Review

Nutritional modulation of xenobiotic metabolism

Caloric restriction: effects on pharmacodynamics

Ronald W. Hart and Angelo Turturro
National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR, USA

Methyl group metabolism and aflatoxin B_1 in hepatocarcinogenesis

Paul M. Newberne and Thomas F. Schrager
Department of Pathology, Boston University School of Medicine, Boston, MA, USA

Metabolic depletion of nutrients by drugs

John N. Hathcock
Experimental Nutrition Branch, Food and Drug Administration, Washington, DC, USA

Comparative studies on the metabolism of food additives: case examples in the safety evaluation of the allylbenzene natural flavors

John Caldwell, J. David Sutton, and Angela J. Howes
Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, London, UK

Introduction

This review focuses upon the overlapping interests of the disciplines of nutrition, toxicology, and pharmacology. Nutrients can alter the metabolic pathways that are responsible for activation and elimination of xenobiotics. Dr. Hart and Dr. Turturro provide as an example the effects of caloric restriction upon xenobiotic metabolism, and Dr. Newberne and Dr. Schrager provide as an example the effects of lipotrope deficiency upon aflatoxin metabolism. In turn, xenobiotics can alter the rates of utilization of nutrients. Dr. Hathcock provides as an example the depletion of sulfur amino acids during the metabolism of acetaminophen. Finally, some constituents of the diet are themselves xenobiotics rather than nutrients. Dr. Caldwell, Dr. Sutton, and Dr. Howes discuss the metabolism of several food flavorings in relation to their potential carcinogenicity.

An understanding of the molecular basis for the effects of nutrients upon biological functions is of signal importance for the application of nutrition to disease prevention and improvement of public health. Nutrients do much more than provide the building blocks and catalysts for growth, development, reproduction, and maintenance. They also determine whether and how we are affected by drugs and toxicologic agents (xenobiotics).

Address reprint requests to Dr. Paul M. Newberne, Department of Pathology, Boston University School of Medicine, Boston, MA 02118, USA.
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Caloric restriction: effects on pharmacodynamics

Introduction

Dietary nutrients modify chemical toxicity. For instance, increasing dietary protein (casein) changes the

LD_{50} of a number of herbicides by a factor of six.[1] However, the most effective positive nutritional modulator of toxicity appears to be caloric restriction (CR). As early as 1908, it was demonstrated that the growth rate of transplanted tumor cells in rodents was inhibited by extreme food restriction.[1] This finding has been repeated in other systems using more moderate underfeeding[2] and numerous studies have demonstrated the inhibitory effect of food restriction on chemically induced carcinogenesis, ([benzo[a]pyrene and methylcholanthrene in the skin; diethylnitrosamine in liver; and dimethylbenz[a]anthrecene in mammary gland]).[3] The incidence of tumors induced by ultraviolet light[4] is also reduced by food restriction. Other diseases have been less studied, but underfeeding has been shown to affect positively survival after viral challenge[5] and to decrease severity of induced end-stage renal pathology.[6] Numerous studies[2,7,8] have also suggested that the most important influence of underfeeding for reducing toxic effects appears to be from diminished total caloric (or energy) intake. Restriction to approximately 60% of ad libitum levels, with the addition of vitamins and minerals to prevent deficiencies, appears to be adequate for long-term survival in a number of different strains of mice and rats[9]; this level of restriction is being used in this study.

Materials and methods

The probject on caloric restriction (PCR) uses four strains of mice and three stains of rats, listed in *Table 1* along with their respective diets. More detailed descriptions of the animals and the diets can be found elsewhere.[9,10] These different strains of rats and mice exhibit different incidences of various spontaneous diseases; this project is evaluating the influence of CR on these spontaneous (historical) disease processes and the observations will be correlated with selected metabolic and other physiological measurements. The B6C3F1 mouse and the F-344 rat are widely used in chronic toxicity studies conducted by the National Cancer Institute and the National Toxicology Program (NCI, NTP); as a result, there is an extensive data base associated with the response of these species and strains to a wide range of toxic substances. The other strains, however, have a much less extensive database; this project will help to expand on information now available and will make the various strains more useful for biological research.

The diets used are the NIH-31, which is fed to all genotypes, as well as special diets (Masoro and Emory-Morse 911A) fed only to diet-comparison cohorts of the B6C3F1 mice and F-344 rats (*Table 1*). Restriction to 60% of the diets consumed by the ad libitum groups start from 14 to 16 weeks of age (except for one diet/genotype started at 6–8 weeks), and continues throughout the study. The diets of the restricted groups are fortified with vitamins and minerals to provide amounts of these nutrients equal to amounts consumed by ad libitum groups. All animals are housed singly and maintained in specific pathogen-free (SPF) barrier conditions. Over the planned 9-year experimental period, the study is expected to produce over 100,000 animals, with as many as 22,000 being maintained at any one time; the animals are already being shared by numerous university based scientists as well as investigators at the National Institutes of Health (NIH) and this practice will be expanded in the future. A few of the preliminary observations on some physiological parameters are presented here; more extensive results are found in the various references in the text and in the list of references.

Table 1 Genotypes and diets[a]

Mouse	Diet	Rat	Diet
B6D2F1	NIH-31	Fischer-344	NIH-31
DBA/2NNia	NIH-31	Fischer-344	Marsoro
B6C3F1	NIH-31	Brown-Norway	NIH-31
C57B1/6NNia	NIH-31	Brown-Norway X Fischer-344	NIH-31
C57B1/6NNia	EM-911A		

[a] Adapted from Turturro and Hart.[10] NIH-31 is the autoclavable form of NIH-06. EM 911A is Emory-Morse 911A developed by the Emory Morse Co., Guilford, CT, USA. Masoro is Masoro Diet C from Ralston Purina.

Results

Based on body weights, animals on the CR study are, indeed, restricted. Data in *Table 2* show the body weight differences at selected ages of C57B1/6 females. This trend, typical for other strains, illustrates that the restriction in mice results in significantly less body weight gain. For the other groups, the final weight as a percentage of ad libitum weight varies with genotype, species, and sex, but has averaged from 30 to 40% of control. In all genotypes examined there has been an increase in age-specific survival, consistent with other studies using CR. These are described in other publications.[10-18]

Cellular metabolic alterations. Intermediary metabolism is central to the processes that occur in a target cell permitting it to metabolize an agent delivered to it, or to interact with an active metabolite. The liver is a good model of target cell metabolism, where many toxins as well as essential nutrients are metabolized to active forms and released into the general circulation or stored. Some of the changes associated with CR occur in the liver and these, in turn, can influence response to toxic substances. For example, gluconeogenesis is significantly enhanced by CR. Enzymes, such as glutamate dehydrogenase (GDH) and glucose-6-phosphatase, may be significantly increased, while those related to glycolysis, such as lactic dehydrogenase (LDH), pyruvate kinase (PK), sorbitol dehydrogenase (SDH), and alcohol dehydrogenase (ADH), are decreased with CR.[10,11] Also decreased were the enzymes associated with lipid metabolism, suggesting perhaps a decreased potential for

