Metabolic and Mechanistic Studies in the Safety Evaluation of *trans*-Cinnamaldehyde

by

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Abstract

Concern over the human safety of *trans*-cinnamaldehyde, a very important food flavour, resulted in the withdrawal of its previously allocated ADI (acceptable daily intake) by the FAO/WHO Joint Expert Committee on Food Additives in 1989. As a contribution to the safety evaluation of this α,ß-unsaturated aldehyde, its metabolism was investigated in rat, mouse and man. ^{14}C-cinnamaldehyde was rapidly excreted (> 70% in the 0-24 h urine) and radio HPLC analysis showed that its metabolism involved principally oxidation to cinnamic acid followed by ß-oxidation, analogous to that of fatty acids, to benzoic acid which was mostly conjugated with glycine as hippuric acid (> 80% urinary ^{14}C). A second pathway of metabolism was conjugation with reduced glutathione (GSH) as indicated by the incorporation of ^{35}S-cysteine (CySH) into minor metabolites when rats and mice were given ^{35}S-CySH and *trans*-cinnamaldehyde. GC with sulfur selective flame ionisation detection and GC-MS showed the presence of mercapturic acids derived from direct conjugation of GSH at the ß-atom of the carbon double bond of cinnamaldehyde and these accounted for 7% of the dose in rat and mouse and 3% in man. The metabolism of cinnamaldehyde in rat, mouse and man was very similar which permits extrapolation of toxicological data across species.

In rat and mouse, the metabolism of cinnamaldehyde was unaltered over the dose range 2-250 mg/kg when given orally. After intraperitoneal (i.p.) administration, there was some evidence of saturation of the ß-oxidation of the side chain. The GSH conjugation pathway was unaffected by dose size and it is interesting to note that rat liver GSH levels were reduced by 26%, to 74% of control, 0.5 h after a dose of 250 mg/kg i.p. and that this returned to control after 2 h.

Studies in rat hepatocytes and rat and mouse liver cytosol in which the concentration of GSH, and the oxidation of cinnamaldehyde to cinnamic acid and its reduction to cinnamyl alcohol were modulated by specific inhibitors, showed that ^{14}C-cinnamaldehyde was bound covalently to protein, but only at high concentrations at which a measure of saturation of metabolism occurred. GSH conjugation protected against binding.

The metabolic capacity of the liver towards cinnamaldehyde by ß-oxidation is large compared with the anticipated human intake and conjugation with GSH constitutes a further detoxication mechanism. These data are discussed in terms of their importance for the human safety of this common natural food flavour.

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Dear Nick, a final word of thanks to you, the thesis is finished, I hope we will live happily ever after.

Dedicated to
Mia and Guus
Hanneke and Pascale

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Abbreviations

AAF	2-acetyl aminofluorene
ADH	alcohol dehydrogenase
ADI	acceptable daily intake
ALDH	aldehyde dehydrogenase
benz. gluc.	benzoyl glucuronide
BSO	*L*-buthionine (S,R)sulfoximine
CA	cinnamaldehyde
CAC	cinnamic acid
CALC	cinnamyl alcohol
CA-NALC	N-acetyl-S-(1-phenyl-propen-3-al)cysteine
CAC-NALC	N-acetyl-S-(1-phenyl-2-carboxyethyl)cysteine
CALC-NALC	N-acetyl-S-(1-phenyl-3-hydroxypropyl)cysteine
CYN	cyanamide
cin. glyc.	cinnamoyl glycine
CySH	cysteine
DEM	diethyl maleate
DMSO	dimethyl sulfoximine
EA	ethyl acrylate
EBSS	Earle's balanced salt solution
ETOH	ethanol
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers' Association
GC-MS	gas chromatography mass spectrometry
GRAS	generally regarded as safe
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
HCB	hexachlorobenzene
HPLC	high performance liquid chromatography
HPPA	3-hydroxy-3-phenylpropionic aicd
IARC	International Agency for Research of Cancer
JECFA	Joint FAO-WHO Expert Committee on Food Additives
LDH	lactate dehydrogenase
lmwt	low molecular weight
MAFF	Ministry of Agriculture, Fisheries and Foods
MBBr	monobromobimane

4 MP	4-methyl pyrazole
MTD	maximum tolerated dose
NALC	N-acetyl-L-cysteine
NALC-CA	N-acetyl-S-(3-phenyl-2-propenyl)cysteine
NCI	National Cancer Institute
NEM	N-ethyl morpholine
NOEL	no observed effect level
NTP	National Toxicology Program
PADI	possible average daily intake
Pr-S-S-Pr	protein mixed disulfides
PrSH	protein thiol groups
S1, S2, S3	unknown sulfur-containing metabolites
SCF	subcellular fractions
SDS	sodium dodecyl sulfate
SIM	selective ion monitoring
TCA	trichloroacetic acid
TLC	thin layer chromatography
TNBS	2,4,6-trinitrobenzene sulfonic acid

Chapter 1

Introduction

1.1 Cinnamaldehyde, an important food flavour

trans-Cinnamaldehyde (3-phenyl-2-propenal) occurs in a very large number of foodstuffs of vegetable origin and is the major constituent of cinnamon oil (up to 90%). It is a potent flavouring agent, with extensive use in beverages, ice-creams, confectionary, baked foods, chewing gums, condiments and meat preparations at concentrations ranging from 8 ppm (ice creams) to 700 ppm (sweets) (Mantovani *et al.*, 1989). It is the second most important flavour in sweet products, after the minty flavours, and its use in the USA in 1987 totalled 0.5 million kg (FEMA, 1987a).

Not much is known about cinnamaldehyde toxicity after ingestion, for it is a traditional product, derived by distillation of cinnamon bark and used throughout history for medicinal purposes and as a natural food flavour. Since 1900 cinnamaldehyde has been synthesized industrially by condensation of acetaldehyde and benzaldehyde. The Flavor and Extract Manufacturers' Association (FEMA) have given cinnamaldehyde GRAS (Generally Regarded as Safe) status (FEMA, 1965c) and it is also approved for food use by the Food and Drug Administration (FDA) of the United States. The Council of Europe (1981) included cinnamaldehyde in the ADI of 1.25 mg/kg for total cinnamyl compounds. The Joint FAO-WHO Expert Committee on Food Additives (JECFA, 1967b) initially set an ADI of 1.25 mg/kg body weight based on the toxic effect level in rats fed a diet containing cinnamaldehyde for 16 weeks (Hagan *et al.*, 1967). However, this was reduced to 0.7 mg/kg in 1984 due to lack of data on its metabolism and pharmacokinetics and carcinogenicity. The ADI was eventually completely withdrawn in 1989 subject to further data being presented. Further studies on cinnamaldehyde requested by JECFA (Anonymous, 1985b) are:

1. Short-term feeding study in a non-rodent species
2. Long-term feeding study to evaluate carcinogenic potential
3. Further metabolic and pharmacokinetic data on cinnamyl compounds

Cinnamaldehyde (Fig. 1.1) has two functional groups, an α,ß-unsaturated carbon bond and an aldehyde group which are sites of metabolism and possible toxicity. Majetti and Suskin (1977) provided evidence to support the idea that the contact sensitization of cinnamaldehyde was mediated *via* a Schiff base adduct formed by reaction of the aldehyde with protein amino groups. Boyland and Chasseaud (1967, 1968) established the reactivity of the carbon double bond towards GSH, but an epoxide intermediate, as is formed with styrene and other α,ß-unsaturated compounds, is thought unlikely (Van Bladeren *et al.*, 1981; Delbressine *et al.*, 1981).

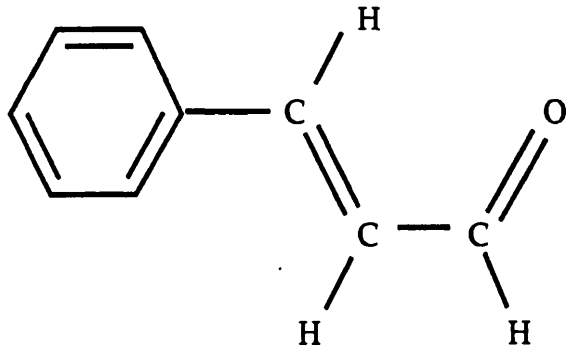


Figure 1.1 Structure of *trans*-cinnamaldehyde

Concern over the safety of cinnamyl compounds as a group, was raised when a National Toxicology Program (NTP) study showed that administration of cinnamyl anthranilate in the diet at 15000 and 30000 ppm caused liver tumours in mice but not in rats (NCI, 1980). Following these findings, JECFA (1981) has requested that cinnamyl

anthranilate be retested to clarify the effects seen in mice. The FDA later prohibited the use of cinnamyl anthranilate in human food (Anonymous, 1985a). Recent studies have related the observed toxicity to the parent ester and greatly reduced the suspicion of cinnamaldehyde and other metabolites (Caldwell, 1992).

However, cinnamaldehyde's structural similarity to a number of toxic compounds such as acrolein and crotonaldehyde and its status as a strong contact sensitizer indicate the necessity of toxicological research and further understanding of its metabolic fate after ingestion. Cinnamaldehyde is currently undergoing NTP testing in the USA (Sapienza *et al.*, 1991). Cinnamaldehyde exposure may be expected to rise in the future due to the increasing consumption of "fast foods" and convenience foods, and public concern over the use of food additives can be expected to rise accordingly.

For a better understanding of the relation between the structure of a chemical and its effect on living organisms and possible adverse effects, a knowledge of the various routes along which it can be metabolized is indispensable. The work presented in this thesis was undertaken with the aim to extend this knowledge as a contribution to the safety evaluation of this important food flavour.

1.2 Food legislation and use of toxicology in safety assessment

1.2.1 Food additives

Over the last several decades, Western societies have undergone many life style changes that have led to an increase in the addition of various substances to food for technological purposes. Processed foods now

represent over 50% of the American diet. For instance, the annual *per capita* consumption of fresh citrus fruits decreased from 32 to 28 lb between 1960 and 1976, whereas the consumption of processed fruit increased from 50 to 90 lb. Soft-drink consumption more than doubled during the same period. Several trends, such as the growth in ready to eat and snack foods, the population shift from rural to urban areas, the interest in ethnic foods, the constant year round supply of seasonal foods and the need for stable and low food prices, have increased the utilization and need for the addition of various substances to food (Hayes and Campbell, 1986). The increased use of food additives, coupled with the public demand for an essentially risk-free food supply, has raised both the scientific and public debate over the safety of these materials added to food.

Currently, there may be as many as 2800 substances used as direct food additives. Food additives can be classified into the following categories: flavourings, natural colorants, synthetic colorants, emulsifiers, thickeners, suspension stabilizers, sweeteners, antioxidants and preservatives. The vast majority of these additives are used only in trace amounts and only a few in large quantities. The FDA has estimated that sucrose, corn syrup, dextrose and salt represent 93% by weight, of the total food additives used. The inclusion of black pepper, caramel, carbon dioxide, citric acid, modified starch, sodium bicarbonate, yeasts and yellow mustard brings this figure up to 95%. It is estimated that the average person consumes 61 lb of food additives a year (Palmer and Matthews, 1986).

An adequate and safe food supply is the concern of every government, but as knowledge of toxicology and the cause of patterns of human diseases increases, as well as the ability to determine with accuracy constituents present only in trace quantities, it is becoming

progressively more difficult to decide just what is safe and what is potentially harmful to the consumer. These factors have created the necessity for a logical, rational and scientific approach to the regulation of these materials in the food supply. Decisions in this area not only impact on public health but also have economic impact on both the food industry and the consumer (Bunyan *et al.*, 1986).

1.2.2 Food legislation

In each society the rise of food trade, increasing the distance between producer and consumer, will necessitate a certain form of regulation. The oldest documented food law was found on an Assyrian clay tablet and contained the same two principles that food legislation is based on today, *i.e.* ensuring the safety and wholesomeness of the food consumed by the community (public health) and preventing fraudulent claims as to its true nature (fair trade). In the UK these two principles are translated into the "General Provisions" of the Food Act (Anonymous, 1984) which provide a basic framework covering all food legislation. They also form the basis of, for instance, the Dutch "Warenwet", a very detailed piece of legislation.

The USA has always been at the forefront of food legislation. The use of food additives is controlled by the Food and Drugs Administration. The role of the FDA is to enforce the Food, Drug and Cosmetics Act originally passed in 1906, which declares unlawful any food that contains "any added poisonous or other added deleterious ingredient which may render such article injurious to health". This act was replaced by the Federal Drug and Cosmetics Act in 1938, which still is the basic legislation, although amended in 1958. The Food Additives Amendment had a three-fold purpose. Firstly, it aimed to ensure the protection of the public health by requiring proof of safety in use of any

food additive. Secondly, it provided regulations of the safe use in foods of substances deemed technologically necessary to improve the food supply and, thirdly, the forbidding for use in food of any substance in any amount whatsoever which is found to induce cancer when ingested by man or animal (the Delaney Clause) (Merill, 1986).

These amendments were of considerable importance as they contained the Delaney Clause, which has resulted in a tremendous increase in toxicological testing, as well as introducing the concept of GRAS. GRAS-status is given to "any substance which is generally recognized among experts qualified by scientific training to evaluate its safety, as having been adequately shown through scientific procedure or experience based upon common use in food, to be safe under the conditions of its intended use". GRAS substances are not subject to restrictive control in the same way as are the defined and permitted food additives. In 1958, the FDA drew up a list of substances used in the manufacture of food dividing food additives into two groups. Those substances whose use in food was GRAS and secondly, substances that either the FDA or the US Department of Agriculture had sanctioned for use in food prior to 1958 (Merill, 1986). Apart from legislating and government bodies, a number of other organisations are involved in food regulation. Cooperation between these and the government bodies is important for exchange of knowledge and a smooth introduction of new regulations. In the USA, FEMA set up an Expert Panel in 1960 which was given the task of accumulating data on the usage of flavouring materials. A list of substances classified as GRAS was published in 1965 (Heath, 1981).

Internationally the use of food additives is regulated by a committee known as the Joint FAO/WHO Experts Committee on Food Additives (JECFA). It is the job of this committee to lay down a flexible

framework for the toxicological evaluation of food additives. The Council of Europe is a body of the United Nations, established in 1949, including all the countries in the European Community (EC) and some more. Like JECFA, this is not a statutory authority, but its advice is influential. The EC initiates legislation to harmonize the different compositial requirements of member states so as to enable food legally produced in one country to be accepted by another, with the purpose of reducing barriers to trade. The European Commission, which initiates Community policy, takes advice on the composition and safety of both food and its ingredients from the Scientific Committee for Food, which was set up in 1974 and is composed of representatives from fifteen member states (Heath, 1981). The Council of Ministers of the EC propose legislation, which has to be adopted by the individual member nations. Legislation within the community is still the responsibility of the individual national governments, although the aim is to come to unified regulations before the end of 1992. Currently the members of the EC differ considerably in their attitudes to food additives and flavourings and the unification is a slow process (Hardinge, 1990).

1.2.3 Flavours

Among the food additives, flavourings take a special place. The use of flavouring ingredients is a long-standing practice both domestically and in the commercial processing of foods. Historically, most flavourings are of natural origin e.g. vanillin from vanilla pods, cinnamaldehyde from cinnamon bark, eugenol from clove buds and anethole from fennel. As society moved towards industrial production of foods, many of these are now replaced by flavourings which simulate their natural counterpart, thus ensuring an uninterrupted supply and a "nature identical product" of high purity (Hall, 1981). In view of the very large number of materials used, their diversity, low usage rate and low total

consumption, as well as their low cost relative to other food ingredients, flavourings have not been subjected to any extensive toxicological evaluation to establish their safety in use either in the short or long term. To illustrate this, the number of permitted food colours in the UK is 45, while 13 preservatives are allowed, compared to thousands of flavourings. Analysis of an apple reveals more than 300 components, while 5000 nature-identical materials are currently available to the industry. The second problem is their usage levels. For a preservative to function it may be present in quantities of 0.2%, while a stabilizer will be present in higher levels. The vast majority of flavouring substances are used in only ppb amounts (Hardinge, 1990).

Flavourings are currently not controlled by a specific statutory instrument. In the USA flavours are included on FEMA and FDA GRAS lists. Between European countries there are differences in opinion concerning the legal status of food flavourings. Ethyl acetate is considered a natural flavour in Italy, nature-identical in Germany and artificial in France. The European Commission first met to discuss this in the mid 1970s and only in 1988 did a council directive appear, covering the approximation of the laws of the member states relating to flavourings for use in foodstuffs and to source materials for their production. This is a framework directive covering labelling, definitions and some general purity criteria. Over the past 20 years the UK Ministry of Agriculture, Fisheries and Foods (MAFF) has considered the problem several times and it has been examined by the ministry advisory committees. Legislation was not enacted however, leaving the group to be covered as stated by the General Provisions of the Food Act (Hardinge, 1990). Recently the first two pieces of legislation have been accepted in the UK (Anonymous, 1992). However, a consensus on the safety evaluation of flavours on the basis of a real health risk posed by this class of additives, has never been

reached.

1.2.4 Risk of food additives

The relation between diet and health has become evident from epidemiological studies. Most studies concentrate on cancer, one of the main causes of death in western societies. The major preventable risk factors that have been identified thus far are tobacco, dietary imbalances, hormones and high dose occupational exposure (Ames and Gold, 1990). An estimated 35% of all cancer deaths in man is related to factors present in our food (Doll and Peto, 1981; Table 1.1). These can be macro as well as micro nutrients, natural toxins or additives. Much information concerning the relation between food and cancer is derived from "migrant studies". Epidemiological studies concerning Japanese who emigrated to Hawaii showed that cancer incidence is mainly determined by environmental factors (Haenszel *et al.*, 1973). The third generation Japanese had a pattern of cancer incidence identical to that of the indigenous Hawaiian population: a low incidence in stomach cancer and a high incidence of colon cancer, reversing the pattern in Japan. The environmental factors influencing the incidence and type of cancer are not so much industrial chemicals, but rather life style and diet, as is shown from research with members of the Mormon church in the United States. The incidence of cancer is much lower among this part of the population than among people who do not follow the same codes of behaviour, such as abstaining from the use of coffee, tea, alcohol, cigarettes and meat (Banbury Report 4, 1981). Recently, the National Cancer Institute (NCI) of the USA published a ten year prospective study of a large population and confirmed earlier investigations that diet was a major factor in the development of colon cancer. Parameters that correlated best with the development of cancer were a high fat diet and low vegetable consumption (Thun *et al.*, 1992).

Table 1.1 Proportion of cancer death attributed to various factors

Factor	% of all cancer death
Diet	35
Tobacco	30
Infection	10
Reproduction/ sexual behaviour	7
Occupation	5
Alcohol	3
Geophysical factors	3
Pollution	2
Medicine and medical procedure	1
Industrial products	< 1
Food additives	< 1

From Doll and Peto, 1981

Although studies indicate that diet is an important risk factor, less than 1% of the cancer deaths were attributed to food additives. As may be seen from Table 1.2, this is not in proportion to the attention this group of chemicals has received from the general public. Table 1.2 lists the six major areas of concern the FDA in the USA has identified with regard to food-related health hazards and compares them to the measure of hazard perceived by the public. Although food additives are judged to be the least significant of these risks, it is this area which has aroused the most public concern (Hall, 1971; Caldwell and Sangster, 1985).

The discrepancy between the public perception of risk and that of scientists is also seen in the assumption that "natural is safe and artificial is toxic". On a scientific basis this holds little value, since artificial additives are generally based on natural compounds with

Table 1.2 Order of priority of six areas of health hazard in relation to diet, in order of significance

FDA priority	Public concern
1. Nutritional excesses or deficiencies	1. Food additives
2. Microbiological contamination	2. Pesticide residues
3. Environmental contamination	3. Environmental contamination
4. Natural toxicants	4. Microbiological contamination
5. Pesticides	5. Natural toxicants
6. Food Additives	

From Hall, 1971

similar toxicities and many of these are present in plant products which form a natural part of our diet. These include solanine in potatoes, peptide mycotoxins or glycosides such as amygdalin and cycasin, while some 12 naturally occurring chemicals important for the flavour characteristics of herbs and spices, among which safrole and allyl isothiocyanate have been identified as posing some degree of toxic risk (Hall, 1973; Ames and Gold, 1990; Ames *et al.*, 1990).

In conclusion, on the basis of epidemiological data the use of food additives, although causing much concern to the consumer, presents only a minor health hazard. However, the increasing use of flavours and our changing diet may introduce certain risk factors which may not show in epidemiological studies. Toxicity studies can identify these active components in our diet, naturally occurring or added, and thus provide a logical approach to hazard identification and safety evaluation.

1.2.5 Role of toxicity studies in safety evaluation

Additives which have not been given GRAS status or are not covered by previous rulings require complex scientific testing before their use is legally permitted in foods. MAFF, the regulatory authority in the UK, and the FDA in the US have laid down rules which require food additives to be tested for potential toxicity and carcinogenicity by performing experiments on laboratory animals. Although there are no standard protocols for testing in the UK and no regulations are in force for the toxicity testing of food flavours, MAFF has promulgated certain guidelines for animal testing, outlining the type of test required for other food additives (MAFF, 1965). In the United States, a formal protocol is laid down by the FDA, which must be satisfied for all proposed food additives (FDA, 1982). This entails the following steps:

1. Definition of test material (often mixtures of chemicals, impurities).
2. Exposure assessment, including the intake level for high level consumers.
3. Acute toxicity. The evaluation of LD_{50} and target organ studies involving single-dose and multiple-dose studies in both rats and mice of both sexes and in one other species, preferably a non-rodent. Depending on the outcome of this, a decision is made to either discontinue testing at this point or continue to the next testing stage.
4. Biotransformation and pharmacokinetic studies, which may yield data useful in ascertaining the quantitative and qualitative characteristics of the compound's absorption, biotransformation and excretion and possibly provide evidence of species differences in these parameters. If studies reveal tissue storage of either the parent compound or its metabolites, this may indicate that the substance is unsuitable as a food additive.
5. Mutagenicity tests. The mutagenic potential of an additive is assessed

in vivo and *in vitro* during short-term studies. Evidence for chemical interactions with DNA may be used as a basic indication that the additive could be carcinogenic. If genetic toxicology studies reveal that the substance is not mutagenic and may not initiate carcinogenesis, the compound may be accepted at this point.

6. Subchronic toxicity studies, including a 90 day feeding study at several doses and study for potential reproductive toxicity and teratogenicity. The test substance may be accepted if consumption levels for high consumers are relatively low and structure activity relationships, genetic toxicology and biotransformation reveal no evidence of carcinogenicity.

7. Chronic toxicity studies to follow the exposure of the test substance over the greater part of the animal's life span, approximately 80 weeks in mice and 2 years in rats. At post-mortem a report on histological examination of all major organs is submitted.

Standard toxicity studies involve dose regimes based on the Maximum Tolerated Dose (MTD) (FDA, 1982). The MTD is defined by the International Agency for Research of Cancer (IARC, 1981) as "the highest dose of the test agent during the chronic study that can be predicted to cause some toxicity when administered for the duration of the study, but will not:

a. induce overt toxicity resulting in appreciable death of cells or organ disfunction,

b. toxic manifestations that are predicted to reduce the life span of the animal other than carcinogenicity and

c. 10% or greater retardation of body weight gain compared with control animals."

The MTD is determined from 30 day and 90 day studies. A typical

dosing for a NTP long term carcinogenicity study presently employs doses of 1/4 MTD, 1/2 MTD and MTD. Other doses used, below the MTD, are generally chosen to help define a No Observed Effect Level (NOEL), where no lesions are produced over the "normal" background response. The NOEL is used to calculate the ADI in man which JECFA (1967a) defined as "the daily dose of a chemical that appears to be without appreciable risk (to man) on the basis of all the known facts at the time". This is the average daily amount of a substance (mg/kg body weight) which is acceptable for life time exposure without causing any damage to health. The ADI is calculated by dividing the NOEL by a large safety factor, usually 100. A factor of 10 allows for the possibility that man is more sensitive than the experimental animals and a further factor of 10 for possible differences between individuals. The difficulties encountered when making these extrapolations are discussed below.

GRAS substances do not require the same rigorous testing because they were included on the list before modern methods and concepts of toxicological evaluation had been developed. In 1968 there was a Presidential directive to the FDA to readdress the status of substances listed as GRAS. In part the review of GRAS substances was sparked by the discovery of the suspected carcinogenicity of the artificial food sweetener cyclamate. This produced concern that other GRAS substances may also have unrecognized toxicities. Still the procedure for GRAS substances is not as rigorous, but it is going from the concept of GRAS substances being "evaluated for lack of evidence of hazard" to the inclusion of these substances as food additives "evaluated for the evidence of safety" (Hayes and Campbell, 1986).

1.2.6 The value of metabolic and pharmacokinetic studies in the safety assessment of food additives

The requirement that food additives newly introduced on to the market be proven safe, together with the need to reevaluate those compounds which have been given temporary GRAS status, has created a demand for toxicological testing greater than the supply. In addition, the length of time and cost of comprehensive testing have become simply impossible to afford. Many million dollars, in each case, have been spent on toxicological work on cyclamates, saccharin, Red No 2 and monosodium glutamate. Yet it is clear that from a regulatory standpoint the safety of these long-used ingredients is far from well established. It is neither possible nor sensible to try and obtain the information needed to assess every imaginable toxic risk associated with every substance. The pursuit of greater safety, therefore, demands the setting of priorities on a rational basis (Cramer *et al.*, 1978). Metabolic and kinetic studies can contribute to this in two ways.

Firstly, comparative metabolic studies provide an insight into the mechanism whereby a compound exerts its toxicity. The toxic response to a chemical does not depend on the absolute dosage, but on the concentration-time profile of the ultimate reactive species, which may be the parent compound or its metabolite(s), at the target site. Biotransformation studies provide information on whether the toxicity of significant metabolites needs to be studied in further detail and can be used to obtain particular information on the role of metabolic activation and detoxication pathways. If biotransformation-pharmacokinetic studies indicate that the substance is biotransformed to metabolites with known toxicity, it will be unnecessary to determine this. However, in cases where metabolites of unknown toxicity are detected further study may be necessary. If metabolic activation to products forming covalent

adducts is encountered, results should be compared with genetic studies. This knowledge thus helps to set sensible limits to investigation and may aid in the prediction of possible risk of related substances.

Secondly, knowledge of metabolism and pharmacokinetics is necessary for a proper interpretation of data from animal experiments for their relevance to man. In some cases the occurrence of the pathway and the formation of toxic metabolites may be species dependent. Examples of such species differences will be discussed later in this thesis. Comparative metabolic data improve the assessment of true exposure on a *trans*-species basis rather than consideration of "dosage" alone. Chemicals administered to animals undergo metabolism to a variable extent and along various metabolic pathways, which may themselves be influenced by animal species, sex, strain as well as by dose size, frequency and route of administration. It is therefore inappropriate to translate dose size across species unless the appropriate metabolic and pharmacokinetic data are available (Wagner, 1992; Monro, 1992).

As described before, toxicity testing involves doses based on the MTD. The reasons for using dose levels which are millions of times the estimated human daily intake of food additives are two-fold. Firstly, large doses are used to increase the poor sensitivity of the test method, so that no outcome however small is missed by the experimenter and to allow statistical evaluation of the toxicity/tumour incidences found in the treated and control animals. Secondly, the administration of smaller doses closer to human exposure levels, which would have much less evident effects, would necessitate the use of a very large number of animals to achieve statistical significance, which in itself is economically prohibitive (ECETOC, 1982).

In such experiments, the assumption is made that the toxic or carcinogenic response to a chemical is linear with dose and that the high administered doses proportionately increase the incidence of toxicity or tumours and (perhaps) shorten the latency period of carcinogenicity. The restriction at this point is that the pharmacokinetics of compounds may differ at high doses from low doses. In many cases, the metabolic profile is determined by the amount administered, as well as the species in question. The use of very high doses in toxicity testing may give metabolic patterns, and therefore biological responses that are unrepresentative of the situation at the actual level of exposure. Therefore, data on the influence of dose levels on metabolism in the test animal should be generated to determine whether adsorption, metabolism or excretion processes may have a threshold. An animal model, apparently suitable at one level of exposure, may be less appropriate at a different level (Monro, 1992).

Thus, both the relevant animal species and dose regime must be identified if one aims to extrapolate from animal toxicity data to the toxicity in man. In interpreting such data, attention should be paid to the similarities of the mechanisms of toxicity in the various animal species, and also to the possibility that toxicity may involve interactions between the parent compound and its metabolites, which may not be the same in all animal species and may be irrelevant for man (JECFA, 1987).

The usefulness of comparative metabolic and kinetic data in humans and in animal species used for testing in the safety assessment of natural food flavours is evident from the study of the natural allylbenzenes. The carcinogenicity of allylbenzenes as a class arises from metabolic activation by hydroxylation at the benzylic (1'-) carbon atom of the side chain, followed by sulfation. The facile loss of O-sulfate results in carbonium ions capable of forming adducts with

DNA, which may be assumed to play a role in tumorigenesis (Boberg *et al.*, 1983). This mechanism is similar to that of safrole, which has now been banned for use as a food additive (Wislocki *et al.*, 1976).

For allylbenzenes it can be shown that the activation reaction is dose dependent and this confounds attempts to extrapolate animal data from carcinogenicity studies where large doses are required to elicit tumours (at least 0.46% (*w/w*) of the diet over at least 12 months), compared to the minute quantities present in the human diet (of the order of 70 µg/*capita*/day). An example is formed by estragole (*p*-methoxyallylbenzene), which is a murine hepatocarcinogen (Miller *et al.*, 1983). Estragole is metabolized along three distinct pathways, 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 (Anthony *et al.*, 1987). With increasing dose, the importance of the O-demethylation pathway decreases and the other pathways, including the formation of the proximate carcinogen 1'-hydroxyestragole, increase. Thus, the exposure to the proximate carcinogen increases disproportionately with dose from 1 to 10%. Human metabolic data show that at a dose of 100 µg (close to the dietary intake levels) 1'-hydroxyestragole excretion accounts for only 0.3% of the dose (Sangster *et al.*, 1987).

Eugenol, although an allylbenzene, is apparently not a carcinogen in rodents (Miller *et al.*, 1983; NTP, 1983), a finding accounted for by its atypical metabolism (Sutton *et al.*, 1985; Sutton, 1986), which involves facile conjugation of the free hydroxyl group with sulfate or glucuronic acid, and consequent rapid elimination. Furthermore, the 1'-hydroxylation is absent and gut microflora reduce the allylic double bond, thereby reducing the reactivity of the benzylic carbon and effectively preventing metabolic activation.

These and similar data illustrate the usefulness of metabolic information in the specific context of the safety assessment of natural food flavours (JECFA, 1987). This type of data not only aids the interpretation of toxicity tests, making it possible to devise soundly based mathematical models for the assessment of the implications for human health of the consumption of allylbenzenes and other dietary toxicants, but also permits the design of more relevant tests. In addition metabolic data are important in the establishment of testing priorities, by directing limited resources towards compounds most likely to constitute human health hazards. It must not be overlooked that many natural flavours consist of closely related chemical series and the establishment of structure-metabolism relationships, as has been achieved with the allylbenzenes, aids in the group assessment of such series without the need for comprehensive testing of individual congeners (Caldwell *et al.*, 1990a,b).

1.3 Biotransformation of xenobiotics

1.3.1 Introduction

Upon entering the body, a xenobiotic may be eliminated unchanged, retained unchanged or undergo spontaneous chemical transformation, but the majority of compounds will undergo biotransformation. The process of biotransformation, in which several enzymes participate, can conveniently be divided in Phase I and Phase II reactions. Phase I reactions involve oxidation, reduction and hydrolysis reactions and Phase II conjugation reactions or syntheses. Phase I reactions generally convert xenobiotic chemicals to more hydrophilic derivatives by unmasking or introducing functional groups such as hydroxyl, sulfhydryl or carboxylic groups. Phase II reactions are conjugations of the xenobiotic or metabolites derived from Phase I with endogenous

molecules such as glucuronic acid, sulfate or GSH. In general these two reactions facilitate transport and enhance elimination *via* renal or biliary routes (Williams, 1959).

The group of cytochrome P450 isoenzymes is the most important Phase I enzyme system. The microsomal P450 enzyme system consists of various cytochrome P450 isoenzymes and NADPH-cytochrome reductase. It is involved in various metabolic reactions. At least three main types of activities can be distinguished namely mono-oxygenase, oxidase and reductive activity. Other Phase I enzymes are aldehyde and alcohol dehydrogenases (ALDH and ADH), important in the metabolism of cinnamaldehyde. The major conjugation reactions are listed in Table 1.3 and of these the glycine, glucuronic acid and GSH conjugation will be discussed later on in greater detail.

Table 1.3 The eight classical conjugation reactions

Reaction	Conjugating agent
A. Reactions involving activated conjugating agents	
Glucuronidation	UDP-glucuronic acid
Glucose conjugation	UDP-glucose
Sulfation	PAPS
Methylation	S-adenosyl methionine
Acetylation	Acetyl CoA
Cyanide detoxication	Sulfane sulfur
B. Reactions involving activated foreign compounds	
GSH conjugation	GSH
Amino acid conjugation	Glycine, Ornithine, Taurine

The main site of metabolism of foreign compounds is the liver although extrahepatic tissues, frequently the site of entry or excretion to or from the body e.g. the skin, lungs, kidneys and gastrointestinal mucosa, also play a role in the metabolism of xenobiotics (Gibson and Skett, 1986 and references therein).

1.3.2 Factors influencing metabolism of xenobiotics

Factors influencing the rate and extent of metabolism *via* Phase I or Phase II reactions *in vivo* can be physicochemical, endogenous or exogenous (Table 1.4). These are important in determining the biological effect of a xenobiotic. The intracellular concentration of a chemical is primarily dependent on dose and physicochemical and structural properties. Since metabolism of most foreign compounds is carried out by enzymes, any factor which can influence the activity of these can alter metabolism.

Table 1.4 Physicochemical, endogenous and exogenous factors affecting the rate and extent of metabolism of xenobiotic chemicals *in vivo*

Physicochemical	Endogenous	Exogenous
Electrophilicity	Age	Dose
Nucleophilicity	Sex	Nutrition
Lipophilicity	Species	Route of administration
Polarity	Strain	Time of day
Protein binding	Pathology	Enzyme inhibition
	Genetic deficiencies	Enzyme induction
	Cofactor availability	

As described in section 1.2 the extrapolation of data from animal toxicity studies, which employ high doses, to the human situation of life-long, low exposure is complicated by any factor that influences the

rates and routes of metabolism of the chemical under study, thereby altering the duration and extent of internal exposure to the chemical and its active metabolites, with possible consequences for toxicity.

1.3.2.1 Species differences in metabolism and toxicity

Species differences in detoxification are most significant in metabolism but differences also occur in absorption, distribution and excretion of foreign compounds. Species differences may occur in absorption due to pH differences in the gastrointestinal tract, in distribution due to differences in plasma binding and bioavailability. Differences in excretion can be due to functional differences in kidney and urine pH, biliary excretion due to species differences in molecular weight thresholds, bile flow and pH of the bile (Timbrell, 1987).

Species differences in drug metabolism reflect differences in the activities of the enzymes responsible for the various transformations. Such variations could arise from differences in the absolute activities of the enzymes or the amounts of any endogenous inhibitors present, or the extent of any reverse reactions which might occur. Species differences occur in both Phase I and II metabolism and can be either quantitative (same metabolic route but different rates) or qualitative (different metabolic routes). Inter-species differences in metabolism are most apparent in the occurrence of conjugation reactions or the relative capacity of these (Caldwell, 1988). Given that species differences do occur, it is possible to suggest that they arise from one or more of three origins.

Firstly, differences in metabolism can be due to deficiencies in certain enzymes and lead to a defect in a metabolic reaction which is otherwise widespread in occurrence. An example is the defect of glucuronidation

in the cat and related species and acetylation of aromatic amines in the dog. The increased toxicity of glucuronidogenic compounds in the cat has been illustrated, while the failure of N-acetylation of aromatic amines in the dog is presumably the reason for increased toxicity of *p*-aminobenzoic acid and various hydrazines in this species (Caldwell, 1978).

Secondly, restricted species occurrences are seen for the particular amino acid (glycine, glutamine, taurine or ornithine) that is used in the conjugation of acids. For benzoic, heterocyclic and cinnamic acids, most species use glycine, which is replaced by ornithine in birds. Of special interest are the reactions which are restricted to primates such as the glutamine conjugation of phenylacetic acid for they present complications in the choice of animal species suitable for testing (Caldwell *et al.*, 1980).

The most common species differences are in rates of metabolism of a compound rather than the particular pathway along which it is metabolized. An example is found in the metabolism of amphetamine which undergoes either aromatic hydroxylation giving 4'-hydroxyamphetamine, or side chain degradation to benzoic acid. The compound is metabolized extensively in most species, but the nature of metabolites formed is variable. In the rat ring hydroxylation predominates, in the guinea pig chain breakdown is the major route, and in other species both routes are significant (Caldwell, 1980, 1986, 1992)

1.3.2.2 Differences in metabolism and toxicity due to differences in dose size

To avoid missing a small effect that would be seen on a large number of

rodents and to allow for the low dose but life time exposure as to flavouring agents in man, standard toxicity studies involve dose regimes based on very high doses often in a more than thousand-fold excess of the estimated human exposure. The assumption is made that the toxic response to a chemical shows a linear relationship with dose. However, at high levels of exposure, normal major pathways of metabolism and the capacities of the various primary mechanisms involved in the absorption, distribution and excretion may become saturated so that secondary mechanisms come into play. This phenomenon is commonly referred to as "metabolic switching" (Feron and Kroes, 1986). Thus, before an extrapolation can be made, data on the influence of dose levels on metabolism in the test animal should be generated to determine whether absorption, metabolism or excretion processes may have a threshold.

The influence of dose size on rates and routes of metabolism and its consequences for toxicity has been discussed earlier for the allylbenzenes. At low dose, the metabolism of these food flavours follows largely the safe metabolic pathway *via* O-demethylation, but this pathway is saturated at higher doses, leading to a disproportionate increase in metabolism *via* the proximate carcinogen, 1'hydroxylation and enhanced toxicity (Caldwell *et al.*, 1990a,b).

The influence of dose size on metabolism and toxicity has been clearly demonstrated for paracetamol, a very safe drug at low doses, but a hepatotoxin at large doses, when primary routes of metabolism become saturated. At low dose paracetamol metabolism occurs mainly *via* sulfation and glucuronide conjugation. A small portion of the dose is metabolized by hepatic mixed function oxidases to a reactive electrophile that is detoxified by conjugation with GSH. When large doses of paracetamol are absorbed the normal detoxication pathways

are saturated and a greater fraction of the dose is metabolized *via* cytochrome P450 which can lead to depletion of hepatic GSH. If this occurs to an appreciable extent the reactive metabolite can bind covalently with cellular macromolecules and this may lead to cell death and tissue necrosis (Mitchell *et al.*, 1975).

When, in contrast saturation of an activating pathway occurs, a single high dose will have a lesser effect than repeated administration of small doses, as in the case of isoniazid. Given in large doses to experimental animals, isoniazid does not cause hepatic necrosis, whereas several smaller doses do, probably because acetylation is saturated at high doses and the drug is metabolized by other routes (Timbrell, 1987).

An example of how dose size can affect disposition, is provided by 1-naphthylacetic acid excretion in the rat. This compound undergoes conjugation with either glycine or glucuronic acid and is subsequently excreted. The glycine conjugate is largely excreted in the urine whilst the glucuronide is secreted in the bile and excreted in the faeces. At low doses, the majority of the dose is conjugated with glycine and so present in the urine, but at higher doses, the glycine conjugation mechanism becomes saturated and a greater proportion of the dose undergoes conjugation with glucuronic acid and is so excreted in the faeces (Dixon *et al.*, 1977).

These examples indicate that information on metabolic switching in the test species should be gathered before metabolism and toxicity data may be extrapolated from high to low dose.

1.3.2.3 Sex differences in metabolism and toxicity

There is strong evidence for the existence of sex-related differences in

susceptibility to environmental and occupational toxic substances. These differences occur in a broad range of species including humans and in response to a large number of substances. In addition, the magnitude of such differences is not trivial and may be more than ten-fold. Routine toxicity tests are therefore performed both in male and female animals. However, regulatory authorities have not yet responded by taking these differences into account when setting safety standards (Calabrese, 1985).

Sex-related differences in toxic susceptibility in humans include examples of epidemiological studies in which men appear more sensitive to cigarette smoking-induced lung cancer (Haenszel and Tauber, 1964) and aflatoxin-induced liver cancer (Shank *et al.*, 1972). Women are more susceptible to agents such as lead (Roels *et al.*, 1979) and lithium (Lloyd *et al.*, 1973) and retain benzene for a significantly longer time (Sato *et al.*, 1975). A biochemical explanation for these differences is often not provided. A well known sex difference is the greater alcohol tolerance in male humans as compared to females. Researchers used autopsied specimens and found no sex differences in liver ADH activity, but a markedly greater renal ADH activity was found in men as compared to women (Ohno *et al.*, 1970).

Studies using laboratory animals show that sex differences occur in metabolism, biliary secretion and excretion (Smith, 1973). A clear example of sex differences in susceptibility is that of the renal toxicity of chloroform in mice, where males are markedly more sensitive than females (Eschenbrenner and Miller, 1945). However, sex differences in metabolism are most apparent in the rat and less in other species. They are mostly due to the influence of sex hormones, and can be reversed by hormone treatment. In general male rats metabolize foreign compounds more rapidly than females, although sex differences in

metabolism depend on substrate. The implication of this for toxicity depends upon whether a compound is detoxified or activated by metabolism. Sex differences in metabolism in the rat include examples of different P450-activities and differences in the rate of glucuronidation and GSH conjugation. In the rat, females are at least 100 times less sensitive to the renal toxicity of decalin than males (Alden *et al.*, 1983) and males display a 21-fold greater activity in the O-dealkylation of coumarin (Kamataki *et al.*, 1980). Most P450-mediated reactions display 3 to 4 times greater activity in the male, such as the hydroxylation of hexobarbital, which is 3.5 times greater in the male (Kato and Gillette, 1965) and accounts for the longer hexobarbital-induced sleeping time in females as compared to males.

D'Amour and Charbonneau (1992) related differences in toxic susceptibility to hexachlorobenzene (HCB) in male and female rats to the metabolism of this compound. HCB induces hepatic porphyria and liver cancer in female rats, whereas toxicity is minimal in male rats. Oxidation of HCB to pentachlorophenol and tetrachlorohydroquinone has been associated with hepatic porphyria, whereas the production of sulfur-containing metabolites appears to represent a detoxication pathway. In male rats detoxication *via* GSH conjugation occurred to a much greater extent than in females, while in females a higher percentage of the dose was excreted as oxidative products. Hepatic GSH and GSH S-transferase (GST) activity (μ-class) were three times higher in male control rats as compared to females and this activity was more inducible by HCB treatment.

1.3.2.4 Influence of route of administration

Dependent on the route of administration a "first pass effect" will occur in the first organ that is reached upon administration. Thus, after oral

administration the majority of the dose would be expected to enter the liver *via* the hepatic portal vein, which links the small intestine with the liver, before entering the general circulation. In some cases hepatic removal of a compound, either by binding to hepatic macromolecules such as ligandin, elimination into the bile and most commonly metabolism, may be so complete that very little of the parent compound reaches the systemic circulation. After i.p. administration a large part of the dose may be eliminated by metabolism in the gut mucosa and the liver and will never reach the general circulation. A large dose given i.p., may reach the liver as a bolus that overwhelms the capacity of the enzymes involved in metabolism, and part of the dose would then return to the circulation and possibly undergo metabolism in other organs.

A wide variety of metabolic reactions are associated with the intestinal tract mucosal cells. The extent to which these occur is influenced by the particular site in the enteric tract, the compound under study and species. It would appear that the Phase II conjugation reactions, particularly those involved with glucuronic acid conjugation and sulfation, are of more significance than the Phase I reactions, which activities are much smaller than those present in the liver. The contribution of the intestinal mucosa in the metabolism of isoprenaline is apparent, it undergoes extensive sulfation when administered orally, but when given intravenously it is found in the urine unchanged and as an O-methyl conjugate, but the sulfate is not found (Caldwell and Smith, 1977).

The role of the intestinal microflora in drug metabolism and its implications in toxicology have been reviewed in detail (Scheline, 1973). The gut flora is capable of effecting a wide range of metabolic reactions some of which appear to have no counterpart in mammalian

tissues. Enterobacterial metabolism is probably of more significance in laboratory animal species than in man, because the gastrointestinal tract of laboratory species such as rat, mouse and guinea pig is extensively colonized along the whole tract, whereas in the case of man this begins from the distal small intestine onwards. Since absorption by passive processes occurs mainly in the proximal small intestine this means that in man most compounds taken by mouth will have been absorbed before coming into contact with the flora in the distal part of the tract. However, with laboratory animal species such as the rat, extensive enterobacterial metabolism of drugs administered orally may occur.

1.3.3 Role of metabolism in toxicity

Phase I and II metabolism will usually result in a more polar product with greater molecular weight, which will facilitate excretion and thus lead to detoxication of the parent compound. However, it has become increasingly clear that many toxicological effects of xenobiotics are mediated through the formation of reactive metabolites ("toxication, "metabolic activation" or "bioactivation"). Bioactivation of chemicals is now known to be catalyzed by almost all of the enzymes involved in the process of biotransformation. For example, conjugation with GSH will mostly convert a xenobiotic to a metabolite less toxic than the parent compound, but the conjugation of dibromoethane with GSH, catalyzed by GSTs results in the formation of a reactive thiiranium ion (Van Bladeren *et al.*, 1980), which is responsible for the carcinogenicity of this compound in rats and mice. Thus, the biological activity of a substance is not dependent on the absolute dose but rather on the concentration of the ultimate reactive species at the target molecules in the cell. Factors important in determining the toxic response following such interaction of the reactive species with the targets are:

1. The nature of the ultimate reactive species - in many cases a reactive intermediate - and the availability of target molecules at the place of formation. Reactive intermediates that have been identified in causing toxicity are electrophilic structures such as epoxides, quinones and nitrenium ions, radicals and other reduced forms of oxygen and reactive oxygen species. Depending on the properties of the electrophiles certain sites in cellular macromolecules will be favoured. One of the most successful theories in this respect is the theory of "Hard and Soft Acids and Bases" (Pearson and Songstad, 1967). This theory predicts that hard electrophiles (low polarizibility and small atomic radii) will preferentially react with nucleophiles, while the soft species (high polarizibility and relatively greater atomic radii) will react best with each other.

2. The role of the target molecules in cell function. Of primary importance in cell function are the energy supply and the integrity of cellular membranes. The cell needs to have sufficient ATP to perform transport, synthesis and repair processes in an adequate way. A reduction in the ATP synthesis, for example as a result of uncoupling of the oxidative phosphorylation, can cause cell death. Other critical targets are enzymes and nucleic acids.

3. The effectiveness of cellular defence mechanisms in detoxifying the active species and repairing initial damage. Detoxication reactions will compete with the reaction of the reactive intermediate with tissue macromolecules and convert them into harmless metabolites that are eliminated from the body. For epoxides, for instance, epoxide hydrolase as well as the GSTs are of major importance in their detoxication. For other electrophiles, in addition to the GSTs, GSH itself and sometimes amino acids such as methionine constitute a first line of defence. The relative concentration of activating and detoxifying

enzyme systems determines the extent of toxicity. Any factor influencing these activities, will influence the toxic response.

1.3.4 Reactive intermediates

Early studies by Brodie *et al.* (1971) and Reid *et al.* (1973) demonstrated the role of drug metabolism in the hepatotoxicity of bromobenzene. The inducer of cytochrome P450, phenobarbitone, increased the severity of the necrosis, whereas inhibitors of this enzyme, such as SKF, decreased toxicity, indicating the importance of the P450 monooxygenase system in the metabolic activation of this compound. The protective role of GSH and hence the electrophilic nature of the reactive metabolite involved was demonstrated by increased liver necrosis in bromobenzene-treated rats after pretreatment with diethyl maleate (DEM), a compound known to drastically reduce GSH levels in the liver (Reid and Krishna, 1973). On the basis of the major urinary metabolites and the correlation of hepatotoxicity with changes in their ratio, hepatotoxicity was ascribed to the bromobenzene-3,4-epoxide (Jerina and Daly, 1974). More recent *in vitro* studies (Buben *et al.*, 1988; Narasimhan *et al.*, 1988) indicate that the quinone product of this (ortho quinone of bromobenzene) is the ultimate reactive intermediate. Quinones can be detoxified by GSH in two ways, either by a GST-catalyzed addition or by reduction in which the quinone acts as an electrophile or oxidator.

Nitrenium ions are an example of electrophilic structures generated by biphasic metabolic activation. 2-Acetyl aminofluorene (AAF) causes liver cancer in rodents and the primary target of this compound is the C-8 position of guanine in DNA. The bioactivation of AAF proceeds *via* oxidation by cytochrome P450 on the N-position and sulfation on the newly introduced hydroxy group, after which desulfation ($OHSO_3$ is a

good leaving group) leads to the formation of a nitrenium ion, which is the ultimate carcinogen (De Baun *et al.*, 1970). Sulfate conjugation is also the activating step in the metabolism of the natural occurring food flavour safrole (Borchert *et al.*, 1973.).

Free radicals are an important group of reactive intermediates. The one electron reduction of many chemicals is catalyzed by the microsomal NADPH-cytochrome P450 reductases. Free radicals can induce a range of effects from membrane damage and inactivation of enzymes to cell death and cancer. The role of radicals in toxicity has been extensively studied in the liver necrosis caused by halomethanes such as the commonly used solvent chloroform (Pohl *et al.*, 1977, 1979; Pohl and Krishna, 1978). The trichloromethyl radical, formed by the reductive activity of P450, causes lipid peroxidation and GSH protects against this lipid peroxidation *in vivo* (Pohl *et al.*, 1981).

Reactive oxygen species include superoxide anions, hydrogen peroxide and hydroxyl radicals, the latter being the most reactive. All these are involved in membrane damage, cardio and neurotoxicity, cellular oxidative stress and cancer (Trush *et al.*, 1982). In the cell these species are formed endogenously as a result of respiration or autooxidation of haem proteins (uncoupling of P450 oxidase activity), but also after exposure to xenobiotics, such as quinones and hydroquinones which undergo redox cycling and lead to one-electron reductions of oxygen. An important example is the herbicide paraquat that causes damage to lung tissue in man (Cagen and Gibson, 1977).

Reactive oxygen species are detoxified by three classes of enzymes; peroxidases are specific for hydrogen peroxide, superoxide dismutases detoxify superoxide anion radicals to O_2 and H_2O_2 and these are in turn detoxified by catalases. Non-enzymatic defence is also

involved in the protection against reactive oxygen species. Important radical scavengers are vitamin C in the cytosol and vitamin E, which is embedded in membranes and protects against lipid peroxidation. During detoxication Vitamin C and E radicals are formed, but these are less reactive than the oxygen species due to resonance stabilization and can be regenerated by reduction by GSH dependent systems. The GSSG (oxidized glutathione) so formed is then regenerated by NADPH dependent GSH reductases (Trush *et al.*, 1982).

1.3.5 Mechanisms of cell death

1.3.5.1 Covalent binding

In 1968, Boyland and Chasseaud suggested that a probable function of GSTs in the cell was to protect important thiol containing enzymes and other cellular proteins. In 1969, these investigators expanded this concept and postulated that a probable function of the GSTs was the protection of cellular constituents from strong electrophilic agents (Boyland and Chasseaud, 1969a). Furthermore the concept was emerging that the toxicity of various chemicals correlated well with the binding of their electrophilic metabolites to macromolecules. Brodie, Gillette and coworkers at the National Institute of Health investigated this using bromobenzene, a model for phenyl-type drugs (Brodie *et al.*, 1971; Reid *et al.*, 1973; Jollow *et al.*, 1974) and the commonly used analgesic paracetamol (Potter *et al.*, 1973; Jollow *et al.*, 1973; Mitchell *et al.*, 1973) and on the basis of their results the following theory was developed. Reactive intermediates can bind covalently to cellular macromolecules such as nucleic acids, proteins, cofactors, lipids and polysaccharides, which can have a number of effects such as reduced energy production, changes in membrane permeability and inhibition of the synthesis of macromolecules. An early effect after an overdose of

bromobenzene or paracetamol *in vivo* is depletion of cellular GSH in the liver. A similar effect is seen *in vitro* at high concentrations and it seemed therefore that the GSH system is important in the detoxication of reactive intermediates of both xenobiotics. GSH protects protein thiol (PrSH) groups in hepatocytes against the attack of toxic metabolites and it was shown that thiol depletion was followed by an elevation of the cytosolic Ca^{2+} concentration. In the cell Ca^{2+} is stored in the endoplasmic reticulum, mitochondria and cytosol and in the transport of Ca^{2+} between these cell compartments, thiol dependent Ca^{2+}/Mg^{2+}-ATP-ases play an important role. From this it was suggested that a disturbance of the Ca^{2+} homeostasis is related to a disturbance in the thiol homeostasis. A disturbance in Ca^{2+} homeostasis can ultimately lead to altered permeability of cellular membranes, which then causes a disruption of the protective compartmentalization of the cell and its environment, release of lysosomal hydrolases and enhanced peroxidation of unsaturated fatty acids.

In conclusion, the sequence of processes leading to cell death is thought to be: Bioactivation to a reactive intermediate --> interaction with cellular macromolecules (covalent binding) --> disturbance of the thiol homeostasis--> disturbance of the Ca^{2+} homeostasis --> activation of lipases and cytomorphological changes ("blebbing") --> cell death.

1.3.5.2 Oxidative interactions

A second model for the relation between reactive intermediates and cell death involves oxidative stress. Oxidative stress occurs when the demand for reducing equivalents exceeds the amount needed for normal functioning of cells. Reducing equivalents are stored in NADPH, GSH, PrSH and vitamins C and E and GSH plays a central role in this

storage system. Oxidative stress can be caused by reactive oxygen species and quinone structures that undergo redox cycling and thus overwhelm the capacity of the defence mechanisms (Orrenius, 1985). In addition to redox cycling, quinones can also directly interact with components of the reductive defence mechanism by oxidation of NADPH and thiols and by conjugation with thiols. In order to distinguish between these two actions, studies were performed with paracetamol and its 3,5-dimethyl analogue. The ultimate reactive intermediate, the quinone imine of paracetamol, undergoes both reduction and conjugation, but the quinone imine of 3,5-dimethyl paracetamol only undergoes reduction. The cytotoxicity of the quinone imine of paracetamol is comparable to that of the parent compound: GSH depletion followed by cell death, whereas the substituted paracetamol and its quinone imine are not cytotoxic. These results indicate the importance of covalent binding of paracetamol metabolites in cell death as compared to redox cycling (Nicotera *et al.*, 1989; Weis *et al.*, 1992). However, other studies showed that prevention of oxidative stress by catechin, an anti-oxidant, prevented paracetamol cytotoxicity and did not influence covalent binding (Albano *et al.*, 1985). Probably, GSH depletion causes those conditions under which "oxidative stress" can be induced in cells. The importance of redox cycling was also shown in 6-hydroxydopamine neurotoxicity, which is associated with the compound's capacity to undergo auto-oxidation, rather than an interaction with thiol groups.

The sequence of events leading to cell death in this case is thought to be: Bioactivation --> GSH depletion --> oxidative stress --> depletion of reducing equivalents --> elevation of Ca^{2+} concentration.

Elevation of Ca^{2+} levels may be achieved in many ways. Firstly, by the Ca^{2+}/Mg^{2+}-ATP-ase, which is activated by oxidation of thiol groups,

but also by mitochondrial damage, caused by oxidation of pyridine nucleotides NAD(P)H, which can lead to an elevation of cytosolic Ca^{2+} by release from mitochondria.

Oxidative stress may lead to other changes in the cell apart from disturbances in Ca^{2+} homeostasis. One example is membrane damage caused by lipid peroxidation of unsaturated fatty acids in cell membranes, endoplasmic reticulum or mitochondria. Lipid peroxidation involves a reaction sequence initiated by an H-atom abstraction from the membrane fatty acids, leading to the ultimate formation of malon(di)aldehyde (Kappus, 1986). However, lipid peroxidation and cell death often coincide so that cause and result can not be distinguished. Many findings, such as the fact that the disulfide reducing agent dithiothreitol protects against toxic effects of paracetamol in hepatocytes, but not against lipid peroxidation, have caused doubt for the causal relationship of lipid peroxidation and cell death (Younes and Siegers, 1981).

1.3.6 Glutathione

In all of the processes described above GSH plays a central role as a conjugating agent, provider of reducing equivalents and as cofactor in GSH peroxidases. The importance of GSH in detoxication of xenobiotics and endobiotics, as well as in physiological processes such as protein function and in regulatory processes, is becoming increasingly clear (Meister, 1988; Reed, 1990). In the next paragraph this will be further discussed. Fig. 1.2 shows pathways involved in GSH metabolism and conjugation.

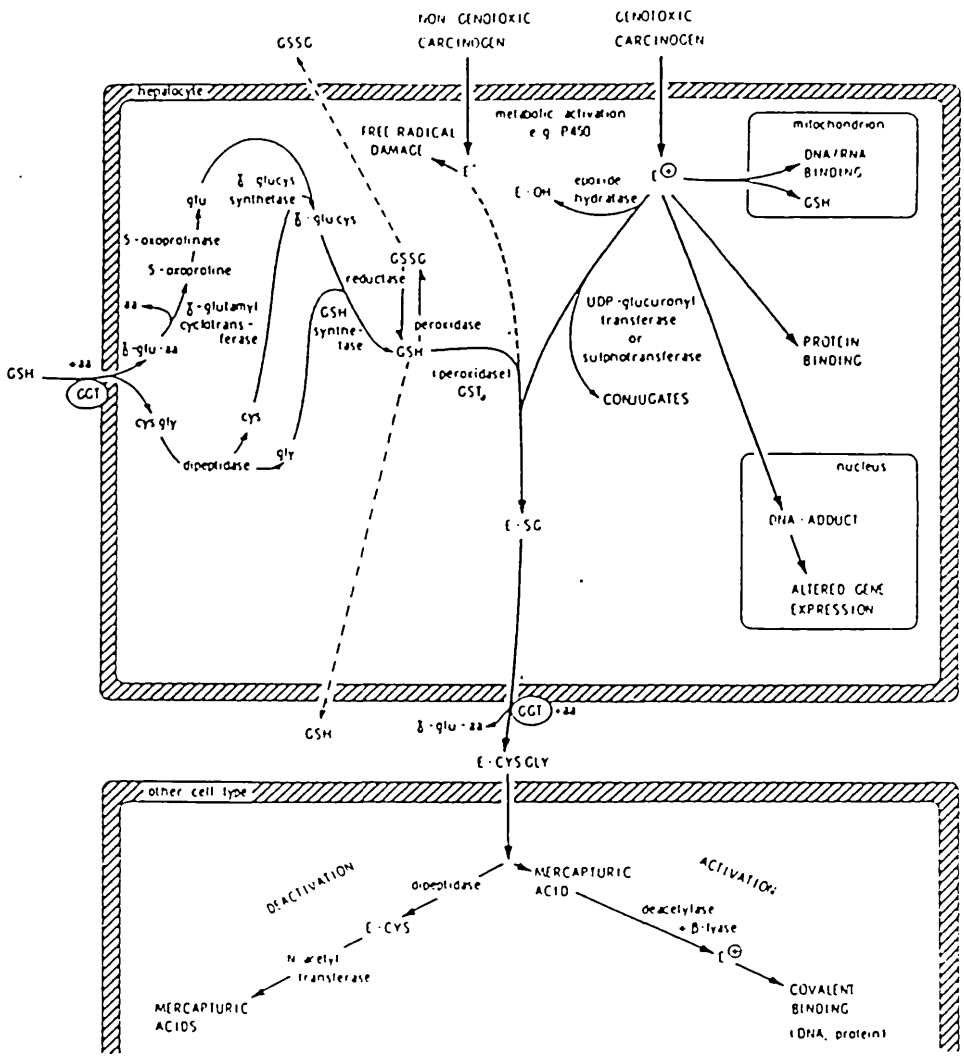


Figure 1.2 A schematic representation of some of the ways in which GSH and GSTs may be involved in a cell's response to a carcinogen or xenobiotic

1.3.6.1 Biosynthesis of glutathione

GSH is present in bacteria, plants and animal tissues as a characteristic component of nearly all living cells. First isolated in a crude from by de Rey-Pailhade in 1888, GSH was obtained crystalline from yeast by Hopkins in 1921 and subsequent studies showed that the molecule was a tripeptide of L-glutamic acid, L-cysteine and glycine (Fig. 1.3). The sulfhydryl group is highly reactive due to its ionization potential and the high electron density of its sulfur atom.

