EPOXIDES

First draft prepared by

Professor I.G. Sipes and Dr A. Mattia

Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Arizona, USA; and US Food and Drug Administration, College Park, Maryland, USA

1. EVALUATION

1.1 Introduction

The Committee evaluated a group of nine epoxide flavouring agents, including ethyl methylphenylglycidate (No. 1577) (Table 1). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p. 170). The Committee previously evaluated two members of the group: ethyl 3-phenylglycidate (No. 1576) was evaluated at the twenty-fifth meeting (Annex 1, reference 56), when no ADI was assigned; ethyl methylphenyl-glycidate (No. 1577) was evaluated at the twenty-eighth meeting, when an ADI of 0–0.5 mg/kg bw was assigned (Annex 1, reference 66).

Five of the nine flavouring agents (Nos 1570–1572, 1574 and 1575) have been reported to occur naturally in fruits (e.g. citrus fruit, currants, mango

Table 1. Summary of results of safety evaluations of epoxides used or proposed for use as flavouring agents

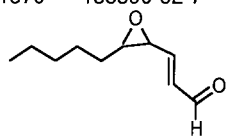
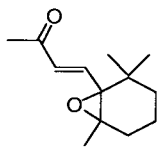
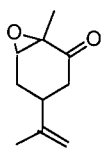
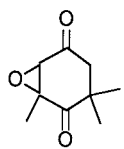
Flavouring agent	No.	CAS no. and structure	Step A3[a] Does intake exceed the threshold for human intake?	Step A4 Is the agent or are its metabolites endogenous?	Step A5 Adequate NOEL for substance or related substance?	Comments	Conclusion based on estimated daily intake
Structural class III							
4,5-Epoxy-(E)-2-decenal	1570	188590-62-7 	No Europe: 0.1[b] USA: 0.2[b]	NR	NR	See note 1	No safety concern (conditional)
β-Ionone epoxide	1571	23267-57-4 	No Europe: 0.09[b] USA: 0.1[b]	NR	NR	See note 1	No safety concern (conditional)
trans-Carvone-5,6-oxide	1572	18383-49-8 	No Europe: 0.01 USA: 0.2	NR	NR	See note 1	No safety concern
Epoxyoxophorone	1573	38284-11-6 	No Europe: 0.1[b] USA: 0.2[b]	NR	NR	See note 1	No safety concern (conditional)

Table 1 (contd)

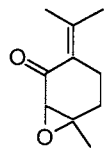
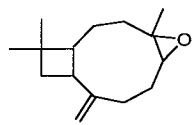
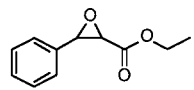
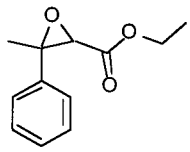
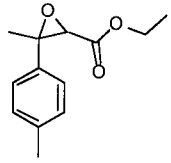
Flavouring agent	No.	CAS no. and structure	Step A3[a] Does intake exceed the threshold for human intake?	Step A4 Is the agent or are its metabolites endogenous?	Step A5 Adequate NOEL for substance or related substance?	Comments	Conclusion based on estimated daily intake
Piperitenone oxide	1574	35178-55-3 	No Europe: 0.01 USA: 0.2	NR	NR	See note 1	No safety concern
β-Caryophyllene oxide	1575	1139-30-6 	No Europe: 0.01 USA: 0.1	NR	NR	See note 1	No safety concern
Ethyl 3-phenylglycidate	1576	121-39-1 	Yes Europe: 114 USA: 96	No	Yes. The NOEL for the related compound, ethylmethylphenyl glycidate, is 35 mg/kg bw per day (Dunnington et al., 1981), which is > 17 000 times the estimated daily intake of ethyl 3-phenyl-glycidate of 2 μg/kg bw in Europe and the USA when used as a flavouring agent.	See note 2	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step A3[a] Does intake exceed the threshold for human intake?	Step A4 Is the agent or are its metabolites endogenous?	Step A5 Adequate NOEL for substance or related substance?	Comments	Conclusion based on estimated daily intake
Ethyl methylphenyl-glycidate	1577	77-83-8 	Yes Europe: 240 USA: 1840	No	Yes. The NOEL is 35 mg/kg bw per day (Dunnington et al., 1981), which is > 8000 and > 1000 times the estimated daily intakes of 4 µg/kg bw in Europe and 31 µg/kg bw in the USA from use as a flavouring agent.	See note 3	An ADI of 0–0.5 mg/kg bw was established for ethyl methylphenyl-glycidate by the Committee at its 28th meeting (Annex 1, reference 66), which was maintained at the present meeting.
Ethyl methyl-*para*-tolylglycidate	1578	74367-97-8 	No Europe: 23 USA: 0.009	NR	NR	See note 4	No safety concern

CAS, Chemical Abstracts Service; ND, no intake data reported; NR, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.
Step 1: All the agents in this group are in structural class III (Cramer et al., 1978).
Step 2: All the agents in this group are expected to be metabolized to innocuous products.

Table 1 (contd)

[a] The threshold for human intake for structural class III is 90 μg/day. All intake values are expressed in μg/day. The combined per capita intakes of flavouring agents in structural class III are 377 μg per day in Europe and 1937 μg per day in the USA.
[b] Intake estimate based on anticipated annual volume of production

Notes:
1. Epoxide hydrolysed via epoxide hydrolase to form vicinal diol, which forms glucuronic acid conjugate and is eliminated in the urine,or the epoxide is directly conjugated with glutathione by glutathione transferase and is eliminated in the urine.
2. The ester group is hydrolysed by carboxyl esterases followed by loss of carbon dioxide and rearrangement to phenacetaldehyde.
3. The ester group is hydrolysed by carboxyl esterases followed by loss of carbon dioxide and rearrangement to 2-phenylpropanal.
4. The ester group is hydrolysed by carboxyl esterases followed by loss of carbon dioxide and rearrangement to para-methyl-2-phenylpropanal.

and guava), beverages (beer) and a wide variety of spices and essential oils (e.g. scotch spearmint oil, celery seed, cinnamon bark and leaf oil, clove stem oil, ginger, peppermint oil, cornmint oil, pepper, thyme, hop oil, calamus, basil, rosemary, lemon balm, sage, pimento leaf, winter savoury, angelica seed oil, German camomile oil and mastic gum oil) (Guth & Grosch, 1990, 1993; Gassenmeier & Schieberle, 1994; Kerler & Grosch, 1997; Buttery & Ling, 1998; Reiners & Grosch, 1998; Buettner & Schieberle, 2001; Nijssen et al., 2003).

1.2 Estimated daily per capita exposure

Annual volumes of production have been reported for six of the nine flavouring agents in this group (Nos 1572 and 1574–1578). For the remaining three substances (1570, 1571 and 1573), anticipated annual volumes of production were given for their proposed use as flavouring agents. The total reported and anticipated annual volume of production of the nine epoxides is about 2600 kg in Europe (International Organization of the Flavor Industry, 1995) and 14 800 kg in the USA (National Academy of Sciences, 1970, 1987; Flavor and Extract Manufacturers Association, 1992; Lucas et al., 1999). About 95% of the total annual reported and anticipated volume in Europe and about 99% of that in the USA are accounted for by ethyl methylphenylglycidate (No. 1577) and ethyl 3-phenylglycidate (No. 1576). The estimated per capita exposure to ethyl methylphenylglycidate is 240 μg/day in Europe and 1840 μg/day in the USA, and that to ethyl 3-phenylglycidate is 114 and 96 μg/day, respectively. The estimated exposure to all the other agents in the group, with very low reported or anticipated annual volumes of production, is 0.01–23 μg/day in Europe and 0.009–0.2 μg/day in the USA (National Academy of Sciences, 1970, 1987; Flavor and Extract Manufacturers Association, 1992; International Organization of the Flavor Industry, 1995; Lucas et al., 1999). The estimated daily per capita exposure to each agent is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

Epoxides are characterized by an oxygen-containing three-membered ring. The inherent strain and polarity of the C–O bond in the epoxide ring are factors

Table 2. Annual volumes of production of epoxides 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		
4,5-Epoxy-(E)-2-decenal (No. 1570)					
Europe[e]	1	0.1	0.002		
USA[e,f]	1	0.2	0.003	+	NA
β-Ionone epoxide (No.. 1571)					
Europe[e]	0.6	000	0.001		
USA[e]	0.6	0.1	0.002	+	NA
trans-Carvone-5,6-oxide (No. 1572)					
Europe[e]	0.1	000	0.0002		
USA[h]	1	0.2	0.003	+	NA
Epoxyoxophorone (No. 1573)					
Europe[e]	1	0.1	0.002		
USA[e]	1	0.2	0.003	–	NA
Piperitenone oxide (No. 1574)					
Europe[e]	0.1	000	0.0002		
USA[g]	1	0.2	0.003	+	NA
β-Caryophyllene oxide (No. 1575)					
Europe[e]	0.1	000	0.0002		
USA	0.9	0.1	0.002	488	542
Ethyl 3-phenylglycidate (No. 1576)					
Europe	799	114	2		
USA	730	96	2	–	NA
Ethyl methylphenylglycidate (No. 1577)					
Europe	1 682	240	4		
USA	13 971	1 840	31	–	NA
Ethyl methyl-*para*-tolylglycidate (No. 1578)					
Europe	158	23	0.4		
USA[g]	0.05	0.009	0.0001	–	NA
Total					
Europe	2 642				
USA	14 779				

NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Maarse et al., 1999), but no quantitative data; –, not reported to occur naturally in foods

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

[b] Intake (µg/person per day) calculated as follows:
[[(annual volume, kg) x (1×10^9 µg/kg)]/[population × survey correction factor × 365 days]], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA; where correction factor = 0.6 for Europe, US National Academy of Sciences surveys, anticipated annual volumes, and annual volume cited by the Flavor and Extract Manufacturers Association of the United States and 0.8 for the Lucas et al. USA survey representing the assumption that only 60% and 80% of the annual flavour volume,

Table 2 (contd)

respectively, was reported in the poundage surveys (National Academy of Sciences, 1970, 1987; International Organization of the Flavor Industry, 1995; Lucas et al., 1999) 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 via 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.

[f] Natural occurrence data reported (Guth & Grosch, 1990; Buttery & Ling, 1993; Guth & Grosch, 1993; Gassenmeier & Schieberle, 1994; Kerler & Grosch, 1997; Buttery & Ling, 1998; Reiners & Grosch, 1998; Buettner & Schieberle, 2001).

[g] Annual volume reported in previous surveys in the USA (National Academy of Sciences, 1970, 1987).

