The FEMA GRAS Assessment of Lactones Used as Flavour Ingredients

T. B. ADAMS[1], D. B. GREER[1], J. DOULL, I. C. MUNRO,
P. NEWBERNE, P. S. PORTOGHESE, R. L. SMITH, B. M. WAGNER,
C. S. WEIL, L. A. WOODS and R. A. FORD[2]

[1]Consultant to the Flavor and Extract Manufacturers' Association, 1620 I Street, NW, Suite 925, Washington, DC 20006 and [2]Research Institute for Fragrance Materials, Hackensack, New Jersey, USA

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FEMA EXPERT PANEL MEMBERS

John Doull, MD, PhD
Department of Pharmacology and Toxicology
University of Kansas Medical Center
Kansas City, Kansas

Jay I. Goodman, PhD
Department of Pharmacology and Toxicology
Michigan State University
East Lansing, Michigan

Ian C. Munro, PhD
CanTox, Inc.
Mississauga, Ontario

Paul M. Newberne, DVM, PhD
Department of Pathology
Boston University
School of Medicine
Boston, Massachusetts

Philip S. Portoghese, PhD
Department of Medicinal Chemistry
University of Minnesota
Minneapolis, Minnesota

Robert L. Smith, PhD
Department of Biochemical and
    Experimental Pharmacology
St Mary's Hospital
University of London
Paddington, London

Bernard M. Wagner, MD
New York University
School of Medicine
New York, New York
Bernard M. Wagner, Associates
Millburn, New Jersey

Carrol S. Weil, MA
Carrol S. Weil, Consultant
Pittsburgh, Pennsylvania

Lauren A. Woods, MD, PhD
Medical College of Virginia
Richmond, Virginia

PART 1—THE FEMA GRAS ASSESSMENT PROGRAM

I. Chemical identity

This summary presents the key scientific data relevant to the safety evaluation of 44 lactones used as flavour ingredients (see Appendix A). Lactones are cyclic esters which form by intramolecular cyclization of the corresponding hydroxycarboxylic acids. Most naturally occurring lactones are either five-membered (γ) or six-membered (δ) rings formed from saturated or unsaturated hydroxylated fatty acids. The group of lactones reviewed here includes those formed from the following:

(1) aliphatic acyclic linear saturated hydroxy-carboxylic acids (19; Nos 1–19)
(2) aliphatic acyclic linear unsaturated hydroxy-carboxylic acids (9; Nos 20–28)
(3) aliphatic acyclic branched-chain hydroxy-carboxylic acids (7; Nos 29–35)
(4) alicyclic and aromatic hydroxycarboxylic acids (9; Nos 36–44)

Abbreviations: ABS = chromosome aberrations; ALP = alkaline phosphatase; ALT = alanine aminotransferase; CHO = Chinese hamster ovary; CoA, = coenzyme A; CR = consumption ratio; CYP450 = cytochrome *P*-450; DNA = deoxyribonucleic acid; FEMA = The Flavor and Extract Manufacturers' Association; GGT = γ-glutamyl transferase; GRAS = generally recognized as safe; LD_{50} = median lethal dose; MLA = mouse lymphoma cells; RLH = rat liver homogenate; SCE = sister chromatid exchanges; UDS = unscheduled DNA synthesis.

II. Exposure

A. Flavour use

Low molecular weight lactones ($<C8$) formed from acyclic hydroxycarboxylic acids (Nos 1–35) generally exhibit a sweet herbaceous aroma accompanied by a sweet caramel-like taste. Higher molecular weight lactones mainly derived from fatty acids exhibit a fatty-, peachy- or coconut-like aroma. Two alicyclic lactones (Nos 37 and 38) derived from side-chain oxidation of menthone (i.e. dehydromenthofur-olactone) and menthol (i.e., mint lactone), respectively, exhibit a peppermint flavour. Two lactones fused to an aromatic ring system, dihydrocoumarin (No. 40) and 6-methylcoumarin (No. 41) have the sweet herbaceous aroma of new-mown hay.

Lactones are usually added as flavour ingredients to foods at levels less than 20 ppm, with a range of 0.05 to 80 ppm. Maximum levels of use are between 0.1 and 500 ppm, but are generally below 50 ppm. Only three substances, γ-valerolactone (148 ppm), 6-hydroxy-3,7-dimethyl octanoic acid lactone (500 ppm) and dihydrocoumarin (165 ppm) are used at levels greater than 100 ppm. The concentrations of aliphatic lactones intentionally added to foods are similar to those which occur naturally in food. For example, low molecular weight aliphatic ($<C9$) lactones are found in berries, fruits and related alcoholic beverages at concentrations of less than 1.0 ppm. Higher molecular weight aliphatic lactones ($>C9$) are present in meat and dairy products, mainly cheese and butter, at less than 20 ppm. Highest concentrations have been detected

in coconut (97 ppm) and strawberry jam (173 ppm) (CIVO–TNO, 1994).

The aliphatic lactones having the highest reported annual volumes of use in industry surveys (NAS, 1970, 1975, 1982 and 1987) are γ- and δ-lactones formed from linear saturated hydroxycarboxylic acids. Lactones derived from aliphatic hydroxycarboxylic acids of carbon chain length from C8 to C12 account for greater than 94% of the reported annual volume in the most recent survey (NAS, 1987). Four (4) γ- and δ-lactones (γ- and δ-decalactones and γ- and δ-dodecalactones) formed from the hydroxylated C10 and C12 fatty acids make up approximately two-thirds of the annual volume (NAS, 1987). Each of these lactones has been detected as a naturally occurring component of more than 30 foods (CIVO–TNO, 1994) and was originally studied as a flavour ingredient for use in margarine.

Dihydrocoumarin (3750 kg/yr) is the only lactone derived from alicyclic or aromatic hydroxycarboxylic acids with a reported annual volume of use greater than 1000 kg (NAS, 1987). Its use as a flavour ingredient has decreased significantly since 1970, from 11,900 kg to 3750 kg. Based on a reported annual volume of 3750 kg (NAS, 1987), the estimated daily per capita intake ("eaters only") of dihydrocoumarin is 12 μg/kg.

B. Natural occurrence

33 ingredients in this group (see Tables B1 and B2) have been reported to occur naturally in traditional foods, at levels between 0.001 and 200 ppm (CIVO–TNO, 1994). Four lactones formed from aliphatic acyclic hydroxycarboxylic acids with significant use as flavour ingredients (i.e. the γ- and δ-lactone of hydroxydecanoic acid and the γ- and δ-lactone of hydroxydodecanoic acid; Nos 10, 11, 15 and 16) are ubiquitous in food occurring mainly in fruits, berries, alcoholic beverages, meats and dairy products. In general, aliphatic lactones exist naturally as mixtures of enantiomers. For instance, γ- and δ-hexalactone and δ-octalactone are present in pineapple in enantiomeric ratios (S/R) of 76/24, 16/84 and 49/51, respectively (Engel et al., 1989). The distribution of chiral γ-lactones in fruit (e.g. pineapple) increases in favour of the R configuration with increasing chain length (Mosandl et al., 1990).

Aliphatic lactones occur naturally at highest concentrations (up to 100 ppm) in foods having a high fat content such as meat, cheese, milk and coconuts. Seven lactones derived from aliphatic acyclic hydroxycarboxylic acids have not yet been reported in food, including three ϵ-lactones (Nos 12, 17 and 32), 5-hydroxy-8-undecenoic acid δ-lactone (No. 25), and three branched-chain aliphatic lactones (Nos 29, 30 and 34). These seven lactones have minimal use as flavour ingredients (<50 kg/yr). Four lactones fused to alicyclic or aromatic ring systems (Nos 36, 40, 41 and 42), including dihydro-

coumarin and 6-methylcoumarin, have not been reported as components of food (see Table B2).

Quantitative natural occurrence data have been reported for 17 lactones formed from aliphatic acyclic hydroxycarboxylic acids and one lactone formed from alicyclic and aromatic hydroxycarboxylic acids (Stofberg and Kirschman, 1985). As represented by the consumption ratio (CR) (Stofberg and Grundschober, 1987; Stofberg and Kirschman, 1985), exposure to all but four of the 18 lactones occurs predominantly from food (i.e. CR > 1; see Tables B1 and B2). Intake of δ-nonalactone (No. 9), γ-decalactone (No. 11), γ-undecalactone (No. 13), and δ-undecalactone (No. 14) predominates from flavour use in dairy and butter flavourings (Lawrence, 1991; White and White, 1991).

III. Metabolism

A. Equilibrium between aliphatic lactones and ring-opened hydroxycarboxylic acids: effect of pH

Lactones are formed in nature by acid-catalysed intramolecular cyclization of γ- or δ-hydroxycarboxylic acids to yield five- or six-membered lactone rings (Fig. 1). In an aqueous environment, a pH-dependent equilibrium is established between the open-chain hydroxycarboxylate anion and the lactone ring. In basic media, the open-chain hydroxycarboxylate anion is favoured while in acidic media, such as in the urine, the lactone form predominates. γ-Valerolactone and γ-hexalactone have been detected in the urine of normal human adults (Zlatkis and Liebich, 1971).

When 4-hydroxybutanoic acid γ-lactone is administered intravenously (Roth and Giarman, 1966) or orally (Guidotti and Ballotti, 1970) to rats, the open-chain 4-hydroxybutanoate anion is formed in the blood and tissues. The half-life for
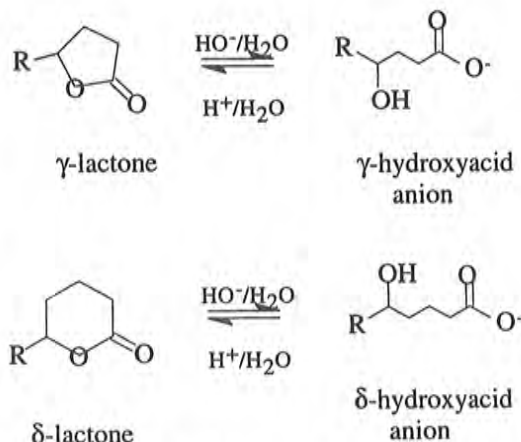

Fig. 1. Equilibrium of γ- and δ-lactone and hydroxycarboxylate anion.

Table 1. *In vitro* lactone hydrolysis data

Lactone	FEMA	Hydrolysis[a] (%)	Time (hr)	Reference
γ-Valerolactone	3103	32 (SIF)	4	Morgareidge, 1962
		(93 (RLH)	1	Morgareidge, 1963
4,4-Dibutyl-γ-butyrolactone	2372	92 (SIF)	1	Morgareidge, 1962
γ-Nonalactone	2781	62-94 (RLH, pH = 7.5)	1	Morgareidge, 1963
		81-88 (RLH, pH = 8.0)	1	Morgareidge, 1963
γ-Undecalactone	3091	58 (SIF)	4	Morgareidge, 1962
		26-40 (RLH, pH = 7.5)	1	Morgareidge, 1963
		45-70 (RLH, pH = 8.0)	1	Morgareidge, 1963
ω-6-Hexadecenlactone	2555	92 (SIF)	0.25	Morgareidge, 1962
		96 (SIF)	1	Morgareidge, 1963

[a]Incubation, 1 mM with simulated intestinal fluid (SIF) or rat liver homogenate (RLH).

the conversion of the lactone ring to the open-chain anion in the blood is less than 1 min. The reaction is catalysed by γ-lactonase, which shows greater activity in the plasma than in the liver or brain (Fishbein and Bessman, 1966). Hydrolysis of γ- and ε-aliphatic lactones and lactones formed from tertiary alcohols have been observed *in vitro* in basic simulated intestinal fluid and rat liver homogenate (see Table 1 and B3).

B. Metabolism of lactones formed from aliphatic acyclic linear saturated and unsaturated hydroxycarboxylic acids (Nos 1–28)

Linear aliphatic hydroxycarboxylic acids are hydrolysed and rapidly oxidized via the fatty acid pathway. Linear saturated 5-hydroxycarboxylic acids formed from δ-lactones are converted, via acetyl coenzyme A, to hydroxythioesters which then undergo β-oxidation and cleavage to yield an acetyl CoA fragment and a new β-hydroxythioester reduced by two carbons. Even-numbered carbon acids continue to be oxidized and cleaved to yield acetyl CoA while odd-numbered carbon acids yield acetyl CoA and propionyl CoA. Acetyl CoA enters the citric acid cycle directly while propionyl CoA is transformed into succinyl CoA which then enters the citric acid cycle. Linear saturated 4- or 6-hydroxycarboxylic acids formed from γ- or ε-lactones participate in the same pathway; however, loss of an acetyl CoA fragment produces an α-hydroxythioester which undergoes α-decarboxylation to yield a linear carboxylic acid and eventually carbon dioxide (Voet and Voet, 1990). In rats and dogs, [14]C₁-γ-decalactone and [14]C₁-γ-dodecalactone are metabolized in a manner similar to [14]C₁-lauric acid with approximately 75% of the labelled ^{14}C being eliminated as carbon dioxide within 48 hr (Fassett, 1961).

