THE CHLORINATED ETHYLENES : THEIR HEPATIC METABOLISM AND CARCINOGENICITY

by

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1. Tetrachloroethylene: Binding and metabolism by hepatic microsomal cytochrome P-450. A. K. Costa and K. M. Ivanetich. Biochem. Pharmacol. 29, 20, 2863 (1980).

3. Vinylidene Chloride: Its metabolism by hepatic microsomal cytochrome P-450 in vitro. A. K. Costa and K. M. Ivanetich. Biochem. Pharmacol. 31, 11, 2083 (1982).

4. The 1,2-dichloroethylenes: Their metabolism by hepatic microsomal cytochrome P-450 in vitro. A. K. Costa and K.M. Ivanetich. Biochem. Pharmacol. 31, 11, 2083 (1982).

5. Limitations on the metyrapone assay for the major phenobarbital inducible form of cytochrome P-450. K. M. Ivanetich, A. K. Costa and T. Brittain. Biochem. Biophys. Res. Commun. 105, 4, 1322 (1982).

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Some of the work reported in this thesis has been presented at scientific meetings:

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2. Mechanisms of chemical carcinogenesis. In vitro investigations of the metabolic activation of chlorinated ethylenes. K. M. Ivanetich, A. K. Costa and I. D. Katz. First South African Conference on Fundamental Molecular and Cellular Cancer Research, April 5-7, 1978, Cape Town.

3. The metabolism and toxicity of perchloroethylene: One avenue in an investigation into the metabolism and toxicity of a series of chlorinated ethylenes. K. M. Ivanetich and A. K. Costa. 4th Congress of South African Biochemical Society, January 1979, Grahamstown.

4. The hepatic metabolism of halogenated anaesthetic agents. A. K. Costa, UCT 150 Academic Festival, December 7, 1979.

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9. The metabolic activation of carcinogenic and non-carcinogenic chloroethylenes by hepatic cytochrome P-450. A. K. Costa and K. M. Ivanetich. 7th Congress of the SA Biochemical Society, January 1983, Stellenbosch, Cape.

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ABSTRACT

Following the observation that vinyl chloride monomer was activated into potentially carcinogenic species by the hepatic cytochrome P-450 enzyme system, widespread interest was generated in the metabolism of the other chlorinated ethylenes. The chlorinated ethylenes which were investigated herein included vinylidene chloride which is used for the manufacture of Saran plastics; cis- and trans-1,2-dichloroethylene and tetrachloroethylene, which are industrial solvents; and trichloroethylene which is a volatile anaesthetic agent.

An investigation of the hepatic metabolism and potential carcinogenicity of the chlorinated ethylenes is reported in this thesis. The hepatic metabolism of these compounds was primarily investigated using two different liver preparations, viz. hepatic microsomal preparations and freshly isolated hepatocytes, although partially purified cytochrome P-450 was used in one case, viz. trichloroethylene. Hepatic microsomes were utilized to investigate Phase I of xenobiotic metabolism by the cytochrome P-450 enzyme system, while the isolated hepatocytes were utilized as a system for assessing the metabolism of the chlorinated ethylenes which might provide a better representation of the *in vivo* situation since these cells contain the enzymes of both Phase I and Phase II of xenobiotic metabolism. Furthermore, the hepatocytes provide a potentially advantageous system for testing chemically-induced DNA repair synthesis in order to measure the carcinogenic potential of chemicals that require activation by the liver.

The chlorinated ethylenes were found to be metabolized by

hepatic cytochrome P-450 as observed from

(i) their production of a Type I difference spectrum with hepatic microsomes;

(ii) their ability to stimulate hepatic microsomal CO-inhibitable NADPH oxidation; and

(iii) the requirements for their conversion to chlorinated metabolites being hepatic microsomes, NADPH-generating system and substrate.

(iv) specific inhibitors of cytochrome P-450 diminished or eliminated metabolite production by hepatic microsomal incubation mixtures.

It was found that the forms of hepatic microsomal cytochrome P-450 elevated by phenobarbital and the forms found in untreated animals, not inducible by phenobarbital or by the polycyclic hydrocarbons, were involved in the metabolism of the chlorinated ethylenes. Cytochrome P-450c, which is induced by pretreatment with polycyclic hydrocarbons, appeared to play no role in the metabolism of any of the chlorinated ethylenes in vitro.

The major metabolites of the chlorinated ethylenes following incubation in the presence of hepatic microsomes and a NADPH-generating system were as follows: Vinylidene chloride, chloroacetate and dichloroacetaldehyde; cis- and trans-1,2-dichloroethylene, dichloroacetaldehyde; trichloroethylene, chloral hydrate; and tetrachloroethylene, trichloroacetate. The abovementioned metabolites were generally found in isolated hepatocytes in the presence of the chlorinated ethylenes, but in some cases, there was extensive secondary metabolism of the major metabolites observed in hepatic microsomes,

e.g. following vinylidene chloride and trans-1,2-dichloroethylene metabolism in isolated hepatocytes, only trace amounts of dichloroacetaldehyde were seen. In isolated hepatocytes, dichloroacetaldehyde was extensively converted to 2,2-dichloroethanol and dichloroacetic acid by the cytosolic alcohol and aldehyde dehydrogenase, respectively. Chloral or its hydrated analogue, chloral hydrate, the sole metabolite of trichloroethylene in hepatic microsomes, was also converted to 2,2,2-trichloroethanol and trichloroacetic acid in the presence of isolated hepatocytes. The issue is, however, further complicated by the observation that the levels of many of the chlorinated metabolites decreased with time in hepatic suspensions; perhaps in part due to their incorporation into physiological pathways, e.g. chloroacetate into chlorocitrate.

It was found that the extent and position of chlorination of the chlorinated ethylenes appears to play a very important role in the hepatic management of the chlorinated ethylenes in the short term, i.e. the rates of metabolite production and which form of cytochrome P-450 is involved; and in the long term, i.e. their carcinogenic potential, for example,

i). Cytochrome P-450b and the form of cytochrome P-450 found in untreated rats (and not inducible by phenobarbital or polycyclic hydrocarbons) play an increasing and decreasing role, respectively, in the metabolism of the chlorinated ethylenes as the extent of chlorination increases.

ii). It was observed that the chlorinated ethylenes with the greater degree of symmetry, e.g. tetrachloroethylene and trans-1,2-dichloroethylene, were metabolized at a lower rate

than those chlorinated ethylenes with relatively fewer degrees of symmetry, e.g. trichloroethylene and cis-1,2-dichloroethylene.

iii). The degree of symmetry also plays a role in the ability of the chlorinated ethylene to "uncouple" the cytochrome P-450 enzyme system. cis-1,2-Dichloroethylene and vinylidene chloride, which each possess relatively fewer degrees of symmetry, act as uncouplers with the subsequent production of H_2O_2, while trans-1,2-dichloroethylene and tetrachloroethylene, with higher degrees of symmetry, do not.

iv). The ability of the chlorinated ethylenes to cause the degradation of cytochrome P-450 is linked to the chemical nature of the major rearrangement product of the proposed epoxide intermediate. The epoxides that rearrange exclusively to aldehydes in hepatic microsomes correspond to chlorinated ethylenes that modify the heme of cytochrome P-450, viz. cis- and trans-1,2-dichloroethylene and trichloroethylene, while the epoxides that rearrange to an acyl chloride or to both an aldehyde and an acyl chloride are from chlorinated ethylenes that do not modify the heme of cytochrome P-450, viz. vinylidene chloride and tetrachloroethylene.

v). The ability of the chlorinated ethylenes to induce DNA repair synthesis in a mammalian test system appears to provide an indication of their carcinogenic potential. Vinylidene chloride, cis-1,2-dichloroethylene and trichloroethylene gave rise to DNA repair in isolated rat hepatocytes, while trans-1,2-dichloroethylene and tetrachloroethylene did not. This ability

shows a positive correlation with a relatively lower degree of symmetry and/or their ability to cause H_2O_2 production.

A good correlation appears to exist between the ability of the chlorinated ethylenes to induce DNA repair in isolated hepatocytes and their reported carcinogenicity in vivo. An exciting observation was that the cis-isomer of the 1,2-dichloroethylenes, both of which are considered to be non-carcinogenic, gave rise to DNA repair, while the trans-isomer did not.

LIST OF ABBREVIATIONS

ADH	: Aldehyde dehydrogenase
$^{\Delta}A_{max}$	: Maximal extent of binding of substrate to enzyme
β -NF	: β-Naphthoflavone
BP	: Benzpyrene
BrUrd	: 5-Bromodeoxyuridine
ca.	: circa
CAA	: Chloroacetaldehyde
CEO	: Chloroethyleneoxide
Ci	: Curies
cpm	: Cycles per minute
CSF	: Cyanide-sensitive factor
DCAA	: Dichloroacetaldehyde
DCA	: Dichloroacetic acid
DCE	: 2,2-Dichloroethanol
cis-DCE	: cis-1,2-Dichloroethylene
trans-DCE	: trans-1,2-Dichloroethylene
DNA	: Deoxyribonucleic acid
DMSO	: Dimethylsulfoxide
dpm	: Disintegrations per minute
EDTA	: Ethylenediaminetetra acetic acid
FCS	: Fetal calf serum
FdUrd	: 5-Fluorodeoxyuridine
G.C.-M.S.	: Gas chromatograph linked to a mass spectrometer
GSH	: Glutathione reduced
HEPES	: N-2-Hydroxyethyl piparazine-N'-2-ethanesulfonic acid
hr	: hour(s)
I.U.	: International units

K_m	:	Michaelis-Menten constant
K_s	:	Spectrally determined dissociation constant
MC	:	3-Methylcholanthrene
min	:	minute(s)
NAD(H)	:	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	:	Nicotinamide adenine dinucleotide phosphate (reduced)
PAPS	:	3'-Phospho-adenosine-5'-phosphosulfate
PB	:	Phenobarbital
PCE	:	Perchloroethylene (tetrachloroethylene)
PCN	:	Pregnenolone-16 α-carbonitrile
ppm	:	Parts per million
PVC	:	Polyvinyl chloride
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
S.D.	:	Standard deviation
sec	:	Second(s)
SKF-525A	:	2-Diethylaminoethyl-2,2-diphenylvalerate
TCA	:	Trichloroacetic acid
TCE	:	Trichloroethylene
Tris	:	Tris(hydroxy methyl)methylamine
UDP-	:	Uridine 5'-diphospho-
UDS	:	Unscheduled DNA synthesis
VC(M)	:	Vinyl chloride (monomer)
VDC	:	Vinylidene chloride
V_{max}	:	Maximum velocity which may be attained by an enzyme-catalyzed reaction
M	:	Molar
mol	:	mole(s)

m- : milli- (10^{-3})

μ- : micro (10^{-6})

n- : nano- (10^{-9})

p- : pico- (10^{-12})

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1. INTRODUCTION

1.1. Xenobiotic Biotransformation

Xenobiotics are substances foreign to the human body, i.e. substances not found endogenously, be they drugs, anaesthetic agents, environmental pollutants, pesticides, carcinogens, etc. It is well accepted that most xenobiotics undergo a variety of metabolic changes *in vivo* before being excreted via routes such as the urine, faeces or exhaled air.

Xenobiotics can be classified in two categories:

(i) Those that are specifically designed to mimic substances normally synthesized and catabolized in the body and therefore are metabolized by the specific enzymes which normally cope with the substrate analogue, and

(ii) those that have no endogenous counterpart and are metabolized by the non-specific enzyme systems known as the xenobiotic* or drug metabolizing enzymes.

Over the last three or four decades, the metabolism of xenobiotics by these non-specific enzyme systems has been extensively investigated. It has been established that the liver contains the highest concentrations and activities of the drug metabolizing enzymes; although to a far lower extent (usually 5% of total), activity is also found in the kidneys, lungs, intestines, blood, skin and virtually every organ and tissue in the body.

The major function of these non-specific enzymes is to render a lipophilic xenobiotic more hydrophilic and therefore easily excretable

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*The term 'drug metabolism' is synonymous with 'metabolism of xenobiotics'; and although it is misleading, the former term is used to a much greater extent than the latter.

via, the urine. This process prevents the harmful effects of the accumulation of the xenobiotic in the fatty tissues of the body.

The catabolism of xenobiotics is implemented in two phases known as Phase I and Phase II [1] of xenobiotic or drug metabolism, as can be seen in Figure 1. A variety of non-specific drug metabolizing enzymes are involved in each of the two phases.

Figure 1: Proposed general pathway of Xenobiotic Metabolism

	Phase I		Phase II	
Hydrophobic compound	⟶	Relatively hydrophilic	⟶	Highly hydrophilic
Non-polar		Polar		Highly polar
Toxic				Non-toxic

Phase I of drug metabolism involves the oxidation of lipophilic, non-polar compounds to relatively hydrophilic or polar metabolites. The latter can be excreted unchanged at this point or can undergo a Phase II reaction, which involves conjugation of the metabolite to a small endogenous polar molecule, e.g. glutathione, glucuronic acid, sulfate, glycine or water [2], to produce a highly hydrophilic metabolite which can be excreted with ease via the kidneys into the urine.

Examples of the types of reactions involved in Phase I and Phase II of drug metabolism, as well as the enzymes catalyzing these phases, are presented in Table I. Phase I reactions are generally catalyzed by cytochrome P-450* but also to a far lesser extent, by other

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*'Cytochrome P-450' is a collective term used for a group of microsomal heme proteins which function as terminal oxidases in drug oxidations.

Table 1: Hepatic Metabolism of Xenobiotics [2,3]

Reaction	Enzyme	Major subcellular location of enzyme
Phase I		
Oxidation of aliphatic and aromatic groups, N-oxidation, sulphoxidation, oxidative dealkylation, epoxidation	Cytochrome P-450	Endoplasmic reticulum
Reduction of azo-, nitro- compounds	Flavin enzymes or cytochrome P-450	Endoplasmic reticulum
Reduction of carbonyl-compounds	Cytochrome P-450	Endoplasmic reticulum
Oxidation and reduction of alcohols, aldehydes and carboxylic acids	Alcohol dehydrogenase, aldehyde dehydrogenase	Cytosol
Oxidation of amines	eg. Monoamine oxidases	Mitochondria
Hydrolysis of esters	Esterases	Cytosol and various organelles
Phase II		
Conjugation with glucuronic acid	UDP-glucuronyl transferases	Endoplasmic reticulum, nuclear envelope
Conjugation with glutathione	Glutathione-S-transferases	Cytosol
Conjugation with sulphate, glycine, other amino acids; acetylation and methylation	Various transferases	Cytosol and various organelles
Hydration of epoxides	Epoxide hydrase	Endoplasmic reticulum

enzymes of more limited substrate specificity. Phase II reactions involve conjugation reactions catalyzed mainly by the glucuronyl- and glutathione-transferases, and by the sulfo-transferases and various other transferases to a lesser extent (see Table I)[2,3].

The net result of Phase I and Phase II of drug metabolism is the formation of non-toxic, hydrophilic, easily excretable metabolite(s) from a toxic, lipophilic parent compound by way of reactions such as oxidations, reductions, hydrolysis or conjugations as shown in Table I. However, the drug metabolizing enzymes can, in some circumstances, give rise to metabolites which are more active (viz. more toxic or carcinogenic) than is the parent compound. For example, vinyl chloride monomer, which is not carcinogenic in its own right, is rendered carcinogenic following metabolic activation by the hepatic microsomal* cytochrome P-450 enzyme system [5]. Further examples are the volatile anaesthetic agent, fluroxene, which is metabolized by the hepatic microsomal cytochrome P-450 to the toxic compound, 2,2,2-trifluoroethanol [6]; and the generally used solvent, carbon tetrachloride, which is thought to be metabolized by hepatic microsomal cytochrome P-450 to $CCl_3^{\cdot}$ and $Cl^{\cdot}$, which are highly reactive free radicals [7].

Although it was thought that there were very few instances where reactive metabolite(s) were formed following a conjugation reaction (Phase II), more and more exceptions to this rule have been discovered. For example, 1,2-dibromoethane and 1,2-dichloroethane

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*Upon homogenization of the liver, the endoplasmic reticulum is fragmented, resulting in the formation of tiny vesicles of endoplasmic reticulum known as microsomes [4]. In this thesis, enzymes bound to the smooth endoplasmic reticulum will be referred to as 'microsomal enzymes'.

are activated through conjugation with glutathione [8,9,10]. The metabolism of paracetamol gives rise to a toxic species following conjugation with glutathione [11]. The glucuronyl- and the sulfo-transferases are involved in the formation of the ultimate carcinogenic species of N-hydroxy-N-acetylaminofluorene, viz. the N-O-glucuronide and N-O-sulfate ester of N-hydroxy-N-acetylaminofluorene [12,13]. Furthermore, the action of microsomal epoxide hydrase in the metabolism of benzpyrene is essential in the pathway leading to the formation of the ultimate carcinogen (viz. the 7,8-diol-9,10-epoxide) (14.15).

Some of the major enzyme systems of drug metabolism will be discussed in more detail, viz:

1.1.1 The cytochrome P-450 drug metabolizing system

1.1.2 The conjugating enzymes

1.1.1 The Cytochrome P-450 Drug Metabolizing System

In 1937 Michel [16] showed that the major site of the conversion of foreign chemicals into more polar derivatives was the liver. However, it was not until 1955 that Brodie et al. [17] intimated that the enzymes causing many of these biotransformations were situated in the endoplasmic reticulum. The existence of a microsomal CO-binding pigment was reported by Klingenberg [18] and by Garfinkel [19] in 1958. However, it was not until 1962 that Omura and Sato [20] demonstrated the haemoprotein nature of the enzymes that effected the biotransformation of foreign compounds. These co-workers named this haemoprotein cytochrome P-450 for the characteristic absorption peak at 450 nm of the ferrous-CO complex [20,21,22].

Under some circumstances, cytochrome P-450 is found to undergo a change to an inactive form, cytochrome P-420, so called because the absorbance maximum of the CO-complex of the ferrohemoprotein is at 420 nm [21,22]. This can reflect denaturation of cytochrome P-450 and/or the dissociation of heme from apo-cytochrome P-450 [22,275].

Cytochrome P-450 is a group of b type cytochromes, each of which consists of iron protoporphyrin IX non-covalently bound to an apo-protein moeity, of molecular weight between 44000 to 54000 daltons. These enzymes are found buried in the deep layers of the lipid portion of the endoplasmic reticulum [4,23].

It is currently thought that cytochrome P-450 evolved to function in the prevention of oxygen toxicity on the advent of atmospheric oxygen, billions of years ago when organisms survived in a reducing, anaerobic atmosphere* [24]. However, with the subsequent adaptive development of aerobic organisms, this protective capability was no longer required, but the presence of cytochrome P-450 was still maintained, perhaps for biosynthetic processes and/or for the degradation of varied complex molecules [24]. It is, however only over the last 50 years or more that the vast potential of this cytochrome P-450 enzyme system has been realized, due to man's exposure to an ever-increasing range and number of foreign chemicals.

The most remarkable features of this cytochrome P-450 enzyme system are:

(i) The broad substrate specificity. This is in part due to

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*It is also possible that the cytochrome P-450 enzyme system could have functioned reductively before free oxygen appeared, i.e. when the earth's atmosphere contained mainly hydrogen, nitrogen, methane, ammonia, carbon monoxide and carbon dioxide [25].

the existence of multiple forms of the enzyme in a given tissue [25,26,27]; however, even single purified forms of cytochrome P-450 often have strikingly broad substrate specificities. Examples of the variety of possible substrates are shown in Table 2 which include compounds which vary from fatty acids and steroids to anaesthetic agents and environmental pollutants (i.e. halogenated hydrocarbons and pesticides).

(ii) The wide variety of reactions that it catalyzes. Cytochrome P-450 is perhaps unique in that it catalyzes both oxydative and reductive reactions. Table 3 [2] provides an indication of the wide variety of reactions catalyzed by this enzyme system in both of these categories.

The multiple forms of cytochrome P-450 differ from each other in their amino acid composition and molecular weight, physiochemical properties and substrate specificity [28]. Since the selective elevation of certain form(s) of cytochrome P-450 by chemicals, i.e. their induction, is a very powerful tool in drug metabolism, this topic will be considered separately in detail in the section entitled 'Induction of the hepatic microsomal enzymes'. Therein will be described the differences in the multiple forms of cytochrome P-450.

The first step in any of the hepatic cytochrome P-450 mediated reactions, be it oxidative or reductive, is the binding of the substrate by ferricytochrome P-450. Therefore the binding of compounds by cytochrome P-450 will initiate the discussion on the metabolism of xenobiotics by cytochrome P-450.

Table 2: Compounds metabolized by Cytochrome P-450

Type	Specific Examples
Pesticides	DDT
Carcinogens	Vinyl chloride; N-acetylaminofluorene
Barbiturates	Hexobarbital; Pentobarbital
Anaesthetics	Fluroxene; Halothane; Enflurane
Fatty Acids	Lauric Acid
Steroids	Testosterone; β-Estradiol
Food additives	BHT
Cannabinoids	THC
Dyes	Aniline derivatives
Chlorinated hydrocarbons	Trichloroethane; Dibromoethane
Polycyclic hydrocarbons	3-Methylcholanthrene; Benzpyrene

Table 3: Metabolism of Drugs by Cytochrome P-450 in Liver Microsomes (2)

Type	Substrate	Product
Oxidation		
Aromatic hydroxylation	Aniline	p-Aminophenol
Aliphatic hydroxylation	Hexobarbital	Hydroxyhexobarbital
Arene oxide formation	Bromobenzene	Bromobenzene epoxide
N-Dealkylation	Aminopyrene	4-Aminoantipyrine
N-Hydroxylation	2-Acetylamino-fluorene	N-Hydroxy-2-acetyl aminofluorene
O-Dealkylation	p-Acetanisidine	p-Hydroxyacetanilide
S-Dealkylation	6-Methylthiopurine	6-Thiopurine
N-Oxidation	Dimethylaniline	Dimethylaniline N-oxide
S-Oxidation	Chloropromazine	Chloropromazine sulfoxide
S-Oxidation	Diaminodiphenyl sulfide	Diaminodiphenyl sulfoxide
Oxidative Dechlorination	Halothane	Trifluoroethanol
Reduction		
Deamination	Amphetamine	Phenylacetone
Desulfuration	O-Ethyl-O-(4-nitro-phenyl) phenylphos-phonothionate	O-Ethyl-O-(p-nitro-phenyl) phenylphos-phate
Dechlorination	Carbon tetra-chloride	Chloroform
Dealkylation of metalloalkanes	Tetraethyl lead	Triethyl lead
Reductive dechlorination	Halothane	2-Chloro-1,1,1-trifluoro-ethyl radical

1.1.1a Binding to hepatic cytochrome P-450

A significant breakthrough in elucidating the possible mechanism of drug oxidations and reductions was the discovery in 1966 that various substrates and inhibitors combine with ferricytochrome P-450 to produce small but significant, characteristic difference spectra [29,30,31,32]. The experimental system comprises a sample cuvette containing the compound under investigation and hepatic microsomes versus a reference cuvette with hepatic microsomes alone. The changes in the absorbance spectrum caused by the binding of compounds to cytochrome P-450 can be categorized as Type I, Type II and Type IR difference spectra [33] (see Figure 2). A Type I difference spectrum arises as a result of the formation of the enzyme-substrate complex while Type II and Type IR difference spectra arise as a result of compounds which are not substrates of cytochrome P-450 binding to sites other than the substrate binding site of the enzyme.

The Type I spectral change is characterized by an absorption peak at ca. 390 nm and a trough at ca. 420 nm [34] (see Figure 2). The increase in absorption at ca. 390 nm corresponds to an increase in the amount of ferricytochrome P-450 in the high spin state upon the binding of the substrate, with the concommitant loss of the low spin state form which absorbs at ca. 420 nm.* In a microsomal preparation, prior to the addition of a substrate, cytochrome P-450 exists in a mixture of spin states. There will be a certain

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*The ferric form of cytochrome P-450 has 5 d electrons. A strong field ligand will cause the electrons to be paired in each of the two lowest energy d orbitals, with one unpaired electron in the next highest orbital, and this is called low spin. A weak field ligand will result in all five electrons being unpaired and this is called high spin [35].

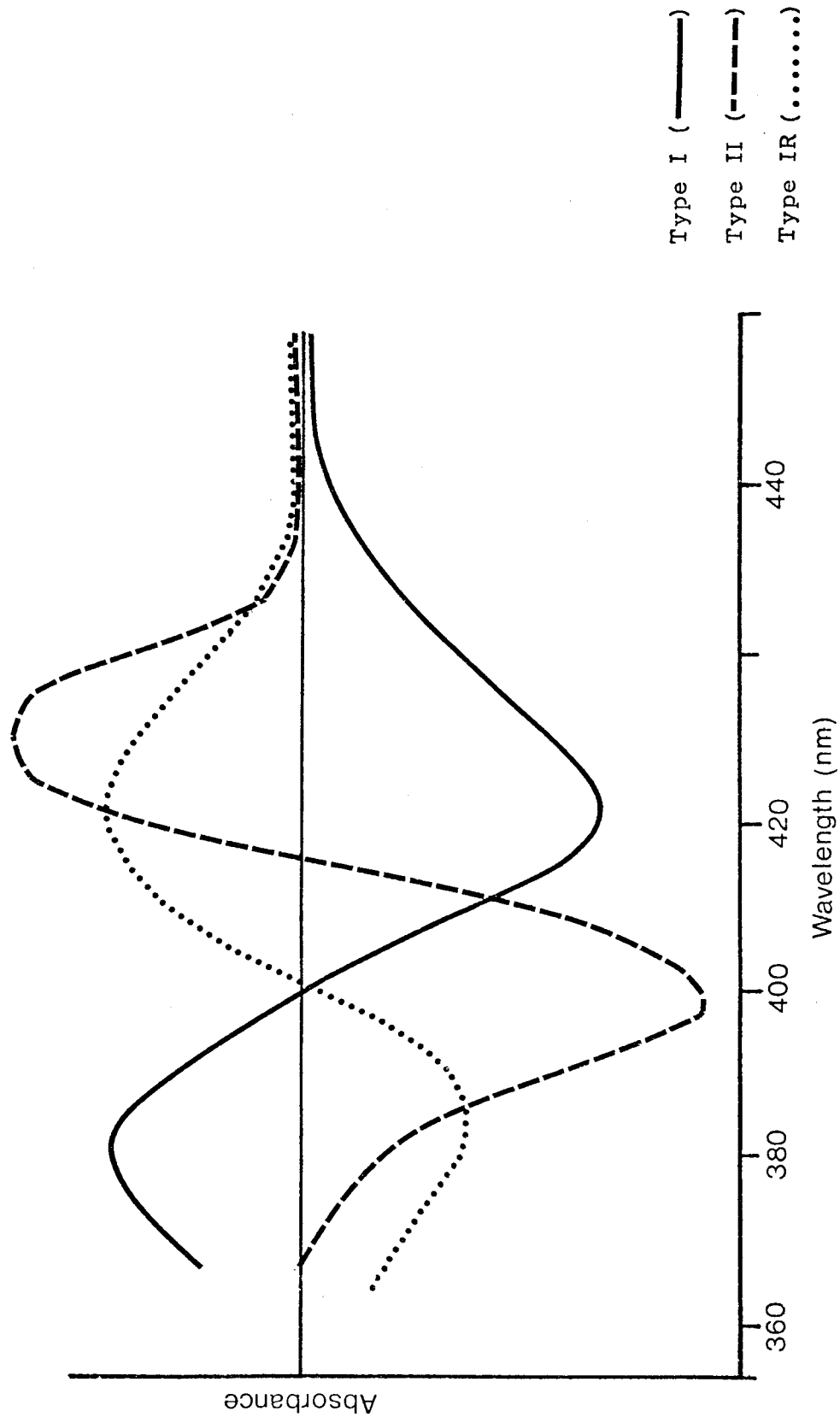

Figure 2: Difference spectra in hepatic microsomes following addition of compound to sample cuvette

percentage of cytochrome P-450 in the high spin state (i.e. *in vivo* high spin form) which may be a reflection of the environment of the cytochrome P-450 iron itself in the vicinity of the heme protein, or may reflect contributions from cytochrome P-450 bound to endogenous compounds. The magnitude of the spectral change following the addition of an exogenous compound depends largely on the *in vivo* spin state of the cytochrome P-450 iron in freshly prepared microsomes [39]. The *in vivo* percentage of high spin or low spin form of the iron of cytochrome P-450 can be changed by pretreatment with inducing agents *in vivo*, e.g. phenobarbital [see under 'Induction of hepatic microsomal enzymes', Section 1.1.3] [35] and provides an explanation for the day-to-day variation seen in a Type I difference spectrum elicited *in vitro* following the addition of a substrate.

The Type I spectrum is thus due to the change in the absorbance of the heme moeity as a consequence of a change in its environment and is not due to a direct interaction between the substrate and heme moeity [40]. This spectral change has been shown to result from the formation of the enzyme-substrate complex, primarily on the basis of the similarity between the Michaelis constants (K_m) and the spectral dissociation constants (K_s) for a wide variety of substrates [33,34]. Thus, there is often a direct correlation between the magnitude of the Type I spectral change, which is substrate and microsomal protein concentration dependant, and the rate of metabolism [41]. Since many substrates of the mixed function oxidase have in common only the property of being lipid-soluble (see Table 4), it is assumed that the active site of the enzyme is in the hydrophobic region of the apoenzyme [29] (see Figure 3). The active substrate binding site and catalytic site

Table 4 : Examples of compounds that bind to hepatic cytochrome P-450 in a Type I, II and IR manner [33,36,37,38,39]

Type of Difference Spectrum	Class (Type) of compound	Specific examples
Type I	Barbiturates	Phenobarbital, Hexobarbital, Amobarbital
	Anaesthetic agents/analgesics	Fluroxene, Halothane, Lidocaine
	Steroids and/or derivatives	Testosterone, β-Estradiol
	Chlorinated hydrocarbons (Environmental pollutants)	Dichlorodiphenyl trichloroethane (DDT), Vinyl chloride tetrachloroethylene, trichloroethylene, Trichloroethane, etc.
	Secondary and tertiary amines	N,N-dimethylaniline, SKF-525 A
	Others	Aminopyrine
Type II	Amines	Aniline, pyridine, nicotine, nicotinamide, p-aminophenol, octylamine-1,2,4-dichloro-6-phenyl phenoxyethylamine hydrochloride
Type IR	Alcohols	Methanol, ethanol, 2-propanol, n-butanol
	Corticosteroids	Cortisol, corticosterone
	Others	Acetone, rotenone, cyclohexanone, phenacetin, acetanilide, cyanide, ethylisocyanide

i.e. oxygen binding site, are thought to be as shown in Figure 6 [42,43]. One further characteristic of Type I compounds is that by altering the environment of the heme they stimulate the reduction of ferricytochrome P-450 [44].

The Type II difference spectrum [33] is due to the formation of a ferrihemochrome, which reflects the binding of a ligand to the heme iron of cytochrome P-450 [33]. This type of spectrum is characterized by the appearance of an absorption peak between 425 and 445 nm and the appearance of the absorption minimum at 395 nm. The peak is a spectral reflection of the interaction between a compound and the cytochrome P-450 heme iron; with the position of the absorption peak being determined by the resulting ferrihemochrome [36] and the trough representing a decrease in the high spin form of cytochrome P-450 (see Figure 2). The interaction of the heme of cytochrome P-450 with any good electron donor, e.g. basic amines, thiols etc., gives rise to a Type II difference spectrum. It has been demonstrated that Type II compounds bind to the same site as does oxygen, viz. the heme iron, which explains how CO and other Type II ligands inhibit cytochrome P-450 dependent reactions, viz. by interfering with oxygen binding [33].

The observation that inhibitors that bind at the catalytic site, i.e. certain Type II ligands, can also affect the binding of substrates to the binding site of cytochrome P-450 and their ensuing metabolism by cytochrome P-450 is explained by the proposal that the catalytic site (or oxygen binding site) and the substrate binding site are thought to be very close to each other [43] (see Figure 3).

Type II compounds are not generally substrates for cytochrome P-450. Only one or two Type II compounds, e.g. aniline [33], pyridine

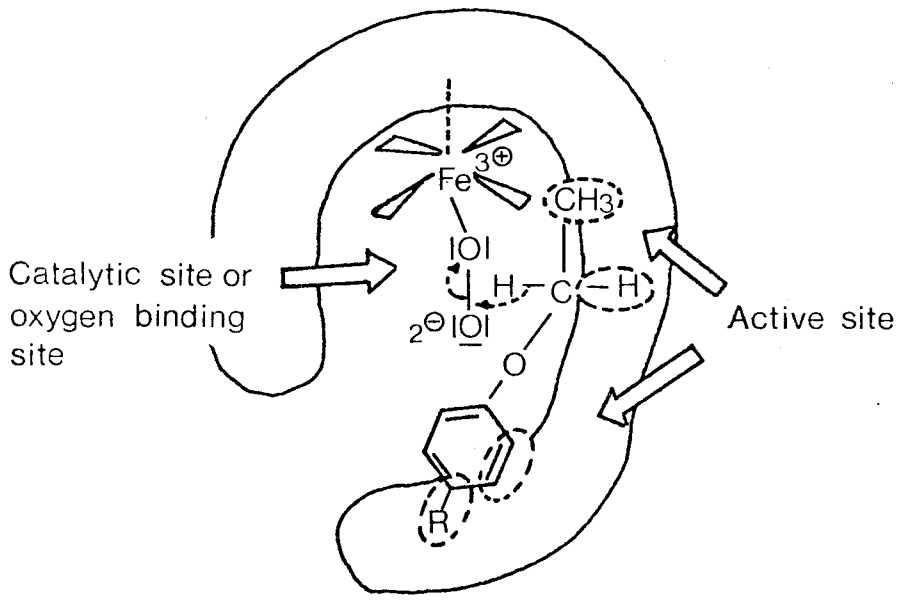

Figure 3: Hypothetical scheme of the transition state of cytochrome P-450-catalyzed mono-oxygenations of Type I compounds (i.e. substrates) (43)

and nicotinamide [45], are known exceptions to this statement and they appear to bind as Type I substrates to a small extent.

The type IR (Reverse Type I) (see Figure 2) difference spectrum is thought to arise as a result of the interaction of selected compounds with a site other than the substrate binding site and heme iron [34]. For example, Type IR compounds do not compete with aniline or carbon monoxide for binding to the heme of ferrocytochrome P-450. The Type IR spectral change is, therefore, the spectral manifestation of interaction of a class of compounds (not very often substrates) with the apo-enzyme component of cytochrome P-450 [46]. As the Type IR difference spectrum is in appearance exactly the opposite of a Type I spectral change, the spectral change appears to arise from a decrease in the high spin state of the iron of cytochrome P-450 (loss of absorbance at ca. 390 nm) and an increase in the low spin state of the heme of cytochrome P-450 (increase of absorbance at ca. 420 nm). Type IR compounds also decrease the activity of NADPH-cytochrome P-450 reductase as there is a decrease in the rate of reduction of the cytochrome P-450-IR complex; the decrease in activity appears to be directly related to the magnitude of spectral change with cytochrome P-450 [36].

Type IR compounds are not often substrates for hepatic cytochrome P-450. When a Type IR compound has been found to be a substrate, it has been found to possess a small Type I binding component, e.g. phenacetin [36]. There are some compounds, e.g. hexobarbital, which at low concentrations bind to hepatic cytochrome P-450 with the production of a Type I difference spectrum, but at

higher concentrations (5-fold increase) elicit a Type IR spectrum.

1.1.1b Oxidative reactions

The oxidative metabolism of a xenobiotic by cytochrome P-450 can be viewed as essentially involving an initial hydroxylation reaction. The overall reaction*, as shown earlier, generally is of the following form [47]:

$$NADPH + H^+ + RCH_3 + O_2 \longrightarrow RCH_2OH + NADP^+ + H_2O$$

Several examples of cytochrome P-450 dependent hydroxylation reactions are shown in Figure 4 [48]. As can be seen, in many cases, the cytochrome P-450 dependent metabolism of a substrate, although involving an initial hydroxylation reaction, yields a final rearrangement product of different structure.

The source of the hydroxyl group for cytochrome P-450 dependent hydroxylations has been shown to be molecular oxygen. One atom of the oxygen molecule is incorporated into the substrate, while the other atom is reduced to water** [50]. The reducing equivalents, which are required for the oxidative metabolism of a substrate by microsomal cytochrome P-450, are most effectively supplied by NADPH, but can also be supplied by NADH, ascorbate and other electron donors to a lesser extent.

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*As this reaction involves the oxidation of the substrate and the oxidation of NADPH, the cytochrome P-450 enzyme system is also referred to as 'the mixed function oxidase' [49].

**Hence, cytochrome P-450 is also known as a 'monooxygenase' enzyme, in contrast to a dioxygenase (synonym: true oxygenase) where both oxygen atoms are incorporated into the substrate.

Figure 4: Oxidation reactions catalyzed by cytochrome P-450 (48)

Aliphatic hydroxylations

$$R\text{-}CH_3 \xrightarrow{OH} R\text{-}CH_2OH + H^+$$

Aromatic hydroxylations

$$NHCOCH_3 \xrightarrow{OH} NHCOCH_3$$

N-dealkylation

$$R\text{-}NH\text{-}CH_3 \xrightarrow{OH} [R\text{-}NH\text{-}CH_2OH] \longrightarrow RNH_2 + CH_2O \text{ (formaldehyde)}$$

O-dealkylation

$$R\text{-}O\text{-}CH_3 \xrightarrow{OH} [R\text{-}O\text{-}CH_2OH] \longrightarrow ROH + CH_2O \text{ (formaldehyde)}$$

Deamination

$$R\text{-}CH(NH_2)\text{-}CH_3 \xrightarrow{OH} [R\text{-}C(OH)(NH_2)\text{-}CH_3] \longrightarrow R\text{-}CO\text{-}CH_3 + NH_3$$

Sulphoxidation

$$R\text{-}S\text{-}R' \xrightarrow{OH} [R\text{-}SOH\text{-}R']^+ \longrightarrow R\text{-}SO\text{-}R' + H^+$$

N-Oxidation

$$(CH_3)_3N \xrightarrow{[OH]} [(CH_3)_3NOH]^+ \longrightarrow (CH_3)_3NO + H^+$$

Some general rules about the site at which xenobiotics are oxidized have been made on the basis of numerous comparative experiments [51]:

(i) π-Systems, either as an isolated double bond or as an aromatic ring system, undergo epoxidation.

(ii) CH-bonds are hydroxylated in the preferential sequence tertiary > secondary > primary. The difference in rates across this series is large.

(iii) Heteroatoms bearing a proton (e.g. > NH or SH) can by hydroxylated or can be oxidized at a lone electron pair, yielding N $\longrightarrow$ O or S $\longrightarrow$ O.

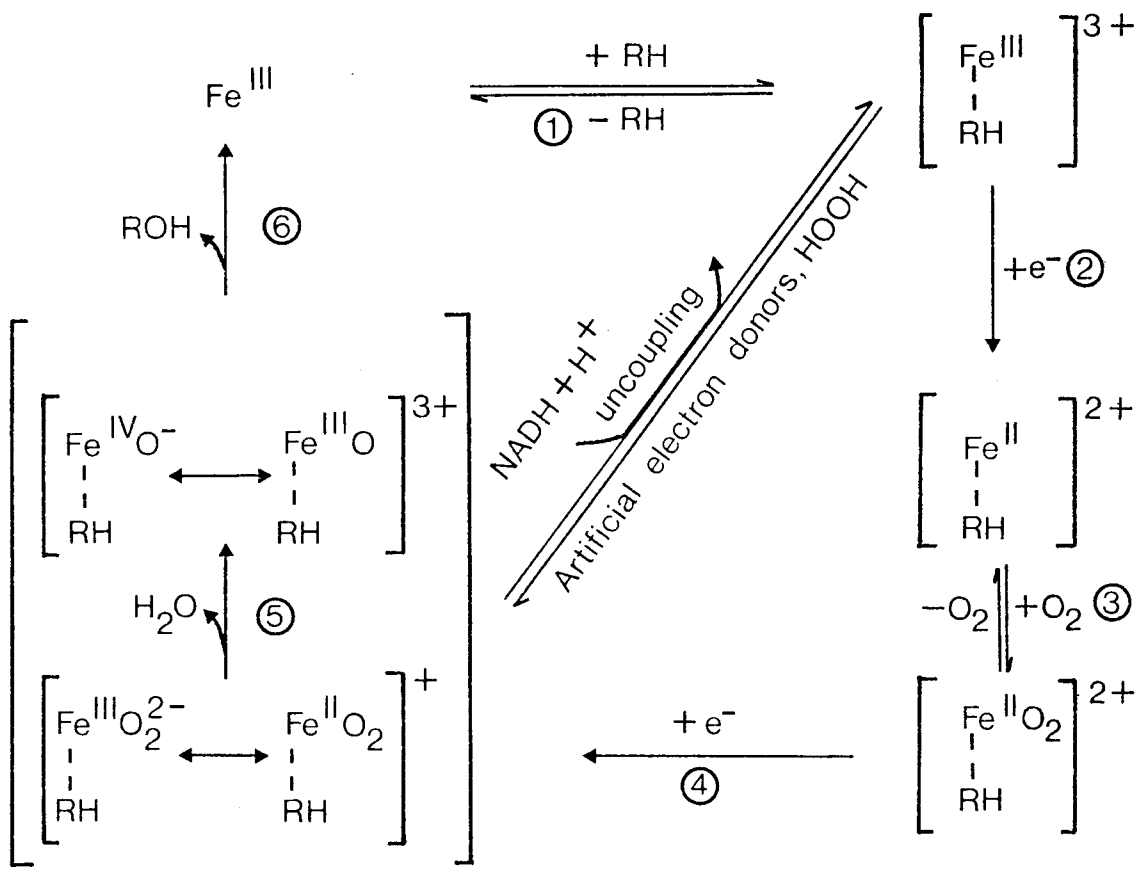
Proposed mechanism of cytochrome P-450 dependent oxidative metabolism of xenobiotics.

Despite the observations that the oxidative metabolism of xenobiotics by cytochrome P-450 is carried out on such a wide variety of substrates and that at least five different species of hepatic cytochrome P-450 exist in a single tissue from one species, the mechanism of the oxidative metabolism of all xenobiotics seems to be identical for all forms of cytochrome P-450 [52].

The mechanism of action of cytochrome P-450 in oxidative drug metabolism is generally thought to include the steps outlined in Figure 5 and presented below. This sequence was proposed initially by Estabrook et al. [53,54] and has subsequently been extended upon by Coon et al. [55] and Ullrich [52] and is summarised as follows:

Step 1: The binding of the substrate to the active site of cytochrome P-450 (viz. Type I binding, as described earlier) is the triggering event for the monooxygenation process [52].

Figure 5: The postulated mechanism of cytochrome P-450 dependent oxidative metabolism (52,55,56)



Fe^{III} - ferric form of cytochrome P-450

Fe^{II} - ferrous form of cytochrome P-450

RH - substrate

ROH - hydroxylated product

The ferric form (Fe^{3+}) of cytochrome P-450, in a low spin form, interacts with a molecule of organic substrate (RH) to form a high spin ferric enzyme – substrate complex (Fe^{3+}– RH).

Step 2: Electrons from NADPH are transferred via NADPH-cytochrome P-450 reductase to the ferri-cytochrome P-450 – substrate complex to form the ferrous enzyme – substrate complex (Fe^{2+}– RH).

Step 3: The ferrous enzyme – substrate complex reacts with molecular oxygen to form a ternary complex (Fe^{2+}– O_2– RH).

Step 4: The oxyferrocytochrome P-450 – substrate complex undergoes a second stage of reduction to yield a superoxide ferrous enzyme – substrate intermediate, which is in resonance with the hydroperoxo-ferric form of the enzyme – substrate complex [56,57]. This second electron may be donated by either NADH via NADH-cytochrome $\underline{b}_5$ reductase and cytochrome $\underline{b}_5$ or by NADPH via NADPH-cytochrome P-450 reductase. The most efficient transfer of the second electron occurs with NADH as electron donor [58]. The reduction with the second electron is the rate limiting step in the case of microsomes from phenobarbital pretreated rats [see under 'Induction'] for the metabolism of substrates such as hexobarbital, but the rate limiting step is an earlier one, viz. the reduction with the first electron (Step 2) prior to oxygen binding, in the case of microsomes from untreated rats for this substrate [53,54].

Step 5: It is postulated that the hydroperoxo-ferric enzyme –

substrate complex decomposes readily by splitting of the O—O bond to produce water and a ferric enzyme-monooxygen species which is in resonance with the ferryl ion complex ($Fe^{4+}O^{-}$); the latter complex is considered to contain the active oxygen or oxenoid species [59].

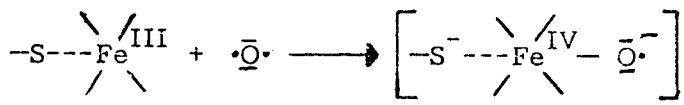
Step 6: In the last step of the reaction the active oxygen is transferred to the substrate and the resulting hydroxylated product is released from cytochrome P-450 with the regeneration of the low spin ferric form of cytochrome P-450. A special situation, however, arises if the substrate cannot react with the active oxygen to form an oxidized product and a phenomenon, known as uncoupling, arises [see section on 'Uncouplers'].

Active oxygen donors for hepatic microsomal cytochrome P-450.

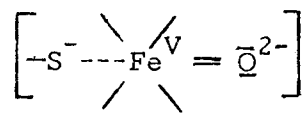
Several years ago it was discovered that hepatic microsomal cytochrome P-450 could catalyze monooxygenations *in vitro* without molecular O_2, or NADPH, provided that certain oxidizing agents were present [52]. Initially, it was shown that hepatic microsomal cytochrome P-450 could catalyze the hydroxylation of various substrates, e.g. drugs, amines, carcinogens and fatty acids in the presence of organic hydrogen peroxides without NADPH and oxygen [60 - 64]. Subsequently sodium periodate, sodium chlorite, cumene hydroperoxide and iodobenzene were also found to support the hydroxylation of several substrates by hepatic microsomal cytochrome P-450 *in vitro* to a similar extent as found with NADPH and organic hydroperoxide [56,62,65]. This finding has been further supported by the obser-

vation that purified hepatic microsomal cytochrome P-450 catalyzes the hydrogen peroxide-dependent hydroxylation of a variety of substrates in the absence of NADPH, NADPH-cytochrome P-450 reductase, and molecular oxygen [66].

Presumably these oxidizing agents can directly provide cytochrome P-450 with the same active oxygen species involved in cytochrome P-450 dependent reactions. These oxidizing agents apparently act as oxene donors using cytochrome P-450 as an oxene transferase. More clear-cut evidence as to the active oxygen species was obtained when it was found that iodosobenzene could act as an oxene donor [65], because the iodoso-group can provide only one oxygen atom for the cytochrome P-450 catalyzed monooxygenations, whereas all the organic hydroperoxides could release an oxygen atom as well as a hydroperoxide anion [52]. This has resulted in the postulation that the active oxygen complex or oxenoid species is an $[FeO]^{3+}$ species with the possible electronic structures of this complex which can be depicted as follows: [52]



OR



Stoichiometry of the hepatic microsomal oxidative metabolism of xenobiotics by cytochrome P-450

As shown earlier, the hydroxylation of drugs (xenobiotics) by hepatic microsomal cytochrome P-450 requires equivalent amounts of

NADPH, oxygen and substrate [see Section 1.1.1b]. Therefore, a 1:1:1 stoichiometry should exist in theory between substrate disappearance (or metabolite production), oxygen consumption and NADPH oxidation [69]. However, experimentally in dealing with hepatic microsomes, the expected stoichiometry for NADPH oxidation or oxygen consumption to metabolite formation was seldom achieved. A marked improvement in achieving the expected stoichiometry is obtained when rates of microsomal NADPH oxidation in the presence of a substrate are corrected for rates in the presence of the substrate under an atmosphere of $CO:O_2$ (80:20, v/v).

The endogenous CO-insensitive NADPH oxidase activity is apparently due to the autoxidation of cytochrome P-450 [55] which results in the formation of H_2O_2 in liver microsomes* [70], the metabolism of endogenous compounds, other microsomal enzymes or the insensitivity of some cytochrome P-450 reactions to CO.

Therefore, subject to correcting for background rates of NADPH oxidation [71], the oxidation of NADPH has provided a convenient <u>in vitro</u> tool for monitoring the total rate of metabolism of a substrate by hepatic microsomal cytochrome P-450. However, one must be aware that even when using this correction, one finds many cases in which the stoichiometry of NADPH oxidized to product formed is not what one would expect [see following section on 'Uncoupling'].

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*The production of H_2O_2 accounts for 50% of the NADPH oxidized and oxygen utilized in the absence of exogenous substrates in phenobarbital pretreated rats [72].

Uncoupling of the hepatic microsomal cytochrome P-450 enzyme system

With regard to the hepatic microsomal cytochrome P-450, the phenomenon of uncoupling refers to the transfer of electrons from NADPH to oxygen without the concomitant oxidation of the substrate [73]. As the oxygen atom is not incorporated into the substrate, oxygen is reduced either to water or to H_2O_2 [28].* Uncouplers (i.e. compounds that give rise to the uncoupling of cytochrome P-450) can be classified as

(i) full uncouplers, or

(ii) partial uncouplers.

Full uncouplers are compounds which bind to hepatic cytochrome P-450 in a Type I manner, but are not hydroxylated. This type of uncoupler is very rare, the sole known example being perfluoro-n-hexane [74], where the C-F bonds totally resist oxidative attack by the active oxygen species [see Figure 5, Steps 5 and 6].

Far more common are partial uncouplers, where a portion of the reducing equivalents is used for substrate hydroxylation and a portion is used for H_2O_2 or H_2O formation, i.e. uncoupling occurs in parallel with hydroxylation. Examples of partial uncouplers are hexobarbital and n-hexane [75]. As more and more substrates are being identified as partial uncouplers of hepatic cytochrome P-450, it would appear that uncoupling is more of a rule than an exception among substrates of microsomal cytochrome P-450. [52].

The mechanism of uncoupling of hepatic microsomal cytochrome P-450 is thought to be as follows:

The uncoupler binds to the active site (Type I binding site)

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*The reduction of the active oxygen species to water or the liberation of the active oxygen species as H_2O_2 indicates a different mechanism of oxygen activation[28].

of ferricytochrome P-450 to yield an enzyme-substrate complex and as a consequence stimulates the reduction of ferri-cytochrome P-450. The reduced cytochrome P-450 substrate complex then binds and activates oxygen. At this point the pathway diverges from that for normal cytochrome P-450 dependent oxidative metabolism and the activated oxygen is released as H_2O_2 without substrate hydroxylation.

1.1.1c Reductive reactions of hepatic cytochrome P-450

The ability of cytochrome P-450 to catalyze reductive reactions was reported as early as 1957 by Fouts and Brodie [76], who observed the anaerobic reduction of the nitro group of chloramphenicol and p-nitrobenzoic acid by liver microsomes. Lipophilic organic azo-compounds and N-oxides have since been reported to be reduced by hepatic cytochrome P-450 to yield the corresponding amines [77,78,79], and a series of halogenated alkanes were found to be reductively dehalogenated. (See Figure 6).

Initially, it was uncertain as to whether hepatic cytochrome P-450 or NADPH-cytochrome P-450 reductase mediated these one electron reductions. However, the observation that CO and metyrapene strikingly inhibited (by > 70%) the anaerobic metabolism of carbon tetrachloride, strongly suggested the participation of cytochrome P-450 [80] [see Section on 'Inhibitors of hepatic microsomal cytochrome P-450']. Subsequently halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was shown to be reductively defluorinated by cytochrome P-450. The reaction was supported by NADPH and inhibited by CO [81] and by using a purified, reconstituted hepatic cytochrome P-450 enzyme system [82].

Research into the reductive mechanism of polyhalogenated aliphatic compounds has resulted in the proposed reaction scheme shown in Figure 6 [81,83].

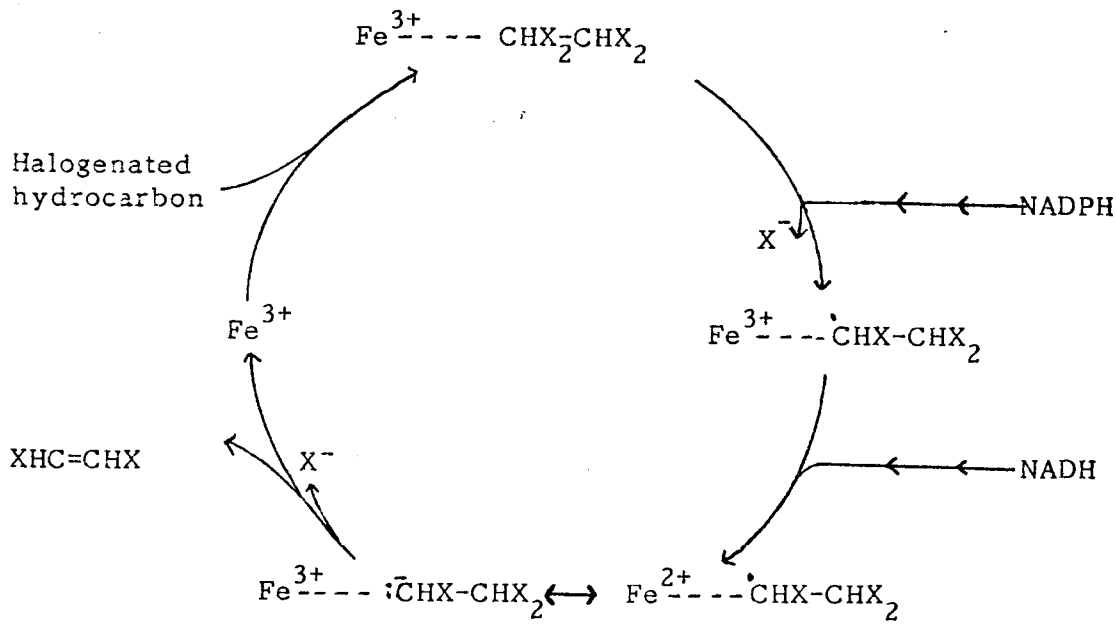
Until very recently it was believed that anaerobic conditions were a prerequisite for hepatic cytochrome P-450 mediated reductive metabolism of substrates, but it was recently found that the microsomal reductive metabolism of pentachloro- and hexachloroethane occurred at normal oxygen tension [84]. This unexpected finding poses the question as to what extent these reductive reactions occur *in vivo*. Relevant to this question is the consideration that under certain conditions, e.g. anaesthesia, oxygen levels may be lower than normal and thus may provide suitable conditions for the reductive capability of hepatic cytochrome P-450.

Intermediates and metabolites of the reductive pathway of certain nitro-containing compounds and polyhalogenated compounds by hepatic cytochrome P-450 are highly reactive and may cause deleterious effects. For example, the one-electron reduction of carbon tetrachloride gives rise to the trichloromethyl radical; which is apparently responsible for the carbon tetrachloride mediated lipid peroxidation and consequent destruction of biological membranes and resulting cellular necrosis [85]. Furthermore, the reductive metabolism of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) gives rise to 2-chloro-1,1,1-trifluorethane which is thought to be responsible for the halothane mediated hepato-toxicity [86].

1.1.1d The electron transport pathways to cytochrome P-450

For both oxidative and reductive metabolism of xenobiotics, cytochrome P-450 requires intermediary electron transport carriers

Figure 6: Proposed mechanism of the microsomal reductive dehalogenation of halogenated hydrocarbons (81,83)



Fe^{2+},Fe^{3+} represents the heme centre of cytochrome P-450

X = Br, Cl or F

H = can be hydrogen or a halogen

Examples of substrates and their respective products of cytochrome P-450 mediated reductive metabolism are halothane, pentachloroethane, and hexachloroethane and 2-chloro-1,1-difluoroethylene, trichloroethylene and tetrachloroethylene, respectively.

in order to accept the reducing equivalents from NADPH and NADH.

Reducing equivalents to cytochrome P-450 can arise from:

(i) NADPH, through the flavoprotein, microsomal NADPH-cytochrome P-450 reductase (also known as NADPH-cytochrome c reductase), and

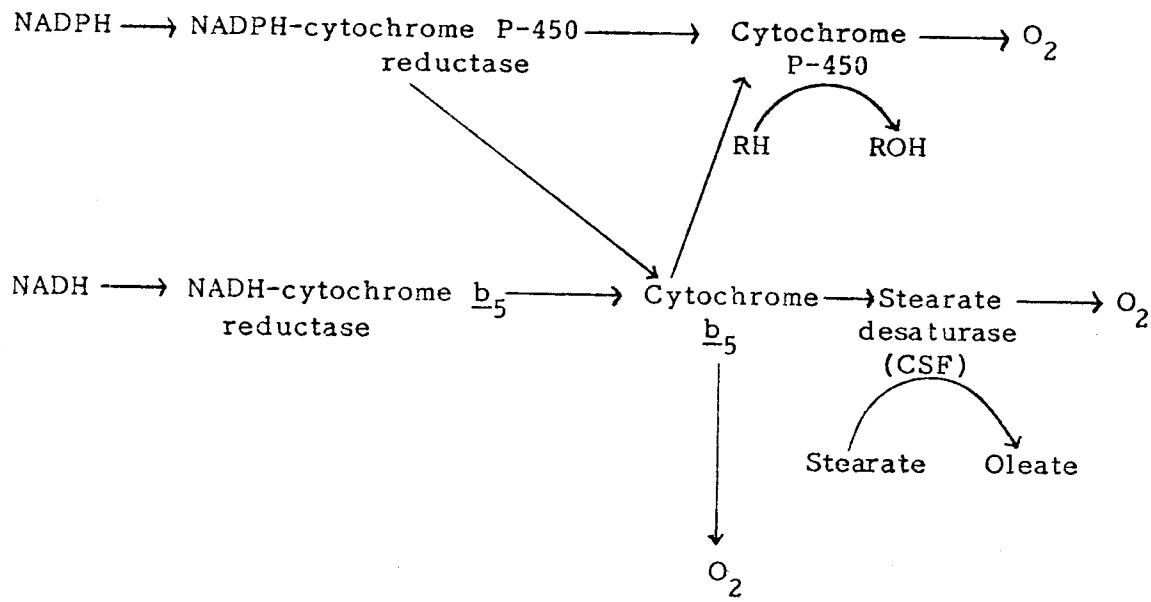
(ii) NADH, via NADH-cytochrome b_5 reductase (also known as NADH-cytochrome c reductase) and cytochrome b_5 (see Figure 7).

The NADPH-dependent electron transfer pathway

NADPH-cytochrome P-450 reductase, which transfers the reducing equivalents from NADPH to cytochrome P-450, is an amphipathic protein with a molecular weight of approximately 80 000 daltons [89]. As the stoichiometric ratio of cytochrome P-450 to NADPH-cytochrome P-450 reductase in the hepatic endoplasmic reticulum is usually 20 : 1, but can even be as high as 40 : 1 [90], it appears that one molecule of the reductase has to serve a large number of molecules of cytochrome P-450 for efficient catalysis [91].

The purified, reconstituted cytochrome P-450 enzyme preparation has been shown to require not only hepatic cytochrome P-450 and NADPH-cytochrome P-450 reductase (for the microsomal electron transfer from NADPH) but also a lipid fraction [92]. Recently, it has been discovered that the microsomal lipid fraction can be replaced by various phospholipids, of which phosphatidyl choline proves to be the most effective [93]. Changes in the lipid fluidity caused by alteration of the ratio of unsaturated to saturated fatty acids can affect the extent of interaction between NADPH-cytochrome P-450 reductase and cytochrome P-450, resulting in decreased or increased electron flow [94]. The lipid fraction does not contain components

Figure 7: Cytochrome P-450 and b_5 electron transport pathways (87, 88)



CSF - Cyanide-sensitive factor

RH - Substrate

ROH - Hydroxylated product

⟶ Indicates electron flow

↷ Indicates metabolism

that readily undergo redox reactions, but appears to be required for obtaining the proper conformation of cytochrome P-450 [93,95,96].

NADH-dependent electron transfer pathway

Hepatic microsomes also contain a NADH-dependent electron transfer pathway which supplies reducing equivalents for the desaturation of fatty acids and other processes [87, 97] (see Figure 7).

Electron flow proceeds from NADH via NADH-cytochrome b_5 reductase and cytochrome b_5 to a number of acceptors, e.g. desaturase enzymes which catalyze the biosynthesis of unsaturated fatty acids in the presence of oxygen.

Stearate desaturase (also known as the cyanide-sensitive factor, CSF) which is a terminal oxidase in the conversion of stearoyl-CoA to oleate, has been found to interact with xenobiotics, e.g. halothane, enflurane and methoxyflurane [98]. It has not yet been established whether any of the other electron acceptors from cytochrome b_5 involved in the biotransformation of endogenous substrates, can also interact with xenobiotics.

It would appear that there is some interaction between the NADH- and NADPH-dependent microsomal electron transfer pathways because, although NADH alone is unable to support most cytochrome P-450 dependent reactions to an appreciable extent (ca. 10%), when both NADH and NADPH are present, a synergistic effect on microsomal hydroxylation is observed. This observation suggests that NADH can donate electrons to the hydroxylation pathway when NADPH is also present, or can prevent the funneling of electrons from NADPH to other pathways [99]. On the latter line, the addition of stearoyl-

CoA to divert the electrons to the fatty acid desaturation pathway, causes the synergistic effect of NADH on drug oxidation to be revoked [58].

NADH plays an important role when the uncoupling of the cytochrome P-450 enzyme system occurs (see Section 1.1.1b). The production of H_2O_2 requires reducing equivalents which are provided by NADPH via NADPH-cytochrome P-450 reductase. If NADPH is present, (which reflects the conditions *in vivo*) an increased electron flow is observed, from NADH via NADH-cytochrome b_5 reductase and cytochrome b_5 to the uncoupled active oxygen [73]. Therefore under these conditions, NADH can save reducing equivalents from NADPH for their normal function on cytochrome P-450 [100].

1.1.2 The conjugating enzymes

The glutathione-, glucuronyl- and sulfo-transferases are the major enzymes in Phase II of drug metabolism. These enzymes all catalyze conjugation reactions, which produce a dramatic change in the solubility of the substrate*, making it highly hydrophilic and easily excretable.