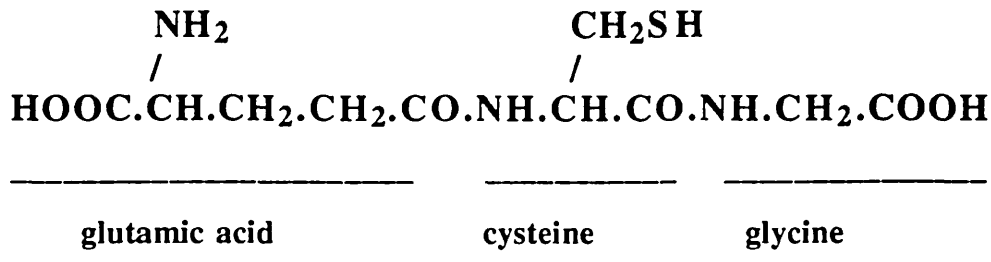


Figure 1.3 Structure of glutathione

The biosynthesis of GSH involves two reactions, catalyzed by two different enzymes. First, a dipeptide is formed from L-glutamate and L-cysteine by glutamylcysteine synthetase, the cytosolic rate limiting enzyme. The tripeptide GSH is then synthesized from γ-glutamylcysteine and glycine *via* GSH synthetase. The synthesis of GSH takes place mainly in the liver. Part of the hepatic GSH will enter the blood plasma. Once it is present in the blood, it can be transferred to other organs (Reed, 1990). Typical concentrations in the liver are in the millimolar range (5-10 mM), concentrations in blood are *ca* 0.5 mM (Cotgreave and Moldeus, 1986).

1.3.6.2 Glutathione as a redox system

As described before, GSH and its oxidized form, GSSG, represent the major thiol redox system of the cell. GSH in the cell is maintained in the reduced state (GSH:GSSG ratio of 250:1) by the enzyme GSH reductase which is coupled to the NADP/NADPH redox pair. GSH protects cells from toxic effects of reactive oxygen species in order to maintain normal membrane integrity and cytoskeletal organization. For instance, hydrogen peroxide and other organic peroxides are reduced by GSH in a GSH peroxidase-catalyzed reaction. This reaction results in the formation of GSSG, which can be reduced by NADPH. NADPH

is formed from NADP at the expense of glucose-6-phosphate *via* the enzyme glucose-6-phosphate dehydrogenase. GSH also provides the reducing equivalents for the maintenance of other low molecular weight thiols, such as cysteine and coenzyme A, *via* the activity of GSH transhydrogenase (thioltransferase) enzymes. Protein mixed disulfides can be reduced *via* this route as well (Reed, 1986).

1.3.6.3 Protein S-thiolation

It used to be thought that a depletion of GSH of more than 40% of control caused a disturbance in the cell great enough to cause cytotoxicity *per se*. Various recent investigations have indicated that PrSHs, more so than non-PrSHs, are critical for the maintenance of cell viability during toxic chemical insult. It is interesting to mention that the α,ß-unsaturated diketone, DEM, can remove essentially all the GSH in hepatocytes in suspension but is not cytotoxic, whereas cinnamaldehyde causes cytotoxicity at concentrations that deplete only 40% of GSH. DEM did not affect PrSH groups, while cytotoxic concentrations of cinnamaldehyde reduced the number of free PrSH groups by *ca* 20% (Swales, 1993). Thiol groups are essential for the activities of many enzymes, including the membrane bound Ca^{2+} translocases. It has been proposed that GSH maintains cell viability *via* the maintenance of membrane PrSH groups, through thiol-disulfide exchange reactions. Increased oxidation of GSH to GSSG *in vivo* can promote protein S-thiolation, leading to the formation of sulfur-bridges between proteins (protein mixed disulfides, Pr-S-S-Pr). In addition to protein disulfides, low molecular weight disulfides may participate in this process such as GSSG, cystine, and cystamine. So far, cellular S-thiolation has been shown to occur with four enzymes, glycogen phosphorylase, creatine kinase, a protein inhibitor and glyceraldehyde 3-

phosphate dehydrogenase, but it may play a role in many other cellular processes such as hormone release and protein synthesis (Cotgreave *et al.*, 1990).

Generally, a 15-30% decrease in PrSH content elicits rapid cell death (Cotgreave *et al.*, 1990 and references therein). However, paracetamol did not cause cytotoxicity even when PrSH was depleted up to 40% of normal values. This indicates that the cytotoxicity of a particular compound may depend not just on the degree of PrSH modification, but on the additional toxic manifestations which the compound may inflict upon the cell. One such determinant is thought to be the subcellular localization of GSH and the different functions of GSH in the various cell compartments. Depletion of GSH in mitochondria in particular has been implicated in cytotoxicity. Meredith and Reed (1982, 1983) showed that the onset of injury in isolated rat hepatocytes by ethacrynic acid correlated well with the depletion of mitochondrial GSH, whereas the cytosolic pool could be depleted without affecting cell viability. The redox status of mitochondrial GSH may influence intramitochondrial PrSH groups and the integrity of mitochondrial membranes and thus influence cellular concentrations of free Ca^{2+}, probably as a result of secondary alterations of mitochondrial membrane sulfhydryl groups that are involved in Ca^{2+} retention. It is believed that homeostasis of thiols and Ca^{2+} in the mitochondria are closely linked together either directly or through the pyridine nucleotides; an imbalance in one could affect the status of the other and diminish cell viability (Reed, 1990). Recently, high levels of GSH have been determined in the nucleus and a role for GSH in the protection against DNA damage and in the regulation of physiological processes, such as the transport of mRNA into the cytoplasm and in nuclear division, has been proposed (Tirmenstein and Reed, 1988; Bellomo, 1992).

1.3.6.4 Glutathione S-transferases

A wide range of electrophilic, lipophilic reactive metabolites can be inactivated by conjugation with GSH (Chasseaud, 1979). This inactivation is mediated by GSTs, primarily located in the cytosolic fraction, but also present in microsomes (Morgenstern *et al.*, 1988) and nuclei (Tirmenstein and Reed, 1988). All substrates for GSTs, also react spontaneously with GSH. The quantitative importance of GSTs in the detoxication of highly reactive metabolites is an area which has received little attention. Often the non-enzymatic reaction is so rapid, that the contribution of the enzyme-mediated reaction is difficult to measure. In *in vitro* systems, CySH is often just as effective as GSH in preventing binding of electrophilic reactive metabolites to macromolecules. This indicates that many reactive metabolites will react spontaneously with sulfhydryl-containing agents, since CySH is not accepted as a substrate by GSTs. It is therefore likely that GSH conjugates formed *in vitro* are a result of spontaneous chemical reactions (Tunek *et al.*, 1980). Boyland and Chasseaud (1968; 1969a) showed that there is no relation between the rate of the spontaneous and enzymatic reaction. DEM, for instance did not react with GSH in buffer, but was one of the best substrates in the presence of cytosol or GST. The K_m of GST enzymes is in the order of 0.1 mM (Ketterer *et al.*, 1988), making them good catalysts even when GSH levels are low *i.e.* the maximum rate is achieved even when liver GSH is 90% depleted (Polhuijs *et al.*, 1992).

The activity of GSTs is normally highest in the liver and these enzymes comprise nearly 5% of the total soluble hepatic protein in the rat and 3% in man (Jakoby and Habig, 1980). Apart from catalyzing addition or substitution reactions of GSH with electrophiles, GSTs may have a further detoxifying function by serving as an irreversible or reversible

binding site for these reactive substances (Ploemen *et al.*, 1991). It is also of interest to note that some GSTs are localized within discrete nuclear regions in the interchromatinic domains. Therefore, one may expect more specific physiological functions of the enzymes and of the S-conjugates in addition to the detoxication function of this class of enzymes (Cotgreave *et al.*, 1990; Corcoran and Ray, 1992).

Because of the central role GSH and GSTs play in many cellular processes, the reaction of a compound with GSH *in vivo* and *in vitro* is a marker for its potential for toxicity. Cinnamaldehyde is known to react with GSH and in relation to the safety evaluation it is therefore important to determine the relative extents to which this and other metabolic pathways contribute to its detoxication.

1.4 Routes of metabolism that may be of importance in the fate of cinnamaldehyde

From the previous paragraph it follows that the pathways along which a compound is metabolized and excreted determine its toxicity and knowledge of these is necessary before a proper safety evaluation of its use can be made. Possible routes of importance in the metabolism of cinnamaldehyde and toxicities associated with reactive intermediates in these pathways for related compounds are discussed below.

Cinnamaldehyde has two structural groups, an α,ß-unsaturated carbon bond and an aldehyde grouping and these are the sites of metabolism and possible toxicity.

1.4.1 Metabolism of aldehydes

1.4.1.1 Oxidation and reduction

Aldehydes are biotransformed in the body by oxidation and reduction. The principal enzyme systems involved in these redox reactions are ADH and aldehyde reductase, which catalyze reductions of aldehydes, and oxidizing systems such as ALDH and aldehyde oxidase.

ADHs are cytosolic enzymes, located primarily in the liver. Some ADH activity is associated with the endoplasmic reticulum. These enzymes catalyze the oxidation of alcohols while reducing NAD+ to NADH and can also perform the reverse reaction, the reduction of aldehydes, using NADH as a reductant. Aldehyde reductases are cytosolic enzymes, which typically use NADPH as a source of reduction equivalents. In the cell the NADH/NAD+ ratio is low, but the NADPH/NADP+ ratio is high. By using NADPH as a cofactor these enzymes achieve reductive reactions in an oxidizing environment (Sladek *et al.*, 1989).

$$CH_3CH_2OH + NAD^+ \xrightarrow{\text{Alcohol Dehydrogenase}} CH_3CHO + NADH + H^+$$

ALDHs oxidize aldehydes to carboxylic acids with NAD+ as cofactor. Isoenzymes of ALDH have been characterized in rat liver cytosol, microsomes and mitochondria. The different ALDH isoenzymes have varying specificities to a wide range of substrates and demonstrate differential inhibition to a number of chemically diverse compounds. In general acetaldehyde is oxidized by mitochondrial ALDH, whereas xenobiotics seem to be substrates for cytosolic and microsomal

forms of ALDH (Siew *et al.*, 1976).

$CH_3CHO + NAD^+ + H_2O$ --------> $CH_3COOH + NADH + H^+$

Aldehyde Dehydrogenase

In mitochondria a specific formaldehyde dehydrogenase enzyme is found which is GSH dependent. The reaction product, a GSH thiol ester, is irreversibly hydrolysed to GSH and formate by a second specific enzyme, S-formylglutathione hydrolase. GSH is not consumed during the reaction, but is released in its reduced form (Koivusalo *et al.*, 1989).

Formaldehyde + GSH + NAD^+ ---> S-formylglutatione + NADH + H^+

Formaldehyde dehydrogenase

In general, oxidation is more important than reduction in the metabolism of aldehydes. The reduction is also reversible, whereas oxidation of the aldehyde to a carboxylic acid is essentially irreversible (Sladek *et al.*, 1989)

Williams (1959) reported that all aromatic aldehydes investigated undergo oxidation in the animal body to the corresponding acids. The enzymatic aspects of this oxidation have not been widely investigated. Liver ALDH is known to oxidize benzaldehyde and salicylaldehyde at a relatively slow rate but other aromatic aldehydes have not been investigated. Flavin-linked aldehyde oxidase from pig liver oxidizes benzaldehyde and salicylaldehyde at a four to five times slower rate than at which it oxidizes acetaldehyde. The oxidation of aromatic aldehydes *in vivo*, however, is usually complete, except in cases of the

phenolic aldehydes which are capable of undergoing the alternative reactions of sulfate and glucuronic acid conjugation at the phenolic hydroxyl group. Reduction of the aldehyde to an alcohol is also a possible reaction but has not yet been observed with aromatic aldehydes.

1.4.1.2 Reaction of aldehydes with thiols and amines

Aldehydes constitute a group of relatively reactive organic compounds characterized by the presence of a carbon-oxygen bond. The oxygen atom is highly polarized and therefore aldehydes have substantial dipole moments. Nucleophilic attack on the carbonyl moiety by thiols and amines results in hemiacetals and Schiff bases respectively. Aldehydes also have reactivity towards these groups in proteins. From the initial adduct of aldehydes with CySH or GSH, a thiazolidine can be formed. Attack of a second thiol or amine on the initial adducts can result in protein-protein, DNA-protein or DNA-DNA cross-linking. For saturated aldehydes the main targets in these molecules are amino groups, for example guanosine. In α,ß-unsaturated aldehydes, such as cinnamaldehyde, the ß-carbon atom of the unsaturated double bond is the prime target for soft nucleophiles like GSH or CySH. The mechanism of reaction appears broadly similar for a range of α,ß-unsaturated species *viz* attack of GS- on the unsaturated double bond to give an adduct, which is then protonated by solvent. In principle this reaction is reversible and the alkylating aldehydes might be released at some other site (Monks *et al.*, 1990). Thus, *in vitro*, aldehydes can react with GSH, cause DNA cross linking and bind to protein amino and thiol groups. Cinnamaldehyde reacts both with amino (Majetti and Suskind, 1977) and thiol (Weibel and Hansen, 1989a,b) groups in proteins *in vitro*.

1.4.1.3 Toxicity of aldehydes

Aldehydes as reactive intermediates have received increasing attention. Muconaldehyde is the putative myelotoxic metabolite of benzene (Goldstein *et al.*, 1981), acetaldehyde is thought to be the reactive species in ethanol toxicity (Weiner *et al.*, 1988). Many aldehydes are formed endogenously, such as lipid peroxidation products of membranes including 2-alkenals and 4-hydroxyalkenals (Esterbauer *et al.*, 1982), for which the body has a number of powerful enzyme systems. Ingestion of aldehydes may however perturb the balance between aldehyde formation and breakdown and raise the concentration of endogenous aldehydes by perturbating the cells redox state and competition with aldehyde metabolizing enzymes (Tipton, 1990).

Feron *et al.* (1991) reviewed the toxicity of aldehydes present in our food and drink water. The α,ß-unsaturated aldehyde, acrolein is irritating and strongly cytotoxic, but there is no convincing evidence that acrolein possesses genotoxic or carcinogenic activity. Crotonaldehyde possesses genotoxic activity; it is an irritant to the respiratory tract of rats and the stomach of rats and mice and hepatotoxic and carcinogenic to rats, producing in this species a variety of non-neoplastic and neoplastic liver lesions including hepatocellular carcinomas, and possibly also tumours in other organs. The authors conclude that although for some aldehydes carcinogenic potential has been demonstrated (formaldehyde, acetaldehyde) and some are cytotoxic (acrolein, crotonaldehyde), in general there is a lack of data for the risk assessment of this group of chemicals and more data on mutagenicity, carcinogenicity and cytotoxicity should be provided. Cinnamaldehyde's structural resemblance to the α,ß-unsaturated aldehydes acrolein and crotonaldehyde necessitates further study of this

compound.

1.4.2 Biotransformation and toxicity of α,ß-unsaturated compounds

1.4.2.1 Glutathione conjugation

As described in the former paragraph, aldehydes may be conjugated with GSH *via* the aldehyde group, to form a hemiacetal. However, α,ß-unsaturated aldehydes such as cinnamaldehyde are most likely to react with GSH *via* a Michael addition at the ß-carbon of the unsaturated double bond (Schauenstein *et al.*, 1977; Esterbauer *et al.*, 1975; Esterbauer *et al.*, 1976). Boyland and Chasseaud (1968) found that cinnamaldehyde reacts with GSH at the same rate as its hemiacetal, which shares the α,ß-unsaturated double bond, but not the aldehyde group, and concluded that therefore the reactivity must reside in the double bond. The GSH conjugation either proceeds spontaneously or catalyzed by the group of GSTs. The family of GSTs consists of many isoenzymes with a broad overlapping substrate specificity. GSTs of man, rat and mouse are generally classified in three groups namely class α (basic), class μ (neutral) and class π (acid) according to their structural and enzymatic properties. Until now, at least ten different GST isoenzymes have been purified from liver, kidney, lung, intestine and heart tissue of the rat, activities being highest in the liver. In man multiple isoenzymes have been isolated from different tissues (Vos *et al.*, 1988). The α and μ class both show remarkable polymorphisms in man. Patterns of the α class isoenzymes vary widely among individuals differing from tissue to tissue within individuals, whereas the μ class isoenzyme, which catalyzes the conjugation of GSH and epoxides of

polycyclic aromatic compounds, are absent in 40-50% of the population. Soluble GSTs function as dimers, with each subunit functioning independently. Boyland and Chasseaud (1968) isolated a form catalyzing the metabolism of α,ß-unsaturated carbonyl compounds from rat liver cytosol and showed that cinnamaldehyde was a good substrate for this enzyme.

1.4.2.2 Mercapturic acid pathway

Due to the high molecular weight and ampholitic character of GSH conjugates elimination in the bile is preferred. So far GSH conjugates have been detected only in bile and not in urine. The biliary pathway may therefore be the only pathway for their elimination. The S-substituted GSH undergoes further metabolism in the bile and in the kidney after resorption from the gastro-intestinal tract by a number of pathways of which the mercapturic acid pathway (Fig. 1.4) is most common.

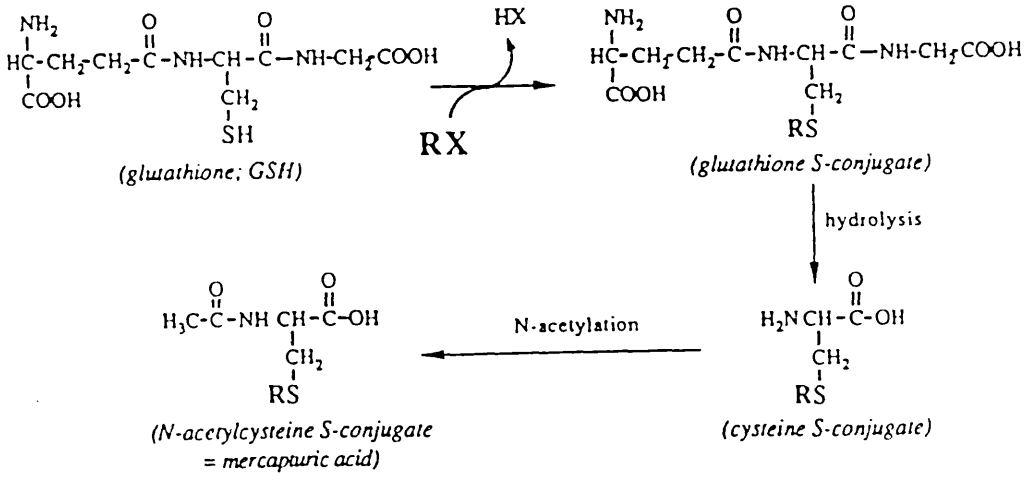


Figure 1.4 The mercapturic acid pathway: initial conjugation to glutathione to an electrophile RX is followed by hydrolysis to the corresponding cysteine

The S-substituted glutathione first loses the γ-glutamyl moiety, under the influence of γ-glutamyl-transpeptidase, and then the glycine residue to give the corresponding S-substituted cysteine by cysteinylglycinase. This is then N-acetylated by N-acetyl transferases and the resulting mercapturic acid (S-substituted N-acetylcysteine) is excreted in the urine (Knight and Young, 1958; Barnes *et al.*, 1959; Bray *et al.*, 1959; Boyland and Chasseaud, 1969b; Wood, 1970).

Fig. 1.4 shows that the GSH conjugate as well as the products of further metabolism are thioethers, which can be defined as organic compounds in which two hydrocarbon moieties are joined by an atom of sulfur, *i.e.* R'-S-R". In recent years, there has been a considerable interest in possible fates of CySH conjugates. In addition to N-acetylation, they may undergo (Fig. 1.5):

a. C-S Cleavage by tissue and intestinal microflora lyases giving rise to alanine and a thiol. The thiols so produced are readily methylated, and the thiomethyl conjugates are frequently oxidized to the corresponding sulfoxides and/or sulfones.
b. Transamination yielding first thiopyruvates, which are then reduced to thiolacetates or decarboxylated to thioacetic (thioglycolic) acids, and
c. Sulfoxidation

Of these pathways the formation of mercapturic acids is quantitatively the most important, but the ß-lyase pathway (b) is now realized to be toxicologically highly significant in a number of cases. These reactions have been reviewed by Jakoby and Stevens (1984), Bakke (1986) and Commandeur and Vermeulen (1990) and are further discussed below.

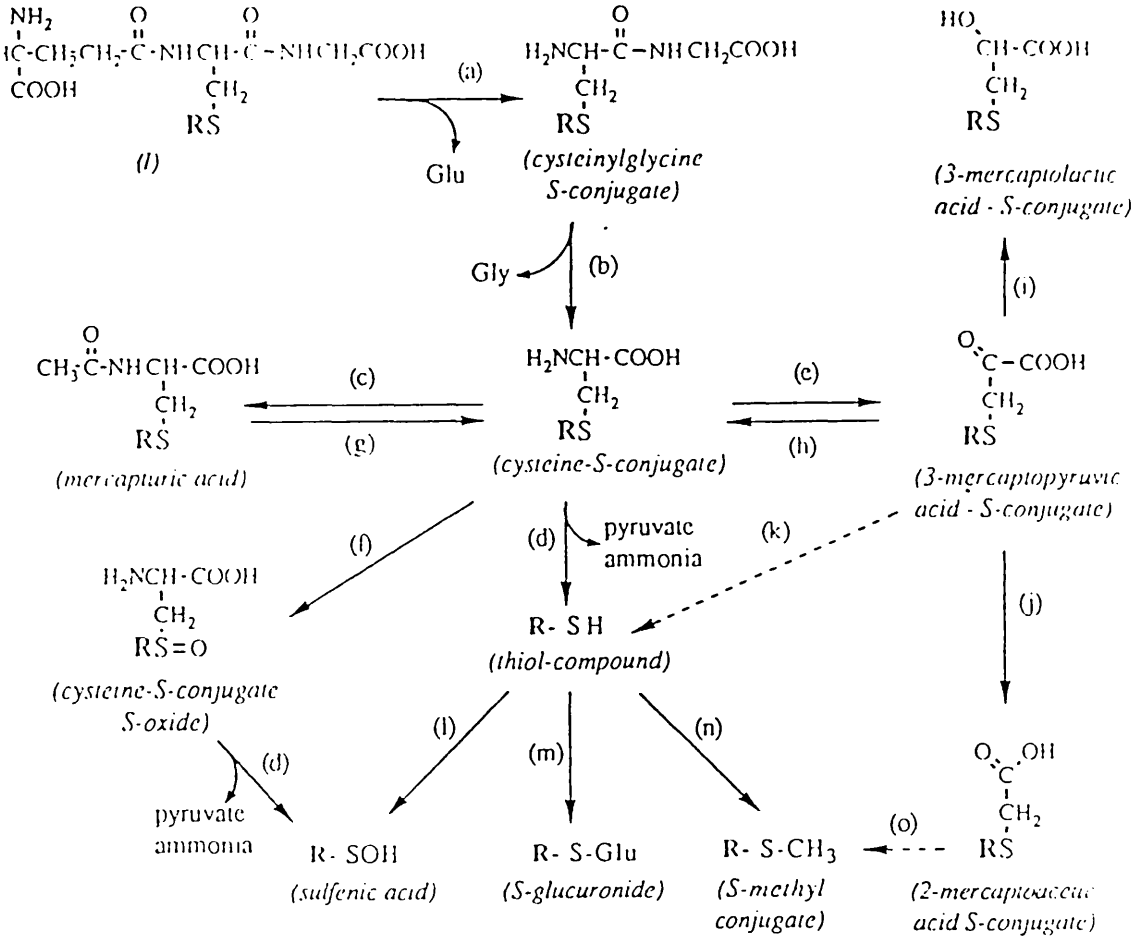


Figure 1.5 Possible routes of catabolism of glutathione S-conjugates *(I)*. Steps are catalyzed by: (a) γ-glutamyl transpeptidase; (b) dipeptidases;cysteinyl glycine dipeptidase and aminopeptidase M; (c) cysteine conjugate N-acetyltransferase; (d) cysteine conjugate ß-lyase; (e) cysteine conjugate transaminase and l-amino acid oxidase; (f) cysteine conjugate S-oxidase; (g) N-deacetylase; (h) transaminases; (i) 3-mercaptopyruvic acid S-conjugate reductase; (j) decarboxylase; (k) unknown; (l) S-oxygenase; (m) UDP-glucuronyl transferase; (n) S-methyl transferase; (o) decarboxylase. S-Methyl conjugates may undergo further metabolism to the corresponding sulfoxides and sulfones
From: Commandeur and Vermeulen, 1990

1.4.2.3 P450-mediated metabolism of α,ß-unsaturated compounds

Apart from a direct addition of GSH to the ß-carbon atom of the double bond, α,ß-unsaturated compounds may also form an epoxide across

the double bond by the action of cytochrome P450 mixed function oxidases. The mercapturic acids derived from the GSH conjugate of the epoxide can be determined in urine as a marker for the presence of this metabolite *in vivo*. Van Bladeren *et al.* (1981) investigated the formation of epoxides using the compounds acrylonitrile, crotonitrile and cinnamonitrile and found that hydroxymercapturic acids accounted for only 33%, 9% and 2% of total mercapturic acids in 0-24 h rat urine respectively. Delbressine *et al.* (1981) did not identify such an epoxide-derived mercapturic acid in urine of rats after cinnamaldehyde. Other acrylates led to the excretion of mercapturic acids in urine of rats, but mass-spectral analysis confirmed that these compounds arose from conjugation with GSH with acrylic esters by a Michael type reaction. No mercapturate esters could be detected from GSH conjugation with epoxy esters (Delbressine *et al.*, 1982). Thus, epoxide formation can be a minor pathway in the metabolism of some α,ß-unsaturated aldehydes, but is most unlikely in the case of cinnamaldehyde.

1.4.2.4 Toxicity associated with glutathione conjugation

In a number of cases it has been demonstrated that GSH conjugation may constitute a bioactivation pathway. The reactive GSH conjugate of 1,2-bromoethane (thiiranium ion) has already been mentioned. Other GSH conjugates require further metabolism, such as S-(1,2-dichlorovinyl)-GSH conjugates, which are metabolized to the corresponding CySH conjugates and bioactivated by renal CySH conjugate ß-lyase to yield cytotoxic or mutagenic metabolites. More than thirty years ago the CySH conjugate was identified as the toxic factor in trichloroethylene-extracted soybean food which caused fatal aplastic anemia in cattle and induced severe nephrotoxicity in rodents

(McKinney *et al.*, 1959). A third group is formed by those GSH conjugates that are in equilibrium with the parent compound. An example is the bladder carcinogen allyl isothiocyanate which, after forming a GSH conjugate in the liver, can be released in the bladder where pH and GSH concentration are different (Igwe, 1986; Monks *et al.*, 1990; Anders *et al.*, 1992). In general, however, these reactions are detoxications since they render electrophilic, non-polar foreign molecules more hydrophilic.

However, the formation of mercapturic acids, also reflects GSH utilization. As has been outlined before, GSH plays a central role in many processes in the cell and a sufficient degree of GSH depletion may therefore lead to toxicity. Only a few reports have tried to correlate thioether excretion with GSH utilisation/depletion and biological or biochemical events (Chasseaud, 1988). A well known example is that of paracetamol, the reactive intermediate (quinone imine) of which is detoxified by GSH conjugation and excreted as mercapturic acid metabolites in the urine. Measurement of mercapturic acids excreted in the urine after different doses of paracetamol indicated that the excretion of the conjugate was reduced after toxic doses of paracetamol and that at the same time hepatic GSH was depleted to about 20% or less of the normal level (Mitchell *et al.*, 1975). The proposed mechanism of paracetamol hepatotoxicity is that GSH conjugation, normally an adequate detoxication pathway, is overloaded after large toxic doses, so that hepatic levels of GSH are reduced more rapidly than they can be replenished. As a result, the reactive metabolite reacts with various hepatic macromolecules and this in some as yet unknown way gives rise to hepatic necrosis.

Measurement of mercapturic acid in the urine as a function of the dose administered may thus be a helpful tool in determining whether liver

GSH levels have reached critical levels of depletion. A number of studies with α,ß-unsaturated compounds have shown the correlation between mercapturic acid (thioether) excretion and hepatic GSH content. Inhalation exposure to vinyl toluene caused a dose-related decrease in hepatic non-PrSH and increased urinary thioether excretion (Heinonen *et al.*, 1982). Van Bladeren *et al.* (1981) found a limit to the amount of mercapturic acids excreted after acrylonitrile administration. A significantly lower percentage of the dose was excreted as mercapturic acids at the high dose in the female rat. A similar phenomenon has been observed in the case of 1,2-dibromoethane and cyclohexene oxide.

1.4.3 Metabolism of carboxylic acids

Cinnamic acid, the oxidation product of cinnamaldehyde, may undergo further metabolism. Carboxylic acids have the potential to undergo conjugation reactions with a variety of small molecular weight compounds such as amino acids or some sugars e.g. glucuronic acid. These are the classical Phase II reactions described by Williams (1959). Williams describes how in general, the aromatic carboxyl group may be excreted combined with glycine as a hippuric acid (aroylglycine) or with glucuronic acid as an ester glucuronide. Hydroxylation of the aromatic nucleus to form phenolic acids may also occur (e.g. conversion of salicylic acid to gentisic acid) but normally this reaction takes place only to a minor extent. Benzoic acid is not decarboxylated since $C_6H_5{}^{14}COOH$ did not yield radioactive CO_2 in expired air. Nutley *et al.* (1993) showed that in the rat and mouse cinnamic acid underwent ß-oxidation, analogous to that of fatty acids, to yield benzoic acid, which was excreted as such or conjugated with glycine or glucuronic acid. These reaction sequences will be discussed below.

1.4.3.1 Glycine conjugation

Glycine conjugation results in the formation of an amide (peptide) bond between the carboxylic acid group of the xenobiotic and the amino group of glycine. The carboxylic group is first activated *via* the synthesis of a high-energy CoA thioester from Coenzyme A and ATP. This is a two step reaction catalyzed by ATP-dependent acid:CoA ligases; the reaction sequence is:

$RCO_2H + ATP \text{ ----> } RCO\sim AMP + PPi$

$RCO\sim AMP + HSCoA \text{ ----> } RCO\sim SCoA + AMP$

The adenylate conjugate apparently only occurs as an enzyme bound intermediate. The conjugation of the CoA derivative with the amino acid such as glycine is then carried out by mitochondrial enzymes known as N-acyltransferases, the reaction being:

$RCO\sim SCoA + H_2NCH_2CO_2H \text{ ----> } RCONHCH_2CO_2H + HSCoA$

Schachter and Taggart (1953, 1954) obtained a soluble protein from pig kidney cortex which was able to synthesize hippuric acid from benzoyl CoA and glycine. Since then other groups have isolated similar proteins from a number of species including man. Enzymes catalyzing this type of reaction vary in their substrate specificity. For example Webster *et al.* (1976) isolated and purified an acyl CoA:glycine N-acyl transferase and an acyl CoA:glutamine N-acyl transferase from the liver mitochondrial fraction of both Rhesus monkey and man. These enzymes are specific for the amino acid required for conjugation. Nandi *et al.* (1979) isolated two enzymes from bovine liver mitochondria. These were CoA:glycine N-acyl transferases specific for benzoyl or phenylacetyl CoA derivatives. It is apparent that organisms may contain

a number of these N-acyl transferase enzymes which can vary in specificity for both the carboxyl CoA derivative and the amino acid required for conjugation. A partially purified aromatic acid:CoA ligase, known as Mahler's enzyme, isolated from bovine liver mitochondria has been shown to conjugate cinnamic acid with Coenzyme A (Killenberg and Webster, 1980).

1.4.3.2 Glucuronic acid conjugation

Glucuronic acid conjugation of aromatic carboxylic acids is an alternative to glycine conjugation. In general glucuronic acid conjugation can be characterized as a low affinity, but high capacity process and it has widespread distribution through species and tissues. This in contrast to glycine conjugation, which is a high affinity, but medium capacity process. The glucuronic acid residue incorporated into the conjugate derives from the nucleotide uridine diphosphate glucuronic acid (UDPGA) and is transferred to the xenobiotic by UDP glucuronyl transferases (UDPGTs). These are membrane bound enzymes, found particularly in the endoplasmic reticulum of the liver. To date, eleven different rat liver isoenzymes have been identified (Mulder *et al.*, 1990). Glucuronide conjugation is important in biliary excretion from two points of view: firstly it makes a non polar compound much more polar and secondly it increases the molecular weight by 176 units. Both factors, polarity and molecular weight, are important for biliary excretion (Smith, 1973). The formation of glucuronides is not limited to the liver, but can also occur extrahepatically, in tissues of the gastrointestinal tract, the kidneys, lungs and spleen (Caldwell, 1980).

1.4.3.3 ß-oxidation of xenobiotic carboxylic acids

The nature of metabolites formed from cinnamic acid supports the idea that it is metabolized by a pathway analogous to the ß-oxidation pathway of fatty acid degradation. This was first suggested from the early results obtained by Knoop (1905) and substantiated by the work of Dakin (1909). These researchers studied independently at about the same time the metabolism of a homologous series of alkylbenzenes in rabbits, cats and dogs and found that the major metabolites of all these compounds was either hippuric or phenaceturic acid. If the alkyl side chain had an even number of carbon atoms then phenaceturic acid was excreted whilst hippuric acid was found if there was an odd number of carbon atoms. From these results, Knoop proposed that fatty acid degradation occurs two carbon atoms at a time and so outlined the sequence of reactions of the ß-oxidation pathway. The reactions involved in ß-oxidation are believed to occur primarily in the liver although many other tissues have been shown to have enzymes capable of carrying out these reactions.

The enzymology of the ß-oxidation is well understood now for fatty acids and xenobiotic carboxylic acids are believed to be substrates for related enzymes, although there are some differences which will be discussed in Chapter 2 (Yamada *et al.*, 1986b). After entering the cell, fatty acids must be converted to their CoA-derivatives. This reaction requires cytoplasmic thiokinase and is similar to the already described conversion before glycine conjugation. The mitochondrial membrane is impermeable to fatty acids and their CoA derivatives and therefore this transport is mediated by a carrier molecule, carnitine. The CoA group of the fatty acid is exchanged for carnitine, thereby forming an extra-mitochondrial complex with carnitine. This reaction is catalyzed by carnitine-acyl CoA transferase. Reversal of the exchange probably

occurs on the inside of the membrane, with the acyl group now being attached to intra-mitochondrial CoA. Inside the mitochondria the fatty acyl CoA is a substrate for a class of enzymes called fatty acyl CoA dehydrogenases, which are flavoproteins having FAD as coenzyme, to yield an unsaturated product. The unsaturated acyl CoA then accepts a molecule of water, a reaction catalyzed by enoyl CoA hydratase, or crotonase. The product L-ß-hydroxyacyl CoA is oxidized by ß-hydroxyacyl CoA dehydrogenase, a reaction requiring NAD+. The final step in the ß-oxidation is the cleavage of the ß-ketoacyl CoA by ß-ketothiolase, which results in the formation of acetyl CoA and a saturated acyl CoA with two fewer carbons than the original substrate. Phenyl substituted xenobiotic fatty acids with a longer side chain will undergo this sequence of oxidation, hydration and oxidation again until a one- or two-carbon residue (Orten and Neuhaus, 1982).

Alternative pathways for fatty acid oxidation are microsomal α-oxidation in mammalian brain and liver and microsomal ω-oxidation in liver, which are both probably P450 mediated (Moody *et al.*, 1992).

1.4.3.4 Toxicity associated with carboxylic acids

Possible mechanisms of toxicity of carboxylic acids arise from their interference with lipid metabolism and most hypotheses have centred around the intermediacy of the xenobiotic acyl CoA esters (Caldwell and Marsh, 1983; Caldwell, 1984, 1985). Conjugation with CoA may lead to inhibition of lipid metabolism or sequestering of CoA, thereby altering the energy and redox status of the cell (Sherratt, 1985). Toxic actions of carboxylic acids include Jamaican vomiting sickness caused by the active principle of the Ackee fruit, hypoglycin A, which is bioactivated *via* lipid metabolism

(Sherratt, 1969) and both the pharmacological (hypolipidaemic) and adverse effects of clofibrate such as peroxisome proliferation in animals (Grasso, 1985). Carboxylic acid such as 4-hydroxyloxybenzoate lead to lasting residues and membrane disturbances due to the formation of hybrid fatty acids (Hutson *et al.*, 1985). A minor pathway in the metabolism of ^{14}C-benzoic acid is the addition of a two-carbon fragment to the carboxyl group. As well as the addition of a single two-carbon unit, yielding products that are excreted in the urine, it is possible that multiple units can be added leading to hybrid fatty acids incorporated in tissues (Caldwell, 1985).

The ability of carboxylic acids to induce peroxisome proliferation in test animals, has received much attention, because it is a characteristic of some chemicals, such as the hypolipidaemic drug clofibrate, which cause serious side effects and even death in man (Reddy, 1990). Cinnamyl compounds as a group of food flavours came under suspicion when the ester cinnamyl anthranilate, a synthetic food flavouring agent, was withdrawn from food use in 1985 due to its murine, but not rat hepatocarcinogenicity. Studies by Caldwell *et al.* (reviewed in Caldwell, 1992) showed cinnamyl anthranilate to be a mouse specific peroxisome proliferator. The presence of the intact ester, which is not fully metabolized at high doses in mice, has been postulated to be the chemical species responsible for the hepatic enzyme induction. Viswalingam *et al.* (1988) studied the effect of cinnamyl alcohol and anthranilic acid, the two components of the ester, and cinnamic acid, believe to be the major metabolite of cinnamyl alcohol, on the hepatic parameters associated with peroxisome proliferation in mice *i.e.* increased liver weight, induction of total microsomal cytochrome P450, lauric acid hydroxylase, CN-insensitive palmitoyl CoA oxidation, cytosolic epoxide hydrolase and a marked increase in peroxisome/mitochondrial ratio, aminopyrine N-demethylase and

ethoxyresorufin O-deethylase. Neither cinnamyl alcohol nor anthranilic acid administered singly or together induced any of these parameters and cinnamic acid also failed to induce a response. The results support the postulation that the intact ester is the chemical species responsible for the peroxisome proliferation in the mouse. Like cinnamic acid, cinnamaldehyde too had no effect upon the hepatic parameters examined. No structure-activity relationship for peroxisome proliferating activity could be drawn from this study and it was concluded that peroxisome proliferation was a phenomenon specific to cinnamyl anthranilate and not a general feature of cinnamyl compounds.

1.5 Toxicity of cinnamaldehyde and related compounds

1.5.1 Occurrence and exposure to cinnamyl compounds

The occurrence, metabolism, toxicity and pharmacology of cinnamyl compounds has been described by Opdyke in various monographs (1974; 1978; 1979) and reviewed by Hoskins (1984). Cinnamyl compounds include cinnamaldehyde, cinnamyl alcohol, cinnamic acid and various cinnamyl and cinnamate esters (esters of cinnamyl alcohol and cinnamic acid respectively) (Fig. 1.5). Cinnamic acid has a central role in plant metabolism, for it is important in plant cell wall synthesis as a lignin precursor (Goodwin and Mercer, 1972). Cinnamic acid and related compounds are thus found throughout the plant kingdom and are therefore natural constituents of the human diet. The progenitor of the name is the spice cinnamon, known and used by humans for at least 4000 years. Cinnamyl compounds are generally only found in trace amounts, although high concentrations are present in some essential oils and oil resins and the plant material from which they are obtained. For instance, cinnamaldehyde is the major constituent (90%) of Cassia oil, extracted from the bark of the small trees of the *Cinnamonum* sp.

(Heath, 1981). Cinnamaldehyde is thought to be formed during fermentation of the bark *via* a reduction sequence from cinnamic acid (Angmor *et al.*, 1979).

The main reasons for humans coming into contact with cinnamyl compounds, other than those trace quantities present in vegetable foods, is their odorous qualities. Cinnamaldehyde is the most commonly used cinnamyl compound. Cinnamic acid is used in flavour compositions to enhance cinnamon flavours and may reach 40 ppm in

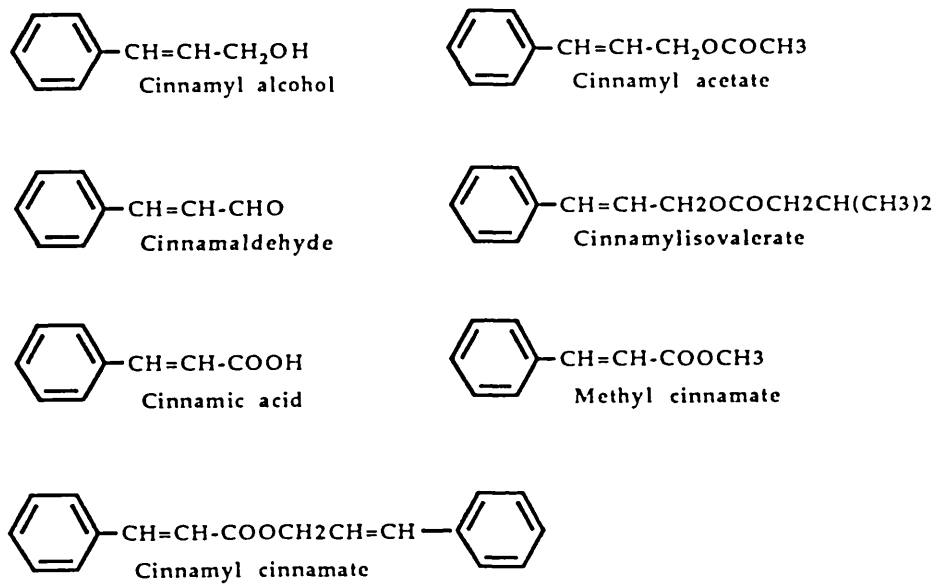


Figure 1.6 Structures of cinnamaldehyde and some related compounds

ice cream (FEMA,1965a, 1987b). In wider use than cinnamic acid are the cinnamate esters, which can either provide spicy or fruity notes in flavour mixtures. Because of the complex nature of most flavours, the individual esters are rarely used and mixtures of esters and other

aromatic agents are commonplace. Cinnamyl alcohol (FEMA, 1965b, 1987c) has a floral balsam-like scent and is mostly used in fragrances, although it also finds its use in the food industry. Cinnamyl alcohol is generally found esterified with various low molecular weight organic acids. Therefore, other sources of exposure to cinnamyl alcohol may include the various cinnamyl esters (both natural and man-made).

1.5.2 Toxicology of cinnamyl compounds

1.5.2.1 Chemical properties

Cinnamaldehyde is a liquid at room temperature which is steam volatile and has low solubility in water. Cinnamic acid is a white crystal (m.p. 133 °C) and cinnamyl alcohol a low-melting solid (m.p. 33 °C).

1.5.2.2 Irritation and sensitization

Research into cinnamaldehyde toxicity mostly concerns its irritating effect on the skin of man, rabbit and guinea pig. It is a strong sensitizer in almost all repeatedly exposed people. Many cases of contact dermatitis and allergy were reported in occupationally exposed persons and others who used cinnamaldehyde in fragrances (Danneman *et al.*, 1983). There is evidence to support the idea that the allergen is a Schiff base formed by reaction of the aldehyde with protein (Majetti and Suskin, 1977). However, when it is present in vegetable oil, it is most often harmless (Opdyke, 1976). This thesis is concerned with the use of cinnamaldehyde as a food flavour and therefore its skin toxicity will not further be mentioned, but it will be discussed in Chapter 10, in relation to the overall safety of the compound. The irritating and sensitizing properties of cinnamyl alcohol when used as a fragrance are less strong than those of cinnamaldehyde (Steltenkamp *et al.*, 1980).

1.5.2.3 Acute toxicity

Toxicity data on cinnamyl compounds other than sensitization are mainly restricted to LD_{50} tests. Cinnamic acid and the cinnamate esters are generally considered to be of low acute toxicity. Reported oral LD_{50} values for cinnamic acid are in excess of 5 mg/kg and they range from 1.5-9.9 g/kg for the various esters commercially available. The acute toxicity (LD_{50}) of cinnamyl alcohol has been estimated at 2.0 g/kg in rats following oral administration.The LD_{50} values for cinnamyl esters range from 2.9 to >5.0 g/kg in rats, mice and guinea pigs after oral administration. Cinnamaldehyde is the most toxic of the cinnamyl compounds, with values ranging from 0.6 to > 2 g/kg in various species, indicating it to be a compound of moderate toxicity.

Toxic symptoms after cinnamaldehyde exposure included neurological and respiratory effects in the rabbit (Friedman and Mai, 1931), depression, diarrhoea and scrawny appearance and death from 2 to 3 h in rats and coma and death from 2 h to 4 days in the guinea pig (Jenner *et al.*, 1964). Pharmacological effects of cinnamaldehyde include sedative action and effects on the central nervous system (Opdyke, 1979). The only reported case of acute toxicity in man is that of a 7 year-old child taking an oral dose of 60 ml (ca 3000 mg/kg) of cinnamon oil. The toxic manifestations included only moderate effects on the GI tract, the central nervous and cardiovascular system (Pilapil, 1989). The child recovered within one day.

1.5.2.4 Subacute toxicity

The MTD, defined as the maximum single dose tolerated by all of a group of five mice after receiving six i.p. injections over a two-week

period, was 0.25 g/kg for cinnamaldehyde, while other cinnamyl compounds were less toxic (Stoner *et al.*, 1973).

A 12-week feeding study (Hagan *et al.*, 1967) using a mixture of five related compounds (cinnamaldehyde, methyl cinnamate, ethyl cinnamate, benzyl cinnamate, cinnamyl cinnamate and α-methyl cinnamaldehyde) at an equivalent cinnamaldehyde dose level of 103.5 mg/kg, showed no adverse effects. A further 12-week oral study in rats using cinnamaldehyde at 58, 114 or 227 mg/kg showed no effects on appearance, behaviour, growth, food consumption, efficiency of food utilization, presence of sugar or albumin in urine, blood haemoglobin, liver and kidney weights, or gross pathology. Rats given cinnamaldehyde for 16 weeks in the diet showed no effects on growth or haematology and macroscopic tissue changes at doses of 1000 and 2500 ppm, equivalent to 50 and 125 mg/kg, but at 10000 ppm (500 mg/kg) slight hepatic swelling in the liver and hyperkeratosis of the squamous portion of the stomach was observed.

At a total dose equivalent of LD_{50} x 5, given over 25 days, no cumulative effects were observed in white rats. When cinnamaldehyde or cinnamic alcohol were administered to rats orally at 0.02 LD_{50} per day for 4 months, no significant effects on blood serum were found after 40 or 140 days (Zaitsev and Rakhmania, 1974).

1.5.2.5 Mutagenicity, genotoxicity and carcinogenicity

Cinnamaldehyde is unequivocally negative in bacterial mutagenicity assays such as the Ames test with various strains of Salmonella, the S. cerevisiae microbial assay and the *E. coli* reversion test (Eder *et al.*, 1980, 1982; Lutz *et al.*, 1982; Neudecker *et al.*, 1983). But, conflicting

results have been obtained for the genotoxic potential of cinnamaldehyde in *in vitro* test systems. Cinnamaldehyde did not increase sister chromatid exchange in ovarian cells of hamsters, but potentiated the effect of an added mutagen (Sasaki *et al.*, 1987) and it induced chromosomal abberations in Chinese hamster fibroblasts (Ishidate *et al.*, 1984). Of more relevance, cinnamaldehyde was negative in the *in vivo* micronucleus test, which detects chromosomal aberrations in rodent bone marrow cells. No effect was seen in male mice at doses of 0-500 mg/kg given i.p. once or in multiples (4-5) with 24 h between injections (Hayashi *et al.*, 1988). Although cinnamaldehyde gave a positive reaction in the *B. subtilis* DNA repair test (Sekizawa and Shibamoto, 1982), it was negative in the unscheduled DNA assay using cultured mammalian cells (rat) (Swales and Caldwell, 1991).

1.5.2.6 Cytotoxicity

A factor that complicates these tests and may account for the anti-bacterial and anti-carcinogenic properties of cinnamaldehyde reported by many authors is the cytotoxicity of the compound, which may affect the survival of the microorganisms and cells (Rutten and Gocke, 1988; De Silva and Shankel, 1987). Recent studies in our laboratory on the toxicity of cinnamaldehyde in rat hepatocytes in suspension (Swales and Caldwell, 1991) showed that cinnamaldehyde caused a dose-dependent cytotoxicity at concentrations of 0.5 mM and above. Cinnamyl alcohol was equipotent albeit with a latency in its effect, whereas cinnamic acid was non-toxic.

1.5.2.7 Teratogenicity

Cinnamaldehyde, at doses up to 1200 mg/kg, was negative in an *in vivo* developmental toxicity assay (Hardin *et al.*, 1987). Mantovani *et al.* (1989) studied the pre-natal toxicity of cinnamaldehyde and found no significant dose-related abnormalities in treated dams or foetuses at doses of 5, 25 and 250 mg/kg.

1.5.2.8 Carcinogenicity

The only cinnamyl compound that has been shown to have any appreciable toxicity is cinnamyl anthranilate. The incidence of primary lung tumours was significantly increased in male and female mice (A/He) dosed i.p. with the MTD (250 mg/kg) or 0.2 MTD three times per week for 8 weeks in tricaprylin (total dose 12.00 or 2.40 g/kg respectively). Similar experiments with cinnamaldehyde and cinnamyl alcohol did not increase the frequency in tumours (Stoner *et al.*, 1973).

These data correlate with the results from the NTP carcinogenicity bioassay of cinnamyl anthranilate. A 103 week study in the B6C3F1 mouse showed dose-related reductions in mean body weight gain in both sexes and a significant dose-related increase in the incidence of hepatocellular carcinomas in both sexes at levels of 15 and 30 g/kg in the diet. An identical study in the Fischer 344 rat showed similar dose-related effects on growth. There was an increased incidence of mineralization and inflammation of the kidneys of treated rats and a non-statistically significant increase in the incidence of pancreatic and kidney tumours. Survivals in the male control group were lower than in experimentally treated animals (64% compared with 80%) (NCI, 1980).

In contrast to cinnamyl anthranilate, administration of cinnamyl alcohol, cinnamic acid and cinnamaldehyde to mice did not induce hepatic enzymes associated with peroxisome proliferation (Viswalingam *et al.*, 1988; Caldwell *et al.*, 1989). These data show that the ability to induce peroxisome proliferation is limited to cinnamyl anthranilate and not shared by other cinnamyl compounds.

1.6 Aims of the present study

The present study was undertaken to provide metabolic and mechanistic data which might contribute to our understanding of the reactivity and possible adverse effects of *trans*-cinnamaldehyde and help in the safety evaluation of this food flavour. From our knowledge so far, cinnamaldehyde is proposed to be metabolized in much the same way as cinnamic acid *via* safe metabolic pathways. In addition, conjugation with GSH might play a role in its detoxication. The following questions will be addressed:

1. What is the relative importance of the two proposed routes of cinnamaldehyde metabolism: oxidation and conjugation with GSH in rat and mouse.

2. How does metabolism vary with dose, route of administration and sex of the animals.

3. How is cinnamaldehyde metabolized in man.

4. What is the nature of any GSH conjugate-derived products formed in rat, mouse and man and their toxicological significance.

5. To what extent does a single dose of cinnamaldehyde deplete non-

PrSHs (GSH and CySH) and PrSH and protein amino groups in rat liver.

Recent studies in our laboratory showed cinnamaldehyde to be toxic towards rat hepatocytes in suspension and indicated a role for sulfhydryl groups in this cytotoxicity. Metabolic studies were undertaken to investigate:

6. The metabolism and protein binding of cinnamaldehyde, cinnamic acid and cinnamyl alcohol in rat hepatocytes in suspension and to determine the nature of the reactive species.

7. The effect of modulation of both oxidative and GSH conjugation pathways on metabolism and protein binding in rat hepatocytes in suspension.

8. The reactivity of cinnamaldehyde towards GSH in buffer and its metabolism and protein binding in the presence of rat and mouse cytosol.

Chapter 2

Pathways of ^{14}C-cinnamaldehyde metabolism in the rat and mouse

2.1 Introduction

trans-Cinnamaldehyde (3-phenyl-2-propenal) has had a long use as a food flavour and animal data show it to be a compound of only moderate toxicity (Opdyke, 1979; Hoskins, 1984). Concern about the safety of cinnamyl compounds in general was raised when cinnamyl anthranilate, the ester of anthranilic acid and cinnamyl alcohol, was found to be a hepatocarcinogen (NCI, 1980). More recent studies indicate that cinnamyl anthranilate is a mouse-specific hepatic peroxisomal proliferator and that this activity is mediated by the intact ester rather than by the products of hydrolysis (Caldwell, 1992). However, the structural similarity of cinnamaldehyde to other reactive and toxic aldehydes such as crotonaldehyde and acrolein and the widespread human exposure to this compound as a result of its occurrence in many foods indicates the necessity of a further understanding of its metabolism and possible mechanisms of toxicity. Structurally, cinnamaldehyde is an α,ß-unsaturated aldehyde, with two reactive groups, an aldehyde and an unsaturated carbon bond and these are the sites of metabolism and possible toxicity. The structures of cinnamaldehyde, crotonaldehyde and acrolein are shown in Fig. 2.1.

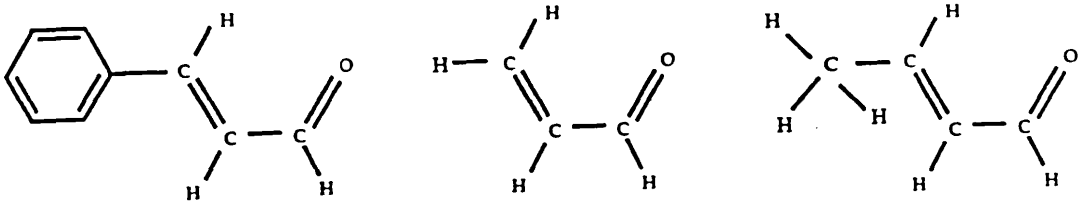


Figure 2.1 Structures of three related α,ß-unsaturated aldehydes
(a) cinnamaldehyde (b) acrolein and (c) crotonaldehyde

The cinnamyl compound which has been most extensively studied is cinnamic acid, beginning with the study of Erdmann and Marchand (1842) who showed that there was an increase in the urinary excretion of hippuric acid after ingestion of this compound by human subjects. Dakin (1909) confirmed this result with more extensive studies in which dogs and cats received large doses of ammonium cinnamate. In addition to hippuric acid, Dakin identified 3-hydroxy-3-phenylpropionic acid, acetophenone and cinnamoyl glycine as urinary metabolites.

Subsequently, various workers have studied cinnamic acid metabolism in a number of species (Raper and Wayne, 1928; Snapper *et al.*, 1940, El Masry *et al.*, 1956; Teuchy and van Sumere, 1971; Fahelbaum and James, 1977). In all cases hippuric acid was the major metabolite. Recently, Nutley *et al.* (1993) showed hippuric acid, benzoyl glucuronide, benzoic acid, acetophenone, 3-hydroxy-3-phenylpropionic acid and cinnamoyl glycine as metabolites of cinnamic acid in rat and mouse by the use of ^{14}C-cinnamic acid. The use of the radiolabel enabled quantitative determination of metabolites, where in previous reports this was not achieved due to the presence of several of the metabolites, such as hippuric acid, as endogenous components in urine.

The metabolism of cinnamaldehyde is much less well understood than that of cinnamic acid. It has been assumed to follow largely the same pathways as that of cinnamic acid after oxidation of the aldehyde by NAD-dependent ALDH (Hoskins, 1984). In addition to oxidation to cinnamic acid, conjugation with GSH may play a role in its metabolism. Cinnamaldehyde, like many other α,ß-unsaturated compounds reacts both spontaneously and enzymatically with GSH

(Boyland and Chasseaud, 1968), causes GSH depletion in rat liver *in vivo* (Boyland and Chasseaud, 1970) and leads to the excretion of mercapturic acids in the urine of rats (Delbressine *et al.*, 1981).

The aim of the present study was the identification and quantitation of metabolites of *trans*-cinnamaldehyde in the rat and mouse using ^{14}C labelled material. A dose of 250 mg/kg cinnamaldehyde was chosen, the MTD in mice (Stoner *et al.*, 1973) which was administered i.p. to ensure that complete absorption occurred.

2.2 Materials and methods

2.2.1 Chemicals

[3-^{14}C] *trans*-Cinnamaldehyde, sp.act. 4.1 mCi/mmol, radiochemical purity 96.8% was a custom synthesis by Amersham International, Amersham, UK. *trans*-Cinnamaldehyde and cinnamyl alcohol were purchased from Aldrich Chemical, Gillingham, Dorset, UK. Hippuric acid, benzoic acid, acetophenone and *trans*-cinnamic acid were obtained from Sigma Chemical Co, Poole, Dorset, UK. 3-Hydroxy-3-phenylpropionic acid (Marsh *et al.* 1982), benzoyl glucuronide, *trans*-cinnamoyl glycine (Nutley *et al.* 1993) and benzylmercapturic acid (Chidgey *et al.* 1986) were samples synthesized and characterized in our laboratories.

2.2.2 Animals and treatment

A group of 4 male Fischer 344 rats (Harlan-OLAC, London; bwt 200 ± 10 g) received a single dose of ^{14}C-cinnamaldehyde (250 mg/kg, 10 µCi/rat in 0.4 ml trioctanoin) by i.p. injection. Further experiments were performed with a group of 6 male CD1 mice (Charles Rivers

Laboratories, Manston, Kent, UK; bwt 27 ± 2 g) which received a single dose of ^{14}C-cinnamaldehyde (250 mg/kg, 12 µCi/mouse in 0.2 ml trioctanoin). Animals were housed individually in glass metabolism cages (rat, Metabowl; mouse, Mini Metabowl, Jencons Ltd., Hemel Hemsted, Herts, UK) with free access to food and water. Urine and faeces were collected on the day prior to the experiment and daily for three days after dosing and were kept at -20°C until analysis. At the end of each 24 h period, the cages were rinsed with *ca* 100 ml ethanol/water 1:1 (*v/v*) and the washings counted for ^{14}C.

2.2.3 Radiochemical techniques

^{14}C was determined in urine and cage washes by liquid scintillation counting (LSC) with a Packard Tricarb 4640 instrument. Quench correction was achieved by reference to an external standard using a standard curve established at regular intervals. Ecoscint (National Diagnostics, Watford, UK) was used as a scintillant.

Faeces were homogenized in distilled water (rat, 30 ml and mouse, 15 ml), in a Stomacher 80 lab blender for 1 min. Aliquots (2 ml) were bleached with 5 M NaOH (1 ml), H_2O_2 (2 ml) and 100 µl *iso*-amylalcohol to control foaming. After 24 h the mixture was neutralized with 200 µl glacial acetic acid, made up to 10 ml with ethanol and heated for 30 min at 60 °C (Caldwell *et al.*, 1972). Aliquots of 0.5 ml were then counted for radioactivity after cooling.

Residual activity in the carcasses (after 72 h) was assayed after alkaline digestion. The frozen carcasses were cut into cubes (*ca* 2 cm^3) and dissolved in 40% (*w/v*) KOH in 80% (*v/v*) aqueous ethanol (1 ml/g carcass). The mixture was kept in the dark until complete dissolution. Aliquots (1 ml) were decolorized by addition of 200 µl H_2O_2 and

100 µl *iso*-amylalcohol and after 2 h samples were neutralised with 200 µl HCl. Aliquots (0.5 ml) were then counted for ^{14}C.

2.2.4 Analysis of urinary metabolites

Prior to HPLC, samples were centrifuged (Heraeus Sepatech Biofuge B, 10 min, 11,000 rpm) to remove particulate matter and aliquots of the supernatant injected directly on to the column. Faecal extracts were prepared by lyophilizing 10 ml aliquots of homogenate and extracting the residue with 3 x 10 ml methanol. The methanol was filtered and evaporated under N_2 to *ca* 0.5 ml before examination by HPLC.

To determine any glucuronidated or sulfated cinnamaldehyde metabolites, aliquots of urine (0.5 ml rat, 0.2 ml mouse) were diluted with an equal volume of 0.2 M acetate buffer pH 5.0 and incubated with an equal volume of Glucurase (ß-glucuronidase 5000 units/ml, *ex* bovine liver, Sigma) or 0.64 mg sulfatase (Type H-1, *ex Helix pomatia*, sp.act. 18000 units/g, Sigma) overnight at 37 °C. To inhibit the ß-glucuronidase activity present in the sulfatase, control incubations were performed with the addition of 0.8 mg D-saccharic acid 1,4-lactone. The reaction was stopped by freezing to -20 °C. Metabolic profiles were compared to those of control samples incubated without enzyme. To determine the presence of any metabolites labile to mild alkali, urine samples were adjusted to pH 10.0 with 1 M NaOH, left to stand at room temperature for 1 h and neutralized with 1 M HCl prior to chromatography (Caldwell and Hutt, 1986).