The metabolic fate of lactones has been extensively studied in animals, including humans. In rats, oral doses greater than 100 mg/kg of γ-butyrolactone (4-hydroxybutanoic acid lactone) were absorbed rapidly and completely from the intestinal tract (Arena and Fung, 1980; Guidotti and Ballotti,

1970; Lettieri and Fung, 1978). The majority of [14]C₁-labelled sodium 4-hydroxybutanoate administered by intravenous injection to rats was recovered as [14]CO₂ within 2.5 hr (Roth and Giarman, 1965). Intermediates formed during β-oxidation of lactones have been detected in humans (Lee, 1977; Walkenstein *et al.*, 1964). (S)-3,4-Dihydroxybutyric acid, glycolic acid and 4-hydroxy-3-oxobutyric acid were present in the urine of humans given a 1.0 g oral dose of γ-butyrolactone (Lee, 1977) (Fig. 2).

γ-Butyrolactone, the only lactone in this group formed from a primary alcohol, may participate in an alternate oxidation pathway. Oxidation of γ-butyrolactone to succinate by alcohol dehydrogenase and succinic semialdehyde dehydrogenase occurs primarily in the liver (Jakoby and Scott, 1959). Succinate then participates in the citric acid cycle (Doherty and Roth, 1978; Lee, 1977; Möhler *et al.*, 1976; Walkenstein *et al.*, 1964).

If the lactone is formed from a linear hydroxycarboxylic acid containing unsaturation, cleavage of acetyl CoA units will continue along the carbon chain until the position of unsaturation is reached. If the unsaturation begins at an odd-numbered carbon, acetyl CoA fragmentation will eventually yield a Δ3-enoyl CoA which is converted to the *trans*-Δ2-enoyl CoA before entering the fatty acid pathway. If unsaturation begins at an even-numbered carbon, acetyl CoA fragmentation yields a Δ2-enoyl CoA product which is a substrate for further fatty acid oxidation. If the stereochemistry of the double bond is *cis*, hydration yields (R)-3-hydroxyacyl CoA which is isomerized to (S)-3-hydroxyacyl CoA by 3-hydroxyacyl CoA epimerase prior to entering into normal fatty acid metabolism (Voet and Voet, 1990).

If the lactone contains a conjugated alkene, glutathione conjugation may occur as a minor metabolic pathway. Liver glutathione levels were significantly reduced following an intraperitoneal injection of 5-hydroxy-2-hexenoic acid δ-lactone (parasorbic acid lactone) to rats (Boyland and Chasseaud, 1970). *In vitro* the reaction is catalysed by glutathione *S*-transferase (Chasseaud, 1979).
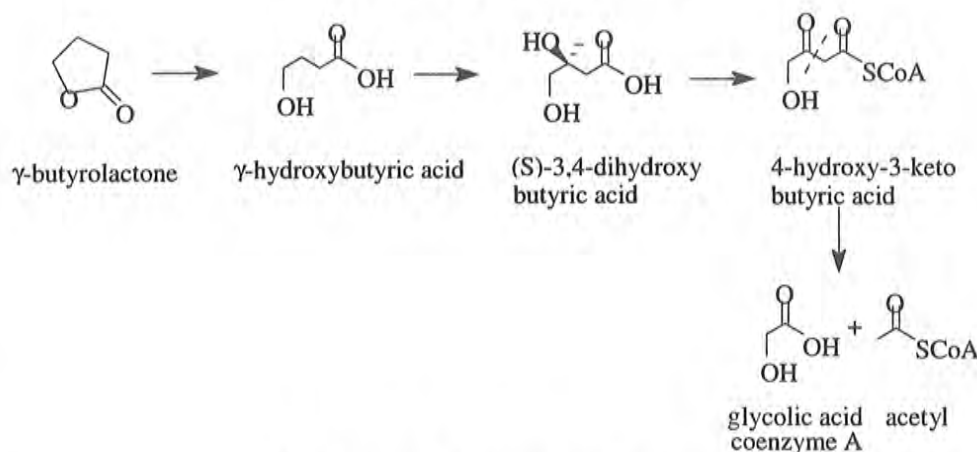
Fig. 2. Metabolism of γ-butyrolactone in humans.

C. Metabolism of lactones formed from aliphatic acyclic branched-chain saturated and unsaturated hydroxycarboxylic acids (Nos 29–35)

Short-chain (<C6) branched aliphatic acyclic hydroxycarboxylic acids may be excreted unchanged as the glucuronic acid conjugate, or undergo α- or β-oxidation followed by cleavage and complete metabolism to CO_2 (Voet and Voet, 1990; Williams, 1959) via the fatty acid pathway and the tricarboxylic acid cycle. Alternatively, as chain length, substitution and lipophilicity increase, the hydroxycarboxylic acid may undergo a combination of ω-, ω-1 and β-oxidation to yield polar hydroxyacid, ketoacid and hydroxydiacid metabolites which may be excreted as the glucuronic acid or sulfate conjugates in the urine and, to a lesser extent, in the faeces (Diliberto et al., 1990). Methyl substituted carboxylic acids are, to some extent, ω-oxidized in animals to form diacids which can be detected in the urine (Williams, 1959). The principal metabolic pathways utilized for detoxication of branched-chain acids are influenced by the position, number, and size of alkyl substituents.

Acids with a methyl substituent located at an even-numbered carbon (e.g. 2-methylpentanoic acid or 4-methyldecanoic acid) are extensively metabolized in the fatty acid pathway to CO_2 via β-oxidation and cleavage of the longer-branched chain. If the methyl group is located at an odd-numbered carbon such as the 3-position, β-oxidation is inhibited and ω-oxidation predominates, primarily leading to polar, acidic metabolites capable of being excreted in the urine (Williams, 1959). Larger alkyl substituents (>C2) located at the α- or β-position inhibit metabolism to CO_2 (Albro, 1975; Deisinger et al., 1994; Deuel, 1957), in which case there is either direct conjugation of the acid with glucuronic acid, or ω-oxidation leading to diacid metabolites which may be conjugated and excreted.

D. Metabolism of lactones formed from hydroxycarboxylic acids fused to alicyclic (4) and aromatic (5) ring systems (Nos 36–44)

1. Lactones fused to alicyclic rings (Nos 36–39). The pathways of metabolism for lactones fused to alicyclic rings depends on the size of the lactone ring (e.g. γ or δ), the degree of unsaturation and type of substitution on the ring system. If the lactone is a δ-lactone fused to an alicyclic ring (e.g. octahydrocoumarin; No. 36), it may be hydrolysed to a carboxylic acid containing an odd number (3) of carbons in the side-chain. β-Oxidation of the side-chain of octahydrocoumarin and cleavage yields 3-hydroxycyclohexanecarboxylic acid which may be subsequently aromatized to a benzoic acid derivative and excreted mainly as the hippuric acid.

In perfused rat liver, cyclohexanecarboxylic acid is primarily metabolized to hippuric acid. Small amounts of cyclohexylcarbonyl glucuronide, hexahydrohippuric acid, 3,4,5,6-tetrahydrohippuric acid, unchanged cyclohexanecarboxylic acid and benzoic acid also were detected (Brewster et al., 1977b). Benzoyl glucuronide was found in the bile of rats as an in vivo metabolite of cyclohexanecarboxylic acid (Brewster et al., 1977a).

If alkyl substituents are present on the alicyclic ring, hydrolysis may be followed by excretion in the urine or oxidation of ring substituents or the ring itself to yield polar hydroxylated metabolites, which may also be excreted primarily in the urine. The lactone ring in dehydromenthofurolactone (No. 37) or mintlactone (No. 38) is expected to hydrolyse to the enolic form of the ketocarboxylic acid, 9-carboxypulegone or 9-carboxymenthol, respectively. The ketocarboxylic acid or hydroxycarboxylic acid may be excreted unchanged as the glucuronic acid conjugate or undergo side-chain oxidation to yield hydroxylated metabolites which may also be excreted. The structurally related detoxication metabolites 3-hydroxymenthofurolactone, its ring-

opened form 9-carboxypulegone, and the hydroxyl-ated metabolites of 9-carboxypulegone have been detected in the urine of rats given pulegone or menthofuran (Madyastha and Raj, 1993).

2. Lactones fused to aromatic rings (Nos 40–44). Unlike aliphatic lactones which are readily hydrolysed to the corresponding hydroxy acids *in vivo* (Oser, 1962 and 1963), fused ring aromatic lactones such as coumarin are relatively stable so that oxidation competes favourably with hydrolysis of the lactone ring. Increased stability may result from extended conjugation of the aromatic ring with the α,β-unsaturated lactone ring present in coumarin derivatives. Despite the similarities in their names, dihydro-coumarin is not a member of the class of aro-matic coumarin derivatives. Dihydrocoumarin lacks α,β-unsaturation in the lactone ring (see Appendix A, Fig. A4), which is a key structural feature to the metabolism of coumarin. Cou-marin is principally metabolized in humans, cynomologus monkeys and baboons by electro-philic substitution (i.e. 7-hydroxylation), and in rats and several other species by epoxidation of the alkene function to form mainly 3-hydroxy-coumarin (e.g. Egan *et al.*, 1990; Van Iersel *et al.*, 1994). The absence of a double bond in dihydrocoumarin precludes the formation of the reactive epoxide and subsequent metabolites. Therefore, the metabolism of dihydrocoumarin would more closely resemble that of an aliphatic δ-lactone than coumarin.

In animals, dihydrocoumarin is expected to hydrolyse to the corresponding ring-opened hydro-xycarboxylic acid, *o*-hydroxyphenylpropionic acid (*o*-HPPA) (Fig. 3). In a report containing limited experimental data (Furuya, 1958), a "considerable dose" of dihydrocoumarin was given orally to rab-bits. Principle urinary metabolites identified by qualitative thin layer chromatography included *o*-HPPA and the glycine conjugate of *o*-HPPA. Minor urinary metabolites included *o*-coumaric

acid, *o*-coumarylglycine, coumarin, 7-hydroxycou-marin and 3-hydroxycoumarin. Dihydrocoumarin undergoes ring opening hydrolysis to *o*-HPPA when incubated with rat caecal extract (Scheline, 1968).

Following hydrolysis, *o*-HPPA may undergo β-oxidation to yield *o*-hydroxybenzoic acid (salicylic acid), which is excreted primarily in the urine unchanged or as the glycine conjugate (Oser, 1962 and 1963) (Fig. 3). In humans, the structurally re-lated acids 3-phenylpropionic acid (Pollitt, 1974) and 3-phenylpropenoic acid (i.e. cinnamic acid) (Williams, 1959) undergo β-oxidation and cleavage to yield benzoic acid which is excreted predomi-nantly in the urine as hippuric acid. In rats and mice, cinnamic acid is also metabolized mainly to hippuric acid (Nutley *et al.*, 1994); in rats, however, *o*-methoxycinnamic acid metabolizes mainly to the β-hydroxy derivative [i.e. 3-hydroxy-3-(*o*-methoxy-phenyl) propionic acid] suggesting that oxygenated substituents at the *ortho* position may inhibit com-plete β-oxidation in rats (Samuelson *et al.*, 1986).

It is anticipated that man will hydrolyse dihydro-coumarin to form the corresponding hydroxyacid, *o*-HPPA. The acid may either be conjugated with glycine prior to excretion or β-oxidized and cleaved to yield *o*-hydroxybenzoic acid (i.e. salicylic acid) (Fig. 3).

Unlike dihydrocoumarin, 6-methylcoumarin (No. 41) is a methyl substituted coumarin ring system which is expected to metabolize via methyl group oxidation to yield the corresponding aromatic car-boxylic acid or coumarin ring oxidation. In the lat-ter case, oxidation of the benzene ring (i.e. 7-hydroxylation) or the lactone ring alkene (i.e. 3,4-epoxidation) is possible. Compared with other metabolic pathways, 6-methylcoumarin is expected to exhibit an increased relative rate of benzene ring hydroxylation at the C7 position associated with the presence of a methyl substituent (Lake *et al.*, 1994a).