It is not imperative that Phase II reactions follow Phase I reactions. In fact Phase II modifications can in some instances provide the first step in the metabolism of a xenobiotic such as with 1,2-dibromoethane, or paracetamol.

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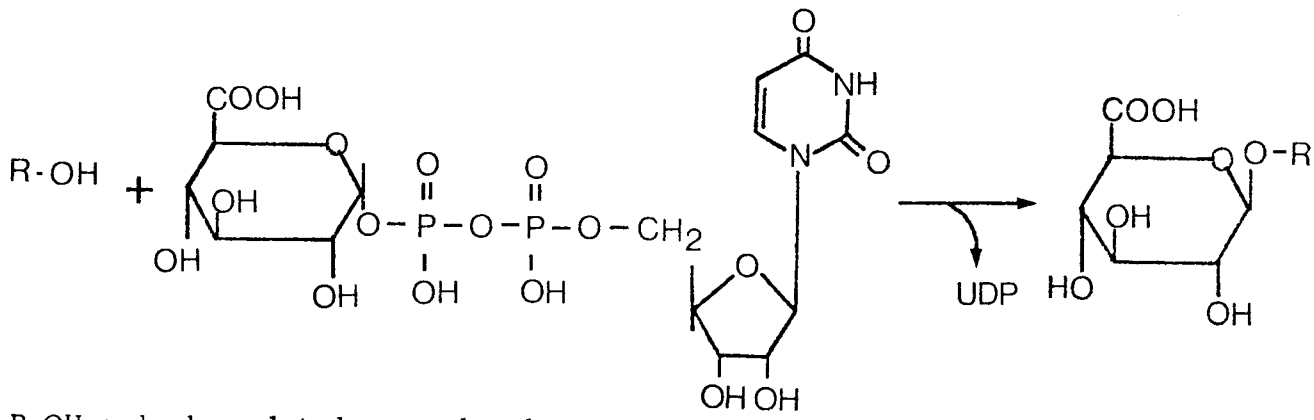
*It must be noted that compounds (intermediates) undergoing Phase II reactions are regarded as second substrates of the conjugating enzymes; the first substrate being the small polar molecule (e.g. glutathione or sulfate) with which the resulting conjugation process occurs.

There are three major types of conjugation, viz, glucuronidation, sulfation, and conjugation with glutathione, and these will be considered in detail below.

1.1.2a Conjugation with glucuronic acid

Glucuronidation involves the metabolic conversion of xenobiotics and of numerous endogenous compounds which contain functional groups, such as -OH, -COOH, $-NH_2$ and -SH, to form glucuronides, which then are excreted from the body in either the urine or the faeces [101]. Glucuronidation followed by excretion is quantitively one of the most important pathways of detoxification in man.

The glucuronidation reaction can be represented as follows[102]:



R-OH = hydroxylated second substrate.

Glucuronidation is catalyzed by the membrane-bound UDP-glucuronyl-transferases which require glucuronic acid as the first substrate. The glucuronic acid must be in the form of the high energy compound, uridine diphosphoglucuronic acid (UDP-glucuronic acid)

The conformation of the product suggests a S_N-2 type of nucleophilic displacement of the UDP-moiety with resulting inversion [103].

The resulting conjugate is more polar than the second substrate, primarily as a result of the introduced carboxyl group (viz. on C-6 of the glucuronic acid), which at the pH of most physiological fluids remains unprotonated and thus allows salt formation and facilitates excretion in either the urine or faeces [103].

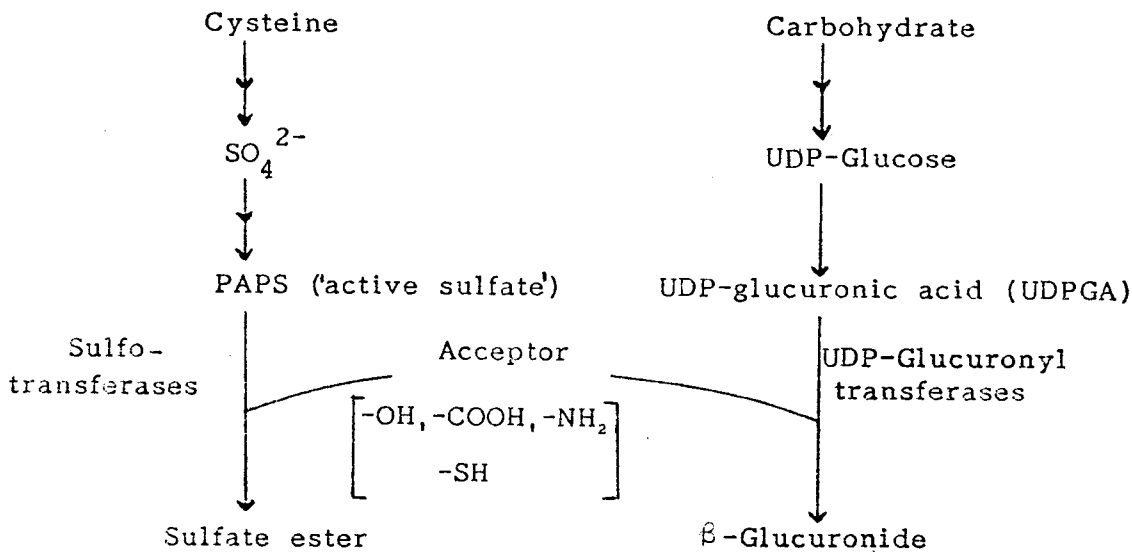
An interesting feature of the glucuronidation reaction is the large structural diversity of the substrates that the glucuronyl transferases accept. This diversity is in part the result of the active site of the UDP-glucuronyl transferase accommodating a wide range of geometric shapes or sizes, and in part of the existence of more than one transferase. Investigations into the latter alternative were previously hampered by the dramatic decrease in UDP-glucuronyl transferase activity *in vitro* following the isolation of microsomes. Activity could be increased by treatment with detergent or phospholipases, but one was unsure of the relevance of results with such preparations were to the *in vivo* situation [104,105]. The glucuronyl transferases have since been isolated and purified, and it has been shown that at least two forms of these transferases exist [106].

1.1.2b Conjugation with sulfate

Sulfate-transferases, which are located in the cytoplasm, often compete with the microsomal glucuronyl transferases for the same substrates although the sulfo-transferases generally show a preferance for aliphatic or phenolic hydoxyl groups. Three forms of aryl sulfo-transferases [107] and three forms of steroid sulfo-transferases [108] have been isolated to homogeneity from rat liver. All of the sulfo-transferases require as first substrate 'active sulfate'

in the form of PAPS (3'-phospho-adenosine 5'-phosphosulfate). Active sulfate is produced *in vivo* from cysteine rather than from sulfate which is poorly absorbed from the gut. Since cysteine is required for other purposes, e.g. protein synthesis and glutathione formation, it is more likely that the production of PAPS can be impaired than the production of UDP-glucuronic acid. The latter is generated from carbohydrate sources [109] (Figure 8).

Figure 8: Formation of UDP-glucuronic acid and PAPS for conjugation [109,110]



1.1.2c The formation of reactive sulfate ester and glucuronide conjugates.

Although conjugation reactions usually detoxify reactive compounds or intermediates, it does happen in some cases, that conjugates prove to be more reactive and toxic than the parent compound, as for example, with the N-O-glucuronide and N-O-sulfate ester of N-hydroxy-N-acetylaminofluorene [12,13]. Of the two conjugates, the sulfate ester is more toxic to the liver and is proposed to be the ultimate carcinogen after the administration of aminofluorene* [12]. Reactive glucuronides are instrumental in kidney and bladder toxicity, apparently from the high concentrations of the conjugates, e.g. the N-O-glucuronide of N-hydroxy-phenacetin, at these sites [101]. Another contributing factor is that the glucuronides are hydrolyzed in the kidneys by the low pH of the urinary fluid or by β-glucuronidase with the release of reactive, electrophilic derivatives. These species can, in turn, bind covalently to DNA, RNA and protein of the bladder epithelium [101].

1.1.2d Conjugation with glutathione

The glutathione-S-transferases catalyze reactions between the thiol group of glutathione (GSH) and a wide variety of compounds which have in common only an electrophilic site and/or region of the molecule that can be regarded as hydrophobic [111]. Although these transferases possess broad and overlapping specificities

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*It must be noted that the balance between glucuronidation and sulfation may be easily altered in vivo, e.g., pretreatment of animals with phenobarbital increases glucuronidation without affecting sulfation [101].

of their second substrates (glutathione is regarded as the first substrate), there appears to be an almost absolute specificity for glutathione as the first (thiol) substrate [112]. The glutathione transferases are regarded as playing a very important role in the detoxification of alkylating agents and xenobiotics by substituting electrophilic attack on glutathione for attack on the nucleophilic sites of cellular macromolecules, such as protein, DNA, RNA [113,114].

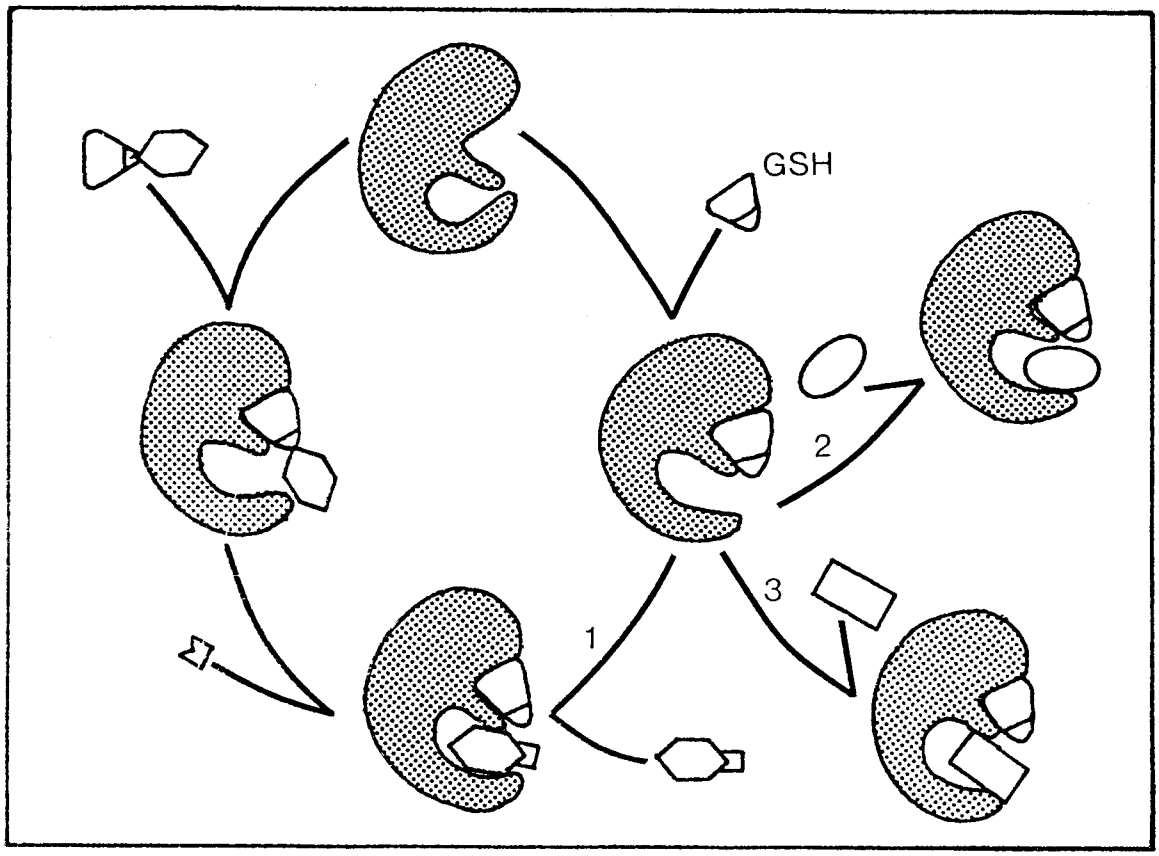
Jakoby et al. [111,115] has labelled the glutathione-S-transferases as a 'triple threat in detoxication' and this is motivated as can be seen in Figure 9.

The three modes of detoxification of the glutathione-S-transferases are briefly as follows:

1. The normal catalytic cycle: the first substrate, glutathione, binds to the enzyme followed by the binding of the second substrate; conjugation occurs and the products are released sequentially. The whole reaction follows a random sequential mechanism [116].
2. Compounds (ligands) that do not contain a sufficiently electrophilic centre to undergo conjugation, bind reversibly to the enzyme. Thus the glutathione transferases can act as putative storage and transport agents. For example, ligandin influences the net uptake of hepatic bilirubin by this process [117].
3. Finally, highly reactive compounds can bind covalently to the glutathione-S-transferases in an irreversible manner, which results in the inactivation of the enzyme.

The glutathione-S-transferases appear to catalyze two main types of reactions with glutathione, i.e. normal catalytic cycle

(i) nucleophilic substitution, e.g. by the conjugation of



= glutathione

= second substrate

= weak ligand

= highly reactive ligand

= conjugate

Figure 9: Diagrammatic illustration of the three modes of detoxification in which the glutathione transferases participate (see text for details) (106).

glutathione with alkyl, aryl or aralkyl halides, thus neutralizing their electrophilic sites by halide displacement;

(ii) nucleophilic addition, as seen by the conjugation of glutathione with epoxides or α,β-unsaturated compounds (114).

Recently, an additional function of the glutathione-S-transferases has been reported. These enzymes are able to promote a nucleophilic attack on electrophilic oxygen by binding organic hydroperoxides and glutathione in close proximity. This has been described as the non-selenium dependent glutathione peroxidase activity of the glutathione-S-transferases (118).

Glutathione conjugates are not excreted as such, but are further modified as follows: The glutathione conjugate undergoes cleavage, losing the γ-glutamyl and then the glycyl residue to produce a cysteine conjugate. The latter mentioned product is acetylated at the amino group of cysteine in an acetyl-CoA linked reaction, to yield a mercapturic acid derivative, which is non-toxic and highly polar, and is excreted via the kidneys (115).

Glutathione conjugation is very important in vivo in view of the high concentration of the glutathione-S-transferases and of glutathione present, particularly in the liver. The glutathione-S-transferases account for 10% of the soluble extractable protein of the liver (111), while the glutathione concentrations in most tissues range between 3 and 10 mM (119). Furthermore, glutathione conjugation does not require the formation of an activated form of the first substrate (114), as does glucuronidation and sulfation. In

many cases glutathione and an electrophilic substrate are able to react non-enzymically at a very slow rate, but this reaction is usually accelerated substantially by the glutathione-S-transferases[120]. It appears that one function of the glutathione-S-transferase active site(s) would be firstly to promote ionization of the sulfhydryl group of glutathione by lowering its pK value of 9.2 and thereby rendering it a more effective nucleophile [112]. A further function is to bind the electrophilic substrate which then can preferentially react with ionized glutathione rather than with other cellular nucleophiles [112].

Six distinct species of glutathione-S-transferases have been found in rat liver cytosol* and have been named in the reverse order of elution from an ion exchange column, viz, glutathione-S-transferases AA, A, B, C, D and E. Glutathione-S-transferase B has been shown to be immunologically indistinguishable from ligandin [123].

1.1.3 Induction of hepatic drug metabolizing enzymes

There are a host of factors that influence the levels of hepatic drug metabolizing enzymes in mammalian species. Firstly, there are great differences in drug metabolic ability between species and between different strains of one species [124,125]. Secondly, factors such as age [126], sex (hormonal differences) [125,126], nutritional status [127], exposure to low temperatures [128], stress [129] and gamma irradiation [130] can affect the levels of drug metabolizing

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*Recently, Morgenstern et al.[121,122] have characterized microsomal (i.e. membrane bound) glutathione-S-transferase activity which previously was thought to be as a result of cytosolic contamination.

enzymes such as cytochrome P-450. Thirdly, it has been found over the last 25 years that there is a vast number (>300) of pharmacologically and chemically unrelated compounds which have been shown to induce drug metabolizing enzymes [37]. Such compounds include a wide variety of xenobiotics such as barbiturates, insecticides, analgesics and polycyclic hydrocarbons.

The term 'enzyme induction' is generally taken to describe the process which increases the rate of synthesis of an enzyme relative to its normal rate of synthesis in the uninduced organism [38]. However, it must be stated clearly that in the field of drug metabolism, the term 'induction' is often used in a loose sense, viz. to describe elevated activity of (a) drug metabolizing enzyme(s) in a treated animal compared to an equivalent control, without reference to the mechanism behind this process.

The specific effects of selected inducing agents on the drug metabolizing enzymes is discussed below:

1.1.3a Phenobarbital

Phenobarbital is the classic example of an inducing agent of drug metabolizing enzymes [131]. This compound causes increased proliferation of the smooth endoplasmic reticulum, increased liver weight and often strikingly increased metabolic ability towards xenobiotics (see Table 5). Administration of phenobarbital leads to:

(a) elevated levels of cytochrome P-450 as well as NADPH-cytochrome P-450 reductase [131];

(b) increased glucuronidation of one group of substrates and not another [132];

(c) increased levels of the glutathione-S-transferases (up to ca. 100% increase) [119].

Table 5: Hepatic parameters altered by phenobarbital and 3-methylcholanthrene induction* (48, 131)

Hepatic activity	Effect of inducer +	
	Phenobarbital	3-Methylcholanthrene
Cytochrome P-450 (P-450b)	+	?
Cytochrome P-448 (P-450c)	?	+
NADPH-cytochrome c reductase	+	0
NADPH-cytochrome P-450 reductase	+	0
Benzamphetamine N-demethylation	+	0
Hexobarbital hydroxylation	+	0
Steroid metabolism	+	0
3,4-Benzpyrene hydroxylation	+	+
Microsomal protein	+	±
Liver weight	+	±

*Hepatic microsomal preparations obtained from pretreated male rats were utilized.

+Key: + = increase;
± = slight increase;
0 = no change.

The form of cytochrome P-450 induced by phenobarbital pretreatment is known as cytochrome P-450b [133] and has the absorption maximum of its reduced CO-complex at 450-451 nm [134] (see Table 7). The levels of the total microsomal cytochrome P-450 can be increased 2- to 3-fold following phenobarbital pretreatment, while the rate of metabolism of selected substrates can be increased by a far greater amount (ca. 7-fold).

1.1.3b 3-Methylcholanthrene

Administration of 3-methylcholanthrene is an example of a polycyclic hydrocarbon inducing agent. Other polycyclic hydrocarbons that are well known as inducers of the drug metabolizing enzymes are β-naphthoflavone and benzpyrene [131].

Unlike phenobarbital treatment, the administration of 3-methylcholanthrene causes a selective stimulation of a few hepatic drug metabolizing enzymes, e.g. one form of cytochrome P-450, UDP-glucuronyl transferases [135] and glutathione-S-transferases [136], but gives rise to little or no increased proliferation of the smooth endoplasmic reticulum (see Table 5). The net result of 3-methylcholanthrene administration is the increased metabolism of relatively few substrates (when compared to phenobarbital administration)[131].

Induction by polycyclic hydrocarbons produce an altered form of cytochrome P-450 called cytochrome P-450c [133] or cytochrome P-448, as its reduced CO-complex has an absorption maximum at ca. 448 nm (see Table 7).

It has been shown that cytochrome P-450c* differs qualita-

*From this point the term 'cytochrome P-450b' and 'cytochrome P-450c' will be used to refer to the specific enzymes induced by phenobarbital and 3-methylcholanthrene (or β-naphthoflavone) respectively, and the term 'cytochrome P-450' will be used for the heterogeneous group of hemoproteins of that name.

Table 6: Effect of inducers on the metabolism of drugs in rat hepatic microsomes* (138,143,251)

Pre-treatment of rats	Benzphetamine N-demethylation	Ethylmorphine N-demethylation	3,4-Benzpyrene hydroxylation	Hexobarbital metabolism
None	100	100	100	100
Phenobarbital	730	450	400	ND
Pregnenolone -16α-carbo-nitrile	350	1000	650	ND
3-Methyl-cholanthrene	90	230	1400	ND
Spironolactone	ND	320	190	220

*Female Sprague-Dawley rats were utilized.

ND = Not Determined.

Table 7: Summary of five hepatic cytochrome P-450 species found in untreated and variously pretreated rats (133,140,250)

Form of cytochrome P-450	Molecular weight (daltons)	CO-binding spectrum (maximum)	Substrates	Inducing agent	N-terminal amino acid	C-terminal amino acid
P-450b	49 000	450	Benzamphetamine N,N-dimethylaniline Parathione	PB	glutamic acid	serine
P-450c	54 000	448	Polycyclic hydrocarbons 7-ethoxycoumarin Zoxazolamine	3-MC BP β-NF	isoleucine	leucine
P-450a	48 000	452	α-hydroxylation of testosterone	PB 3-MC	methionine	methionine
P-450	51 000	449-450	N-demethylation of ethyl morphine and aminopyrine	None PCN	-	-
P-450d	52 000	447	Poor catalytic activity before or after removal of the bound isosafrole metabolite	Iso-safrole	-	-

Abbreviations are: PB = phenobarbital; 3-MC = 3-Methylcholanthrene; BP = Benzpyrene; PCN = Pregnenolone-16α-carbonitrile; β-NF = β-naphthoflavone

tively from cytochrome P-450b, or the major form of cytochrome P-450 found in untreated rats, which is not elevated by either 3-methylcholanthrene or phenobarbital [131]. The substrate specificities of cytochrome P-450b and P-450c differ dramatically (see Tables 5, 6 and 7) from each other and from the substrate specificity of the major uninducible form of cytochrome P-450 found in untreated rats. The mechanism of induction of cytochrome P-450 is currently under investigation. The current proposal is that polycyclic hydrocarbons and phenobarbital interact at different cellular sites to bring about the induction of different forms of hepatic cytochrome P-450.

1.1.3c Pregnenolone-16α-carbonitrile (3 β-hydroxy-20-oxo-5 pregnene-16 α-carbonitrile)

This steroid derivative induces hepatic cytochrome P-450, enhances NADPH-cytochrome P-450 reductase activity*, causes proliferation of the smooth endoplasmic reticulum and an increment in liver weight [137].

That the form of cytochrome P-450 induced by pregnenolone-16 α-carbonitrile might be unique (different) was shown by Conney et al. [138] (Table 6), who demonstrated a significant difference in the substrate specificity of hepatic microsomes from pregnenolone-16 α-carbonitrile-treated rats for a variety of substrates compared to microsomes from untreated and variously pretreated rats.

Although there is a striking similarity between certain

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*While phenobarbital appears to produce a uniform induction of the reductase within the liver lobule, pregnenolone-16α-carbonitrile treatment results in greater induction of the reductase in the periportal hepatocytes [139].

properties of hepatic cytochrome P-450b and the form of cytochrome P-450 induced by pregnenolone-16α-carbonitrile, e.g. the identical spectral change as a result of their respective reduced CO-complexes [138], it has been shown by chromatography and polyacrylamide gel electrophoresis [140] that the form of cytochrome P-450 induced by pregnenolone-16α-carbonitrile is a different isoenzyme from hepatic cytochrome P-450b and P-450c.

1.1.3d Spironolactone

Spironolactone is a steroidal aldosterone antagonist that is widely used as a diuretic and anti-hypertensive [141,142]. Spironolactone pretreatment of rats results in an induction of hepatic microsomal NADPH-cytochrome P-450 reductase and an increase in the rate of reduction of the cytochrome P-450 substrate complex. In contrast to phenobarbital and 3-methylcholanthrene induction, spironolactone treatment results in decreased levels of hepatic cytochrome P-450 [143,144]. However, it appears that spironolactone may alter the mixture of forms of cytochrome P-450 present in hepatic microsomes since the substrate specificity of cytochrome P-450 is altered following spironolactone induction (see Table 6). Furthermore, spironolactone induction appears to be sex-dependent, with induction being greater in female than in male rats [143]. Pretreatment with this steroid has been found to prevent the toxicity of a variety of xenobiotics and carcinogens [145,146,147,148] presumably by lowering the levels of hepatic microsomal cytochrome P-450.

1.1.3e Untreated rats

The two major forms of cytochrome P-450 which are not induced

by agents such as phenobarbital, 3-methylcholanthrene or pregnenolone-16α-carbonitrile, have been purified from hepatic microsomes from untreated rats [148]. The M_r of the apoprotein moeities of these two forms of cytochrome P-450 is ca. 52 000 and the absorption maxima of the reduced CO-complex are 450 nm and 450.8 nm. Neither form is very active in catalyzing the metabolism of exogenous substrates.

1.1.3f The role of the multiple forms of cytochrome P-450 in the liver

The irrefutable presence of multiple forms of cytochrome P-450 in the liver raises the question why nature has supplied mammalian systems with several enzymes with a reasonable degree of specificity and not one relatively non-specific enzyme. Two possible advantages are as follows [52]:

- Firstly, an enzyme with a reasonable degree of specificity is more efficient in metabolizing low levels of toxic environmental compounds like the polycyclic hydrocarbons.
- Secondly, it is more economical for the cell rapidly to produce an enzyme which effectively metabolizes a xenobiotic present in the organism, than to synthesize large amounts of a non-specific and less efficient enzyme.

1.1.4 Inhibitors of the hepatic microsomal cytochrome P-450 system

Numerous substances are known to inhibit the metabolism of xenobiotics by liver microsomal cytochrome P-450 *in vitro* and *in vivo*. Generally, there are two major ways in which an inhibitor

can affect the enzymatic activity of cytochrome P-450 [43]:

(i) The inhibitor may bind to the substrate binding site and therefore compete with the substrate for binding to the enzyme (viz. competitive inhibition). This can occur with either alternate substrates, viz. compounds which elicit a Type I difference spectrum (see Table 4); or with 'dead-end inhibitors' such as perfluoro-n-hexane [74].

(ii) The inhibitor may bind as a ligand to the catalytic site, viz,. the iron atom [43] of cytochrome P-450 and thus interfere with oxygen binding (non-competitive inhibition) (see Figure 10). Any Type II compound competes with oxygen binding. One particularly effective and widely used inhibitor of oxygen binding is CO.

Some of the most potent inhibitors of cytochrome P-450 mediated reactions are those that bind to both the substrate binding site and the catalytic site (see Figure 10 and compare Figure 3). An example of this type of inhibitor is metyrapone [153,154], which inhibits binding of a substrate to the substrate binding site and of oxygen to the catalytic site, thus inhibiting the metabolism of a wide variety of substrates by cytochrome P-450. Metyrapone was originally though to be a specific inhibitor for the phenobarbital-inducible form of cytochrome P-450, viz. cytochrome P-450b, but recently has been shown to interact also with the forms of the enzyme induced by 3-methylcholanthrene and pregnenolone-16α-carbonitrile [155].

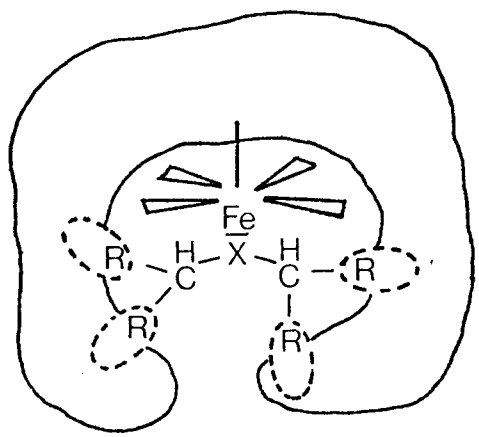

Figure 10: Possible mechanism of interaction of inhibitors with the active and catalytic sites of cytochrome P-450 (43) (see Figure 3).

A further type of inhibition of cytochrome P-450 mediated reactions involves compounds which are substrates of hepatic cytochrome P-450, but where a metabolic intermediate or a product of the cytochrome P-450 mediated reaction does not leave the enzyme, giving rise to a 'metabolic intermediate complex' (MI complex) [156] and thus preventing the enzyme from catalyzing further reactions. The MI complex, the formation of which requires NADPH and oxygen, is probably due to a ligand interaction of the metabolic intermediate with the heme of cytochrome P-450. Type I compounds can inhibit or reverse the formation of the metabolic intermediate complex, while Type II compounds cannot [156]. Compounds, e.g. isosafrole, that can form a MI complex, initially give rise to competitive inhibition due to their binding to cytochrome P-450, but later proceed to inhibit the enzyme in a non-competitive manner as a result of the interaction of the metabolic intermediate with the heme moiety of cytochrome P-450 [156].

Finally, in contrast to the more conventional types of inhibitors mentioned above, a new type of inhibitor has been observed which binds irreversibly to the active site of cytochrome P-450. This process causes the apparent degradation of cytochrome P-450, viz. the loss of this enzyme in the spectral assay for cytochrome P-450 (see Section 2). These inhibitors apparently modify the heme but not the apoprotein of this enzyme. Examples of compounds with the above-mentioned effect on hepatic microsomal cytochrome P-450 are 1,1,1-trichloropropane-2,3-oxide (TCPO) [157], vinyl chloride [158], fluroxene and allyl-iso-propylacetamide [283].

1.2 Drug metabolism and its relationship to chemical carcinogenesis

Recently it has been proposed that up to 80% of the cases of human cancer result from exposure to chemical carcinogens present in the environment. Despite their vast chemical differences, such carcinogens can be divided into two distinct categories based primarily on their chemical reactivity [160] and mode of action, viz:

(i) carcinogens such as methyl methanesulfonate [161] whose chemical structure allows them to act directly on their intracellular targets; and

(ii) chemicals (procarcinogens), e.g. the fungal aflatoxins, that require metabolism to a form (the ultimate carcinogen) which can react with the intracellular targets [162]. The aflatoxins are produced by Aspergillus flavus and are activated to a carcinogenic epoxide derivative by hepatic cytochrome P-450 [163]. In Figure 11 is a reaction scheme of the activation of aflatoxin b_1 by the hepatic microsomal cytochrome P-450 and the binding of this species to cellular macromolecules.

1.2.1 The relationship between chemical-mediated carcinogenicity, mutagenicity and toxicity

The question of the initiation of chemical carcinogenesis* has resulted in a mechanistic classification of chemical carcinogens, viz. the genotoxic carcinogens which bind (usually covalently) to DNA and the epigenetic carcinogens which are characterized by

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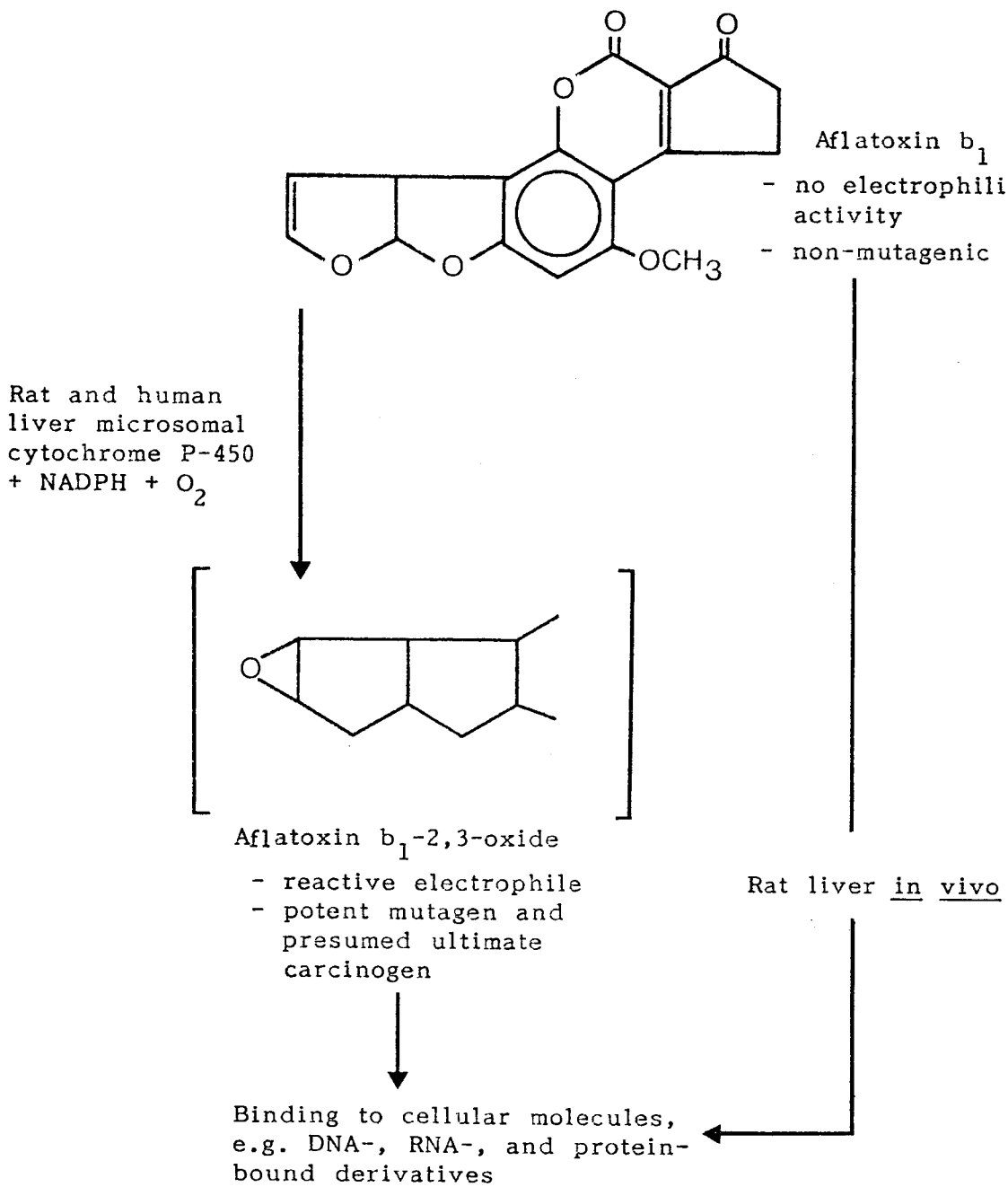
*Carcinogenesis represents the production of an increased incidence of malignant tumours in an intact organism.

Figure 11: Hepatic metabolism of Aflatoxin b_1



no direct interaction with DNA [164]. Chemical carcinogenesis brought about by the genotoxic mechanism is currently favoured and will be considered for the purpose of this thesis.

Damage of the cell's hereditary material, DNA, leads to carcinogenesis [165,166,167]. DNA, which constitutes a very small portion of the chemical components of the cell, is the memory of the cell. Therefore, damage to the DNA will affect the future of the cell, whereas damage to proteins and other cellular constituents will have far less serious consequences. It is thought that carcinogenesis may be initiated by the binding of reactive electrophiles to DNA [162,168,169,170], while toxicity may be reflected by the binding of electrophiles to protein and/or RNA.

1.2.2 Binding to DNA

The site and type of binding of a reactive species (electrophile) to DNA is thought to ultimately determine the carcinogenic potential of the chemical (or metabolite).

(i) Site of binding

Alkylation may occur at a number of different sites on DNA, viz. N-1, N-3 and N-7 of adenine; the N-3, N-7 and O-6 of guanine; the N-3 and O-2 of cytosine; and N-3, O-4 and O-2 of thymine, as well as sites on the deoxyribose and phosphate moieties [171]. So far, it has been found that many alkylating agents react principally (85%) at the N-7 of guanine [172]. However, it appears that binding to the O-6 of guanine is more closely related to the carcinogenic potential of an electrophile than is binding to the N-7 of guanine [172].

(ii) Type of binding

The stability of the resulting DNA adduct, i.e. the rate of repair (dealkylation), also plays a large role in determining carcinogenic potency. DNA repair can be defined [173] as any enzyme-mediated modification of damaged DNA which constitutes part or all of a process for the elimination of the damage from DNA [173]. DNA repair can be measured by the incorporation of DNA precursors following exposure to the test chemical. However, the fact that DNA repair is enzymatically mediated and therefore a saturable process [174] as well as the fact that repair is never totally efficient, can result in the ensuing DNA replication yielding an undamaged double helix, but one bearing a different coded message to the original DNA. The resulting changes in the DNA are known as mutations*; the process being mutagenesis. Therefore induction of chemical carcinogenesis can in part be related to the efficiency of the repair process.

DNA damage, although it is essential in the initiation of chemical carcinogenesis, does not usually in itself lead to the occurance of cancer; additional factors are required in the promotion of cellular events leading up to the first transformation and successive stages as can be seen in Figure 12 [175].

Therefore, chemicals (or metabolites) that give rise to DNA damage and repair are only potential carcinogens.

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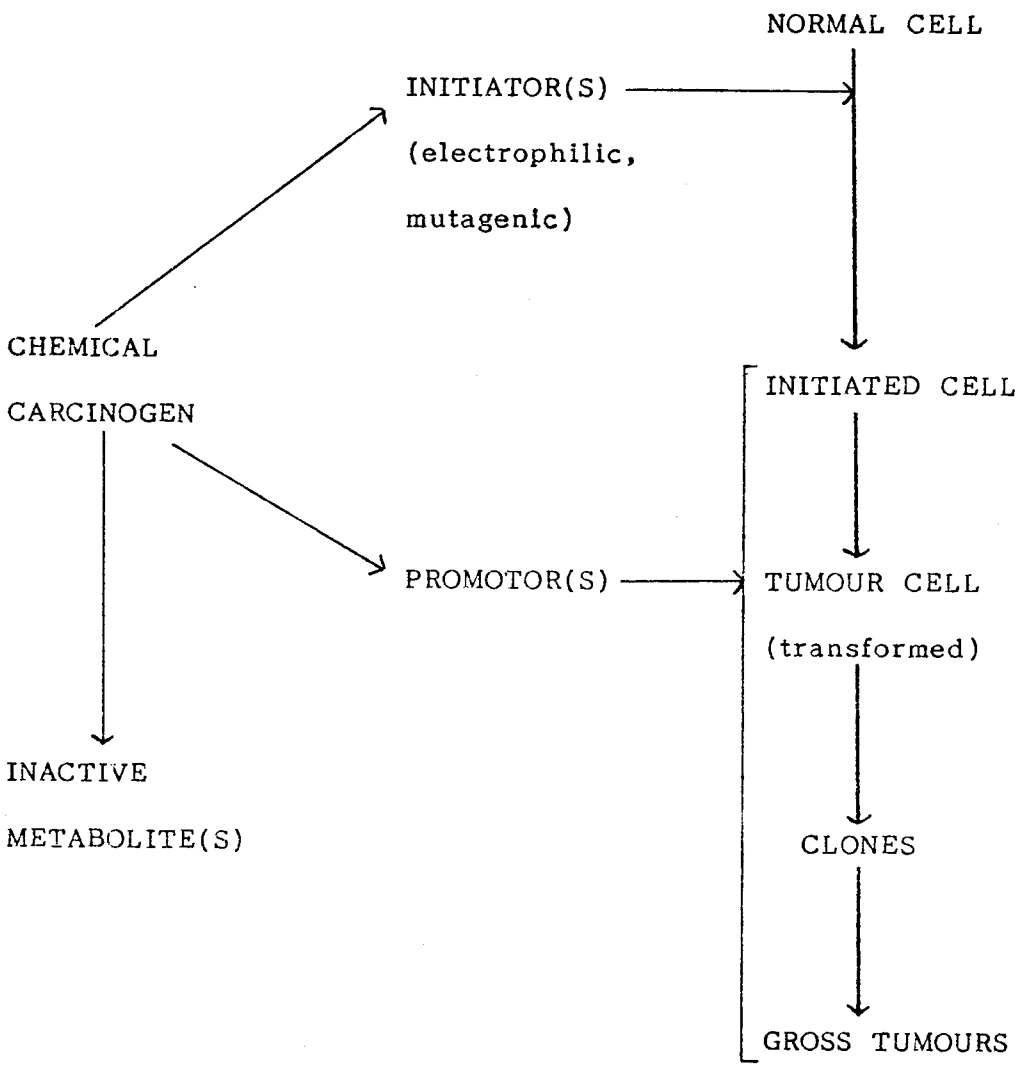
*The genotoxic mechanism is also known as the mutational mechanism while the epigenetic mechanism is called the non-mutation mechanism.

Figure 12: A simplified scheme for the steps in the induction of cancer by a chemical [175]



1.2.3 Methods for screening chemical carcinogens

Animal tests for the carcinogenicity of a chemical are time consuming and expensive. Even when these tests are feasible, manufacturers need fast, inexpensive methods for identifying potential carcinogens before new products are brought onto the market. Therefore, a simple, quick and inexpensive method for screening potential carcinogens is required. For this purpose, mutagenicity assays have proved of some value. [As noted earlier, the ability to induce a mutation implies that a chemical (or metabolite) is usually an electrophile and therefore has a reasonable probability of being carcinogenic [176]].

The best known of the mutagenicity assays is the Ames Test which was developed by Ames and his associates in 1971, using a bacterial cell culture system *in vitro*. The ability of the compound under investigation to produce mutations in a series of cell lines is taken as a measure of its carcinogenic potential. In some cases the assay medium is supplemented with preparations of hepatic microsomes [177,178] in order to more accurately detect procarcinogens which are activated by hepatic enzymes. The Ames Test has a fairly good success rate, but also has the disadvantage that when microsomal fractions are added to cell cultures as a source of monooxygenase activity, the active metabolites (electrophiles) are produced outside the cell and can result in erroneous results [179].

Consequently, an improvement in the testing for the mutagenic potential of a chemical would be the use of liver cells *invitro*, since the liver, which is the target organ of many carcinogens, contains all the enzymes necessary for the metabolism of procarcinogens to the ultimate carcinogen, as well as enzymes involved in the detoxification

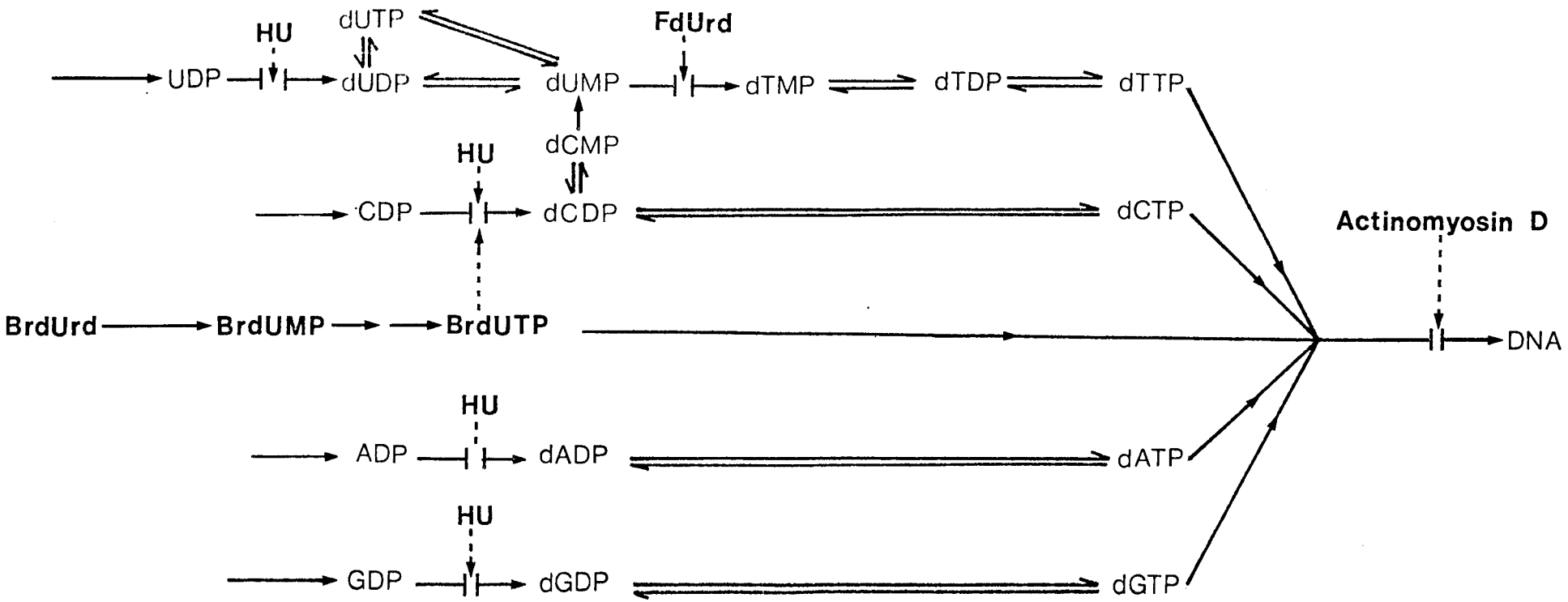
of electrophilic intermediates. The hepatocytes provide an excellent system as all factors (e.g. protein, RNA, etc) competing with DNA for the electrophile, are roughly present as *in vivo*.

Since the activity of the xenobiotic metabolizing enzymes rapidly declines in cultures of hepatocytes (the levels of cytochrome P-450 drop by 80% after 24 hours)[180], primary maintenance cultures of hepatocytes supplemented with a complex mixture of hormones, fatty acids and δ-aminolevulinic acid or nicotinamide[161], which maintain xenobiotic metabolism capabilities, provide a better system. However, the consequences of these additions are not known.

Perhaps, therefore, a more advantageous system for the screening of possible carcinogens would be freshly isolated hepatocytes. Since DNA repair is one of the first biological effects which can be observed after carcinogen treatment, it would provide a possible method for screening carcinogens using freshly isolated liver cells.

A major problem immediately arises, viz. how can DNA repair synthesis be differentiated from normal conservative DNA replication. This problem can be overcome by the use of hydroxyurea which inhibits the conversion of the nucleotides to their deoxyribose analogues, as can be seen in Figure 13. DNA repair can then be estimated in isolated rat hepatocytes by either autoradiographic determination of unscheduled DNA synthesis (UDS)[74,181,182,183], or by measurement of the incorporation of radioactive thymidine into DNA[182,184]. The bromodeoxyuridine-CsCl gradient method, which appears to be the most reliable technique for the detection of DNA repair synthesis in mammalian cell cultures[185], has been successfully adapted for freshly isolated hepatocytes[186]. However,

Figure 13: Approximate steps in the pathways of synthesis for precursors of DNA at which various inhibitors act (192,193,194)



Abbreviations: FdUrd = Fluorodeoxyuridine;
BrdUrd = Bromodeoxyuridine;
HU = Hydroxyurea.

as it was recently demonstrated that in the presence of microsomal enzymes, hydroxyurea gives rise to DNA repair [187,188], fluorodeoxyuridine (FdUdr) was utilized to block the conversion of deoxyuridinemonophosphate (dUMP) to deoxythymidinemonophosphate (dTMP). This results in the incorporation of heavy bromodeoxyuridinetriphosphate (BrdUTP) into DNA during S-phase of semi-conservative replication [189] (see Figure 13). Following addition of the potential carcinogen in the presence of [^{3}H]-deoxycytidine, de novo synthesis can thus be measured by separating the heavy-labelled DNA (semi-conservative replication) from the normal density DNA (repair synthesis) by CsCl [190] and monitoring the incorporation of radioactivity into the light DNA strands, e.g. see Figure 14 [191].

1.3 The Chlorinated Ethylenes

When it was discovered a few years ago that long-term workers in the polyvinylchloride (PVC) plastic industry developed angiosarcoma, a very rare form of liver cancer, great interest and concern was evoked as to the biotransformation of vinyl chloride monomer (VCM) and PVC by mammalian systems. VCM, the basic constituent of PVC plastics, was found to be mutagenic in vitro and carcinogenic and hepatotoxic in vivo [195,196]. The deleterious effects of vinyl chloride monomer (VCM) were found to require the biotransformation of VCM by the hepatic cytochrome P-450 and did not result from the parent compound itself [197,198,199,200].

As a result, research has progressed to the related analogues of VCM, viz. the series of chlorinated ethylenes, which are often widely used in industry, agriculture and medicine (see Table 8).

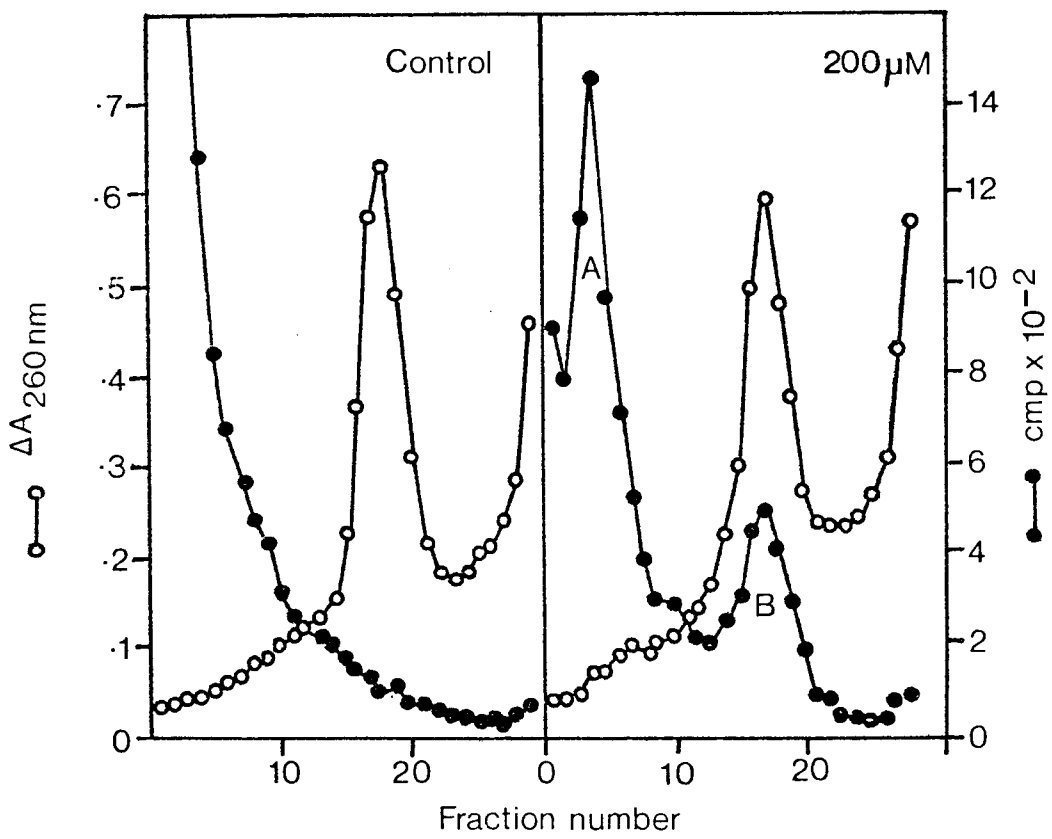
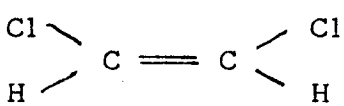
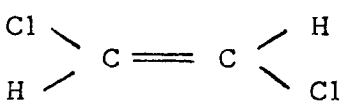
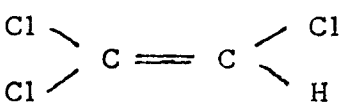
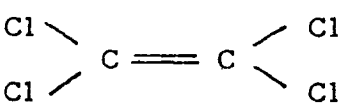


Figure 14: Induction of repair synthesis in rat hepatocytes with 5-diazouracil (200 μM).
Repair synthesis is the radioactivity peak (B) (● —— ●) banding with parental DNA (o —— o) (coincident with the UV absorbance peak)[191].
A = Semi-conservative synthesis.
B = Repair synthesis.

Table 8: The chlorinated ethylenes and their uses

Chlorinated ethylene	Name (abbreviation)	General uses
	Vinyl chloride monomer (VCM)	Manufacture of PVC plastics (205)
	Vinylidene chloride (VDC) 1,1-dichloroethylene	The manufacture of Saran-type plastics (159)
	cis-1,2-Dichloroethylene (cis-1,2-DCE)	Industrial solvents for fats, oils, waxes and particularly for rubber (159)
	trans-1,2-Dichloroethylene (trans-1,2-DCE)	
	Trichloroethylene (TCE)	Dry-cleaning solvent, metal degreasing agent, anaesthetic agent and analgesic, for decaffeinating tea and coffee (201,202)
	Perchloroethylene (PCE) Tetrachloroethylene	Dry-cleaning and metal degreasing agent, for fabric finishing (203,204)

1.3.1 Vinyl chloride monomer (VCM)

Vinyl chloride monomer was produced at an annual rate of three billion kilograms in one year in the United States alone, for the manufacture of PVC plastics [205].

In view of its carcinogenicity, extensive research has been done on the biotransformation of vinyl chloride *in vivo* and *in vitro*. A summary of the metabolism of vinyl chloride appears in Figure 15 [206].

The toxic effects of vinyl chloride monomer appear to depend on its metabolism by hepatic microsomal cytochrome P-450. The hepatic transformation of vinyl chloride gives rise to a decrease in the levels of cytochrome P-450 *in vitro** [158] and the production of chloroethylene oxide [207] and its non-enzymic rearrangement to chloroacetaldehyde [211]. Both these metabolites are capable of binding cellular cofactors, protein, RNA and DNA. The products of the modification of cellular DNA by vinyl chloride metabolites include $1,N^6$-ethenodesoxyadenosine, $3,N^4$-ethenodesoxycytidine and N-7-oxyethyldesoxyguanosine [212,213,214].

Although the pre-carcinogen vinyl chloride is converted to an ultimate carcinogen by the cytochrome P-450 system of the hepatocytes, it is interesting to note that the carcinogenic effect of this chemical is expressed in the sinusoidal cells of the liver, which

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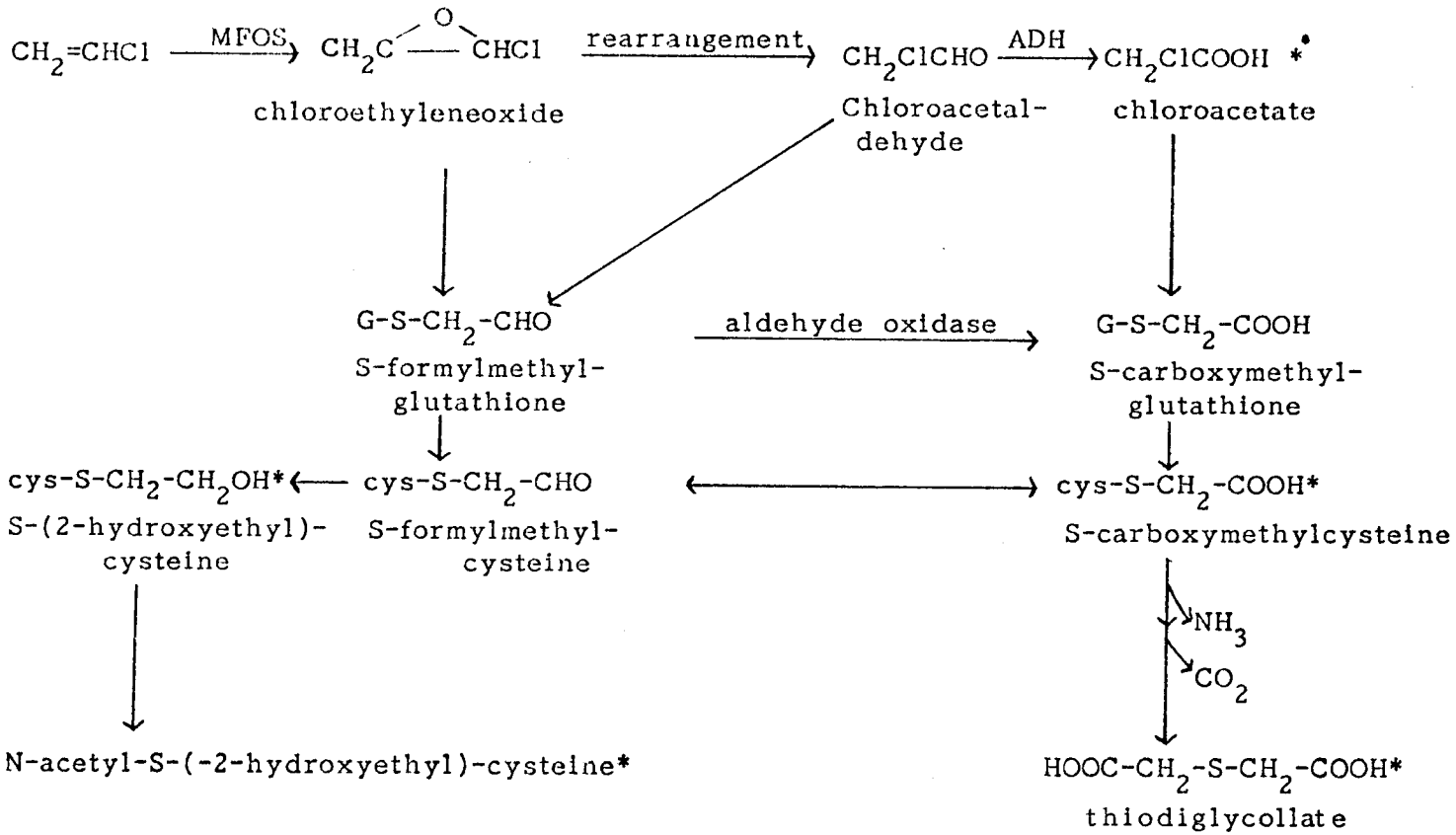
*Other microsomal enzymes, e.g. cytochrome b_5 and NADPH-cytochrome P-450 reductase, are unaffected following vinyl chloride treatment in hepatic microsomes from uninduced or induced animals [158].

Figure 15: The major metabolic pathway of vinyl chloride

(158,206,207,208,209,210)



* identified in vivo

• identified in vitro

themselves only have very low capacities for oxidative metabolism of xenobiotics [215]. Recent results [216] suggest that this phenomenon reflects the ability of the ultimate carcinogen to travel from the hepatocyte where it is produced, to the sinusoidal cells, where its carcinogenicity is expressed as a result of the inability of the sinusoidal cell to detoxify reactive species. The hepatocyte, in contrast, is abundantly endowed with detoxifying systems and is thus protected from the carcinogenic effects of the reactive metabolites of vinyl chloride, even though it provides the site of production of these metabolites.

1.3.2 Vinylidene Chloride (1,1-dichloroethylene)

Widespread interest has arisen in this compound, as to its toxic, mutagenic and carcinogenic potential, as a consequence of its close structural similarity to vinyl chloride.

Vinylidene chloride has been found to be toxic *in vivo*. The toxicity of vinylidene chloride *in vivo* is unaffected or decreased following the induction of hepatic cytochrome P-450, but is increased following the depletion of hepatic GSH [217,218,219]. In addition, vinylidene chloride has been found to be mutagenic in the presence of liver activating enzymes (post mitochondrial supernatant, S_9 fraction) [211,220,221,222,223], and carcinogenic in rats and mice, although sex, species and strain of the experimental animal affect its carcinogenic potential [224,225,226,227]. The mutagenic and carcinogenic effects of vinylidene chloride are thought to be produced by reactive metabolites of the parent compound such as dichloroethylene oxide or chloroacetyl chloride [211,222]. Neither of these compounds has, however, been identified as a

metabolite of vinylidene chloride *in vivo* or *in vitro**. The only metabolites of vinylidene chloride that have been identified are monochloroacetate, conjugates, thiodiglycollic acid and related products; all found to be urinary metabolites of vinylidene chloride [228,229,230]. All other intermediates in the proposed metabolic pathway for vinylidene chloride have not been identified (see Figure 16).

The metabolism of vinylidene chloride has been proposed to involve hepatic microsomal cytochrome P-450 on the basis of the observation that the conversion of vinylidene chloride to monochloroacetate and an unknown metabolite is catalyzed by hepatic post-mitochondrial supernatant and NADPH-generating system [230]. There was no other available information on the metabolism of vinylidene chloride when this investigation was initiated.

1.3.3 *cis*- and *trans*-1,2-Dichloroethylene

The deleterious effects of the 1,2-dichloroethylenes have not been extensively investigated. However, these compounds are reportedly hepatotoxic to laboratory rats [232], but are not mutagenic in the presence or absence of liver activating enzymes and are not known to be carcinogenic in laboratory animals [221].

The metabolism of the 1,2-dichloroethylenes has not been studied extensively *in vivo* or *in vitro*, but the following pathway for their *in vivo* metabolism has been proposed solely on the basis of identifying 2,2-dichloroethanol and dichloroacetic acid in perfused liver [211,230] (see Figure 17).

It would appear that the hepatic microsomal cytochrome P-450

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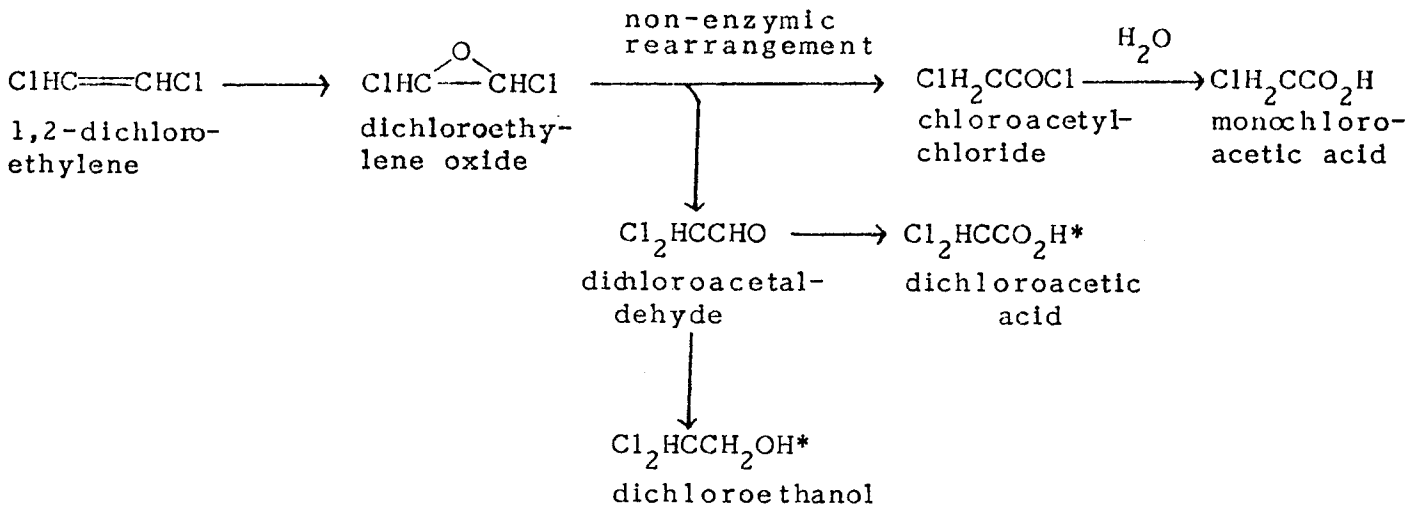
*Uehleke *et al.* [231] demonstrated spectral evidence for the formation of 1,1-dichloroethylene-oxide as an intermediate in the hepatic metabolism of 1,1-dichloroethylene.