2.2.5 HPLC analysis

Gradient elution radio HPLC used Waters 5001 and M6000A pumps (Millipore, Waters Associates, Millford, USA) a Waters 712 WISP

autoinjector, a Waters M441 absorbance detector, set at 254 nm, and an Isoflo II on-line solid phase radioactivity detector (Nuclear Enterprises Ltd., Edinburgh, UK), the whole under the control of Ramona software (Raytek, Sheffield, UK) run on a Tandon 286 P.C. The column used was a 250 x 4.6 mm S5 ODS2-column (Hichrom Ltd., Reading, UK). With gradient elution, solvent A was water and solvent B acetonitrile, both containing 0.1% acetic acid. The gradient comprised 100% A for 5 min, then to 20% B over 15 min, to 30% B over 20 min and then to 100% B over 10 min. These final conditions were held for 10 min, each segment was linear and the flow rate was 1 ml/min throughout.

2.3 Results

2.3.1 Excretion balance in rat and mouse

The recovery of ^{14}C in rat and mouse urine and faeces is presented in Table 2.1. Following i.p. administration of 250 mg/kg ^{14}C-cinnamaldehyde to rats the total recovery after 72 h was 94%, with the bulk (75%) in the 0-24 h urine. In mice, 81% of the dose was recovered in the 0-24 h urine and the total recovery was 94% in 3 days.

2.3.2 Metabolism of cinnamaldehyde in the rat

A typical HPLC profile for 0-24 h rat urine after administration of ^{14}C-cinnamaldehyde is shown in Fig. 2.2A. The major urinary metabolite in the rat (79% of ^{14}C in 0-24 h urine) coeluted with hippuric acid and benzoyl glucuronide standards. After ß-glucuronidase treatment, this peak was reduced by 7% with a concomitant increase in the benzoic acid peak, indicating that benzoyl glucuronide accounts for 7% of 0-24 h urinary ^{14}C. Other minor metabolites corresponded in HPLC

retention time to benzoic acid, cinnamoyl glycine and 3-hydroxy-3-phenylpropionic acid. Traces of cinnamyl alcohol, cinnamic acid and cinnamaldehyde were found (respectively 0.6, 0.9 and 0.4%) only after storage (freezing and thawing) of urine. A number of very small unknown peaks (each <1%) were detected with retention times from 15 to 18 and 20 to 27 min, none of which was affected by alkali or sulfatase treatment. Two new metabolites, S1 and S2, with retention times 27.4 and 28.4 min, not eluting with any available standards each accounted for *ca* 3% of 0-24 h urinary metabolism.

In the 24-48 and 48-72 h urine, hippuric acid was the only detectable metabolite. Analysis of faecal samples showed the presence of metabolites eluting around 30 min, possibly cinnamoyl glycine, cinnamoyl glucuronide, benzoic acid or hippuric acid, most likely due to contamination of faeces with urine.

2.3.3 Metabolism of cinnamaldehyde in the mouse

A typical HPLC profile for the 0-24 h urine of mice is presented in Fig. 2.2B. The major urinary metabolite in 0-24 h mouse urine coeluted with hippuric acid and benzoyl glucuronide. Treatment with ß-glucuronidase showed that these accounted for 72% and 2% respectively. The second largest metabolite (8% of metabolites in 0-24 h) coeluted with cinnamoyl glycine and 3-hydroxy-3-phenylpropionic acid (5%) was also present. Traces of cinnamyl alcohol and cinnamaldehyde were found (0.4 and 0.2% respectively) but only after storage of urine. Three unknown metabolites were found accounting for between 1.5 and 3% each. Two of these had the same retention times as S1 and S2 in the rat with the third, S3, eluting later around 32 min.

HPLC analysis of 24-48 h and 48-72 h mouse urine showed that hippuric acid was the only metabolite. Faecal extracts contained metabolites with the same retention times as those in rat faeces.

A quantitative comparison of the fate of ^{14}C-cinnamaldehyde in rat and mouse is presented in Table 2.2.

2.4 Discussion

The results presented here show that the principal routes of metabolism of cinnamaldehyde are similar to those of cinnamic acid (Nutley *et al.*, 1993).

In both rat and mouse the elimination of cinnamaldehyde is essentially quantitive and after 72 h less than 3% of dose remains in the carcass. Tissue distribution studies as part of NTP testing (Sapienza *et al.*, 1991) showed no specific residue sites. Cinnamaldehyde is excreted rapidly, with 80% of the dose in the rat and 87% in the mouse being excreted in the first 24 h after dosing. Elimination occurs predominantly *via* the urine and recovery of ^{14}C in faeces was low and variable and was at least in part attributed to contamination of faeces with urine. Elimination in the mouse is slightly but significantly (χ^2 test, $p<0.05$) faster than in the rat, but the very similar relative excretion in urine and faeces does not suggest any differences in disposition between rats and mice.

Cinnamaldehyde is completely metabolized in both rat and mouse, with no parent compound excreted. A metabolic map consistent with the findings presented in this Chapter is shown in Fig. 2.3. Hippuric acid is the major metabolite (72% both in rat and mouse) and is suggested to

arise from oxidation of the aldehyde to cinnamic acid and further metabolism *via* ß-oxidation of the side chain followed by glycine conjugation to yield hippuric acid. This is analogous with the sequence in the catabolism of fatty acids, which was studied by Knoop (1905) and Dakin (1908) with the use of cinnamic and other ω-phenyl fatty acids. Xenobiotic carboxylic acids which enter the cell are converted to their acyl-CoA thioesters (1) and undergo the various steps of the ß-oxidation pathway as these high energy intermediates (Caldwell, 1984). Cinnamoyl CoA undergoes either conjugation with glycine by an N-acyl transferase (2), a reaction mostly seen in the mouse, or the addition of water across the double bond to yield 3-hydroxy-3-phenylpropionyl CoA in the first step of ß-oxidation (3). The enzymatic activity responsible for this reaction is enoyl CoA hydratase and has been detected in both hepatic mitochondria and peroxisomes. This CoA may be cleaved by hydrolytic enzymes known as thiolases to release 3-hydroxy-3-phenylpropionic acid (4), but the vast majority is oxidized by the action of 3-hydroxy fatty acyl CoA dehydrogenase losing 2 hydrogen atoms to NAD to give 3-keto-3-phenylpropionyl CoA (5). Acetophenone, the decarboxylation product of this intermediate (6), was not detected in rat or mouse urine (although it has been reported as a metabolite of cinnamic acid (Nutley *et al.*, 1993). 3-Keto-3-phenylpropionyl CoA then loses a two carbon acetyl moiety, presumably by reaction with CoA, yielding benzoyl CoA and acetyl CoA (7). Benzoyl CoA is then in turn conjugated with glycine giving hippuric acid (8), or hydrolysed to free benzoic acid, excreted as such (9) or after glucuronic acid conjugation (10).

The need for the conversion of cinnamic acid, just like fatty acids, to its CoA ester derivative before undergoing further metabolism is suggested from the results of Ranganathan and Ramasarma (1971, 1974). These

authors incubated rat liver homogenate with *p*-hydroxycinnamic acid and found that the addition of ATP was necessary for the formation of *p*-hydroxybenzoic acid. Comparison of the ß-oxidation of fatty acids with that of *p*-hydroxycinnamic acid showed similarities but, also some differences. When cinnamate was included in the incubation it had the ability to inhibit the formation of *p*-hydroxybenzoic acid, but left the oxidation of octanoic acid unaffected. This suggests that cinnamic acid and *p*-hydroxycinnamic acid may be metabolized by the same enzyme system, whereas (short-chain) fatty acids are metabolized by a different system. One possibility is the involvement of peroxisomes, which metabolize long-chain fatty acids, rather than mitochondria, which catalyze the metabolism of short-chain fatty acids, in the ß-oxidation of xenobiotic acyl CoA derivatives (Reddy and Lalwani, 1983; Yamada *et al.*, 1984 and Yamada *et al.*, 1986a,b). An indication for this was the finding that the metabolism of *p*-hydroxycinnamic acid to *p*-hydroxybenzoic acid was insensitive to inhibition by cyanide, an inhibitor of mitochondrial, but not peroxisomal fatty acid metabolism (Lazarow and Deduve, 1976) and that clofibric acid inhibited *p*-coumaric acid metabolism although it increased octanoic acid oxidation. Clofibric acid is the active metabolite of the hypolipidaemic agent clofibrate (Cayen, 1983). A well known property of the majority of hypolipidaemic agents is their ability to induce the proliferation of hepatic parenchymal cell peroxisomes (Reddy, 1990). Evidence such as this suggests that *p*-coumaric acid and, by analogy, cinnamic acid may be metabolized by hepatic peroxisomes.

The main features of the pattern of oxidative metabolites of cinnamaldehyde in the rat and mouse were very similar, with the exception of cinnamoyl glycine which was only important in the mouse. This results possibly from a greater affinity of mouse N-acyltransferase for cinnamic acid or a greater capacity of competing

metabolism *via* ß-oxidation in the rat.

Many minor unknown metabolites were formed after administration of cinnamaldehyde, but none accounted for more than 1-2% of dose. These might be derived from metabolism at the benzene ring: Delbressine *et al.* (1982) found *p*-hydroxycinnamic acid as a metabolite of cinnamate esters *in vitro* and the experiments of Bhatia *et al.* (1977) showed that several phenolic metabolites including *p*-hydroxycinnamic acid formed from cinnamate esters in rats.

In addition to the oxidative metabolites, administration of cinnamaldehyde to rats and mice also led to the excretion of metabolites, not identified as urinary metabolites of cinnamic acid (Nutley *et al.*, 1993). Two new metabolites were found in the rat and the same two and an additional third in the mouse. Together these accounted for 6-7% of dose in both species. In Chapter 5 it will be shown that these are mercapturic acids derived from direct conjugation of cinnamaldehyde with GSH and thus form a second, minor route in the metabolism of cinnamaldehyde, the major route being oxidative metabolism analogous to the ß-oxidation of fatty acids.

Table 2.1

Elimination of ^{14}C in urine and faeces of F344 rats and CD1 mice given 250 mg/kg ^{14}C-cinnamaldehyde i.p.*

	0-24 h	24-48 h	48-72 h	0-72 h
rat				
urine	75.0 ± 14.8	7.5 ± 1.7	3.4 ± 1.3	85.9 ± 14.9
faeces	6.1 ± 5.7	1.4 ± 0.5	0.4 ± 0.2	7.9 ± 5.8
total	81.1 ± 9.8	8.9 ± 1.4	3.8 ± 1.3	93.9 ± 9.8
mouse				
urine	81.2 ± 11.5	5.5 ± 3.6	0.9 ± 0.2	87.6 ± 14.6
faeces	5.8 ± 5.1	0.3 ± 0.1	0.1 ± 0.1	6.3 ± 4.9
total	87.0 ± 10.9	5.8 ± 3.6	1.1 ± 0.2	93.8 ± 15.7

* Figures are means ± S.D. % of dose excreted in each time period, n=4 rats, n=6, mice.

Figure 2.2

Typical radio HPLC profile of 0-24 h urine after 250 mg/kg ^{14}C-cinnamaldehyde i.p. (a) rat and (b) mouse

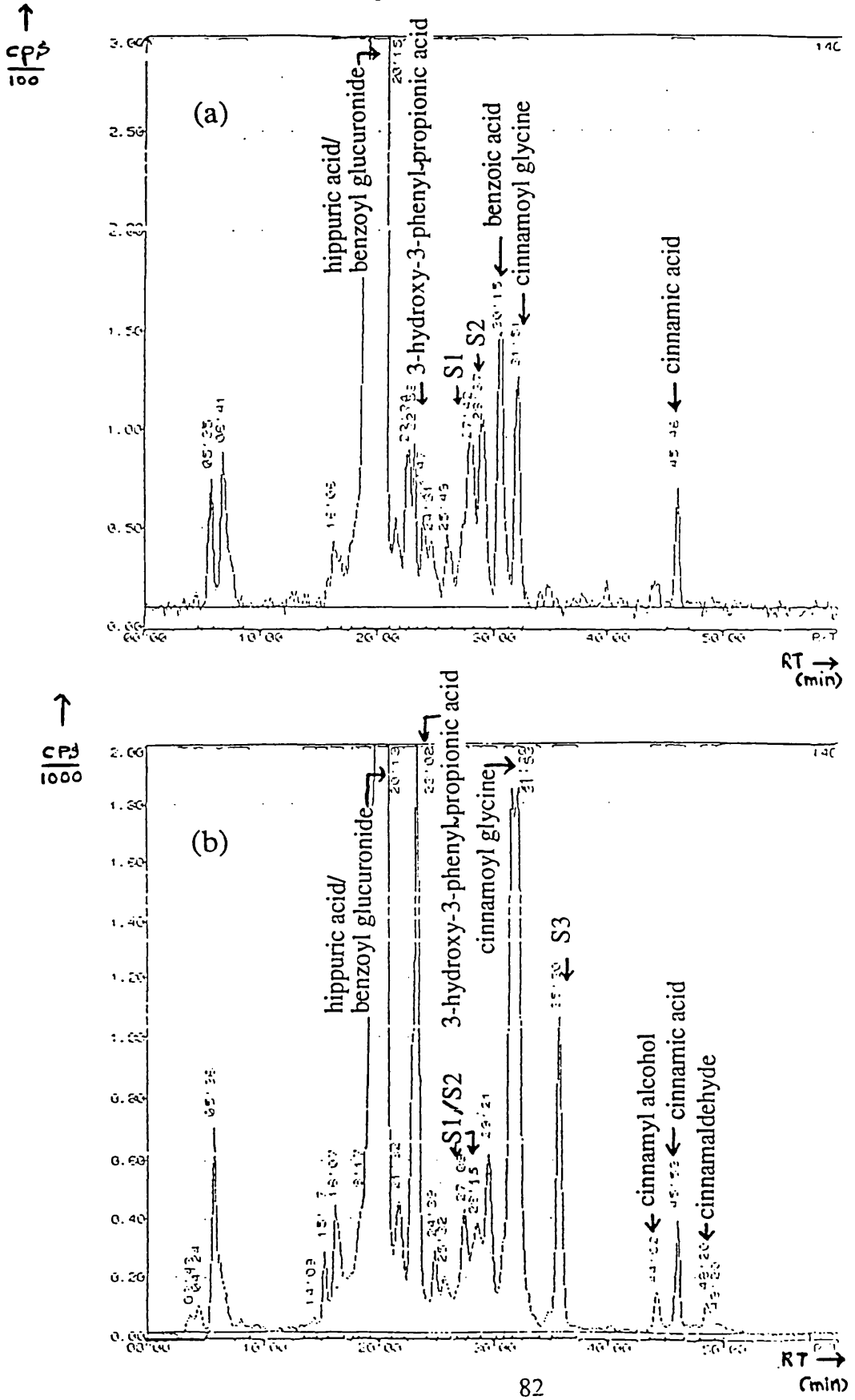


Table 2.2

Metabolites of ^{14}C-cinnamaldehyde in the 0-24 h urine of rats and mice given 250 mg/kg i.p.

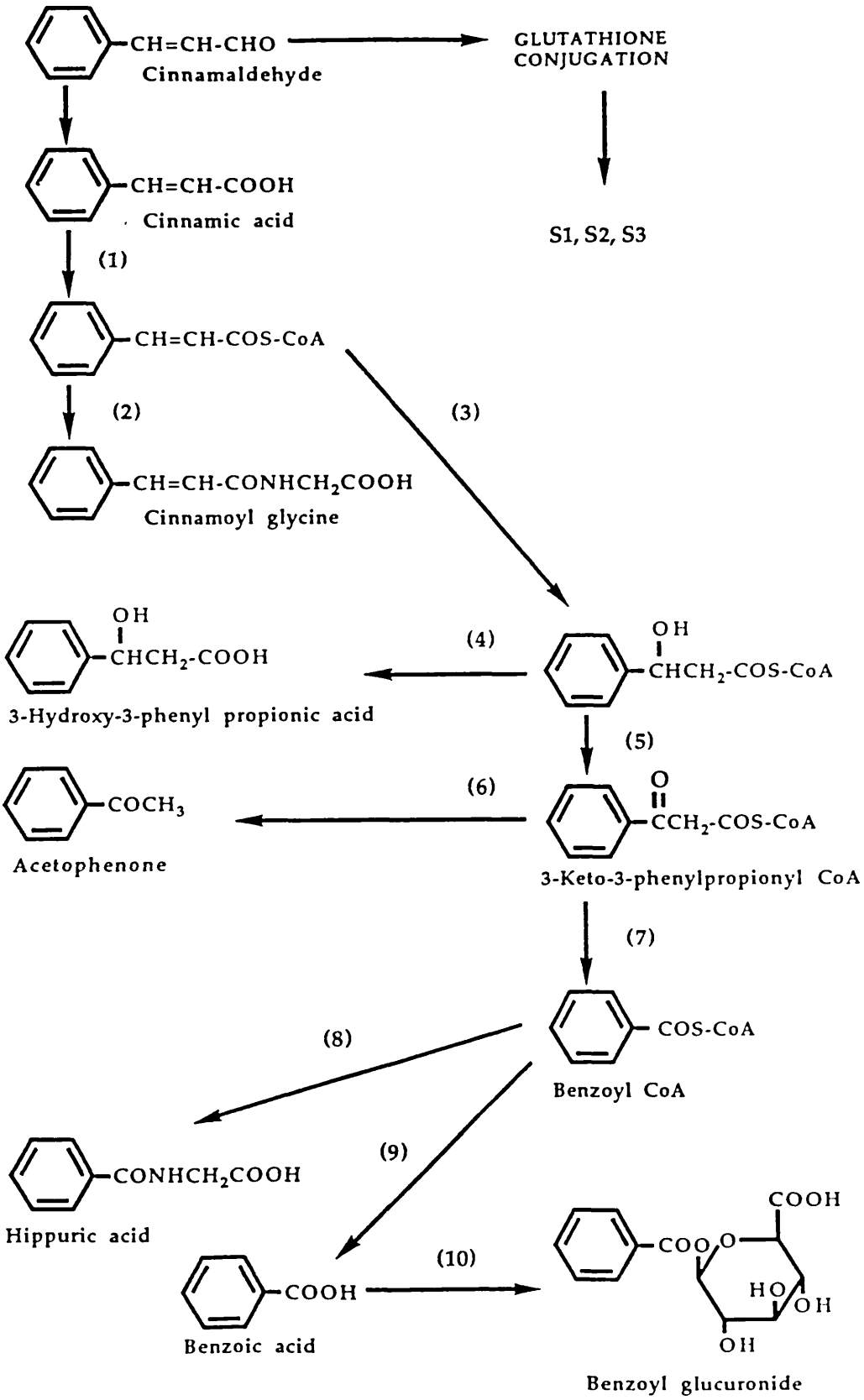
Metabolite	rat	mouse
	^{14}C *	^{14}C *
hippuric acid	71.6 ± 1.0	72.0 ± 5.8
benzoyl glucuronide	7.0 ± 0.5	2.1 ± 1.1
HPPA	2.1 ± 0.7	4.5 ± 1.9
cinnamoyl glycine	2.7 ± 0.5	8.2 ± 1.3
benzoic acid	4.7 ± 0.6	-
S1	3.1 ± 0.5	1.6 ± 0.9
S2	2.7 ± 0.2	1.8 ± 0.9
S3	-	3.0 ± 0.5
cinnamyl alcohol	0.6 ± 0.5	0.4 ± 0.2
cinnamic acid	0.9 ± 0.6	-
cinnamaldehyde	0.4 ± 0.3	0.2 ± 0.1
other**	4.5 ± 1.3	7.0 ± 0.6

* ^{14}C-cinnamaldehyde metabolites expressed as a percentage of the total recovery of ^{14}C after radio HPLC analysis of 0-24 h urine. Figures are means ± S.D., n=4 rats, n=6 mice.

** More than one peak, metabolites eluting closely together, individual metabolites account for less than 1% of dose.

Figure 2.3

The proposed metabolism of cinnamaldehyde in rat and mouse



Chapter 3

Influence of dose size, sex and route of administration on the metabolism of ^{14}C-cinnamaldehyde in rats and mice

3.1 Introduction

The complete safety evaluation of a chemical such as a food additive must consider its metabolism and pharmacokinetics. Data gained from these studies give insight into the toxic mechanism of a compound, which is needed for the proper interpretation of animal studies to the human situation. After ingestion, chemicals may variously be accumulated, excreted or metabolized to more polar, water soluble compounds, leading to detoxication or possibly bioactivation. The toxic response will therefore not be dependent on the absolute dosage, but on the concentration-time profile of the ultimate reactive species, which may be the parent compound or a metabolite (Monro, 1992). Among the most important factors that affect rates and pathways of metabolism and thus the toxicity of xenobiotics, are dose size, species, sex of the animals and route of administration. These are studied in this Chapter for cinnamaldehyde.

The value of comparative metabolic studies in the safety evaluation of food flavours is illustrated by the case of cinnamyl anthranilate, which was banned after an NCI test found it to be a mouse hepatocarcinogen. Mechanistic studies of ^{14}C-cinnamyl anthranilate indicated the intact ester as the reactive species (Viswalingam, 1988; Caldwell *et al.*, 1989). In agreement with this, cinnamyl anthranilate was completely metabolized to hippuric and benzoic acid in the rat at a dose of 250 mg/kg, but in mouse urine in addition to these, unchanged cinnamyl anthranilate was detected at doses of 5 mg/kg and above (Keyhanfar, 1991a). Metabolism of cinnamyl anthranilate in human volunteers (Keyhanfar, 1991b) was comparable to that in the rat, but not the mouse, suggesting that peroxisome proliferation, and thus, presumably, cancer induction, is not to be expected in man. Thus, species differences in the rate of metabolism of cinnamyl anthranilate

accounted for differences in hepatocarcinogenesis and provided a rational basis for the safety evaluation of this compound.

Apart from possible differences in metabolism between species, the extrapolation of data from very high dose experiments in animals to the low exposure in man is further complicated by the fact that primary mechanisms in absorption, metabolism and distribution can become saturated at the high doses used in toxicity tests. For example the flavouring agent estragole is metabolically handled by the "safe" metabolic pathways of O-demethylation and oxidation of the side chain at low levels of exposure, but at higher levels the utilisation of these primary pathways declines and the metabolism is progressively switched to the 1'-hydroxylation pathway, leading to the formation of the primate carcinogen 1'-hydroxyestragole (Anthony *et al.*, 1987). Results obtained from high dose animal studies with estragole are thus not representative for human use in low quantities. This type of metabolic switching is not confined to estragole but occurs also with related compounds such as *p*-propylanisole (Sangster *et al.*, 1983) and anethole (Sangster *et al.*, 1984) although these do not share the metabolic activation by 1'-hydroxylation.

In this Chapter a comparison is made between oral and i.p. routes of administration to give insight in the role of absorption, which may be incomplete after oral administration, and the contribution of microbial metabolism before the systemic circulation is reached. An i.p. injection, on the other hand, delivers the complete dose into the animal and reaches the liver as a bolus, thereby perhaps overwhelming primary routes of metabolism (Caldwell and Smith, 1977). Toxicity tests are performed after oral administration, by gavage or in the diet, which is relevant for human exposure to cinnamyl compounds *via* the food, whereas i.p. injection may be used for convenience and when high

levels in the food cause it to be unpalatable.

Routine toxicity testing is performed in both sexes and information on sex differences in metabolism is important when assessing the extent to which uncertainty factors may be used in the regulatory process. Cinnamaldehyde metabolism was therefore examined in both males and females. Sex differences in metabolism are most apparent in the laboratory rat and less in other species (Calabrese, 1985). In general male rats are known to metabolize foreign compounds more rapidly than females. Sex differences in metabolism in the rat include examples of differences in the rate of glucuronidation and GSH conjugation, which may be relevant to cinnamaldehyde. An example of how sex differences in metabolism determine toxic susceptibility is the different response of male and female rats to hexachlorobenzene (HCB). HCB induces hepatic porphyria and liver cancer in female rats, whereas toxicity is minimal in male rats. D'Amour and Charbonneau (1992) studied the sex-related differences in HCB toxicity and metabolism and found that the detoxication *via* GSH conjugation was greater in male than females.

In this Chapter the fate of cinnamaldehyde was examined in male and female rats and mice after i.p or oral administration of 250 mg/kg, the MTD, and 2 mg/kg, the lowest dose possible given the specific activity of our ^{14}C-cinnamaldehyde, and near the expected human daily intake. In Chapter 2 it was shown that ^{14}C-cinnamaldehyde metabolism in rats and mice largely follows that of cinnamic acid, with hippuric acid as the major metabolite, but that in addition conjugation occurs with GSH, leading to the excretion of sulfur containing metabolites (S1, S2, S3) in the urine (Chapter 5). In particular the nature and extent of variation in the fate of GSH conjugates is important as a variety of toxicological consequences have been attributed to these metabolites.

3.2 Materials and methods

3.2.1 Chemicals

The chemicals used in this study are all as described in Chapter 2

3.2.2 Animals and dosing

Fischer 344 rats were purchased from Charles River Laboratories, UK and allowed free access to food and water. Groups of four male rats (bwt 186.8 ± 1.8 g) received a single i.p. or oral dose of 250 mg/kg or a single i.p. dose of 2 mg/kg (10 μCi/rat in 0.4 ml trioctanoin). Female rats (bwt 169.7 ± 3.7 g) received i.p. cinnamaldehyde doses of 250 mg/kg (10 μCi in 0.4 ml trioctanoin) or 2 mg/kg (11 μCi in 0.4 ml trioctanoin).

Groups of six male CD1 mice (bwt 23.7 ± 0.7 g, Charles River Laboratories, UK) received single i.p. or oral doses of 250 mg/kg or a single i.p. dose of 2 mg/kg cinnamaldehyde (10 μCi in 0.2 ml trioctanoin). Female mice (bwt 23.8 ± 0.7 g) received i.p. doses of 250 mg/kg (10 μCi, 0.2 ml trioctanoin) or 2 mg/kg (11 μCi, 0.2 ml trioctanoin).

3.2.3 Radiochemical techniques

Urine and faeces were collected for 3 days. Quantitation of ^{14}C in urine, faeces, cage washes and carcasses and identification of cinnamaldehyde metabolites was achieved by radio HPLC analysis as described in Chapter 2.

3.3 Results

3.3.1 Recovery of ^{14}C in urine and faeces of rats

An essentially complete recovery of administered ^{14}C was achieved in all experiments and a summary is given in Table 3.1. Recoveries of ^{14}C in urine and faeces 72 h after 250 or 2 mg/kg i.p. was 102% and 99% of dose respectively in the male rat, with 96% recovered at the high and 99% at the low dose in the female rat. After oral administration of 250 mg/kg to male rats, 104% of dose was recovered. The total recovery of ^{14}C did not significantly differ with dose, sex or route of administration. Less than 2% of dose was recovered in the carcass after 72 h.

After administration of cinnamaldehyde to rats, the excretion of ^{14}C was rapid in all cases, but slightly, though significantly (χ^2-test) faster in the male rat at low dose and after oral administration. The bulk of the dose administered (> 70%) was found in the 0-24 h urine and excretion *via* the faeces was some 7% on the first day. None of the factors studied significantly influenced the relative importance of the urinary or faecal route of excretion. The similar percentage of dose found eliminated *via* the faeces after i.p. and oral administration indicates that absorption from the intestinal tract is complete.

3.3.2 Metabolic profile in the rat

The metabolic profile of ^{14}C-cinnamaldehyde in the rat and the influence of dose size, sex and administration on the formation of metabolites is shown in Fig. 3.1. Hippuric acid formation is shown in Table 3.3.

The metabolic profiles at high and low doses in male rat are similar (Fig. 3.1A and 3.1B) showing only minor quantitative differences. When the dose administered is reduced from 250 mg/kg to 2 mg/kg, less of the minor metabolites (e.g. benzoyl glucuronide, 3-hydroxy-3-phenylpropionic acid) were formed while hippuric acid remains the major metabolite (73% at high and 85% at low dose respectively). The two thioethers, S1 and S2, were formed to the same extent independent of dose size (7% of urinary ^{14}C).

Comparison of the male and female rat at high dose (Fig 3.1A and 3.1C) shows that hippuric acid is the major metabolite in both sexes (73% of 0-24 h urinary metabolites in males, 84% in the females). Minor metabolites (e.g. benzoyl glucuronide) were formed to a lesser extent in the female. The two sulfur metabolites, S1 and S2, were also found in the female and formed to the same extent as in the male rat. When the dose was reduced from 250 mg/kg to 2 mg/kg in female rats (Fig. 3.1C and 3.1D) similar changes were observed as in male rats. At the low dose less 3-hydroxy-3-phenylpropionic acid and other oxidative metabolites were formed, with the exception of one new metabolite which accounted for α 2.5% of the total radioactivity. The formation of metabolites S1 and S2 did not significantly differ at the two dose levels.

Comparison of i.p. and oral administration (Fig. 3.1A and 3.1E) of 250 mg/kg cinnamaldehyde to male rats shows a simpler profile after oral dosing, more comparable to the profile seen at a low i.p. dose. Significantly more hippuric acid (73% i.p. and 87% p.o.) and less 3-hydroxy-3-phenylpropionic acid and other oxidative metabolites (e.g. benzoyl glucuronide) were formed after oral administration, but S1 and S2 accounted for α 6% of urinary ^{14}C after both routes of administration.

3.3.3 Recovery of ^{14}C in urine and faeces of mice

A quantitative recovery of administered ^{14}C was achieved in all experiments and total recoveries in urine and faeces are shown in Table 3.2. Recoveries after 72 h were > 93% of radioactivity administered and did not significantly vary with dose, sex or route of administration. Less than 2% of dose was recovered in the carcass.

Cinnamaldehyde was excreted rapidly, mainly in the 0-24 h urine (> 71%), while faecal excretion ranged from 6% to 15% and was probably due to contamination of faeces with urine. The excretion rate and the relative amount of ^{14}C eliminated *via* urine and faeces is very similar at both dose levels, sexes and routes of administration, but slightly slower after a high dose given i.p.

3.3.4 Metabolic profile in the mouse

The metabolic profile of ^{14}C-cinnamaldehyde in the mouse and the influence of dose size, sex and route of administration is presented in Fig. 3.2. Hippuric acid formation is shown in Table 3.3

When the dose was reduced from 250 mg/kg to 2 mg/kg in the male mouse (Fig. 3.2A and 3.2B) slight changes in the metabolic profile occurred. At both dose levels hippuric acid accounted for 72% of metabolism in 0-24 h urine. Other oxidative metabolites amounted to 20% at high dose and 19% at low dose, but changes occurred in the relative extent to which each metabolite was formed. At high dose, less cinnamoyl glycine (8% v 11% at low dose) and benzoic acid (0% *vs* 3%), but more of the other oxidative metabolites, e.g. 3-hydroxy-3-phenylpropionic acid (5% *vs* 2% at low dose) was excreted. At the low dose level, the GSH conjugation pathway accounted for 9% of

metabolism as compared to 6% at the higher dose. This was due to a decline in the formation of all three mercapturates S1, S2 and S3 with increasing dose, which did not achieve statistical significance.

Comparison of the metabolism in the male and female mouse at high dose (Fig. 3.2A and 3.2C) shows the same trend as in the rat. Hippuric acid, the major metabolite, is formed to the same extent (72% in males and 71% in females). 3-Hydroxy-3-phenylpropionic acid is formed to the same extent (5% in males and 4% in females), but especially the cinnamoyl glycine formation is greater in the male than the female animal (8% in male *vs* 4% in female). More benzoyl glucuronide is formed in the female than the male mouse. There was no difference in the formation of the three mercapturic acids S1, S2 and S3 between the sexes (6% in both) although metabolites S1 and S2 were formed to a greater extent in the females with rather less S3 present.

When the dose administered to female mice was reduced from 250 mg/kg to 2 mg/kg (Fig. 3.2C and 3.2D), similar changes were observed to those seen in males (Fig. 3.2A and 3.2B). Hippuric acid formation remained the same (71% *vs* 72% at the low dose). Less 3-hydroxy-3-phenylpropionic acid and other oxidative metabolites were formed at low dose with the exception of cinnamoyl glycine and benzoic acid, which were present to a greater extent at low dose. The extent of mercapturic acid formation was 6% at the high dose and 5% at the low dose.

Comparison of i.p. and oral administration (Fig. 3.2A and 3.2E) at the high dose in the male mouse shows a profile after oral dosing that is more like the low dose i.p. profile. After oral administration slightly more hippuric acid (75% *vs* 72%) and cinnamoyl glycine (15% *vs* 8%) and less 3-hydroxy-3-phenylpropionic acid and other oxidative

metabolites are formed. The sulfur metabolites S1, S2 and S3 accounted for 6% of 0-24 h urinary metabolites after both i.p. and oral administration.

3.3.5 Faecal and 24-72 h urine samples

Faecal and 24-48 h and 48-72 h urine samples of rats and mice were examined for the presence of S1, S2 or S3, but these were not detected. The only urinary metabolite was hippuric acid, while the faecal metabolites were hippuric acid (most likely from contamination with urine) and a number of metabolites eluting about 30 min.

3.4 Discussion

The results presented show that ^{14}C-cinnamaldehyde is completely eliminated from the body in 72 h and less than 2% of the dose remains in the carcass in both species. Elimination of radioactivity is slightly quicker in the male animals at 2 mg/kg and after oral administration as compared to i.p. administration of 250 mg/kg. The elimination of cinnamaldehyde was slightly more rapid in the mouse than in the rat, which is consistent with the general finding that smaller animals have a higher metabolic capacity (Feron *et al.*, 1990). In both rats and mice cinnamaldehyde is excreted predominantly in the urine, with a minor fraction of ^{14}C found in the faeces. The relative elimination in urine and faeces is not affected by dose, sex or species or route, indicating that an oral dose of cinnamaldehyde is completely absorbed.

Analysis of urine samples showed that cinnamaldehyde is completely metabolized in both rat and mouse, with no parent compound excreted even at the high dose level. As described before (Chapter 2), the metabolism of cinnamaldehyde largely follows that of cinnamic acid,

with ß-oxidation of the side chain to benzoic acid and subsequent conjugation with glycine to yield hippuric acid. In addition to ß-oxidation, cinnamaldehyde undergoes conjugation with GSH (Chapter 5) and this forms a minor pathway accounting for 5-9 % of 0-24 h urinary metabolites.

The metabolic profile of cinnamaldehyde in the 0-24 h urine of rats was largely similar in each experiment. The relative importance of the two metabolic pathways, oxidation and GSH conjugation, was unaltered by dose size, sex or route of administration. Quantitative differences occurred within the oxidative pathway, but were minor. Hippuric acid was the major metabolite, accounting for 76 to 88% of dose. In general, the capacity of the ß-oxidation pathway was large compared to the very high dose given in the experiments described here. However, at the high dose a breakthrough was seen with the limited capacity of glycine N transferase to conjugate benzoic acid, leading to the excretion of greater proportions of the dose as benzoyl glucuronide and free benzoic acid.

In the mouse, as in the rat, neither the excretion nor the metabolic profile changed with any of the factors examined: Hippuric acid accounted for 71% to 75% and the mercapturic acids for 6-9% of metabolism. However, contrary to the results in the rat, the mouse shows interesting changes in the amount of cinnamoyl glycine excreted, a metabolite only found in trace amounts in the rat. The presence of this metabolite suggests that compared to the rat, the mouse glycine N-acyl transferase has a higher affinity for cinnamoyl CoA. The formation of cinnamoyl glycine falls with increasing dose in male and female mice, suggesting that the glycine conjugation has a low capacity. The limited capacity of the glycine conjugation mechanism, first reported by Quick (1931), is well known. Early evidence suggested that this arose from

the limited availability of free glycine, but more recently it has been suggested that the kinetic properties of the enzymes involved may also be responsible (Caldwell *et al.*, 1980). The increasing role of glucuronic acid conjugation relative to glycine conjugation as dose size increases has been reported many times in the metabolism of carboxylic acids (Bray *et al.*, 1951; Bridges *et al.*, 1970; Nutley *et al.*, 1993)

The amount of hippuric acid as a percentage of dose is unaltered by dose size, but an increase is seen in other oxidative metabolites, 3-hydroxy-3-phenylpropionic acid, benzoyl glucuronide and minor unknowns, suggesting that some saturation of the ß-oxidation pathway occurs, leading to the hydrolysis of the intermediate CoA esters and their subsequent excretion in the urine. The effect of different routes of administration, i.p. as compared to oral, suggests a measure of saturation in oxidative metabolism after i.p. administration when a bolus dose reaches the liver, leading to an slight decrease in the rate of elimination and change in metabolic pattern.

The excretion of the thioether metabolites of cinnamaldehyde was unaltered by dose size or route of administration, perhaps indicating that the initial oxidation to cinnamic acid has not become saturated, which would be expected to lead to a higher percentage of dose excreted as sulfur containing metabolites, and indicating that GSH levels have not been fully depleted, which would result in a fall in the percentage of mercapturates excreted as has been demonstrated for paracetamol (Mitchell *et al.*, 1975). The nature of the urinary sulfur metabolites and the effect of a single high dose of cinnamaldehyde on liver GSH were further examined in Chapters 5 and 9.

Sex differences in the fate of cinnamaldehyde in rats and mice were minor. In general the metabolic profile was simpler in the female as

compared to the male. The most marked difference was in the formation of cinnamoyl glycine, which was higher in the male than female mouse.

The information gained from this study of ^{14}C-cinnamaldehyde in rat and mouse is that metabolism is largely unaffected by any of the factors examined. Metabolites seen after a high dose given i.p. are not novel, but rather reflect increased excretion rates of metabolites observed at low dose. However, some adverse effects were seen on the animals' health. Rats and mice appeared distressed, ate little and excreted concentrated urine, effects noted in toxicity studies with cinnamaldehyde (Friedmann and Mai, 1931; Jenner *et al.*, 1964; Hagan *et al.*, 1967) and these were absent after dosing vehicle only, 2 mg/kg i.p. and 250 mg/kg by gavage. The high dose of 250 mg/kg cinnamaldehyde was chosen in these metabolism studies for it was the MTD reported for mice, and likely to be used in toxicity studies, whereas the low dose of 2 mg/kg approximates the estimated human exposure to this food flavour. On the basis of metabolism, it thus seems that extrapolation from very high to low dose is valid when cinnamaldehyde is administered orally, but effects seen at 250 mg/kg given i.p. may not be representative for human life-long, low exposure *via* the diet.

Table 3.1

Elimination of ^{14}C in urine and faeces of F344 rats given ^{14}C-cinnamaldehyde* and the influence of dose size, sex and route of administration

	0-24 h	24-48 h	48-72 h	0-72 h
Male high i.p.				
urine	85.0 ± 6.1	7.9 ± 2.8	4.7 ± 1.3	97.5 ± 4.0
faeces	0.8 ± 0.5	2.9 ± 2.0	0.9 ± 0.3	4.5 ± 2.5
total	85.8 ± 6.0	10.7 ± 2.1	5.6 ± 1.4	102.0 ± 5.8
Male low i.p.				
urine	81.0 ± 0.8	5.7 ± 6.2	3.4 ± 2.1	90.1 ± 6.9
faeces	7.5 ± 4.5	1.2 ± 0.5	0.3 ± 0.2	9.0 ± 4.3
total	88.5 ± 4.0	6.9 ± 6.0	3.7 ± 2.3	99.1 ± 4.1
Female high i.p.				
urine	70.1 ± 11.2	8.7 ± 4.4	3.4 ± 1.8	82.1 ± 16.7
faeces	8.8 ± 4.4	4.4 ± 0.8	0.9 ± 0.6	14.1 ± 6.4
total	78.8 ± 5.0	13.1 ± 4.6	4.3 ± 2.4	96.2 ± 11.0
Female low i.p.				
urine	81.4 ± 5.5	3.7 ± 2.3	1.9 ± 1.1	87.0 ± 4.0
faeces	8.0 ± 7.3	1.7 ± 1.2	2.6 ± 1.4	12.3 ± 6.6
total	89.4 ± 11.7	5.4 ± 3.0	4.5 ± 2.4	99.2 ± 10.3
Male high oral				
urine	91.0 ± 7.5	2.0 ± 0.4	0.6 ± 0.3	93.6 ± 7.6
faeces	7.3 ± 2.4	1.9 ± 0.9	0.3 ± 0.1	9.5 ± 2.4
total	98.3 ± 7.3	3.9 ± 1.0	0.9 ± 0.3	103.1 ± 6.8

* Figures are means ± S.D. % dose excreted in each time period, n=4.

Table 3.2

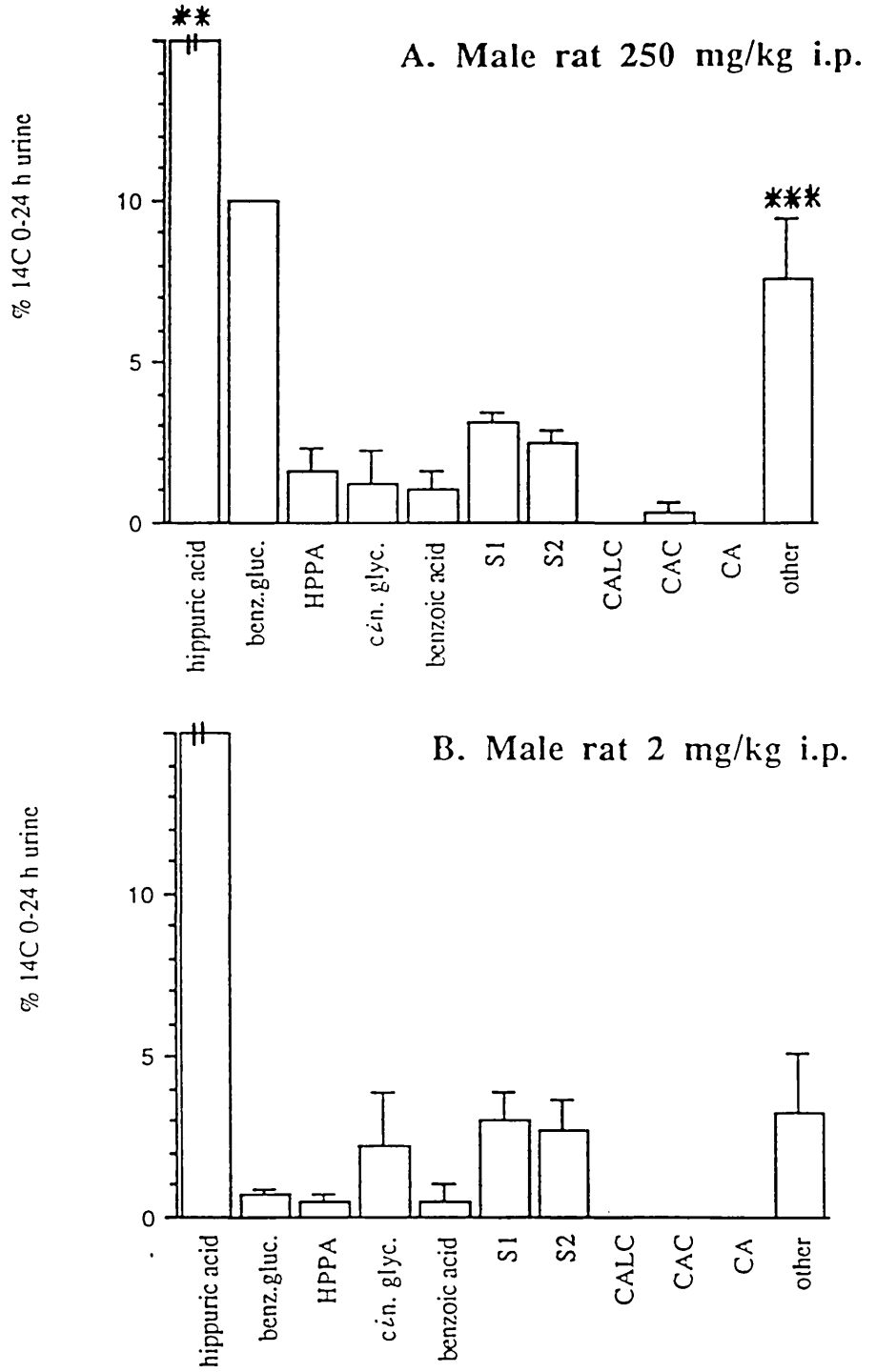
Elimination of ^{14}C in urine and faeces of CD1 mice given ^{14}C-cinnamaldehyde* and the influence of dose size, sex and route of administration

	0-24 h	24-48 h	48-72 h	0-72 h
Male high i.p.				
urine	81.2 ± 11.5	5.5 ± 3.6	0.9 ± 0.2	87.6 ± 14.6
faeces	5.8 ± 5.1	0.3 ± 0.1	0.1 ± 0.1	6.3 ± 4.9
total	87.0 ± 10.9	5.8 ± 3.6	1.1 ± 0.2	93.8 ± 14.7
Male low i.p.				
urine	85.8 ± 19.2	4.2 ± 3.0	3.0 ± 1.8	93.0 ± 17.2
faeces	8.6 ± 5.1	0.8 ± 0.7	0.5 ± 0.6	9.9 ± 5.9
total	94.3 ± 14.7	5.0 ± 3.6	3.6 ± 2.3	102.9 ± 11.5
Female high i.p.				
urine	78.7 ± 3.4	7.8 ± 4.6	0.7 ± 0.8	87.3 ± 7.3
faeces	10.1 ± 4.6	0.4 ± 0.3	0.1 ± 0.2	10.6 ± 4.4
total	88.8 ± 3.0	8.2 ± 4.9	0.9 ± 0.8	97.8 ± 6.4
Female low i.p.				
urine	71.0 ± 17.7	4.8 ± 3.7	1.1 ± 0.5	77.0 ± 18.0
faeces	15.5 ± 16.0	0.5 ± 0.2	0.2 ± 0.1	15.8 ± 16.0
total	86.3 ± 13.4	5.3 ± 3.5	1.3 ± 0.5	92.9 ± 14.3
Male high oral				
urine	84.2 ± 11.6	4.3 ± 4.9	1.6 ± 1.5	90.0 ± 11.0
faeces	8.3 ± 7.8	0.5 ± 0.2	0.1 ± 0.0	8.9 ± 7.7
total	92.5 ± 10.8	4.8 ± 4.9	1.7 ± 1.5	98.9 ± 7.1

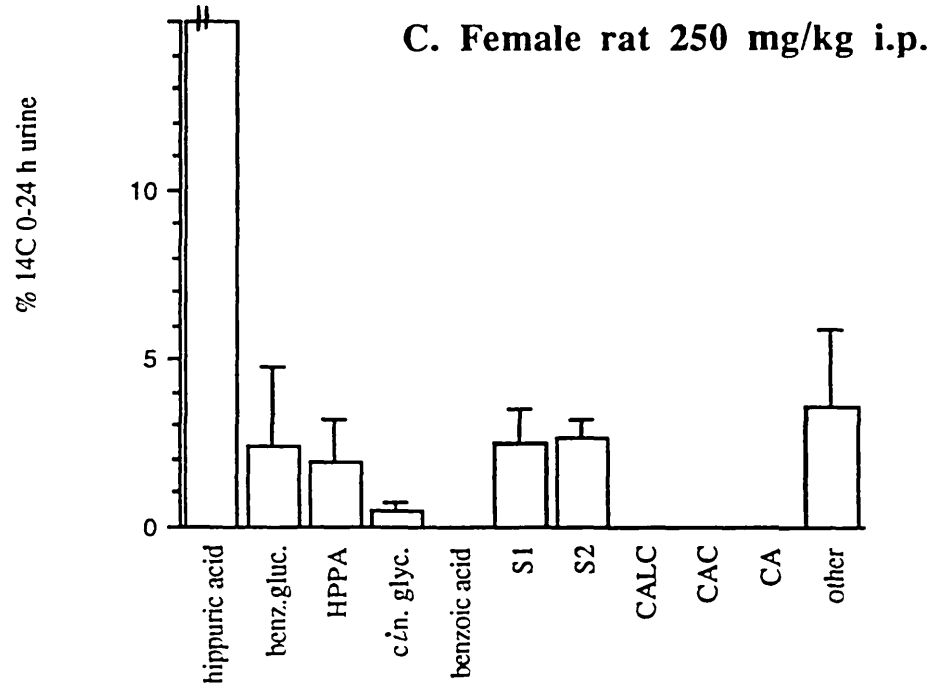
* Figures are means ± S.D. % dose excreted in each time period, n=6.

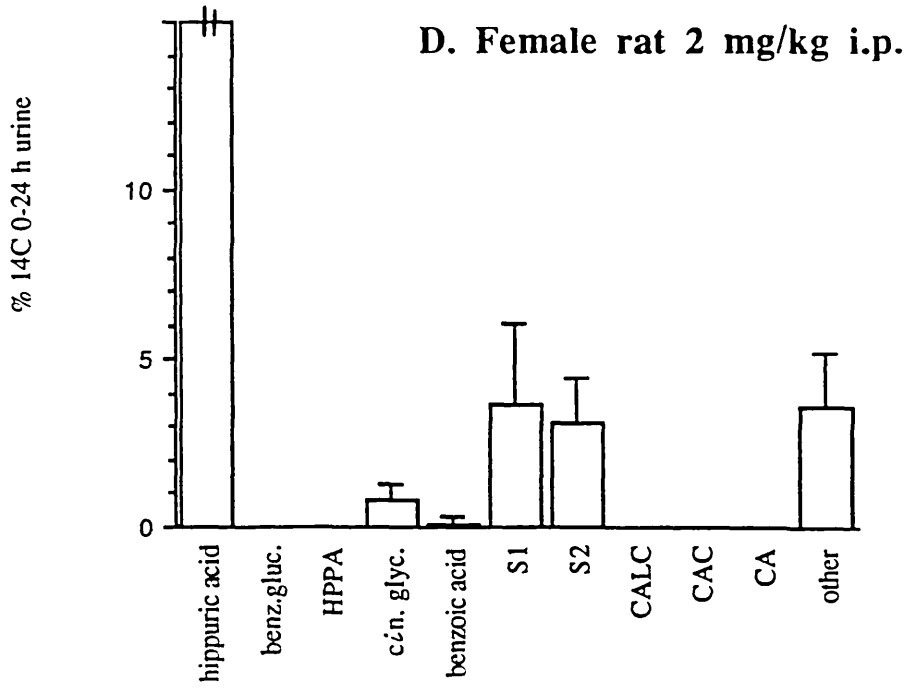
Figure 3.1

Metabolic profile of ^{14}C-cinnamaldehyde in F344 rat 0-24 h urine*
Influence of dose size, sex and route of administration



* ^{14}C-cinnamaldehyde metabolites expressed as percentage of total ^{14}C recovered after radio HPLC analysis of 0-24 h urine.
** See Table 3.3 for % hippuric acid.
*** other = more than one metabolite, each accounting for less than 1-2% of urinary ^{14}C.


C. Female rat 250 mg/kg i.p.
% 14C 0-24 h urine
10
5
0
hippuric acid
benz.gluc.
HPPA
c.in. glyc.
benzoic acid
S1
S2
CALC
CAC
CA
other


D. Female rat 2 mg/kg i.p.
% 14C 0-24 h urine
10
5
0
hippuric acid
benz.gluc.
HPPA
c.in. glyc.
benzoic acid
S1
S2
CALC
CAC
CA
other

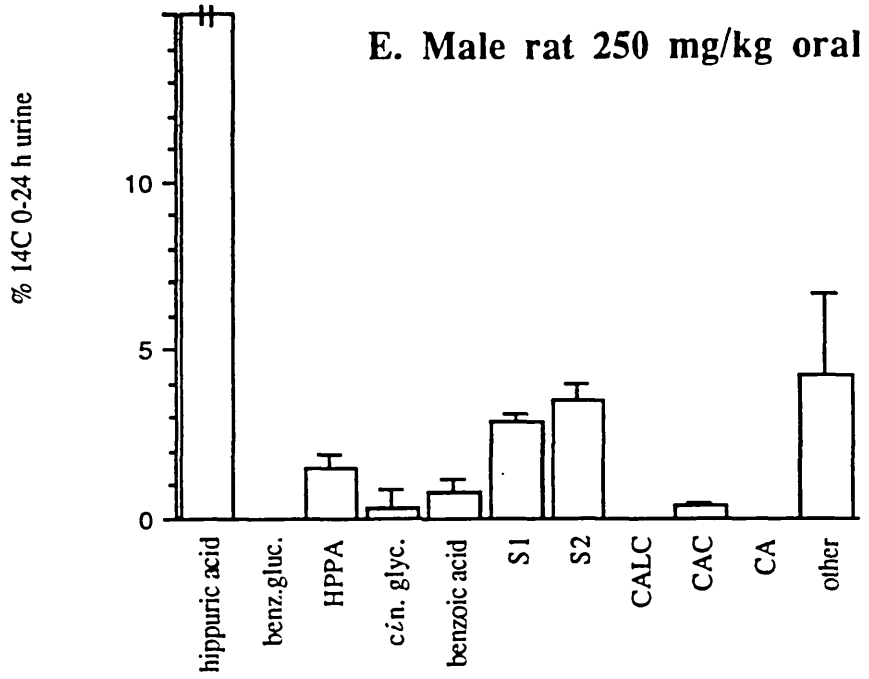
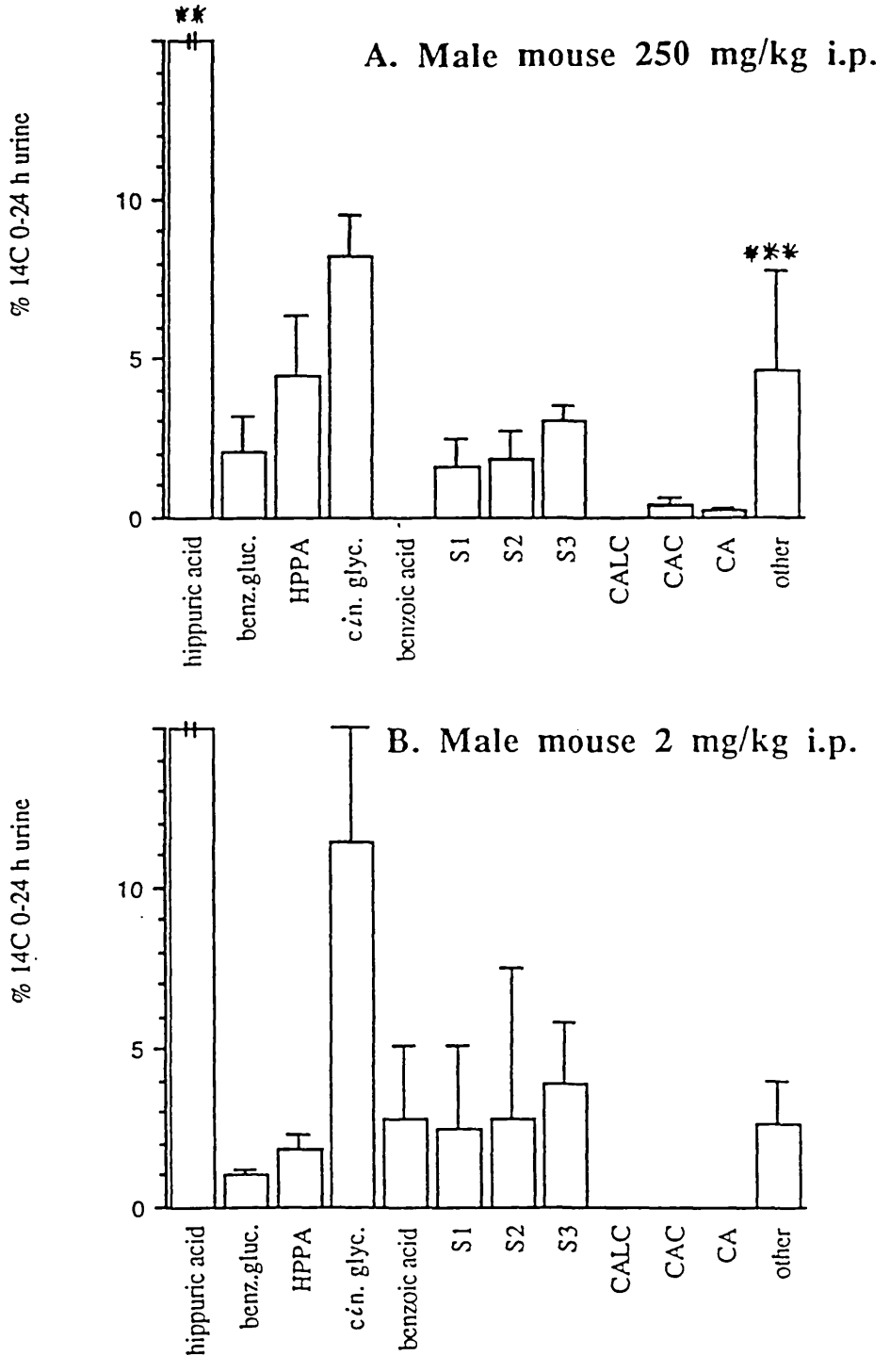
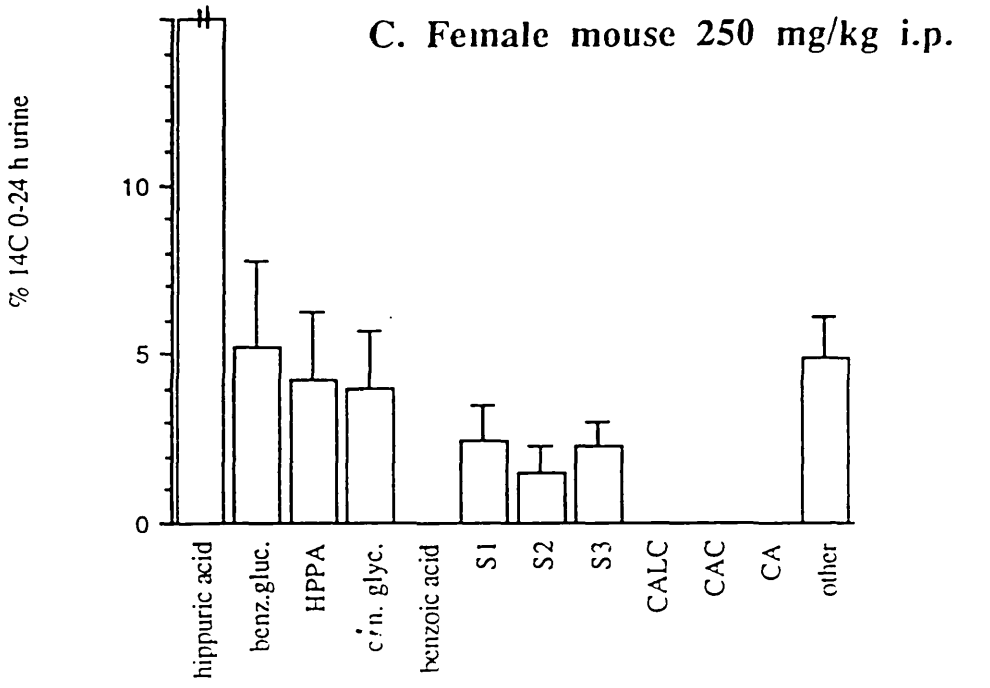

E. Male rat 250 mg/kg oral
% 14C 0-24 h urine
10
5
0
hippuric acid
benz.gluc.
HPPA
cin. glyc.
benzoic acid
S1
S2
CALC
CAC
CA
other

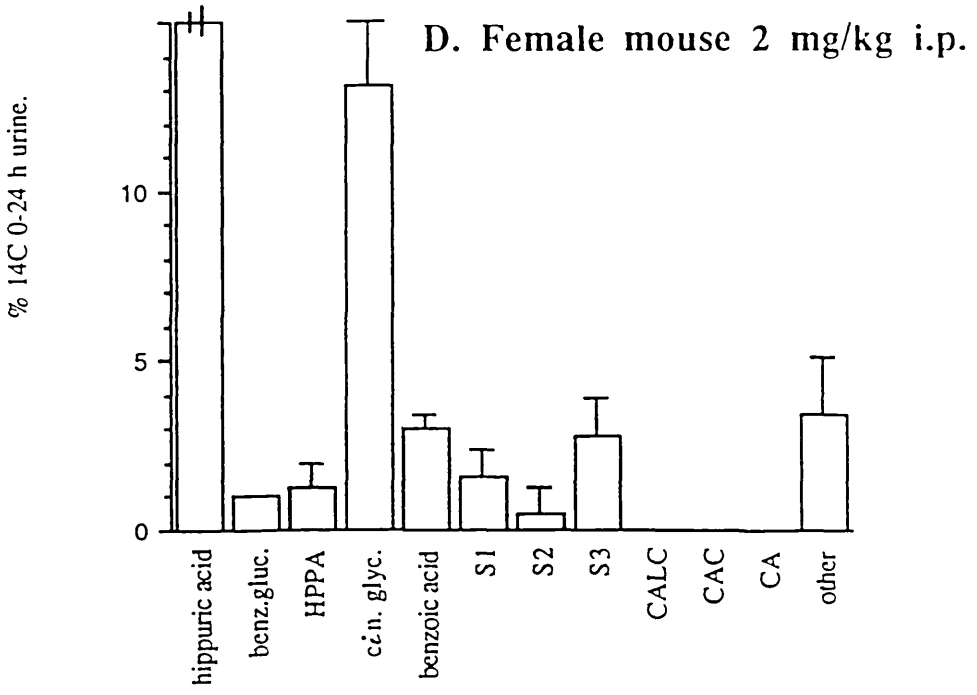
Figure 3.2

Metabolic profile of ^{14}C-cinnamaldehyde in CD1 mice 0-24 h urine.* Influence of dose size, sex and route of administration



* ^{14}C-cinnamaldehyde metabolites expressed as percentage of total ^{14}C recovered after radio HPLC analysis of 0-24 h urine.
** See Table 3.3 for % hippuric acid.
*** other = more than one metabolite, each accounting for less than 1-2% of urinary ^{14}C.