[h] Annual volume cited by the Flavor and Extract Manufacturers Association (1992)

that promote its cleavage in the presence of suitable nucleophiles. They undergo chemical hydrolysis in gastrointestinal fluids. In vivo, epoxide hydrolase, which has been identified in the cytosol (Gill et al., 1974), endoplasmic reticulum (microsomes), mitochondria (Oesch et al., 1970) and nuclei (Bresnick et al., 1977) of liver and to some extent kidney cells, catalyses epoxide ring cleavage by water to yield vicinal *trans*-diols. The diols are then excreted primarily in the urine unchanged or as glucuronic acid or sulfate conjugates. Alternatively, epoxides can be conjugated with glutathione (GSH), mediated by glutathione *S*-transferase (GST), to yield the corresponding mercapturic acid conjugates, which also are excreted in the urine.

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 all nine flavouring agents (Nos 1570–1578) to structural class III (Cramer et al., 1978).

Step 2. All the flavouring agents in this group can be predicted to be metabolized to innocuous products. The evaluation of all the agents in this group therefore proceeded via the A-side of the Procedure.

Step A3. The estimated daily exposure to seven of the nine flavouring agents (Nos 1570–1575 and 1578) is below the threshold of concern for structural class III (90 mg per day). One substance (No. 1578) is reported to be used as a flavouring agent in Europe and the USA, and three others (Nos 1572, 1574 and 1575) are reported to be used in one region only. The remaining three substances (Nos 1570, 1571 and 1573) are only proposed for use as flavouring agents. According to the Procedure, the use of these seven flavouring agents and the estimated exposure raise no safety concern; however, less uncertain exposure estimates are needed for those flavouring agents for which only anticipated volume data were available (Nos 1570, 1571 and 1573).

The estimated daily per capita exposure to the remaining two substances, ethyl 3-phenylglycidate (No. 1576) and ethyl methylphenylglycidate (No. 1577), for which annual volumes of production were reported, exceed the threshold of concern for structural class III (90 mg per day). The per capita exposure to ethyl 3-phenylglycidate (No. 1576) is 114 μg/day in Europe and 96 μg/day in the USA, and that of ethyl methylphenylglycidate (No. 1577) is 240 μg/day in Europe and 1840 μg/ day in the USA. Accordingly, the evaluation of these agents proceeded to step A4 of the Procedure.

Step A4. These two agents and their metabolites are not endogenous. Accordingly, the evaluation of this agent proceeded to step A5.

Step A5. At its twenty-eighth meeting, the Committee established an ADI of 0–0.5 mg/kg bw for ethyl methylphenylglycidate on the basis of the results of a long-term study (Dunnington, 1981), in which the NOEL was 35 mg/kg bw per day. This NOEL is more than 8000 times the estimated daily intake of 4 μg/kg bw in Europe and more than 1000 times that of 31 μg/kg bw in the USA. This NOEL is more than 17 000 times the estimated intake of the related substance, ethyl 3-phenylglycidate, from its use as a flavouring agent in Europe and in the USA (both 2 mg/kg bw per day). The Committee therefore concluded that these flavouring agents would not present a safety concern at the estimated daily intakes.

The intake considerations and other information used to evaluate the nine epoxides according to the procedure are summarized in Table 1.

1.5 Consideration of secondary components

One member of this group of flavouring agents, 4,5-epoxy-(E)-2-decenal (No. 1570), has an assay value of < 95%. The secondary component, 4-5-epoxy-(Z)-2-decenal, is expected to have the same metabolic fate as the E isomer. It was therefore considered not to present a safety concern at the estimated levels of intake.

1.6 Consideration of combined exposure from use as flavouring agents

In the unlikely event that all nine flavouring agents in this group were to be consumed concurrently on a daily basis, the estimated combined exposure would exceed the human exposure threshold for class III (90 μg per person per day); however, all nine agents are expected to be efficiently metabolized at the exposure levels estimated from their use as flavouring agents. Specifically, epoxides primarily undergo epoxide hydrolase-catalysed ring cleavage, resulting in the production of vicinal *trans*-diols, which are subsequently excreted predominantly in the urine unchanged or as glucuronic acid or sulfate conjugates. In an alternative pathway of metabolism, epoxides can undergo conjugation with GSH to yield the corresponding mercapturic acid conjugates, which are also excreted in urine. Theoretically, therefore, simultaneous consumption of the epoxides (especially *trans*-epoxides) at sufficiently high concentrations could result in depletion of GSH; however, under normal conditions, intracellular GSH concentrations (1–10 mmol/l) (Armstrong, 1987, 1991) can be replenished and are sufficient to detoxify the concentrations of epoxides resulting from their use as flavouring agents. Moreover, additional cytoprotection is provided by the hydrolytic activity of epoxide hydrolase. Therefore, at the exposure levels resulting from use of the nine epoxides evaluated in this group as flavouring agents and due to the constant replenishment of GSH by biosynthesis, combined exposure to these flavouring agents would not present a safety concern.

1.7 Conclusions

The Committee maintained the previously established ADI of 0–0.5 mg/kg bw for ethyl methylphenylglycidate (No. 1577). It concluded that use of the flavouring agents in this group of epoxides would not present a safety concern at the estimated intakes. For three flavouring agents (Nos 1570, 1571 and 1573), the evaluation was made conditional because the estimated daily intakes were based on anticipated annual volumes of production. The conclusions of the safety evaluations of these three agents will be revoked if use levels or poundage data are not provided by December 2007. The Committee noted that the available data on the toxicity and metabolism of these epoxides are consistent with the safety evaluation made with the procedure.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The relevant background information summarizes the key scientific data applicable to the safety evaluation of nine epoxide flavouring agents.

2.2 Additional considerations on intake

Quantitative data on natural occurrence and a consumption ratio have been reported for β-caryophyllene epoxide (No. 1575), which show that it is consumed predominantly from traditional foods (i.e., consumption ratio > 1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987).

2.3 Biological data

2.3.1 Biochemical data

(a) Hydrolysis

The three phenylglycidate esters (Nos 1576, 1577 and 1578) are anticipated to undergo ester hydrolysis and ring-opening epoxide hydrolysis to varying degrees in gastric juice and intestinal fluid. Hydrolysis of phenylglycidate esters is mediated by classes of enzymes known as carboxylesterases, which occur in most tissues (Heymann, 1980; Anders, 1989) but predominate in the liver (Heymann, 1980). Ester hydrolysis yields the aliphatic alcohol and corresponding phenylglycidic acid. In a study of hydrolysis in vitro, the *cis* and *trans* isomers of ethyl methylphenylglycidate (No. 1577) and 'commercial' ethyl methylphenylglycidate (consisting of equal amounts of the two isomers) were each incubated with artificial gastric juice (pH, about 1.2). After 5 h, the percentage ester hydrolysis was 15% for *cis*-, 7.1 for *trans*- and 11.9% for commercial ethyl methylphenylglycidate. Incubation of the same materials for 5 h in artificial intestinal fluid (pH 7.5) resulted in 17.7% ester hydrolysis for *cis*-, 6.6% for *trans*- and 14% for commercial material (Morgareidge, 1962). In a follow-up study, hydrolysis of the ester group of ethyl 3-phenylglycidate (No. 1576) was tested in rat liver homogenate. The percentage hydrolysis of 50, 100 or 200 mg of ethyl 3-phenylglycidate was 32%, 48% and 56% at pH 7.5 and 90%, 73% and 67% at pH 8.0, respectively (Morgareidge, 1963).

In the acidic environment of the stomach, the epoxide functional group is itself susceptible to acid-catalysed hydrolytic ring opening, to yield the corresponding vicinal diol. As diols are more polar than the corresponding epoxides, hydrolysis in gastric fluid might be associated with decreased absorption of epoxides. Accordingly, oxirane ring (epoxide ring) breakdown was measured in artificial gastric juice. The *cis* isomer of ethyl methylphenylglycidate (No. 1577) resulted in about 80% epoxide hydrolysis within the first hour, and this level remained constant up to 5 h. The *trans* isomer showed 76% epoxide hydrolysis at 5 h, and the commercial material showed 65% loss. When *cis*-, *trans*- and commercial ethyl methylphenylglycidate were incubated in artificial intestinal fluid for 5 h, 75%, 31% and 72% epoxide degradation was found, respectively. The observation of extensive enzymatic epoxide hydrolysis in gastric juice supports the conclusion that orally administered epoxides undergo ring-opening hydrolysis before absorption (Morgareidge, 1962).

Cyclohexane oxide, a structurally related substance, was incubated in a series of aqueous solutions with pH ranging from 1 to 7, designed to replicate the acidic conditions of the stomach. After the 4-h incubation period, no intact cyclohexane oxide was detected in the acidic solutions (pH 1–3). Instead, cyclohexane oxide was completely converted to cyclohexane-1,2-diol. Cyclohexane oxide was determined to be most stable at pH 7. When incubated in plasma for 24 h, cyclohexane oxide was stable during the first 2 h; however, by 4 h, 80% had been hydrolysed to the diol, and within 8–12 h no detectable epoxide remained (Sauer et al., 1997).

These results indicate that both the ester and epoxide functional groups are labile under the conditions present in the gastrointestinal tract. Hydrolysis of these functional groups yields polar metabolites (i.e. diols from epoxides and diol carboxylic acid derivatives from glycidate esters), which can be absorbed from the

gastrointestinal tract and are subsequently excreted either unchanged or in conjugated form.

(b) Absorption, distribution and elimination

In mice and rats, orally administered aliphatic epoxides are rapidly absorbed from the gastrointestinal tract and extensively metabolized to polar metabolites, which are conjugated and eliminated, primarily in the urine.

Terpene epoxides: Cyclohexane oxide (related substance)

Cyclohexane oxide, a structurally related compound, is rapidly absorbed, metabolized and excreted in the urine. Measurement of the volume of distribution showed limited distribution of cyclohexane oxide to body tissues. It is presumably hydrolysed in the gastrointestinal tract to yield a diol, which is absorbed, conjugated in the liver and excreted, primarily in the urine.

Male JVC Fischer 344 rats were given 50 mg/kg bw (120 μCi/ml) of ^{14}C-cyclohexane oxide by intravenous injection. Urine was collected 6, 12, 24 and 48 h after injection, and faeces were collected at 24 and 48 h. Blood samples were taken from a jugular cannula at several times up to 24 h after administration. The 24-h urine showed that > 70% of the radioactivity had been eliminated. A small amount (~ 2.4%) of ^{14}C-cyclohexane oxide was detected in faeces, and about 7% of the radiolabel was exhaled. At 24 h, little radioactivity (~ 1.5%) was retained in the tissues. The initial (3 min) plasma concentration of ^{14}C-cyclohexane oxide (~ 200 μg/ml) declined to below the limit of detection (1 μg/ml) within 1 h. The disposition in plasma was characteristic of a linear two-compartment model, with initial and terminal disposition half-lives of 2.3 ± 0.6 min and 19.3 ± 1.6 min, respectively. The mean residence time was short (14.5 ± 0.5 min). The average apparent steady-state volume of distribution (0.44 ± 0.08 l/kg) and the systemic clearance (31.3 ± 0.5 l/kg·min) were indicative of rapid distribution and clearance of ^{14}C-cyclohexane oxide (Sauer et al., 1997).

