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The Assessment of Carcinogenic Hazard for Human Beings Exposed to Methylene Chloride

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THE ASSESSMENT OF CARCINOGENIC HAZARD FOR HUMAN BEINGS EXPOSED TO METHYLENE CHLORIDE

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THE ASSESSMENT OF CARCINOGENIC HAZARD FOR HUMAN BEINGS EXPOSED TO METHYLENE CHLORIDE

1. SUMMARY

An increased incidence of lung and liver cancer was observed in mice exposed by inhalation to high concentrations of methylene chloride for two years in studies performed for the USA National Toxicology Programme. This response was not seen in rats or hamsters exposed to similar dose levels by inhalation, nor in mice exposed to lower concentrations in drinking water. Rats exposed at the higher atmospheric concentrations had an increased incidence of benign mammary tumours that was not seen in mice or hamsters of either sex. These studies have raised widespread concern over the potential of this chemical to cause cancer in humans. A considerable amount of data now exists from which the relevance of the animal studies to human exposure can be evaluated. Data derived from metabolic and pharmacokinetic studies provide clear evidence that the mouse differs substantially from rat, the hamster, and man with respect to the rate and relative importance of the two pathways of methylene chloride metabolism. These studies provide an explanation for the species difference in carcinogenicity at high dose levels and also the lack of carcinogenicity in mice exposed at low dose levels in drinking water although the exact mechanism by which methylene chloride causes cancer in mice remains unclear. Although mutagenic in some prokaryotes the complete lack of genotoxicity in 'in vitro' mammalian gene mutation assays and 'in vivo' mammalian tests, including those for DNA damage and chromosomal effects, indicate that methylene chloride is not genotoxic 'in vivo'. It is probable that methylene chloride affects a later stage of carcinogenic process and that it accelerates the development of tumours which occur spontaneously incidence in the B6C3F1 mouse used in these studies. The data on which these conclusions are based are reviewed, together with the carcinogenicity and other relevant studies on methylene chloride in laboratory animals. An evaluation of the relevance of the findings in animal studies to human exposure is presented and conclusions are drawn which suggest the results of the carcinogenicity studies in mice are not relevant to human exposure to methylene chloride.

2. INTRODUCTION

Methylene chloride has a variety of important uses. Its characteristic as a solvent has led to its use in paint strippers, in the decaffeination of coffee, as an industrial cleaning agent and as a process solvent for industrial products. It is also used as a component of aerosol propellant mixtures. As a result of these applications a large number of people may be exposed to methylene chloride for short or long periods.

Since the recent review of the toxicity of methylene chloride by ECETOC (1984), the National Toxicology Program has reported carcinogenic activity in mice and rats (NTP, 1986) which has given rise to concern about its safe use.

Methylene chloride is one of a number of chlorinated alkanes and alkenes which induce cancer in mice, particularly of the liver, but do not have a similar effect in rats or other species. The high level of susceptibility of the mouse to the carcinogenic (particularly hepatocarcinogenic) effects of these compounds present a major challenge to the assessment of hazard to man. Its other toxic properties are relatively unimportant at the exposure levels encountered during normal use although its metabolism to carbon monoxide (CO), resulting in the production of carboxyhaemoglobin has been observed in exposed populations and is the basis on which current occupational exposure limits are set.

An important principle in the analysis of hazard, risk and benefit is that they should be analysed as objectively and scientifically as possible so that those in regulatory positions and those considering the overall social consequences of regulation have the best possible base from which to make their decisions. Methylene chloride provides a challenging opportunity for the scientific approach to hazard assessment. It has been tested for genotoxic effects in mutagenicity studies in prokaryotic and eukaryotic microorganisms, mammalian cells in culture and *in vivo*

mammalian studies. Chronic toxicity and carcinogenicity studies in 3 species and careful analysis of its metabolism and pharmacokinetics in rats and mice and in rat, mouse, hamster and human tissues in vitro provide an unusually broad range of data of relevance to risk assessment.