C. Female mouse 250 mg/kg i.p.
% 14C 0-24 h urine
10
5
0
hippuric acid
benz.gluc.
HPPA
cin. glyc.
benzoic acid
S1
S2
S3
CALC
CAC
CA
other


D. Female mouse 2 mg/kg i.p.
% 14C 0-24 h urine.
10
5
0
hippuric acid
benz.gluc.
HPPA
cin. glyc.
benzoic acid
S1
S2
S3
CALC
CAC
CA
other

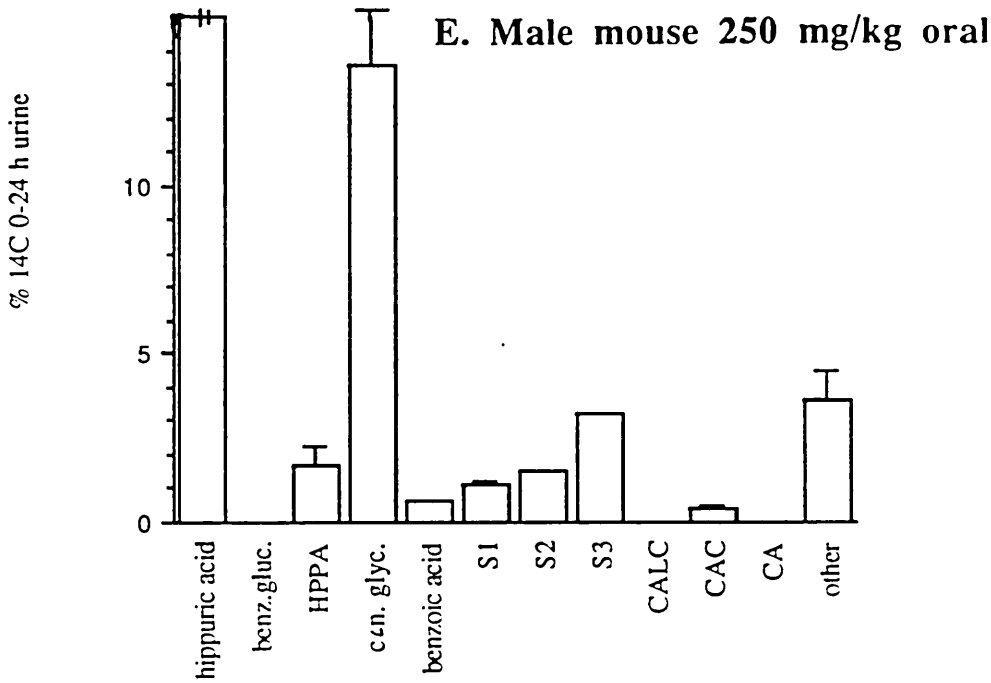

E. Male mouse 250 mg/kg oral
% 14C 0-24 h urine
10
5
0
hippuric acid
benz.gluc.
HPPA
c.n. glyc.
benzoic acid
S1
S2
S3
CALC
CAC
CA
other

Table 3.3

% ^{14}C in 0-24 h urine excreted as hippuric acid in rat and mouse and the influence of dose size, sex and route of administration

rat	male i.p.	female i.p.	male oral
high	73.1 ± 2.1	84.4 ± 6.6	86.9 ± 2.6
low	85.1 ± 3.5	88.2 ± 2.2	
mouse			
high	72.0 ± 5.8	71.4 ± 4.8	74.5 ± 2.5
low	71.8 ± 8.2	71.7 ± 2.3	

* Figures are means ± S.D. % ^{14}C recovered after radio HPLC analysis, n=4 rats, n=6 mice.

Chapter 4

The metabolic disposition of ^{14}C-cinnamaldehyde in human volunteers

4.1 Introduction

Presently used procedures for assessing the toxic risk of food additives to man principally involve long term feeding studies in rodent species such as the mouse and rat. The difficulty of predicting human risk from animal studies has already been discussed in Chapters 1 and 3. These arise from high dose-low dose extrapolation and species differences in absorption, distribution, routes and rates of metabolism, the contribution of the gut microflora and from differences in excretion.

An example where the relevance of animal data for the human situation has been questioned is the ban on the use of the food flavour coumarin. Coumarin elicits species differences both in metabolism and toxicity. In man and the baboon, coumarin is extensively metabolized to 7-hydroxy-coumarin (Shilling *et al.*, 1969; Gangolli *et al.*, 1974), whereas in several other species, including the rat, hamster, dog and rabbit, 7-hydroxylation constitutes only a minor metabolic pathway (Cohen, 1979). The major metabolic pathway in the rat appears to involve an initial 3-hydroxylation reaction with subsequent opening of the lactone ring and further metabolism to *o*-hydroxyphenylacetic acid (Kaighen and Williams, 1961; Cohen, 1979). The administration of single doses of coumarin to rats produces centrilobular bile duct lesions (Cohen, 1979; Evans *et al.*, 1979). However, there is no evidence for adverse effects in man (Cohen, 1979). Although the mechanism of coumarin toxicity in the rat has not yet been identified, the differences in metabolism between rat and man do not justify extrapolation of toxicological data across species. Equally, the ban on the sweetener saccharin has fuelled the debate on the appropriateness of the traditional rat and mouse bioassays which employ high doses unrepresentative of the human situation of low life-long exposure.

In the experiment described in this Chapter, a dose of 0.7 mg/kg was chosen, the ADI advised by JECFA before it was withdrawn in 1989. JECFA first set an ADI of 1.25 mg/kg (1967b) based on the toxic effect level in rats fed a diet containing cinnamaldehyde for 16 weeks (Hagan *et al.*, 1967). In 1984 the ADI was reduced to 0.7 mg/kg unless and until further data were presented on metabolism and pharmacokinetics and carcinogenicity, and eventually withdrawn in 1989 when these data were not forthcoming. This illustrates that ADIs are not based on true risk or exposure. An estimate of human exposure is however difficult. Often an approximation is achieved by total tonnage used by industries, which for cinnamaldehyde was 0.5 million kg in the USA in 1970. The same figure was reported for cinnamaldehyde use in 1987, not reflecting the increase in real consumption as a result of the increased use of processed foods. This may be accounted for by the rationalisation in the process industry, which has led to more efficient use of materials (FEMA, 1978, 1987a). A better estimate is given by analysis of the diet of a large group of people, but this is hard to obtain. The last reported PADI (possible average daily intake) for cinnamaldehyde calculated in this way was 179.2 mg (FEMA, 1978). A further difficulty determining the true level of exposure of a population is the difference in diet between individuals. For instance, the average per capita consumption of saccharin in 1972 was estimated as 23 mg/kg/day, but the major consuming population consisted of low calorie soft drink consumers, whose average consumption was 389 mg/kg/day (Hayes and Campbell, 1986). A high user group of cinnamaldehyde *i.e.* soft drink and convenience meal consumers could easily have a daily intake of 200 mg (3 mg/kg), whereas others would hardly be exposed (FEMA, 1978).

The risk presented by foodstuffs depends not only on individual differences in intake, but also on individual differences in response,

which are greater in humans than in genetically identical laboratory animals kept under standard conditions. Differences in susceptibility can be due to disease, diet, lack of vitamins, GSH status and idiosyncratic reactions, such as immunological hypersensitivity (Calabrese, 1983). Genetically determined differences in amount and rate of metabolism (polymorphisms) in man are known, such as the absence of the alicyclic oxidation of the anti-hypertensive drug debrisoquine, which is associated with a heightened sensitivity to this drug in deficient individuals (Timbrell, 1987). In relation to the metabolism of cinnamaldehyde it is of interest to mention that the GST iso-enzyme μ is deficient in 50% of the population (Vos *et al.*, 1988).

The metabolism of cinnamaldehyde has not previously been studied in man, but reports exist on the metabolism of benzoic acid (Smith and Williams, 1974), cinnamic acid (Snapper *et al.*, 1940; Hoskins *et al.*, 1984) and sodium cinnamate (Snapper and Saltzman, 1948, 1949), which was used as a test of liver function by measuring the excretion of hippuric acid and benzoyl glucuronide in healthy subjects and patients (Saltzman and Caraway, 1953; Quarto di Paolo and Bertolini, 1963). These studies show that the metabolism of cinnamic acid in man is comparable to that in rodents, with the exception of cinnamoyl glucuronide which is found in human urine after ingestion of large doses, but not in urine of rats or mice (Nutley *et al.*, 1993). However, in addition to the common metabolites of cinnamaldehyde and cinnamic acid, a second pathway *via* GSH conjugation plays a role in the metabolism of cinnamaldehyde in rat and mouse. It is therefore of interest to study the relative importance of these two routes of metabolism in man. In the study described below two volunteers received 0.7 mg/kg ^{14}C-cinnamaldehyde.

4.2 Materials and methods

4.2.1 Chemicals

All chemicals used were as described in Chapter 2.

4.2.2 Human study

Two healthy non-smoking male volunteers, ages 44 and 57, abstained from alcohol 24 h before and during the experiment, but kept to their normal diet. Control urine was collected for 24 h the day before the study. At 9.00 a.m. a single oral dose of 50 mg (α 0.7 mg/kg) 10 µCi ^{14}C-cinnamaldehyde was taken in a gelatine capsule with water. Urine samples were collected as voided until 100% recovery was obtained.

4.2.3 Scintillation counting

Urine volumes at each time period were measured and samples assayed for ^{14}C by liquid scintillation counting as described in Chapter 2. Urine was stored at -20 °C until further analysis.

4.2.4 Analysis of urinary metabolites by radio HPLC

Pooled 0-24 h urine samples were freeze dried and taken up in a small volume of 50% (*v/v*) methanol/water. Samples were centrifuged (11,000 rpm, 10 min, Heraeus Sepatech, Biofuge B) and supernatants analyzed by radio HPLC as described in Chapter 2.

Alternatively, urine samples were adjusted to pH 5.0 by addition of an equal volume of 0.2 M pH 5.0 acetate buffer and extracted with 2 x 5 volumes of diethyl ether. The ether extracts were combined, dried on

Na_2SO_4 overnight, evaporated to dryness under N_2 and taken up in 50% methanol/ water before radio HPLC analysis. To the remaining aqueous phase Glucurase (ß-glucuronidase 5000 units/ml, *ex* bovine liver, Sigma) or 0.64 mg sulfatase (Type H-1, *ex Helix pomatia*, sp.act. 18000 units/g, Sigma) was added in an equal volume, and the whole incubated at 37 °C overnight. To inhibit the ß-glucuronidase activity present in the sulfatase, control incubations were performed with the addition of 0.8 mg D-saccharic acid 1,4-lactone. The reaction was stopped by freezing to -20 °C. Samples were then extracted with ether and the ether phase prepared for radio HPLC analysis as described above. The aqueous phase was brought to pH 1.0 by addition of concentrated HCl and again extracted with ether and analyzed by HPLC. Recoveries of ^{14}C at each extraction step were calculated by measuring volumes and radioactivity in each phase. Metabolic profiles were compared to those of control samples incubated without enzyme. To determine the presence of any metabolites labile to mild alkali, urine samples were adjusted to pH 10.0 with 1 M NaOH, left to stand at room temperature for 1 h and neutralized with 1 M HCl prior to ether extraction and chromatography.

4.3 Results

4.3.1 Excretion of ^{14}C

The elimination of ^{14}C-cinnamaldehyde by human volunteers was rapid, with 85% present in the 0-2 h urine and 100% recovered in 8 h (Fig. 4.1) for both subjects. No ^{14}C was detected in the 8-24 h urine.

4.3.2 Metabolism of ^{14}C-cinnamaldehyde

A typical HPLC trace for the 0-24 h urine of a human volunteer after 0.7 mg/kg ^{14}C-cinnamaldehyde is presented in Fig. 4.2. HPLC analysis (Table 4.1) showed cinnamaldehyde to be completely metabolized and the major urinary metabolite was hippuric acid (93.1 and 93.1% of 0-24 h urine). 3-Hydroxy-3-phenylpropionic acid and benzoic acid were minor metabolites. The presence of small amounts of benzoic acid was demonstrated in one of the subjects after ß-glucuronidase treatment indicating the excretion of benzoyl glucuronide. No increase was seen in cinnamic acid after enzyme treatment, thus excluding the presence of cinnamoyl glucuronide.

Two unknown metabolites had retention times similar to the sulfur containing metabolites, S1 and S2, found in rats and mice when ^{35}S-CySH and non-labelled cinnamaldehyde were coadministered (Chapter 5) and these accounted for 3% of metabolism in both subjects.

4.4 Discussion

^{14}C-*trans*-Cinnamaldehyde is readily absorbed from the gastro-intestinal tract when administered orally to human volunteers, as shown by its rapid and extensive elimination in the urine as various metabolites. A recovery of 100% of the administered dose was achieved in 8 h.

trans-Cinnamaldehyde was completely metabolized with no parent compound excreted. The major metabolite (93% in both volunteers) was hippuric acid, the glycine conjugate of benzoic acid, accompanied by 3-hydroxy-3-phenylpropionic acid and benzoic acid, metabolites both formed *via* oxidation of the aldehyde to its acid and subsequent ß-

oxidation of the side chain. Metabolism in man resembles that in rodents and proceeds mainly *via* ß-oxidation analogous to that of fatty acids. Cinnamoyl glycine, which was formed up to 20% in mice at low doses (Chapter 3), was not found as a metabolite of cinnamaldehyde in man consistent with findings of other authors who studied human metabolism of cinnamic acid (Snapper and Saltzman, 1949).

After treatment of urine with ß-glucuronidase a small increase was seen in the benzoic acid peak in one subject (subject 1), indicating the formation of a trace of benzoyl glucuronide, which has been reported as a minor metabolite of cinnamic acid in man (Snapper *et al.*, 1940; Snapper and Saltzman, 1949). Upon treatment with ß-glucuronidase, cinnamic acid was not formed, showing that cinnamoyl glucuronide was not a metabolite of cinnamaldehyde in man after 0.7 mg/kg, although it was reported by Snapper *et al.* (1940) and Hoskins *et al.* (1984) as a metabolite of cinnamic acid in man after large doses. Cinnamoyl glucuronide was not identified by Nutley *et al.* (1993) as a metabolite of cinnamic acid in rat or mouse, nor as a metabolite of cinnamaldehyde in rat or mouse urine (Chapters 2 and 3) by any of the tests used to show the presence of benzoyl glucuronide. The molecular weight of cinnamoyl glucuronide (324) is such that it is possible this metabolite was formed and excreted in bile of rodents, but in the urine of man, where the molecular weight threshold for biliary excretion is higher than in rodents (Smith, 1973).

Two further metabolites (ca 3% of metabolism in each volunteer) coeluted with the sulfur metabolites detected in the rat and mouse after administration of ^{35}S-CySH and cinnamaldehyde (Chapter 5). Analysis of urine by GC-MS and GC with sulfur selective detection (Chapter 5) shows the presence of two mercapturic acids in the urine of human volunteers after 0.7 mg/kg cinnamaldehyde and in rat urine and it is

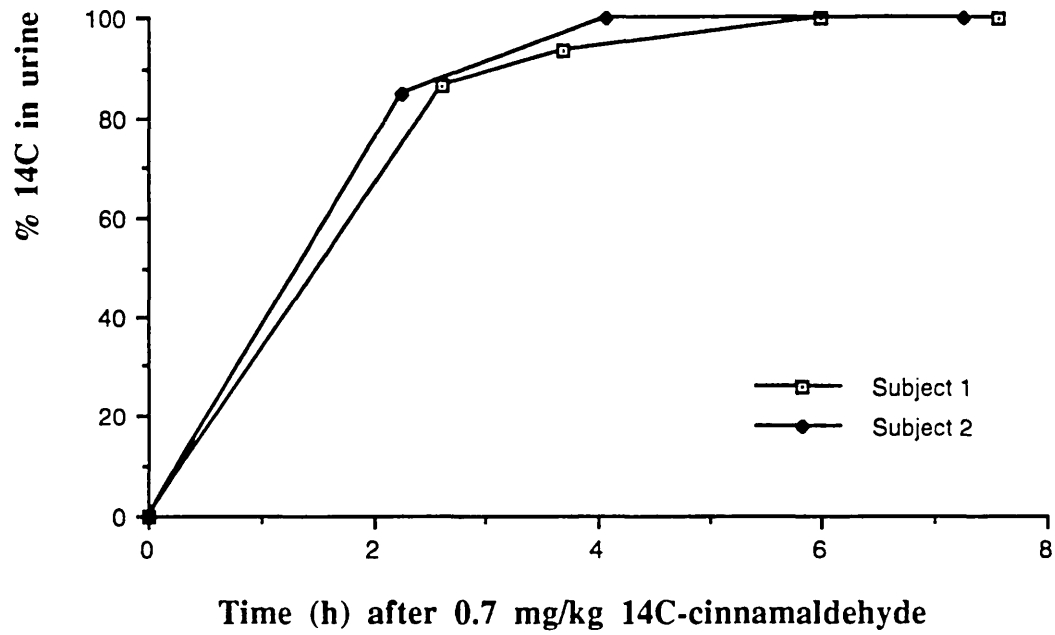
proposed that these are the metabolites found in this experiment. This suggests that, apart from oxidative metabolism, there is a second pathway for the elimination of cinnamaldehyde in man *via* conjugation with GSH. This pathway accounts for 3% of metabolism, compared to 7% in rat and mouse. The third sulfur containing metabolite, found in the mouse after ^{35}S-CySH and cinnamaldehyde, was not detected in human urine.

If the human fate of cinnamaldehyde is compared to its disposition in animals, it is seen that the pattern of elimination of ^{14}C in man, resembles that in rodents, with complete recovery of ^{14}C in urine in man and very low excretion in the faeces of animals even at high dose. However, the rate of elimination in man is faster, with the bulk of the dose equivalent to normal dietary levels being excreted in the urine within 8 h. In contrast, the elimination of a similar proportion of high doses by animals takes up to 72 h. The more rapid elimination of cinnamaldehyde is reflected in a higher proportion of the dose excreted as hippuric acid in man as compared to rodents.

A knowledge of comparative patterns of the metabolism and disposition of test compounds in animals and man is important in the safety assessment of chemicals. Although such data are now quite commonly available with reference to candidate drugs, it is still unusual to have this information about food additives. Comparison of the present results with those previously obtained in rats and mice indicates that the qualitative aspects of the pathways of metabolism are similar in rodents and man. Thus, man is exposed to an array of metabolites comparable to that in animals used in toxicity tests of cinnamaldehyde. The relative minor importance of GSH conjugation and the nature of mercapturic acids formed, as will be discussed in Chapter 5, seems to be similar in rat, mouse and man and would permit extrapolation across species.

Figure 4.1

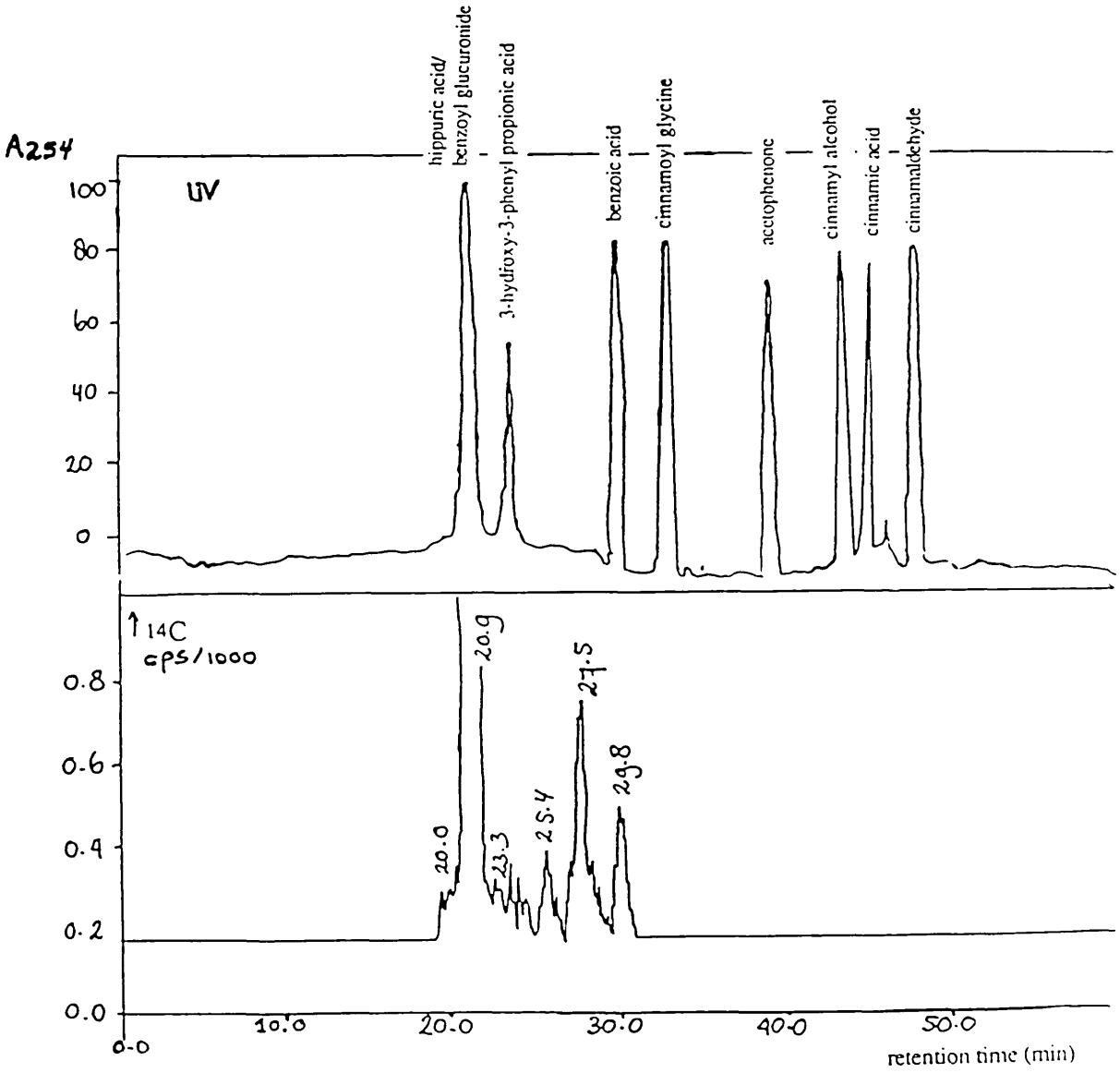
Elimination of ^{14}C in urine of human volunteers given 0.7 mg/kg ^{14}C-cinnamaldehyde



*Accumulative data % of dose excreted in each time period.

Figure 4.2

Radio HPLC trace of 0-24 h urine of a human volunteer given 0.7 mg/kg ^{14}C-cinnamaldehyde by mouth*



* Comparison with UV retention times of metabolite standards

Table 4.1

Metabolic profile of ^{14}C-cinnamaldehyde in the 0-24 h urine of human volunteers after 0.7 mg/kg

Metabolite	subject 1	subject 2
hippuric acid	93.1	93.1
benzoyl glucuronide	0.6	n.d
HPPA	1.2	1.5
unknown (25.2 min)	n.d	1.1
cinnamoyl glycine	n.d	n.d
S1	3.0	2.1
S2	**	1.0
benzoic acid	1.3	1.1
cinnamoyl alcohol	n.d	n.d
cinnamic acid	n.d	n.d
cinnamaldehyde	n.d	n.d
-----	----	----
total	99.2%	100%

* ^{14}C-cinnamaldehyde metabolites expressed as a percentage of the total recovery of ^{14}C after radio HPLC analysis of 0-24 h urine.

** coelution with S1.

Chapter 5

The role of glutathione conjugation in the metabolism of *trans*-cinnamaldehyde in rat, mouse and man

5.1 Introduction

In Chapters 2 and 3 the metabolism of ^{14}C-cinnamaldehyde was studied in rats and mice together with its dependency on dose, sex and route of administration. Metabolites included hippuric acid, benzoic acid, 3-hydroxy-3-phenylpropionic acid and benzoyl glucuronide, with hippuric acid as the major metabolite, all resulting from metabolism *via* a ß-oxidation pathway analogous to that of fatty-acids and in the mouse considerable amounts of cinnamoyl glycine were found. ^{14}C-Cinnamaldehyde metabolism thus largely resembles that of ^{14}C-cinnamic acid (Nutley*et al.*, 1993), but in addition to the common metabolites of cinnamaldehyde and cinnamic acid, two new metabolites were found in the rat and the same two and an additional third in the mouse accounting together for 7% of 0-24 h urinary metabolites. Cinnamaldehyde metabolism in man (Chapter 4) is similar to that in the rat and mouse mainly involving ß-oxidation, while two metabolites corresponding in retention times to the new metabolites in the rat accounted for 3% of dose. Literature suggests that conjugation with GSH may play a role in cinnamaldehyde metabolism *in vivo* (Boyland and Chasseaud, 1970; Delbressine *et al.*, 1981), but the relative importance of the GSH conjugation and ß-oxidation pathway in the detoxification of cinnamaldehyde is not known.

Toxicologically the extent to which a GSH-conjugation pathway is involved in cinnamaldehyde metabolism is of interest. Cinnamaldehyde is reactive *in vitro* towards protein thiol (Weibel and Hansen, 1989) and amino (Majetti and Suskind, 1973) groups and GSH conjugation protects against such damage, but a sufficient degree of GSH depletion may lead to toxicity. The formation of mercapturic acids in the urine, reflects GSH utilization and measurement of these metabolites as a function of the dose administered may thus be a helpful tool in

determining whether liver GSH levels have reached critical levels of depletion (Chasseaud, 1988).

Secondly, the nature of mercapturic acid metabolites in the urine provides information about the reactive species in the GSH conjugation. α,ß-Unsaturated compounds may react with GSH either by a direct addition of GSH to the ß-carbon atom of the double bond or after an epoxide is formed across the double bond by the action of cytochrome P450 mixed function oxidases. The mercapturic acids derived from such epoxide-GSH conjugates can be determined in urine and serve as a marker for the presence of these electrophilic metabolites *in vivo* (Van Bladeren *et al.*, 1981). Furthermore, although GSH conjugation and the subsequent formation of mercapturic acids generally leads to detoxication, facilitating the rapid elimination of chemicals from the body, some GSH conjugates can undergo extensive further metabolism. These pathways in GSH conjugate metabolism have been outlined in Chapter 1 and a number of toxicological consequences have been attributed to the various metabolites (Commandeur and Vermeulen, 1990).

The aim of the present study was the identification of possible metabolites of *trans*-cinnamaldehyde in rat, mouse and man derived from conjugation with GSH. To investigate the presence of these metabolites, an experiment was performed in which animals were dosed with the GSH precursor ^{35}S-CySH, followed by unlabelled cinnamaldehyde. Of the GSH precursors, glycine, glutamic acid and CySH, CySH is most effectively recovered as ^{35}S-labelled GSH conjugates (Lauterburg and Mitchell, 1981). CySH is not expected to raise the GSH concentration because this amino acid is rapidly metabolized (Meister, 1983). The urinary metabolic profile, corrected

for the control profile obtained before administration of cinnamaldehyde, was compared to that obtained in rats and mice administered with ^{14}C-cinnamaldehyde (Chapter 2). Metabolites detected in both experiments must have been derived from conjugation of cinnamaldehyde with GSH. The urine of rats and mice (250 mg/kg cinnamaldehyde i.p.) and human volunteers (0.7 mg/kg cinnamaldehyde by mouth) was also examined by GC with flame photometric sulfur detection and with GC-MS to establish the nature of the sulfur metabolites by comparison with synthesized standards.

5.2 Materials and Methods

5.2.1 Chemicals

L-[^{35}S]CySH, sp.act. 1300 mCi/mmol was purchased from Amersham International, Amersham, UK. Chemicals used in the synthesis of the mercapturic acids were purchased from Janssen, Beerse, Belgium. All other chemicals were obtained as reported in Chapter 2.

5.2.2 Synthesis of mercapturic acids

N-acetyl-S-(1-phenyl-propen-3-al)cysteine (the mercapturic acid of cinnamaldehyde, CA-NALC). This was prepared by addition of 2.5 g cinnamaldehyde (CA) to a stirred, cooled solution of 2.5 g N-acetyl-L-cysteine (NALC) in 50 ml diethyl formamide in the presence of 10 ml triethylamine. The mixture was kept under N_2 and left at room temperature for 3 days. The progress of the reaction was followed by examination of samples of the reaction mixture by silica gel TLC, which was developed with 70% n-propanol/water. NALC and NALC conjugates were detected by spraying with 0.1 M K_2CrO_7/acetic acid

(1:1) followed by 0.1 M $AgNO_3$ (Knight and Young, 1958). When no NALC remained according to TLC and GC analysis of methylated samples, the product was evaporated *in vacuo*, dried by addition of 2 x 50 ml toluene and again evaporation *in vacuo*. Ethyl acetate was added to the remaining oil and methylated samples were analyzed by GC-MS. From mass and NMR spectra it was concluded that the major reaction product was the methyl ester of CA-NALC and that no other mercapturate was present (Fig. 5.1A)

N-acetyl-S-(1-phenyl-3-hydroxypropyl)cysteine (the mercapturic acid of cinnamic alcohol, CALC-NALC). A sample of the reaction mixture of CA-NALC as described above was left at room temperature under N_2 for 3 days and then taken up in ethyl acetate. An aliquot of the reducing agent $NaBH_4$ was added and after 30 min, when the evolution of gas had ceased, the reaction mixture was filtered, evaporated *in vacuo* and dried with toluene. GC-MS confirmed that no CA-NALC remained. The mass spectrum of the major product was identical to that described by Delbressine *et al.* (1981) for the methyl ester of CALC-NALC, but the dimethyl ester of CALC-NALC was also found (Fig. 5.1B and C).

N-acetyl-S-(1-phenyl-3-carboxypropyl)cysteine (the mercapturic acid of cinnamic acid, CAC-NALC) was prepared by addition of 2.5 g cinnamic acid (CAC) and 2.5 g NALC to 50 ml of diethyl formamide and 10 ml triethylamine under N_2 and refluxing for 5 h. After cooling, the reaction mixture was evaporated *in vacuo* and dried with toluene. GC-MS analysis of methylated samples showed that CAC-NALC was the major product. The mass spectrum was identical to that described by Delbressine *et al.* (1981) for the methyl ester of CAC-NALC, although both CAC and NALC remained in the reaction

medium (Fig. 5.1D).

N-acetyl-S-(3-phenyl-2-propenyl)cysteine (the mercapturic acid of cinnamaldehyde on the aldehyde position, NALC-CA) **methyl ester.** This was synthesized as described by Delbressine *et al.* (1981). The desired ester was obtained as white crystals. The mass spectrum (Fig. 5.1E) was identical to that described by Delbressine *et al.* (1981).

5.2.3 Human study

Six healthy volunteers (2 female, 4 male) received a capsule containing 50 mg (ca 0.7 mg/kg) cinnamaldehyde which was taken by mouth. Urine was collected for up to 5 h after ingestion. Urine passed before the start of the experiment served as a control.

5.2.4 Rats and mice

A group of 4 male Fischer 344 (Harlan-OLAC, London; bwt 200 ± 10 g) or Wistar (Harlan, CPB, Zeist, The Netherlands, bwt 200 ± 10 g) rats received a single dose of 250 mg/kg cinnamaldehyde (in 0.4 ml trioctanoin) by i.p. injection. A group of 4 male Fischer 344 rats was dosed with ^{35}S-CySH (i.p., 56 µCi/rat in 0.3 ml trioctanoin). After 24 h a second dose of ^{35}S-CySH was given directly followed by a single dose of 250 mg/kg *trans*-cinnamaldehyde i.p. (in 0.4 ml trioctanoin). Further experiments were performed with groups of 6 male CD1 mice (Charles Rivers Laboratories, Manston, Kent, UK; bwt 27 ± 2 g) which received either a single dose of cinnamaldehyde (250 mg/kg in 0.2 ml trioctanoin) or two doses of ^{35}S-CySH (i.p., 2 x 25 µCi in 0.2 ml trioctanoin) followed by a single dose of 250 mg/kg cinnamaldehyde with the same timing as for the rats.

Animals were housed individually in glass metabolism cages (rat, Metabowl; mouse, Mini Metabowl, Jencons Ltd., Hemel Hemstead, Herts, UK) with free access to food and water. Urine and faeces were collected on the day prior to the experiment and daily for three days after dosing and kept at -20 °C until analysis. At the end of each 24 h period, the cages were rinsed with *ca* 100 ml 50% (*v/v*) ethanol/water and the washings counted for ^{35}S.

5.2.5 Radiochemical techniques

^{35}S was determined in rat and mouse urine, faeces and cage washes by scintillation counting and urinary and faecal metabolites were analyzed by HPLC as described in Chapters 2 and 3.

5.2.6 Preparation of samples for GC and GC-MS

To 200 µl of rat and mouse 0-24 h urine and 10% of the total human urine was added benzylmercapturic acid as internal standard (40 µg rats, 20 µg mice and 100 µg man). Samples were adjusted to pH < 2 by the addition of 3 N HCl and extracted twice with ethyl acetate by vortex mixing for 1 min and centrifugation at 4,000 rpm, 4 °C for 20 min. The combined ethyl acetate layers were evaporated to dryness under N_2 and methylated with diazomethane.

5.2.7 Methylation with diazomethane

Diazomethane was prepared by addition of 50% (*w/v*) KOH to dimethyl nitrozourea in ether. Diazomethane was added until no decolorization occurred and left for 1 h to ensure complete methylation. The ether was evaporated and the dry samples taken up in a small volume of ethyl acetate.

5.2.8 Gas chromatography with sulfur selective detection

Detection of sulfur containing metabolites in urine was achieved with an HP 5890 Series 2 gas chromatograph equipped with a Packard model 906 flame-photometric detector (GC-FPD, S-mode) and a ball-valve solid injector and an HP 3390A integrator. A CP Sil 19 CB column (25 m, 0.2 mm i.d., d_f 0.20 µm) was used. Injector and detector temperature were both 250 °C. The initial temperature was 50 °C (1 min), rising at 20 °C/min to 170 °C, held at 170 °C for 3 min, rising again at 10 °C/min to 250 °C and these final conditions were held for 10 min. Helium was used as a carrier gas, with a flow rate of 1 ml/min. In the detector the hydrogen flow rate was 142 ml/min and the air flow rates were 55 and 165 ml/min. Retention times were 20.3 and 20.5 min for CAC-NALC, 22.2 min for CALC-NALC, 24.0 for CA-NALC and 25.0 for NALC-CA.

5.2.9 Gas chromatography-mass spectrometry

GC-MS analysis was carried out on an HP 5890/MSD system. A CP Sil 19 column (25 m, 0.2 mm i.d., d_f 0.20 µm) was used. The operation temperatures were 280 °C (split injector and ion source) and electron impact ionization was performed at an electron energy of 70 eV. Helium was used as carrier gas (flow rate 1 ml/min) and isobutane as a reagent gas for chemical ionisation. The column temperature was programmed from 80 °C (1 min) rising to 280 °C at 20 °C/min. Retention times of the compounds under these conditions were 11.7 min for benzylmercapturic acid, 12.8 min for dimethyl CALC-NALC and 13.2 for monomethyl CAC-NALC, 13.4 min for CA-NALC and 13.6 min for monomethyl CALC-NALC. Mercapturic acids in urine of rats, mice and man after cinnamaldehyde were analyzed using selective ion monitoring (SIM) of ten characteristic ions, *m/z* 176, 208, 234,

248, 252, 264, 266, 293, 339, 323 with an acquisition time of 60 μsec for each ion. These ions were chosen on the basis of the expected fragments from the mercapturic acids, the spectra published by Delbressine *et al.* (1981) and the fragmentation of the methylated standards synthesized here. Aliquots of 2 μl were injected with a solid injector.

5.3 Results

5.3.1 Metabolites of cinnamaldehyde in rats after ^{35}S-cysteine

When animals were dosed with ^{35}S-CySH together with unlabelled *trans*-cinnamaldehyde, two new metabolites were seen which were not present in the urine of rats dosed with ^{35}S-CySH only. Their retention times (27.4 and 28.4 min) corresponded to the two unknowns, S1 and S2, found previously in experiments with ^{14}C-cinnamaldehyde (Chapters 2 and 3). The relative proportion of these two metabolites was 1:1 (Table 5.1).

In the 24-48 and 48-72 h urine there were no ^{35}S metabolites not found in urine from animals dosed with ^{35}S-CySH only. Analysis of faecal samples failed to show the presence of these metabolites.

5.3.2 Metabolites of cinnamaldehyde in mice after ^{35}S-cysteine

When cinnamaldehyde was given to animals pretreated with ^{35}S-CySH three metabolites in mouse urine were found that contained sulfur (Table 5.1). Two of these had the same retention times as S1 and S2 in the rat with the third, S3, eluting later around 32 min. S1, S2 and S3,

were formed in a ratio 1:1:1.2 and coeluted with the unknowns previously detected as metabolites of ^{14}C-cinnamaldehyde (Chapters 2 and 3).

HPLC analysis of 24-48 h and 48-72 h mouse urine and of faecal extracts showed that no sulfur containing metabolites of cinnamaldehyde were present in these samples.

5.3.3 Analysis of rat urine by sulfur selective detection

After administration of 250 mg/kg cinnamaldehyde to male F344 rats, two metabolites that were absent from control urine were detected by GC with sulfur selective detection (Fig. 5.2A and B). These cochromatographed with the synthesized methyl esters of CALC-NALC and CAC-NALC and were formed in an average ratio of 3:1 (peak area), CALC-NALC being the major metabolite. Both enantiomers of both metabolites were formed and these were seen as individual peaks after GC. The enantiomers of both CALC-NALC and CAC-NALC were formed in a 2:1 ratio.

When the same experiments were performed with Wistar rats, CALC-NALC and CAC-NALC were formed in a ratio 4:1, whereas the enantiomers of both CAC-NALC and CALC-NALC were found in a 1:1 ratio.

5.3.4 Analysis of human urine by sulfur selective detection

After cinnamaldehyde two metabolites were found in human urine absent from control urine (Fig. 5.3A and B). The predominant metabolite was CALC-NALC, which was formed in an average 11:1 ratio to CAC-NALC. The ratio of the enantiomers of CALC-NALC and

CAC-NALC was 5:1 for both, showing a preference for one of the two enantiomers.

5.3.5 GC-MS using selective ion monitoring

The presence of CALC-NALC and CAC-NALC in the urine of rat (Fig. 5.4) and human volunteers (Fig. 5.6) after cinnamaldehyde was confirmed by GC-MS analysis using a selective ion monitoring method involving scanning of ten characteristic ions, *m/z* 176, 208, 234, 248, 252, 264, 266, 293, 339, 323. Methyl esters of the mercapturic acids in the urine were identical to those from CALC-NALC and CAC-NALC (Fig. 5.1B,C and D). Neither CA-NALC (Fig. 5.1A) nor the mercapturic acid or cinnamaldehyde derived from conjugation with GSH on the aldehyde group (Fig. 5.1E) were present in urine.

CALC-NALC and CAC-NALC were metabolites of cinnamaldehyde in mouse urine (Fig. 5.5). There was twice as much CALC-NALC as CAC-NALC.

5.4 Discussion

The results presented in this Chapter show that next to the principal route of metabolism *via* ß-oxidation, a second route operates involving direct conjugation of cinnamaldehyde with GSH at its unsaturated double bond.

Administration of cinnamaldehyde leads to the excretion of ^{35}S containing metabolites in the urine of rats and mice and these coeluted with previously detected unknown metabolites of ^{14}C-cinnamaldehyde (Chapters 2 and 3) and were formed in similar ratios in experiments with both labels. In the rat, two such metabolites were present with a

third also found in the mouse, together accounting for 6-7% of urinary metabolism in both species, showing that conjugation with GSH is a minor pathway in the metabolism of cinnamaldehyde. In man two metabolites with identical retention times to those containing ^{35}S in the rat, accounted for 3% of metabolism. ^{35}S-containing metabolites were only found in 0-24 h rat and mouse urine and were not specifically excreted at later time indicating that no extensive enterohepatic circulation occurs. The sulfur containing metabolites were not found in faeces, which is important as it shows that faeces are not a route for their elimination.

The presence of sulfur metabolites in urine of rats, mice and human volunteers was confirmed using GC-MS with selective ion monitoring and GC with sulfur selective detection. The major sulfur metabolite in all three species was CALC-NALC, the mercapturic acid of cinnamyl alcohol, and smaller amounts of CAC-NALC, the mercapturic acid of cinnamic acid, were also found. No mercapturic acids of cinnamaldehyde that derived either from conjugation at the carbon double bond with retention of the aldehyde group or from conjugation at the aldehyde group were detected. These results compare to those presented by Delbressine *et al.* (1981), who found CALC-NALC and CAC-NALC as the only metabolites of cinnamaldehyde after dosage of 250 mg/kg. i.p., daily 5 days a week for 2 weeks to female Wistar rats. In an experiment (results not shown) in which rats and mice were given BSO (*L*-Buthionine (S,R)Sulfoximine), an inhibitor of GSH synthesis, 1 h prior to an oral dose of 250 mg/kg ^{14}C-cinnamaldehyde there was a significant reduction in the formation of all sulfur metabolites, leaving the formation of other metabolites unaffected. This further shows that S1, S2 and also S3, although the identity of this metabolite could not be determined with GC-MS and GC-S detection, were derived from conjugation with GSH. The nature of the mercapturic acid metabolites

in rat and man would permit extrapolation across species.

On the basis of their chemical structure α,ß-unsaturated compounds may react with GSH in two ways; by direct reaction *via* a Michael addition to the ß-carbon atom at the double bond, or by reaction with an epoxide formed across the double bond by the action of cytochrome P450 mixed function oxidases to form a hydroxymercapturic acid. Van Bladeren *et al.* (1981) investigated the possibility of both routes of conjugation using the compounds acrylonitrile, crotonitrile and cinnamonitrile and found that hydroxymercapturic acids accounted for respectively 33%, 9% and 2% of the total mercapturic acids in the 0-24 h urine of rats. The mass spectra of the methyl esters of the cinnamaldehyde mercapturic acids found as urinary metabolites in rat, mouse and man indicate that these are derived from direct addition of GSH at the ß-carbon atom of the unsaturated double bond. This is in agreement with the fact that cinnamaldehyde reacts with GSH in buffer in the absence of any metabolic activating system (Boyland and Chasseaud, 1967; Swales, 1993; Chapter 8).

Studies in buffer, cytosol and hepatocytes show that cinnamic acid does not react with GSH and that cinnamyl alcohol is reactive only after conversion to the aldehyde (Swales, 1993). The mercapturic acids found *in vivo* must thus have been derived from conjugation of cinnamaldehyde with GSH *via* its carbon double bond, followed by reduction or oxidation of the aldehyde group. The GSH conjugate of acrolein (Mitchell and Petersen, 1989) has been shown to be a substrate for cytosolic ADH and microsomal ALDH, leading to the formation of the mercapturic acid of allyl alcohol as the major metabolite in urine. Any GSH acrolein adduct escaping the reductive pathway of metabolism could diffuse into the mitochondria to undergo oxidation,

as a minor route. A similar metabolism could explain the formation of crotonaldehyde mercapturates (Gray and Barnsley, 1971) and the cinnamaldehyde mercapturates in which the aldehyde group was reduced or oxidized (Fig. 5.7).

Further evidence of the aldehyde as the reactive species in the GSH conjugation was provided by experiments by Delbressine *et al.* (1981). Administration of 125 mg/kg cinnamyl alcohol to female Wistar rats led to the formation of a mercapturic acid in the urine, which accounted for 8.8% of dose according to the thioether test of Seutter-Berlage *et al.* (1979). However, pretreatment of rats with pyrazole, an ADH inhibitor, 0.5 h before administration of cinnamyl alcohol (125 mg/kg) lowered the thioether excretion significantly to 3.3 ± 1.4% (n=4) of dose. Cinnamic acid showed no increase in thioether excretion. Delbressine (1981) proposes that the aldehyde is an intermediate in the conversion of the alcohol to its mercapturic acid, which is in agreement with the reduction in thioether excretion after inhibition of ADH by pyrazole. Other authors have presented evidence for similar mechanisms in order to explain the formation of mercapturic acids from crotyl alcohol (Gray and Barnsley, 1971) and allyl alcohol (Kaye, 1973).

Seutter-Berlage *et al.* (1981) found that certain aromatic aldehydes are metabolized to mercapturic acids *via* the corresponding alcohols. These alcohols are converted to sulfate esters which react with GSH (Clapp and Young, 1970; Kaye, 1974; Chidgey *et al.*, 1986). Like Delbressine *et al.* (1981), N-acetyl-S-(3-phenyl-2-propenyl)cysteine could not be detected as a metabolite of cinnamaldehyde, thus ruling out a metabolic sequence involving reduction to cinnamyl alcohol, sulfation and GSH conjugation. Experiments in which rats were dosed with pyrazole 0.5 h prior to ^{14}C-cinnamaldehyde (results not shown), showed no alteration

in the excretion of S1, S2 or S3 in rat and mouse, thus excluding cinnamyl alcohol as an intermediate in the formation of these mercapturates.

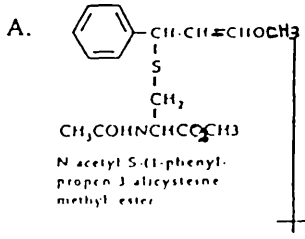
The finding of sulfur-containing metabolites in the urine shows that conjugation with GSH plays a part in the detoxification of cinnamaldehyde but inversely means that cinnamaldehyde reacts as an electrophilic species *in vivo* and could therefore pose a threat to PrSH groups and other macromolecules once extensive depletion of GSH has occurred. In view of the safety evaluation of this food flavour, it is of interest that GSH conjugation is only a minor route in the metabolism of cinnamaldehyde (3% in man, 6-7% in rat and mouse) and that in rat and mouse this pathway is independent of dose; the excretion of mercapturic acid metabolites does not decrease with dose, perhaps indicating that liver GSH levels, even at the high dose are not extensively depleted. Experiments in Chapter 9 in which liver GSH was measured after a single dose of cinnamaldehyde were designed to further examine this.

Figure 5.1

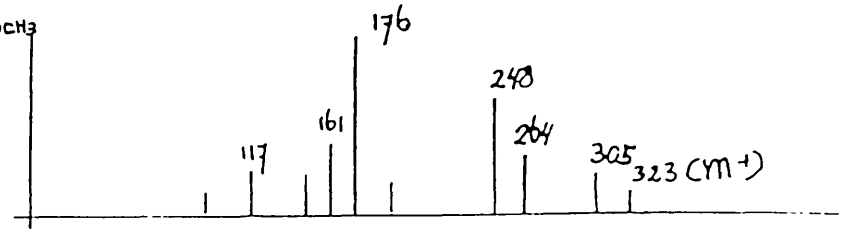
GC-MS total ion chromatograms of methylated mercapturic acid standards

GC-MS retention time 13.4 min

A.

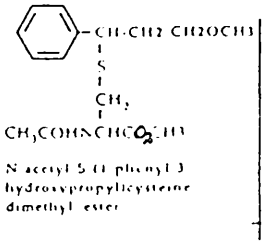


N-acetyl S-(1-phenyl-propen-3-yl)cysteine methyl ester

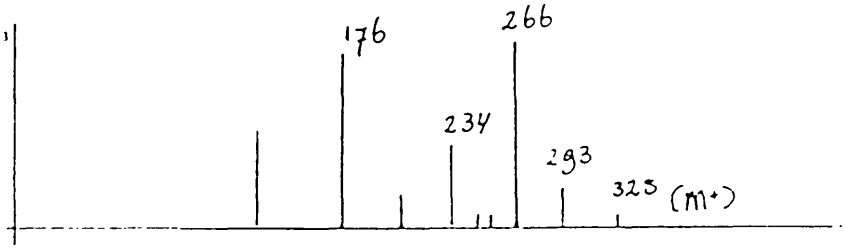


GC-MS retention time 12.8 min

B.

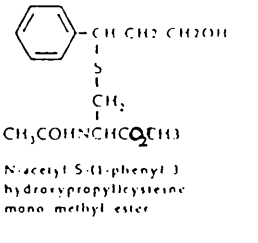


N-acetyl S-(1-phenyl-3-hydroxypropyl)cysteine dimethyl ester

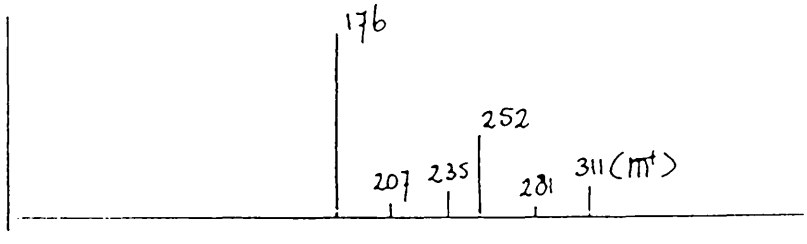


GC-MS retention time 13.6 min

C.

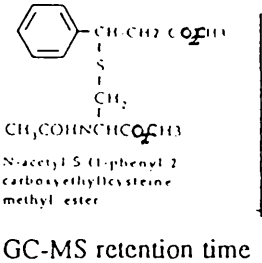


N-acetyl S-(1-phenyl-3-hydroxypropyl)cysteine mono methyl ester

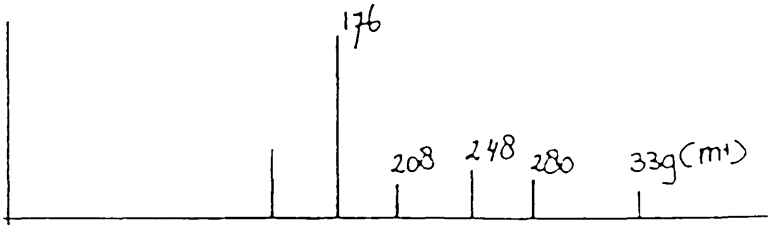


GC-MS retention time 13.2 min

D.

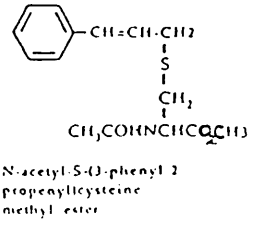


N-acetyl S-(1-phenyl-2-carboxyethyl)cysteine methyl ester



GC-MS retention time 13.3 min

E.



N-acetyl-S-(3-phenyl-2-propenyl)cysteine methyl ester

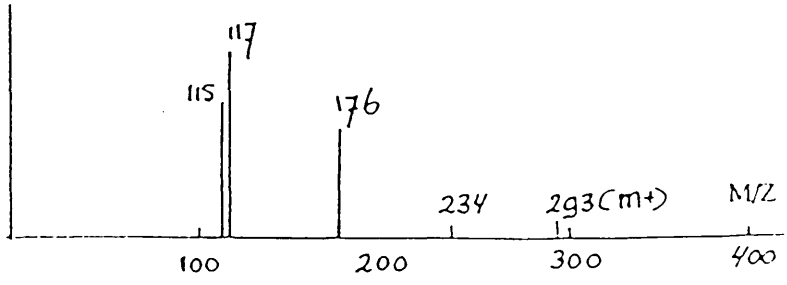


Table 5.1

Sulfur-containing metabolites of *trans*-cinnamaldehyde in the 0-24 h urine of rats and mice given 250 mg/kg i.p. Comparison of metabolites after ^{35}S-CySH and *trans*-cinnamaldehyde and after ^{14}C-cinnamaldehyde

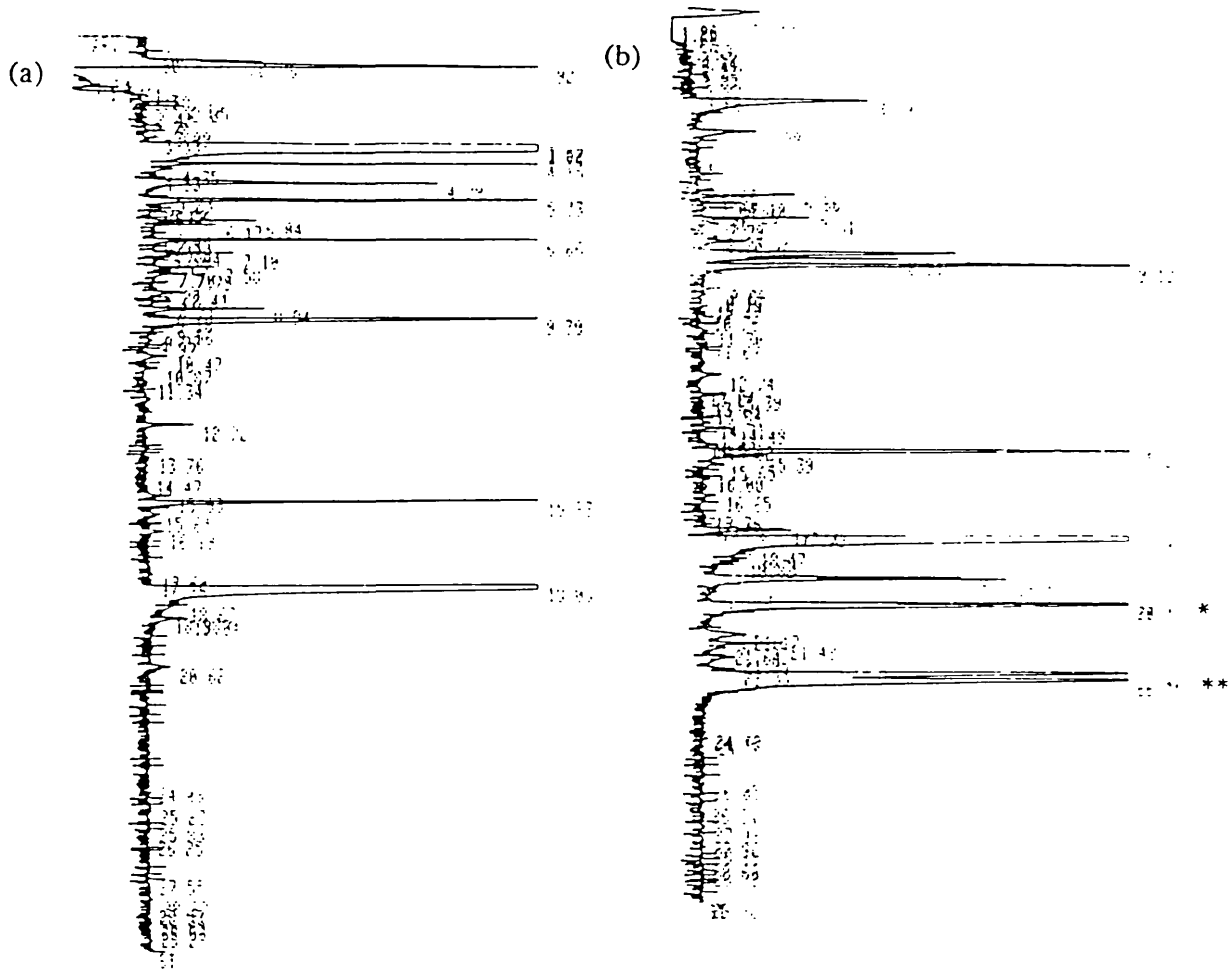
Metabolite	rat		mouse	
	^{14}C*	^{35}S**	^{14}C*	^{35}S**
S1	3.1 ± 0.5	1	1.6 ± 0.9	1
S2	2.7 ± 0.2	1	1.8 ± 0.9	1
S3	-	-	3.0 ± 0.5	1.2

* ^{14}C-cinnamaldehyde metabolites expressed as % of total recovery of ^{14}C after radio HPLC analysis of 0-24 h urine. Figures are means ± S.D., n=4 rats, n=6 mice.

** Relative formation of ^{35}S-metabolites absent form control urine as compared to S1 (=1).

Figure 5.2

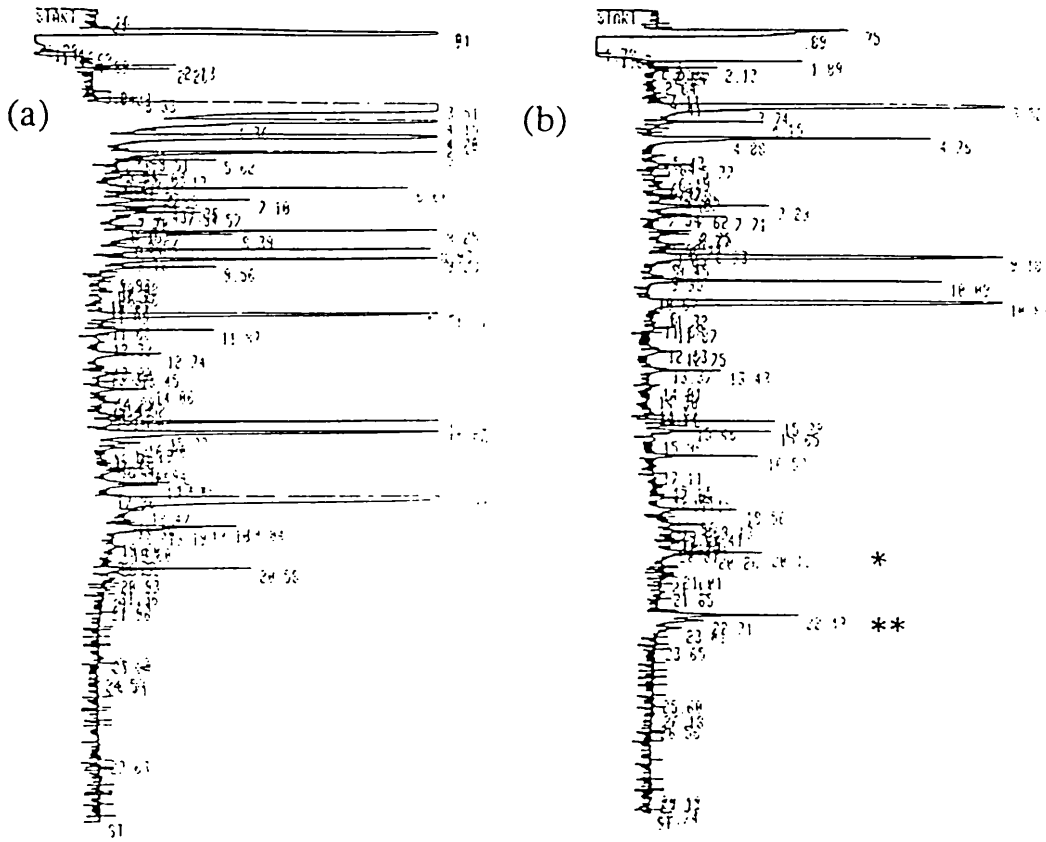
Typical GC-S trace of 0-24 h urine of F344 rat (a) control urine and (b) given 250 mg/kg *trans*-cinnamaldehyde i.p.



