from records of the population-based Cancer Surveillance System (CSS)/ (SEER) Program; cases were white women born after 1944 who were diagnosed between 1983 and 1990 and who resided in three counties of western Washington	960 control subjects identified through random digit dialing; frequency matched on age (within 5 years)	Interviews	*Hair coloring use* None Any use Any rinse Any semi-permanent dye use Any permanent dye use Any bleach then dye	 315 529 92 172 282 69	 1.0 1.3 (1.0–1.6) 1.7 (1.2–2.5) 1.4 (1.0–1.8) 1.2 (1.0–1.6) 2.5 (1.6–3.9)	Age, parity, weight in kg, family history of breast cancer in first degree relative	

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Zheng *et al.* (2002) Connecticut, USA 1994–1997	608 cases of histologically-confirmed breast cancer; between 1994 and 1997; aged 30–80 years; with no previous diagnosis of cancer, with the exception of non-melanoma skin cancer; cases had breast-related surgery at the Yale-New Haven Hospital (YNHH), in New Haven County, or who were residents of Tolland County, Connecticut	609 population-based controls were recruited using RDD methods, from Health Care Finance Administration files or from computerized files of patients who had breast surgery but were histologically confirmed to be without breast cancer.	Interview	**Hair dye use** *Permanent dye use* Never Ever *Semi-permanent dye use* Never Ever	 163 237 163 102	 1.0 0.9 (0.7–1.2) 1.0 1.2 (0.9–1.8)	Age, race and at age at menopause, study site	

Table 2.9 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Petro-Nustas *et al*. (2002) Jordan 1996	100 Jordanian women with breast cancer listed in the Jordan Cancer Registry	100 controls were identified through a convenience proportionate sample based on the percentages of cases from each area of the country; controls were selected to match the cases based on age, parity, level of education, and place of residence	Interview	**Hair dye use** *Within 5 years of diagnosis* No Yes	 5 95	 1.0 8.62 (3.33–22.28)	History of cancer in the family, trauma to the breast, environmental and pollutant factors, abnormal breast size, breast cancer screening, reproductive history, lifestyle	Increased risk of breast cancer among older women.

BBD, benign breast disease; CI, confidence interval; OR, odds ratio; RDD, random-digit dialling

for 0, 5, 10 and 15 years were, respectively, 1.08, 1.31, 1.58 and 1.44 (none significantly different from 1.0). A significant relationship ($P = 0.01$) was noted between a measure of cumulative hair-dye use (number of years times frequency per year) and breast cancer. This relationship also held if the analysis was limited to cases in which the patient herself had responded to the telephone interview. Among women who had used hair dyes 10 years before developing breast cancer, the relationship held only for women at low risk (as assessed from the distribution of a multivariate confounder score) and for those 50–79 years old. [The Working Group noted that the use of a multivariate confounder score for the control of confounding may produce misleading results.]

To follow-up on these findings, Koenig *et al.* (1991) carried out a case–control study of 398 women with breast cancer and 790 controls identified at the same screening centre. For ever-use, the adjusted OR was 0.8 (95% CI, 0.6–1.1), and there was no trend with increased use.

Stavraky *et al.* (1979) compared 50 breast-cancer cases at a cancer-treatment centre with 100 hospitalized controls in London, Ontario, Canada and 35 breast-cancer cases with 70 neighbourhood controls in Toronto, Ontario, Canada, with respect to hair-dye use. The ORs for breast cancer from use of permanent hair dyes (at any time) were 1.3 (95% CI, 0.6–2.5) in London and 1.1 (0.5–2.4) in Toronto. Further statistical analyses, allowing for smoking habits, family history of cancer and age at first birth, showed no significant relationship between hair-dye use and breast-cancer incidence.

Nasca *et al.* (1980) reported a study of 118 patients with breast cancer and 233 controls matched to the patients by age and county of residence (115 matched triplets and three matched pairs) in upper New York State, USA. In the study overall, there was no significant association between breast cancer and use of permanent or semi-permanent dyes (OR, 1.11), nor was an increase in risk seen with increasing numbers of times hair dyes were used or with increasing time since first use. The authors commented that women who dyed their hair to change its colour, as distinct from those who dyed their hair to mask greyness, had a significantly increased risk for breast cancer (OR, 3.13; 95% CI, 1.50–6.54). In this group, there was a significant trend towards increasing risk with increasing numbers of exposures to hair dyes. Examination of risk for hair-dye use in subgroups of women defined by other risk factors for breast cancer showed an OR of 4.5 (95% CI, 1.20–16.78) for women with a past history of benign breast disease, an OR of 1.75 ($P = 0.03$, one-tailed test) for 12 or more years of schooling, and an OR of 3.33 (95% CI, 1.10–10.85) for women aged 40–49 years; the OR was near unity for all other age groups. These effects appeared to be independent of one another and were not explained by confounding by past pregnancy, age at first pregnancy, history of artificial menopause or age at menarche. The authors stressed that the associations observed in the subgroups should be considered as newly generated hypotheses, requiring further testing. In a larger, subsequent study (Nasca *et al.*, 1990) (reported as an abstract) of 1617 cases of breast cancer in New York State and 1617 controls, these authors found no relationship with hair-dye use (OR, 1.04; 95% CI, 0.90–1.21), no significant difference in the ORs for

women with a history of benign breast disease (1.15; 95% CI, 0.86–1.53) and those without (0.98; 95% CI, 0.83–1.16) and no association with duration of hair-dye use.

Wynder and Goodman (1983) carried out a hospital-based case–control study of 401 cases of breast cancer in New York City in 1979–1981. No association was found with hair-dye use (OR, 1.02; 95% CI, 0.78–1.32) and there was no dose–response relationship.

Cook *et al.* (1999) carried out a case–control study in Western Washington (USA) using the Cancer Surveillance System (CSS) to recruit 844 cases of breast cancer (747 invasive and 97 *in situ*). The 960 controls were frequency-matched to cases by age. The aim was to evaluate use of hair colouring and hair-spray application and breast-cancer risk among women in their reproductive age. An increased risk was identified for ever-use of hair dyes (OR, 1.3; 95% CI, 1.0–1.6) adjusted by age, parity, weight and history of breast cancer in first-degree relatives. No relation to breast cancer risk was found for hair-spray application.

In 2002, Petro-Nustas *et al.* evaluated exposure to chemical hair dyes and the risk for breast cancer among Jordanian women. One hundred breast-cancer cases identified through the Jordan cancer registry and 100 population-based controls were included, matched to age, parity, education level and place of residence. A high prevalence in the use of chemical hair dyes was observed among cases compared with controls (95% *vs* 51%) with an eightfold increased risk for breast cancer among users (OR, 8.62; 95% CI, 3.33–22.28). Information about frequency, colour, type and years of exposure was not available.

Zheng *et al.* in 2002 carried out a case–control study in Connecticut, USA to evaluate personal use of hair dyes and risk for breast cancer. A total of 608 incident breast-cancer cases and 609 population controls were included. No increase in risk associated with personal use of hair dyes was observed (OR, 0.9; 95% CI, 0.7–1.2) after adjustment for age, race, study site and age at menopause. Detailed information on duration, frequency, type and colour allowed quantitative study of risk; none of the ORs related to these factors was statistically significant.

A meta-analysis by Takkouche *et al.* (2005) included 12 case–control and two cohort studies, and reached an overall risk estimate for breast cancer of 1.04 (95% CI, 0.98–1.09) with use of hair dyes. When restricted to a population-based framework, the analysis showed an OR of 1.12 (95% CI, 1.01–1.23). No association with use of permanent dyes (OR, 1.00; 95% CI, 0.94–1.05) or intensity of exposure (OR, 0.99; 95% CI, 0.89–1.11) was identified under the assumptions of the fixed effects model, and no differences in estimates were found under the hypothesis of random effects.

(c) Lymphatic and haematopoietic cancers (Table 2.10)

In a further report of the study of Stavraky *et al.* (1979) in Canada, these authors found no significant increase in risk for leukaemia or lymphoma (70 cases) (Stavraky *et al.* (1981). [The Working Group noted that it was not possible to distinguish different haematopoietic malignancies.]

Table 2.10. Case–control studies of lymphatic haematopoietic cancer and personal hair dye use

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Stavraky *et al*. (1981) Ontario, Canada 1976–1979	A total of 70 cases of lymphoma and leukaemia were identified from two hospital clinics located in Toronto and London, Ontario, Canada; cases were identified between 1976 and 1979	In London, Ontario, 314 hospital controls were identified from women hospitalized with illnesses other than cancer; in Toronto, Ontario, 470 neighborhood controls were identified; in both cities 2 controls were matched for each case	Interview	**Use of semi-permanent and permanent hair dyes** *Toronto* Yes *London* Yes	 45 25	 0.7 (0.3–1.6) 1.2 (0.4–3.8)	Oral contraceptives, smoking, use of hair spray	
Cantor *et al*. (1988) Iowa and Minessota, USA 1980–1983	578 cases of leukaemia and 622 cases of non-Hodgkin lymphoma living in Iowa and Minnesota between 1980-1983; cases were male, aged >30 years	1245 controls were selected from the general population and frequency matched to cases on state of residence, five-year age category, and vital status; controls were selected using random digit dialing methods or using a random listing provided by the Federal Health Care Financing Administration.	Interview	**Use of hair dyes** *Leukaemia* Never used Ever used *Non-Hodgkin lymphoma* Never used Ever used	 534 43 569 53	 1.0 1.8 (1.1–2.7) 1.0 2.0 (1.3–3.0)	Age, state of residence	

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Zahm *et al*. (1992) Nebraska, USA 1983–1986	650 histologically confirmed cases of non-Hodgkin lymphoma, Hodgkin's disease, multiple myeloma, chronic lymphocytic leukaemia were identified Nebraska Lymphoma Study Group and area hospitals; White men and women aged >21 years	1655 controls were selected from residents of the same 66-county area as the cases; 3: 1 frequency matched by race, sex, vital status, and age(± 2 years)	Interview	**Use of hair colouring products**			Age	
				NHL				
				Female				
				Never used	74	1.0		
				Ever used	106	1.5 (1.1–2.2)		
				Male				
				Never used	190	1.0		
				Ever used	11	0.8 (0.4–1.6)		
				HD				
				Female				
				Never used	19	1.0		
				Ever used	16	1.7 (0.7–4.0)		
				Male				
				Never used	32	1.0		
				Ever used	3	1.7 (0.4–6.3)		
				MM				
				Female				
				Never used	14	1.0		
				Ever used	24	1.8 (0.9–3.7)		
				Male				
				Never used	27	1.0		
				Ever used	4	1.8 (0.5–5.7)		
				CLL				
				Female				
				Never used	10	1.0		
				Ever used	9	1.0 (0.3–2.6)		
				Male				
				Never used	34	1.0		
				Ever used	3	1.0 (0.1–28.6)		

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Brown *et al.* (1992) Iowa, USA 1981–1984	173 histologically confirmed cases of multiple myeloma; White men; aged ≥30; diagnosed between 1981 and 1984; cases were identified from the Iowa Health Registry	650 population-based White male control subjects without lymphatic or hematopoietic cancer; identified from three sources: random-digit dialing, Medicare records provided by the Health Care Financing Administration and state death certificate files; frequency matched by 5-year age group and vital status at time of interview	Interview	*Hair dye use* Never Ever Used <1 year or <1 time /month Used ≥1 year or ≥1 time /month	 159 14 10 4	 1.0 1.9 (1.0–3.6) 1.5 (0.7–3.3) 4.3 (0.9–19.7)	Vital status (alive, dead), age	Analysis restricted to Whites. Lack of detailed questions on hair dyes usage.

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Herrinton *et al*. (1994) USA 1977–1981	689 case patients aged <82 years with incident multiple myeloma; diagnosed from 1977 to 1981; identified through cancer registries participating in the Surveillance, Epidemiology, and End Results program (SEER) in four geographic areas	1681 control subjects, matched on age, sex, and race; identified using area sampling methods and random-digit dialing	Interview	**Ever regularly used hair dyes** *Men* No Yes *Women* No Yes	 343 17 205 114	 1.0 1.3 (0.7–2.3) 1.0 1.1 (0.83–1.5)	Age, race, study center, educational attainment	No information about type and colouring
Markovic-Denic *et al*. (1995) Yugoslavia, 1989	130 patients with histologically confirmed chronic lymphocytic leukaemia (CLL); selected from Departments of Hematology, Faculty of Medicine in Belgrade and Nis	130 control patients treated at the Department of Orthopedics and Traumatology, Faculty of Medicine in Belgrade and Nis; matched on age (2 years), sex, place of residence and area of residence	Interview	*Hair dye use* Yes	 11	 1.97 (1.08–3.59)	Age, sex, place of residence	

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Mele *et al*. (1995) Italy 1986–1990	Cases were aged ≥15-year with newly diagnosed leukaemias or pre-leukaemias (refractory anemias with excess of blasts, RAEB) between 1986 and 1990	Controls were recruited in the region of the study hospital during the study period from among outpatients with no hematologic malignancy, and they were seen in the same hospitals in which the cases had been identified	Standard questionnaire	**Hair dye use** *APL* No Yes *Other AML* No Yes	 [25] [9] [169] [47]	 1.0 1.5 (0.6–3.7) 1.0 0.8 (0.5–1.3)	Age, sex, education, residence outside the study town	
Mele *et al*. (1996) Italy 1986–1990	39 cases aged >20 years or older with newly diagnosed ET between 1986 and 1990 in each hospital, diagnoses were verified by chart review	156 controls; outpatients without neoplastic hernatologic disorders in the same hospitals as identified cases; frequency matched to the patients (4:l) after stratification by hospital, sex, age (+ 1 year), and closest diagnosis date	Standard questionnaire	**Use of hair dyes** *Ever used* No Yes *Duration of dark color use* <10 years >10 years	 [24] [15] [33] [6]	 1.0 1.5 (0.7–3.2) 1.0 5.3 (1.4–19.9)	Education, living outside the study area	

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Ido *et al*. (1996) Japan 1992–93	116 (69 male and 47 female) cases of myelodysplastic syndromes (MDS) were selected from the patients treated at 32 hospitals between 1992 and 1993.	116 hospital-based controls matched on age (within 5 years), sex, and hospital was recruited	Self-administered questionnaire	*Hair dye use* No Yes	 78 38	 1.0 1.77 (0.90–3.49)	Cigarette smoking, alcohol drinking, exposure to organic solvents	
Nagata *et al*. (1999) Japan 1995–1996	111 cases of MDS treated at 28 institutes in Japan, between 1995 and 1996, cases diagnosed within 3 years prior to the date of the survey; aged between 20 and 74 years old	830 neighborhood controls recruited from residents in the same prefecture of cases; aged 20–74 years old	Questionnaire	*Hair dye use* No hair dye use Ever used *Duration of use (years)* 9 10+	 75 34 17 16	 1.0 1.99 (1.17–3.38) 1.58 (0.83–3.00) 2.99 (1.43–6.24)	Age, sex, living area (prefecture)	No information about colour and type

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Holly *et al*. (1998) USA 1991–1995	1593 patients recently diagnosed with non-Hodgkin lymphoma were identified and interviewed; between age 21 and 74 years within 1 month of primary diagnosis from hospitals in the 6 San Francisco Bay area counties	2515 population-based controls were identified with random-digit dialing; frequency matched to cases on gender, by age within 5 years and by county of residence	Interview	**Hair dye use** *Men* Never used Ever used *Women* Never used Ever used	 348 37 143 185	 1.0 1.3 (0.86–2.00) 1.0 1.0 (0.77–1.30)	Age (for women); sexual preference, age (for men)	Study period was from 1988-1995. Questions about hair dyes use were included in the questionnaire in 1991

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Miligi *et al*. (1999) Italy	1183 newly diagnosed cases of non-Hodgkin lymphomas (ICD IX 200, 202), Hodgkin disease (ICD IX 201), leukaemias (ICD IX 204–208), and multiple myeloma (ICD IX 203), aged 20–74 years old who resided in the areas under study.	828 controls randomly selected from the general population; aged 20–74 residing in each of the areas under study; stratified by sex and 5-year groups; 1:1 matched to cases for NHL and CLL only	Interviews	**Hair dye use** *Ever used* NHL & CLL No Yes MM No Yes Leukaemia No Yes HD No Yes *Use of dark permanent hair dye* No Yes	622	 1.0 1.0 (0.8–1.2) 1.0 0.8 (0.5–1.2) 1.0 0.9 (0.7–1.3) 1.0 0.7 (0.5–1.1) 1.0 2.00 (1.1–3.8)	Age	Analyses restricted to women.