This report summarizes and evaluates the experimental data available on the metabolism and pharmacokinetics, mutagenicity and carcinogenicity of methylene chloride with a view to assessing its potential hazard to human health. In the first section the metabolic pathways in in vitro experimental systems and in laboratory animals and man are reviewed. The results of mutagenicity and carcinogenicity experiments are reviewed and evaluated in terms of the knowledge of the metabolism of methylene chloride. The comparative studies on pharmacokinetics of methylene chloride in rats and mice and in rat, mouse, hamster and human tissues are reviewed and the importance of metabolism by the glutathione pathway identified. Finally the assessment of human hazard is approached by considering the suitability of the mouse as a model for human hazard assessment. A numerical risk assessment is not presented as sufficient data is not yet available to allow a satisfactory quantitative assessment. Further studies are in progress to define the complex relationship between dose and the pathways of methylene chloride metabolism.

3. METABOLIC PATHWAYS

Experiments in laboratory animals and humans have shown that methylene chloride is rapidly absorbed through the lungs and distributed throughout the body reaching all organs, including the brain, and crossing the placental barrier to the foetus (Winneke and Fodor, 1976; Schwetz et al, 1975). It has a particular affinity for body fat, concentrations reaching 7 to 8-fold higher than those in other tissues (Savolainen et al, 1977, 1981). Following oral or inhalational exposure the greater portion of the dose is exhaled unchanged (Riley et al, 1966; McKenna and Zempel, 1981; McKenna et al, 1982). The lungs are also the major route of elimination of methylene chloride metabolites with only small percentages of the dose being eliminated in urine and faeces (Di Vincenzo and Hamilton, 1975).

Methylene chloride and the other dihalomethanes are unique in being the only class of industrial chemicals known to be metabolised to carbon monoxide. This metabolic pathway, first discovered in man (Stewart et al, 1972), results in elevated levels of carboxyhaemoglobin and in increased levels of carbon monoxide in expired air. Subsequent studies in experimental animals and in man established that this pathway is rate limited by enzyme saturation so that at high doses the levels of carboxyhaemoglobin became constant and independent of dose (Rodkey and Collison, 1977). Later experiments in animals using radiolabelled methylene chloride identified carbon dioxide as the other major metabolite (DiVincenzo and Hamilton, 1975). Although carbon dioxide is a known metabolite of carbon monoxide (Fenn, 1970) the amount of carbon dioxide formed from the monoxide was thought unlikely to account for the levels found during exposure to methylene chloride. This suggested the presence of a second pathway, which was subsequently confirmed in experimental animals, but has not been established in man.

Confirmation of the presence of two metabolic pathways was obtained from in vitro experiments using liver fractions, homogenates, slices and hepatocytes, mainly from the rat. The primary reaction, first described by Kubic and Anders (1975) appears to be an oxidative dehalogenation giving carbon monoxide and chloride ion. The reaction is catalysed by rat liver microsomal fractions and is dependent upon NADPH and molecular oxygen. The presence of a binding spectrum and the outcome of studies using metabolic inhibitors and inducers confirmed the involvement of the cytochrome P-450 mixed function oxidase system. The highest activity was found in liver microsomes which were five-fold more active than lung microsomes and thirty-fold more active than kidney microsomes. The proposed mechanism involves rearrangement of the primary hydroxylation product to formyl chloride followed by decomposition to carbon monoxide (Fig 1) (Kubic and Anders, 1978).

Although the transient intermediates have not been isolated or identified their formation is consistent with the enzyme involved and the products formed.

The other metabolic pathway occurring in rat liver is localised in the soluble (cytosolic) fraction (Ahmed and Anders, 1976, 1978). It does not require oxygen but is dependent upon glutathione and a glutathione-S-transferase enzyme, the products in vitro being formaldehyde and chloride ion. The rapid and almost quantitative conversion of formaldehyde to formic acid and then carbon dioxide known to occur in vivo (Neely, 1964) is consistent with this pathway being the source of carbon dioxide exhaled after exposure to methylene chloride. The intermediates involved in the metabolism of methylene chloride to formaldehyde are unknown, but the nature of the enzyme involved and the dependence upon glutathione suggest that S-chloromethylglutathione is formed and rapidly hydrolysed and degraded to glutathione and formaldehyde (Ahmed and Anders, 1978)(Fig 1).