Figure 17: Proposed metabolic pathway of the 1,2-dichloroethylenes in vivo



*Metabolites of the 1,2-dichloroethylenes which have been identified from perfused rat liver (211).

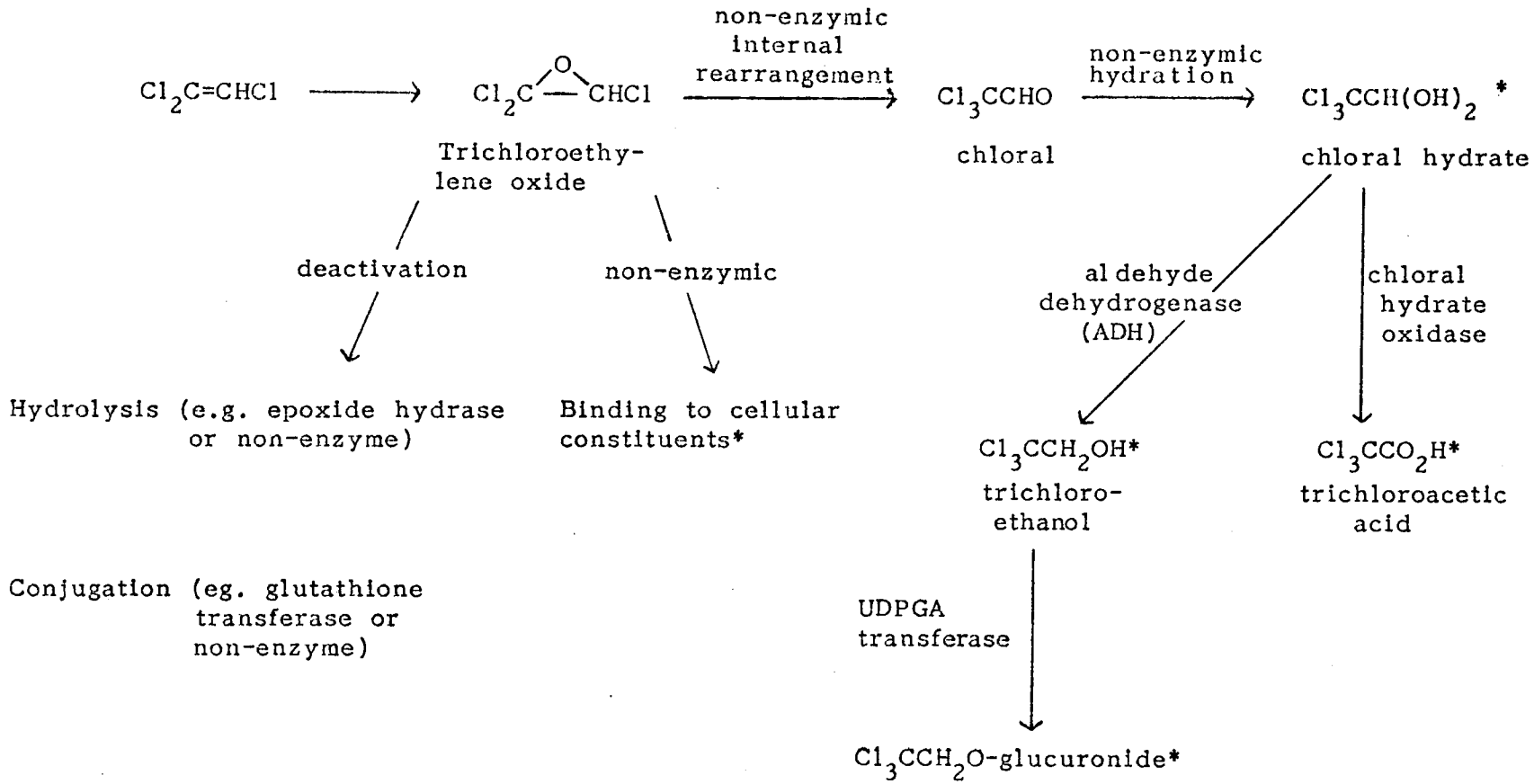
enzyme system may be involved in the metabolism of the 1,2-dichloroethylenes: The 1,2-dichloroethylenes have been proposed to inhibit the demethylation of aminopyrine *in vitro* and the metabolism of hexobarbital *in vivo*, apparently by competing for the binding to the active site of cytochrome P-450 [233,234]. Furthermore, the 1,2-dichloroethylenes have been reported to be metabolized by hepatic post-mitochondrial supernatant plus NADPH to an unknown metabolite, which is not monochloroacetate [230].

1.3.4 Trichloroethylene

Exposure to trichloroethylene has been reported to result in a variety of disorders, including central nervous system depression, hepatotoxicity and nephrotoxicity [200,201]. In addition, trichloroethylene is mutagenic in the modified Ames test in the presence of liver activating enzymes [221,222] and is carcinogenic in mice, but not in rats [200,213 but also see 235]. The deleterious effects of trichloroethylene may result from reactive metabolites of trichloroethylene, since there is a direct correlation between the extent of metabolism of trichloroethylene and its hepatotoxicity[236,237,238]. The reactive metabolite presumed to be responsible for these effects is trichloroethylene-oxide [222,236,238] (see Figure 18). The actual existence of an epoxide metabolite of trichloroethylene was proposed by Powell [239] as early as 1945 to explain the observation that urinary trichloroacetate was produced from trichloroethylene *in vivo*.

The enzyme system which catalyzes the primary oxidation of trichloroethylene to its first readily isolable metabolite, chloral hydrate, appears to be the hepatic microsomal mixed function oxidase system [239,240,242] (see Figure 18). This reaction is

Figure 18: Proposed pathways for the metabolism of trichloroethylene [236,240,241]



*Isolated metabolites.

presumed to proceed via an initial enzymic conversion of trichloroethylene to trichloroethylene-oxide (epoxide)* and a subsequent non-enzymic internal rearrangement of the epoxide to chloral hydrate [236,240,244]. The epoxide can undergo alternative reactions with cellular macrocmolecules, which might lead to toxic effects (?carcinogenicity and mutagenicity), or with small molecules, such as glutathione or water, in a detoxification reaction [236,238].

1.3.5 Tetrachloroethylene (Perchloroethylene)

As a consequence of its non-inflammability and low vapour pressure, tetrachloroethylene is often used industrially in preference to trichloroethylene [204,245]. Although tetrachloroethylene is generally regarded as being of low toxicity, toxic effects such as liver impairment, central nervous system depression and disturbance of the peripheral nervous system have been reported in humans following exposure to tetrachloroethylene [203,204]. Tetrachloroethylene was found to be not mutagenic with the modified Ames test in the presence of activating enzymes (S_9 fraction) [221,222], but to be weakly carcinogenic in some mammalian species [203,246].

Tetrachloroethylene is retained unchanged within the body for relatively long periods of time: the half-life of tetrachloroethylene

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*There is some controversy as to whether an epoxide is an intermediate in the metabolism of trichloroethylene by hepatic cytochrome P-450. Uehleke et al.[231] reported that trichloroethylene forms a complex with reduced cytochrome P-450, after the formation of metabolite intermediate(s), which is evidenced by difference absorption at 452 nm and which is assumed to be the corresponding epoxide. Miller and Guengerich, however, have questioned the formation of an epoxide intermediate in the hepatic metabolism of trichloroethylene and proposed a trichloroethylene-oxygenated P-450 transition state for the reaction, rather than trichloroethyleneoxide [243].

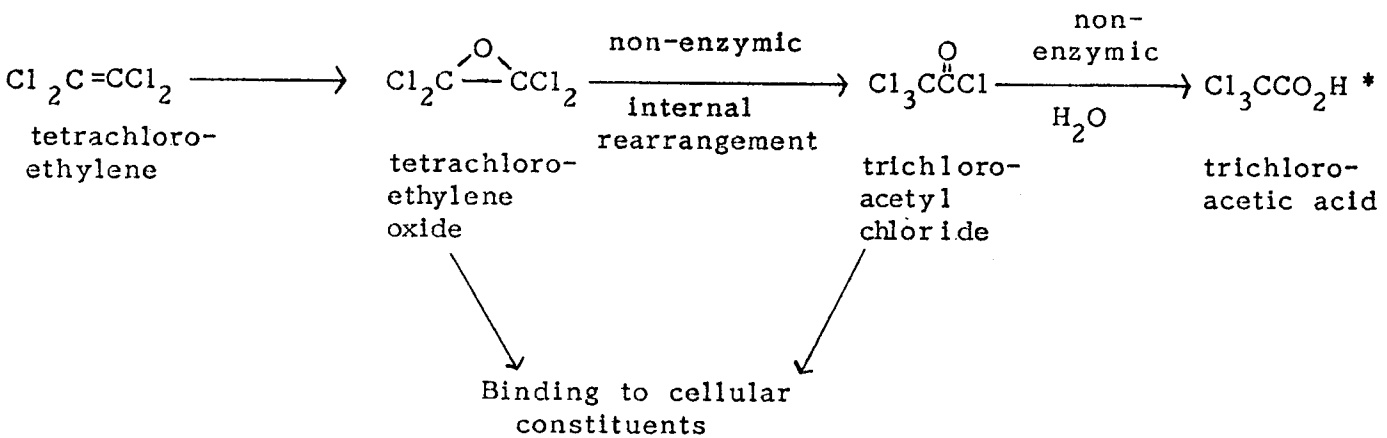
is approximately 144 hours, whereas that of trichloroethylene is 44 hours [247]. The longer retention time of tetrachloroethylene in the body presumably arises, in part, from the inability of mammalian species extensively and rapidly to metabolize this lipophilic compound and, in part, from its high lipid solubility. In rats 98% of the dose of tetrachloroethylene is excreted unchanged, whereas only 72 - 85% of the dosage of trichloroethylene administered is excreted unchanged [244]. Similarly, in isolated, perfused rat liver, only 10 - 15% of the total tetrachloroethylene taken up by the liver is metabolized, whereas 82 - 100% of the trichloroethylene taken up is metabolized, even though the uptake of trichloroethylene is 7-fold greater than that of tetrachloroethylene [211].

The major urinary metabolites of tetrachloroethylene *in vivo* have been shown to be trichloroacetic acide and chloride ion, with 2,2,2-trichloroethanol being a minor metabolite [237,244,245,248]. Trichloroacetate is also the major metabolite of tetrachloroethylene in perfused rat liver, with the minor metabolite (ca. 25%) being the trichloroacetyl moeity covalently bound to cellular constituents [211].

The accepted pathway for the production of trichloroacetic acid from tetrachloroethylene is shown in Figure 19. This pathway was proposed by Powell in 1945 [239] and subsequently supported by the results of Daniel [137] and others [211,221] (see Figure 7).

The first step in this pathway appears to be catalyzed by the hepatic cytochrome P-450 system. Tetrachloroethylene is known to bind to the substrate binding site of hepatic microsomal cytochrome P-450 *in vitro*, and a direct relation has been demonstrated between

Figure 19: Proposed pathway for the metabolism of tetrachloroethylene *in vivo*



*identified metabolite *in vivo*.

the level of hepatic microsomal cytochromes P-450 and the extent of metabolism of tetrachloroethylene in vivo [237,249].

1.4 General aims of the research project

The major objective of this research project was to investigate the hepatic biotransformation of the analogues of the hepatocarcinogen, vinyl chloride, viz. the chlorinated ethylenes, in an attempt to assess the following:

- firstly, is there a relationship between structure (or chlorine substitution) and type and extent of metabolism?
- secondly, is there a correlation between the metabolites formed and the hepatotoxic, mutagenic and/or carcinogenic effects of the chlorinated ethylenes? This latter aspect is of particular importance in that it presents an endeavour to acquire an insight into the question of the initiation of chemical carcinogenesis.

Since extensive research has already been done on vinyl chloride [158, 207-216], the major part of this thesis has concentrated on the hepatic management of the di-, tri- and tetrachlorinated ethylenes.

This thesis covers a detailed investigation of the following:

(i) the interaction of the chlorinated ethylenes with hepatic microsomal cytochrome P-450 in vitro. This involved a detailed study of the binding and metabolism of the chlorinated ethylenes by hepatic cytochrome P-450 as well as the effect that inducing agents exerted on the abovementioned parameters. Furthermore, the effects of the metabolism of the chlorinated ethylenes on the levels and activity of hepatic microsomal

cytochrome P-450 and on various other hepatic microsomal enzymes was monitored.

(ii) The metabolism of the chlorinated ethylenes by freshly isolated hepatocyte preparations was investigated. The hepatocytes were obtained from variously pretreated rats, in an attempt to better assess the extent and type of metabolism of the chlorinated ethylenes in vivo.

(iii) The mutagenic potential of the chlorinated ethylenes was assessed by their ability to stimulate DNA repair in isolated hepatocytes. This is an attempt to measure the potential carcinogenicity of these compounds.

2. EXPERIMENTAL

2.1 Materials

2.1.1 Chemicals for pretreatment of animals

Sodium phenobarbital and β-naphthoflavone were obtained from Maybaker, Port Elizabeth, R.S.A. and the Aldrich Chemical Co., Milwaukee, WI, U.S.A., respectively. Pregnenolone 16α-carbonitrile and spironolactone were gifts from Searle Laboratories, Chicago, IL , U.S.A. 3-Methylcholanthrene was purchased from Eastman-Kodak Co. N.Y., U.S.A.

2.1.2 Chlorinated ethylenes

Vinyl chloride was initially purchased from Matheson Ltd, U.S.A. and subsequently generously donated by AECI, Johannesburg, R.S.A. Pure cis- and trans-1,2-dichloroethylele were from Ferak, Berlin, FDR, and Fluka AG, Buchs, Switzerland, respectively, The mixture of cis- and trans-1,2-dichloroethylene (30:70); vinylidene chloride; trichloroethylene and tetrachloroethylene, were from Merck Chemicals, Darmstadt, FDR.

2.1.3 Possible metabolites

2-Chloro-, 2,2-dichloro- and 2,2,2-trichloroethanol, chloral hydrate and mono-, di- and trichloroacetic acid were purchased from Merck Chemicals, Darmstadt, FDR. Chloroacetaldehyde was obtained from Fluka AG, Buchs, Switzerland, but was redistilled prior to use and stored as a 50% aqueous solution in an amber bottle. Dichloroacetaldehyde was prepared from freshly distilled chloral according to the method of Swietoslawski and Silowieki [252]. Stock solutions of dichloroacetaldehyde in water were prepared immediately

after distillation of the freshly prepared dichloroacetaldehyde in order to prevent polymerization. The acetaldehyde content of the stock solutions was confirmed by the method of Malhotra and Anand [273].

2.1.4 Inhibitors

SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) and metyrapone [2-methyl-1,2-bis(3'-pyridyl)-1-propanone] were gifts from Smith Kline and French Laboratories, Isando, R.S.A. and Ciba-Geigy Ltd., Basle, Switzerland, respectively.

2.1.5 Supplies for gas liquid chromatography

Chromosorb 101, Carbowax 20 M and Chromosorb W (80/100 mesh) were supplied by Johns-Manville, Denver, Colorado, U.S.A.

2.1.6 Chemicals for microsomal assays

NADH, NADPH, NADP, cytochrome c, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Miles Laboratories, R.S.A. Horse liver alcohol dehydrogenase was from Sigma Chemical Co, St Louis, MO, U.S.A. Benzpyrene and 4-dimethylamino-antipyrene were also purchased from Sigma Chemical Co, St Louis, MO, U.S.A. Ethoxyresofurin and resofurin were obtained from the Pierce Chemical Co, Rockford, IL, and Eastman-Kodak Co, Rochester, NY, U.S.A. p-Nitroanisole (Eastman-Kodak) was recrystallized from petroleum ether prior to use. All other chemicals were analytical grade reagents.

Cylinders of carbon monoxide, nitrogen and oxygen were

purchased from Afrox Ltd, R.S.A. Water, which was distilled or distilled and deionized, was regularly checked for the presence of pyrogens at the State Vaccine Institute, Pinelands, R.S.A.

2.1.7 Chemicals for hepatocytes

Collagenase (Type I V), HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and sulphatase (Type VI) were obtained from Sigma Chemical Co, St Louis, MO, U.S.A. Dry Leibowitz-15 (L-15) Medium, a product of Flow Laboratories, Scotland, was purchased from Tauber and Corsen, Cape Town, R.S.A., β-glucuronidase was from Miles Laboratories, Cape Town, R.S.A. and CO_2:O_2 (5:95) was supplied by Afrox Ltd., R.S.A. Heparin was supplied by Glaxo (Pty) Ltd., Wadeville, R.S.A., and Sagatal (sodium pentobarbitone) was obtained from Maybaker, Port Elizabeth, R.S.A.

2.1.8 Chemicals for DNA repair assay

5-Fluoro- and 5-bromodeoxyuridine were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. [5-^{3}H]-deoxycytidine was purchased from Amersham International, Buckinghamshire, U.K. Calf thymus DNA and proteinase K were supplied by Miles Laboratories, Cape Town, R.S.A. and Merck Chemicals, Darmstadt, FDR, respectively.

2.1.9 Partially purified cytochrome P-450

Partially purified cytochrome P-450 was isolated from the hepatic microsomes of phenobarbital-induced rats, as described by van der Hoeven and Coon [254]. This preparation consists of a mixture of forms of cytochrome P-450 free from other microsomal components,

viz. NADPH-cytochrome c reductase and cytochrome b_5.

2.1.10 Instruments for spectral assays

For most spectral studies, a Beckman 5230 UV-visible scanning spectrophotometer or a Pye-Unicam SP 1800 UV-visible scanning spectrophotometer were utilized. For certain assays, e.g. determination of microsomal protein, as indicated in the methods, a Gilford UV-visible single beam spectrophotometer was used.

2.2 Methods

2.2.1 Treatment of animals

Male Long-Evans rats (180 - 220 g) were used for all experments. Animals were permitted free access to Epol laboratory chow (protein min 20%; fat 2.5%; fibre max 6%; calcium 1.4%; phosphorus 0.7%) obtained from Epol Ltd., Goodwood, Cape, R.S.A., and water unless otherwise indicated.

Induction of drug metabolizing enzymes was accomplished by intraperitoneal injection of sodium phenobarbital (80 mg/kg/day in 0.9% sterile saline); 3-methylcholanthrene (40 mg/kg/day in corn oil); pregnenolone-16α-carbonitrile (50 mg/kg/day in corn oil); or spironolactone (90 mg/kg/day, twice a day) on each of three consecutive days [255,256]. β-Naphthoflavone was administered as a single injection of 80 mg/kg in corn oil 36 hours prior to killing the animals [257]. All animals were fasted 16 hr and were then sacrificed by cervical fracture.

Rats were treated with vinyl chloride gas as follows: Phenobarbital treated rats, in groups of three or four, were placed on a fenestrated floor raised eight centimetres from the base of a perspex anaesthetic chamber (30x30x60 cm); the space below being filled with standard sodalime. They were exposed to concentrations of vinyl chloride gas of 3 000 or 20 000 ppm in oxygen for six hours. The inlet and exhaust ports were at opposite sides of the chamber. The control animals were exposed to oxygen alone in an identical chamber. Animals were fasted for a minimum of 14 hr after exposure to vinyl chloride and/or oxygen and were then sacrificed.

2.2.2 Preparation of hepatic microsomes

Livers were removed from animals immediately after sacrifice, homogenized in 3 volumes of 0.15 M KCl-0.02 M Tris-HCl, pH 7.4, at 4°C and subsequently treated according to the method of Holzmann and Carr [258]: cell debris, nuclei and mitochondria were spun down at 9000 g for 20 min. The post-mitochondrial supernatant was spun at 105 000 g for one hr, and the resultant microsomal pellet was resuspended in 0.15 M KCl-0.02 M Tris-HCl, pH 7.4, and further centrifuged at 105 000 g for 45 min.

Microsomal protein concentration was determined by the method of Lowry *et al.* [259] as modified by Chaykin [260] using bovine serum albumin as standard. Final absorbances were measured at 600 nm in a Gilford spectrophotometer. The microsomal suspension was diluted to the required protein concentration (normally 2 mg microsomal protein/ml) with 0.02 M Tris-HCl, pH 7.4, unless otherwise indicated.

2.2.3 Addition of the chlorinated ethylenes to hepatic microsomes

2.2.3a For Difference Spectra

Suspensions of hepatic microsomes (2 mg protein/ml) were divided equally between two 1-cm pathlength cuvettes. One of the chlorinated ethylenes (neat or as an ethanolic solution) was introduced below the surface of the microsomal suspension in the sample cuvette by means of a Hamilton syringe. The cuvette was immediately stoppered and vortex mixed for 30 sec (for trichloroethylene, cis- and trans-1,2-dichloroethylene, vinylidene chloride and vinyl chloride) or for 90 sec for tetrachloroethylene, in order to disperse and solubilize the chlorinated ethylene. Vortex mixing for shorter time-intervals did not result in the full production of the difference spectrum. Reference cuvettes contained microsomal suspension only and were not vortex mixed. The magnitude of the resultant difference spectrum for each of the abovementioned compounds was measured as the difference in absorbance between the peak at ca. 386-390 nm and the trough at ca. 418-420 nm. The reported magnitude of the difference spectrum has been corrected for any intrinsic difference in absorbance of the microsomal suspension at these wavelengths. In the case of tetrachloroethylene, where the sample cuvette had to be vortex mixed for 90 sec, an additional correction was made for absorbance differences between 386 nm and 416 nm that arose as a result of the vortex mixing of microsomal suspensions in the absence of tetrachloroethylene (see Section 3.1.4). In no case was any sample vortex mixed for more than 90 sec. All difference spectra were recorded at room temperature.

2.2.3b Vinyl chloride

For in vitro experiments with vinyl chloride, vinyl chloride gas was dissolved in absolute ethanol and introduced into the microsomal suspensions with a pre-cooled, gas-tight Hamilton µl syringe. Reaction mixtures contained vinyl chloride (0-30 mM), NADPH-generating system [303] (see 2.2.5), EDTA (0.2 mM) and hepatic microsomes from phenobarbital pretreated rats (2 mg protein/ml) in 0.02 M Tris-HCl, pH 7.4, were incubated in sealed 10 ml hypodermic vials at 30° with shaking at 60 cycles per min. At the end of the incubation period, the gas-tight seals were removed and vinyl chloride was blown off by bubbling with nitrogen for 5 min at 4°.

2.2.4 NADPH oxidation

The rates of NADPH oxidation were determined in the presence of each of the chlorinated ethylenes, NADPH and hepatic microsomes as follows: Equal quantities of microsomal suspension (2 mg microsomal protein/ml) were divided between two 1-cm path-length cuvettes. The chlorinated ethylene was introduced into the sample cuvette as described for the difference spectra (Section 2.2.3a) and the reaction was initiated by the addition of NADPH to give a final concentration of 0.18 mM. NADPH oxidation was monitored spectrally at 340 nm using a thermostatted cell compartment adjacent to the photomultiplier. The extinction coefficient for NADPH at 340 nm is 6.2 mM^{-1} cm^{-1}. Correction was made for background rates of endogenous NADPH oxidation in the presence of the chlorinated ethylene in an atmosphere of $CO:O_2$ (80:20; v/v) except in the case of trichloroethylene [71] (see Section 3.2.3).

Carbon monoxide and oxygen flows were controlled by Matheson Gas Products model 7600 flowmeters. The mixture of gases was allowed to bubble through the microsomal suspension for 30 sec at 20 ml per min prior to the addition of the substrate and cofactor.

2.2.5 Incubation systems for the metabolism of the chlorinated ethylenes using hepatic microsomal suspensions

The reaction mixtures for the metabolism of the chlorinated ethylenes comprised hepatic microsomal suspension (2 mg protein/ml), NADPH-generating system (NADP, 0.4 mM; glucose-6-phosphate, 7.5 mM; glucose-6-phosphate dehydrogenase, 0.5 U/ml; $MgCl_2$, 5 mM; nicotinamide, 1 mM; and EDTA, 0.2 mM, final concentrations)[303] and varying amounts of chlorinated ethylene, in 0.02 M Tris-HCl, pH 7.4, unless otherwise indicated. The air space above the incubation mixture constituted ca. 20% of the total volume (4 ml) of the stoppered reaction vial.

The hepatic microsomes were preincubated at 30° for 2-3 min, the compound was added (as described for obtaining difference spectra) and vortex mixed for 30 sec (90 sec for tetrachloroethylene). The reaction was initiated by the addition of the NADPH-generating system plus EDTA, and reaction mixtures were incubated with shaking (60 cycles/min) at 30° in a Gallenkamp thermostatted water bath for the times indicated. Termination of the reaction was achieved by the addition of 100 µl 2N H_2SO_4 (33.3 mM, final concentration) and 100 µl 0.3 M Na_2WO_4 (10 mM, final concentration) per 3 ml of microsomal suspension [261]. The precipitated protein was removed by centrifugation at 1000 g in a MSE-Mistral 6 L

centrifuge for 10 min. The amounts of the chlorinated metabolites of the chlorinated ethylenes in the supernatant or extracts thereof were determined by the following procedures:

2.2.5a Fujiwara Assay

Some of the chlorinated metabolites, viz. chloral hydrate, 2,2,2-trichloroethanol and trichloroacetic acid, were assayed spectrally by the modified Fujiwara assay of Leibman and Hindman (261). The basis of the Fujiwara reaction is the remarkable ease with which the pyridine ring can be split when heated in the presence of alkali and some organic halo-compounds. The reaction results in the formation of a crimson colour which has an absorption maximum at 530 nm. This spectral determination, utilized to monitor the presence of the trichlorinated species mentioned above (as metabolic products of tetra- and trichloroethylene) is insensitive to dichloroacetaldehyde, 2,2-dichloroethanol, and mono- and dichloroacetic acid (metabolic products of the dichlorinated ethylenes). The assay was performed exactly as described by Leibman and Hindman (261) except that after centrifugation, the supernatant fraction was bubbled with medical air or nitrogen for 10 min to displace trichloroethylene (BP_{760} 86.7°), which interfered with the assay.

2.2.5b Spectral assay for 2,2,2-trichloroethanol

Levels of 2,2,2-trichloroethanol produced were determined spectrally by the modified Fujiwara method of Friedman and Cooper (262, but see 261). 2,2,2-Trichloroethanol reacts with pyridine

upon heating in alkali to form a chromaphore with an absorbance maximum at 440 nm. The absorbance maximum of 440 nm is not produced by trichloroacetic acid or chloral hydrate when treated in an identical manner.

2.2.5c Assay of 2,2,2-trichloroethanol (from trichloroethylene and tetrachloroethylene) in the presence of hepatic microsomes, by gas-liquid chromatography

The detection of 2,2,2-trichloroethanol by gas-liquid chromatography was achieved using a 2 m x 6 mm copper column of 10% di-iso-decylphthalate on acid-washed Chromosorb P. This column will be referred to as Column I in Sections 2 and 3. Gas-liquid chromatography experiments were performed on a Pye-Unicam GCV gas-liquid chromatograph with a flame ionization detector. Injector, column and detector temperatures were 230°, 130° and 250°, respectively. Aliquots of microsomal suspension, following incubation with trichloroethylene or tetrachloroethylene in the presence of a NADPH-generating system, were injected directly into the gas-liquid chromatograph. Trichloroethylene, tetrachloroethylene, and 2,2,2-trichloroethanol eluted at 130, 150 and 580 sec, respectively. For the detection of 2,2,2-trichloroethanol using an electron capture detector, see Section 2.2.5e. The latter method was used for monitoring 2,2,2-trichloroethanol production from trichloroethylene and tetrachloroethylene in the presence of isolated viable hepatocytes (see Section 2.2.8).

2.2.5d Assay of chlorinated aldehydes and chlorinated acetic acids by gas-liquid chromatography

The presence of mono- and dichloroacetaldehyde and mono-, di- and trichloroacetic acid in reaction mixtures was monitored using a 2m x 6 mm glass column of Chromosorb 101 [263]. This column will be referred to as Column II in Sections 2 and 3.

Injector, column and detector temperatures were 220°, 200° and 250°, respectively. A Packard 428 gas-liquid chromatograph with a ^{63}Ni electron capture detector was utilized for these experiments.

Following incubation, precipitation and removal of protein in the microsomal suspensions (see 2.2.5), the supernatant was extracted with 4 ml of diethyl ether. The ether layer was dried with anhydrous Na_2SO_4. The methyl esters of the chlorinated carboxylic acid(s) present in the ether layer were prepared by bubbling with diazomethane under a stream of N_2 at room temperature. The retention times of metabolites and substrates on Column II were as follows: methyl monochloroacetate, 348 sec; methyl dichloroacetate, 550 sec; methyl trichloroacetate, 800 sec; monochloroacetaldehyde, 145 sec; and dichloroacetaldehyde, 210 sec. The chlorinated acetaldehydes were unaffected by diazomethane methylation.

2.2.5e Assay of chlorinated ethanols and chloral hydrate by gas-liquid chromatography

To monitor levels of chlorinated ethanol production as well as that of chloral hydrate, a 3 m x 6 mm glass column of 10%

Carbowax 20 M on Chromosorb W (80/100 mesh) was utilized (same gas-liquid chromatograph and detector as in Section 2.2.5d). This column will be referred to as Column III in Sections 2 and 3. Injector, column and detector temperatures were 200°, 170° and 230°, respectively.

In the case of the production of mono- and dichlorinated ethanols from the dichlorinated ethylenes, incubation mixtures comprised of 15 ml of hepatic microsomes at 6 mg microsomal protein per ml. After precipitation and removal of the protein as described earlier, the supernatant fraction was extracted with 5 ml of ethyl acetate. The organic phase was removed, dried with anhydrous Na_2SO_4 and analyzed by gas-liquid chromatography.

In the case of the production of chloral hydrate, incubation mixtures of 3 ml (2 mg protein/ml) of which the protein was removed, were extracted into 4 ml of diethyl ether. The ether layer was dried with anhydrous Na_2SO_4 and analyzed by gas-liquid chromatography. Retention times were as follows: 2-chloroethanol, 137 sec; 2,2-dichloroethanol, 270 sec; 2,2,2-trichloroethanol, 450 sec; and chloral hydrate, 215 sec.

For all gas-liquid chromatographic investigations, standards were prepared from analytical grade reagents or freshly prepared chemicals which were added to hepatic microsomes (or hepatocytes, see 2.2.8) and treated exactly as were the incubation mixtures. Peak areas were calculated by a Pye-Unicam DP 88 computing integrator.

2.2.5f Covalently bound chlorinated acetyl moieties

Reaction mixtures, containing a chlorinated ethylene, NADPH-generating system, EDTA and hepatic microsomes (2 mg protein/ml) from phenobarbital induced rats, which had been previously incubated at 30°, were treated with 20% H_2SO_4 for 16 hr at 100° [211], Methyl esters of the released acids were assayed by gas-liquid chromatography as described above.

2.2.6 Enzyme Assays

2.2.6a Cytochrome P-450

The concentration of microsomal cytochrome P-450 was determined from the difference spectrum of CO-ferrocytochrome P-450 vs. ferrocytochrome P-450 according to the method of Omura and Sato [21], using an extinction coefficient of 91 cm^{-1} mM^{-1} for the difference in absorbance between 450 nm and 490 nm. It should be noted that total type P-450 cytochromes are measured by this method. The average levels of hepatic microsomal cytochrome P-450 in microsomes from untreated and variously pretreated rats are as follows (in nmol per mg microsomal protein): untreated, 0.9±0.2; β-naphthoflavone, 1.3±0.1; pregnenolone-16α-carbonitrile, 1.6±0.2; spironolactone, 0.8±0.1; 3-methylcholanthrene, 1.4±0.1; and phenobarbital, 2.3±0.4.

The amount of the phenobarbital inducible form of cytochrome P-450 present in hepatic microsomes from phenobarbital pretreated rats was estimated as the metyrapone-ferrocytochrome P-450 complex. Metyrapone (1 mM, final concentration) was added to the microsomal suspension in the sample cuvette and dithionite

was added to both sample and reference cuvette. An extinction coefficient of 52 mM^{-1} cm^{-1} was used for the difference in absorbance between 446 and 490 nm [280 but see 155].

2.2.6b Microsomal heme

Microsomal heme was determined spectrally as the reduced pyridine hemochrome according to the method of Omura and Sato [21] using an extinction coefficient of 32.4 mM^{-1} cm^{-1} for the difference in absorbance between 557 nm and 575 nm.

2.2.6c Cytochrome $\underline{b}_5$

The levels of cytochrome $\underline{b}_5$ were measured spectrally (in the presence of 0.24 mM NADH) as the difference in absorbance at 424 and 409 nm of ferrocytochrome $\underline{b}_5$ vs. ferricytochrome $\underline{b}_5$ ($\varepsilon_{424-409\ nm}$ 185 mM^{-1} cm^{-1}) [264].

2.2.6d NADPH-cytochrome c (P-450) reductase

The activity of NADPH-cytochrome c reductase was determined from the increase in the levels of ferrocytochrome c at 550 nm ($\varepsilon_{550\ nm}$ 21.1 mM^{-1} cm^{-1}) according to the method of Omura and Takesue [264]. One unit of enzyme is that which reduces 1 µmol cytochrome c per min.

2.2.6e Reduced glutathione (GSH)

GSH levels were assayed fluorimetrically by the method of Cohn and Lyle [265]. The level of glutathione present in liver

microsomal suspensions was assessed before and after treatment with the chlorinated ethylenes. Following the incubations, the protein was precipitated with 25% metaphosphoric acid (3.6%, final concentration), and the precipitate was removed by subsequent centrifugation. The GSH present in the supernatant was reacted with o-phthalaldehyde at pH 8.3 yielding a highly fluorescent product. Standard solutions of GSH and blanks of water were similarly treated to incubation mixtures, and following removal of the protein, treated with o-phthalaldehyde at pH 8.3. A Perkin-Elmer fluorescence spectrophotometer 203 was utilized in measuring the fluorescence at 420 nm resulting from excitation at 365 nm.

2.2.6f Glucose-6-phosphatase

The activity of glucose-6-phosphatase was determined according to the method of Nordlie and Arion [266] by measuring the inorganic phosphate released during incubation of glucose-6-phosphate (20 mM) with hepatic microsomes (1.3 mg microsomal protein/ml 0.02 M Tris-HCl, pH 7.4). In as much as the NADPH-generating system (see 2.2.5) contains glucose-6-phosphate, an isocitrate dehydrogenase NADPH-generating system was used for this assay. It comprised $MgCl_2$, 10 mM; EDTA, 0.2 mM; isocitrate, 6.4 mM; isocitrate dehydrogenase 0.01 U/ml and NADP, 0.4 mM; final concentrations. [279].

The reaction was allowed to proceed for 20 min at 30°, with shaking at 60 cycles per min, and was terminated by addition of 10% trichloroacetic acid (TCA). Blanks and standards containing

microsomal suspension and glucose-6-phosphate in microsomal suspension, respectively, were also treated with 10% TCA. Following precipitation, the protein was removed by centrifugation at 1800 g in a MSE Mistral 6 L centrifuge for 5 min. The supernatant was assayed for inorganic phosphate by the method of King [267]. Blanks and standards for the inorganic phosphate assay were water and potassium dihydrogen phosphate, respectively. The absorbance at 660 nm was read in a Gilford spectrophotometer.

2.2.6g p-Nitroanisole O-demethylase

The O-demethylation of p-nitroanisole was measured by a modification of the method of Netter and Seidel [269]. A 2 mM solution of p-nitroanisole in 0.02 M Tris-HCl, pH 7.4, was prepared by heating to 60°. The stock solution of p-nitroanisole was kept at 37° for the duration of the assay. Microsomal suspension (0.6 mg protein/ml, final concentration) and p-nitroanisole (0.6 mM, final concentration) in 0.02 M Tris-HCl, pH 7.4 in a total volume of 3.0 ml, were equilibrated to 37°. The reaction was initiated by the addition of NADPH-generating system and initial reaction rates were recorded for about 5 min at 420 nm against a reference without NADPH-generating system.

2.2.6h Benzpyrene 3-Hydroxylase

The hydroxylation of benzpyrene was measured spectrophotometrically by the method of Prough et al. [270]. 2.5 ml of microsomal suspension containing 80 µM benzpyrene was equilibrated to 30° in cuvettes. 100 µl NADH (200 µM, final concentration) was

then added to both cuvettes and the reaction was initiated with the addition of 50 µl NADPH (100 µM final concentration) to the sample cuvette. The absorbance changes at 454 nm and 428 nm were recorded with time, and initial rates were calculated for the change in absorbance at 428 nm relative to 454 nm.

2.2.6i Ethoxyresofurin deethylase

The deethylation of ethoxyresofurin was measured by the fluorimetric assay of Burke and Mayer [271]. Reaction mixtures contained hepatic microsomes (1.6 mg protein/ml), and ethoxyresofurin (10-50 µM) plus NADPH (0.18 mM). Excitation and emission wavelengths were 510 nm and 586 nm. A stock solution of resofurin in methanol was used to standardize the fluorimeter. Experiments were carried out on a Perkin-Elmer fluorescence spectrophotometer 203 linked to a Pye-Unicam AR Linear Recorder.

2.2.6j Aminopyrine N-demethylase

The demethylation of aminopyrine was measured according to the method of Mazel [272]. 3 ml of microsomal suspension, 0.1 ml of aminopyrine (5 mM, final concentration), 0.1 ml of semicarbazide (4.5 mM, final concentration) and NADPH-generating system, were incubated at 37° for 30 min, with shaking at 60 cycles per min. The reaction was quenched by the addition of 1 ml of 15% zinc sulphate. 5 min later, 1 ml of saturated aqueous barium hydroxide was added. The precipitated protein was removed by centrifugation at 1000 g in a MSE Mistral 6 L centrifuge. 2.5 ml of the supernatant was used for the determination of formaldehyde by the

Hantzsch reaction as described by Nash [273]: 1.0 ml of the Nash reagent was added to the supernatant (2.5 ml) and the colour was allowed to develop at 60° for 30 min. The absorbance was read at 415 nm. A standard curve was constructed from solutions containing 0.5 - 4.0 µg formaldehyde per ml of supernatant obtained from hepatic microsomes alone, as described above. The standards were assayed in an identical manner to the incubation mixtures.

2.2.7 Other assays.

2.2.7a H_2O_2 production

Reaction mixtures for H_2O_2 assay contained microsomes from phenobarbital induced rats (2 mg protein/ml), NADPH-generating system, EDTA (0.1 mM)*, sodium azide (0.2 mM) and the chlorinated ethylene, incubated at 30° with shaking for 5 to 10 min. A parallel control was run containing hepatic microsomes from phenobarbital induced rats, EDTA (0.1 mM) and a NADPH-generating system. Aliquots (1.5 ml) of the incubation mixture were added to 1.5 ml of 5% TCA (2,5%, final concentration) and the resulting suspension was centrifuged at 1000 g in a MSE Mistral 6 L centrifuge for 10 min to remove the protein. Hydrogen peroxide (H_2O_2) was assayed by the method of Hildebrandt et al. [268] with the following precautions: To 2 ml of supernatant, ferro-ammonium sulfate (1.0 mM, final concentration) was added and potassium thiocyanate (0.13 M, final concentration) was added 2 min later. The absorbance of the ferric thiocyanate complex was monitored at 480 nm, exactly 4 min after the addition of potassium thiocyanate, in a Beckman UV-visible spectrophotometer.

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*Concentrations of EDTA above 0.1 mM interfered with this assay.

2.2.7b Artificial electron donors

The ability of artificial electron donors to support the metabolism of the chlorinated ethylenes by partially purified cytochrome P-450 (see Section 2.1.9) was assessed in incubation mixtures containing (where indicated) the chlorinated ethylene, partially purified cytochrome P-450 from phenobarbital-induced rat liver microsomes (ca. 2 μM, final concentration) and $NaClO_2$ (5 mM final concentration), H_2O_2 (10 mM, final concentration) or $NaIO_4$ (7.5 mM, final concentration) in 0.02 M Tris-HCl, pH 7.4. Incubations were carried out at 30° with shaking (60 cycles/min). Reaction mixtures were assayed at time zero and at 10 min for metabolite formation.

2.2.7c Degradation of microsomal cytochrome P-450 and heme

Degradation of microsomal cytochrome P-450 and heme were assessed as follows: Incubation of microsomal suspension in the presence of the chlorinated ethylene, NADPH-generating system and EDTA (0.2 mM, final concentration) were carried out for 15 - 30 min, and the concentrations of cytochrome P-450 and heme were determined as described in Sections 2.2.6a* and 2.2.6b, respectively. The reference cuvettes contained incubated microsomes alone, i.e. the chlorinated ethylene and NADPH-generating system were absent from reference incubation mixtures.

2.2.7d Inhibitors

Inhibitors were in all cases added to microsomal suspensions

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*All reported values are relative to zero time samples of hepatic microsomes plus the chlorinated ethylene. The addition of NADPH-generating system to the zero time determinations did not affect the $\Delta A_{450\ nm - 490\ nm}$.

prior to the addition of the chlorinated ethylene and NADPH-generating system. SKF-525A and metyrapone were added to microsomal suspensions at final concentrations of 200 mM and 2.3 mM, respectively. After addition of the inhibitor, the microsomal suspensions were stoppered and mixed before any further additions were made. In the case of inhibition with CO, $CO:O_2$ (80:20; v/v) was bubbled through the incubation mixture for 30 sec at a flow rate of 20 ml per min.

2.2.8 Preparation of hepatocytes

Viable hepatocytes were obtained by a modification of the perfusion method of Seglen [274], as follows: (see attached photographs).

1. The rat is anaesthetized with 0.4 ml of pentobarbital (60 mg/ml).

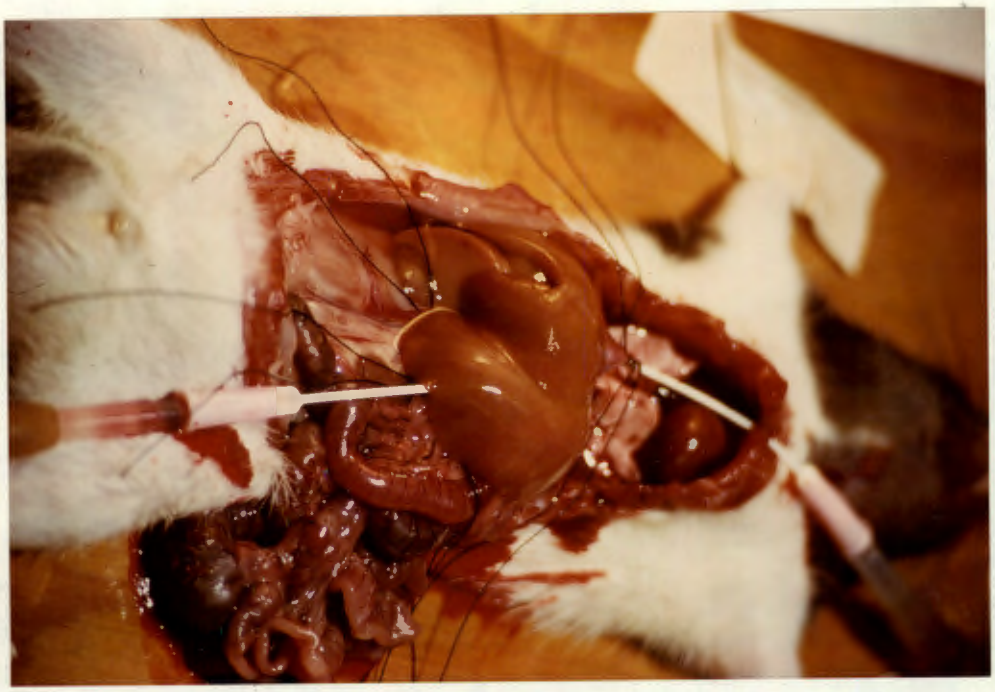
2. 0.2 ml heparin (5000 U/ml) is injected into one of the tail veins (the tail veins become obvious by immersing the tail in warm water for ca. 5 min).

3. The rat is strapped to a dissecting board, the abdomen opened and the intestines pushed aside to reveal the inferior vena cava and the portal vein.

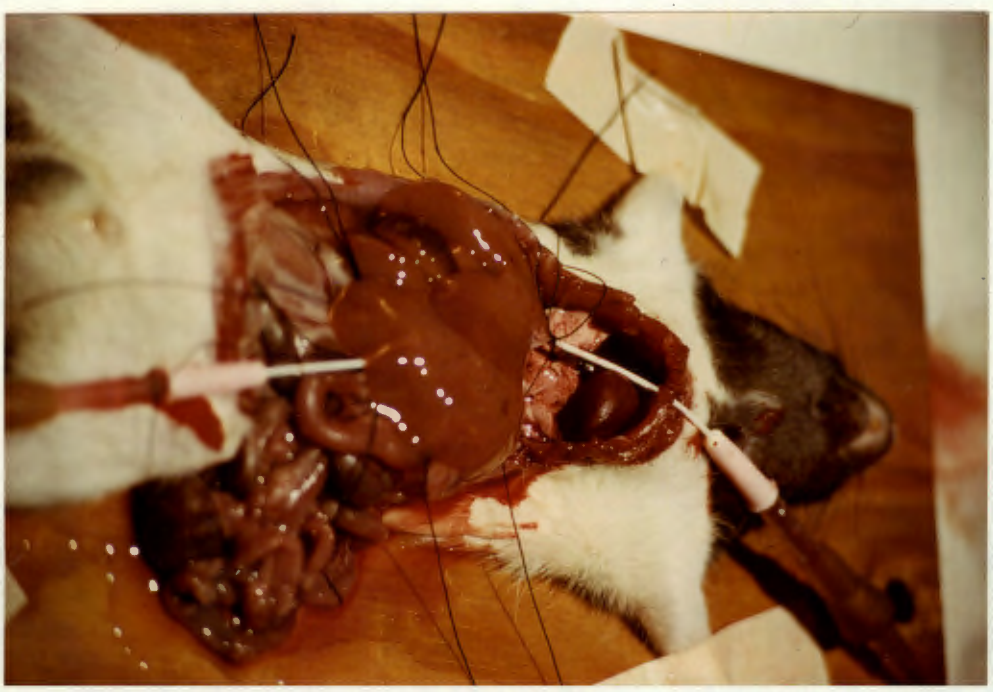
4. A subsidiary vein of the portal vein (just to the right of the portal vein; ca. one-third from the top of the exposed portion of the portal vein) and the bile duct are ligated and cut off.

5. The portal vein is ligated and a cannula inserted quickly just above the ligature. The cannula is then well secured.

6. Pre-perfusion buffer (0.14 mol NaCl, 6.6 mmol KCl and 12 mmol HEPES made up to 1 litre with distilled water and adjusted to pH 7.4 with 1 M NaOH) which is oxygenated by bubbling with $O_2:CO_2$ (95:5)



Step 7: A well blanched liver following cannulation.



Step 10: Definite signs of disintegration of the liver following collagenase treatment.

for 10 min at 37 - 39° prior to use, is passed through with the aid of a peristaltic pump (Gilson Minipuls 2) at a rate of 5 - 10 ml per min.

7. Immediately, the thoracic wall is cut open and the inferior vena cava is cannulated above the diaphragm as quickly as possible.

8. The flow speed of the pre-perfusion buffer through the liver is increased to 50 - 60 ml per min and this speed is maintained for ca. 10 min or until the pre-perfusion buffer (400 ml) is finished.

9. 0.5% Collagenase is dissolved in the perfusion buffer (3.9 g NaCl, 0.5 g KCl, 0.7 g $CaCl_2.2H_2O$ and 24 g HEPES dissolved in 1 litre of distilled water and adjusted to pH 7.6 with 1 M NaOH). The perfusion buffer is oxygenated as was the pre-perfusion buffer in Step 6 but prior to the addition of collagenase. The collagenase solution is then pumped through the liver at 50 - 60 ml per min for 10 - 15 min. The liver is kept warm by having a 100W lamp suspended 20 cm above it.

10. When the liver shows very definite signs of disintegration, the perfusion is terminated and the liver is removed to a small beaker. The liver capsule is ruptured and the cells are suspended in oxygenated ice-cold washing buffer (8.3 g NaCl, 0.5 g KCl, 0.18 g $CaCl_2.2H_2O$ and 2.4 g HEPES made up to 1 litre and pH 7.4 with 1 M NaOH) and spun down at 50 g for 45 sec.

11. The cells are washed three times with washing buffer as in Step 10; the last resuspension being to a known volume of ca. 80 ml per liver in washing buffer (to calculate cell yield).

2.2.8a Determination of the viability of the isolated hepatocytes.

The viability of the hepatocytes was determined by the exclusion of 4.2 mM trypan blue in water. 0.1 ml of trypan blue (1.2 mM, final concentration) was added to 0.25 ml of hepatocytes (ca. $4\text{-}5\text{x}10^6$ cells per ml at 0° in oxygenated washing buffer), and the cell count and cell viability were ascertained by counting, using a haemocytometer. The preparation of hepatocytes was used for experimentation only if 85% or more of the cells excluded trypan blue (except for the preliminary experiments reported in Table 34). The concentration of the hepatocytes was adjusted to $2\text{x}10^6$ viable cells per ml in Leibowitz-15 medium, that had been bubbled with CO_2:O_2 (5:95; v/v) and supplemented with fetal calf serum (FCS) (3%, final concentration), penicillin (100 IU/ml) and streptomycin (100 µg/ml). All experiments were performed with hepatocyte suspensions of $2\text{x}10^6$ viable cells per ml in Leibowitz-15 medium within 5 hr of preparation.

2.2.8b Cytochrome P-450

The levels of hepatic cytochrome P-450 in hepatocytes, were determined as described in Section 2.2.6a, on cells sonicated for 30 sec using a Branson Model B-12 Sonifier.

2.2.8c Metabolism of the chlorinated ethylenes

Investigations into the metabolism of the chlorinated ethylenes using hepatocyte suspensions were performed using 50 ml Erlenmeyer flasks each containing 5 ml of hepatocyte suspension

($2x10^6$ cells/ml). Incubations of hepatocyte suspensions of volumes greater than 5 ml (in 50 ml Erlenmeyer flasks) resulted in decreased viability of the cells with time.

The chlorinated ethylenes were introduced into the hepatocyte suspensions with the aid of a Hamilton syringe as a 1 : 1 ethanolic solution (for tri- and tetrachloroethylene) and as a 1 : 2 ethanolic solution (for the dichlorinated ethylenes) as no vortex mixing of intact cells is permissable. The flasks were stoppered with air-tight rubber septa and the cells were prevented from settling to the bottom of the flasks during incubation by rotation of the flasks at 30 revolutions per min at an angle of 35°. All incubations were carried out at 37°.

Otherwise, incubations of hepatocytes with the chlorinated ethylenes were identical to those performed on hepatic microsomal suspensions (see Section 2.2.5), except that the hepatocyte preparations did not require the addition of a NADPH-generating system. Following removal of the protein by the addition of 200 µl 2N H_2SO_4 (40 mM, final concentration) and 200 µl 0.3 M Na_2WO_4 (12 mM, final concentration) and subsequent centrifugation, the supernatant was used directly for the modified Fujiwara assay [261] or was extracted with 2 - 4 ml of diethyl ether for gas liquid chromatographic analysis using Column II or Column III.

2.2.8d Conjugated metabolites

Hepatocytes ($2x10^6$ cells/ml), isolated from phenobarbital pretreated animals, were incubated with the chlorinated ethylenes for periods of time in which the production of identified, free metabolites were linear. These incubation mixtures were then

treated with β-glucuronidase (2500 U/ml or 10000 U/ml, final concentration) or arylsulfatase (375 U/ml, final concentration) [281] and were further incubated at 37° overnight, with shaking at 60 cycles per min, in a Gallenkamp thermostatted waterbath. The following morning the protein was precipitated and removed by centrifugation (see Section 2.2.8c). The supernatant was extracted with 2 ml of diethyl ether and analyzed by gas-liquid chromatography using Column II. Alternatively, H_2SO_4 (6.25 M, final concentration) was added to reaction mixtures and the resultant suspension was placed in a boiling water bath for 30 min [282]. After the hydrolyzed sample had cooled, free metabolites were extracted with 10 ml of diethyl ether or iso-octane, dried and analyzed as above.

2.2.8e Covalently bound chloroacetyl moieties

Following incubation of the chlorinated ethylenes with viable hepatocytes isolated from phenobarbital pretreated animals, the hepatocyte suspension was treated with an equal volume of 20% H_2SO_4 at 100° for 6 hr to measure chloroacetyl moieties covalently bound to cellular constituents.

2.2.9 Assay for DNA repair

DNA repair caused by the metabolism of the chlorinated ethylenes in freshly isolated hepatocytes was assessed by the method of Andrae [186]. Benzpyrene (20 μM, final concentration) and DMSO (0.01%, final concentration) were used as a positive and negative control, respectively [186]. The method is as follows: Incubation of hepatocytes (2 ml at $2x10^6$ cells/ml) was performed in Leibowitz-

15 medium supplemented with glutamine (2 mM, final concentration) and streptomycin (100 µg/ml) in rotating flasks (30 rpm) at 37°. After preincubation for 1 hr with bromodeoxyuridine (BrUrd) (50 µM, final concentration) and fluorodeoxyruidine (FrUrd) (10 µM, final concentration), the cells were resuspended in fresh medium, composed as described above. The test compound dissolved in dimethylsulfoxide and 20 µCi [5-^{3}H]-deoxycytidine (specific activity 24 Ci/nmol) were added to the cell suspension and the incubation was continued for 2.5 hr. The reaction was terminated by the addition of 5 ml of ice-cold buffer solution (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, pH 7.5). The cells were spun down at 1000 g for 5 min at O° and washed once, as above. The cell pellet was frozen at -20°. The frozen pellet was lyzed by resuspension in 3.3 ml of 0.5% sodium dodecylsulfate and subsequently digested with proteinase K (50 µg/ml, final concentration) for 2 hr at 50° according to the method of Smith et al.[185]. 2.0 ml of the aqueous layer containing the nucleic acids was added to 8.15 g CsCl and the solution was brought by the addition of ca. 4 ml of 0.1 M K_2HPO_4, pH 12.5, to a refractive index of 1.4066-1.4068 (Bellingham and Stanley refractometer). The solution was centrifuged at 37000 rpm (20°) for 40 hr in a Beckman L8-70 Ultracentrifuge using stainless steel centrifuge tubes. With the aid of a Buchler Auto Densi-Flow IIC Gradient Remover, linked to a LKB Ultrorac Fraction Collector, ca. 30 equal fractions of ca. 0.3 ml were collected from each gradient. After dilution of each fraction with 0.4 ml of 0.1 M Tris-HCl, pH 4.0, 0.25 ml of each fraction was removed and mixed with 0.4 ml of distilled H_2O with the aid of a Gilson Model 400 diluter dispenser.

The absorbance at 260 nm was monitored for these samples. The remaining part of each fraction was mixed with 10 ug of calf thymus DNA in 1.5 ml of 0.1 M K_2HPO_4, pH 12.5. The DNA was precipitated with 10% TCA and applied to Whatman GF/C glass fibre filters (2.5 cm). The filters were washed using 5% TCA and 6% sodium pyrophosphate in 5% TCA, dried and counted with 5 ml of Beckman Ready-Solv EP scintillator in a Beckman LS 9000 liquid scintillation counter.

2.3 Calculations and Statistical Analysis

Reported values are means ± standard deviations (S.D.) for assays in triplicate or quadruplicate on two or more preparations of microsomes or hepatocytes, unless otherwise indicated. Binding (K_s) and Michaelis (K_m) constants, maximal extents of binding (ΔA_{max}) and maximal rates of metabolism (V_{max}) were calculated from Hanes and Eadie-Hofstee plots. In these plots, ΔA represents the difference in absorbance between peak and trough observed in difference spectral studies; and v represents the initial rate of the reaction under investigation. A Texas SR-51-II calculator was used to determine the intercepts on the x-axis and the y-axis, the slope of the plots as well as the correlation coefficients*.

The Student's t-test was utilized to calculate significant differences between means. A difference was considered to be significant with $P < 0.01$ and probably significant with $P < 0.05$.

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*The correlation coefficient for each plot drawn is as shown in the accompanying figure legend.

3. RESULTS

Results obtained from the binding of the chloroethylenes to hepatic cytochrome P-450 as well as their stimulation of CO-inhibitable NADPH oxidation will be presented in the first section of the results.

Subsequently, the results of the interaction of the chlorinated ethylenes with the microsomal drug metabolizing enzymes will be dealt with compound by compound in the order of increasing number of chlorine substituents.

3.1 Binding of the chlorinated ethylenes to hepatic microsomal cytochrome P-450 in vitro.

Vinylidene chloride, cis- and trans-1,2-dichloroethylene, trichloroethylene and tetrachloroethylene each bound to hepatic microsomal cytochrome P-450, resulting in the production of Type I difference spectrum (λ_{max} = 386 nm; λ_{min} = 416 nm) in accordance with an initial report by Pelkonen and Vainio that trichloroethylene and tetrachloroethylene produced a Type I difference spectrum with hepatic microsomes [249].

The effects of induction of different forms of cytochrome P-450 on the binding constants (K_s) and maximum extents of binding (ΔA_{max} and ΔA_{max} per nmol cytochrome P-450) for the chlorinated ethylenes are as follows:

3.1.1 Vinylidene chloride

Hanes plots of the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 were in all cases biphasic and were characterized by two K_s values and two corresponding ΔA_{max} values

(see Figures 20, 21 and 22). The effect of inducing agents for different forms of cytochrome P-450 on the binding constants (K_s) and the maximal extents of binding (ΔA_{max}) for the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 is shown in Table 9. The K_s values for the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 were not significantly altered by induction except that the K_s value for the low affinity site for the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 was significantly decreased following phenobarbital induction. The ΔA_{max} values were unaffected or decreased following β-naphthoflavone induction, and increased following phenobarbital induction. However, ΔA_{max} per nmol cytochrome P-450 were either unaffected or decreased following both types of induction (see Table 9).

3.1.2 cis- and trans-1,2-Dichloroethylene

Hanes plots of the binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450 were also biphasic (see e.g. Figures 23 - 26), and therefore two binding constants (K_s) and two corresponding values for the maximal extents of binding (ΔA_{max}) were calculated for the binding of each of the 1,2-dichloroethylenes to hepatic cytochrome P-450 in microsomes from untreated and pretreated rats. The effect of induction of different forms of hepatic microsomal cytochrome P-450 on the values of K_s and ΔA_{max} for the binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450 is shown in Table 10.

The K_s values for the binding of a mixture of cis- and trans-1,2-dichloroethylene as well as trans-1,2-dichloroethylene to

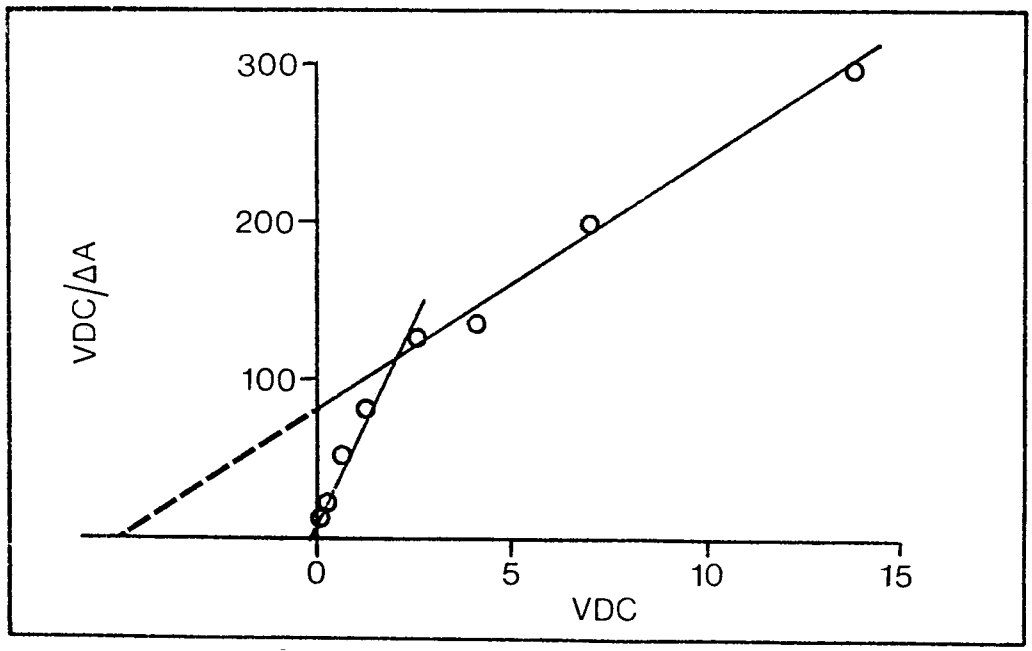

Figure 20: Hanes plot of the binding of vinylidene chloride to hepatic cytochrome P-450 in microsomes from β-naphthoflavone treated rats. Vinylidene chloride (VDC), mM; ΔA, $A_{386\ nm}-A_{418\ nm}$. Correlation coefficient for high affinity site = 0.975; low affinity site = 0.953.