Table 2 Mean body weights for female C57B1/6 mice on NIH-31

Age (weeks)	Ad libitum weight (g)	Restricted
14	22.8	22.7
30	25.2	22.8
50	28.1	21.1
90	28.8	22.5

Body weights are means taken from a cohort of 20 ad libitum and restricted animals.

fatty acid epoxide formation (*Table 3*).[13,14] A number of studies have measured the mutagenic potential of fatty acid epoxides, and found them to be potent mutagens in the Ames assay system. Taken together, the results of enzyme studies in the restricted rodent suggest that under conditions of restriction:

1. Metabolism becomes more efficient, geared toward increased production of blood sugar.
2. This altered metabolism reduces glycolysis, important to tumor cells, thereby reducing the potential for transformation and growth.
3. Fatty acid metabolism, and its resultant mutagenic epoxides are reduced.

Among other observations of significance, the P_{450}-dependent monooxygenase system is stimulated[12] and hepatic microsomal testosterone 6β-hydroxylase, lauric acid 12-hydroxylase, and 4-nitrophenol hydroxylase activities are increased by CR. This suggests that the cytochrome P_{450} isozymes may be induced by decreasing caloric intake, but this remains to be documented. Activities for nonspecific monooxygenases, such as aminopyrene-*N*-demethylase, were not affected by CR, but 7-ethoxycoumarin-*O*-deethylase was markedly elevated by CR. Other isozymes were variably affected by CR, as described in referenced publications.[10-13]

A result of some of the changes associated with CR is a reduction in the activation of aflatoxin, a human carcinogen, to its putative carcinogenic form. After a single-dose administration of labeled aflatoxin, hepatic binding was decreased to less than 50% per milligram DNA, and to even less per total liver.[14] Moreover, CR has recently been shown to reduce the incidence of aflatoxin-induced carcinogenesis.

Macromolecular interactions. DNA repair, as measured by unscheduled DNA synthesis (UDS), is enhanced at least 50% by CR.[15] A capacity to stimulate DNA repair by nutritional modulation has some exciting possibilities in mitigating adverse effects of exposure to genotoxic agents.

Cellular replication and gene expression. Cellular replication and the expression of genes associated with cellular proliferation are accepted as prerequisites for tumor formation. Preliminary observations in our CR studies reveal little if any change in the proliferative rate of the cells in the liver or bone marrow with restricted caloric intake. This, combined with results described above for aflatoxin, indicate that, although it has been assumed that CR affects tumorigenesis by altering cellular proliferation, current information does not necessarily support this assumption.

Although proliferation may not be affected by CR, the induction of changes consistent with tumorigenesis may be. One such biomarker is an oncogene, c-*Myc*, which, like other oncogenes, is associated with control of cellular proliferation. Preliminary observations suggest that under conditions of CR there is inhibition of expression of c-*Myc* gene,[16] which fits with the influence of CR on aflatoxin carcinogenesis.[15]

Conclusions

Caloric restriction significantly alters the pharmacodynamics of metabolic changes associated with tumor induction and reduces the incidence of a number of tumor types. This nutritional modification of toxicity may be important to understanding the relationship between nutrition and response to toxic substances, to further identify appropriate biomarkers of both carcinogenesis and aging, and to aid in the prevention of adverse effects of environmental toxicants.

Comments and statements made in the above paragraphs are supported by data in the references listed at the end of the review.

Methyl group metabolism and aflatoxin B_1 in hepatocarcinogenesis

The classical research of a few scientists during the period from 1920 to 1950 identified the profound influence of methyl group metabolism on cell growth and differentiation. Although the implications of the results of the work of these dedicated individuals were

Table 3 Effects of caloric restriction on intermediary metabolic functions of the liver

Enzyme	Increase	Decrease	No change
Gluconeogenic			
Glutamate dehydrogenase	X		
glucose-6-phosphatase	X		
amino acid oxidase			X
Glycolytic			
alcohol dehydrogenase		X	
lactate dehydrogenase		X	
sorbitol dehydrogenase		X	
pyruvate kinase		X	
Lipid enzymes			
Fatty acid synthetase			X
glycerol kinase		X	
Malic enzyme		X	
Cytochrome P_{450}			
P_{450}	X		
B-5	X		
Cyt. C reductase	X		
Conjugating enzymes			
glutathione-S-transferase			
N-acetyltransferase	X		
UPD-glucuronyl transferase			
2-aminophenol	X		
bilirubin	X		

not fully appreciated at the time, the keen insight of these pioneers laid the groundwork for today's intense interest in lipotropic factors (choline, methionine, folate, and vitamin B_{12}) and the effects these nutrients have on biological mechanisms, including xenobiotic metabolism and susceptibility to carcinogenesis (see Newberne[19] for a review).

Lipotropes are an important class of nutrients which are essential to a large number of metabolic processes, in particular the synthesis of nucleic acids, proteins, and cell membranes. They are essential to cell proliferation and for the maintenance of tissue integrity.[20-25] The members of this class of nutrients interact extensively with each other and other nutrients in the metabolism of 1-carbon units.[24,26,27]

The original observation of Copeland and Salmon[25] that rats fed diets deficient in methyl groups developed malignant liver cell neoplasms raised interesting questions regarding susceptibility to cancer. The observations of Copeland and Salmon were repeated in a number of laboratories[26-30] and it was further shown that the deficit also enhanced hepatocarcinogenesis initiated by a number of structurally diverse chemicals.[31-33] *Table 4* lists results of studies that document the influence of methyl group deficiency on cancer induced in rodents.

Materials and methods

A number of strains of rats were used in the various studies alluded to in this paper. In general, outbred Sprague–Dawley rats and the inbred Fischer 344 strains have been used in most of our studies. They were fed semipurified diets based on casein/peanut meal, soybean protein and refined carbohydrates, or purified amino acids and carbohydrates, along with appropriate vitamins and minerals. These diets are all marginal in methionine and choline; vitamin B_{12} and folate were varied according to design protocol. The diets were fed from weaning until termination of the study, which usually occurred between 18 and 24 months, in the case of carcinogenesis studies, or earlier for other types of investigations such as metabolic evaluations.

Specific biochemical methodology has been described in the papers listed in the references. Histopathological evaluation was done according to standard techniques, also described in the listed references.[34]

Table 4 Influence of methyl group deficiency on rodent chemical carcinogenesis observed in the same laboratory[a]

Carcinogen	Tumor site	% Rats with tumors	
		Control	Methyl deprived
Aflatoxin B_1	Liver	15	87
Diethylnitrosamine	Liver	70	80
Dibutylnitrosamine	Liver	24	64
	Bladder	84	80
Dimethylnitrosamine	Liver	28	27
	Kidney	16	3
N-2-Fluoreny acetamide	Liver	19	41
	Mammary	80	79
N-[4-(5-Nitro-2-furyl)-2-thaizolyl]	Bladder	53	61
7,12-Dimethyl benzanthracene	Mammary	48	15
1,2-Dimethyl hydrazine	Colon	86	100

[a] Data abridged from Reference 33.