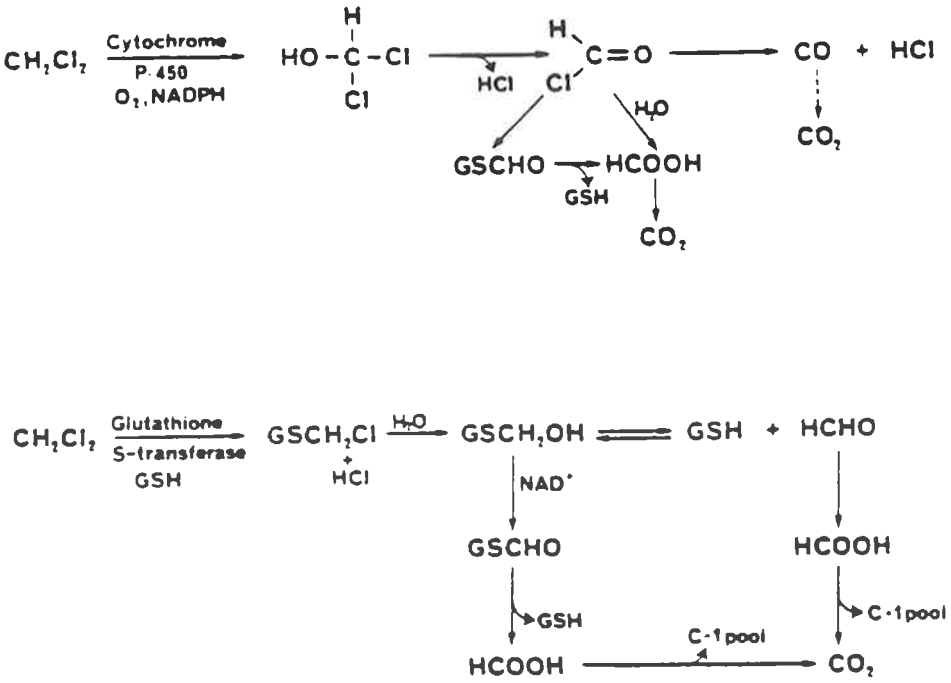

Fig 1. The proposed pathways of methylene chloride metabolism . (Adapted from Anders et al, 1977 and Gargas et al, 1986).

The chemistry of the S-chloromethyl thioethers (Bohme et al, 1949) and the lack of depletion of glutathione during this reaction are consistent with these conjugates being extremely transient. Formaldehyde, in addition to its metabolism to carbon dioxide, becomes incorporated into the C-1 metabolic pool via formic acid. Therefore exposure to radiolabelled methylene chloride results in the incorporation of radioactivity into macromolecules including nucleic acids.

At first sight it might appear that the relative molar amounts of carbon monoxide and carbon dioxide exhaled in vivo provide an index of the activity of the two metabolic pathways. Recent studies using metabolic inhibitors suggest that significant amounts of carbon dioxide are also derived from the oxidative cytochrome P-450 pathway (Gargas et al, 1986; Reitz et al, (1986). This is consistent with either hydrolysis of formyl chloride to formic acid or with formyl chloride reacting with glutathione to form S-formyl glutathione. The rapid enzymic and chemical breakdown of this conjugate (Uotila and Koivusalo, 1974a,b) would yield formic acid and hence carbon dioxide. Thus a quantitative correlation between the amount of carbon monoxide and carbon dioxide exhaled and the activity of the two pathways no longer appears to be valid.