Groups of four male JVC Fischer 344 rats and groups of four female B6C3F$_1$ mice were given 10 or 100 mg/kg bw of ^{14}C-cyclohexane oxide (50 μCi) by gavage. Urine was collected 6, 12, 24 and 48 h after administration, and faeces were collected at 24 and 48 h. Blood samples were collected from the rats through a jugular cannula at several times up to 48 h after dosing. Mice were killed at each time and blood was collected from the posterior vena cava. Most of the radiolabel was recovered in urine collected over 48 h from both species (76–93% for rats and 74–82% for mice). The faeces of rats and mice contained 1.9–2.2% and 4.0–5.3% of the administered radiolabel, respectively. The amount of exhaled radioactivity was consistent with or below the background level (~ 1.4%). No parent compound was detected in the plasma of either species during the sampling period. Plasma samples from rats had peak cyclohexane-1,2-diol concentrations of 24 μg/ml 6 h after the dose of 10 mg/kg bw and 34 μg/ml 1 h after the dose of 100 mg/kg bw. In mice, the peak plasma level of cyclohexane-1,2-diol (24.5 μg/ml) was attained 2 h after the higher dose. The diol was not detected in plasma samples obtained from mice given 10 mg/kg bw of ^{14}C-cyclohexane oxide. Oxidation of the rat tissue samples showed that little (0.7%) residual radioactivity was retained. The authors reported similar results for the mice, but no quantitative data were cited (Sauer et al., 1997).

(c) Metabolism

Epoxides are three-membered rings containing an oxygen atom. The inherent ring strain and the polarity of the C–O bond in the epoxide ring promote cleavage of the three-membered ring in the presence of suitable nucleophiles. In vivo, epoxide hydrolase, which has been identified in cytosol (Gill et al., 1974), endoplasmic reticulum (microsomes), mitochondria (Oesch et al., 1970) and nuclei (Bresnick et al., 1977) of liver and to a more limited extent kidney, catalyses epoxide ring cleavage by water to yield vicinal *trans* diols. Alternatively, GST present in the cytosol catalyses ring cleavage by GSH to yield *trans*-thioalcohol conjugates (Jakoby, 1978).

In an evaluation of the potential effect of modulators of epoxide metabolism on the cytotoxicity of *trans*-anethole in isolated rat hepatocytes, hydrolysis of the *trans*-anethole epoxide (anethole 1,2-epoxide) was shown to be mediated primarily by cytosolic epoxide hydrolase, rather than by microsomal epoxide hydrolase, as inhibition of microsomal epoxide hydrolase failed to increase the cytotoxic effects of *trans*-anethole (Marshall & Caldwell, 1992). Chemically induced depletion of GSH levels was also associated with an increase in *trans*-anethole-mediated cytotoxicity. In contrast to the relatively rapid increase in *trans*-anethole-induced cytotoxicity after inhibition of the cytosolic epoxide hydrolase, inhibition of de novo GSH synthesis was associated with a delayed increase in cytotoxicity, indicating that reaction of the *trans*-anethole epoxide with GSH is a slower pathway. This finding emphasizes the importance of the epoxide hydrolase detoxification pathway (Marshall & Caldwell, 1992, 1996).

The reaction of chiral or achiral epoxides with nucleophiles is associated with stereochemical consequences. If the epoxide ring contains one or two (e.g. *cis* and *trans* isomers of methyl 3-phenylglycidate; see below) chiral centres, ring-opening by water or GSH can lead to a mixture of enantiomers or diastereomers, respectively (Bruice et al., 1976). For instance, reaction of the achiral cyclohexene oxide with water yields racemic *trans*-1,2-cyclohexanediol. In some cases, enzymatic catalysis involves regio-selective ring-opening. The metabolism of *cis*-methyl 3-phenylglycidate with GSH involves a selective attack of GSH on the C2 position (i.e. the carbon bearing the carboxy ester functional group) of methyl 3-phenylglycidate.

Epoxide hydrolase

Epoxide hydrolase, as the name denotes, is responsible for the hydrolysis of epoxides and the resulting formation of the corresponding *trans*-1,2-diols. In a series of studies, 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene and *cis*-epoxy-methyl stearate were used as substrates to determine the distribution of soluble (cytosolic) epoxide hydrolase activity in mammalian organs and tissues (Gill & Hammock, 1980). The epoxide hydrolase activity of the soluble and microsomal fractions in various organs of male Swiss-Webster mice and female New Zealand rabbits are shown in Table 3.

Males of four strains of mice (Swiss-Webster, BALB, AKR and C57B1) had similar cytosolic liver epoxide hydrolase activities (1200 ± 46, 1300 ± 490, 1700 ± 270 and 1700 ± 140 pmol/min per mg protein). The epoxide hydrolase activity in the soluble fraction of liver was markedly lower in male Sprague-Dawley rats, however (40 ± 7.1 pmol/min per mg protein). The authors therefore not only

Table 3. Epoxide hydrolase activity of microsomal and soluble fractions from various organs of male Swiss-Webster mice and female New Zealand rabbits

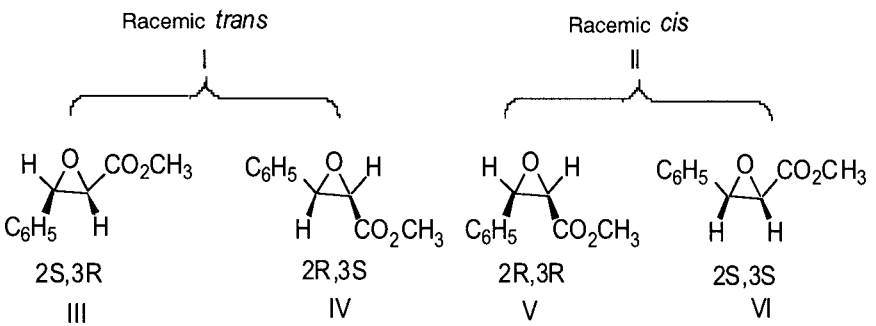
Organ	Fraction	Specific activity (pmol/min per mg protein)	
		Male Swiss-Webster mouse	Female New Zealand rabbit
Liver	Microsomes	130 ± 58	Not assayed
	Soluble	1200 ± 350	1400 ± 270
Kidney	Microsomes	59 ± 2.1	Not assayed
	Soluble	670 ± 93	940 ± 140
Lung	Microsomes	Not detectable	Not assayed
	Soluble	77 ± 26	120 ± 36
Testes	Microsomes	Not detectable	Not assayed
	Soluble	58 ± 34	Not assayed
Spleen	Microsomes	Not detectable	Not assayed
	Soluble	12 ± 9	46 ± 10
Duodenum	Microsomes	Not assayed	Not assayed
	Soluble	Not assayed	310 ± 150
Colon	Microsomes	Not assayed	Not assayed
	Soluble	Not assayed	150 ± 6.3
Muscle	Microsomes	Not assayed	Not assayed
	Soluble	Not assayed	250 ± 47

From Gill and Hammock (1980); 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-trans-2-octene was used as the substrate

observed variation in the specific activity of the soluble cytosolic epoxide hydrolase between species but also found sex- and strain-specific differences (Gill & Hammock, 1980).

Another important route of detoxication for epoxides is conjugation with GSH to yield mercapturic acid metabolites. Glycidic esters are a special class of epoxide, characterized by the presence of a strong electron-withdrawing alkoxy-

Figure 1. Structures and absolute configurations of optically active* trans- *and* cis-*methyl epoxycinnamate (3-phenyl-2,3-epoxypropanoate) and the urinary metabolites isolated from rats



From Rietveld et al. (1988)

carbonyl substituent. To investigate stereochemical effects associated with the metabolism of glycidate esters, racemic *cis*- or *trans*-methyl 3-phenylglycidate (i.e. 3-phenyl-2,3-epoxypropanoate I and II; see Figure 1) were administered intraperitoneally in propylene glycol to four adult male Wistar rats as a single dose of 0.7 mmol/kg bw (125 mg/kg bw). The first 24-h urine samples showed a significant increase in the amount of urinary thioether excretion ($p < 0.05$) expressed as SH equivalents. The racemic mixture obtained from the cis isomer caused a greater increase in SH equivalents (approximately 42 μmol) than the trans mixture (24 μmol) or the vehicle (16 μmol). GSH depletion by each of the four epoxide isomers (II, III, IV and V) and the racemic mixtures (I and II) was measured in rat liver homogenate (S9) at 37 °C and pH 7.4. The cis-epoxy esters were more efficient than the trans isomers in depleting GSH from the reaction mixture, compound V being the most active. In addition, the epoxy esters containing the R configuration at position 3 were more effective in depleting GSH than were the three S stereoisomers. The depletion of the initial GSH amount was 19 ± 2%, 50 ± 2%, 26 ± 2%, 10 ± 4%, 72 ± 3% and 41 ± 2% for compounds I, II, III, IV, V and VI, respectively (Rietveld et al., 1988).

In a longer evaluation, four adult male Wistar rats received intraperitoneal injections of 0.7 mmol/kg bw (125 mg/kg bw) of *cis*- (II) or *trans*-methyl 3-phenylglycidate (I), 5 days per week for 2 weeks. Urine was collected and the ether extracts pooled and analysed for the corresponding mercapturic acid derivatives. The *cis*- and *trans*-racemic mixtures and the mercapturic acid derivatives resulted from regiospecific opening of the epoxide ring at the α-carbon adjacent to the carboxylate group, with a preference for the R configuration at the β-carbon. Some epoxides have been reported to be *N*-alkylating agents, which, at high cellular concentrations, can react with proteins or DNA. The *N*-alkylating potential of *trans*- and *cis*-3-phenylglycidate esters (I and II, respectively) was investigated in vitro with 4-(*para*-nitrobenzyl)pyridine. The racemic *trans*-epoxy ester had greater alkylating ability than the racemic *cis*-epoxy ester (Rietveld et al., 1988).

The difference between the *cis* and *trans* isomers in alkylating activity is consistent with the greater mutagenic potential of the *trans* isomer (see below). These results are also consistent with the fact that the *cis* isomer is more readily detoxicated by GSH conjugation, leaving relatively higher concentrations of the *trans* isomer for *N*-alkylation reactions in vivo with nitrogen-containing substrates of low relative molecular mass, such as proteins and DNA.