Results

As noted above in *Table 4*, research conducted in our laboratory illustrates the influence of methyl group deficiency in the promotion of chemically induced carcinogenesis. This work was done in a single laboratory under identical conditions of handling and housing. These observations raised important questions and quickly led to attempts to determine the mechanisms(s) by which methyl group deficiency resulted in an enhancement of neoplasia. The deranged lipid metabolism and accumulation of fat in the tissues of deficient animals suggested that lipid peroxidation and tissue injury from free radicals might be involved. Data from our laboratory[34,35] and from Ghoshal et al.[36] suggested that there was increased peroxidation and free radical formation and that antitoxidants offered some protection. Injury of critical macromolecules by free radicals, generated when methyl groups are deficient, may be significant in initiation or promotion of hepatocellular carcinogenesis in human liver cancer as well as in our animal model.[37-39] The livers of patients with hepatocellular cancer as well as of alcoholic patients are fatty and often cirrhotic, characteristics considered to be involved in the carcinogenesis process. This area of methyl group metabolism, including effects of antioxidants, is currently under intense investigation.[40]

Since most chemical carcinogens require metabolic activation by enzyme systems, an interesting possibility for methyl group metabolic effects might be via changes in xenobiotic metabolism. Earlier work in our laboratory had shown that lipotrope deficiency had a significant protective effect on the acute toxicity of aflatoxin B_1 (AFB_1).[37] The protective effect was shown to be associated with modification in the activity of liver mixed function oxidases, responsible for AFB_1 activation/deactivation.[38] Collaborative work with Campbell[39] had demonstrated further that hepatic mixed function oxidases were involved in in vivo covalent binding of AFB_1 to liver DNA in rats deficient in methyl groups.

The ultimate carcinogenic form of aflatoxin is thought to be the 8,9-oxide, which binds predominately to the N^7 atom of guanine in DNA. It also binds to RNA and protein.[40] In addition to binding to macromolecules, the AFB_1 8,9-oxide undergoes hydrolysis to aflatoxin 8,9-dihydrodiol and conjugation with glutathione to form the aflatoxin–glutathione conjugate.[40-42] The work of Kensler et al.[43] demonstrated

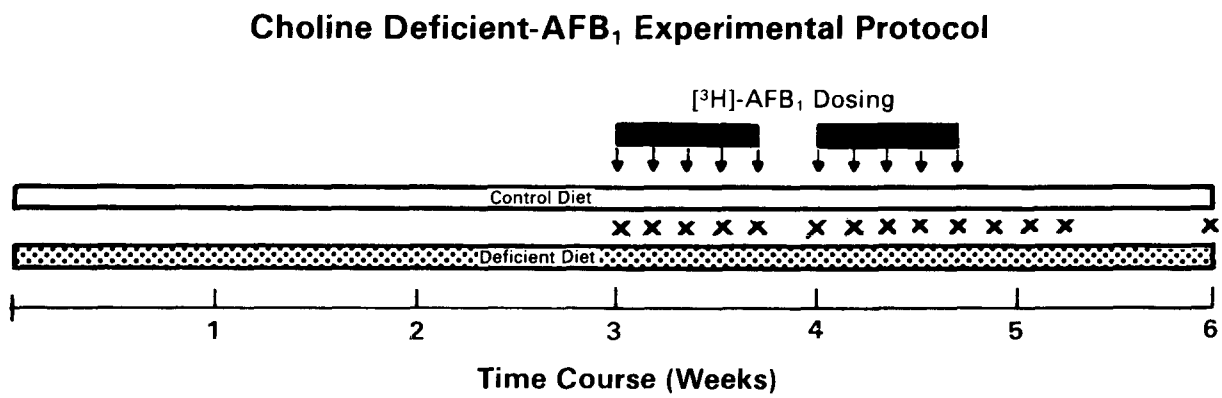

Figure 1 Time course for treatment with AFB_1. Note the two dosing periods of five daily doses each. The **X** denotes time of taking samples for adduct formation.

that the induction of enzymes, important to aflatoxin B_1 detoxification, by the antitoxidant ethoxyquin protects against aflatoxin-induced hepatic cancer, further confirming nutrient effects on metabolism. The antioxidant also reduces AFB_1–DNA adduct formation and hastens DNA repair resulting from AFB_1 injury. We have examined the effects of methyl group deficiency on AFB_1–DNA adduct formation and removal in rat liver.[44] Rats examined for AFB_1–DNA adducts each day during a carcinogenic 10-day dosing schedule reveal a significant influence of methyl groups on formation and removal of adducts (*Figures 1* and *2*). Rats were given AFB_1 on a schedule that does not result in tumorigenesis, (a single dose of AFB_1). At 2 or 24 h following administration of the single dose, there were no significant differences in total aflatoxin–DNA adduct formation in rats fed choline-deficient or control diets. Since choline deficiency exerts such a dramatic enhancing effect on tumor burden and time to first tumor following multiple-dose AFB_1 exposure, we conducted a study with multiple dosing, as shown in *Figure 1*. The rats were fed either the choline-deficient or the supplemented control diet for 21 days and then dosed daily, 5 days a week for 2 weeks. After each dosing, 4–6 rats were killed and their livers were examined for AFB_1–DNA adducts. *Figure 2* shows the temporal pattern of overall binding during and after

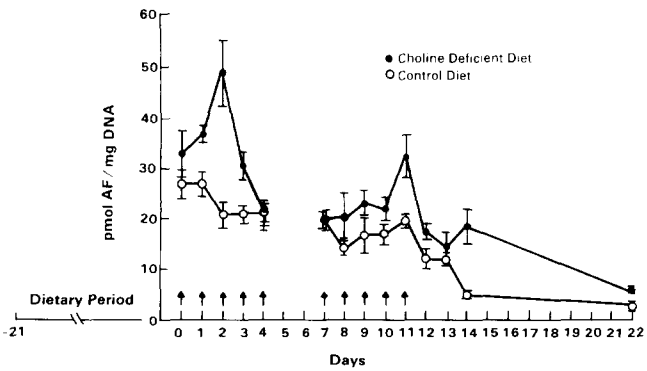

Figure 2 AFB_1-DNA adducts formed during successive days of exposure to AFB_1 and fed a control (○) or methyl deficient (●) diet.

dosing with the carcinogen. Binding of AFB_1 to DNA of control rats peaked after the first 2 days by dosing and then decreased during the following 3 days of the first week. During the second week of dosing, binding was somewhat reduced compared to the first week, suggesting metabolic adjustment.