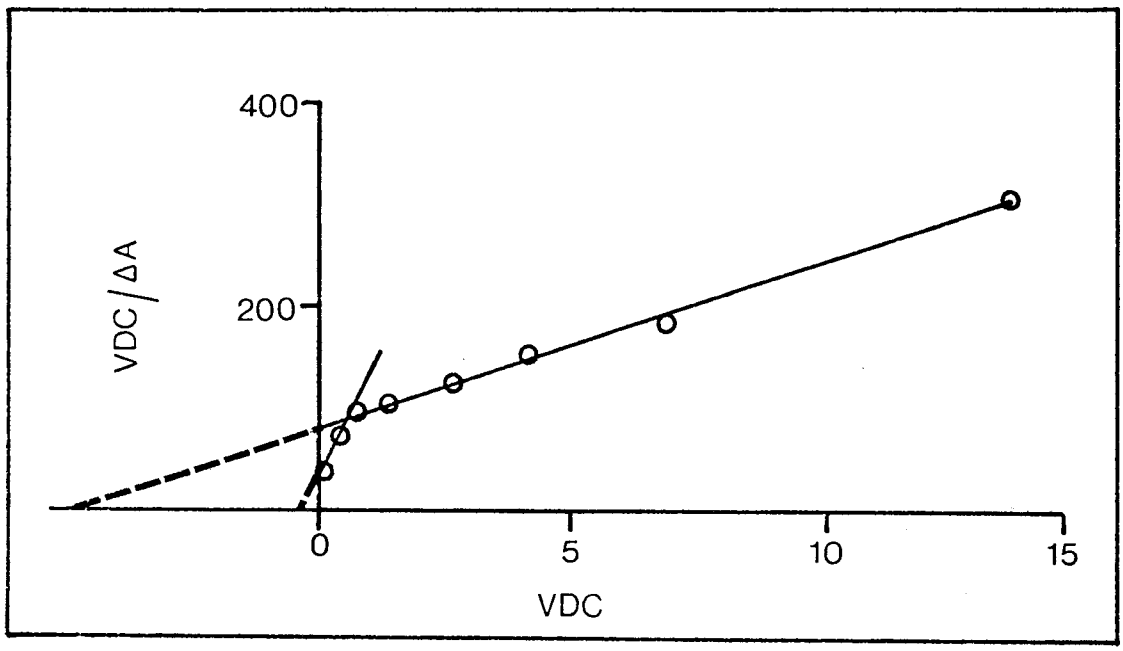

Figure 21: Hanes plot of the binding of vinylidene chloride to hepatic cytochrome P-450 in microsomes from untreated rats. Vinylidene chloride (VDC), mM; ΔA, $A_{386nm}-A_{418nm}$. Correlation coefficient for high affinity site = 0.999; low affinity site = 0.946.

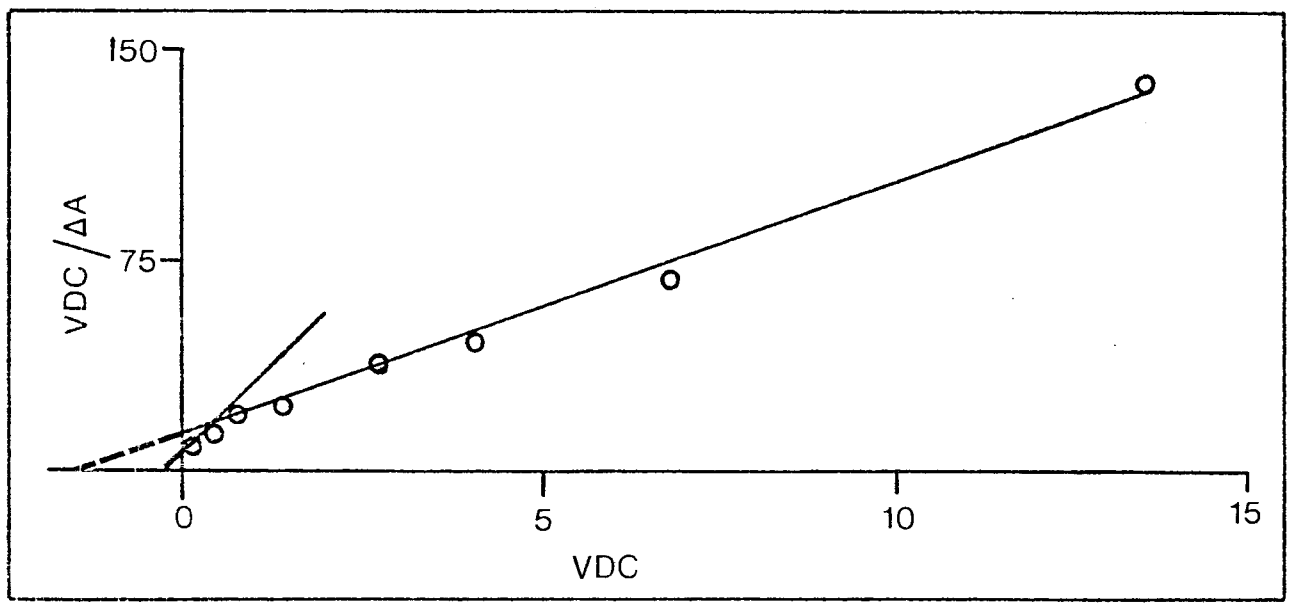

Figure 22: Hanes plot of the binding of vinylidene chloride to hepatic cytochrome P-450 in microsomes from phenobarbital treated animals. Vinylidene chloride (VDC), mM; ΔA, $A_{386nm}-A_{418nm}$. Correlation coefficient for high affinity site = 0.974; low affinity site = 0.998.

Table 9: Binding of vinylidene chloride by hepatic microsomal cytochrome P-450*

Inducing agent	K_s (mM)		$10^2 \Delta A_{max}$ ($A_{386nm}-A_{418nm}$)		$10^2 \Delta A_{max}$/nmol cytochrome P-450	
None	0.22±0.06	5.8±1.3	1.3±0.5	7.2±1.7	1.4±0.6	8.0±1.9
β-Naphthoflavone	0.19±0.06	4.8±0.6	1.1±0.6	4.5±1.4	0.9±0.5	3.8±1.2+
Phenobarbital	0.26±0.01	1.6±0.1+	4.0±0.4+	11.4±0.1+	1.9±0.2	5.4±0.1+

*Values reported are means ± S.D. for experiments performed in triplicate with three or more preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.3.

+Differs from corresponding value for microsomes from untreated rats, $P < 0.01$.

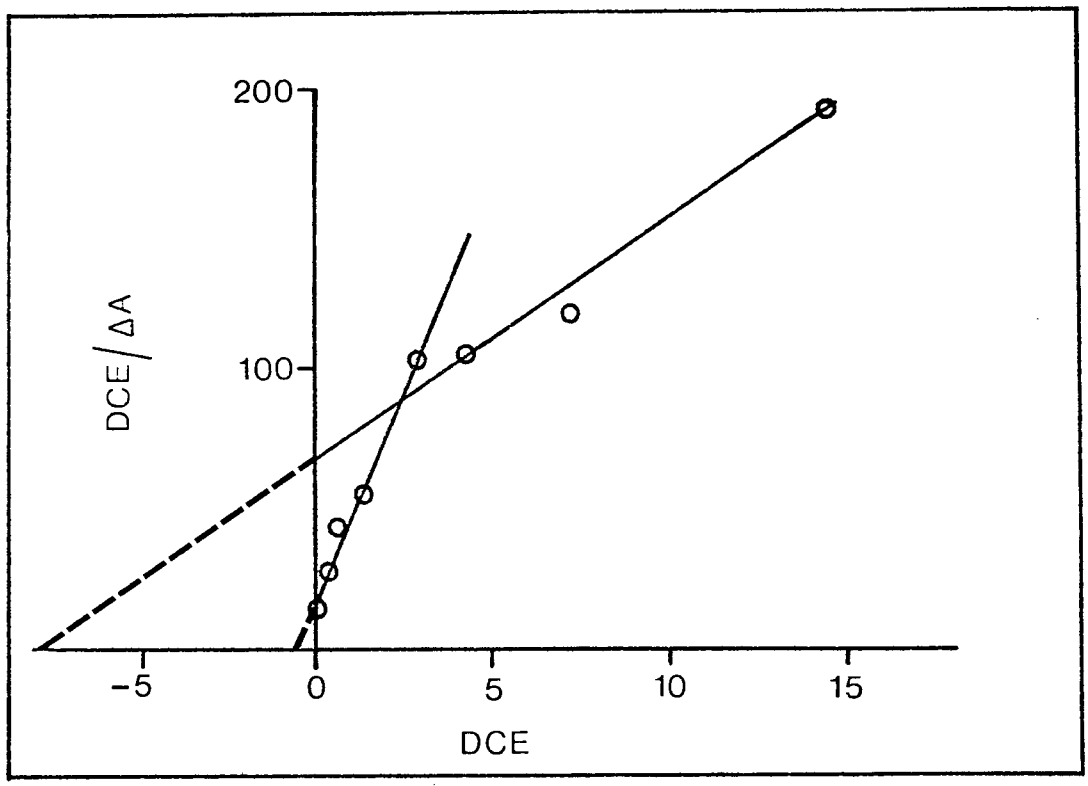

Figure 23: Hanes plot of the binding of cis-1,2-dichloroethylene to cytochrome P-450 in hepatic microsomes from phenobarbital treated rats. cis-1,2-Dichloroethylene (DCE), mM; ΔA, $A_{386nm}-A_{418nm}$. Correlation coefficient for high affinity site = 0.987; low affinity site = 0.995.

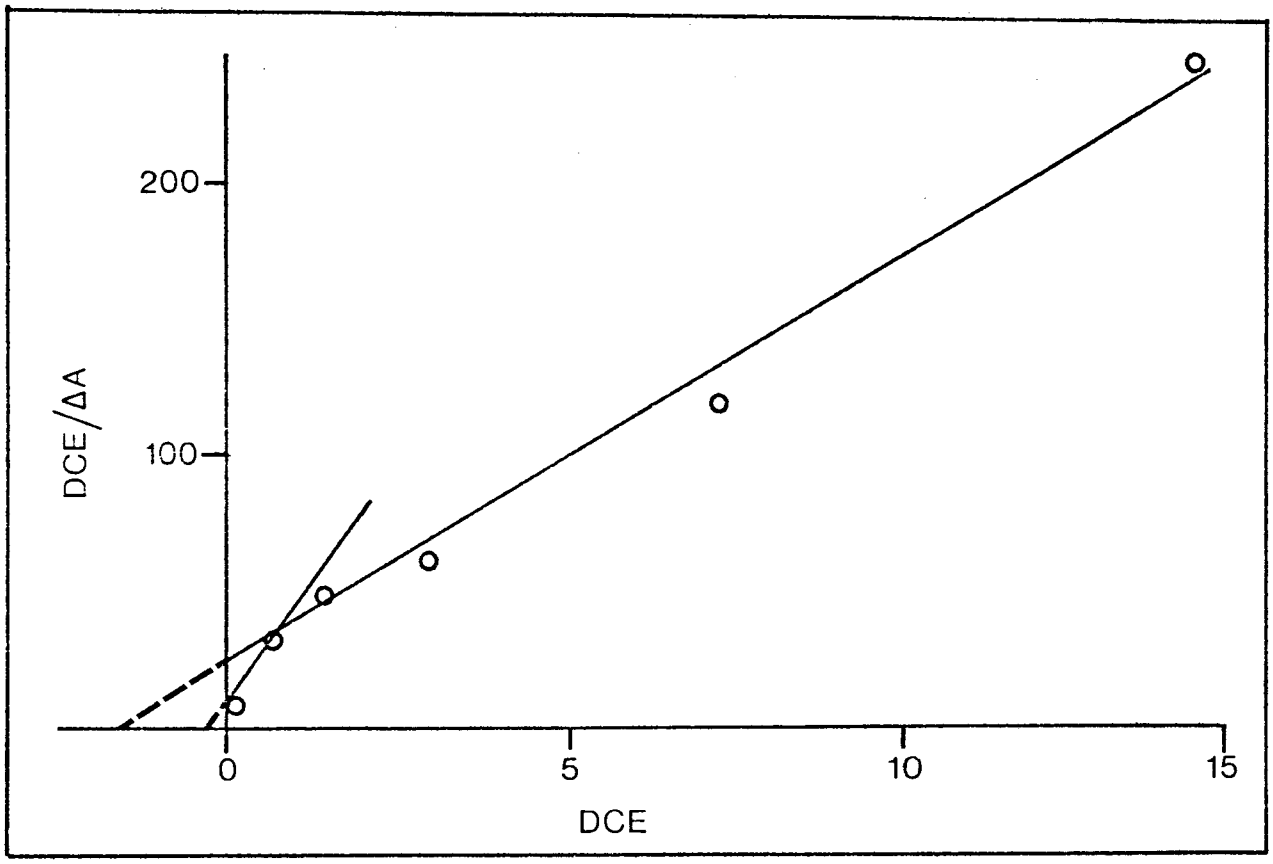

Figure 24: Hanes plot of the binding of trans-1,2-dichloroethylene to hepatic cytochrome P-450 in microsomes from phenobarbital treated rats. trans-1,2-Dichloroethylene (DCE), mM; ΔA, $A_{386nm}-A_{418nm}$. Correlation coefficient for high affinity site = 0.989; low affinity site = 0.996.

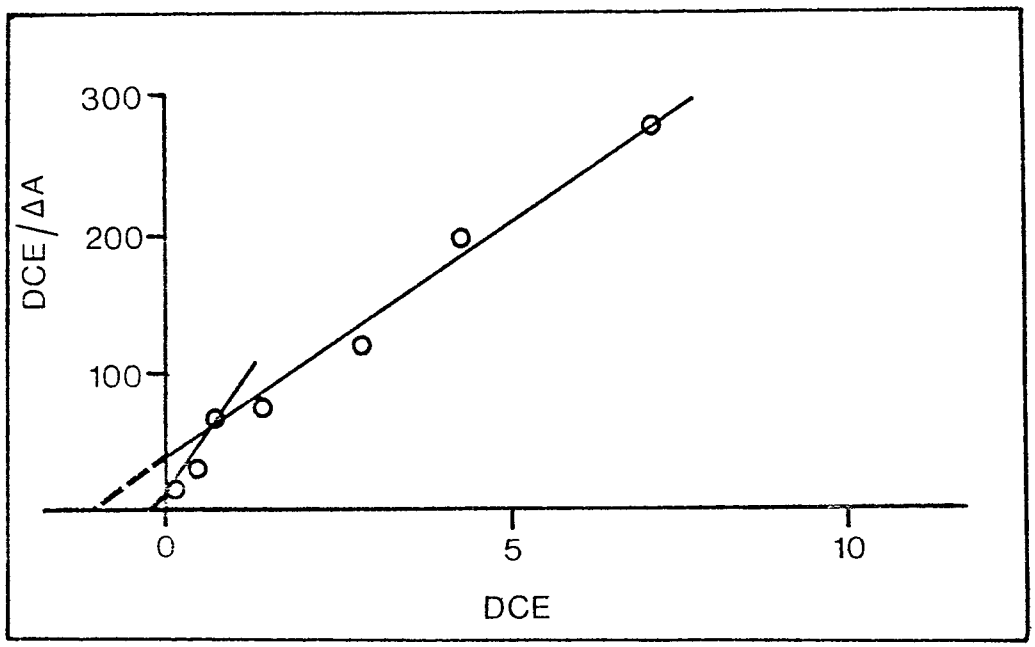

Figure 25: Hanes plot of the binding of the mixture of cis- and trans-1,2-dichloroethylene (30:70) to hepatic cytochrome P-450 in microsomes from β-naphthoflavone treated rats. cis- and trans-1,2-Dichloroethylene (DCE), mM; ΔA, $A_{386\ nm}-A_{418\ nm}$. Correlation coefficient for high affinity site = 0.952; low affinity site = 0.992.

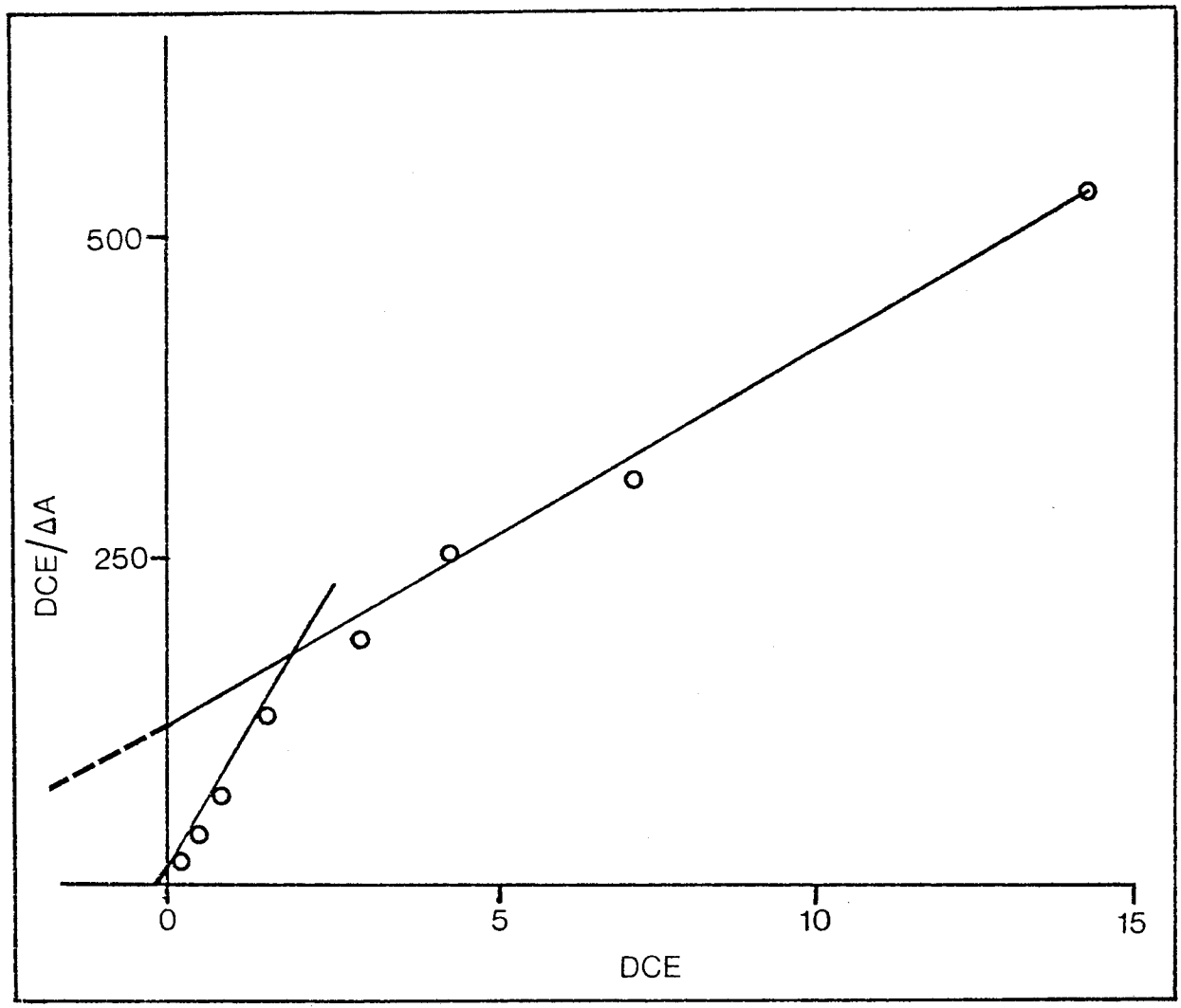

Figure 26: Hanes plot of the binding of the mixture of cis- and trans-1,2-dichloroethylene (30:70) to hepatic cytochrome P-450 in microsomes from untreated rats. cis- and trans-1,2-Dichloroethylene (DCE), mM; ΔA, $A_{386\ nm}-A_{418\ nm}$. Correlation coefficient for high affinity site = 0.999; low affinity site = 0.997.

Table 10: The binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450*

Inducing agent	Cis- and trans-1,2-dichloroethylene (30:70)				Trans-1,2-dichloroethylene				Cis-1,2-dichloroethylene			
	K_s (mM)		$10^2 \Delta A_{max}$		K_s (mM)		$10^2 \Delta A_{max}$		K_s (mM)		$10^2 \Delta A_{max}$	
None	0.2±.1	4.1±.3	2.0±0.5 (2.2±0.6)	4.0±.8 (4.4±.9)	0.4±.1	8.2±3.9	1.3±.1 (1.6±.1)	4.9±1.0 (5.6±1.1)	ND	ND	ND	ND
β NF	0.3±.1	5.2±.8	1.0±0.3$^{\neq}$ (0.7±0.2^{+})	3.3±.3 (2.2±.2^{+})	0.6±.2	8.6±0.8	1.6±.1 (1.3±.1$^{\neq}$)	6.5±0.7$^{\neq}$ (5.2±0.6)	ND	ND	ND	ND
PB	0.3±.1	2.2±.3^{+}	3.6±1.2^{+} (1.7±.6)	6.8±.2^{+} (3.2±.1)	0.4±.2	2.5±1.3^{+}	3.9±.8^{+} (1.8±.4)	8.9±3.2^{+} (4.0±1.5)	0.4±.1	7.7±.2	2.8±.4 (1.5±.2)	9.0±.6 (5.2±.4)

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes except for results reported for pure cis-1,2-dichloroethylene where results were in triplicate from a single preparation of hepatic microsomes. Experimental details are as described in Section 2.2.3. Values in parentheses are per nmol cytochrome P-450. Abbreviations used are ND = not determined; β NF = β-Naphthoflavone; PB = Phenobarbital.

$^{+}$Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

$^{\neq}$Probably differs from corresponding value for microsomes from untreated rats, $P < 0.05$.

hepatic microsomal cytochrome P-450 were not altered following β-naphthoflavone induction. Following phenobarbital treatment, there was no effect on K_s for the high affinity site but there was a significant decrease in the K_s for the low affinity site for the mixture of isomers and for the pure trans- isomer.

The values of ΔA_{max} and ΔA_{max} per nmol cytochrome P-450 for the mixture of isomers and for trans-1,2-dichloroethylene were generally decreased following β-naphthoflavone treatment. In contrast, ΔA_{max} for the mixture of isomers and for pure trans-1,2-dichloroethylene was generally elevated by phenobarbital induction, while ΔA_{max} per nmol cytochrome P-450 was not. For each type of induction, the values of K_s and ΔA_{max} were within a factor of two for the mixture of isomers and for pure cis- and trans-1,2-dichloroethylene, except for the K_s for the low affinity site for the cis-isomer which, following phenobarbital induction, was three-fold greater than the corresponding values for the mixture of isomers or pure trans-1,2-dichloroethylene (Table 10).

3.1.3 Trichloroethylene

As can be seen in Figures 27 and 28, Hanes plots of the binding of trichloroethylene to hepatic microsomal cytochrome P-450 were monophasic in hepatic microsomes from untreated and phenobarbital pretreated rats (compare to vinylidene chloride, Section 3.1.1). Hanes plots for the binding of trichloroethylene to hepatic microsomal cytochrome P-450 in hepatic microsomes from spironolactone and 3-methylcholanthrene pretreated rats were also monophasic (data not shown).

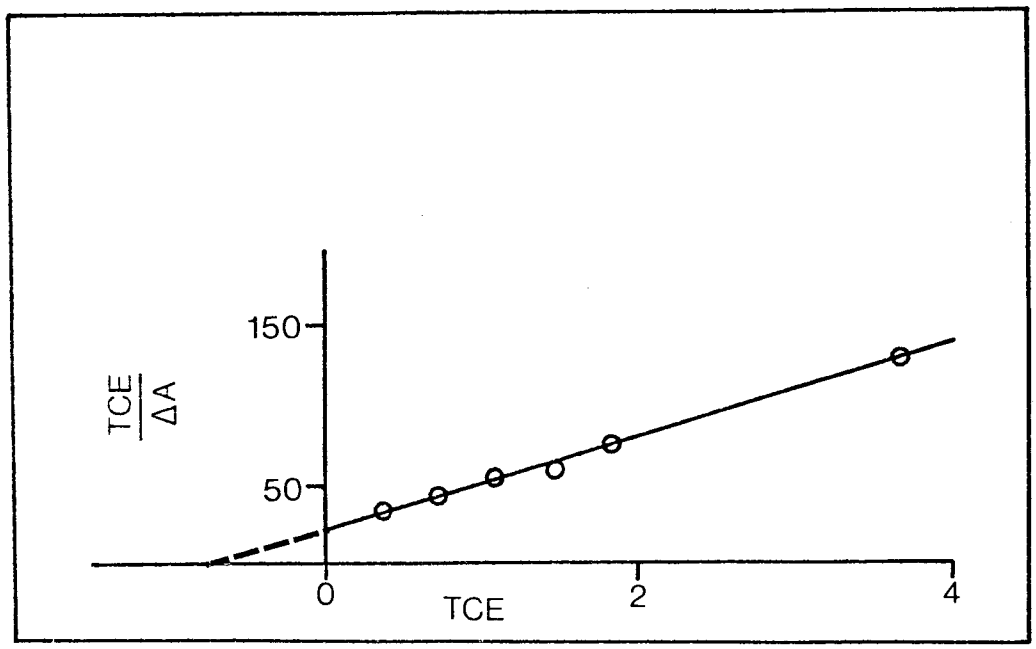

Figure 27: Hanes plot for the binding of trichloroethylene to hepatic cytochrome P-450 in microsomes from untreated rats. Trichloroethylene (TCE), mM; ΔA, $A_{388nm}-A_{419nm}$. Correlation coefficient = 0.998.

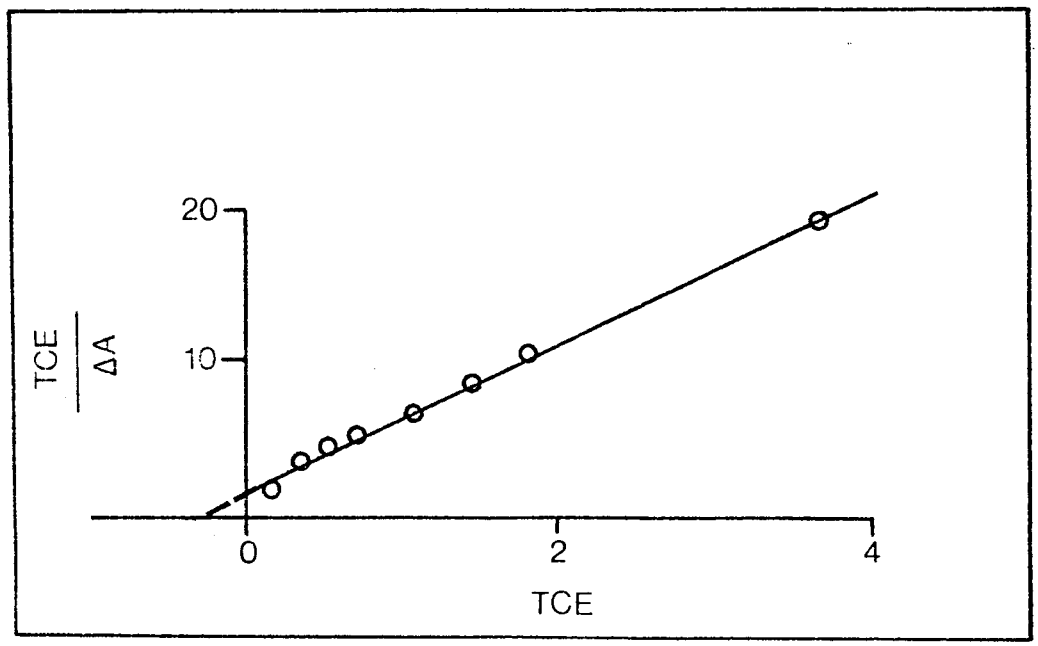

Figure 28: Hanes plot for the binding of trichloroethylene to hepatic cytochrome P-450 in microsomes from phenobarbital treated animals. Trichloroethylene (TCE), mM; ΔA, $A_{388nm}-A_{419nm}$. Correlation coefficient = 0.999.

The effects of inducing agents for different forms of cytochrome P-450 on the binding constants (K_s) and the maximum extents of binding (ΔA_{max}) for the binding of trichloroethylene to hepatic microsomal cytochrome P-450 are shown in Table 11. The K_s values for the binding of trichloroethylene to hepatic microsomal cytochrome P-450 were not altered by prior induction with 3-methylcholanthrene, spironolactone or phenobarbital. The values of ΔA_{max} and ΔA_{max} per nmol of cytochrome P-450 were increased following phenobarbital induction ($P < 0.01$), but not following 3-methylcholanthrene or spironolactone induction ($P > 0.1$)

Furthermore, trichloroethylene also bound to partially purified cytochrome P-450, isolated from the hepatic microsomes of phenobarbital-induced rats, with the production of a Type I difference spectrum (λ_{max} = 388 nm; λ_{min} = 419 nm). The K_s values obtained from Hanes plots for the binding of trichloroethylene to phenobarbital induced partially purified cytochrome P-450 (see Figures 29 and 30) were 1.85 ± 0.1 mM and 1.38 ± 0.11 mM for the enzyme suspended in 0.02 M Tris-HCl, pH 7.4, and for the glycerol solubilized enzyme, respectively.

3.1.4 Tetrachloroethylene

Unlike the other chlorinated ethylenes, tetrachloroethylene required vortex mixing for 90 sec to attain optimal dispersion in the microsomal suspension (see, e.g. Figure 31). Vortex mixing tetrachloroethylene for 90 sec was therefore utilized for microsomes from untreated as well as for microsomes from variously pretreated animals. In no case was any sample vortex mixed for longer than

Table 11. Effect of induction on the binding of trichloroethylene by hepatic microsomal cytochrome P-450*

Induction	Cytochrome P-450 (nmoles/mg microsomal protein)	K_s (mM)	$10^2 \Delta A_{max}$ ($A_{386}-A_{416}$)	$10^2 \Delta A_{max}$/ nmol cytochrome P-450
None	0.93±.10	0.69±.39	3.4±0.8	3.6±1.0
3-Methylcholanthrene	1.40±.04	0.86±.36	3.9±0.7	2.8±0.5
Spironolactone	0.78±.12	0.54±.28	3.2±1.2	4.1±1.0
Phenobarbital	2.54±.69	0.63±.14	18.0±4.3+	7.1±1.0+

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.3.

+Differs from corresponding value for uninduced microsomes, $P < 0.01$

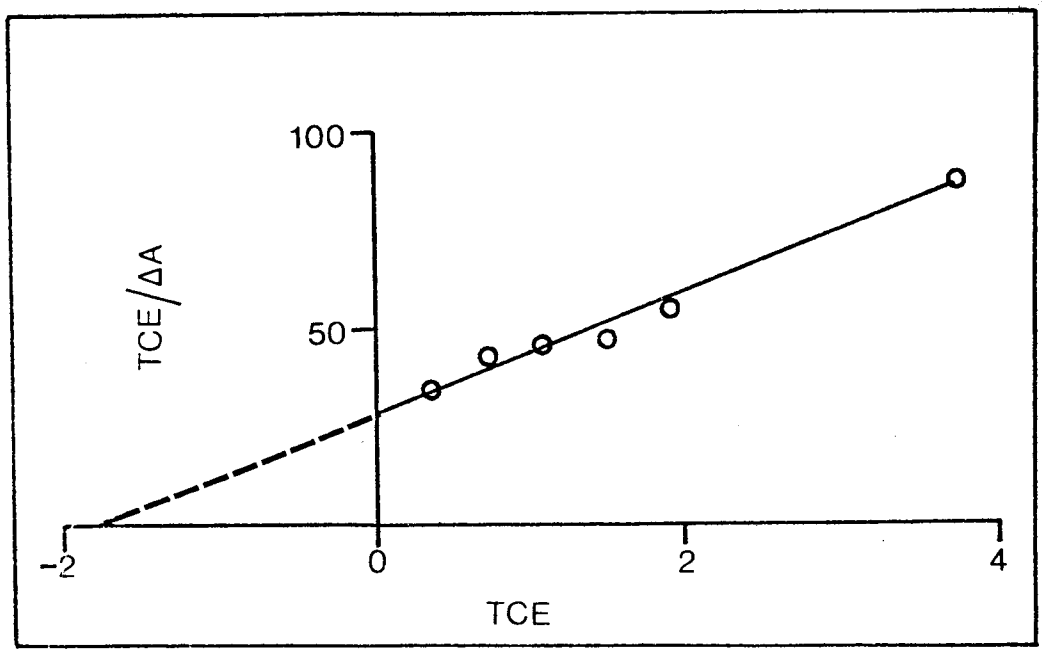

Figure 29: Hanes plot of the binding of trichloroethylene to partially purified cytochrome P-450 obtained from hepatic microsomes from phenobarbital treated animals. Partially purified cytochrome P-450 was suspended in 0.02 M Tris-HCl, pH 7.4. Trichloroethylene (TCE), mM; ΔA, $A_{388nm}-A_{419nm}$. Correlation coefficient = 0.990.

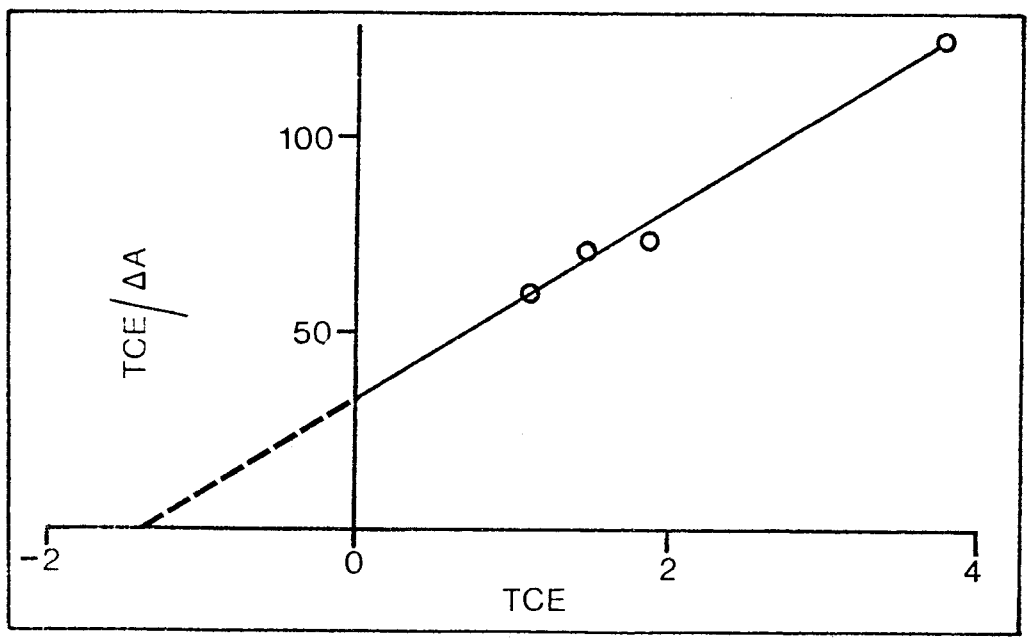

Figure 30: Hanes plot of the binding of trichloroethylene to partially purified cytochrome P-450 obtained from hepatic microsomes from phenobarbital treated rats. Partially purified hepatic cytochrome P-450 was solubilized in glycerol. Trichloroethylene (TCE), mM; ΔA, $A_{388nm}-A_{419nm}$. Correlation coefficient = 0.994.

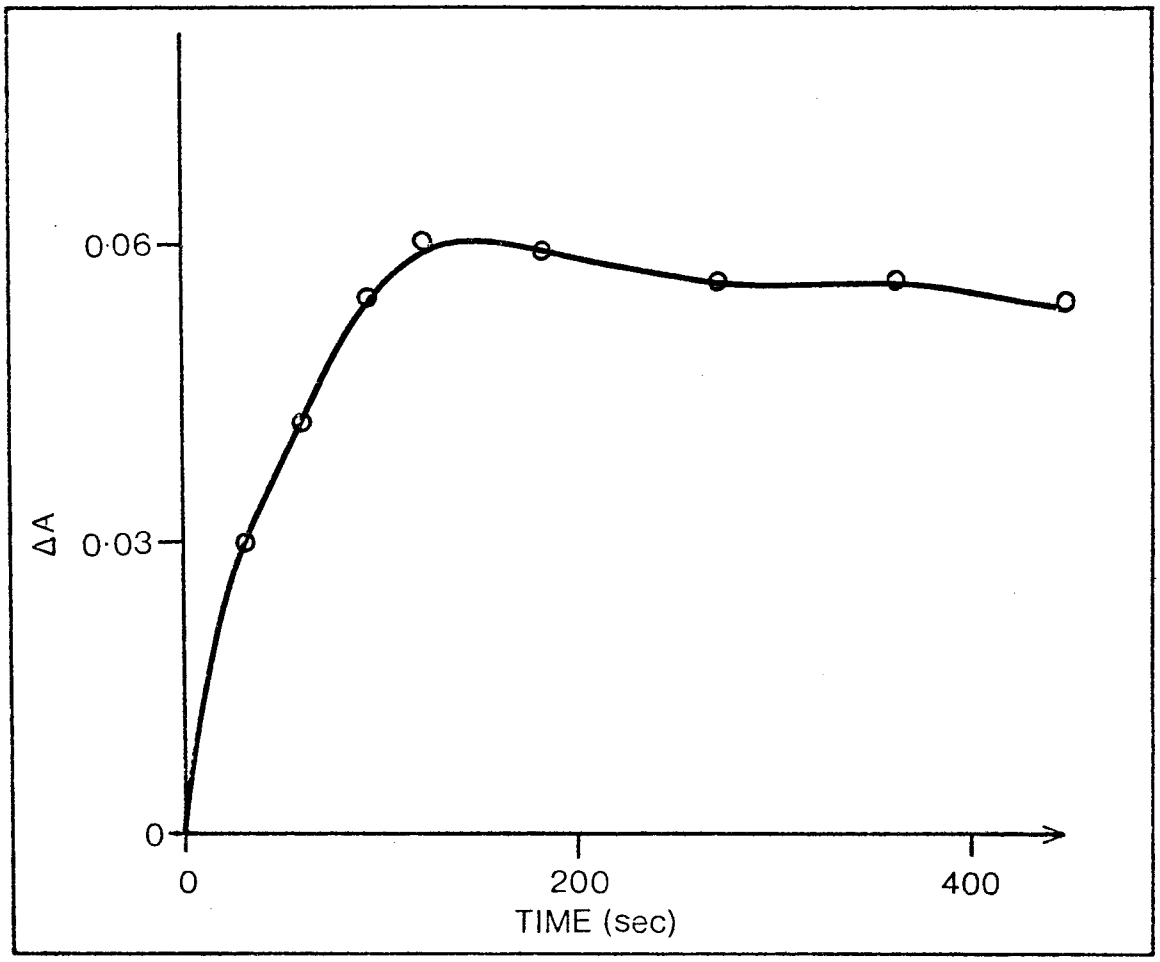

Figure 31: Binding of tetrachloroethylene to hepatic cytochrome P-450 in microsomes from untreated rats as a function of time. Δ A, $A_{388nm}-A_{418nm}$; Time, sec. / vortexing

90 sec. (viz. only one single addition of a compound was made to any sample of microsomal suspension). Each reported ΔA value has been corrected for the small decrease in absorbance at ca. 418 nm, which arose from vortex mixing for 90 sec. of microsomal suspension in the sample cuvette without any added compound. The loss of absorbance at 418 nm apparently arose from the denaturation of the hepatic microsomal cytochrome P-450 to the inactive form, cytochrome P-420. If a comparison is made with the initial levels of hepatic microsomal cytochrome P-450, vortex mixing of microsomes from untreated, β-naphthoflavone and phenobarbital treated animals resulted in 20%, 13% and 5% losses, respectively, whether or not tetrachloroethylene was present (Table 33).

As found with the binding of trichloroethylene to hepatic microsomal cytochrome P-450, Hanes plot of the binding of tetrachloroethylene to hepatic microsomal cytochrome P-450 were monophasic for microsomes from untreated and variously pretreated rats (see, e.g. Figures 32 and 33). The effect of induction of different forms of cytochrome P-450 on the spectral binding constants (K_s) and the maximum extents of binding (ΔA_{max}) of tetrachloroethylene are presented in Table 12. It can be seen that the induction of different forms of cytochrome P-450 with β-naphthoflavone or phenobarbital did not affect K_s, while induction with pregnenolone-16α-carbonitrile significantly increased K_s. The maximum extent of spectral binding of tetrachloroethylene (ΔA_{max}) was increased by both pregnenolone-16α-carbonitrile and phenobarbital, with the latter compound having the greater effect. ΔA_{max} per nmol cytochrome P-450, however, was unaffected following all three types of induction (see Table 12).

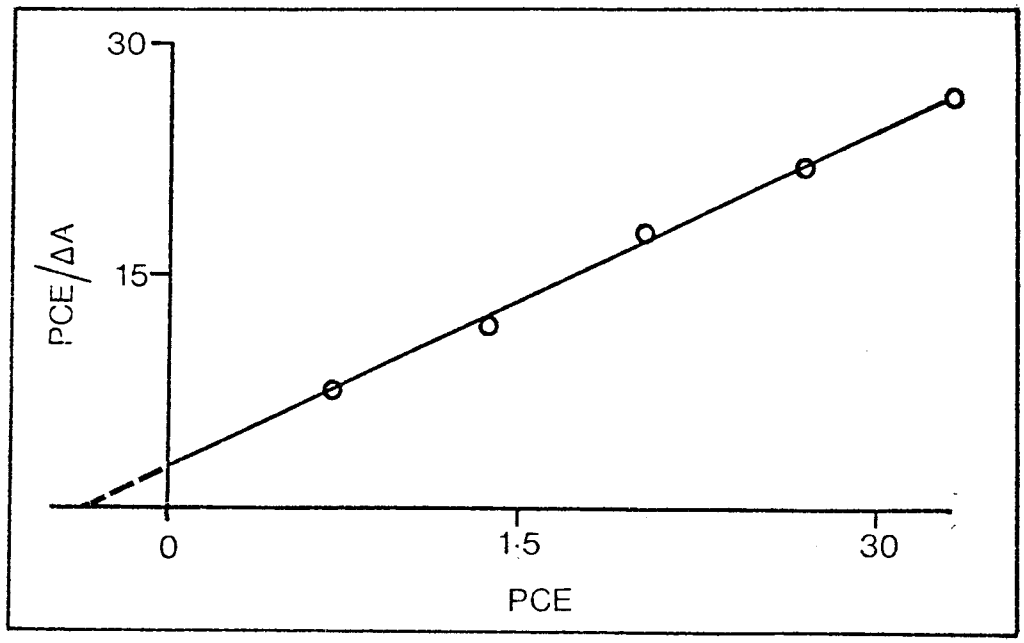

Figure 32: Monophasic Hanes plot for the binding of tetrachloroethylene to hepatic cytochrome P-450 in microsomes from phenobarbital treated rats. Tetrachloroethylene (PCE), mM; Δ A, $A_{388nm}-A_{418nm}$. Correlation coefficient = 0.998.

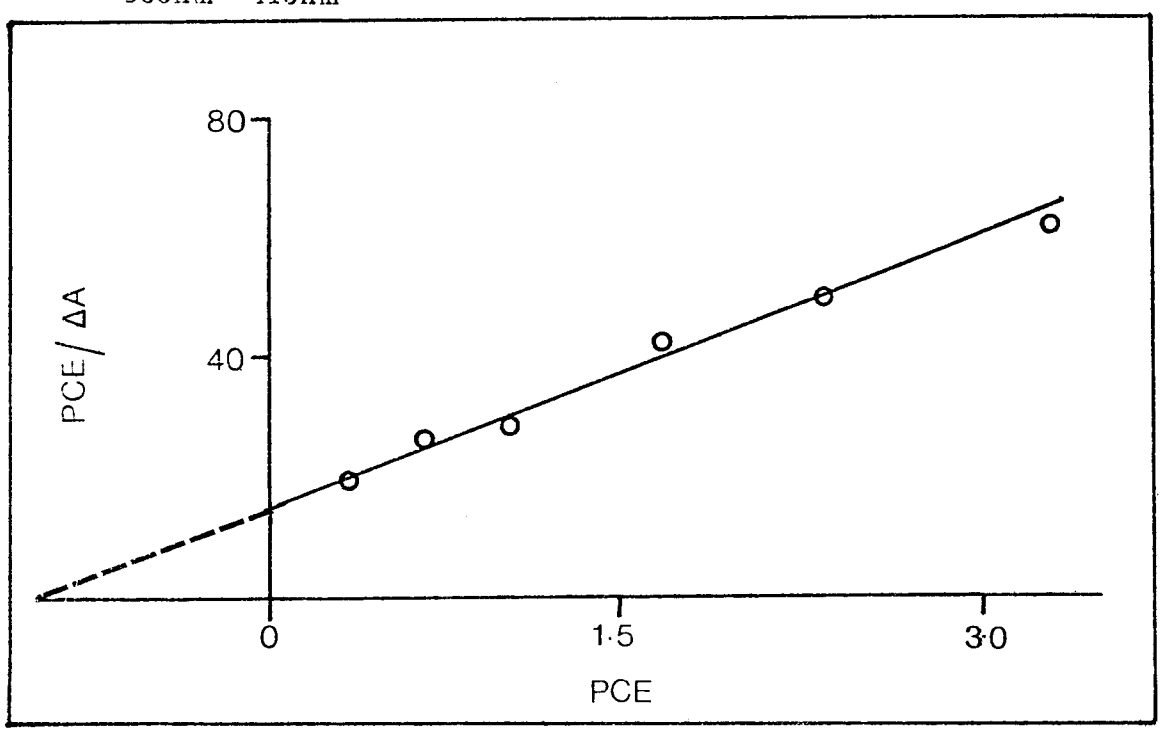

Figure 33: Monophasic Hanes plot for the binding of tetrachloroethylene to hepatic cytochrome P-450 in microsomes from pregnenolone-16α-carbonitrile pretreated rats. Tetrachloroethylene (PCE), mM; Δ A, $A_{388nm}-A_{418nm}$. Correlation coefficient = 0.978.

Table 12: Effect of induction on the binding of tetrachloroethylene by hepatic microsomal cytochrome P-450*

Induction	Cytochrome P-450 (nmol/mg microsomal protein)	K_s (mM)	$10^2 \Delta A_{max}$ ($A_{386nm}-A_{418nm}$)	$10^2 \Delta A_{max}$/nmol cytochrome P-450
None	0.75±0.05	0.43±0.05	5,8±0.8	4.4±0.6
β-Naphthoflavone	1.01±0.03	0.39±0.03	4.5±0.6	4.5±0.6
Pregnenolone-16α-carbonitrile	1.56±0.16	1.03±0.10^{+}	7.7±0.6$^{\neq}$	4.9±0.5
Phenobarbital	2.53±0.28	0.43±0.03	11.4±0.9^{+}	4.5±0.7

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.3.

$^{+}$Differs significantly from value for uninduced microsomes, $P < 0.01$.

$^{\neq}$Probably differs from value for uninduced microsomes, $P < 0.05$.

Tetrachloroethylene bound to partially purified cytochrome P-450 from phenobarbital induced rats with the production of the characteristic Type I difference spectrum. The K_s values calculated for the binding of tetrachloroethylene to the phenobarbital induced partially purified form of cytochrome P-450 were 3.25 ± 0.15 and 2.88 ± 0.12 mM for the enzyme suspended in 0.02 M Tris HCl, pH 7.4, and for the glycerol solubilized enzyme, respectively (see Hanes plots for the binding of tetrachloroethylene to partially purified cytochrome P-450 in Figures 34 and 35).

3.2 Hepatic microsomal NADPH oxidation

The rates of hepatic microsomal CO-inhibitable NADPH oxidation were found to be stimulated in vitro by each one of the chlorinated ethylenes in hepatic microsomes from untreated and pretreated animals. Detailed results of the chloroethylene-stimulated NADPH oxidation, which presumably to some extent reflects the oxidation of NADPH which accompanies the metabolism of the chlorinated ethylenes by hepatic microsomal cytochrome P-450, are as follows:

3.2.1 Vinylidene chloride

The rate of vinylidene chloride stimulated CO-inhibitable NADPH oxidation (see Table 13) was not significantly altered following β-naphthoflavone induction but was significantly elevated following phenobarbital induction. However, if one looks at the vinylidene chloride stimulated NADPH oxidation per nmol cytochrome P-450, no alteration is found following β-naphthoflavone or phenobarbital induction (see Table 13).

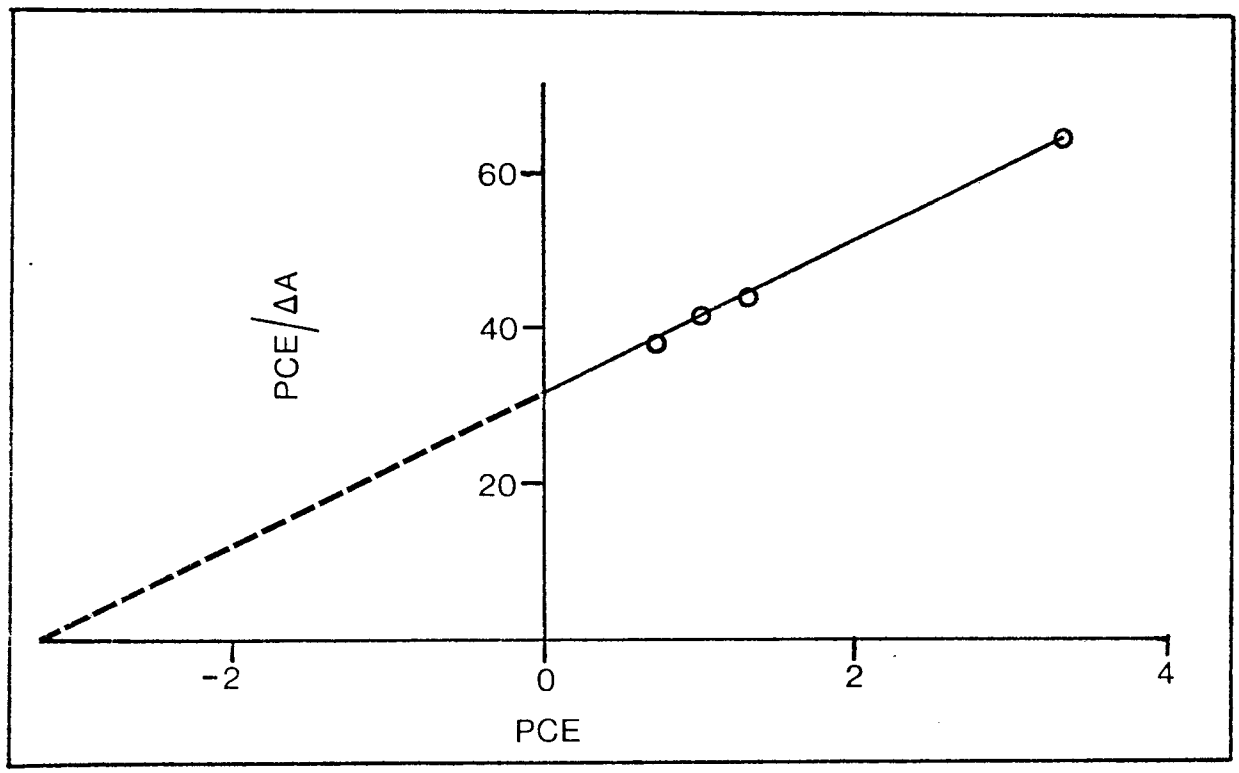

Figure 34: Hanes plot for the binding of tetrachloroethylene to partially purified cytochrome obtained from hepatic microsomes from phenobarbital treated rats. Partially purified hepatic cytochrome P-450 was suspended in 0.02 M Tris-HCl, pH 7.4. Tetrachloroethylene (PCE), mM; Δ A, $A_{388nm}-A_{418nm}$. Correlation coefficient = 0.999.

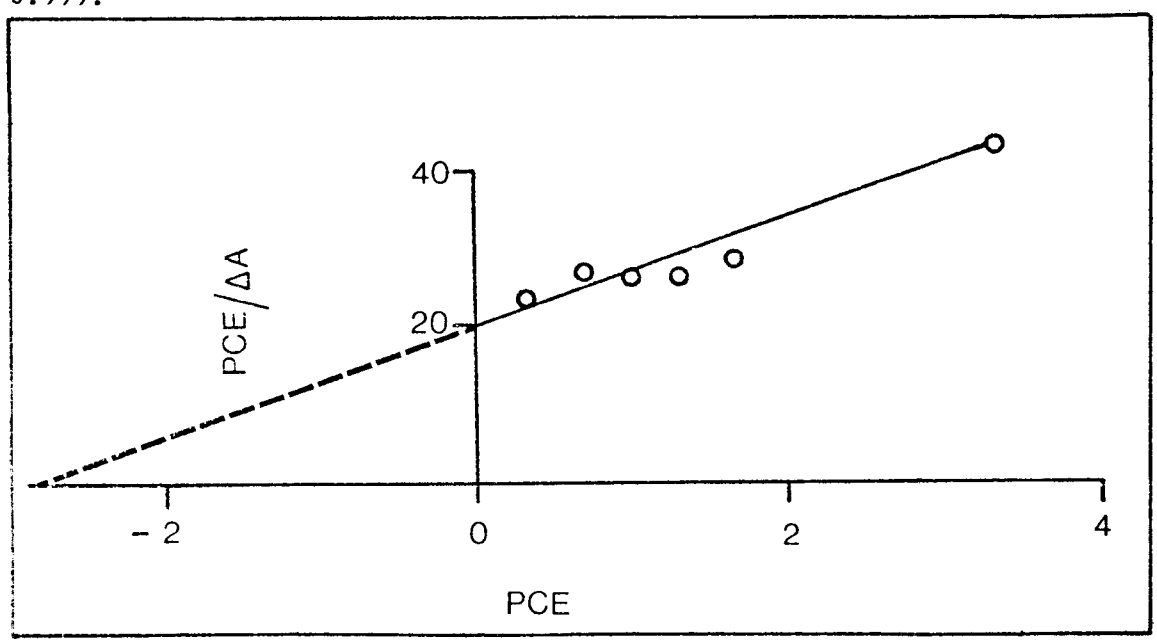

Figure 35: Hanes plot for the binding of tetrachloroethylene to partially purified cytochrome P-450 obtained from hepatic microsomes from phenobarbital pretreated animals. Partially purified hepatic cytochrome P-450 was solubilized in glycerol. Tetrachloroethylene (PCE), mM; Δ A, $A_{388\ nm}-A_{418\ nm}$. Correlation coefficient = 0.960.

Table 13: Stimulation of CO-inhibitable hepatic microsomal NADPH oxidation by vinylidene chloride (6.9 mM)*

Inducing Agent	Cytochrome P-450 (nmol/mg microsomal protein)	CO-inhibitable NADPH oxidation	
		(nmol/min/mg microsomal protein)	(nmol/min/nmol cytochrome P-450
None	0.9±0.2	1.6±0.6	1.8±0.7
β-Naphthoflavone	1.2±0.1+	1.1±0.6	0.9±0.5
Phenobarbital	2.1±0.1+	3.1±0.8+	1.5±0.4

*Values reported are means ± S.D. for experiments performed in triplicate on three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.4.

+Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

3.2.2 cis- and trans-1,2-Dichloroethylene

The effect of induction on the cis- and trans-1,2-dichloroethylene-stimulated NADPH oxidation can be seen in Table 14. Following β-naphthoflavone induction, there was no significant effect on the rate of CO-inhibitable NADPH oxidation per mg microsomal protein or per nmol cytochrome P-450 for either the mixture of isomers or for trans-1,2-dichloroethylene, while following phenobarbital induction, the rate of CO-inhibitable NADPH oxidation was increased per mg of microsomal protein but not per nmol cytochrome P-450. The rates of CO-inhibitable NADPH oxidation in the presence of the mixture of isomers or of pure cis- or trans-1,2-dichloroethylene were, for each type of induction, identical (see Table 14).

3.2.3 Trichloroethylene

The effect of induction on the trichloroethylene-stimulated NADPH oxidation can be seen in Table 15. Induction with phenobarbital resulted in significantly increased rates of trichloroethylene-stimulated NADPH oxidation per min per mg microsomal protein, while induction with spironolactone and 3-methylcholanthrene did not (Table 15). In addition, the rates of trichloroethylene-stimulated NADPH oxidation per min per nmol cytochrome P-450 were not altered by prior treatment with inducing agents (Table 15). Rates of trichloroethylene-stimulated NADPH oxidation are not corrected for endogenous (background) NADPH oxidation (viz. CO-uninhibitable rate) since CO differentially affected NADPH oxidation and metabolite production from trichloroethylene (see Table 29).

Table 14: Effect of inducing agents on the stimulation of hepatic microsomal CO-inhibitable NADPH oxidation by the 1,2-dichloroethylenes*

Inducing Agent	CO-inhibitable NADPH oxidation (nmol/min/mg microsomal protein)		
	cis- and trans-DCE (30 : 70)	trans-DCE	cis-DCE
None	1.1±0.3 (1.2±0.3)	1.0±0.3 (1.1±0.3)	ND
β-Naphthoflavone	1.0±0.2 (0.9±0.2)	1.3±0.1 (1.2±0.1)	ND
Phenobarbital	3.4±0.8+ (1.6±0.4)	3.1±1.1+ (1.5±0.5)	3.0±0.3 (1.4±0.1)

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.4. Values in brackets are in nmol/min/nmol cytochrome P-450.

Abbreviations used are: DCE = 1,2-dichloroethylene; ND = not determined.

+Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

Table 15: Effect of induction on the rates of trichloroethylene-stimulated NADPH oxidation mediated by hepatic cytochrome P-450*

Induction	NADPH oxidation+	
	nmol/min/mg microsomal protein	nmol/min/nmol cytochrome P-450
None	1.70±0.59	1.73±0.56
3-Methylcholanthrene	2.25±0.10	1.61±0.11
Spironolactone	1.73±0.45	2.22±0.46
Phenobarbital	4.57±1.28≠	1.80±0.20

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.4, except that results are not corrected for background rates in presence of CO : O_2 (80 : 20, v/v).

+In the presence of 7.5mM trichloroethylene

≠Differs significantly from the corresponding value for microsomes from uninduced rats, $P < 0.01$.

3.2.4 Tetrachloroethylene

The effect of induction on the tetrachloroethylene-stimulated CO-inhibitable NADPH oxidation can be seen in Table 16. Induction with phenobarbital resulted in a marked increase in tetrachloroethylene stimulated CO-inhibitable NADPH oxidation per mg microsomal protein and per nmol cytochrome P-450, whereas induction with β-naphthoflavone or pregnenolone-16α-carbonitrile had no effect on this process, either per mg microsomal protein or per nmol cytochrome P-450 (Table 16).

3.3 The metabolism of the chlorinated ethylenes by hepatic microsomal suspensions

3.3.1 Metabolism of vinylidene chloride by hepatic microsomal cytochrome P-450.

The 2-chloro- and 2,2-dichloro- derivatives of ethanol, acetaldehyde and acetic acid, were investigated as possible metabolites of vinylidene chloride by hepatic microsomal cytochrome P-450. Incubation mixtures contained vinylidene chloride (6.9 mM), hepatic microsomes from phenobarbital induced microsomes (4 or 6 mg protein/ml), EDTA (0.2 mM) and NADPH-generating system. The amounts of 2-chloroethanol, chloroacetaldehyde and dichloroacetic acid produced from these incubation mixtures were below their limits of detection, viz:

2-chloroethanol <16.2 nmol/mg protein/30 min;

chloroacetaldehyde <0.06 nmol/mg protein/30 min; and

dichloroacetic acid <0.08 nmol/mg protein/30 min.

Low levels of 2,2-dichloroethanol (0.22 nmol/mg protein/20

Table 16: Effect of induction on the tetrachloroethylene-stimulated CO-inhibitable NADPH oxidation in hepatic microsomes*

Inducing Agent	Cytochrome P-450 (nmol/mg microsomal protein)	CO-inhibitable NADPH oxidation+	
		nmol/min/mg microsomal protein	nmol/min/nmol cytochrome P-450
None	0.89±0.06	1.1±0.2	1.2±0.1
β-Naphthoflavone	1.03±0.01	0.8±0.2	0.8±0.2
Pregnenolone-16α-carbonitrile	1.50±0.11	1.2±0.4	0.8±0.2
Phenobarbital	2.32±0.32	5.8±0.8≠	2.5±0.5 ≠

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.4.

+Measured in the presence of 3.3 mM tetrachloroethylene.

≠Differs significantly from value for uninduced microsomes, $P < 0.01$.

min) were initially found in incubation mixtures. However, following redistillation of the reagent grade vinylidene chloride, no 2,2-dichloroethanol (limit of detection 0.15 nmol/mg protein/20 min) was produced, suggesting that its appearance was due to metabolism of a contaminant of the commercially available vinylidene chloride, possibly such as of 1,1-dichloroethane [276].

Following the extraction of acidified reaction mixtures and methylation with diazomethane, one of the metabolites of vinylidene chloride chromatographed identically to methyl chloroacetate. Neutralization of the reaction mixture prior to methylation, or the omission of the methylation reaction from the isolation procedure, eliminated the observed chromatographic peak. Using a Varian Aerograph 2700 gas-liquid chromatograph linked to a Varian MAT 311A mass spectrometer, the mass spectrum of the methylated reaction product was found to be identical to that of an authentic sample of methyl chloroacetate: significant peaks at 108, 77, 59 and 49 corresponded to the $ClCH_2COOCH_3^+$, $ClCH_2CO^+$, $COOCH_3^+$ and $ClCH_2^+$ segments, respectively (see Figure 36).