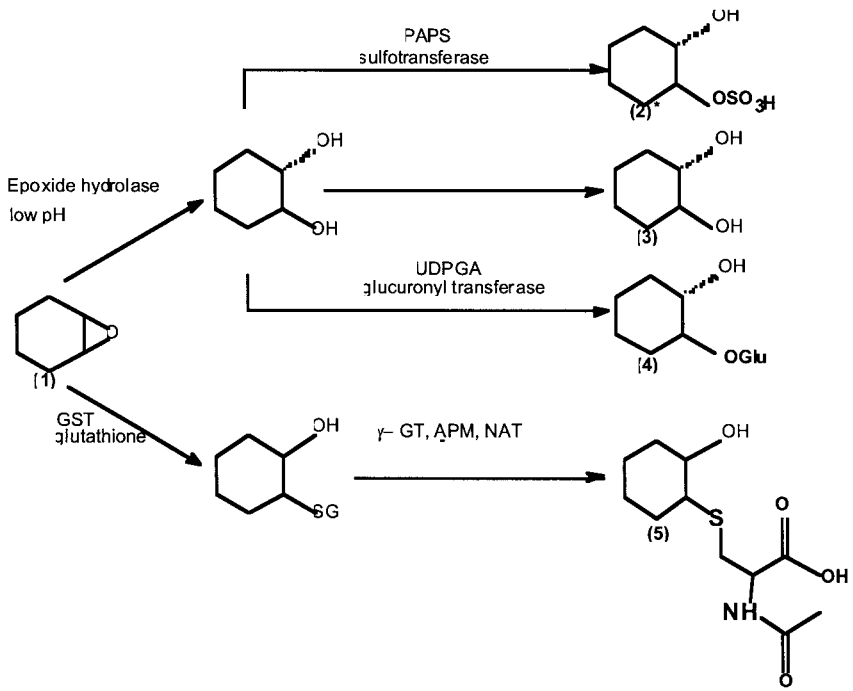
Terpene epoxides

Studies on the metabolism and pharmacokinetics of the aliphatic epoxides indicate that these agents, like the glycidate esters discussed above, undergo detoxication by GSH conjugation mediated by GST or 1,2-diol formation mediated by epoxide hydrolase (see Figure 2).

Caryophyllene oxide (No. 6)

In six male rabbits given 12 g of caryophyllene-5,6-oxide in 0.02% Tween 80 by gavage, about 60% of a total of 3.31 g of neutral metabolites eliminated in 72-h urine was recovered as (10*S*)-(−)-14-hydroxycaryophyllene-5,6-oxide (Asakawa et al., 1981, 1986).

Figure 2. Metabolic fate of cyclohexane oxide in rats and mice



From Sauer et al. (1997)
APM, aminopeptidase; γ-GT, γ-glutamyltransferase; GST, glutathione *S*-transferase; NAT, *N*-acetyltransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; UDPGA, UDP-glucuronic acid
(1) cyclohexane oxide; (2) cyclohexane-1,2-diol-*O*-sulfate; (3) cyclohexane-1,2-diol; (4) cyclohexane-1,2-diol-*O*-glucuronide; and (5) *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine
* Metabolite found only in $B6C3F_1$ mice

In a study of enzyme induction, 20 mg of caryophyllene oxide were administered to female A/J mice in cottonseed oil by gavage once every 2 days for 6 days. Statistically significant increases in GST activity were seen in the liver and small bowel mucosa (2.13 ± 0.58 and 1.20 ± 0.70 μmol/min per mg protein, respectively; 0.74 ± 0.18 and 0.25 ± 0.12 μmol/min per mg protein in vehicle controls). The forestomachs did not have more GST activity (0.59 ± 0.10 μmol/min per mg protein) than those of controls (0.62 ± 0.12 μmol/min per mg protein) (Zheng et al., 1992).

Cyclohexane oxide (related substance)

Cyclohexane oxide primarily undergoes hydrolysis at the low pH encountered in the stomach of mice and rats to yield the *trans*-1,2-cyclohexanediol. After hydrolysis, the resulting cyclohexane-1,2-diol is absorbed and is conjugated with

glucuronic acid. Additionally, mice excrete the 1,2-diol unchanged or conjugated with sulfate (see Figure 2). To a lesser extent, cyclohexane oxide reacts with GSH and is excreted as the mercapturic acid conjugate in the urine.

In a study to determine the ring carbon configuration (*cis* or *trans*) of a series of epoxide compounds in vitro, epoxides of cyclopentane, cyclohexane, cycloheptane, cyclooctane and cyclodecane formed the respective 1,2-diols by acid hydrolysis to varying degrees. In contrast, the cyclododecane epoxide was unreactive to acid hydrolysis. As *cis*-epoxides are hydrolysed more readily than *trans*-epoxides, all the epoxides tested, with the exception of cyclododecane epoxide, were in the *cis* configuration. When the same group of alicyclic epoxides was incubated with mouse liver microsomal epoxide hydrolase, hydration of the epoxide moieties was observed at low levels. Although hydrolase activity was reported to be very low with cyclopentane and cyclohexane epoxide, it increased with the size of the ring, such that maximal activity was obtained with cycloheptane epoxide (2.3 nmol/min per mg protein), declining somewhat with cyclooctane epoxide (1.0 nmol/min per mg protein). Mouse liver cytosolic (soluble) epoxide hydrolase showed the greatest activity for compounds containing a medium-sized ring, particularly cyclodecane epoxide (1.7 nmol/min per mg protein), and very low levels of activity or no activity for other epoxides (Magdalou & Hammock, 1988).

In groups of three or four male Fischer 344 rats, administration of cyclohexane oxide by gavage (100 mg/kg bw) or intravenous injection (50 mg/kg bw) resulted in urinary excretion of 12.5% and 13.2% of the administered dose as *trans*-cyclohexane-1,2-diol, 39.6% and 27.2% as a glucuronic acid conjugate of *trans*-cyclohexane-1,2-diol and 18.5% and 21.9% of the administered dose as a mercapturic acid conjugate (*N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine), respectively. In female B6C3F$_1$ mice, the urinary metabolic profile after administration of cyclohexane oxide (100 mg/kg bw) by gavage was similar to that in rats, except that a sulfate conjugate of *trans*-cyclohexane-1,2-diol was also identified (i.e. 8.2% cyclohexane-1,2-diol-*O*-sulfate, 8.5% cyclohexane-1,2-diol, 22.9% cyclohexane-1,2-diol-*O*-glucuronide and 17% *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine) (Sauer et al., 1997). The metabolic fate of cyclohexane oxide in Fischer 344 rats and B6C3F$_1$ mice is illustrated in Figure 2.

Male Wistar rats were given 0–1.2 mmol (≤ 118 mg or 621 mg/kg bw) of cyclohexane oxide intraperitoneally or via a cannulated jugular vein. The 48-h urine contained a mercapturic acid conjugate, *N*-acetyl-*S*-*trans*-2-hydroxycyclohexyl-L-cysteine. At doses ≤ 0.5 mmol (≤ 263 mg/kg bw) of cyclohexane oxide, the increase in urinary excretion of the mercapturic acid derivative was directly related to the administered dose. At higher doses (> 0.5 mmol), the amount of mercapturic acid conjugate excreted in the urine remained constant at 21% of the original dose. Formation of GSH conjugates by glutathione *S*-epoxide transferase is limited by the supply of free GSH, which is reported to be present in the rat liver at a concentration of about 9 mmol/l (Moron et al., 1978; Pessayre et al., 1979). Therefore, at high doses of cyclohexane oxide, the cellular GSH supply is depleted and de novo synthesis is inadequate to meet the demand (van Bladeren et al., 1981). It should be noted, however, that the intake of epoxides from their use as flavouring agents is significantly lower than the doses that were reported to saturate GSH after administration of cyclohexane oxide to rats. Consequently, GSH depletion is not expected to result from the use of epoxides as flavouring agents.

Groups of four to eight rats (strain unspecified) were given water suspensions of cyclopentane oxide (3.8 mmol/kg bw), cyclohexane oxide (3.0 mmol/kg bw) or cycloheptane oxide (1.5 mmol/kg bw) by stomach tube. Two hours after administration, the total GSH levels in the liver were 166, 82 and 96 mg/100 g liver with cyclopentane oxide, cyclohexane oxide and cycloheptane oxide, respectively, indicating a decrease in rat liver GSH from the control value (186 mg/100 g liver). The urine of rats and rabbits, collected at unspecified times, contained both *cis* and *trans* mecapturic acid derivatives after administration of cyclopentane oxide, cyclohexane oxide and cycloheptane oxide by gavage. These derivatives were *cis*- and *trans*-(2-hydroxycyclopentyl) mercapturic acid, *cis*-(2-hydroxycyclohexyl) mercapturic acid and trace amounts of *trans*-(2-hydroxycyclohexyl) mercapturic acid and *cis*- and *trans*-(2-hydroxycycloheptyl) mercapturic acid. The main metabolites identified in rabbit urine were the corresponding glucuronic acid conjugates of cyclopentane diol (15%), cyclohexane diol (37%) and cycloheptane diol (16%) (James et al., 1971). No data were given on glucuronic acid conjugation.

Limonene epoxide (related substance)

Limonene epoxide, a structurally related substance, was administered at a dose of 0.47 ml/kg bw to 10 adult male Long-Evans rats by intraperitoneal injection daily for 5 days. The urine contained limonene-1,2-diol as the only metabolite. Incubation of limonene epoxide with rat liver microsomes in vitro yielded the same metabolite (Regan et al., 1980). In another study, 2 mmol/l limonene 1,2-epoxide incubated with rat liver microsomes obtained from male Wistar rats yielded the corresponding limonene-1,2-diol at a rate of 0.6 nmol/mg protein per min (Watabe et al., 1980). When limonene 1,2-epoxide was incubated with cytosolic and microsomal mouse liver epoxide hydrolase, the initial rate of reaction was 134 pmol/min per mg tissue equivalent for the microsomal fraction; the rate was not determined for the cytosolic fraction (Hammock & Hasegawa, 1983). Incubation of limonene-1,2-epoxide with mouse liver microsomes resulted in formation of the 1,2-diol at a similar rate (10.6 nmol/min per mg protein). No hydration of limonene-1,2-epoxide by mouse liver cytosol was detected (Gill et al., 1983).

Thus, glycidate epoxides and alicyclic epoxides (including terpene) are readily detoxicated via two pathways, hydrolysis and GSH conjugation. Spontaneous hydrolysis can occur in the stomach or in tissues to form the corresponding *trans*-1,2-diols. Enzymatic hydrolysis is mediated by tissue epoxide hydrolases. The diols are then excreted primarily in the urine unchanged or as glucuronic acid or sulfate conjugates. Conjugation with GSH, mediated by GST, yields the corresponding mercapturic acid conjugates, which are also excreted in the urine. High doses of epoxides (especially *trans*-epoxides) can deplete GSH. Secondary *N*-alkylation reactions with nitrogen-containing biomolecules can then occur; however, as discussed above, GSH depletion is not expected to occur after consumption of epoxides at levels resulting from their use as flavouring agents.

