MISCELLANEOUS NITROGEN-CONTAINING SUBSTANCES

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 16 flavouring agents (Table 1) by the Procedure for the Safety Evaluation of flavouring Agents (see Figure 1, p. 170). This group comprised five structurally related isothiocyanates (Nos 1560–1564) that included allyl isothiocyanate (No. 1560); six alkylated oxazole analogues (Nos 1553–1557 and 1569); two methylated oxazoline analogues (Nos 1558–1559); two pyrimidines (Nos 1565–1566); and one pyrazole (No. 1568). None of these agents has previously been evaluated by the Committee. Fourteen of the 16 substances (Nos 1553–1565 and 1569) have been reported to occur naturally in foods and

Table 1. Summary of the results of safety evaluations of miscellaneous nitrogen-containing substances

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
Structural class II							
Trimethyloxazole	1553	20662-84-4	No Europe: 1[b] USA: 1[b]	Yes. The NOEL of 2.3 mg/kg bw per day for the related substance 2-ethyl-4,5-dimethyl-oxazole (Griffiths et al., 1979) is 115 000 times the estimated daily intake of trimethyloxazole of 0.02 µg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern (conditional)
2,5-Dimethyl-4-ethyloxazole	1554	30408-61-8	No Europe: 0.2[b] USA: 0.2[b]	Yes. The NOEL of 2.3 mg/kg bw per day for the related substance 2-ethyl-4,5-dimethyloxazole (Griffiths et al., 1979) is 575 000 times the estimated daily intake 2,5-dimethyl-4-ethyloxazole of 0.004 µg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern (conditional)

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
2-Ethyl-4,5-dimethyloxazole	1555	53833-30-0	No Europe: 0.03 USA: 0.7[b]	Yes. The NOEL of 2.3 mg/kg bw per day (Griffiths et al., 1979) is 4 600 000 and 230 000 times the estimated daily intake of 2-ethyl-4,5-dimethyloxazole of 0.0005 µg/kg bw in Europe and 0.01 µg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 3 (conditional)	No safety concern
2-Isobutyl-4,5-dimethyloxazole	1556	26131-91-9	No Europe: 0.2[b] USA: 0.2[b]	Yes. The NOEL of 2.3 mg/kg bw per day for the related substance 2-ethyl-4,5-dimethyloxazole (Griffiths et al., 1979) is 575 000 times the estimated daily intake of 2-isobutyl-4,5-dimethyloxazole of 0.004 µg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern (conditional)

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
2-Methyl-4,5-benzo-oxazole	1557	95-21-6	No Europe: 0.1[b] USA: ND	Yes. The NOEL of 2.3 mg/kg bw per day for the related substance 2-ethyl-4,5-dimethyloxazole (Griffiths et al., 1979) is 1 150 000 times the estimated daily intake of 2-methyl-4,5-benzo-oxazole of 0.002 μg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 1,2	No safety concern (conditional)
2,4-Dimethyl-3-oxazoline	1558	77311-02-5	No Europe: 0.07[b] USA: ND	Yes. The NOEL of 41 mg/kg bw per day for the related substance 2,4,5-trimethyl-Δ-3-oxazoline (Morgareidge,1972) is 41 000 000 times the estimated daily intake of 2,4-dimethyl-3-oxazoline of 0.001 μg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern (conditional)

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
2,4,5-Trimethyl-Δ3-oxazoline	1559	22694-96-8	No Europe: 0.04 USA: 0.01	Yes. The NOEL of 41 mg/kg bw per day (Morgareidge, 1972) is approximately 60 000 000 and 205 000 times the estimated daily intake of 2,4,5-trimethyl-Δ-3-oxazoline of 0.0007 μg/kg bw in Europe and 0.0002 μg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern
Allyl isothiocyanate	1560	57-06-7	Yes Europe: 1502 USA: 133		Yes. The NOEL of 12 mg/kg bw per day (National Toxicology Program, 1982) is > 400 and > 5000 times the estimated daily intake of allyl isothiocyanate of 25 μg/kg bw in Europe and 2.2 μg/kg bw in the USA, when used as a flavouringagent.	See notes 4 and 5	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
Butyl isothio-cyanate	1561	592-82-5	No Europe: 2[b] USA: ND	Yes. The NOEL of 12 mg/kg bw per day for the related substance allyl isothiocyanate (National Toxicology Program, 1982) is 400 000 times the estimated daily intake of butyl isothiocyanate of 0.03 μg/kg bw in Europe, when used as a flavouring agent.	N/R	See notes 4 and 5	No safety concern (conditional)
Benzyl isothio-cyanate	1562	622-78-6	No Europe: 1[b] USA: 0.4[b]	Yes. The NOEL of 5 mg/kg bw per day for the related substance phenethyl isothiocyanate (Ogawa et al., 2001) is 250 000 and about 700 000 times the estimated daily intake of benzyl isothiocyanate of 0.02 μg/kg bw in Europe and 0.007 μg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 4	No safety concern (conditional)

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
Phenethyl isothiocyanate	1563	2257-09-2	No Europe: 0.4[b] USA: 0.5[b]	Yes. The NOEL of 5 mg/kg bw per day (Ogawa et al., 2001) is 700 000 and about 600 000 times the estimated daily intake of phenethyl isothiocyanate of 0.007 μg/kg bw in Europe and 0.008 μg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 4	No safety concern (conditional)
3-Methylthiopropyl isothiocyanate	1564	505-79-3	No Europe: 13 USA: 52	Yes. The NOEL of 30 mg/kg bw per day (Harper et al., 1961) is 150 000 and 30 000 times the estimated daily intake of 3-methylthiopropyl isothiocyanate of 0.2 μg/kg bw in Europe and 0.9 μg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 4	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
4-Acetyl-2-methyl-pyrimidine	1565	67860-38-2	No Europe: ND USA: 0.01	Yes. The NOEL of 1 mg/kg bw per day (Peano, 1981) is 5 000 000 times the estimated daily intake of 4-acetyl-2-methylpyrimidine of 0.0002 μg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 4	No safety concern
4,5-Dimethyl-2-propyloxazole	1569	53833-32-2	No Europe: 0.1[b] USA: 0.1[b]	Yes. The NOEL of 2.3 mg/kg bw per day for the related substance 2-ethyl-4,5-dimethyloxazole (Griffiths et al., 1979) is 1 150 000 times the estimated daily intake of 4,5-dimethyl-2-propyloxazole of 0.002 μg/kg bw in both Europe and the USA, when used as a flavouring agent.	N/R	See note 3	No safety concern (conditional)

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step B3[a] Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for the flavouring agent or related substance?	Are additional data available to perform a safety evaluation for substances with an intake exceeding the threshold of concern?	Comments	Conclusion based on estimated daily intake
Structural class III							
5,7-Dihydro-2-methylthieno(3,4-*d*)-pyrimidine	1566	36267-71-7	No Europe: ND USA: 0.4	Yes. The NOEL of 6.6 mg/kg bw per day (Shellenberger, 1970) is 1 100 000 times the estimated daily intake of 5,7-dihydro-2-methylthieno-(3,4-*d*)pyrimidine of 0.006 µg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 2	No safety concern
1-Phenyl-3- or -5-propylpyrazole	1568	65504-93-0	No Europe: ND USA: 0.2	Yes. The NOEL of 25 mg/kg bw per day (Posternak et al., 1969) is about 6 000 000 times the estimated daily intake of 1-phenyl-3- or -5-propylpyrazole of 0.004 µg/kg bw in the USA, when used as a flavouring agent.	N/R	See note 2	No safety concern

Table 1 (contd)

CAS, Chemical Abstracts Service; ND, no intake data reported; N/R, not required for evaluation because consumption of the substance was determined not to exceed the threshold of concern at Step B3 of the Procedure
Step 1: Fourteen flavouring agents are in structural class II (Nos 1553–1565 and 1569), and two are in structural class III (Nos 1566 and 1568).
Step 2: None of the miscellaneous nitrogen derivatives (Nos 1553–1566, 1568 and 1569) can be predicted to be metabolized to innocuous products.

[a] The thresholds for concern for structural classes II and III are 540 and 90 μg/day, respectively. All intake values are expressed in μg per day. The combined per capita intake of the flavouring agents in structural class II is 1520 μg per day in Europe and 188 μg per day in the USA. The combined per capita intake of the flavouring agents in structural class III is 0.6 μg per day in the USA (no intake data reported for Europe).
[b] Intake estimate based on anticipated annual volume of production

Notes:
1. Predicted to be absorbed rapidly, followed by ring cleavage and excretion in the urine
2. Predicted to be readily absorbed, followed by ring hydroxylation and excretion in the urine
3. Predicted to be readily absorbed, followed by side-chain oxidation and excretion in the urine
4. Readily absorbed, principally conjugated with glutathione, followed by formation of mercapturic acid conjugate and excretion in the urine
5. Hydrolysis followed by excretion in the urine

have been detected in a variety of vegetables, cooked meats, cocoa, coffee, pineapple and papaya (Nijssen et al., 2003).

1.2 Estimated daily per capita exposure

Annual volumes of production have been reported for six of the 16 flavouring agents in this group (Nos 1559, 1560, 1564–1566 and 1568). For the remaining 10 substances (Nos 1553–1558, 1561–1563 and 1569), anticipated annual volumes of production have been given for their proposed use as flavouring agents. The total reported and anticipated annual volume of production of the 16 flavouring agents in this group is about 10 700 kg in Europe (International Organization of the Flavor Industry, 1995) and 1400 kg in the USA (National Academy of Sciences, 1982; Lucas et al., 1999). More than 98% of the total reported and anticipated annual volume of production in Europe and more than 70% in the USA is accounted for by one substance in this group, allyl isothiocyanate (No. 1560). The estimated per capita exposure to this substance is about 1500 μg/day in Europe and 130 μg/day in the USA. The per capita exposure to all the other flavouring agents in the group is 0.03–13 μg/day in Europe and 0.01–52 μg/day in the USA (National Academy of Sciences, 1982; International Organization of the Flavor Industry, 1995; Lucas et al., 1999), most of the values being at the lower end of the range. The estimated daily per capita exposure to each agent in Europe and the USA is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

Data on structurally related substances indicate that oxazoles, oxazolines, pyrimidines and pyrazoles will be rapidly absorbed, metabolized and excreted in the urine. The metabolism of oxazoles involves two pathways: oxazole ring cleavage and, if the ring is properly substituted, ring hydroxylation. The presence of a substituent at the 2-position tends to stabilize the oxazole ring.

Isothiocyanates are readily absorbed and distributed to all the main tissues in rodents, peak concentrations in the tissues being achieved 2–8 h after dosing. At comparable doses, there are clear sex- and species-specific differences in the distribution, metabolism and excretion of substituted isothiocyanates.

Metabolic studies in humans, mice and rats indicate that isothiocyanates react readily with reduced glutathione (GSH) to form a conjugate as the principal metabolite and that the reaction is catalysed enzymatically by glutathione *S*-transferase enzymes and non-enzymatically (at a slower rate), both reactions occurring in a pH-dependent equilibrium. The isothiocyanate–GSH conjugates formed are subsequently excreted into bile, and corresponding *N*-acetylcysteine adducts appear as the major metabolite in urine. A key element of isothiocyanate metabolism is the highly electrophilic, reactive central carbon in the group, as it drives Michael addition reactions with *N*-, *O*- or *S*-based nucleophiles (e.g. GSH), giving rise to the relatively stable but reversible conjugates implicated in its toxicity. In humans and rats, aromatic isothiocyanates are metabolized mainly to the corresponding mercapturic acid conjugates, which subsequently hydrolyse to the corresponding cysteine conjugates as the major urinary metabolites. The lability of glutathione conjugates under the conditions in the rodent bladder can lead to formation of unconjugated, 'free' isothiocyanate and GSH. The presence of free isothiocyanates can increase irritation of the rat bladder epithelium. In rabbits, mice and guinea-

Table 2. Annual volumes of production of miscellaneous nitrogen derivatives used or proposed for use as flavouring agents in Europe and the USA

Agent (No.)	Reported[a] / anticipated annual volume (kg)	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
Trimethyloxazole (1553)					
Europe[e]	10	1	000		
USA[e]	8	1	000	+	NA
2,5-Dimethyl-4-ethyloxazole (1554)					
Europe[e]	1.5	0.2	0.004		
USA[e]	1.3	0.2	0.004	+	NA
2-Ethyl-4,5-dimethyloxazole (1555)					
Europe	0.2	000	0.0005		
USAe	4	0.7	000	+	NA
2-Isobutyl-4,5-dimethyloxazole (1556)					
Europe[e]	1.5	0.2	0.004		
USA[e]	1.3	0.2	0.004	+	NA
2-Methyl-4,5-benzo-oxazole (1557)					
Europe[e]	0.7	0.1	0.002		
USA	N/R	ND	ND	+	NA
2,4-Dimethyl-3-oxazoline (1558)					
Europe[e]	0.5	000	0.001		
USA	N/R	ND	ND	+	NA
2,4,5-Trimethyl-Δ3-oxazoline (1559)					
Europe	0.3	000	0.0007		
USA	0.09	000	0.0002	+	NA
Allyl isothiocyanate (1560)					
Europe	10 524	1502	25		
USA	1 007	133	2.2	91 825.2	91
Butyl isothiocyanate (1561)					
Europe[e]	11	2	000		
USA	N/R	ND	ND	+	NA
Benzyl isothiocyanate (1562)					
Europe[e]	7.0	1	000		
USA[e]	2.5	0.4	0.007	+	NA
Phenethyl isothiocyanate (1563)					
Europe[e]	2.8	0.4	0.007		
USA[e]	2.8	0.5	0.008	+	NA

Table 2 (contd)

Agent (No.)	Reported[a] / anticipated annual volume (kg)	Intake[b] µg/day	Intake[b] µg/kg bw per day	Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
3-Methylthiopropyl isothiocyanate (1564)					
Europe	89	13	0.2		
USA	395	52	0.9	110.4	0.3
4-Acetyl-2-methylpyrimidine (1565)					
Europe	N/R	ND	ND		
USA	0.09	000	0.0002	+	NA
5,7-Dihydro-2-methylthieno(3,4-*d*)pyrimidine (1566)					
Europe	N/R	ND	ND		
USA	2.7	0.4	0.006	–	NA
1-Phenyl-3- or -5-propylpyrazole (1568)					
Europe	N/R	ND	ND		
USA[f]	1.4	0.2	0.004	–	NA
4,5-Dimethyl-2-propyloxazole (1569)					
Europe[e]	1.0	0.1	0.002		
USA[e]	0.8	0.1	0.002	+	NA
Total					
Europe	10 650				
USA	1 427				

NA, not available; ND, no intake data reported; + reported to occur naturally in foods (Njissen et al., 2003), but no quantitative data; –. not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1970, 1982, 1987)

[b] Intake (µg/person per day) calculated as follows: [(annual volume, kg) x (1 x 10^9 µg/kg)/(population x survey correction factor x 365 days)], where population (10%, 'eaters only') = 32 x 10^6 for Europe and 26 x 10^6 for the USA; where survey correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual flavour volume, respectively, was reported in poundage surveys (International Organization of the Flavor Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1982) or in the anticipated annual volume.
Intake (µg/kg bw per day) calculated as follows: [(µg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg and Grundschober (1987)

[d] The consumption ratio is calculated as follows: (annual consumption from food, kg)/(most recent reported volume as a flavouring substance, kg)

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. National surveys (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999), if applicable, revealed no reported use as a flavour agent.

[f] Annual volume reported in previous surveys in the USA (National Academy of Sciences, 1970, 1982)

pigs, however, the cysteine conjugate is hydrolysed and then undergoes transamination and cyclization to form a substituted thiazolidine-2-thione as the main urinary metabolite.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

In applying the Procedure to flavouring agents for which both a reported and an anticipated volume of production were given, the Committee based its evaluation on the reported volume of production if the exposure estimated from it exceeded the exposure estimated from the anticipated volume of production and applied no conditions to its decision on safety. If the exposure estimated from the anticipated volume of production exceeded the exposure estimated from the reported volume of production, the Committee based its evaluation on the anticipated volume of production but considered its decision on safety to be 'conditional', pending receipt of information on use levels or poundage data by December 2007. In applying the Procedure to flavouring agents for which only anticipated volumes of production were given, the decision was likewise made conditional.

Step 1. In applying the Procedure, the Committee assigned 14 agents in this group (Nos 1553–1565 and 1569) to structural class II and the remaining two agents (Nos 1566 and 1568) to structural class III (Cramer et al., 1978).

Step 2. None of the flavouring agents in this group can be predicted to be metabolized to innocuous products. The evaluation of all flavouring agents in this group therefore proceeded via the B-side of the procedure.

Step B3. The estimated daily per capita exposure in Europe and the USA of 13 of the flavouring agents in structural class II (Nos 1553–1559, 1561–1565 and 1569) and both of the flavouring agents in structural class III (Nos 1566 and 1568) is below the threshold of concern for their respective class (i.e. class II, 540 μg/day; class III, 90 μg/day). Accordingly, the evaluation of these 15 agents proceeded to Step B4. The estimated per capita exposure to one of the agents in structural class II, allyl isothiocyanate (No. 1560), which is about 1500 μg/day in Europe and 130 μg/day in the USA, exceeds the threshold of concern (540 μg/day) for its class. In accordance with the Procedure, more extensive data are needed to evaluate the safety of flavouring agents exposure to which exceeds the threshold of concern for their structural class at Step B3. Additional data on allyl isothiocyanate were therefore considered (see below).

Step B4. For 2-ethyl-4,5-dimethyloxazole (No. 1555), the NOEL of 2.3 mg/kg bw per day in a 91-day study in rats treated by gavage (Griffiths et al., 1979) is 4 600 000 times the estimated intake from its use as flavouring agent in Europe (0.0005 μg/kg bw per day) and 230 000 times the estimated intake from its proposed use in the USA (0.01 μg/kg bw per day).