A chromatographic peak (on Column II) with a retention time equal to dichloroacetaldehyde was also produced following the incubation of vinylidene chloride with hepatic microsomes, NADPH-generating system and EDTA. The Fehling's test for aldehydes was positive for extracts of reaction mixtures containing this peak, and the application of this test to reaction mixtures eliminated this peak from gas liquid chromatograms. Because of its volatility, this metabolite was lost during attempts to concentrate ether extracts of reaction mixtures for assay by GC-MS. Therefore, the following treatment for the oxidation of aldehydes to carboxylic acids was

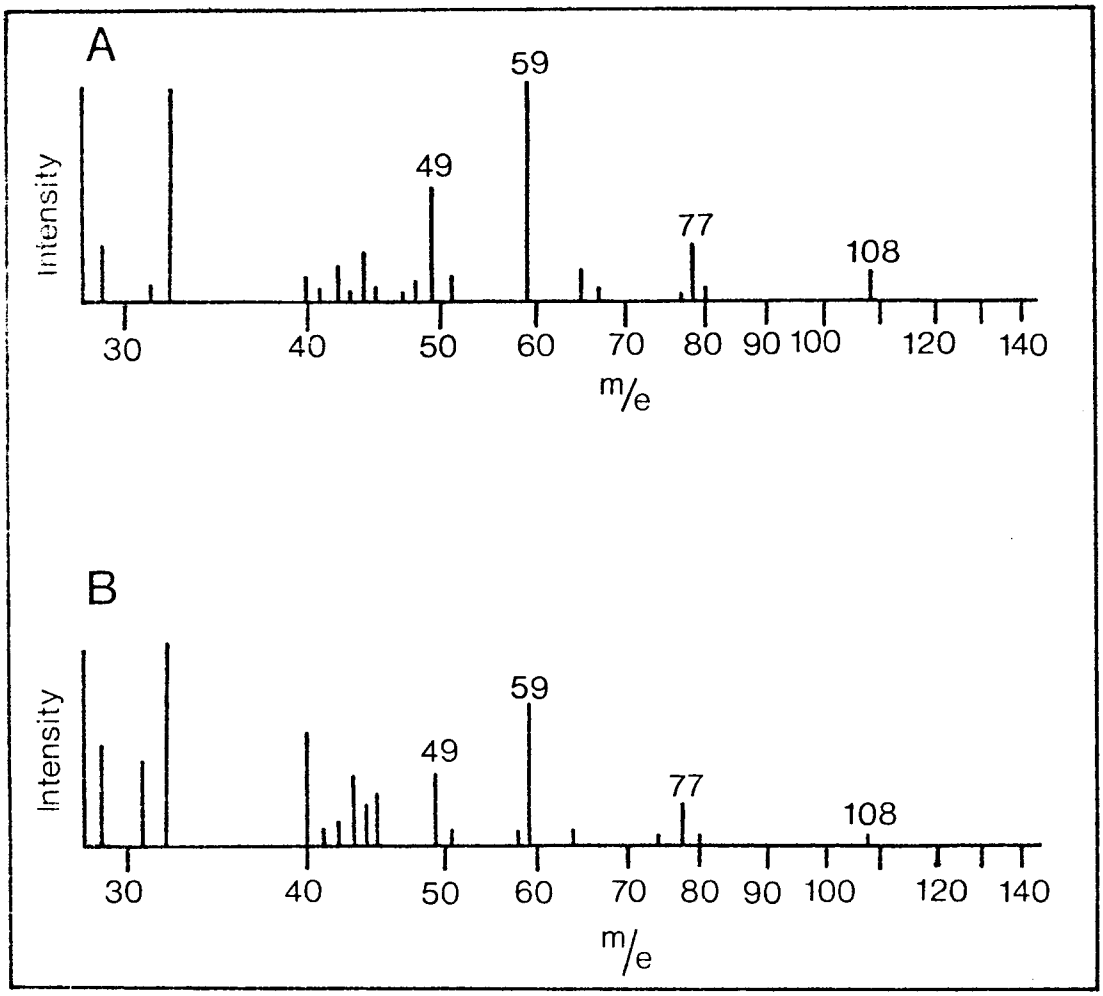

Figure 36: Mass spectra for authentic samples of methyl chloroacetate (A) and of the methylated reaction product of the metabolism of vinylidene chloride by hepatic microsomes from phenobarbital treated rats (B).

applied: The reaction mixture (15 ml) was treated with 0.2 ml NaOH (10%, w/v) plus 10 ml of potassium permanganate (0.1 M), acidified with H_2SO_4 (2 M), and then an aqueous solution of sodium bisulfate (5%, w/v) was added until all colour was lost [277]. Protein was removed by centrifugation, and the supernatant was extracted with 10 ml diethyl ether. This treatment eliminated this chromatographic peak (on Column II) with the retention time of dichloroacetaldehyde (210 sec). Following methylation with diazomethane, a new peak appeared at 550 sec, which corresponded to the retention time of methyl dichloroacetate.

In no case were any of the above chlorinated metabolites measureable in extracts of incubation mixtures of hepatic microsomes plus NADPH-generating system and EDTA or of hepatic microsomes plus vinylidene chloride.

3.3.1a Kinetics of metabolite production.

The conversion of vinylidene chloride to monochloroacetate was linear for 30 min for incubation mixtures containing hepatic microsomes (4 mg protein/ml) from phenobarbital induced rats, vinylidene chloride (6.9 mM), EDTA (0.2 mM) and a NADPH-generating system (Figure 37). The rates of monochloroacetate production per mg microsomal protein per 20 min were identical whether incubation mixtures of hepatic microsomes from phenobarbital induced rats at concentrations of 2 or 4 mg protein per ml were utilized. The levels of monochloroacetate produced for microsomes from untreated, β-naphthoflavone and phenobarbital treated rats can be seen in Table 17. Monochloroacetate production was unaffected following β-naphthoflavone induction (either per mg microsomal protein or per

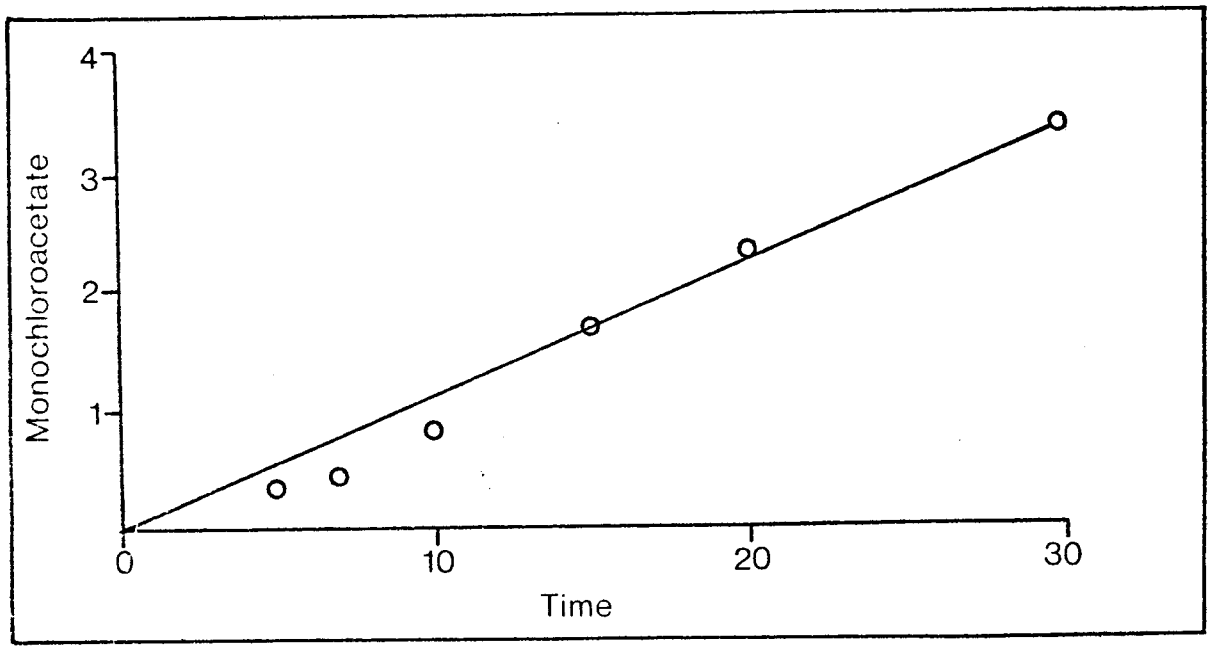

Figure 37: The production of monochloroacetate, from vinylidene chloride by hepatic microsomes (4 mg protein/ml) from phenobarbital pretreated animals, as a function of time.
Monochloroacetate, nmol/mg microsomal protein; Time, min.

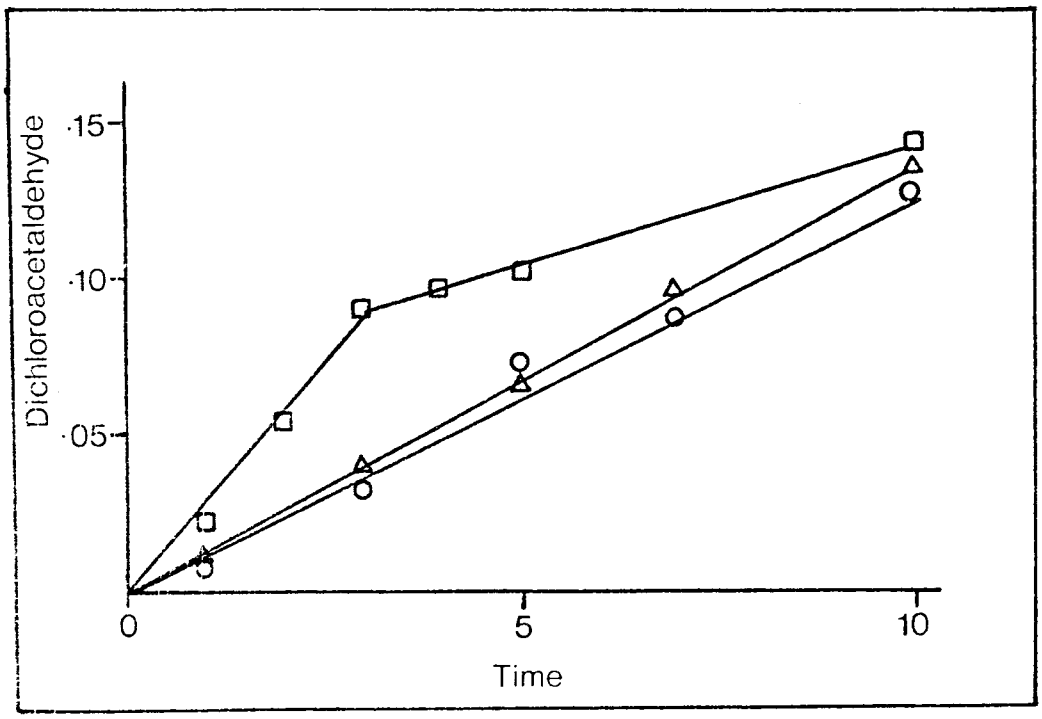

Figure 38: The effect of inducing agents on the production of dichloroacetaldehyde from vinylidene chloride as a function of time for microsomes from untreated (O), β-naphthoflavone treated (Δ) and phenobarbital treated (□) rats.
Dichloroacetaldehyde, nmol/mg microsomal protein; Time, min.

Table 17: The conversion of vinylidene chloride to monochloroacetate by hepatic microsomal cytochrome P-450*

Inducing Agent	Levels of monocholoroacetate produced	
	nmol/mg microsomal protein/20 min	nmol/nmol cytochrome P-450/20 min
None	1.32±0.13	1.59±0.16
β-Naphthoflavone	1.57±0.18	1.34±0.15
Phenobarbital	1.85±0.14+	0.69±0.05+

*Values reported are means ± S.D. for experiments performed in triplicate with two or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.5.

+Differs significantly from values obtained for microsomes from untreated rats, $P < 0.01$.

nmol cytochrome P-450). Although the levels of monochloroacetate produced per mg microsomal protein were significantly elevated following phenobarbital treatment (Table 17), a significant decrease was seen in the monochloroacetate production per nmol cytochrome P-450 following phenobarbital pretreatment (Table 17).

The production of dichloroacetaldehyde from vinylidene chloride in incubation mixtures prepared exactly as described above was linear for 3 min in microsomes from phenobarbital induced rats and for 10 min for microsomes from untreated or β-naphthoflavone induced rats (Figure 38).

3.3.1b K_m and V_{max} for the metabolism of vinylidene chloride

A K_m of 0.16 ± 0.02 mM and a V_{max} of 1.85 ± 0.14 nmol per mg microsomal protein per 20 min (0.69 ± 0.05 nmol per nmol cytochrome P-450 per 20 min) were calculated from Hanes plots for monochloroacetate production from vinylidene chloride by hepatic microsomes from phenobarbital pretreated rats (Figure 39).

The metabolism of vinylidene chloride to dichloroacetaldehyde gave rise to biphasic Hanes plots regardless of the pretreatment of the rats from which the hepatic microsomes were isolated (see, e.g. Figures 40 and 41).

The K_m for the high affinity site for the production of dichloroacetaldehyde was unaffected by β-naphthoflavone and phenobarbital treatment, while the K_m for the low affinity site was significantly increased by both of these inducing agents (Table 18). Neither inducing agent significantly affected either of the V_{max} values per mg microsomal protein (Table 18). The values of V_{max} per nmol cytochrome P-450 were not affected by β-naphthoflavone induction, but were decreased

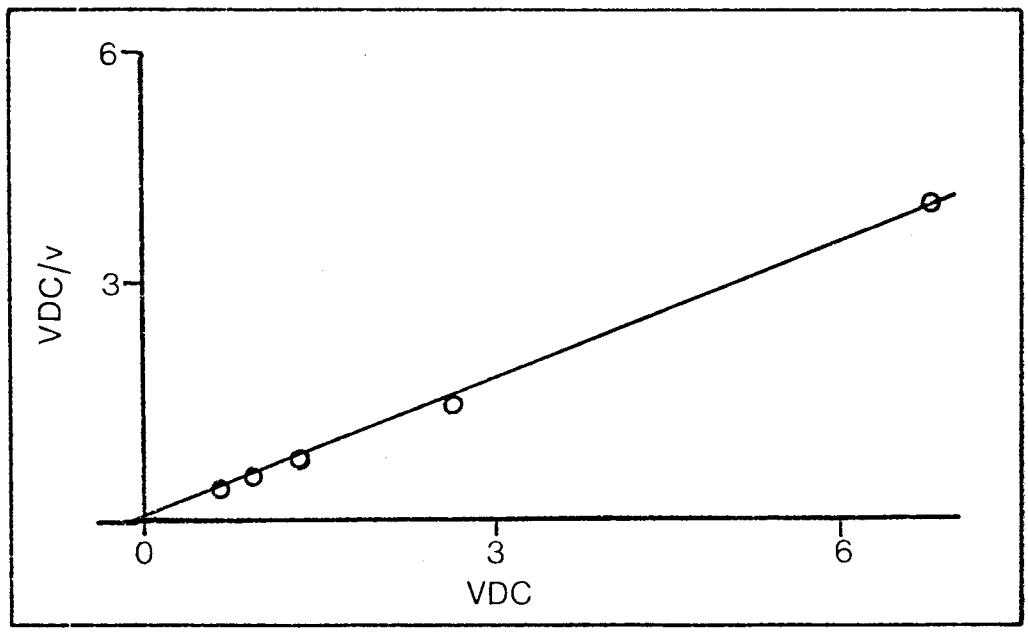

Figure 39: Hanes plot for the production of monochloroacetate from vinylidene chloride by microsomes from phenobarbital pretreated rats. Vinylidene chloride (VDC), mM; v, nmol monochloroacetate/mg microsomal protein/20 min.
Correlation coefficient = 0.999.

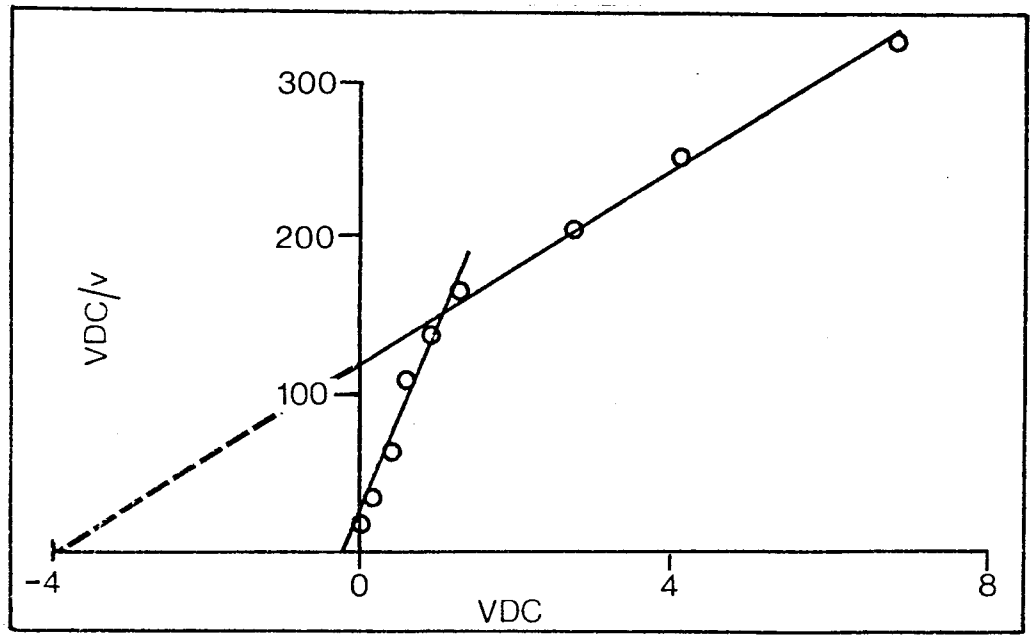

Figure 40: Hanes plot for the production of dichloroacetaldehyde from vinylidene chloride by microsomes from phenobarbital pretreated rats. Vinylidene chloride (VDC), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/ min.
Correlation coefficient for high affinity site = 0.998; low affinity site = 0.969.

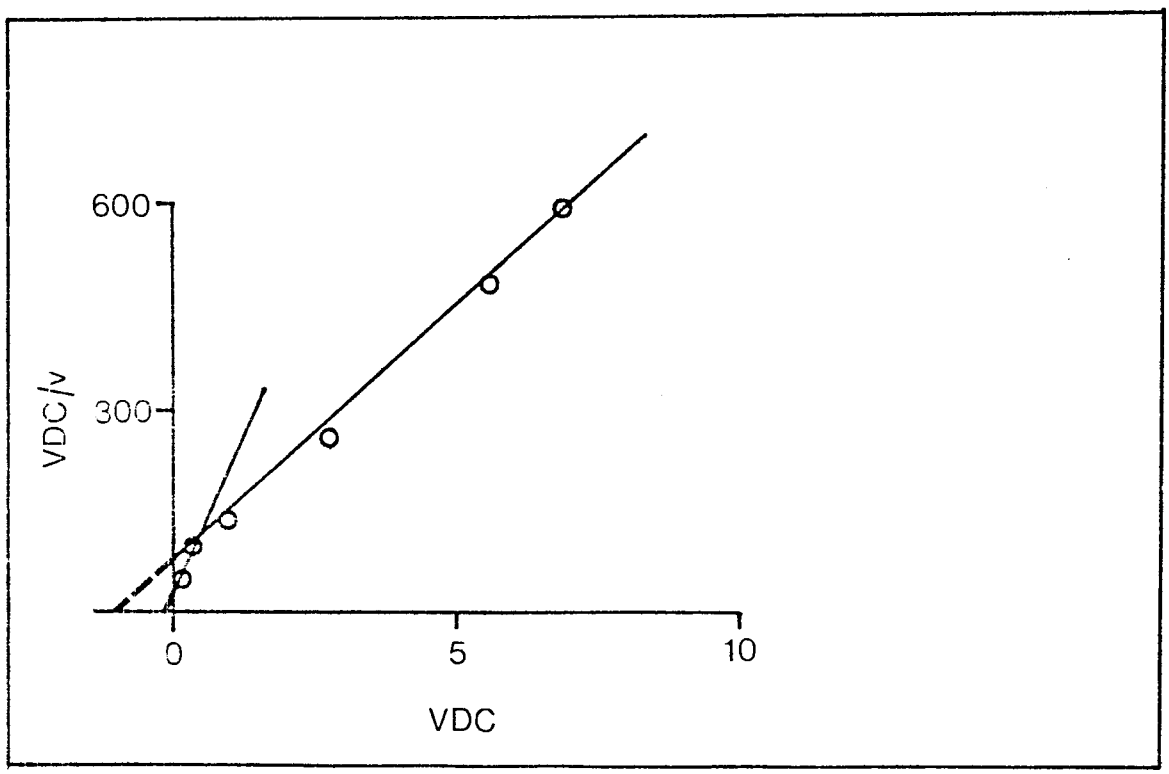

Figure 41: Hanes plot for the production of dichloroacetaldehyde from vinylidene chloride by hepatic microsomes from untreated rats. Vinylidene chloride (VDC), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/ min.
Correlation coefficient for high affinity site = 1.; low affinity site = 0.999.

Table 18: Effect of induction on the conversion of vinylidene chloride to dichloroacetaldehyde by hepatic microsomal cytochrome P-450*

Inducing Agent	K_m (mM)		V_{max} nmol/mg microsomal protein/min		V_{max} nmol/nmol cytochrome P-450/min	
None	0.17±0.05	0.59±0.05	0.025±0.011	0.052±0.010	0.023±0.011	0.048±0.009
β-Naphthoflavone	0.17±0.04	2.2±0.2+	0.034±0.011	0.070±0.011	0.024±0.008	0.057±0.008
Phenobarbital	0.21±0.02	2.9±1.0+	0.019±0.002	0.054±0.010	0.007±0.001	0.021±0.003+

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are described in Section 2.2.5.

+Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

by 2- to 3-fold following phenobarbital induction.

3.3.1c NADH as electron donor for the hepatic microsomal metabolism of vinylidene chloride

Incubation of reaction mixtures containing hepatic microsomes from phenobarbital treated rats, vinylidene chloride (6.9 mM), EDTA (0.2 mM) and NADH (2 mM) resulted in the production of 0.23 nmol monochloroacetate per mg microsomal protein per 20 min and 0.03 nmol dichloroacetaldehyde per mg microsomal protein per 3 min, corresponding to 13% and 19% of the rates of production of these metabolites from vinylidene chloride with NADPH as the electron donor.

3.3.1d Effect of inhibitors on vinylidene chloride metabolism

The inhibitors of hepatic microsomal cytochrome P-450, viz. SKF-525 A (200 mM), $CO:O_2$ (80:20; v/v) and metyrapone (2.33 mM) inhibited the production of monochloroacetate and of dichloroacetaldehyde (Table 19) to varying extents in reaction mixtures containing vinylidene chloride (6.9 mM), hepatic microsomes (4 mg protein/ml) from phenobarbital pretreated rats, EDTA (0.2 mM) and NADPH-generating system. For each inhibitor investigated, the extent of inhibition of monochloroacetate production was equivalent to the extent of inhibition of dichloroacetaldehyde production, e.g. SKF-525 A inhibited both monochloroacetate production and dichloroacetaldehyde production by 68%.

3.3.1e Assay for the covalently bound chlorinated acetyl moieties.

The treatment of reaction mixtures containing vinylidene

Table 19: Effect of inhibitors on the production of monochloroacetate and dichloroacetaldehyde from vinylidene chloride in the presence of hepatic microsomes*

Additions	Percentage inhibition of production of Monochloroacetate +	Dichloroacetaldehyde ≠
-	0	0
$CO:O_2$ (80:20, v/v)	66 ± 8	60 ± 1
Metyrapone (2.3 mM)	32 ± 3	31 ± 4
SKF-525A (200 mM)	68 ± 10	68 ± 1

*Values are means ± S.D. of experiments performed in triplicate on at least two different preparations of hepatic microsomes from phenobarbital pre-treated animals, where initial rates, in the absence of inhibitors, were 1.69±0.05 nmol monochloroacetate/mg protein/20 min and 0.21 nmol dichloroacetaldehyde/mg protein/3 min.

+Incubations were for 20 min at 30°.

≠Incubations were for 3 min at 30°.

chloride, NADPH-generating system, EDTA and hepatic microsomes (4 mg protein/ml) from phenobarbital induced rats, which had been incubated at 30° for 30 min, with 20% H_2SO_4 for 16 hr at 100° [211] resulted in no significant increase in the levels of monochloroacetic acid or dichloroacetic acid.

3.3.1f Hepatic microsomal H_2O_2 production

Incubation for 5 min at 30° of hepatic microsomes (2 mg protein/ml) from phenobarbital treated rats, NADPH-generating system, EDTA (0.1 mM), sodium azide (0.2 mM) and vinylidene chloride (6.9 mM) resulted in the production of 3.2 ± 1.6 nmol H_2O_2 per mg protein per min. The above figures are corrected for the production of H_2O_2 seen in the presence of hepatic microsomes plus NADPH-generating system and EDTA of ca. 1 nmole per mg protein per min.

3.3.1g Effect of vinylidene chloride on the levels of hepatic microsomal cytochrome P-450 and heme

Incubation of vinylidene chloride (6.9 mM), hepatic microsomes, EDTA (0.2 mM) and a NADPH-generating system resulted in no significant alteration in the levels of cytochrome P-450 or heme in hepatic microsomes from untreated and β-naphthoflavone or phenobarbital treated rats (Table 20).

Since other chlorinated ethylenes decrease hepatic microsomal cytochrome P-450 levels, e.g. vinyl chloride [158], it was thought that vinylidene chloride might have been lost during the incubation procedure due to its low boiling point of 32°. The above experiments were therefore repeated using sealed, gas-tight hyperdermic vials and delivering the chilled vinylidene chloride into the sealed

Table 20: Effect of vinylidene chloride on the levels of hepatic microsomal cytochrome P-450 and heme*

Induction	Vinylidene chloride (mM)	Cytochrome P-450 (30 min / O min)	Loss	Heme (30 min / 0 min)	Loss
None	0	0.79 ±0.04 / 0.83±0.08	0.01	1.40±0.11 / 1.44±0.07	0.04
	6.9	0.67 ±0.03 / 0.77±0.07	0.10	1.32±0.07 / 1.43±0.11	0.11
β-Naphthoflavone	0	1.14±0.14 / 1.24±0.06	0.10	1.76±0.31 / 1.84±0.38	0.08
	6.9	1.15±0.11 / 1.05±0.18	0.10	1.68±0.23 / 1.80±0.32	0.12
Phenobarbital	0	1.99±0.25 / 2.10±0.28	0.11	2.69±0.28 / 2.84±0.31	0.15
	6.9	1.87±0.32 / 1.99±0.20	0.12	2.48±0.32 / 2.73±0.27	0.25

*Values reported are means ± S.D. for experiments performed in triplicate with at least three different preparations of hepatic microsomes. Incubation mixtures (3 ml) contained hepatic microsomes (2 mg protein/ml), NADPH-generating system, EDTA (0.2 mM) and where indicated, vinylidene chloride (6.9 mM). Losses of cytochrome P-450 and heme in nmol/mg microsomal protein are reported for samples incubated at 30° for 30 min with shaking, relative to unincubated samples.

vials with an ice-cooled, gas-tight Hamilton syringe. This precaution did not affect the above results, viz. vinylidene chloride still had no significant effect on the levels of hepatic microsomal cytochrome P-450 or on the hepatic cytochrome P-450 dependant metabolism of vinylidene chloride in vitro (see Section 3.3.1a).

3.3.2 Metabolism of the 1,2-dichloroethylenes by hepatic microsomal cytochrome P-450

As with vinylidene chloride, the 2-chloro- and 2,2-dichloro-derivatives of ethanol, acetaldehyde and acetic acid, were investigated as possible metabolites of pure cis- and trans-1,2-dichloroethylene. Incubation mixtures contained the dichloroethylene (7.2 mM), hepatic microsomes from phenobarbital treated rats, EDTA, and NADPH-generating system. 2-Chloroethanol, chloroacetaldehyde and chloroacetic acid were not produced in significant amounts from these incubation mixtures*, the limits of detection being 1.0, 0.06 and 0.15 nmol per mg microsomal protein per 30 min, respectively. 2,2-Dichloroethanol and dichloroacetaldehyde were produced in measurable amounts in incubation mixtures containing either cis- or trans-1,2-dichloroethylene. The rates of production of 2,2-dichloroethanol were 1.2 ± 0.1 and 0.32 ± 0.04 nmol per mg microsomal protein per 20 min from the cis- and trans- isomers, respectively. The identification of 2,2-dichloroethanol was based on its retention time for gas liquid chromatography on Column III being identical to an authentic sample of 2,2-dichloroethanol. In addition,

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*2-Chloroethanol and chloroacetic acid production were monitored on Columns II and III respectively, while chloroacetaldehyde production was monitored on Column II.

the peak was converted by acetyl chloride treatment [278] to one that chromatographed identically to an authentic sample of acetyl 2,2-dichloroethanol on Column II. The rates of production of 2,2-dichloroethanol were not diminished if freshly redistilled samples of cis- and trans-1,2-dichloroethylene were utilized in incubation mixtures. It was not possible to obtain metabolic parameters for the production of 2,2-dichloroethanol due to the low amounts produced under the conditions of our experiments and due to the relative insensitivity of the electron capture detector to this product.

Dichloroacetaldehyde was identified in extracts of reaction mixtures by its retention time for gas liquid chromatography using Column II and by the application of the Fehling's test for aldehydes. This test was positive for extracts of reacting mixtures, and its application to extracts eliminated the peak attributed to dichloroacetaldehyde from gas liquid chromatograms. Furthermore, the metabolite proposed to be dichloroacetaldehyde was oxidized by the method for the oxidation of aldehydes to carboxylic acids (see Section 3.3.1). This treatment eliminated the chromatographic peak with the retention time of dichloroacetaldehyde (210 sec on Column II) and following methylation with diazomethane, resulted in the appearance of a new peak at 550 sec, which corresponds to the retention time of methyl dichloroacetate (on Column II).

Dichloroacetic acid (0.88 nmol/mg protein/20 min) was found to be a metabolite of trans-1,2-dichloroethylene on the basis of its retention time, following methylation, being identical to that of methyl dichloroacetate on two gas liquid chromatography columns (Column II and Column III), and the

peak being eliminated by the neutralization of reaction mixtures prior to methylation, or by the omission of the methylation reaction.

Following incubation of cis-1,2-dichloroethylene with hepatic microsomes in the presence of NADPH-generating system and EDTA, and subsequent treatment overnight with 20% H_2SO_4 at 100° [211], significant levels of dichloroacetic acid (identified as above) were produced (viz. 5.4 nmol/mg protein/20 min), where only traces of dichloroacetic acid (< 0.1 nmol/mg protein/20 min) were found in incubation mixtures before hydrolysis or in hydrolyzed but not incubated reaction mixtures. CO, metyrapone and SKF-525A had no effect on the production of the covalently bound dichloroacetyl moiety, and it was subsequently found that the omission of the NADPH-generating system from incubation mixtures did not diminish the rate of formation of covalently bound dichloroacetate. These results indicate that the covalently bound dichloroacetate was produced as a function of time, but that its production was not the result of a cytochrome P-450 dependent reaction.

3.3.2a Kinetics of dichloroacetaldehyde production

The conversion of cis- or trans-1,2-dichloroethylene to dichloroacetaldehyde was linear for 10 min in microsomes from untreated or β-naphthoflavone induced rats and for at least 3 min with hepatic microsomes from phenobarbital induced rats (see e.g. Figure 42). The rates of dichloroacetaldehyde production per mg microsomal protein were identical whether incubation mixtures of hepatic microsomes at concentrations of 2 or 4 mg protein per ml were utilized.

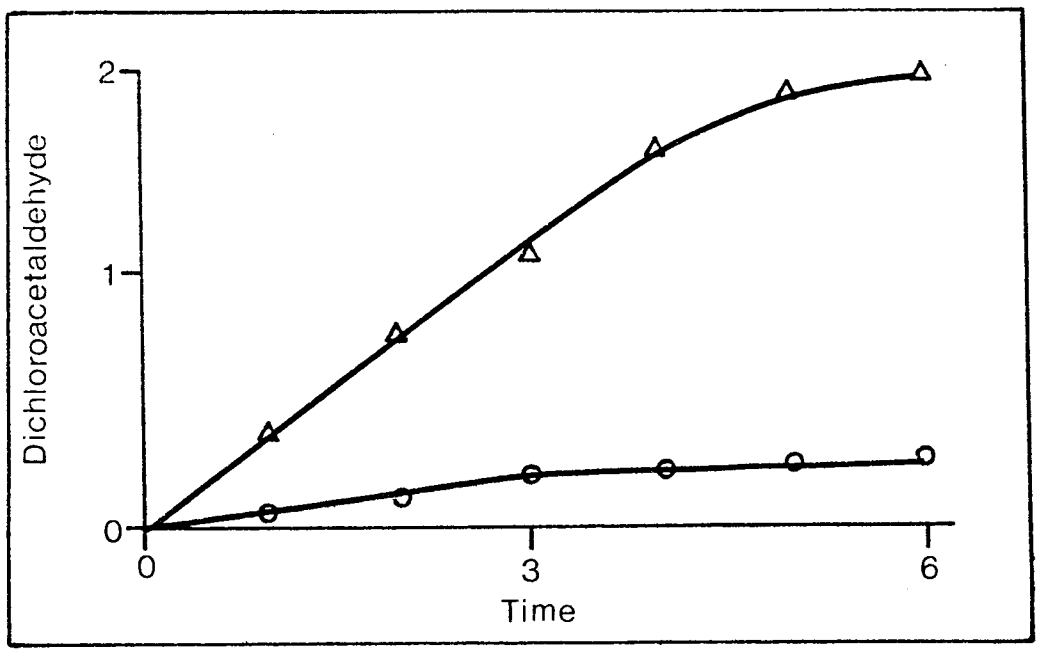

Figure 42: Production of dichloroacetaldehyde from cis- and trans-1,2-dichloroethylene as a function of time. Dichloroacetaldehyde, nmol /mg microsomal protein; Time, min. Reaction mixtures contained hepatic microsomes (4 mg protein/ml) from phenobarbital treated rats, NADPH-generating system, EDTA (0.2 mM) and cis- (Δ) or trans- (O)-1,2-dichloroethylene (7.2 mM).

3.3.2b K_m and V_{max} for dichloroacetaldehyde production.

The metabolism of cis-1,2-dichloroethylene to dichloroacetaldehyde gave rise to biphasic Hanes plots regardless of the pretreatment of the rats from which the hepatic microsomes were isolated (see Figures 43 - 45). However, for the metabolism of trans-1,2-dichloroethylene to dichloroacetaldehyde, biphasic Hanes plots only arose following phenobarbital induction (see Figure 46). In microsomes from untreated and β-naphthoflavone induced rats, the conversion of trans-1,2-dichloroethylene to dichloroacetaldehyde gave rise to monophasic Hanes plots (see Figures 47 and 48). For cis- and trans-1,2-dichloroethylene, β-naphthoflavone induction did not significantly affect the values of K_m or of V_{max} per nmol cytochrome P-450 except for the low affinity K_m for the cis-isomer, but generally resulted in an increase in V_{max} per mg microsomal protein. Phenobarbital induction generally resulted in increases in the value of K_m and V_{max} per mg microsomal protein, but did not significantly affect V_{max} per nmol cytochrome P-450. For each type of pretreatment, V_{max} (either per mg microsomal protein or per nmol cytochrome P-450) was significantly greater for cis- than for trans-1,2-dichloroethylene ($P < 0.01$) (Table 21).

3.3.2c Effect of inhibitors of cytochrome P-450 on the metabolism of cis- and trans-1,2-dichloroethylene.

CO, metyrapone and/or SKF-525A effectively inhibited the metabolism of cis- and trans-1,2-dichloroethylene to 2,2-dichloroethanol and dichloroacetaldehyde (Table 22). Metyrapone was the least effective inhibitor of the three for the conversion of the cis-isomer to dichloroacetaldehyde but was the most effective inhibitor

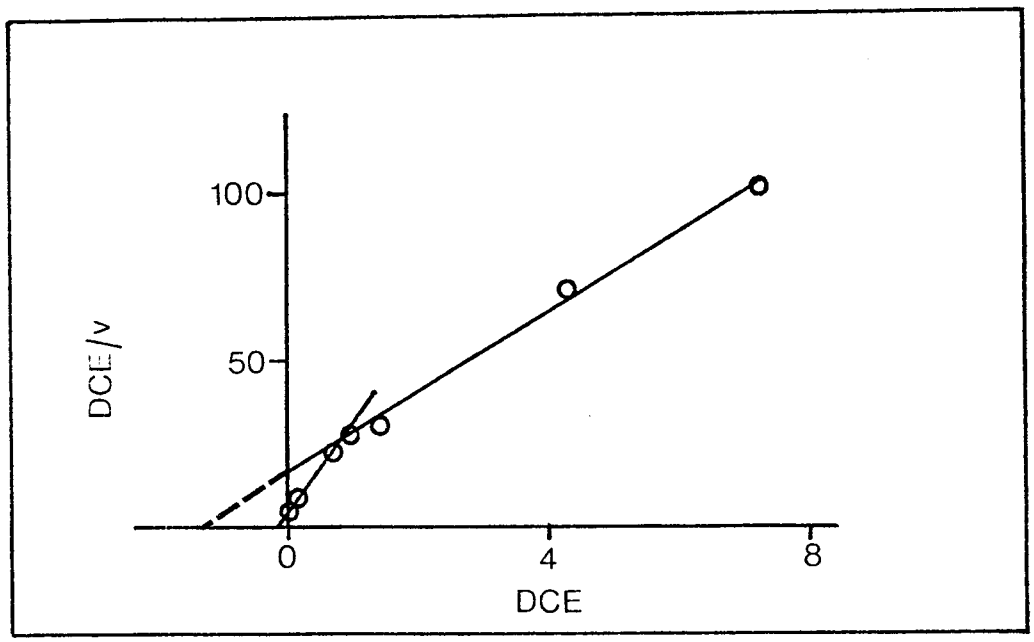

Figure 43: Hanes plot of the production of dichloroacetaldehyde from cis-1,2-dichloroethylene by hepatic microsomes from β-naphthoflavone treated rats. cis-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/min. Correlation coefficient for high affinity site = 0.995; low affinity site = 0.997.

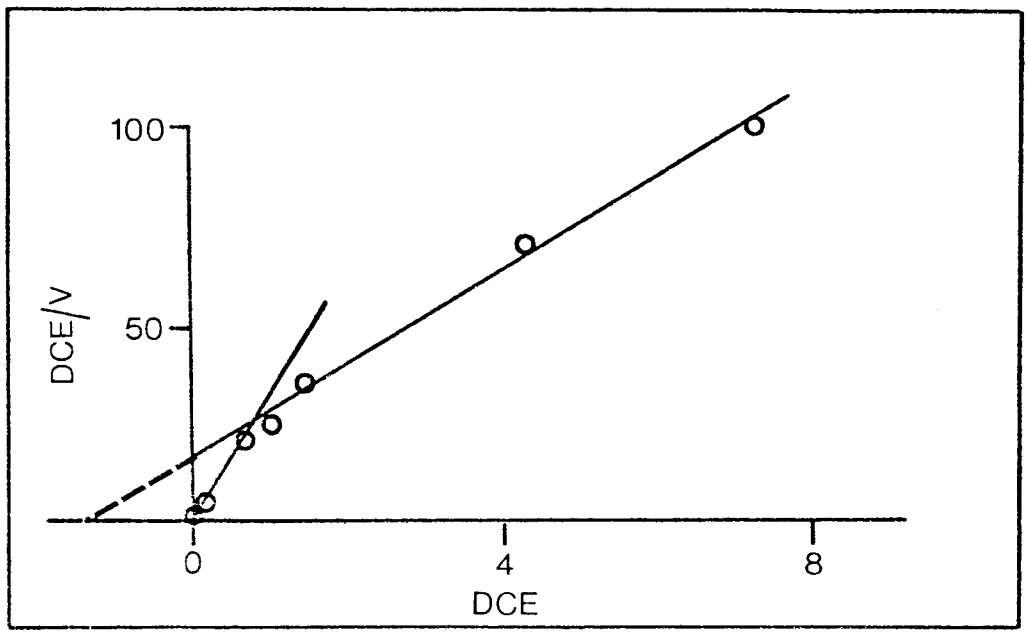

Figure 44: Hanes plot of the production of dichloroacetaldehyde from cis-1,2-dichloroethylene by hepatic microsomes from untreated rats. cis-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/min.
Correlation coefficient for high affinity site = 0.999; low affinity site = 0.993.

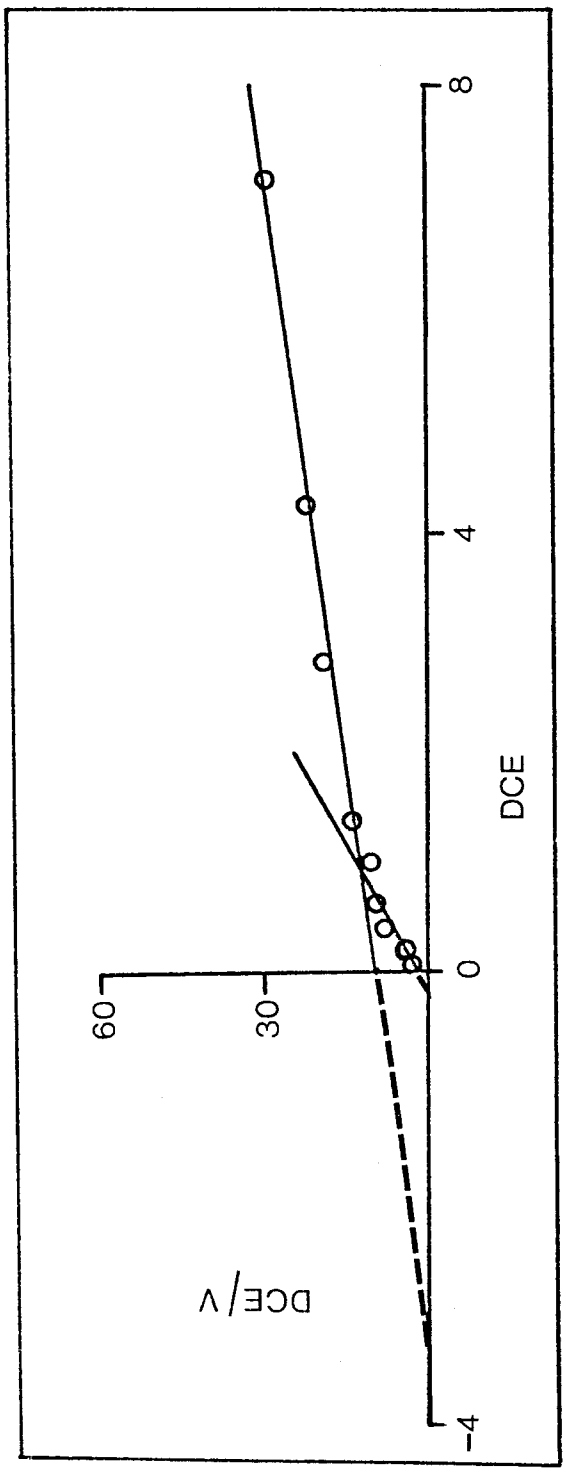

Figure 45: Hanes plot of the production of dichloroacetaldehyde from cis-1,2-dichloroethylene by hepatic microsomes from phenobarbital pre-treated rats. cis-1,2-Dichloroethylene (DCE), mM; v, nmol dichloro-acetaldehyde/mg microsomal protein/min. Correlation coefficient for high affinity site = 0.974; low affinity site = 0.981.

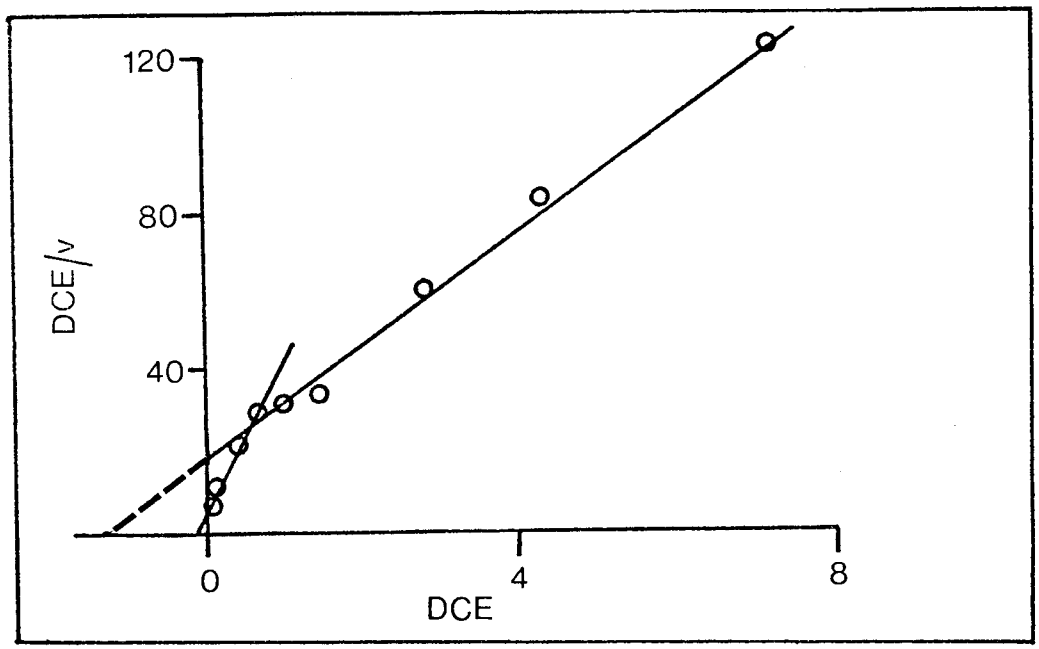

Figure 46: Hanes plot of the production of dichloroacetaldehyde from trans-1,2-dichloroethylene by hepatic microsomes from phenobarbital treated rats.
trans-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/min.
Correlation coefficient for high affinity site = 0.999; low affinity site = 0.992.

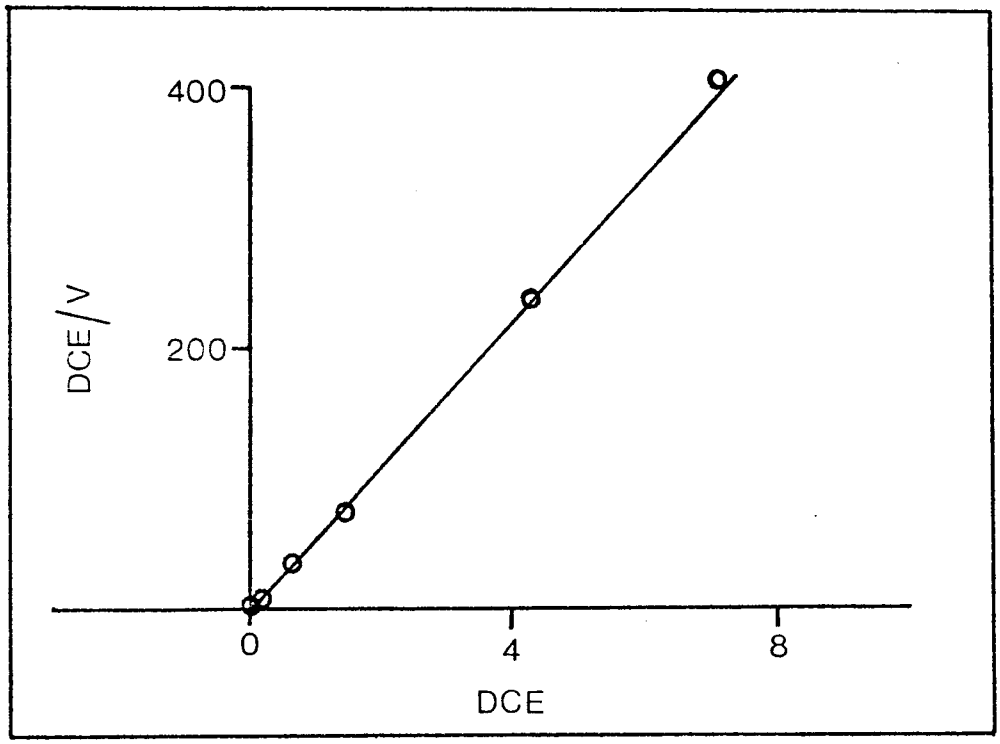

Figure 47: Hanes plot of the production of dichloroacetaldehyde from trans-1,2-dichloroethylene by hepatic microsomes from β-naphthoflavone treated rats. trans-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/min. Correlation coefficient = 0.998.

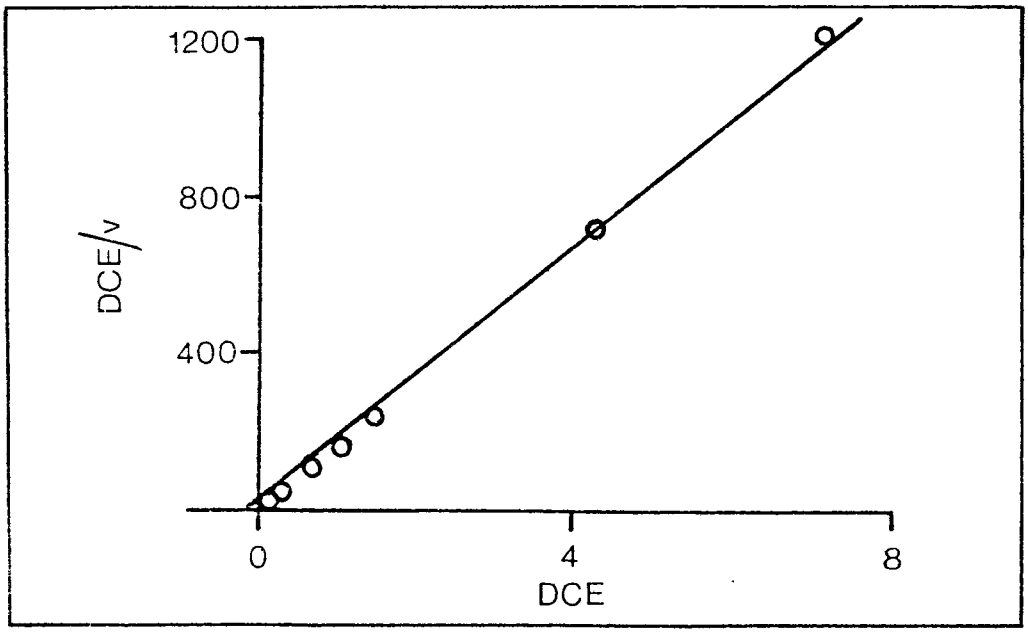

Figure 48: Hanes plot of the production of dichloroacetaldehyde from trans-1,2-dichloroethylene by hepatic microsomes from untreated rats. trans-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde/mg microsomal protein/min.
Correlation coefficient = 0.999.

Table 21: Effect of inducing agents on the hepatic microsomal conversion of cis- and trans-1,2-dichloroethylene to dichloroacetaldehyde*

Inducing Agent	trans-1,2-Dichloroethylene				cis-1,2-Dichloroethylene			
	K_m (mM)		V_{max} (nmol/min/mg prot.)		K_m (mM)		V_{max} (nmol/min/mg prot.)	
None	0.06 ± 0.01		0.006 ± 0.001		0.04±0.01	1.3±0.2	0.03±0.01	0.07±0.02
			(0.007 ± 0.001)				(0.03±0.01)	(0.08±0.02)
β-Naphtho-flavone	0.08 ± 0.01		0.016 ± 0.003+		0.14±0.05+	2.2±0.9	0.05±0.01	0.14±0.06+
			(0.009 ± 0.002)				(0.03±0.01)	(0.08±0.04)
Pheno-barbital	0.15±0.06+	1.66±0.32+	0.023±0.003+	0.067±0.006+	0.26±0.14+	2.1±1.2	0.11±0.03+	0.30±0.11+
			(0.007±0.001)	(0.020±0.002+)			(0.03±0.01)	(0.09±0.03)

*Values reported are means ± S.D. for experiments performed in triplicate with two or three different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.5. Abbreviation used is, prot., protein.

+Differs significantly from the corresponding value for microsomes from untreated rats, $P < 0.01$.

Table 22: Effect of inhibitors on the metabolism of cis- and trans-1,2-dichloroethylene by hepatic microsomal cytochrome P-450*

Compound	Inhibitor	% Inhibition of	
		Dichloroacetaldehyde production	2,2-Dichloroethanol production
cis-1,2-Dichloroethylene	$CO:O_2$ (80:20, v/v)	47 ± 6	38 ± 3
	Metyrapone (2.3 mM)	37 ± 3	ND
	SKF-525A (200 mM)	53 ± 6	ND
trans-1,2-Dichloroethylene	$CO:O_2$ (80:20, v/v)	38 ± 4	29 ± 2
	Metyrapone (2.3 mM)	63 ± 3	62 ± 2
	SKF-525A (200 mM)	29 ± 4	ND

*Values reported are means ± S.D. for experiments performed in duplicate or triplicate with two different preparations of hepatic microsomes from phenobarbital pretreated rats. Experimental details are given in Section 2.2.7d.

Abbreviation used is, ND = not determined.

of the conversion of the trans- isomer to this metabolite ($P < 0.01$). For each inhibitor investigated, the extent of inhibition of 2,2-dichloroethanol production was similar to the extent of inhibition of dichloroacetaldehyde production.

3.3.2d Effect of the 1,2-dichloroethylenes on hepatic microsomal H_2O_2 production.

Incubation of cis- or trans-1,2-dichloroethylene (7.2 mM), hepatic microsomes (2 mg protein/ml) from phenobarbital treated rats, NADPH-generating system, EDTA (0.1 mM) and sodium azide (0.2 mM) for 5 min at 30° with shaking at 60 cycles per min resulted in 2.33 nmol H_2O_2 per min per mg microsomal protein from the cis- isomer and none from the trans- isomer, relative to incubation mixtures in which the 1,2-dichloroethylene was absent. Both cis- or trans-1,2-dichloroethylene (7.2 mM) caused a 10% decrease in H_2O_2 levels present over 5 min when a known amount of H_2O_2 was added to incubation mixtures. The reported levels of H_2O_2 produced have been corrected for this loss and also as described in Section 3.3.1f.

3.3.2e Effect of the 1,2-dichloroethylenes on the levels of hepatic microsomal cytochrome P-450.

The effect of a mixture of cis- and trans-1,2-dichloroethylene on the levels of hepatic microsomal cytochrome P-450 and heme are shown in Table 23. No appreciable degradation of hepatic microsomal cytochrome P-450 or heme was observed in the absence of the 1,2-dichloroethylene or of the NADPH-generating system. Incubation for 15 min of the dichloroethylene with hepatic microsomes and NADPH-

Table 23: Effect of a mixture of cis- and trans-1,2-dichloroethylene (30:70) on the levels of hepatic microsomal cytochrome P-450*

Inducing Agent	1,2-DCE (mM)	Cytochrome P-450[+] level at 15 min / initial level	Heme[+] level at 15 min / initial level	Loss Heme[+≠] / Loss Cytochrome P-450
None	0	0.79±0.04 / 0.82±0.08	1.40±0.11 / 1.44±0.04	
	7.2	0.45±0.03 / 0.72±0.05	1.14±0.07 / 1.43±0.11	0.24 / 0.23
β-Naphthoflavone	0	1.14±0.14 / 1.24±0.06	1.76±0.31 / 1.84±0.09	
	7.2	0.60±0.09 / 1.11±0.09	1.4 ±0.23 / 1.76±0.03	0.28 / 0.41
Phenobarbital	0	1.99±0.25 / 2.10±0.28	2.69±0.28 / 2.84±0.31	
	7.2	0.85±0.05 / 1.79±0.10	1.68±0.23 / 2.75±0.27	0.91 / 0.83

*Values reported are means ± S.D. for experiments performed in triplicate with at least three different preparations of hepatic microsomes. Incubation mixtures (3 ml) contained: hepatic microsomes (2 mg protein/ml), NADPH-generating system, EDTA (0.2 mM) and a mixture of cis- and trans-1,2-dichloroethylene (7.2 mM). Losses are reported for samples incubated for 15 min at 30° with shaking relative to zero time samples of identical composition. Abbreviation used: 1,2-DCE = 1,2-dichloroethylene.

[+]In nmol /mg of microsomal protein.

[≠]Corrected for background losses seen in incubation mixtures not containing 1,2-dichloroethylene.

generating system and EDTA however resulted in significant losses of both hepatic microsomal cytochrome P-450 and heme. In these incubation mixtures, the losses of cytochrome P-450 were approximately equivalent to the losses of heme for each type of induction. The total losses of microsomal P-450 and heme were far more pronounced for microsomes from phenobarbital induced rats than from untreated or β-naphthoflavone treated rats (Table 23). Both CO and metyrapone inhibited the degradation of cytochrome P-450 by the 1,2-dichloroethylenes in hepatic microsomes from phenobarbital treated rats (see Table 24). NADH (1 mM) did not measurably support the 1,2-dichloroethylene mediated degradation of cytochrome P-450 in microsomes from phenobarbital induced rats.

Following incubation in the presence of hepatic microsomes from untreated rats (4 mg/ml), NADPH-generating system and EDTA, pure trans-1,2-dichloroethylene decreased the levels of hepatic microsomal cytochrome P-450 to a greater extent than did pure cis-1,2-dichloroethylene (Table 25).

Similar results were found for microsomes from phenobarbital treated rats (2 mg protein/ml), where the losses of cytochrome P-450 were 1.7 ± 0.18 and 1.52 ± 0.30 nmol cytochrome P-450 per 15 min for the pure cis- and trans-isomers respectively. (These losses are comparable to the losses seen with the mixture of isomers ($P > 0.1$) (Table 24).

Most of the degradation studies carried out on the 1,2-dichlorinated ethylenes were performed using the mixture of cis- and trans 1,2-dichloroethylene (30:70), where the lesser effect of cis-1,2-dichloroethylene is presumably masked by the greater

Table 24: Effect of inhibitors and NADH on the in vitro degradation of hepatic microsomal cytochrome P-450 by cis- and trans-1,2-dichloroethylene*

Inducing Agent	1,2-DCE (mM)	Other additions	Cytochrome P-450		
			levels at/initial[+] 15 min levels	Loss[+§]	% Inhibition of loss
Phenobarbital	O	-	3.04±0.13/3.1±0.28	-	
	7.2	-	1.28±0.21/3.02±0.28	1.58	
	7.2	$CO:O_2$ (80:20, v/v)	1.5 ±0.19/2.83±0.35	1.17	26
	7.2	Metyrapone (2.3 mM)	1.83±0.22/2.57±0.35	0.58	63
	7.2	NADH (0.6 mM)[≠]	1.98±0.19/2.53±0.26	0.39	
	O	NADH (0.6 mM)[≠]	2.09±0.13/2.46±0.12	0.21	

*Values reported are means ± S.D. for experiments performed in triplicate on two different preparations of hepatic microsomes from phenobarbital pretreated animals. Incubation mixtures were as described in Table 23.

[+]In nmol/mg of microsomal protein.

[≠]NADPH-generating system omitted from incubation mixtures.

[§]Corrected for background losses seen in incubation mixtures not containing 1,2-dichloroethylene.

Table 25: Effect of pure cis-1,2-dichloroethylene, pure trans-1,2-dichloroethylene and the mixture of cis- and trans-1,2-dichloroethylene (30:70) on the levels of hepatic microsomal cytochrome P-450*

Additions	(mM)	Cytochrome P-450+ 15 min	Cytochrome P-450+ 0 min	Loss cytochrome P-450+ ≠	Percentage loss ≠
-	-	1.53±0.04	1.66±0.01		
trans-1,2-DCE	7.2	0.52±0.04	1.46±0.05	0.81	57
cis-1,2-DCE	7.2	0.94±0.02	1.64±0.02	0.57	35
cis- and trans-1,2-DCE (30:70)	7.2	0.59±0.02	1.46±0.01	0.74	52

*Values reported are means ± S.D. for experiments performed in triplicate on one preparation of hepatic microsomes from untreated rats. Incubation mixtures (3 ml) contained hepatic microsomes (4 mg protein/ml), and otherwise conditions were identical to those in Table 23.

+In nmol/mg of microsomal protein.

≠Corrected for background losses seen in incubation mixtures not containing 1,2-dichloroethylene.

amounts and effectiveness of trans-1,2-dichloroethylene present in the mixture.

3.3.3 Metabolism of trichloroethylene by hepatic microsomal cytochrome P-450.

In the absence of NADPH-generating system or trichloroethylene, measurable amounts of Fujiwara positive material were not formed in hepatic microsomal incubation mixtures. In the presence of trichloroethylene, NADPH-generating system, EDTA and hepatic microsomes, the production of chlorinated metabolites as assayed by the modified Fujiwara method [261] was linear for 5 min for microsomes from uninduced or induced rats (Figure 49). This incubation time was utilized in all further experiments in which the metabolism of trichloroethylene was monitored by this method.

Chloral hydrate was identified as the sole volatile metabolite of trichloroethylene by gas liquid chromatography (see Section 2.2.5c). The amounts of chloral hydrate produced by microsomes from phenobarbital induced rats of 6.2± 0.3 nmol per min per mg microsomal protein were identical to the amounts of chloral hydrate produced from trichloroethylene, as measured by the modified Fujiwara assay (Table 26). The levels of chloral hydrate were therefore assayed routinely using the latter rapid method. Measurable amounts of trichloroacetic acid or of 2,2,2-trichloroethanol were not produced from trichloroethylene in any incubation mixtures as assessed by gas liquid chromatography.

Hanes plots were linear for the production of chloral hydrate from trichloroethylene when added to hepatic microsomes in the

Table 26: Effect of induction on the metabolism of trichloroethylene to chloral hydrate by hepatic microsomal cytochrome P-450*

Induction	Chloral hydrate production		
	K_m (mM)	V_{max} (nmol/min/mg microsomal protein)	V_{max} (nmol/min/nmol cytochrome P-450)
None	0.62 ± 0.45	1.55 ± 0.65	1.58 ± 0.66
3-Methylcholanthrene	0.45 ± 0.02	2.00 ± 0.57	1.43 ± 0.41
Spironolactone	0.71 ± 0.02	2.00 ± 0.20	2.56 ± 0.21^{+}
Phenobarbital	0.48 ± 0.30	5.80 ± 0.70^{+}	2.28 ± 0.28$^{\neq}$

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.5.

$^{+}$Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

$^{\neq}$Probably differs from corresponding value obtained from uninduced rats, $P < 0.05$.