The choline-deficient diet did not significantly alter AFB_1 binding after the first dose of AFB_1; however, after the second dose, binding levels increased significantly and this pattern was sustained during the remainder of the 2-week dosing period. Furthermore, in the control rats AFB_1–DNA binding fell 30% after the end of dosing and an additional 50% during the next 48 h. In the deficient rats binding peaked after the third dose at 2.5 times control values, and remained significantly higher than control values for the second week of dosing. Twenty-two days after the start of dosing, total adducts in the livers of deficient rats were significantly higher than in those of controls.

In this study the influence of nutrients on the binding and removal of AFB_1–DNA adducts was clearly demonstrated. The deficient diet resulted in a significantly reduced rate of removal of adducts. However, this effect was observed only after the second and subsequent doses, not after a single dose. Since the formation of adducts is putatively a potential carcinogenic effect, this increased adduct formation and reduced rate of removal in choline deficiency may help to explain the enhancing effect of hepatocarcinogenesis in methyl deficient rats. The rats in this study would have developed liver tumors had the animals been allowed to live longer. These observations, and the fact that the activity of cytochrome P_{450} isozymes is decreased in the choline-deficient rat, support the hypothesis that metabolism of the carcinogen is significantly modified by choline deficiency.[45,46] The deficiency may shift the balance of AFB_1 metabolism from the less toxic hydroxylated metabolite to the more reactive epoxide, a putative carcinogen or procarcinogen.

Metabolic depletion of nutrients by drugs

Nutrition and drug interactions may interfere with drug actions or they may disrupt nutritional processes

Table 5 Effects of prolonged acetaminophen ingestion on methionine functions

Indicator	Effect	Reference
Growth rate	Decreased	73, 79, 80
Protein synthesis	Decreased	80
Cysteine (free), liver	Decreased	80
GSH, liver	Decreased	53, 72, 80, 81
L-[^{14}C]Phenylalanine oxidation	Increased	81
N-Methylnicotinamine, urine	Decreased	80
Creatine, muscle	No change	80
Creatinine, plasma	No change	80
Creatinine, urine	Decreased	80

and functions in a number of ways. The antinutritional impact of drugs involve several mechanisms including (1) appetite suppression, (2) chemical destruction or binding of a nutrient in the gastrointestinal tract, (3) inhibition of intestinal absorption, (4) altered nutrient transport, (5) inhibition of nutrient activation, (6) inhibition of nutrient function, (7) increased nutrient catabolism, and (8) increased nutrient excretion. Many pharmacologically active substances, notably alcohol, have multiple mechanisms for decreasing nutritional status.[47,48] Drug-induced increases in nutrient catabolism may be mediated through either phase I or phase II reactions. Phase I reactions add or expose functional groups of molecule, through oxidation, reduction, or hydrolysis, preparing the chemical to be directly excreted or for phase II-conjugation with glutathionine or sulfate or by acetylation, or via amino conjugates or mercapturic acid, followed by excretion. Illustrations are barbiturate induction of microsomal enzymes that hydroxylate vitamin D and acetaminophen (ACAP) conjugation by sulfate and glutathione (GSH).[49] *Table 5* lists effects of ACAP on a number of methionine functions.

Most studies of interactions between dietary methionine and ACAP metabolism and toxicity have involved prolonged methionine (and cystine) restriction and acute ACAP administration.[50,51] The decreases in available sulfate and GSH during sulfur amino acid deficiency lead to decreased ACAP conjugation, increased formation of ACAP adducts to macromolecules, and increased hepatotoxicity.[52,53] Similar effects of methionine/cystine restriction have been observed for other toxicants.[50,54,55]

Interactions of methionine and acetaminophen

ACAP is metabolized by three pathways, as noted in *Figure 3*. Two of these are direct phase II conjugations that produce ACAP-*O*-sulfate and ACAP-*O*-glucuronide.[56] The third pathway involves a cytochrome P_{450}-dependent oxidation[56,57] that produces an electrophilic product, possibly an ACAP quinonimide,[56] and, usually, conjugation of this reactant by GSH.[58] The conjugate is converted before excretion to the corresponding mercapturic acid.[58] The unconjugated electrophile reacts with macromolecules, leading to toxicity.

A therapeutic dose of ACAP in mice is partitioned, approximately: 45% ACAP-sulfate, 35% ACAP-glucuronide, 4% ACAP-mercapturic acid, and 12% free ACAP.[56,59] The dose partition in humans is similar but with a large variability.[60,61] With increasing dose, the fraction excreted as the sulfate decreases but the total amount excreted as ACAP-sulfate increases. Also with increasing dose, the amount and fraction excreted as GSH-derived conjugates increases in mice.[51,52] The degree of compensatory increase in uridinediphosphoglucuronic acid (UDPGA) transferase activity appears to depend on the species, diet and substrate.[56,59,61-63] The influence of these factors on UDPGA transferase activity has not been adequately assessed with ACAP as the substrate.

The sulfur amino acids are required for several processes in detoxification of foreign compounds. These functions include not only sulfation and GSH conjugation, but also cysteine conjugation, metallothionein binding of heavy metals and alkylating agents, and protein synthesis for enzymes and other proteins involved in these processes and in the mixed-function oxidase catalyzed activation of ACAP.[52,53,64] Methionine deficiency increases the toxic potencies and interactions of ACAP and alcohol.[50,51] Several sulfur compounds, including methionine *S*-adenosylmethionine, *N*-acetylcysteine, and sulfate, are effective oral therapies in acute ACAP intoxication.[59,60]

Metabolic production and excretion of sulfate are decreased in fasting[65] and in protein deficiency[66] in the rat and in anorexia nervosa[67] and kwashiorkor[68] in humans. Acute ACAP administration to rats causes temporary depletion of endogenous sulfate,[69-71] but pro-

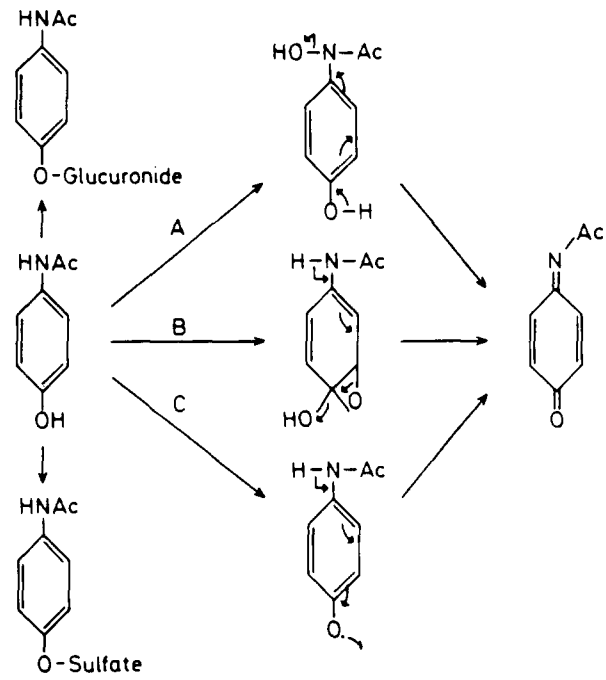

Figure 3 The three pathways for acetaminophen metabolism. Two are phase II and the third involves P_{450}-dependent oxidation.