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Björk *et al*. (2001) Southern Sweden 1976–1993	255 adult patients with Ph+CML from southern Sweden, cytogenetically analyzed between 1976–1993 at the Department of Clinical Genetics, Lund, Sweden	Population-based controls 3:1 matched on sex, age, and county of living from the study population of southern Sweden at the calendar year each case was diagnosed; controls selected by the Swedish national bureau of statistics (Statistics Sweden)	Structured telephone interview	*Regular use of personal hair dye* No Yes Uncertain	 195 25 6	 1.0 0.35 (0.18–0.68) NR		
Schroeder *et al*. (2002) Iowa and Minessota USA 1980–1982	68 t(14;18)-positive and 114-negative cases of non-Hodgkin's lymphoma identified through the National Cancer Institute's Factors Affecting Rural Men (FARM) study	1245 population-based controls; white men aged <30 years without hemolymphatic cancer; identified through the FARM study; frequency-matched to cases on state, vital status, and age within 5-year age groups	Structured in-person interviews	Any hair dye use	26	0.9 (0.4–2.1)	Age, vital status, age	70% of cases were not classifiable

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Zhang *et al*. (2004) Connecticut USA 1995–2001	601 histologically confirmed cases of incident, non-Hodgkin lymphoma in Connecticut, diagnosed between 1995 and 2001; cases were women aged 21–84 years at diagnosis with no previous diagnosis of cancer, with the exception of non-melanoma skin cancer; cases identified through the Yale Cancer Center's Rapid Case Ascertainment Shared Resource (RCA).	717 population-based controls with Connecticut addresses were recruited using random digit dialing methods or Health Care Finance Administration files; frequency matched on age (+5 years)	Interviews	*Hair dye use* Never used Ever used Started use before 1980 Started use after 1980	 152 449 295 154	 1.0 1.1 (0.9–1.5) 1.3 (1.0–1.8) 0.9 (0.7–1.2)	Age, family history of NHL in first-degree relative	Increased risk for follicular and B-Cell lymphoma who used permanent hair dyes (OR=1.9; 1.1–3.2 and OR=1.6; 1.2–2.3, respectively)

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Rauscher *et al*. (2004) USA and Canada 1986–1989	769 incident cases of adult acute leukaemia were recruited through Cancer and Leukaemia Group B (CALGB), a multi-institutional cooperative cancer treatment group located throughout the United States and Canada; between 1986 and 1989	623 population-based controls selected using a two-stage random digit dialing procedure; controls identified within 6 months of case accrual; frequency matched to cases by age in 10-year intervals, sex, race and region of residence within the United States and Canada	Interview	*Hair dye use* No use Any use Exclusive permanent dye use	 584 185 87	 1.0 1.3 (0.99–1.8) 1.6 (1.1–2.4)	Age, race, sex, geographic region, education	.
Benavente *et al*. (2005) Spain 1998–2002	574 incident cases of lymphoma recruited at four centres in Spain served by three pathology departments: Barcelona, Tortosa-Reus, and Madrid; between 1998 and 2002	616 hospital-based controls were frequency matched to cases by age, sex, and hospital; selected from admission lists excluding hospitalizations for cancer, organ transplant, systemic infection, or severe immunosuppression.	Structured interview	**Hair dye use** *All lymphomas* Never used Ever used *CLL* Use of permanent dye Started use before 1980	 395 179 35 27	 1.0 1.2 (0.9–1.7) 3.4 (1.4–7.8) 3.5 (1.5–7.8) .	Age, sex, center of recruitment, house ownership	Significant linear trend for years exposed among CLL cases

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Tavani *et al*. (2005) Northern Italy 1985–1997	158 patients with histologically confirmed incident HD, 446 with NHL, 141 with MM, 221 with STS; between 1985 and 1997; aged 14–79 years	1295 hospital-based control patients with acute non-neoplastic conditions; aged 17–79 years	Interviews	**Any use of hair dye** *HD* Never Ever *NHL* Never Ever *MM* Never Ever *STS* Never Ever *Use of semi-permanent dyes* Never Ever	 129 26 336 93 97 36 166 39 110 23	 1.0 0.68 (0.4–1.18) 1.0 1.03 (0.73–1.44) 1.0 1.17 (0.7–1.97) 1.0 0.73 (0.45–1.17) 1.0 1.78 (1.02–3.12)	Age, sex, area of residence, education, smoking	No information about duration, shade, age at first use
de Sanjosé *et al*. (2006) Spain, Germany, France, Ireland, Finland, Italy, Czech Republic 1998–2003	2 302 incident lymphoma cases with an initial diagnosis of lymphoid malignancy between 1998 and 2003;	2 417 controls were identified from the general population from census lists or were recruited from the same hospitals as the cases; controls were matched to the cases by age (+5 years), gender, and study center	Structured face-to-face interview	*Lymphoma* Never use Ever use *Used prior to 1980* Never use Ever use *CLL* Never use Ever use	 1436 866 1436 340 280 127	 1.0 1.19 (1.01–1.53) 1.0 1.37 (1.09–1.72) 1.0 1.43 (1.01–2.03)	Age, sex, center of recruitment, house ownership	The risk of lymphoma increased with increasing years of using dark hair dyes

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Morton *et al*. (2007) USA 1988–2000	1321 cases with a histologically confirmed, first primary diagnosis of NHL; aged 20–74 years, diagnosed between 1998 and 2000; identified among residents of four SEER registries in Iowa, Los Angeles County, metropolitan Detroit and metropolitan Seattle	1057 population-based controls were selected from residents of the same four SEER areas; frequency matching to the cases by age (within 5-year age groups), sex, race and SEER area.	In person interviews	**Use of any hair dye** *Men* Yes *Women* Yes *Use of permanent intense colors for more than 15 years* Yes	 113 509 17	 0.9 (0.6–1.2) 1.2 (0.9–1.6) 3.9 (1.2–12.5)	Age, race, SEER area	.

Table 2.10 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Chiu *et al*. (2007) Eastern Nebraska, USA 1983–1986	426 cases of NHL, Hodgkin's disease, multiple myeloma, and chronic lymphocytic leukaemia diagnosed between 1983 and 1986 among White men and women aged 21 years or older who resided in one of 66 counties in eastern Nebraska	1655 population-based controls without hematopoietic cancer; randomly selected from the same 66-county area; 3:1 frequency matched on sex, vital status, and age (5-year age groups)	Telephone interviews	**Any use of hair dye products** *t(14;18)-negative*			Age, type of respondent, farming status	Analysis restricted to 175 cases of NHL; ORs for t(14;18)-positive and t(14;18)-negative NHL did not differ significantly. Information on exposures from proxies for about 40% of cases and controls
				Men				
				Never	39	1.0		
				Ever	4	1.2 (0.4–3.4)		
				Women				
				Never	31	1.0		
				Ever	33	1.1 (0.6–1.8)		
				Semi-permanent	24	1.1 (0.6–1.8)		
				Permanent	15	1.4 (0.7–2.7)		

APL, acute polymyelocytic leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myelogenous leukaemia; HD, Hodgkin disease; ICD-IX, International Classification of Diseases, Volume 9; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndromes; MM, multiple myeloma; OR, odds ratio; SEER, Surveillance Epidemiology and End Results programme; STS, soft tissue sarcoma

In a hospital-based case–control study (101 matched pairs) of acute non-lymphocytic leukaemia in the Baltimore (USA) area, published only as an abstract, Markowitz *et al.* (1985) found a significant positive association with hair-dye use (OR, 3.1). There was, however, no difference between regular use (at least once a year) (OR, 2.7) and less frequent use (OR, 2.2).

Cantor *et al.* (1988) carried out a population-based case–control study of hair-dye use among 578 men with leukaemia, 622 with non-Hodgkin lymphoma and 1245 population controls in Iowa and Minnesota, USA, in 1980–83. Significantly increased ORs were found for leukaemia (OR, 1.8; 95% CI, 1.1–2.7) and non-Hodgkin lymphoma (OR, 2.0; 1.3–3.0) in association with personal use or other potential exposure to hair tints, any hair colouring product or hair dyes. The authors stated that the ORs did not substantially change after exclusion of the 10 men with other potential exposure to hair-colouring products (e.g., occupational exposure), but detailed results were not presented. [The Working Group noted that, although the authors suggested an increased risk with increasing extent of hair-dye use, examination of the paper could not verify this.]

A population-based case–control study carried out in eastern Nebraska, USA, during 1983–1986 investigated use of hair-colouring products among a total of 201 male and 184 female cases of non-Hodgkin lymphoma, 35 male and 35 female cases of Hodgkin disease, 32 male and 40 female cases of multiple myeloma, 37 male and 19 female cases of chronic lymphocytic leukaemia, and 725 male and 707 female residential controls who could be interviewed (Zahm *et al.*, 1992). Telephone interviews were conducted with cases, controls or their next of kin; response rates were 81–96% for cases and 84% for controls. Among women, use of any hair-colouring product was associated with an increased risk for non-Hodgkin lymphoma (OR, 1.5; 95% CI, 1.1–2.2), Hodgkin disease (OR, 1.7; 0.7–4.0) and multiple myeloma (OR, 1.8; 0.9–3.7), and women who used permanent hair dyes had high ORs for all three neoplasms (non-Hodgkin lymphoma, (OR, 1.7; 95% CI, 1.1–2.8), Hodgkin disease (OR, 3.0, 95% CI, 1.1–7.9) and multiple myeloma (OR, 2.8; 95% CI, 1.1–7.1); all $P < 0.05$). For non-Hodgkin lymphoma and multiple myeloma, the risks were highest among women who used dark permanent dyes. Long duration and early age at first use tended to increase the risk among men, use of any hair-colouring product was associated with nonsignificantly increased ORs for Hodgkin disease (OR, 1.7), and multiple myeloma (OR, 1.8), on the basis of three and four exposed cases, respectively; no increase was found for non-Hodgkin lymphoma (OR, 0.8). Use of any hair dye was not associated with chronic lymphocytic leukaemia in either women or men (OR, 1.0).

A population-based case–control study of 173 white men with multiple myeloma and 650 controls was carried out in Iowa, USA. The risk for multiple myeloma was significantly elevated (OR, 1.9; 95% CI, 1.0–3.6; 14 exposed cases) among users of hair dyes. For men who had used hair dyes for one year or more at a frequency or one or more times per month, the OR was 4.3 (95% CI, 0.9–19.7; four exposed cases) (Brown *et al.*, 1992).

Herrinton *et al.* (1994) carried out a case–control study to evaluate the relation between both personal exposure to hair dyes and the risk for multiple myeloma (MM) in the USA. Six hundred eighty-nine MM cases and 1681 controls were included. Among women, no significant increase in risk was observed for personal use (OR, 1.1; 95% CI, 0.83–1.5). Results for males were also non-significant and based on small numbers of exposed cases. Information about colour and type was not collected.

Mele *et al.* (1995) carried out a case–control study in Italy to evaluate exposure to hair dyes and risk for acute promyelocytic leukaemia (APL) and acute myeloid leukaemia (AML). A total of 254 cases and 1161 hospital controls were included. No significant associations were found for APL (OR, 1.5; 95% CI, 0.6–3.7) or AML (OR, 0.8; 95% CI, 0.5–1.3). No data were available for colour or frequency.

In 1996, Ido *et al.* carried out a case–control study in Japan. A total of 116 myelodysplastic syndromes (MDS) cases from among hospital members of the Idiopathic Disorders Organs Research Committee, and 116 hospital controls were included to evaluate exposure to hair dyes. An increased risk for MDS was observed among users of hair-dye products (OR, 1.77; 95% CI, 0.90–3.49), although it did not reach statistical significance. The odds ratio among women was 2.50 (95% CI, 0.97–6.41). Although information about duration and frequency of hair-dye use was collected, no results about these associations were reported.

Mele *et al.* (1996) carried out another case–control study in Italy. Thirty-nine thrombocythemia cases and 156 hospital controls were included to evaluate both personal use of hair dye and occupational exposure. The prevalence of hair-dye exposure among cases was 38.2%. A non-significantly increased risk was reported (OR, 1.5; 95% CI, 0.7–3.2), although association between dark hair-dye use and increased risk was statistically significant (OR, 5.3; 95% CI, 1.4–19.9) when the duration of exposure exceeded 10 years.

Nagata *et al.* (1999) evaluated the risk for myelodysplastic syndromes (MDS) and personal use of hair dyes using a case–control study carried out in Japan. A total of 111 MDS cases and 830 controls randomly selected from the same prefecture as the cases responded to a health questionnaire. The risk for MDS for ever having used hair dyes was 1.99 (95% CI, 1.17–3.38). A statistically significant trend was observed in risk with increasing years of exposure and number of hair-dye applications ever used.

Holly *et al.* (1998) carried out a case–control study in the San Francisco Bay area (USA) to evaluate the association of hair-dye use and the risk for non-Hodgkin lymphoma (NHL). A total of 713 NHL cases and 1604 population controls were asked about type, colour, number of years of exposure for each product, age at first and last use, and frequency of use. Stratified results by gender were reported. No increases in risk were found for ever-use of hair dyes (OR, 1.00; 95% CI, 0.77–1.30 for women and OR, 1.3; 95% CI, 0.86–2.00 for men) or for years of use, lifetime frequency of use, colour or type.

Miligi *et al.* (1999) carried out a case–control study in Italy to study the role of hair-dye exposure and occupation as a hairdresser in the etiology of haematolymphopoietic malignancies. A total of 1170 cases (611 NHL, 260 leukaemia, 165 Hodgkin lymphoma, and 134 multiple myeloma) and 828 population controls were enrolled. The overall

estimation of OR for ever-exposure to hair dyes was not reported, but for none of the studied diseases was a significant risk observed. Among those women who had used dark permanent products, an increased in risk was observed for leukaemia (OR, 2.0; 95% CI, 1.1–3.8).

In 2001, Björk *et al.* conducted a case–control study in Southern Sweden to evaluate the risk for Philadelphia chromosome-positive chronic myeloid leukaemia associated with the regular use of hair dyes. Among the 226 cases included, the prevalence of exposure to hair dyes was 11.1%, whereas that among the 251 population controls was 26.4%. The estimated risk for ever-use of hair dyes was less among cases compared with controls (OR, 0.35; 95% CI, 0.18–0.68). Information about dose-response was not provided.