The NOEL for 2-ethyl-4,5-dimethyloxazole is also appropriate for the structurally related agents trimethyloxazole (No. 1553), 2,5-dimethyl-4-

ethyloxazole (No. 1554), 2-isobutyl-4,5-dimethyloxazole (No. 1556), 2-methyl-4,5-benzo-oxazole (No. 1557) and 4,5-dimethyl-2-propyloxazole (No. 1569), all of which are oxazole analogues and as such are expected to be metabolized via similar metabolic pathways. The NOEL of 2.3 mg/kg bw per day is 115 000 times the estimated intake of trimethyloxazole from its proposed use as a flavouring agent in both Europe and the USA (0.02 μg/kg bw per day), 575 000 times the estimated intakes of 2,5-dimethyl-4-ethyloxazole and 2-isobutyl-4,5-dimethyloxazole from their proposed use as flavouring agents in both Europe and the USA (0.004 μg/kg bw per day) and 1 500 000 times the estimated intakes of 2-methyl-4,5-benzo-oxazole and 4,5-dimethyl-2-propyloxazole from their proposed use as flavouring agents in both Europe and the USA (0.002 μg/kg bw per day).

The NOEL of 41 mg/kg bw per day for 2,4,5-trimethyl-Δ3-oxazoline (No. 1559) from a 90-day feeding study in rats (Morgareidge, 1972) is approximately 60 000,000 times the estimated intake of this substance from its use as flavouring agent in Europe (0.0007 μg/kg bw per day) and 205 000 000 times that in the USA (0.0002 μg/kg bw per day).

The NOEL for 2,4,5-trimethyl-Δ3-oxazoline is also appropriate for the structurally related agent 2,4-dimethyl-3-oxazoline (No. 1558), as this is an oxazoline analogue and is therefore expected to be metabolized via similar metabolic pathways. The NOEL of 41 mg/kg bw per day is 41 000 000 times the estimated intake of 2,4-dimethyl-3-oxazoline from its proposed use as a flavouring agent in Europe (0.001 μg/kg bw per day).

Although no NOEL is available for butyl isothiocyanate (No. 1561), the NOEL of 12 mg/kg bw per day for the structurally related agent allyl isothiocyanate (No. 1560; see below) is also appropriate for butyl isothiocyanate, as they are both isothiocyanates, which will be metabolized via similar metabolic pathways. This NOEL is 400 000 times the estimated intake of butyl isothiocyanate from its proposed use as a flavouring agent in Europe (0.03 μg/kg bw per day).

Although no NOEL is available for benzyl isothiocyanate (No. 1562), the NOEL of 5 mg/kg bw per day for the structurally related agent phenethyl isothiocyanate is also appropriate for benzyl isothiocyanate, as both are isothiocyanates, which will be metabolized via similar metabolic pathways. This NOEL is 250 000 times the estimated intake of this substance from its proposed use as a flavouring agent in Europe (0.02 μg/kg bw per day) and about 700 000 times that in the USA (0.007 μg/kg bw per day).

For phenethyl isothiocyanate (No. 1563), the NOEL of 5 mg/kg bw per day in a 91-day feeding study in rats (Ogawa et al., 2001) is approximately 700 000 times the estimated intake of this substance from its proposed use as a flavouring agent in Europe (0.007 μg/kg bw per day) and about 600 000 times that in the USA (0.008 μg/kg bw per day).

For 3-methylthiopropyl isothiocyanate (No. 1564), the NOEL of 30 mg/kg bw per day in an 84-day feeding study in rats (Harper et al., 1961) is 150 000 times the estimated intake of this substance from its use as a

flavouring agent in Europe (0.2 μg/kg bw per day) and about 30 000 times that in the USA (0.9 μg/kg bw per day).

For 4-acetyl-2-methylpyrimidine (No. 1565), the NOEL of 1 mg/kg bw per day in a 91-day study in rats treated by gavage (Peano, 1981) is 5 000 000 times the estimated intake of this substance from its use as a flavouring agent in the USA (0.0002 μg/kg bw per day).

For 5,7-dihydro-2-methylthieno(3,4-d)pyrimidine (No. 1566), the NOEL of 6.6 mg/kg bw per day in a 90-day feeding study in rats (Shellenberger, 1970) is 1 100 000 times the estimated intake of this substance from its use as a flavouring agent in the USA (0.006 μg/kg bw per day).

For 1-phenyl-3- or -5-propylpyrazole (No. 1568), the NOEL of 25 mg/kg bw per day in a 90-day feeding study in rats (Posternak et al., 1969) is about 6 000 000 times the estimated intake of this substance from its use as flavouring agent in the USA (0.004 μg/kg bw per day).

The Committee concluded that the margins between the estimated daily exposure to the five substances reported to be used as flavouring agents (Nos 1559, 1564–1566 and 1568) and the NOELs for these agents were adequate, and that their use would not present a safety concern. The Committee also concluded that the margins between the exposure estimated from anticipated annual volumes of production for the other 10 substances proposed for use as flavouring agents (Nos 1553–1558, 1561–1563 and 1569) and the NOELs for these agents were adequate. Although their use would raise no safety concern at estimated exposure, less uncertain estimates of exposure are required.

The exposure considerations and other information used to evaluate these miscellaneous nitrogen-containing flavouring agents are summarized in Table 2.

1.5 Consideration of flavouring agents with high exposure evaluated on the B-side of the Procedure

As stipulated in the Procedure, more extensive data on metabolism and toxicity were considered to complete the safety evaluation of allyl isothiocyanate (No. 1560), as the exposure level estimated from use of this compound as a flavouring agent in Europe exceeded the threshold of concern for structural class II (540 μg/ person per day).

Short-term and long-term studies

Several short-term studies of toxicity were conducted in rats and mice given allyl isothiocyanate orally. Furthermore, allyl isothiocyanate was tested by the National Toxicology Program in a series of short-term studies of toxicity and long-term studies of carcinogenicity in both laboratory species.

In a 20-day study, groups of five weanling Osborne-Mendel rats of each sex were given allyl isothiocyanate in corn oil at a dose of 0 (vehicle control), 20 or 50 mg/kg bw per day. Macroscopically, the non-glandular part of the stomach was thickened with occasional roughening of the lining at both doses. Minor inflammatory foci were reported in the livers of rats at the higher dose (Hagan et al., 1967).

Groups of male outbred Shoe:WIST rats (number of animals per group not specified) were given allyl isothiocyanate at a dose of 0 (paraffin oil vehicle control),

10, 20 or 40 mg/kg bw per day, 5 days per week for up to 6 weeks by gavage. Hepatohistopathology showed diffuse ballooning of centrilobular hepatocytes in some rats at the highest dose. The kidneys of rats at this dose and at the middle dose showed dilatation of distal tubules and increased desquamation (Lewerenz et al., 1988a).

In a 9-week study conducted to examine the possible effects of allyl isothiocyanate on growth, groups of four weanling rats (strain not specified) were fed basal diet (control) or basal diet with 0.1% allyl isothiocyanate for 5 weeks. This dietary level was calculated to provide an average daily intake of 100 mg/kg bw (Food & Drug Administration, 1993). During weeks 6–9, the rats were given allyl isothiocyanate by gavage daily at the dose in food (about 100 mg/kg bw). Only animals treated by gavage had lower body-weight gains than controls (statistics not reported) (Ahmad et al., 1966).

A dose-range-finding study was conducted in mice by the National Toxicology Program. Groups of five B6C3F$_1$ mice of each sex were given allyl isothiocyanate in corn oil by gavage at a dose of 3, 6, 12, 25 or 50 mg/kg bw per day for 14 days. One male at 50 mg/kg bw per day died. No dose-dependent change in body-weight gain was found. Four of the five males and all females at the highest dose showed thickened areas of mucosa in the non-glandular region of the stomach, and four males and one female had a thickened urinary bladder wall (National Toxicology Program, 1982).

In a 13-week study, groups of 10 B6C3F$_1$ mice of each sex were given allyl isothiocyanate by gavage at a dose of 0 (vehicle control), 1.5, 3, 6, 12 or 25 mg/kg bw per day on 5 days per week. The mean body weights of treated and control animals were comparable, and no gross or histological changes were reported at any dose (National Toxicology Program, 1982).

In a study of carcinogenicity, groups of 50 male and 50 female B6C3F$_1$ mice received allyl isothiocyanate by gavage at a dose of 0 (vehicle control), 12 or 25 mg/kg bw per day, 5 days per week for 103 weeks. Many of the female mice that died before week 104 had suppurative inflammation of the peritoneum, uterus or multiple organs, suggesting generalized infection. The final mean body weights of treated and control animals were comparable (statistics not reported). The incidence of primary tumours was not increased in treated mice. Male mice showed a statistically significant, dose-related increase in cytoplasmic vacuolization in the liver (control, 2/49; low dose, 8/49; high dose, 13/50). The severity of this lesion was similar in the three groups. Most of the vacuoles were centrilobular and all contained fat. The authors concluded that allyl isothiocyanate was not carcinogenic (National Toxicology Program, 1982).

A dose-range finding study was conducted in groups of five Fischer 344/N rats of each sex given allyl isothiocyanate in corn oil by gavage at a dose of 25, 50, 100, 200 or 400 mg/kg bw per day for 14 days. Clinical signs, including inactivity and ruffled fur, were observed at all doses, the severity increasing with dose. All animals at the two highest doses died before the end of the study. Reduced body weights were observed at 100 mg/kg bw per day. Gross necropsy revealed a thickened mucosal surface of the stomach and adhesion of the stomach to the peritoneum in treated animals (National Toxicology Program, 1982).

Groups of 10 Fischer 344/N rats of each sex were given allyl isothiocyanate by gavage at a dose of 0 (vehicle control), 1.5, 3, 6, 12 or 25 mg/kg bw per day, 5 days per week for 13 weeks. All animals survived to scheduled termination with no

clinical signs of toxicity. No significant changes in body weight and no gross or histological changes were reported at any dose (National Toxicology Program, 1982).

In a carcinogenesis bioassay, groups of 50 male and 50 female Fischer 344/N rats received allyl isothiocyanate by gavage at a dose of 0 (vehicle control), 12 or 25 mg/kg bw per day, 5 days per week for 103 weeks. The mean body weights of male rats at the higher dose were lower than those of controls throughout the study. No clinical signs of toxicity were reported. Survival (58–74%) was comparable in all groups, including controls. The incidence of subcutaneous fibrosarcomas was increased in females at the higher dose, with a significant positive trend. The incidence of undifferentiated leukaemia was increased over that in controls in treated male rats at both doses: control, 2/50; low dose, 6/50; high dose, 8/50. Males at the higher dose also had a significantly increased incidence of transitional-cell papillomas of the urinary bladder: control, 0/49; high dose, 4/49. Epithelial hyperplasia of the urinary bladder also occurred in males, with a significant overall trend at the higher dose. Hyperplasia was not observed in animals with papillomas. The authors concluded that, under the conditions of the bioassay, allyl isothiocyanate was carcinogenic in male rats, causing transitional-cell papillomas of the urinary bladder, but that the evidence for an association between treatment and subcutaneous fibrosarcomas in female rats was equivocal (National Toxicology Program, 1982).

Genotoxicity

Numerous tests for genotoxicity were reported. The studies indicated mixed results in vitro, but mainly negative results (8 of 10 studies) in vivo. The two studies with positive results showed only weak activity.

Mode of action

In rats given allyl isothiocyanate at an LD_{50} of 112 mg/kg bw by stomach tube, marked irritation of the lungs and gastrointestinal tract was reported (Anderson & Hurwitz, 1953). In mice, the sensitizing effects of allyl isothiocyanates on the skin correlated with a perturbation in the ratio of skin GSH to glutathione disulfide, suggesting that the substance might induce oxidative stress in mouse skin epithelia (Schmidt & Chung, 1993).

An underlying concept in the hypothesis for the mode of toxic action of allyl isothiocyanate is the chemical reactivity of the highly electrophilic central carbon in the isothiocyanate group (–N=C=S). It can efficiently undergo Michael addition reactions with *N*-, *O*- or *S*-based nucleophiles such as the thiol of GSH. The reaction gives rise to relatively stable but reversible GSH adducts, which have been implicated in its cytotoxicity in hepatocytes in vitro (Bruggeman et al., 1986). Free and conjugated forms of allyl isothiocyanate are in equilibrium, allowing for the presence of as much as 15–20% free allyl isothiocyanate (Bollard et al., 1997). The position of the equilibrium can be shifted in favour of the free form under conditions of low GSH concentration and alkaline pH (Bruggeman et al., 1986), which may exist in the urinary bladder. Therefore, increased absorption of free allyl isothiocyanate might occur in the bladder epithelium.

Data on the metabolism and disposition of allyl isothiocyanate show clear sex- and species-specific differences. Mice excrete essentially all orally administered

allyl ^{14}C-isothiocyanate within 96 h, while rats retain up to 20% in the carcass over the same interval. Blood concentrations of radioactivity returned to background levels within 96 h in mice but persisted up to 240 h in rats. In a separate experiment, mice excreted more than 80% of an administered dose of allyl ^{14}C-isothiocyanate in the urine within 3 days, while rats excreted only 55%, indicating that rats selectively retain thiocyanate ion (Bollard et al., 1997). Studies in rats showed that females produce twice as much urine as males (Ioannou et al., 1984; Bollard et al., 1997), so that the male rat bladder is exposed to higher concentrations of allyl isothiocyanate and its metabolites than the female rat bladder.

The histopathological evidence of male bladder epithelial hyperplasia reported in the study of the National Toxicology Program (1982) correlates with the biochemical evidence that the male rat bladder is subjected to longer exposure to high concentrations of possible irritants such as allyl isothiocyanate or its metabolites.

Both the qualitative and the quantitative aspects of the molecular disposition of allyl isothiocyanate and its associated toxicological sequelae have been relatively well defined in studies in mammals and are similar to those reported for other irritating substances in the urinary bladder (Cohen & Ellwein, 1990). Epithelial-cell papillomas are benign lesions on luminal surfaces.

On the basis of the observations that allyl isothiocyanate has strong irritant and cytotoxic properties, is considered not to be genotoxic in vivo and induces sex- and species-specific benign transitional-cell papillomas only in male rats, it is highly probable that allyl isothiocyanate operates through a secondary non-genotoxic mechanism, like other irritating bladder carcinogens, such as sodium saccharin (Cohen & Ellwein, 1990).

The two factors that distinguish human exposure to allyl isothiocyanate from that of rats are daily intake and bladder function. Rats in the 2-year study (National Toxicology Program, 1982) were given 25 000 μg/kg bw daily, while human intake from use of allyl isothiocyanate as a flavouring agent is approximately 1000 times less (25 μg/kg bw per day). Furthermore, humans excrete about 1500 ml of urine per day. Therefore, the potential concentration of allyl isothiocyanate or its metabolites in the human bladder is orders of magnitude lower than that required to induce hyperplasia and papillomas in rats. As no toxicity was observed in the bladder at a dose of 12 000 μg/kg bw per day in the study in rats, it is highly unlikely that this mode of action of carcinogenicity would operate in humans.

The NOEL for allyl isothiocyanate in the 2-year study in rats was 12 mg/kg bw per day. This NOEL is more than 400 times the estimated daily intake of allyl isothiocyanate when used as a flavouring agent in Europe (25 μg/kg bw per day) and more than 5000 times that in the USA (2.2 μg/kg bw per day). Therefore, on the basis of the additional data on toxicity, the Committee concluded that allyl isothiocyanate (No. 1560) would not be expected to present a safety concern at estimated current intake (Table 2).

1.6 Consideration of secondary components

One member of this group of flavouring agents, 2,4,5-trimethyl-Δ3-oxazoline (No. 1559), has an assay value of < 95%. The secondary component in this substance, trimethyloxazole (No. 1553), was evaluated at the present meeting, where present levels of intake were considered to present no safety concern.

1.7 Consideration of combined exposure from use as flavouring agents

In the unlikely event that all 14 agents in structural class II were to be consumed concurrently on a daily basis, the estimated combined exposure would exceed the human exposure threshold for class II (540 μg per person per day). More than 98% of the total combined estimated exposure in Europe (1520 μg/kg bw per day) is accounted for by allyl isothiocyanate (No. 1560), for which toxicity data are available that adequately support the safety of this substance at the exposure level estimated from its use as a flavouring agent. In the unlikely event that both agents in structural class III were to be consumed concurrently on a daily basis, the estimated combined exposure would not exceed the human exposure threshold for class III (90 μg per person per day). Overall evaluation of the data indicates that combined exposure would not raise concern about safety.

1.8 Conclusions

The Committee concluded that use of the miscellaneous nitrogen-containing flavouring agents would not present a safety concern at the estimated daily exposure. For 10 flavouring agents (Nos 1553–1558, 1561–1563 and 1569), the evaluation was conditional because the estimated exposure was based on anticipated annual volumes of production. The conclusions of the safety evaluations of these agents will be revoked if use levels or poundage data are not provided before December 2007. The Committee noted that the available data on the toxicity and metabolism of these miscellaneous nitrogen-containing flavouring agents were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 16 miscellaneous nitrogen derivatives (see Table 1). The group comprises eight alkyl-substituted oxazole and oxazoline analogues (Nos 1553–1557 and 1569), five alkyl- or aryl-substituted isothiocyanate analogues (Nos 1560–1564), two pyrimidine analogues (Nos 1565 and 1566) and one alkyl-substituted pyrazole (No. 1568). The four sub-groups are structurally related and therefore have similar metabolic and toxicological profiles.

2.2 Additional considerations on exposure

Oxazoles and oxazolines are formed by the Strecker reaction during the frying of chicken or potatoes or roasting of meats, peanuts and coffee (Nijssen et al., 2003). At elevated temperatures, amino acids condense with α-dicarbonyl compounds to form unstable hydroxyimines, which then cyclize to produce substituted oxazoles and oxazolines. Naturally occurring oxazoles are detectable as flavours at extremely low concentrations. For instance, 2,4,5-trimethyloxazole and 2,4,5-trimethyl-3-oxazoline impart a boiled beef aroma at concentrations of about 0.5 ppb and 1 ppm, respectively (Mussinan et al., 1976). At similar levels, pyrimidines such

as 4-acetyl-2-methylpyrimidine impart a roasted, grilled or roasted nut flavour (Ohloff & Flament, 1978).