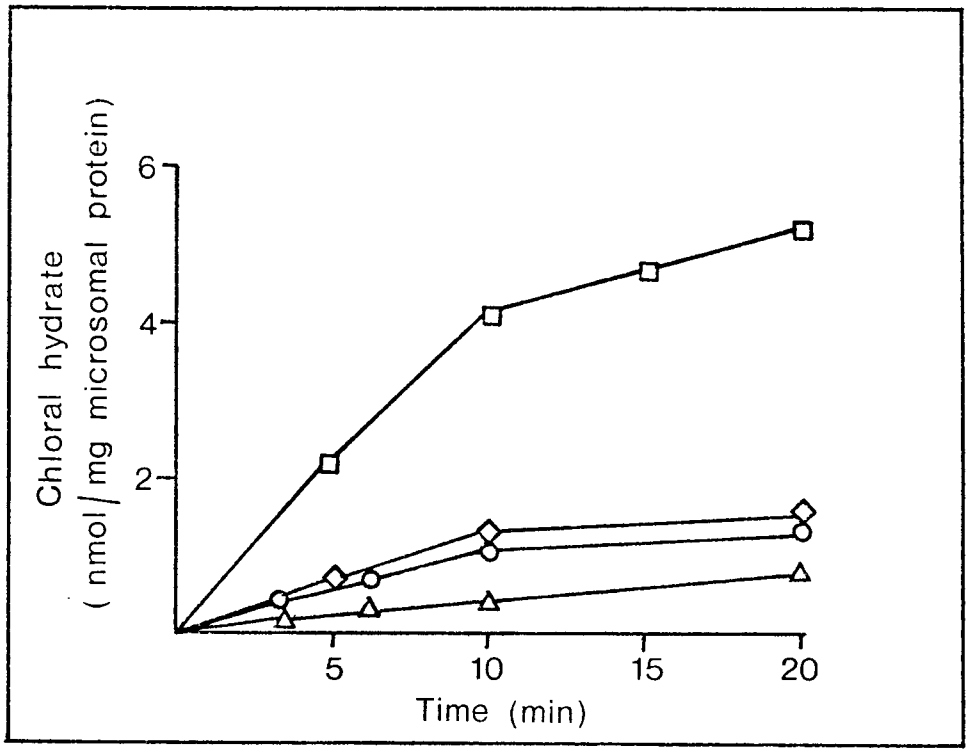

Figure 49: The effect of inducing agents on the production of chloral hydrate from trichloroethylene as a function of time for microsomes from untreated (O), 3-methylcholanthrene treated (△), spironolactone treated (◇) and phenobarbital treated (□) rats. Chloral hydrate, nmol/mg microsomal protein; Time, min.

presence of an NADPH-generating system and EDTA (0.2 mM) (see e.g. Figures 50 and 51). The K_m values for the conversion of trichloroethylene to chloral hydrate were unaffected by the induction of different forms of cytochrome P-450 (Table 26). The V_{max} values for trichloroethylene were unaffected by induction with spironolactone or 3-methylcholanthrene but were increased following phenobarbital induction (Table 26). In contrast, V_{max} per nmol cytochrome P-450 was increased by both pregnenolone-16α-carbonitrile and phenobarbital. Chloral hydrate production was not corrected for $CO:O_2$ (80:20) background rates (see Section 3.2.3).

NADH (1 mM) was found to support the metabolism of trichloroethylene by phenobarbital induced hepatic microsomes to the extent of 0.39 nmol chloral hydrate formed per mg microsomal protein per min, which represents approximately 13% of the rate of production of chloral hydrate seen in the presence of trichloroethylene and NADPH-generating system.

3.3.3a Metabolism of trichloroethylene by partially purified cytochrome P-450.

Incubation for 10 min of trichloroethylene, plus either H_2O_2, $NaIO_4$, $NaClO_2$ or cytochrome P-450 resulted in no appreciable increase of absorbance (0.00 ± 0.04) in the modified Fujiwara assay, relative to zero time samples. Following incubation of trichloroethylene, partially purified cytochrome P-450 and $NaClO_2$ or H_2O_2, but not $NaIO_4$, appreciable conversion of trichloroethylene to a Fujiwara positive product was observed. Absorbances at 530 nm of 0.12, 0.18 and -0.02, respectively, were obtained for these artificial electron donors, the values of which are equivalent to 1.8, 2.8 and zero

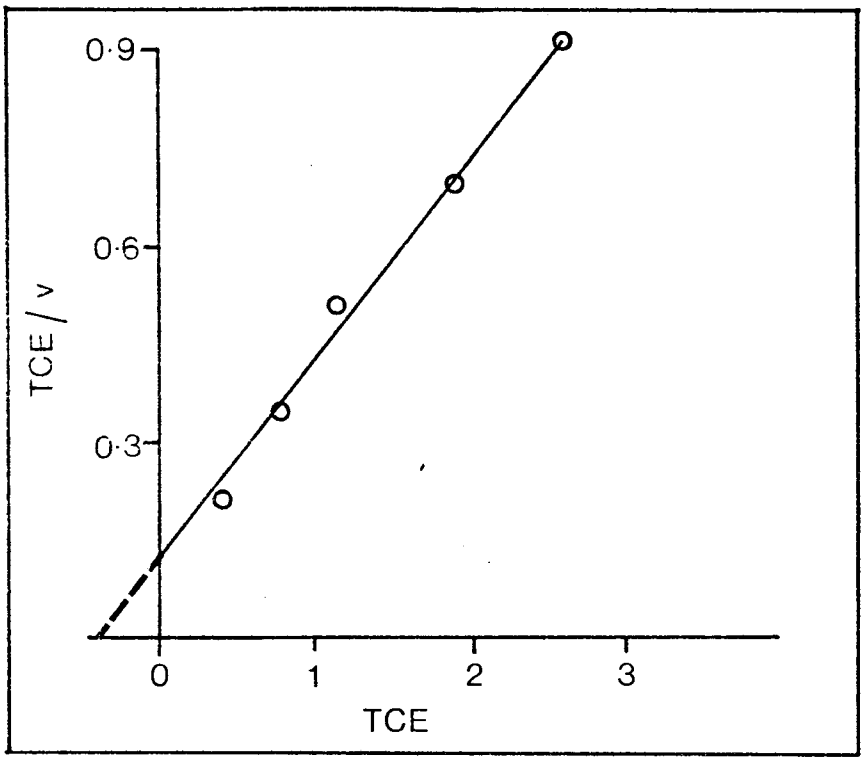

Figure 50: Hanes plot for the production of chloral hydrate from trichloroethylene by hepatic microsomes from untreated rats. Trichloroethylene (TCE), mM; v, nmol chloral hydrate/mg microsomal protein/5 min. Correlation coefficient = 0.995.

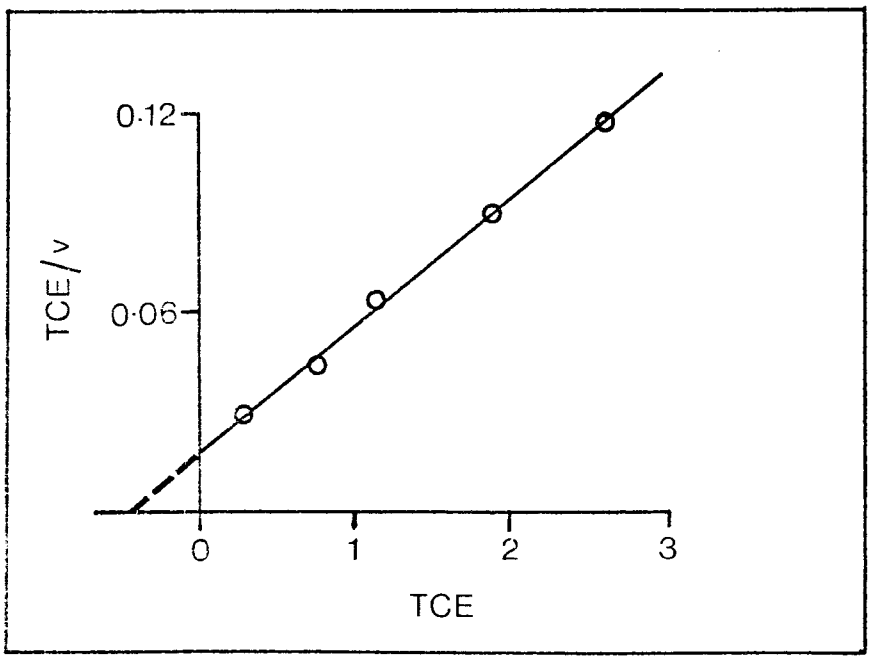

Figure 51: Hanes plot for the production of chloral hydrate from trichloroethylene by hepatic microsomes from phenobarbital treated rats. Trichloroethylene (TCE), mM; v, nmol chloral hydrate/mg microsomal protein/5 min. Correlation coefficient = 0.998.

nmol chloral hydrate per 10 min per nmol cytochrome P-450.

3.3.3b The effect of trichloroethylene on the levels of hepatic microsomal enzymes.

The effects of trichloroethylene on the levels of hepatic microsomal cytochrome P-450, heme, cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase are shown in Tables 27 and 28. Incubation of trichloroethylene, hepatic microsomes, EDTA and NADPH-generating system resulted in decreases in the levels of cytochrome P-450 and heme for each type of induction. No appreciable degradation of cytochrome P-450 or heme was observed in the absence of trichloroethylene or NADPH-generating system. The losses of cytochrome P-450 were approximately equivalent to the losses of heme for each type of induction. Reduced glutathione plus liver post-microsomal supernatant — which contains a mixture of the glutathione transferases — did not inhibit the degradation of cytochrome P-450 in uninduced or phenobarbital induced microsomes. NADH did not measurably support the degradation of cytochrome P-450 in phenobarbital induced microsomes (Table 27).

The levels of cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase were not altered following incubation of uninduced or induced microsomes in the presence of trichloroethylene and NADPH-generating system (Table 28). The activity of glucose-6-phosphatase in phenobarbital induced microsomes was not significantly affected by incubation of hepatic microsomes (2 mg protein/ml), an isocitrate dehydrogenase NADPH-generating system [279], EDTA (0.2 mM) and trichloroethylene (7.5 mM). The activity of glucose-6-phosphatase was 0.15 ± 0.049 and 0.13 ± 0.055 µmoles P_i per min per mg protein

Table 27: Effect of trichloroethylene on the levels of hepatic microsomal cytochrome P-450 and heme in vitro*

Induction	TCE	Other Additions	Cytochrome P-450+ (30 min/0 min)	Cytochrome P-450+ Loss+	Heme+ (30 min/0 min)	Heme+ Loss+	Loss Heme +≠ / Loss cytochrome P-450
None	-	-	0.89±0.04/0.94±0.09	0.05	1.53±0.06/1.66±0.08	0.13	
	+	-	0.67±0.07/0.88±0.08	0.21	1.27±0.22/1.58±0.12	0.29	0.19/0.16
	-	GSH/SN	0.90±0.03/0.99±0.05	0.09			
	+	GSH/SN	0.72±0.03/0.92±0.03	0.20			
MC	-	-	1.35±0.15/1.35±0.15	0.00	2.08±0.20/2.16±0.22	0.08	
	+	-	1.12±0.09/1.32±0.11	0.21	1.81±0.21/2.10±0.21	0.29	0.25/0.21
	+	-x	1.30±0.06/1.33±0.03	0.03			
SP	-	-	0.62±0.10/0.67±0.10	0.05	1.50±0.02/1.57±0.06	0.07	
	+	-	0.41±0.03/0.59±0.02	0.18	1.26±0.04/1.54±0.02	0.28	0.21/0.13
PB	-	-	1.74±0.21/1.87±0.11	0.13	2.35±0.17/2.58±0.08	0.23	
	+	-	1.38±0.28/1.76±0.18	0.38	1.88±0.02/2.56±0.14	0.62	0.39/0.25
	+	-x	1.77±0.09/1.77±0.05	0.00			
	+	NADHx	1.59±0.10/1.64±0.08	0.05			
	-	GSH/SN	1.89±0.07/1.90±0.02	0.01			
	+	GSH/SN	1.62±0.06/2.03±0.11	0.41			

*Values reported are means ± S.D. for experiments performed in triplicate for at least three different preparations of hepatic microsomes. Incubation mixtures (3 ml) contained hepatic microsomes (2 mg protein/ml), NADPH-generating system, 0.2 mM EDTA and, where indicated, 7.5 mM trichloroethylene, 1 mM glutathione plus 10 ul rat liver post-microsomal supernatant, or 0.6 mM NADH. Losses are reported for samples incubated for 30 min at 30° with shaking relative to unincubated samples. Abbreviations used are: TCE = trichloroethylene; MC = 3-methylcholanthrene; SP = spironolactone; PB = phenobarbital; GSH = reduced glutathione; SN = rat liver post-microsomal supernatant.

+In nmol/mg microsomal protein.

≠Corrected for losses of heme and cytochrome P-450 following incubation of hepatic microsomes, NADPH-generating system and EDTA.

xNADPH-generating system was omitted from incubation mixture.

Table 28: Effect of trichloroethylene on the levels of microsomal cytochrome b_5 and NADPH-cytochrome c reductase in vitro*

Induction	TCE	Cytochrome b_5 + (30 min/0 min)	NADPH-cytochrome c reductase ≠ (30 min/0 min)
None	-	0.44±0.04/0.40±0.06	0.056±0.004/0.058±0.005
	+	0.42±0.05/0.37±0.03	0.058±0.006/0.057±0.005
3-Methylcholanthrene	-	0.57±0.05/0.54±0.07	0.044±0.003/0.045±0.002
	+	0.53±0.05/0.50±0.06	0.046±0.005/0.043±0.004
Spironolactone	-	0.36±0.01/0.33±0.01	0.076±0.009/0.075±0.003
	+	0.39±0.02/0.32±0.01	0.076±0.004/0.076±0.005
Phenobarbital	-	0.54±0.02/0.47±0.03	0.085±0.016/0.083±0.012
	+	0.53±0.05/0.42±0.04	0.078±0.017/0.080±0.014

Experimental conditions are as described in Table 27.

Abbreviation used: TCE = trichloroethylene.

+In nmol/mg microsomal protein.

≠In units/mg microsomal protein.

at time zero and after 15 min incubation, whether or not trichloroethylene was present in the incubation mixture.

3.3.3c Effect of inhibitors on the interaction of trichloroethylene with hepatic microsomal cytochrome P-450.

The effect of inhibitors of cytochrome P-450 on the interaction of trichloroethylene with this group of enzymes is shown in Table 29. Metyrapone and SKF-525A were equivalent as inhibitors of the binding and metabolism of trichloroethylene and they inhibited each of these processes to a similar extent. Metyrapone and SKF-525A inhibited the degradation of cytochrome P-450 to a greater extent than the binding and metabolism of trichloroethylene.

In contrast, CO inhibited the metabolism of trichloroethylene and the degradation of cytochrome P-450 to approximately equivalent extents.

3.3.4 Metabolism of tetrachloroethylene by hepatic microsomal cytochrome P-450.

3.3.4a Identification of the major metabolite of tetrachloroethylene.

The structure of the major chlorinated metabolite of tetrachloroethylene from hepatic microsomal cytochrome P-450 was elucidated as follows: Reaction mixtures containing hepatic microsomes, NADPH (or NADPH-generating system), EDTA and tetrachloroethylene were incubated for 30 min. Assays for 2,2,2-trichloroethanol, chloral hydrate and trichloroacetate were performed on incubation mixtures or extracts thereof. No appreci-

Table 29: Effect of inhibitors on the interaction of trichloroethylene with hepatic microsomal cytochrome P-450*

Additions	Binding		NADPH oxidation		Chloral hydrate production		Degradation of Cytochrome P-450	
	$10^2\Delta A$/nmol cytochrome P-450	%	nmol/min/nmol cytochrome P-450	%	nmol/min/nmol cytochrome P-450	%	nmol/mg microsomal protein at 15 min.	% degradation
None	6.9±0.5	100	1.75±0.09	100	2.52±0.13	100	1.88±0.09	22
SKF-525 A (200 mM)	2.3±0.1	33	ND	ND	0.73±0.09	29	2.36±0.04	0
Metyrapone (2.33 mM)	1.6±0.2	23	0.58±0.03	33	0.78±0.11	31	2.35±0.07	0
$CO:O_2$ (80:20, v/v)	ND	ND	0.54±0.03	31	0.36±0.07	14	2.25±0.08	5

*Means ± S.D. are reported for experiments performed in triplicate with two to three different preparations of hepatic microsomes. Experiments were performed with phenobarbital induced microsomes as described in Section 2.2. In all cases, the inhibitors were added before the addition of the trichloroethylene (7.5 mM, final concentration). For binding studies, the inhibitors were added to both the sample and the reference cuvettes.

Abbreviation: ND = not determined.

able amounts of 2,2,2-trichloroethanol were detected in incubation mixtures by the method of Friedman and Cooper [262] (limit of detection at 440 nm, ca. 0.5 nmol/ml). Furthermore, assaying by gas liquid chromatography failed to demonstrate measurable levels of volatile metabolites, including 2,2,2-trichloroethanol, with the limit of detection of the latter compound being below 0.5 nmol per ml.

In order to assay for chloral hydrate, reaction mixtures were incubated for 30 min as described in Section 2.2.5. At the end of the incubation period, horse liver alcohol dehydrogenase (0.33 U/ml) and NADH (1 mM) were added to reaction mixture which was then incubated for a further 30 min. Final incubation mixtures were assayed by gas liquid chromatography for 2,2,2-trichloroethanol, which would have been formed from the reduction of chloral hydrate by alcohol dehydrogenase. Under these conditions, no appreciable amount of trichloroethanol (< 0.5 nmol/ml) was found.

Trichloroacetic acid, as assayed by method B of Leibman and Hindman, was apparently found not to be present in incubation mixtures. It was subsequently observed that the NADPH-generating system interfered with method B (but not with method A) of this assay with incubation mixtures or with authentic samples of trichloroacetic acid (see Table 30).

The identity of the major product of tetrachloroethylene was shown by infra-red spectroscopy and gas liquid chromatography to be trichloroacetic acid. The production of trichloroacetic acid was confirmed by infra-red spectroscopy as follows: Following incubation, microsomal protein was precipitated with HCl (2N, final concentration) and the precipitate was removed by centrifugation as

Table 30 : Effect of NADPH-generating system on Procedures A and B for the detection of trichloroacetic acid by the modified Fujiwara assay of Leibman and Hindman*(261)

Additions to hepatic microsomes	NADPH-generating system	OD_{530} (corrected)+	
		Procedure A	Procedure B
TCA (1.7mM)	-	0.124 ± 0.012	0.147 ± 0.011
TCA (1.7 mM)	+	0.127 ± 0.008	0.015 ± 0.004
TCA (1.7 mM) + PCE (3.3 mM)	-	0.126 ± 0.019	0.144 ± 0.018
TCA (1.7 mM) + PCE (3.3 mM)≠	+	0.121 ± 0.014	0.016 ± 0.005

*Values are means ± S.D. for determinations done in triplicate on one preparation of hepatic microsomes (2 mg protein/ml). No incubations were performed. The protein was precipitated as described in Section 2.2.5.

Abbreviations used are: TCA = trichloroacetic acid; PCE = tetrachloroethylene.

+Corrected for background absorbances of microsomal suspension alone at 530 nm.

≠Tetrachloroethylene was added after the precipitants (2N H_2SO_4 and 0.3M $NaWO_4$) in order to prevent the initiation of hepatic metabolism of tetrachloroethylene with the NADPH-generating system present.

described earlier (see Section 2.2.5). The supernatant was extracted thrice with diethyl ether. The ether layers were pooled and subsequently extracted with 0.1 N NaOH. The aqueous alkaline extracts were pooled, acidified to pH2 with 7.5 M HCl and extracted repeatedly with diethyl ether. The ether was evaporated at 25° under a stream of N_2. The infra-red spectrum of the reaction product isolated from incubation mixtures was identical to that of an authentic sample of trichloroacetic acid which had been taken through the same extraction procedure (see Figure 52). Notably, the distinctive peaks characteristic of carboxylic acids (3300 cm^{-1}) and of the carbonyl group (1740 cm^{-1}) were present in both spectra.

Methyl trichloroacetate was found to be present in methylated extracts of incubation mixtures by gas liquid chromatography. The amounts of methyl trichloroacetate produced from phenobarbital induced microsomes of 0.26 ± 0.01 nmol per min per mg microsomal protein compared well with the value of 0.19 ± 0.05 nmol trichloroacetic acid per min per mg microsonal protein found to be produced in incubation mixtures using the modified Fujiwara assay of Leibman and Hindman [261]. Therefore, for all further experiments monitoring trichloroacetic acid production from tetrachloroethylene, the modified Fujiwara assay was used.

Using procedure A of the modified Fujiwara assay of Leibman and Hindman [261], the conversion of tetrachloroethylene to trichloroacetic acid was shown to be linear for 30 min from microsomes from uninduced rats. For microsomes from β-naphthoflavone, pregnenolone-16α-carbonitrile and phenobarbital induced rats, the metabolism of tetrachloroethylene was linear for 15, 20 and 7 min, respectively (Figure 53). These incubation times were utilized for

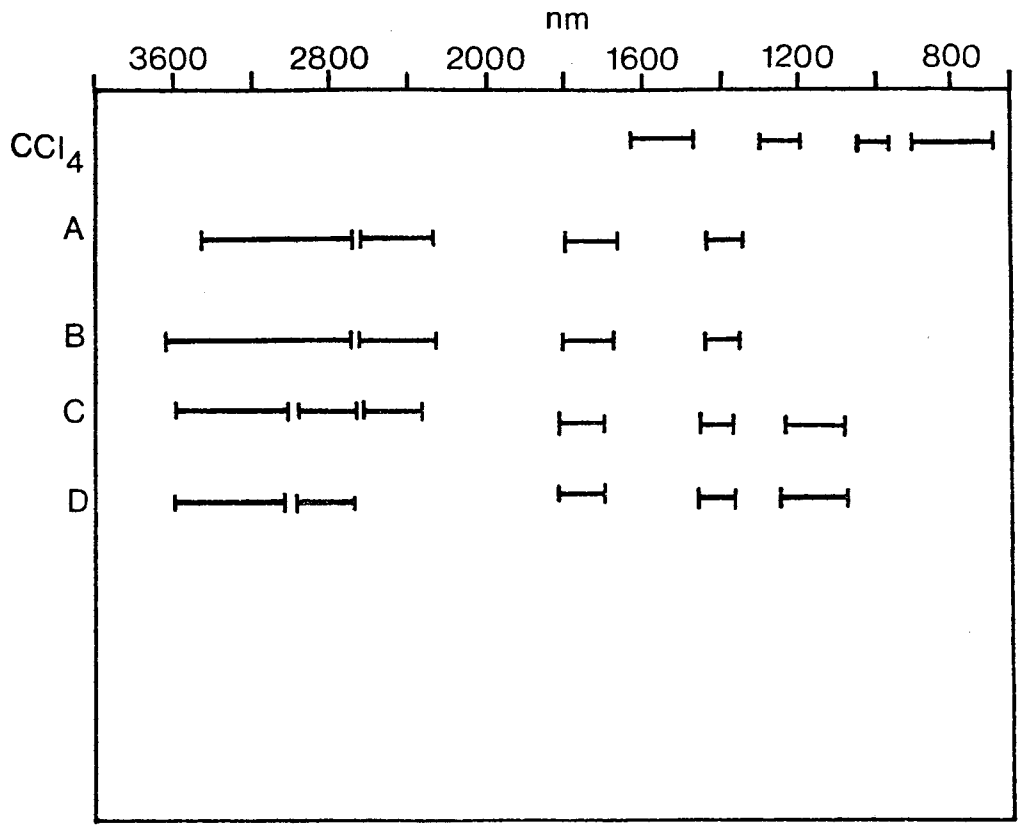

Figure 52: Infrared spectra of an authentic sample of trichloroacetic acid (A), authentic sample of trichloroacetic acid extracted from hepatic microsomes (B), and the reaction product of tetrachloroethylene in the presence of hepatic microsomes from phenobarbital treated rats and a NADPH-generating system (C and D). See text for procedure.

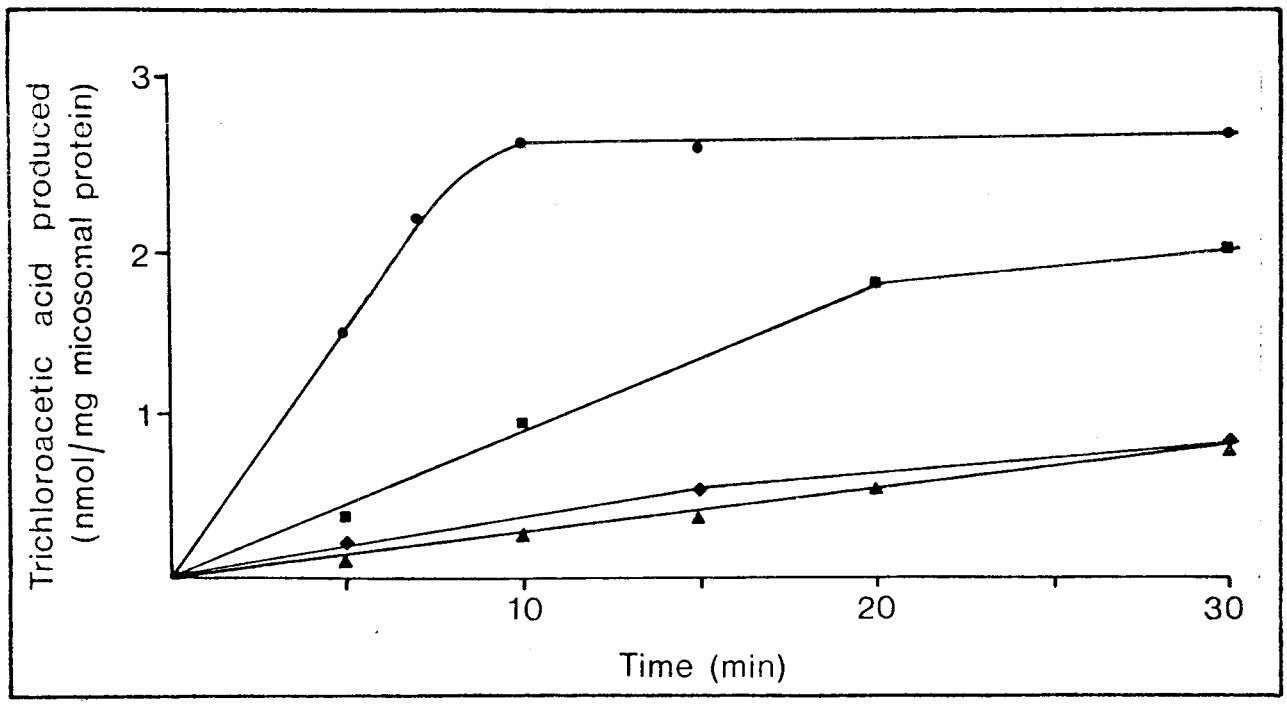

Figure 53: The effect of inducing agents on the production of trichloroacetic acid from tetrachloroethylene as a function of time for microsomes from untreated (▲), β-naphthoflavone treated (◆), pregeneolone-16α-carbonitrile treated (■) and phenobarbital treated (●) rats. Trichloroacetic acid, nmol /mg microsomal protein; Time, min.

all further experiments in which the metabolism of tetrachloroethylene was assessed by the method of Leibman and Hindman [261].

Hanes plots for the conversion of tetrachloroethylene to trichloroacetic acid by hepatic microsomal cytochrome P-450 were linear (see Figures 54 - 57). The effects of inducing agents for different forms of cytochrome P-450 on the Michaelis constants (K_m) and the maximum rates of metabolism (V_{max}) for tetrachloroethylene, as assessed by the modified Fujiwara assay, are shown in Table 31 . K_m for tetrachloroethylene was decreased following phenobarbital induction and increased following induction with pregnenolone-16α-carbonitrile. Neither K_m nor V_{max} was significantly affected by induction with β-naphthoflavone. V_{max} per mg microsomal protein and V_{max} per nmol cytochrome P-450 was increased following induction with pregnenolone-16α-carbonitrile and phenobarbital.

3.3.4b Effects of inhibitors on the binding and metabolism of tetrachloroethylene.

The effects of three inhibitors of cytochrome P-450 on the binding and metabolism of tetrachloroethylene are presented in Table 32. Metyrapone, CO and SKF-525 A each inhibited the binding, NADPH oxidation and metabolism of tetrachloroethylene in the presence of hepatic microsomes. Metyrapone and CO were equivalent as inhibitors of these processes, while SKF-525 A was much less effective. Each of the inhibitors was found to inhibit the three processes to approximately the same extent.

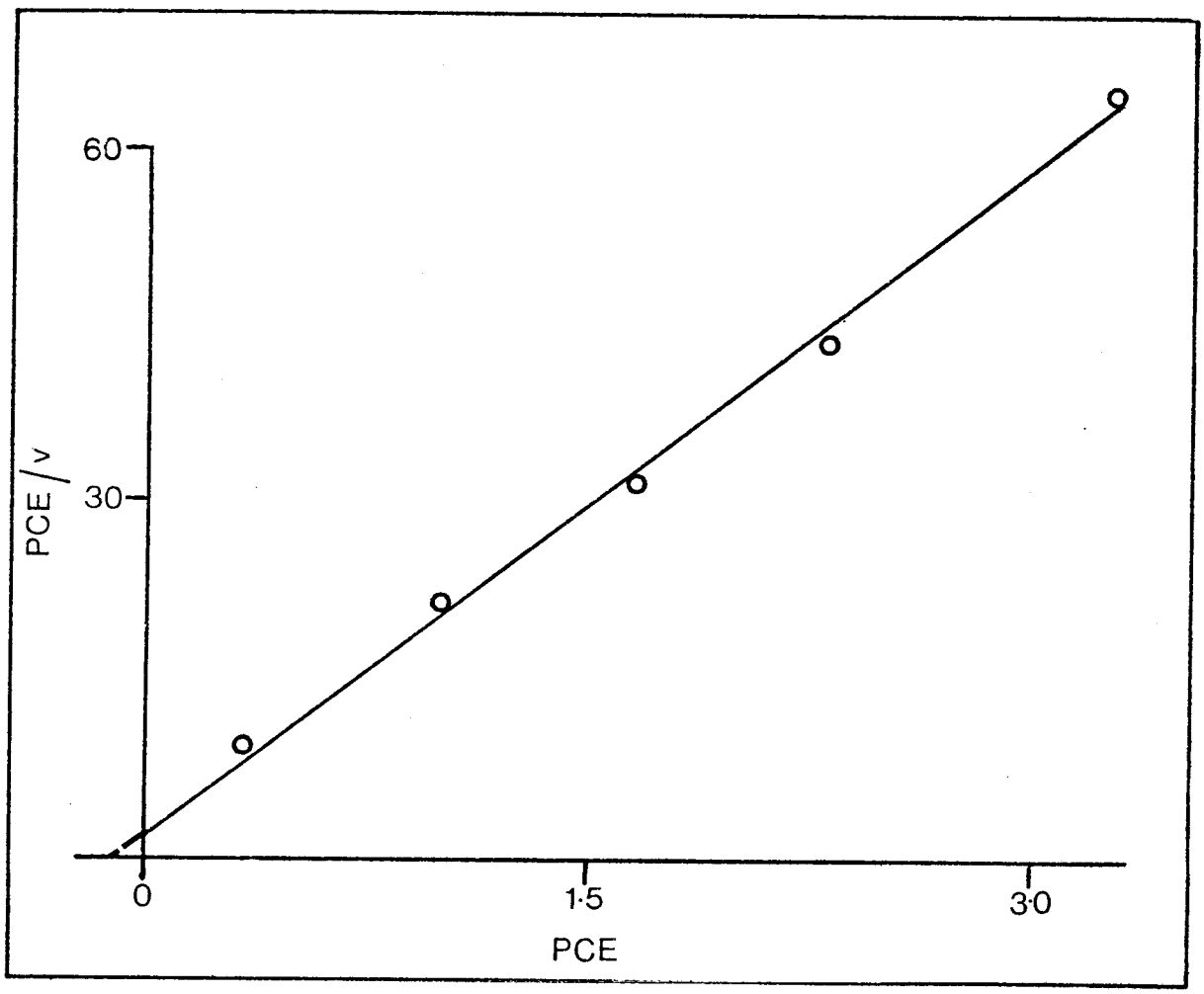

Figure 54: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene by hepatic microsomes from β-naphthoflavone treated rats. Tetrachloroethylene (PCE), mM; v, nmol trichloroacetic acid/mg microsomal protein/min.
Correlation coefficient = 0.998.

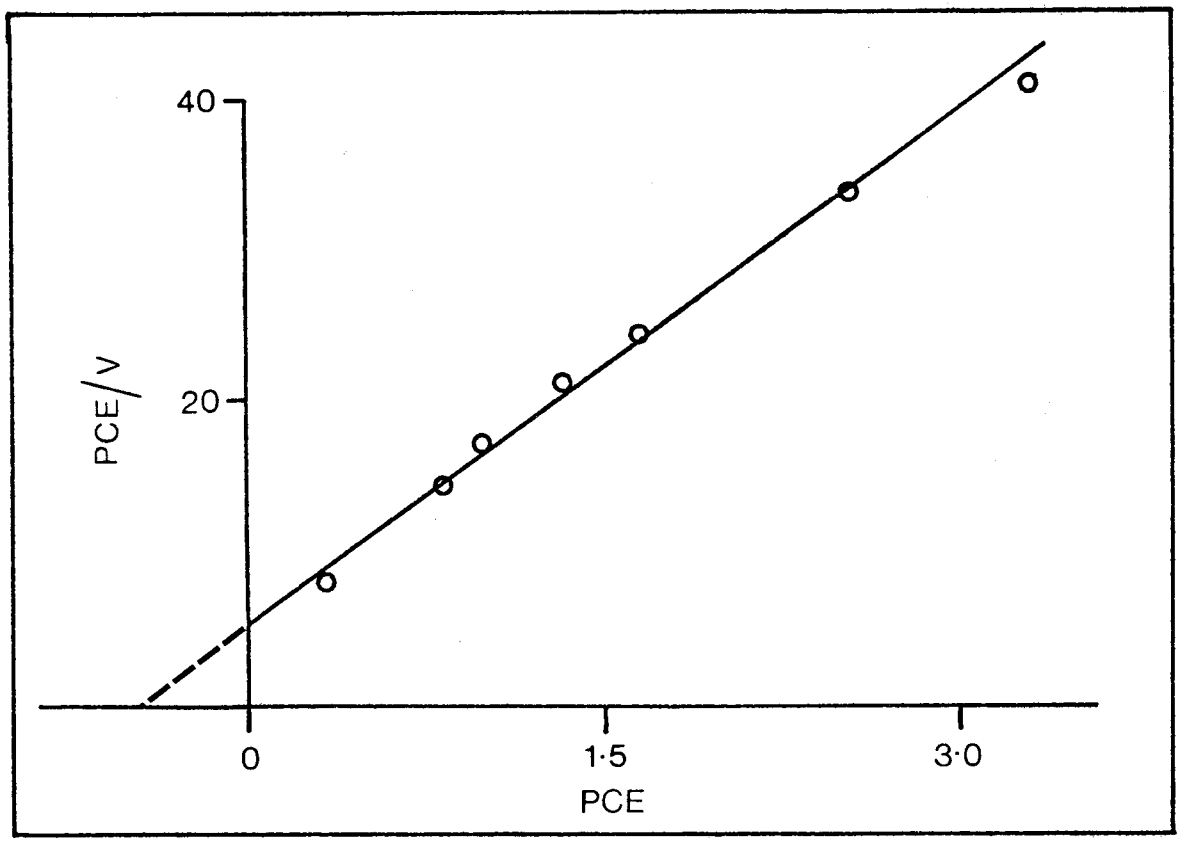

Figure 55: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene by hepatic microsomes from untreated rats. Tetrachloroethylene (PCE), mM; v, nmol trichloroacetic acid/mg microsomal protein/min. Correlation coefficient = 0.990.

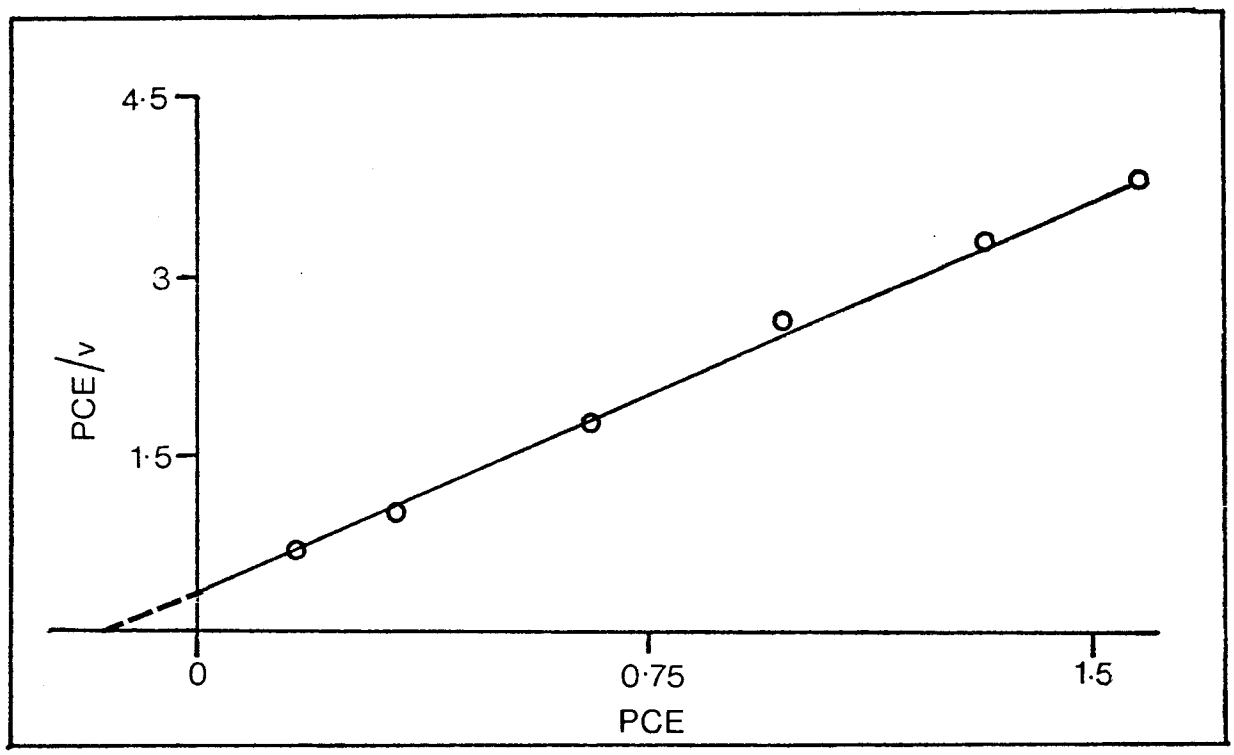

Figure 56: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene by hepatic microsomes from phenobarbital treated rats. Tetrachloroethylene (PCE), mM; v, nmol trichloroacetic acid/mg microsomal protein/min. Correlation coefficient = 0.998.

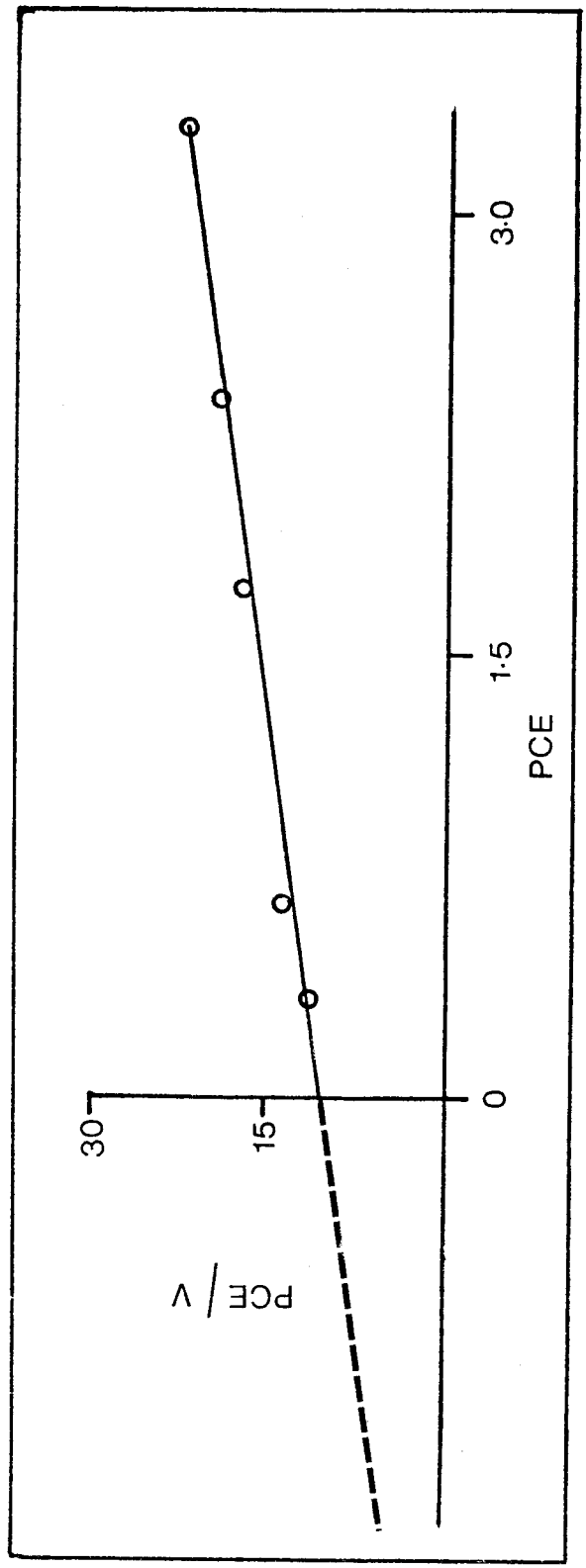

Figure 57: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene by hepatic microsomes from pregnenolone-16α-carbonitrile treated rats. Tetrachloroethylene (PCE), mM; v, nmol trichloroacetic acid/mg microsomal protein/min.
Correlation coefficient = 0.997.

Table 31: Effect of induction on the metabolism of tetrachloroethylene to trichloroacetic acid by hepatic microsomal cytochrome P-450*

Induction	K_m (mM)	V_{max}	
		nmol TCA/min/mg microsomal protein	nmol TCA/min/nmol cytochrome P-450
None	1.1±0.8	0.041±0.003	0.046±0.004
β-Naphthoflavone	0.5±0.5 ≠	0.056±0.014	0.055±0.014
Pregnenolone-16α-carbonitrile	2.4±0.8 +	0.24±0.11 +	0.16±0.09 +
Phenobarbital	0.2±0.1 +	0.44±0.05 +	0.19±0.02 +

*Values reported are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in Section 2.2.5. Abbreviation used is: TCA = trichloroacetic acid.

+Differs significantly from value for uninduced microsomes, $P < 0.01$

≠Probably differs from corresponding value for uninduced microsomes, $P < 0.05$.

Table 32: Effect of inhibitors on the interaction of tetrachloroethylene with hepatic microsomal cytochrome P-450*

Additions	Binding (%)	NADPH Oxidation (%)	Trichloroacetate production (%)
None	100	100	100
SKF-525 A (200 mM)	81±3	ND	67±8
Metyrapone (2.33 mM)	20±4	35±6	25±7
$CO:O_2$ (80:20), v/v)	ND	25±12	25±10

*Means ± S.D. are reported for experiments performed in triplicate with two to three different preparations of hepatic microsomes. Experiments were performed with phenobarbital induced microsomes as described in Section 2.2.5. In all cases, the inhibitors were added before the tetrachloroethylene (3.3 mM, final concentration). For binding studies, the inhibitors were added to both the sample and the reference cuvettes.

Values in the absence of inhibitors were as follows: Binding, $A_{386\ nm} - A_{418\ nm}$, 0.10/nmol cytochrome P-450; NADPH oxidation, 2.04 nmol/min/nmol cytochrome P-450; Trichloroacetate production, 0.113 nmol/min/nmol cytochrome P-450.

Abbreviation used: ND = not determined.

3.3.4c Assay for covalently bound trichloroacetyl moeities.

The treatment of reaction mixtures (containing tetrachloroethylene, NADPH-generating system, EDTA and hepatic microsomes from phenobarbital induced rats) which had been previously incubated at 30° for 30 min, with H_2SO_4 (20% H_2SO_4 at 100° for 6 hr) [211] resulted in significantly ($P < 0.001$) increased levels of trichloroacetic acid. The levels of trichloroacetate before and after incubation with H_2SO_4 were 1.01 ± 0.04 and 1.81 ± 0.19 nmol trichloroacetic acid per 30 min per nmol cytochrome P-450.

3.3.4d Effects of tetrachloroethylene on hepatic microsomal enzymes heme and GSH.

Incubation of hepatic microsomes from untreated and variously pretreated rats in the presence of NADPH-generating system, EDTA and tetrachloroethylene did not result in any appreciable alteration in the levels of hepatic microsomal cytochrome P-450, cytochrome b_5, heme and NADPH-cytochrome c reductase (Table 33). Incubations as above, with microsomes from phenobarbital pretreated animals, but in the presence of GSH, were without effect on the levels of GSH. The levels of GSH at zero time and after 15 min incubation were 0.05 ± 0.008 and 0.04 ± 0.008 mM for incubations in the presence and absence of 3.3 mM tetrachloroethylene ($P > 0.1$).

Levels of glucose-6-phosphatase were also unaffected in the presence of tetrachloroethylene (3.3 mM), hepatic microsomes from phenobarbital pretreated animals, EDTA (0.2 mM) and an isocitrate dehydrogenase NADPH-generating system ($P > 0.1$) [279]. Glucose-6-phosphatase activity was 0.15 ± 0.01 and 0.12 ± 0.01 umoles P_i per min per mg microsomal protein at time zero and 15 min incubation,

Table 33: Effect of tetrachloroethylene on the levels of hepatic microsomal components*

Induction	Tetrachloroethylene (mM)	Cytochrome P-450[+] (Loss/Initial level)	Heme[+] (Loss/Initial level)	Cytochrome b_5[+] (30 min/0 min)	NADPH-cytochrome c reductase[≠] (30 min/0 min)
None	0	0.127/0.81	0.12/1.20	0.47±.04/0.42±.01	0.049±.017/0.045±.015
	3.3	0.095/0.65	0.05/1.15	0.44±.03/0.40±.01	0.051±.016/0.048±.017
β-Naphthoflavone	0	0.13/1.17	0.13/1.18	0.45±.09/0.37±.08	0.061±.005/0.057±.008
	3.3	0.13/1.02	0.14/1.15	0.42±.07/0.36±.09	0.058±.005/0.058±.009
Phenobarbital	0	0.28/2.44	0.27/2.21	0.56±.08/0.46±.07	0.091±.009/0.094±.007
	3.3	0.39/2.35	0.33/2.14	0.45±.05/0.37±.06	0.082±.003/0.088±.009

*Values are means ± S.D. for experiments performed in triplicate with three different preparations of hepatic microsomes. Incubation mixtures (3 ml) contained hepatic microsomes (2 mg protein/ml), EDTA (0.2 mM), NADPH-generating system and, where indicated, 3.3 mM tetrachloroethylene. Incubations were for 30 min.

[+]In nmol/mg microsomal protein.

[≠]In U/mg microsomal protein.

whether or not tetrachloroethylene was present in the incubation mixture.

3.4 Metabolism of the chlorinated ethylenes by isolated hepatocytes

3.4.1 Results of pretreatment of animals for experimentation using isolated hepatocytes

Isolation of hepatocytes was not satisfactorily achieved when animals had been pretreated with 3-methylcholanthrene and β-naphthoflavone. This was apparently due to administration of these two inducing agents in corn oil. On account of the large amounts of corn oil deposited in the abdominal cavity (below the diaphragm) of these animals, perfusion of the liver was very tricky and a uniform blanching of the liver seldom occurred. Therefore only untreated rats and phenobarbital (in 0.9% saline) pretreated rats were used for studies using isolated hepatocytes.

It must be noted that results were obtained in triplicate from each of three different hepatocyte preparations (unless otherwise indicated). Hanes plots for the production of chlorinated metabolites from the chlorinated ethylenes (i.e. Figures) are given for experiments performed in triplicate on one set of hepatocytes, which provided a good representative of the results obtained from three different hepatocyte preparations.

3.4.2 Metabolism of the chlorinated ethylenes using hepatocyte preparations

The effect of increasing concentrations of vinylidene chloride on the viability of hepatocytes isolated from phenobarbital pretreated animals is shown in Figure 58. The viability of the cells decreased for ca. 30 min and remained constant following incubation

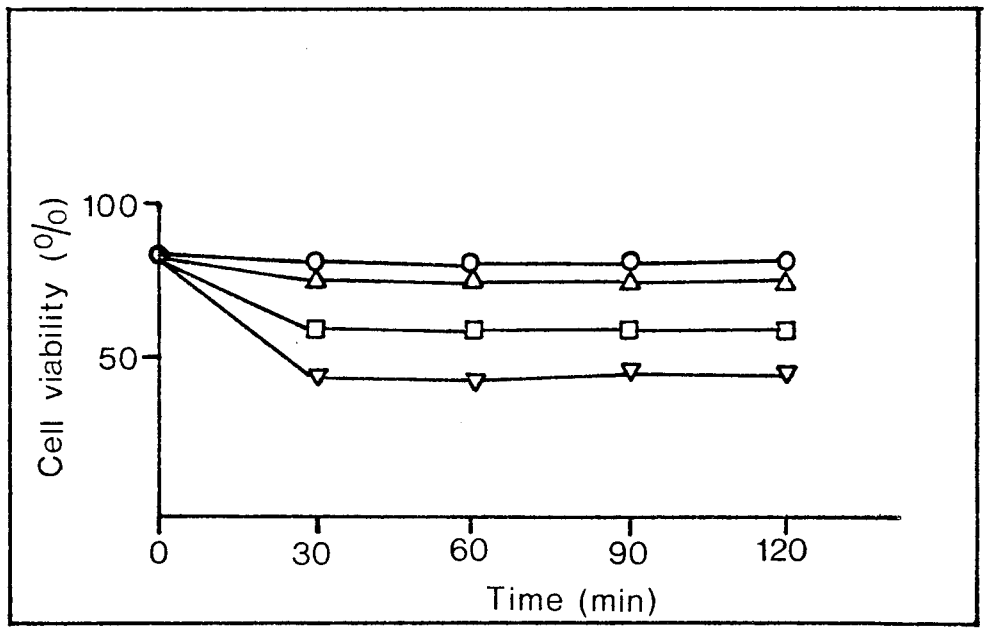

Figure 58: Effect of varying concentrations of vinylidene chloride with time on the viability of isolated hepatocyte preparations from a phenobarbital pretreated animal. Vinylidene chloride concentration in mM; 0.4 (O); 2.1 (Δ); 4.1 (□); 6.2 (▽).

periods of between 30 and 120 min (see Figure 58). The same phenomenon was seen when experiments were performed with the other chlorinated ethylenes. The effect of the concentration of the chlorinated ethylenes on the viability of the isolated viable hepatocytes after 30 min is summarized in Table 34. Ethanol alone (1 mM, final concentration) did not affect cell viability over 120 min (Data not shown).

3.4.2a The metabolism of the dichlorinated ethylenes

The conversion of the dichlorinated ethylenes (vinylidene chloride, 2.1 mM, cis- (4.3 mM) and trans-1,2-dichloroethylene, 4.3 mM) to mono- and dichlorinated metabolites was assessed with isolated hepatocyte suspensions (2 x 10^6 cells/ml) from phenobarbital pretreated rats. The chlorinated metabolites produced were assayed by gas liquid chromatography using Column II. Since the metabolites produced had all been positively identified in hepatic microsomal suspensions earlier using a second column and/or by specific chemical tests (see 3.3.1 and 3.3.2), it was not deemed necessary to repeat this confirmation with incubations from hepatocyte suspensions.

The metabolism of vinylidene chloride (2.1 mM) in the presence of isolated hepatocytes from phenobarbital pretreated rats resulted in the production of monochloroacetic acid (0.001±0.0002 nmol/10^6 cells/min) and dichloroacetic acid (0.015±0.01 nmol/10^6 cells/min) (see Table 35) and traces of dichloroacetaldehyde and 2,2-dichloroethanol. Measurable levels of the following were not produced from vinylidene chloride — 2-chloroethanol and monochloroacetaldehyde with detection limits of $<$12.2 nmol/10^6 cells/30 min and 0.4 nmol/10^6 cells/30 min, respectively.

Table 34: Effect of concentration of the chlorinated ethylenes with time on the viability of hepatocytes isolated from phenobarbital-induced rats*

Additions	mM	% Viability § after 30 min
Vinylidene chloride+	0.4	80
	2.1¶	78
	4.1	60
	6.2	45
1,2-Dichloroethylene+	0.4	81
	2.2	80
	4.3¶	80
	21.6	52
Trichloroethylene≠	0.56	81
	2.8¶	79
	5.6	43
Tetrachloroethylene≠	2.5¶	79
	5.0	39

*Values reported were performed in duplicate on one preparation of hepatocytes (2 x 10^6 cells/ml). Incubations were performed at 37° (30 rpm) with the flasks at an agle of 35°.

+Chlorinated ethylene : ethanol in the ratio of 1 : 2.

≠Chlorinated ethylene : ethanol in the ratio of 1 : 1.

§82% of hepatocytes on isolation excluded trypan blue.

¶ mM concentrations used for all further experimentation.

Table 35: The metabolism of vinylidene chloride, cis- and trans-1,2-dichloroethylene by isolated, viable hepatocytes*

Chlorinated ethylene	Metabolite production (nmol/10^6 cells/10 min)			
	2,2-Dichloro-ethanol	Monochloroacetic acid	Dichloroacetic acid	Dichloroacetaldehyde
Vinylidene chloride (2.1 mM)	Traces	0.011 ± 0.002	0.15 ± 0.08	Traces
cis-1,2-dichloro-ethylene (4.3 mM)	2.4 ± 0.20	ND	0.3 ± 0.11	0.04 ± 0.01
trans-1,2-dichloro-ethylene(4.3 mM)	Traces	ND	0.05 ± 0.01	Traces

*Values are means ± S.D. for experiments performed in triplicate on two different preparations of isolated hepatocytes from phenobarbital induced rats.

ND = Not detectable.

The metabolism of trans-1,2-dichloroethylene (4.1 mM) in isolated hepatocytes gave rise to dichloroacetic acid (0.05 ± 0.01 nmol/10^6 cells/10 min) and traces of dichloroacetaldehyde and 2,2-dichloroethanol (see Table 35). 2-Chloroethanol, chloroacetaldehyde (for detection limits, see above) and monochloroacetic acid (limit of detection < 0.05 nmol/10^6 cells/30 min) were not detected as metabolites of trans-1,2-dichloroethylene in isolated hepatocytes.

The metabolism of cis-1,2-dichloroethylene resulted in the production of 2,2-dichloroethanol (0.24 ± 0.20 nmol/10^6 cells/min), dichloroacetaldehyde (0.004 ± 0.01 nmol/10^6 cells/ min) and dichloroacetic acid (0.03 ± 0.11 nmol/10^6 cells/min) (see Table 35). No other chlorinated metabolites were produced in measurable amounts (see detection limits above).

For the conversion of cis-1,2-dichloroethylene to each of its major chlorinated metabolites, time courses for metabolite production, Michaelis constants (K_m) and maximal rate of metabolite production (V_{max}) were assessed.

While the production of 2,2-dichloroethanol and dichloroacetic acid was linear for 25-30 min, the production of dichloroacetaldehyde was linear for only 10 min. Therefore further metabolic studies with cis-1,2-dichloroethylene in isolated hepatocyte suspensions were carried out for 10 min.

Hanes plots of the production of 2,2-dichloroethanol, dichloroacetic acid and dichloroacetaldehyde from cis-1,2-dichloroethylene were all monophasic (see Figures 59 — 61). As can be seen from Table 36, the apparent K_m values for the production of 2,2-dichloroethanol and dichloroacetaldehyde were similar ($P > 0.1$), while the apparent K_m for dichloroacetic acid production was significantly lower ($P < 0.01$).

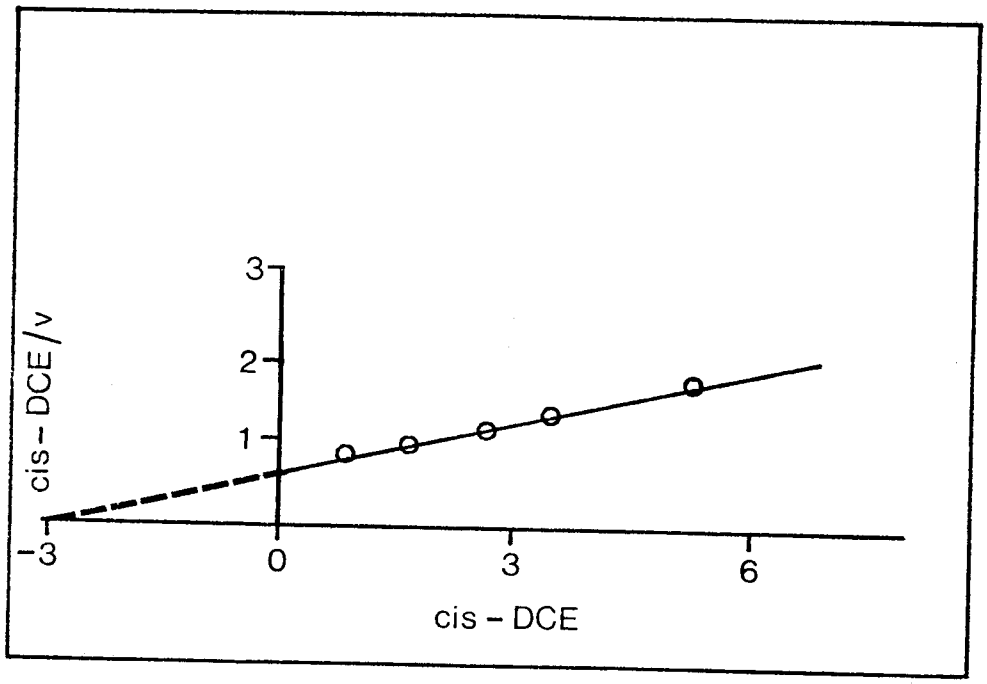

Figure 59: Hanes plot for the production of 2,2-dichloroethanol from cis-1,2-dichloroethylene in hepatocytes isolated from a phenobarbital pretreated rat. cis-1,2-Dichloroethylene (DCE), mM; v, nmol 2,2-dichloroethanol /2x10^6 cells/10 min. Correlation coefficient = 0.985.

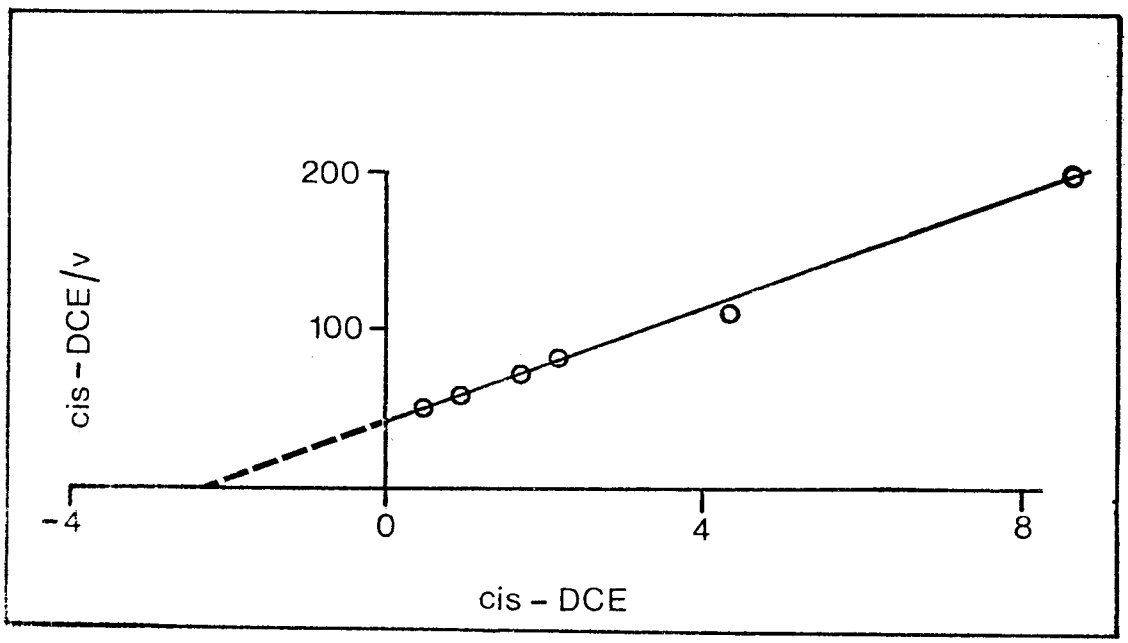

Figure 60: Hanes plot for the production of dichloroacetaldehyde from cis-1,2-dichloroethylene in hepatocytes isolated from a phenobarbital pretreated rat. cis-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetaldehyde /2x10^6 cells/10 min. Correlation coefficient = 0.997.