Schroeder *et al.* (2002) carried out a population-based NHL study in Iowa and Minnesota (USA) to evaluate non-occupational exposures. A total of 622 cases and 1245 controls were included. The study also considered the presence of the t(14;18) translocation in the tumours. An increased risk for NHL with ever-use of hair dyes was observed independently of the t(14;18) translocation (OR, 2.0; 95% CI, 1.3–3.4). An excess in risk was observed both among subjects with the t(14;18) translocation (OR, 1.8; 95% CI, 0.9–3.7) and without the translocation (OR, 2.1; 95% CI, 1.3–3.4). A large proportion of NHL (> 70%) could not be evaluated in histological subtypes due to lack of histological information.

Pu *et al.* (2003) carried out a case–control study (114 cases and 114 controls) on haematological cancer and reported increased risks associated with ever-use of hair dyes (OR, 3.3; 95% CI, 1.59–7.18) and with increasing frequency of use (OR, 3.28; 95% CI, 1.99–7.34). [The Working Group noticed that the study did not provide enough methodological and statistical details to fully evaluate the results; these data are not included in Table 2.10].

Rauscher *et al.* (2004) carried out a population-based acute leukaemia study evaluating the risk from hair-dye exposure. A total of 769 cases and 623 controls were interviewed in the USA and Canada. A slight increase in risk was observed for those ever exposed to hair dyes (OR, 1.3; 95% CI, 0.99–1.80). In addition, the use of permanent dye only, for 15 years or more, was associated with an increased acute leukaemia risk (OR, 1.9; 95% CI, 1.1–3.6). The OR was marginally elevated for use of permanent dyes that had started in the 1970s (OR, 1.7; 95% CI, 0.98–3.0).

Zhang *et al.* (2004) carried out a case–control study in Connecticut, USA to evaluate the association between hair dyes and risk for NHL among females. A total of 601 cases and 717 population controls were included. The OR associated with hair-dye use was 1.1 (95% CI, 0.9–1.5). Additionally, no evidence of a dose-response with the total number of applications, duration of use, years since first use, colour or type was observed. However, the OR for permanent dark-colour users for more than 25 years was 2.1 (95% CI, 1.0–4.0) and it was 1.2 (95% CI, 0.6–2.1) if the duration of use was less than 15 years. Considering lifetime dose, those with more than 200 applications had an OR of 1.7 (95% CI, 1.0–2.8). An increase in risk was observed for those starting use before 1980 (OR, 1.3; 95% CI, 1.0–1.8). By NHL subtypes, a significant increase in risk was seen for follicular

lymphoma and B-Cell NHL among women who used permanent hair-colouring products (OR for follicular lymphoma, 1.9; 95% CI, 1.2–3.2; OR for B-cell NHL, 1.6; 95% CI , 1.2–2.3).

Benavente *et al.* (2005) carried out a case–control study in Spain to evaluate the risk for lymphoma associated with hair-dye exposure. A total of 574 lymphoma cases and 616 hospital controls were included in the study. The prevalence of hair-dye exposure was 79% among females and 8% among males. A non-significant increase in risk was observed for ever-use (OR, 1.2; 95% CI, 0.9–1.7). No association was observed for colour, type, frequency, or lifetime dose. By specific lymphoma subtypes, a twofold increase in risk for ever-use was observed for CLL (95% CI, 1.1–4.7). A significant linear trend by years of use was also observed for CLL ($P = 0.04$) among those exposed for 10 years or less (OR, 1.1; 95% CI, 0.4–3.0), from 11 to 24 years of use (OR, 3.2; 95% CI, 1.3–8.2) and for 24 years or more (OR, 3.7; 95% CI, 1.5–8.9). In this group of cases, those who started hair-dye use before 1980 had a 3.5-fold increased risk (95% CI, 1.5–7.8) compared with non-users. Use of dark colour and permanent hair-dye types were both associated with an increased risk for CLL (OR, 2.3; 95% CI, 1.1–4.9 for dark colour and OR, 3.4; 95% CI, 1.4–7.8 for permanent).

Tavani *et al.* (2005) carried out a case–control study in northern Italy aimed at evaluating the relationship between lymphoid neoplasm and tissue sarcoma and use of hair dyes. A total of 966 cases and 1295 hospital controls were interviewed. No significant association with hair-dye use was seen for any of the lymphoma subtypes. An increased risk for multiple myeloma among semi-permanent dye users was observed (OR, 1.78; 95% CI, 1.02–3.12). Information about colour was not included in the study.

De Sanjosé *et al.* (2006) carried out a multicentric case-control study including seven countries in Europe. A total of 2302 lymphoma cases and 2417 controls were pooled to study the relationship between hair dyes and the risk for lymphoma. A slight increase in risk for lymphoma was observed for ever-use of hair dyes (OR, 1.19; 95% CI, 1.00–1.41). The risk for lymphoma for those starting use before 1980 was 1.37 (95% CI, 1.09–1.72). Among all lymphoma categories, a statistically significant increase in risk for CLL was seen (OR, 1.43; 95% CI, 1.01–2.03). The risk for lymphoma increased with the number of years of use of dark hair dyes (*P* for linear trend, 0.07): four years or less: OR, 1.11 (95% CI, 0.84–1.46); from 4.5 to 13 years: OR, 1.28 (95% CI, 0.96–1.70) and for 14 years or more: OR, 1.45 (95% CI, 1.09–1.94). This study included the data of Benavente *et al.* (2005), but sensitivity analysis showed that results were not explained by any of the individual studies.

Morton *et al.* (2007) carried out a population-based NHL study in the USA among 1321 cases and 1057 controls, examining NHL risk in relation to reported hair-dye use and genetic variation in *NAT1* and *NAT2*. No increased in risk was observed for ever-exposure among either males or females (OR, 1.2; 95% CI, 0.9–1.6). The OR for females exposed to 100 or more lifetime applications was 1.4 (95% CI, 1.0–2.0). Among females, a fourfold increase in risk was observed for those using permanent and intense tones for more than 15 years before 1980 (OR, 3.9; 95% CI, 1.2–12.5). Concerning *NAT1* and

NAT2 variation, an increase in risk was observed for the use of dark or intense permanent hair dyes before 1980 among women if they had the NAT2 rapid/intermediate phenotype (OR, 3.3; 95% CI, 1.3–8.6) or if they had one or two copies of the *NAT*10* allele (OR, 3.0; 95% CI, 1.1–8.1).

Chiu *et al.* (2007) carried out a case–control study in Eastern Nebraska (USA) to evaluate the relationship between personal use of hair dyes and the risk for t(14;18)-defined subtypes of Non-Hodgkin lymphoma. A total of 385 cases and 1432 population controls were interviewed. No relation was observed for ever-use of hair dyes and the risk for NHL for either t(14;18)-negative or -positive translocation cases.

A meta-analysis by Takkouche *et al.* (2005) included 40 studies on non-Hodgkin lymphoma, Hodgkin lymphoma and multiple myeloma. All haematopoietic cancers were significantly increased among ever-users of hair dyes (RR, 1.13; 95% CI, 1.06–1.20). The combined OR estimated for permanent hair-dye use was 1.14 (95% CI, 0.99–1.29) and for intensive use it was 1.12 (95% CI, 0.98–1.28). The different sensitivity analyses provided a range of estimates from 0.87 for Hodgkin-lymphoma studies to 1.57 for those studies including men only. No evaluation of the period of exposure was included in this analysis.

(d) Childhood cancer (see Table 2.11; only studies since 1993 are shown in the Table)

Kramer *et al.* (1987) reported a matched case–control study of maternal exposures during pregnancy and neuroblastoma diagnosed during the period 1970–1979 in the Greater Delaware Valley, USA. Of the 181 cases identified, 139 met the eligibility criteria, and interviews were completed with 104 case families (75%). Control subjects were selected by random-digit dialling and were matched with cases on age, race and the first five digits of their telephone number at the time of diagnosis; the response rate among those eligible was 57% (101 of 177). In addition, the authors compared 86 patients who had at least one sibling with a randomly selected sibling. Mothers were asked about six main exposures, specified for hypothesis testing, and about a variety of other exposures, including the use of hair-colouring products. The OR associated with maternal exposure to hair dye was 3.00 (90% CI, 1.64–5.48; one-sided *P*-value, 0.002; 36 discordant pairs) in comparison with controls selected by telephone and 2.20 (90% CI, 0.93–5.22; one-sided *P*-value, 0.07; 16 discordant pairs) in comparison with siblings.

Bunin *et al.* (1987) conducted a case–control study of Wilms' tumour diagnosed in children under 15 during the period 1970–1983 in the Greater Philadelphia (USA) area in relation to use of hair dyes by their mothers during pregnancy. Of 152 white cases, 28 were ineligible for a variety of reasons. Interviews were completed with the parents of 88 (71%) of the 124 eligible cases and 88 of 159 (55%) controls, on average 10 years after the relevant pregnancy. For Wilms tumour overall, the OR associated with maternal hair-dye use was 3.6 (95% CI, 1.4–10.2; based on 32 discordant pairs). A total of 68 cases could be classified as genetic (26 cases) (if they were bilateral or had nephroblastomatosis) or non-genetic (42 cases) (if they were unilateral without nephroblastomatosis or

Table 2.11. Case–control studies of childhood cancer and personal hair dye use

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Olshan *et al*. (1993) USA,Canada 1984–86	200 cases were identified throught the National Wilms Tumor Study (NWTS) between 1984 and 1986; aged ≤15 years	200 control subjects 1:1 matched on age (± 2 years) and geographical area; controls identified through a modified random digit dialing procedure	Interview	**Hair dye use during pregnancy** *Wilms Tumor* No Yes	 180 20	 1.0 1.37 (0.66–2.85)	Household income and father's education	Discrepancy between risk estimated for cases diagnosed < 2 years old in results section and in discussion section (OR = 1.23, 95% CI = 0.16 –9.45 and OR = 2.92, 95% CI = 0.91 –9.33, for results and discussion, respectively)

Table 2.11 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Bunin *et al*. (1994) USA, Canada 1986–89	322 cases with astrocystic glioma or PNET were identified through the Children Cancer group; eligible cases were diagnosed before 6 years of age between 1986 and 1989	321 controls; 1:1 matched on race, birth year, telephone area code and prefix; controls selected through random digit dialing procedure	Interview	**Use of hair dyes during pregnancy** *Astrocystoma* Not used Colouring products Permanent procucts *PNET* Not used Colouring products Permanent procucts	 305 16 59 305 16 64	 1.0 0.7 (0.3–1.6) 0.9 (0.5–1.5) 1.0 1.1 (0.4–2.6) 1.2 (.07–2.0)	Income level	

Table 2.11 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Holly *et al*. (2002) San Francisco, Seattle, Los Angeles 1984–1991	540 cases were recruited from three areas in California and Washington state; aged <20 years at the time of diagnosis between 1984 and 1991 and had a benign or malignant primary tumour of the brain, cranial nerves or cranial meninges of any histological type. USA cancer registry	Random digit dialing telephone methods were used to select control subjects without CBT who were frequency matched to patients by sex and birth year in Seattle and San Francisco and individually matched on sex and birth year in Los Angeles	Interview	**Use of hair dye during pregnancy** *Astrocystic tumor* Never used Ever used *PNET* Never used Ever used *Other glioma* Never used Ever used	 265 40 94 12 113 12	 1.0 1.0 (0.69–1.5) 1.0 0.97 (0.51–1.9) 1.0 0.76 (0.40–1.4)	Child age and sex	

Table 2.11 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
McCall *et al*. (2005) USA, Canada 1992–94	538 cases identified through hospitals participating in the Children's Cancer Group or the Pediatric Oncology Group between 1992 and 1994; aged <19 years with newly diagnosed neuroblastoma	504 controls were identified through random-digit dialing; controls were 1:1 matched based on date of birth (±6 months for cases <3 years old and ±1 year for cases >3-years old) and telephone number	Interview	**Maternal hair dye use the month before and/or during pregnancy** *Any hair dye use* Unexposed Exposed *Permanent hair dye use* Unexposed Exposed *Temporary hair dye use* Unexposed Exposed	 124 410 98 436 33 501	 1.0 1.6 (1.2–2.2) 1.0 1.4 (1.0–2.0) 1.0 2.0 (1.1–3.7)	Child's age	Risk persists when restricted to exposure during pregnancy. No differences by age at diagnosis. Stronger effect among smokers for any and permanent hair dye

Table 2.11 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Exposed cases	OR (95% CI)	Adjustment factors	Comments
Chen *et al*. (2006) USA 1993–2001	273 cases were recruited through the Children's Oncology Group (COG); aged <15 years newly diagnosed with a GCT (germinoma (dysgerminoma/seminoma/germinoma), embryonal carcinoma, yolk sac tumor, choriocarcinoma, immature teratoma, and mixed GCT between 1993 and 2001	418 controls were selected by random-digit dialing; frequency matched on the child's sex, year of birth ±1 year and geographic location at diagnosis. The matching ratio was 1:2 for males and 1:1 for females	Interview	**Maternal hair dye use** *Total* Exposed *Boys* Exposed *Girls* Exposed	 79 30 49	 1.3 (1.0–1.7) 1.4 (0.9–2.3) 1.2 (0.9–1.7)	Child gender, age, maternal education, race, age at index pregnancy and family income	

CBT, childhood brain tumours; CI, confidence interval; GCT, germ-cell tumours; OR, odds ratio; PNET, primitive neuroectodermal tumour

a Wilms tumour-associated congenital anomaly). The OR associated with maternal use of hair-colouring agents was 5.5 (95% CI, 1.0–71.9; on the basis of 13 discordant pairs out of 42) for non-genetic cases and 3.3 (95% CI, 0.7–22.1; on the basis of 13 out of 26 discordant pairs) for genetic cases. The ORs associated with exposure to hair dyes were similar for an interval of 2–10 years and for an interval of 11–24 years between pregnancy and interview.

Kuijten *et al.* (1990), in an earlier report of the study of Kuijten *et al.* (1992), found no association between astrocytoma and maternal use of hair-colouring products during pregnancy (OR, 0.9; 95% CI, 0.4–1.8; 37 discordant pairs).

Olshan *et al.* (1993) carried out a case–control study in the USA and Canada using the National Wilms Tumor Study to recruit 200 cases of Wilms tumours to evaluate parental exposures during pregnancy, including hair-colouring products. Cases were matched to 233 controls living in the same neighbourhood and having the same age, selected using random-digit dialling. A non-significant increased risk was observed for ever-use of hair dyes during pregnancy (OR, 1.37; 95% CI, 0.66–2.85). There is a discrepancy in this paper between the risk estimated for cases diagnosed younger than 2 years in the Results section and in the Discussion section (OR, 1.23; 95% CI, 0.16–9.45 and OR, 2.92; 95% CI, 0.91–9.33, for Results and Discussion sections, respectively). The study had a moderate inclusion rate (61% of the selected cases and 52% of the controls) and exposure was assessed by interview.