The four isothiocyanates in this group occur naturally in cruciferous vegetables such as horseradish, cabbage, mustard, cauliflower, Brussels sprouts, watercress, turnip, rutabaga, radish, endive and kohlrabi (Nijssen et al., 2003). Isothiocyanates form when the glucosinolate precursors (e.g. sinigrin) are hydrolysed by thioglucosidase (e.g. myrosinase), which is released when the plant material is disrupted or by microbes present in the human gut. In the hydrolysis reaction, glucose and sulfate are formed, with an unstable aglycone that undergoes rearrangement to a thiocyanate, isothiocyanate and nitrile, depending on the hydrolytic conditions and prior treatment of the plant material (see Figure 1) (Ettlinger & Kjaer, 1968). Each of the isothiocyanates in this group is formed from a specific glucosinolate present in different members of the Cruciferae plant family. Sinigrin, the glucosinolate source of allyl isothiocyanate, occurs in the head, leaves and buds of cabbage, kale, Brussels sprouts, cauliflower and broccoli and in the roots of horseradish, rapeseed and mustard. Gluconasturtiin, the source of 2-phenethyl isothiocyanate, is present in the roots of turnips, horseradish and Argentine rapeseed and in the leaves of watercress. Glucocochicarin, the glucosinolate source of butyl isothiocyanate, is found in cabbage, Brussels sprouts, watercress and *wasabi*. Glucotropaeolin, the source of benzyl isothiocyanate, occurs naturally in papaya, horseradish, watercress, garden cress, endive and rutabaga. Glucoibervirin, the glucosinolate source of 3-methylthiopropyl isothiocyanate, is present in cabbage, cauliflower, kohlrabi, horseradish and *wasabi*.

The key flavouring agent, allyl isothiocyanate, occurs in a wide range of raw and cooked Brassicaceae species at levels up to parts per million (e.g. 2.9 ppm in raw cabbage). Much higher levels have been measured in horseradish, *wasabi* and mustard (up to 9000 ppm in horseradish and 9585 ppm in *wasabi*) (Nijssen et al., 2003). Allyl isothiocyanate is responsible for the sharpness of these products and has both irritating and vesicating properties.

Figure 1. Formation of isothiocyanates

Fourteen of the 16 flavouring agents in this group have been reported to occur naturally in foods (Stofberg & Grundschober, 1987; Nijssen et al., 2003). Production volumes and intake values for each flavouring agent in this group are shown in Table 2.

2.3 Biological data

2.3.1 Biochemical data

(a) Absorption, distribution and excretion

Oxazoles, oxazolines, pyrimidines and pyrazoles

Few data are available on the absorption, distribution and excretion of the heterocyclic nitrogen derivatives in this group. Those that exist and data on other structurally related substances indicate that these substances are rapidly absorbed, metabolized and excreted in the urine. The majority of a dose of 1000 mg of 2-methyl-4,5-benzo-oxazole (No. 1557) or unsubstituted benzoxazole given orally to rabbits (2–3 kg bw) by stomach tube was excreted as glucuronic acid and sulfate conjugates in the urine within 24 h (Bray et al., 1952). Within 75 h, an oral dose of 400 mg of a 4,5-diphenyloxazole analogue [4,5-diphenyl-2-bis(2-hydroxylethyl)amino-oxazole] given to five healthy volunteers was excreted mainly as the parent compound and as polar metabolites in the urine, either unchanged or conjugated with glucuronic acid and sulfate (Maurer & Kleff, 1988). In six rabbits, most an oral dose of 1000 mg (2–3 kg bw) of 4,6-dimethylpyrimidine was absorbed, metabolized and excreted as polar acidic metabolites in the urine within 24 h (Bray et al., 1951).

Isothiocyanates

Given the importance of allyl isothiocyanate as the principal flavour ingredient in horseradish and mustard oil and the current interest in the anti-carcinogenic properties of phenethyl isothiocyanate and other arylalkyl isothiocyanates (Wattenberg, 1977; Morse et al., 1989a,b), extensive data on the pharmacokinetics and metabolism of this group of compounds are available. Isothiocyanates are readily absorbed and distributed to all major tissues in rodents, with peak levels achieved 2–8 h after dosing. At comparable doses, there are clear sex- and species-specific differences in the distribution, metabolism and excretion of substituted isothiocyanates.

Allyl isothiocyanate (No. 1560)

Dietary administration of allyl isothicyanate was reported to produce transitional-cell papillomas and epithelial hyperplasia in the urinary bladders of male Fischer 344 rats only (National Toxicology Program, 1982). In a study to determine the origin of species- and sex-specific benign bladder tumours in male rats, urine, faecal matter and expired CO_2 were collected from Fischer 344 rats and B6C3F$_1$ mice given ^{14}C-allyl isothiocyanate at 2.5 or 25 mg/kg bw orally or 25 mg/kg bw by intravenous injection. Most of the radioactivity was detected in urine (73–87% of the administered dose) from both species, regardless of the route of administration, after 72 h. Faecal excretion accounted for 3–6% of the oral dose and 1–2% of the

intravenous dose. At 24 h, 12–14% of the oral dose was detected in expired CO_2 of rats. The tissue distribution was determined after intravenous administration at times ranging from 15 min to 3 days after exposure: radioactivity was detected in blood, liver, kidneys and urinary bladder of both species at all doses. High levels of radioactivity were detected in the blood and kidneys of rats 15 and 45 min after dosage and in the liver and kidneys of mice 15 min after dosage. The highest levels of radioactivity were detected in the urinary bladder of male rats and male mice. After intravenous injection, there was about 18 times more ^{14}C-allyl isothiocyanate-derived radioactivity in male rat bladder than in female rat bladder. As the female rat bladder produces almost twice the volume of urine as that of male rats, the authors concluded that the concentration of potential irritants, allyl isothiocyanate or its metabolites, is greater in the urinary bladder of males than females (Ioannou et al., 1984).

In a similar study, conducted to examine sex and species differences, Fischer 344 rats and B6C3F_1 mice of each sex were given 2.5 or 25 mg/kg bw of radiolabelled allyl isothiocyanate orally, and urine and faeces were collected daily for 4 days. The animals were placed in metabolism cages for collection of exhaled CO_2. Most of the radioactivity was detected in the urine of both species, mice excreting significantly more of the administered dose (about 80%) than rats (about 55%). Between 10% and 18% of the administered radioactivity was detected in expired CO_2 and faeces of both rats and mice. About 20% of the administered dose remained in the carcass of rats and only 2–5% in the carcass of mice. Blood samples collected from both species showed peak blood radioactivity about 3 h after dosage, indicating that allyl isothiocyanate was rapidly absorbed. Analysis of radioactivity in blood samples drawn over 240 h indicated that allyl isothiocyanate underwent biphasic elimination, with an initial rapid phase of excretion dominating on the first day, followed by a slower phase. The half-life of allyl isothiocyante in the blood during the first phase of excretion was 15 h for mice and 37 h for rats, and that during the second, slower phase of excretion was 56 h for mice and 140 h for rats. This indicates that long-lived metabolites are retained in the blood. The levels of radioactivity that were detectable after 240 h in rats were no longer found after 96 h in mice, indicating that mice eliminated radioactivity from the blood more rapidly than rats.

In the same study, the tissue distribution of ^{14}C-allyl isothiocyanate was evaluated. Rats were given 25 mg/kg bw of the test substance orally and killed 0.33, 0.66, 1, 1.5, 2 and 6 h thereafter for removal of liver, kidneys, spleen, brain and urinary bladder. Radioactivity was detected in all organs at all times, with the highest level in the urinary bladder, followed by kidneys, liver, spleen and brain. The amount of radioactivity detected in the urinary bladder was similar in the two sexes at the first two sampling times but was significantly higher in males than females between 1 and 2 h. By 6 h, the difference in the level of radioactivity in the bladder between male and female rats was no longer significant owing to large inter-animal variation. Biliary excretion was also examined in this study. Cannulated male and female Fischer 344 rats given 2.5 mg/kg bw of ^{14}C-allyl isothiocyanate intravenously excreted the compound into the bile at a slow, steady rate, 12.8 ± 2.9% and 8.1 ± 2.4% of the dose passing through the bile duct of males and females, respectively, within 6 h of administration. The main peak detected by high-performance liquid chromatography (HPLC) was not identified but was not comparable to inorganic thiocyanate or allylthiocarbamoylmercapturic acid, the identified urinary metabolites of allyl

isothiocyanate; the authors suggested that the peak was due to a GSH conjugate. A comparison with the amount of radioactivity excreted into the faeces in the experiment described above suggested some enterohepatic recirculation of ^{14}C-allyl isothiocyanate or its metabolites in rats (Bollard et al., 1997).

To examine the effect of age on the metabolism and excretion of allyl isothiocyanate, male Fischer rats aged 3, 16 and 27 months were given 25 mg/kg bw of ^{14}C-allyl isothiocyanate orally, and urine, faeces and expired $^{14}CO_2$ and volatiles were collected for 72 h. Radioactivity was excreted primarily in the urine, in similar amounts by each age group (67–79% of the administered dose). In the 3- and 16-month-old animals, faecal excretion comprised 12–14% of the administered radioactivity but was significantly lower in the oldest group (about 6% of the dose). Similarly, expired $^{14}CO_2$ represented 10% of the administered allyl isothiocyanate in the two younger groups, while this route of elimination represented a slightly lower percentage in the oldest group (about 8%). Expired volatiles represented 4%, 2% and 7% of the allyl isothiocyanate dose in the 3-, 16- and 27-month-old groups, with a significant difference reported between the two older groups. Overall, the authors reported that age had no effect on the total excretion of radioactivity.

In the same study, to determine biliary excretion and bile flow rates, cannulated Fischer rats aged 3, 16 and 27 months were given 10 mg/kg bw of radiolabelled allyl isothiocyanate by intravenous injection into the femoral vein. Biliary excretion of radioactivity was highest in the 16-month-old rats (36.8 ± 2.16% of the dose) after 6 h and lowest in the 27-month-old rats (14.5 ± 2.5% of the dose). The bile flow rates were 53 ± 7, 42 ± 3 and 35 ± 5 mg bile per min/kg bw in the three groups, respectively. Although a significant decrease in bile flow seemed to correlate with increasing age, there was no corresponding decrease in the rate or level of overall elimination of radioactivity (Borghoff & Birnbaum, 1986).

In a study of the elimination of allyl isothiocyanate metabolites, volunteers received 10 or 20 g of mustard, which is known to contain 0.453 mg/g of allyl isothiocyante. Within 2 or 4 h of consumption, the levels of urinary metabolites identified as cysteine conjugates of allyl isothiocyanate reached 44.2–63.4% of the original dose of allyl isothiocyanate (Jiao et al., 1994).

Phenethyl isothiocyanate (No. 1563)

The endogenous mean plasma level of isothiocyanates in 23 non-dietary controlled volunteers was reported to be 413 ± 193 nmol/l in phenethyl isothiocyanate equivalents (Liebes et al., 2001).

In a phase-I clinical trial, three volunteers were given a single oral dose of 40 mg phenethyl isothiocyanate, and blood samples were collected at baseline and at 7.5, 15, 30 and 45 min and at 1, 1.5, 2, 3, 4, 6, 8, and 24 h after administration. In a single-compartment non-linear model of oral absorption for analysis of the pharmacokinetics, the area under the curve was 10.5 ± 1.61 μmol/l × h, clearance was 236 ± 36.8 ml/m^2 per min, the maximum plasma concentration was 1.04 ± 0.22 μmol/l, the elimination half-life was 3.7 ± 1.3 h and the time to maximum plasma concentration was 4.6 ± 0.7 h (Liebes et al., 2001).

Groups of three male Fischer 344 rats were given a single dose of 50 μmol/kg bw (3.71 μCi/μmol) of ^{14}C-phenethyl isothiocyanate (about 8.2 mg/kg bw) by gavage and killed after 0.5, 1, 2, 4, 8, 24 or 48 h. Rats to be killed at 24 and 48 h were

maintained in metabolism chambers for collection of urine, faeces and expired $^{14}CO_2$ at 8, 24 and 48 h. Rats that were killed at earlier times were maintained in metabolism cages for collection of urine and faeces 2, 4 and 8 h after dosing. Blood and tissue samples were taken for analysis after termination. Radioactivity in whole blood peaked at 2.9 h, at a concentration of 18.8 nmol/ml blood. In a two-compartment model for calculating pharmacokinetics in whole blood, the b elimination half-life of ^{14}C-phenethyl isothiocyanate was 21.7 h, and the area under the curve was 328.1 ± 106.9 nmol/ml × h. Radiolabelled phenethyl isothiocyanate was detected in several organs, listed from the highest maximum concentration: gastrointestinal tract (stomach, small intestine, caecum and colon), kidneys, liver, nasal mucosa, lungs, pancreas, spleen, oesophagus, heart and brain. At 24 h after administration, expired $^{14}CO_2$ represented 0.1% of the dose (0.039 ± 0.007 μCi). After 48 h, excretion of radiolabel in the urine and faeces represented 88.7 ± 2.2% and 9.9 ± 1.9% of the dose, respectively, most of the urinary excretion occurring within 24 h of dosing (Conaway et al., 1999).

Groups of three female A/J mice were given 5 μmol ^{14}C-phenethyl isothiocyanate per mouse (2 μCi per mouse; about 816 μg per mouse) by gavage and killed 1, 2, 4, 8, 24, 48 or 72 h after dosage. After sacrifice, various tissues were removed for analysis of the distribution of ^{14}C-phenethyl isothiocyanate, and the urine and faeces from each group were collected. Radioactivity was detected in the spleen, liver, lung, kidney and brain within 1 h of administration and in the heart after 2 h. The liver, kidney and lung had the highest levels of radioactivity, with peaks occurring 2–8 h after dosage in the liver and lung and peaks maintained for 1–8 h after dosage in the kidneys. Low levels of radioactivity were still detectable in these three tissues 72 h after administration. Excretion of radioactivity in urine and faeces after 24 h represented 36.6% and 12.0% of the dose, respectively. By 72 h, urinary and faecal excretion represented 55.2% and 23.3% of the dose, respectively (Eklind et al., 1990).

(b) Metabolism

Oxazoles, pyrimidines and pyrazoles

The metabolism of oxazoles follows two pathways: oxazole ring cleavage and, if the ring is properly substituted, ring hydroxylation. Within 75 h, an oral dose of 400 mg of a 4,5-diphenyloxazole analogue (4,5-diphenyl-2-bis(2-hydroxyethyl)-amino-oxazole) given to five healthy volunteers was metabolized by aromatic ring hydroxylation or hydrolytic oxazole ring cleavage to polar metabolites, which were excreted in the urine either unchanged or conjugated (Maurer & Kleff, 1988). Urine collected from rats after intravenous administration of 200 mg/kg bw of an alkyl-substituted oxazole analogue used as an antibiotic (flucloxacillin) showed side-chain oxidation of the alkyl substituent (Everett et al., 1989).

The presence of a substituent at the 2 position might stabilize the oxazole ring. For example, most of a dose of 1000 mg of benzoxazole given to eight rabbits (2–3 kg bw; equivalent to 500 mg/kg bw) by gavage was excreted in the urine as *ortho*-aminophenol, the oxazole ring cleavage product, and was found in the urine as aminophenol conjugates (*ortho*-aminophenylsulfate, *ortho*-aminophenylglucuronide and acylated aminophenol). When 1000 mg of 2-methyl-4,5-benzo-oxazole (No. 1557) were given orally to rabbits at the same dose by gavage, about 32% was

hydroxylated and excreted in the urine as glucuronide and sulfate conjugates, without rupture of the oxazole ring; lesser amounts were excreted as the oxazole ring cleavage products. When 500 mg/kg bw of 2-phenylbenzoxazole were administered to rabbits by gavage, no oxazole ring cleavage metabolite was detected in the urine. All the glucuronic acid or sulfate conjugated metabolites of the parent compounds and unchanged parent compound were excreted in urine within 24 h (Bray et al., 1952).

In six rabbits, most an oral dose of 1000 mg of 4,6-dimethylpyrimidine (2–3 kg bw) was metabolized to 4-methylpyrimidine-6-carboxylic acid, which was excreted in the urine as ether-soluble acid (55%), ethereal sulfate (8%) and ester glucuronide (2%) within 24 h (Bray et al., 1951). It is concluded that oxazole, pyrimidine and pyrazole flavouring agents are metabolized to polar products, which are excreted mainly in the urine.

Allyl isothiocyanate (No. 1560)

Metabolic studies in humans, mice and rats indicate that allyl isothiocyanate reacts readily with GSH by nucleophilic attack on the central carbon of the isothiocyanate group to form the GSH dithiocarbamate conjugate product as the principal metabolite. The reaction is catalysed enzymatically by glutathione *S*-transferase as well as non-enzymatically (at a slower rate), both reactions occurring in a pH-dependent equilibrium. Allyl dithiocarbamate conjugates are subsequently modified in a series of enzymatic steps, leading to urinary excretion of the mercapturic acid *N*-acetyl-*S*-(*N*-allylthiocarbamoyl)-L-cysteine metabolite (allyl thiocarbamoylmercapturate) (Mennicke et al., 1983; Ioannou et al., 1984; Borghoff & Birnbaum, 1986; Bruggeman et al., 1986; Jiao et al., 1994; Bollard et al., 1997). The stability of GSH conjugates under the conditions in the rodent bladder might lead to equilibrium with 'unconjugated free' isothiocyanate and GSH (Bollard et al., 1997). Thus, the presence of free isothiocyanates might irritate the rat bladder epithelium.

After ingestion of 10 or 20 g of mustard containing 0.453 mg/g of allyl isothiocyante by four volunteers, about half of the allyl isothiocyanate present in the mustard was excreted in urine as allyl thiocarbamoylmercapturate 12 h later. Most of the metabolite was excreted within 8 h, and no allyl isothiocyanate metabolites were detected in urine after 12 h. The amount of conjugated allyl isothiocyanate detected increased with the dose of mustard: at 10 g mustard consumed, 5.4 ± 1.7 mg of conjugate were detected in the urine; at 20 g of mustard, 12.8 ± 2.0 mg of conjugate were detected (Jiao et al., 1994).

After Fischer 344 rats and B6C3F$_1$ mice were given 25 mg/kg bw of ^{14}C-allyl isothiocyanate intravenously, metabolites were determined in bile (rats only), urine and faeces. The major urinary metabolite (24-h urine sample) identified in rats by nuclear magnetic resonance spectroscopy was allyl thiocarbamoylmercapturate, derived from GSH conjugation, which accounted for up to 82% of the administered dose. An unidentified metabolite was the major urinary metabolite in female mice, representing 59% of the administered dose, whereas four unidentified metabolites, each comprising 20–29% of the administered dose were the primary urinary metabolites in male mice. Unchanged allyl isothiocyanate was detected in trace amounts in rats (0.3–0.4%) and male mice (1.9%). The same unidentified metabolite constituted 100% of the allyl isothiocyanate metabolites detected in the faeces of all rodents. Four unidentified metabolites were detected in the bile (6-h sample) of

male rats but only two in the bile of female rats (Ioannou et al., 1984).