longed administration of ACAP does not decrease plasma sulfate levels,[72] suggesting that with prolonged ACAP administration there is an increased rate of sulfate production. Malnutrition is associated with decreased ACAP conjugation in the rat[51,66] and in the mouse[52] and with increase ACAP hepatotoxicity in mice[50] and humans.[67]

Stoichiometry of methionine and acetaminophen relationship

Because ACAP is often ingested in multiple-gram daily dosages, its prolonged consumption has the unusual potential to induce methionine deficiency through depletion of sulfate and GSH.[73] ACAP is recommended in daily dosages up to 4 g[74] and up to 50% of this dose is conjugated by sulfate or GSH.[52] ACAP intake and metabolism under these conditions represent 13.2 mmol/day of sulfate plus GSH depletion. Virtually all GSH must be derived from methionine or cystine metabolism. Most sulfate in human urine is derived from sulfur amino acid oxidation.[75,76] Therefore, ACAP conjugation by sulfate and GSH requires stoichiometric amounts of sulfur amino acid metabolism.

The adult requirement for methionine plus cystine is 13 mg/kg/day,[77] or 5.9 mmol/day for a 60-kg person. As an arbitrarily selected population category probably having low methionine/cystine intake, the 25th percentile of protein intake in the 65- to 74-year-old female is 36 g/day.[78] The calculated total sulfur amino acid intake is 10.9 mmol/day, if the probably generous assumption is made that the protein sources are as follows: meat, 50%; milk, 12.5%; egg, 12.5%; and wheat, 25%.

In summary, the potential demand for sulfur metabolites (sulfate and GSH) to conjugate the maximum recommended intake of ACAP (13.2 mmol/day) exceeds a generous estimate of the 25th percentile sulfur amino acid intake by the 65- to 74-year-old female (10.9 mmol/day) and exceeds by a greater margin the sulfur amino acid maintenance requirement in a 60-kg individual (5.9 mmol/day). It is quantitatively plausible, therefore, that chronic ACAP usage could induce methionine deficiency if sulfur amino acid intake is not sufficient to meet both the maintenance requirement and the additional demand for ACAP conjugation by sulfate and GSH.

Acetaminophen-induced methionine deficiency

Studies with rodents have shown that prolonged ACAP administration can exaggerate methionine deficiency or induce it if dietary sulfur amino acids are marginal. Prolonged ACAP administration induces methionine deficiency in methionine-marginal animals, as indicated by decreased growth,[72,73,79] inhibited protein synthesis, decreases in plasma-free cysteine, and in excretion of creatinine and *N*-methylnicotinamide.[80] These are accompanied by prolonged decreases in tissue GSH levels.[52,72,80] Although prolonged ACAP treatment decreased creatinine excretion, plasma levels of creatinine and muscle creatine do not appear to be affected.[80] Also, dietary ACAP treatment increased the oxidation of [^{14}C]phenylalanine, indicating its increased availability for oxidation due to ACAP-induced methionine deficiency.[81] These effects were generally prevented when methionine was fed at 200% of the requirement rather than the marginally adequate 100% of the requirement level, even though the high methionine groups were pair-fed to the lowest food intake group (marginal methionine with highest ACAP intake).[81]

Decreased growth with biochemical corroboration is not sufficient to demonstate that methionine deficiency has been produced; decreased growth could be due to possible direct growth-inhibiting effects of ACAP. The altered primary indicators of methionine status at ACAP levels not generating hepatotoxicity demonstrate that methionine deficiency can be induced by ACAP when sulfur amino acid intakes are marginally adequate.[81] The effects of prolonged ACAP administration on indicators of methionine function are summarized in *Table 5*.

Summary

The increased utilization of sulfate and GSH in detoxification of prolonged ACAP consumption imposes methionine deficiency in mice when the sulfur amino acid intake is marginal. It seems possible that in humans chronically consuming large amounts of ACAP, conjugation of the drug may increase methionine needs for sulfate and GSH sufficient to cause methionine deficiency, particularly when intake is marginal; that is, the methionine requirement under those conditions is increased.

Comparative studies on the metabolism of food: case examples in the safety evaluation of the allylbenzene natural flavors

Introduction

It has been estimated that up to 60% of all human cancers are diet-related[82]; as a consequence of the greatly increased interest in the long-term safety of the diet, much attention has recently been focused on the food additive chemicals the diet contains. There are more than 30,000 compounds that provide the flavors of natural foods, some 3,000 of which are encountered on a regular basis. The amount of toxicological information available on these materials varies from compound to compound, but it is apparent that, at least under normal conditions of use, they are essentially devoid of acute toxicity. However, concern remains over their long-term safety. Results of recently performed rodent carcinogenicity studies on quantitatively important flavor compounds, largely under the auspices of the U.S. National Toxicology Progran (NTP), have involved the administration of maximum tolerated doses (MTD) essentially over the lifetime of the animals. Under these conditions a number of flavors have exhibited carcinogenic activity: *d*-limo-

nene,[83] allyl isothiocyanate,[84] estragole and methyleugenol,[85] and anethole.[86]

Attempting to extrapolate animal toxicity data to indicate human risk is subject to a number of difficulties. This is especially the case with the studies referred to above. The administration of doses enormously in excess of human consumption of flavors leads to results that are difficult if not impossible to interpret, based solely on tumor incidence and frequency. Thus, there is a need for a more effective basis for the interpretation of the significance of such findings for very low-dose human exposure. This has been addressed in a recent publication of the Joint WHO/FAO Expert Committee on Food Additives.[87]

Over the last 20 years it has become increasingly evident that metabolic and mechanistic studies help to bridge the gap in our knowledge of toxicology by providing information on a number of matters critical to the evaluation of toxicity, including the following:

1. The definition of metabolic pathways, notably those involving metabolic activation.[88,89] These processes are critical determinants of the nature, intensity, and duration of biological activity; variations in metabolism and disposition both between and within animal species are confounding factors in interspecies extrapolations of toxicity from animals to humans.
2. Mechanism-based short-term indices of genotoxicity, a matter of great importance with putative weak carcinogens such as some of the flavors.[90]
3. The assessment of enzyme induction that can result from repeated administration of xenobiotics.[91,92] Changes induced by these processes can lead in as yet uncertain ways to hyperplastic and even neoplastic changes to the organs involved, generally the liver.[93]

This review considers the contributions of metabolic and kinetic studies in animal species used in carcinogenicity testing, and compares these to similar studies in humans in attempts to evaluate the safety of natural food flavors. This will be exemplified by case studies of a number of allylbenzene congeners, a group of compounds that pose important toxicological problems and that indicate the ways in which metabolic knowledge can improve the basis for decision making in their safety evaluation.