There are three transient reactive intermediates in the metabolism of methylene chloride. Two of them, formyl chloride and S-chloromethyl-glutathione, are assumed to be present on the basis of knowledge of the metabolic pathways; the third, formaldehyde, has been identified in vitro. All three have the reactivity necessary to bind covalently to macromolecules. Of these S-chloromethyl glutathione is potentially the most potent alkylating agent, a conclusion based on the known reactivity of the halothioethers (Bohme et al, 1949), structural similarities to the mutagenic glutathione conjugates of the 1,2 dihaloethanes and on the outcome of several studies using different liver fractions in the Salmonella mutation assay (Green 1983; Jongen et al, 1982). Formyl chloride is highly unstable existing chemically only at low temperatures (-80°C) in inert solvents (Staab and Datta, 1964). Formaldehyde is a common metabolic product in vivo which is efficiently metabolised in the

liver to formic acid. The endogenous formation and metabolism of formaldehyde occurs at a high rate and the additional formaldehyde derived from methylene chloride would be metabolised by the same efficient pathways. It is therefore unlikely that formaldehyde formed intracellularly, where there is already a substantial metabolic pool, will act as a carcinogen.

The capacity of these intermediates to act as alkylating agents appears to be limited, presumably because of their unstable nature and extremely short half-lives (Bohme et al, 1949; Staab and Datta, 1964). Covalent binding of reactive intermediates to cellular macromolecules has been investigated in several studies and evidence of binding is confined to protein and lipid and does not involve nucleic acids (Anders et al, 1977; Cunningham et al, 1981). The interpretation of these studies, which detected only limited binding, may be confounded by incorporation of radioactivity into the macromolecule through the C-1 pool. Thus the presence of radioactivity in proteins and lipids after administration of ^{14}C-methylene chloride may be due to binding or to incorporation of ^{14}C via the C-1 pool or a combination of both.

4. MUTAGENIC ACTIVITY

A volatile compound, such as methylene chloride, presents considerable problems to the experimentalist using in vitro methods because of the difficulty of controlling the exposure levels. Negative results may be due to inadequacy of techniques of maintaining the concentration of methylene chloride over a suitable period of time. In addition extremely high concentrations, which are not representative of in vivo circumstances, may produce results which are irrelevant to assessment of hazard in vivo. These factors influencing dosimetry must be taken into account when assessing the results from in vitro mutagenicity studies.

(a) Gene Mutation

Methylene chloride is mutagenic when tested using the Ames protocol in Salmonella typhimurium strains TA98 (Gocke et al, 1981; Kirwin et al, 1980) TA100 (Gocke et al, 1981; Green, 1983; Jongen et al, 1978) and in TA1535 (Nestmann et al, 1980). Positive results were observed at atmospheric concentrations above 6000ppm (Table 1) following exposure in air tight containers appropriate to experiments on volatile chemicals.

The mutagenic activity of methylene chloride in Salmonella is observed both with and without the presence of a metabolic activation system. The addition of S9 produced only a small increase in the mutagenic activity of methylene chloride (Simmon and Tardiff, 1977) when compared with chlorofluoromethane (Green, 1983) and the cytosolic fraction was more active than the microsomal fraction in this respect (Green, 1983). Further work by Jongen (1984) confirms that after 72 hours exposure there was only a slight enhancement of mutagenic activity by S9, but that there was a larger increase in mutagenicity at 6 hours, although the amount of the increase (148%) was still substantially less than that observed with other mutagens which require metabolic activation.

Methylene chloride is metabolised by Salmonella to CO and CO_2 (Green, 1983) and it is likely that the mutagenic activity seen in Salmonella follows the conversion of methylene chloride to an active metabolite by bacterial enzymes. The relatively small increase in mutagenic activity produced by rat-liver post-mitochondrial supernatant is predominantly the consequence of cytosolic enzyme activation, particularly glutathione conjugation catalysed by cytosolic glutathione-S-transferase. The direct reaction of glutathione with methylene chloride in the mutagenicity assay only produced a very small enhancement of mutagenicity (Jongen et al, 1982). The relatively small enhancement of

mutagenic activity produced by liver fractions and particularly the microsomal fraction containing cytochrome P-450 enzymes, is likely to be due to the instability of the postulated reactive intermediates, particularly formyl chloride, which has an extremely short half-life. When produced by bacterial metabolism of methylene chloride the mutagenic activity results because of the close proximity of bacterial DNA to the metabolising enzymes where the active metabolites are generated.