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for four of the nine substances in this group (Table 4). In rats, the LD_{50} values ranged from 2450 mg/kg bw for trans-

Table 4. Results of studies of acute oral toxicity with epoxides

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1572	*trans*-Carvone-5,6-oxide	Rat	2450	Moreno (1978)
1575	Caryophyllene oxide	Rat	> 5000	Moreno (1979a)
1575	Caryophyllene oxide	Rat; M	> 5000	Moreno (1979b)
1576	Ethyl 3-phenylglycidate	Rat	< 5000	Shelanski & Moldovan (1973)
1577	Ethyl methylphenylglycidate	Rat	5000	Levenstein (1976)
1577	Ethyl methylphenylglycidate	Rat; M, F	5470	Jenner et al. (1964)
1577	Ethyl methylphenylglycidate	Rat	5470	Bär & Griepentrog (1967)
1577	Ethyl methylphenylglycidate	Guinea-pig; M, F	4050	Jenner et al. (1964)

M, male; F, female; NR, not reported

carvone-5,6-oxide (No. 1572) to > 5470 mg/kg bw for ethyl methylphenylglycidate (No. 1577), indicating that the acute toxicity of epoxides given orally is very low (Jenner et al., 1964; Bär & Griepentrog, 1967; Shelanski & Moldovan, 1973; Levenstein, 1976; Moreno, 1978, 1979a,b). In guinea-pigs, an LD_{50} value of 4050 mg/kg bw was reported for ethyl methylphenylglycidate (No. 1577), also indicating low acute toxicity (Jenner et al., 1964).

The results of short- and long-term studies of toxicity with representative epoxides in experimental animals are summarized in Table 5 and described below.

(b) Short-term studies of toxicity

Ethyl methylphenylglycidate (No. 1577)

From 1957, a series of studies was conducted in which ethyl methylphenylglycidate was administered at repeated doses to rodents for up to 1 year. inconsistent effects were found on target organs. Although no effects were reported in a 12-week study in which rats were estimated to have ingested 24–29 mg/kg bw per day of ethyl methylphenylglycidate (Oser, 1957) or in a 1-year study in which ethyl methylphenylglycidate was given in the diet at a concentration of 2500 ppm (about 125 mg/kg bw per day) (Hagan et al., 1967), growth retardation and testicular atrophy were reported in a 16-week study at a dietary concentration of 10 000 ppm ethyl methyl phenylglycidate (about 1000 mg/kg bw per day) (Hagan et al., 1967). Furthermore, variations indicative of neuropathy, including hind-limb paralysis and degenerative changes of the sciatic nerve, were reported in two 2-year studies in which ethyl methylphenylglycidate was given to rats in the diet at concentrations ≥ 5000 ppm (about 250 mg/kg bw per day) (Bär & Griepentrog, 1967; Griepentrog, 1969). Some of the earlier studies, however, suffered from limited test protocols (Bär & Griepentrog, 1967; Griepentrog, 1969) or did not provide analytical data on the test substance (Oser, 1957; Bär & Griepentrog, 1967; Hagan et al., 1967; Griepentrog, 1969). Consequently, two more recent, traditional studies (Mason et al., 1978; Dunnington et al., 1981) of up to 2 years' duration were performed with ethyl methylphenylglycidate, partly to resolve the inconsistent toxicological effects.

Table 5. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with epoxides administered orally

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							
1577	Ethyl methylphenylglycidate[c]	Rat; M, F	1/24	Diet	84 (12 weeks)	M: > 24[d] F: > 2[d]	Oser (1957)
1577	Ethyl methylphenylglycidate	Rat; M, F	3/30	Diet	105 (15 weeks)	50	Mason et al. (1978)
1577	Ethyl methylphenylglycidate	Rat; M,F	1/10	Diet	112 (16 weeks)	< 1000	Hagan et al. (1967)
Long-term studies							
1577	Ethyl methylphenylglycidate	Rat; M,F	1/10	Diet	365 (1 year)	> 125[d]	Hagan et al. (1967)
1577	Ethyl methylphenylglycidate	Rat; M, F	3/96	Diet	730 (2 years)	M: 35 F: 60	Dunnington et al. (1981)
1577	Ethyl methylphenylglycidate	Rat; M, F	4/10-30	Diet	730 (2 years)	< 50	Griepentrog (1969)
1577	Ethyl methylphenylglycidate	Rat; M, F	2/10–20	Diet	730 (2 years)	50	Bär & Griepentrog (1967)

M, male; F, female

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] The test material consisted of a flavour mixture of ethylbenzoate, isobutyl benzoate, benzyl acetate, benzyl butyrate, ethyl methylphenylglycidate and glycidate M-116 blended in proportion to the use levels reported for the individual flavouring compounds (0.15, 25, 18.7, 25, 25 and 25 ppm, respectively). The resulting blend, containing therefore approximately 21% ethyl methylphenylglycidate, was incorporated in the test diet to provide a daily dose of 100 mg/kg bw per day of the flavour mixture. On the basis of an actual mean intakes of 115 and 137 mg/kg bw per day of the flavour mixture by male and female rats, respectively, the average daily intakes of ethyl methylphenylglycidate were about 24 and 29 mg/kg bw per day for male and female rats, respectively.

[d] 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 effects. The actual NOEL might be higher.

Rats

Groups of 12 male and 12 female rats (strain unspecified) were fed a diet for 12 weeks designed to provide a dose of 100 mg/kg bw per day of a flavour mixture consisting of a series of aromatic esters blended at concentrations proportional to their reported use levels: ethyl benzoate, 0.15 ppm; isobutyl benzoate, 25 ppm; benzyl acetate, 18.7 ppm; benzyl butyrate, 25 ppm; ethyl methylphenylglycidate (No. 1577), 25 ppm; and glycidate M-116, 25 ppm. The actual mean intakes of the flavour mixture were reported to be 115 and 137 mg/kg bw per day for male and female rats, respectively. On the basis of the concentration at which ethyl methylphenylglycidate was incorporated into the flavour mixture (about 21%), the mean daily intakes of the flavour mixture corresponded to average intakes of about 24 and 29 mg/kg bw per day of ethyl methylphenylglycidate for male and female rats, respectively. Concurrent controls (12 of each sex) received the basal diet. Daily observations of physical appearance and behaviour, weekly measurements of body-weight gain and food intake, and calculation of food use efficiency revealed no significant differences between test and control animals. At the end of the treatment period, analysis of urine for glucose and albumin revealed elevated albumin values in all test and control males and some control females. In the absence of a difference between the frequency or the intensity of albumin levels in test and control groups, the increase was not attributed to treatment. Haemoglobin levels determined at week 12 were comparable in test and control animals. With the exception of pulmonary disease in some control animals, the results of necropsy were unremarkable. No variations were reported in the absolute or relative weights of the liver or kidneys of test animals in comparison with controls. The authors concluded that no adverse effects were associated with administration of the the flavour mixture in the diet (Oser, 1957).

Groups of five weanling Osborne-Mendel rats of each sex were given diets containing ethyl methylphenylglycidate (No. 8) at a concentration of 0 or 10 000 ppm for 16 weeks, corresponding to an estimated daily intake of about 1000 mg/kg bw per day (Food & Drug Administration, 1993). Weekly observation of general appearance and measurements of body weight and food intake revealed reduced body-weight gain for treated males in comparison with controls. Examination of white and red cell counts, haemoglobin level and erythrocyte volume fraction at study completion showed no remarkable findings. At necropsy at the end of the study, the liver, kidneys, spleen, heart and testes were weighed and examined histopathologically, and the abdominal and thoracic viscera and the bone, bone marrow and muscle of one hind leg were examined microscopically. With the exception of testicular atrophy in treated males (statistical significance not specified), no other weight variations or pathological abnormalities were observed (Hagan et al., 1967).

Groups of 15 Sprague-Dawley rats of each sex were maintained on diets containing 0, 0.02, 0.1 or 0.5% ethyl methylphenylglycidate (No. 1577) for 15 weeks. The test substance was 99% pure and had well defined specifications. On the basis of measurements of body weight and food and water intake on days 0 (first treatment day), 22, 48 and 93, the daily intake of ethyl methylphenylglycidate at each dietary concentration ranged from 29 to 9 mg/kg bw (0.02%), 154 to 49 mg/kg bw (0.1%) and 774 to 213 mg/kg bw (0.5%) for males and 31 to 11 mg/kg bw (0.02%), 154 to 65 mg/kg bw (0.1%) and 823 to 274 (0.5%) mg/kg bw for females.

Additionally, satellite groups were maintained on diets containing 0, 0.1 or 0.5% ethyl methylphenylglycidate for 2 or 6 weeks.

Weekly measurements of body weight and food and water consumption revealed a transient (from day 29 to day 62 inclusively) increase in body weight for males on the diet containing 0.02% ethyl methylphenylglycidate. Renal function tests (i.e., specific gravity and 2- and 6-h volume output after water loading and during water deprivation, respectively) were conducted in weeks 2, 6 and 13, while urine components (blood, bile salts, proteins, ketones, glucose and microscopic constituents) were analysed during the last week of treatment. In week 2, but not in week 13, males at 0.5% produced urine of higher specific gravity and in smaller quantities during the 2-h urinary collection period (neither result was statistically significant) and had a 50% smaller 6-h urine volume than controls. The results were considered to be indicative of a mild impairment in the urine diluting and concentrating ability of the animals. Haematological examination on week 6 revealed a significant reduction in the white cell count in males at 0.5% in comparison with controls. No haematological variations were observed in week 15. Statistically significant variations in clinical chemistry consisted of decreased aspartate aminotransferase activity in females at 0.5% in week 15.

Statistically significant differences in organ weights seen in weeks 2 and 6 consisted of increased relative kidney weights in males at 0.5% and decreased relative stomach weights in males at 0.1%. At the end of the study, the relative liver and kidney weights were significantly increased in males at 0.5%. In females at 0.5%, the relative weights of the liver, spleen, kidneys, stomach, small intestine and empty caecum were significantly increased at the end of the study. Additionally, females at 0.02% showed an increased relative weight of the small intestine in comparison with controls. No statistically significant differences in the incidence of histopathological changes in any of these tissues or any of the other tissues examined (brain, heart, full caecum, adrenals, gonads, pituitary and thyroid gland) were observed between test and control animals after the 15-week treatment period. Histological examination of kidneys from all males that appeared to have impaired renal function in week 2 of the study revealed areas of dense lymphocytic infiltration surrounding the tubules and glomeruli in the cortical area. The cells in these areas appeared pale and swollen. In the lumina of a few tubules, desquamated cells were identified, some of which appeared to be necrotic. Slight thickening of the Bowman capsule was observed in areas of dense lymphocytic infiltration. Although these changes are consistent with early-stage nephrosis, similar variations were observed in two control males, in one control female and in two females at 0.5%. Similar lesions were seen four control males and two at 0.5% and in one female at this dietary concentration in week 6. In week 15, kidney lesions consistent with early signs of nephrosis were reported in 2/12 control males and 2/15 at 0.5% and in 1/14 female controls. As these kidney lesions occurred in both control and test animals, the authors did not consider the occurrence of nephrosis to be related to administration of ethyl methylphenylglycidate; however, although the increase in relative kidney weights in both males and females at 0.5% in week 15 was not accompanied by impaired renal function or by statistically significant incidences of histopathological changes, the authors concluded that the enlargement of the kidneys was treatment-related.