In a more recent study at the same doses, urine samples taken over 96 h from Fischer 344 rats and B6C3F$_1$ mice given 2.5 or 25 mg/kg bw of allyl ^{14}C-isothiocyanate orally showed three peaks in HPLC analysis, which were identified as inorganic thiocyanate, allyl thiocarbamoylmercapturic acid and allyl thiocarbamoylcysteine in both species. At the higher dose, thiocyanate output increased at the expense of allyl thiocarbamoylmercapturic acid in the rats and of allyl thiocarbamoylmercapturic acid and allyl thiocarbamoylcysteine in the mouse. In mice, up to 80% of the radioactivity detected in the urine was associated with ^{14}C-thiocyanate ion, whereas in rats about 75% was associated with the mercapturic acid conjugate. The authors concluded that hydrolysis of allyl isothiocyanate is the primary metabolic pathway in mice and that GSH conjugation predominates in rats. The authors noted a discrepancy between the number of metabolites identified in the urine (three) and the number identified in an earlier investigation (six) by Ioannou et al. (1984) and concluded that it was because the radiolabel was placed on the isothiocyanate moiety in the present study and on the more reactive, allyl moiety in the study of Ioannou et al. (1984). The larger number of metabolites identified by Ioannou et al. (1984) was thought to arise from subsequent oxidative metabolism of the allyl moiety (Bollard et al., 1997).

Twenty-four-hour urine samples collected from male Wistar rats given a single oral dose of 10 mg of allyl isothiocyanate (30–50 mg/kg bw) contained 40–50% of the administered dose as the mercapturic acid conjugate allyl thiocarbamoylmercapturate (Mennicke et al., 1983).

Male Fischer rats aged 3, 16 or 27 months were given 25 mg/kg bw of ^{14}C-allyl isothiocyanate orally. The main metabolite identified in urine was allyl thiocarbamoylmercapturate, and the percentage excreted in urine did not differ significantly by age group. A minor unidentified metabolite was detected in the two older groups only. Bile samples taken from cannulated Fischer rats aged 3, 16 and 27 months given 10 mg/kg bw of radiolabelled allyl isothiocyanate by intravenous injection showed similar metabolic profiles for the three age groups (metabolites not identified). The only exception was that allyl isothiocyanate was not detected in the oldest rats but at 8% of the administered dose in 3-month-old rats and 11% of the dose in 16-month-old rats 30 min after injection. The authors also assessed the GSH content of the livers of untreated rats of various ages to determine the potential for allyl isothiocyanate conjugation. Rats aged 2.5 and 3 months had a slight, but significantly lower GSH content in the liver and total GSH than rats aged 12 months and older. The authors concluded that the main metabolites of allyl isothiocyanate are produced by GSH conjugation and mercapturic acid formation in rat urine, as measured by allyl isothiocyanate-derived radioactivity. The formation of these major metabolites did not vary with age, while the occurrence of minor metabolites did (Borghoff & Birnbaum, 1986).

The potential effect of allyl isothiocyanate on blood clotting and prothrombin time was examined in two groups of four rats (strain and sex not specified) given 2 mg/kg bw orally. Before treatment and 4 h after treatment, blood samples were collected, and clotting and prothrombin times were determined. Prothrombin time was not affected by treatment; however, clotting times were decreased by 37% after consumption of allyl isothiocyanate. The same authors examined the possible effect of allyl isothiocyanate on plasma constituents and liver and kidney enzymes. A group

of four rats (sex and strain not specified) received 0 or 2 mg/kg bw of allyl isothiocyanate orally. The animals were killed 4 h after treatment, blood was collected from the heart, and the liver and kidneys were removed and homogenized. The total protein, total lipid and cholesterol values in plasma were similar in control and treated animals, showing no statistically significant differences, although lipid constituents tended to be slightly increased in treated animals. Renal D-amino acid oxidase and hepatic xanthine oxidase activities were significantly lower in treated animals than in controls, whereas hepatic succinic dehydrogenase activity was significantly increased (Ahmad et al., 1966).

In a follow-up study, allyl isothiocyanate was administered to rats either intraperitoneally or in the diet, and its effect on hepatic xanthine oxidase was examined. Groups of four rats (sex and strain not specified) were given 0 (vehicle control) or 0.5 mg/ml of allyl isothiocyanate daily by intraperitoneal injection or 0 (basal diet) or 0.3% in the diet. This dietary level was calculated to provide an average daily intake of 300 mg/kg bw (Food & Drug Administration, 1993), and the intraperitoneal injections provided 3–11 mg/kg bw. Both treatments were given for 30 days. On day 31, all the animals were killed, and their livers were removed and assayed for enzyme activity. Intraperitoneal and oral administration decreased xanthine oxidase activity by 92% and 59%, respectively, when compared with controls (statistics not reported). The authors speculated that allyl isothiocyanate binds strongly to xanthine oxidase and therefore blocks the access of xanthine and hypoxanthine (the normal substrates for xanthine oxidase) to the active site of the enzyme (Ahmad & Muztar, 1972).

Male outbred (Shoe:WIST) rats were given 0 (vehicle control), 50, 100 or 150 mg/kg bw of allyl isothiocyanate by gavage daily for 3 days and were killed 24 h after the last dose. Blood samples were taken, and the liver was removed, weighed and homogenized for analysis of enzyme activity. Body weights were significantly decreased in rats receiving 100 or 150 mg/kg bw per day. The absolute liver weight was significantly increased at the highest dose, and the relative liver weights were significantly increased at all doses. The protein concentration of the supernatant fraction of the liver homogenate was unaffected by treatment. Allyl isothiocyanate significantly decreased liver monooxygenase activities (aminopyrine-*N*-demethylation, *para*-nitroanisole-*O*-demethylation and aniline-*para*-hydroxylation) in all treated groups in a dose-dependent manner. Serum aspartate aminotransferase activity was significantly increased at the two higher doses, but alanine aminotransferase activity was unaffected by treatment (Lewerenz et al., 1988b).

Allyl isothiocyanate given to rats in the diet at a concentration of 0.1% for 30 days significantly decreased the blood levels of uric acid and glucose when compared with controls. This dietary level was calculated to provide an average daily intake of 100 mg/kg bw (Food & Drug Administration, 1993). The urinary concentrations of uric acid and glucose and the urine volume 24 h after treatment were significantly increased over control values (Huque & Ahmad, 1975).

Butyl isothiocyanate (No. 1561)

Twenty-four-hour urine samples collected from male Wistar rats (weighing 200–300 g) given a single oral dose of 10 mg of butyl isothiocyanate (about 40 mg/kg bw) contained 10–20% of the dose as the mercapturic acid conjugate, allyl thiocarbamoylmercapturate (Mennicke et al., 1983).

Benzyl isothiocyanate (No. 1562)

Aromatic isothiocyanate flavouring agents are also metabolized primarily to mercapturic acid conjugates. In humans and rats, aromatic isothiocyanates were metabolized mainly to the corresponding mercapturic acid conjugates, which subsequently hydrolysed to the corresponding cysteine conjugate and are excreted as the major urinary metabolite (see Figure 2). In rabbits, mice and guinea-pigs, however, the cysteine conjugate is hydrolysed and then undergoes transamination and cyclization to form a substituted thiazolidine-2-thione as the principal urinary metabolite.

Six male volunteers ingested 14.4 mg of encapsulated benzyl isothiocyanate 1–2 h after eating, and urine was collected at 2-h intervals for up to 24 h. The major urinary metabolite was identified by comparative chromatography as the mercapturic acid conjugate, allyl thiocarbamoylmercapturate (53.7 ± 5.9% of dose), which was detectable up to 10 h after dosage. The urinary concentration of this conjugate peaked 2–6 h after dosage in most of the men. The authors postulated that benzyl isothiocyanate conjugates with GSH and is readily excreted through the kidneys as the mercapturic acid conjugate (Mennicke et al., 1988).

Oral administration of 10 mg benzyl isothiocyanate or 20 mg of its cysteine conjugate, *S*-(*N*-benzylthiocarbamoyl)-L-cysteine, to male guinea-pigs resulted in urinary excretion of 4-hydroxy-4-carboxy-3-benzylthizolidin-2-thione, representing 23.3 ± 2.8% and 33.1 ± 4.0% of the dose, respectively. The *N*-acetylcysteine conjugate of benzyl isothiocyanate was detected only in trace amounts. Similar results were reported after administration of benzyl isothiocyanate (5 mg orally) and cysteine (12 mg orally; 20 mg intravenously), GSH (50 mg orally; 20 mg intravenously), cysteinylglycine (50 mg orally; 20 mg intravenously) or *N*-acetylcysteine (50 mg orally) conjugates of benzyl isothiocyanate (Görler et al., 1982).

Phenethyl isothiocyanate (No. 1563)

Female A/J mice were given 5 or 25 μmol per mouse of unlabelled phenethyl isothiocyanate by gavage (about 816 and 4081 μg per mouse), and their urine was collected for 72 h for HPLC analysis. Three metabolites accounting for > 80% of the urinary metabolites were detected: an unidentified metabolite (9–12.2%), 4-hydroxy-4-carboxy-3-phenethylthiazolidine-2-thione (46.8–58%) and allyl thiocarbamoyl-mercapturate (17.4–27.6%). About 10% of the ingested phenethyl isothiocyanate was excreted as the cyclic mercaptopyruvic acid conjugate and 25% as allyl

Figure 2. Metabolism of aromatic isothiocyanates

Aromatic isothiocyanate
R = phenyl or phenethyl

Major metabolite in humans and rats

Transamination
Cyclization

Major metabolite in mice, rabbits and guinea-pigs

thiocarbamoylmercapturate. The authors cited the study of Görler et al. (1982) to explain the formation of the cyclic mercaptopyruvic acid conjugate in vivo, which was thought to be initiated by enzymatic hydrolysis of the GSH conjugate to the cysteine conjugate. Transamination of the cysteine conjugate yields an *S*-substituted mercaptopyruvate that enolizes and immediately cyclizes to form a substituted thiazolidine-2-thione (Eklind et al., 1990).

Phenethyl isothiocyanate administered by gavage at a dose of 163 mg/kg bw per day to eight male rats previously given ethanol orally for 38 days prevented an ethanol-induced increase in cytochrome P450 2E1 apoprotein and decreased the catalytic activity and the amount of mRNA to levels lower than those in controls. Administration of phenethyl isothiocyanate was also associated with a significant increase in blood acetaldehyde levels over those of rats receiving ethanol only. After intraperitoneal administration to rats at a dose of 0.2 or 1 mmol/kg bw (32.6 and 163.2 mg/kg bw, respectively), phenethyl isothiocyanate inhibited total liver aldehyde dehydrogenase activity, but not alcohol or lactate dehydrogenase activities, by more than 25% and 70%, respectively. This effect persisted for at least 24 h after administration (Lindros et al., 1995).

In conclusion, isothiocyanates are readily absorbed, conjugated with GSH and excreted, predominantly in the urine as mercapturic acid derivatives. It has been suggested (Bollard et al., 1997) that at high doses (e.g. 25 mg/kg bw per day) of allyl isothiocyanate, the position of the equilibrium between the GSH conjugated and unconjugated forms shifts towards formation of 'unconjugated free' isothiocyanate or isothiocyanate metabolites, especially in the bladder of male rats. These irritants would enhance toxicity in bladder epithelium with prolonged exposure (see below). Given that female rats produce twice the volume of urine of male rats, the dilution would significantly reduce the exposure of females. This conclusion is supported by the observation that bladder toxicity is observed only in male rats.

2.3.2 Toxicological studies

The toxicological studies are summarized below according to duration, flavouring agent and then species. In order to preserve the continuity of the studies performed within the National Toxicology Program, however, the short-term studies of toxicity and the carcinogenicity studies are both discussed in the section on long-term studies in the sequence in which they were conducted.

(a) Acute toxicity

Oral LD_{50} values have been reported for three of the 16 agents in this group (see Table 3). The LD_{50} of 2,4,5-trimethyl-Δ3-oxazoline (No. 1559) is 4840 mg/kg bw in mice (Shellenberger & Gough, 1971); that of allyl isothiocyanate (No. 1560) is 112–490 mg/kg bw in rats and 310 mg/kg bw in mice (Anderson & Hurwitz, 1953; Jenner et al., 1964; Vernot et al., 1977), and that of 3-methylthiopropyl isothiocyanate (No. 1564) is 540 mg/kg bw (range, 485–600 kg bw) in rats (Harper & Ginn, 1964).

(b) Short-term studies of toxicity

The results of short-term studies with representative miscellaneous nitrogen derivatives are summarized in Table 4 and are described below. Short-term studies have been performed with oxazole (No. 1555), oxazoline (No. 1559), pyrimidine

Table 3. Results of studies for acute toxicity with orally administered miscellaneous nitrogen derivatives

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1559	2,4,5-trimethyl-Δ3-oxazoline	Mice; M, F	4840	Shellenberger & Gough (1971)
1560	Allyl isothiocyanate	Rat; F	112	Anderson & Hurwitz (1953)
1560	Allyl isothiocyanate	Rat; M, F	339	Jenner et al. (1964)
1560	Allyl isothiocyanate	Rat; M	490	Vernot et al. (1977)
1560	Allyl isothiocyanate	Mice; M	310	Vernot et al. (1977)
1564	3-Methylthiopropyl isothiocyanate	Rat; M, F	540	Harper & Ginn (1964)

M, male; F, female

(Nos 1565 and 1566) and pyrazole (No. 1568) analogues and with four (Nos 1560 and 1562–1564) of the five substituted isothiocyanates.

2-Ethyl-4,5-dimethyloxazole (No. 1555)

Groups of 16 Sprague-Dawley rats of each sex were given 0 or 2.3 mg/kg bw per day of 2-ethyl-4,5-dimethyloxazole by gavage for 13 weeks. The animals were observed daily for clinical signs of toxicity, and body weight and food consumption were measured weekly. At 4 weeks, blood samples were taken for analysis from eight rats of each sex per group. At 13 weeks, blood samples were taken, the animals were killed and necropsied, organs were removed and weighed (liver, spleen, kidney, heart, brain, testes, uterus, prostate, adrenals and hypophysis), and selected tissues were prepared for microscopic examination. No differences in clinical signs of toxicity, body weight changes or food consumption were found between dosed and control animals. No deaths occurred during the study. Clinical chemistry showed a statistically significant decrease in blood urea nitrogen ($p < 0.001$) in treated males at 4 weeks but not at 13 weeks. At 13 weeks, treated males had statistically significant lower serum bilirubin values ($p < 0.005$) than controls. No other changes in clinical chemistry parameters were reported. No effects on haematological end-points were reported at 4 weeks or in treated males at 13 weeks; however, treated females had significantly reduced erythrocyte volume fractions ($p < 0.01$), red blood cell counts ($p < 0.01$) and haemoglobin concentration ($p < 0.005$) at 13 weeks. Necropsy revealed no significant gross or microscopic changes. The absolute organ weights of treated and control animals were similar; however, on a percentage body weight basis, the liver and heart of treated males weighed significantly ($p < 0.01$) more than those of controls. There were no accompanying histopathological changes in these organs, and the authors concluded that the minimal haematological and clinical chemistry changes did not represent toxic effects (Griffiths et al., 1979).

2,4,5-Trimethyl-Δ3-oxazoline (No. 1559)

Groups of 15 male and 15 female Wistar-derived rats were fed diets containing 2,4,5-trimethyl-Δ3-oxazoline at a concentration that provided an average intake of

Table 4. Results of short-term and long-term studies of toxicity with miscellaneous nitrogen derivatives

No.	Substance	Species; sex	No. test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL mg/kg bw per day)	Reference
Short-term studies							
1555	2-Ethyl-4,5-dimethyloxazole	Rat; M, F	1/32	Gavage	91	2.3[c]	Griffiths et al. (1979)
1559	2,4,5-Trimethyl-Δ3-oxazoline	Rat; M, F	1/30	Diet	90	41[c]	Morgareidge (1972)
1560	Allyl isothiocyanate	Rat; M, F	2/10	Gavage	20	< 20	Hagan et al. (1967)
1560	Allyl isothiocyanate	Rat; M	3/NR	Gavage	≤ 42	20	Lewerenz et al. (1988a)
1560	Allyl isothiocyanate	Rat; NR	1/4	Gavage[d]	28	<100	Ahmad et al. (1966)
1560	Allyl isothiocyanate	Rat; NR	1/4	Diet	35	100[c]	Ahmad et al. (1966)
1560	Allyl isothiocyanate	Rat; M, F	3/10	Diet	182	500[c]	Hagan et al. (1967)
1563	Phenethyl isothiocyanate	Rat; M	1/12	Diet	≤ 686	65.2[c]	Chung et al. (1996)
1563	Phenethyl isothiocyanate	Rat; M	2/6	Diet	91	56[c,e]	Gray et al. (1995)
1563	Phenethyl isothiocyanate	Rat; M	2/6	Diet	91	< 8[f]	Gray et al. (1995)
1563	Phenethyl isothiocyanate	Rat; M	3/15	Diet	224	5	Ogawa et al. (2001)
1564	3-Methylthiopropyl isothiocyanate	Rat; M	3/10	Diet	84	30[c]	Harper et al. (1961)
1565	4-Acetyl-2-methylpyrimidine	Rat; M, F	1/32	Gavage	91	1[c]	Peano (1981)
1566	5,7-Dihydro-2-methylthieno(3,4-*d*)-pyrimidine	Rat; M, F	1/46	Diet	90	M: 6.60[c] F: 6.71[c]	Shellenberger (1970)
1568	1-Phenyl-3- or -5-propylpyrazole	Rat; M, F	1/20–32	Diet	90	M: 26.07[c] F: 24.81[c]	Posternak et al. (1969)
Long-term studies							
1560	Allyl isothiocyanate	Mouse; M, F	5/10	Gavage	14	25	National Toxicology Program (1982)
1560	Allyl isothiocyanate	Mouse; M, F	5/20	Gavage	91	25c	National Toxicology Program (1982)
1560	Allyl isothiocyanate	Mouse; M, F	2/100	Gavage	721	25[c,g]	National Toxicology Program (1982)

Table 4 (contd)

No.	Substance	Species; sex	No. test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL mg/kg bw per day)	Reference
1560	Allyl isothiocyanate	Rat; M, F	5/10	Gavage	14	25	National Toxicology Program (1982)
1560	Allyl isothiocyanate	Rat; M, F	5/20	Gavage	91	25[c]	National Toxicology Program (1982)
1560	Allyl isothiocyanate	Rat; M, F	2/100	Gavage	721	12	National Toxicology Program (1982)

M, male; F, female; N/R, not reported

[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Study performed with either a single dose or multiple doses that had no adverse effect. The value is therefore not a true NOEL, but is the highest dose tested that had no adverse effect. The actual NOEL might be higher.
[d] Animals previously given test substance in feed for 5 weeks
[e] Rats were given AIN-76A purified diet containing phenethyl isothiocyanate at a concentration of 0, 0.75 or 6.0 mmol/kg for 13 weeks (corresponding to 0, 7 and 56 mg/kg bw per day, respectively). While control animals had significantly increased hepatic lipid accumulation, significant reductions in hepatocyte lipid content were observed in treated groups.
[f] Rats were given Wayne cereal-based rodent meal containing phenethyl isothiocyanate at a concentration of 0, 0.75 or 6.0 mmol/kg for 13 weeks (corresponding to 0, 8 and 67 mg/kg bw per day, respectively). Dose-related reductions in hepatocyte, lipid droplet, peroxisome and mitochondrial volumes were observed in treated groups compared with controls.
[g] Significant dose-related increase in cytoplasmic vacuolization of the liver in male mice; severity similar in control and treated groups; most vacuoles were centrilobular, and all contained fat.

about 41 mg/kg bw per day for 90 days. Control animals received 444 mg/kg bw per day of beef tallow in the diet. Rats were observed daily for clinical signs of toxicity, and body weights and food consumption were recorded weekly. At weeks 6 and 12, blood and urine samples were taken from eight rats of each sex per group for haematological, clinical chemistry and urine analyses. After 90 days, the rats were killed, the livers and kidneys were removed and weighed, and the rats were necropsied. Multiple tissues from eight rats of each sex per group and the liver and kidneys from the remaining animals in each group were examined histopathologically. No differences in body weight, food consumption or haematological, clinical chemistry or urine parameters were found between treated and control rats, and no gross or histopathological changes attributable to treatment were noted (Morgareidge, 1972).