The demethylated analogue coumarin is rapidly absorbed and metabolized in humans (Cohen,
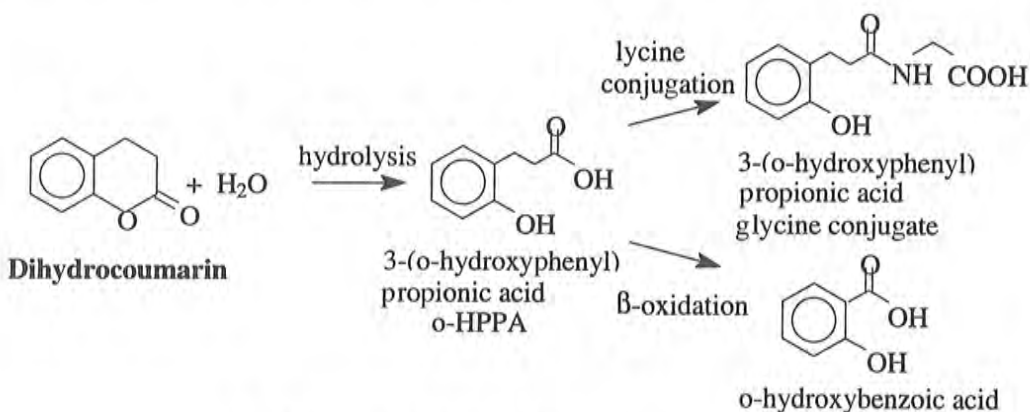

Fig. 3. Proposed metabolism of dihydrocoumarin in humans.

1979). 7-Hydroxycoumarin is the principle metabolite in humans and other primates (Cholerton et al., 1992; Egan et al., 1990; Rautio et al., 1992; Shilling et al., 1969; Waller and Chasseaud, 1981) formed by first pass metabolism in the liver (van Iersel et al., 1994). 7-Hydroxycoumarin is then rapidly conjugated with glucuronic acid (Ritschel et al., 1977) and excreted in the urine. 7-Hydroxylation of coumarin is catalysed by cytochrome P450 isoenzymes 2A6 (CYP2A6) and 2B6 (CYP2B6). Gene subfamilies CYP2A and CYP2B, which are expressed to form these enzymes have been isolated from human hepatic mRNA derived from chromosome 19 (Forrester et al., 1992; Miles et al., 1990). Volunteers excreted varying levels of 7-hydroxycoumarin (Cholerton et al., 1992; Rautio et al., 1992; Van Iersel et al., 1994) suggesting that some inter-individual variation may exist for gene expression of CYP2A6 (Miles et al., 1990; Pearce et al., 1992; Yamano et al., 1990; Yamazaki et al., 1992).

In the alternate 3-hydroxylation pathway, which predominates in rats, coumarin is oxidized via a 3,4-epoxide intermediate to yield mainly o-hydroxyphenylacetic acid (o-HPPA). This pathway may compete favourably in humans at high substrate concentrations (Fentem and Fry, 1992; van Iersel et al., 1994), presumably when CYP2A6 approaches saturation or when gene expression limits production of CYP2A6. o-Hydroxyphenylacetic acid (o-HPPA) accounted for only 1–6% of a 200 mg dose of coumarin given to humans (Shilling et al., 1969). In rats, coumarin is predominantly oxidized to o-HPPA, presumably via a 3,4-epoxide which in part reacts with glutathione at the α-position (Huwer et al., 1991). Additionally, the epoxide may bind covalently to cytoplasmic proteins (Lake et al., 1989; Peters et al., 1991), form a 3,4-diol, or rearrange to yield 3-hydroxycoumarin (Lewis et al., 1994).

It is anticipated that humans will metabolize 6-methylcoumarin via methyl group oxidation to the corresponding benzoic acid derivative which may also be readily excreted. Alternatively, 6-methylcoumarin may undergo ring hydroxylation to form the corresponding 7-hydroxy metabolite followed by excretion as the glucuronic acid conjugate. Metabolism via the 3,4-epoxide at high dose levels constitute, at most, a minor pathway, even in individuals exhibiting decreased activity of CYP2A6.

Phthalides are lactones formed from intramolecular cyclization of o-hydroxyphenylacetic acid. Hydrolysis of α-alkenyl substituted phthalides (Nos 42 and 43) would yield the enol form of the corresponding ketocarboxylic acid. The benzoic acid moiety may conjugate with glycine and be excreted mainly as the hippurate, and the ketone function may be reduced to the corresponding alcohol and be excreted as the glucuronic acid conjugate. In animals, mitochondrial acid:CoA ligases activate benzoates to form an intermediate CoA thioester which reacts with the amino acids glycine and glutamine. The resulting glycine conjugate of benzoic acid is efficiently removed from circulation during a single pass through the kidney (Killenberg and Webster, 1980). The phenylketone produced by hydrolysis may be reduced to the corresponding alcohol by aromatic ketone reductase (Felsted and Bachur, 1980). Reduction of other aromatic ketones (e.g. acetophenone) occurs stereoselectively (Culp and McMahon, 1968). However, it is difficult to predict stereospecificity of the reaction since aromatic ketone reductase consists of multiple enzymes with different absolute stereospecific or stereocatalytic properties. It is anticipated that humans will hydrolyse phthalides to their corresponding ketoacids, which may form their corresponding hippurate and be excreted in the urine or undergo ketone reduction to yield an alcohol which may be excreted as the corresponding glucuronic acid conjugate.

IV. Toxicology

A. Acute oral toxicity

In general, lactones formed from aliphatic acyclic hydroxycarboxylic acids (Nos 1–35) exhibit very low acute oral toxicity. Oral LD_{50} values greater than 1000 mg/kg have been reported for 28 of the 35 lactones (see Table B3) in this group. The majority of LD_{50} values are greater than 5000 mg/kg. The four lactones (Nos 36–39) fused to alicyclic rings have LD_{50} values which range from a low of 530 mg/kg for mintlactone (No. 37) to more than 5000 mg/kg for sclareolide (No. 39). The five lactones fused to benzene rings (Nos 40–44) have LD_{50} values greater than 1000 mg/kg (see Table B4).

B. Subchronic and chronic oral toxicity studies

Lactones formed from 6 linear saturated, two linear unsaturated, and two branched-chain aliphatic acyclic hydroxycarboxylic acids have been subjected to oral subchronic or chronic toxicity studies in rodents (see Section IV, B.1–B.5 below and Table B3). γ-Butyrolactone has been used therapeutically in humans as a sedative (Boncinelli et al., 1971; Hunter et al., 1971; Laborit et al., 1960; Mamelak et al., 1986), in the treatment of alcohol dependency (Ferrara et al., 1992), and was the subject of a recent 2-year carcinogenicity bioassay (NTP, 1992).

One alicyclic lactone dehydromenthofurolactone has also been subjected to an oral subchronic study (Table B4). Subchronic and chronic toxicity studies have been performed for dihydrocoumarin and 6-methylcoumarin while a single subchronic study exists for 3-propylidenephthalide (see Sections IV, B.6–B.8 below and Table B4). Dihydrocoumarin has also been the subject of a recent 2-year carcinogenicity bioassay (NTP, 1992). In all of these

studies, no-adverse-effect levels were at least 1000 times, and in most cases at least 10,000 times the respective daily *per capita* intakes ("eaters only") from use of these lactones as flavour ingredients.

1. Lactones formed from aliphatic acyclic linear saturated hydroxycarboxylic acids (Nos 1–19)

Subchronic and/or chronic studies have been performed using γ-lactones formed from C4, C5, C9 and C11 linear saturated hydroxycarboxylic acids (Table B3). No dose-related effects were reported when rats were maintained on diets providing an average daily intake of 49 mg γ-valerolactone/kg for 90 days (Oser *et al.*, 1965) or 500 mg/kg for 14 wk (Hagan *et al.*, 1967); 62.8 mg γ-nonalactone/kg for 90 days (Oser *et al.*, 1965) or 250 mg/kg for 1.5 to 2 years (Bar and Griepentrog, 1967); 16.5 mg γ-undecalactone/kg for 90 days (Oser *et al.*, 1965), 166 mg/kg for 130 days (Galea, 1965), or 250 mg/kg for 1.5 to 2 years (Bar and Griepentrog, 1967). No effects were observed in a 49-week study in rats or a 38-week study in dogs when a mixture of δ-lactones (δ-decalactone and δ-dodecalactone) was added to the diet of test animals. The mixture provided average daily intakes of δ-decalactone and δ-dodecalactone of 150 mg/kg and 300 mg/kg, respectively, to rats, and 75 mg/kg and 150 mg/kg, respectively to dogs (Wilson, 1961a). No adverse effects were reported when γ-butyrolactone was administered daily by gavage for 13 wk to mice or rats at a dose level of 112 mg/kg or 131 mg/kg, respectively (NTP, 1992). The intake levels that produced no effects in subchronic studies are greater than 10,000 times the daily *per capita* intake ("eaters only") from use of the respective lactone as a flavour ingredient or from exposure as a natural component of food.

A mixture composed primarily of aliphatic carboxylic acids and lactones was added to the diet of a limited number of rats (four males, three females) at a level calculated to result in the average daily intake of 100 mg γ-butyrolactone/kg and 32 mg/kg each for δ-octalactone, γ-nonalactone, γ-decalactone, γ-undecalactone and γ-dodecalactone for 4 to 6 months (Wilson, 1961b). Five succeeding generations were maintained on the same dietary regimen. Histopathological examinations of all treated animals revealed no effects. Although the validity of the results of the study are of limited value based on the small number of animals in the study (D. Fassett, unpublished report to RIFM, 1961), the results are consistent with those in feeding studies for individual lactone components reported above (i.e. γ-butyrolactone, γ-nonalactone, γ-decalactone).

2. Lactones formed from aliphatic acyclic linear unsaturated hydroxycarboxylic acids (Nos 20–28)

Lactones derived from the linear unsaturated carboxylic acids, 4-hydroxy-3-pentenoic acid or 5-hydroxy-2,4-decadienoic acid have been added to the diet of rats for 90 days at levels calculated to provide an average daily intake of 17.4 mg/kg (Shellenberger, 1971) or 12.1 mg/kg, respectively (Cox, 1974). The only effect reported in either study was an increase in absolute liver and kidney weights in animals fed 4-hydroxy-3-pentenoic acid lactone. However, relative organ weights were unchanged and there was no evidence of histopathology in any organs.

4-Hydroxy-3-pentenoic acid is the enolic form of laevulinic acid (4-ketopentanoic acid) which would form on *in vivo* hydrolysis of the lactone. Humans have been given oral doses of approximately 40 or 80 mg/kg of laevulinic acid in fruit juice daily for 30 days. Based on biweekly measurements of physical well-being, urinalysis and clinical chemistry parameters no effects were reported (Tischer, 1942). These intake levels that resulted in no adverse effects are greater than 100,000 times the daily *per capita* intake ("eaters only") of 4-hydroxy-3-pentenoic acid lactone (0.08 μg/kg) or 5-hydroxy-2,4-decadienoic acid lactone (0.002 μg/kg) from use as flavour ingredients.

3. Lactones formed from aliphatic acyclic branched-chain hydroxycarboxylic acids (Nos 29–35)

No effects were reported when the branched-chain γ-lactone 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone (No. 34) was fed to rats at levels calculated to provide average daily intakes of 1.29 mg/kg (males) or 1.47 mg/kg (females) for 90 days (Posternak *et al.*, 1969). No toxic or carcinogenic effects were observed when rats were maintained on diets containing 4,5-dimethyl-3-hydroxy-2,5-dihydrofuran-2-one (No. 35) at levels calculated to provide an average daily intake up to 46 mg/kg for 13 wk (Mundy and Kirkby, 1971) or 1 year (Mundy and Kirkby, 1973). The intake levels that produced no effects are greater than 10,000 times the daily *per capita* intake ("eaters only") of 0.1 mg/kg from use of 5-ethyl-3,4-dihydroxy-3-methyl-2-pentenoic acid lactone or 0.002 μg/kg 5-methyl-3,4-dihydroxy-3-methyl-2-pentenoic acid lactone from use as flavour ingredients.