In order to evaluate any compound-related neurological effects, as reported in an earlier study (Griepentrog, 1969), a Rotarod was used to assess the

coordinated motor activity of animals during treatment. No difference in performance was seen between test and control animals. Furthermore, histological examination of the sciatic and brachial nerves showed no evidence of neurological changes.

The authors concluded that the study provided no evidence of compound-related testicular atrophy (Hagan et al., 1967) or neurological toxicity (Bär & Greipentrog, 1967; Griepentrog, 1969), which had been described in earlier studies. On the basis of the changes in relative organ weights observed in both males and females at the 0.5% level, the authors concluded that 0.1% was the no-observable-adverse-effect level. For both males and females, depending on the time during the study, the dietary level of 0.1% was reported to correspond to a daily intake of ethyl methylphenylglycidate of 50–150 mg/kg bw per day (Mason et al., 1978).

Cyclohexane epoxide (related substance)

Mice

In a 28-day study, groups of five male and five female B6C3F$_1$ mice were given 0 (vehicle control), 6.25, 12.5, 25, 50 or 100 mg/kg bw of cyclohexane oxide in 0.5% methylcellulose by gavage on 5 days per week. The mice were weighed on day 1, then once a week and at the end of the study. At scheduled necropsy, the liver, thymus, right kidney, right testicle, heart and lungs were excised and weighed. Histopathological examination was limited to the heart, ovary and forestomach. No significant differences between treated animals and controls were found in body or organ weights. Necropsy revealed a few gross lesions; no compound-related pathological alterations were observed at microscopic examination (Sauer et al., 1997).

Rats

In a 28-day study, groups of five male and five female JVC Fischer 344 rats were given 0 (vehicle control), 6.25, 12.5, 25, 50 or 100 mg/kg bw of cyclohexane oxide in 0.5% methylcellulose by gavage on 5 days per week. The rats were weighed on day 1, then once a week and at the end of the study. At scheduled necropsy, the liver, thymus, right kidney, right testicle, heart and lungs were excised and weighed. Histopathological examination was limited to the heart, ovary and forestomach. No significant differences between treated animals and controls were found in body or organ weights. The authors noted, however, that the lung weights of treated females (dose not specified) tended to be increased; however, owing to large variance in the data, the result was not statistically significant. Necropsy revealed a few gross lesions; no compound-related pathological alterations were observed at microscopic examination (Sauer et al., 1997).

(c) Long-term studies of toxicity and carcinogenicity

Ethyl methylphenylglycidate (No. 1577)

In a 1-year study, groups of five weanling Osborne-Mendel rats of each sex were given diets containing ethyl methylphenylglycidate at a concentration of 2500 ppm [about 125 mg/kg bw per day (Food & Drug Administration, 1993)]. Weekly observations of general appearance and measurements of body weight and food intake showed no differences between test and control animals.

Haematological examinations performed at 3, 6 and 12 months for white and red cell counts, haemoglobin level and erythrocyte volume fraction gave normal values. At necropsy, the liver, kidneys, spleen, heart and testes were weighed, and these organs, as well as the abdominal and thoracic viscera and the bone, bone marrow and muscle of one hind leg were preserved for histopathological examination. No significant differences in organ weights were reported, and the histopathological findings were unremarkable (Hagan et al., 1967).

In a 2-year study, with limited reporting of data, equal numbers of male and female white rats (strain unspecified) were given diets supplemented with 0, 0.1% or 0.5% of ethyl methylphenylglycidate [approximately 0, 50 and 250 mg/kg bw per day in rats, respectively (Food & Drug Administration, 1993)]. The appearance, behaviour and body-weight gain of the animals were monitored throughout the study at unspecified intervals. All animals, including those that died before the end of the study, were necropsied and the liver, kidneys, adrenals, heart, spleen, pancreas, brain and any tumours were examined histopathologically. With the exception of a slight reduction in the survival of animals (7/10 at the end of the study), no adverse effects were reported at the lower dietary concentration. At 0.5%, survival was significantly affected (14/20 rats alive at 1 year, 1/20 at 1.5 years and 0/20 at 2 years), and a reduction in body-weight gain was reported. Paralysis of the rear extremities was observed, and histopathological examination revealed degeneration of the sciatic nerves in animals at 0.5%. No further details were provided (Bär & Griepentrog, 1967).

In a similar 2-year study conducted at the same laboratory, groups of five male and five female white rats (strain unspecified) were maintained on diets containing 0, 0.1% or 0.5% ethyl methylphenylglycidate [approximately 50 and 250 mg/kg bw per day, respectively (Food & Drug Administration, 1993)]. In a follow-up investigation, also of 2 years' duration, groups of 10 rats of each sex were given diets containing 0, 0.1% or 0.6% ethyl methylphenylglycidate [approximately 50 and 300 mg/kg bw per day, respectively (Food & Drug Administration, 1993)]. In yet another 1-year study, groups of 15 rats of each sex received diets containing 0 or 0.35% ethyl methylphenylglycidate [approximately 175 mg/kg bw per day (Food & Drug Administration, 1993)]. A transient reduction in body weights was observed in rats at 0.6%, which was accompanied by a decrease in food intake. The authors commented that the effect might have been due to the unpalatability of the strongly flavoured food.

All animals were necropsied, and the liver, spleen, heart, kidneys and brain were examined histologically. In view of the previous finding of compound-related nerve damage, the sciatic nerve and spinal marrow from all animals that were necropsied and the sciatic nerve from moribund animals killed before the end of the study were also examined. In some cases, the lumbar nerve root was also evaluated histopathologcially. Animals in all treated groups showed some degree of paralysis of the posterior extremities, the paralysis being more pronounced at higher dietary levels of ethyl methylphenylglycidate. In particular, paralysis was rare at 0.1%, and the incidence (22%) of histological changes (demyelination of nerve fibres and, in some cases, destruction of the axis cylinder) in the sciatic nerve was low; at concentrations of 0.35%, 0.5% and 0.6%, however, paralysis and changes in the sciatic nerve were seen in 22% of animals at 0.1% in the diet, 70% at 0.35%, 65% at 0.5% and 60% at 0.6% ethyl methylphenylglycidate. Histological changes were also observed in the lumbar nerve root, including Waller

degeneration with dissolution of myelin in the form of marrow balls and lumps in animals with only mild demyelination. No other gross or microscopic changes were observed (Griepentrog, 1969).

In the most recent 2-year study, groups of 48 weanling Wistar-derived rats of each sex, housed four per cage, were maintained on diets containing 0, 0.02%, 0.1% or 0.5% ethyl methylphenylglycidate. On the basis of body weights and food intake, the approximate daily intake of ethyl methylphenylglycidate at 0.02%, 0.1% and 0.5% in the diet was estimated to range from 23 to 10 mg/kg bw, 114 to 29 mg/kg bw and 559 to 145 mg/kg bw for males and 23 to 10 mg/kg bw, 114 to 50 mg/kg bw and 562 to 248 mg/kg bw for females, respectively. Cumulative mortality records showed no significant differences in the survival of treated and control animals. Although the number of deaths among males and females at 0.5% was lower than in the control group, the difference did not reach statistical significance.

Daily observations of test and control group showed no abnormalities. Regular measurements of body weights and food and water intake, at various intervals throughout the study, revealed significantly lower body weights (up to week 92) for females at the highest dietary level (0.5%) ($p < 0.05$), but the body weight variations were less than 10% and were associated with a slight reduction in food intake. Increased water consumption was noted in males and females at the lowest dose (0.02%) and in females at 0.1%. Motor activity was evaluated in weeks 2, 15, 21, 62 and 84 by the Rotarod method (Mason et al., 1978). In contrast to the results of earlier studies (Bär & Griepentrog, 1967; Hagan et al., 1967; Griepentrog, 1969), there was no evidence of neuropathy in treated groups.

Haematological examinations (haemoglobin, packed-cell volume and erythrocyte and total leukocyte counts) on 10 animals of each sex in the control, 0.1 and 0.5% groups in weeks 16, 24 and 54 and on all groups at the end of the study revealed a transient increase in leukocyte count in males at 0.5%; however, at the end of the 2-year treatment period, the haematological values were similar in all groups. Clinical chemistry determinations revealed no statistically significant differences between males at 0.02% and controls; however, at 0.5%, statistically significant variations were seen, including reduced aspartate aminotransferase activity in males and females, elevated glucose levels in males and decreased and increased urea levels in females and males, respectively, in comparison with controls. At 12, 24, 54 and 79 weeks, renal function tests and urine analyses were conducted on animals in the control, 0.02% (weeks 12 and 24 only), 0.1% and 0.5% groups. Although some differences in urinary volume output were seen between test and control animals, no consistent effects were reported.

At necropsy, the relative brain, heart and pituitary weights were found to be significantly lower in males at 0.02% but not in those at higher dietary concentrations. Females at 0.02% had significantly lowere relative weights of empty caeca, and those at 0.1% had significantly lower relative pituitary weights. No weight differences were reported at the highest dietary concentration of ethyl methylphenylglycidate.