Allyl isothiocyanate (No. 1560)

Groups of five weanling Osborne-Mendel rats of each sex were given 0 (vehicle control), 20 or 50 mg/kg bw per day of allyl isothiocyanate in corn oil by stomach tube for 20 days. Macroscopically, the non-glandular part of the stomach was thickened, with occasional roughening of the lining at both doses. Microscopically, these lesions were accompanied by slight-to-moderate epithelial hyperplasia with acute-to-subacute ulcers in all rats at the higher dose and in 50% of those at the lower dose. Minor inflammatory foci were reported in the livers of rats at the higher dose. No further details were provided (Hagan et al., 1967).

Groups of male outbred rats (Shoe:WIST) (number of animals per group not specified) were given 0 (paraffin oil vehicle control), 10, 20 or 40 mg/kg bw per day of allyl isothiocyanate, 5 days per week for up to 6 weeks by gavage. Body weight and food intake were recorded weekly, and haematological, clinical biochemistry and organ weight analyses were carried out at intervals of 7 or 14 days. The urine of treated rats was analysed after 5 weeks. The rats were subjected to complete necropsy and histopathological examination at the end of 6 weeks. Body-weight gain was significantly reduced at weeks 5 ($p < 0.05$) and 6 ($p < 0.01$) in rats at the highest dose. Food consumption was significantly increased in rats at the lowest dose in weeks 1 and 2 but was significantly reduced in animals at the intermediate dose at week 6 and in rats at the highest dose in weeks 1 and 6. At 2 weeks, there was a significantly increased percentage of neutrophils and a significantly decreased percentage of lymphocytes in blood samples from rats at the highest dose; however, these percentages returned to normal after 3 weeks. When tested over 4 weeks, the absolute thymus weight was significantly decreased in animals at the highest dose in weeks 1 and 2, and the absolute liver and adrenal weights were significantly increased in all treated animals at week 3. By week 4, all the absolute organ weights were comparable to those of controls. The relative thymus weight was also decreased in animals at the highest dose at week 2, and the relative liver weight was significantly increased in all treated animals at week 1 and in animals at the two higher doses at week 3; the relative adrenal weight was significantly increased in animals at the highest dose at week 1 and in those at the intermediate dose at week 3. The relative organ weights were also comparable to those of controls by week 4. The absolute and relative thyroid weights in treated animals up to 4 weeks were comparable to those of controls. Blood samples taken up to 4 weeks showed significant hypoglycaemia in animals at the highest dose at weeks 2 and 4 and a significant decrease

in serum globulin at week 2, but not at weeks 3 or 4. Urine samples taken at 5 weeks showed a significantly decreased urine volume at the highest dose, significantly decreased specific gravity at all doses, and significantly increased aspartate aminotransferase activity at the highest dose. Microscopically, the livers of some rats at the highest dose showed diffuse ballooning of hepatocytes in the centrilobular region. The kidneys of rats at the highest dose and most of those at the intermediate dose showed dilatation of the distal tubules and increased desquamation (Lewerenz et al., 1988b).

In a 9-week study conducted to examine the possible effects of allyl isothiocyanate on growth, groups of four weanling rats (strain not specified) were fed basal diet containing 0 or 0.1% allyl isothiocyanate for 5 weeks. This dietary level was calculated to provide an average daily intake of 100 mg/kg bw (Food & Drug Administration, 1993). During weeks 6–9, rats were gavaged daily with the amount of allyl isothiocyanate calculated to be received daily in food (approximately 100 mg/kg bw). During the initial 5 weeks, when the rats received ally isothiocyanate in the diet, there was no significant difference in body-weight changes between control and treated animals. After week 6, when the animals were given daily bolus doses by gavage, treated animals had decreased body-weight gain (statistics not reported) (Ahmad et al., 1966).

Groups of five weanling Osborne-Mendel rats of each sex were fed diets containing allyl isothiocyanate at a concentration of 0, 1000, 2500 or 10 000 ppm for 26 weeks without adverse effects. These dietary levels were calculated to provide average daily intakes of 0, 50, 125 and 500 mg/kg bw, respectively (Food & Drug Administration, 1993). No additional details were available (Hagan et al., 1967).

Benzyl isothiocyanate (No. 1562)

Groups of 15 male rats (Shoe:WIST) were given 0 (vehicle control), 50, 100 or 200 mg/kg bw per day of benzyl isothiocyanate in sunflower oil by gavage for 4 weeks. Animals were observed daily for appearance, behaviour and any clinical signs of toxicity; body weights were recorded daily, and food consumption was recorded weekly. After 1, 2 and 4 weeks of treatment, blood samples were taken for haematological analysis, whereas serum samples for clinical chemistry were collected only in week 4. Urine samples were collected over 16 h after 1, 2 and 3 weeks of treatment. After 4 weeks of treatment, all animals were killed and necropsied, and several major organs were removed from 10 animals per group and weighed, while several tissue samples from a range of organs were taken from five animals per group for microscopic examination. One rat at 50 mg/kg bw per day died as a result of a gavaging error. No other premature deaths occurred, and there were no clinical signs of toxicity. Body-weight gain was significantly reduced in a dose-dependent manner in all groups of treated rats as compared with controls. Food consumption was significantly reduced at 200 mg/kg bw per day during weeks 1–3 and at 100 mg/kg bw per day during weeks 1 and 2 when compared with controls; however, no significant variations in food consumption were observed at week 4. The reductions in body-weight gain were accompanied by decreased food use efficiency at the two higher doses. In rats at the highest dose, statistically significant variations were observed in all the haematological parameters tested (haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, mean

corpuscular volume, platelets and total and differential white blood cell counts) at some point during the study; however, only mean corpuscular haemoglobin (decreased), total white blood cell count (decreased), neutrophil count (increased) and leukocyte count (decreased) were significantly different from control values after 4 weeks of treatment. At week 2, the levels of eosinophils and leukocytes were significantly lower and those of neutrophil levels significantly higher in animals at the intermediate dose than in controls; however, after 4 weeks of treatment, the values were comparable. The only significant haematological finding at the lowest dose was an increase in total white blood cell count after 4 weeks of treatment, which contrasts with the finding of decreased total white blood cells in animals at the highest dose at the same time. After 4 weeks of treatment, statistically significant, dose-dependent increases in serum cholesterol levels were seen at all doses, whereas serum triglycerides were significantly decreased only at the highest dose. Urine volume was decreased at each dose, reaching statistical significance after 1, 2 and 3 weeks of treatment at the highest dose and after 3 weeks at the lowest dose. Urinary protein was significantly increased, although not in a dose-dependent manner, after 2 and 3 weeks of treatment in animals at the two higher doses. The urinary activity of lactate dehydrogenase was significantly increased in all treated animals, but the activities were higher in animals at the lowest dose than in those at the highest dose. The absolute weights of the brain, pituitary, thyroid, thymus, heart, liver, spleen, kidneys and testes were significantly reduced in rats at the highest dose, whereas the absolute adrenal weights were significantly increased in these animals. In rats at the intermediate dose, the absolute weights of the brain, thymus, heart, spleen and kidney were significantly reduced. At the lowest dose, the relative weights of the thyroid and adrenals were significantly increased. At the two higher doses, the relative (to body weight) weights of the brain, pituitary, adrenals and testes were significantly increased, whereas the relative thymus weight was significantly decreased. At the highest dose, the relative weights of the thyroid and kidney were significantly increased and that of the spleen was significantly decreased. The relative liver weight was significantly increased at all doses. Gross examination revealed peritonitis accompanied by adhesions in the abdominal cavity of 6/15 animals at the highest dose and 1/15 at the intermediate dose. Enlargement of the mesenteric lymph nodes was reported at the two higher doses (intermediate dose, 2/15; highest dose, 6/15). Catarrhal enteritis was seen in 3/15 rats at the highest dose. Microscopic examination revealed proliferation of the epithelium of the bile duct at each dose: control, 0/5; lowest dose, 3/5; intermediate dose, 3/5; highest dose 5/5. Additionally, 60–100% of the five rats at the highest dose whose organs were examined microscopically had lymphadenitis of the mesenteric lymph nodes, chronic catarrhal ileitis and cloudy swelling of hepatocytes. The lowest-observable-effect level (LOEL) was 50 mg/kg bw per day (Lewerenz et al., 1992).

Although no NOEL is available for benzyl isothiocyanate (No. 1562), the NOEL of 5 mg/kg bw per day (Ogawa et al., 2001) for the structurally related agent phenethyl isothiocyanate is also appropriate for benzyl isothiocyanate, as they are both isothiocyanates, which are metabolized through similar metabolic pathways. This NOEL is 250 000 times the estimated intake of this substance from its proposed use as a flavouring agent in Europe (0.02 μg/kg bw per day) and about 700 000 times the estimated intake from its proposed use as a flavouring agent in the USA (0.007 μg/kg bw per day).

Phenethyl isothiocyanate (No. 1563)

A bioassay of chemopreventive efficacy was conducted in groups of 12 male Fischer 344 rats given diets containing phenethyl isothiocyanate at a concentration of 0 or 1304 ppm for up to 98 weeks. This dietary level was calculated to provide an average daily intake of 65.2 mg/kg bw (Food & Drug Administration, 1993). According to the authors, the concentration of phenethyl isothiocyanate used in the study represented 80% of the maximum tolerated dose, which was determined to be 1630 ppm [about 81.5 mg/kg bw per day (Food & Drug Administration, 1993)]. During the study, food consumption and body weights were recorded weekly for the first 20 weeks and then once every month for the remainder of the study. The study was terminated after 98 weeks, when about 80% mortality was observed, and rats were subjected to gross necropsy examination. No significant differences were observed between control and treated rats in body weight, food consumption, survival rate or tumour incidence in the lungs, liver, nasal cavity, pancreas and testes during the study (Chung et al., 1996).

To investigate the observation in previous studies of marked hepatic lipidosis, particularly in the periportal region of the liver, in rats fed AIN-76A purified diet, groups of six male Fischer 344 rats were fed either AIN-76A purified diet or Wayne rodent meal (cereal-based) containing phenethyl isothiocyanate at a concentration of 0, 0.75 or 6.0 mmol/kg for 13 weeks. The average daily intakes of phenethyl isothiocyanate were 0, 7 and 56 mg/kg bw from the AIN-76A diet and 0, 8 and 67 mg/kg bw from the Wayne diet. Animals were observed daily for clinical signs of toxicity, and body weight and food consumption were recorded weekly (results not reported). At the end of the study, the animals were killed and their livers were removed for weighing and gross and microscopic examination. No significant effects on absolute liver weight were reported. All animals (including controls) fed the AIN-76A diet showed markedly more ($p < 0.05$) hepatic lipid accumulation than those fed the Wayne diet. Dietary phenethyl isothiocyanate significantly ($p < 0.05$) reduced the hepatocyte lipid content in animals on the AIN-76A diet but significantly ($p < 0.05$) increased the lipid content in animals fed the Wayne diet, in comparison with controls. Significant ($p < 0.05$) and to some extent dose-related reductions in hepatocyte, lipid droplet, peroxisome and mitochondrial volumes were reported in rats given phenethyl isothiocyanate in the Wayne diet. The authors suggested that the lipid reduction was a result of inhibition of lipid synthesis or induction of lipid mobility to reduce the lipid content. Binding of phenethyl isothiocyanate to membrane proteins would result in reduced protein synthesis and altered lipid metabolism and excretory and secretory functions of hepatocytes. The authors reported that in an unpublished study in Fischer 344 rats given an NIH-07 diet containing 0 or 3–15 mmol/kg diet of phenethyl isothiocyanate, no liver toxicity was observed in control or treated rats. They concluded that, under experimental conditions in which any hepatotoxicity could be ascertained, phenethyl isothiocyanate did not appear to be toxic and that, with respect to the liver, phenethyl isothiocyanate could be tolerated at doses that far exceeded those that have effective chemopreventive activity (Gray et al., 1995).

As part of a study of chemoprevention in rat urinary bladder and liver, groups of 15 male Fischer 344 rats were fed diets containing phenethyl isothiocyanate at a concentration of 0, 0.01, 0.05 or 0.1% for 32 weeks. These dietary levels were calculated to provide average daily intakes of 0, 5, 25 and 50 mg/kg bw, respectively

(Food & Drug Administration, 1993). All animals were killed at the end of the 32-week feeding period, and their livers and urinary bladders were excised for weighing and analysis. Eight rats from each group received an intraperitoneal injection of 100 mg/kg bw bromodeoxyuridine 1 h before sacrifice. When compared with controls, no significant alterations in body weights were seen in treated rats at the end of the study. The relative liver and urinary bladder weights were increased in a dose-dependent manner and were significantly increased in rats at 25 and 50 mg/kg bw per day. No histopathological effects on the liver were observed in treated rats, but histopathological examination of the urinary bladder showed statistically significant, dose-dependent increases in the incidences of simple hyperplasia (control, 0/15; lowest dose, 1/15; intermediate dose, 15/15; highest dose, 15/15), papillary or nodular hyperplasia (control, 0/15; lowest dose, 0/15; intermediate dose, 15/15; highest dose, 15/15) and dysplasia (control, 0/15; lowest dose, 0/15; intermediate dose, 8/15; highest dose, 12/15). There were no significant differences between treated and control groups in the incidences of urinary bladder papillomas, transitional-cell carcinomas or squamous-cell carcinomas. Relative to controls, rats at the highest dose had significantly increased bromodeoxyuridine labelling indices in normal mucosal tissue, papillary or nodular hyperplasia and dysplasia of the urinary bladder (Ogawa et al., 2001).

3-Methylthiopropyl isothiocyanate (No. 1564)

Groups of 10 male rats were fed diets containing 0, 0.6, 60 or 600 ppm of 3-methylthiopropyl isothiocyanate for 12 weeks, calculated to provide average daily intakes of 0, 0.03, 3 and 30 mg/kg bw, respectively (Food & Drug Administration, 1993). Weekly food consumption was calculated, and body weights were recorded at weeks 1, 2, 4, 6, 8 and 12. All surviving rats were killed after 12 weeks and necropsied. All animals survived to scheduled termination. The food consumption and body-weight gain of treated animals were similar to control values. Treatment had no effect on organ weights, and no histological changes were reported (Harper et al., 1961).

4-Acetyl-2-methylpyrimidine (No. 1565)

Groups of 16 Sprague-Dawley Charles River CD (SD) BR rats of each sex were given 0 (vehicle control) or 1 mg/kg bw per day of 4-acetyl-2-methylpyrimidine in corn oil by gavage for 13 weeks. The rats were observed daily for clinical signs of toxicity, and body weights and food consumption were recorded weekly. At weeks 4 and 13, blood samples were taken from eight rats of each sex per group for analysis. After 13 weeks, the rats were killed and necropsied. All animals survived to scheduled necropsy with no clinical signs of toxicity. Initially, all animals had diarrhoea, which was considered to be a result of the volume of corn oil used (10 ml/kg bw); when the volume was reduced (5 ml/kg bw) after 20 days, the diarrhoea stopped. No differences in body weight or food consumption were reported between treated and control rats. No effects on haematological end-points were reported at week 4. At week 13, treated males had a significantly increased mean corpuscular haemoglobin concentration, total bilirubin and alkaline phosphatase activity and significantly reduced mean corpuscular volume compared with controls. Treated females had

significantly reduced erythrocyte counts and alkaline phosphatase activity; however, the individual values for these animals remained within the normal physiological ranges for this species. The absolute organ weights of treated animals of each sex and the relative organ weights of treated males were similar to control values. In females, the relative weights of the liver, kidney and heart were significantly decreased, but the reductions were not accompanied by histopathological changes. Furthermore, the individual values for relative organ weights in treated female rats remained within the normal physiological ranges. No histological changes attributable to treatment were reported (Peano, 1981).