The allylbenzenes are an important group of flavor constituents. They are found in a wide range of herb and spice oils. Quantitatively, the most important members of the series are eugenol (clove), estragole (basil, tarragon), and the apiols (dill, parsley).[94] Interest in this series arises from the finding in the early 1960s that safrole was an hepatocarcinogen in rodents,[95] which led to its withdrawal as a specific food additive in the United States. Subsequently, a number of other congeners have been shown to be rodent carcinogens, for example, estragole, methyleugenol,[85] and, most recently, anethole.[86] Three congeners will be discussed specifically here: estragole, eugenol, and

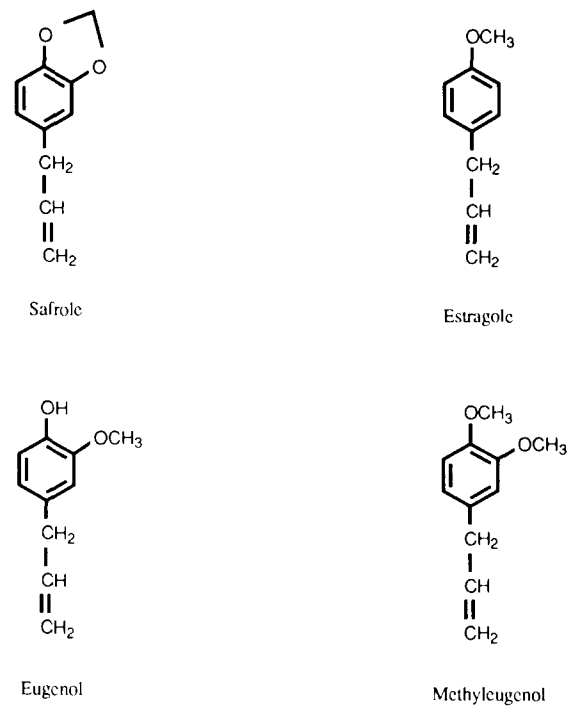

Figure 4 Chemical structures of the allylbenzenes safrole, estragole, eugenol, and methyleugenol, important flavoring materials.

anethole. Their structures, together with that of safrole, are shown in *Figure 4*.

It is now understood that the carcinogenicity of the allylbenzenes, as a class, arises from metabolic activation by hydroxylation at the benzylic (1′-)carbon atom of the side chain, followed by sulfation (*Figure 5*). The facile loss of *O*-sulfate results in carbonium ions able to form adducts with DNA, which are presumed to play a role in tumorigenesis.[96]

Metabolic studies thus have a critical role to play for this class of compounds by delineating factors influencing their metabolic activation and, presumably, carcinogenic potential, such as chemical structure, dose size, and animal species. In the cases of the allylbenzenes, the dose variable assumes considerable importance, owing to the discrepancy between the very large dose required to elicit tumors in animal carcinogenicity studies (at least 0.46% w/w of the diet for at least 12 months administration) with the minute quantities present in the human diet (on the order of 70 μg/person/day). A comparison of animal and human exposures for estragole, eugenol, anethole, and methyleugenol is presented in *Table 6*. This substantial dose discrepancy confounds attempts to extrapolate animal data to the human situation. Thus, knowledge of metabolic patterns in rodents and humans is critical for the interpretation of existing animal carcinogenicity data which may provide a basis for the assessment of human risk and for the design of more appropriate tests.

Estragole

Estragole (*p*-methoxyallylbenzene) is a murine hepatocarcinogen[85] that is metabolized along three distinct

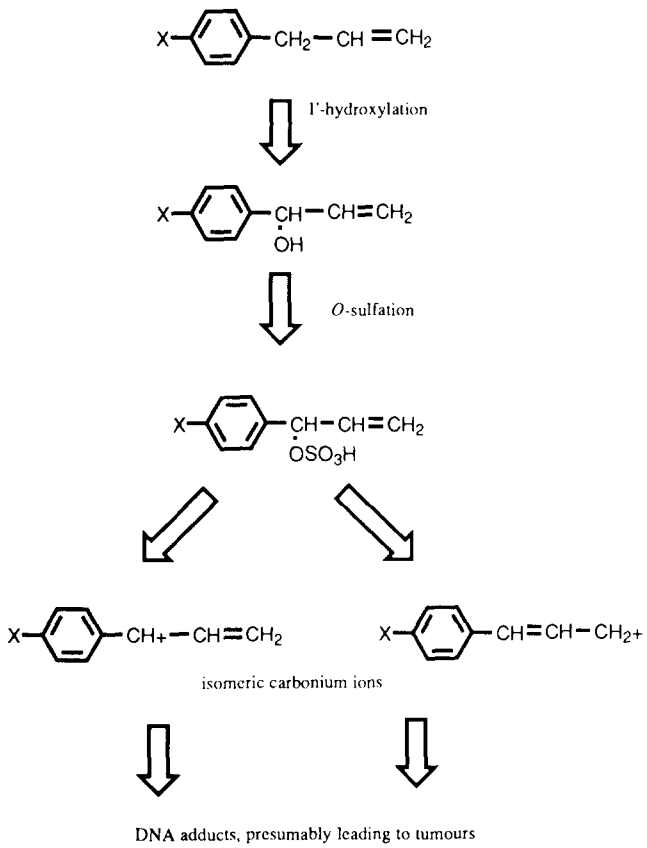

Figure 5 Metabolic activation of allylbenzenes as a class. Hydroxylation of the benzylic carbon atom followed by sulfation and, ultimately, formation of DNA adducts.