Histopathological examination revealed a dose-dependent increase in the incidence (18/39; $p < 0.05$) of focal fatty vacuolation of the liver in males at 0.5% and liver necrosis, albeit not dose-dependent, at 0.1% (9/39; $p < 0.01$) in comparison with the control group (9/37 and 1/39, respectively); however, the extent and severity of the lesions was comparable to those in control males, and no significant hepatic abnormalities were found in females. The authors noted that fatty vacuolization

occurs commonly in ageing male rats (Burek, 1978). As female rats showed no statistically significant increase in the incidence of fatty vacuolation and the lesions in affected males were similar in appearance and severity to those of control animals, the authors concluded that it was unlikely that the liver variations were related to treatment. The incidence of dilatation of the pancreatic duct in males at 0.5% (13/39) and in females at 0.02% (7/44) was statistically significant ($p < 0.001$); however, in view of the lack of a dose-dependent response in females and as atrophy of the pancreas is considered to be a common finding in ageing rats (Burek, 1978), the authors concluded that the pancreatic variations were incidental and unrelated to treatment. The increased incidences of lymph-node-sinus dilatation (5/38) ($p < 0.05$) and adrenal hyperplastic nodules (8/32) ($p < 0.05$) in males at the 0.5% dietary concentration also were reported to be common findings in ageing rats (Berg, 1967; Burek, 1978). Moreover, dilatation of the lymph node sinus was also found in a control female. Statistically significant increases in the incidences of pituitary adenomas were found in females and of testicular interstitial-cell adenomas in males at 0.02% and 0.5%. There was, however, no statistical difference in the overall incidence of benign or malignant tumours between treated and control animals. Moreover, the authors noted that spontaneous tumours of the pituitary are particularly common in ageing rats, with incidences as high as 50–70% in control rats (Andersson, 1969; Ito et al., 1972). The authors noted that the values were comparable with the incidence rates typically encountered in their laboratory for this strain of rat (50–72%). Therefore, they suggested that the relatively low incidence (42%) of tumours in the control rats in this study was probably responsible for the statistically significant difference in tumour incidence between test and control animals.

Likewise, interstitial-cell tumours are the commonest testicular tumours in male rats (Guerin, 1954; Davey & Moloney, 1970). The incidence in control Wistar rats is reported to be as high as 10% (Evans et al. 1977). In view of the lack of a dose-dependent increase in the incidence of these neoplasms (controls, 5.4%; 0.02% diet, 22%; 0.1% diet, 2.7%; 0.5% diet, 23%) and the fact that tumours were present in animals necropsied at the end of the study and not in those that died before the end of the study, the authors concluded that the distribution of testicular tumours was a reflection of variations in the normal spontaneous occurrence of this type of tumour in rats and was not related to administration of the test substance. The incidence of testicular adenomas in control male rats used in National Toxicology Program dietary studies is remarkably high (89.1%; range, 74–98%) (Haseman et al., 1998).

In view of the variations in body weight and the increased incidence of histological changes in the liver, pancreas, adrenal glands and lymph nodes at the highest dietary level, the NOEL was 0.1%, corresponding to an estimated daily intake of 35 mg/kg bw per day of ethyl methylphenylglycidate (Dunnington et al., 1981).

(f) Genotoxicity

Three representative epoxides in this group (Nos 1575, 1576 and 1577) and a series of structurally related glycidic and cycloaliphatic epoxides have been tested for mutagenicity and genotoxicity. The results of these tests are summarized in Table 6 and described below.

Table 6. Studies of genotoxicity with epoxides used as flavouring agents

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
In vitro						
1575	Caryophyllene oxide	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538	0, 10, 100, 1000 or 10 000 µg/plate	Negative[a,b]	Richold et al. (1979)
1575	Caryophyllene oxide	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	0, 10, 50, 100 or 500 µg/plate	Negative[a,c]	Richold et al. (1979)
1576	Ethyl 3-phenyl-glycidate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 10–500 µg/plate	Negative[d,e]	Canter et al. (1986)
1576	Ethyl 3-phenyl-glycidate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 10–2200 µg/plate	Negative[f,g]	Canter et al. (1986)
1576	Ethyl 3-phenyl-glycidate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 80, 400 or 2000 µg/plate	Negative[d,h] Negative/ weakly positive[f,i]	Tilch & Elias (1984)
1576	Ethyl 3-phenyl-glycidate digest[j]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 400, 2000 or 10 000 µg/plate	Negative[a]	Tilch & Elias (1984)
1576	Ethyl 3-phenyl glycidate	Reverse mutation	*S. typhimurium* TA100	0, 200, 500, 1000 or 2000 µg/ml	Negative/ positive[a,k]	Voogd et al. (1981)
1576	Ethyl 3-phenyl-glycidate	Reverse mutation	*S. typhimurium* TA100, TA98	0, 25, 50, 100, 250, 500, 1000, 2500 or 5000 µg/plate	Negative/ positive[d,i] Positive[l]	Wagner & Walton (1999)
1576	Ethyl 3-phenyl-glycidate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	≤ 4000 µg/plate	Negative/ positive[a,i]	Wild et al. (1983)
1576	Ethyl 3-phenyl-glycidate	SOS chromotest	*E. coli* PQ37	0, 0.01, 0.03, 0.1, 0.3 or 1.0 mmol/l (0, 1.9, 5.8, 19.2, 57.7 or 192.2 µg/ml)	Negative[a]	von der Hude et al. (1990a)
1576	Ethyl 3-phenyl-glycidate	Sister chromatid exchange	Chinese hamster ovary-K1-BH4 cells	0 or 103 µg/ml (0.103 mg/ml)	Positive[a]	Tilch & Elias (1984)

Table 6 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1576	Ethyl 3-phenyl-glycidate digest[j]	Sister chromatid exchange	Chinese hamster ovary-K1-BH4 cells	0 or 3280 µg/ml (3.28 mg/ml)	Negative[a]	Tilch & Elias (1984)
1576	Ethyl 3-phenyl-glycidate	Sister chromatid exchange	Chinese hamster V79 cells	0, 0.078, 0.16, 0.32, 0.63, 1.25 or 2.5 mmol/l (0, 15, 30.8, 61.5, 121.1, 240.3 or 480.5 µg/ml)	Positive[l,m]	von der Hude et al. (1991)
1577	Ethyl methylphenyl-glycidate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 100–10 000 µg/plate	Negative[a,n]	Canter et al. (1986)
1577	Ethyl methylphenyl-glycidate	Reverse mutation	*S. typhimurium* TA100	0, 200, 500, 1000, 2000 or 5000 µg/ml	Negative[a]	Voogd et al. (1981)
1577	Ethyl methylphenyl-glycidate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	≤ 3600 µg/plate	Negative[a]	Wild et al. (1983)
1577	Ethyl methylphenyl-glycidate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	≤ 1200 µg/plate	Negative[a,o]	Wild et al. (1983)
1577	Ethyl methylphenyl-glycidate	Sister chromatid exchange	Chinese hamster ovary cells W B1	0, 16, 50 or 160 mg/ml 0, 16, 50, 160 or 500 mg/ml	Positive[d] Negative[f]	Galloway et al. (1987)
1577	Ethyl methylphenyl-glycidate	Chromosomal aberration	Chinese hamster ovary cells W B1	0, 50, 160 or 500 mg/ml	Positive[d] Equivocal[f,p]	Galloway et al. (1987)
In vivo						
1576	Ethyl 3-phenyl-glycidate	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster* (480.5 µg/ml)	0 or 2.5 mmol/l	Negative[q]	Wild et al. (1983)
1576	Ethyl 3-phenyl-glycidate	Micronucleus induction	NMRI mice	0, 577, 961 or 1538 mg/kg bw[r]	Negative	Wild et al. (1983)
1577	Ethyl methylphenyl-glycidate	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster*	0 or 10 mmol/l (2062.4 µg/ml)[s]	Weakly positive	Wild et al. (1983)

Table 6 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1577	Ethyl methylphenyl-glycidate	Micronucleus induction	NMRI mice	0, 619, 1237 or 1856 mg/kg bw[r]	Negative	Wild et al. (1983)

[a] With or without S9 activation
[b] Cytotoxicity observed at 1000 and 10 000 μg/plate in *S. typhimurium* TA1535, TA1537 and TA1538
[c] Cytotoxicity at 500 μg/plate in *S. typhimurium* TA100 and TA98; results for strain TA1538 not reported with metabolic activation because of sample contamination
[d] Without S9 activation
[e] Cytotoxicity at highest dose in all strains tested
[f] With S9 activation.
[g] Cytotoxicity at 1600 and 2200 μg/plate in all strains tested
[h] Cytotoxicity at 2000 μg/plate in *S. typhimurium* TA98, TA1535 and TA1538
[i] Mutagenic only in *S. typhimurium* TA100
[j] Pepsin and pancreatin digest of ethyl 3-phenylglycidate; used to simulate mammalian digestion
[k] Positive only at 1000 and 2000 μg/ml
[l] Precipitation and cytotoxicity at 2.5 mmol/l (480.5 μg/ml)
[m] Absence or presence of metabolic activation not specified
[n] Cytotoxicity at 10 000 μg/plate in *S. typhimurium* strain TA100
[o] Pre-incubation method
[p] p value reported as < 0.003 in the trend test; however, none of the results was significant at $p \leq 0.05$ by the Dunnett method.
[q] Statistically significant increase in only one of three experiments and only in the first of three broods; cumulative number of mutations in all three experiments with first brood not statistically significantly different from controls
[r] Single intraperitoneal doses
[s] Statistically significant increase in number of mutations in only one of four experiments and only in the first of three broods; cumulative number of mutations in all four experiments with first brood significantly increased over controls

In vitro

In standard assays for reverse mutation in *Salmonella typhimurium*, ethyl methylphenylglycidate (No. 1577) and caryophyllene oxide (No. 1575) were consistently non-mutagenic in strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations up to 10 000 mg/plate, with and without metabolic activation (S9) (Richold et al., 1979; Voogd et al., 1981; Wild et al., 1983; Canter et al., 1986). Ethyl 3-phenylglycidate (No. 1576) gave inconsistent results in this test: at ≤ 4000 μg/plate, it did not induce reverse mutations in *S. typhimurium* TA1535, TA1537 or TA1538 with or without metabolic activation (Wild et al., 1983; Tilch & Elias, 1984; Canter et al., 1986); however, negative results were reported in several studies in TA98 incubated with ethyl 3-phenylglycidate at concentrations ≤ 4000 μg/plate, irrespective of metabolic activation (Wild et al., 1983; Tilch & Elias, 1984; Canter et al., 1986; Wagner & Walton 1999), whereas increased mutagenicity was found in TA98 with metabolic activation (≤ 5000 μg/plate). In the absence of metabolic activation, the frequency of revertants was comparable to that of controls (Wagner & Walton, 1999).

Although Canter et al. (1986) reported negative results in TA100 incubated with ethyl 3-phenylglycidate at ≤ 2200 μg/plate with and without metabolic activation, positive results were reported with and without metabolic activation in other studies (Voogd et al., 1981; Wild et al., 1983; Tilch & Elias, 1984; Wagner & Walton, 1999). In the study of Voogd et al. (1981), positive results were found only at the two highest concentrations (1000 and 2000 μg/ml), with and without metabolic activation. Only a weak mutagenic response was reported by Tilch and Elias (1984) and only in the presence of metabolic activation. In an assay in which the mutagenicity of a pepsin and pancreatin digest of ethyl 3-phenylglycidate (≤ 10 000 μg/plate), simulating mammalian digestion, was investigated in several strains of *S. typhimurium* including TA100, no increase in the mutagenic response was observed (Tilch & Elias, 1984).