4. Results of 2-year NTP bioassay with γ-butyrolactone

A chronic study of γ-butyrolactone (4-hydroxybutanoic acid lactone, No. 1) was conducted by the National Toxicology Program (NTP, 1992). The standard NTP protocol was used with F344 rats and B6C3F₁ mice. Groups of 50 male rats were administered γ-butyrolactone in corn oil by gavage in doses of 0, 112 and 225 mg/kg/day, 5 days per week, for 2 years. The same dosage schedule and sample preparation were used for female rats administered 0, 225 and 450 mg/kg/day, and for both sexes of mice administered 0, 262 and 525 mg/kg/day. Decreased body weight was reported in the high dose group of female rats and all of the test

mice. Increased mortality among the high-dose group of male mice was partially attributed to fighting on emergence from 4-hydroxybutanoic acid lactone sedation. There was no difference in survival between control and treated female mice.

Observations included statistically significant dose-related decreases in the incidence of mononuclear cell leukaemia in male rats; mammary and pituitary gland cysts and mammary gland fibroadenomas in female rats; and hepatocellular neoplasms in male mice. Male mice exhibited a statistically significant increase in the incidence of adrenal medulla hyperplasia in the low-dose group only, and an increase in the incidence of benign or malignant pheochromocytoma. The latter was not statistically significant ($P = 0.092$) and occurred only at the low dose. The lack of a dose–response relationship for these effects was postulated (NTP, 1992) to be due to the increased mortality of the high dose mice due to fighting, which prevented a greater portion of the group from receiving sample doses for a duration which would be sufficient for tumour development.

The NTP report concluded the following: "*Under the conditions of these 2-year gavage studies, there was no evidence of carcinogenicity of γ-butyrolactone in male F344 rats given 112 or 225 mg/kg or in female rats given 225 or 450 mg/kg in corn oil. There was equivocal evidence of carcinogenic activity γ-butyrolactone in male B6C3F₁ mice based on marginally increased incidences of adrenal medulla pheochromocytomas and hyperplasia in the low-dose group. The sensitivity of the study in male mice to detect a carcinogenic effect was reduced by the low survival of the high-dose group associated with fighting. There was no evidence of carcinogenic activity of γ-butyrolactone in female B6C3F₁ mice receiving 262 or 525 mg/kg in corn oil.*"

The NTP hypothesized that the incidence of benign and malignant pheochromocytomas in male mice would have been statistically significant ($P < 0.05$) at both dose levels if a sufficient number of animals in the high-dose group had survived long term. However, if a comparison is made between the control and treated animals that survived longer than the period of onset of neoplasms (i.e. > 582 days), there is no evidence to support the NTP hypothesis. All of the reported adrenocortical adenomas (three) and benign (five) and malignant (one) pheochromocytomas were identified in low-dose male mice surviving at least 582 days. The percentage of each group surviving at least 582 days of the study is given in Table 2. The incidence (4.6%) of pheochromocytomas observed in the control and high dose groups of male B6C3F₁ mice receiving a corn oil vehicle by gavage is within the historical range of 0–6% (NTP, 1992). Thus, taking into account the survival rates in groups of control and treated male mice, it is unlikely that the incidence of the tumours is related to the administered dose of the γ-butyrolactone.

No record is available for the onset of adrenal medulla hyperplasia. However, an estimate of the incidence of hyperplasia in animals surviving at least 582 days reveals that the occurrence of hyperplasia is increased in the low-dose (9/36) and high-dose (4/24) groups, but again there is no dose–response correlation (Table 2).

In evaluating the relevance of these results to human safety, the following factors should be considered: First, there is no difference in the incidence of pheochromocytomas in control and high-dose male mice surviving at least 582 days. The increased incidence of adrenal hyperplasia in treated male mice surviving 528 days was not accompanied by evidence of a significant dose-related increase in neoplasms of the adrenal medulla. In addition, there was no evidence of carcinogenicity in male and female F344 rats or B6C3F₁ female mice (NTP, 1992) and in a second carcinogenicity study in

Table 2. Summary of neoplastic and non-neoplastic effects in B6C3F₁ male mice[a]

Lesion	Dose groups:		
	0 mg/kg/day	262 mg/kg/day	525 mg/kg/day
Pheochromocytoma incidence/total number mice	2/48	6/50	1/50
(%)	(4.2%) $P = 0.472$	(12%) $P = 0.092$	(2.0%) $P = 0.592$
Pheochromocytoma incidence/total number mice surviving > 582 days (%)	2/43 (4.7%)	6/36 (17%)	1/24 (4.2%)
Adrenal medulla hyperplasia incidence/total number mice	2/48	9/50[a]	4/50
(%)	(4.2%) $P = 0.071$	(18%) $P = 0.011$	(8.0%) $P = 0.191$
Adrenal medulla hyperplasia incidence/total number mice surviving more than 582 days (%)	2/43 (4.7%)	9/36 (25%)	4/24 (17%)

[a]Historical incidence for 2-year NTP corn oil gavage studies with vehicle control groups (mean ± standard deviation): 18/582 (3.1% ± 1.8%), range 0%–6%.
[b]Significantly different ($P < 0.05$) from the control group by logistic regression.

which six rats were administered a 2-mg dose of γ-butyrolactone by subcutaneous injection twice weekly for 104 weeks (Dickens and Jones, 1965).

Secondly, there is also no evidence to suggest that humans are subject to the type of adrenal medullary proliferative disease that is observed in laboratory rodent assays (Roe and Baer, 1985). In man, adrenal medullary proliferative disease is extremely rare (Symington, 1969). Furthermore, there is no evidence of any relationship between hyperadrenalism and the incidence of pheochromo-cytomas in humans.

Thirdly, based on the results of more than 50 genotoxicity studies (see Section IV, B.5 below), there is compelling evidence that γ-butyrolactone is not genotoxic. Fourthly, the ring-opened form of γ-butyrolactone is endogenous in humans as a metabolite of γ-amino butyrate (Roth *et al.*, 1980). Therefore, the results of the NTP bioassay are not relevant to the safety evaluation of low levels of intake of γ-butyrolactone from use as a flavour ingredient in humans.

5. Genetic toxicology

a. γ-Butyrolactone. No effects were reported in any of 16 mutagenicity studies of 4-hydroxybuta-noic acid lactone using *Salmonella typhimurium* (Aeschbacher *et al.*, 1989; Baker and Bonin, 1981; Brooks and Dean, 1981; Garner *et al.*, 1981; Haworth *et al.*, 1983; Hubbard *et al.*, 1981; Ichinot-subo *et al.*, 1981; Katz *et al.*, 1981; Loquet *et al.*, 1981; MacDonald, 1981; Nagao and Takahashi, 1981; NTP, 1992; Rowland and Severn, 1981; Sim-mon and Sheperd, 1981; Trueman, 1981; Venitt and Crofton-Sleigh, 1981) and one using *Escherichia coli* (Venitt and Crofton-Sleigh, 1981). Concentrations of up to 10,000 μg/plate or up to 1.3 mmol (also 10 mg/ml) of the lactone were used in a variety of vehicles including DMSO, water, benzene and etha-nol. Variations on the standard Ames assays per-formed in the above studies include preincubation, gene mutation induction, and S-9 metabolic acti-vation. One study using up to 10,000 μg/plate in DMSO with and without S-9 activation did show positive results; however, the result was not sup-ported by a retest using newer strains of bacteria (Richold and Jones, 1981). A fluctuation test, also conducted with *S. typhimurium* with and without S-9 and rat hepatocytes, also showed no effects at doses up to 500 μg/ml in DMSO (Hubbard *et al.*, 1981).

Of 36 applicable genetoxicity studies (two were considered not applicable due to false positive rates in controls), 30 reported no effects. The latter include sex-linked recessive lethal mutation tests (Foureman *et al.*, 1994; NTP, 1992), sister chroma-tid exchange and chromosomal aberration assays in Chinese hamster ovary cells and *Saccharomyces cer-evisiae* (Kassinova *et al.*, 1981; NTP, 1992), unscheduled DNA synthesis *in vitro* using adult

human HeLa S3 cells with and without S-9 acti-vation (Martin and McDermid, 1981), and human FL cell NAD content (Yingnian *et al.*, 1990).

Positive effects in six studies occurred at the high-est dose levels or in non-standard assays for which interpretation is difficult. These results include: weak inhibition of *E. coli* DNA polymerase present only in a liquid suspension method with and with-out S-9 activation and in disc diffusion method without S-9 (Rosenkranz *et al.*, 1981); mitotic gene conversion induction in *S. cerevisiae* in which no effects were reported at 750 μg/ml in EtOH, but some effects were seen at 500 μg/ml in DMSO (Sharp and Parry, 1981); a positive result in the BHK-21 hamster kidney cell transformation assay with rat S-9 at 25–250 μg/ml, but with no effects at lower concentrations (Styles, 1981); a displacement of endoplasmic reticulum-polysomes in rat liver in DMSO at a concentration of 25 mg/litre (Fey *et al.*, 1981); lethal expression of gal genes under the con-trol of "lambda prophage repressors" in DMSO with and without S-9 in a non-standard strain of *E. coli.* (SA 500) at cytotoxic concentrations (Dambly *et al.*, 1981); and sperm mortality in mice following intraperitoneal injection of 1.0 ml/kg for 5 days, but no abnormal sperm at 0.1–1.0 ml/kg (Topham, 1981).

The FEMA Expert Panel reviewed genotoxicity data and concluded, on the basis of the weight of evidence, that γ-butyrolactone (4-hydroxybutanoic acid lactone) is not mutagenic and that isolated positive results performed in non-standard assays at high solution concentrations are not compelling evi-dence of genotoxic potential. The negative response in repeated *Salmonella* mutagenicity assays (Ashby and Tenant, 1991) supports the Panel's conclusion that exposure to 4-hydroxybutanoic acid lactone exhibits little potential for interaction with DNA.

b. Other aliphatic lactones (Nos 2–33). No muta-genic effects were reported in standard Ames tests using strains (TA98, TA100, TA102, TA1535 and TA1537) of *S. typhimurium* with δ-hexalactone (Kawachi *et al.*, 1981), γ-heptalactone (Heck *et al.* 1989), γ-nonalactone (Heck *et al.*, 1989), γ-undeca-lactone (Fujita and Sasaki, 1987; Ishidate *et al.*, 1984; Yoo, 1986), ω-pentadecalactone (Aeschbacher *et al.*, 1989), and 1,4-dodec-6-enolactone (Watanabe and Kinosaki, 1990). γ-Heptalactone did not induce an increase in unscheduled DNA synthesis when concentrations of 3000 μg/ml were incubated with rat hepatocytes (Heck *et al.* 1989).

Aliphatic lactones exhibit equivocal results in cytogenetic assays [sister chromatid exchange (SCE) and chromosomal aberration (ABS) assays] and in mutation assays using cultured mouse lymphoma cells. In a SCE assay, δ-hexalactone does not induce sister chromatid exchanges in hamster lung fibro-blast cells (Kawachi *et al.*, 1981). In an ABS assay, it induced an increase in aberrations in hamster lung fibroblast cells, but no induction was observed

in human embryo fibroblast cells or rat bone marrow cells (Kawachi *et al.*, 1981). γ-Undecalactone does not induce ABS when concentrations up to 500 mg/ml were incubated with hamster lung fibroblast cells (Ishidate *et al.*, 1984; Yoo, 1986). In the standard mouse lymphoma (MLA) assay, γ-nonalactone did not exhibit mutagenic activity with metabolic activation, but exhibited a positive mutagenic response at concentrations greater than 600 ml/ml without activation (Heck *et al.*, 1989).

The authors of the MLA study (Heck *et al.*, 1989) and others (Brusick, 1986) have emphasized that the positive results in the ABS and MLA assays may be artefacts resulting from changes in culture pH and osmolality. Treatment with high dose levels of substances with the potential to alter acidity or osmolality may induce a significant increase in mutant frequencies or aberrations in these assays. Often the results are inconsistent with the results of other genotoxicity assays (i.e. AMS and UDS) (Heck *et al.*, 1989).