Table 6 Comparison of human dietary intakes and rodent carcinogenicity dose regimens for selected allylbenzenes

Allylbenzene	Human intake (μg)[a]	Rodent carcinogenicity dose (% in diet)
Estragole	70	0.46% for 12 months (mouse)[b]
Eugenol	70	0.6% for 2 years (mouse, male rat)[c]
		1.25 for 2 years (female rat)[c]
Anethole	65	1.0% for 2 years (rat)[d]
		0.46% for 12 months (mouse)[b]
Methyleugenol	6	0.46% for 12 months (mouse)[b]

[a] Reference 94.
[b] Reference 85.
[c] Reference 99.
[d] Reference 86.

pathways, as shown in *Figure 6*, namely, oxidative *O*-demethylation, resulting in the exhalation of $^{14}CO_2$ when [*methoxy*-^{14}C]estragole is administered, and 1-hydroxylation and epoxidation of the allylic side chain.[97] The relative extents of these pathways in rats and mice vary considerably with dose size over the range of 0.05–1000 mg/kg, which encompasses doses close to the human consumption up to those used in carcinogenicity tests. As the dose is increased in the mouse the extent of $^{14}CO_2$ exhalation falls, from 62% of dose in 72 h at 0.5 mg/kg to 37% at 1000 mg/kg with a 32% compensatory increase in the urinary recovery of ^{14}C from 28 to 60% over the same dose range.[97] The increased urinary recovery is principally accounted for by an increase in the urinary elimination of the glucuronide of the proximate carcinogen 1'-hydroxyestragole. This compound is a minor (<1% of dose) metabolite at the lowest dose, but increases in significance to become a major (<10%) metabolite at the rodent carcinogenic dose level.[97] Thus, exposure to the proximate carcinogenic metabolite increases disproportionately with dose, which becomes more striking when the data are expressed in molar terms. We are fortunate in the case of estragole to have human metabolic data, which show that at a dose of 100 μg (close to dietary intake levels), 1'-hydroxyestragole excretion accounts for only 0.3% of dose.[98] *Table 7* summarizes these data, and shows that while the ratio between the murine carcinogenic dose and human dietary intake is 500,000, the exposure ratio to the proximate carcinogen, based upon its urinary elimination, is in excess of 13,000,000.

Eugenol

Eugenol, although an allylbenzene, is apparently not a carcinogen in rodents,[85,99] a finding accounted for by its atypical metabolism[100,101] by pathways (*Figure 7*) that involve (1) facile conjugation of the free hydroxyl group with sulfate or glucuronic acid, and consequent

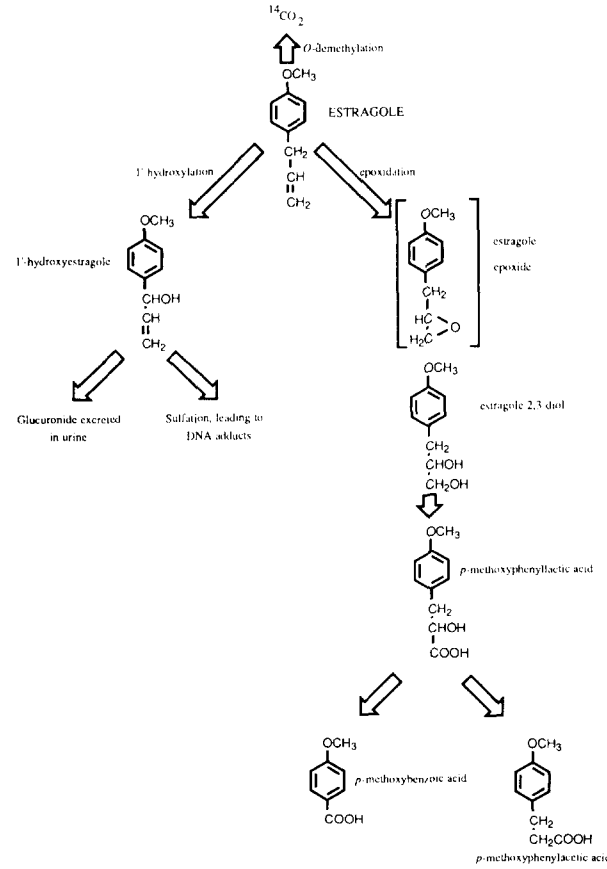

Figure 6 Activation and metabolism of estragole.

Table 7 Dose and species variations in the formation of 1′-hydroxyestragole[a]

Species and dose (mg/kg)	% Dose	Output (nmol/kg/day)
Rat		
0.05	1.3	4.5
5	3.0	1,020
500	11.4	387,600
1000	13.7	931,600
Mouse		
0.05	1.3	4.5
5	2.1	714
500	7.8	265,390
1000	9.4	639,200
Human		
0.001		0.02

[a] Dose ratio murine carcinogenic/human diet, 500,000; exposure ratio for 1-hydroxyestragole, 13,269,500. Data drawn from References 97 and 98.

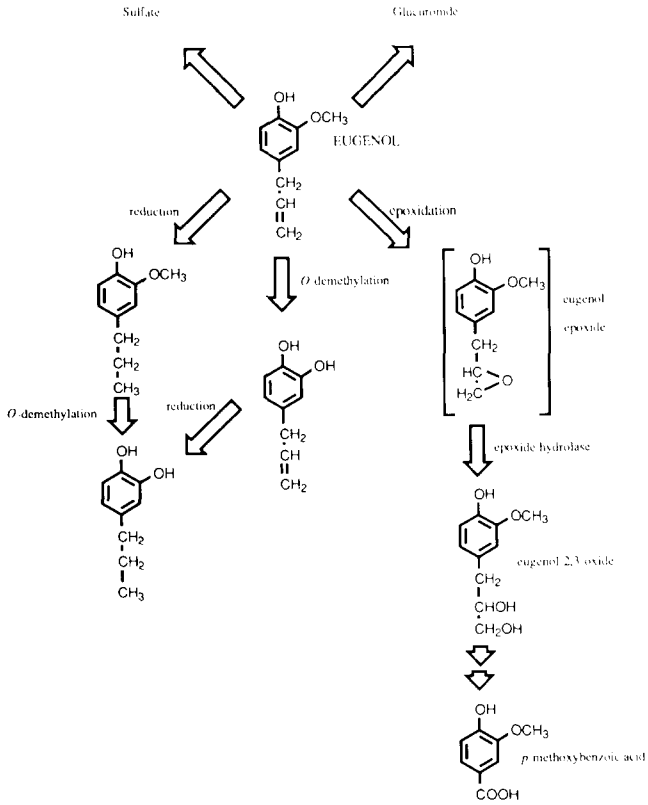

Figure 7 Metabolism of eugenol.

rapid elimination, (2) absence of 1′-hydroxylation, and (3) reduction of the allylic double bond by the gut microflora, thereby reducing the reactivity of the benzylic carbon and effectively preventing metabolic activation. Rats and mice differ in the relative extents of the double-bond reduction, which is more extensive in the rat. In both species, the major impact of increasing dose size up to the doses used in carcinogenicity tests is to shift the conjugation pattern away from sulfate at low doses toward glucuronic acid at the higest doses. The human metabolism of a low dose of eugenol involves principally glucuronidation (50% of dose) with a further 20% as the sulfate conjugate. Reduction of the side-chain double bond did not occur, while only 6% of the dose was accounted for as oxidation products. Studies using induction of unscheduled DNA synthesis (UDS) as a marker[102] are further clarifying the role of the allylbenzene in carcinogenesis. *Table 8* presents a summary of these dose and species variations in the fate of eugenol, which indicates that its fate in the rodents given high doses closely approximates that in the human subjects receiving the normal dietary intake. This information, together with the absence of metabolic activation by 1′-hydroxylation in either rodents or humans, suggests that, given the resources required for such tests, eugenol need not have been subjected to an expensive, time-consuming formal carcinogenicity bioassay.