* CAC-NALC ** CALC-NALC

Figure 5.3

Typical GC-S trace of 0-4 h urine of human volunteers (a) control urine and (b) given 0.7 mg/kg *trans*-cinnamaldehyde



* CAC-NALC ** CALC-NALC

Figure 5.4

Typical GC-MS profiles of F344 rat urine (a) total ion chromatogram of blank urine, (b) total ion chromatogram of 0- 24 h urine after 250 mg/kg cinnamaldehyde i.p. and (c) ion 176 chromatogram of 0-24 h urine after 250 mg/kg

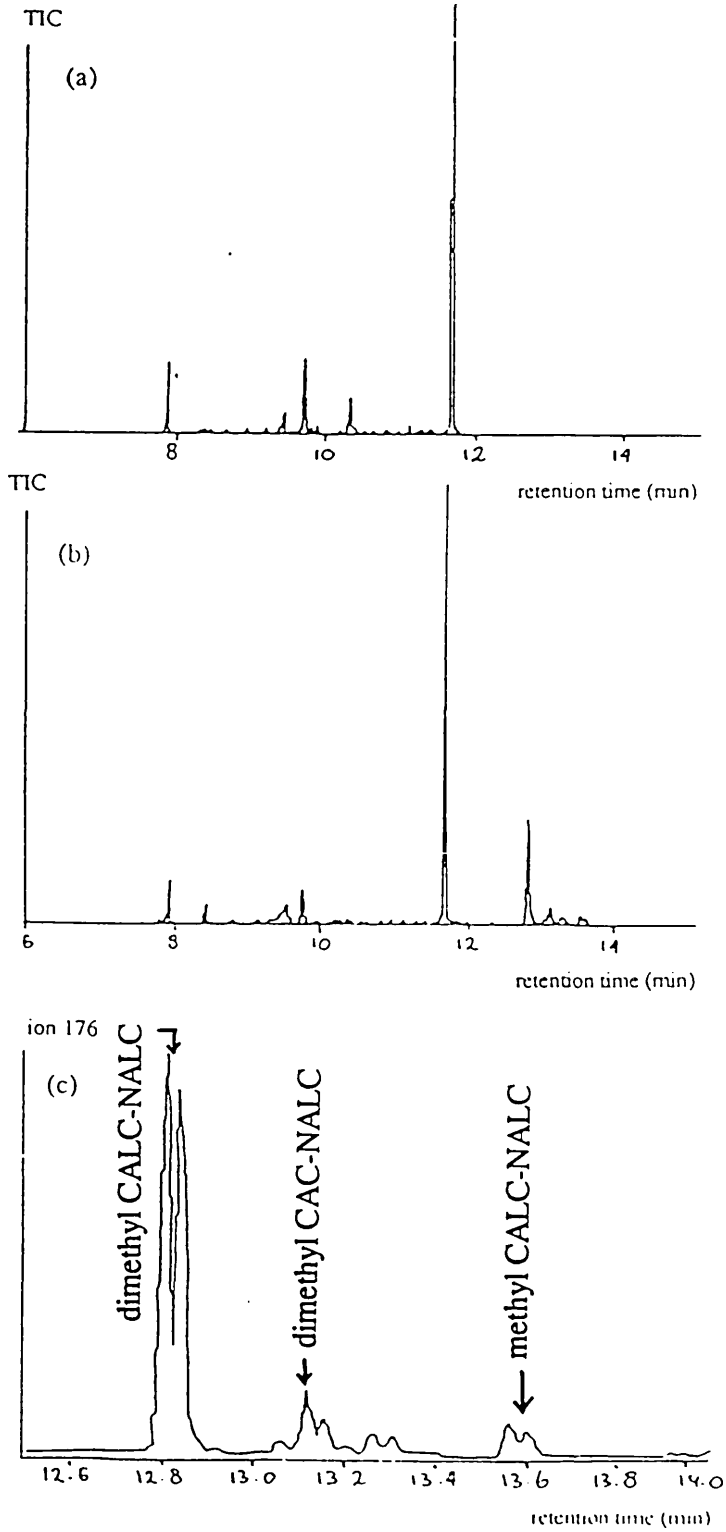


Figure 5.5

Typical GC-MS profiles of CD1 mouse urine (a) total ion chromatogram of blank urine, (b) total ion chromatogram of 0- 24 h urine after 250 mg/kg cinnamaldehyde i.p. and (c) ion 176 chromatogram of 0-24 h urine after 250 mg/kg

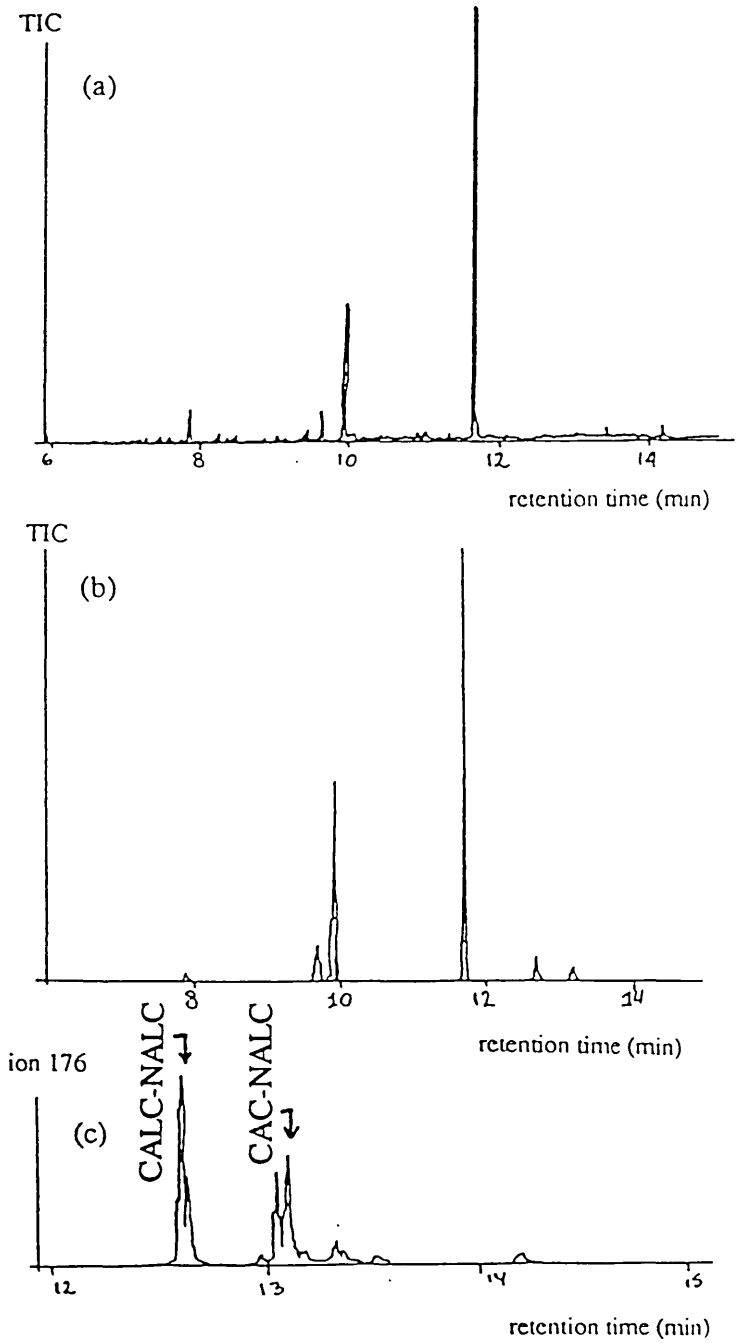


Figure 5.6

Typical GC-MS profiles of human urine (a) total ion chromatogram of blank urine, (b) total ion chromatogram of 0-4 h urine after 0.7 mg/kg cinnamaldehyde and (c) ion 176 chromatogram of 0-4 h urine after 0.7 mg/kg

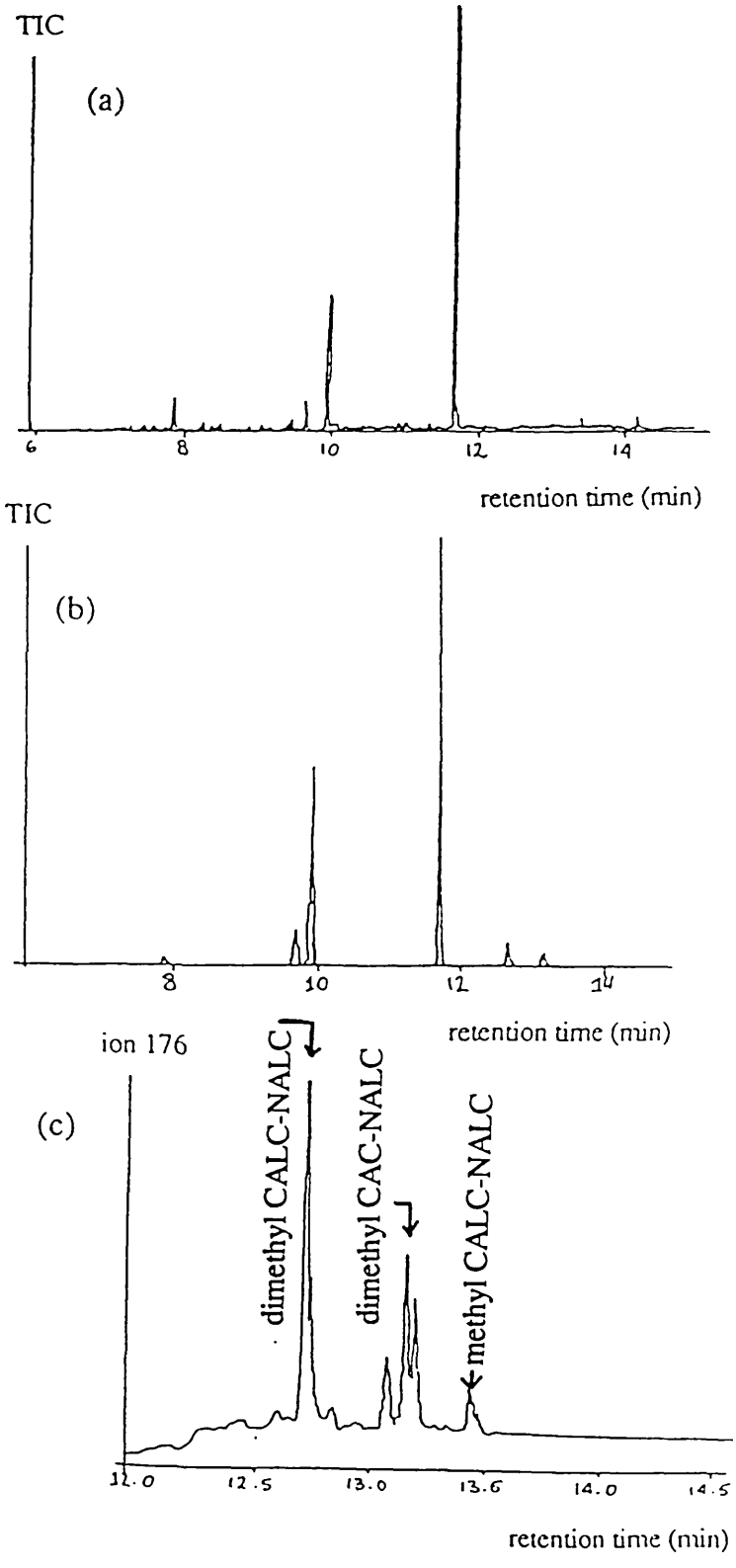
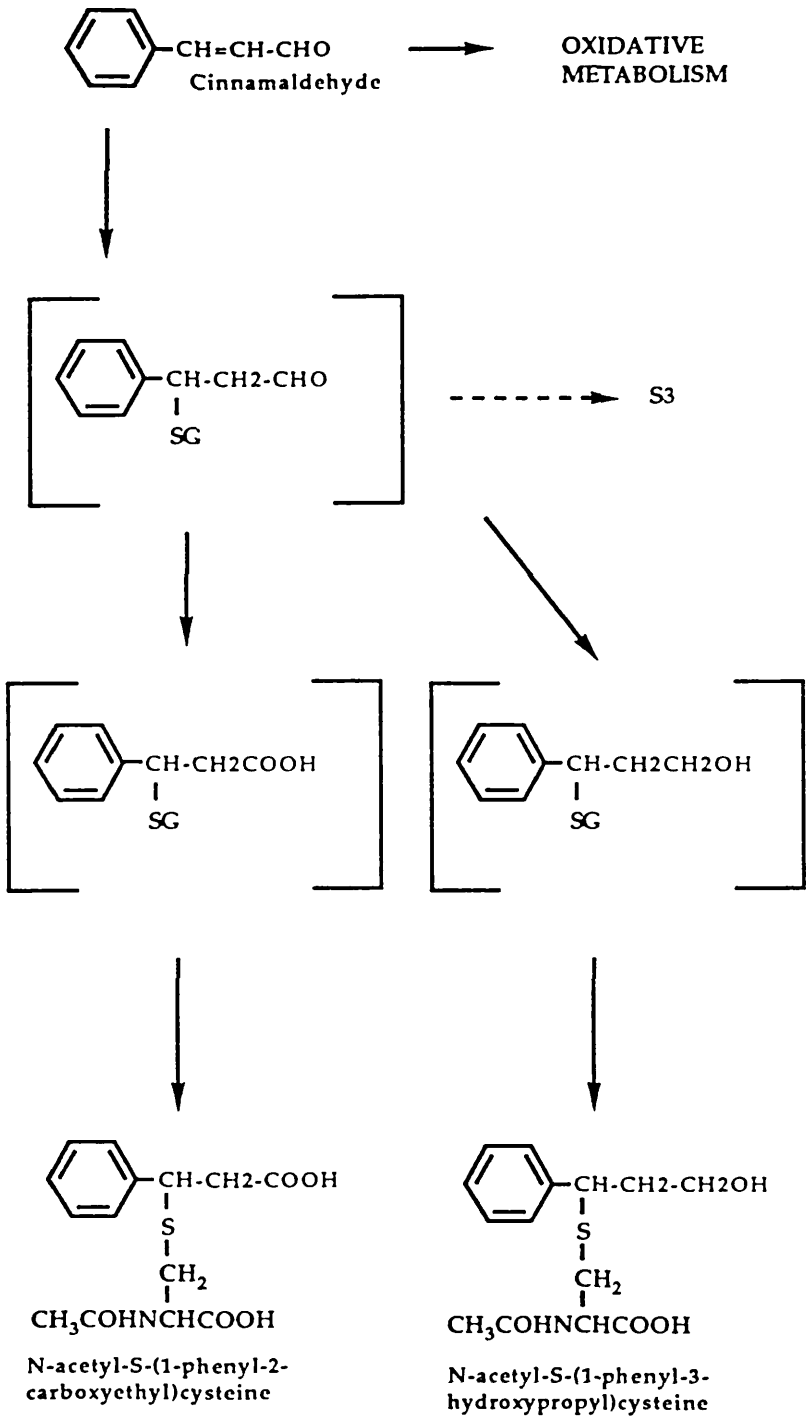


Figure 5.7 The proposed metabolism of cinnamaldehyde to sulfur-containing metabolites in rat, mouse and man



Chapter 6

Metabolism and protein binding of ^{14}C-cinnamaldehyde, ^{14}C-cinnamic acid and ^{14}C-cinnamyl alcohol in rat and mouse hepatocytes in suspension

6.1 Introduction

The metabolism of cinnamaldehyde in rat, mouse and man (Chapters 2, 3 and 4) mainly involves oxidation to cinnamic acid leading to hippuric acid as the major metabolite excreted in the urine, but a minor percentage of dose undergoes direct conjugation with GSH by an addition to the ß-carbon of the unsaturated bond to yield the mercapturic acids N-acetyl-S-(1-phenyl-3-hydroxypropyl)cysteine and N-acetyl-S-(1-phenyl-2-carboxy-ethyl)cysteine (Chapter 5). The finding of mercapturic acid metabolites of cinnamaldehyde in the urine of rats, mice and man shows the contribution of GSH conjugation to its elimination *in vivo* and also provides evidence for the *in vivo* reactivity of this α,ß-unsaturated aldehyde towards thiols. It has been demonstrated that cinnamaldehyde has *in vitro* reactivity towards PrSH groups of bovine serum albumin (BSA) (Weibel and Hansen, 1989a,b) as well as towards protein amino groups (Majetti and Suskind, 1977; Zaugg *et al.*, 1977). Recent investigations in our laboratory (Swales, 1993) indicate a role for sulfhydryl components, both non-PrSH (GSH and CySH) and PrSH, in the toxicity of cinnamaldehyde towards rat hepatocytes in suspension. High concentrations of cinnamaldehyde severely depleted cellular GSH and CySH and this was followed by the loss of free PrSH groups and at later time by cytotoxicity.

Hepatocytes are a particularly suitable test system to study metabolism and a series of factors affecting GSH conjugation: cinnamaldehyde concentration, intracellular GSH and competitive metabolism *via* oxidation to cinnamic acid and possible reduction to cinnamic alcohol. To investigate the interplay of these factors ^{14}C-cinnamaldehyde was incubated in F344 rat hepatocytes in suspension, metabolites were analyzed by radio HPLC and covalent binding of ^{14}C to cellular

macromolecules was determined. Leakage of lactate dehydrogenase (LDH) from the cells was an indicator for cytotoxicity and cellular GSH were measured to compare results to those of Swales (1993), which showed that cinnamaldehyde caused concentration dependent GSH depletion and cytotoxicity in rat hepatocytes in suspension. At concentrations up to 0.5 mM, depletion of GSH did not lead to cell death and repletion of cellular GSH to control levels occurred. At concentrations of 1 mM and higher depletion of GSH was followed by cytotoxicity.

In the experiments described below covalent binding of radiolabel to hepatocyte macromolecules was used as a parameter of toxicity. As described in Chapter 1, binding of reactive chemicals to cellular molecules can lead to many events: reduced energy production, changes in membrane permeability, inhibition of synthesis of functional molecules and by binding to nucleic acids, a compound can exert a mutagenic and carcinogenic action. Little is known about the implications of covalent binding to cofactors, lipids and polysaccharides. Covalent interactions are believed to be involved in liver, kidney, lung and bone marrow toxicity. Reid and Krishna (1973) found that for a number of halogenated benzenes the severity of liver necrosis correlated with the amount of covalent binding to liver macromolecules. Bioactivation of chlorobenzene by cytochrome P450 causes binding to DNA, RNA and proteins in liver, kidney and lung and interaction between metabolites of chlorobenzene and synthetic polyribonucleotides also occurred *in vitro* (Grilli *et al.*, 1985). Covalent binding is often found to coincide with toxicity but in most cases it is not known whether binding is the cause, result or by-product of toxicity *in vivo*. For instance, the reactive metabolite of carbon tetrachloride binds covalently to phospholipids and proteins in the liver. For a long time covalent binding to proteins was measured, though by now it is

generally accepted that the real target molecules are phospholipids in the endoplasmic reticulum. In spite of the fact that the events were not causally related, covalent binding provided a good parameter for toxicity. Changes in the covalent binding to protein were always paralleled by changes that occurred in the covalent binding to phospholipids and the degree of toxicity (Gillette, 1974). Thus, although the relation between covalent binding and toxicity was not causal, covalent binding was predictive for toxicity.

The present study will investigate the metabolism of ^{14}C-cinnamaldehyde, ^{14}C-cinnamic acid and ^{14}C-cinnamyl alcohol in rat and of ^{14}C-cinnamaldehyde in mouse hepatocytes in suspension and concentrate on the reactive species in the ^{14}C binding to cellular macromolecules. The highest non-cytotoxic cinnamaldehyde concentration, 0.5 mM served as a median concentration to study the metabolism of these cinnamyl compounds.

6.2 Materials and methods

6.2.1 Chemicals

[3-^{14}C]*trans*-Cinnamaldehyde, sp.act. 4.1 mCi/mmol, radiochemical purity 96.8% (a custom synthesis) and [3-^{14}C]*trans*-cinnamic acid, sp.act. 10 mCi/mmol, radiochemical purity 98% were purchased from Amersham International, Amersham, UK. [3-^{14}C]*trans*-Cinnamyl alcohol, sp.act. 0.2 mCi/mmol, radiochemical purity > 99% was synthesized and characterized in our laboratory (Keyhanfar, 1991b). Earle's Balanced Salt Solution (EBSS) was purchased from Gibco BRL, Paisley, Scotland and *o*-phthalaldehyde (OPA) from Fluka AG, Buchs, Switzerland. All other chemicals were of the highest grade commercially available from Sigma or Aldrich Chemical Co. Ltd., UK.

6.2.2 Standard hepatocyte incubations

Hepatocytes derived from male Fischer 344 rats (150-300 g) or CD1 mice (20-30 g) by a two step collagenase perfusion (Howes *et al.*, 1986) were suspended at 10^6 viable cells/ml in EBSS and treated with ^{14}C-cinnamaldehyde (1 µCi/ml), ^{14}C-cinnamic acid (1 µCi) or ^{14}C-cinnamyl alcohol (0.2 µCi) added in DMSO to 0.5% of the final incubation volume or DMSO only. Cells were incubated at 37 °C under an atmosphere of 95% O_2/ 5% CO_2 and samples were taken at various time points up to 6 h. Initial viabilities were between 80 and 95% as determined by trypan blue exclusion.

6.2.3 Termination of metabolism

Before termination of metabolism, samples (2 x 0.5 ml) were taken for the determination of LDH leakage and GSH and assayed as described below. To the remainder of the incubating tube an equal volume of 0.5 ml ice cold 5% (*v/v*) perchloric acid (PCA) or 50% methanol was added to terminate metabolism. Tubes were spun (2,000 rpm, 15 min, Centaur, MSE, Fisons, Crawley, UK) and the supernatant pipetted off. From the supernatant a 50 µl sample was taken for recovery of radioactivity and 100 µl for radio HPLC analysis as described in Chapter 2. Pellets were kept for protein binding studies described below.

6.2.4 Glutathione assay

After centrifugation of samples (150 *g*, 3 min), the supernatant was removed and 0.25 ml 5% PCA was added to the remaining pellet. Samples were kept on ice before snap freezing in liquid nitrogen and stored at -70 °C until further analysis. Total non-PrSH was determined

fluorimetrically according to Hissen and Hilf (1976). Samples were defrosted at 0 °C and centrifuged at 3,010 *g* for 15 min to remove protein precipitates. A 100 µl aliquot of supernatant was made up to a final volume of 2 ml in 0.1 M phosphate buffer pH 8 containing 5 mM EDTA and 0.1 mM KOH to neutralize the PCA and samples were kept on ice. OPA (100 µl of a 1 mg/ml solution in methanol) was added and the mixture incubated for 15 min at 22 °C. Fluorescence was measured at an excitation wavelength of 350 nm and emission of 420 nm using a Baird spectrofluorimeter (Fluoricord, Talbot Scientific Ltd, Alderley Edge, UK). GSH concentrations were calculated with reference to a standard curve which was linear over the range 0-14 nmol GSH.

6.2.5 Lactate dehydrogenase leakage

Cytotoxicity after various incubation times was measured by LDH activity in cells and medium due to leakage of this enzyme from the cells caused by membrane disruption. After sedimentation of the cells (150 *g*, 3 min) the medium was removed and taken into a clean Eppendorf tube. The remaining cells were lysed in a solution of 1% Triton-X-100 (0.5 ml) in phosphate buffered saline and stored at 4 °C for no more than 2 days. Samples were centrifuged at 1,500 rpm for 3 min before analysis. LDH activity in cells and medium was determined by the enzymic conversion of pyruvate to lactate measured by the disappearance of cofactor NADH at 340 nm using a modification of the method by Jauregui *et al.* (1981). Duplicate samples (100 µl) were taken of medium and lysate and added to 800 µl 85 mM Tris HCl pH 7.4 in the presence of 0.46 mM NADH. After a 15-20 min preincubation, the reaction was initiated by the addition of substrate (100 µl 1.4 mM pyruvate). The average reaction rate was measured using a Shimadzu MPS 2000 spectrophotometer (V.A. Howe, Oxon, UK) with the cell holder at 37 °C.

6.2.6 Protein binding

To the pellets, 5 ml 5% *(w/v)* trichloroacetic acid (TCA) was added and tubes were left on ice for 30 min. Protein and liquid were separated by centrifugation at 2000 rpm for 10 min. Pellets were subsequently washed with 2 x 5 ml 5% TCA and at least 5 times with 5 ml 80% methanol. To ensure that extraction was complete, samples were taken for scintillation counting until no radio activity could be detected in the liquid of all last wash fractions. Then, all liquid was drained off and samples were left overnight to dry. To the dry samples 1 ml 1 M NaOH was added and the protein was digested overnight at 37 °C. When samples were fully digested, 1 ml H_2O was added and aliquots were taken for scintillation counting (3 x 200 µl). Recovery of protein after washing was determined according to Lowry *et al.* (1951) as described below. ^{14}C Binding was expressed as nmol ^{14}C/mg protein and corrected for the loss during washing.

6.2.7 Protein assay

Protein contents were measured according to Lowry *et al.* (1951). Reagent A was 2% *(w/v)* Na_2CO_3 in 0.1 M NaOH, reagent B1 was 0.5% *(w/v)* $CuSO_4.5H_2O$ and reagent B2 was 1% *(w/v)* NaKtartrate. These were mixed in a ratio of A:B1:B2 = 100:1:1 (reagent C). Reagent D was commercially available Folin-Ciocalteu's phenol reagent diluted 1:1 with water. Samples containing 5-100 µg protein were made up to a volume of 200 µl with water. To this 1 ml reagent C was added and tubes were left for at least 10 min. Then 100 µl Reagent D was added, tubes were mixed and placed in the dark for 30 min until colour had developed. Absorbances were read at 720 nm using a Shimadzu MPS 2000 spectrophotometer and protein contents were determined with

reference to a BSA standard curve.

6.3 Results

6.3.1 Cell survival and GSH depletion

In all experiments cellular GSH and cytotoxicity were comparable to parallel experiments described by Swales (1993). Cell viability was not reduced by cinnamaldehyde as compared to control cells incubated with DMSO only, at concentrations up to 2 mM in the first 4 h of incubation. Concentrations of 5 mM and above were cytotoxic even at 1 h. The highest non-toxic concentration was 0.5 mM (no increases in LDH leakage compared to control over the 6 h incubation period), which caused a rapid depletion of GSH to 70% of control levels at 0.5 h and maximal depletion to 60% of control levels (40% depletion) at 1.5 h, after which time repletion occurred. Cinnamyl alcohol was less cytotoxic than cinnamaldehyde at all concentrations and depleted GSH only after a latent period of 0.5 h. Cinnamic acid was slightly cytotoxic at 5 mM but did not cause GSH depletion other than by cytotoxicity.

6.3.2 Metabolism of 0.5 mM ^{14}C-cinnamaldehyde

Incubation of hepatocytes with 0.5 mM ^{14}C-cinnamaldehyde gave rise both to the formation of polar metabolites and to covalent binding of ^{14}C to hepatocyte protein (Fig. 6.1A). Cinnamaldehyde metabolism, expressed as the sum of all polar metabolites, was 97% after 0.5 h and complete after 1 h of incubation, when no parent compound was detected. Radio HPLC analysis of incubation mixtures revealed a number of metabolites. By comparison with known cinnamaldehyde metabolite standards (Fig. 6.1B), ^{14}C-cinnamaldehyde was

metabolized to cinnamic acid and at later time to benzoic acid and hippuric acid. Cinnamic alcohol was only present in the first hour of incubation. In addition, many minor metabolites (each accounting for less than 1 or 2% of metabolism) were formed at later incubation times, coeluting with 3-hydroxy-3-phenylpropionic acid, sulfu\r-containing metabolites S1 and S2 and possibly *p*-hydroxycinnamic acid. Covalent binding of ^{14}C-cinnamaldehyde, defined as non-extractable ^{14}C after exhaustive washing, accounted for less than 1% of the substrate concentration. This binding was not related to the formation of polar cinnamaldehyde metabolite(s), but was maximal at 0 h, decreasing with incubation time until it stabilized after 2 h at 0.3% of total ^{14}C.

6.3.3 Variation of the cinnamaldehyde concentration

When the conversion of ^{14}C-cinnamaldehyde was measured as a function of the substrate concentration, the nature of polar metabolites did not change (Fig. 6.2B). But, whereas cinnamaldehyde was completely metabolized within 1 h at 0.5 mM, its metabolism at 1 mM and higher became saturated and measurable amounts of the parent compound remained present in the incubation medium. Binding of ^{14}C to cellular macromolecules occurred at all concentrations, but was markedly enhanced at concentrations of 1 mM and higher (Fig. 6.2A)

6.3.4 Metabolism of ^{14}C-cinnamic acid

Metabolism of 0.5 mM ^{14}C-cinnamic acid to polar metabolites was slower than that of cinnamaldehyde: after 6 h of incubation 57% of the substrate remained present (Fig. 6.3A). In hepatocyte incubations ^{14}C-cinnamic acid was metabolized to benzoic-, hippuric- and 3-hydroxy-3-phenyl-3-propionic acid and a few minor unknowns (Fig. 6.3B). Cinnamaldehyde or cinnamyl alcohol were not found as metabolites of

^{14}C-cinnamic acid. No qualitative differences in the metabolite profile were seen over the concentration range 0.02 to 5 mM (Not shown).

"Binding" of ^{14}C to hepatocyte protein accounted for only 0.02% of the substrate concentration and was unchanged over the 0-6 h incubation time (Fig. 6.3A) or with variation of the substrate concentration from 0.02 mM to 5 mM ^{14}C-cinnamic acid (Fig. 6.3C), suggesting that this was due to ^{14}C label trapped in the hepatocyte protein and not related to the presence of cinnamic acid or the formation of metabolites.

6.3.5 Metabolism of ^{14}C-cinnamyl alcohol

Hepatocyte conversion of 0.5 mM ^{14}C-cinnamyl alcohol was complete after 6 h of incubation (Fig. 6.4B). Metabolites were cinnamic acid, benzoic acid and hippuric acid. No cinnamaldehyde was found at any stage and a GSH conjugate was not detected. Protein binding (Fig. 6.4A) was high in comparison to that seen in incubations with cinnamaldehyde. Results show that binding of ^{14}C is metabolism related; 0.5% of ^{14}C at 0 h, increasing to 3.8% at 1 h, after which it decreases with incubation time until stabilising at 0.8% of ^{14}C after 4 h.

6.3.6 Metabolism of ^{14}C-cinnamaldehyde in mouse hepatocytes

Metabolism of 0.5 mM cinnamaldehyde in mouse hepatocytes (Fig. 6.5A) was slower than in the rat. Complete metabolism was reached only after 4 h of incubation. As in the rat, covalent binding in mouse hepatocytes was partly reversible with incubation time, amounting to 0.2% of dose after 2 h.

Metabolites formed (Fig. 6.5B) were the same as in rat hepatocytes:

Cinnamyl alcohol, cinnamic acid, benzoic acid and hippuric acid and a number of smaller metabolites. Compared to rat hepatocytes, oxidation to cinnamic acid was less important in the initial stages of metabolism and reduction to cinnamic alcohol occurred to a greater extent. An unknown metabolite accounted for 2-6% of ^{14}C. This metabolite had the same retention time as the ^{14}C-containing product formed when ^{14}C-cinnamaldehyde and GSH were incubated in buffer, possibly a GSH conjugate.

6.4 Discussion

Results presented in this Chapter show that the major route of ^{14}C-cinnamaldehyde metabolism in rat hepatocytes is its oxidation to cinnamic acid, with some reduction to cinnamyl alcohol. When hepatocytes were incubated with ^{14}C-cinnamic acid, no cinnamaldehyde or cinnamyl alcohol were formed showing that the oxidation step is irreversible. In contrast, the oxidation of cinnamyl alcohol to cinnamaldehyde appears to be reversible, for in experiments with ^{14}C-cinnamaldehyde, cinnamyl alcohol was a metabolite and in incubations with ^{14}C-cinnamyl alcohol, although no intermediate aldehyde was detected, cinnamic acid was a metabolite. A schematic representation for the proposed interrelationship of cinnamyl compounds is presented in Fig. 6.6. These findings are in general agreement with our knowledge of carbonyl metabolism (Weiner and Flynn, 1985).

^{14}C-Cinnamaldehyde is rapidly metabolized in hepatocytes which is in good agreement with its rapid elimination in rat urine as various metabolites (Chapters 2 and 3). The pathways in the metabolism of cinnamaldehyde in hepatocytes resemble those in the rat, oxidation to cinnamic acid and ß-oxidation of the side chain to benzoic acid, which

is excreted as such or after conjugation with glycine (hippuric acid). After 6 h the cinnamic acid formed in the incubation medium was completely further metabolized to benzoic and hippuric acid, in good agreement with the finding that cinnamic acid is not excreted in rat urine. *In vivo*, hippuric acid is by far the major metabolite of cinnamaldehyde (> 70%) and benzoic acid is a very minor metabolite (< 5%), whereas in hepatocytes benzoic acid remains the greater of the two. Glycine conjugation in hepatocytes is possibly limited by the more limited availability of cofactors (e.g. CoA) and glycine conjugation *in vivo* might take place in other organs than the liver e.g. in the kidney (Caldwell, 1984). The half-life of cinnamyl alcohol in hepatocytes is short and this metabolite is not found excreted in urine of rats (Chapters 2 and 3).

Boyland and Chasseaud (1967, 1968) have shown that cinnamaldehyde reacts spontaneously and enzymatically with GSH *in vitro*. Recent studies in our laboratories (Swales, 1993) showed that cinnamaldehyde caused dose-dependent depletion of GSH in buffer pH 8.0 and rat hepatocytes in suspension, while cinnamyl alcohol did not react in buffer, but did deplete GSH in hepatocytes. Cinnamic acid caused no reduction in GSH levels in buffer or hepatocytes. These data are in agreement with the proposed metabolic interrelationships of the cinnamyl compounds (Fig. 6.6) and show that cinnamaldehyde is the cinnamyl compound reactive towards GSH. Cinnamyl alcohol requires metabolic activation to cinnamaldehyde in order to react with GSH. The conversion of cinnamaldehyde to cinnamic acid is irreversible and therefore no depletion of GSH is observed when hepatocytes are incubated with cinnamic acid.

In rats, cinnamaldehyde leads to the excretion of mercapturic acids in the urine (Delbressine, 1981; Chapter 5), showing that the depletion of

GSH caused by cinnamaldehyde *in vivo* (Boyland and Chasseaud, 1970) is at least partly due to the formation of a conjugate. Boyland and Chasseaud (1967) studied the *in vitro* metabolism of cinnamaldehyde in the presence of GSH by rat liver supernatant and TLC analysis of the reaction mixture after 1 h incubation revealed a single sulfur containing spot that was ninhydrin positive. These experiments showed that cinnamaldehyde caused loss of GSH from the reaction medium at least partly by conjugation with GSH and not just by formation of oxidized GSH. In Chapter 8, ^{14}C-cinnamaldehyde was incubated with GSH in the presence of cytosol or in buffer. Analysis by radio HPLC showed a ^{14}C-metabolite with a retention time of 20 min compared to 46 min for cinnamaldehyde, the formation of which was dependent on both the GSH and cinnamaldehyde concentration. This GSH conjugate was not detected as a metabolite in rat hepatocyte incubations, even at concentrations where in parallel experiments cinnamaldehyde depleted cells of GSH to 60% of control (Swales, 1993), although the formation of GSH conjugates has been reported in hepatocytes (Moldeus *et al.*, 1978). In the study described in this Chapter the identification of the conjugate is hindered since the expected peak would account for only 2% of the cinnamaldehyde concentration; Control GSH levels in hepatocytes were 25 nmol/10^6 cells and each incubation contained 10^6 cells/ml. The maximal depletion achieved by 0.5 mM (500 nmol/ml) cinnamaldehyde was to 60% of control, which equals 10 nmol/25 nmol. Assuming that cinnamaldehyde and GSH react in a 1:1 ratio, this will also involve 10 nmol cinnamaldehyde, which is 2% of the 500 nmol cinnamaldehyde present per incubation.

The rates of metabolism of ^{14}C-cinnamaldehyde, ^{14}C-cinnamic acid and ^{14}C-cinnamyl alcohol were different. ^{14}C-Cinnamaldehyde was rapidly metabolized in 1-2 h and the cinnamic acid formed was completely

metabolized in 6 h, showing a great metabolic capacity for cinnamic acid metabolism in hepatocytes. However, when cells were incubated with a non cytotoxic concentration of ^{14}C-cinnamic acid, this remained present up to 53% after 6 h. Similarly, ^{14}C-cinnamyl alcohol metabolism in hepatocytes took 6 h until completion. A number of factors could contribute to this. Uptake of the lipophilic aldehyde into the cell will occur *via* diffusion whereas the acid and perhaps the alcohol are ionized at physiological pH, hindering their uptake into the cell. Secondly, once in the cell a more lipophilic chemical like cinnamaldehyde could get to different places within the cell. Thirdly, metabolism of cinnamaldehyde could be facilitated by binding to GSH or ligandin. Other researchers reported that the complex of cinnamaldehyde with BSA releases cinnamic acid and cinnamyl alcohol (Weibel and Hansen, 1989a,b).

In addition to the conversion to polar metabolites, incubation of hepatocytes with ^{14}C-cinnamaldehyde leads to covalent binding of ^{14}C to cellular macromolecules. Interestingly, this binding was not associated with the formation of cinnamaldehyde metabolite(s), but was dependent on the presence of the parent compound. The fact that incubation of hepatocytes with ^{14}C-cinnamic acid did not cause covalent binding implies that oxidation of ^{14}C-cinnamaldehyde to cinnamic acid is not responsible for the binding observed. Incubation with ^{14}C-cinnamyl alcohol led to metabolism-related binding suggesting a metabolite of ^{14}C-cinnamyl alcohol as the reactive species. The concentration-dependency of the ^{14}C binding is in agreement with cinnamaldehyde being the reactive species. At 1 mM ^{14}C-cinnamaldehyde, when metabolism becomes saturated, routes of metabolism do not change, but binding of ^{14}C to macromolecules is enhanced. The results pointing at cinnamaldehyde as the reactive species are in agreement with the *in vitro* reactivity of cinnamaldehyde

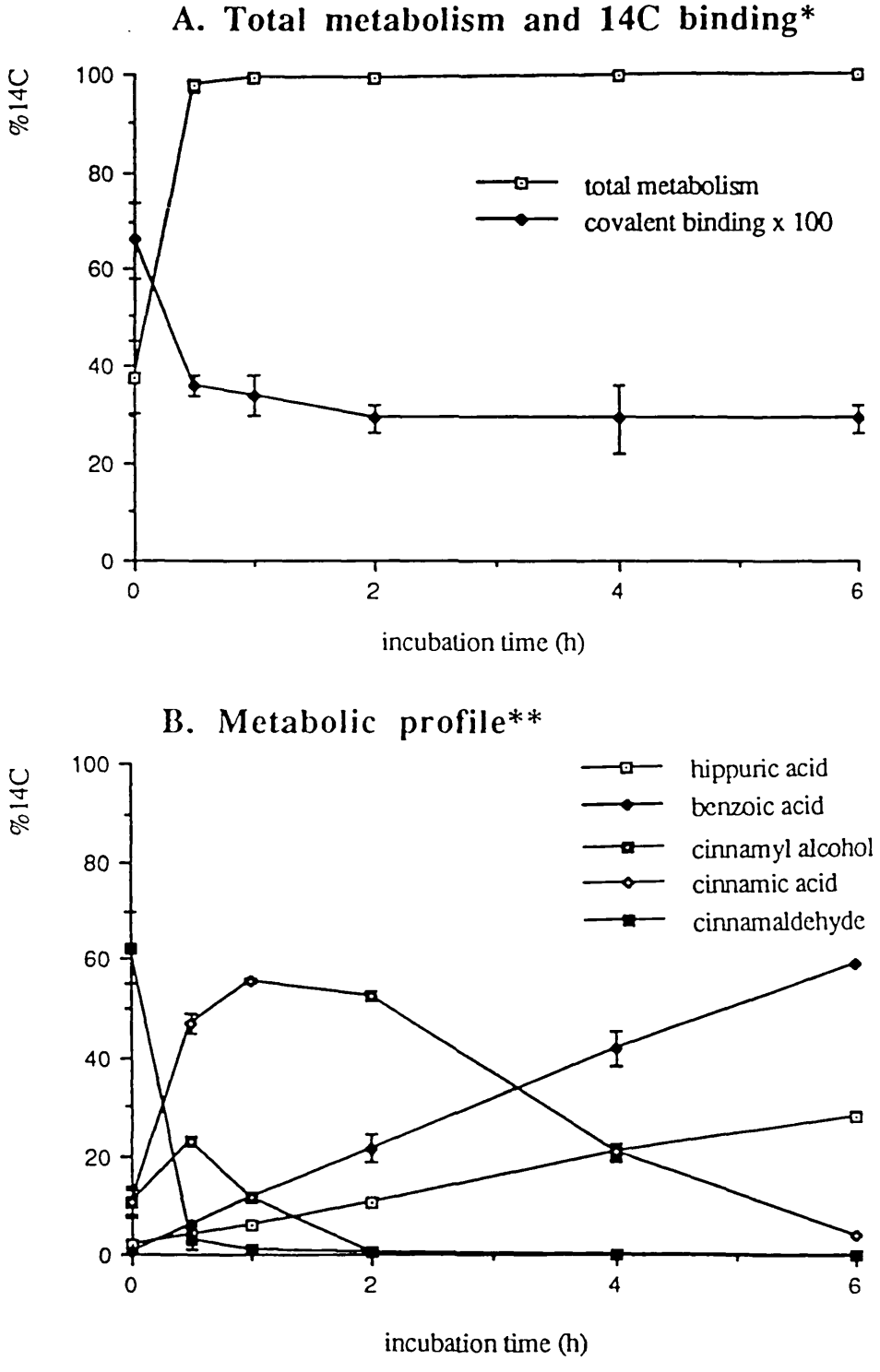
towards PrSH (Weibel and Hansen, 1989a,b) and protein amino groups (Majetti and Suskind, 1977). The interesting feature of this binding is that it is partly reversible. Reversible binding is known for both PrSHs and protein-Schiff base conjugates.

There is a clear threshold concentration of 1 mM for saturation of metabolism and enhanced protein binding and this coincides with the threshold described by Swales (1993) for enhanced GSH depletion followed at later time by cell death. As mentioned in the introduction of this Chapter, whether the occurrence of covalent binding and GSH depletion are causal in toxicity can not be concluded before the ultimate target for toxicity within the cell is identified. It has often been questioned whether covalent binding measured in biological systems is the most relevant toxicological parameter. Reactive intermediates can bind to non-critical macromolecules and such an interaction is of little or no toxicological significance. Sometimes there is no correlation between the amount of covalent binding *in vitro* and the toxicity observed *in vivo*. Conditions that enhanced the *in vitro* covalent binding of *p*-bromophenol, such as treatment with phenobarbitone and the absence of GSH, did not cause toxicity *in vivo* and the chemically reactive metabolites of *p*-bromophenol therefore are not thought to play a part in bromobenzene toxicity *in vivo* (Monks *et al.*, 1984a,b,c). To understand the role of covalent binding in chemical toxicity, the specificity of the covalent binding has to be investigated (Dent and Sun, 1981). In this respect both the place of de/activation in relation to the target molecules and the structure of metabolites are important (Jergil *et al.*, 1981). However, results from the study presented here combined with those of Swales (1993) allow the conclusion that ^{14}C-binding and GSH depletion, if not causal, are at least indicative for the cytotoxicity of cinnamaldehyde to rat hepatocytes in suspension. In conclusion, ^{14}C-cinnamaldehyde was rapidly metabolized by hepatocytes to various

polar metabolites and caused enhanced covalent binding only after saturation of its metabolism at high concentrations.

Figure 6.1

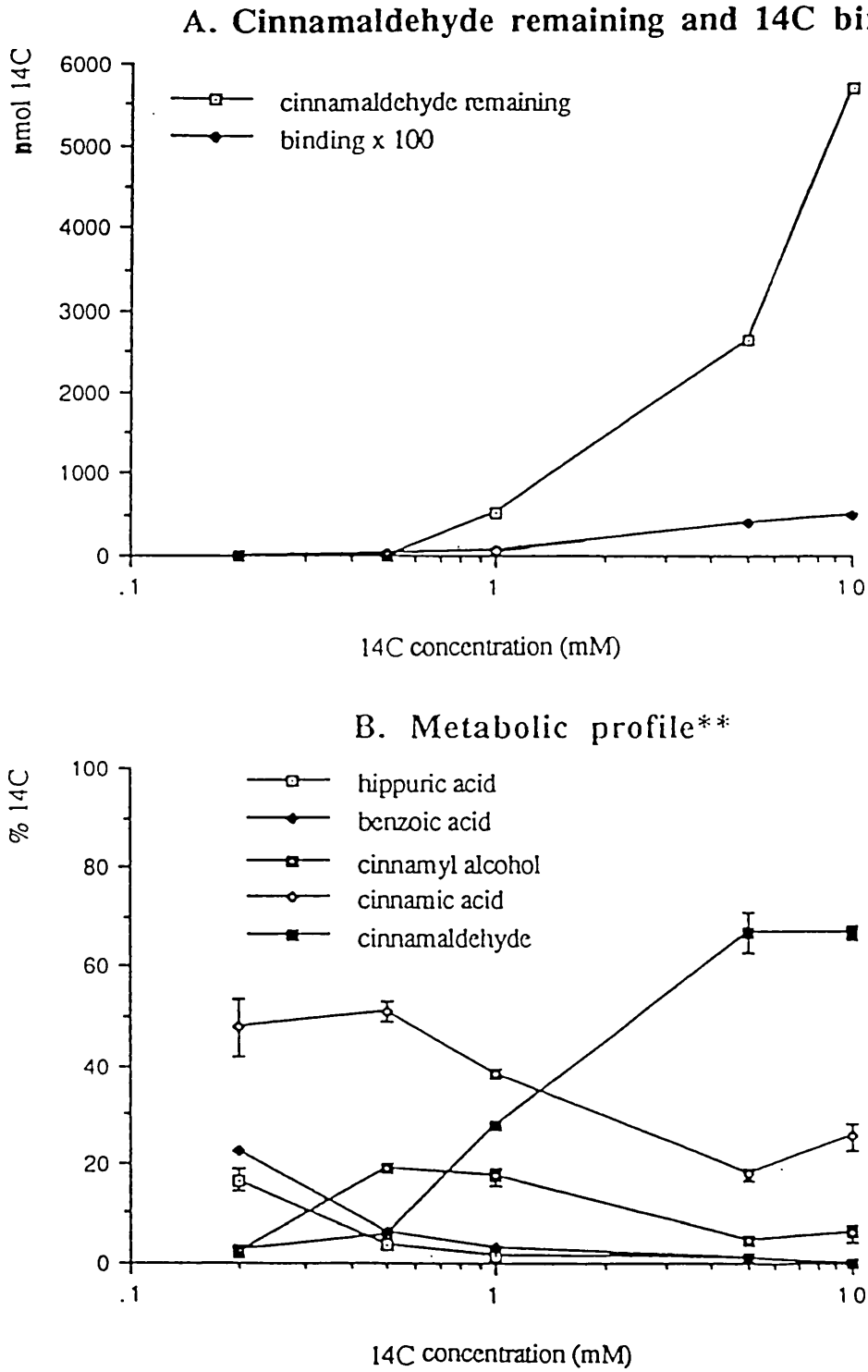
Metabolism of 0.5 mM ^{14}C-cinnamaldehyde by F344 rat hepatocytes in suspension as a function of the incubation time



* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

Figure 6.2

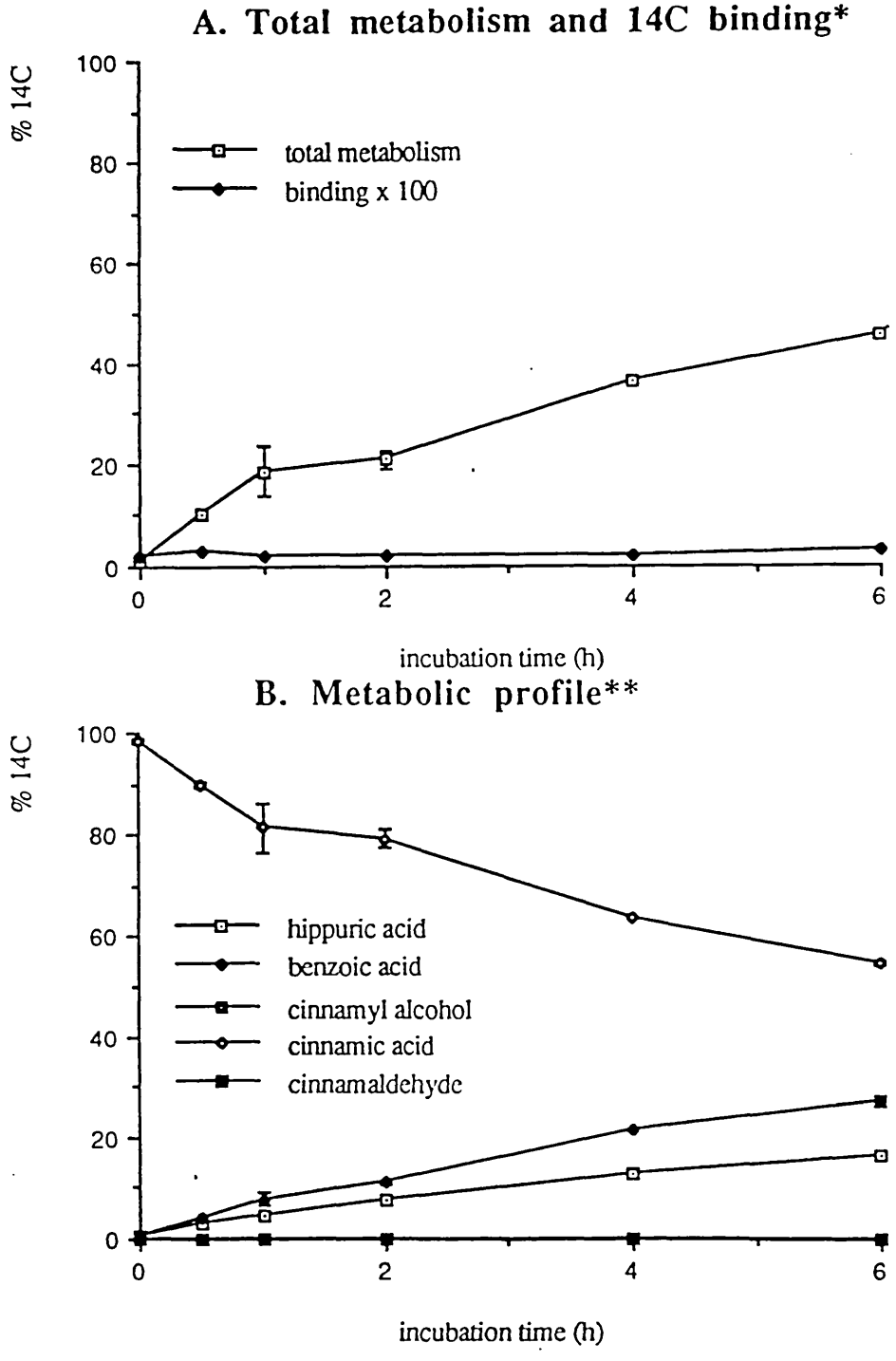
Metabolism of ^{14}C-cinnamaldehyde by F344 rat hepatocytes in suspension as a function of the substrate concentration



* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

Figure 6.3

Metabolism and covalent binding by ^{14}C-cinnamic acid in F344 rat hepatocytes in suspension, (a-b) metabolism of 0.5 mM cinnamic acid as a function of the incubation time and (c) metabolism of cinnamic acid as a function of the substrate concentration



* Total metabolism was calculated as the sum of ^{14}C-cinnamic acid metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamic acid and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

C. Cinnamic acid remaining and 14C binding*

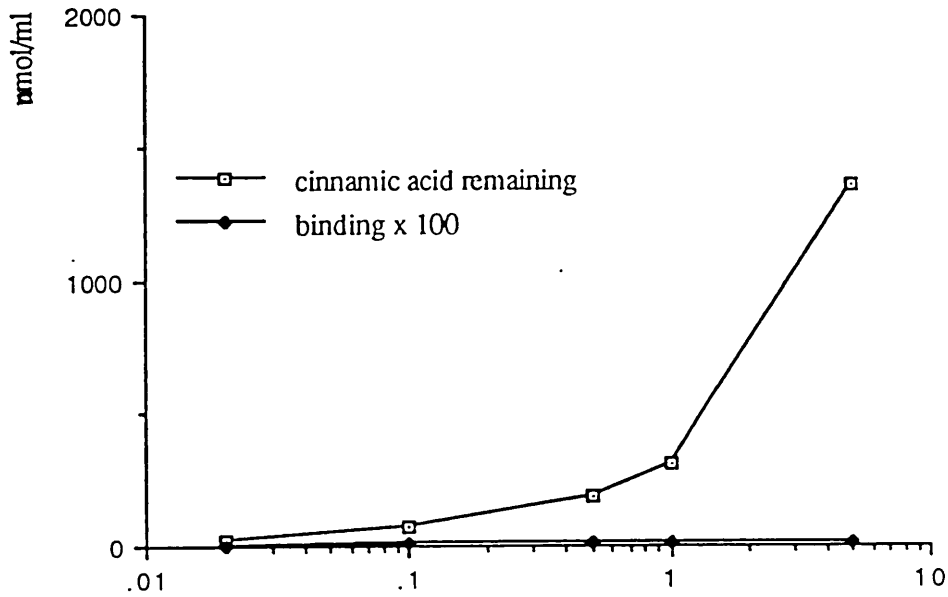
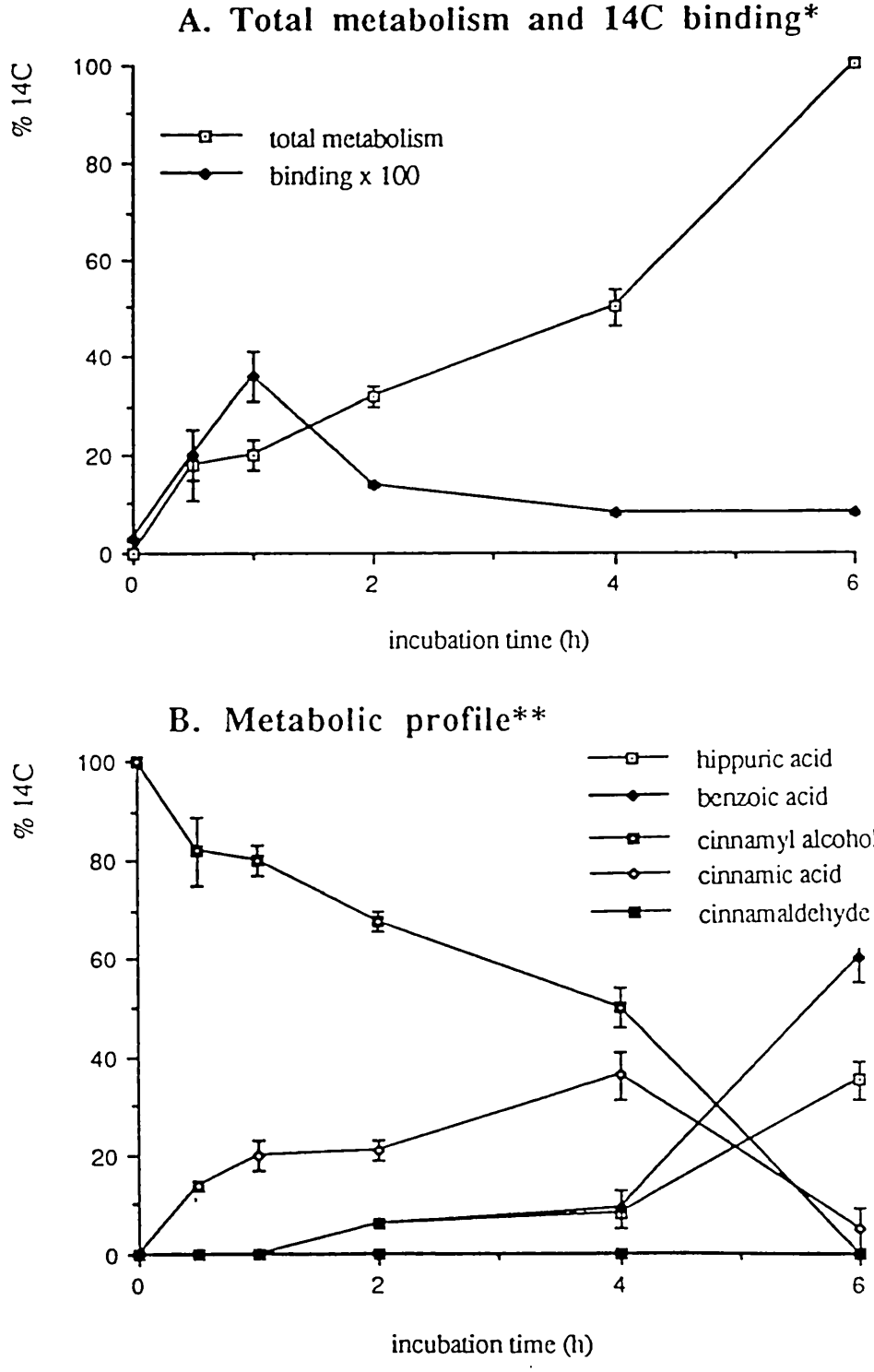


Figure 6.4

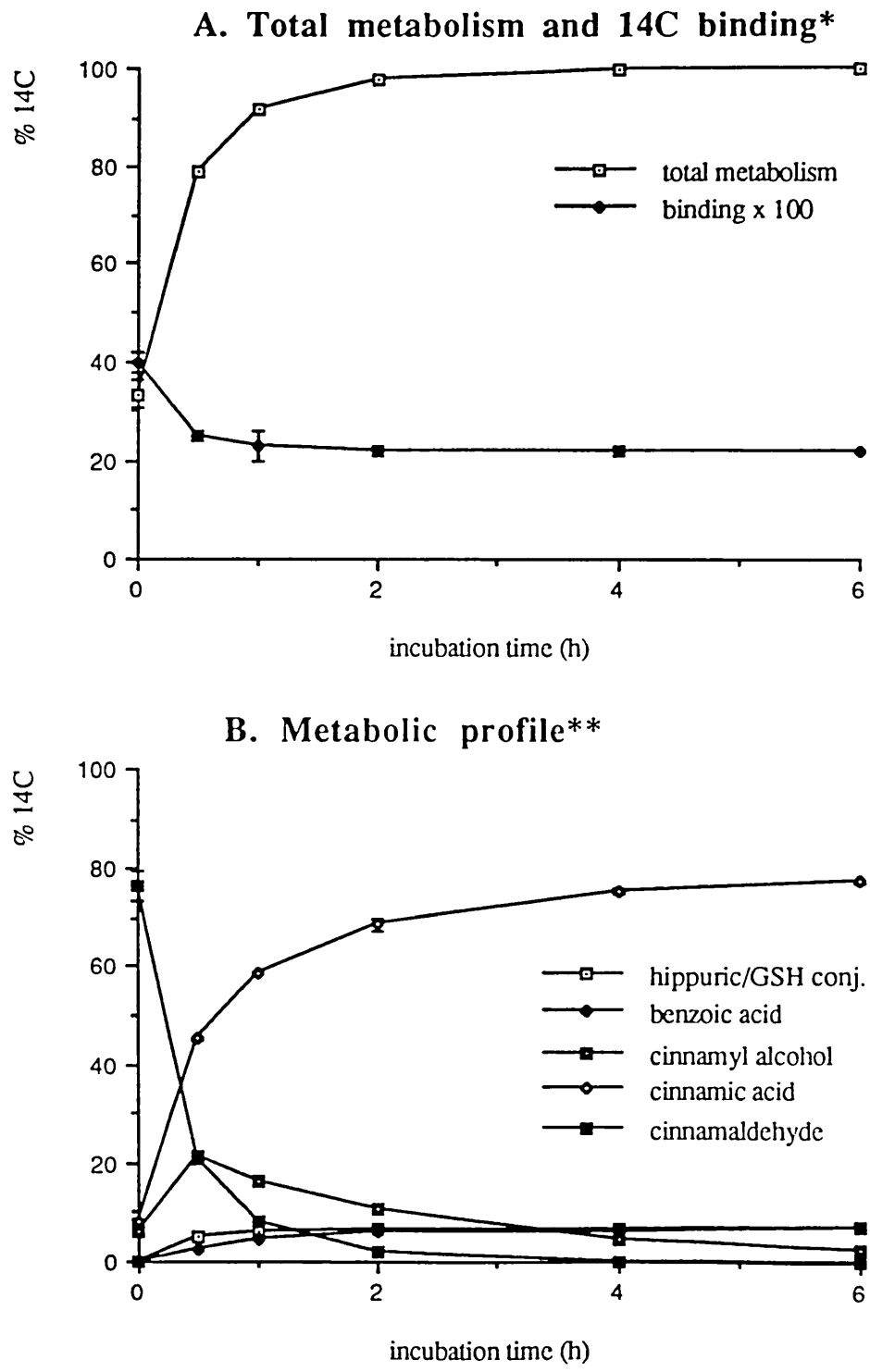
Metabolism of ^{14}C-cinnamyl alcohol by F344 rat hepatocytes in suspension as a function of the incubation time



* Total metabolism was calculated as the sum of ^{14}C-cinnamyl alcohol metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamyl alcohol and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

Figure 6.5

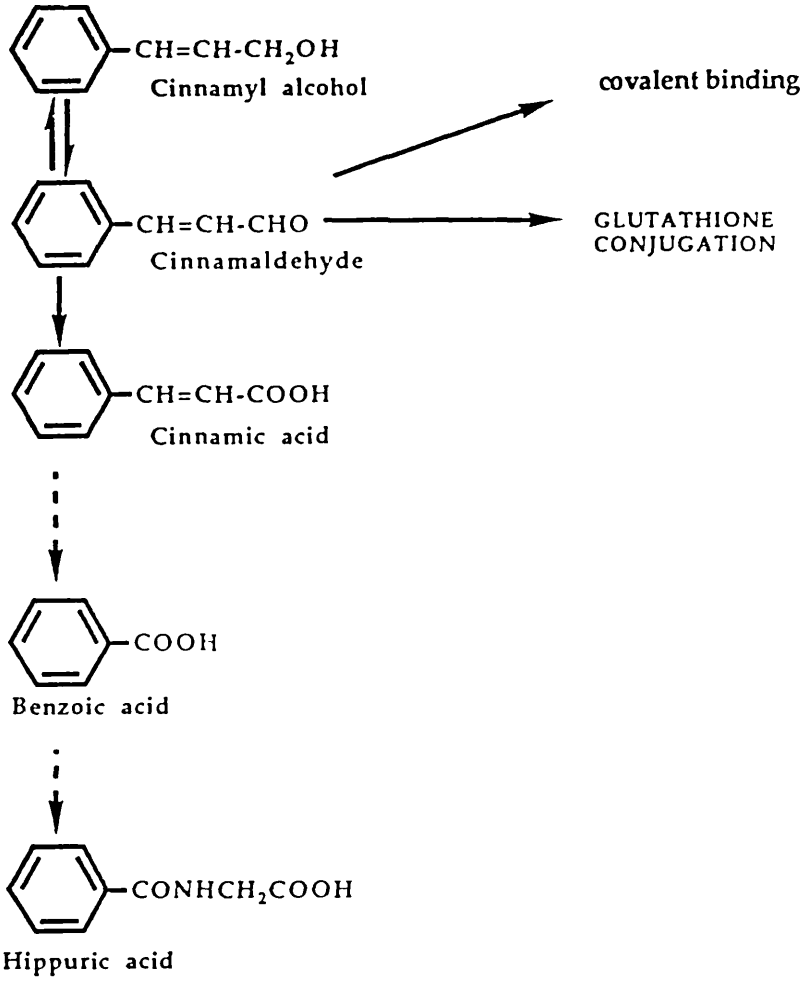
Metabolism of 0.5 mM ^{14}C-cinnamaldehyde by CD1 mouse hepatocytes in suspension as a function of the incubation time



* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation.
** ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

Figure 6.6

Proposed metabolism of cinnamaldehyde by rat and mouse hepatocytes in suspension



Chapter 7

Manipulation of ^{14}C-cinnamaldehyde metabolism in rat hepatocytes in suspension and the effect on protein binding

7.1 Introduction

In Chapter 6 it was demonstrated that the major metabolic route of ^{14}C-cinnamaldehyde in rat hepatocytes is irreversible oxidation to cinnamic acid, with some reversible reduction to cinnamyl alcohol. In addition to the conversion to polar metabolites, incubation of hepatocytes with ^{14}C-cinnamaldehyde led to covalent binding of ^{14}C to cellular macromolecules and this binding was not associated with the formation of cinnamaldehyde metabolite(s), but depended on the presence of the parent compound. Incubation of hepatocytes with ^{14}C-cinnamic acid did not cause covalent binding, implying that oxidation of ^{14}C-cinnamaldehyde to cinnamic acid is not responsible for the binding observed. Incubation with ^{14}C-cinnamyl alcohol led to metabolism-related binding and points therefore at a metabolite of ^{14}C-cinnamyl alcohol as the reactive species. The concentration dependency of the ^{14}C binding is in agreement with cinnamaldehyde being the reactive species. At 1 mM when ^{14}C-cinnamaldehyde metabolism becomes saturated and cinnamaldehyde remains present, routes of metabolism do not change, but binding of ^{14}C to macromolecules is enhanced.