The mutagenic activity of methylene chloride has been studied in a variety of other microbial systems. Positive results have been reported in E. coli WU361089 (tyrosine prototrophy) and in E. coli K49 (prophage induction) but no mutagenicity was observed in E. coli Sd-4 (forward mutation)(Osterman-Golkar et al, 1983). The authors point out that these results are only qualitative. Negative results were also reported in E. coli Sd-4 by Turtoczky and Ehrenberg (1969) and in the B. subtilis rec assay (Kanada and Uyeta, 1978).

The findings in studies using various strains of yeast (Saccharomyces cerevisiae) demonstrate the importance of metabolic activation of methylene chloride in microorganisms. Experiments with strain D4 (used to monitor gene conversion) and D7 (for mitotic conversion and gene conversion) where methylene chloride exposure was for 4 hours produced a "marginally" positive result. However, a one-hour exposure of strain D7 resulted in a positive response (Callen et al, 1980), under conditions of log-phase growth when cytochrome P-450 enzyme levels were up to 5 times higher than in strain D4, which provided a negative response. The mutagenic results in D7 occurred at toxic doses (360,000ppm) in which survival of the yeast cells was reduced to 42%. In a further mitotic recombination assay using strain D3 negative results were reported but few details were given (Simmon et al, 1977).

The results of mammalian cell gene mutation assays do not indicate mutagenic activity with methylene chloride. Negative results were reported in the mouse lymphoma assay (L5178Y/thymidine kinase locus) (Thilagar et al, 1984) and in CHO and V79 cells (HGPRT locus) (Jongen et al, 1981) at doses of up to 5% equivalent to 10,000ppm (Table 1).

In vivo gene mutation assays with methylene chloride have not been reported.

(b) Chromosomal Effects

Studies on chromosome morphology in mammalian cells in culture have shown that methylene chloride is clastogenic. Chromosome alterations were observed after exposure to methylene chloride in CHO cells (Thilagar and Kumaroo, 1983) human lymphocytes and L5178Y mouse lymphoma cells (Thilagar et al, 1984) with or without metabolic activation. Chromatid damage and chromosomal exchanges were observed but there was no increase in sister-chromatid exchanges (SCE) in any of the cell types. A small (but statistically significant) increase in SCEs, which was more than double the control value but was without clear evidence of a dose response, was observed in Chinese hamster V79 cells (Jongen et al, 1981). This response was observed with and without the addition of a metabolic activation system and did not increase in time or when the dose was increased to toxic levels. [In CHO cells a dose-related and statistically significant ($p<0.01$) increase in SCEs was observed at a concentration of 7% after 24 hours exposure (McCarroll et al, 1983). Exposure of shorter duration (2, 4 or 10 hours) was without effect. These data cannot be evaluated as they are reported in abstract form only].

Chromosomal mutation assays in Drosophila have also given conflicting results. Negative findings were reported by Filippova et al (1967), Abrahamson and Valencia (1980) and Kramers et al (1983) in the sex linked recessive lethal test. A study in

Drosophila using the BASC test (Muller-5, Bar White Apricot Scute test) for recessive lethal mutations in which adults were fed 125 or 620mM methylene chloride resulted in a marginal increase in sex linked recessive lethals (Gocke et al, 1981). This study may not be reliable because control values from different solvent treatments were pooled and because the results on methylene chloride were not repeated when a more strongly positive response in an experiment with zinc sulphate was shown to be in error. The increase in recessive lethals occurred only in the first breed, was significant only when results from the 2 doses were pooled and were only double the control value.