*5,7-Dihydro-2-methylthieno(3,4-*d*)pyrimidine (No. 1566)*

In a 90-day study, groups of 23 weanling Sprague-Dawley rats of each sex were fed diets containing 5,7-dihydro-2-methylthieno(3,4-*d*)pyrimidine at concentrations that provided average intakes of 6.60 and 6.71 mg/kg bw per day for males and females, respectively. Controls were fed basal diet. Animals were observed daily for clinical signs of toxicity, and body weights and food consumption were recorded weekly. At weeks 6 and 13, urine was collected from eight rats of each sex in each group for analysis. At week 6, eight rats of each sex in each group were killed for blood sampling and analysis. At week 13, the remaining 15 rats of each sex per group were killed and necropsied. Before sacrifice, blood samples were taken from eight rats of each sex per group. There were no clinical signs of toxicity, and the body weights and food consumption of treated rats were similar to those of controls. There were no consistent, significant differences from controls in haematological, clinical chemistry, and urine analyses or in organ weights. All the values were within the expected ranges for rats of this age and strain. Histopathological examination revealed hyperaemic lung tissue in some animals (both treated and control), indicative of a chronic mild respiratory condition that is not unusual in this strain. Additionally, some animals (both treated and controls) had mild, pale, discoloured or mottled areas on the liver in a randomly distributed low incidence. One control male had an abscess in the liver, and one treated male had a small hepatic cyst. Overall, no adverse effects were observed (Shellenberger, 1970).

1-Phenyl-3- or -5-propylpyrazole (No. 1568)

Groups of 10–16 Charles River CD rats of each sex were fed a diet containing 1-phenyl-3- or -5-propylpyrazole at concentrations that provided average daily intakes 26.1 and 24.8 mg/kg bw for males and females, respectively, for 90 days. Body weight and food consumption were recorded weekly, and food use efficiency was calculated. Haematological end-points and blood urea were determined for half the animals at week 7 and for all animals at week 13. At necropsy, gross and histological examinations were conducted on a wide range of organs, and the liver and kidneys were removed and weighed. No compound-related effects on body weight, food consumption, food use efficiency, haematological and clinical chemistry (i.e. blood urea) end-points, organ weights or histopathological appearance were reported (Posternak et al., 1969).

(c) Long-term studies of toxicity and carcinogenicity

Allyl isothiocyanate (No. 1560)

Mice

Groups of five $B6C3F_1$ mice of each sex were gavaged with 3, 6, 12, 25 or 50 mg/kg bw per day of allyl isothiocyanate in corn oil for 14 days. No control groups were used. Animals were observed twice daily and weighed on days 1 and 15. On days 17–31, the surviving animals were killed and examined grossly. No clinical signs of toxicity were observed. One male at 50 mg/kg bw per day died on day 15. No dose-dependent changes were seen between groups in body-weight gain, which varied from 3% to 16%. At gross necropsy, four males and all females at the highest dose showed a thickened area of mucosa in the non-glandular region of the stomach, and four males and one female at this dose had a thickened urinary bladder wall (National Toxicology Program, 1982).

Groups of 10 $B6C3F_1$ mice of each sex were given 0 (vehicle control), 1.5, 3, 6, 12 or 25 mg/kg bw per day of allyl isothiocyanate by gavage, 5 days per week for 13 weeks. Animals were observed twice daily for mortality, morbidity and clinical signs of toxicity. On days 92–96, surviving animals were killed and necropsied. Animals found dead or moribund were also necropsied. Various tissues, masses and gross lesions from controls and animals at 25 mg/kg bw per day were examined histologically. No compound-related deaths or clinical signs of toxicity were reported. One male at 1.5 mg/kg bw per day and one at 12 mg/kg bw per day and two females at 3 mg/kg bw per day and each at 6 and 12 mg/kg bw per day died as a result of gavaging errors. The mean body weights of treated animals were comparable to those of controls. No gross or histological changes were reported at any dose (National Toxicology Program, 1982).

In a bioassay for carcinogenesis, groups of 50 male and 50 female $B6C3F_1$ mice received 0 (vehicle control), 12 or 25 mg/kg bw per day of allyl isothiocyanate by gavage, 5 days per week for 103 weeks. The animals were observed twice daily for morbidity and deaths, and clinical signs of toxicity and body weights were recorded every 4 weeks. During weeks 104–106, the surviving animals were killed and necropsied. Animals found dead were also necropsied, and those found moribund were killed and necropsied. A selection of tissues, masses and gross lesions from all groups were examined histologically. Survival was comparable in all the groups, although that of control females was consistently lower than the survival of treated groups after week 40. One control male, six at the lowest dose and seven at the highest dose and one female at the highest dose died as a result of gavaging errors. Many of the female mice that died before week 104 (control, 13/34; lowest dose, 6/25; highest dose, 12/30) had suppurative inflammation of the peritoneum, uterus or multiple organs, suggesting generalized infection. The mean body weights of mice of each sex at the highest dose were higher than those of controls for most of the study; however, the final mean body weights of treated and control animals were comparable (statistics not reported). The incidence of primary tumours was not increased in treated mice. Male mice showed a statistically significant ($p < 0.05$), dose-related increase in cytoplasmic vacuolization in the liver (control, 2/49; lowest dose, 8/49; highest dose, 13/50). The severity of this lesion was similar in all three groups. Most of the vacuoles were centrilobular, and all contained fat. The authors

concluded that allyl isothiocyanate was not carcinogenic (National Toxicology Program, 1982).

Rats

Groups of five Fischer 344/N rats of each sex were given 25, 50, 100, 200 or 400 mg/kg bw per day of allyl isothiocyanate in corn oil by gavage for 14 days. No control groups were used. Animals were observed twice daily and were weighed on days 1 and 15. On days 16–17, the surviving animals were killed and underwent gross necropsy. Clinical signs, including inactivity and ruffled fur, were observed at all doses, the severity increasing with dose; all animals at the two higher doses died before the end of the study. Animals at 100 mg/kg bw per day gained less weight than those at 25 or 50 mg/kg bw per day. Gross necropsy revealed a thickened mucosal surface of the stomach in animals at 50–400 mg/kg bw per day. Male rats given 50–400 mg/kg bw per day and female rats given 100–400 mg/kg bw per day also had adhesion of the stomach to the peritoneum (National Toxicology Program, 1982).

Groups of 10 Fischer 344/N rats of each sex were given 0 (vehicle control), 1.5, 3, 6, 12 or 25 mg/kg bw per day allyl isothiocyanate by gavage, 5 days per week for 13 weeks. The animals were observed twice daily for deaths, morbidity and clinical signs of toxicity. On days 92–96, the surviving animals were killed and necropsied. Animals found dead were also necropsied, and those found moribund were killed and necropsied. Various tissues, masses and gross lesions from controls and rats at 25 mg/kg bw per day were examined histologically. All animals survived to scheduled termination, with no clinical signs of toxicity. The mean body weights of treated animals were comparable to those of controls. No gross or histological changes were found at any dose (National Toxicology Program, 1982).

In a bioassay for carcinogenesis, groups of 50 male and 50 female Fischer 344 rats received 0 (vehicle control), 12 or 25 mg/kg bw per day allyl isothiocyanate by gavage, 5 days per week for 103 weeks. The animals were observed twice daily for morbidity and deaths, and clinical signs of toxicity and body weights were recorded every 4 weeks. During weeks 104–106, the surviving animals were killed and necropsied. Animals found dead were also necropsied, and those found moribund were killed and necropsied. A selection of tissues, masses and gross lesions from all groups were examined histologically. The mean body weights of males at 25 mg/kg bw per day were lower than those of controls throughout the study, whereas the mean body weights of treated females were higher in the second half of the study (statistics not reported). No clinical signs of toxicity were reported. One male and two females at 12 mg/kg bw per day and one male at 25 mg/kg bw per day died as a result of gavaging errors. Survival (58–74%) was comparable in all groups (including controls). The incidence of subcutaneous tissue fibrosarcomas was increased in females at the higher dose, with a significant trend ($p < 0.05$); however, the increase was not statistically significant in comparison with controls (control, 0/50; lower dose, 0/50; higher dose, 3/50). The incidence of undifferentiated leukaemia was increased, with a significant trend ($p < 0.05$), in treated male rats, and at the higher dose the incidence was significantly ($p < 0.05$) greater than in controls (control, 2/50; lower dose, 6/50; higher dose, 8/50). Also in male rats, the incidence of transitional-cell papillomas of the urinary bladder showed a significant trend ($p < 0.05$; control, 0/49; lower dose, 2/49; higher dose, 4/49), but the incidence at the lower dose was not

statistically significant. One female at the higher dose also had this lesion, but the incidence was not statistically significant. Epithelial hyperplasia of the urinary bladder occurred in males with a significant ($p < 0.05$) overall trend and was significantly increased at the higher dose (control, 0/49; lower dose, 1/49; higher dose, 6/49). Hyperplasia was not seen in animals with papillomas. The incidence of non-neoplastic lesions of the eye (retinopathy and cataract) was significantly increased in males at the higher dose and females at the lower dose; however, the increased incidence correlated with cage placement. The authors concluded that, under the conditions of the bioassay, allyl isothiocyanate was carcinogenic for male rats, causing transitional-cell papillomas of the urinary bladder, but that the evidence that allyl isothiocyanate caused subcutaneous fibrosarcomas in female rats was equivocal (National Toxicology Program, 1982).

Concusions

Isothiocyanates are strong irritants in epithelial tissue and often cause contact dermatitis. In rats given an LD_{50} dose of 112 mg/kg bw of allyl isothiocyanate by stomach tube, marked irritation of the lungs and gastrointestinal tract was reported (Anderson & Hurwitz, 1953). Fischer 344 rats and $B6C3F_1$ mice of each sex given 50 mg/kg bw of allyl isothiocyanates by gavage for 14 consecutive days had stomach damage and thickening of the urinary bladder epithelium, especially male rats (National Toxicology Program, 1982). In mice, the dermal sensitizing effects of allyl isothiocyanates have been correlated with a change in the ratio of GSH:glutathione disulfide in skin, suggesting that the substance induces oxidative stress in mouse skin epithelium (Schmidt & Chung, 1993). On the basis of the evidence that allyl isothiocyanate has strong irritant and cytotoxic properties, the observation that it is not genotoxic in vivo (see below) and the report that it induces benign transitional-cell papillomas only in male rats, this substance probably operates through a secondary non-genotoxic mechanism, as has been established for other irritating bladder carcinogens, such as sodium saccharin (Cohen & Ellwein, 1990).

Studies on the metabolism and disposition of allyl isothiocyanate (see section 2.3.1 above) show clear sex- and species-specific differences. Mice excreted essentially all of an oral dose of allyl ^{14}C-isothiocyanate within 96 h, while rats retained up to 20% in the carcass during the same interval. Blood radioactivity returned to background levels within 96 h in mice but within up to 240 h in rats. In a separate experiment, mice excreted more than 80% of a dose of allyl ^{14}C-isothiocyanate in the urine within 3 days, while rats excreted only 55%, indicating that rats selectively retain thiocyanate ion (Bollard et al., 1997). These results support the conclusion that rats are more heavily exposed to allyl isothiocyanate and its metabolites than mice.

Female rats produce twice as much urine as male rats (Ioannou et al., 1984; Bollard et al., 1997), strengthening the conclusion that the male rat bladder is exposed to higher concentrations of allyl isothiocyanate and its metabolites than the female rat bladder. The observation that radioactivity levels from allyl ^{14}C-isothiocyanate measured in the urinary bladder 1, 1.5 and 2 h after dosing were significantly higher in male than in female rats supports this conclusion (Bollard et al., 1997).

The thiol conjugates of allyl isothiocyanates are potentially toxic, given their inherent instability. An equilibrium exists between unconjugated and conjugated forms of allyl isothiocyanate, which allows for the presence of as much as 15–20% of

unconjugated allyl isothiocyanate (Bollard et al., 1997). The equilibrium can, however, be shifted in favour of the unconjugated form in the presence of a low GSH concentration and an alkaline pH (Bruggeman et al., 1986), conditions that might exist in the urinary bladder of rats that feed at night. Therefore, increased absorption of free allyl isothiocyanate might occur in the bladder epithelium. The histopathological evidence of epithelial hyperplasia in the male bladder (National Toxicology Program, 1982) correlates with the biochemical evidence that the male rat bladder is exposed to high levels of possible irritants, including allyl isothiocyanate and its metabolites. Both the qualitative and quantitative aspects of the molecular disposition of allyl isothiocyanate and its associated toxicological sequelae in mammals have been relatively well defined and are similar to those reported for other irritating substances in the urinary bladder (Cohen & Ellwein, 1990). Epithelial cell papillomas are benign lesions on luminal surfaces. Apparently, exposure to high concentrations of an irritating material is the likely source of regenerative hyperplasia and subsequent benign papillomas in the male rat bladder. When male rats are given repeated high doses of allyl isothiocyanate, it is metabolized by GSH conjugation. When the conjugate accumulates in the male rat bladder, it either irritates the bladder epithelium or dissociates into free allyl isothiocyanate, which also acts as an irritant.

The relevance of the appearance of benign bladder tumours to potential carcinogenic targets in humans has been the subject of much investigation (Tennant et al., 1986; Ashby & Tennant, 1988; Cohen & Ellwein, 1990). Ashby and Tennant (1988) concluded from a detailed analysis of the National Toxicology Program database on carcinogens that genotoxicity was not the primary determinant of the carcinogenicity of allyl isothiocyanate. A working group convened by the International Agency for Research on Cancer (1985) concluded that the evidence for the carcinogenicity of allyl isothiocyanate in rodents was limited, as benign tumours were induced only in male rats in a single tissue. On the basis of the conclusion that papillomas are induced by a non-genotoxic mechanism, the relevance of the occurrence of these tumours to human health must be evaluated in the context of the conditions of exposure in the experiments and those experienced by humans during intake of allyl isothiocyanates as a flavouring substance.

The two factors that distinguish the exposure of humans to allyl isothiocyanate from that of rats are daily intake and the exposure of the bladder. The rats in the 2-year study of the National Toxicology Program (1982) were exposed to 25 000 μg/kg bw daily, while human intake from use of allyl isothiocyanate as a flavouring substance is approximately 1000 times less (25 μg/kg bw per day). This lower level of exposure and the fact that humans excrete about 1500 ml of urine per day mean that the concentration of allyl isothiocyanate, or its metabolites, in the human bladder would be orders of magnitude lower than that required to induce hyperplasia and papillomas in rats. Given that no bladder toxicity was observed at the lowest dose of 12 000 μg/kg bw per day in the 2-year study of the National Toxicology Program (1982), it is unlikely that this secondary mechanism of carcinogenicity operates in humans.

(e) Genotoxicity

Four representative miscellaneous nitrogen derivatives (Nos 1560, 1561, 1562 and 1563) have been tested for genotoxicity. The results of these tests are summarized in Table 5 and described below.

Table 5. Studies of genotoxicity with miscellaneous nitrogen derivatives

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
In vitro						
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA97, TA102	0.001–0.1 mg/plate	Negative[a]	Fujita & Sasaki (1987)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA98, TA1535, TA1537	1–1000 µg/plate	Negative[a]	Mortelmans et al. (1986)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA1535, TA1536, TA1537, 1538	0, 100, 200 or 300 µg/plate	Negative[b,c]	Yamaguchi (1980)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA97, TA98, TA100	1 mg/ml	Negative[a,d]	Azizan & Blevins (1995)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA98	0, 100, 200 or 300 µg/plate	Weakly positive[b,c]	Yamaguchi (1980)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	1–1000 µg/plate	Equivocal[a,e]	Mortelmans et al. (1986)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	≤ 100 µg/plate	Positive[b]	Yamaguchi (1980)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	50 µg/ml	Positive[b]	Brooks et al. (1984)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	0.025–2.5 µl/ml (0.026–2.6 mg/ml)[f]	Negative[a]	Eder et al. (1980)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	NR	Weakly positive[b,g]	Eder et al. (1982)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	NR	Negative[h]	Eder et al. (1982)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	≤ 0.5 µg/plate	Negative[b]	Neudecker & Henschler (1985)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	≤ 0.5 µg/plate	Negative/ positive[h,i,j]	Neudecker & Henschler (1985)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA98, TA100	0.05–500 µg/plate	Negative[a]	Kasamaki et al. (1982)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 µg/plate	Positive[b,k]	Kassie & Knasmüller (2000)
1560	Allyl isothiocyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 µg/plate	Negative[h,k]	Kassie & Knasmüller (2000)
1560	Allyl isothiocyanate	Reverse mutation	*E. coli* WP 67 (trp uvrA polA)	0.1, 1 or 3 mmol/l (9.9, 99 or 297 µg/ml)[l]	Negative[b,m]	Říhová (1982)

Table 5 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1560	Allyl isothiocyanate	Reverse mutation	*E. coli* WP 67 (trp uvrA polA)	1,3 or 5 mmol/l (99, 297 or 496 µg/ml)[l]	Positive[h,n]	Ríhová (1982)
1560	Allyl isothiocyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr+/rec+/Lac–)	≤ 25 µg/ml	Positive[b]	Kassie & Knasmüller (2000)
1560	Allyl isothiocyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr$^+$/rec$^+$/Lac$^-$)	≤ 25 µg/ml	Negative[h]	Kassie & Knasmüller (2000)
1560	Allyl isothiocyanate	DNA repair	*Bacillus subtilis* H17 (rec$^+$) and M45 (rec$^-$)	20 µg/disc	Negative[b]	Oda et al. (1979)
1560	Allyl isothiocyanate	Forward mutation	Mouse lymphoma L5178Y cells	0.2, 0.4, 0.6, 0.8 or 1.0 µg/ml	Positive[b,o,p]	McGregor et al. (1988)
1560	Allyl isothiocyanate	Forward mutation	Mouse lymphoma L5178Y cells	0.4, 0.8, 1.0, 1.2, 1.4 or 1.6 µg/ml	Positive[b,o,p]	McGregor et al. (1988)
1560	Allyl isothiocyanate	Sister chromatid exchange	Chinese hamster ovary cells	0.1–0.5 µg/ml	Negative[b]	Galloway et al. (1987)
1560	Allyl isothiocyanate	Sister chromatid exchange	Chinese hamster ovary cells	0.16–1.6 µg/ml	Positive[h]	Galloway et al. (1987)
1560	Allyl isothiocyanate	Sister chromatid exchange	Chinese hamster ovary cells	2.4, 2.7 or 3.0 µg/ml	Negative[b,m]	Musk et al. (1995)
1560	Allyl isothiocyanate	Chromosomal aberrations	Chinese hamster ovary cells	0.5–5 µg/ml	Weakly positive[a]	Galloway et al. (1987)
1560	Allyl isothiocyanate	Chromosomal aberrations	Chinese hamster ovary cells	≤ 5 nmol/l (0.496 ng/ml)[m]	Positive[b]	Kasamaki et al. (1982)
1560	Allyl isothiocyanate	Chromosomal aberrations	Chinese hamster ovary cells	1–10 nmol/l (0.099–0.99 ng/ml)[m]	Positive[b,o]	Kasamaki & Urasawa (1985)
1560	Allyl isothiocyanate	Chromosomal aberrations	Chinese hamster ovary cells	2.4, 2.7 or 3.0 µg/mll	Negative[b,m]	Musk et al. (1995)
1560	Allyl isothiocyanate	Chromosomal aberrations	SV40-transformed Indian muntjac cells	0.2, 0.4 or 0.8 µg/mll	Negative[b,m]	Musk & Johnson (1993)
1560	Allyl isothiocyanate	Micronucleus induction	Human Hep G2 cells	≤ 5 µg/ml	Weakly positive[m]	Kassie & Knasmüller (2000)