Bunin *et al.* (1994) carried out a case–control study, in the USA and Canada, based on the Children's Cancer Group to evaluate several exposures occurring during the pregnancy and breastfeeding periods. The exposures included hair dyes, hair sprays and makeup in association with astrocytic glioma ($n = 155$) and primitive neuroectodermal tumours (PNET) ($n = 166$) diagnosed before age 6. Controls ($n = 321$) were selected via random-digit dialling and matched to the cases by neighbourhood, race and age. No association was identified for astrocytoma with use of make-up (OR, 1.2; 95% CI, 0.7–1.9), hair spray (OR, 1.2; 95% CI, 0.7–2.0), hair colouring (OR, 0.7; 95% CI, 0.3–1.6) or use of permanent hair dye (OR, 0.9; 95% CI, 0.5–1.5) or for PNET (OR for make–up, 0.8; 95% CI, 0.5–1.2; for hairspray, 1.0; 95% CI, 0.6–1.7; for hair-colouring products, 1.1; 95% CI, 0.4–2.6; for permanent hair-dye use 1.2; 95% CI, 0.7.-2.0). The analysis was fully adjusted for income level for the astrocytoma analysis. Participation was around 65%, and exposure was assessed by interview.

McCredie *et al.* (1994) conducted a case–control study of incident primary malignant brain tumours diagnosed during 1985–1989 in children below 14 years old in Sydney, Australia, and evaluated several hypotheses among which ever-use of hair dyes by the mothers. No association was shown (OR, 0.8; 95% CI, 0.4–1.6).

Holly *et al.* (2002) carried out a case–control study to evaluate childhood brain tumours (CBT) and maternal use of hair colouring one month before pregnancy and during pregnancy. The study used cancer registries from Los Angeles County, the San Francisco Bay area and the Seattle area, USA. It included 540 biological mothers of CBT children diagnosed during 1984–1991 and 801 control mothers matched to cases by birth

year and sex of the index child. Exposure was assessed through interview. A non-statistically significant increase in risk was identified for those mothers using exclusively semi-permanent dyes one month before pregnancy or during the first trimester of pregnancy (OR, 2.5; 95% CI, 0.58–10.0). Exclusive use of permanent dyes, temporary dyes or hair darkeners was not related to an increased risk. Exclusive use of semi-permanent dyes was related to an increased risk (OR, 2.0; 95% CI, 0.83–4.7) for users before or during pregnancy. No differences by histological tumour type were observed. Participation rates were over 70% for cases and controls.

McCall *et al.* (2005) carried out a case–control study on neuroblastoma in association with exposure to hair-dye use before and during pregnancy. The study recruited 538 cases from the USA and Canadian Children's Cancer Group or the Pediatric Oncology Group. A total of 504 controls were selected using telephone random-digit sampling. Controls were matched to the cases by sex, year of birth and neighbourhood. Participation rates were over 70% for both cases and controls. Use of temporary hair dyes was associated with a twofold increased risk for neuroblastoma (OR, 2.0; 95% CI, 1.1–3.7). Use of hair dyes in the month before pregnancy showed a 60% increase in risk (OR, 1.6; 95% CI, 1.2–2.2). Ever smokers were more likely to have an increased risk for neuroblastoma in their offspring if mothers used hair dyes (OR, 2.2; 95% CI, 1.3–3.8) or permanent dyes (OR, 2.2; 95% CI, 1.2–4.1). No dose-responses were reported for permanent dyes.

Chen *et al.* (2006) reported a case–control study that explored the association between childhood germ-cell tumours and hair-dye use by the mother before and during pregnancy and during breastfeeding, among 273 cases diagnosed between 1993 and 2001 from the Children's Oncology Group in the USA. A total of 418 controls were selected via random-digit dialling and matched to the cases by sex, age and geographical area. Maternal use of hair dyes one month before pregnancy was related to a 1.7-fold increased risk for boys (95% CI, 1.0–2.8). For mothers who used hair dyes during breastfeeding the overall OR was 1.5 (95% CI, 1.0–2.2) and 1.7 among girls (95% CI, 1.1–2.6).

(e) Other cancer sites

Ahlbom *et al.* (1986) carried out a case–control study in Stockholm and Uppsala, Sweden, of 78 patients with astrocytoma diagnosed in 1980–1981, 197 hospital controls (with meningioma, pituitary adenoma or cerebral aneurysm) and 92 population controls. The ORs for the 23 astrocytoma patients who had dyed their hair were 0.8 (95% CI, 0.4–1.8) relative to 83 hospital controls and 1.5 (95% CI, 0.6–3.7) when compared with 46 population controls who had dyed their hair.

Burch *et al.* (1987) found that significantly more adults with brain cancer diagnosed in Canada in 1977–1981 than hospital controls reported having used hair dye or hair spray (OR, 1.96; $P = 0.013$; 43/22 discordant pairs). No data by histology were provided, but 133 of 215 were astrocytomas and 67 of 215 had a glioma or related histology.

Heineman *et al.* (2005) evaluated the risk for glioma in the female white population of Nebraska associated to use of hair dyes. Cases were diagnosed between 1988 and 1993, and controls were retrieved from a previous study, were population-based and were

matched to the case by age. Adjustment by year of birth and educational level was done throughout the study. Questions about exposures from hair-dye use before 1985 were assessed by personal or proxy interview (79% of the cases). The study identified an increased risk for glioma for ever-use of hair dye (OR, 1.7; 95% CI, 1.0–2.9) and a 2.4-fold risk from use of permanent hair dyes (95% CI, 1.3–4.5). The risk for glioblastoma multiforme increased 4.9-fold (95% CI, 1.6–15.7) with exposure to dark colours for those reporting use during 20 years or less.

Bluhm *et al.* (2007) explored the association between use of synthetic hair dyes and risk for brain tumours in a hospital-based case–control study, including 489 patients with glioma, 197 with meningioma, 96 with acoustic neuroma, and 799 controls. The study, carried out in 1994–1998, identified no increase in risk for these tumours from ever-use among exposed women or exposed men, including in evaluations of frequency, lifetime number of doses, or colour. There was only one positive association linked to use of brown colour for more than 20 years for all glioma cases and use of red colour during less than 20 years. Both ORs were stronger for acoustic neuroma. [The Working Group was concerned about data stratification resulting in very small numbers and high ORs]

A meta-analysis by Takkouche *et al.* (2005) included 15 studies on a wide range of cancer sites other than breast, bladder or non-Hodgkin lymphoma. The pooled RR was 1.83 (95% CI, 1.16–2.89) for brain cancer; 1.71 (95% CI, 1.15–2.53) for ovarian cancer; 0.74 (95% CI, 0.51–1.07) for skin cancer; and 0.89 (95% CI, 0.53–1.90) for cervical cancer.

Stavraky *et al.* (1981), in a case–control study, found no significant increase in crude or adjusted risks for cancer of the cervix (38 cases), cancer of the ovary (58 cases), cancer of the lung (70 cases), cancers of the kidney and bladder (35 cases) or endometrial cancers (36 cases) among ever-users of hair-colouring agents in either Toronto or London, Ontario, Canada.

Holman & Armstrong (1983) examined hair-dye use in a population-based case–control study of 511 patients with malignant melanoma and individually matched controls in Western Australia in 1980–1981. No relationship was found with ever-use of permanent hair dyes. The ORs obtained from a conditional logistic regression analysis with adjustment for solar exposure, reaction to sunlight and hair colour (Armstrong & Holman, 1985) for 86 cases of Hutchinson's melanotic freckle associated with use of semi-permanent and temporary dyes were: never used, 1.00; used 1–9 times, 1.5 (95% CI, 0.3–6.8); used ≥ 10 times, 3.3 (95% CI, 1.0–11.5; *P* for trend, 0.05). The OR for Hutchinson's melanotic freckle in relation to use of permanent dyes was not elevated. [The Working Group noted that the number of exposed subjects was not reported.]

Osterlind *et al.* (1988a,b) found a negative association with use of permanent or semi-permanent hair dyes among women with malignant melanoma in Denmark in 1982–1985 (OR for hair dye use, 0.6; 95% CI, 0.5–0.9; 136 exposed cases). Cases of Hutchinson's melanotic freckle were not included in this population-based study.

Spitz *et al.* (1990) examined hair dye use in a case–control study of 37 male and 27 female patients with salivary gland cancer in Texas, USA, in the period 1985–1989.

Controls were patients with other malignancies. Among ever-users of hair dyes, an increased OR was found for women (OR, 4.1; 95% CI, 1.5–11.5; 14 cases). There was no difference between female cases and controls with respect to frequency of use, except that the OR for use during more than 15 years (OR, 3.5; 95% CI, 0.9–12.8) was higher than that for shorter duration of use (OR, 2.3; 95% CI, 0.9–6.2).

2.2.3 *Pooled analysis* (Tables 2.12–2.16)

A recent analysis (Zhang *et al.*, 2008) pooled original data from four previously reported case–control studies that were part of the International Lymphoma Epidemiology Consortium (InterLymph), including a total of 4461 NHL cases (2123 men and 2338 women) and 5799 controls (2837 men and 2962 women) to investigate the relationship between personal hair-dye use and risk for NHL. Three studies were from the USA, the Connecticut Women's NHL Study, the National Cancer Institute (NCI)/Surveillance, Epidemiology, and End Results (SEER) MultiCenter Case-Control Study (NCI/SEER), and the Epidemiology of NHL Study from the University of California at San Francisco (UCSF). The three US studies collectively represent a total of six sites from the SEER programme (Connecticut, San Francisco-Oakland, Iowa, Detroit, Seattle-Puget Sound, and Los Angeles). The other case–control study, from Europe (the EpiLymph International Case-Control Study of Lymphomas), represents geographic sites from six countries (Czech Republic, France, Germany, Ireland, Italy, and Spain). Each study collected detailed information on hair-dye use (including duration of use, total number of applications, year of use, and type and colour of hair-dye) and included histologically-confirmed incident NHL cases. Among women, 75% of the cases and 70% of the controls reported ever having used hair dyes. An increased risk for NHL was observed among women who started using hair dyes before 1980, compared with non-users (OR, 1.3; 95% CI, 1.1–1.4). After stratification by NHL subtype, hair-dye use was associated with an increased risk for follicular lymphoma (FL) and chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) but not other NHL subtypes. The increased risk for FL (OR, 1.4; 95% CI, 1.1–1.9) and CLL/SLL (OR, 1.5; 95% CI, 1.1–2.0) associated with hair-dye use was mainly observed among women who started use before 1980, with a significant trend in risk with duration of use ($P < 0.01$, 0.02 respectively). For women who began using the products in 1980 or later, a higher risk for FL was limited to users of dark-coloured hair dyes (OR, 1.5; 95% CI, 1.1–2.0) with ORs of 1.5 (95% CI, 1.1–2.1) for permanent dark-coloured hair dyes and 1.7 (95% CI, 1.1–2.4) for non-permanent dark-coloured dyes. Among men, approximately 10% of cases and 10% of controls had ever used hair dyes. Risk for NHL was not associated with hair-dye use before or after 1980 among men. The results indicate that personal hair-dye use may play a role in the risk for FL and CLL/SLL in women who started use before 1980, and that an increased risk for FL in women starting use in 1980 or later cannot be excluded.

Table 2.12. Characteristics of case–control studies included in pooled analysis

Reference, study, period[†]	Location	Year	Age (years)	Cases (n=4461)[*]			Controls (n=5799)[*]			
				n	Source	Participation rate (%)	Matching Criteria	n	Participation rate (%)	Source
Zhang *et al*. (2004) Yale	Connecticut, USA	1996–2000	21–84	601	Population-based	72	Frequency matched by age within 5-year groups	717	RDD: 69 CMMS: 47	<65 years: RDD, >=65 years: random, selection from CMMS files
Morton *et al*. (2007) NCI-SEER	Detroit, Los Angeles, Seattle, and Iowa State, USA	1998–2001	20–74	1321 (1319)	Population-based	76	Frequency matched by age within 5-year groups, sex, and study site	1057 (1056)	52	<65 years:RDD, >=65 years:random, selection from CMMS files
Holly *et al*. (1998) UCSF	San Francisco Bay Area, CA, USA	1988–1993	21–74	1304 (837)	Population-based	72	Frequency matched by 5-year age groups, sex, and county of residence within 5 years	2402 (1609)	78	RDD and random selection from CMMS files for >=65 years old

Table 2.12 (contd)

Reference, study, period[†]	Location	Year	Age (years)	Cases (n=4461)[*]			Controls (n=5799)[*]			
				n	Source	Participation Rate (%)	Matching Criteria	n	Participation Rate (%)	Source
De Sanjosé *et al*. (2006) EpiLymph	Italy, Spain, Germany, France, Finland, Ireland, and Czech Republic	1998–2003	18+	2480 (1704)	Population-based (Italy, Germany), Hospital-based (Spain, France, Finland, Ireland, Czech Republic)	87	Age, sex, geographical area	2540 (2417)	75	Population-based (Italy, Germany), hospital-based (Spain, France, Finland, Ireland, Czech Republic)

From Zhang *et al*. (2008)

[†] Yale = Yale University Connecticut Women's NHL Study; NCI/SEER = National Cancer Institute (NCI)/Surveillance, Epidemiology, and End Results (SEER) Multi-Center Case-Control Study; UCSF = the Epidemiology of NHL Study from the University of California at San Francisco; EpiLymph = European multi-center case-control study coordinated by the International Agency for Research on Cancer.

CMMS, Centers for Medicare and Medicaid Service; RDD, random digit dialing.

[*] The numbers of cases and controls shown in parentheses are reported in the respective cited reference.

Table 2.13. Characteristics of non-Hodgkin lymphoma cases and controls

Characteristics	Cases (n=4461)		Controls (n=5799)	
	#	%	#	%
Study center				
Yale	601	13.5	717	12.4
NCI/SEER	1319	29.5	1056	18.2
UCSF	837	18.8	1609	27.7
EpiLymph	1704	38.2	2417	41.7
Gender				
Male	2123	47.6	2837	48.9
Female	2338	52.4	2962	51.1
Race				
White	4108	92.1	5347	92.2
Black	175	3.9	248	4.3
Others	178	4.0	204	3.5
Age (years)				
<30	147	3.3	309	5.3
30–39	362	8.1	599	10.3
40–49	644	14.4	798	13.8
50–59	971	21.8	1183	20.4
60–69	1330	29.8	1631	28.1
70–79	912	20.5	1159	20.0
80+	95	2.1	120	2.1
NHL subtype				
DLBCL	1543	34.6		
Follicular lymphoma	908	20.3		
CLL/SLL	736	16.5		
Marginal zone lymphoma	146	3.3		
T-cell lymphoma	298	6.7		
Others	830	18.6		

From Zhang *et al.* (2008)
DLBCL = diffuse large B-cell lymphoma ; CLL/SLL = small lymphocytic lymphoma/chronic lymphocytic leukemia; NHL = Non-Hodgkin lymphoma; NCI/SEER = National Cancer Institute/Surveillance, Epidemiology and End Results; Epylymph = European multicenter case-control study coordinated by the International Agency for Research on Cancer

Table 2.14. Risk of non-Hodgkin lymphoma and hair-dye use by year started and gender