Although no conjugate of cinnamaldehyde with GSH was detected in the studies in Chapter 6, parallel studies (Swales, 1993) have shown that cinnamaldehyde causes dose dependent depletion of GSH in rat hepatocytes in suspension and the *in vivo* studies described in Chapters 2, 3, 4 and 5 show that conjugation with GSH forms a route of cinnamaldehyde metabolism *in vivo* in rat, mouse and man. Many investigations have shown that GSH and GSTs form a protective mechanism against chemical induced cytotoxicity (see Chapter 1). Thus the ability of cinnamaldehyde to react with GSH is of toxicological significance. To study the importance of GSH, it is interesting to alter the thiol status of the cell and note changes in toxicity.

To reduce initial GSH levels cells can be treated with DEM (diethyl maleate), an α,ß-unsaturated ester which is conjugated with GSH in a GST-catalyzed reaction (Boyland and Chasseaud, 1967; 1970). DEM rapidly depletes hepatocytes of GSH without causing toxicity (Swales, 1993). To prevent resynthesis of GSH during the course of the incubation, BSO (*L*-buthionine (S,R)sulfoximine) can be added together with DEM. BSO is a potent and selective inhibitor of γ-glutamyl cysteine synthetase (Griffith and Meister, 1979; Meister, 1983), the rate limiting enzyme in the synthesis of GSH, with which it forms an irreversible complex. DEM and BSO have been widely used as tools in toxicity studies and have for instance been shown to increase the severity of the toxicity of bromobenzene, paracetamol and 4-ipomeanol and eugenol *in vitro* and *in vivo* (Mitchell *et al.*, 1973; Jollow *et al.*, 1974; Boyd and Burka, 1978; Mizutani *et al.*, 1991). In contrast, pretreatment of cells with BSO and DEM protects against 1-naphthylisocyanate toxicity, showing that GSH conjugation forms an activating pathway in the case of this chemical *in vitro* (Carpenter-Deyo *et al.*, 1991).

It is difficult to elevate normal GSH levels by stimulating GSH biosynthesis, since GSH is a feed back inhibitor of the first step of its own synthesis. Small increases in GSH levels can however be achieved by administration of CySH precursors such as NALC (Issels *et al.*, 1988; Traber *et al.*, 1992). NALC has been successful in the treatment of paracetamol poisoning (Prescott *et al.*, 1977) and inhibited the metabolic activation of pro-carcinogens such as aflatoxin B_1, AAF and cyclophosphamide (De Flora *et al.*, 1985).

In the metabolism of cinnamaldehyde it would be of interest to study the importance of the oxidation of cinnamaldehyde to cinnamic acid.

This pathway can be inhibited by cyanamide, an inhibitor of ALDHs and of acetaldehyde (Weiner *et al.*, 1988) and acrolein (Rikans, 1987) metabolism. Pyrazole, or its precursor 4-methyl pyrazole, an inhibitor of ADH and of ethanol metabolism can be employed to inhibit the reduction of cinnamaldehyde to its alcohol. Pyrazole inhibits ADHs by forming a complex with the enzyme and NAD+, with pyrazole occupying the alcohol binding site (Theorell and Yonetani, 1963; Puntarulo and Cederbaum, 1988). Ethanol was added to the hepatocyte incubations as a competing substrate for the dehydrogenase enzymes. Oxidation of ethanol to acetaldehyde will reduce cofactor NAD+ to NADH, thereby facilitating reductive metabolism of cinnamaldehyde to its alcohol.

In this Chapter experiments in rat hepatocytes in suspension are described in which both the oxidation and GSH conjugation of cinnamaldehyde are selectively altered and the effect on ^{14}C-binding is investigated. Metabolism was manipulated by the addition of inhibitors of ALDH and ADH (cyanamide and 4-methyl pyrazole), the competitive substrate ethanol, and combined treatment with DEM and BSO to deplete initial GSH and prevent resynthesis, while initial GSH levels were boosted by NALC. Experiments were performed at 0.5 mM, a non-cytotoxic concentration and at 1 mM, a concentration marginally cytotoxic in control cells.

7.2 Materials and Methods

7.2.1 Chemicals

DEM was purchased from Aldrich Chemical Co., Gillingham, Dorset, UK. BSO, NALC, cyanamide and 4-methyl pyrazole were obtained from Sigma Chemical Co., Ltd., St Louis, USA. All other chemicals

were as described in Chapter 6.

7.2.2 Hepatocyte incubations

Standard hepatocyte incubations were performed as described in Chapter 6. DEM (360 μM) and BSO (2.5 mM) were added to the cell suspension 1 h before the addition of cinnamaldehyde. NALC (2 mM) was added 1 h prior to the experiment. Cyanamide (1 mM) or 4-methyl pyrazole (1 mM) were added to cell suspensions 0.5 h before the addition of ^{14}C-cinnamaldehyde and ethanol (0.2%) was added together with cinnamaldehyde. Control incubations were performed without modulators in the presence of cinnamaldehyde only and with DMSO only.

7.2.3 Glutathione, lactate dehydrogenase leakage, radio HPLC analysis and binding of ^{14}C

GSH, LDH leakage and binding of ^{14}C to hepatocyte macromolecules were determined as described in Chapter 6. Radio HPLC analysis of supernatants was achieved as described in Chapter 2.

7.3 Results

7.3.1 Cell survival and GSH depletion

In all experiments cell viability was not reduced as compared to control cells incubated with DMSO only over the first 3 h after addition of cinnamaldehyde. Depletion of GSH was comparable to that in parallel experiments described by Swales (1993). Pretreatment of cells with DEM and BSO reduced initial GSH levels to 30% of control (60% depletion) and NALC increased initial GSH to 190% of control. Other

inhibitors did not affect GSH over the first 2 h of incubation.

7.3.2 Effect of DEM and BSO on ^{14}C-cinnamaldehyde metabolism

The metabolism of 0.5 mM ^{14}C-cinnamaldehyde in control cells and in cells treated with DEM and BSO, in which initial GSH values were reduced to 30% of control, is shown in Fig. 7.1. No qualitative differences were observed in the metabolic profile (Fig. 7.1B), but cinnamaldehyde and cinnamyl alcohol, which are metabolized in 1 to 2 h in control cells, remained present in small amounts for up to 4 h in treated hepatocytes.

The protein binding profile after DEM and BSO showed the same biphasic plot as in control cells, with binding being partly reversible, maximal at 0 h and decreasing with incubation time until stabilising after 2 h, after which time some ^{14}C remained irreversibly bound during the 6 h of the experiment. This irreversible binding was 2.5-fold higher in DEM and BSO treated cells than in control cells (Fig. 7.1A) (t-test, $p<0.001$).

The effects of DEM and BSO on metabolism and covalent binding, were further examined by varying the ^{14}C-cinnamaldehyde concentration (Fig. 7.2). As described in Chapter 6, in control cells 0.5 mM ^{14}C-cinnamaldehyde is completely metabolized within 1 h, but after DEM and BSO, metabolism was inhibited and 10% of the parent compound remained in the incubation medium even at 0.1 mM cinnamaldehyde (Fig. 7.2A). This was paralleled by an increase in ^{14}C binding in treated cells at every substrate concentration (500% of control at 0.1 mM and 5% increase at 10 mM) (t-test, $p<0.002$; Fig. 7.2B).

7.3.3 Ethanol as a cosubstrate in ^{14}C-cinnamaldehyde metabolism

The presence of ethanol in hepatocyte incubations did not influence the overall conversion rate of ^{14}C-cinnamaldehyde to polar metabolites (Fig. 7.3A), but reductive metabolism to cinnamyl alcohol was enhanced at the cost of oxidation to cinnamic acid. Cinnamyl alcohol was formed to 30% maximum compared to 10% in control incubations, and remained in the incubation medium for up to 4 h, whereas it is fully metabolized in 2 h in control cells (Fig. 7.3B).

The presence of ethanol in the incubation medium altered both the initial binding of ^{14}C to hepatocyte macromolecules, which was reduced from 0.5% in control cells to 0.2% (0.5 h) and caused a small, but statistically significant (t-test, $p<0.02$) reduction in the irreversible binding (6 h) from 0.22 to 0.14% (fig. 7.3A).

7.3.4 Modulators of cinnamaldehyde metabolism

The effect of a number of modulators on ^{14}C-cinnamaldehyde metabolism (Fig. 7.5) and ^{14}C binding (Fig. 7.4) was studied in a 1 h incubation at the non-cytotoxic concentration of 0.5 mM and at 1 mM which is cytotoxic after 3 h of incubation.

Pretreament of cells with DEM and BSO inhibited the metabolism of ^{14}C-cinnamaldehyde to cinnamic acid and to cinnamyl alcohol, causing a reduction in the overall conversion to 64% and 67% of control at 0.5 and 1 mM respectively. DEM and BSO significantly ($p<0.01$) enhanced protein binding to 147% of control at 0.5 mM and marginally significantly ($p<0.05$) to 143% of control at 1 mM.

Inclusion of ethanol in the incubation medium increased ^{14}C-cinnamaldehyde metabolism to cinnamyl alcohol at 0.5 mM and at 1 mM. Total metabolism was 117% of control at 0.5 mM ($p=0$) and unaffected at 1 mM ($p>0.1$), while ethanol reduced ^{14}C binding to 76% at 0.5 mM ($p<0.05$) and in contrast enhanced binding to 114% of control at 1 mM, although this did not reach statistical significance ($p>0.2$).

The ALDH inhibitor, cyanamide, caused a 75% inhibition in the metabolism of ^{14}C-cinnamaldehyde to cinnamic acid, but also reduced the formation of cinnamyl alcohol to 50% of control. Cyanamide inhibited the total metabolism of cinnamaldehyde to polar products to 43% at 0.5 mM and to 65% of control at 1 mM, while protein binding was significantly enhanced to 213% ($p<0.01$) and marginally significantly to 151% ($p<0.05$) of control respectively. Treatment of cells with both cyanamide and ethanol was a summation of the effect of the two compounds on their own; The oxidation to cinnamic acid was inhibited and reduction to cinnamyl alcohol enhanced. Total metabolism was 80% of control at 0.5 mM and 72% at 1 mM, although relatively more cinnamaldehyde was converted to cinnamyl alcohol at the higher substrate concentration. Protein binding was enhanced at 0.5 mM (123%, $p<0.1$), but not at 1 mM (106%, $p>0.6$).

The ADH inhibitor, 4-methyl pyrazole inhibited cinnamyl alcohol formation to *ca* 30% of control, but also inhibited metabolism to cinnamic acid. The overall effect was that metabolism was reduced (83% and 68% of control) and covalent binding enhanced to 137% ($p<0.01$) and 116% ($p<0.05$) of control at 0.5 and 1 mM respectively.

After pretreatment of cells with NALC, a metabolite with a retention time of *ca* 20 min, coeluting with the GSH conjugate of

cinnamaldehyde (Chapter 8) accounted for α 30% of ^{14}C, whereas this metabolite was formed to less than 3% in control incubations. NALC inhibited the oxidation of cinnamaldehyde to cinnamic acid, but the reduction to cinnamyl alcohol was unaffected. The overall metabolism after NALC was significantly enhanced to 153% and 165% of control at 0.5 mM ($p<0.01$) and 1 mM ($p=0$) respectively. However, enhanced metabolism did not reduce covalent binding at 0.5 mM (104%, $p= 0.6$) and significantly increased binding to 136% of control at 1 mM ($p<0.01$).

7.4 Discussion

The results presented in this Chapter show that all manipulations of ^{14}C-cinnamaldehyde metabolism which resulted in a prolonged presence of cinnamaldehyde in the cell, enhance ^{14}C-binding to cellular macromolecules and this confirms that the parent compound is the reactive species in the observed binding.

In Chapter 6 it was demonstrated that a threshold exists for the saturation of cinnamaldehyde metabolism. Concentrations of up to 0.5 mM were rapidly metabolized in 1 h, but at 1 mM and higher the parent compound remained present. Depletion of initial cellular GSH with DEM and BSO shifted the threshold value for saturation of metabolism towards lower substrate concentrations and enhanced protein binding. After treatment with DEM and BSO, the parent compound remained present after 1 h of incubation even at 0.2 mM, concomitant with a 5-fold enhancement of ^{14}C binding at this concentration. Swales (1993) showed that combined treatment of cells with BSO and DEM caused a concentration of 0.5 mM cinnamaldehyde, which is non-cytotoxic in control cells, to become cytotoxic after 3 h. BSO and DEM pretreatment also further increased the cytotoxicity of 1 mM cinnamaldehyde from

20 to 40% after 3 h of incubation. Thus, the lower concentration at which cytotoxicity is observed in these experiments coincides with the lowering of the threshold concentration for saturation of metabolism and enhanced protein binding. In the same way it has been demonstrated (Jollow, 1980) that the cytotoxicity of paracetamol is preceded by the depletion of cellular GSH and that predepletion of GSH by BSO and DEM enhances this toxicity.

DEM and BSO not only depleted GSH, but also reduced metabolism *via* the oxidative pathway to cinnamic acid. This can not be attributed to toxicity of the modulators, because incubation of these compounds did not reduce the viability of hepatocytes in suspension over the first 3 hours as measured by LDH leakage. DEM has been reported to have a number of side-effects apart from the depletion of GSH. DEM administration to rats (Lauterburg and Mitchell, 1982) influenced sulfur metabolism by depleting CySH and methionine, the cellular sources of PAPS, the cofactor in sulfation. When rats treated with DEM, were dosed with acetaminophen i.v. the formation of the GSH conjugate was not only reduced, but sulfation and glucuronidation, the two alternative pathways, were also inhibited as compared to control. DEM is also known to affect certain enzyme activities e.g. it stimulates haem oxygenase and it causes choleresis in rats (Gallinsky, 1986). However, in the case of cinnamaldehyde these routes of metabolism are not relevant and coaddition of BSO uncouples GSH metabolism in the cell from that of other sulfur compounds. In fact it is not surprising, given the critical role of GSH in the cell, that changes in other aspects of cinnamaldehyde metabolism are observed. The absence of GSH alters the redox status of the cell and could for instance cause oxidation of the thiol group in the active site of the ALDH enzyme (Cotgreave *et al.*, 1990). GSH also serves as a cofactor in the metabolism of formaldehyde by GSH-dependent formaldehyde dehydrogenases

(Koivusalo *et al.*, 1989) and it has recently been suggested that this enzyme activity could be responsible for the metabolism of other aldehydes such as acrolein (Mitchell and Petersen, 1989). A third function of GSH could be transport of cinnamaldehyde in the cell. Douglas (1988) describes how the formation of a hemithioacetal of GSH with methylglyoxal purchases time for the glyoxalase system to encounter the aldehyde and act on it, thus contributing to its detoxication. Based on the data presented in this Chapter it is still possible to attribute the potentiating effect of BSO and DEM on the ^{14}C-cinnamaldehyde-related binding to the absence of GSH, either directly or indirectly, and to conclude that the prolonged presence of the parent compound enhances covalent binding.

The fact that predepletion of cellular GSH by BSO and DEM enhanced protein binding indicates a role for GSH in the cell against irreversible protein binding. However, pretreatment of cells with NALC, which caused a two-fold increase in initial GSH, did not protect against ^{14}C-binding, although it increased total metabolism by formation of a cinnamaldehyde-GSH conjugate. This is not consistent with the effect of other modulators, where total metabolism and covalent binding correlated reciprocally. But these results do tie in with data of Swales (1993) which show that in spite of higher initial GSH after NALC, 0.5 mM cinnamaldehyde depleted GSH to the same extent in treated and control cells (both to 60% of control values) and did not alter the time of onset or the extent of cytotoxicity.

The effect of the competing substrate ethanol is interesting and relevant with regard to the use of cinnamaldehyde as a flavouring in alcoholic beverages. The ADH-dependent oxidation of ethanol directs the metabolism of cinnamaldehyde towards reduction to cinnamyl alcohol. This can be understood from the cofactor requirements of the

dehydrogenase enzymes. In the cell the ratio of NAD+/NADH is high, favouring oxidative metabolism rather than reduction. The oxidation of ethanol by ADHs results in the formation of NADH, providing the reducing equivalents for the reduction of cinnamaldehyde. Similar findings have been reported by Goon *et al.* (1992). The metabolism of *trans*,*trans*-muconaldehyde in the presence of both ALDH and ADH and cofactors NAD(P)+ or NAD(P)H, led to the formation of reductive as well as oxidative metabolites, with one aldehyde group reduced and the other oxidized, also suggesting cycling of cofactor, such as NAD+ formed from NADH during ADH-mediated metabolism which can be utilized by ALDH and *vice versa.* Coaddition of cinnamaldehyde and ethanol reduced the amount of covalent binding at 0.5 mM, the highest non-cytotoxic concentration. Thus, a shift in metabolism towards cinnamyl alcohol is protective against protein binding and it is as though by the slow release of cinnamaldehyde *via* its alcohol the cell is able to cope better. The threshold phenomenon is indicated again by the fact that 0.2% ethanol is protective at 0.5 mM, but not at 1 mM cinnamaldehyde once the threshold for saturation has been surpassed.

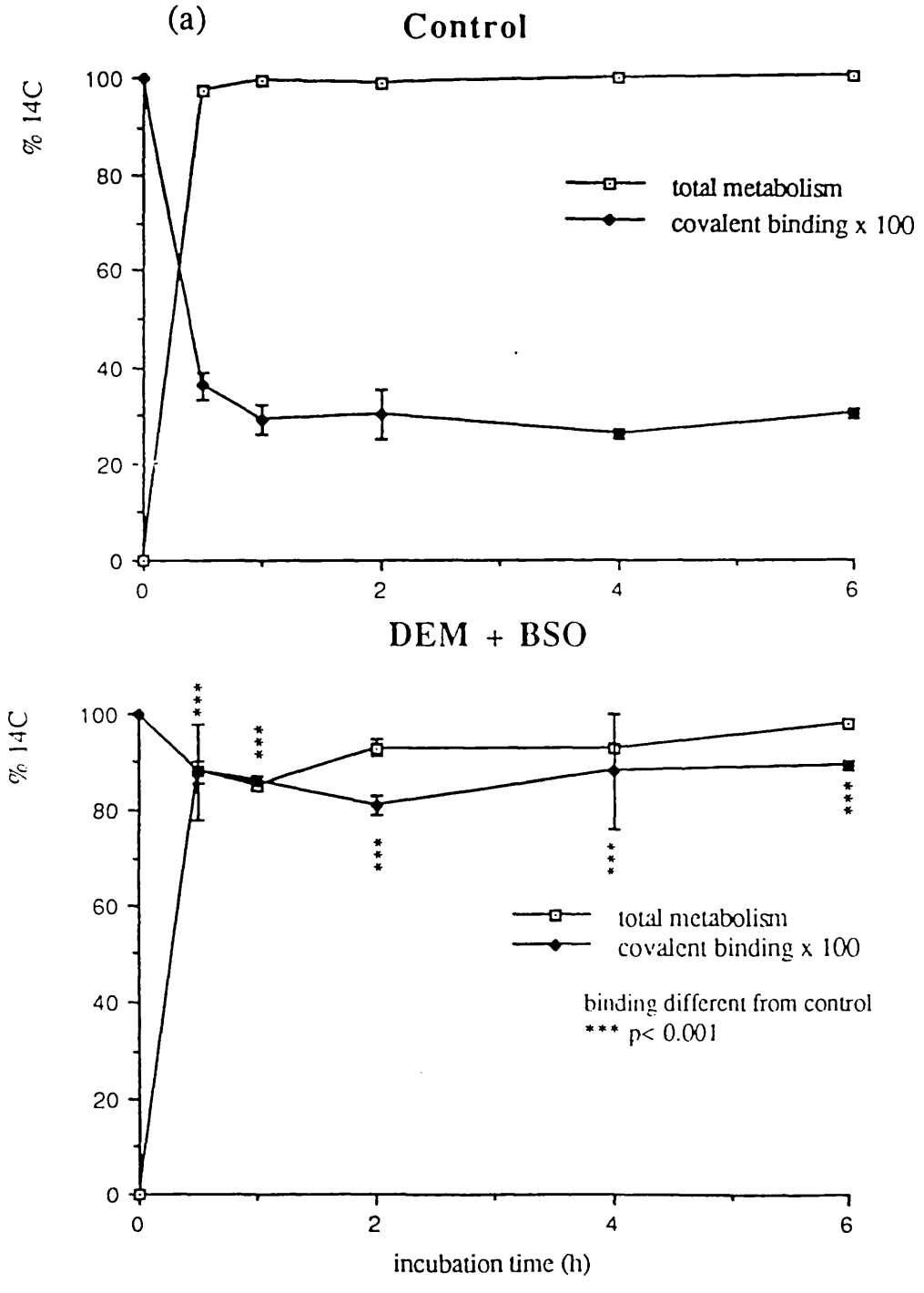
The existence of a threshold is also observed in the effect of cyanamide and 4-methyl pyrazole. These inhibitors of dehydrogenase enzymes enhanced ^{14}C binding due to inhibition of metabolism resulting in a prolonged presence of cinnamaldehyde as compared to control incubations, but had relatively more effect at the low than the high substrate concentration, where metabolism was already saturated. The results presented here show the value of metabolism studies in the interpretation of the effect of modulators of metabolism, for although a compound may be known to inhibit certain enzymes, inhibition may not be complete and the addition of a compound may have an effect on other aspects of metabolism. The inhibitory effect of cyanamide on ALDH (even in a 1 h incubation) was not complete, for 25% of

the control activity was still present, neither was it specific for this dehydrogenase, it also inhibited the formation of cinnamyl alcohol to 60% of control. 4-Methyl pyrazole inhibited ADH to 45% of control, but also inhibited the formation of cinnamic acid to 70% of control. Knowing the extent to which inhibition of the various routes of metabolism occurred, makes the conclusion possible that their overall effect was the inhibition of total metabolism and enhancement of covalent binding, showing that cinnamaldehyde is the reactive species in the ^{14}C binding.

In conclusion, manipulation of metabolism led to similar changes in ^{14}C binding, GSH depletion and ultimately in cytotoxicity, suggesting that cinnamaldehyde is the reactive species in all three events. Although, as explained in Chapter 6, enhanced covalent binding is possibly not causal in the cytotoxicity observed in parallel experiments (Swales, 1993), it is indicative for this toxicity. ^{14}C-Cinnamaldehyde is rapidly metabolized by hepatocytes to various polar metabolites and leads to enhanced ^{14}C binding only after saturation of its metabolism at high concentrations or after inhibition of its metabolism by various modulators or predepletion of cellular GSH. Conversely, the inclusion of ethanol in the incubation medium enhances metabolism and protects against ^{14}C binding.

Figure 7.1

The effect of DEM and BSO on (a) total metabolism and ^{14}C binding* and (b) the metabolic profile**of 0.5 mM ^{14}C-cinnamaldehyde in F344 rat hepatocytes in suspension as a function of the incubation time.



* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

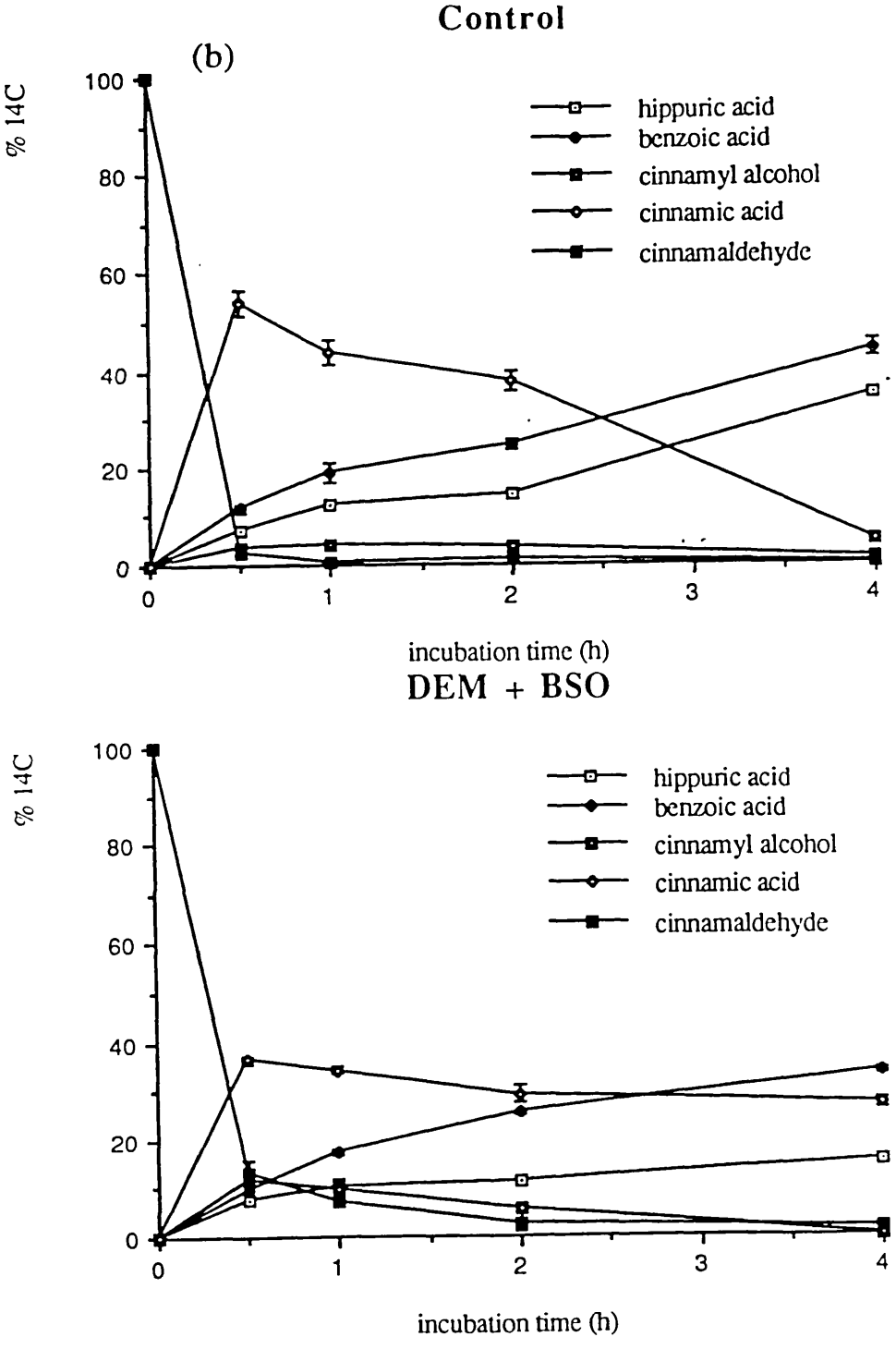
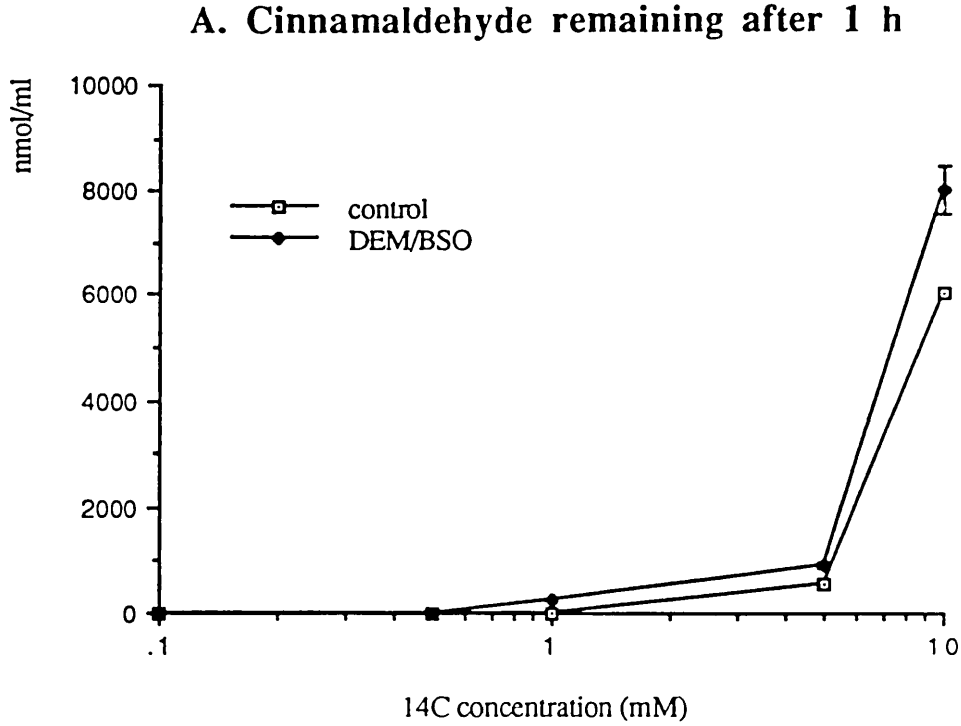
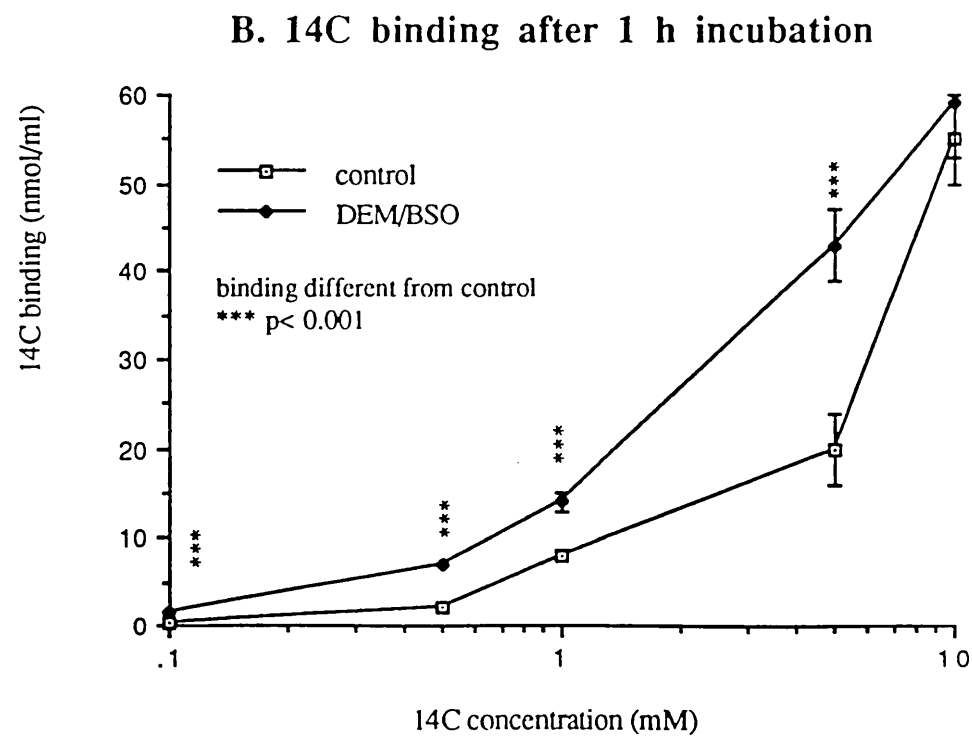

Control
(b)
% 14C
100
80
60
40
20
0
0
1
2
3
4
hippuric acid
benzoic acid
cinnamyl alcohol
cinnamic acid
cinnamaldehyde
incubation time (h)
DEM + BSO
% 14C
100
80
60
40
20
0
0
1
2
3
4
hippuric acid
benzoic acid
cinnamyl alcohol
cinnamic acid
cinnamaldehyde
incubation time (h)

Figure 7.2

The effect of DEM and BSO on (a) cinnamaldehyde remaining* and (b) ^{14}C binding** of ^{14}C-cinnamaldehyde in F344 rat hepatocytes in suspension as a function of the substrate concentration





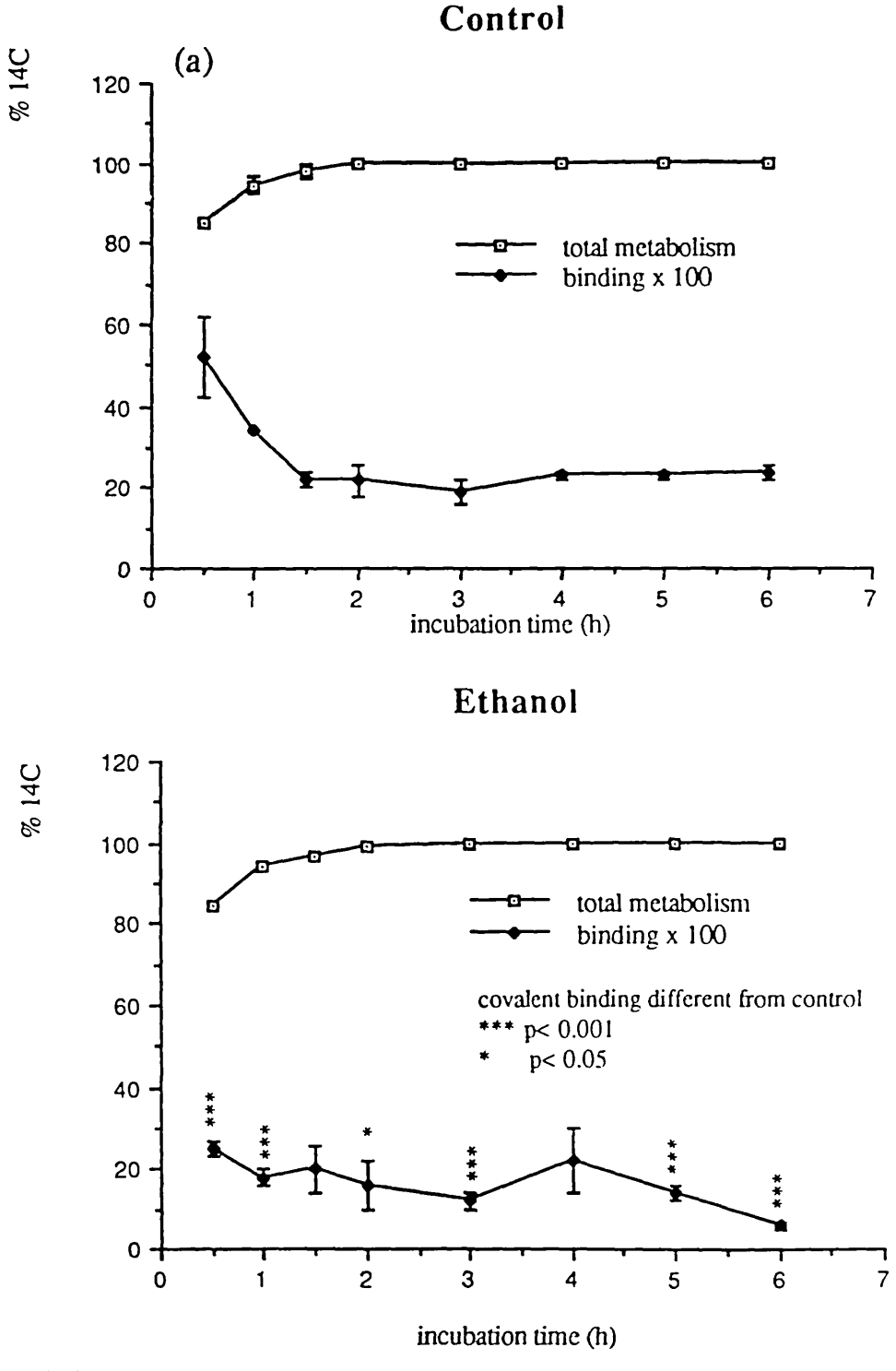
* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis

** ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation.

Figures are means ± S.D., n=3.

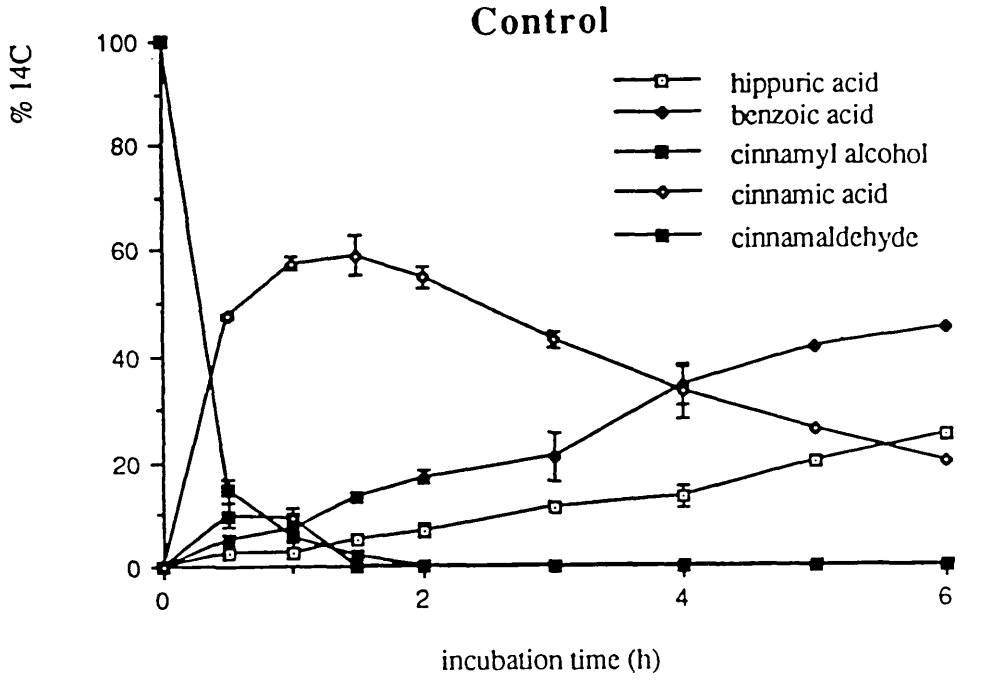
Figure 7.3

The effect of 0.2% ethanol on (a) total metabolism and ^{14}C binding* and (b) metabolic profile** of 0.5 mM ^{14}C-cinnamaldehyde in F344 rat hepatocytes in suspension as a function of the incubation time



* Total metabolism was calculated as the sum of ^{14}C-cinnamaldehyde metabolites as % of total ^{14}C after radio HPLC analysis and ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation. Figures are means ± S.D., n=3.
** ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

(b)


Control
% 14C
100
80
60
40
20
0
0
2
4
6
incubation time (h)
hippuric acid
benzoic acid
cinnamyl alcohol
cinnamic acid
cinnamaldehyde

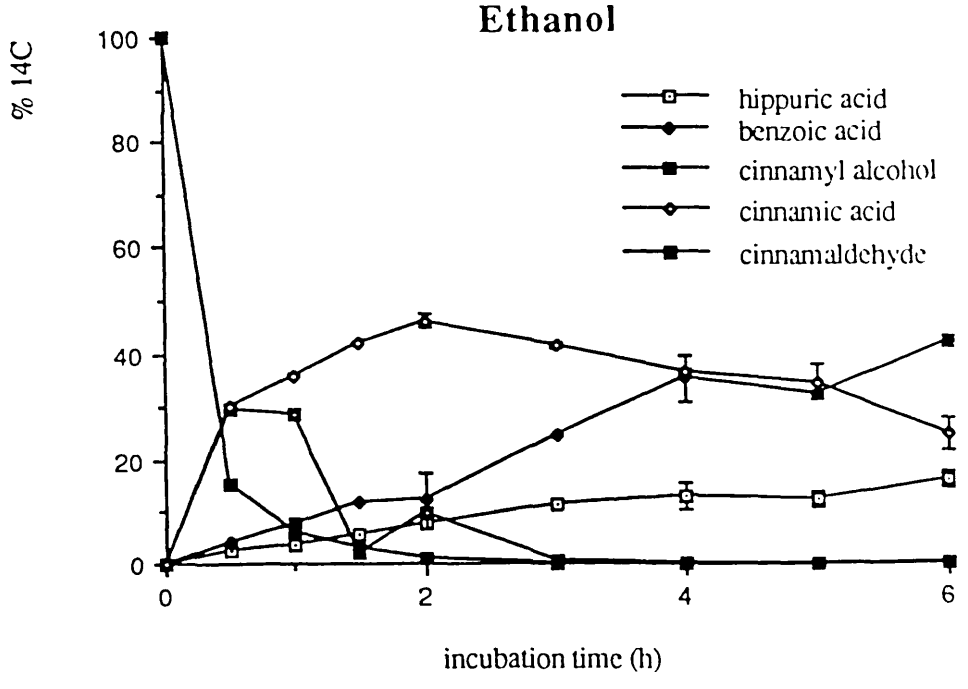
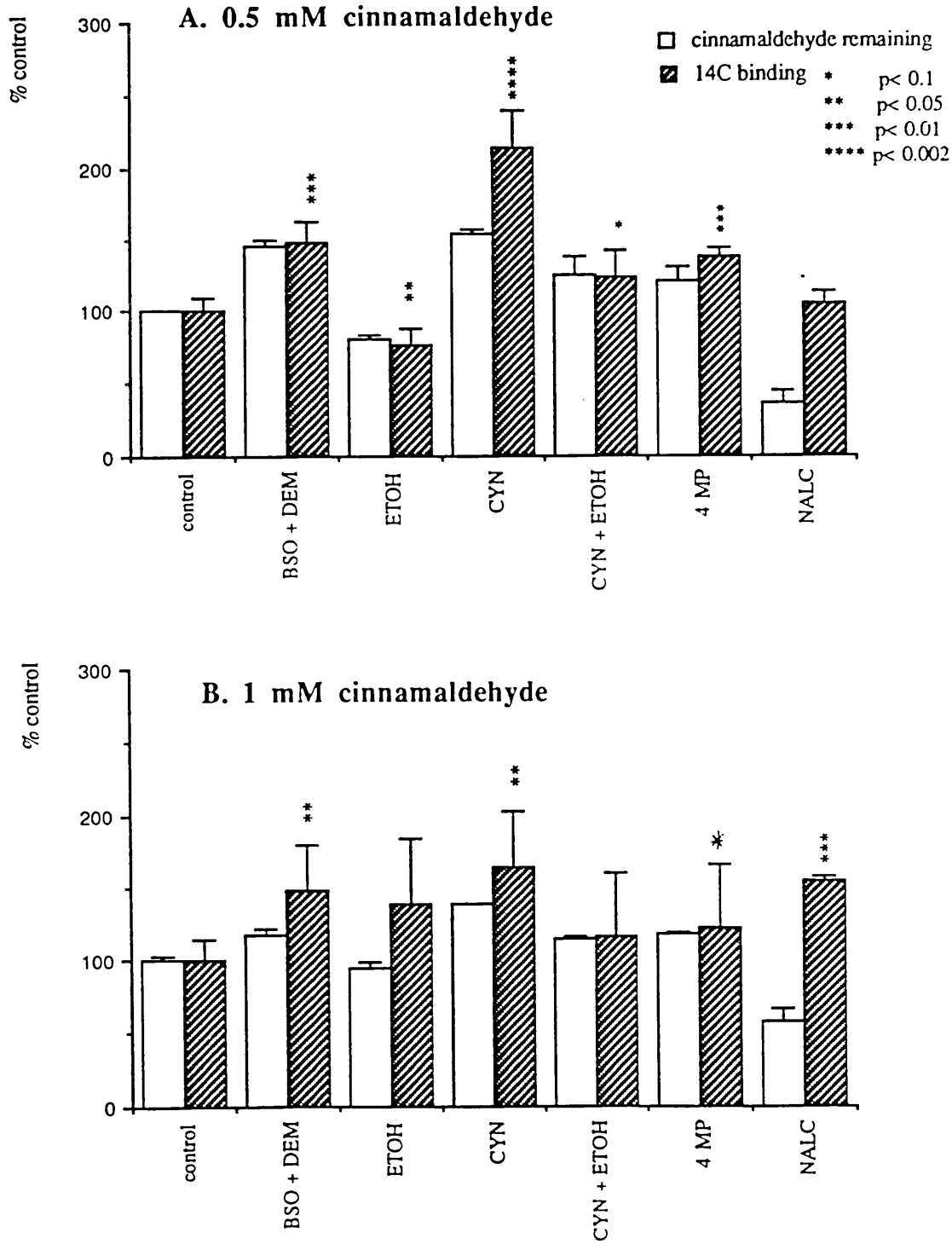

Ethanol
% 14C
100
80
60
40
20
0
0
2
4
6
incubation time (h)
hippuric acid
benzoic acid
cinnamyl alcohol
cinnamic acid
cinnamaldehyde

Figure 7.4

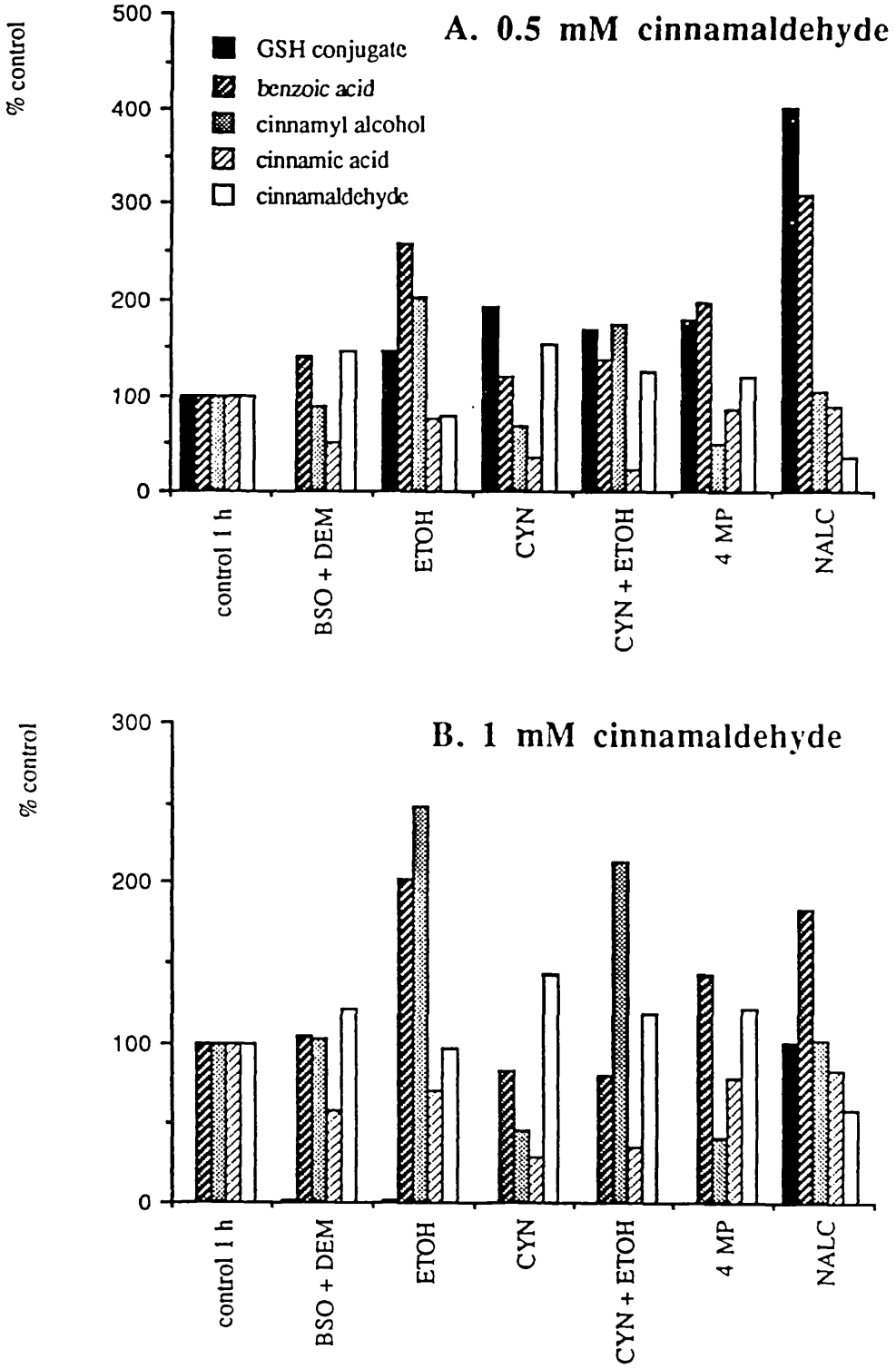
The effect of modulators on cinnamaldehyde remaining after 1 h* and ^{14}C binding** of (a) 0.5 mM and (b) 1 mM ^{14}C-cinnamaldehyde in F344 rat hepatocytes in suspension



* ^{14}C-cinnamaldehyde as % of total ^{14}C after radio HPLC analysis
** ^{14}C binding was unextractable ^{14}C expressed as % of substrate incubation.
Figures are means ± S.D., n=3.

Figure 7.5

The effect of modulators on the metabolic profile* of (a) 0.5 mM and (b) 1 mM ^{14}C-cinnamaldehyde in F344 rat hepatocytes in suspension



* ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis.

Chapter 8

Metabolism and protein binding of ^{14}C-cinnamaldehyde in rat and mouse cytosol

8.1 Introduction

The results obtained in Chapter 7 combined with those of Swales (1993) indicate that GSH protects against both the covalent binding and cytotoxicity of ^{14}C-cinnamaldehyde in hepatocytes, but increased initial GSH did not reduce binding nor cytotoxicity, and it is thus relevant to study the role of GSH in the metabolism of ^{14}C-cinnamaldehyde further. *In vivo* studies showed that cinnamaldehyde is conjugated with GSH by a direct addition across the carbon double bond (Chapter 5). In the case of some α,ß-unsaturated compounds this does not lead to detoxication since the reactive parent compound can still be released from the conjugate e.g. allyl isothiocyanate (Bruggeman *et al.*, 1986; Temmink *et al.*, 1986), although the only example of a reversible Michael adduct actually playing a role in a toxic effect is that of the veterinary drug furazolidone (Vroomen *et al.*, 1987, 1988). On addition of GSH to incubation mixtures of furazolidone with liver microsomes, conjugates were formed from a cyano-substituted, α,ß-unsaturated ketone. These conjugates could alkylate proteins and were found to be mutagenic. GSH conjugates (hemiacetals) of formaldehyde and acetaldehyde can also fall apart and reform the aldehyde, but these can also go on to react with a second molecule of GSH or perhaps with nitrogen nucleophiles in DNA (Fennell *et al.*, 1988).

As mentioned in Chapter 7, GSH may have additional functions such as its action as a cofactor in the oxidation of formaldehyde, where it is not the aldehyde, but its hemiacetal that is oxidized by ALDH (Koivusalo *et al.*, 1989). α,ß-Unsaturated aldehydes such as acrolein are not substrates for the NAD+ dependent ALDHs, but are inhibitors of these enzymes, perhaps as a result of covalent binding to the active site of the enzyme. Studies of human ALDH have identified an

essential, potentially susceptible sulfhydryl group associated with the active site of the enzyme to which acrolein having a highly active ß-carbon could bind (Mitchell and Petersen, 1988). The oxidation of acrolein by microsomal ALDH and reduction by cytosolic ADH only seems to take place after conjugation with GSH (Mitchell and Petersen, 1989). It has been proposed that this mechanism, similar to that of formaldehyde oxidation, is more general than previously thought (Burk, 1992). Kubo and Armstrong (1989) suggested that GSTs enable the protonation of the carbonyl group (of the incipient enolate) in the transition state in the formation of a GSH conjugate, thereby not only catalyzing GSH conjugation, but also serving as an aldehyde dehydrogenase. If the enzyme activities described operate for cinnamaldehyde, its oxidation would be enhanced in the presence of GSH.

Results obtained in Chapters 2, 3 and 5 showed that mercapturic acids accounted for 7% of cinnamaldehyde metabolism in the rat and a concentration of 0.5 mM cinnamaldehyde depletes cellular GSH to 60% of control in rat hepatocytes in suspension (Swales, 1993). It was therefore expected to find a GSH conjugate in hepatocyte incubations. However, the formation of this conjugate involves only 2% of the substrate (cinnamaldehyde), which hinders the selective study of this metabolite. The results in Chapters 6 and 7 show that the oxidative capacity of hepatocytes is too high to study the characteristics of the GSH conjugation. For instance, pretreatment of cells with DEM and BSO to deplete initial cellular GSH, did not reduce the formation of any of the minor metabolites. Therefore experiments in this Chapter were performed in buffer, to determine the spontaneous reactivity of cinnamaldehyde towards GSH, CySH and NALC, and in rat and mouse liver cytosol, which also makes it possible to distinguish between enzymatic and non-enzymatic conversion. By adding or

withholding cofactors NADPH and NAD+ the cofactor dependency of the ADH and ALDH-mediated metabolism can be studied. Comparison of rat and mouse cytosolic metabolism could provide an enzymatic basis for species differences.

8.2 Methods

8.2.1 Chemicals

NADPH, NAD+, GSH, CySH and NALC were obtained from Sigma, Chemical Co., Poole, Dorset, UK. All other chemicals were obtained as previously described.

8.2.2 Preparation of hepatic subcellular fractions

Male F344 rats (*ca* 200 g) and male CD1 mice (*ca* 25 g) were weighed and sacrificed by cervical dislocation. Livers were removed and rinsed with ice cold 0.1 M phosphate buffer pH 7.4, blotted on Whatman No.1 filter paper and weighed. Livers were perfused with ice cold 0.9% (*w/v*) NaCl, minced with scissors and homogenized in 3 volumes 0.1 M phosphate buffer pH 7.4 (Potter Homogenizer, Braun, Melsungen A.G., Germany, 10 return strokes). The homogenate was centrifuged at 10,000 *g*, 4 °C for 30 min using a Sorvall RC5B centrifuge to obtain the post-mitochondrial supernatants and subsequently at 100,000 *g*, 4 °C for 60 min using a Sorvall Combi Ultracentrifuge. The supernatant (cytosol) was removed, aliquots taken for the determination of protein content according to Lowry *et al.* (1951) as described in Chapter 6 and the remainder was used immediately for cytosolic incubations or stored at -70 °C.

8.2.3 Standard cytosolic incubations

Standard incubation mixtures contained 1 mg/ml rat or mouse liver cytosolic protein, 1 mM NADPH or NAD+, 1 mM ^{14}C-cinnamaldehyde (1 μCi/ml) and 1 mM GSH and were made up to a final volume of 1 ml in 0.1 M phosphate buffer pH 7.4 containing 5 mM $MgCl_2$. After preincubations at 37 °C for 2 min, the reaction was started by addition ^{14}C-cinnamaldehyde in DMSO (5 μl). Reactions were terminated after 30 min by the addition of 1 ml ice cold methanol, vortex mixing and placing tubes on ice.

8.2.4 HPLC analysis and determination of ^{14}C binding

After centrifugation (2,000 rpm, 10 min, Heraeus, Sepatech Biofuge B) 20 μl samples of the supernatant were taken for radio HPLC analysis and 50 μl aliquots for scintillation counting to determine the ^{14}C recovery as described in Chapter 2. Covalent binding of ^{14}C-cinnamaldehyde or its metabolites to cytosolic protein was determined by resuspending the pellet in 5 ml of 5% TCA by vortex mixing. This was left on ice for 30 min. After centrifugation (2,000 rpm, 15 min) the supernatant was discarded and the pellet washed with 5% TCA and 80% methanol/water as described in Chapter 6.

8.3 Results

8.3.1 Conjugation of ^{14}C-cinnamaldehyde with thiols in buffer

When ^{14}C-cinnamaldehyde was incubated with GSH in buffer at pH 7.4 a single ^{14}C-containing metabolite was detected by radio HPLC with a retention time of *ca* 20 min compared to a retention time of 48

min for cinnamaldehyde. The formation of this metabolite was linear with both ^{14}C-cinnamaldehyde (Fig. 8.2A) and GSH concentrations (Fig. 8.3A), suggesting that this was a GSH conjugate of ^{14}C-cinnamaldehyde. The reaction of 5 mM GSH and 1 mM cinnamaldehyde in buffer yielded *ca* 40% of the ^{14}C-cinnamaldehyde concentration as this conjugate. When the reaction was followed in time (Fig. 8.1A) this was showing an initial rapid reaction and slower over the rest of the 30 min time-course. The reaction did not reach completion, but rather an equilibrium was achieved, since ^{14}C-cinnamaldehyde remained present even when incubated with a five-fold excess of GSH.

After incubation of ^{14}C-cinnamaldehyde with CySH in buffer at pH 7.4 radio HPLC analysis showed a single ^{14}C-containing metabolite, with a retention time of 21 min, the formation of which was linear with both cinnamaldehyde and CySH concentrations. Comparison of the reactivity of cinnamaldehyde towards GSH (Fig. 8.1A) and CySH (Fig. 8.1B) showed that reaction with CySH was more extensive: When 5 mM CySH and 1 mM ^{14}C-cinnamaldehyde were incubated *ca* 65% of ^{14}C was recovered as the CySH conjugate. The reaction of ^{14}C-cinnamaldehyde with NALC led to the formation of a ^{14}C-containing metabolite, with a retention time of *ca* 30 min. The formation of this metabolite was linear with cinnamaldehyde (Fig. 8.2C) and NALC (Fig. 8.3C) concentrations. The reactivity of cinnamaldehyde towards NALC was less than that towards GSH, for only 30% of ^{14}C was converted to the NALC conjugate when 5 mM NALC and 1 mM ^{14}C-cinnamaldehyde were incubated (Fig. 8.1C), compared to 46% with GSH (Fig. 8.1A) and 65% with CySH (Fig. 8.1B).

8.3.2 Metabolism of ^{14}C-cinnamaldehyde by mouse liver cytosolic fraction as a function of the protein concentration

In the presence of mouse liver cytosolic fraction, 20% of 0.5 mM ^{14}C-cinnamaldehyde was metabolized to cinnamic acid and this conversion was linear with the cytosolic protein concentration (Fig. 8.4A). When an equimolar amount of GSH was added (Fig. 8.4C) a product was formed with an identical retention time to that described in section 8.3.1 when ^{14}C-cinnamaldehyde and GSH were incubated in buffer suggesting that this was a GSH conjugate of ^{14}C-cinnamaldehyde. The formation of this product was enhanced by the presence of cytosol as compared to buffer (Fig. 8.3A; 0 mg protein). The formation of cinnamic acid was unaffected by the addition of 0.5 mM GSH.