Table 5 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1560	Allyl isothiocyanate	Unscheduled DNA synthesis	Human HeLa S3 cells	NR[q]	Negative	Schiffmann et al. (1983)
1561	Butyl isothiocyanate	Reverse mutation	*S. typhimurium* TA100	≤ 100 μg/plate	Positive[b]	Yamaguchi (1980)
1562	Benzyl isothio-cyanate	Reverse mutation	*S. typhimurium* TA100	≤ 150 μg/plate	Positive[b,q]	Yamaguchi (1980)
1562	Benzyl isothio-cyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 μg/plate	Positive[b,k]	Kassie et al. (1999)
1562	Benzyl isothio-cyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 μg/plate	Negative[h,k]	Kassie et al. (1999)
1562	Benzyl isothio-cyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr$^+$/rec$^+$/Lac$^-$)	≤ 5.5 μg/ml	Positive[b,m]	Kassie et al. (1999)
1562	Benzyl isothio-cyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr$^+$/rec$^+$/Lac$^-$)	≤ 5.5 μg/ml	Negative[h,m]	Kassie et al. (1999)
1562	Benzyl isothio-cyanate	Chromosomal aberrations	SV40-transformed Indian muntjac cells	0.22, 0.44 or 0.88 μg/ml	Positive[b,m]	Musk & Johnson (1993)
1562	Benzyl isothio-cyanate	Micronucleus induction	Human Hep G2 cells	1 to 4 μg/ml	Positive[m]	Kassie et al. (1999)
1563	Phenethyl isothio-cyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 μg/plate	Positive[b,k]	Kassie & Knasmüller (2000)
1563	Phenethyl isothio-cyanate	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 200 μg/plate	Negative[h,k]	Kassie & Knasmüller (2000)
1563	Phenethyl isothio-cyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr$^+$/rec$^+$/Lac$^-$)	≤ 25 μg/ml	Positive[b]	Kassie & Knasmüller (2000)
1563	Phenethyl isothio-cyanate	DNA repair	*E. coli* 343/753 (uvrB/recA/Lac$^-$), 343/765 (uvr$^+$/rec$^+$/Lac$^-$)	≤ 25 μg/ml	Negative[h]	Kassie & Knasmüller (2000)
1563	Phenethyl isothio-cyanate	Sister chromatid exchange	Chinese hamster ovary cells	0.6, 0.9 or 1.2 μg/ml	Positive[b,m,r]	Musk et al. (1995)
1563	Phenethyl isothio-cyanate	Chromosomal aberrations	Chinese hamster ovary cells	0.6, 0.9 or 1.2 μg/ml	Positive[b,m,s]	Musk et al. (1995)

Table 5 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1563	Phenethyl isothio-cyanate	Chromosomal aberrations	SV40-transformed Indian muntjac cells	0.44, 0.88 or 1.32 µg/ml	Positive[b,m]	Musk & Johnson (1993)
1563	Phenethyl isothio-cyanate	Micronucleus induction	Human Hep G2 cells	≤ 5 µg/ml	Positive[m]	Kassie & Knasmüller (2000)
In vivo						
1560	Allyl isothiocyanate	Sex-linked recessive lethal mutations	*Drosophila melanogaster* (male)	NR[t]	Weakly positive	Auerbach & Robson (1947)
1560	Allyl isothiocyanate	Sex-linked recessive lethal mutations	*Drosophila melanogaster* (male)	650 ppm[u]	Negative	Valencia et al. (1985)
1560	Allyl isothiocyanate	Sex-linked recessive lethal mutations	*Drosophila melanogaster* (male)	54 ppm[u]	Negative	Zimmering et al. (1989)
1560	Allyl isothiocyanate	Sex-linked recessive lethal mutations	*Drosophila melanogaster* (male)	700 ppm[v]	Negative	Valencia et al. (1985)
1560	Allyl isothiocyanate	Chromosome breaks	*Drosophila melanogaster*	NR[t]	Negative	Auerbach & Robson (1947)
1560	Allyl isothiocyanate	Host-mediated DNA repair	Swiss albino mouse (*E. coli* 343/753 ($uvrB/recA/Lac^-$) and 343/765 ($uvr^+/rec^+/Lac^-$))	90 or 270 mg/kg bw[w]	Positive[x]	Kassie & Knasmüller (2000)
1560	Allyl isothiocyanate	Micronucleus induction	Male B6C3F_1 mouse (bone marrow)	37.5, 75 or 150 mg/kg bw per day[y]	Negative	Shelby et al. (1993)
1560	Allyl isothiocyanate	Micronucleus induction	Male outbred (Shoe:WIST) rat (bone marrow)	10, 20 or 40 mg/kg bw per day[z]	Negative	Lewerenz et al. (1988a)
1560	Allyl isothiocyanate	Unscheduled DNA synthesis	Male Hsd/Ola Sprague-Dawley rat (hepatocytes)	37.5 or 125 mg/kg bw[aa]	Negative	Bechtel et al. (1998)
1560	Allyl isothiocyanate	Dominant lethal mutations	Male ICR/Ha Swiss mouse	3.8 or 19 mg/kg bw[bb]	Negative	Epstein et al. (1972)
1562	Benzyl isothio-cyanate	Host-mediated DNA repair	Swiss albino mouse (*E. coli* 343/753 ($uvrB/recA/Lac^+$), 343/765 ($uvr^+/rec^+/Lac^-$))	30, 90 or 270 mg/kg bw[w]	Positive[cc]	Kassie et al. (1999)

Table 5 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1562	Benzyl isothiocyanate	Unscheduled DNA synthesis	Male Fischer 344 rat (hepatocytes)	400 ppm[dd] (40 mg/kg bw per day)	Negative	Sugie et al. (1993)
1562	Benzyl isothiocyanate	Replicative DNA synthesis	Male Fischer 344 rat (hepatocytes)	400 ppm[dd] (40 mg/kg bw per day)	Negative	Sugie et al. (1993)
1563	Phenethyl isothiocyanate	Host-mediated DNA repair	Swiss albino mouse (*E. coli* 343/753 (uvrB/recA/Lac$^+$) and 343/765 (uvr$^+$/rec$^+$/Lac$^-$))	90 or 270 mg/kg bw[w]	Negative	Kassie & Knasmüller (2000)
1563	Phenethyl isothiocyanate	Chromosomal aberrations	Male Swiss albino mouse (bone marrow)	1 µmol/kg bw per day[ee] (0.16 mg/kg bw per day)[ff]	Negative	Sen et al. (1996)

NR, not reported; S9, 9000 x *g* rat liver microsomal fraction

[a] With and without S9
[b] Without S9
[c] Cytotoxic at 200 and 300 µg/plate
[d] Pre-incubation method (60 and 120 min)
[e] Tested in two different laboratories: in one, at concentrations ≤ 400 µg/plate, with weakly positive results (increases less than twofold greater than control values but dose-dependent) with or without metabolic activation, with cytotoxicity at highest dose; in the other, tested at concentrations ≤ 1000 µg/plate with and without metabolic activation; cytotoxicity at highest concentration. All other results consistently negative
[f] Calculated from density of 1.02 g/ml
[g] Borderline significance
[h] With S9
[i] Pre-incubation method (20, 80 and 120 min); negative after 20-min pre-incubation; positive after 80- and 120-min pre-incubation
[j] Although positive with S9, greater mutagenic potential at lower doses of S9 mix
[k] Cytotoxic at > 100 µg/plate
[l] Calculated from relative molecular mass = 99.15 g/mol
[m] Cytotoxic at all concentrations
[n] Cytotoxic at ≥ 3 mmol/l
[o] Cytotoxic at highest concentration
[p] Lethal at highest concentrations

Table 5 (contd)

[q] Authors found result positive, but number of revertants was not twice the number of spontaneous revertants in controls
[r] Significant increase in sister chromatid exchange only at highest dose
[s] Positive at 0.9 and 1.2 µg/ml
[t] Dose applied as a 'pure' spray every 10 s for a total of 9 min
[u] In feed
[v] Injection
[w] Mixture of indicator bacteria injected into tail vein followed by gavage with test compound
[x] Significant decrease in relative survival rates of *E. coli* strains in liver, lung and colon at 90 mg/kg bw per day and in all organs at 270 mg/kg bw per day
[y] Intraperitoneal injections over 3 consecutive days
[z] Gavage for 6 weeks
[aa] Single gavage dose tested at 2 and 14 h
[bb] Single intraperitoneal injection
[cc] At two highest doses
[dd] In diet for 1 week
[ee] Gavage for 7 days
[ff] Calculated from relative molecular mass = 163.24 g/mol

In vitro

The results of standard and modified assays for reverse mutation (Ames tests) conducted with allyl isothiocyanate (No. 1560) were not consistent. In *Salmonella typhimurium* strains TA97, TA102, TA1535, TA1536, TA1537 and 1538, no evidence of mutagenicity was found with or without metabolic activation at concentrations up to 1000 μg/plate or 1 mg/ml of allyl isothiocyanate (Yamaguchi, 1980; Mortelmans et al., 1986; Fujita & Sasaki, 1987; Azizan & Blevins, 1995); however, mixed results were reported when allyl isothiocyanate was tested in *S. typhimurium* strains TA100 and TA98 (Eder et al., 1980; Yamaguchi, 1980; Eder et al., 1982; Kasamaki et al., 1982; Brooks et al., 1984; Neudecker & Henschler, 1985; Mortelmans et al., 1986; Kassie & Knasmüller, 2000). At concentrations of up to 500 μg/plate with and without metabolic activation, allyl isothiocyanate did not increase the number of revertants in TA100 in comparison with controls (Kasamaki et al., 1982). Mortelmans et al. (1986) reported the results for allyl isothiocyanate tested in two different laboratories. While in one laboratory allyl isothiocyanate gave weakly positive results (i.e. increases less than twofold greater than control values but dose-dependent) at concentrations up to 400 μg/plate with and without metabolic activation, in the other laboratory concentrations up to 1000 μg/plate in the presence and absence of metabolic activation did not induce a significant increase in the number of revertants relative to controls. In both laboratories, cytotoxicity was reported at the highest concentrations tested.

Allyl isothiocyanate induced reverse mutation in *S. typhimurium* strain TA100 without metabolic activation at concentrations up to 50 μg/plate or 100 μg/ml (Yamaguchi, 1980; Eder et al., 1982; Brooks et al., 1984), with cytotoxicity at higher concentrations (Yamaguchi, 1980). Although Eder et al. (1982) reported weak mutagenic activity of borderline significance for allyl isothiocyanate (concentrations not specified) in *S. typhimurium* TA100 in the absence of metabolic activation, no mutagenicity was observed with metabolic activation. Moreover, in an earlier study, Eder et al. (1980) observed no mutagenic potential in TA100 with or without metabolic activation at concentrations up to 2.6 mg/ml. Similarly, in the absence of metabolic activation, allyl isothiocyanate caused a dose-dependent increase in the number of His^+ revertants in *S. typhimurium* TA100 at concentrations up to 100 μg/plate (1 μmol/plate), with significant toxicity at the highest concentration of 200 μg/plate; however, after addition of metabolic activation, the revertant frequency did not differ from that of controls (Kassie & Knasmüller, 2000).

In the presence of metabolic activation, pre-incubation time was shown to be a significant determinant of the mutagenicity of allyl isothiocyanate in *S. typhimurium* strain TA100 (Neudecker & Henschler, 1985). While allyl isothiocyanate was not mutagenic in a modified Ames assay with a pre-incubation time of 20 min and with metabolic activation, longer pre-incubation times (80 and 120 min) resulted in a time-dependent increase in the number of revertants; however, allyl isothiocyanate was more mutagenic at lower concentrations of the S9 mix. Without metabolic activation, allyl isothiocyanate did not increase the number of revertants, regardless of pre-incubation time. In a follow-up study conducted by Azizan and Blevins (1995), however, in which allyl isothiocyanate was tested at concentrations up to 1 mg/ml in *S. typhimurium* TA100 with and without metabolic activation in the modified Ames assay with pre-incubation times up to 120 min, negative results were reported.

Although allyl isothiocyanate was mutagenic in *S. typhimurium* TA98 without metabolic activation at concentrations up to 100 μg/plate (Yamaguchi, 1980; Kassie & Knasmüller, 2000), no increase in the number of revertants was reported in these studies in the presence of metabolic activation (Kassie & Knasmüller, 2000) or in several other studies in which allyl isothiocyanate was tested at concentrations up to 1000 μg/plate or 1 mg/ml with and without metabolic activation, even when the pre-incubation time was up to 120 min (Kasamaki et al., 1982; Mortelmans et al., 1986; Azizan & Blevins, 1995).

Butyl isothiocyanate and benzyl isothiocyanate also induced reverse mutation in *S. typhimurium* strains TA98 and TA100 at concentrations up to 200 μg/plate without metabolic activation (Yamaguchi, 1980; Kassie et al., 1999). In the presence of metabolic activation, benzyl isothiocyanate gave negative results (butyl isothiocyanate was not re-evaluated with S9) (Kassie et al., 1999).

Kassie and Knasmüller (2000) also evaluated the potential mutagenicity of phenethyl isothiocyanate in *S. typhimurium* TA100. Like allyl isothiocyanate, phenethyl isothiocyanate at a concentration of 100 μg/plate induced a dose-dependent increase in the number of revertants in the absence, but not in the presence, of metabolic activation.

In *Escherichia coli* WP 67 (trp uvrA polA), allyl isothiocyanate at concentrations up to 5 mmol/l (496 μg/ml) induced reverse mutations only in the presence of metabolic activation after an incubation of 120 min. In an assay without metabolic activation, concentrations of 0.1, 1 and 3 mmol/l were all highly cytotoxic, whereas with metabolic activation marked cytotoxicty was apparent at concentrations ≥ 3 mmol/l. The authors noted that the degree of mutagenicity was related to the source and protein content of the metabolic activation mix: microsomal fractions from the liver of phenobarbital-treated rats, goats and monkeys were more active at a lower protein content than those obtained from mice and hamsters (Říhová, 1982).

The extent of reparable DNA damage induced by isothiocyanates was measured by comparing the viability of two *E. coli* strains that differ in their DNA repair capacity (343/753 and 343/765). There was no evidence of DNA damage when concentrations of up to 25 μg/ml of allyl isothiocyanate or phenethyl isothiocyanate (Kassie & Knasmüller, 2000) or concentrations of up to 5.5 μg/ml of benzyl isothiocyanate (No. 1562) (Kassie et al., 1999) were incubated with *E. coli* strains 343/753 (uvrB/recA/Lac$^+$) and 343/765 (uvr$^+$/rec$^+$/Lac$^-$) in the presence of Aroclor 1254-induced S9 mix. In contrast, without metabolic activation, allyl isothiocyanate and phenethyl isothiocyanate strongly reduced the viability of the repair-deficient strain in a concentration-dependent manner after a 2-h incubation (Kassie & Knasmüller, 2000). Similarly, benzyl isothiocyanate gave positive results without metabolic activation, inducing a > 50% reduction in the relative survival rate at 1.5 μg/ml (Kassie et al., 1999).

In order to evaluate the protective effect of non-enzymatic protein binding and amino and sulfhydryl group reactions with respect to the apparent reduction in mutagenic activity after addition of S9, allyl isothiocyanate and phenethyl isothiocyanate were incubated with two *E. coli* indicator strains with or without bovine serum or human saliva for 2 h. Both bovine serum and human saliva reduced the differential DNA damage induced by the test substances in a concentration-dependent manner. The genotoxic effects of both substances were nearly completely attenuated by bovine serum at 12 ng/ml, whereas 150 μl of human saliva added to the test

mixtures reduced reparable DNA damage by approximately 60% and 40% for allyl isothiocyanate and phenethyl isothiocyanate, respectively (Kassie & Knasmüller, 2000). Similar results were reported with benzyl isothiocyanate (Kassie et al., 1999). Bovine serum at 9 mg/ml completely abolished the mutagenic effects of benzyl isothiocyanate, whereas human saliva and gastric juice reduced the effects by 40% and 45%, respectively. The presence of radical scavengers (i.e. vitamin E, β-carotene, vitamin C and sodium benzoate) also reduced the differential DNA damage caused by allyl isothiocyanate, phenethyl isothiocyanate and benzyl isothiocyanate (Kassie et al., 1999; Kassie & Knasmüller, 2000). On the basis of these results, the authors suggested that mechanisms such as liver detoxication, non-enzymatic protein binding and preferential reaction with proteins provide protection against isothiocyanates in vivo (Kassie & Knasmüller, 2000).

In the rec assay, which is a method of detecting DNA damaging activity by differences in growth inhibition zones in *Bacillus subtilis* H17 and M45, allyl isothiocyanate at a concentration of 20 μg/disc gave negative results (Oda et al., 1979).

Allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate have been tested in mammalian cell lines. Allyl isothiocyanate induced forward mutation in two trials at concentrations up to 1.6 μg/m in L5178Y tk^{+}/tk^{-} mouse lymphoma cells without metabolic activation; however, the mutagenic responses were accompanied by relatively high toxicity, with a relative total growth of 15% at the lowest effective concentration of 0.4 μg/ml. Furthermore, allyl isothiocyanate was lethal at the highest concentration tested in each experiment, 1.0 μg/ml and 1.6 μg/ml (McGregor et al., 1988).

Allyl isothiocyanate did not significantly increase the frequency of sister chromatid exchange in Chinese hamster ovary cells without metabolic activation at concentrations of 0.1–0.5 μg/ml; however, the frequency was increased at concentrations of 0.16–1.6 μg/ml after addition of S9. Likewise, no statistically significant increase in the number of sister chromatid exchanges was observed in the absence of metabolic activation in Chinese hamster ovary cells at concentrations up to 3.0 μg/ml, but it was cytotoxic at all concentrations (Galloway et al., 1987).