	Total				Women				Men			
	Controls	Cases	OR (95%CI)	p	Controls	Cases	OR (95%CI)	p	Controls	Cases	OR (95%CI)	p
Hair-Dye Use												
Never	3432	2433			874	576			2558	1857		
Ever	2365	1915	1.0(0.9–1.2)	0.38	2087	1711	1.1(1.0–1.3)	0.04	278	204	0.9(0.7–1.1)	0.38
Type												
Permanent	1561	1217	1.0(0.9–1.2)	0.60	1433	1131	1.1(1.0–1.3)	0.09	128	86	0.9(0.6–1.2)	0.33
Non-permanent	997	854	1.1(1.0–1.3)	0.11	884	765	1.2(1.0–1.4)	0.02	113	89	1.1(0.8–1.4)	0.72
Color												
Dark color	1405	1158	1.0(0.9–1.1)	0.82	1252	1033	1.1(1.0–1.3)	0.12	153	125	0.9(0.7–1.1)	0.27
Light color	939	763	1.0(0.9–1.2)	0.54	879	734	1.1(1.0–1.3)	0.07	60	29	0.7(0.4–1.1)	0.15
Type & Color												
Permanent dark	898	697	1.0(0.9–1.1)	0.95	813	637	1.1(0.9–1.3)	0.22	85	60	0.8(0.6–1.2)	0.27
Permanent light	715	551	1.1(0.9–1.2)	0.35	671	530	1.2(1.0–1.4)	0.07	44	21	0.8(0.5–1.4)	0.48
Non-permanent dark	580	565	1.1(1.0–1.3)	0.17	525	507	1.2(1.0–1.5)	0.02	55	58	1.0(0.7–1.5)	0.87
Non-permanent light	158	144	1.0(0.8–1.3)	0.74	153	142	1.2(0.9–1.5)	0.20	5	2	0.5(0.1–2.6)	0.42
Started Use Before 1980												
Never	3432	2433			874	576			2558	1857		
Ever	1169	1053	1.2(1.0–1.3)	0.01	1102	997	1.3(1.1–1.4)	<0.01	67	56	1.0(0.7–1.5)	0.90
Type												
Permanent	778	678	1.2(1.0–1.4)	0.02	746	658	1.3(1.1–1.5)	<0.01	32	20	0.8(0.4–1.4)	0.45
Non-permanent	444	392	1.2(1.0–1.4)	0.06	413	365	1.2(1.0–1.5)	0.02	31	27	1.3(0.7–2.2)	0.41
Color												
Dark color	644	579	1.1(1.0–1.3)	0.07	607	547	1.2(1.1–1.5)	0.01	37	32	0.9(0.6–1.5)	0.69
Light color	479	462	1.2(1.1–1.5)	<0.01	469	457	1.3(1.1–1.6)	<0.01	10	5	0.7(0.2–2.2)	0.58
Type & Color												
Permanent dark	435	372	1.2(1.0–1.4)	0.05	413	356	1.3(1.0–1.5)	0.01	22	16	0.9(0.4–1.7)	0.67
Permanent light	363	328	1.3(1.1–1.5)	<0.01	354	326	1.3(1.1–1.6)	<0.01	9	2	0.4(0.1–2.0)	0.28
Non-permanent dark	226	232	1.1(0.9–1.4)	0.28	213	217	1.2(1.0–1.6)	0.06	13	15	1.1(0.5–2.3)	0.89
Non-permanent light	72	76	1.2(0.8–1.7)	0.32	72	76	1.3(0.9–1.9)	0.12	0	0		

Table 2.14 (contd)

	Total				Women				Men			
	Controls	Cases	OR (95%CI)	p	Controls	Cases	OR (95%CI)	p	Controls	Cases	OR (95%CI)	p
Started Use in 1980 or Later												
Never	3432	2433			874	576			2558	1857		
Ever	1157	844	1.0(0.9–1.1)	0.67	966	703	1.1(0.9–1.2)	0.40	191	141	0.9(0.7–1.1)	0.32
Type												
Permanent	709	480	0.9(0.8–1.1)	0.35	616	415	1.0(0.9–1.2)	0.86	93	65	0.9(0.6–1.3)	0.51
Non-permanent	486	401	1.1(0.9–1.3)	0.23	406	342	1.2(1.0–1.5)	0.05	80	59	1.0(0.7–1.4)	0.85
Color												
Dark color	716	548	1.0(0.8–1.1)	0.76	604	459	1.1(0.9–1.3)	0.32	112	89	0.8(0.6–1.1)	0.24
Light color	390	262	0.9(0.8–1.1)	0.35	341	238	1.0(0.8–1.3)	0.83	49	24	0.7(0.4–1.2)	0.20
Type & Color												
Permanent dark	421	288	0.9(0.8–1.1)	0.29	362	246	1.0(0.8–1.2)	0.97	59	42	0.8(0.5–1.2)	0.32
Permanent light	288	185	0.9(0.8–1.2)	0.58	254	166	1.0(0.8–1.3)	0.88	34	19	0.9(0.5–1.7)	0.84
Non-permanent dark	314	294	1.2(1.0–1.4)	0.13	272	254	1.3(1.0–1.6)	0.02	42	40	1.0(0.6–1.5)	0.84
Non-permanent light	71	52	0.9(0.6–1.3)	0.65	66	50	1.1(0.7–1.6)	0.77	5	2	0.5(0.1–2.6)	0.42

From Zhang *et al.* (2008)

Table 2.15. Risk of non-Hodgkin lymphoma and hair-dye use by NHL subtype and year started hair dye use among women

	DLBCL			Follicular			CLL/SLL			MZL			T-cell		
	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p
Hair-Dye Use															
Never	224			117			80			20			40		
Ever	564	1.0(0.9–1.2)	0.69	400	1.3(1.0–1.6)	0.02	244	1.3(1.0–1.6)	0.10	74	1.1(0.7–1.9)	0.69	109	1.0(0.7–1.5)	0.91
Type															
Permanent	351	1.0(0.8–1.2)	0.64	274	1.3(1.1–1.7)	0.02	163	1.2(0.9–1.6)	0.15	52	1.2(0.7–2.1)	0.42	65	0.9(0.6–1.4)	0.61
Non-permanent	261	1.1(0.9–1.4)	0.36	180	1.3(1.0–1.7)	0.03	101	1.3(0.9–1.7)	0.14	32	1.0(0.6–1.8)	0.97	58	1.3(0.8–2.0)	0.24
Color															
Dark color	314	0.9(0.8–1.2)	0.61	249	1.3(1.0–1.7)	0.02	148	1.2(0.9–1.7)	0.14	53	1.2(0.7–2.0)	0.57	65	1.0(0.6–1.4)	0.83
Light color	251	1.1(0.9–1.3)	0.4	173	1.3(1.0–1.7)	0.06	104	1.3(0.9–1.8)	0.12	29	1.0(0.6–1.8)	0.96	40	0.9(0.6–1.4)	0.58
Type & Color															
Permanent dark	185	0.9(0.7–1.1)	0.24	164	1.4(1.1–1.8)	0.01	96	1.3(0.9–1.7)	0.14	35	1.4(0.8–2.4)	0.3	38	0.9(0.6–1.4)	0.68
Permanent light	175	1.0(0.8–1.3)	0.76	124	1.3(1.0–1.7)	0.05	75	1.3(0.9–1.8)	0.18	22	1.3(0.7–2.5)	0.4	30	0.9(0.6–1.5)	0.69
Non-permanent dark	155	1.1(0.9–1.4)	0.43	118	1.4(1.0–1.8)	0.03	64	1.3(0.9–1.8)	0.18	24	1.0(0.5–1.8)	0.96	35	1.1(0.7–1.8)	0.61
Non-permanent light	45	1.1(0.8–1.6)	0.57	31	1.2(0.8–1.9)	0.34	27	1.7(1.0–2.7)	0.04	7	1.1(0.4–2.6)	0.86	11	1.3(0.7–2.7)	0.44
Started Use Before 1980															
Never	224			117			80			20			40		
Ever	311	1.1(0.9–1.3)	0.47	236	1.4(1.1–1.9)	<0.01	159	1.5(1.1–2.0)	<0.01	47	1.1(0.7–2.0)	0.64	55	1.0(0.6–1.5)	0.93
Type															
Permanent	204	1.1(0.9–1.3)	0.57	151	1.4(1.1–1.9)	<0.01	105	1.5(1.1–2.0)	0.01	33	1.4(0.8–2.4)	0.3	36	1.0(0.6–1.5)	0.9
Non-permanent	114	1.0(0.8–1.3)	0.78	84	1.4(1.0–1.9)	0.05	56	1.5(1.0–2.1)	0.04	18	1.1(0.6–2.1)	0.79	23	1.1(0.7–2.0)	0.62
Color															
Dark color	161	1.0(0.8–1.3)	0.94	124	1.4(1.0–1.8)	0.02	90	1.5(1.1–2.1)	0.01	31	1.3(0.7–2.3)	0.4	33	1.0(0.6–1.7)	0.86
Light color	145	1.2(0.9–1.5)	0.17	115	1.6(1.2–2.1)	<0.01	73	1.6(1.1–2.2)	<0.01	21	1.2(0.6–2.3)	0.59	19	0.8(0.4–1.4)	0.38

Table 2.15 (contd)

	DLBCL			Follicular			CLL/SLL			MZL			T-cell		
	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p	Cases	OR (95%CI)	p
Started Use Before 1980 (contd.)															
Type & Color															
Permanent dark	111	1.0(0.8–1.3)	0.77	80	1.4(1.0–1.9)	0.03	58	1.5(1.0–2.2)	0.03	21	1.6(0.8–3.0)	0.15	23	1.2(0.7–2.0)	0.58
Permanent light	102	1.1(0.9–1.5)	0.32	80	1.6(1.2–2.2)	<0.01	51	1.5(1.0–2.2)	0.03	15	1.5(0.8–3.1)	0.23	14	0.8(0.4–1.5)	0.5
Non-permanent dark	58	1.0(0.7–1.4)	0.99	44	1.2(0.8–1.8)	0.34	36	1.6(1.0–2.5)	0.03	12	1.0(0.5–2.1)	0.94	13	1.0(0.5–1.9)	1
Non-permanent light	19	1.0(0.6–1.7)	1	20	1.7(1.0–3.0)	0.05	15	1.9(1.1–3.6)	0.03	4	1.1(0.4–3.3)	0.9	5	1.2(0.4–3.2)	0.72
Started Use in 1980 or Later															
Never	224			117			80			20			40		
Ever	246	1.0(0.8–1.2)	0.89	163	1.3(1.0–1.7)	0.07	84	1.1(0.8–1.5)	0.63	27	1.0(0.6–1.9)	0.93	52	1.0(0.7–1.6)	0.93
Type															
Permanent	128	0.8(0.6–1.1)	0.12	104	1.3(1.0–1.8)	0.06	50	1.1(0.7–1.6)	0.8	18	1.2(0.6–2.5)	0.52	29	0.9(0.5–1.5)	0.66
Non-permanent	124	1.1(0.9–1.5)	0.3	80	1.4(1.0–2.0)	0.03	41	1.3(0.8–1.9)	0.24	12	0.9(0.4–2.0)	0.85	33	1.5(0.9–2.6)	0.09
Color															
Dark color	149	0.9(0.7–1.2)	0.65	114	1.5(1.1–2.0)	0.02	54	1.1(0.8–1.6)	0.6	22	1.2(0.6–2.2)	0.66	31	0.9(0.6–1.6)	0.82
Light color	88	1.0(0.7–1.3)	0.98	51	1.1(0.8–1.6)	0.58	28	1.1(0.7–1.7)	0.75	5	0.6(0.2–1.6)	0.28	20	1.1(0.6–1.9)	0.8
Type & Color															
Permanent dark	69	0.7(0.5–1.0)	0.05	69	1.5(1.1–2.1)	0.02	32	1.1(0.7–1.8)	0.62	14	1.4(0.7–2.9)	0.36	14	0.7(0.4–1.4)	0.35
Permanent light	60	1.0(0.7–1.3)	0.77	38	1.2(0.8–1.8)	0.35	17	0.9(0.5–1.7)	0.82	3	0.6(0.2–2.2)	0.45	16	1.2(0.6–2.2)	0.62
Non-permanent dark	85	1.2(0.9–1.6)	0.28	61	1.7(1.1–2.4)	<0.01	27	1.3(0.8–2.1)	0.35	11	1.0(0.5–2.3)	0.9	22	1.4(0.8–2.5)	0.23
Non-permanent light	19	1.1(0.6–1.9)	0.75	9	1.0(0.5–2.1)	0.98	11	1.8(0.9–3.7)	0.09	1	0.5(0.1–4.0)	0.52	5	1.4(0.5–3.8)	0.48

From Zhang *et al.* (2008)
DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MZ, marginal-zone lymphoma; T-cell, T-cell lymphoma.
Adjusted for age (continuous), race (white, black, others), and study site.

Table 2.16. Associations between hair dye use and risk of follicular lymphoma and CLL/SLL among women by duration, frequency, and total applications

	Follicular lymphoma						SLL/CLL					
	Overall		Started use before 1980		Started use in 1980 or later		Overall		Started use before 1980		Started use in 1980 or later	
	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
DURATION (in years)												
Any hair-dye use												
<8	1.2(0.9–1.6)	0.23	1.4(0.9–2.1)	0.11	1.2(0.9–1.7)	0.21	1.2(0.8–1.6)	0.40	1.5(1.0–2.4)	0.08	1.1(0.7–1.7)	0.61
8–19	1.3(1.0–1.7)	0.09	1.3(0.9–1.9)	0.11	1.4(1.0–1.9)	0.08	1.3(0.9–1.8)	0.16	1.6(1.0–2.4)	0.03	1.2(0.8–1.8)	0.46
20+	1.5(1.1–1.9)	0.01	1.5(1.1–2.0)	<0.01	1.5(0.6–4.0)	0.41	1.3(1.0–1.8)	0.08	1.5(1.1–2.0)	0.02	0.3(0.0–2.0)	0.20
p for trend	0.01		<0.01		0.06		0.07		0.02		0.90	
Type												
Permanent												
<8	1.3(0.9–1.7)	0.13	1.6(1.0–2.5)	0.03	1.2(0.8–1.7)	0.35	1.0(0.7–1.5)	0.96	1.2(0.7–2.2)	0.53	1.0(0.6–1.6)	0.95
8–19	1.3(1.0–1.8)	0.08	1.2(0.8–1.8)	0.44	1.5(1.0–2.2)	0.03	1.3(0.9–1.9)	0.17	1.5(0.9–2.5)	0.09	1.2(0.7–2.0)	0.43
20+	1.4(1.1–1.9)	0.02	1.5(1.1–2.0)	0.01	1.9(0.6–5.6)	0.26	1.4(1.0–2.0)	0.05	1.5(1.1–2.2)	0.02	0.4(0.1–3.3)	0.42
p for trend	0.02		0.02		0.02		0.03		0.01		0.75	
Non-permanent												
<8	1.3(0.9–1.9)	0.14	1.2(0.7–1.9)	0.54	1.5(0.9–2.4)	0.09	1.0(0.6–1.7)	0.94	1.1(0.6–2.1)	0.81	1.1(0.6–2.3)	0.71
8–19	1.3(0.8–2.1)	0.31	1.5(0.7–3.2)	0.28	1.3(0.7–2.4)	0.49	2.0(1.1–3.4)	0.02	2.3(1.0–5.2)	0.04	1.9(0.9–3.9)	0.08
20+	1.3(0.9–1.7)	0.12	1.3(0.9–2.0)	0.13	1.4(0.9–2.2)	0.13	1.2(0.8–1.8)	0.34	1.6(1.0–2.5)	0.03	0.9(0.5–1.7)	0.84
p for trend	0.11		0.13		0.10		0.15		0.02		0.90	
Color												
Dark color												
<8	1.3(1.0–1.8)	0.05	1.3(0.8–2.0)	0.27	1.4(1.0–2.1)	0.05	1.2(0.8–1.7)	0.43	1.3(0.8–2.3)	0.29	1.1(0.7–1.8)	0.59
8–19	1.2(0.9–1.7)	0.22	1.0(0.6–1.7)	0.87	1.6(1.1–2.3)	0.02	1.2(0.8–1.8)	0.28	1.6(0.9–2.7)	0.08	1.2(0.7–2.0)	0.46
20+	1.5(1.1–2.2)	0.01	1.6(1.1–2.3)	0.01	1.5(0.4–5.2)	0.53	1.4(1.0–2.1)	0.08	1.6(1.1–2.4)	0.02	0.4(0.1–3.3)	0.42
p for trend	0.02		0.01		0.04		0.08		0.01		0.74	