The results of *rec*-assays with aliphatic lactones also are equivocal. δ-Hexalactone was negative in a *rec*-assay using *Bacillus subtilis* (Kawachi *et al.*, 1981). γ-Undecalactone (Kuroda, 1984; Yoo, 1986), δ-undecalactone (Kuroda *et al.*, 1984) and γ-nonalactone (Yoo, 1986) exhibited differences in growth inhibition zones between strains M45 (*rec⁻*) and H17 (*rec⁺*) of *B. subtilis* in the spore *rec*-assay. However, γ-undecalactone and δ-undecalactone failed to show any mutagenic activity using a similar test with the same bacterial strains (*B. subtilis* H17 and M45) (Oda *et al.*, 1978). 1,4-Dodec-6-enolactone was not mutagenic when inoculated with *E. coli* at concentrations of 500 μg/plate (Watanabe and Kinosaki, 1990). γ-Undecalactone exhibited antimutagenic activity in a *rec*-assay using *E. coli* WP uvrA (trp⁻) pretreated with AF-2 or MNNG (Yoo, 1986).

The only *in vivo* genotoxicity study with an aliphatic lactone other than γ-butyrolactone was a single intraperitoneal injection of 2000 mg γ-undecalactone/kg to male ddy mice. No increase in micronucleated polychromatic erythrocytes in B6C3F₁ mouse bone marrow cells was observed (Hayashi *et al.*, 1988).

Based on the results of the battery [i.e. AMS(−), UDS (−), SCE(−), ABS(+/−) and MLA (+/−)] of *in vitro* genotoxicity tests and the evidence that neither γ-butyrolactone nor γ-undecalactone were genotoxic *in vivo* in the mouse micronucleus assay or sex-linked lethal assay, it is concluded that the group of aliphatic lactones formed from straight- and branched-chain aliphatic hydroxycarboxylic acids is not genotoxic.

6. Lactones fused to alicyclic ring systems (Nos 36–39)

The alicyclic lactone formed from the enol form of 9-carboxymenthone dehydromenthofurolactone

(No. 38) has been added to the diet of rats at levels providing 1.0, 10.0 and 100.0 mg/kg daily for 13 weeks (Voss *et al.*, 1985) (see Table B6). Slight to mild hyperkeratosis and epithelial thickening of the oesophageal mucosa and squamous forestomach were reported in mid- and high-dose animals. Increased relative liver weights were reported in the mid- and high-dose groups. As absolute liver weights were unchanged, the increased relative organ weights were attributed to lower body weight gains associated with decreased palatability of the test diet. The 1.0 mg/kg no-effect level is greater than 1000 times the anticipated daily *per capita* intake ("eaters only") of 0.7 mg/kg from use of dehydromenthofurolactone as a flavour ingredient.

7. Lactones fused to aromatic ring systems (Nos 40–44)

Subchronic and chronic oral studies are available for 6-methylcoumarin and dihydrocoumarin, and a 90-day subchronic study has been performed with the phthalide derivative 3-propylidenephthalide (see Table B4). No adverse effects were reported when 3-propylidenephthalide was added to the basal diet of rats providing an average daily intake of 5.4 mg/kg for males and 6.5 mg/kg for females for 90 days (Posternak *et al.*, 1969). The 5.4 or 6.5 mg/kg/day dose level that produced no effects in rats is greater than 100,000 times the daily *per capita* intake ("eaters only") of 0.05 μg/kg from use of 3-propylidenephthalide as a flavour ingredient.

No adverse effects were reported when rats were maintained on diets containing 6-methylcoumarin (No. 42) at levels up to 10,000 ppm for 14 weeks (Hagan *et al.*, 1967). The dietary level of 10,000 ppm is calculated to provide an average daily intake of 500 mg/kg (FDA, 1993). For 2 years, rats were maintained on diets containing 6-methylcoumarin (Hagan *et al.*, 1967) at levels calculated to provide an average daily intake of up to 750 mg/kg (FDA, 1993). Depression in growth rates was noted in males provided 375 mg/kg/day. At 750 mg/kg/day, growth depression was severe in males and moderate in females. Hepatic effects at 750 mg/kg/day included fatty metamorphosis, slight bile duct proliferation and focal telangiectasis. No adverse effects were observed at intake levels up to 175 mg/kg/day.

6-Methylcoumarin was administered to dogs by capsule at a daily dose level of 150 mg/kg, 6 days per week for 39 days. Moderate to severe hepatitis and atrophy of skeletal muscle was reported (Hagan *et al.*, 1967). No adverse effects were observed when dogs were given 50 mg 6-methylcoumarin/kg by capsule, 6 days/week for 2 years (Hagan *et al.*, 1967).

The mechanism of hepatotoxicity of coumarin and related coumarin derivatives, including 6-methylcoumarin, has been studied in rats (Lake *et al.*, 1994b). For 13 weeks rats were fed diets containing 0.5 and 0.75% coumarin or 0.82% 6-

methylcoumarin (equimolar to the 0.75% dietary level of coumarin). Dose levels of 6-methylcoumarin are calculated (FDA, 1993) to provide an average daily intake of 410 mg/kg. Although relative liver weights and γ-glutamyl transferase were increased for all treated groups, only coumarin-treated animals exhibited increased levels of plasma aminotransferase. Histopathological examination of coumarin-treated animals revealed vacuolation of centrilobular hepatocytes, bile duct hyperplasia and cholangiofibrosis, particularly in rats fed the 0.75% diet. No increase in plasma aminotransaminase levels and no bile duct hyperplasia or cholangiofibrosis were observed in animals maintained on the 6-methylcoumarin diet, and only a slight vacuolation of hepatocytes were observed in a minority of animals. 6-Methylcoumarin treatment increased mixed function oxidase activity (i.e. 7-ethoxycoumarin *O*-deethylase activity), which supports other reports that 6-methylcoumarin can induce hepatic cytochrome *P*-450 activity in the rat (Feuer, 1974). 6-Methylcoumarin did not markedly effect the activities of carnitine acetyltransferase and palmitoyl-CoA oxidation, indicating that 6-methylcoumarin is unlikely to be a rodent liver peroxisome proliferator (Lake and Lewis, 1993; Lock *et al.*, 1989).

The 175 mg/kg/day intake level in the 2-year rat study and the 50 mg/kg/day dose level in the 2-year dog study that produced no adverse effects are at least 50,000 times the daily *per capita* intake ("eaters only") of 1 μg/kg from use of 6-methylcoumarin as a flavour ingredient.

No adverse effects were reported when rats were administered up to 10,000 ppm dihydrocoumarin (No. 40) in the diet for 14 weeks (Hagan *et al.*, 1967). This dietary level is calculated to provide an average daily intake of 500 mg/kg (FDA, 1993). No adverse effects were reported when dihydrocoumarin was added to the diet of rats at a level providing 110 mg/kg/day for 12 weeks (Trubeck Labs, 1957) and to dogs at levels of 50 and 150 mg/kg/day for 2 years (Hagan *et al.*, 1967).

Groups of male and female rats were administered dihydrocoumarin in corn oil by gavage 5 days per week for 13 weeks providing dose levels of 75, 150, 300, 600 or 1200 mg/kg/day (NTP, 1993). No adverse effects were observed at the 75 and 150 mg/kg/day levels. Increased absolute and relative liver and kidney weights were reported in both sexes at the 600 mg/kg/day and 1200 mg/kg/day dose levels. Hepatocellular hypertrophy was reported at the three highest dose levels. Male and female rats receiving 600 mg/kg/day for up to 2 years exhibited elevated alkaline phosphatase and alanine aminotransferase activity. Rats receiving 300 mg/kg/day or greater for 13 weeks, and 300 or 600 mg/kg/day for periods up to 2 years exhibited a mild anticoagulant effect. A companion gavage study in mice given 100, 200, 400, 800 and 1600 mg/kg/day resulted in increased absolute liver weights in both

sexes and increased relative kidney weights in males only at the 1600 mg/kg/day level. Dihydrocoumarin administration to mice at dose levels less than 800 mg/kg/day for up to 2 years produced no change in hepatic enzyme activities and resulted in no treatment-related lesions.

A recent subchronic study in rats demonstrates the different hepatotoxic potential of coumarin and dihydrocoumarin (Lake *et al.*, 1994). For 13 weeks rats were fed diets containing 0.5 and 0.75% coumarin or 0.76% dihydrocoumarin (equimolar to the 0.75% dietary level of coumarin). Although relative liver weights were increased for all treated groups, histopathological examination of dihydrocoumarin-treated animals revealed no abnormalities. Coumarin-treated animals exhibited increased plasma amino acid transferase levels which were accompanied by vacuolation of centrilobular hepatocytes, bile duct hyperplasia and cholangiofibrosis, particularly in rats fed the 0.75% diet. Dihydrocoumarin did not markedly affect the activities of carnitine acetyltransferase and palmitoyl-CoA oxidation, indicating that dihydrocoumarin is unlikely to be a rodent liver peroxisome proliferator (Lake and Lewis, 1993; Lock *et al.*, 1989). The dietary level of 0.76% dihydrocoumarin that produced no significant effects is calculated (FDA, 1993) to provide an average daily intake of 380 mg/kg. The dose levels of 150 mg/kg/day by gavage and 380 mg/kg/day in the diet that produced no adverse effects are greater than 10,000 times the daily *per capita* intake ("eaters only") of 12 mg/kg* from use of dihydrocoumarin as a flavour ingredient.

8. Results of the 2-year NTP bioassay with 3,4-dihydrocoumarin

a. Effects on growth, survival, clinical findings, haematology and clinical chemistry. A chronic study of dihydrocoumarin was conducted by the National Toxicology Program (NTP, 1993a). The standard NTP protocol was used with F344/N rats and B6C3F₁ mice of both sexes. Rats and mice received dihydrocoumarin in corn oil by gavage 5 days per week for 103 weeks, providing dose levels of 0, 150, 300 or 600 mg/kg to rats and 0, 200, 400 or 800 mg/kg to mice. In a dose-related manner, decreased survival rates were reported only in male rats at all dose levels with the majority of deaths occurring after week 92. The decreased survival was attributed to a progressive degenerative nephropathy leading to renal failure. Typically, the severity of nephropathy increased with dose level. The mean body weight gain of high-dose males was significantly lower (5% to 10%) than that of controls.

In a related stop-exposure evaluation, a group of 40 male rats received 600 mg/kg body weight dihydrocoumarin by gavage for 9 months. 20 rats were sacrificed after 9 months and the remaining rats (20), which were designated the 9-month stop-exposure group, received only the corn oil vehicle

until death or the end of the study. A second group of male rats (30) received 600 mg/kg body weight dihydrocoumarin by gavage. 10 of these rats were sacrificed at 15 months and the remaining rats (20), which were designated the 15-month stop-exposure group, received only the corn oil vehicle until death or the end of the study. Groups of 20 and 10 control rats receiving only the corn oil vehicle by gavage were sacrificed at 9 and 15 months, respectively. To determine the progression or regression of chemically-related lesions during the recovery period, neoplastic and non-neoplastic lesions in the 9- and 15-month stop-exposure groups were compared with those of the groups sacrificed at 9 and 15 months, respectively.

The incidence of nephropathy in the stop-exposure groups was not statistically different from that of the control group or groups sacrificed at 9 or 15 months, but the nephropathy was more severe. The observation that the process is irreversible and that nephropathy appears in all groups of control and treated male rats supports the conclusion that it is a progressive degenerative disease associated with the ageing process, especially in male F344/N rats. This background level of kidney disease may make the male F344/N rat particularly susceptible to any chemically-induced nephrotoxicity. The sex- and species-specific nature of the nephrotoxic effect was confirmed by the observations that female rats and mice of both sexes exhibited no significant change in survival rates, mean body weight changes, and no clinical findings of renal toxicity related to the administration of dihydrocoumarin.

Haematology and clinical chemistry performed at the 15-month interim evaluation revealed statistically significant increased serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), sorbitol dehydrogenase or γ-glutamyl transferase (GGT) in male rats at 300 and 600 mg/kg. Increased ALP and GGT were reported for female rats at 600 mg/kg. Only the ALT level (approx. four times greater than control values) in male rats at 600 mg/kg may be considered to be biologically significant for this species. Although significant changes in the activity of this enzyme are normally associated with altered liver function, the increase in ALT was not accompanied by evidence of histopathology. There was no significant difference in haematology or clinical chemistry parameters for mice at any dose level. Administration of 3,4-dihydrocoumarin in the 2-year chronic study did not produce coumarin like-induced hepatotoxicity in either rats or mice.

b. Pathological findings

(1) Renal neoplasms in male rats: Nephropathy was manifest in both sexes of control and dosed rats, but was more severe in animals that received the test compound. The incidence of nephropathy

was greater in male rats than female rats, indicating a greater susceptibility of males to chronic nephropathy.