A significant increase in the frequency of chromosomal aberrations was observed in Chinese hamster ovary cells at concentrations up to 0.99 ng/ml of allyl isothiocyanate (10 nmol/l) without metabolic activation (Kasamaki et al., 1982; Kasamaki & Urasawa, 1985). In a study with higher concentrations (0.5–5 μg/ml), however, the number of chromosomal aberrations was only weakly increased in comparison with controls, with or without metabolic activation (Galloway et al., 1987). In another study, allyl isothiocyanate did not significantly increase the incidence of chromosomal aberrations in the absence of metabolic activation in Chinese hamster ovary cells at concentrations of 2.7–3.0 μg/ml, all of which were cytotoxic. In contrast, phenethyl isothiocyanate significantly increased the frequency of both chromosomal aberrations and sister chromatid exchanges in these cells at cytotoxic concentrations of 0.9 and 1.2 μg/ml, without metabolic activation (Musk et al., 1995).

SV40-transformed Indian muntjac cells were incubated with up to 0.8 μg/ml allyl isothiocyanate, up to 0.88 μg/ml benzyl isothiocyanate or up to 1.32 μg/ml phenethyl isothiocyanate for 24 h and examined for chromosomal aberrations. All three isothiocyanates were markedly cytotoxic and significantly reduced clonal survival. At the highest concentrations tested, the mitotic indices were reduced by

approximately 75%. Allyl isothiocyanate did not induce aberrations, whereas benzyl isothiocyanate and phenethyl isothiocyanate induced significant, concentration-dependent increases in the numbers of chromatid gaps or breaks and rearrangements (Musk & Johnson, 1993).

Allyl isothiocyanate did not induce unscheduled DNA synthesis in human HeLa S3 cells (concentrations not specified). The authors noted, however, that while allyl isothiocyanate was not clastogenic it was significantly cytotoxic at concentrations at which other allylic compounds increased unscheduled DNA synthesis (Schiffman et al., 1983). Incubation of up to 5 μg/ml of phenethyl isothiocyanate (Kassie & Knasmüller, 2000) or 4 μg/ml of benzyl isothiocyanate (Kassie et al., 1999) with human Hep G2 cells significantly increased the number of micronuclei, with reduced cell viability at all concentrations. Phenethyl isothiocyanate had the strongest effect, as reflected by a threefold increase in micronucleus frequency at 3 μg/ml. Concentrations up to 5 μg/ml of allyl isothiocyanate only weakly increased the incidence of micronuclei in comparison with the effect of phenethyl isothiocyanate.

In vivo

A low but statistically significant increase in the number of sex-linked recessive lethal mutations was observed in male *Drosophila melanogaster* exposed to a spray of 'pure' allyl isothiocyanate (No. 1560) at 10-s intervals during an 8–9-min exposure (specific dose not reported) in comparison with controls (Auerbach & Robson, 1947). In several other assays, allyl isothiocyanate did not increase the frequency of sex-linked recessive lethal mutation in male *Drosophila* given concentrations up to 650 ppm in feeding trials (Auerbach & Robson, 1947; Zimmering et al., 1989) or given the compound by injection at a concentration of 700 ppm (Valencia et al., 1985). Furthermore, allyl isothiocyanate did not increase the number of chromosome breaks in first-generation female *Drosophila* flies obtained after mating males treated with the allyl isothiocyanate spray with untreated females (Auerbach & Robson, 1947).

In a host-mediated assay for differential DNA repair in vivo, groups of six male Swiss albino mice were injected with 4–8 × 10^9 viable cells of a mixture of *E. coli* strains 343/753 (uvrB/recA/Lac$^+$) and 343/765 (uvr$^+$/rec$^+$/Lac$^-$), differing in DNA repair potential, into the lateral tail vein after a 24-h fast. Immediately after the injection, the mice were gavaged with 90 or 270 mg/kg bw of allyl isothiocyanate or phenethyl isothiocyanate and were killed 2 h later. The liver, lung, kidneys, stomach and colon were removed, homogenized and suspended in phosphate-buffered saline; the suspensions were incubated on neutral red agar plates for 12 h at 37 °C and kept at room temperature for an additional 12 h. The survival rates of the two *E. coli* strains were counted. Phenethyl isothiocyanate did not change the survival rates of the indicator cells, whereas allyl isothiocyanate significantly decreased the relative survival rates of both strains in liver, lung and colon at the lower dose and in all organs at the higher dose in comparison with untreated controls (Kassie & Knasmüller, 2000).

In a similar host-mediated assay, in which male Swiss albino mice were given benzyl isothiocyanate at a dose of 30, 90 or 270 mg/kg bw by gavage after injection of a mixture of two *E. coli* strains (343/753 and 343/765), dose-dependent differential DNA damage was reported in the repair-deficient strain at the two higher doses (Kassie et al., 1999).

The clastogenicity of allyl isothiocyanate was evaluated in assays for micronucleus formation in mouse and rat bone marrow. Allyl isothiocyanate administered to male B6C3F$_1$ mice at a dose of 37.5, 75 or 150 mg/kg bw per day by intraperitoneal injection for 3 days did not significantly increase the number of micronuclei in bone-marrow cells sampled 24 h after the last treatment over the number in controls (Shelby et al., 1993). Similarly, the percentages of polychromatic erythrocytes and erythrocytes with micronuclei in the femoral marrow of male outbred (Shoe:WIST) rats were not affected by gavage with 0, 10, 20 or 40 mg/kg bw per day allyl isothiocyanate for 6 weeks (Lewerenz et al., 1988a).

In groups of four male Hsd/Ola Sprague-Dawley rats, allyl isothiocyanate, administered once by gavage at a dose of 37.5 or 125 mg/kg bw, did not induce unscheduled DNA synthesis in hepatocytes (examined by autoradiography) 2 and 14 h after treatment. Although a significant increase in gross nuclear and cytoplasmic grain counts was observed at the higher dose at the later sampling time, this was not considered to be unscheduled DNA synthesis as there was no increased net nuclear grain count (Bechtel et al., 1998).

In an assay for dominant lethal mutation, allyl isothiocyanate administered as a single intraperitoneal injection at a dose of 3.8 or 19 mg/kg bw to male ICR/Ha Swiss mice did not increase the incidence of early fetal deaths or pre-implantation losses above control limits in females mated with the treated males over a period of 8 weeks (Epstein et al., 1972).

As part of a study designed to examine the effect of benzyl isothiocyanate on unscheduled and replicative DNA synthesis induced by exposure to known carcinogens, groups of young male Fischer 344 rats were fed benzyl isothiocyanate in the diet at a concentration of 0 or 400 ppm for 1 week. This dietary level was calculated to provide an average daily intake of about 40 mg/kg bw (Food & Drug Administration, 1993). In addition to the four groups of rats exposed to the known carcinogens, a fifth group received the vehicle, dimethyl sulfoxide, as a control. The animals were killed, and hepatocytes were isolated and examined for unscheduled and replicative DNA synthesis at the end of treatment. Generally, dietary benzyl isothiocyanate significantly reduced the occurrence of unscheduled DNA synthesis in hepatocytes treated with three of the carcinogens in comparison with carcinogen-treated cells obtained from rats on basal diet. In solvent-treated control cells, no effect on unscheduled DNA synthesis was observed in hepatocytes obtained from benzyl isothiocyanate-treated animals. Furthermore, in comparison with hepatocytes obtained from controls on basal diet, dietary benzyl isothiocyanate reduced the levels of replicative DNA synthesis in carcinogen- and solvent control-treated cells (Sugie et al., 1993).

In male Swiss albino mice given phenethyl isothiocyanate (No. 1563) by gavage at a dose of 1 μmol/kg bw per day (about 0.16 mg/kg bw per day) for 7 days, the number of chromosomal aberrations in bone-marrow cells of treated mice was comparable to that in controls (Sen et al., 1996).

Conclusion

In *S. typhimurium* strain TA100 in the absence of metabolic activation, both positive and negative results were reported with allyl isothiocyanante, usually at or near cytotoxic concentrations. In the presence of metabolic activation, however, except in one study (Neudecker & Henschler, 1985), the results were consistently

negative, even in a modified Ames assay with an extended pre-incubation period. In the absence of metabolic activation, benzyl isothiocyanate, butyl isothiocyanate and phenethyl isothiocyanate were also mutagenic in *S. typhimurium* TA100; with metabolic activation, however, neither benzyl isothiocyanate nor phenethyl isothiocyanate was active (butyl isothiocyanate was not tested). Likewise, in *S. typhimurium* TA98, positive results were observed with allyl isothicyanante and benzyl isothiocyanate only in the absence of metabolic activation. No mutagenicity was observed in a variety of other tester strains (TA97, TA102, TA1535, TA1536, TA1537 and TA1538) with and without metabolic activation. Similarly, in *E. coli*, the isothiocyanates induced DNA repair only in the absence of S9.

Although allyl isothiocyanate induced forward mutation in L5178Y tk^{+}/tk^{-} mouse lymphoma cells without metabolic activation in two trials, the effects were accompanied by relatively high levels of toxicity, and allyl isothiocyanate was lethal at the highest concentrations tested in each trial, 1.0 and 1.6 μg/ml.

Equivocal evidence for sister chromatid exchange, chromosomal aberrations and micronuclei was found in assays in eukaryotic test systems, including human cells, in vitro with and without metabolic activation. The conditions used in most of these studies provided the opportunity for either direct interaction of the isothiocyanates with DNA or indirect formation of DNA adducts due to oxidative stress and subsequent cytotoxicity. On the basis of the metabolism of isothiocyanates, high concentrations of isothiocyanates in vitro are anticipated to deplete GSH levels, leading to the release of nucleocytolytic enzymes that induce DNA fragmentation, cellular damage and apoptosis.

In the late 1980s, researchers began studying the test conditions (e.g. osmalitiy, ionic strength, low pH) that could increase the frequency of chromosomal aberrations and micronuclei in the absence of any direct effect on DNA (Zajac-Kaye & Ts'o, 1984; Brusick, 1986; Bradley et al., 1987; Galloway et al. 1987; Seeberg et al., 1988; Morita et al., 1989; Scott et al., 1991). More recent research indicates that extreme culture conditions (hypo- and hyper-osmolality and high pH) induce apoptosis and necrosis, leading to DNA fragmentation, thus producing false-positive responses in assays for clastogenicity (Meintieres & Marzin, 2004).

Apoptosis is a type of cell death that occurs under physiological conditions or external stimuli, such as DNA-damaging agents, growth factor deprivation or receptor triggering. The mechanism of formation of apoptotic cells includes activation of cysteine proteases (caspases), leading to increased mitochondrial permeability, release of cytochrome c, DNA cleavage and redistribution of phosphatidyl serine by the outer layers of the cell membrane, which enhances binding of cells to phagocytes. DNA cleavage, due to irreversible activation of endonucleases, is followed by chromatin condensation and oligonucleosomal fragmentation resulting from double-strand cleavage of DNA in nucleosomal linker regions (Saraste & Pulkki, 2000). During chromatin condensation, the nucleus can split into a number of dense micronuclei. Fragmented DNA and chromatin condensation due to apoptotic events are not easily distinguished from a direct action of a specific chemical. Therefore, evidence for micronucleus formation and chromosomal aberrations must be evaluated in the context of the potential for apoptosis to occur under the test conditions.

In vivo, allyl isothiocyanate and benzyl isothiocyanate gave positive results in *E. coli* in a host-mediated assay for DNA repair in mice, whereas phenethyl

isothiocyanate did not. In *Drosophila*, allyl isothiocyanate gave predominantly results for the induction of sex-linked recessive lethal mutations and chromosomal breaks. Furthermore, in studies with allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate, consistently negative results for micronucleus formation, unscheduled DNA synthesis, replicative DNA synthesis and chromosomal aberrations were found in mice and rats after oral administration (diet or gavage) at doses up to 150 mg/kg bw per day for up to 6 weeks.

Overall, the results of the tests for genotoxicity in vitro were mixed, but the results of 8 of 10 studies in vivo were negative.

(f) Other relevant studies

Developmental and reproductive toxicity

Allyl isothiocyanate (No. 1560)

In a study of the teratogenic potential of 16 compounds, groups of 5–10 pregnant Wistar rats were given a single oral dose of 60 mg/kg bw of allyl isothiocyanate by stomach tube on day 12 or 13 of gestation. Control dams received an equivalent volume of vehicle (5 ml/kg). On day 22 of gestation, the dams were killed, and individual litter weight, litter size and numbers of deciduomas and corpora lutea were determined. Fetal anomalies were recorded during skeletal and visceral examinations. Allyl isothiocyanate was among 15 compounds that were not teratogenic at doses that were well tolerated by the dams. All the observed fetal anomalies, such as non-fusion of the fifth sternebra, wavy ribs, retarded ossification and a fourteenth rib, were considered by the study authors to be minor. In an attempt to elicit a teratogenic response, allyl isothiocyanate was administered to pregnant rats at a dose of 120 mg/kg bw, which resulted in the deaths of some dams (Ruddick et al., 1976).

Benzyl isothiocyanate (No. 1562)

To examine the effect of pre- and post-implantation exposure to benzyl isothiocyanate on pregnancy outcomes, groups of seven or eight pregnant Sprague-Dawley rats were given 0 (vehicle control), 12.5, 25 or 50 mg/kg bw per day of benzyl isothiocyanate by gavage on days 1–5 or 7–13 of gestation. The dams were observed for vaginal bleeding and signs of clinical toxicity. Body weights were recorded on days 1, 5, 10 and 16 of gestation for rats treated before implantation (days 1–5) and on days 7, 11, 15 and 20 of gestation for rats treated after implantation (days 7–13). Rats treated before implantation were killed on day 16 of gestation, and those treated after implantation were killed on day 20 of gestation. At necropsy, the numbers of implantation sites, viable fetuses and fetal resorptions and the weights of viable fetuses and their placentas and of maternal liver, kidney and spleen were recorded. The fetuses were examined for external malformations. Clinical signs of toxicity, including hypoactivity, lethargy, ruffled fur, perinasal staining, piloerection and hunched posture, were observed in all treated rats. A dose-dependent decrease in maternal body weights was seen during both treatment periods, although this finding was not statistically significant. Food consumption was not measured in this study, but reduced food intake might have contributed to this effect. In both groups, the relative weights of the maternal liver, kidney and spleen were comparable for

treated and control dams. No vaginal bleeding was observed, but two dams treated before implantation and one treated after implantation died immediately after treatment at the highest dose. An increased number of fetal resorptions was found in rats treated before and after implantation. Although these findings were not statistically significant, the increase in rats treated before implantation was dose-dependent. There was no significant difference in the number of implantation sites in treated animals and controls, and no toxicologically important observations were made in fetuses during external examinations. The weights of fetuses of rats treated after implantation at the two higher doses were significantly lower than those of controls, and the placental weights of all rats treated after implantation were significantly lower than those of controls. This finding could not be related to the number of fetuses, as the numbers of viable fetuses in treated and control dams were not significantly different. The authors concluded that benzyl isothiocyanate did not cause significant pre- or post-implantation fetal loss in pregnant rats. It induced low fetal and placental weights in animals treated after implantation but at doses that were toxic to the dams (Adebiyi et al., 2004).

Thyroid toxicity

Groups of six male Wistar rats were given 2.5 or 5.0 mg of allyl isothiocyanate in 3 ml distilled water by stomach tube for 60 days, resulting in doses (on the basis of final body weights) equivalent to 8.4 and 17.0 mg/kg bw per day, respectively. The doses were selcted on the basis of the authors' estimate of equivalence to the amount of total mustard oils contained in 40 g of cabbage, the approximate amount consumed daily by rats. Five control animals received 3 ml distilled water under the same conditions. Thyroid weights were significantly increased ($p < 0.05$) in rats receiving the higher dose. An increase in thyroid weight was also seen in rats receiving the lower dose, but the increase was not statistically significant. The absolute and relative total iodine contents of the thyroid were not significantly altered by treatment. Serum protein-bound iodine was significantly reduced in rats receiving the lower dose ($p < 0.02$) or the higher dose ($p < 0.01$) in comparison with controls (Langer & Stolc, 1965).

Groups of three or four male Wistar rats (weighing 200 g) received 2 or 4 mg of allyl isothiocyanate in 2 ml distilled water by stomach tube, equivalent to 10 and 20 mg/kg bw, respectively. Control animals received 2 ml distilled water only. One hour after administration, each rat was injected intraperitoneally with carrier-free ^{131}I (0.5 μCi), and the rats were killed 2 or 4 h later. The thyroids were dissected, and the radioactivity they contained was measured with a scintillation counter to quantify radioiodine uptake. Uptake was significantly depressed in rats treated with the lower ($p < 0.05$) or the higher dose ($p < 0.01$) when compared with control. The author suggested that the antithyroid effect of allyl isothiocyanate is due to formation of thiohydantoin derivatives in vivo by a chemical reaction of allyl isothiocyanate with amino acids and peptides. This reaction would initially form thiocarbamyl derivatives, which would subsequently yield thiohydantoin derivatives by spontaneous cyclization (see Figure 3). Several thiohydantoin derivatives have been shown to have antithyroid activity (Langer, 1964).

In another study on the goitrogenic effects of allyl isothiocyanate, male Wistar rats (weighing 110–130 g) received 2 or 6 mg of allyl isothiocyanate (equivalent to 18.2 and 55.0 mg/kg bw per day, respectively) in 3 ml distilled water by stomach

Figure 3. Possible mechanism for isothiocyanate-induced antithyroidal activity

R1—N=C=S (Isothiocyanate) + HO2C—CH—NH2 R2 (Amino acid)

R1—NH HO2C S CH-NH R2 Thiocarbamyl derivative

Propyl thio uracil (clinically used thyroid depressant)

Thiohydantoin

tube for 50 or 20 days, respectively. Control animals received 3 ml distilled water. All rats were maintained on a low-iodine diet supplemented with carrots. The thyroid weights and total thyroid iodine in the group of rats receiving 55.0 mg/kg bw per day for 20 days were significantly higher than control values ($p < 0.05$). The radioactivity level in the thyroid was higher than in controls, but this finding was not statistically significant. Serum protein-bound iodine also did not differ significantly from the control value. In rats receiving the lower dose for 50 days, the thyroid weight and iodine level were not significantly different from those of control animals (Langer, 1964).

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