The presence of NADPH (Fig. 8.4B) led to a protein concentration-dependent formation of cinnamyl alcohol (maximal 42%). A ^{14}C-cinnamaldehyde-GSH conjugate was formed with GSH present in the cytosol (0.05 nmol/mg protein), which accounted for 20% of ^{14}C at the highest cytosol concentration, while the total conversion was 86%. Coaddition of NADPH and 0.5 mM GSH (Fig. 8.4D) did not influence the oxidation of cinnamaldehyde to cinnamic acid, but the formation of cinnamyl alcohol was reduced due to competing metabolism via GSH conjugation, which accounted for ca 30% of ^{14}C compared to 20% in control incubations.

Incubation of cytosolic preparations with ^{14}C-cinnamaldehyde led to the recovery of 1 to 5% of ^{14}C (3 to 12 nmol ^{14}C/mg protein) covalently bound to cytosolic protein (Fig. 8.4E) and this binding was linear with the amount of cytosolic protein present. NADPH protected against protein binding. Addition of an equimolar amount of 0.5 mM GSH, although not effective on its own, further reduced binding in

combination with NADPH slightly. Correlation coefficients for ^{14}C-cinnamaldehyde remaining in the medium after 30 min and ^{14}C binding were 0.79 for 0.5 mg cytosol and 0.87 and 0.95 for 1 and 2 mg cytosol respectively.

8.3.3 Metabolism of ^{14}C-cinnamaldehyde as a function of substrate concentration

Incubations containing 1 mg cytosolic protein and various concentrations of ^{14}C-cinnamaldehyde showed a substrate concentration-dependent formation of cinnamic acid and cinnamyl alcohol in the presence of NADPH and 4 mM GSH (Fig. 8.5A). At low cinnamaldehyde concentrations, the amount of GSH conjugate formed in the presence of cytosol was less than in buffer (Fig. 8.2A) due to competing metabolism to cinnamyl alcohol and cinnamic acid, but at high concentrations when the reduction of cinnamaldehyde to its alcohol and oxidation to its acid were saturated the GSH conjugate was formed to a comparable extent in buffer and in the presence of cytosol (Fig. 8.5A) accounting for α 40% of ^{14}C.

Binding of ^{14}C to cytosolic protein (Fig. 8.5B) was linear with the amount of ^{14}C-cinnamaldehyde remaining in the incubation medium after 30 min and ranged from 4 to 71 nmol (0.6-2.7% of ^{14}C). 4 mM GSH protected against binding at 0.5 and 1 mM, but not 5 mM cinnamaldehyde. NADPH reduced binding at 0.5 and 1 mM cinnamaldehyde and coaddition of GSH further reduced this. The correlation coefficient between ^{14}C cinnamaldehyde remaining after 30 min and ^{14}C binding was 0.86; 0.98 and -0.03 at 0.5 mm, 1.0 mM and 5 mM respectively, showing that enhanced metabolism protected against binding of ^{14}C at 0.5 and 1 mM, but not at 5 mM cinnamaldehyde.

8.3.4 Comparison of ^{14}C-cinnamaldehyde metabolism in rat and mouse liver cytosol

A comparison of the metabolism of 1 mM ^{14}C-cinnamaldehyde in rat and mouse cytosol is shown in Table. 8.1. Cinnamic acid was formed without the addition of cofactor, slightly more (9.6 vs. 8.4%) in the mouse than in the rat. Upon addition of NAD+, the oxidation of cinnamaldehyde to cinnamic acid and its reduction to cinnamyl alcohol were enhanced. Oxidative and reductive metabolism were greater in the rat than in the mouse. An equimolar amount of GSH led to a greater formation of a cinnamaldehyde-GSH conjugate in the mouse than in the rat, while the oxidation to cinnamic acid was unaffected by GSH. Coaddition of NAD+ and 1 mM GSH enhanced GSH conjugation in the mouse and in the rat.

Binding of ^{14}C (nmol/mg protein) was very similar in rat and mouse cytosol. Both 1 mM NAD+ and 1 mM GSH reduced covalent binding of 1 mM ^{14}C-cinnamaldehyde.Correlation coefficients between ^{14}C-cinnamaldehyde remaining after 30 min and covalent binding were 0.88 in mouse and 0.94 in rat cytosolic incubations.

8.4 Discussion

The results presented in this Chapter show that ^{14}C-cinnamaldehyde reacts rapidly with GSH both enzymatically and non-enzymatically and reacts non-enzymatically with CySH and NALC. Cinnamaldehyde is metabolized by cytosolic enzymes to cinnamic acid and cinnamyl alcohol. The amount of ^{14}C binding to cytosolic protein correlates with the presence of the parent compound. Binding increases with increasing cinnamaldehyde concentration and is reduced by the addition of cofactors which enhance metabolism. GSH protects against ^{14}C

binding to the extent that it increases metabolism by formation of a GSH conjugate with cinnamaldehyde. These results confirm those obtained in hepatocytes (Chapters 6 and 7) and point at cinnamaldehyde as the reactive species in GSH conjugation and protein binding.

In buffer, ^{14}C-cinnamaldehyde reacts spontaneously with CySH, GSH and NALC and the reactivity towards these thiols is greatest for CySH and least for NALC, which correlates with the order reported by Moon and Pack (1983). For all three thiols this reaction is biphasic, rapid for the first 5 min of incubation, followed by a much slower reaction over the rest of the 30 min time-course. The amount of substrate recovered as ^{14}C-conjugate was linear both with thiol and cinnamaldehyde concentrations, but even when thiol was in vast excess, ^{14}C-cinnamaldehyde remained present after 30 min incubation, showing that an equilibrium was reached. When equimolar amounts of GSH and ^{14}C-cinnamaldehyde were incubated, only 5% of ^{14}C was recovered as the conjugate and a 10-fold excess was needed to deplete 50% of the cinnamaldehyde present. Conjugation with GSH was enhanced in the presence of cytosol. Overall these results indicate that the reactivity of cinnamaldehyde towards GSH is much less than that of some other α,ß-unsaturated compounds such as DEM (Boyland and Chasseaud, 1968; Swales, 1993). This is reflected in the fact that only 6-7% of dose was recovered as mercapturic acids *in vivo* even at a dose of 250 mg/kg when cinnamaldehyde (400 µmol/ 200 g rat) was in *ca* 7-fold excess of liver GSH (60 µmol) (Chapters 2,3 and 9). Similarly, *in vitro* in rat hepatocytes in suspension a 20-fold excess of cinnamaldehyde (500 nmol/ml *vs* 25 nmol/ml GSH) only caused a 30% depletion of control GSH levels (Swales, 1993). These results show that the minor involvement of GSH conjugation in the metabolism of cinnamaldehyde *in vivo* and in hepatocytes is due to its relative low reactivity towards

GSH, together with the large capacity of liver enzymes to metabolize cinnamaldehyde *via* the competing pathways of oxidation and reduction.

Recently, there has been some doubt as to which enzyme systems, cytosolic, microsomal or mitochondrial, are responsible for the oxidation and reduction of α,ß-unsaturated aldehydes. The inability of NAD+ dependent cytosolic and mitochondrial ALDHs to oxidize some α,ß-unsaturated compounds has been implicated in their toxicity. Smith and Packer (1972) reported that cinnamaldehyde, acrolein and crotonaldehyde were not substrates for NAD+ dependent mitochondrial ALDHs. Although citral was a substrate for NADH dependent cytosolic ADHs, it was not believed to be a substrate for mitochondrial or cytosolic ALDHs, but rather it became bound to the enzyme (Boyer and Petersen, 1990). This binding differed from that of acrolein or malondialdehyde, in that it did not interfere with the NAD+ binding site of the enzymes and that the effect was reversible. As mentioned before, acrolein might interact with the active site of the ALDH which contains a thiol group (Mitchell and Petersen, 1988). In the present experiments however, it was shown that cinnamaldehyde unlike acrolein is a substrate for NAD+ dependent cytosolic enzymes. Furthermore results from Chapter 7 showed that cinnamaldehyde was rapidly oxidized to cinnamic acid by rat hepatocytes in suspension and that oxidation could be inhibited by cyanamide, an inhibitor of acetaldehyde metabolism and ALDHs. A further difference between the oxidation of acrolein and cinnamaldehyde is the fact that addition, even of large amounts of GSH, to the incubation mixture did not affect the conversion of cinnamaldehyde to cinnamic acid, whereas a GSH conjugate was thought a necessary intermediate in the oxidation of acrolein by a proposed GSH-dependent ALDH. In view of the safety evaluation of

cinnamaldehyde this is very important, for these differences in metabolism between cinnamaldehyde and related aldehydes such as acrolein and citral, which are toxic and have *in vitro* mutagenic properties, make the occurrence of similar adverse affects for cinnamaldehyde unlikely.

In cytosol the reduction of cinnamaldehyde to cinnamyl alcohol was greater than its oxidation to cinnamic acid, whereas in hepatocytes oxidation was favoured (Chapters 6 and 7). This shows that reductive enzymes are present to a large extent in rat and mouse liver, but that the redox status of the cell determines that metabolism will be directed towards oxidation. In addition to cytosolic ALDHs, microsomal ALDH enzymes possibly contribute to the oxidation of cinnamaldehyde in hepatocytes.

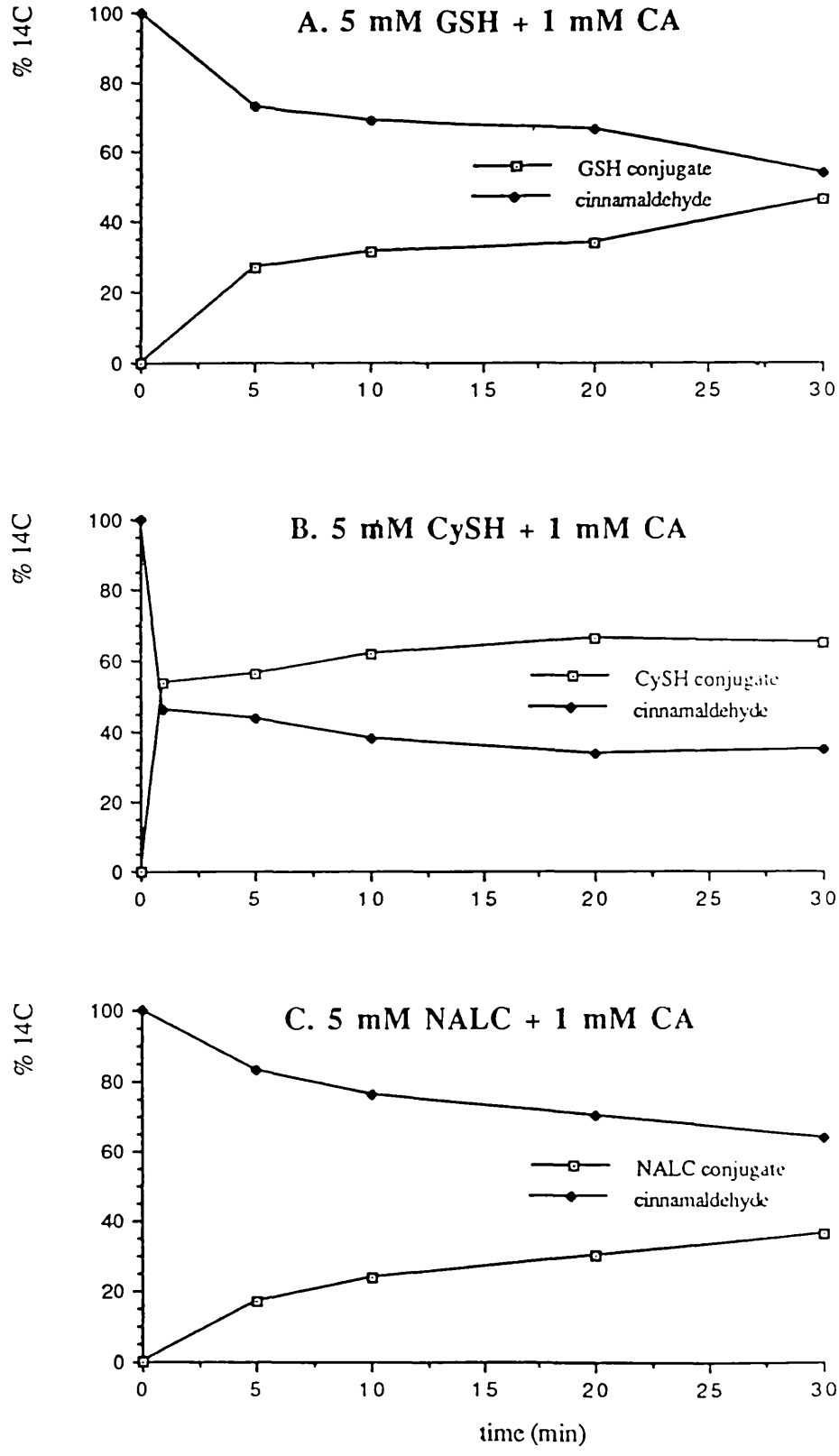
Comparison of the cytosolic metabolism of cinnamaldehyde between mouse and rat shows a slightly slower oxidative and reductive metabolism in the mouse, but more GSH conjugation than in the rat. The overall rate of metabolism is very similar in mouse and rat cytosol and this is reflected in a similar amount of protein binding, *ca* 2% of ^{14}C in both species. Although rat cytosol had a higher reductive capacity than mouse cytosol, the greater formation of cinnamyl alcohol in mouse hepatocytes might be explained by the slower competing oxidative metabolism. Possibly the lower rate of oxidative metabolism in the mouse accounts for the higher *in vivo* formation of cinnamoyl glycine in this species as compared to ß-oxidation products in the rat. GSH conjugation in mouse cytosol is much higher (ca 27%) than in rat cytosol (4%). These results compare with the somewhat greater formation (less than 6% of metabolism) of GSH conjugate in mouse hepatocytes as compared to the rat (possibly 2%). In rat urine *in vivo* two sulfur containing metabolites were found and in mouse urine the

same two and an additional third, however the sum of sulfur containing metabolites was 6-7% in both species.

Summarizing the results it can be concluded that cinnamaldehyde reacts spontaneously with GSH in buffer at pH 7.4 to form a cinnamaldehyde-GSH conjugate. This conjugation is linear with both cinnamaldehyde and GSH concentrations and reaches an equilibrium. At low GSH concentrations conjugation is entirely dependent on the presence of cytosol. GSH protects against binding of ^{14}C to cytosolic macromolecules to the extent that it increases total metabolism by conjugate formation. Differences in the cytosolic metabolism of cinnamaldehyde between rat and mouse are small; in the rat, enzyme-catalyzed oxidative and reductive metabolism is slightly greater, while in the mouse GSH conjugation is more important.

Figure 8.1

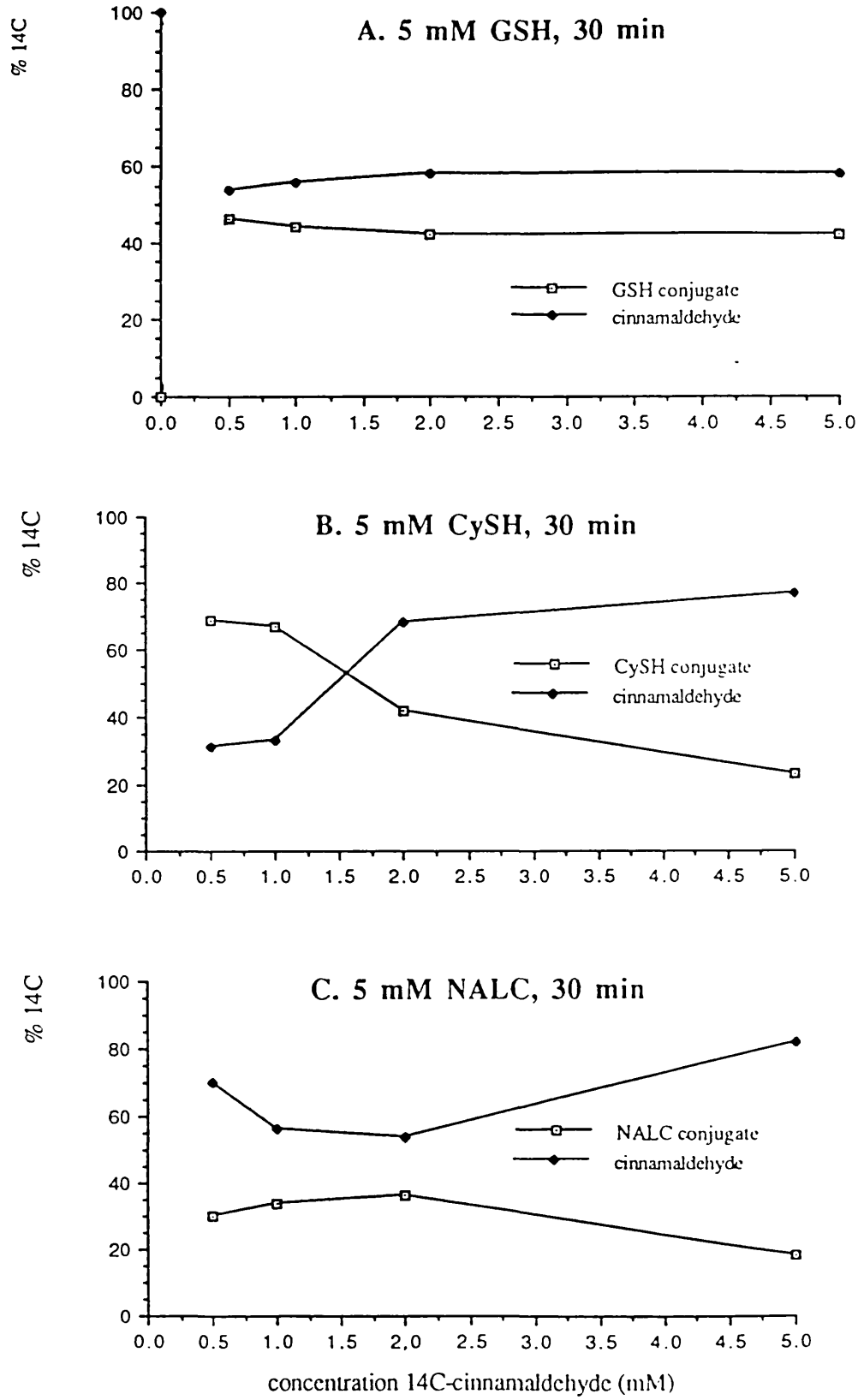
Reaction of 1 mM ^{14}C-cinnamaldehyde and 5 mM thiol in buffer pH 7.4 as a function of the incubation time*



* % ^{14}C recovered as ^{14}C-cinnamaldehyde or ^{14}C-cinnamaldehyde-thiol conjugate. Figures are means ± S.D., n=3.

Figure 8.2

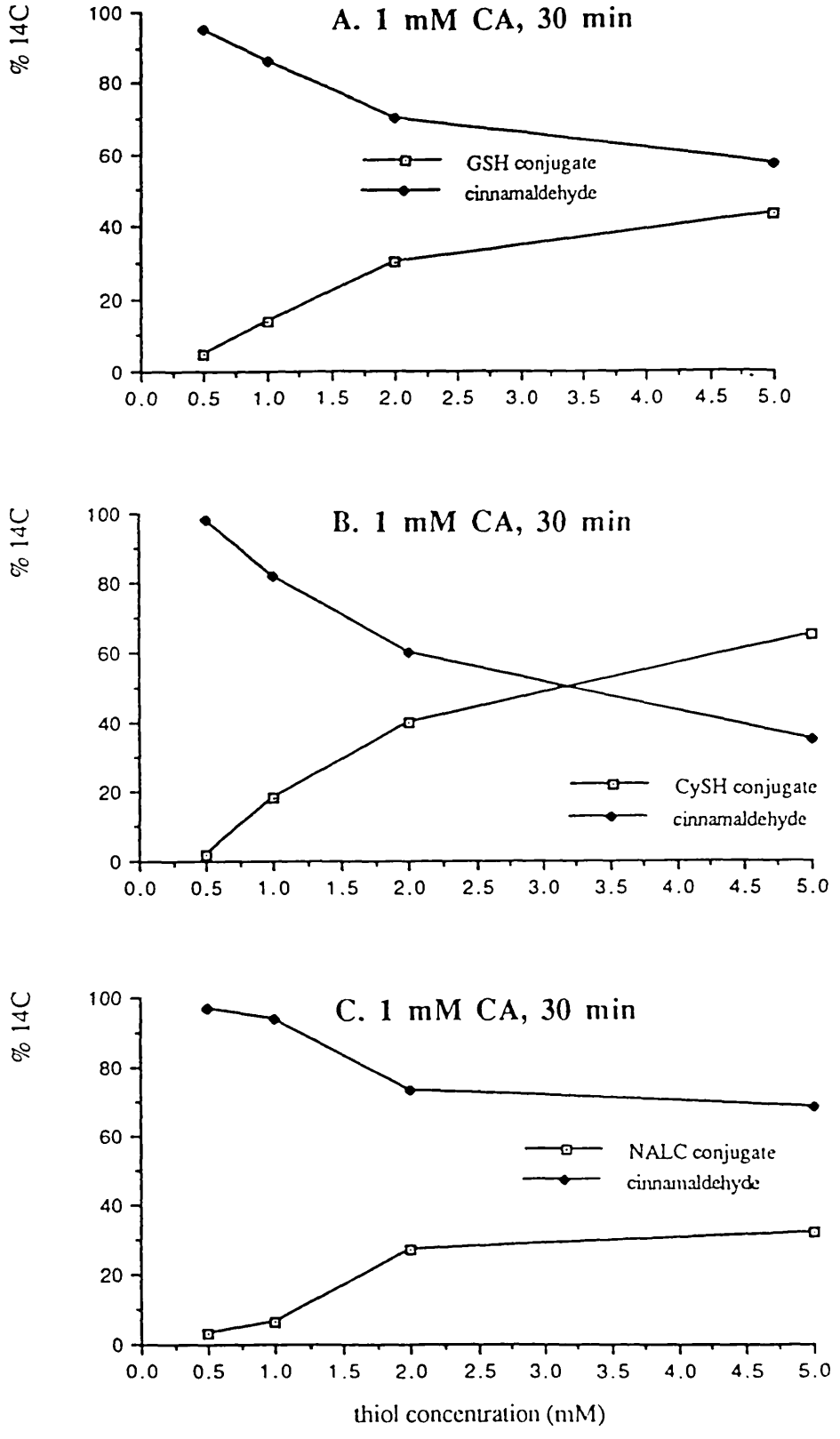
Reaction of ^{14}C-cinnamaldehyde and 5 mM thiol in buffer pH 7.4 as a function of the cinnamaldehyde concentration*



* % ^{14}C recovered as ^{14}C-cinnamaldehyde or ^{14}C-cinnamaldehyde-thiol conjugate. Figures are means ± S.D., n=3.

Figure 8.3

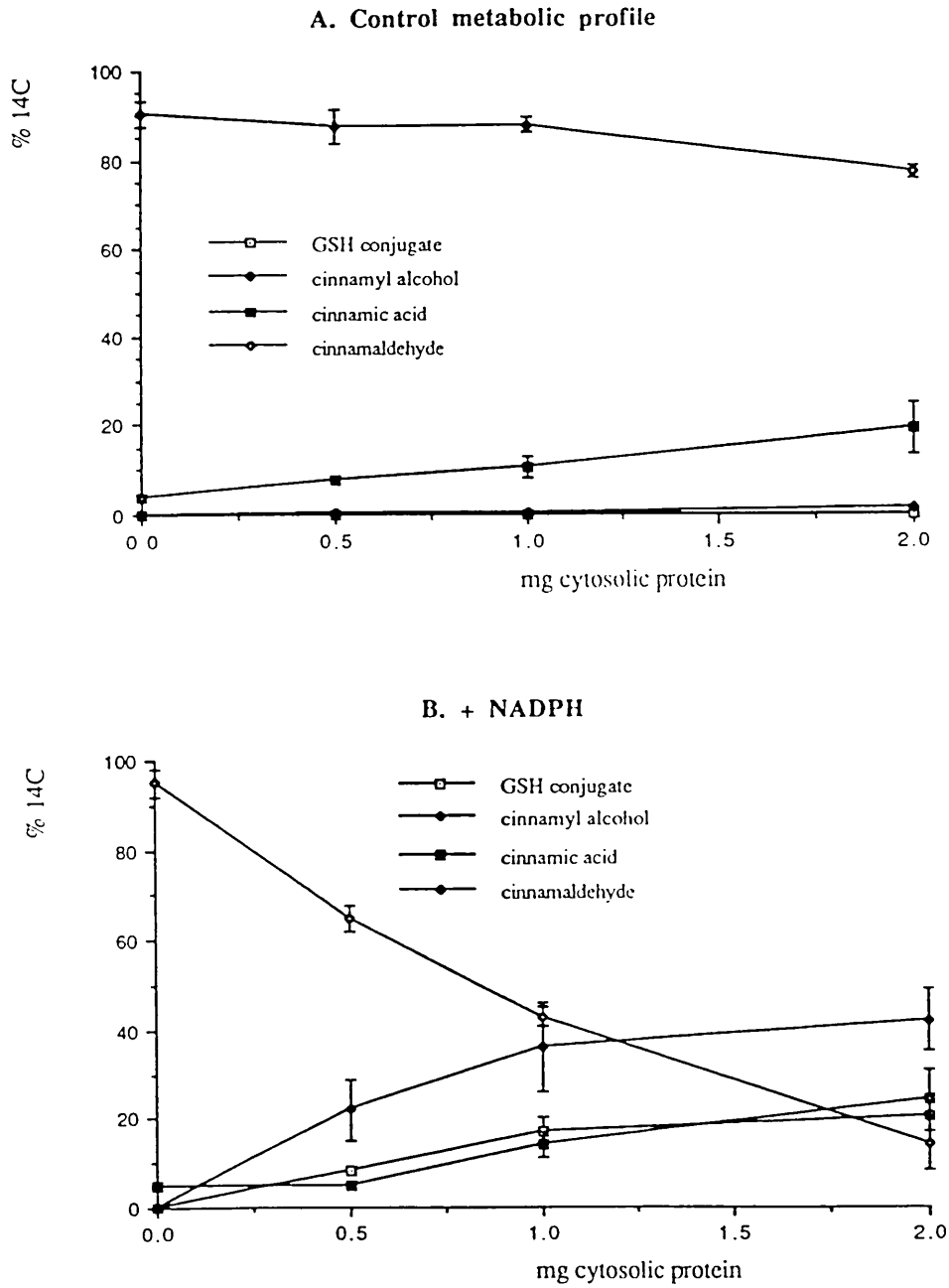
Reaction of 1 mM ^{14}C-cinnamaldehyde and thiol in buffer pH 7.4 as a function of the thiol concentration*



* % ^{14}C recovered as ^{14}C-cinnamaldehyde or ^{14}C-cinnamaldehyde-thiol conjugate. Figures are means ± S.D., n=3.

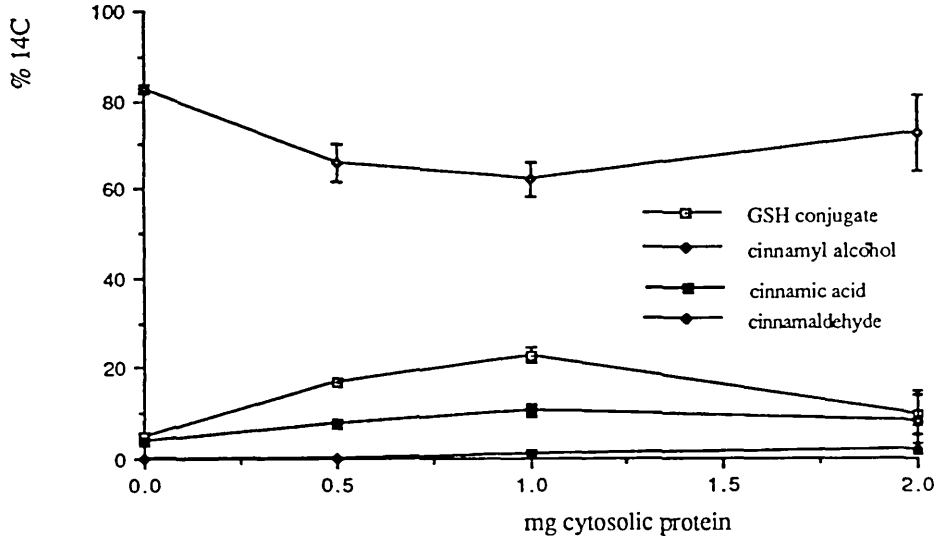
Figure 8.4

Metabolism of 0.5 mM ^{14}C-cinnamaldehyde by CD1 mouse liver cytosol as a function of the protein concentration and the effect of modulation of metabolism on (a-d) metabolic profile* and (e) ^{14}C binding**

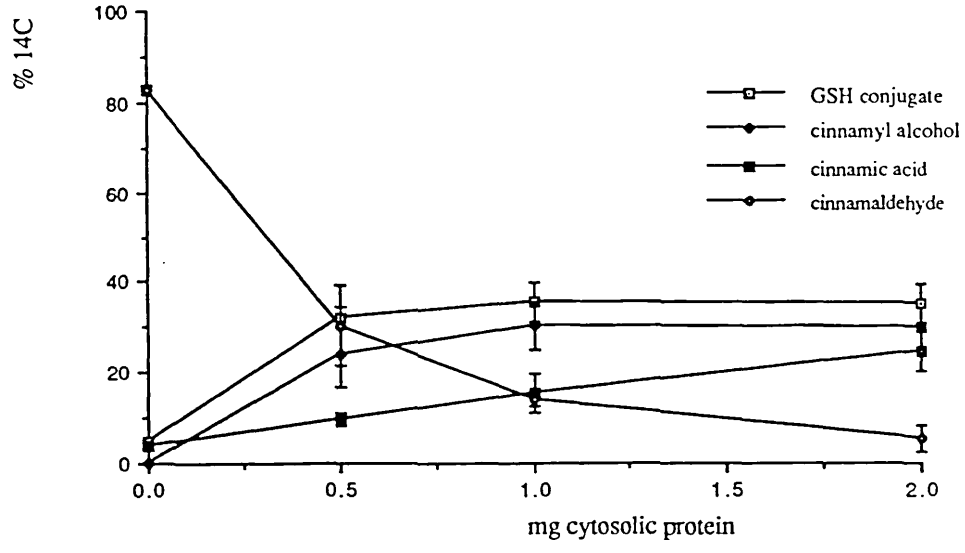


* ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis. Figures are means ± S.D., n=3.
** ^{14}C binding was determined as unextractable ^{14}C expressed as % of substrate

C. + 0.5 mM GSH



D. + NADPH + GSH



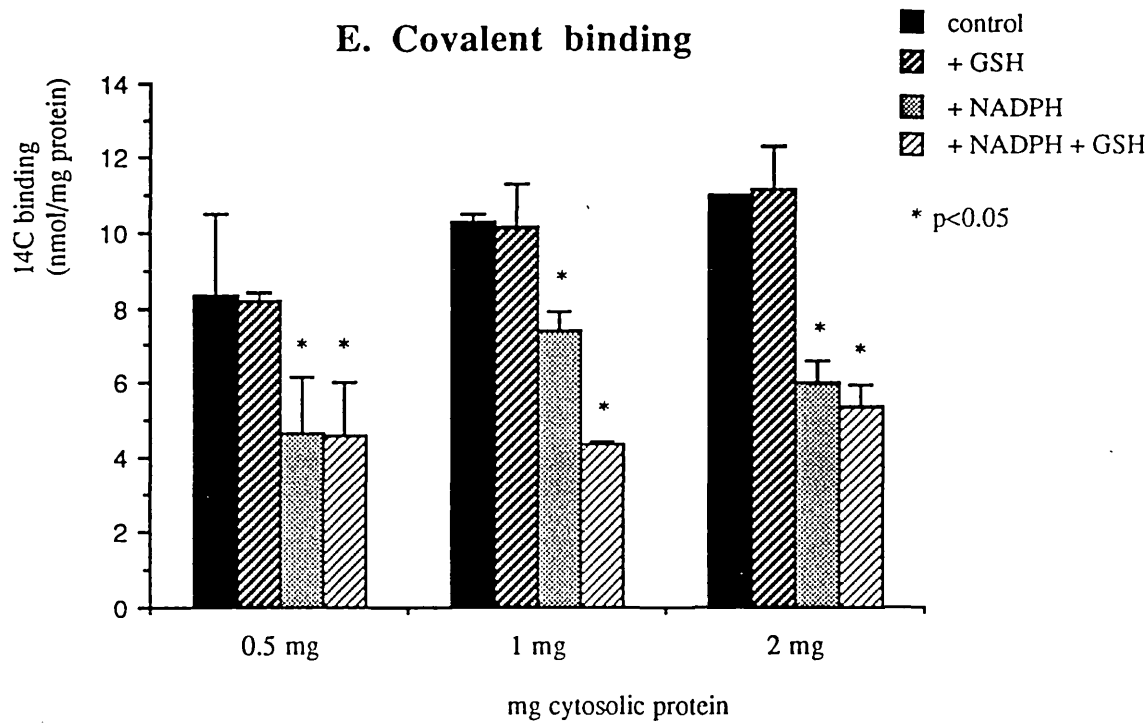
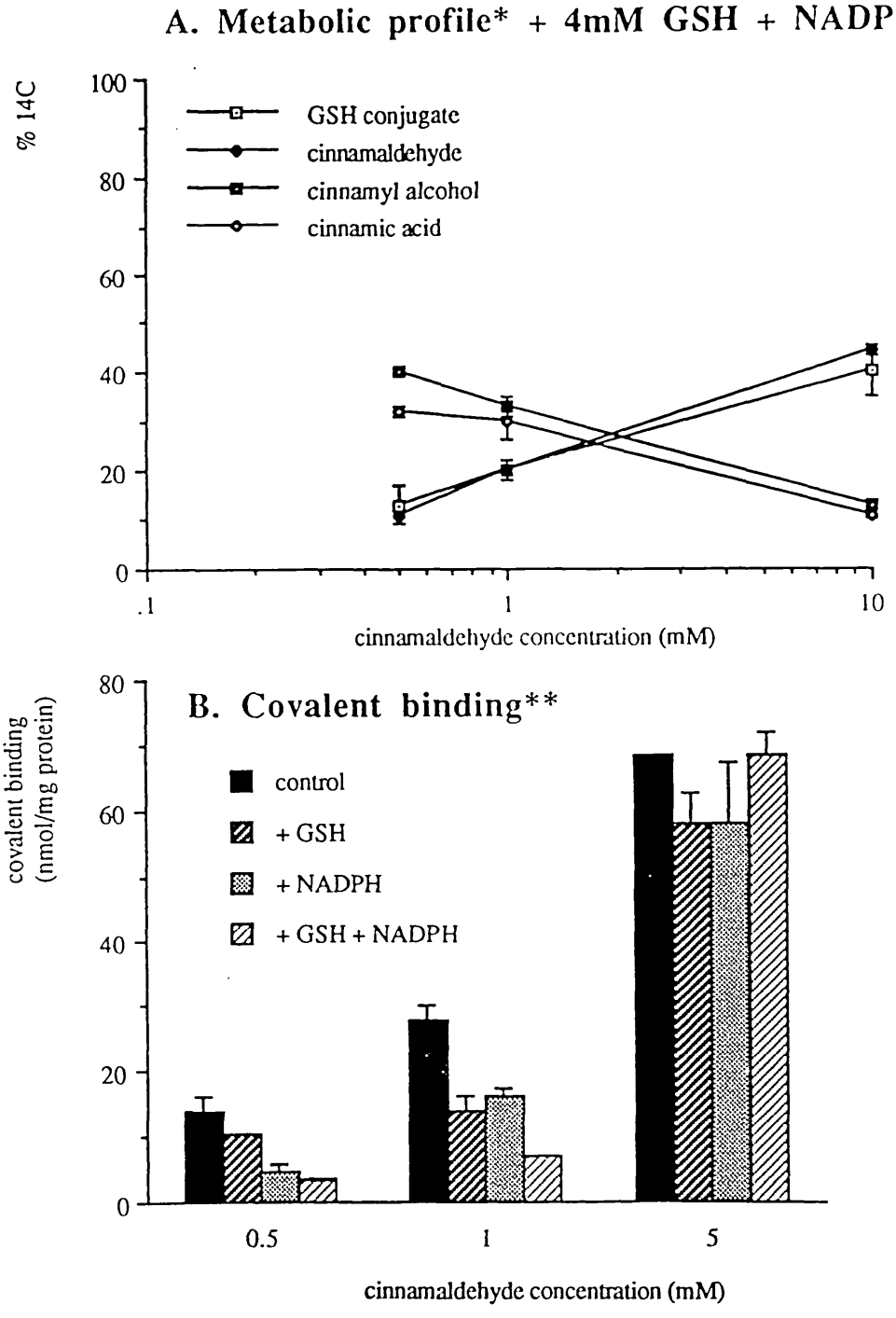

E. Covalent binding
control
+ GSH
+ NADPH
+ NADPH + GSH
* p<0.05
14C binding
(nmol/mg protein)
14
12
10
8
6
4
2
0
*
*
*
*
*
*
0.5 mg
1 mg
2 mg
mg cytosolic protein

Figure 8.5

Metabolism of ^{14}C-cinnamaldehyde by CD1 mouse liver cytosol as a function of the cinnamaldehyde concentration, (a) metabolic* profile in the presence of NADPH and 4 mM GSH and (b) ^{14}C binding** to cytosolic protein after various modulations of metabolism



* ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis. Figures are means ± S.D., n=3.
** ^{14}C binding was determined as unextractable ^{14}C expressed as % of substrate

Table 8.1

Metabolism and ^{14}C binding of 1 mM ^{14}C-cinnamaldehyde in incubations with liver cytosolic fractions of CD1 mouse and F344 rat*

	GSH conj	CALC	CAC	CA	^{14}Cb**
<u>mouse</u>					
control	-	0.4 ± 0.5	9.6 ± 3.6	84.3 ± 3.6	2.4 ± 0.6
+ GSH	23.8 ± 7.2	-	9.4 ± 3.9	65.8 ± 5.9	1.5 ± 0.4
+ NAD	-	7.3 ± 0.4	13.1 ± 3.9	75.3 ± 6.9	1.5 ± 0.2
+ GSH/NAD	27.2 ± 1.9	6.8 ± 0.4	11.8 ± 1.5	47.3 ± 5.9	0.9 ± 0.6
<u>rat</u>					
control	-	0.6 ± 0.8	8.4 ± 3.5	86.9 ± 1.4	2.2 ± 0.3
+ GSH	2.4 ± 1.7	0.3 ± 0.5	8.4 ± 3.5	86.0 ± 3.0	1.5 ± 1.1
+ NAD	-	13.4 ± 2.9	20.5 ± 3.5	62.4 ± 5.2	1.6 ± 0.3
+ GSH/NAD	8.7 ± 4.7	9.5 ± 4.0	20.7 ± 2.5	54.3 ± 9.9	1.1 ± 0.3

* ^{14}C-cinnamaldehyde and ^{14}C metabolites as % of total ^{14}C after radio HPLC analysis per mg cytosolic protein (Figures are means ± S.D., n=3).
** ^{14}C binding was determined as unextractable ^{14}C expressed as % of substrate incubation per mg cytosolic protein.

Chapter 9

Depletion of F344 rat liver glutathione by *trans*-cinnamaldehyde

9.1 Introduction

The studies presented in this thesis as a contribution to the safety evaluation of cinnamaldehyde, have concentrated on the role of GSH in the metabolism and possible toxicity of this food flavour. It was shown in Chapters 2, 3 and 5 that the metabolism of cinnamaldehyde in the rat involves conjugation with GSH as a minor pathway (7%) and that the relative importance of this pathway compared to oxidation to cinnamic acid was unaltered by dose size, sex or route of administration. This forms a good indication that liver GSH is not largely depleted after a single dose of cinnamaldehyde up to 250 mg/kg. In rat hepatocytes in suspension, however, it was demonstrated that cinnamaldehyde depletes GSH in a dose-dependent manner and that the extent of depletion is indicative for cytotoxicity at later times (Swales, 1993).

For a long time it was believed that a depletion of GSH to less than 60% of control, would lead to a disturbance in the cell great enough to cause cytotoxicity *per se*. Various recent investigations have however indicated that PrSHs, more so than non-PrSHs, are critical for the maintenance of cell viability during toxic chemical insult. Thiol groups are essential for the activities of many enzymes, including the membrane bound Ca^{2+}-translocases, and it has been proposed that GSH maintains cell viability *via* the maintenance of membrane PrSH groups through thiol-disulfide exchange reactions (Cotgreave *et al.*, 1990). It is therefore of interest to measure PrSH levels in rat liver after cinnamaldehyde in addition to GSH and CySH. Generally, a 15-30% decrease in PrSH content elicits rapid cell death (Cotgreave *et al.*, 1990 and references therein). However, exceptions do arise. Paracetamol has been shown not to cause cytotoxicity even when PrSHs were depleted up to 40% of normal values, indicating that the cytotoxicity of a particular compound may depend not just on the degree of PrSH

modification, but on the additional toxic manifestations which the compound may inflict upon the cell. One such determinant is the subcellular localisation of GSH. Different functions have been ascribed to the various thiol stores within the cell (see Chapter 1) with the mitochondrial pool being the most critical in the determination of cytotoxicity. It was therefore of interest to study binding of cinnamaldehyde to protein groups in liver whole homogenates and also in subcellular fractions.

In Chapters 6 and 7 it was shown that ^{14}C-cinnamaldehyde binds covalently to cellular macromolecules in rat hepatocytes in suspension at high concentrations when its metabolism becomes saturated. Parallel studies by Swales (1993) showed that cinnamaldehyde depletes PrSH groups in rat hepatocytes. This effect was seen after GSH had become depleted and was followed by cell death. In contrast to cinnamaldehyde, high doses of DEM essentially removed all the GSH in the cell, but did not affect PrSH groups and were not cytotoxic. These results indicate a relation between PrSH depletion and cell death. Targets for cinnamaldehyde *in vitro* are next to PrSHs (Weibel and Hansen, 1989a,b), which react by 1,4-addition to the unsaturated bond or perhaps with the aldehyde to form a thioacetal, also protein amino groups (Majetti and Suskin, 1977), which form a Schiff's base with cinnamaldehyde's aldehyde group (Zaugg *et al.*, 1977).

In the study described here, rats were initially given the highest dose of cinnamaldehyde used in the metabolism studies described in Chapters 2 and 3 (250 mg/kg), administered orally since human exposure to this food flavour will mostly occur *via* the diet. GSH, CySH and PrSH and amino groups were measured, but no significant depletion in any of these parameters was found, although in metabolism studies 7% of dose was recovered as mercapturic acids, indicating GSH utilisation.

To examine the limits of the liver capacity to replete GSH, higher doses of 375 and 500 mg/kg were administered orally and i.p., since Boyland and Chasseaud (1970) reported that 550 mg/kg cinnamaldehyde given i.p. reduced rat liver GSH levels by 35% after 0.5 h and 70% after 2 h.

9.2 Materials and Methods

9.2.1 Chemicals

Monobromobimane (MBBr) was obtained from Calbiochem-Behring, La Jolla, USA. All oxidized and reduced low molecular weight (lmwt) thiols, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and BSA were obtained from Sigma, St. Louis, USA. N-ethyl morpholine (NEM) was obtained from Fluka A.G. Buchs, Switzerland. Folin-Ciocalteu's phenol reagent, Triton X-100 and sodium dodecyl sulfate (SDS) were purchased from Merck, Poole, Dorset, UK.

9.2.2 Animals and dosing

Groups of three male F344 rats (Harlan-Olac Ltd., Bicester, Oxon; 150-260 g bwt) received a single dose of either dosing vehicle only (0.4 ml trioctanoin) or 250, 375 or 500 mg/kg cinnamaldehyde by gavage or i.p. between 10.00 am and 12.00 am. After 0.5 h or 2 h rats were killed by cervical dislocation.

9.2.3 MBBr derivatization procedure

Livers were perfused *in situ* with *ca* 10 ml 50 mM Tris 0.154 mM KCl buffer pH 7.4 to flush out excess blood before removal and weighing. A *ca* 4 g portion was minced with scissors and homogenized (Potter Homogenizer, Braun, Melsungen A.G., Germany) in 30 ml ice cold

Tris/KCl buffer pH 7.4. Aliquots of tissue homogenate (1.5 ml) were mixed with 1.5 ml Tris/KCl buffer pH 8.0 containing 8 mM MBBr and 50 mM NEM. MBBr was predissolved in a minimum amount of acetonitrile for it is relatively insoluble in water. Samples were left to stand for 5 min in the dark. An aliquot of 0.5 ml was taken for the determination of GSH by HPLC as described below. Subcellular fractions were prepared from MBBr-treated whole homogenate by differential centrifugation.

9.2.4 Differential centrifugation

Subcellular fractions (SCF) of cytosol, mitochondria, microsomes and nuclei and plasma membrane were prepared as shown in Fig. 9.1 (adapted from Cotgreave *et al.*, 1988) using a Sorvall OTD-Combi Ultracentrifuge (Du Pont, Delaware, USA) and a Sorvall RC-5B Refrigerated Superspeed Centrifuge.

9.2.5 HPLC separation of lmwt thiol-MBBr adducts

Aliquots of whole homogenate or cytosol (100 µl) were acidified by the addition of 10 µl 100% TCA. Precipitated protein was removed by centrifugation at 11,000 *g* for 3 min. Aliquots of the supernatant (20 µl) were applied to the HPLC column for the determination of lmwt thiol-MBBr adducts according to Cotgreave and Moldeus (1986). The column used was an RP ODS 5 µm (4.6 mm x 75 mm, Merck, Poole, Dorset, UK). Separation was achieved with an LC 6A pump and a Waters SSV solvent select valve flow controller, a CR 6A computing integrator and fluorescence detection with a Model RF 535 detector ($_{ex}$394 nm, $_{em}$480 nm) (all from Shimadzu, supplied by Dyson Instruments Ltd., Houghton-le-Spring, Tyne and Wear, UK). Samples were introduced on to the column with a Waters Associates

(Northwich, UK) WISP Model 712B autoinjector and eluted with solvent A, 9% (*v/v*) aqueous acetonitrile containing 0.25% (*v/v*) acetic acid, brought to pH 3.7 and solvent B, 75% (*v/v*) acetonitrile in water. The following elution profile was used: Isocratic conditions (100% A) for 7 min, stepping directly to 100% B for 4 min, followed by an immediate return to initial conditions for reequilibration (7 min). The flow rate was 1 ml/min throughout. The amounts of GSH and CySH in whole homogenates and subcellular fractions were determined by comparison with a standard curve containing 0-70 μM GSH or CySH.

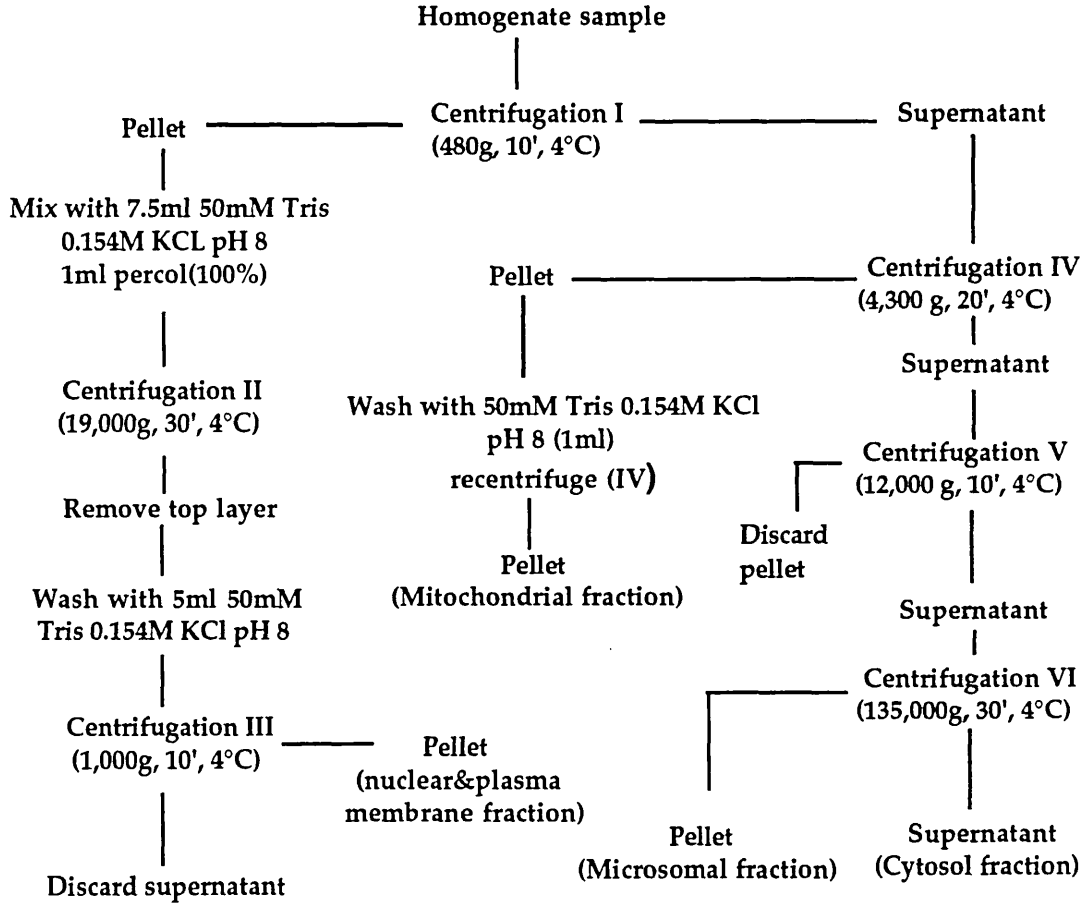


Figure 9.1 A schematic representation of the subcellular fractionation of rat liver whole homogenate

9.2.6 Sample preparation for protein sulfhydryl assay

Samples of SCFs were extracted by methanol/chloroform/water phase partitioning according to Wessel and Flüge (1984), to ensure denaturation of the protein and removal of lipids from the samples. Methanol (0.4 ml) was added to aliquots of 0.1 ml and samples were vortexed and centrifuged (10 s at 9,000 *g*, Heraeus, Sepatech, Biofuge B). Then chloroform (0.1 ml) was added and the samples vortexed and centrifuged again (10 s at 9,000 *g*). Distilled water (0.3 ml) was added and the samples vortexed and centrifuged for 10 s at 9,000 *g*. The supernatant was removed and a second 0.4 ml methanol added to the remaining interphase of protein and chloroform. Samples were mixed and centrifuged for 2 min at 9,000 *g*. The supernatant was removed and the pellet washed four times with methanol to remove unreacted MBBr and lmwt MBBr adducts with thiols such as GSH and CySH. Protein was then dissolved in 100 µl Krebs-Henseleit buffer containing 5 mM EGTA and 2% SDS *(w/v)*.

9.2.7 Determination of protein sulfhydryl groups

PrSHs in subcellular fractions were determined as described by Cotgreave and Moldeus (1986). Protein samples (10 µl) were diluted to a final volume of 3 ml in distilled water and PrSHs measured fluorometrically with a Shimadzu RF 540 spectrofluorometer ($_{ex}$394 nm, $_{em}$480 nm). PrSH concentrations were determined with reference to a BSA standard curve (0-100 µg MBBr-treated BSA, 0-10 nmol, 1-100 µM thiol equivalents). Results were adjusted for protein content assayed according to Lowry *et al.* (1951) as described in Chapter 6.

9.2.8 Protein amino group determination

Protein amino groups (lysine, histidine, arginine) were determined according to Habeeb (1966). Samples of whole homogenate and subcellular fractions were diluted in water to *ca* 0.25 mg/ml in a final volume of 1 ml. To this 1 ml of 4% $NaHCO_3$ was added and tubes mixed. After addition of 1 ml 0.1% TNBS tubes were mixed again. Samples were incubated for 2 h at 40 °C in the dark. Before terminating the reaction 1 ml 5% SDS (*w/v*) was added to keep proteins in solution, the whole was mixed, 0.5 ml 1 M HCl added and the absorbance read at λ 335 nm, using a Shimadzu MPS 2000 spectrophotometer. A BSA standard curve was constructed and the assay was linear up to 1 mg/ml BSA and at least up to 0.5 mg/ml of protein in whole homogenates or subcellular fractions. Alternatively, samples were washed with 3 x 1 ml methanol to remove any non-protein amino acids and taken up in 0.5 ml 2% SDS for determination of amino groups as described above.

9.3. Results

9.3.1 Glutathione and cysteine in liver homogenates

GSH and CySH levels in liver homogenates 0.5 h after an oral or i.p. dose of 250, 375 or 500 mg/kg cinnamaldehyde or dosing vehicle only are presented in Fig. 9.2 and 9.3.

Control GSH levels in rat liver whole homogenates were 5798 nmol/g liver. An oral dose of 250 mg/kg cinnamaldehyde did not significantly reduced GSH, but 375 and 500 mg/kg depleted GSH to 84 and 82% of control respectively ($p < 0.05$, t-test; Fig. 9.2A). Control CySH levels were 135 nmol/g liver, *ca* 40 times less than GSH. An oral dose of 250

mg/kg cinnamaldehyde had no significant effect on hepatic CySH (Fig. 9.3A), while 375 mg/kg marginally significantly increased CySH to 135% of control, and levels fell again slightly to 89% at 500 mg/kg, which was not significantly different from control.

When administered i.p., 250, 375 and 500 mg/kg cinnamaldehyde depleted liver GSH to 74, 70 and 68% of control respectively (Fig. 9.2B). Values were significantly different from control ($p < 0.05$, t-test), although the depletion was not dose dependent (values were not significantly different from each other). In contrast to GSH, CySH levels in rat liver homogenates were not affected after 250 mg/kg i.p. At 375 and 500 mg/kg, CySH decreased with increasing dose to 88 and 69% of control, but this did not achieve statistical significance (Fig. 9.3B).

9.3.2 Time dependency of glutathione and cysteine depletion

Fig. 9.4A shows liver GSH levels of rats at various times (0 h, 0.5 h and 2 h) after a single dose of 0, 250 or 500 mg/kg cinnamaldehyde administered i.p. As described above, 250 and 500 mg/kg i.p. both depleted liver GSH after 0.5 h to 74 and 70% of control respectively. After 2 h liver GSH had returned to 92% of control at 250 mg/kg, but at 500 mg/kg, GSH levels fell as low as 29% of control (t-test, $p<0.02$ significance from control). A dose of 250 mg/kg did not significantly affect CySH at both times measured. CySH was reduced 0.5 h after 500 mg/kg Cinnamaldehyde i.p. but levels had returned to control (102%) after 2 h (Fig. 9.4B).

9.3.3 Thiol status of the cytosolic fraction

GSH in liver cytosolic fractions of control animals was 21 nmol/mg protein compared to 31 nmol/mg protein in whole liver homogenate (=5800 nmol/g liver). Cytosolic GSH (Fig. 9.5A) was not dose-dependently depleted after oral administration of cinnamaldehyde at 250, 375 or 500 mg/kg, in contrast with the slight reductions in whole homogenates.

When cinnamaldehyde was given i.p. (Fig. 9.5B), cytosolic GSH was dose-dependently decreased to 74, 62 and 50% of control respectively at 250, 375 and 500 mg/kg, which reached significance at the two highest doses, reflecting the depletion seen in whole homogenate. At 250 mg/kg i.p. cytosolic GSH returned to control levels (106% of control) after 2 h, but a dose of 500 mg/kg further depleted GSH to 29% of control, as in the whole homogenates (Fig. 9.6).

Cytosolic CySH was not affected by dose, route of administration or time after dosing (results not shown), which reflects results in whole homogenate

9.3.4 Protein sulfhydryls in subcellular fractions

PrSH groups (Table 9.1) in control liver fractions were in the order cytosol > microsomal > mitochondrial > nuclei and plasma membrane. Cytosolic PrSHs were 48 nmol/mg protein and levels were 46, 32 and 19 nmol/mg protein for the microsomal, mitochondrial and nuclei and plasma membrane fraction respectively. PrSHs in the various fractions were unaltered at any of the doses, with the exception of PrSHs in the nuclei and plasma membrane fractions which were significantly increased after an oral dose of 375, but not at 500 mg/kg, and an

increase in mitochondrial PrSHs 0.5 h after 250, 375 and 500 mg/kg i.p., but there was no dose-dependency in these effects.

9.3.5 Protein amino groups in whole homogenate and subcellular fractions

Protein amino groups (BSA equivalents/mg protein) in liver whole homogenates were not significantly affected by any of the cinnamaldehyde doses (Fig. 9.7A).

Oral and i.p. doses of cinnamaldehyde did not affect protein amino groups detected in the mitochondrial or cytosolic fraction (Fig. 9.7C and D). A slight but significant reduction ($p<0.05$) was seen in the nuclei and plasma membrane fraction 2 h after 500 mg/kg i.p. (Fig. 9.7B) and in the microsomal fraction 0.5 h after 250 or 375, but not 500 mg/kg i.p. (Fig. 9.7E) However, an overall pattern in the reduction of amino groups could not be discerned.

9.3.6 Observations

Oral administration of 0, 250, 375 or 500 mg/kg cinnamaldehyde caused no toxic or pharmacological effects in rats after 0.5 h or 2 h. However, i.p. administration caused immediate dose-dependent toxic effects, e.g. laboured respiration and ataxia. At the high dose the effect of cinnamaldehyde was that of an anaesthetic. Animals injected with trioctanoin only were unaffected. Livers of rats receiving 500 mg/kg i.p. were darkly coloured after 2 h (toxic shock) indicating that animals were dying.

9.4 Discussion

Although oral doses of cinnamaldehyde up to 500 mg/kg were well tolerated by the animals, signs of toxicity comparable to those described in the literature were observed after i.p. administration. Jenner *et al.* (1964) reported that rats given toxic doses of cinnamaldehyde developed depression, diarrhoea and a scrawny appearance and died within 2-3 h, while in guinea pigs, coma was followed by death within 2 h to 4 days. A rabbit given 0.5 g/kg i.p. (Friedmann and Mai, 1931) showed restlessness, shortness of breath, convulsions, twitchings and disturbance of balance, concentrated urine was passed every 2-3 min and other neurological and respiratory symptoms were observed. Pharmacological symptoms reported after cinnamaldehyde include sedative effects (Opdyke, 1979). Reported LD_{50} values for cinnamaldehyde in various rodent species range from 0.5 to > 2 g/kg when administered orally and from 0.2 to 2.3 g/kg after i.p. dosing, showing it to be a compound of only moderate toxicity after both routes of administration (Opdyke, 1979). However, Stoner *et al.* (1973) determined the MTD as 250 mg/kg, much lower than would be expected from the LD_{50}. Stoner's results compare to the present report and the high LD_{50} values after i.p. administration seem to be overestimates, probably because results were derived from extrapolation from low dose not taking the steep dose-response of this compound into account. In the experiments described in this Chapter, 250 mg/kg i.p. caused only very slight toxicity, but 500 mg/kg was almost lethal. In view of the differences in oral and i.p. dosing, toxicity tests in the safety evaluation of cinnamaldehyde should not be performed by i.p. administration for this is not representative for the human situation of (low) oral exposure.

Control values for GSH (5800 nmol/g liver) as assessed with the MBBr assay compare well with those reported by other authors using this assay for male Sprague Dawley rats (Cotgreave and Moldeus, 1986) and other methods (Boyland and Chasseaud, 1970 plus references therein). CySH values determined (85 nmol/g liver) were slightly higher than those (62 nmol/g liver) reported by Cotgreave and Moldeus (1986).

In metabolism studies a constant 7% of dose is recovered as mercapturic acids in the urine of rats (Chapters 2, 3 and 5) and theoretically 7% of a 250 mg/kg dose could deplete *ca* 50% of liver GSH if no repletion occurs. In this Chapter it is shown that this extent of depletion is approximated only after i.p. dosing when a bolus dose reaches the liver and depletes GSH to 67% of control, while the same dose given orally did not reduce liver GSH levels. Similarly, depletion at 375 and 500 mg/kg was less marked after an oral dose as compared to i.p. which illustrates the differences between oral and i.p. dosing also seen in metabolism (Chapter 3). However, even when GSH was depleted after 250 mg/kg i.p. this was readily reversible and levels had returned to control values in 2 h, indicating the large capacity of the liver to resynthesize GSH. Thus, both the high metabolic capacity of the liver to oxidize cinnamaldehyde to cinnamic acid, accounting for 93% of metabolism at doses up to 250 mg/kg (Chapters 2 and 3), and the liver capacity to replete GSH contribute to the detoxication of cinnamaldehyde *in vivo*.