In vivo mutagenicity assays have been reported in both mice and rats. Large doses of methylene chloride (two doses of 425, 850 and 1700mg/kg) given intraperitoneally to mice produced a negative response in the micronucleus test (Gocke et al, 1981). Doses of up to 4000mg/kg by gavage (the maximum tolerated dose) were also negative in the micronucleus assay in C57B16J mice (Sheldon et al, 1987). No increase in chromosomal aberrations was observed in rat bone marrow cells after 6 months exposure of 6hrs per day, 5 days per week to 500, 1000 or 3500ppm (Burek et al, 1984).

(c) Miscellaneous Test Systems

Unscheduled DNA synthesis (UDS) is a measure of reparative (rather than replicative) synthesis consequent on damage to DNA. UDS, measured as the uptake of tritiated thymidine into non-dividing cells, can be estimated in both in vitro and in vivo systems. Concentrations of up to 16mM methylene chloride failed to induce UDS in cultured rat hepatocytes although some reduction in replicative DNA synthesis occurred at the higher doses (Andrae and Wolff, 1983). A "marginal" positive result was reported in a primary rat hepatocyte UDS assay, but no details are available (Thilagar et al, 1984). Concentrations of up to 5% methylene chloride failed to induce UDS in human fibroblasts (AH cells) or hamster V79 cells (Jongen et al, 1981). In these

latter experiments non-specific inhibition of replicative DNA synthesis was observed in both cell lines probably due to a metabolic block of synthesis. Methylene chloride did not induce UDS in human lymphocytes in culture at doses up to 10µl/ml in the presence or absence of rat liver S-9 (Perocco and Prodi, 1981) and under experimental conditions in which other volatile compounds showed positive results.

Methylene chloride failed to induce unscheduled DNA synthesis (UDS) in male AP:Alpk rats when administered by gavage in corn oil at dose levels up to 1g/kg. Sampling times of 4 or 12h following exposure were evaluated (Ashby and Trueman, 1987). Similarly, negative responses were observed in male Fischer 344 rats and B6C3F1 mice exposed to 2000 or 4000ppm of methylene chloride by inhalation for periods up to 6h (Ashby and Trueman, 1987, Trueman et al, 1986). In concomitant experiments with B6C3F1 mice, using similar inhalation protocols, methylene chloride gave limited, but statistically significant evidence of the induction of mitosis in the liver 36h after exposure (Lefevre and Ashby, 1986). Further experiments are in progress to establish if this is a biologically significant effect (Ashby et al, unpublished).

Several studies have investigated the potential of methylene chloride and its metabolites to bind covalently to DNA. DNA has been isolated from the livers and salivary glands of rats and hamsters exposed to 3500ppm of C-14 methylene chloride (Schumann, 1984) and from the livers and lungs of mice and rats exposed to 4000ppm (Green et al, 1987a). In both studies the DNA was hydrolysed and analysed by chromatography to distinguish between alkylations of DNA and incorporation of radioactivity through the C-1 pool. No evidence of alkylation was found; both studies having the power to detect one alkylation in 10^6 nucleotides. These observations confirmed those of earlier in vitro studies (Anders et al, 1977, Cunningham et al, 1981) which also failed to detect DNA binding but were able to measure binding to proteins and lipids.

Methylene chloride has been tested for its ability to induce transformation in a variety of cell systems. Negative results were obtained in C3H-10T ½ CL8 mouse cells at 10µl/ml and in BALB/C-3T3 mouse cells at 0.01%. After positive results had been obtained with one sample of methylene chloride, repeated negative results were obtained with a higher purity sample in the Fischer rat embryo cell line inducible for leukaemia virus transformation (Broome and Sivak, 1986). Methylene chloride, however, significantly enhanced the viral transformation frequency by SA7 virus in a dose-related manner (Hatch et al, 1983). This response may well relate to the chromosome lesions induced by methylene chloride, because it has been shown that damage to the integrity of the host DNA in this sytem enhances viral transformation of the cells by the SA7 virus (Broome and Sivak, 1986).

(d) Evaluation

There is an extensive data base on which to assess the mutagenicity of methylene chloride. In all in vitro systems, it is essential that appropriate conditions are used to take account of the volatility of methylene chloride. Under appropriate exposure conditions (see Table 1) there is evidence of mutagenic activity only in specific assay systems.