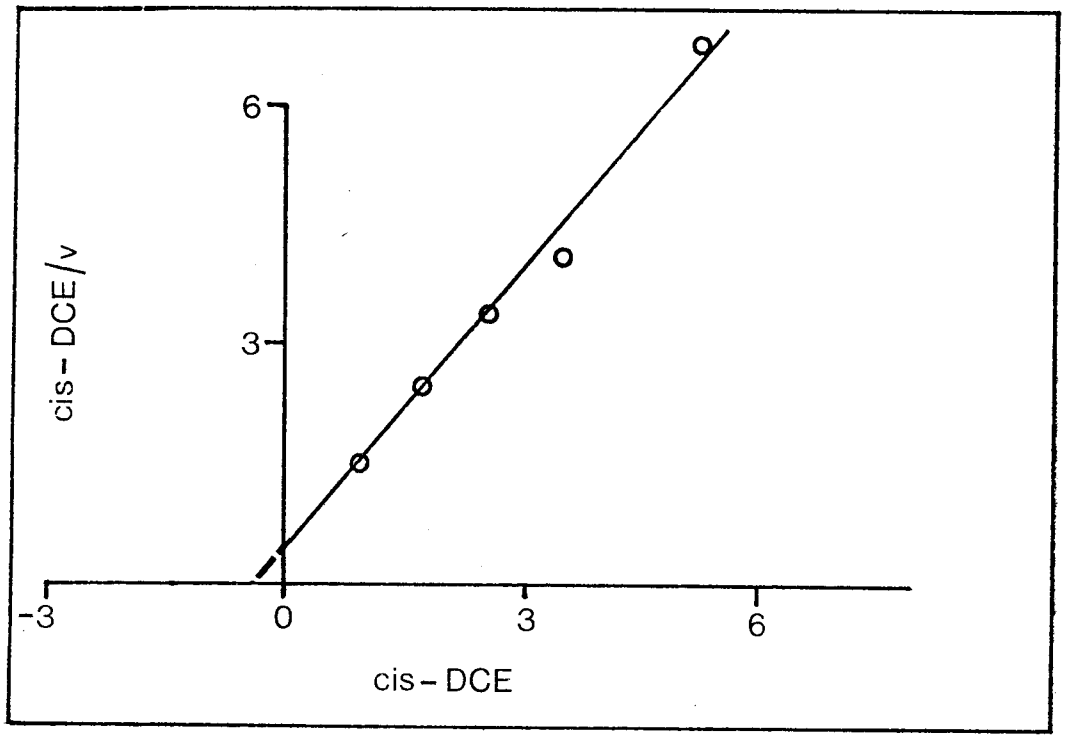

Figure 61: Hanes plot for the production of dichloroacetic acid from cis-1,2-dichloroethylene in hepatocytes isolated from a phenobarbital pretreated rat. cis-1,2-Dichloroethylene (DCE), mM; v, nmol dichloroacetic acid/$2x10^6$ cells/10 min. Correlation coefficient = 0.991.

Table 36: The K_m and V_{max} values for the production of chlorinated metabolites from cis-1,2-dichloroethylene in isolated hepatocytes from phenobarbital treated rats*

Metabolite	K_m (mM)	V_{max} (pmol/10^6 cells/min)
Dichloroacetaldehyde	2.15 ± 0.09	3.6 ± 0.7
2,2-Dichloroethanol	2.55 ± 1.20	240 ± 20
Dichloroacetic acid	0.67 ± 0.30	27 ± 11

*Values reported are means ± S.D. for experiments performed in triplicate with three different preparations of hepatocytes. Experimental conditions are as described in 2.2.8c.

The V_{max} values obtained for the three products differ, with the V_{max} value for 2,2-dichloroethanol production being 10-fold higher and 100-fold higher than the V_{max} values obtained for the production of dichloroacetic acid and dichloroacetaldehyde respectively. 2,2-Dichloroethanol is, therefore, the major metabolite of cis-1,2-dichloroethylene metabolism in isolated hepatocytes from phenobarbital-treated rats (Table 36).

3.4.2b Metabolism of trichloroethylene

Chloral hydrate was identified as the major metabolite of trichloroethylene, by gas-liquid chromatography using Column III (see Methods 2.2.5). The rate of production of chloral hydrate was found to be 2.3 ± 0.5 nmol/10^6 cells/min. Traces of 2,2,2-trichloroethanol (viz. 0.3 ± 0.02 nmol/10^6 cells/min) and trichloroacetate (viz. 15 pmol/10^6 cells/min) were also found to be produced from trichloroethylene in isolated hepatocytes.

The amounts of chloral hydrate produced from hepatocytes isolated from phenobarbital pretreated rats as assessed by gas-liquid chromatography (viz 2.3 ± 0.51 nmol chloral hydrate/10^6 cells/min) were comparable to amounts of chloral hydrate produced from trichloroethylene as measured by the modified Fujiwara assay [261] (viz 2.7 ± 0.3 nmol chloral hydrate/10^6 cells/min) (Table 37) ($P > 0.1$); therefore the modified Fujiwara assay was used for all further studies of chloral hydrate production, viz., the time course and concentration dependence of the reaction, the latter being to

Table 37: The metabolism of trichloroethylene using isolated, viable hepatocytes*

Inducing agent	K_m (mM)		V_{max} (nmol CH/10^6 cells/min)	
None	1.90 ± 0.14	12.6 ± 2.5	1.0 ± 0.1	0.002 ± 0.0001
Phenobarbital	1.30 ± 0.04+	NOT CALCULATED	2.7 ± 0.8+	NOT CALCULATED

*Values reported are means ± S.D. for experiments performed in triplicate with two different preparations of hepatocytes. Experimental conditions are as described in 2.2.8c. Abbreviation used: CH = Chloral hydrate.

+Differs significantly from value obtained from hepatocytes isolated from untreated animals, $P < 0.01$.

establish the Michaelis constant (K_m) and V_{max} values in isolated hepatocytes.

The production of chloral hydrate from trichloroethylene (2.8 mM) was linear for 30 min and for 5 min for hepatocyte preparations isolated from untreated and phenobarbital pretreated animals, respectively. These incubation times were utilized in all further experiments, when assessing the metabolism of trichloroethylene.

Hanes plots for the conversion of trichloroethylene to chloral hydrate were biphasic for hepatocytes isolated from untreated rats and for hepatocytes isolated from phenobarbital pretreated animals are shown in Figures 62 and 63 .

The K_m value for the cytochrome P-450 dependant conversion of trichloroethylene to chloral hydrate following phenobarbital pretreatment is significantly decreased compared to K_m values found in hepatocytes for untreated animals. Since the low affinity K_m value obtained with hepatocytes from untreated rats (Table 37) is approximately four times greater than the maximum permissible concentration of trichloroethylene in hepatocytes (Table 34) and is associated with an extremely low V_{max} value, it would be insignificant for cytochrome P-450 dependant metabolism in vivo.

3.4.2c Metabolism of tetrachloroethylene

The production of chlorinated metabolites from tetrachloroethylene was linear for 60 min for preparations of hepatocytes isolated from untreated and phenobarbital treated rats as measured by the spectral Fujiwara assay [261].

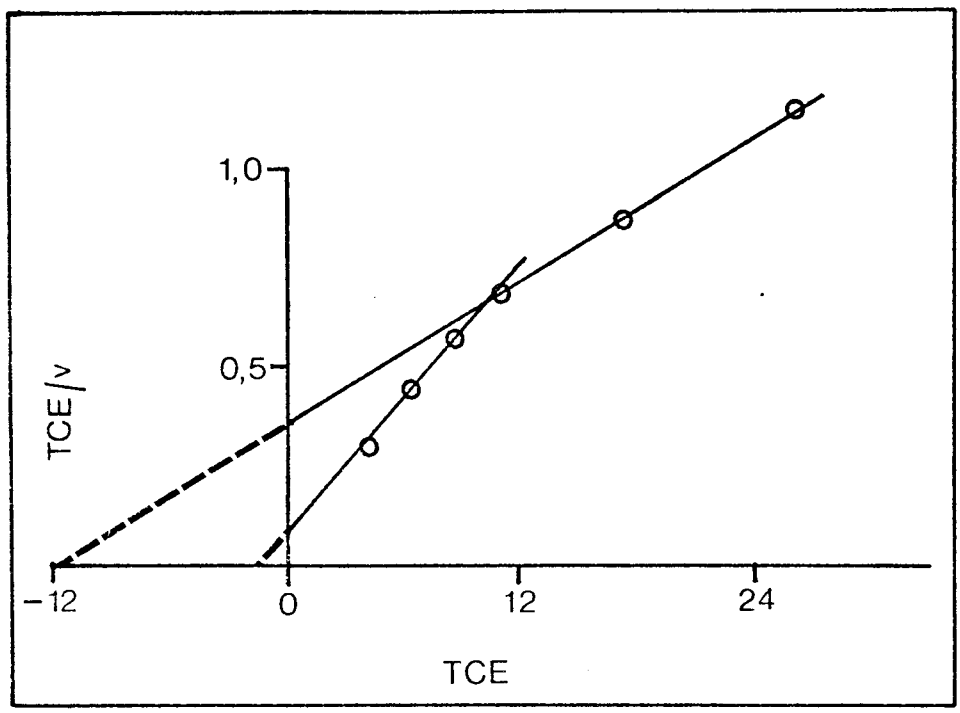

Figure 62: Hanes plot for the production of chloral hydrate from trichloroethylene in isolated hepatocytes from an untreated rat. Trichloroethylene (TCE), mM; v, nmol chloral hydrate/2x10^6 cells/20 min. Correlation coefficient for high affinity site = 0.998; low affinity site = 0.999

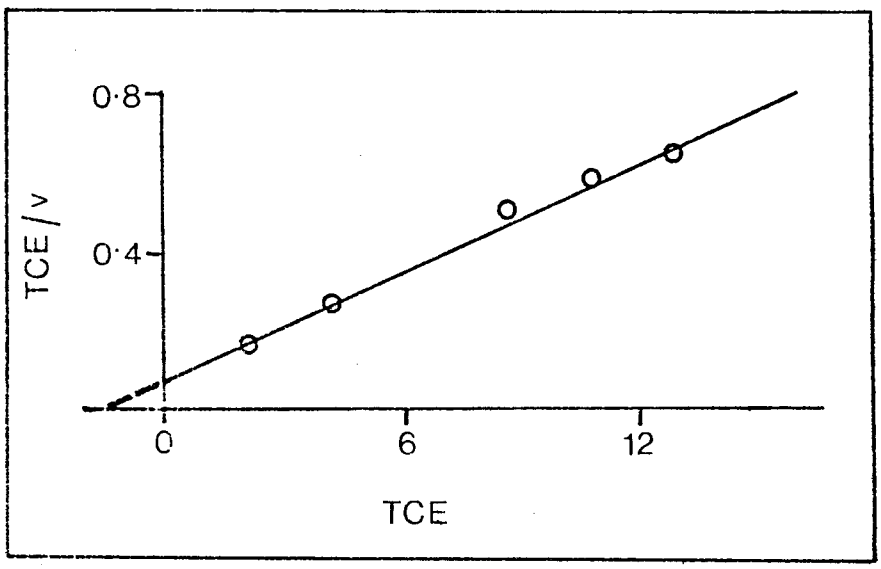

Figure 63: Hanes plot for the production of chloral hydrate from trichloroethylene in the presence of isolated hepatocytes from a phenobarbital pretreated rat. Trichloroethylene (TCE), mM; v, nmol chloral hydrate/2x10^6 cells/5 min. Correlation coefficient = 0.992.

Trichloroacetic acid was identified as the sole metabolite of tetrachloroethylene in the presence of hepatocytes, by gas-liquid chromatography using Column II (see Section 2.2.5d). The amount of trichloroacetate produced from tetrachloroethylene, in the presence of hepatocytes from phenobarbital treated animals, as monitored by gas-liquid chromatography (viz 0.074 ± 0.01 nmol trichloroacetic acid/10^6 cells/min) was identical to results from the spectral Fujiwara assay (viz. 0.098 ± 0.03 nmol trichloroacetic acid/ 10^6 cells/min) (see Table 38). The modified Fujiwara assay was therefore used in all further studies of trichloroacetate production.

Hanes plots of the production of trichloroacetate from tetrachloroethylene were monophasic for hepatocyte suspensions from untreated and phenobarbital pretreated rats (see Figures 64 and 65). Phenobarbital pretreatment did not affect the K_m for the conversion of tetrachloroethylene to trichloroacetate, but did increase the value of V_{max} by ca. 10-fold.

3.4.3 Effect of the chlorinated ethylenes on the levels of cytochrome P-450

The profound effect of cis- and trans-1,2-dichloroethylene and trichloroethylene on the levels of hepatic microsomal cytochrome P-450 *in vitro* was not seen when these compounds were incubated with freshly isolated hepatocyte suspensions (see Table 39).

The incubation of the mixture of cis- and trans-1,2-dichloroethylene (6.9 mM) or trichloroethylene (7.5 mM) with hepatic microsomes from phenobarbital-treated animals and a NADPH-generating system resulted in 42 and 20 percent loss of hepatic microsomal cytochrome P-450 (see 3.3.2 and 3.3.3), respectively. However,

Table 38: The metabolism of tetrachloroethylene in isolated, viable hepatocytes*

Inducing agent	Trichloroacetic acid production	
	K_m (mM)	V_{max} (p mol/10^6 cells/min)
None	0.64 ± 0.37	9.5 ± 0.4
Phenobarbital	0.28 ± 0.04	98 ± 3+

*Values are means ± S.D. on experiments performed in triplicate on two different preparations of isolated hepatocytes. Experimental conditions as in 2.2.8c.

+Differs significantly from value obtained with hepatocytes from untreated animals, $P < 0.01$.

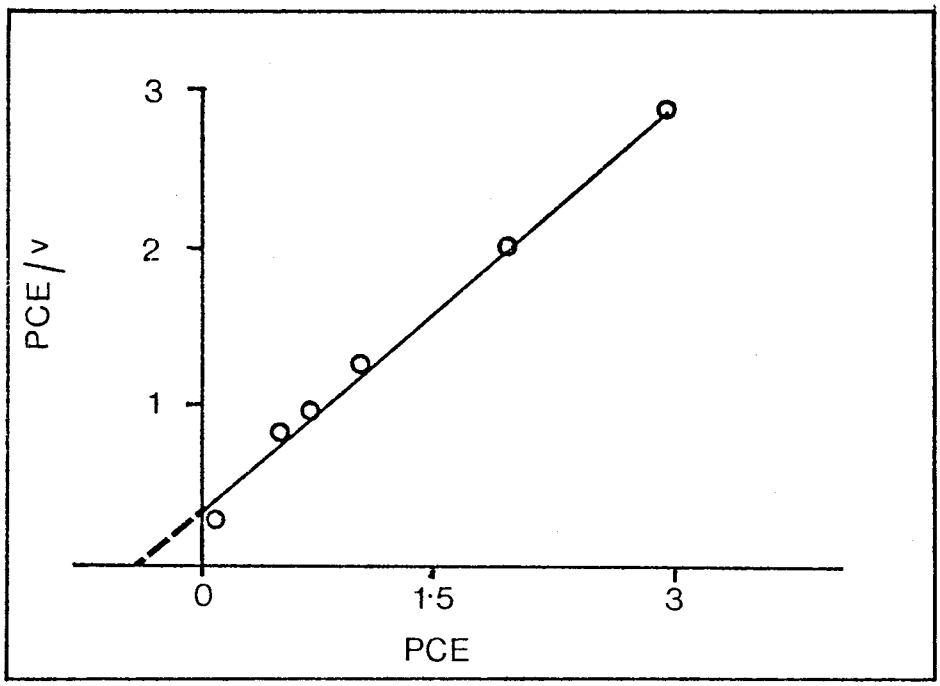

Figure 64: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene in the presence of isolated hepatocytes from an untreated rat. Tetrachloroethylene (PCE), mM; v, nmol trichloroacetate/$2x10^6$ cells/60 min. Correlation coefficient = 0.995.

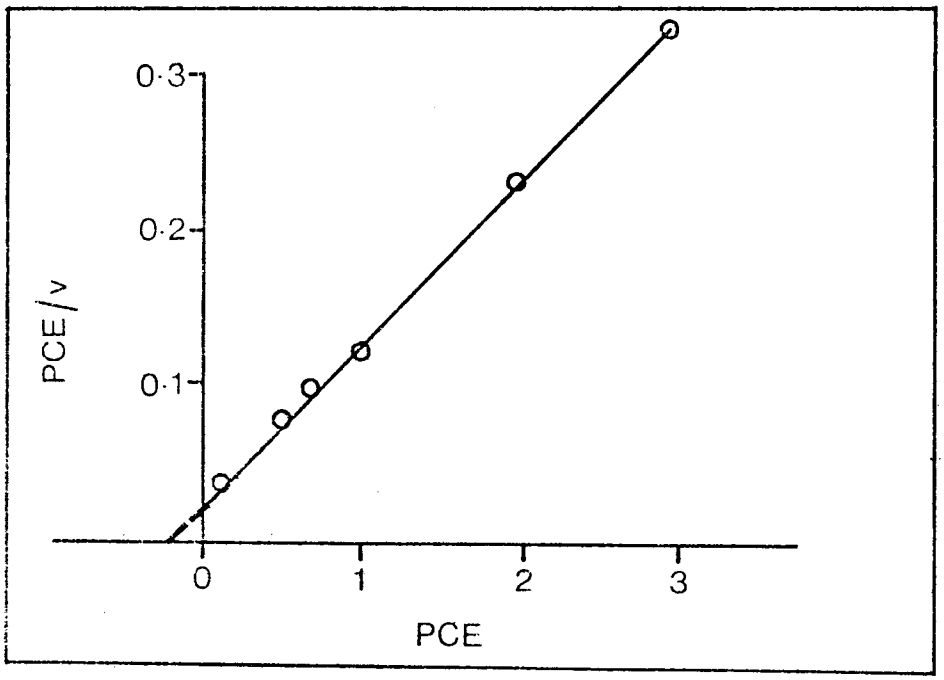

Figure 65: Hanes plot for the production of trichloroacetic acid from tetrachloroethylene in isolated hepatocytes from a phenobarbital pretreated rat. Tetrachloroethylene (PCE), mM: v, nmol trichloroacetate/$2x10^6$ cells/60 min. Correlation coefficient = 0.999.

Table 39: Effect of cis- and trans-1,2-dichloroethylene and trichloroethylene on the levels of hepatic cytochrome P-450 in freshly isolated hepatocytes* and hepatic microsomes+

Chlorinated ethylene	Cytochrome P-450 (nmol/10^6 cells)	% degradation of cytochrome P-450	
		Hepatocytes	Hepatic microsomes
None	0.81 ± 0.01	-	-
cis- and trans-1,2-Dichloroethylene	0.80 ± 0.07	1.3	42≠
Trichloroethylene	0.78 ± 0.04	3.7	20§

*Values are means ± S.D. for experiments performed in triplicate on one preparation of isolated hepatocytes from a phenobarbital pretreated rat. cis- and trans-1,2-Dichloroethylene, 4.3 mM; Trichloroethylene, 2.8 mM. Incubations were at 37°, with rotation, for 60 min. Initial levels of hepatic cytochrome P-450 were 0.82 nmol/10^6 cells.

+Hepatic microsomes from phenobarbital pretreated animals in the presence of a NADPH-generating system, cis- and trans-1,2-dichloroethylene (7.2 mM) or trichloroethylene (7.5 mM).

≠Incubations were for 15 min. Initial levels of cytochrome P-450 were 2.10±0.28 nmol/mg microsomal protein (see Table 23).

§Incubations were for 30 min. Initial levels of cytochrome P-450 were 1.87±0.11 nmol/mg microsomal protein (see Table 27).

when the mixture of cis- and trans-1,2-dichloroethylene (4.3 mM), or when trichloroethylene (2.8 mM), was added to hepatocyte preparations from phenobarbital-treated animals, no significant loss of hepatic cytochrome P-450 was seen, ($P > 0.1$) (see Table 39).

3.4.4 The fate of authentic samples of chlorinated metabolites in hepatocyte suspensions

The effect of incubation with hepatocytes from phenobarbital pretreated rats on the levels of authentic samples of chlorinated metabolites,* viz. mono-, di- and trichloroacetate, dichloroacetaldehyde and chloral hydrate, are shown in Figures 66 - 69. The levels of mono- and di-chloroacetic acid (42 μM and 3 μM, initial concentrations, respectively) decreased rapidly for ca. 10 min and then decreased at a slower rate until negligible amounts were left at 60 min (see Figure 66).

In contrast, the level of trichloroacetic acid (10 μM, initial concentration) was not affected with time (see Figure 66). The levels of dichloroacetaldehyde (1.9 μM, initial concentration) decreased rapidly for ca. 5 min and then at a slower rate until approximately 12% was left after 60 min (Figure 67). A concomittant increase in the levels of dichloroacetic acid and 2,2-dichloroethanol was seen accompanying the disappearance of dichloroacetaldehyde. The levels of dichloroacetate and dichloroethanol increased for 10 min and then decreased with time to negligible levels at 60 min (Figure 68). The levels of chloral hydrate (5 μM, initial concen-

*The concentration of each authentic sample added to isolated hepatocytes was 2-3 fold higher than the concentration used to standardize the gas-liquid chromatograph, yet still in the linear portion of the standard curve.

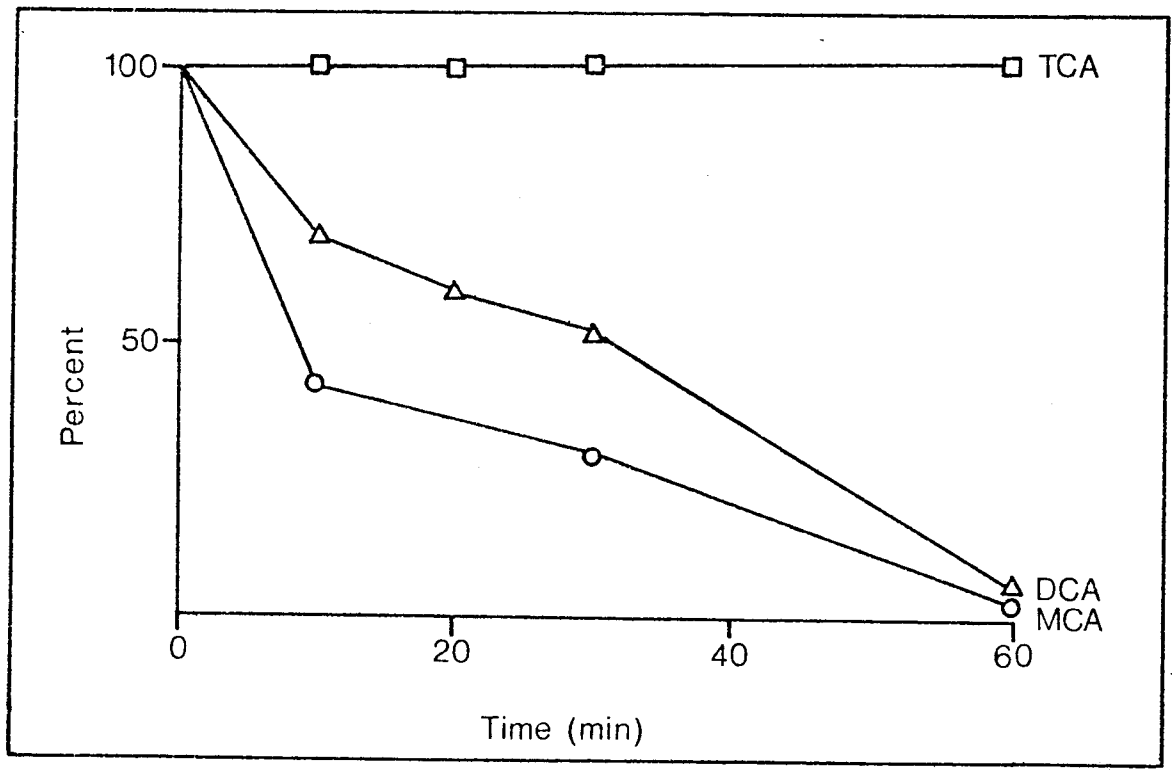

Figure 66: The effect of time on the levels of authentic samples of mono-, di- and tri-chloroacetic acid in hepatocytes isolated from a phenobarbital pretreated rat. Monochloroacetic acid (O); Dichloroacetic acid (Δ); Trichloroacetic acid (□); Time, min. Abbreviations used: MCA, monochloroacetic acid; DCA, dichloroacetic acid; TCA, trichloroacetic acid.

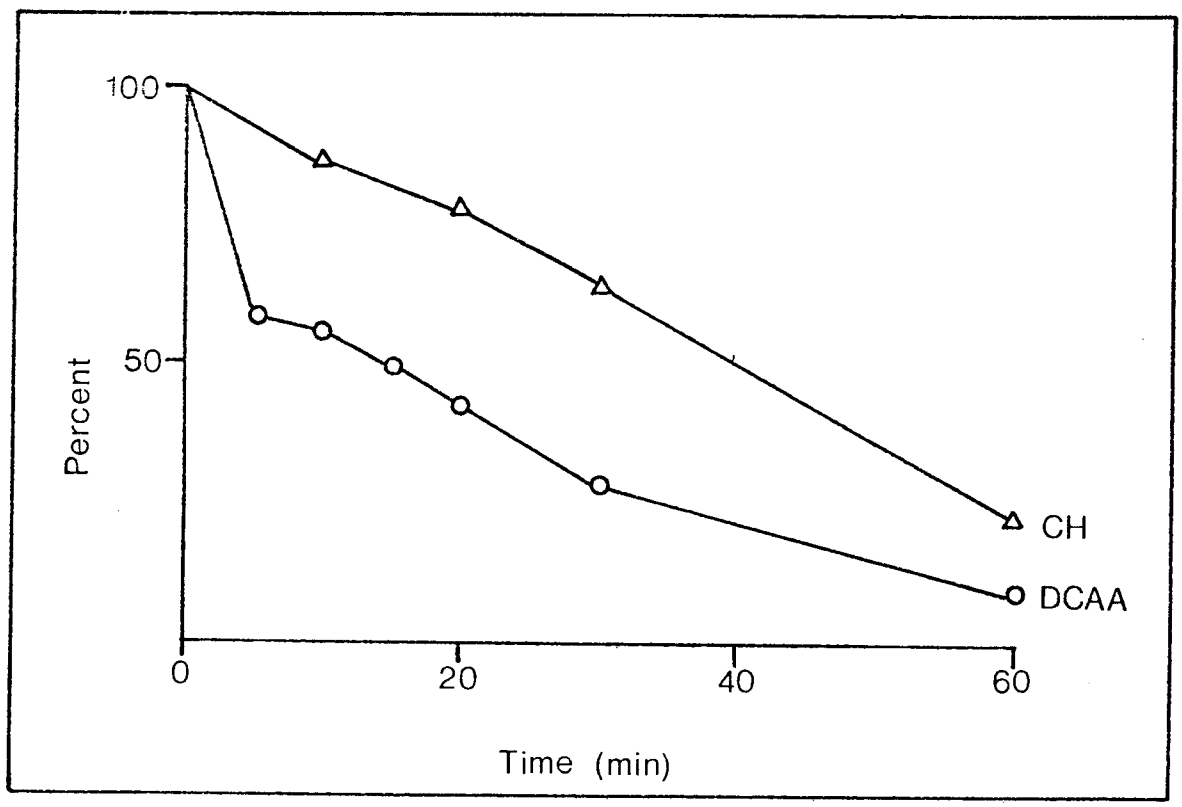

Figure 67: The effect of time on the levels of authentic samples of dichloroacetaldehyde and chloral hydrate in hepatocytes isolated from a phenobarbital pretreated rat. Dichloroacetaldehyde (O); Chloral hydrate (Δ); Time, min. Abbreviations used: DCA, dichloroacetaldehyde; CH, chloral hydrate.

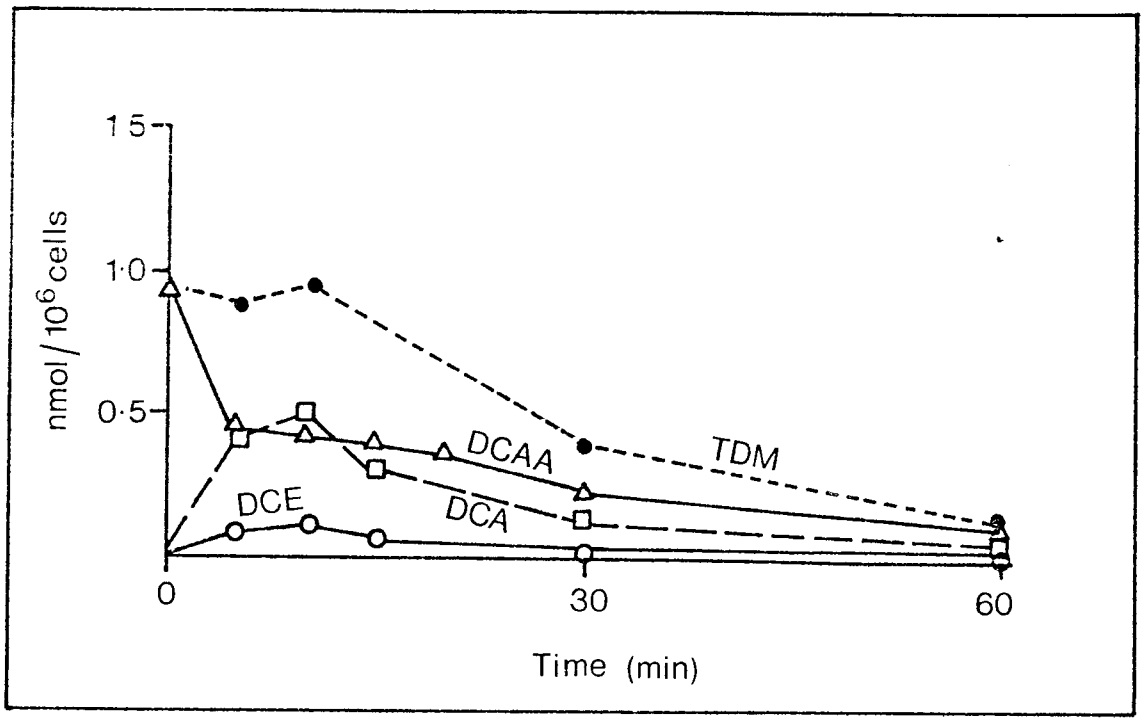

Figure 68: The conversion of dichloroacetaldehyde to 2,2-dichloroethanol and dichloroacetic acid in hepatocytes isolated from a phenobarbital pretreated rat. Dichloroacetaldehyde (Δ); 2,2-Dichloroethanol (O); Dichloroacetic acid (□); Time, min. Abbreviations used: DCE, 2,2-dichloroethanol; DCA, dichloroacetic acid; DCAA, dichloroacetaldehyde; TDM, total dichlorinated metabolites, viz. the sum of DCE, DCAA and DCA.

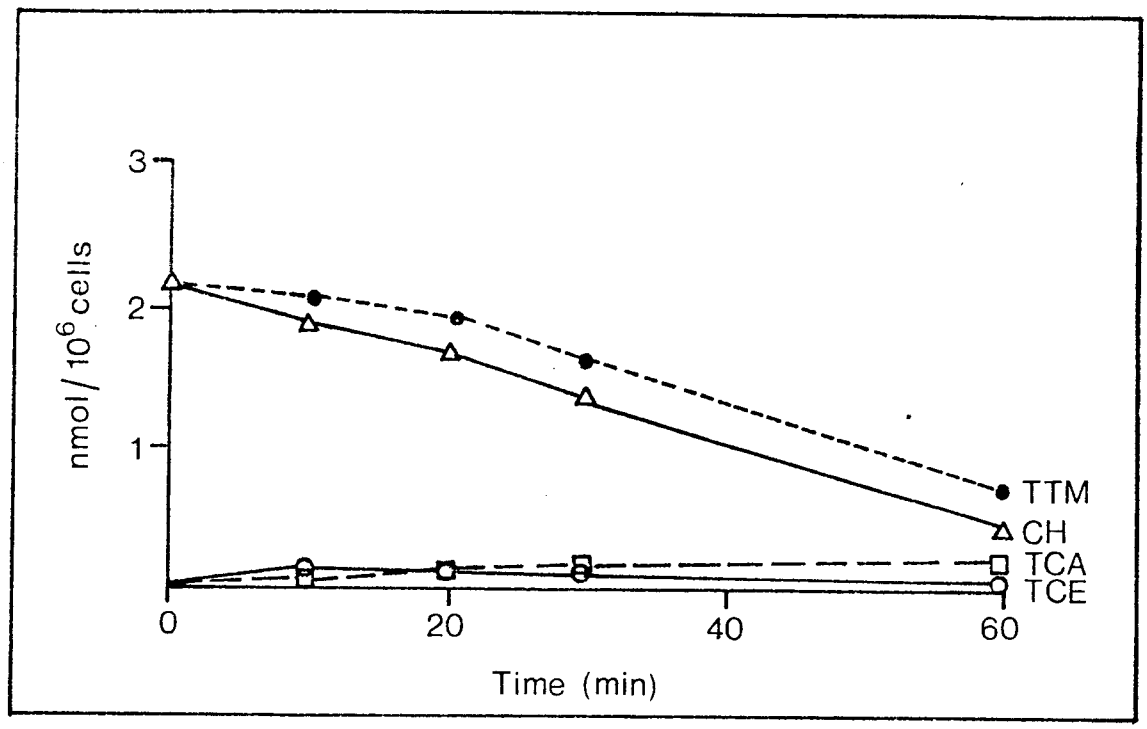

Figure 69: The conversion of chloral hydrate to 2,2,2-trichloroethanol and trichloroacetic acid in hepatocytes isolated from a phenobarbital pretreated rat. Chloral hydrate (Δ); 2,2,2-Trichloroethanol (O); Trichloroacetic acid (□); Time, min. Abbreviations used: TCE, 2,2,2-trichloroethanol; TCA, trichloroacetic acid; CH, chloral hydrate; TTM, total trichlorinated metabolites, viz. the sum of TCE, CH and TCA.

tration), decreased in an approximately linear manner with time, with 23% remaining after 60 min (Figure 67). Concomittant with this decrease in chloral hydrate was the appearance of small amounts of 2,2,2-trichloroethanol and trichloroacetic acid. Trichloroacetic acid levels increased steadily over the 60 min time interval investigated (Figure 69).

When the levels of total di- and trichlorinated metabolites were monitored, with time, it appeared that the levels of both di- and trichlorinated metabolites remain approximately constant for 10 min and then gradually decrease with time.

3.4.5 Assay for covalently bound chlorinated acetyl moieties

The treatment of hepatocyte suspensions (containing the chlorinated ethylene) from phenobarbital induced rats, which had been previously incubated at 37° for 30 min (vinylidene chloride, cis- and trans-1,2-dichloroethylene and trichloroethylene) or 60 min (tetrachloroethylene) with H_2SO_4 (20% H_2SO_4 for 6 hr at 100°) [211] resulted in no additional formation of mono-, di- or trichloroacetate from any of the chlorinated ethylenes.

3.4.6 Assay for possible glucuronide and sulfate conjugates of chlorinated metabolites

The treatment of hepatocyte suspensions (incubations performed as in 3.5.5) with β-glucuronidase (2 500 U/ml or 10 000 U/ml), (for β-glucuronides); with arylsulfatase (374 U/ml) (for sulfate esters) or with 6.25 M H_2SO_4, final concentration, (for both glucuronides and sulfate esters) resulted in no increase in the conversion of the chlorinated ethylenes to chlorinated acetic acids or chlorinated alcohols.

3.5 The ability of the chlorinated ethylenes to induce DNA repair in the presence of hepatocytes obtained from phenobarbital pretreated rats

Vinylidene chloride (2.1 mM), cis-1,2-dichloroethylene (4.3 mM), and trichloroethylene (2.8 mM) caused DNA repair in isolated rat hepatocytes while trans-1,2-dichloroethylene (4.3 mM) and tetrachloroethylene (2.5 mM) did not. This effect of these compounds in the induction of DNA repair synthesis as demonstrated by the gradient profiles is shown in Figure 70.

Benzpyrene (20 µM, final concentration), a known carcinogen, was utilized as a positive control, while DMSO (0.01%, final concentration) or ethanol (0.001%, final concentration) were used as negative controls. Repair synthesis was identified by the radioactive peak banding with parental DNA (coincident with the UV-absorbance peak). In the absence of repair synthesis, no such correlating peak was observed.

3.6 Vinyl chloride : Kinetics of the degradation of hepatic cytochrome P-450

As vinyl chloride has been extensively studied *in vivo* and *in vitro*, investigations into its metabolism have not been repeated here (see Section 1.3.1.) However, an investigation was launched as to the kinetics of the degradation of hepatic cytochrome P-450 by vinyl chloride *in vitro* and *in vivo*. Since these results do not fit in with the rest of the work done on the metabolism of the chlorinated ethylenes *in vitro*, the results obtained will be presented and discussed below.

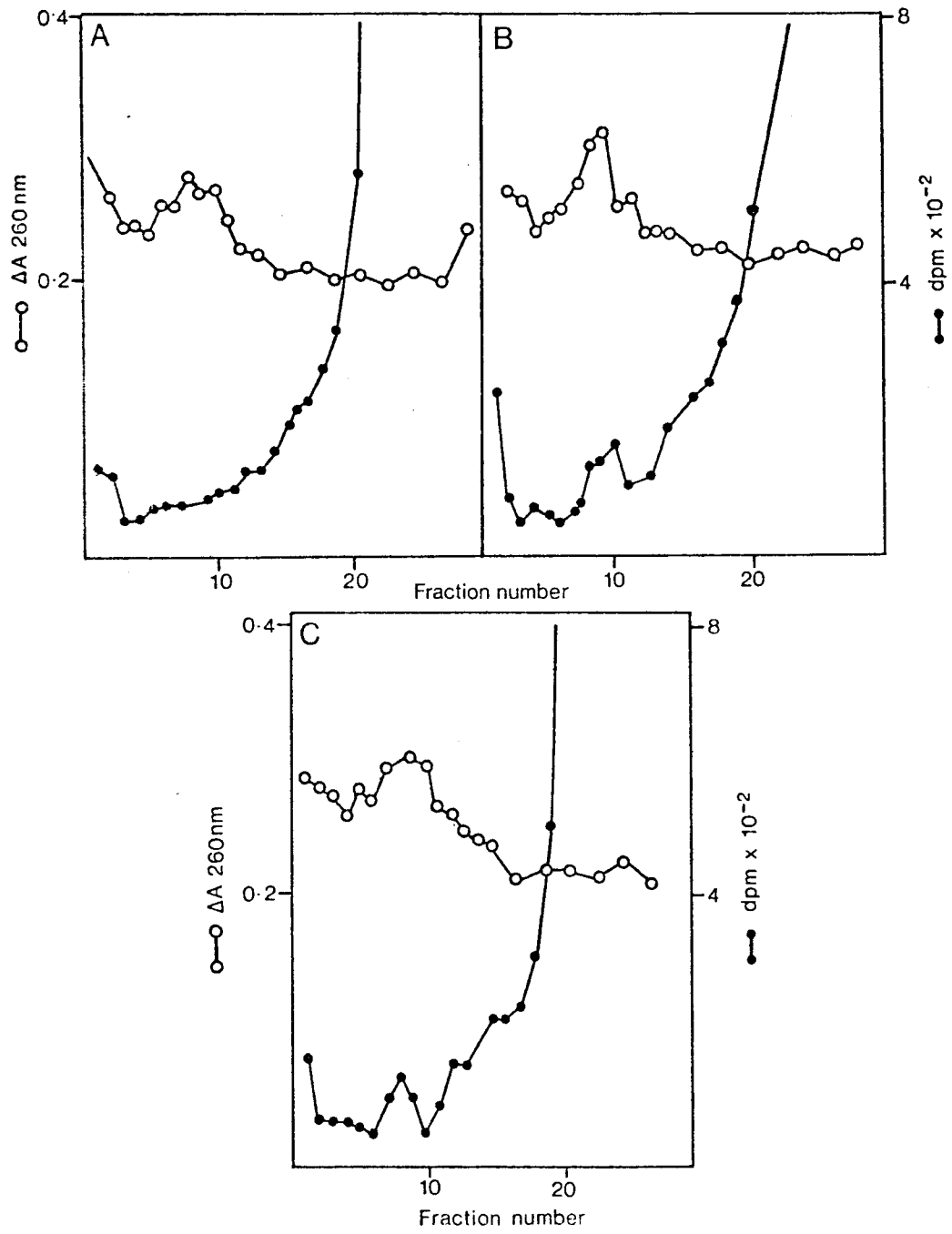

Figure 70: Induction of DNA repair synthesis in rat hepatocytes from phenobarbital treated animals, by the chlorinated ethylenes. After preincubation with FdUrd and BrUrd, cells were labelled for 2.5 hr with 10μ Ci [5-^{3}H] deoxycytidine/ml in the presence of (A) DMSO (control); (B) Benzpyrene (20 μ M); (C) Trichloroethylene (2.8 mM); (D) Tetrachloroethylene (2.5 mM); (E) trans-1,2-Dichloroethylene (4.1 mM); (F) Vinylidene chloride (2.1 mM) and (G) cis-1,2-Dichloroethylene (4.1 mM). Cells were lyzed, digested with proteinase K and centrifuged to equilibrium in alkaline CsCl as described in 2.2.9. Results are presented for one set of hepatocytes. Identical results were obtained with a second set of hepatocytes from a phenobarbital treated rat.

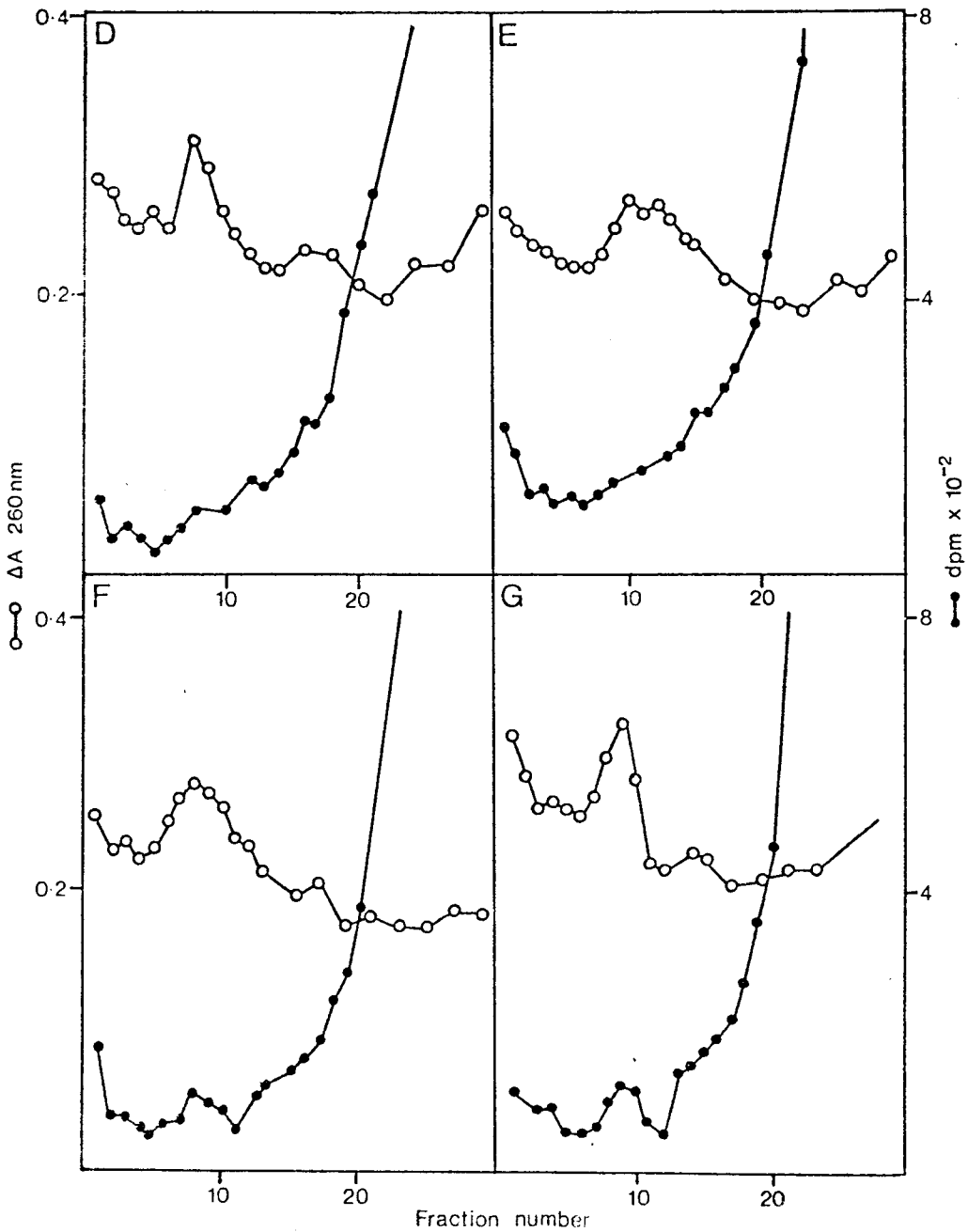

Figure 70 continued.

Following incubation for 15 min of vinyl chloride, NADPH-generating system, EDTA and hepatic microsomes from phenobarbital treated rats, significant and equivalent decreases (ca. 0.5 nmol/mg microsomal protein) in the levels of hepatic microsomal cytochrome P-450 and heme were seen (Table 40), as reported earlier (158). No significant loss of cytochrome P-450 or heme was observed if either the vinyl chloride or the NADPH-generating system was omitted from the reaction mixture (data not shown). The time course for the degradation of hepatic microsomal cytochrome P-450 in the presence of vinyl chloride is shown in Figure 71. The losses with time of hepatic microsomal heme were approximately equivalent to the losses of hepatic microsomal cytochrome P-450 shown in Figure 71; the losses of heme at 5, 10, 15 and 30 min were 0.3, 0.5, 0.8 and 1.1 nmol heme per mg microsomal protein, respectively. As found for all other xenobiotics studied, the degradation of cytochrome P-450 rarely proceeds to over 60% completion (157, 158, 283).

The effect of varying concentrations of vinyl chloride on the levels of cytochrome P-450 in incubation mixtures containing hepatic microsomes from phenobarbital treated rats, EDTA and NADPH-generating system is shown in Figure 72. Half maximal loss of cytochrome P-450 was observed at approximately 0.8 mM vinyl chloride.

The effect of vinyl chloride on multiple forms of cytochrome P-450 *in vitro* are shown in Table 40. Vinyl chloride affected the phenobarbital inducible form of the enzyme *in vitro*, as qualitatively estimated by the metyrapone complex with ferrocytochrome P-450 (280 but see 155) and by aminopyrine demethylase activity. In addition, vinyl

Table 40: Effect of vinyl chloride on the levels of cytochrome P-450 and on associated activities in hepatic microsomes from phenobarbital treated rats in vitro*

Activity measured*	% Activity after 15 min for+	
	Vinyl Chloride	Vinyl Chloride plus NADPH-gs.
Cytochrome P-450	95±5	65±7
metyrapone-Ferro Complex	105±5	64±9
Benzpyrene-3-hydroxylase	128±15≠	35±12≠
Aminopyrine demethylase	87±6	67±4
p-Nitroanisole demethylase	100±5	46±10≠
Ethoxyresofurin deethylase	85±10	52±3 ≠

*Incubation mixtures contained 3 ml of hepatic microsomes (2 mg protein/ml) in 0.02 M Tris HCl, pH 7.4; vinyl chloride (30 mM); EDTA (0.2 mM) and NADPH-generating system (305). Percentage cytochrome P-450 content was relative to samples comprised as above but not incubated. Initial levels of cytochrome P-450 were 2.8 ± 0.2 nmol/mg microsomal protein. Initial activities in nmol/mg microsomal protein/min were: benzpyrene-3-hydroxylase, 0.3; aminopyrine demethylase, 9.0; p-nitroanisole demethylase, 5.0; ethoxyresofurin deethylase, 0.2.

+Hepatic microsomes, EDTA and additions as shown. % Activity is reported relative to incubation mixtures containing hepatic microsomes, NADPH-generating system and EDTA.

≠Differs significantly from value for cytochrome P-450, $P < 0.01$.

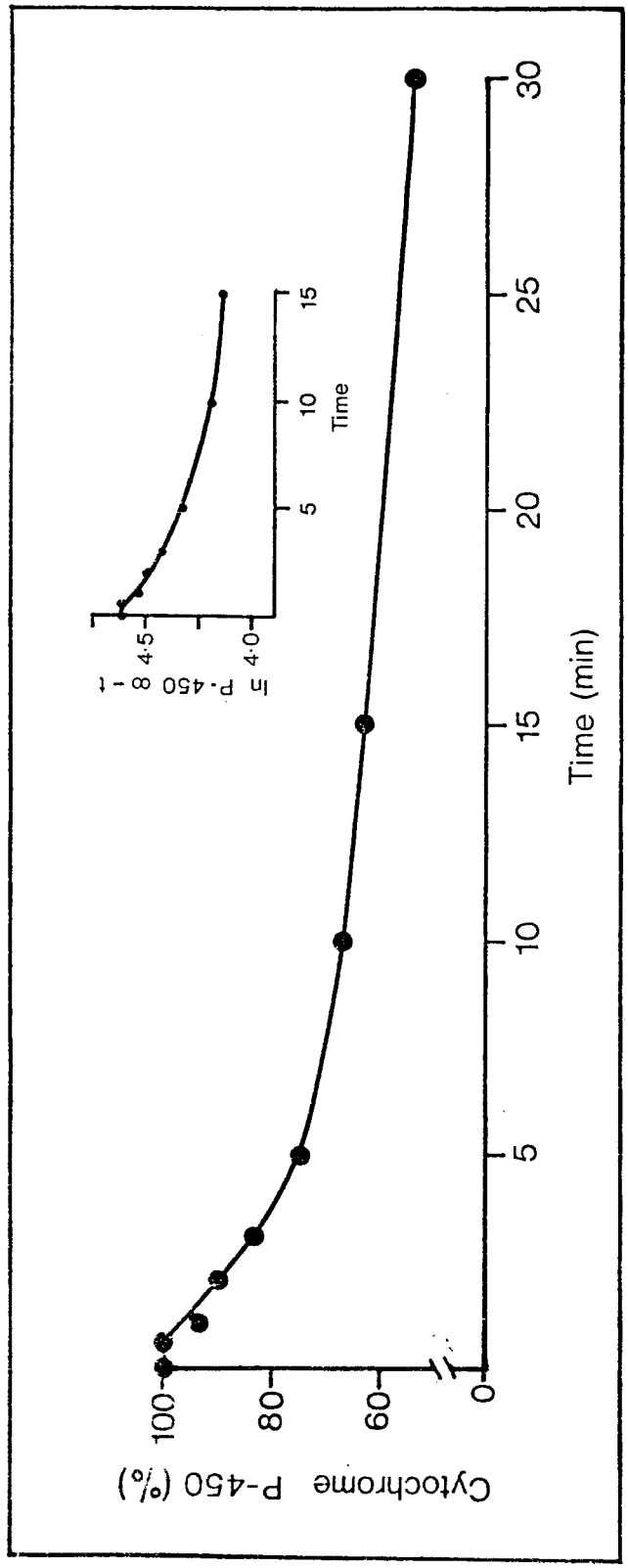

Figure 71: Effect of reaction time on the vinyl chloride mediated loss of cytochrome P-450 in hepatic microsomes from phenobarbital treated rats in vitro. Experimental conditions are as in Table 40.

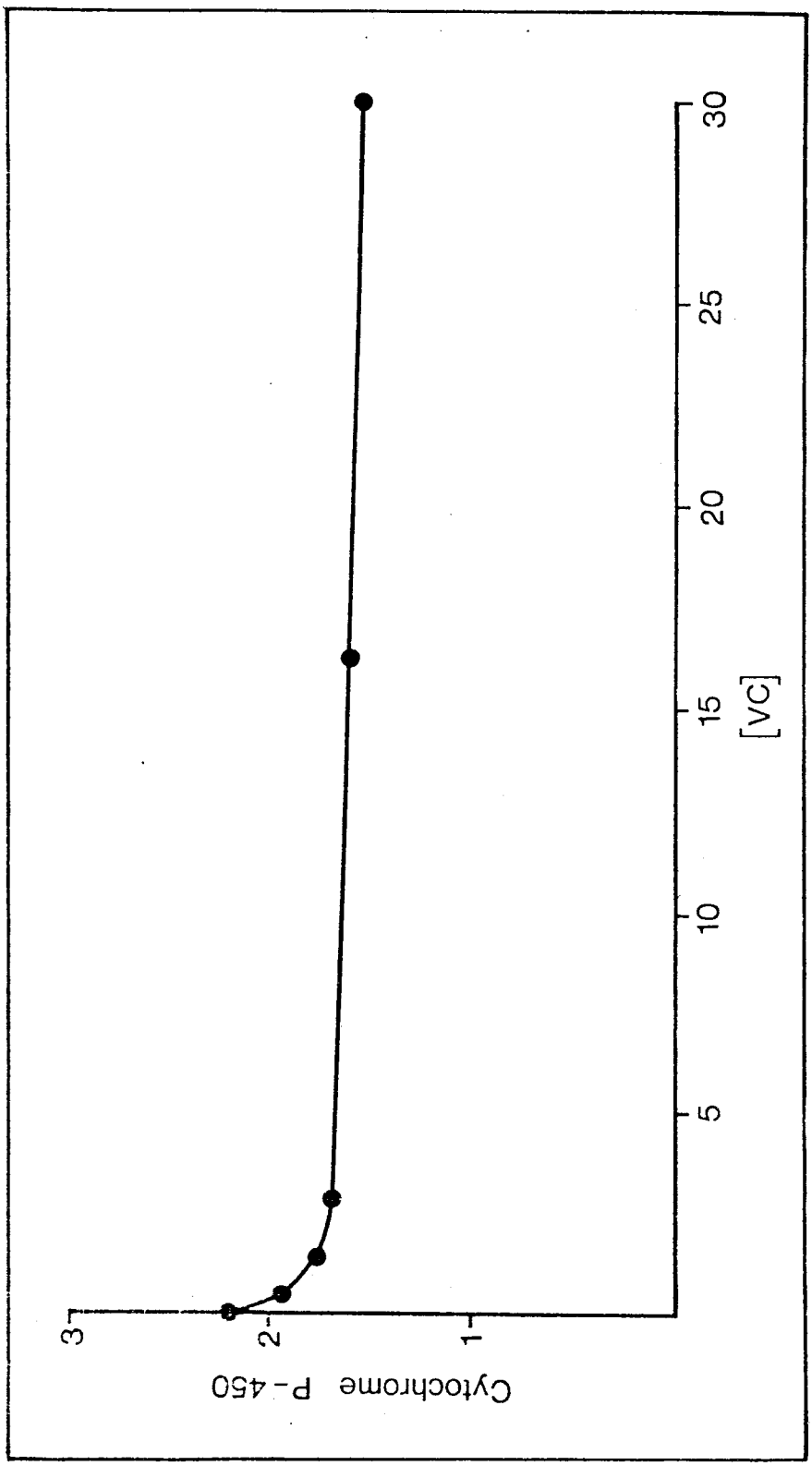

Figure 72: Effects of varying concentrations of vinyl chloride on the levels of hepatic cytochrome P-450 in microsomes from phenobarbital treated rats. Cytochrome P-450 in nmol/mg microsomal protein.

chloride decreased the activities of benzpyrene 3-hydroxylase and ethoxyresofurin deethylase which are associated with polycyclic hydrocarbon inducible forms of the enzyme. Thus, multiple forms of cytochrome P-450 appear to be affected by vinyl chloride in microsomes from phenobarbital treated rats in vitro, in particular the forms of the enzyme elevated by polycyclic hydrocarbons and by phenobarbital itself.

Table 41: Effect of vinyl chloride on the levels of cytochrome P-450 and on associated activities in hepatic microsomes from phenobarbital treated rats in vivo*

Activity measured	% Activity after 6 hr exposure of vinyl chloride at+			
	3000 ppm	20000 ppm		
		Day 1	Day 2	Day 3
Cytochrome P-450	46	81	72	57
metyrapone-Ferro Complex	43	88	69	56
Benzpyrene-3-hydroxylase	ND	88	88	ND
Aminopyrine demethylase	90 ≠	89	94 ≠	84 ≠
p-Nitroanisole demethylase	52	81	67	61
Ethoxyresofurin deethylase	47	ND	ND	ND

*Experiments were performed on hepatic microsomes (2 mg protein/ml) obtained from phenobarbital pretreated rats which had been exposed for 6 hr to 3000 or 20000 ppm vinyl chloride (see Methods 2.2.1) and oxygen. Control animals (phenobarbital pretreated) were exposed to oxygen alone. Initial levels of cytochrome P-450 were 3.18±0.79 nmol/mg microsomal protein. Initial activities are as in Table 40. Loss of Heme/loss of cytochrome P-450 in nmol/mg microsomal protein were 1.77/2.16, 1.00/0.77 and 1.44/1.57 for 3000 ppm, Day 2 and Day 3 at 20000 ppm, respectively.

+% Activity is reported relative to results obtained with animals exposed to oxygen alone.

≠Differs significantly from the value obtained for cytochrome P-450, $P < 0.01$.

The effects of vinyl chloride on multiple forms of cytochrome P-450 *in vivo* are shown in Table 41. Vinyl chloride (3 000 ppm or 20 000 ppm) affected the different forms of cytochrome P-450 to similar extents, with the exception of aminopyrine demethylase, on which it seemed to have little, if any, significant effect. It would therefore appear that, as *in vitro*, the reactive species, generated from the hepatic metabolism of vinyl chloride by cytochrome P-450, are capable of reacting with multiple forms of cytochrome P-450.

The effect of the metabolism of vinyl chloride on the activities of different forms of cytochrome P-450 provides one possible explanation for the observation that a dose of vinyl chloride provides protection against the carcinogenicity of a second dose [197]; e.g. by decreasing the levels of the carcinogen activating enzyme cytochrome P-450.

4

DISCUSSION

That hepatic cytochrome P-450 is involved in the metabolism of the chlorinated ethylenes is shown by the results presented herein. For all of the chloroethylenes studied, these results confirm earlier reports which implicated the involvement of hepatic cytochrome P-450 in their metabolism, viz. cis-1,2- and trans-1,2-dichloroethylene [230], vinylidene chloride [230], trichloroethylene [238,240,242], and tetrachloroethylene [230].

That cytochrome P-450 catalyzes the first step in the metabolism of all the chlorinated ethylenes is supported by the following evidence*:

1. Each of the chlorinated ethylenes bound to the active site of the enzyme as evidenced by the production of a Type I difference spectrum (Section 3.1) and by the stimulation of CO-inhibitable NADPH oxidation (Section 3.2) in hepatic microsomes.
2. Chlorinated metabolites were produced during the aerobic incubation of hepatic microsomes, NADPH-generating system, EDTA and the chlorinated ethylene; with the omission of any component eliminating metabolite production (Section 3.3).
3. The inhibitors of cytochrome P-450- CO, metyrapone and/or SKF-525A [286] inhibited the production of chlorinated metabolites in the presence of hepatic microsomes and NADPH-generating system (Section 3.3). Furthermore, NADH supported the metabolism of vinylidene chloride and trichloroethylene at 13 - 15% of the rate at which NADPH did (Section 3.3.1c and section 3.3.3b).

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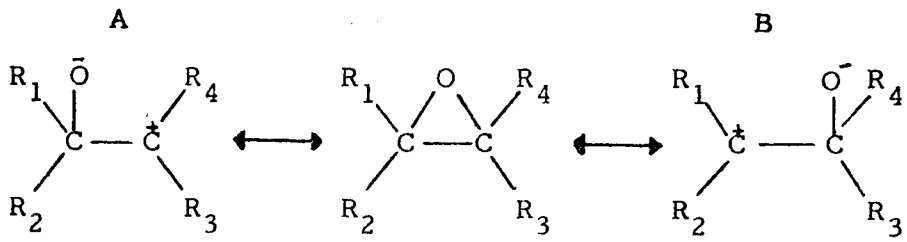
*for all but vinyl chloride.

With the aid of inducing agents, such as β-naphthoflavone and phenobarbital, to elevate the levels of specific forms of hepatic cytochrome P-450, the role that the different forms of the enzyme play in the binding and metabolism of the chlorinated ethylenes in hepatic microsomes, was investigated.

The first step in the metabolism of all the chlorinated ethylenes involves the proposed formation of an epoxide; of which the observed reactivity and breakdown products differ depending on the degree and position of chlorination. It has been proposed that on the active site of hepatic cytochrome P-450, the epoxides can exist as Zwitterionic analogues*, viz. A or B.



However, for the purpose of this thesis, I will refer to these reactive intermediates as epoxides, which is consistent with most other proposals for the metabolic pathways for the chlorinated ethylenes.

It is anticipated that the chlorinated epoxides, once generated, can undergo a variety of reactions [206,288,289]. The high degree of strain of the epoxides renders them short-lived intermediates, which will rapidly undergo either internal rearrangements or

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*These species were proposed by Ortiz de Montellano et al. in order to explain the structures of the adducts of some xenobiotics with the heme of cytochrome P-450[287].

reactions with cellular constituents, e.g.

1. intramolecular rearrangement via a chloride or hydride shift to yield acyl chlorides and aldehydes;
2. reaction with nucleophilic cellular constituents. e.g. macromolecules such as cellular protein, RNA, DNA or low molecular species such as glutathione;
3. hydrolysis to diols, with and without the catalytic activity of epoxide hydrase.

Intramolecular rearrangement to chlorinated acyl chlorides or acetaldehydes is regarded as a very important deactivation mechanism [289], since the proposed epoxides are held responsible for the deleterious effects of the chlorinated ethylenes (e.g. their hepatotoxicity, mutagenicity and carcinogenicity), by many workers.

4.1 Vinylidene chloride

The form of cytochrome P-450 elevated by β-naphthoflavone, viz. cytochrome P-450c does not appear to bind or metabolize vinylidene chloride since β-naphthoflavone induction did not affect the values of K_s and did not alter or decreased ΔA_{max} and ΔA_{max} per nmol cytochrome P-450 for the binding of vinylidene chloride. In addition, the rates of conversion of vinylidene chloride to chloroacetate and dichloroacetaldehyde per mg microsomal protein and per nmol cytochrome P-450 were not affected following β-naphthoflavone induction (Tables 9, 17 and 18).