Table 2.16 (contd)

	Follicular Lymphoma						SLL/CLL					
	Overall		Started use before 1980		Started use in 1980 or later		Overall		Started use before 1980		Started use in 1980 or later	
	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
DURATION (in years)												
Light color												
<8	1.2(0.9–1.7)	0.22	1.6(1.1–2.6)	0.03	1.2(0.8–1.9)	0.41	1.0(0.7–1.7)	0.84	1.2(0.6–2.2)	0.66	1.1(0.6–2.0)	0.82
8–19	1.1(0.8–1.6)	0.56	1.7(1.1–2.8)	0.03	0.9(0.5–1.6)	0.69	1.2(0.7–1.8)	0.50	1.7(0.9–3.1)	0.08	1.2(0.7–2.2)	0.55
20+	1.5(1.1–2.1)	0.02	1.5(1.1–2.1)	0.02	2.7(0.7–10.1)	0.13	1.6(1.1–2.4)	0.01	1.7(1.2–2.6)	0.01	–	
p for trend	0.02		0.01		0.60		0.02		<0.01		0.91	
FREQUENCY (# of applications per year)												
Any hair-dye use												
<5	1.3(1.0–1.7)	0.09	1.4(1.0–2.0)	0.05	1.3(0.9–1.8)	0.21	1.1(0.7–1.5)	0.78	1.2(0.8–1.9)	0.42	1.0(0.6–1.7)	0.86
5–8	1.2(0.9–1.6)	0.17	1.4(1.0–2.0)	0.05	1.1(0.8–1.7)	0.49	1.2(0.8–1.7)	0.30	1.7(1.2–2.5)	0.01	0.7(0.4–1.3)	0.27
9+	1.3(1.0–1.8)	0.03	1.4(1.0–1.9)	0.04	1.5(1.0–2.2)	0.04	1.6(1.1–2.1)	0.01	1.7(1.2–2.4)	<0.01	1.6(1.0–2.5)	0.04
p for trend	0.05		0.03		0.06		<0.01		<0.01		0.19	
NUMBER OF TOTAL APPLICATIONS												
Any hair-dye use												
<31	1.2(0.9–1.6)	0.20	1.4(0.9–2.0)	0.10	1.2(0.9–1.7)	0.25	1.1(0.8–1.6)	0.58	1.3(0.8–2.1)	0.22	1.1(0.7–1.7)	0.77
31–138	1.2(0.9–1.6)	0.15	1.2(0.9–1.8)	0.24	1.4(1.0–1.9)	0.08	1.2(0.8–1.7)	0.31	1.6(1.1–2.5)	0.02	0.9(0.6–1.5)	0.75
139+	1.4(1.1–1.9)	0.01	1.5(1.1–2.0)	0.01	1.3(0.8–2.3)	0.33	1.5(1.1–2.1)	0.01	1.6(1.1–2.2)	0.01	1.8(1.0–3.2)	0.04
p for trend	0.02		0.01		0.07		0.01		<0.01		0.24	

From Zhang *et al.* (2008)
CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma
Adjusted for age (continuous), race (white, black, others), and study sites

3. Studies of Cancer in Experimental Animals

3.1 Occupational use of hair dyes: Hair-dye formulations and some of their components

3.1.1 *Skin application*

(a) Mouse

Groups of 50 male and 50 female Swiss Webster mice, 6–8 weeks of age, received applications of one of three oxidation (permanent) hair-dye formulations, PP-7588, PP-7586 or PP-7585 (all three contained 2,5-toluenediamine sulfate, *para*-phenylenediamine and resorcinol; PP-7586 also contained 2,4-diaminoanisole sulfate, PP-7585 contained *meta*-phenylenediamine, and PP-7588 contained 2,4-toluene-diamine) [see reference for additional details on composition], mixed with an equal volume of 6% hydrogen peroxide just before use; 0.05 mL of the mixture in acetone was applied to the shaved skin of the interscapular region. Controls were given acetone or were left untreated. For each formulation and for the vehicle control, one group was treated once weekly and another group once every other week for 18 months. Survival at 18 months varied from 58% to 80%. No sign of systemic toxicity was found in any of the dye-treated groups. Average body weights were comparable in all groups throughout the study. The incidence of tumours at all sites, including lung tumours, was not statistically different between treated and control groups. No skin tumours were observed at the site of application (Burnett *et al.*, 1975).

The chronic toxicity of 2,4-toluenediamine (2,4-TDA) alone [purity not specified] or in combination with a hair-dye complex (2,5-toluenediamine, *para*-phenylenediamine, and resorcinol) [see reference for additional details on composition] was studied in groups of 28 male and 28 female Swiss Webster mice, 4–7 weeks of age, and weighing 15–20 g by use of a skin-painting technique, whereby a 6% solution of the study materials in a water/isopropanol solvent were mixed with equal volumes of either 6% peroxide or distilled water at doses of 50, 150 or 1500 μg 2,4-TDA per week. Additional groups of vehicle and untreated controls were used. No information on survival was provided. The predominant neoplasms seen in these mice were primary pulmonary adenomas and adenocarcinomas. Skin neoplasms were seen in most groups of mice, including untreated controls. Statistical analysis of the incidences of skin neoplasms among the various groups of mice did not show any significant differences. The 2,4-TDA, alone or mixed with the hair-dye complex, did not produce any abnormal proliferation and maturation of the squamous epithelium of the skin (Giles *et al.*, 1976). [The Working Group noted the lack of information on survival, and the use of water as the skin-painting solvent.]

Groups of 26 male and 22 female DBAf and 26 male and 26 female strain-A mice, 6–7 weeks of age, received skin applications of 0.4 mL (reduced to 0.2 mL at 24 weeks for

DBAf mice) of a 10% solution of a commercially available semi-permanent hair dye ('GS'), containing, among other constituents [unspecified], 1,4-diamino-2-nitrobenzene (2-nitro-*para*-phenylene-diamine) and 1,2-diamino-4-nitrobenzene (4-nitro-*ortho*-phenylenediamine) in 50% aqueous acetone twice a week on the clipped dorsal skin. Groups of 16 male and 16 female control mice of each strain received applications of acetone alone. When the experiment was terminated at 80 weeks, four (18%) lymphomas and six (27%) tumours of the reproductive tract (four ovarian cystadenomas and two uterine fibrosarcomas) had developed in the 22 treated female DBAf mice within 37–80 weeks, and one (4%) lymphoma had developed by week 26 among the 26 treated males. In control DBAf mice, 1 lymphoma and 1 lung adenoma were found in females and 1 hepatoma in males. No difference was observed in the incidence of lymphomas or liver or lung tumours between treated and control strain-A mice. No skin tumour at the site of application was observed in either strain. Of the treated animals, 27 DBAf mice and 32 strain-A mice survived 60–80 weeks without tumours (Searle & Jones, 1977). [The Working Group noted that this study was conducted with a low concentration of the formulation used in this study.]

In the same study, groups of 17 male and 15 female DBAf and 16 male and 16 female strain-A mice, 6–7 weeks of age, received skin applications of 0.4 mL (reduced to 0.2 mL at 24 weeks for DBAf mice) of a 10% solution of a commercially available semi-permanent hair dye ("RB"), containing, among other constituents [unspecified], 4-amino-2-nitrophenol and CI Acid Black 107, in 50% aqueous acetone twice a week on the clipped dorsal skin. The experiment was terminated at 80 weeks. No significant difference was observed in the incidence of tumours at any site between treated and control animals of either strain, and no skin tumour at the site of application was observed in either strain (Searle & Jones, 1977). [The Working Group noted the low concentration of the formulation used in this study.]

In an interim report of the Searle and Jones (1977) study mentioned above, it was noted that the tumours arose consistently earlier in the treated than in the control mice, notably in the DBAf strain, in which the first lymphoma was detected after 26 weeks. Apart from the early appearance, there was also an increased tumour incidence in this strain, due mainly to uterine and ovarian tumours that were not seen in the control group (Venitt & Searle, 1976).

Twelve groups of 50 male and 50 female random-bred Swiss Webster mice, 6–8 weeks of age, were exposed dermally, once per week for 21–23 months, to different hair-dye formulations by placing a 0.05-mL sample on a 1-cm^2 area of the interscapular region, which had been clipped free of hair 24 hours before treatment. Nine oxidative hair dyes (7401, 7402, 7403, 7404, 7405, 7406, P-21, P-25, and P-26) were mixed with an equal volume of 6% hydrogen peroxide and 0.025 ml was applied within 15 minutes. Three semi-permanent hair-dye formulations (P-22, P-23, and P-24) were applied in 0.05 ml within 30 minutes after opening the bottle. In an earlier study, the composition of these hair-dye formulations was given in an extensive table (Burnett *et al.*, 1976). Three groups of 50 males and 50 females served as controls that had their backs shaved but were not

further treated. After seven and nine months, groups of 10 females and 10 males were randomly selected from each group, necropsied and examined by histopathology. The experiment was terminated after 21–23 months. The treatments had no effect on survival. The incidences of skin tumours and liver and lung lymphomas were not greater than in control mice (Burnett *et al.*, 1980).

Groups of 60 male and 60 female Swiss Webster mice, eight weeks of age, received topical applications of two oxidative and 12 non-oxidative hair-dye formulations supplied by five cosmetic companies. The composition of the hair-dye formulations was described. Two groups of 60 male and 60 female Swiss Webster mice served as controls. Each oxidative formulation was applied at 0.05 mL/mouse once weekly for 20 months. Each non-oxidative dye was applied at a dose of 0.05 mL per mouse three times weekly for 20 months. The mice were shaved 24 hours before treatment as needed. Control animals were shaved only and received no treatment. The application of hair dyes did not have an adverse effect on average body-weight gain or survival of any group. There was no significant increase in the incidence of malignant lymphomas in male mice or in liver hemangiomas or lung adenomas of both sexes in these studies. Significant increases in numbers of malignant lymphomas over those in one of the untreated control groups were observed in female mice in the hair dye-formulation groups treated with formulation 7602A (19/60 (32%) treated *vs* 7/60 (12%) control, $P < 0.01$), formulation 7605 (18/60 (30%) treated *vs* 7/60 (12%) control, $P < 0.05$) and formulation 7610 (23/60 (38%) treated *vs* 7/60 (12%) control, $P < 0.01$). The authors pointed out that the incidence in each treated group is not significantly different from the incidence in the second control group in this study, and is within the range of control values previously reported for this strain of mouse. They concluded that these tumours were probably not treatment-related (Jacobs *et al.*, 1984).

(b) Rat

Groups of 50 male and 50 female Sprague-Dawley rats, approximately 14 weeks of age, received topical applications of 0.5 mL of permanent hair-dye mixtures containing [purity unspecified] either 4% *para*-toluenediamine or 3% *para*-toluenediamine, 0.75% resorcinol and 0.75% *meta*-diaminoanisole in vehicle solution (4% Tylose HT, 0.5% sodium sulfite, 8.5–13% ammonia (25%), 3.7% ammonium sulfate or as formed by neutralization and deionized water to 100.0%), with 6% hydrogen peroxide added immediately before use on a 3-cm^2 area of shaved dorsal skin twice a week for two years. The animals were then observed for a further six months. Control groups of 25 males and 25 females of the same strain and age received topical applications of 0.5 mL vehicle alone, to which 6% hydrogen peroxide was added immediately before use. Another group of 50 males and 50 females of the same strain served as untreated controls. No difference in survival was observed between treated, vehicle control and untreated control groups. The skin at the application site, and the liver, kidney, lung and gross lesions were studied histologically. No skin tumour was observed at the site of application, and there was no

significant difference in the incidence of tumours, including those of the skin, between treated, vehicle control and untreated control groups (Kinkel & Holzmann, 1973).

Groups of 10 male and 10 female Wistar rats weighing 120–140 g received topical applications of 0.5 mL oxidized *para*-phenylenediamine [purity unspecified] (1:1 mixture of 5% *para*-phenylenediamine and 2% ammonium hydroxide) and 6% hydrogen peroxide on shaved dorsal skin once a week for 18 months. Control rats were shaved and treated with the corresponding vehicle. Treated and control groups did not differ significantly in body weight gain or survival. All surviving rats were killed after 21 months. Treated rats had a significantly increased incidence of mammary tumours (5/10 (50%); $P < 0.05$ [incidental tumour test]) in comparison with female vehicle-controls (0/9). The first mammary tumour observed was a fibrosarcoma, which occurred at week 47; the others were three adenomas and one fibroadenoma. No skin tumour was observed at the site of application (Rojanapo *et al.*, 1986).

Groups of 60 male and 60 female Sprague-Dawley rats (aged 6–8 weeks) received topical applications of an oxidative hair-dye formulation (7406) containing 0.5% 2-amino-5-nitrophenol, 4.0% *para*-phenylenediamine, 0.7% *para*-aminophenol, 2.0% 4-chlororesorcinol, 5.0% oleic acid, 15.0% isopropanol, 0.2% sodium sulfite, 6.0% ammonia and water to 100%. The formulation was diluted in an equal volume of 6% hydrogen peroxide before application, and 0.5 mL were applied to a shaved area of the back (approx. 2.5 cm in diameter) twice a week up to week 117. Three separate, similarly treated, concurrent control groups of 60 rats received applications of the vehicle alone. Mean body weights and survival were similar in treated and control groups. No skin tumours were observed. The incidence of pituitary adenomas was increased in females in comparison with all three control groups (45/51 (88%) *vs* 34/50 (65%), 36/51 (71%) and 35/50 (70%); $P < 0.05$, χ^2-test). The incidence of mammary gland adenomas was increased in comparison with one control group (6/53 (11%) *vs* 0/49; $P < 0.05$, χ^2-test). The authors concluded that the tumours were probably not treatment-related (Burnett & Goldenthal, 1988).

In the same study, groups of 60 male and female Sprague-Dawley rats, 6–8 weeks of age, received topical applications of an oxidative hair-dye formulation (7405) containing 0.4% 2-amino-4-nitrophenol, 6.0% 2,5-diaminoanisole sulfate, 2.0% resorcinol, 0.3% *ortho*-aminophenol, 5.0% oleic acid, 3.0% isopropanol, 0.2% sodium sulfite, 6.0% ammonia (29%) and water to 100%. The formulation was diluted in an equal volume of 6% hydrogen peroxide, and 0.5 mL was applied to a shaved area of the back (approx. 2.5 cm in diameter) twice a week up to week 117. Mean body weights and survival were similar in treated and control groups. No skin tumours were observed. The incidence of pituitary adenomas was increased in males in comparison with one of the control groups 35/52 (67%) *vs* 14/49; $P < 0.01$; χ^2-test), and no increase in the incidence of tumours at any other site was observed in treated rats compared with control animals. The authors concluded that the tumours were probably not treatment-related (Burnett & Goldenthal, 1988).