Microscopic examination of combined single and step sections of the kidneys of male rats revealed a statistically significant, dose-related increase in renal tubule hyperplasia (control, 1/50; 150 mg/kg, 5/48; 300 mg/kg, 6/47; 600 mg/kg, 8/50). The incidence of renal tubule adenomas in the treated groups of male rats was not significantly different ($P < 0.05$) than the control group (control,1/50; 150 mg/kg, 1/48; 300 mg/kg, 3/47; 600 mg/kg, 6/50), but the total incidence of renal tubule adenomas in all groups of treated male rats (10/145) and in the high-dose group (6/50) exceeds the range in NTP historical controls (8/1019) (Table 3). Renal transitional cell carcinomas were reported in two male rats succumbing early in the study (i.e. days 444 and 528) at the 600 mg/kg dose level. There was no evidence of transitional cell carcinomas in the majority (86%) of males surviving longer than 528 days. The incidence of renal tubule hyperplasia and renal tubule adenomas in stop-exposure groups and 2-year treatment group [600 mg/kg dose level: 9 months, 1/2 (hyperplasia/adenomas); 15 months,4/2; 24 months, 8/6] increased with the duration of treatment. However, analysis of step sections revealed no additional evidence of treatment-related response of the renal transitional cells. There was no increased incidence of malignant renal tubule neoplasms in any group of treated male or female rats.

(2) Renal neoplasms in female rats and male and female mice: The incidence of renal nephropathy in control and treated female rats was significantly less than in males. No significant differences in the incidence of renal tubule hyperplasia or renal tubule adenoma was observed between control and treated groups of female rats. The incidence of renal focal hyperplasia and adenomas in treated and control groups of male mice were not significantly different, and the incidence of renal tubule neoplasms was low and not dose related (controls 0/50; 200 mg/kg, 1/51; 400 mg/kg, 2/51; 800 mg/kg, 1/49) (Table 3). There was no incidence of renal tubule hyperplasia or renal tubule neoplasms in female mice.

The NTP report concluded the following: "*Under the conditions of these 2-year gavage studies, there was some evidence of carcinogenicity of 3,4-dihydrocoumarin in male F344 rats based on the increased incidence of renal tubule adenomas and focal hyperplasia. The transitional cell carcinomas in two 600 mg/kg males may also have been chemical related. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in female F344 rats receiving 150, 300 or 600 mg/kg. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in male B6C3F1 mice receiving 200, 400 or 800 mg/kg.*"

Chronic renal nephropathy and an increased incidence of focal hyperplasia and renal tubule neo-

Table 3. Incidences of renal neoplasms associated with administration of dihydrocoumarin to rats and mice by gavage for 2 years

		Control	150 mg/kg	300 mg/kg	600 mg/kg
1.	Male rats				
	Nephropathy	50/50	47/48	47/47	47/50
	Renal tubule hyperplasia[a]	0/50	5/48*	6/47*	8/50**
	Renal tubule adenoma[a]	1/50	1/48	3/47	6/50*
2.	Female rats				
	Nephropathy	20/50	20/49	37/49**	31/49**
	Renal tubule hyperplasia[a]	0/50	0/49	0/49	2/49
	Renal tubule adenoma[a]	1/50	1/49	1/49	0/49

		Control	200 mg/kg	400 mg/kg	800 mg/kg
3.	Male mice				
	Nephropathy	45/50	46/51	45/51	43/49
	Renal tubule hyperplasia[a]	0/50	1/51	3/51	1/49
	Renal tubule adenoma[a]	0/50	0/51	2/51	1/49
	Renal tubule adenoma or carcinoma[a]	0/50	1/51	2/51	2/49

*Significantly different ($P < 0.05$) from control group by Fisher's exact test (15 month interim), logistics regression test (2-year study) or Mann–Whitney U test.
**Significantly different ($P < 0.01$) from the control group.
[a]Combined single and step sections.

plasms were reported when dihydrocoumarin was administered to male rats by corn oil gavage for 2 years. Although treated female rats also exhibited increased nephropathy at the two highest dose levels, they did not experience a significant increase in either renal tubule hyperplasia or renal tubule neoplasms. The incidence of renal tubule hyperplasia and neoplasms in male mice were not statistically significant and not dose related. Female mice did not exhibit any renal neoplasms. Additionally, no renal histopathology was observed in dietary studies providing similar dose levels of dihydrocoumarin to rats or dogs for periods up to 2 years (Hagan *et al.*, 1967; Trubeck Labs, 1957). Therefore, the renal hyperplastic and neoplastic effects are sex and species specific and occur only at high chronic levels of exposure.

The metabolic fate of dihydrocoumarin at high dose levels may confound the interpretation of the renal effects in male rats. If dihydrocoumarin metabolizes to high endogenous levels of *o*-hydroxybenzoic acid (i.e. salicyclic acid), the metabolite may produce a unique renal toxicity observed with chronic exposure to analgesics. The renal toxicity is characterized by renal medullary and papillary necrosis (Tarloff and Goldstein, 1995). The non-neoplasic lesions of the kidney observed for chronic intake of high dose levels of dihydrocoumarin and analgesics such as acetaminophen (NTP, 1993b) in 2-year bioassays show marked similarities.

The male rat kidney has been shown to be a unique target organ for the carcinogenic effects of a variety of chemical substances (Burdock *et al.*, 1990; EPA, 1991). The FEMA Expert Panel concludes that the renal effects of dihydrocoumarin in the male rat are, indeed, a species- and sex specific phenomena, and in all probability, reflect the sensitivity of the male rat kidney to chronic progressive nephropathy, focal hyperplasia, and specific tumorigenic responses. The observed histopathology suggests that the hyperplastic and neoplastic effects

are not associated with the accumulation of $\alpha_{2\mu}$-globulin in proximal convoluted renal tubules. The relationship of the 600 mg/kg dose level of dihydrocoumarin to the report of transitional cell carcinomas in two male rats is unclear. It was reported that longer-lived males showed no evidence of renal transitional cell changes at any dose level.

(3) Forestomach effects in rats: A significant increase in the incidence of forestomach ulcers in male rats was reported at the end of the study, but not at the 15-month interim evaluation. An increased incidence of ulcers, squamous hyperplasia and chronic inflammation occurred at all dose levels, but the effects were not related to dose. Female rats exhibited no significant forestomach effects. Two neoplasms (a squamous cell papilloma and carcinoma) reported at the 600 mg/kg dose level were not attributed to chemical administration.

(4) Hepatocellular neoplasms in mice: Neoplastic and non-neoplastic lesions associated with administration of dihydrocoumarin to mice developed principally in the liver (Table 4). There was a significant increase in the incidence of hepatocellular adenomas (control, 10/51; 200 mg/kg, 20/50; 400 mg/kg, 22/50; 800 mg/kg, 20/52) and combined hepatocellular adenomas and carcinomas (control, 13/51; 200 mg/kg, 21/50; 400 mg/kg, 25/50; 800 mg/kg, 24/52) in female mice compared with that of the control group, but the incidence of neoplasms was not dose related (Table 4). The amount of time required for onset of neoplasms in the high-dose group was shorter than that for lower-dose groups. No increased incidence of malignant neoplasms of the liver was observed in treated female mice.

The incidence of hepatocellular neoplasms in treated and control male mice was greater than in treated and control female mice, indicating the increased susceptibility of the male mouse liver to neoplastic changes. However, the incidence of hepa-

Table 4. Incidences of hepatocellular neoplasms associated with administrationof dihydrocoumarin to mice by gavage for 2 years

		Control	200 mg/kg	400 mg/kg	800 mg/kg
1.	Male mice				
	Hepatocellular adenoma	29/50	23/51	36/51	31/50
	Hepatocellular carcinoma	11/50	11/51	11/51	6/50
	Hepatoblastoma	0/50	0/51	0/51	2/50
	Combined rates[a]	36/50 (72%)	30/51 (59%)	40/51 (78%)	34/50 (68%)
2.	Female mice				
	Hepatocellular adenoma	10/51	20/50	22/50	20/52
	Hepatocellular carcinoma	3/51	2/50	4/50	6/52
	Combined rates[b]	13/51 (25%)	21/50 (42%)	25/50 (50%)	24/52 (46%)

[a]Historical incidence for 2-year corn oil gavage studies with vehicle control groups (mean ± SD): 370/901 (41.1 ± 15.5%); range 14% to 72%.
[b]Historical incidence: 129/898 (14.4% ± 8.1%); range 2% to 34%.

tocellular adenomas (control, 29/50; 200 mg/kg, 23/51; 400 mg/kg, 36/51; 800 mg/kg, 31/50) and carcinomas (control, 11/50; 200 mg/kg, 11/51; 400 mg/kg, 11/51; 800 mg/kg, 6/50) in treated male mice was not significantly different from that of the control group. There was no evidence of an increased incidence of malignant neoplasms of the liver in either male or female mice.

The NTP report concluded the following: "*There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in male B6C3F₁ mice receiving 200, 400, or 800 mg/kg. There was some evidence of carcinogenic activity in female B6C3F₁ mice based on increased incidences of hepatocellular adenoma, and hepatocellular adenoma and carcinoma (combined).*"

The primary neoplastic effects observed in mice in the NTP study were associated with the liver. The high incidence of hepatocellular adenomas and carcinomas in both control and treated groups of male and female mice demonstrate the sensitivity of the B6C3F₁ mouse liver to neoplastic responses. The high incidence of hepatocellular adenoma was not significantly different between treated and control groups of male mice (control, 29/50; 200 mg/kg, 23/51; 400 mg/kg, 36/51; 800 mg/kg, 31/50). The observation that the incidence of adenomas in control males was greater than in any group of treated females demonstrates the increased sensitivity of the male mouse liver. The incidence of hepatocellular adenomas in all groups of treated female mice were significantly greater than the control group, but the response was not dose related (control, 10/51; 200 mg/kg, 20/50; 400 mg/kg, 22/50; 800 mg/kg, 20/52). The incidence of hepatocellular carcinomas was not significantly different between treated and control groups of male mice (control, 11/50; 200 mg/kg, 11/51; 400 mg/kg, 11/51; 800 mg/kg, 6/50), but again the incidence of carcinomas in control males was greater than in any group of treated females. The lower incidence of hepatocellular carcinomas in groups of treated female mice was not significantly different from that of the control group (3/51, 2/50, 4/50, 6/52). These neoplastic responses are consistent with the historically high levels of background hepatocellular neoplasms in B6C3F₁ mice (Maronpot and Boorman,1982; Maronpot et al., 1986).

The FEMA Expert Panel considers that the observations of hepatic neoplasms in the NTP mouse bioassay are not relevant to the safety of dihydrocoumarin in humans at low levels of intake from use as a flavour ingredient. This conclusion is based on the high incidence of spontaneous hepatocellular neoplasms (adenomas and carcinomas) in the strain of mice studied, the absence of consistent dose–response data, the lack of hepatocellular neoplastic effects in rats, and the relatively high dose levels administered as compared with intake levels from use as a flavour ingredient. Additionally, based on the metabolism of structurally related substances, humans may metabolize low intake levels of dihydrocoumarin via a different principal pathway (i.e. β-oxidation and cleavage) than the pathway (i.e. β-hydroxylation) used by rodents (Samuelson et al., 1986) at high intake levels.

9. Genetic toxicology of 3,4-dihydrocoumarin

Dihydrocoumarin was not mutagenic for S. typhimurium strains (TA98, TA100, TA1535 and TA1537) with or without S-9 metabolic activation (Haworth et al., 1983; Heck et al., 1989; NTP, 1993; Prival et al., 1982). Dihydrocoumarin concentrations of 1400 mg/ml were reported to be mutagenic for mouse lymphoma L5178TK + /− only in the presence of S-9 activation (Heck et al., 1989).

In a cytogenetic test, effective dose levels of 50 to 300 mg dihydrocoumarin/ml induced a dose-related increase in sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells in the absence of S-9. In the presence of S-9, an increase in SCE was observed only at the highest concentrations (1600 and 2000 mg/ml) in each of two tests. Dihydrocoumarin did not induce chromosomal aberrations in CHO at concentrations up to 500 mg/ml without S-9 and up to 1600 mg/ml with S-9 (NTP, 1993a).

No increase in the frequency of micronucleated erythrocytes was reported in peripheral blood samples obtained from male and female mice after 13 weeks at dose levels up to 800 mg/kg (NTP, 1993a).