Methylene chloride is mutagenic in prokaryotic micro-organisms with or without mammalian liver metabolism (Salmonella and E. coli). The evidence (from the stable isotope effect and metabolism by Salmonella) strongly points to the conclusion that positive results are consequent on metabolism of methylene chloride by bacterial enzymes to formyl chloride or S-chloromethyl glutathione, both very short-lived proximate mutagens. In eukaryotic microorganisms (yeast) negative or weakly positive results were obtained, the positive results being in the strain (D7) when survival was poor.

The results of studies of gene mutation and UDS in mammalian cells were uniformly negative. In mammalian cells, the only positive findings are induction of chromosomal aberrations in a variety of cells (human, hamster and mouse). As can be seen from Table 1, the concentrations used in the in vitro chromosomal studies were exceptionally high (up to 185,000ppm) in comparison with doses used in both in vitro and in vivo mutagenicity assays. Recent work on aprotic organic solvents has shown that similarly high concentrations can interfere with tubulin condensation in yeast (Zimmerman et al, 1985); a similar non-DNA specific mechanism may explain the positive results in these in vitro chromosomal assays with methylene chloride. However, negative or equivocal results which do not meet the normal criteria for a positive result, were obtained for SCE induction in these cells.

Studies of the mutagenicity of methylene chloride in whole animal systems also provide evidence of a lack of an effect. In Drosophila, a single unsubstantiated positive result was observed which failed to meet the normal criteria for a positive result. In mammals there is no evidence of mutagenic activity in a mouse bone marrow micronucleus assay, in circulating peripheral lymphocytes of rats, in UDS in mice or rats, or from DNA binding studies in lung of mice or rats, liver of mice, rats or hamsters, salivary gland of rats and hamsters. This is a fairly comprehensive range of in vivo assays at toxic or near toxic doses which provides convincing evidence of the absence of mutagenicity (or of interaction with DNA) of methylene chloride in mammals.

TABLE 1

RESULTS FROM MUTAGENICITY ASSAYS OF METHYLENE CHLORIDE

Test System	Result	Concentration (ppm)	Reference
MICROBIAL			
Salmonella typhimurium	+	6,000[a]	Jongen et al, 1982
(TA100, 98, 1535)	+	10,000	Green, 1983
	+	6,000	Gocke et al,1981
	+	Not known	Kirwin et al, 1980
	+	Not known	Nestmann et al, 1980
E. coli			
Sd4	-	Not known	Osterman-Golkar et al, 1983
WU361089 Tyr	+	Not known	Osterman-Golkar et al, 1983
K39	+	Not known	Osterman-Golkar et al, 1983
SACCHAROMYCES CEREVISIAE			
D3	-	Not known	Simmon et al, 1977
D4	-	Not known	Callen et al, 1980
D7	+	360,000	Callen et al, 1980
MAMMALIAN CELLS POINT MUTATIONS			
CHO/HGPRT	-	50,000[b]	Jongen et al, 1981
VT9/HGPRT	-	50,000	Jongen et al, 1981
L5178Y/TK	-	Not known	Thilagar et al, 1984

TABLE 1 - continued

RESULTS FROM MUTAGENICITY ASSAYS OF METHYLENE CHLORIDE

Test System	Result	Concentration (ppm)	Reference
CHROMOSOMAL EFFECTS			
In Vitro			
CHO cells	+	185,000	Thilager and Kumaroo, 1983
Human peripheral lymphocytes	+	Not known	Thilager et al, 1984
L5178Y	+	Not known	Thilager et al, 1984
SCE			
CHO cells	-	185,000	Thilagar and Kumaroo, 1983
CHO cells	±	70,000	McCarroll et al, 1983
Human lymphocytes	-	Not known	Thilagar et al, 1984
L5178Y	-	Not known	Thilagar et al, 1984
V79	±	10,000[a]	Jongen et al, 1981
In Vivo			
Drosophila	-	Not known	Filippova et al, 1967
Drosophila	-	Not known	Abrahamson and Valencia, 1980
Drosophila	±	Close to LD 50	Golke et al, 1981
Drosophila	-	5,500	Kramers et al, 1983
Rat bone marrow chromosomes	-	6 months x 3500ppm	Burek et al, 1984
Mouse micronucleus	-	2x1700mg/kg	Gocke et al, 1981
Mouse micronucleus	-	1x4000mg/kg	Sheldon et al, 1987