In the same study, groups of 60 male and female Sprague-Dawley rats, 6–8 weeks of age, received topical applications of an oxidative hair-dye formulation (7403) containing 6.0% *para*-toluenediamine sulfate, 0.7% *meta*-aminophenol, 1.0% *para*-aminophenol, 0.25% 4-nitro-*ortho*-phenylene diamine, 0.50% 1-naphtol, 15% oleic acid, 10% isopropanol, 4.5% glycerine, 9.0% propylene glycol, 0.2% sodium sulphite, 9.0% ammonia and water to 100%. The formulation was diluted in an equal volume of 6% hydrogen peroxide before application, and 0.5 mL was applied to a shaved area of the back (approx. 2.5 cm in diameter) twice a week until week 117. Mean body weights and survival were similar in treated and control groups. The incidence of mammary gland adenomas was increased in comparison with one control group (10/52 (19%) *vs* 0/49; $P < 0.01$, χ^2-test). There was no significant increase in the incidence of tumours at any site, and no skin tumour was observed. The authors concluded that the tumours were probably not treatment-related (Burnett & Goldenthal, 1988). [The Working Group wondered why the tumours were assumed not to be treatment-related.]

3.1.2 *Subcutaneous injection*

(a) Rat

Groups of 10 male and 10 female rats weighing 120–140 g received subcutaneous injections of 0.5 mL oxidized *para*-phenylenediamine (5% *para*-phenylenediamine in 2% ammonium hydroxide and 1.8% sodium chloride) in an equal volume of 6% hydrogen peroxide in the hip area every other week for 18 months. Controls were injected similarly with vehicle only. There was no significant difference between treated and control groups in body-weight gain or survival. All survivors were killed after 21 months. The incidence of mammary lesions [duct ectasia or adenosis] was significantly increased (4/7 (57%); $P < 0.05$ incidental tumour test) in females in comparison with vehicle controls (0/10). Two uterine tumours, an adenocarcinoma and an endometrial polyp were observed in females; no such tumours were observed in controls. Two sarcomas [not otherwise classified] at the injection site and two lipomas were also observed in treated animals (Rojanapo *et al.*, 1986). [The Working Group noted the small number of animals used in this study.]

3.2 Personal use of hair dyes

No data were available to the Working Group

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

No data were available to the Working Group.

4.2 Genetic and related effects

4.2.1 *Occupational use of hair dyes*

(a) Humans

Chromosomal aberrations in peripheral lymphocytes were examined in a study of 60 professional hair colourists (28 men, 28.4 ± 9.4 years old; 32 women, 23.3 ± 5.1 years old) and 36 control subjects matched for age and sex (17 men, 28.1 ± 7.3 years old; 19 women, 25.3 ± 6.5 years old) (Kirkland *et al.*, 1978) in the United Kingdom. Information was recorded on smoking habits, alcohol consumption, medical history, use of medicinal drugs and drugs of abuse, infections, vaccinations and exposure to X-rays. Details of occupational exposure to hair dyes were also collected: women had done an average of 11 000 permanent and 5000 semipermanent hair-tinting operations and men had done 15 000 permanent and 6000 semi-permanent operations, over periods ranging from 1 to 15 years. Blood samples were taken at the time of interview, but the time since the last hair-tint application (to themselves or clients) was not recorded. Among the chromosomal aberrations, more gaps were found per cell among female tinters than in controls (0.065 *vs* 0.048; $P < 0.02$) but not among male tinters (0.064 *vs* 0.063). The number of breaks per cell (assumed from the observed aberrations) was not altered among women (0.028 *vs* 0.031) but was lower among men (0.034 *vs* 0.047; $P < 0.05$). After exclusion of subjects exposed to high doses of diagnostic X-rays or who recently had viral infections these differences disappeared (breaks in tinters *vs* controls: women, 0.023 *vs* 0.027; men, 0.036 *vs* 0.038). Reallocation of this smaller set of subjects according to whether or not their own hair was dyed revealed that the number of breaks per cell was higher among women who dyed their hair (dyed *vs* not dyed, 0.031 *vs* 0.018; $P < 0.02$) and lower among men who dyed their hair (0.023 *vs* 0.044; $P < 0.01$). The women had given themselves an average of 90 permanent and 10 semi-permanent tints and the men an average of 30 permanent tints [semi-permanent tints not indicated] over a period similar to their occupational exposure. The authors stated that there was no association between chromosomal damage and the duration and/or frequency of hair dyeing in the women. [The Working Group noted the absence of data to substantiate this statement.] They reported that 20/23 female and 11/18 male tinters wore protective gloves for all applications of permanent and semi-permanent hair tints and deduced that most of the subjects would receive greater exposure to hair-dye components when their own hair was treated. The finding that the number of breaks per cell was lower among men who dyed their hair than among those who did not was explained by the age difference between the group with tinted hair (22.7 ± 5.1 years, $n = 10$) and the group with non-tinted hair (31.8 ± 10.1, $n = 17$). Kirkland *et al.* (1978) based their argument on the observation of Brown *et al.* (1965) that there was much less chromosomal damage of all types in 48-hour blood cultures from men aged 15–24 than from men aged 25–34, whereas there was no difference among women in these age ranges.

Babish *et al.*, (1991) conducted a study in New York State, USA, on cosmetologists (91 women, 7 men) who were occupationally exposed to a wide range of chemicals, including hair dyes, and who had reported a prevalence of skin rashes that was twice as high as that of a control group of 87 female dental personnel (29% *vs* 15%). The two groups were matched for median age, smoking status and proportion of subjects (13–16%) who had had their hair permed or dyed within seven days of the study. At the end of a normal working day, subjects from each group provided a urine sample, which was later concentrated and tested for mutagenicity in *S. typhimurium* TA100 in the presence and absence of S9. In the presence of S9, there was no difference between the groups, but in tests conducted without S9 the frequency of mutagenic urine samples was 15% higher among cosmetologists (39%) than among dental personnel (24%). Multivariate analysis, with adjustment for age and smoking habits, revealed an OR of 2.0 (95% CI, 1.1–3.8) for the presence of urinary mutagens in cosmetologists compared with dental personnel. [The Working Group noted the inadequate reporting of the results.]

Sardaş *et al.* (1997) assessed the cytogenic effects of occupational exposure to oxidation hair dyes by using three assays in professional hair colourists. The assays were analysis of sister chromatid exchange (SCE) in circulating lymphocytes to evaluate the interchange of DNA-replication products at apparently homologous chromosomal loci, the single-cell gel electrophoresis assay to detect the presence of DNA strand-breaks/alkali-labile damage, and the Ames assay with *Salmonella typhimurium* strain TA98 to detect the mutagenicity of the urine. The ability of these assays to detect genetic damage caused by oxidation hair dyes compared with closely matched controls produced the following findings: (i) The SCE assay could not detect an effect in lymphocytes of exposed subjects from whom complete data were obtained. However, subjects (controls and exposed) with a history of smoking had a slightly higher frequency of SCE than the non-smokers in both groups. (ii) The extent of DNA migration (single-cell gel electrophoresis assay) did not distinguish between the samples of exposed and control subjects. Like the SCE results, the exposed smokers and control smokers showed a greater proportion of damaged lymphocytes with apparent change in migration of DNA in the single-cell gel electrophoresis assay. (iii) No clear differences in the mutagenic activity of the urine samples were observed between the exposed and control subjects.

(b) Experimental systems

Wang *et al.* (1991) showed that of 169 commercial oxidative-type (hydrogen peroxide) hair-dye formulations, 150 (89%) were mutagenic in the *Salmonella* mutagenicity assay. Of the 18 components of these hair dyes, nine showed various degrees of mutagenicity: 2,4-diaminoanisole, 4-nitro-*ortho*-phenylenediamine, 2-nitro-*para*-phenylenediamine, 2,5-diaminoanisole, 2-amino-5-nitrophenol, *meta*-phenylene-diamine, *ortho*-phenylenediamine, 2-amino-4-nitrophenol, and 2,5-diaminotoluene. Three hair-dye components (*para*-phenylenediamine, 2,5-diaminotoluene and 2,5-diamino-anisole) became strongly mutagenic after oxidation by hydrogen peroxide: the mutagenic product of *para*-phenylenediamine was identified as the known trimer Bandrowski's base.

[The Working Group considered formation of this mutagenic product unlikely to happen under practical hair-dyeing conditions.] 2,4-Diaminotoluene, a hair-dye component, was also mutagenic: this compound has been shown to be a carcinogen in rats and is used in large amounts in the polyurethane-foam industry. [The Working Group noted that this compound has been taken off the market in the early 1970s] (Ames *et al.*, 1975).

A total of 13 commercial hair-dye products made in China were tested for mutagenicity in two short-term assays, the *Salmonella typhimurium* mutagenicity test in strains TA98 and TA100 and the in-vivo micronucleus assay with mouse bone-marrow polychromatic erythrocytes. The results showed that the 13 hair dyes were not mutagenic in strains TA98 and TA100 with or without S9. In the micronucleus test, no effect was observed (Wang *et al.*, 1991).

Albano *et al.* (1982) found that of 25 commercial permanent hair-dye formulations containing *para*-phenylenediamine, resorcinol and aminophenols incubated with hydrogen peroxide, 12 were mutagenic to *S. typhimurium* TA98 only in the presence of S9. Without the addition of hydrogen peroxide, mutagenicity was reduced for three dyes and disappeared for three others. Four of six formulations, with degrees of mutagenicity varying from zero to high, administered topically with 3% hydrogen peroxide to male rats, induced urine that was mutagenic to *S. typhimurium* TA98 in the presence of S9.

Ferguson *et al.* (1990) tested 40 products, chosen from among 12 brands of commercially available hair colourants used in New Zealand, for mutagenicity in *S. typhimurium* TA98 and TA100 without S9; activators were added when recommended. Twenty-three of these products were mutagenic in one or both strains. When 10 mutagenic hair-dye preparations were tested in the presence of the drug verapamil, used for treating cardiac conditions (Ferguson & Baguley, 1988), the mutagenic activity of four was decreased and that of two was increased (Ferguson *et al.*, 1990).

Watanabe *et al.* (1990) found that two of four commercial hair-dye formulations containing phenylenediamines and aminophenols (two of which also contained 2,5-diaminophenol), when oxidized with 6% hydrogen peroxide, were mutagenic to *S. typhimurium* TA98 in the presence of Kanechlor 500-induced S9. When toxicity was reduced by adsorbing bactericidal products on blue rayon, peroxide treatment increased the mutagenicity of all preparations to different extents; in the two preparations with markedly increased mutagenicity, activity was attributed to the oxidation of *meta*-phenylenediamine to 2,7-diaminophenazine, itself a potent mutagen.

Ammenheuser and Warren (1979) applied two commercial oxidative hair-colouring products at 10–30 mL, both with and without hydrogen peroxide, to the backs of male Sprague-Dawley rats [number unspecified]. Both colourants contained 1,4-diamino-2-nitrobenzene and 1,2-diamino-4-nitrobenzene (4-nitro-*ortho*-phenylenediamine). The solutions were left on the hair for 20 min and then removed by shampooing and rinsing. Urine was collected before and every 24 hours after product application for four days and tested on *S. typhimurium* TA1538, the volumes of urine applied to each plate varying from 3.4 to 11.5% of the total volume. Urine samples collected during the first 24 hours from rats treated with either of the preparations were mutagenic (two to three times

background); no significant mutagenicity was observed in urine samples collected two to four days after application. Prior reaction with hydrogen peroxide had little or no effect on the mutagenicity of the urine.

Stamberg *et al.* (1979) tested henna and its active colouring ingredient, 2-hydroxy-1,4-naphthoquinone, for mutagenicity in *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538. Henna was not mutagenic to any strain, but 2-hydroxy-1,4-naphthoquinone was mutagenic to TA98, in the absence of S9 only.

Matsuki *et al.* (1981) isolated 2-amino-5-methoxy-2′(or 3′)-methylindamine and 2-amino-5-methoxy-2′(or 3′)-methylindoaniline from an oxidative reaction mixture of 2,5-diaminotoluene and 2,4-diaminotoluene. These compounds were found to be highly mutagenic to *S. typhimurium* TA98 in the presence of an exogenous metabolic system.

Burnett *et al.* (1981), in a study of heritable translocation, painted groups of 25 male Sprague-Dawley CD rats twice weekly for 10 weeks on the shaved dorsal skin with 0.5 mL of a semi-permanent dye formulation (comprising base ingredients plus 0.12% CI Disperse Blue 1, 0.04% CI Disperse Black 9, 0.01% HC Red No. 3, 0.21% HC Yellow No. 3, 0.50% HC Blue No. 1, 0.06% Acid Orange No. 3, 0.07% CI Disperse Violet No. 11 and 0.01% HC Yellow No. 2), or with 0.5 mL of an oxidative dye formulation (comprising base ingredients plus 2.2% *para*-phenylenediamine, 3.1% *N,N*-bis(2-hydroxyethyl)-*para*-phenylenediamine sulfate, 1.0% resorcinol and *meta*-aminophenol, mixed 1:1 with 6% hydrogen peroxide just before use). The animals were then mated with untreated female rats. Male F_1 progeny were subsequently mated with other untreated females, and the resulting pregnancies were arrested at day 16 of gestation. No difference in average litter size or frequency of successful matings at the F_1 mating was seen between controls and the two exposed groups. Furthermore, there was no effect on the number of live fetuses, implantations or resorptions at the F_2 (total litters analysed: 275 controls, 261 in the oxidation dye group and 271 in the semi-permanent dye group).

4.2.2 *Personal use of hair dyes*

Hofer *et al.* (1983) studied chromosomal aberrations in lymphocytes from six women and four men who volunteered to have their hair dyed and a similar group of 10 controls matched for age (men: hair-dyed, 35.7 ± 6.7; controls, 30.8 ± 6.4; women: hair-dyed, 30.3 ± 5.7; controls, 35.0 ± 5.8). Records were taken of smoking habits, alcohol consumption and medical drug use and, during the experiment, exposure to X-rays, illness and vaccinations. There were more smokers in the test group. None of the volunteers had used hair dyes or shades for at least one year before entering the study, and the control group did not use hair colourants during the study. The treated group had their hair dyed 13 times at intervals of three to six weeks with commercial preparations containing mixtures of aminotoluenes, aminophenols and hydroxybenzenes and, in some cases, naphthol, as active ingredients; the colouring product used was chosen according to each subject's hair colour, and the same material was used throughout the study. The colouring preparations were mixed (1:1) with 3–6% hydrogen peroxide. Nine blood samples were

taken: three weeks before the first treatment (control sample), 24 hours after a sham dyeing (no dye or hydrogen peroxide) and 24 hours after each of the first three and last four dyeing procedures. No difference was observed between the control and treated groups in the percentage of cells with one or more structural aberrations (excluding gaps) before treatment, after sham-dyeing or after treatment. Subdivision of the groups according to sex revealed no difference. A significant increase in aberration rate with age was observed among the male but not the female subjects. Neither smoking nor X-ray exposure had an effect. In conjunction with this study, sister chromatid exchange was examined in peripheral lymphocytes; no evidence was found of an effect on the frequency (Turanitz *et al.*, 1983).

Kirkland *et al.* (1981) studied sister chromatid exchange in the peripheral lymphocytes of a small group of volunteers comprising 13 women and one man immediately before and 6 hours and seven days after one normal application of four semi-permanent and 10 permanent hair dyes, all of which were mutagenic to *Salmonella typhimurium* TA1538 and TA98 in the presence of metabolic activation. There was no consistent increase in the frequency of sister chromatid exchange per cell.