The FEMA Expert Panel reviewed genotoxicity data and concluded, on the basis of the weight of evidence, that dihydrocoumarin was not mutagenic

and that the isolated positive results from an SCE-CHO assay performed at high solution concentrations of dihydrocoumarin were not compelling evidence of a genotoxic potential. The negative response in the *Salmonella* mutagenicity assay (Ashby and Tenant, 1991) supports the Panel's conclusion that exposure to dihydrocoumarin exhibits little potential for interaction with DNA.

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Appendix A

Fig. A1. Aliphatic lactones formed from aliphatic acyclic linear saturated hydroxycarboxylic acids (Nos 1–19).

1. 4-Hydroxybutanoic acid lactone
   (FEMA No. 3291, γ-Butyrolactone)
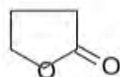
2. γ-Valerolactone
   (FEMA No.3103)
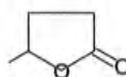
3. γ-Hexalactone
   (FEMA No. 2556)
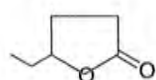
4. δ-Hexalactone
   (FEMA No. 3167)
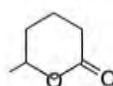
5. γ-Heptalactone
   (FEMA No. 2539)
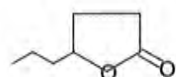
6. γ-Octalactone
   (FEMA No. 2796)
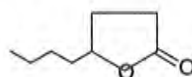
7. δ-Octalactone
   (FEMA No. 3214)
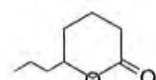
8. γ-Nonalactone
   (FEMA No. 2781)
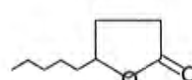
9. Hydroxynonanoic acid δ-lactone
   (FEMA No. 3356, δ-Nonalactone)
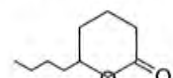
10. γ-Decalactone
    (FEMA No. 2360)
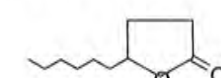
Fig. A1—*Continued overleaf*

Fig. A1—*contd*

11. δ-Decalactone
    (FEMA No. 2361)
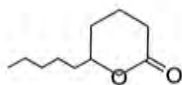
12. ε-Decalactone
    (FEMA No. 3613)
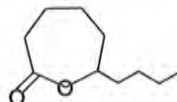
13. γ-Undecalactone
    (FEMA No. 3091)
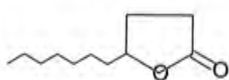
14. 5-Hydroxyundecanoic acid lactone
    (FEMA No. 3294, δ-Undecalactone)
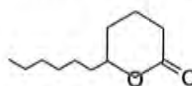
15. γ–Dodecalactone
    (FEMA No. 2400)
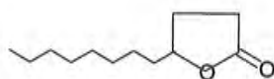
16. δ-Dodecalactone
    (FEMA No. 2401)
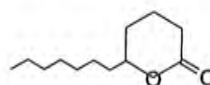
17. ε-Dodecalactone
    (FEMA No. 3610)
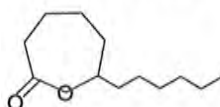
18. δ-Tetradecalactone
    (FEMA No. 3590)
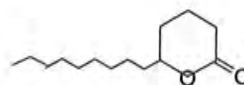
19. ω-Pentadecalactone
    (FEMA 2840)
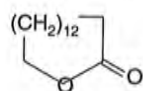
Fig. A2. Lactones formed from aliphatic acyclic linear unsaturated hydroxycarboxylic acids (Nos 20–28).
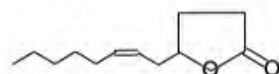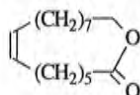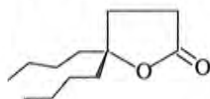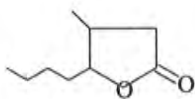
20. 4-Hydroxy-3-pentenoic acid lactone
    (FEMA No. 3293)
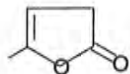
21. 5-Hydroxy-2-decenoic acid δ-lactone
    (FEMA No. 3744)
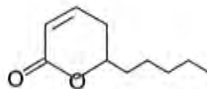
22. 5-Hydroxy-7-decenoic acid δ-lactone
    (FEMA No. 3745)
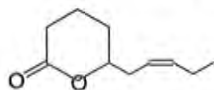
23. 5-Hydroxy-2,4-decadienoic acid δ-lactone
    (FEMA No. 3696)
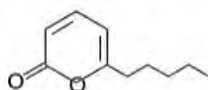
24. Massoia bark oil
    (FEMA No. 3747)

25. 5-Hydroxy-8- undecenoic acid δ-lactone
    (FEMA No. 3758)
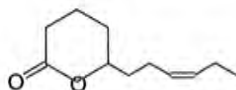
26. 5-Hydroxy-2-dodecenoic acid lactone
    (FEMA No. 3802)
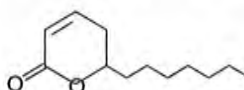
27. (Z)-4-Hydroxy-6-dodecenoic acid lactone
    (FEMA No. 3780)

28. ω-6-Hexadecenlactone
    (FEMA No. 2555)

Fig. A3. Lactones formed from aliphatic acyclic branched-chain hydroxycarboxylic acids (Nos 29–35).
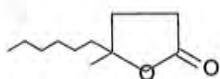
29. 4,4-Dibutyl- γ-butyrolactone
    (FEMA No. 2372)

30. 3-Heptyl-5-methyl-2(3H)-furanone
    (FEMA No. 3350)
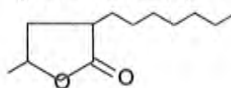
31. 4-Hydroxy-3-methyloctanoic acid lactone
lactone
    (FEMA No. 3803)

32. 6-Hydroxy-3,7- dimethyloctanoic acid
    (FEMA No. 3355)

33. γ-Methyldecalactone
    (FEMA No. 3786)
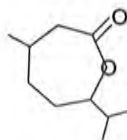
34. 5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone
    (FEMA No. 3153)

35. 4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one
    (FEMA No. 3634)
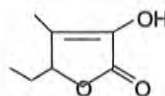
Fig. A4. Aliphatic lactones formed from alicyclic and aromatic hydroxycarboxylic acids (Nos 36–44).

36. Octahydrocoumarin
   (FEMA No. 3791)
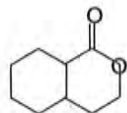
37. Mintlactone
   (FEMA No. 3764)
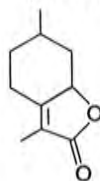
38. Dehydromenthofurolactone
   (FEMA No. 3755)
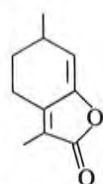
39. Sclareolide
   (FEMA No. 3794)
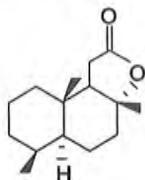
40. Dihydrocoumarin
   (FEMA No. 2381)
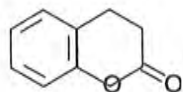
41. 6-Methylcoumarin
   (FEMA No. 2699)

42. 3-Propylidenephthalide
   (FEMA No. 2952)
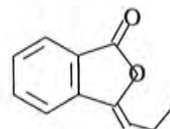
43. 3-Butylidenephthalide
   (FEMA No. 3333)
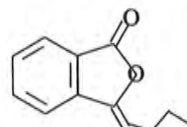
44. 3-n-Butylphthalide
   (FEMA No. 3334)
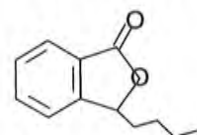
Appendix B

Table B1. Summary of exposure data for lactones formed from aliphatic acyclic hydroxycarboxylic acids

Lactones formed from aliphatic acyclic saturated and unsaturated hydroxycarboxylic acids

	Substance	FEMA No.	Annual volume 1982 (kg)	Annual volume 1987 (kg)	Most recent per capita intake[1] (μg/kg bw/day)	Natural occurrence in food[2]	Consumption ratio[3]
1.	4-Hydroxybutanoic acid lactone (γ-butyrolactone)	3291	15	508	2	+++	>50
2.	γ-Valerolactone	3103	80	300	1	+++	>100
3.	γ-Hexalactone	2556	110	100	0.3	+++	>5
4.	δ-Hexalactone	3167	43	13	0.04	++	>50
5.	γ-Heptalactone	2539	300	220	0.7	+++	>10
6.	γ-Octalactone	2796	771	476	1.5	+++	>5
7.	δ-Octalactone	3214	50	90	0.3	+++	>10
8.	γ-Nonalactone	2781	3120	2460	8	+++	>1
9.	Hydroxynonanoic acid, δ-lactone (δ-nonalactone)	3356	24	60	0.2	+++	0.3
10.	γ-Decalactone	2360	649	1910	6	+++	>1
11.	δ-Decalactone	2361	4810	9840	31	+++	0.6
12.	ε-Decalactone	3613	9	<0.01	0.03	NR	NA
13.	γ-Undecalactone	3091	3090	2880	9	+++	0.02
14.	5-Hydroxyundecanoic acid lactone (δ-undecalactone)	3294	617	925	3	++	0.2
15.	γ-Dodecalactone	2400	390	590	2	+++	>1
16.	δ-Dodecalactone	2401	5760	5990	19	+++	>1
17.	ε-Dodecalactone	3610	0.5	0.9	0.003	NR	NA
18.	δ-Tetradecalactone	3590	0.9	13	0.04	++	>500
19.	ω-Pentadecalactone	2840	34	270	0.9	+	NR
20.	4-Hydroxy-3-pentenoic acid lactone	3293	6	25	0.08	+	NR
21.	5-Hydroxy-2-decenoic acid δ-lactone	3744	NR	0.5	0.002	+	NR
22.	5-Hydroxy-7-decenoic acid δ-lactone	3745	NR	0.5	0.002	+	>100
23.	5-Hydroxy-2,4-decadienoic acid δ-lactone	3696	NR	0.5	0.002	+	NR
24.	Massoia bark oil	3747	NR	11	0.03	+	NR
25.	5-Hydroxy-8-undecenoic acid δ-lactone	3758	NR	45	0.14	NR	NA
26.	5-Hydroxy-2-dodecenoic acid δ-lactone	3802	NR	450	1.4	+	NR
27.	(Z)-4-Hydroxy-6-dodecenoic acid lactone	3780	NR	45	0.14	+	NR
28.	ω-6-Hexadecenlactone	2555	46	0.5	0.002	+	NR
29.	4,4-Dibutyl-γ-butyrolactone	2372	0.8	0.5	0.002	NR	NA
30.	3-Heptyldihydro-5-methyl-2(3H)-furanone	3350	2	0.5	0.002	NR	NA
31.	4-Hydroxy-3-methyloctanoic acid γ-lactone	3803	NR	45	0.14	++	NA
32.	6-Hydroxy-3,7-dimethyloctanoic acid lactone	3355	0.2	<0.01	0.0006	NR	NA
33.	γ-Methyldecalactone	3786	NR	225	0.7	+	NR
34.	5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	3153	168	32	0.10	NR	NA
35.	4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one	3634	0.2	0.5	0.002	+	NR

NR = not reported   NA = not applicable

[1]Intake (μg/kg body weight/day) calculated as follows: [[(annual volume, kg) × (1 × 10^9 μg/kg)]/[population × 0.6 × 60 kg body weight × 365 days]]. where population (10%, "eaters only") = 24 × 10^6, 0.6 represents the assumption that only 60% of the flavour volume was reported in the survey (NAS, 1987).

[2]Substances occurring in more than 10, six to 10, and one to five foods are given the respective designations, + + +, + +, and + as reported in CIVO, 1994.

[3]Consumption ratios were calculated using the method by Stofberg and Kirschman, 1985 and data by Stofberg and Grundschober, 1987.

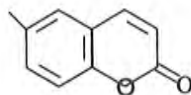Table B2. Summary of exposure data for lactones formed from alicyclic and aromatic hydroxycarboxylic acids

	Substance	FEMA No.	Annual volume 1982 (kg)	Annual volume 1987 (kg)	Most recent per capita intake[1] (μg/kg bw/day)	Natural occurrence in food[2]	Consumption ratio[3]
36.	Octahydrocoumarin	3791	NR	910	3	NR	NR
37.	Mintlactone	3764	NR	225	0.7	+	NR
38.	Dehydromenthofurolactone	3755	NR	225	0.7	+	NR
39.	Sclareolide	3794	NR	225	0.7	+	NR
40.	Dihydrocoumarin	2381	4510	3750	12	NR	NR
41.	6-Methylcoumarin	2699	1770	380	1	NR	NR
42.	3-Propylidenephthalide	2952	38	15	0.05	NR	NR
43.	3-Butylidenephthalide	3333	5	3	<0.01	+	NR
44.	3-n-Butylphthalide	3334	3	<0.01	<0.01	+	>500

NR = not reported

[1]Intake (μg/kg body weight/day) calculated as follows: [[(annual volume, kg) × (1 × 10^9 μg/kg)]/[population × 0.6 × 60 kg body weight × 365 days]], where population (10%, "eaters only") = 24 × 10^6; 0.6 represents the assumption that only 60% of the flavour volume was reported in the survey (NAS, 1987).
[2]Substances occurring in more than 10, six to 10, and one to five foods are given the respective designations, + + +, + +, + + and + as reported in CIVO, 1994.
[3]Consumption ratios were calculated using the method by Stofberg and Kirschman, 1985 and data by Stofberg and Grundschober, 1987.