Anethole

The allylbenzene congener *trans*-anethole (a structural isomer of estragole) is an important food flavor, occurring in fennel, anise, and dill. It is widely consumed in sweet and savory foods as well as in alcoholic and nonalcoholic anise beverages.[94] Although it has a long history of safe human use,[103] a recent long-term toxicity and carcinogenicity study in rats has shown it to cause a small but significant increase in the incidence of hepatocellular carcinoma in female rats given 1% in the diet for 121 weeks.[86] The incidence was within the range of historical controls of this strain in the testing laboratory, and there was no comparable effect in males. Data available in the mouse are very strongly suggestive that anethole is not a carcinogen in this species. A number of studies have shown that anethole is not genotoxic in bacterial or mammalian cells,[86] nor does it induce UDS in male or female rat hepatocytes under conditions where estragole has significant activity.[104]

Table 8 Dose and species variations in the metabolic rate of eugenol[a]

Species and dose (mg/kg)	% Dose excreted as:			
	Sulfate	Glucuronide	Reduced metabolites	Others
Rat				
0.5	34	18	19	15
0	34	21	12	18
50	30	14	21	16
500	27	46	0	11
1000	18	57	0	11
Mouse				
0.5	48	32	0	12
5	25	50	1	9
250	15	49	2	14
500	14	51	2	14
Human				
0.01 mg/kg	20	50	0	6

[a] Data drawn from References 96 and 97.

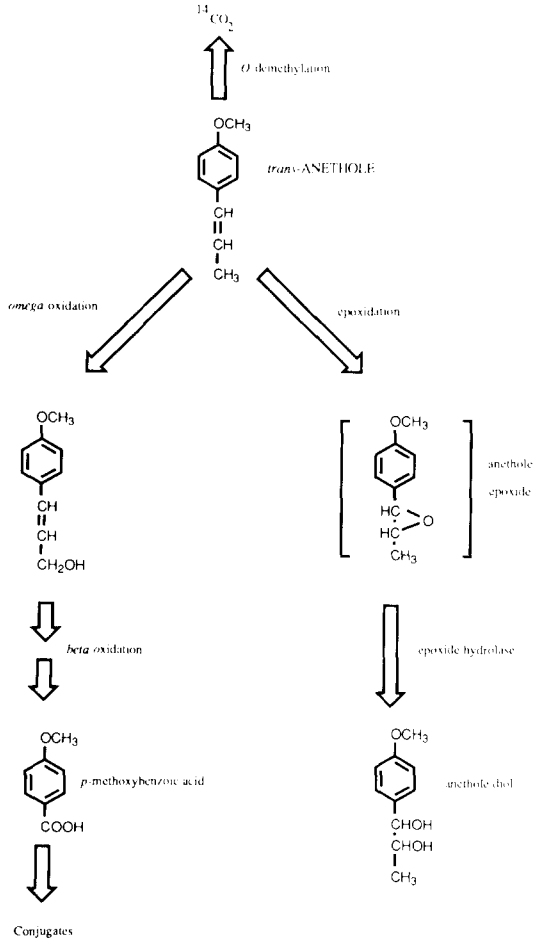

Figure 8 Metabolism of anethole.

The metabolism of anethole proceeds along three pathways (*Figure 8*), namely *O*-demethylation, omega-oxidation, and epoxidation of the side-chain double bond.[105] *O*-Demethylation leads to the excretion of conjugates of the phenol so produced, while omega-oxidation results in the excretion of *p*-methoxybenzoic acid, principally as its conjugates. Epoxidation of the double bond leads to a diol. Comparative studies have shown that mice and humans preferentially metabolize the side chain of anethole by omega-oxidation, while in the rat the epoxidation route predominates.[98,105,106] Furthermore, in rats and mice, as the dose is increased, the extent of *O*-demethylation falls, and the rate of elimination decreases, with less of the material eliminated 0–24 h after dosing and more in the 24- to 48-h collections.[107]

In contrast, the human disposition of anethole is independent of dose size, over the range from 1 mg, representative of its use as a flavor, to 250 mg, the amount present in 6–7 servings of an anise beverage.[106]

It is now possible to correlate the metabolic and toxicological findings with *trans*-anethole in both rats and humans as a function of the dose administered. These data are summarized in *Table 9*. The diols are very minor or undetectable metabolites in humans at all doses tested, whereas in the rat as the dose is increased even within the dose range used by humans these metabolites become increasingly significant. The chronic toxicity studies have shown that 140 mg/kg per day for 121 weeks represent a no observed effect level (NOEL) and at this dose at least 10% of the chemical is converted to diols in the rat. At 280 mg/kg/day there occurs hepatocellular hyperplasia, presenting a histological pattern resembling that seen after the administration of an enzyme inducer, whereas the highest dose tested, 550 mg/kg/day, as we have seen already, causes a low incidence of hepatocellular carcinoma, in only female rats. The highest dose used in metabolic studies is 1500 mg/kg and at this dose the diol pathway accounts for 15% of the dose. It is thus clear that there occurs an important and relevant variation in the fate of anethole as a function of species and dose size, which invalidates any direct extrapolation of the high-dose animal carcinogenicity data to the low-dose human dietary exposure to anethole.

Concluding comments

It seems clear that food additives, as well as nutrients can influence the outcome of interaction between chemicals and biological systems. These various studies illustrate the usefulness of metabolic and mechanistic information for the overall safety assessment of natural food flavors. The results not only aid in the interpretation of toxicity tests, permitting the elabora-

Table 9 Correlation of principal metabolic and toxicological findings with dose size in rat and humans exposed to anethole[a]

Dose (mg/kg)	Chronic toxicity	Metabolism	
		Rat	Human
0.015			1% of dose as diols
0.05		2% of dose as diols	
0.8			No diols detected
4			No diols detected
5		4% of dose as diols	
50		10% of dose as diols	
140	No effect		
280	Hepatocellular hyperplasia and other changes, resembling enzyme induction		
550	Low incidence (in historical control range) of hepatocellular carcinoma in female rats		
1500		15% of dose as diols	

[a] Data drawn from References 86, 100, and 101.

tion of metabolically and mechanistically sound mathematical models for the assessment of the human health implications of allylbenzene consumption, but also can contribute to the design of more relevant tests. Since many natural flavors occur in series that are structurally closely related chemically, metabolic and mechanistic studies can provide a sound basis for the group assessment of such series, without comprehensive testing of all individual congeners.

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Comparative studies on the metabolism of food additives: case examples in the safety evaluation of the allylbenzene natural flavors. Supported by the Flavor and Extract Manufacturers' Association of the USA, and Pernod-Ricard, Paris, France.

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