In *Escherichia coli* PQ37, ethyl 3-phenylglycidate (No. 1576) was not mutagenic in the SOS Chromotest with or without metabolic activation (von der Hude et al., 1990a).

Ethyl 3-phenylglycidate (No. 1576) was clastogenic in Chinese hamster ovary (103 μg/ml) and V79 cells (480.5 μg/ml) with and without metabolic activation (Tilch & Elias, 1984; von der Hude et al., 1991); however, when ethyl 3-phenylglycidate was pre-treated with artificial digestive juices conducive to the formation of the corresponding diol and, subsequently, incubated with Chinese hamster ovary cells, no sister chromatid exchange was reported at concentrations ≤ 3280 μg/ml (Tilch & Elias, 1983).

Ethyl methylphenylglycidate (No. 1577) at ≤ 160 μg/ml induced a significant increase in the number of sister chromatid exchanges when tested in the absence of rat liver S9; however, no increase in sister chromatid exchange frequency was observed in the presence of metabolic activation (≤ 500 μg/ml). Similarly, ethyl methylphenylglycidate at ≤ 500 μg/ml induced chromosomal aberrations without metabolic activation. Although aberrations also occurred in the presence of S9, the results were considered equivocal owing to the lack of statistical significance in the Dunnett test (Galloway et al., 1987).

In order to assess further the potential mutagenicity and genotoxicity of the epoxides, the results of a number of assays performed with structurally related

glycidic and cycloaliphatic epoxide compounds were reviewed. The structurally related glycidic ester, racemic *cis*-methyl epoxycinnamate (II; see Figure 1) at ≤ 15 000 μg/plate was not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with or without metabolic activation in the plate incorporation test. In contrast, racemic *trans*-methyl epoxycinnamate (I; see Figure 1) at ≤ 15 000 μg/plate was mutagenic in *S. typhimurium* strains TA1535, TA1537, TA1538 and TA100, but not TA98, without metabolic activation. With metabolic activation, no significant increases in mutagenic activity were observed in any of the tester strains. When the individual diastereomers of *cis*- (V and VI; see Figure 1) and *trans*-methyl epoxycinnamate (III and IV; see Figure 1) were incubated at 1500 or 3000 μg/plate with *S. typhimurium* TA100 in the absence of metabolic activation, the *trans* isomers showed the greatest mutagenicity, compound IV being the most active (IV < III < VI < V) (Rietveld et al., 1988). The authors correlated these results with the increased *N*-alkylating potential of *trans* isomers discussed above.

The potential genotoxicity of several structurally related aliphatic cyclic epoxides was also investigated in the Ames assay and the SOS Chromotest. Although cyclopentane oxide and cyclohexane oxide were mutagenic in the Ames test, neither was active in the SOS Chromotest. Furthermore, cyclooctane oxide, cyclododecane oxide, (–)-2,3-epoxypinane and (+)-limonene oxide gave uniformly negative results in both assays (Basler et al., 1989). No other experimental details were provided in this abstract.

A series of cycloaliphatic epoxides was evaluated in the Ames assay with the standard battery of *S. typhimurium* strains (TA98, TA100, TA1535, TA1537 and TA1538) without metabolic activation. After incubation with cyclopentane oxide, cyclohexane oxide or norbornane oxide at 15–60 μmol/l, significant increases in the number of reverse mutations were observed only in TA100 and TA1535 in comparison with controls (Frantz & Sinsheimer, 1981). In another study, cyclohexane oxide also gave negative results in the standard Ames plate assay, but a significant increase in the number of reverse mutations was observed in *S. typhimurium* TA100 in a liquid test system (0.33–10 mmol/l) (Turchi et al., 1981). In a comparison of the potential mutagenicity of cycloalkane epoxides with increasingly expanding ring sizes (cyclopentane oxide, cyclohexane oxide, cycloheptane oxide, cyclooctane oxide and cyclododecane oxide) in *S. typhimurium* TA1535 and TA100 (12 μmol/plate), statistically significant mutagenic responses were obtained with cyclopentane oxide and cyclohexane oxide. Moreover, a slight but statistically significant increase in revertant frequency was observed with cycloheptane oxide, although the increase occurred in the presence of marked cytotoxicity. The mutation frequency with cyclooctane oxide was comparable to the spontaneous background levels. No viable colonies were detected after incubation of either *S. typhimurium* tester strain with cyclododecane oxide. Generally, therefore, the genotoxic potential of cycloaliphatic epoxides appears to be related inversely to the ring size; however, compounds with increasing ring sizes were shown to be increasingly cytotoxic. Consequently, specially constructed base-pair mutagen-detector *S. typhimurium* strains (TA92, TA1950 and TA2410), which have a normal lipopolysaccharide cell-wall coating as opposed to the more permeable coating of the TA100 and TA1535 strains, were used in an attempt to separate the mutagenic response from the confounding toxicity. The ratios of

mutagenicity to relative toxicity observed after incubation of the normally coated strains with cyclohexane oxide, cyclooctane oxide and cyclododecane oxide were comparable to those observed in the more permeable strains, indicating that the increase in mutation frequency was not due to the concomitant cytotoxicity (Frantz & Sinsheimer, 1981).

In eukaryotic V79 Chinese hamster cells, a weak but concentration-dependent increase in the occurrence of sister chromatid exchanges was observed in the presence of epoxycyclopentane and epoxycyclohexane. In contrast, V79 Chinese hamster cells incubated with epoxycyclooctane, epoxycyclododecane or (+)-limonene oxide showed no increase in sister chromatid exchange (von der Hude et al., 1991). Significant increases in mutant frequencies were reported when cyclohexane oxide (≤ 5 mmol/l) was incubated with V79 Chinese hamster cells. Cyclohexane oxide (10 mmol/l) also increased the micronucleus frequency and the number of chromosomal aberrations (bridges and lagging chromosomes) in Chinese hamster cells (Turchi et al., 1981).

1,2-Epoxyoctane, 1,2-epoxydecane, epoxycyclooctane, epoxycyclododecane, (+)-limonene oxide, α-pinane oxide and *cis*-2,3-epoxysuccinic acid did not induce unscheduled DNA synthesis in primary rat hepatocytes (von der Hude et al., 1990b).

In vivo

The potential of ethyl methylphenylglycidate (No. 1577) and ethyl 3-phenylglycidate (No. 1576) to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster* was studied in the Basc test. The mutation frequency was significantly increased in flies after 3 days' exposure to 2.5 or 10 mmol/l solutions of ethyl 3-phenylglycidate (480 μg/ml) or ethyl methylphenylglycidate (2062.4 g/ml), respectively; however, the increases were statistically significant in only one of the experiments conducted with each compound and only in the first of three broods tested. Although the isolated increase in mutation frequency observed with ethyl 3-phenylglycidate did not affect the overall number of sex-linked recessive lethal mutations in brood 1, the total number of mutations in brood 1 after exposure to ethyl methylphenylglycidate was significantly greater than in controls. The authors concluded that ethyl methylphenylglycidate is only weakly mutagenic in *Drosophila* (Wild et al., 1983).

The frequencies of micronucleated bone-marrow erythrocytes obtained from groups of four male and four female NMRI mice 30 h after administration of a single intraperitoneal dose of 619, 1237 or 1856 mg/kg bw of ethyl methylphenylglycidate (No. 1577) or 577, 961 or 1538 mg/kg bw of ethyl 3-phenylglycidate (No. 1576) were comparable to those in the corresponding controls (Wild et al., 1983).

Conclusion

The genotoxic potential of glycidate and alicyclic epoxides was studied in several standard assays in bacteria and mammalian cells in vitro. In the Ames assay for reverse mutation, both caryophyllene oxide and ethyl methylphenylglycidate gave unequivocally negative results with and without metabolic activation in a series of standard *S. typhimurium* tester strains. Ethyl 3-phenylglycidate gave positive results only in *S. typhimurium* strains TA100 and TA98 and mainly in the

presence of metabolic activation; however, a digest of ethyl 3-phenylglycidate had no mutagenic potential in *S. typhimurium* TA100 or TA98 with or without metabolic activation. Negative results were reported with *cis*-methyl epoxycinnamate, a structurally related glycidic ester, in the standard battery of *S. typhimurium* tester strains, but *trans*-methyl epoxycinnamate was mutagenic in TA1535, TA1537, TA1538 and TA100, only in the absence of metabolic activation. Cyclopentane and cyclohexane oxide induced reverse mutation in *S. typhymurium* strains TA1535 and TA100. Although, cycloaliphatic epoxides of larger ring size were generally less mutagenic activity than the smaller-ring epoxides, marked cytotoxicity was seen. Uniformly negative results were obtained in the SOS Chromotest in *E. coli*. Although clastogenic activity was reported in mammalian cell lines with most glycidate and alicyclic epoxides, increased sister chromatid exchange frequencies were observed with ethyl methylphenylglycidate only in the absence of metabolic activation. Likewise, chromosomal aberrations were reported in Chinese hamster ovary cells incubated with ethyl methylphenylglycidate or cyclohexane oxide, but the results with ethyl methylphenylglycidate were equivocal when S9 activation was incorporated into the assay. No unscheduled DNA synthesis was found with a series of epoxyalkanes and alicyclic epoxides.

In vivo, ethyl methylphenylglycidate had slight potential to induce sex-linked recessive lethal mutations in *Drosophila*, but ethyl 3-phenylglycidate showed no activity. Ethyl 3-phenylglycidate and ethyl methylphenylglycidate did not induce micronucleus formation in mice given single intraperitoneal doses.

Epoxides are naturally occurring substances that are also added to food as flavouring agents. The principal epoxide used in this way is ethyl methylphenylglycidate. Studies on the metabolism of glycidate and alicyclic (including terpene) epoxides indicate that these compounds are readily and adequately detoxicated in animals via two pathways, GSH conjugation and hydrolysis in the gastrointestinal tract or other tissues, followed by glucuronic acid or sulfate conjugation in the liver. Although glycidate and alicyclic epoxides had some genotoxic potential in standard assays in vitro, the results of assays for genotoxicity in mammals in vivo were negative. Furthermore, a number of long-term studies with dietary administration provided no evidence of carcinogenic potential. Several long-term studies with repeated doses of ethyl methylphenylglycidate showed no carcinogenicity at intake levels that were orders of magnitude higher than the intake of epoxides added as flavouring agents. The NOEL in the 2-year bioassay of ethyl methylphenylglycidate was 35 mg/kg bw per day. This intake level is > 1100 times the daily per capita intake ('eaters only') of 0.031 mg/kg bw per day from use of ethyl methylphenylglycidate as a flavouring agent.

The known pathways of metabolic detoxication, the lack of evidence of carcinogenicity in long-term feeding studies and the lack of genotoxic potential in vivo indicate that it is unlikely that epoxides pose a significant genotoxic risk to humans under the conditions of their use as flavouring agents.

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