Table B3. Summary of metabolism and toxicity data for lactones formed from aliphatic acyclic hydroxycarboxylic acids

	Substance	FEMA No.	Metabolic data[i] *in vitro/in vivo*	Oral LD$_{50}$, mg/kg bw	Repeated dose study[2] NOAEL, mg/kg bw/day (duration)	Repeated dose study for a structurally related substances NOAEL, mg/kg bw/day (duration)
1.	4-Hydroxybutanoic acid lactone (γ-butyrolactone)	3291	+[3]/−	1245[4], 1260[5], 500–700[6]	225 (male rats, 2 yr)[7], 450 (female rats, 2 yr)[7], 525 (female mice, 2 yr)[7], 112 (rats, 13 wk)[7], 131 (mice, 13 wks)[7]	NA
2.	γ-Valerolactone	3103	+[8]/+[9,10]	>5000[11], 2600[12], 9240[12]	49.0 (males) (90 d)[13], 51.1 (females) (90 d)[13], 500 (14 wks)[14]	NA
3.	γ-Hexalactone	2556	+[7]/−	>5000[15]	NR	62.8 (90 d)[16], 72.5 (90 d)[16]
4.	δ-Hexalactone	3167	−/−	13,100[17]	NR	150 (49 wk)[18], 75 (38 wk)[18]
5.	γ-Heptalactone	2539	−/−	NR	NR	250 (1.5–2 yr)[19], 72.5 (90 d)[16]
6.	γ-Octalactone	2796	−/−	>5000[20]	NR	250 (1.5–2 yr)[19], 72.5 (90 d)[16]
7.	δ-Octalactone	3214	−/−	>5000[21]	NR	150 (49 wk)[18], 75 (38 wk)[18]
8.	γ-Nonalactone	2781	−/+[10]	9780[22], 3440[23], 6600[23]	62.8 (90 d)[16], 72.5 (90 d)[16], 250 (1.5–2 yr)[19]	NA
9.	Hydroxynonanoic acid, δ-lactone (δ-nonalactone)	3356	−/−	NR	NR	150 (49 wk)[18], 75 (38 wk)[18]
10.	γ-Decalactone	2360	−/+[10]	>5000[24]	NR	62.8 (90 d)[16], 72.5 (38 wk)[16]
11.	δ-Decalactone	2361	−/−	>5000[25]	150 (49 wk)[18], 75 (38 wk)[18]	NA
12.	ε-Decalactone	3613	−/−	5252	NR	150 (49 wk)[18], 75 (38 wk)[18]
13.	γ-Undecalactone	3091	+[9,10]/−	18,500[22]	16.5 (90 d)[16], 166 (130 d)[26], 250 (1.5–2 yr)[19]	—
14.	5-Hydroxyundecanoic acid	3294	−/−	>5000[24]	NR	150 (49 wk)[18], 75 (38 wk)[18], 250 (1.5–2 yr)[19]
15.	γ-Dodecalactone	2400	−/+[18,27]	>5000[20]	NR	14.6 (90 d)[16], 6.7 (90 d)[16], 166 (130 d)[26], 250 (1.5–2 yr)[19]
16.	δ-Dodecalactone	2401	−/−	>5000[15]	300 (49 wk)[xviii], 150 (38 wk)[18]	—
17.	ε-Dodecalactone	3610	−/−	7898[30]	NR	300 (49 wk)[18], 150 (38 wk)[18]

No.	Name					
18.	δ-Tetradecalactone	3590	-/-	NR	NR	300 (49 wk)[18]
19.	ω-Pentadecalactone	2840	-/-	>5000[28]	NR	300 (49 wk)[18] 150 (38 wk)[18]
20.	4-Hydroxy-3-pentenoic acid lactone	3293	-/-	2820[29] 2800[30]	17.4 (90 d)[21]	46 (13 wk/1 yr)[32,33,34] 80 (30 d)[35]
21.	5-Hydroxy-2-decenoic acid δ-lactone	3744	-/-	3300[36] 3730[36]	NR	12.10 (90 d)[37]
22.	5-Hydroxy-7-decenoic acid δ-lactone	3745	-/-	>5000[37]	NR	12.10 (90 d)[36]
23.	5-Hydroxy-2,4-decadienoic acid δ-lactone	3696	-/-	1600[26]	12.10 (90 d)[37]	—
24.	Massoia bark oil	3747	-/-	5000[26] 3831[39]	NR	12.10 (90 d)[37]
25.	5-Hydroxy-8-undecenoic acid δ-lactone	3758	-/-	>5000[40]	NR	12.10 (90 d)[37]
26.	5-Hydroxy-2-dodecenoic acid δ-lactone	3802	-/-	>2500[41]	12 (14 d)[42]	12.10 (90 d)[37]
27.	(Z)-4-Hydroxy-6-dodecenoic acid lactone	3780	-/-	>5000[43]	NR	17.4 (90 d)[31] 46 (13 wk/1 yr)[32,33,34]
28.	ω-6-Hexadecenlactone	2555	-/-	>5000[44]	NR	46 (13 wk/1 yr)[32,33,34]
29.	4,4-Dibutyl-γ-butyrolactone	2372	-/+[9]	NR	NR	250 (1.5-2 yr)[19] 72.5 (90 d)[16]
30.	3-Heptyldihydro-5-methyl-2(3H)-furanone	3350	-/-	NR	NR	14.6 (90 d)[16] 16.5 (90 d)[16] 166 (130 d)[26] 250 (1.5-2 yr)[19]
31.	5-Hydroxy-3-methylocatanoic acid γ-lactone	3803	-/+[45]	NR	NR	250 (1.5-2 yr)[19]
32.	6-Hydroxy-3.7-dimethyloctanoic acid lactone	3355	-/-	>5000[46] 3550[47]	NR	300 (49 wk)[18] 150 (38 wk)[18]
33.	γ-Methyldecalactone	3786	-/-	>5000[48]	NR	14.6 (90 d)[16] 16.5 (90 d)[16] 166 (130 d)[26] 250 (1.5-2 yr)[19]
34.	5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	3153	-/-	NR	1.29 (males) (90 d)[49] 1.47 (females) (90 d)[49]	NR
35.	4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one	3643	-/-	2360[50]	46 (13 wk)[32]/1 yr[32]	NR

NR = none reported   NA = not applicable, since studies on the parent substance have been reported

[1] A "+" indicates that metabolic data has been reported for the specific flavour ingredient. A "-" indicates that metabolic data for the specific flavour ingredient has not been reported and metabolic fate is presumed based on data from structurally related substances.

[2] The no-observed-adverse-effect level (NOAEL) is reported for oral administration of the substance either in food or in the drinking water, unless noted. Abbreviatons used for "Duration": d = days; m = month(s); wk = week(s); yr = year(s).

[3] Arena and Fung, 1980; Bessman et al., 1953; Bessman and Fishbein, 1963; Doherty and Roth, 1978, Fishbein and Bessman, 1964 and 1966; Guidotti and Ballotti, 1970; Jakoby and Scott, 1959; Lee, 1977; Lettieri and Fung, 1978; Möhler et al., 1976; Roth and Giarman, 1965, 1966 and 1969; Snead et al., 1989; Walkenstein et al., 1964. [4]Schafer, 1985; [5]Hampel and Hapke, 1968; [6]Freifeld and Hort, 1966; [7]NTP, 1992; [8]Zlatkis and Liebich, 1971; [9]Morgareidge, 1962; [10]Morgareidge, 1963; [11]Moreno, 1978; [12]Deichmann et al., 1945; [13]Oser et al., 1965; [14]Hagan et al., 1967; [15]Moreno, 1977; [16]Oser et al., 1965; [17]Smyth et al., 1969; [18]Wilson, 1961a; [19]Bar and Griepentrog, 1967; [20]Moreno, 1974; [21]Moreno, 1977; [22]Jenner et al., 1964; [23]Moreno, 1972; [24]Moreno, 1975a; [25]Levenstein, 1975; [26]Galea, 1965; [27]Fassett, 1961; [28]Levenstein, 1974; [29]Shellenberger, 1973; [30]Moran et al., 1980; [31]Shellenberger, 1971; [32]Mundy and Kirkby, 1971; [33]Mundy and Kirkby, 1973; [34]FDA, 1993; [35]Tischer, 1942; [36]Piccirillo and Hartman, 1980; [37]Cox et al., 1974; [38]Bioassay Systems Corp., 1984; [39]Reagan and Becci, 1984; [40]Moreno, 1980; [41]Roubier, 1991; [42]Ponnock, 1992; [43]Watanabe and Kinosaki, 1990; [44]Wohl, 1974; [45]Emberger, 1994; [46]Moreno, 1978; [47]Affiliated Medical Enterprises, 1970; [48]Moreno, 1976; [49]Posternak et al., 1969; [50]Edwards, 1970.

Table B4. Summary of metabolism and toxicity data for lactones formed from alicyclic and aromatic hydroxycarboxylic acids

	Substance	FEMA No.	Metabolic data[1] *in vitro/in vivo*	Oral LD$_{50}$, mg/kg body weight	Repeated dose study[2] NOAEL, mg/kg body weight/day (duration)	Repeated dose study for a structurally related substance NOAEL, mg/kg body weight/day (duration)
36.	Octahydrocoumarin	3791	−/−	3900[3]	NR	50 (27–28 wk)[4] 125 (13 wk/1 yr)[5,6]
37.	Mintlactone	3764	−/−	530[7]	NR	1.0 (313 wk)[8]
38.	Dehydromenthofurolactone	3755	−/−	1970[9] 2092[10] 2440[10]	1.0 (13 wk)[8]	NA
39.	Sclareolide	3794	−/−	>5000[11]	NR	50 (27–28 wk)[4] 125 (13 wk/1 yr)[5,6]
40.	3,4-Dihydrocoumarin	2381	+[12]/+[13]	1010–1760[14]	380 (13 wk)[15] 150 (13 wk)[16] 150 (2 yr)[6] 500 (14 wk)[6]	NA
41.	6-Methylcoumarin	2699	−/−	465–3050[17]	500 (13 wk)[6] 50 (2 yr)[6] 175 (2 yr)[6]	NA
42.	3-Propylidenephthalide	2952	−/−	1650[18]	5.4 (males) (90 d)[19] 6.5 (females) (90 d)[19]	NA
43.	3-Butylidenephthalide	3333	−/−	1850–2420[20]	NR	5.4–6.5 (90 d)[19]
44.	3-Butylphthalide	3334	−/−	1850–2450[21]	NR	5.4–6.5 (90 d)[19]

NR = none reported　NA = not applicable, since studies on the parent substance have been reported

[1]A "+" indicates that metabolic data has been reported for the specific flavour ingredient. A "−" indicates that metabolic data for the specific flavour ingredient has not been reported and metabolic fate is presumed based on data from structurally related substances.

[2]The no-observed-adverse-effect level (NOAEL) is reported for oral administration of the substance either in food or in the drinking water, unless noted. Abbreviatons used for "Duration": d = days; m = month(s); wk = week(s); yr = year(s).

[3]Moreno, 1978; [4]Bar and Griepentrog, 1967; [5]Paynter, 1957; [6]Hagan, 1967; [7]Collier and Driscoll, 1982; [8]Voss *et al.*, 1985; [9]Collier, 1982; [10]Reagan and Becci, 1984; [11]Lewis and Palanker, 1979; [12]Scheline, 1968; [13]Furuya, 1958; [14]Levenstein, 1953; Jenner *et al.*, Moreno, 1972; [15]Lake *et al.*, 1994; [16]NTP, 1993; [17]Levenstein, 1953; Feuer, 1974; Moreno, 1975; [19]Posternak *et al.*, 1969; [20]Moreno, 1980; Posternak, 1965; [21]Moreno, 1976; Pellmont, 1970.