In contrast to the reversible depletion after 250 mg/kg i.p., GSH was irreversibly depleted at a toxic dose of 500 mg/kg i.p., when levels were reduced from 68% after 0.5 h (32% depleted) to 29% of control in 2 h (79% depleted), in good agreement with the previous report by Boyland and Chasseaud (1970). Cinnamaldehyde toxicity and its

effects on liver GSH both point at a steep dose-response curve, with a threshold value of 250 mg/kg i.p. above which effects are irreversible. Very steep dose response curves have also been reported for an other class of α,ß-unsaturated compounds, ß-chlorovinyl ketones and aldehydes (Riemer *et al.*, 1980). The i.p. doses that caused toxicity and GSH depletion *in vivo* correlated with equivalent doses *in vitro* in rat hepatocytes in suspension (Chapter 6; Swales, 1993). In rat hepatocytes in suspension 0.5 mM cinnamaldehyde, did not cause cytotoxicity, but doses of 1 mM and higher (equivalent to 250 mg/kg) were very toxic. These data may suggest that just like *in vitro* a cytotoxic threshold exists above which metabolism becomes saturated and GSH severely depleted, followed by cell death. In rat hepatocytes, GSH depletion precedes toxicity, indicating that these are separate events and might be causally related. However, in the whole animal these events can not be distinguished and the failure to replenish GSH is more likely to have been a result of toxicity and not a cause.

When changes in CySH are compared to those in GSH, it is seen that both CySH and GSH were unaffected by 250 mg/kg cinnamaldehyde given orally. After an oral dose of 375 mg/kg and 250 mg/kg i.p., when GSH became depleted CySH was initially slightly increased above control but at higher doses a dose dependent reduction in CySH was observed, which was even below control at 500 mg/kg i.p. It seems that the first response of the liver to the GSH depletion caused by cinnamaldehyde is a slight increase in CySH, but that at higher doses CySH is depleted as a result of GSH depletion. The same effect is observed *in vitro*, where GSH depletion is followed at later times by CySH depletion (Swales, 1993). Fluctuations in GSH are much greater than in CySH, which cannot be accounted for by the chemical reactivity of cinnamaldehyde towards either of these thiols, because in buffer

incubations of cinnamaldehyde with high concentrations of CySH or GSH, the spontaneous reaction occurs faster and to a greater extent with CySH than with GSH (Chapter 8; Moon and Pack, 1983). However, liver GSH concentrations are 40 times higher than CySH concentrations and conjugation with GSH could in addition be GST-mediated. Furthermore, CySH is very toxic and its intra-cellular concentration is tightly regulated by homeostatic control mechanisms (Meister, 1988) which might explain why fluctuations in GSH can be much greater than those in CySH.

PrSH groups in subcellular fractions of control rats are in the same order of magnitude as reported by Cotgreave *et al.* (1988) for subcellular fractions of hepatocytes derivatized with MBBr, although differences between fractions of whole liver homogenates were not as marked as in those derived from hepatocytes. Binding of cinnamaldehyde to protein would be expected to reduce the number of available binding sites for the dyes MBBr or TNBS used in the protein assays and thereby cause a decrease in the absorption measured, as was demonstrated for cytotoxic cinnamaldehyde concentrations *in vitro* (Swales, 1993). An increase in absorption could be explained if cinnamaldehyde disrupts or denatures proteins which then exposes PrSH or amino groups that were formerly inaccessible. In the present experiment in the intact rat, the number of protein groups neither increased nor decreased in whole homogenate or subcellular fractions. A small specific binding may not be detected when measuring total PrSH or amino groups and it is the specificity of binding to critical macromolecules in the cell that is important in determining toxicity . However, additional SDS-PAGE of protein fractions (Swales, 1993) showed that cinnamaldehyde did not alter the intensity of fluorescence of any of the protein bands.

In conclusion, results in this Chapter indicate that next to the large capacity of the liver to oxidize cinnamaldehyde to cinnamic acid, the liver capacity to resynthesize the GSH required for detoxification of cinnamaldehyde via GSH conjugation is sufficient for doses up to the MTD (250 mg/kg). Depletion of protein groups observed in the hepatocyte model system (Chapters 6 and 7; Swales, 1993) were not seen *in vivo*, indicating that GSH protects against this binding in the intact rat.

Figure 9.2

GSH (nmol/g liver ± S.D., n=3) in rat liver whole homogenate 0.5 h after various (a) oral and (b) i.p. doses of *trans*-cinnamaldehyde

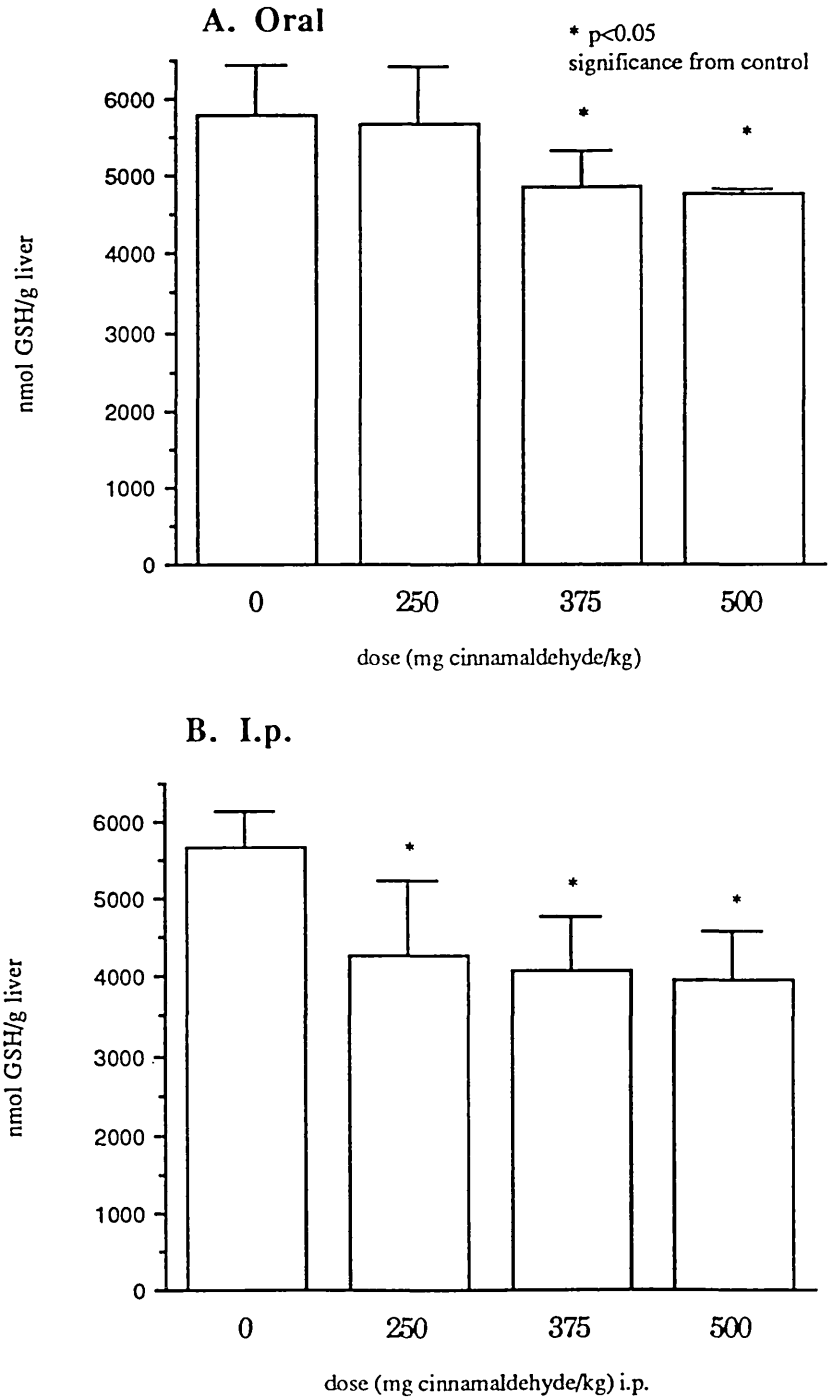


Figure 9.3

CySH (nmol/g liver ± S.D., n=3) in rat liver whole homogenate 0.5 h after various (a) oral and (b) i.p. doses of *trans*-cinnamaldehyde

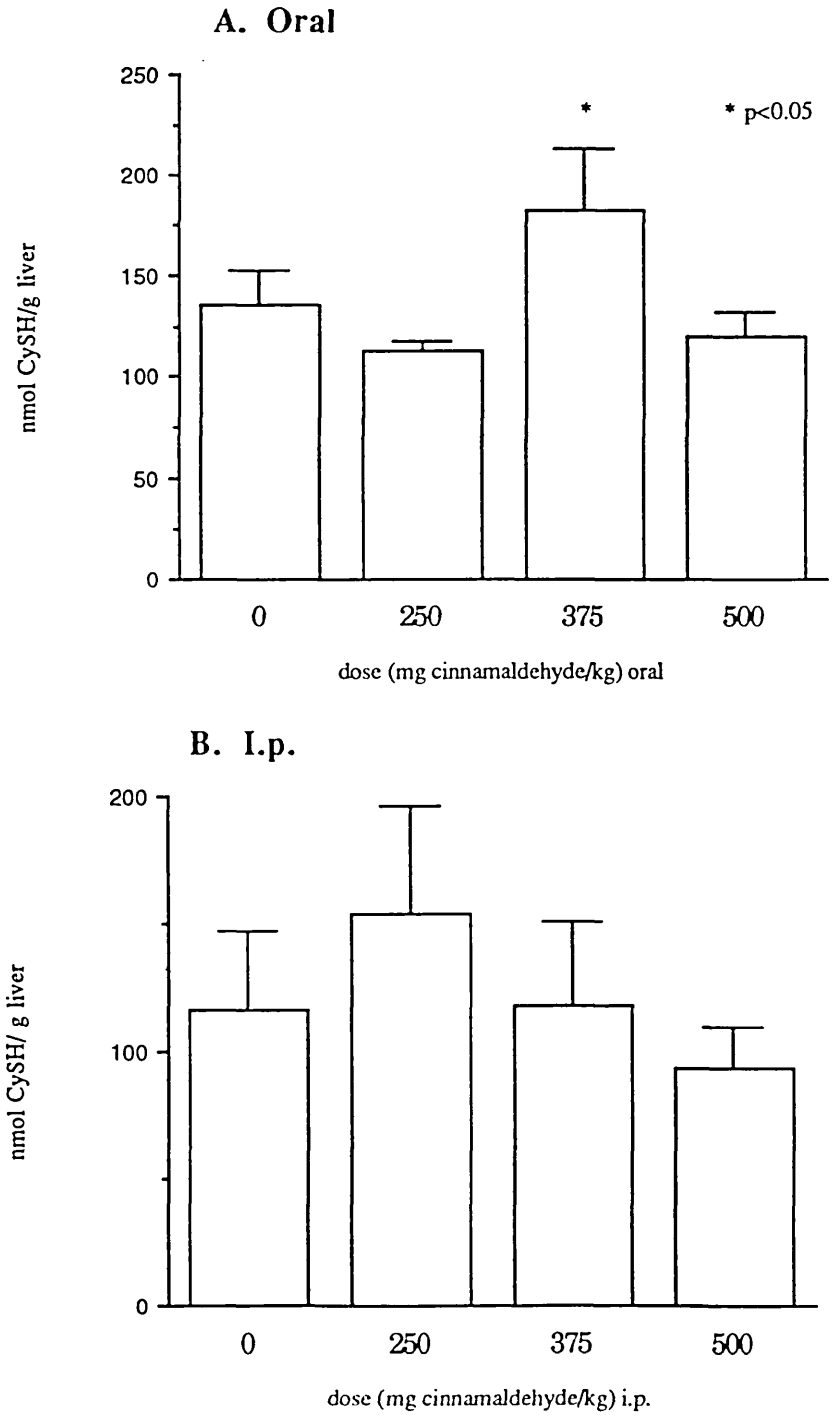


Figure 9.4

Time course of (a) GSH and (b) CySH depletion (nmol/g liver ± S.D., n=3) in rat liver whole homogenate after 250 and 500 mg/kg *trans*-cinnamaldehyde i.p.

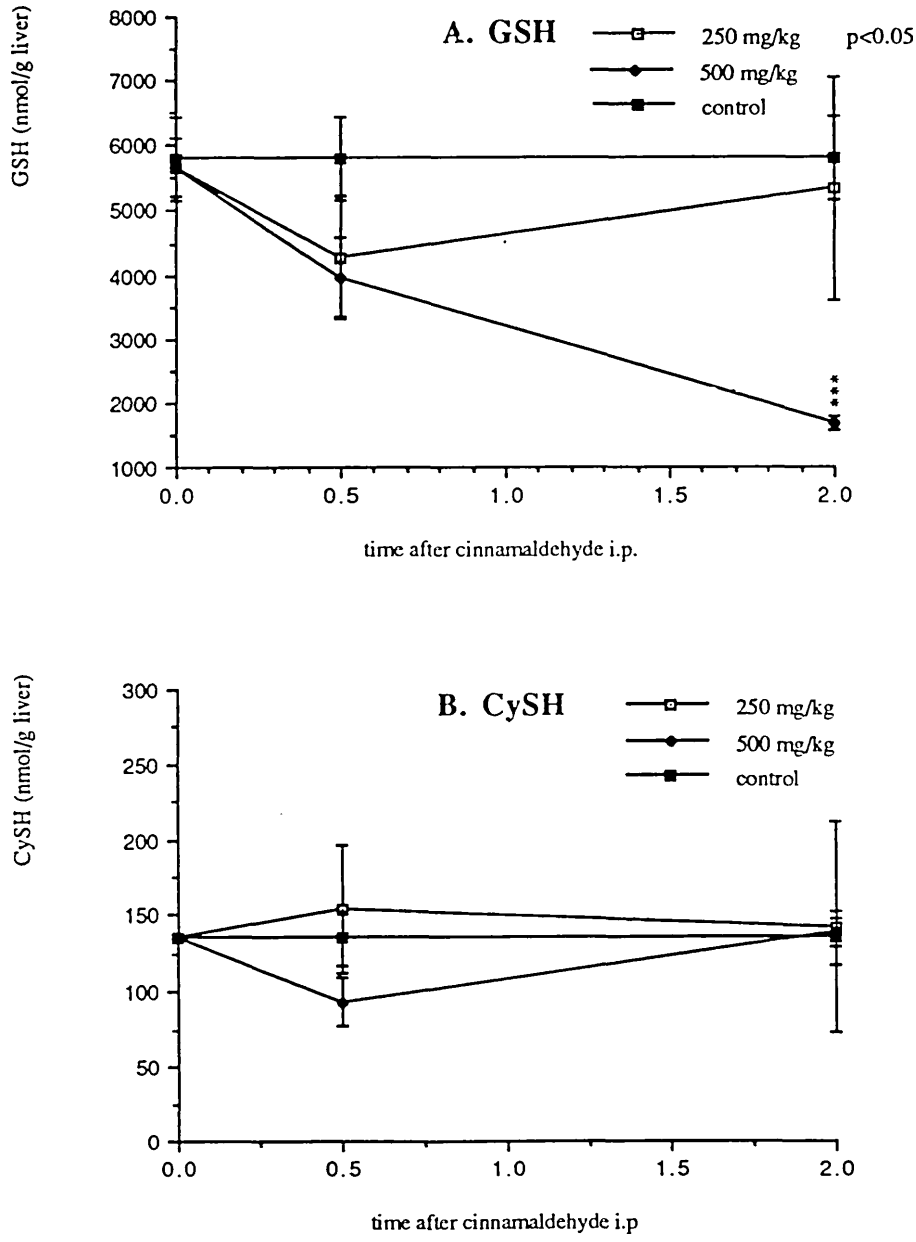


Figure 9.5

GSH (nmol/mg protein ± S.D., n=3) in rat liver cytosolic fraction 0.5 h after various (a) oral and (b) i.p. doses of *trans*-cinnamaldehyde

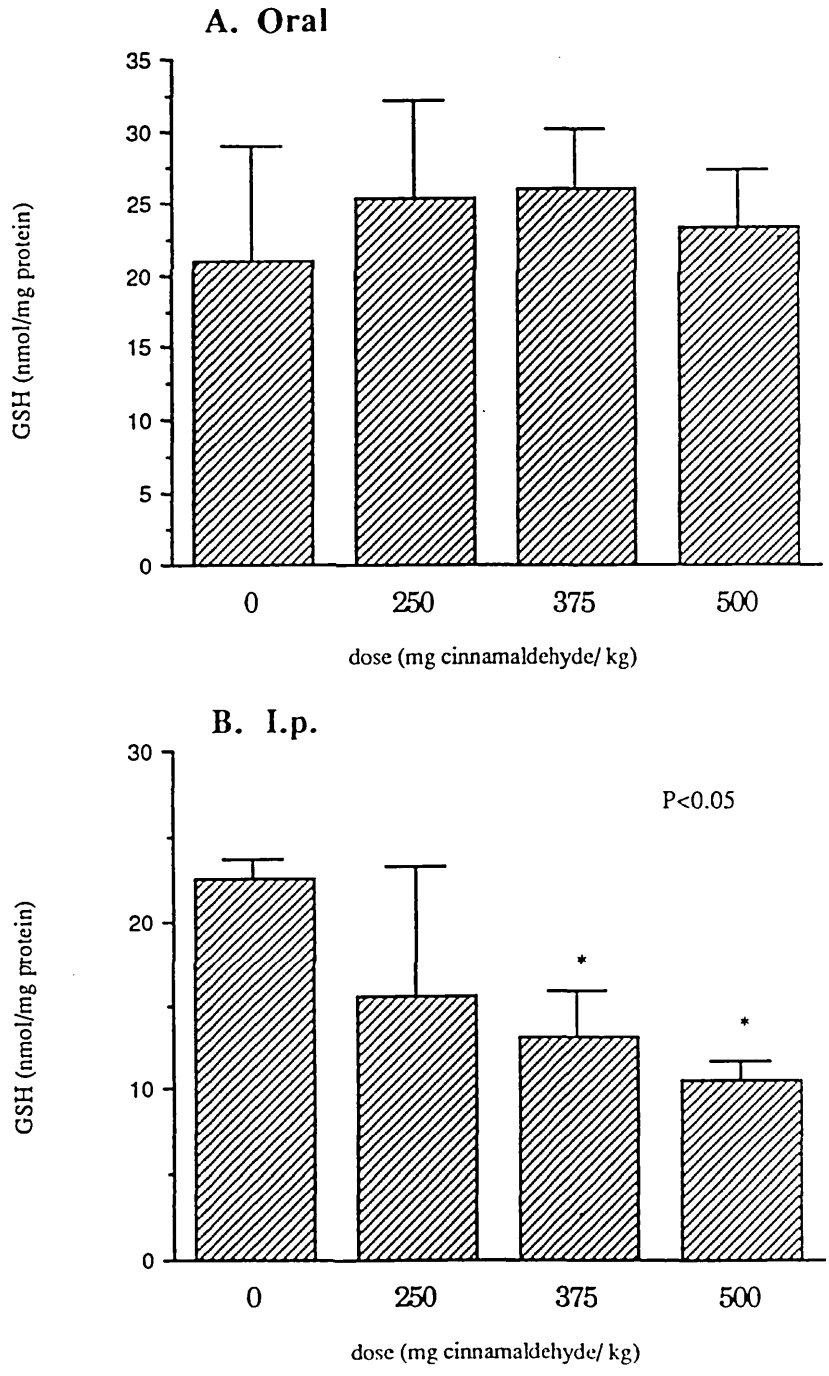


Figure 9.6

Time course of GSH (nmol/mg protein ± S.D., n=3) depletion in rat liver cytosolic fraction after 250 and 500 mg/kg *trans*-cinnamaldehyde i.p.

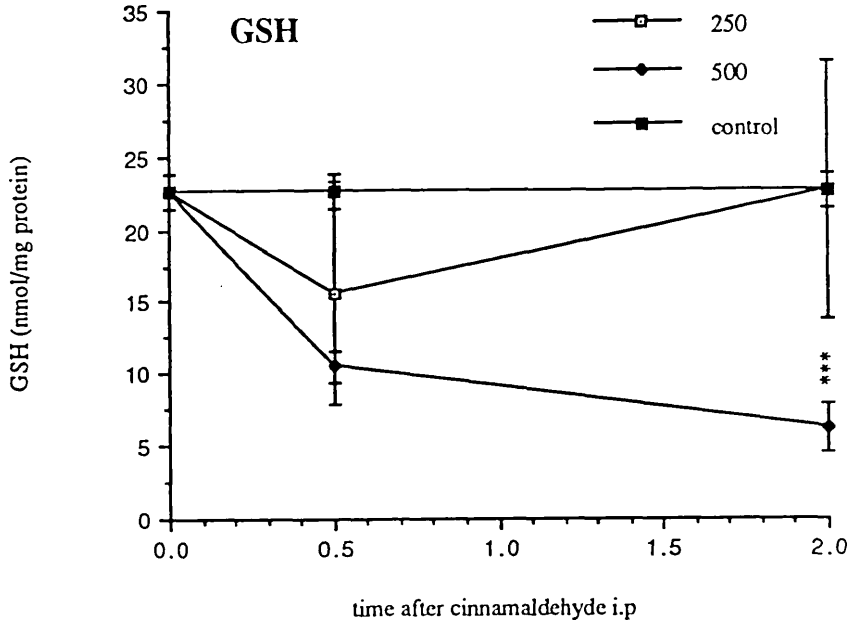


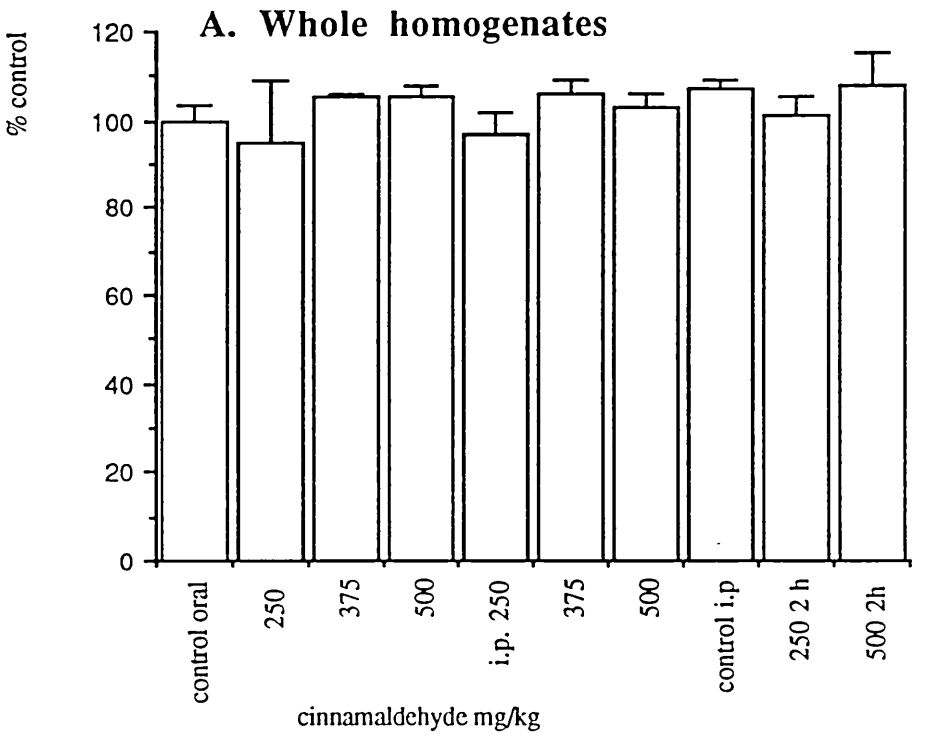
Table 9.1

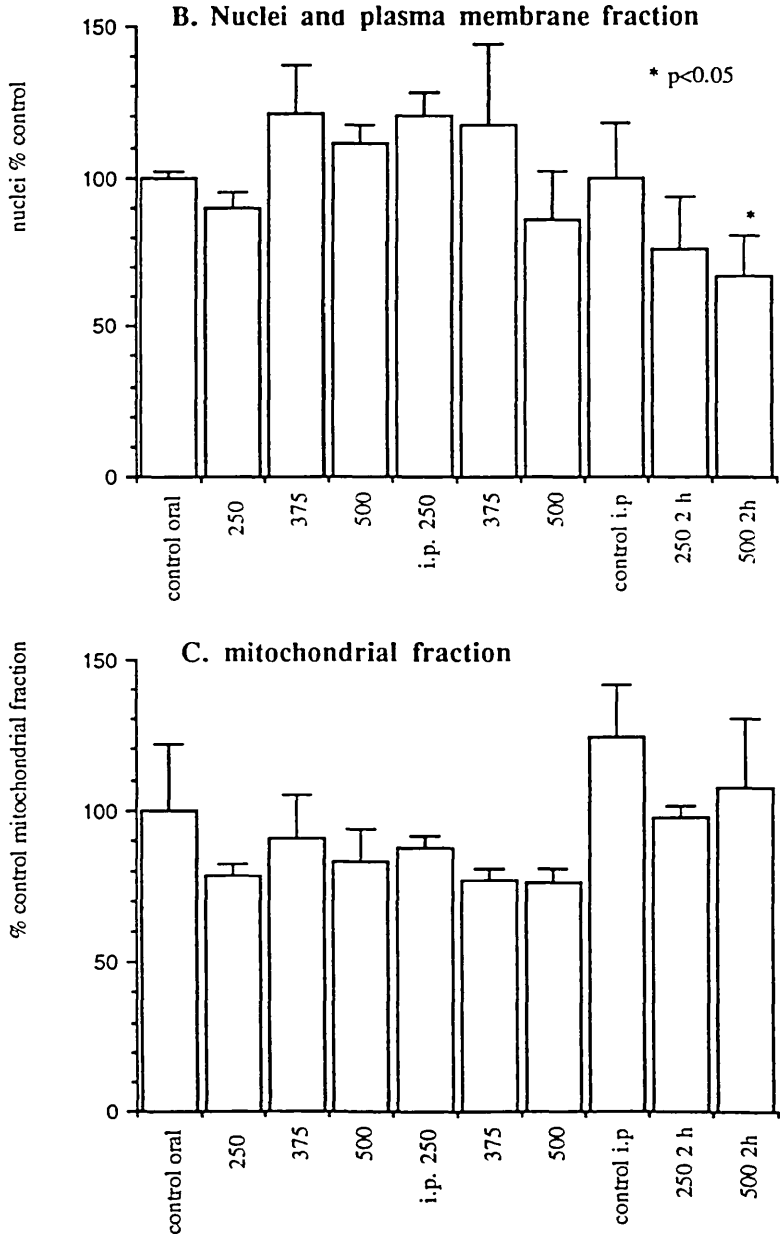
Protein sulfhydryl groups (nmol/mg protein ± S.D., n=3) in subcellular fractions of rat liver after various doses of cinnamaldehyde

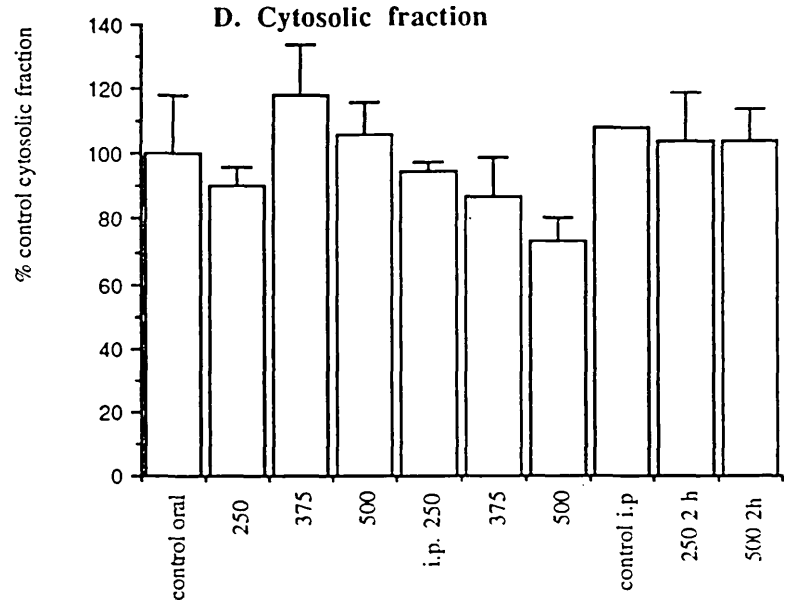
	cytosol	microsomes	mitochondria	cell membr/ nuclei
oral 0.5 h				
0	48.2 ± 7.9	46.0 ± 2.6	31.5 ± 5.6	18.6 ± 2.1
250	53.6 ± 1.7	44.7 ± 8.5	36.2 ± 0.9	18.4 ± 2.7
375	49.7 ± 10.0	42.7 ± 9.1	30.5 ± 1.0	26.6 ± 1.1
500	44.2 ± 7.8	43.0 ± 2.2	28.1 ± 1.1	22.7 ± 1.9
i.p. 0.5 h				
0	56.0 ± 9.3	34.6 ± 2.6	27.0 ± 1.9	23.9 ± 4.4
250	53.9 ± 4.9	33.7 ± 4.4	44.7 ± 6.6	29.6 ± 5.1
375	57.2 ± 4.3	32.8 ± 1.3	43.1 ± 1.9	26.1 ± 1.5
500	60.7 ± 10.6	38.0 ± 9.2	40.0 ± 4.5	26.4 ± 2.5
i.p. 2 h				
0	56.0 ± 9.3	34.6 ± 2.6	27.0 ± 1.9	23.9 ± 4.4
250	48.1 ± 5.3	39.6 ± 10.0	28.8 ± 6.4	24.7 ± 4.7
500	58.2 ± 5.9	24.6 ± 8.0	21.1 ± 5.8	22.2 ± 4.2

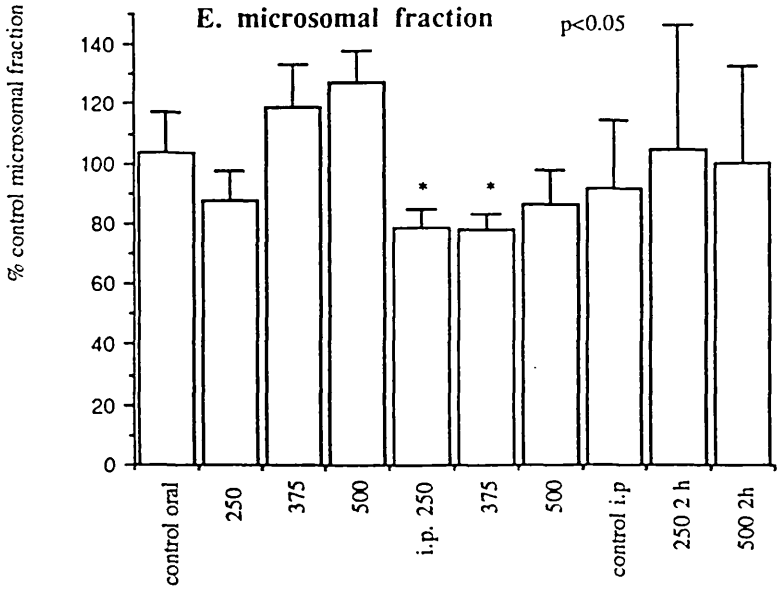
Figure 9.7

Protein amino groups in (a) whole homogenates and (b-e) subcellular fractions of rat liver after *trans*-cinnamaldehyde as a % of control




B. Nuclei and plasma membrane fraction
nuclei % control
* p<0.05
150
100
50
0
control oral
250
375
500
i.p. 250
375
500
control i.p
250 2 h
500 2h
C. mitochondrial fraction
% control mitochondrial fraction
150
100
50
0
control oral
250
375
500
i.p. 250
375
500
control i.p
250 2 h
500 2h


D. Cytosolic fraction
% control cytosolic fraction
140
120
100
80
60
40
20
0
control oral
250
375
500
i.p. 250
375
500
control i.p
250 2 h
500 2h


E. microsomal fraction
p<0.05
% control microsomal fraction
140
120
100
80
60
40
20
0
*
*
control oral
250
375
500
i.p. 250
375
500
control i.p
250 2 h
500 2h

Chapter 10

Discussion

10.1 Summary of results

The inadequacy of animal data relevant to the judgement of the human safety of *trans*-cinnamaldehyde, a very important flavouring agent, caused the withdrawal of its ADI of 0.7 mg/kg in 1989. The work described was undertaken to provide metabolic and mechanistic data as a contribution in the safety evaluation of this α,ß-unsaturated aldehyde.

In Chapter 2 the metabolism of *trans*-[3-^{14}C]cinnamaldehyde was investigated in rats and mice at a dose level of 250 mg/kg. Some 94% of the administered dose was recovered in the excreta in 72 h in both species with the majority (75-81%) present in the 0-24 h urine. Urinary metabolites were identified by their chromatographic characteristics using radio HPLC. In both species the major urinary metabolite was hippuric acid accompanied by 3-hydroxy-3-phenylpropionic acid, benzoic acid and benzoyl glucuronide. One metabolite, the glycine conjugate of cinnamic acid, was formed to a considerable extent only in the mouse. These findings show that cinnamaldehyde metabolism largely follows that of cinnamic acid and are in agreement with the operation of a ß-oxidation pathway analogous to that of fatty acids. Apart from the metabolites that cinnamaldehyde and cinnamic acid have in common, 7% of 0-24 h urinary ^{14}C was accounted for by two new metabolites in the rat and three in the mouse. In Chapter 5, it was shown that these form a second pathway of cinnamaldehyde metabolism *via* conjugation with GSH.

In Chapter 3, the influence of dose size, route of administration and sex on the metabolism in rats and mice was studied. ^{14}C-Cinnamaldehyde was metabolized rapidly and completely in both species at dose levels of 2 mg/kg, near to the estimated daily intake, up to 250 mg/kg, the

MTD. No unchanged cinnamaldehyde was excreted in the urine of rats or mice over this dose range. The main route of elimination was oxidation to cinnamic acid and ß-oxidation followed by conjugation with glycine to yield hippuric acid. There is some evidence for the saturation of this ß-elimination pathway: at high doses after i.p. administration, the intermediate CoA-esters are hydrolysed and a higher proportion of 3-hydroxy-3-phenylpropionic acid and benzoic acid are excreted in the urine as such. The glycine transferase capacity towards cinnamic acid (only seen in mouse and greater in the male than female mouse) becomes saturated at high dose after i.p. administration. In contrast to i.p. dosing, the metabolism of cinnamaldehyde when given orally, is unaltered by dose size. Metabolites derived from conjugation of cinnamaldehyde with GSH, two in the rat and three in the mouse, accounted for *ca* 7% of metabolism, and this was independent of dose size, route of administration or sex of the animals. These results indicate that total excretion and metabolic profile are largely unaffected by the factors studied. When given orally the metabolic profile did not change and extrapolation from high to low dose is permitted.

After studying ^{14}C-cinnamaldehyde metabolism in rats and mice, its fate was examined in man (Chapter 4). Two volunteers received 0.7 mg/kg ^{14}C-cinnamaldehyde. The elimination of radioactivity in the urine was assayed by scintillation counting. HPLC analysis showed cinnamaldehyde to be completely metabolized and the major urinary metabolite was hippuric acid, accompanied by 3-hydroxy-3-phenylpropionic acid, benzoic acid and a trace of benzoyl glucuronide. Two unknown metabolites coeluting with sulfur-containing metabolites in rat urine accounted together for 3% of dose. These results show that cinnamaldehyde metabolism in man resembles that in rat and mouse, mainly involving oxidation of its side chain analogous to the ß-oxidation of fatty acids, while GSH conjugation is a minor pathway.

In Chapter 5, it is shown that the new metabolites found as products of ^{14}C-cinnamaldehyde in rats, mice and man are derived from conjugation with GSH. An experiment using ^{35}S-CySH and non-labelled cinnamaldehyde revealed the presence of sulfur in two metabolites in rat and three in mouse urine. These corresponded in HPLC retention time and relative peak height to previously detected unknown metabolites of ^{14}C-cinnamaldehyde. The presence of N-acetyl-S-(1-phenyl-3-hydroxypropyl)cysteine and N-acetyl-S-(1-phenyl-2-carboxyethyl)cysteine in urine of human volunteers (0.7 mg/kg cinnamaldehyde) and in rats and mice (250 mg/kg cinnamaldehyde) was confirmed using GC with flame photometric sulfur-selective detection and GC-MS with selective ion monitoring. These metabolites arise from direct addition of GSH to the double bond of the α,ß-unsaturated aldehyde and show that cinnamaldehyde metabolism involves next to oxidation also GSH conjugation as a minor metabolic pathway.

In Chapter 6, the conversion of ^{14}C-cinnamaldehyde and covalent binding of ^{14}C was examined *in vitro* in rat hepatocyte suspension. Cinnamaldehyde undergoes oxidation to cinnamic acid and subsequently to benzoic acid and hippuric acid or reversible reduction to cinnamyl alcohol. 0.5 mM ^{14}C-cinnamaldehyde was rapidly metabolized (97% in 1 h), but at concentrations of 1 mM and higher metabolism was saturated and the parent compound remained present. Binding of ^{14}C to cellular macromolecules was not related to the formation of metabolites, but highest at 0 h and partly reversible with incubation time, and enhanced after saturation of metabolism, pointing at cinnamaldehyde as the reactive species. ^{14}C-Cinnamic acid is metabolized to benzoic and hippuric acid and did not cause ^{14}C

binding. ^{14}C-Cinnamyl alcohol is metabolized to cinnamic acid, benzoic acid and hippuric acid and binding of ^{14}C was related to ^{14}C-cinnamoyl alcohol metabolism. The threshold of 1 mM for saturation of metabolism and covalent binding correlates well with cinnamaldehyde's ability to cause enhanced GSH depletion and cytotoxicity at the same concentration (Swales, 1993) and it is concluded that all three events are due to the parent compound *per se*.

The use of modulators of ^{14}C-cinnamaldehyde metabolism in hepatocytes in Chapter 7 confirmed that ^{14}C-cinnamaldehyde is the reactive species in ^{14}C binding. Combined treatment with BSO and DEM was used to reduce, and NALC to boost, initial GSH levels in the cell. Cyanamide was employed as an inhibitor of its oxidation to cinnamic acid and 4-methyl pyrazole and ethanol to modify reductive metabolism to cinnamyl alcohol. All manipulations leading to a prolonged presence of the aldehyde in the cell enhanced binding. Intracellular GSH has a protective effect, for depletion of initial GSH levels enhances covalent binding. However, increased initial GSH after NALC did not reduce this binding.

In Chapter 8, it is shown that 1 mM ^{14}C-cinnamaldehyde reacts rapidly with CySH > GSH > NALC in buffer to form a ^{14}C-containing product. This reaction is not complete, but an equilibrium between conjugate and free reactants is reached. Lower concentrations of cinnamaldehyde and GSH require the presence of cytosol to form the same products. The conversion of cinnamaldehyde to cinnamic acid was NAD+ dependent, the reduction to cinnamyl alcohol was NAD(P)H dependent. All factors that enhanced total cinnamaldehyde metabolism reduced binding of ^{14}C to cytosolic macromolecules. Addition of GSH did not influence oxidative or reductive metabolism, but the formation of a GSH conjugate protected against binding. A

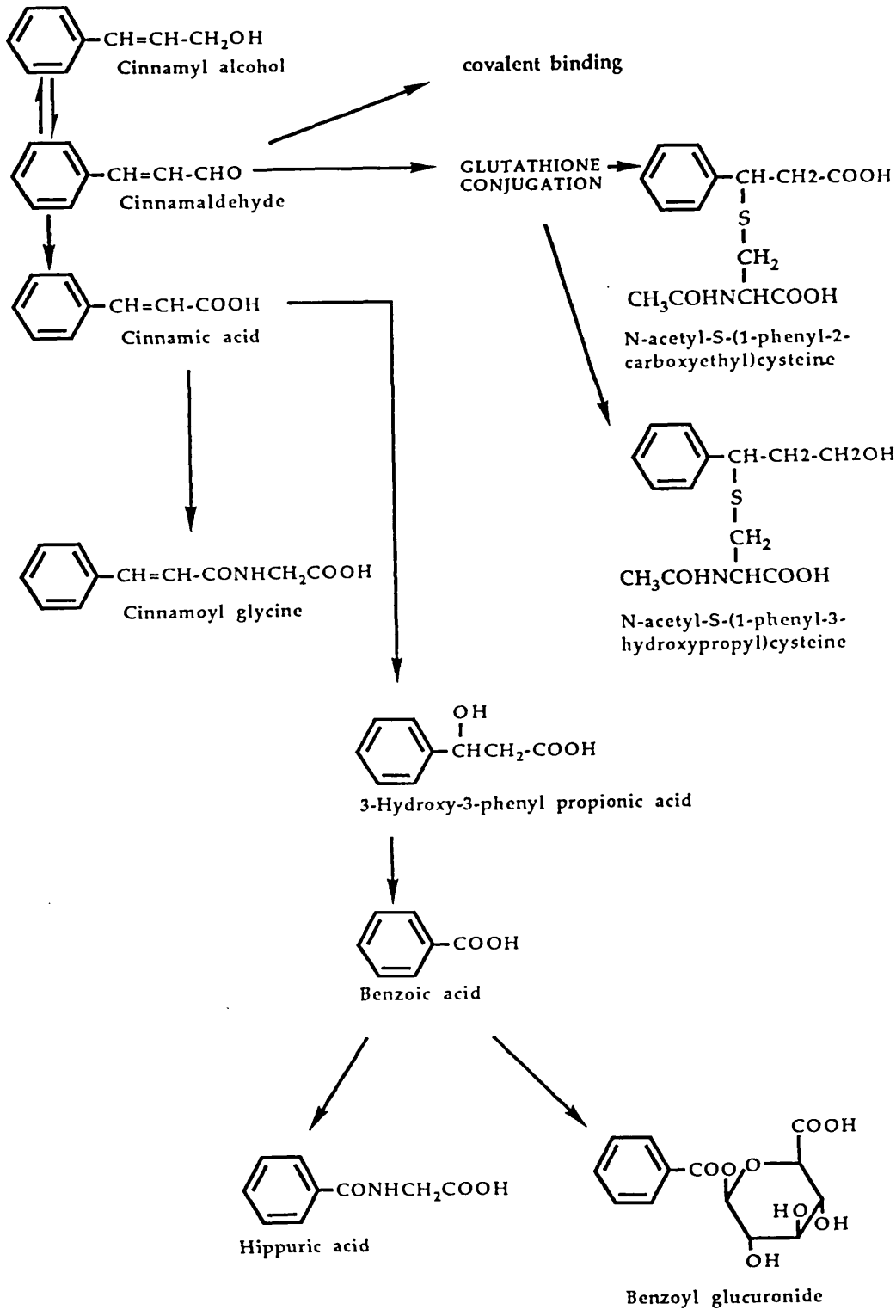
species difference was seen in that the mouse had a higher capacity for GSH conjugation, whereas oxidation was greater in rat cytosol.

The ability of cinnamaldehyde to reduce hepatic GSH levels in rat was examined in Chapter 9. Cinnamaldehyde administered to rats caused no significant depletion of liver GSH after oral dosing of 250, 375 or 500 mg/kg, doses more than 500 times the anticipated human exposure in high user groups of this food flavour. After i.p. dosing 250 mg/kg depleted GSH to 74% of control and this depletion was reversible for after 2 h levels had returned to 92% of control. However, a dose of 500 mg/kg was acutely toxic (neurological effects), and GSH levels were reduced to 68% of control after 0.5 h and further reduced to 29% of control in 2 h. The difference between oral and i.p. dosing indicates that toxicity assays in the safety evaluation of this compound should only be performed by oral administration. Protein groups in subcellular fractions were unaffected by cinnamaldehyde, in spite of reduced GSH levels, indicating that the reaction of cinnamaldehyde with GSH serves as a protection against binding to liver protein amino and PrSH groups *in vivo*.

10.2 Discussion

The metabolic and mechanistic studies of cinnamaldehyde presented in this thesis were undertaken as a contribution to the safety evaluation of this important food flavour. As has been outlined in Chapter 1, flavours have not been subject to extensive testing, but the finding that the cinnamyl compound cinnamyl anthranilate was a hepatocarcinogen in the mouse cast suspicion on other cinnamyl compounds and this, together with cinnamaldehyde's resemblance to toxic α,ß-unsaturated aldehydes such as acrolein and crotonaldehyde and its extensive use,

Figure 10.1 The proposed metabolism of *trans*-cinnamaldehyde in rat, mouse and man



caused JECFA to call for further data on its metabolism and pharmacokinetics and carcinogenicity. Metabolic studies are essential in the design and evaluation of toxicity tests and can help in priority setting and the good use of limited resources in the testing of an ever growing amount of factors in our diet that may pose some degree of risk.

In rat and mouse an oral or i.p. dose of cinnamaldehyde up to 250 mg/kg is rapidly excreted from the body (Chapters 2 and 3) and in human volunteers a dose of 0.7 mg/kg is completely recovered in the urine within 8 h (Chapter 4). There is no indication for residue formation, in agreement with a recent NTP study, where no specific residue sites were found (Sapienza *et al.*, 1991). This is an important finding, for cinnamaldehyde covalently binds to proteins *in vitro* (Chapters 6, 7 and 8) and some CoA derivatives of xenobiotic fatty acids can be incorporated into membranes leading to disturbances of physiological function (Caldwell, 1984).

Cinnamaldehyde is rapidly metabolized *via* two pathways as shown in Fig. 10.1. Oxidation of its side chain to metabolites with known toxicity is the major route. The first step in this is NAD+ dependent oxidation to cinnamic acid as demonstrated in rat and mouse cytosolic incubations (Chapter 8). Cinnamaldehyde metabolism in cytosol does not resemble that described for acrolein, crotonaldehyde (Patel *et al.*, 1980, 1983, 1984; Mitchell and Petersen, 1989) or citral (Boyer and Petersen, 1989), which are believed not to be substrates for cytosolic NAD+ dependent ALDHs, but so reactive that they bind to these enzymes and thus inhibit their own conversion. Cinnamic acid metabolism has been extensively studied (Nutley *et al.*, 1993) and proceeds *via* a ß-oxidation pathway analogous to that of fatty acids. Minor metabolites of cinnamaldehyde such as 3-hydroxy-3-

phenylpropionic acid and benzoic acid provided evidence that the same pathway operates for cinnamaldehyde in rat, mouse and man (Chapters 2, 3 and 4). Also in intact cells *in vitro* (Chapters 6 and 7) cinnamaldehyde is rapidly metabolized *via* oxidation to cinnamic acid, with no aldehyde remaining. Although one member of the group of cinnamyl compounds, cinnamyl anthranilate is a peroxisome proliferator in the mouse, cinnamaldehyde or cinnamic acid have been shown not to alter liver parameters associated with peroxisome proliferation in studies where cinnamyl anthranilate gave a positive result (reviewed in Caldwell, 1992). Metabolism to cinnamic acid can therefore be seen as a high-capacity detoxication pathway. In hepatocytes (Chapters 6 and 7) it was shown that reversible reduction to cinnamyl alcohol also contributes to cinnamaldehyde detoxication.

A second pathway, accounting for a minor portion of dose, is conjugation with GSH. The structures of the mercapturic acids recovered in the urine of rat, mouse and man indicate that these are derived from direct conjugation of GSH at the ß-atom of the double bond (Chapter 5). This is important for it excludes the formation of an epoxide across the double bond, which after subsequent conjugation with GSH, would have led to the excretion of hydroxymercapturic acids in the urine (Van Bladeren *et al.*, 1981). Metabolism by reduction of the aldehyde to its alcohol, followed by sulfation on the hydroxyl group which is then displaced by GSH is also excluded. This pathway has been suggested in the metabolism of benzaldehyde where the sulfate has been indicated as a reactive intermediate, leading to covalent binding to cellular macromolecules (Seutter-Berlage *et al.*, 1981). Apart from the two mercapturic acids identified, no other sulfur-containing metabolites are found in rat urine after coadministration of ^{35}S-CySH and cinnamaldehyde. These results confirm those of Delbressine *et al.* (1981) in female Wistar rats and extend our knowledge to F344 rats

and CD1 mice of both sexes and to man. In the mouse a third sulfur-containing metabolite could not be identified.

It is interesting that after GSH conjugation, the aldehyde is retained and may still be reactive, although the loss of the unsaturated bond and the greater molecular weight and higher hydrophilicity do not favour its reactivity towards protein. Studies with cytosol (Chapter 8) and hepatocytes (Chapter 7), in which initial GSH was manipulated, show that GSH protects against binding of ^{14}C-cinnamaldehyde to the extent that it increases metabolism by the formation of a GSH conjugate. Furthermore, no mercapturic acid in which the aldehyde group is retained is excreted in the urine, showing that the initial GSH conjugate is completely further metabolized by reduction and to a lesser extent, oxidation of the aldehyde group (Chapter 5). GSH conjugates are substrates for ADHs and ALDHs and this has been reported for the conjugate of acrolein and crotonaldehyde (Mitchell and Petersen, 1989; Gray and Barnsley, 1971). In conclusion, reaction with GSH abolishes the reactivity of the α,ß-unsaturated aldehyde, cinnamaldehyde towards protein.

Thus, the nature of mercapturic acids formed indicates that GSH conjugation represents a detoxication pathway. Still, the extent of conjugation is important, for a substantial degree of depletion of liver GSH could lead to cytotoxicity. Only 3% of dose is recovered as sulfur-containing metabolites in man (Chapter 4) and 7% in rat and mouse (Chapters 2 and 3), independent of route of administration, sex of the animals and, most importantly, independent of dose. The formation of mercapturic acid metabolites does not fall with increasing dose, indicating that the availability of GSH has not become limited. Neither does the excretion of mercapturic acids increase disproportionably with

dose, which might indicate that other pathways have become saturated and metabolism rechannelled *via* GSH conjugation. Such is the case for paracetamol, where when glucuronidation and sulfation become limiting the relative rate of P450-mediated metabolism to the quinone imine increases. The imine reacts with GSH and leads to an increased excretion of the corresponding mercapturic acids in the urine. At even higher paracetamol doses, liver GSH becomes depleted and the relative excretion of mercapturic acid metabolites falls again (Mitchell *et al.*, 1975). A dose of 250 mg/kg cinnamaldehyde could theoretically cause a maximal depletion of 50% of liver GSH assuming that a 1:1 conjugate is formed and no repletion occurs. However, cinnamaldehyde given orally does not significantly deplete liver GSH in the rat and an i.p. dose of 250 mg/kg depletes GSH to 65% of control (35% depletion) after 0.5 h, but levels recover in 2 h. Most importantly, neither PrSH nor protein amino groups are affected at any of these doses (Chapter 9). This shows that, in addition to the high oxidative capacity, the ability of the liver to synthesize GSH is sufficient to cope with doses up to the MTD and that GSH protects against the binding of cinnamaldehyde to tissue macromolecules.

Doses of cinnamaldehyde up to 500 mg/kg given orally do not cause any adverse effects on rats (Chapter 9). When a dose of 250 mg/kg is given i.p., however, slight neurological effects are observed, both in mice and rats (Chapters 2, 3 and 9). At high doses, cinnamaldehyde acts as an anaesthetic, a nonspecific effect seen for many lipophilic compounds and solvents, while a dose of 500 mg/kg i.p. is almost lethal (Chapter 9). The difference in effect between oral and i.p. routes of administration indicates the importance of the liver and first pass metabolism in determining cinnamaldehyde's acute toxicity. These results are in agreement with the low oral availability of the compound (Yuan *et al.*, 1992) and point at the parent compound as causing the

adverse effects. When testing cinnamaldehyde for toxicity it is therefore important to choose the appropriate route *i.e.* oral dosing by gavage or, even more relevant for the human situation, *via* the diet.

In contrast to the intact rat, where PrSH and protein amino groups are not affected even after doses of cinnamaldehyde that largely depleted liver GSH, equivalent concentrations are cytotoxic in the *in vitro* model system (Chapter 6; Swales, 1993). In rat hepatocytes in suspension metabolism becomes saturated above a threshold concentration of 1 mM, leading to the prolonged presence of cinnamaldehyde in the cell and enhanced ^{14}C binding (Chapters 6 and 7). Parallel studies of Swales (1993) show that at the same concentration GSH becomes severely depleted, followed by CySH and the loss of PrSHs and at later time by cytotoxicity. These results suggest a function of sulfhydryl groups and protein binding in the toxicity of cinnamaldehyde to hepatocytes.

Comparison of the metabolism and ^{14}C binding of cinnamyl alcohol, cinnamaldehyde and cinnamic acid indicates that cinnamaldehyde is the reactive species in the ^{14}C binding (Chapter 6). Further studies in which ^{14}C-cinnamaldehyde metabolism is manipulated confirm this. Inhibition of metabolism by cyanamide or 4-methyl pyrazole or predepletion of GSH enhances binding, while induction of metabolism by ethanol has a protective effect (Chapter 7). The possibility exists that after saturation of metabolism a minor toxic route becomes disproportionably important, an example of which is the increased 1'-hydroxylation of estragole when primary routes of metabolism are saturated (reviewed in Caldwell *et al.*, 1990). After saturation of cinnamaldehyde metabolism, however, there are no changes in the metabolic profile other than the greater amount of cinnamaldehyde remaining present in the incubation medium. It is conceivable though

that the formation of an alternative metabolite accounts for less than 1% of ^{14}C and perhaps goes unnoticed by HPLC detection, but is enough to be responsible for the 1% binding observed in the hepatocyte incubations. However, when binding is studied as a function of the incubation time, this is highest at 0 h and therefore not metabolism-dependent but only related to the parent compound, cinnamaldehyde (Chapter 6). Still, the role of this binding in the cytotoxicity of cinnamaldehyde remains to be investigated. The conclusion so far is that changes in metabolism and ^{14}C binding were at least indicative for changes in GSH depletion and cytotoxicity.

Dornish *et al.* (1989) showed that cinnamaldehyde interacts with cells in two distinct ways. These authors studied cell inactivation of ovary cells from the human cell line NHIK 3025 by the platinum compound *cis*-DDP, a DNA damaging agent used in the treatment of cancer, and compared the influence of several aldehydes in potentiating or inhibiting this effect. The action of cinnamaldehyde was dual. At concentrations up to 1 mM cinnamaldehyde potentiated *cis*-DDP toxicity by depleting GSH, while cinnamic acid and cinnamyl alcohol had no effect. A structure activity relationship was found between the potentiating effect of cinnamaldehyde derivatives on *cis*-DDP toxicity and the reactivity of the double bond towards cellular sulfhydryls (Dornish *et al.*, 1989). In contrast, at concentrations higher than 1 mM, cinnamaldehyde protected against the action of *cis*-DDP by inhibiting its uptake into the cell due to the formation of a Schiff base with protein amino groups on the outside of the cellular membrane. By comparison with other aromatic aldehydes, such as benzaldehyde, the authors concluded that the longevity of the Schiff base was important for the inhibition of *cis*-DDP uptake and that this was dependent on both the aldehyde and the aromatic ring (Dornish and Petterson, 1985). The fact that neither cinnamic acid nor cinnamyl alcohol are effective shows that both the

aldehyde and the unsaturated carbon bond are important in the interaction of cinnamaldehyde with cells. The aldehyde can form a Schiff base, which may have accounted for the reversible binding of ^{14}C in rat hepatocytes in suspension (Chapters 6 and 7), while the α,ß-unsaturated double bond makes cinnamaldehyde a typical weak electrophile that undergoes GSH conjugation, spontaneously or catalyzed by GSTs as was demonstrated *in vivo* (Chapter 5), in rat hepatocytes in suspension (Chapters 6 and 7) and in cytosol (Chapter 8). The third functional group is the aromatic ring, which contributes to the hydrophobicity of the compound. The 1 mM threshold in the potentiating/ protecting effect of cinnamaldehyde on *cis*-DDP toxicity correlates very well with the threshold for enhanced ^{14}C binding, GSH depletion and cytotoxicity. In conclusion, cinnamaldehyde is reactive and cytotoxic but this effect is only seen at relative high concentrations after its metabolism has become saturated. The reactivity of α,ß-unsaturated aldehydes and ketones towards GSH, PrSH and amino groups forms an interesting field for further study and is relevant since many chemicals of this class are encountered in the diet and the environment (Portoghese *et al.*, 1989; Feron *et al.*, 1990).

JECFA has requested further data on cinnamaldehyde carcinogenicity before its ADI can be continued. However cinnamaldehyde's reactivity towards soft-nucleophiles such as CySH, GSH, NALC (Chapter 8) and PrSH groups in rat hepatocytes in suspension and the lack of reactivity towards DNA in the UDS assay in cultured rat hepatocytes (Swales, 1993) makes a genotoxic mechanism of action very unlikely. Furthermore, its cytotoxicity will obscure test results from *in vitro* systems and its acute toxicity when given i.p. at doses higher than 250 mg/kg makes effects seen in *in vivo* assays unrepresentative for the human situation of oral low exposure. A carcinogenicity assay should

be very carefully designed to avoid these errors. The fact that the cinnamaldehyde *per se* is the reactive species in the observed covalent binding and cytotoxicity *in vitro*, indicates that toxicities of cinnamaldehyde, if any, will be contact site toxicities, skin after occupational exposure or when it is present in fragrances, which is well documented (Hoskins, 1984) and stomach when ingested *via* the food.

A possible risk to these contact sites is cinnamaldehyde's cytotoxicity. Although the metabolic capacity of the liver is very high, other organs may have a lower capacity for cinnamaldehyde metabolism and as a result be more susceptible. Recently, there has been a lot of attention for cytotoxic and irritating chemicals, such as the α,ß-unsaturated carbonyl compound ethyl acrylate (EA) and the phenolic anti-oxidant butylated hydroxyanisole (BHA), which cause forestomach carcinomas in rats but are negative in many genotoxicity tests (Clayson, 1990). The theory is that repeated irritation leads to cell repair/proliferation. In an early study (Hagan *et al.*, 1967), rats given cinnamaldehyde in the diet at levels up to 1% (500 mg/kg) showed effects only at the highest exposure level, which were slight swelling of hepatic cells and hyperkeratinosis in the forestomach. In comparison, EA was an irritant to the stomach at 20 mg/kg and causes forestomach tumours at 200 mg/kg. Large doses (1 g/kg) of BHA cause an early inflammatory reaction in the forestomach. *In vitro* the lipophilic and structural properties of BHA cause the compound to become easily inserted in the cell membrane and disrupt mitochondrial function leading to a decrease in ATP, influx of Ca^{2+} and subsequently cell death, and these events have been implicated in its toxicity (Thompson and Moldeus, 1988). Its action might also be *via* redox cycling after GST-mediated activation (Van Ommen *et al.*, 1992), but there is no indication that this mechanism occurs with cinnamaldehyde. Clayson *et al.* (1990)

reviewed the significance of induced forestomach tumours in rats and concluded that most substances cause effects which are readily reversible and only occur when administered in the diet at high dosages that are well in excess of the MTD and are unlikely to produce tumours in humans under the presently allowed levels of exposure *via* the food supply. Further, it is difficult to assess the importance of this information to species such as humans who do not possess a forestomach.

In a rational approach to the safety evaluation of cinnamaldehyde as a food flavour *in vitro* studies could be designed to examine the significance of the various rates of delivery to tissues versus the rates of metabolic detoxication. Important is that activities of cinnamaldehyde metabolizing enzymes, ADHs, ALDHs and GSTs, or the local concentration of GSH and non-critical binding proteins may be less in contact site organs than in the liver. By determining enzyme activities and the extent of protein binding in whole homogenates of contact site tissues compared to the liver and combining these data with the known GSH concentrations and capacities of the organs to resynthesize GSH, preferably by comparison with other aldehydes and α,ß-unsaturated compounds, the relative susceptibility of these organs to cinnamaldehyde toxicity can be estimated and thus serve as a basis for *in vivo* tests. In this way Frederick *et al.* (1992) designed a physiologically based pharmacokinetic-pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate. With the use of the model they were able to calculate the delivered dose and correlate this with the incidence of contact site toxicity (oedema, inflammation, ulceration and hyperplasia) and predict the lack of toxicity in tissues remote from the dosing site.

10.3 Final Conclusions

1. In view of the safety of *trans*-cinnamaldehyde as a food flavour, the most important conclusion following from the studies presented in this thesis is that cinnamaldehyde is rapidly metabolized *via* safe metabolic pathways and that the metabolic capacity of rat, mouse and man is very large in comparison with the anticipated daily intake *via* the diet. The metabolic profile is comparable between the three species examined, rat, mouse and man, and over the dose range tested and allows extrapolation of data from high dose animal tests to the human situation of low exposure.

2. From studies with hepatocytes in suspension and cytosolic preparations it follows that the parent compound, cinnamaldehyde is the reactive species in the covalent binding of ^{14}C to cellular macromolecules. Changes in covalent binding, were predictive for changes in GSH depletion and cytotoxicity. Toxicities of cinnamaldehyde, if any, will therefore be contact site toxicities, skin in occupationally exposed persons and stomach after ingestion via the food, and further research should concentrate here.

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