In a study in the USA involving 30 women aged 45–65 years, Burnett *et al.*, (1979) determined mutagenicity in urine specimens collected before and during a 24-hour period immediately after application of dark shades of several hair-colouring products containing high levels of dyes and dye intermediates. Many of the women had used hair dyes regularly for over 20 years. Concentrated (XAD-2 resin) urine samples did not increase the number of reverse mutations in *S. typhimurium* TA1538 in the presence of an exogenous metabolic system from rat liver (S9). [The Working Group noted the inadequate reporting of the results.]

Espinoza *et al.* (2008) evaluated micronuclei in urothelial cells to determine the possible association between genetic damage and use of hair dyes in 128 Spanish women. In addition, 72 women who participated as controls in a bladder cancer case–control study in Spain were included as controls. To avoid any kind of bias, only those cells with a typical morphology corresponding to the urothelial cells were scored. The mean micronucleus frequency in the overall study group was 9.72 ± 0.82 micronclei/1000 cells and did not vary by hair-dye exposure: 9.90 micronuclei/1000 cells (± 0.78) observed in women using hair dyes during the preceding month *vs* 9.50 micronuclei/1000 cells (± 2.45) observed in women who did not use hair dye in the preceding month. Use of hair dyes in recent months was associated with a higher frequency of micronuclei, but the association was not statistically significant ($P = 0.536$).

4.3 Mechanistic considerations

Hair dyes are difficult to evaluate as a group. On the one hand, the exposure situation is different for hairdressers and personal users, and on the other hand it seems particularly difficult to obtain information about a causal relationship. Significant associations can hardly be expected from epidemiological investigations. The experience with aromatic

amines somehow directed the attention from the beginning towards genotoxicity, and towards the bladder as the target tissue. Since bladder tumours are not uncommon in the general population, one would expect the tumour incidence to increase significantly only if exposures to hair dye-derived agents are relatively high, i.e. significantly above the background of other "bladder-specific" agents, for instance from smoking. Moreover, since bladder tumours seem to be the result of genotoxic as well as acute toxic effects, it is difficult to assess how (frequently changing) hair-dye components and other exogenous factors contribute to various biological effects. A monocausal approach seems to be particularly questionable in this case. The heterogeneous results of the many epidemiological studies reflect this dilemma. Bolt & Golka (2007) concluded from an extensive review of the literature that there seems to be no relevant bladder-cancer risk associated with the use of hair dyes that are available today. In other words, the potency is considered to be low. On the other hand, the epidemiological results from studies among hairdressers indicate that—with all reservations—the potential of occupational exposure to contribute to the overall cancer risk cannot be excluded.

More recently, other possible target tissues have been taken into account. Ambrosone *et al.* (2007) looked for effect markers of aromatic and heterocyclic amines as well as polycyclic aromatic hydrocarbons in exfoliated breast ductal epithelial cells from breast milk as etiological factors for breast-cancer risk. DNA adducts for each class were detected, and the presence of 4-aminobiphenyl adducts was associated with the use of hair-colouring products. Although this finding does not prove a causal relationship, it underlines the view that environmental exposures contribute to lesions in many tissues and thereby add to the risk. The suspicion of a relationship between hair dyes and cancer developed when some of the components were found to be genotoxic in in-vitro tests, and subsequently also carcinogenic in rodents after oral administration. The results were negative however, if the amines or commercial mixtures containing carcinogenic components were tested via topical application. Although the latter route of administration is more analogous to the current use of hair dyes, the other experiments indicate a carcinogenic potential under certain circumstances. If comparable exposure conditions would be strenuously required in carcinogen testing, the relevance of the results of many if not most animal experiments becomes questionable. However, it is important to keep in mind the difference between hazard and risk.

A particular aspect with permanent hair colourants is that the colourant is generated by oxidation of an amine and the structure of the oxidation product is normally not known from the peer-reviewed literature. Primary intermediates are for instance *para*-phenylenediamine or *para*-aminophenol, and couplers are *meta*-aminophenols or *meta*-hydroxyphenols. In the presence of peroxide, the primary intermediate and the coupler react with each other and coloured oligomers are formed. By itself, *para*-phenylene-diamine is only weakly genotoxic, but a mutagenic trimer (Bandrowski's base) is formed under oxidizing conditions. Moreover, additional activating pathways must be considered with diamines and phenolic amines: quinoid structures are involved, which react with cellular proteins. In this context it is interesting to note that *para*-phenylendiamine is an

established contact sensitizer, which indicates that relevant amounts penetrate the skin and are biologically active. The incidence of contact sensitization is increased particularly among beauticians and hairdressers (Iorizzo *et al*., 2002).

In the light of the above, it seems difficult to differentiate between different exposure situations on the basis if epidemiological observations. If there are hazardous components involved in exposure to hair colourants, they would probably contribute to the background exposure of aromatic amines. For practical purposes, and in line with the ALARA principle, established carcinogens should be avoided, omitted or exchanged for less hazardous components, and biological effect markers should be used to establish to what extent the professional handling of hair dyes and their personal use contribute to the background or total load of aromatic amine exposure.

5. Summary of Data Reported

5.1 Exposure data

5.1.1 *Hair Dyes: Production, use, occupational exposure and exposure after personal use*

Modern hair dyes may be classified as a) oxidative (permanent) hair dyes, b) semi-permanent and c) temporary dyes. Oxidative hair dyes represent approximately 80% of the market and consist of colourless primary intermediates (*para*-substituted aromatic amines) and couplers (*meta*-substituted aromatic amines and other compounds) that, in the presence of peroxide, form the dye by a chemical reaction. The concentration of oxidative hair-dye ingredients is approximately proportional to their degree of shade: dark colours tend to contain the highest concentrations of colouring ingredients (up to 3% primary intermediates during use) whereas light shades (blond) contain lower concentrations. Semi-permanent (direct) hair dyes contain colour molecules of low molecular weight, such as nitro-phenols, nitro-aminophenols and nitro-phenylene-diamines. Temporary dyes contain direct hair dyes of high molecular weight, such as azo, triphenylamine and indamine colourants. A worldwide survey in 2005 showed the presence of 50 ingredients in oxidative, 43 in semi-permanent and 88 ingredients in temporary hair dyes. The most frequently used oxidative hair-dye ingredients are *para*-phenylenediamine, resorcinol, 2,5-diaminotoluene, *para*- and *meta*-aminophenol, 4-amino-2-hydroxytoluene, 4-amino-*meta*-cresol and 2-methyl-5-hydroxyethylamino-phenol. The majority of oxidative hair-dye ingredients have been on the market since the 1930s. Most semi-permanent or temporary hair dyes have a much more limited use.

Occupational exposure studies found no or only traces of hair-dye ingredients in the air of hair salons, whereas measurable amounts were detected on hairdressers' hands. The major occupational exposure pathway appears to be via skin contact, followed by dermal absorption. The same exposure pathway applies to personal (consumer) use of hair dyes.

Hair dyes have been subject to regulations in many countries, and the number of substances permitted for use in hair dyes has been restricted during the past 40 years; in 2007, 135 individual ingredients were no longer allowed in the European Union for use in hair dyes.

5.2 Human carcinogenicity data

5.2.1 *Professional use of hair colourants*

The Working Group reviewed the literature on cancer at several sites in hairdressers, barbers and beauticians. Many additional studies have been published since the previous review in 1993 (IARC Monograph 57). These include several case–control studies and a few cohort studies. Most data from cohort studies derive from linkage between census data and cancer registries in Scandinavian countries, with limited potential to adjust for confounding by important correlates of cancer risk, e.g., lifestyle and reproductive factors. The Working Group noted that the evidence mainly concerned exposures that occurred before 1980s, and often much earlier.

(a) Bladder cancer

The cohort studies indicated an increased risk for cancer of the urinary bladder among male hairdressers, but not among female hairdressers. In a large Scandinavian cohort of hairdressers, barbers, beauticians and other related workers identified in the 1970 census and followed-up for 20 years, there was a significant 50% increase in risk for bladder cancer in men and a non-significant 10% decrease in risk in women. Allowance for smoking was generally not possible, although results for lung cancer suggest that higher exposure to tobacco in hairdressers could not totally account for the bladder cancer excess.

More than 20 case–control studies were available for evaluation. Most of these, including three of the larger studies, reported increased risks in the range of 1.3–1.7 in male hairdressers. A pooled analysis of 11 studies conducted in six European countries found no significant increase in risk among male or female hairdressers. Overall, risks appeared generally lower for women than for men. The number of exposed subjects was generally small, and did not allow a reliable assessment of the risk by duration and period of exposure.

In view of the consistent yet modest increase in risk reported in studies of hairdressers and barbers, especially men, and in the absence of solid data on the relation between duration and period of exposure, the Working Group concluded that there was *limited evidence* of an increased risk for bladder cancer in hairdressers.

(b) Haematological malignancies

With regards to cancers of the haematological system, the heterogeneity in the diseases included and the differences in the classification used often hampered

comparison between the results of different studies. Although one cohort study of barbers among male US veterans and an Italian case–control study reported significant increases in risk for multiple myeloma based on only few exposed cases, these results were not replicated in other studies. In the large Scandinavian cohort, no excess was found in either sex for multiple myeloma or for other haematological malignancies.

(c) Breast cancer

Many studies on breast cancer, including the largest case–control and cohort studies, did not show any increased risk for breast cancer associated with professional use of hair colourants.

(d) Childhood cancers

One cohort study and five case–control studies investigated the risk for childhood cancers in the offspring of hairdressers and barbers. Although some positive associations were reported, an overall evaluation is difficult because of the different sites and/or histologies investigated in various studies and the problems in the identification of the relevant period of exposure (before or around conception, or during pregnancy).

(e) Other sites

(i) *Ovarian cancer*

A modest increase in risk for ovarian cancer was reported in two cohorts of US cosmetologists and Scandinavian hairdressers, which was significant only in the latter study. The excess in risk appeared stronger in—or limited to—women exposed in earlier periods. No case–control study was available for evaluation. The lack of adjustment for potential confounders, especially reproductive history and oral contraceptive use, does not allow confounding to be ruled out.

(ii) *Lung cancer*

Small increases in lung cancer risk of the order of 20–40% were found in most cohort studies, which did not, however, adequately adjust for smoking. A higher prevalence of smokers among hairdressers than in the general population was reported in Scandinavia and the USA. No informative case–control study was available for evaluation. The Working Group concluded that tobacco smoking cannot be excluded as a likely cause of the modest excess in lung cancer observed in hairdressers.

5.2.2 *Personal use of hair colourants*

The Working Group revised and evaluated the epidemiological evidence of an association between cancer at several sites and personal use of hair dyes.

(a) Bladder cancer

Several studies with contradictory results have been published. Increased risks for bladder cancer were reported in two studies in the USA, while no association was found in three larger studies, two from the USA and one from Spain. These recent studies had similar characteristics in design and methodology.

One study from the USA suggested an increased risk for bladder cancer among users of hair colourants, in particular among those who exclusively used permanent hair dyes. Further, this study showed that exclusive use of permanent dyes among subjects with slow acetylation (*NAT2* genotype) or among CYP1A2 slow metabolizers was associated with an increased risk for bladder cancer. The other studies did not confirm these results. In the Spanish study, there was no indication of an increased risk for bladder cancer associated with the *NAT2* genotype, but there was a non-significant association with *NAT1*10*. The available cohort studies were largely negative for bladder cancer. The available meta-analyses did not show an association.

The Working Group considered that the available evidence for cancer of the bladder was overall *inadequate*.

(b) Haematological malignancies

The results for this tumour type were difficult to interpret: many different malignancies are involved, and many of the studies do not provide analyses for the different disease entities. Historically, the results have been inconsistent in identifying an increased risk. While cohort studies were largely negative for haematological malignancies, the results of case–control studies varied greatly. In those that showed an increased risk, the increase tended to be moderate. A recent pooled analysis was evaluated with particular interest because it was a large study evaluating hair-dye exposure in relation to single lymphoma entities including case–control data derived from Canada, the USA and six countries in Europe. The study showed an overall increased risk of 1.1 among women who were regular users, and of 1.3 among those women who had started regular hair-dye use before 1980. The risk was consistently elevated for follicular lymphoma and chronic lymphocytic leukaemia, but not for other types. For these lymphoma subtypes, the risk did not vary by intensity, years of use or type of exposure, remaining generally of the order 1.2–1.4. When the period of first use was considered, the increased risk for chronic lymphocytic leukaemia was mainly observed among those who started use before 1980, with a statistically significant increase in risk among those reporting use for more than 20 years. For follicular lymphoma, increased risks were observed throughout the two study periods. Overall, the Working Group considered this evidence to be *inadequate*.

(c) Breast cancer

For breast cancer the Working Group considered the evidence as *inadequate* based on several studies, none of which except one showed an association.

(d) Childhood cancers

For childhood cancers, the studies evaluated dealt with childhood brain tumours and Wilms tumours. The Working Group discussed in depth the potential biases and study limitations that could explain some of the increased risks observed for some brain tumours, and considered that some of the reported associations could not be simply explained by recall bias, as mothers may not have known about this hypothesis at the time of the studies. The Working Group considered that the evidence presented in the studies was *inadequate*.

(e) Other sites

Only a few studies were available to the Working Group, and no evaluation was made.

5.3 Animal carcinogenicity data

Various commercially available hair-dye formulations and various laboratory preparations of hair dyes were tested for carcinogenicity in mice or rats by skin application in 11 studies and by subcutaneous injection in a single study in rats.

In three studies by skin painting in mice, all using different formulations, increased incidences of lymphomas were observed in female mice compared with concurrent controls, for five different formulations. The increased incidences were not significant when compared with historical controls. In three studies by skin painting in rats, a significant increase in the incidence of mammary adenomas in females was observed for two formulations, and a significant increase in pituitary adenomas was seen in females for one formulation and in males for a different formulation. In the single subcutaneous injection study in rats, an increased incidence in mammary and uterine tumours was observed. The other studies either showed no increased incidence of tumours at any site or were inadequate for evaluation.

5.4 Other relevant data

Studies that investigated the induction of chromosomal aberrations in peripheral blood lymphocytes of professional hair-colourists or in volunteers who had their hair dyed reported no effect. The same is true for two studies that investigated sister chromatid exchange in lymphocytes of hairdressers. Two studies on the mutagenicity of urine collected from hair-dye users and cosmetologists were inadequate for evaluation. One study assessed sister chromatid exchange, DNA breakage (measured by single-cell gel electrophoresis) in lymphocytes and mutagenicity in urine in professional hair-colourists. No effect was seen for any of these three endpoints. A study on micronucleus formation in hair-dye users did not show a difference with non-user controls.

In an early study, 90% of a large number of commercial oxidative hair-dye formulations were mutagenic in bacteria. In later studies, this percentage dropped to 0–50%. The hair-colouring product henna did not show bacterial mutagenicity in one study. When tested separately, its active ingredient was mutagenic.

The urine of rats skin-painted with oxidative hair-colouring products was mutagenic when collected during the first 24 hours. The mutagenicity disappeared afterwards.

No effects were seen in a heritable translocation assay in rats skin-painted twice weekly for ten weeks with a semi-permanent dye formulation.

6. Evaluation

6.1 Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of occupational exposures as a hairdresser or barber.

There is *inadequate evidence* in humans for the carcinogenicity of personal use of hair colourants.

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of hair colourants.

6.3 Overall evaluation

Occupational exposures as a hairdresser or barber are *probably carcinogenic to humans (Group 2A)*.

Personal use of hair colourants is *not classifiable as to its carcinogenicity to humans (Group 3)*.

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