The phenobarbital inducible form of cytochrome P-450, (cytochrome P-450b), appears to play a minor role in the binding and metabolism of vinylidene chloride. Phenobarbital induction significantly increased ΔA_{max} and the rate of monochloroacetate

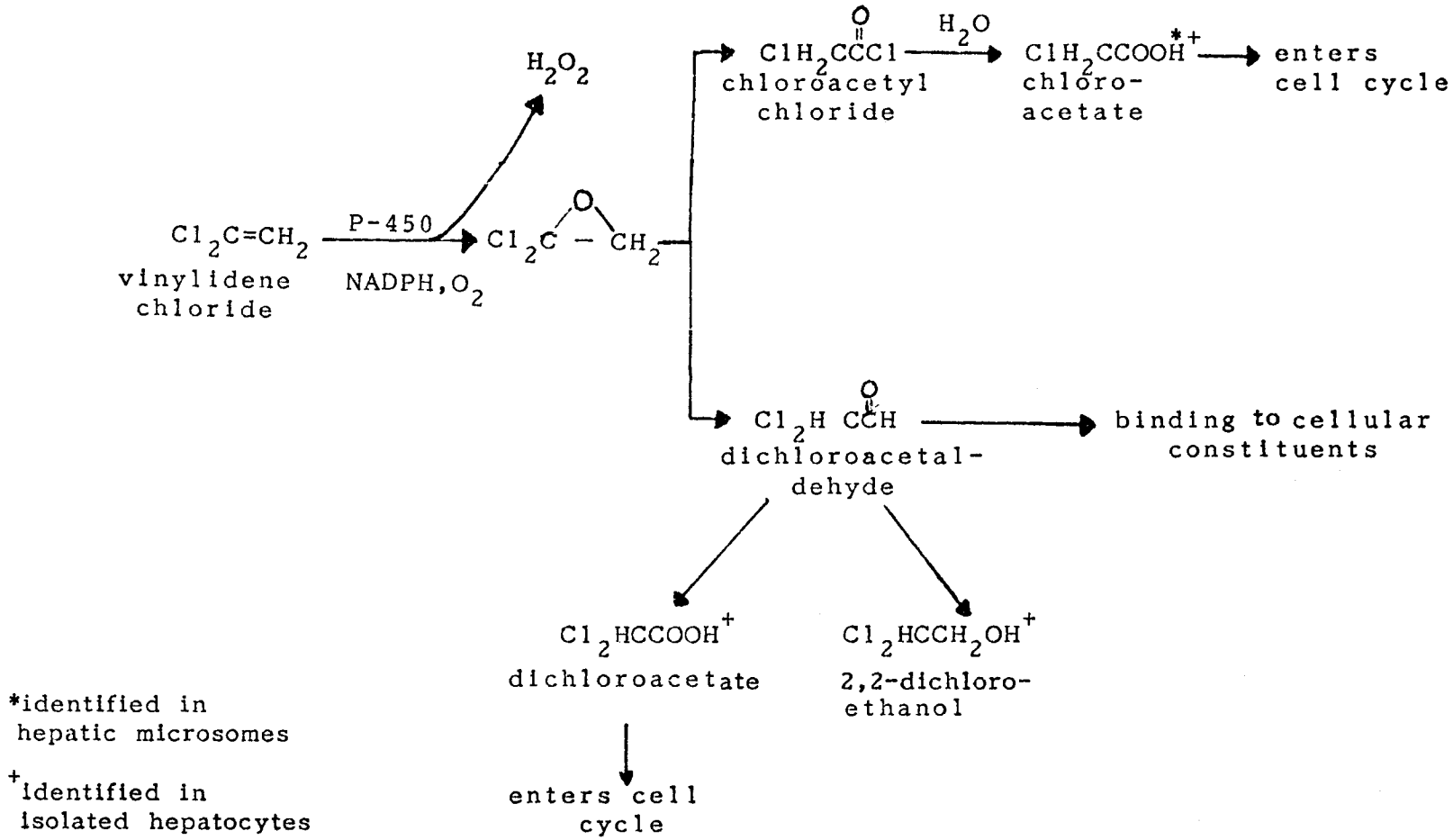
production per mg microsomal protein, but did not affect or decreased the values of these parameters per nmol cytochrome P-450 (Tables 9 and 17). The conversion of vinylidene chloride to dichloroacetaldehyde does not appear to be catalyzed by the phenobarbital inducible form of cytochrome P-450 since phenobarbital induction did not affect the rate of dichloroacetaldehyde production per mg microsomal protein and decreased its rate of production per nmol cytochrome P-450 by 2 to 3 fold (Table 18).

It is proposed that one or more of the forms of cytochrome P-450 present in microsomes from untreated rats may be more efficient in the metabolism of vinylidene chloride to chloroacetate and particularly to dichloroacetaldehyde than is the phenobarbital inducible form of the enzyme (See Section 3.3.1).

The proposed metabolic pathways for vinylidene chloride in vitro can be seen in Figure 73. The pathways shown represent a modification of the earlier proposed pathways (e.g. Leibman and Ortiz[230]), on the basis of the results presented herein of our investigations into the metabolism of vinylidene chloride by hepatic microsomes and hepatocytes (compare to Figure 16 in Section 1.3.2).

The first step in the conversion of vinylidene chloride to its chlorinated metabolites is catalyzed by cytochrome P-450, presumably via the formation of an epoxide (or analogue thereof). In the case of 1,1-dichloroethyleneoxide, since both electron withdrawing chlorines are attached to one carbon, the resulting epoxide is highly unstable and thus rearranges rapidly [222] (Figure 73). In hepatic microsomes, the rearrangement of the epoxide via a chlorine shift to the chloroacetyl chloride or via a hydride shift to dichloroacetaldehyde appear to occur at comparable rates (i.e.

Figure 73: Metabolic pathway for vinylidene chloride as modified from previously postulated pathways[221, 230]



V_{max} for dichloroacetaldehyde formation = 0.028 nmol/nmol cytochrome P-450/min (Table 18) and V_{max} for monochloroacetate formation = 0.034 nmol/nmol cytochrome P-450/min (Table 17).) This is in contrast to what is expected and to the results found with the epoxides of the other chlorinated ethylenes where a chlorine shift is favoured over a hydride shift.

The results reported herein have provided the first unequivocal identification of dichloroacetaldehyde as a metabolite of vinylidene chloride *in vitro* or *in vivo**. The identification of dichloroacetaldehyde as a metabolite of vinylidene chloride from the hepatic microsomal cytochrome P-450 enzyme system is fully consistent with the proposed pathways for the metabolism of vinylidene chloride *in vivo* (see Figures 16 and 73). It is anticipated that dichloroacetaldehyde may undergo a series of reactions, such as Schiff base formation with protein side chains, or oxidative or reductive reactions as seen with hepatocytes. Dichloroacetic acid and traces of 2,2-dichloroethanol; reaction products presumably of the interaction of aldehyde dehydrogenase and alcohol dehydrogenase, respectively, with dichloroacetaldehyde, have been found following incubation of vinylidene chloride in hepatocyte suspensions (Figure 68). That dichloroacetaldehyde undergoes further reaction is supported by the very small amounts of dichloroacetaldehyde observed in hepatocyte suspensions following incubation with vinylidene chloride, as well as by the very short time period for which dichloroacetaldehyde

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*The unidentified volatile halogenated metabolite of vinylidene chloride reported by Leibman and Ortiz to be produced by post-mitochondrial supernatant and NADPH-generating system *in vitro* [230] would be expected to be dichloroacetaldehyde.

production was linear with microsomes from phenobarbital treated rats *in vitro*, viz. 3 min, relative to the 30 min time period over which chloroacetate production was linear (Section 3.3.1a). An ability of dichloroacetaldehyde to undergo side reactions might in part explain why this compound has not been identified as a metabolite of vinylidene chloride *in vivo*.

In hepatocyte suspensions, besides the observed dichlorinated metabolites, small amounts of monochloroacetate were found following the addition of vinylidene chloride (see 3.5.2a). The low levels of monochloroacetate detected 0.068 nmol/10^6 cells/60 min) is consistent with the finding that monochloroacetate disappears from incubation mixtures with time in the presence of viable hepatocyte suspensions (Section 3.4.2a) (Figure 66). No concomitant appearance of chloroacetaldehyde (limit of detection < 0.4 nmol/10^6 cells/30 min) or 2-chloroethanol (limit of detection <12.2 nmol/10^6 cells/30 min) was seen, while losses of chloroacetate were 37 nmol per 10^6 cells per 60 min. Furthermore, no bound monochloroacetyl moeities were detected following acid hydrolysis (20% H_2SO_4 at 100° for 6 hr). These findings together with the finding that the metabolism of vinylidene chloride by hepatic cytochrome P-450 did not result in decreased levels of microsomal cytochrome P-450 or heme *in vitro** (Table 20) suggest that the severe hepatotoxicity found following exposure to vinylidene chloride may be as a result of chloroacetate being metabolically converted to chlorocitrate which, like fluorocitrate (from fluoroacetate) [290,298], acts as an inhibitor of aconitase.

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*This observation is consistent with the findings of Reynolds *et al.* [217] that the endoplasmic reticulum is spared following exposure of rats to vinylidene chloride.

The proposed interference of chloroacetate with the citric acid cycle which was first suggested by Jaeger in 1977 [291], could explain the high toxicity of vinylidene chloride relative to the other chlorinated ethylenes, none of which give rise to monochloroacetate* [237,242].

Another possible cause for the deleterious effects of vinylidene chloride *in vivo* could be the ability of vinylidene chloride to partially uncouple the cytochrome P-450 enzyme system. This is supported by the observation that the rates of vinylidene chloride stimulated CO-inhibitable NADPH oxidation greatly exceeded the rates of metabolite production.

In addition, vinylidene chloride stimulated the rate of hepatic microsomal H_2O_2 production (Tables 13,17 and 18). The H_2O_2 produced, if not broken down, e.g. by catalase, could give rise to lipid peroxidation and consequent cellular damage [292].

Phenobarbital pretreatment which decreases the toxicity of vinylidene chloride *in vivo*, would be expected to enhance the metabolism of vinylidene chloride *in vivo* as a consequence of its ability to enhance the proliferation of the endoplasmic reticulum.** This situation is consistent with the proposal of Reynolds *et al.* [217]

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*Dichloroacetate, like difluoroacetate, can also enter the cell cycle but is not toxic.[294]

**Phenobarbital pretreatment, which slightly increased per mg microsomal protein the cytochrome P-450 mediated conversion of vinylidene chloride to monochloroacetate but not to dichloroacetaldehyde *in vitro* (see Tables 17 and 18), would be expected to increase the rate of conversion of vinylidene chloride to both of these metabolites *in vivo* as a consequence of the ability of phenobarbital to increase the proliferation of the endoplasmic reticulum *in vivo* [131].

that the toxicity of vinylidene chloride is a property of the parent compound and not of a metabolite thereof. However, it is also possible that it is a metabolite of vinylidene chloride which mediates its toxicity, whether that be e.g. a chlorinated intermediate or H_2O_2, provided that the detoxification of reactive metabolites of vinylidene chloride is enhanced by phenobarbital to a greater extent than is the metabolic activation of vinylidene chloride.

4.2 cis- and trans-1,2-Dichloroethylene

It would appear that cis- and trans-1,2-dichloroethylene are bound and metabolized by several forms of hepatic microsomal cytochrome P-450, including the forms induced by β-naphthoflavone and phenobarbital and those found in the liver of untreated rats but not elevated by either of the above agents. Cytochrome P-450c, which is induced by β-naphthoflavone, appears to play a slight, but significant, role in the binding and metabolism of cis- and trans-1,2-dichloroethylene. Although following β-naphthoflavone treatment the values of ΔA_{max} and ΔA_{max} per nmol cytochrome P-450 were generally decreased for the mixture of isomers and for trans-1,2-dichloroethylene, the value of ΔA_{max} for the low affinity site for the binding of trans-1,2-dichloroethylene was slightly increased (Table 10). Furthermore, although β-naphthoflavone treatment did not significantly affect the rates of hepatic microsomal CO-inhibitable NADPH oxidation in the presence of the mixture of isomers or of trans-1,2-dichloroethylene or affect V_{max} per nmol cytochrome P-450 for the conversion of cis- or trans-1,2-dichloroethylene to dichloroacetaldehyde, it did significantly increase the

maximum rates of dichloroacetaldehyde production per mg of microsomal protein from both isomers (Tables 14 and 21).

The form of cytochrome P-450 elevated by phenobarbital, cytochrome P-450b, appears to play a significant role in the binding and metabolism of cis- and trans-1,2-dichloroethylene: Phenobarbital pretreatment significantly increased ΔA_{max} and the rate of NADPH oxidation per mg microsomal protein for both the mixture of isomers and for trans-1,2-dichloroethylene; and the values of these parameters were comparable to those for cis-1,2-dichloroethylene (Tables 10 and 14). Furthermore, phenobarbital pretreatment increased the values of V_{max} per mg microsomal protein for the conversion of cis- and trans-1,2-dichloroethylene to dichloroacetaldehyde (Table 21). Metyrapone, which preferentially interacts with the phenobarbital inducible form of cytochrome P-450 (293,280 but see 155) under the conditions of our experiment, significantly inhibited the conversion of cis- and particularly trans-1,2-dichloroethylene to chlorinated metabolites (Table 22).

Finally, one or more of the multiple forms of cytochrome P-450 found in liver microsomes from untreated rats, but not elevated by β-naphthoflavone or phenobarbital, appears to bind and metabolize the 1,2-dichloroethylenes since per nmol of cytochrome P-450 the values of ΔA_{max} and V_{max} for the 1,2-dichloroethylenes were generally not elevated by either inducing agent relative to the values obtained with microsomes from untreated rats (Tables 10 and 21).

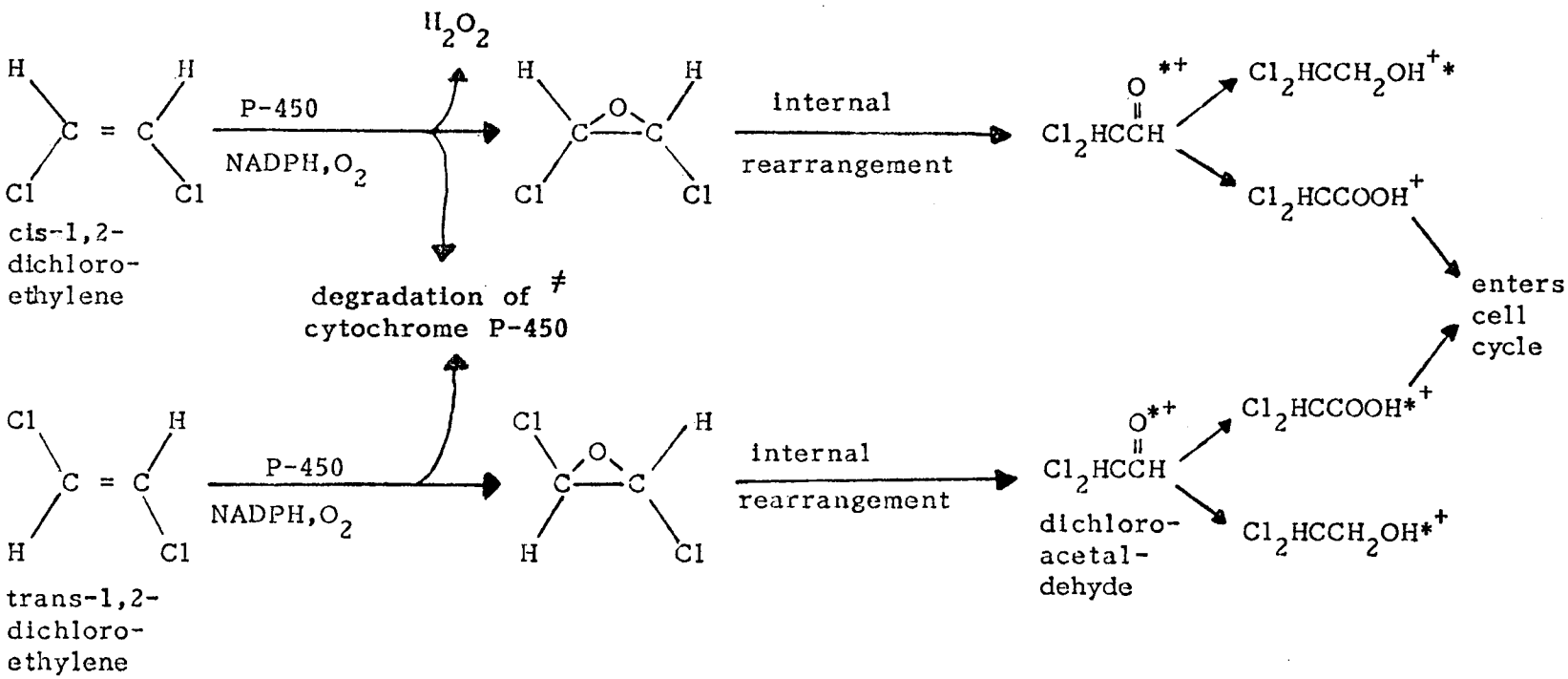
The reaction scheme for the metabolism of cis- and trans-1,2-dichloroethylene <u>in vitro</u> can be seen in Figure 74. It represents the metabolic pathway postulated by Leibman and Ortiz[230] (see Figure 17).

which has been modified to accommodate the results presented herein.

The first step in the metabolism of the 1,2-dichloroethylenes is catalyzed by cytochrome P-450 and proceeds presumably via the formation of an epoxide, e.g. 1,2-dichloroethyleneoxide (Figure 74). Since the 1,2-dichloroethylenes are symmetrically substituted the resulting expoxide is relatively more stable than 1,1-dichloroethyleneoxide [221]. Since the dichlorinated derivatives are the major products of the metabolism of the 1,2-dichloroethylenes in hepatic microsomes and hepatocytes, it is proposed that the non-enzymic rearrangement of the dichlorinated epoxide produced by the cytochrome P-450 enzyme system favours a chlorine shift to yield dichloroacetaldehyde rather than a hydride shift to yield chloroacetyl chloride. The identification of dichloroacetaldehyde as a metabolite of cis- and trans-1,2-dichloroethylene from hepatic microsomal cytochrome P-450 provides the first confirmation in any _in vitro_ or _in vivo_ system that this proposed metabolite is produced from the 1,2-dichloroethylenes (Section 3.3.2). The presence of dichloroacetaldehyde from cis-1,2-dichloroethylene and traces thereof from trans-1,2-dichloroethylene, in isolated hepatocytes, confirms the results found in hepatic microsomes (Section 3.4.2a). It is anticipated that dichloroacetaldehyde is the unidentified metabolite reported by Leibman and Ortiz [230] to be produced from cis- and trans-1,2-dichloroethylene by post-mitochondrial supernatant plus NADPH.

Hanes plots of the production of dichloroacetaldehyde from cis-1,2-dichloroethylene in hepatocytes from phenobarbital treated rats are monophasic and not biphasic as found in hepatic microsomes

Figure 74: Metabolic pathway for cis- and trans-1,2-dichloroethylene as modified from postulated pathways (e.g. 230)



*identified in hepatic microsomes

+isolated in isolated hepatocytes

≠degradation of cytochrome P-450 not seen in hepatocytes

(from untreated and variously pretreated rats). If the production of dichloroacetaldehyde does occur at two different rates in hepatocytes, it is possibly masked by the action of the dehydrogenases present in the cytosol of the liver cell which further convert dichloroacetaldehyde to dichloroacetic acid and 2,2-dichloroethanol. It would be expected that the masking effect occurs with the relatively slow rate of dichloroacetaldehyde production from cis-1,2-dichloroethylene in hepatic microsomes (i.e. rate of production of dichloroacetaldehyde with a low V_{max} and corresponding high K_m value – Table 21). In this case, the dichloroacetaldehyde as it is produced would be immediately attacked by the dehydrogenases. This is as was seen in isolated hepatocyte suspensions from phenobarbital pretreated animals, where the conversion of cis-1,2-dichloroethylene to dichloroacetaldehyde exhibited a K_m value (2.15 mM) that was identical to the low affinity K_m value obtained in hepatic microsomes (Tables 21 and 36).

Dichloroacetaldehyde (generated from the metabolism of the 1,2-dichloroethylenes) is converted to 2,2-dichloroethanol and/or dichloroacetic acid in hepatic microsomes (see 3.3.2), hepatocytes (3.4.2a) and in perfused rat liver [211]. The observation is consistent with the finding that in the presence of hepatocytes (from a phenobarbital pretreated rat) the levels of an authentic sample of dichloroacetaldehyde decreased as a function of time, with a concomitant increase in the levels of dichloroacetic acid and 2,2-dichloroethanol (Figure 68) (Section 3.4.4). The metabolism of dichloroacetaldehyde could possibly be catalyzed by the alcohol dehydrogenase and aldehyde dehydrogenase enzymes present in the cytosol. An analogous reaction also occurs in hepatic microsomes

where dichloroacetaldehyde is also reduced to 2,2-dichloroethanol, which is presumably catalyzed by an alcohol dehydrogenase contaminant of the microsomal preparation.*

The apparent discrepancies in the metabolic patterns of cis- and trans-1,2-dichloroethylene in isolated hepatocytes appear to be explainable on the basis of the observed apparent K_m** and V_{max} values for the conversion of dichloroacetaldehyde to 2,2-dichloroethanol and dichloroacetic acid in hepatocytes. When relatively large amounts of dichloroacetaldehyde were generated (i.e. following cis-1,2-dichloroethylene metabolism) the main metabolite observed was 2,2-dichloroethanol. This is consistent with the finding that, despite the higher catalytic activity of aldehyde dehydrogenase for dichloroacetaldehyde (lower K_m value, Table 36), the V_{max} value observed for the conversion of dichloroacetaldehyde to 2,2-dichloroethanol was 10-fold higher than that for the conversion of dichloroacetaldehyde to dichloroacetic acid (Table 36). However, when dichloroacetaldehyde is produced in very low quantities, such as from trans-1,2-dichloroethylene, the dehydrogenase with the lower apparent K_m (i.e. higher catalytic activity) viz. aldehyde dehydrogenase, will take precedence in the conversion of dichloroacetaldehyde to less toxic species.

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*A similar conclusion has been drawn from the observation that chloroacetaldehyde is reduced to 2-chloroethanol in the presence of hepatic microsomes and NADPH [8].

**The terminology "apparent K_m" is necessary as dichloroacetic acid and 2,2-dichloroethanol are secondary metabolites of cis-1,2-dichloroethylene.

It would appear that in hepatic microsomes cis- and trans-1,2-dichloroethylene may be converted by cytochrome P-450 to other metabolites besides 2,2-dichloroethanol and dichloroacetaldehyde (and dichloroacetate in the case of trans-1,2-dichloroethylene) or may be partial uncouplers of cytochrome P-450 [74] since the rates of dichloroethylene stimulated hepatic microsomal CO-inhibitable NADPH oxidation exceeded the rates of metabolite production by hepatic microsomes for both isomers (Tables 14 and 21). If metabolites other than 2,2-dichloroethanol and dichloroacetaldehyde are produced from the 1,2-dichloroethylenes by hepatic microsomal cytochrome P-450, they do not include 2-chloroethanol, chloroacetaldehyde or chloroacetic acid (see Section 3.3.2). However, it is possible that a portion of the dichloroacetaldehyde produced from the 1,2-dichloroethylenes is bound covalently to microsomal or buffer constituents and thus was not measured by gas-liquid chromatography. This alternative is supported by the very short time period, ca. 3 min and 10 min, over which the production of dichloroacetaldehyde was linear with microsomes and hepatocytes, respectively, from phenobarbital treated rats (Figure 42). The alternate proposal, viz. that the 1,2-dichloroethylenes act as partial uncouplers of hepatic microsomal cytochrome P-450, is consistent with the observation that the rate of H_2O_2 production in the presence of cis-1,2-dichloroethylene and hepatic microsomes was equal to the rate of cis-1,2-dichloroethylene stimulated CO-inhibitable NADPH oxidation (Table 14). Even though measurable amounts of H_2O_2 were not produced by hepatic microsomes in the presence of trans-1,2-dichloroethylene, this compound could possibly

uncouple the cytochrome P-450 enzyme system, with the production of H_2O, as was found with perfluorohexane [74], instead of the production of H_2O_2. Further experimentation is required to elucidate this aspect of the interaction of trans-1,2-dichloroethylene with hepatic microsomal cytochrome P-450.

It would appear that the cytochrome P-450 dependent metabolism of cis- and trans-1,2-dichloroethylene may be the rate limiting step in their metabolism *in vivo*: hepatic microsomal cytochrome P-450 metabolizes cis-1,2-dichloroethylene at a four-fold greater rate than trans-1,2-dichloroethylene *in vitro* (Table 21), and the zero order rate of elimination of the cis-isomer *in vivo* is four-fold greater than that of the trans-isomer [295]. In addition, cis-1,2-dichloroethylene is metabolized at a much greater rate by isolated hepatocytes than is trans-1,2-dichloroethylene (50x) (Table 35).

Cis- and trans-1,2-dichloroethylene appear to be converted by the hepatic microsomal cytochrome P-450 enzyme system to reactive metabolite(s) which can modify the heme moiety of hepatic microsomal cytochrome P-450: incubation of either isomer of the 1,2-dichloroethylenes, hepatic microsomes and NADPH-generating system resulted in the loss of microsomal cytochrome P-450 and heme, with the omission of any component of the incubation mixture eliminating the effect, and inhibitors of cytochrome P-450 decreasing the effect (Table 23). Furthermore, the losses of cytochrome P-450 were virtually eliminated if NADH, which is not an effective electron donor for cytochrome P-450, was substituted for the NADPH-generating system (Table 23). Since the loss of heme is equivalent to the loss of

cytochrome P-450 for the mixture of cis- and trans-1,2-dichloroethylene, it would appear that the reactive species generated by the biotransformation of cis- and trans-1,2-dichloroethylene by hepatic cytochrome P-450 modify the heme moiety of this enzyme. The reactive metabolite(s) mediating the loss of cytochrome P-450 might, for example, be the Zwitterionic species of 1,2-dichloroethylene oxide (see e.g. 287,295).

It would appear that effects previously attributed to the inhibition of cytochrome P-450 by cis- and trans-1,2-dichloroethylene, viz. the ability of the 1,2-dichloroethylenes to diminish the activity of aminopyrine demethylase in vitro and to increase hexobarbital sleeping time in vivo (233,234) might, in part, reflect the ability of metabolites of the 1,2-dichloroethylenes to modify the heme moiety at the active site of cytochrome P-450, rather than the reversible inhibition of this enzyme by the 1,2-dichlorinated ethylenes.

The lack of degradation of cytochrome P-450 seen following the addition of cis- and trans-1,2-dichloroethylene to hepatocyte preparations may reflect a reconstitution of intact cytochrome P-450 by endogenous heme plus apo-cytochrome P-450. For a full explanation of this possibility see discussion on the degradation of cytochrome P-450 by trichloroethylene.

4.3 Trichloroethylene

That a mixed function oxidase of post-mitochondrial supernatant catalyzes the metabolism of trichloroethylene to chloral hydrate was first demonstrated by Leibman and co-workers (240, 242,296). The results presented herein provide conclusive evidence

for the ability of hepatic microsomal cytochrome P-450 to bind and metabolize trichloroethylene. Trichloroethylene bound to the substrate binding site of partially purified cytochrome P-450 and artificial active oxygen donors, $NaClO_2$ and H_2O_2, supported the metabolism of trichloroethylene in this system (see 3.3.3a) to chloral hydrate, which is the major metabolite of trichloroethylene in vitro and in vivo [297] (Figure 75).

It would appear that the form of cytochrome P-450 induced by phenobarbital is the primary form of the enzyme which binds and metabolizes trichloroethylene. This conclusion is drawn from the observation that the binding constants (K_s) and Michaelis constants (K_m) for the interaction of trichloroethylene with hepatic microsomal cytochrome P-450 were not altered by induction of different forms of cytochrome P-450 while the maximum extents of binding and metabolism (ΔA_{max}, V_{max} per mg microsomal protein and per nmol cytochrome P-450) of trichloroethylene were increased following phenobarbital induction but not following 3-methylcholanthrene induction (Tables 11 and 26). The form of cytochrome P-450 induced by spironolactone appears also to play a role in the metabolism of trichloroethylene as there is an increase in the V_{max} per nmol cytochrome P-450 following spironolactone treatment* (Table 11). The lack of effect of induction with 3-methylcholanthrene on the parameters for the binding and metabolism of trichloroethylene by hepatic microsomes (Tables 11 and 26) indicates that cytochrome P-450c may not interact with trichloroethylene.

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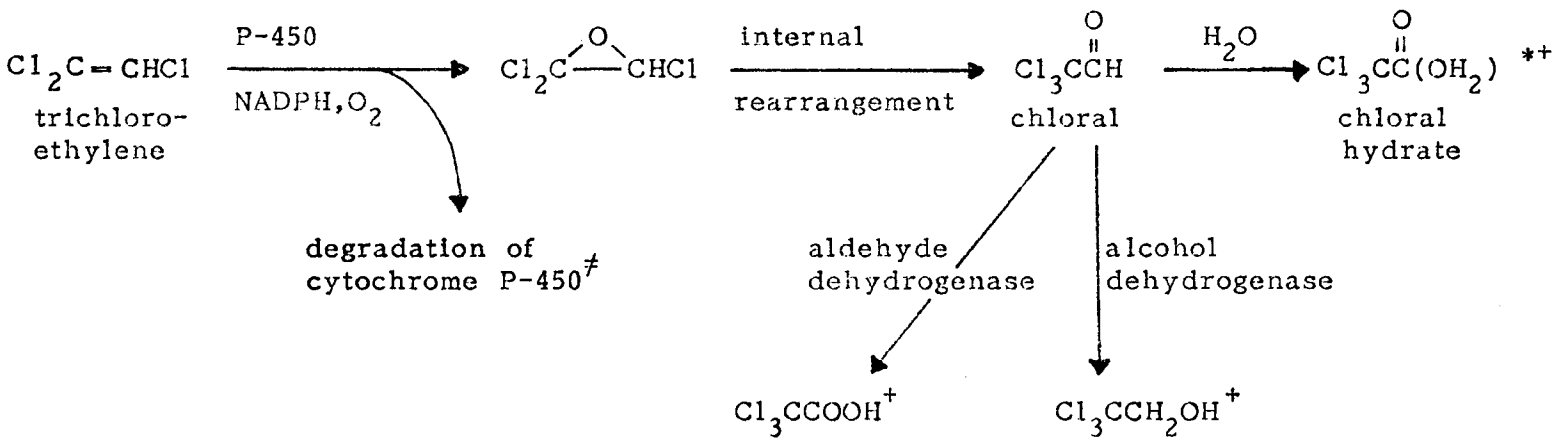
*Spironolactone pretreatment of animals has been found to result in changes in the composition of the mixture of forms of cytochrome P-450 present in hepatic microsomes, but does not result in an overall increase in the levels of total type P-450 cytochromes per mg microsomal protein (Section 1.1.3d).

Figure 75: Metabolic pathway of trichloroethylene as modified from previously postulated pathways (e.g. 238,240,242)



*Identified in hepatic microsomes

+Identified in isolated hepatocytes

≠degradation of cytochrome P-450 not seen in isolated hepatocytes

The proposed pathway for the metabolism of trichloroethylene can be seen in Figure 75. As the metabolism of trichloroethylene has been quite extensively studied in vitro, the metabolic pathway (Figure 75) as found in the literature (e.g 238,240,242) has been only slightly modified.

The first step in the metabolism of trichloroethylene presumably proceeds via a trichloroethylene oxide intermediate (see later)*. In aqueous solution, this epoxide rearranges thermally in vitro primarily to dichloroacetyl chloride (288). In vivo, however, the primary product of the trichloroethylene metabolism is chloral hydrate**. Specifically, it has been proposed that the trivalent iron atom of cytochrome P-450 exerts an influence on the observed intramolecular rearrangement to chloroacetaldehyde and chloral (299).

The chloral, once formed, is hydrated to give rise to chloral hydrate which is the major metabolite of trichloroethylene

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*Although epoxide intermediates have been proposed by most workers, it has been suggested by Miller and Guengerich (243) that the metabolism of trichloroethylene proceeds via a transition state in which trichloroethylene is bound to an activated oxygen of cytochrome P-450. Thus the cytochrome P-450 catalyzed oxidation of trichloroethylene to chloral is thought to involve chlorine migration within an oxygenated trichloroethylene-cytochrome P-450 transition state and not via trichloroethyleneoxide (243).

** A similar discrepancy has been observed for vinyl chloride, where the proposed epoxide intermediate rearranges primarily to acetyl chloride in water in vitro, but the primary product from hepatic microsomal cytochrome P-450 is chloroacetaldehyde (298). It has been proposed that in both cases, viz. the epoxides of vinyl chloride and of trichloroethylene, the rearrangement seen in vivo predominates because of the presence of Lewis acids.

in hepatic microsomes and hepatocytes in vitro. This non-enzymic pathway appears to be favoured over the conversion of chloral to 2,2,2-trichloroethanol or trichloroacetic acid. For example, in isolated hepatocytes, where chloral hydrate is produced at a rate of 2.8 nmol per 10^6 cells per min, only 0.3 nmol 2,2,2-trichloroethanol per 10^6 cells per min and 15 pmol trichloroacetic acid per 10^6 cells per min are produced (see 3.4.2b).

The addition of an authentic sample of chloral hydrate to isolated hepatocytes resulted in a decrease in the levels of chloral hydrate with time. A small portion of the chloral hydrate was converted to 2,2,2-trichloroethanol and to trichloroacetic acid (Figure 69). It appears that the affinity or V_{max} values of the aldehyde and alcohol dehydrogenases for chloral (or the hydrated analogue thereof) may be far lower than for other chlorinated aldehydes, e.g. dichloroacetaldehyde. This is consistent with the observation that the decrease in the levels of chloral hydrate with time was not nearly as dramatic as the decrease seen in the levels of dichloroacetaldehyde during the same time interval (see Figures 68 and 69).

The K_s and K_m values reported herein for the interaction of trichloroethylene with hepatic microsomal cytochrome P-450 are two- to twenty-fold lower than the K_m values for the conversion of trichloroethylene to chloral hydrate by hepatocyte suspensions and 10 to 90 fold greater than the K_m values reported by post-mitochondrial supernatant [242,247]. These discrepancies may reflect the different liver preparations used or perhaps reflect the further metabolism of chloral hydrate to 2,2,2-trichloroethanol and trichloroacetate in hepatocytes and presumably with post-mitochondrial supernatant [242,247] while chloral hydrate was the sole chlorinated metabolite

of trichloroethylene with isolated hepatic microsomes (Section 2.2.4b). The observation that the K_s for the binding of trichloroethylene in hepatic microsomes is equivalent to K_m for the metabolism of trichloroethylene in hepatic microsomes for each type of induction indicates that the binding of trichloroethylene is rapid, compared to the rate limiting step of the reaction.* The inability of spironolactone — which elevates the levels of NADPH-cytochrome P-450 reductase per mg microsomal protein [143]** — to increase significantly the rate of metabolism of trichloroethylene per mg microsomal protein (Table 26) ($P > 0.1$) indicates that the rate limiting step in the metabolism of trichloroethylene by cytochrome P-450 is subsequent to the reduction of the cytochrome P-450 - substrate complex.

The K_m and V_{max} values reported in this manuscript for the metabolism of trichloroethylene to chloral hydrate by hepatic microsomes (Table 26) are similar to those reported by Traylor et al. for the production of CO from trichloroethylene by hepatic microsomal cytochrome P-450 [300].

It appears that trichloroethylene is, indirectly, capable of degrading cytochrome P-450 in hepatic microsomes without affecting the levels of other hepatic microsomal enzymes, viz. cytochrome b_5, NADPH-cytochrome c reductase (Tables 27 and 28) and glucose-6-phosphatase (see Section 3.3.3b). In as much as the levels of

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*In addition, interestingly, the K_s value obtained for the binding of trichloroethylene to partially purified cytochrome P-450 from phenobarbital treated rats is within experimental error of the K_m value for the production of chloral hydrate from trichloroethylene in isolated hepatocytes (Section 3.1.3).

**An increase in the V_{max} per mg microsomal protein (and not per nmol cytochrome P-450) following spironolactone treatment would suggest that the reduction of cytochrome P-450 is rate-limiting in the metabolism of trichloroethylene.

microsomal heme and cytochrome P-450 were decreased to similar extents by trichloroethylene (Table 27), it would appear that trichloroethylene modifies the heme moiety of cytochrome P-450.

The degradation of the heme of cytochrome P-450 by trichloroethylene *in vitro* appears to require the metabolic activation of trichloroethylene by cytochrome P-450 since the reaction required NADPH and was not supported by NADH (Table 27). Furthermore, metyrapone, SKF-525A and CO, which inhibited the metabolism of trichloroethylene, also inhibited the degradation of cytochrome P-450 by trichloroethylene (Table 29). In addition, each type of induction affected the metabolism of trichloroethylene by hepatic microsomal cytochrome P-450 and the degradation of the heme of cytochrome P-450 by trichloroethylene to similar extents in hepatic microsomes *in vitro* (Tables 26 and 27) and *in vivo* [301]. This is in contrast to the situation with fluroxene, a compound which is also known to degrade the heme moiety of cytochrome P-450 *in vitro* [303]. For fluroxene, inducing agents for different forms of cytochrome P-450 differentially affect its metabolism by cytochrome P-450 relative to its degradation of cytochrome P-450 [302].

The trichloroethylene-mediated degradation of hepatic cytochrome P-450, seen in hepatic microsomes, has been observed *in vivo* [304], but was not observed following the incubation of trichloroethylene with isolated hepatocytes (see Section 3.4.3). The apparent lack of effect of trichloroethylene on cytochrome P-450 in isolated hepatocytes may be due to the dissociation of the modified or degraded heme moiety from the apo-cytochrome P-450 and the subsequent binding of heme from the hepatic heme pool to

the apoprotein to reconstitute intact cytochrome P-450. A similar phenomenon has been observed with hepatic post-mitochondrial supernatant (but not with hepatic microsomes) for AIA (allyl-*iso*-propylacetamide) and fluroxene [see e.g. 305].

The effects of inducing agents on the metabolism and metabolic activation of trichloroethylene by hepatic microsomal cytochrome P-450 *in vitro* compare well with their effects on the metabolism and hepatotoxicity of trichloroethylene *in vivo*. Phenobarbital increases the metabolism of trichloroethylene, the degradation of cytochrome P-450 by trichloroethylene [242,247] (Tables 26 and 27) and the binding of metabolites of trichloroethylene to macromolecules *in vitro* [236] as well as enhancing the metabolism and toxicity of trichloroethylene *in vivo* and the degradation of cytochrome P-450 by trichloroethylene *in vivo* [236-238,304,306]. Induction by spironolactone or 3-methylcholanthrene does not or only slightly increases the metabolism of trichloroethylene and the trichloroethylene-mediated degradation of cytochrome P-450 *in vitro* (Tables 26 and 27) and similarly affects the metabolism and hepatotoxicity of trichloroethylene *in vivo* [237,306]. The excellent correlation between the effects of inducing agents on the metabolism and the metabolic activation of trichloroethylene *in vitro* and *in vivo* indicate that the metabolism of trichloroethylene by the phenobarbital inducible form of cytochrome P-450 is the first and possibly the rate limiting step in the metabolism of trichloroethylene *in vivo* and in the production of the toxic effects observed following exposure to trichloroethylene.

4.4 Tetrachloroethylene

Different forms of hepatic microsomal cytochrome P-450 appear to bind and metabolize tetrachloroethylene to differing extents. Cytochrome P-450c does not appear to bind or metabolize tetrachloroethylene measurably, in as much as induction with β-naphthoflavone did not affect the values of K_s, K_m, ΔA_{max} or V_{max} per mg protein and per nmol cytochrome P-450 for tetrachloroethylene (Tables 12 and 31). In addition, SKF-525A, which is an effective inhibitor of cytochrome P-450c [293], was a relatively poor inhibitor of the binding and metabolism of tetrachloroethylene (Table 32).

At least two distinct forms of cytochrome P-450 — those induced by pregnenolone-16α-carbonitrile and by phenobarbital — appear to bind and metabolize tetrachloroethylene. Induction with either of these agents resulted in alterations in K_s and/or K_m and in increased ΔA_{max} and/or V_{max} per mg microsomal protein and per nmol cytochrome P-450 values for the binding and metabolism of tetrachloroethylene (Tables 12 and 31). Furthermore, metyrapone, which is a relatively specific inhibitor of the phenobarbital-induced form of cytochrome P-450 under the conditions of our experiments [293, but see 280,155] was an effective inhibitor of the spectral binding and metabolism of tetrachloroethylene by hepatic microsomes from phenobarbital induced rats (Table 32).

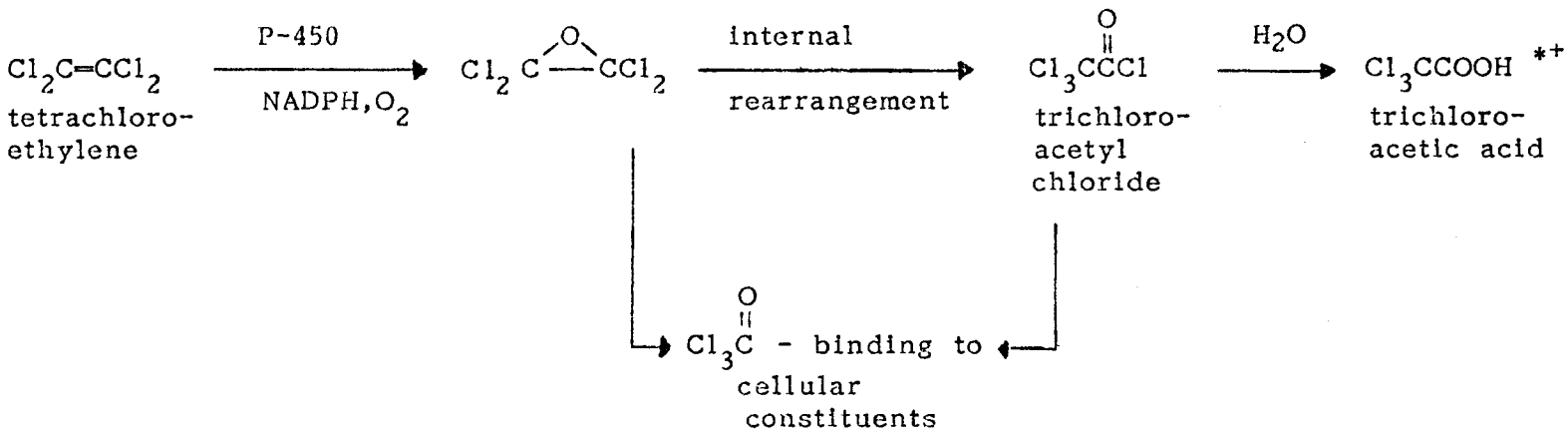
The form of cytochrome P-450 induced by phenobarbital, viz. cytochrome P-450b, appears to metabolize tetrachloroethylene more effectively than any other form of the enzyme. Although phenobarbital and pregnenolone-16α-carbonitrile enhanced V_{max} per nmol cytochrome P-450 for the metabolism of tetrachloroethylene

to similar extents, the K_m for tetrachloroethylene was 10-fold lower for phenobarbital induction than for pregnenolone-16α-carbonitrile induction (Table 31).

The V_{max} per mg protein per min or per 10^6 cells per min values were, in both liver preparations, increased 10-fold following phenobarbital pretreatment compared to untreated rats (Tables 31 and 38) thus providing further confirmation that cytochrome P-450b is instrumental in the metabolism of tetrachloroethylene. The K_m values for the metabolism of tetrachloroethylene to trichloroacetic acid in untreated and phenobarbital treated rats in hepatic microsomes were identical to those for hepatocyte suspensions, for similarly treated rats (e.g. controls and phenobarbital induced) (Tables 31 and 38) indicating that entry of tetrachloroethylene into the cell does not slow its metabolism by cytochrome P-450.

The metabolic pathways for tetrachloroethylene can be seen in Figure 76 exactly as postulated by other research workers (e.g. 211) (compare Figure 19). Cytochrome P-450, which catalyzes the first step in the metabolism of tetrachloroethylene, presumably to an epoxide, appears to be the sole enzyme involved in the conversion of tetrachloroethylene to free trichloroacetate and to the trichloroacetyl moiety covalently bound to cellular constituents (as seen with hepatic microsomes). Trichloroacetate was the sole metabolite found in both hepatic microsomes and in isolated hepatocytes (Table 42), and is the major metabolite of tetrachloroethylene *in vivo* and with perfused liver *in situ* (211,244,245,247). Since small amounts of 2,2,2-trichloroethanol were seen following exposure to tetrachloroethylene *in vivo* (245), but not *in vitro*, it would

Figure 76: Metabolic pathway of tetrachloroethylene (211)



*Identified metabolite in hepatic microsomes

+Identified metabolite in hepatocytes

appear that the reaction is too slow for this minor metabolite to be observed *in vitro*.

The interaction of tetrachloroethylene with hepatic microsomal cytochrome P-450 also resulted in the production of trichloroacetyl derivative(s) of cellular constituents. These covalently bound trichloroacetyl moieties are probably in ester or amide linkage to alcohol or amino functional groups of cellular constituents and presumably arise in a non-enzymic reaction of the epoxide or acyl chloride derivatives of tetrachloroethylene with cellular constituents. In hepatocytes, however, no bound trichloroacetyl derivatives were found (see Section 3.4.5), an observation which is inconsistent with the findings in hepatic microsomes (Section 3.3.4c) and in perfused rat liver [211]. As the experiment was repeated several times with isolated hepatocytes, the reason for this discrepancy is not known. A possible explanation for this finding is the formation of thioester linkages such as glutathione conjugates or mercapturic acid derivatives of the trichloroacetyl moieties. These conjugates could possibly be resistant to acid hydrolysis (6.25 M at 100° for 30 min) and thus not measured under our experimental conditions.

The metabolism of tetrachloroethylene, unlike that of trichloroethylene, did not affect the levels of hepatic microsomal cytochrome P-450 or heme *in vitro* (Figure 33) (see 3.3.4d).

In general, it was observed that the relative rates of metabolite production from the chlorinated ethylenes in hepatocytes differed from those found

in hepatic microsomes (Table 42). This is explainable as follows: Hepatic microsomes mainly contain the enzymes involved in Phase I of drug metabolism, while the hepatocytes contain both the toxifying and detoxifying enzymes of Phase I and Phase II of drug metabolism. Therefore, the conjugating enzymes present, for example, in the cytosol, could further convert the metabolites, from the cytochrome P-450 enzyme system, to conjugates or secondary metabolites, resulting in different patterns of metabolites to those found in hepatic microsomes. For example, dichloroacetaldehyde was a major metabolite for all three dichlorinated ethylenes in hepatic microsomes. However, in isolated hepatocytes measurable amounts were seen only following cis-1,2-dichloroethylene metabolism (the dichloroethylene with the greatest rate of metabolism). Cytosolic aldehyde and alcohol dehydrogenase convert the dichloroacetaldehyde, as it is generated, to the corresponding acid and alcohol.

Another factor contributing to the discrepancy found between rates of metabolism and metabolite production following the metabolism of the chlorinated ethylenes in hepatic microsomes and isolated hepatocytes is the observation that the levels of each chlorinated metabolite, with the exception of trichloroacetic acid, when added to isolated hepatocytes, disappeared with time (Figures 66 - 69).

What does, however, become apparent, when investigating the metabolism of the chlorinated ethylenes in hepatic microsomes

Table 42: Comparison of metabolite production from the chlorinated ethylenes in hepatic microsomes and isolated hepatocyte preparations*

Chlorinated Ethylene	Hepatic microsomes		Hepatocyte suspensions	
	Metabolite	Rate+	Metabolite	Rate≠
Vinylidene chloride	{Monochloroacetate, Dichloroacetaldehyde}	0.2	{Dichloroacetaldehyde, 2,2-Dichloroethanol}	0.02
cis-1,2-Dichloroethylene	{Dichloroacetaldehyde, 2,2-Dichloroethanol}	0.47	{2,2-Dichloroethanol, Dichloroacetic acid, Dichloroacetaldehyde}	0.27
trans-1,2-Dichloroethylene	{Dichloroacetaldehyde, 2,2-Dichloroethanol, Dichloroacetic acid}	0.1	{2,2-Dichloroehtanol, Dichloroacetic acid}	0.05
Trichloroethylene	Chloral hydrate	5.8	{Chloral hydrate, 2,2,2-trichloroethanol}	3.1
Tetrachloroethylene	Trichloroacetate	0.5	Trichloroacetate	0.1

*All animals utilized were phenobarbital pretreated.

+nmol/mg microsomal protein/min.

≠nmol/10^6 cells/min.

and in hepatocytes is that, although hepatic microsomes have the advantage of providing a means of being able to investigate Phase I metabolism of the chlorinated ethylenes, it does not aid in establishing the major metabolic products in vivo (and could lead to erroneous conclusions). A good example is the production of dichloroacetaldehyde as the major metabolite of all three dichlorinated ethylenes in hepatic microsomes, while this toxic metabolite is only observed in measurable quantities following cis-1,2-dichloroethylene metabolism in isolated hepatocytes. Studies with hepatic microsomes, therefore, do not provide a good model for the in vivo situation, but when studied in conjunction with freshly isolated hepatocytes*, place Phase I of drug metabolism in the correct context.

4.5 The hepatic management of the chlorinated ethylenes and its consequences

It appears that the extent and position of chlorine substitution in the chlorinated ethylenes determine several important aspects of their management by the liver, including the following:

i) The role that different forms of cytochrome P-450 play in the binding and metabolism of the chlorinated ethylenes.

ii) The rates of metabolism of the chlorinated ethylenes.

iii) The ability of the chlorinated ethylenes to act as "uncouplers" of hepatic cytochrome P-450.

iv) The ability of the chlorinated ethylenes to degrade hepatic

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*Levels of cytochrome P-450 drop by 80% after 24 hr[180].

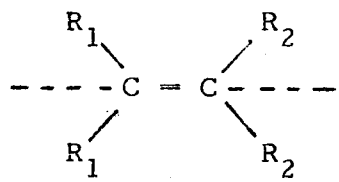
microsomal cytochrome P-450.

v) The reactivity of the resulting epoxides (or analogues) of the chlorinated ethylenes.

vi) The ability of the chlorinated ethylenes to generate species which induce DNA repair.

It would appear that the extent of chlorination, in part, determines which form of cytochrome P-450 accepts the chlorinated ethylenes as substrates. For example, the form(s) of cytochrome P-450 found in untreated rats, but not increased following phenobarbital pretreatment, play an increasing role in the metabolism of the chlorinated ethylenes as extent of chlorination decreases (Table 43). In contrast, the form of cytochrome P-450 induced by phenobarbital, cytochrome P-450b, plays an increasing role in their metabolism as the extent of chlorination increases.

The symmetry of the chloroethylene molecules appears to play an important role in determining their relative rates of metabolism by cytochrome P-450. Each one of the chlorinated ethylenes possesses a plane of symmetry in the plane of the paper. Furthermore, they can possess a plane of symmetry along the double bond, viz.



or a plane of symmetry across the double bond, viz:

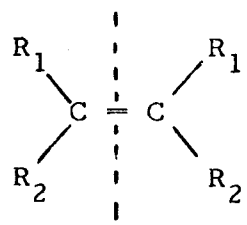
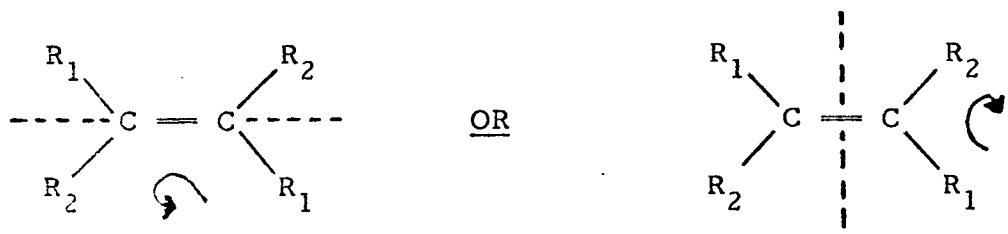


Table 43: The forms of cytochrome P-450 involved in the metabolism of the chlorinated ethylenes as observed in hepatic microsomes

Chlorinated Ethylene	Form of cytochrome P-450 involved in the metabolism
Vinylidene chloride	One or two forms in untreated animals, cytochrome P-450b*
cis-1,2-Dichloroethylene	One or two forms in untreated animals, cytochrome P-450b*
trans-1,2-Dichloroethylene	One or two forms in untreated animals, cytochrome P-450b*
Trichloroethylene	Cytochrome P-450b
Tetrachloroethylene	Cytochrome P-450b

*Minor role

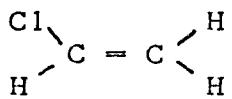
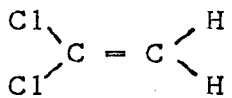
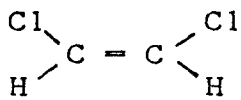
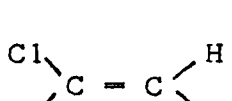
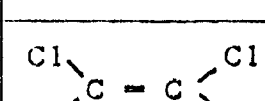
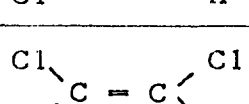
The last two mentioned planes of symmetry can also be symmetrical upon rotation through 180°, viz.:



Decreasing degrees of symmetry seems to result in increased rates of metabolism by hepatic cytochrome P-450. Trans-1,2-dichloroethylene, which possesses three planes of symmetry (two with rotation) (see Table 44) has the lowest rate of conversion by cytochrome P-450 to chlorinated metabolites in hepatic microsomal preparations (see Table 45). Accordingly, tetrachloroethylene, with three degrees of symmetry, is metabolized by hepatic microsomes at a tenth of the rate found with trichloroethylene (with one degree of symmetry) (Table 45). In contrast, trichloroethylene is the only chlorinated ethylene, besides vinyl chloride, which has one plane of symmetry (Table 44) and is metabolized extensively by cytochrome P-450 in hepatic microsomes (and isolated hepatocytes).

Trichloroethylene is also the only member of the series of chlorinated ethylenes where its rate of metabolism equals its rate of stimulated NADPH oxidation. That this does not occur with the other chlorinated ethylenes is supported by the observation that cis-1,2-dichloroethylene and vinylidene chloride are partial uncouplers of hepatic cytochrome P-450 as evidenced by their stimulated H_2O_2 production. These findings are also explainable on the basis of the planes of symmetry, viz. vinylidene chloride and cis-1,2-dichloroethylene, which each posses two planes of symmetry (Table 44), act as uncouplers and give rise to H_2O_2 (Table 47), while trans-1,2-dichloroethylene and tetrachloroethylene, which each

Table 44: Planes of symmetry of the chlorinated ethylenes

Chlorinated Ethylene	Structure	Planes of symmetry*	Total
Vinyl chloride		None	1
Vinylidene chloride		Along the double bond	2
cis-1,2-Dichloroethylene		Across the double bond	2
trans-1,2-Dichloroethylene		(i) Across the double bond, with rotation (ii) Along the double bond, with rotation	3
Trichloroethylene		None	1
Tetrachloroethylene		(i) Along the double bond (ii) Across the double bond	3

*In addition to the plane of symmetry in the plane of the paper inherent to the carbon-carbon double bond for each chlorinated ethylene making the molecule planar.

Table 45: Relationship between the metabolism of the chlorinated ethylenes and the ability to degrade hepatic microsomal cytochrome P-450 in hepatic microsomes in vitro

Chlorinated Ethylenes	Metabolism by cytochrome P-450* Products	Rate+	Ability to degrade hepatic microsomal cytochrome P-450	Ability to induce DNA repair in hepatocytes
Vinylidene chloride	{Monochloroacetate, Dichloroacetaldehyde}	0.2	No	+
cis-1,2-Dichloroethylene	{Dichloroacetaldehyde, 2,2-Dichloroethanol}	0.47	Yes	+
trans-1,2-Dichloroethylene	{Dichloroacetaldehyde, 2,2-Dichloroethanol}	0.1	Yes	-
Trichloroethylene	Chloral hydrate	5.8	Yes	+
Tetrachloroethylene	Trichloroacetate	0.5	No	-

*Experiments were carried out using hepatic microsomes from phenobarbital treated rats.

+Expressed in nmol/min/mg microsomal protein.

possess an additional plane of symmetry (Table 44), do not give rise to H_2O_2 production. Trans-1,2-dichloroethylene and tetrachloroethylene may. however, also be partial uncouplers of cytochrome P-450 and give rise to the production of H_2O*, which would also indicate a role for the degree of symmetry in the metabolic consequences of the chlorinated ethylenes.

The metabolism of the chlorinated ethylenes, in some cases, leads to the destruction of cytochrome P-450 in hepatic microsomes. The abilities of the chlorinated ethylenes to modify the heme moiety of cytochrome P-450 do not however correlate with their rates of metabolism by hepatic microsomal cytochrome P-450 in vitro (see Table 45). Cis- and trans-1,2-Dichloroethylene and trichloroethylene drastically decreased the levels of hepatic microsomal cytochrome P-450 while vinylidene chloride and tetrachloroethylene did not (Table 45). The rates of metabolism in hepatic microsomes vary between the highest observed with trichloroethylene to the lowest seen with trans-1,2-dichloroethylene (both of which were found to degrade hepatic microsomal cytochrome P-450). This ability of reactive metabolites of the chlorinated ethylenes to modify the heme of cytochrome P-450 does, however, appear to correlate with the major rearrangement products of the epoxide intermediates: epoxides that rearrange predominantly to aldehydes in vitro are from compounds that cause the modification of the heme moiety of cytochrome P-450 (viz. cis- and trans-1,2-dichloroethylene and trichloroethylene — Table 45)

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*The production of H_2O as the product of the "uncoupling" of the cytochrome P-450 enzyme system, indicates a different mechanism for oxygen activation (see Section 1.1.1b).

while epoxides that rearrange mainly to acyl chlorides or to both acyl chlorides and aldehydes are from compounds that do not modify the heme moiety of cytochrome P-450 *in vitro*, viz. vinylidene chloride and tetrachloroethylene. This conclusion is in full agreement with the sixth and and most widely studied of the chlorinated ethylenes, viz. vinyl chloride, which causes the degradation of cytochrome P-450 (see 3.6) and the epoxide of vinyl chloride rearranges mainly to chloroacetaldehyde *in vitro* (211).

DNA repair (or unscheduled DNA synthesis) provides evidence of the binding of reactive species to DNA and the subsequent correcting mechanism of the cell in an attempt to prevent a genetic mutation. As DNA repair is seldom totally efficient in excising all the damaged regions, it can be regarded as an indication of the chemicals' ability to induce cancer (see 1.2.1). The advantages that the monitoring of DNA repair has over other mutagenicity-testing systems are that DNA repair is being tested for in the cells of the target organ where the toxifying and detoxifying enzymes are present roughly as they are found *in vivo* (see 1.2).

Of the chlorinated ethylenes, vinylidene chloride, cis-1,2-dichloroethylene and trichloroethylene were found to induce DNA repair while trans-1,2-dichloroethylene and tetrachloroethylene did not (Figure 70, Tables 45 and 46). The ability of cis-1,2-dichloroethylene to induce DNA repair in hepatocytes is in contrast to its lack of mutagenicity *in vitro* [96], and the lack of conclusive evidence for its carcinogenicity in rats *in vivo*.

That DNA repair was induced by vinylidene chloride and not trans-1,2-dichloroethylene emphasizes yet again the influence that the spatial arrangement of the chlorine substituents around the C—C double bond exert on the short-term effects (i.e. rates of metabolism and metabolite production) and the long-term effects (i.e. cancer) of the chlorinated ethylenes. Aside from cis-1,2-dichloroethylene, a good correlation exists between the ability of the other chlorinated ethylenes, viz. vinylidene chloride, trichloroethylene and tetrachloroethylene, to induce DNA repair synthesis in hepatocytes isolated from phenobarbital pretreated animals and their reported carcinogenicity in vivo (Table 46).

A possible explanation for the observed DNA repair by certain members of the series of chlorinated ethylenes could be as a direct result of their ability to "uncouple" cytochrome P-450 with the production of H_2O_2. H_2O_2 and/or other active oxygen species have been shown to give rise to DNA repair (187,307). However, from results with hepatic microsomes, the cytochrome P-450 dependent metabolism of cis-1,2-dichloroethylene and vinylidene chloride gave rise to H_2O_2 production but the metabolism of trichloroethylene did not. However, if the rates of production of H_2O_2 from cis-1,2-dichloroethylene and vinylidene chloride were regarded as rates of metabolite production (Table 47), a positive correlation would exist between rates of metabolite production and the ability to induce DNA repair as trans-1,2-dichloroethylene and tetrachloroethylene do not give rise to H_2O_2 production.

It would appear that for the chlorinated ethylenes to give

Table 46: The ability of the chlorinated ethylenes to induce DNA repair

Chlorinated Ethylene	Ability to induce DNA repair in hepatocytes *	Carcinogenicity (References)
Vinylidene chloride	+	+ (224-227)
cis-1,2-Dichloroethylene	+	- (221)
trans-1,2-Dichloroethylene	-	- (221)
Trichloroethylene	+	+ (200,213)
Tetrachloroethylene	-	- (203,246)

*See Figure 70.

Table 47: The rates of H_2O_2 and chlorinated metabolite production from the chlorinated ethylenes in hepatic microsomes*

Chlorinated Ethylene	Rates of H_2O_2 formation nmol/min/mg protein	Rates of chlorinated metabolite production nmol/mg microsomal protein	Ability to induce DNA repair
Vinylidene chloride	3.2	0.2	+
cis-1,2-Dichloroethylene	2.33	0.47	+
trans-1,2-Dichloroethylene	-	0.1	-
Trichloroethylene	-	5.8	+
Tetrachloroethylene	-	0.5	-

*These values are obtained from experiments performed with hepatic microsomal suspensions from phenobarbital pretreated animals.

rise to DNA repair, they must satisfy one of the following two requirements:

1. Through the action of hepatic cytochrome P-450, an asymmetrically substituted epoxide must be generated, e.g. by vinylidene chloride and trichloroethylene.
2. Through the uncoupling of hepatic cytochrome P-450, H_2O_2 is generated. e.g. by cis-1,2-dichloroethylene and vinylidene chloride.

Finally, we would like to propose that the DNA repair method utilized in these studies is inexpensive and rapid compared to most other techniques for assessing carcinogenicity (see Section 1.2). The DNA repair method utilized has to date been tested on relatively few xenobiotics, and further extensive studies are required before its reliability as a measure of carcinogenic potential can be assessed.

An endeavour to adapt this method to testing DNA repair in freshly isolated human liver cells is presently underway in our laboratory. This method of testing DNA repair will therefore meet almost all the reservations about the relevance of the intermediary mutagenicity/carcinogenicity assays to the human situation.

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