TABLE 1 - continued

RESULTS FROM MUTAGENICITY ASSAYS OF METHYLENE CHLORIDE

Test System	Result	Concentration (ppm)	Reference
UDS			
In Vitro			
Rat hepatocyte	-	4,000	Ashby and Trueman, 1987
Rat hepatocyte	-	>70,000	Andrae and Wolff, 1983
Hamster V79	-	50,000	Jongen et al, 1981
Human AH	-	50,000	Jongen et al, 1981
Human lymphocyte	-	Not known	Perocco and Prodi, 1981
In Vivo			
Rat liver	-	4,000	Ashby and Trueman, 1987
Mouse liver	-	4,000	Ashby and Trueman, 1987
In Vivo DNA Binding			
Hamster salivary gland	-	3,500	Schumann et al, 1984
Hamster liver	-	3,500	Schumann et al, 1984
Rat salivary gland	-	3,500	Schumann et al, 1984
Mouse liver	-	4,000	Green et al, 1987a
Mouse lung	-	4,000	Green et al, 1987a
Rat liver	- -	4,000 3,500	Green et al, 1987a Schumann et al, 1984
Rat lung	-	4,000	Green et al, 1987a

a. Minimum concentration for a positive result.
b. Maximum concentration for a negative result.

The concentrations have been expressed in ppm to allow a comparison between studies. Where not stated directly, such ppm values have been calculated from the data available, assuming complete volatilisation of methylene chloride into the headspace (at 37°C), and that the concentrations are less than that giving a saturated vapour concentration.

5. ANIMAL CARCINOGENICITY EXPERIMENTS

Methylene chloride has been administered to animals in 7 long-term carcinogenicity studies in rats, mice and hamsters. The salient features of 5 of the studies are given in Table 2.

In the first Dow study (Burek et al, 1984) there was an increased incidence of sarcomas of the ventral mid-cervical area in the region of the salivary gland in the 3500ppm group of male Sprague-Dawley rats. Careful examination of the data on these neoplasms shows that, while the neoplasms are all of mesenchymal origin, they represent a variety of tumour types (Table 3). It may therefore be inappropriate to group them together for the purpose of statistical analysis. The fact that no neoplastic response was reported at this site in any of the other studies that have been carried out tends to confirm that the observation in the experiment by Burek et al (1984) was probably not a reproducible methylene chloride-related effect. In the same experiment, however, a concentration related increase in the number of benign mammary neoplasms per rat was observed in female rats but there was no increase in the number of rats with these neoplasms.

The second Dow inhalation study (Nitschke et al, 1982) at lower concentrations confirmed in general the results of the first study. There was no statistically significant increase in malignant tumours of any type (including sarcomas of the mid-cervical region) although there was an increase in benign mammary tumours. In the National Coffee Association drinking water study in F344 rats (Serota et al, 1986a) which had doses up to 250mg/kg/day, no increase in the incidence of any neoplasms was observed. Essentially similar results were observed in the NTP inhalation study in F344 rats, although the number of female rats with benign mammary neoplasms, as well as the number of neoplasms per rat, were increased in a concentration-related fashion. There was also a slight increase in the incidence of leukaemia in female rats against a background of over 30% incidence in the control groups; a small increase of this type in a frequently occurring tumour is unlikely to indicate a carcinogenic hazard to man.

In mice different results were obtained in the two studies reported. There was no increase in the incidence of neoplasms in the drinking water study even at 250mg/kg/day, the top dose used in the National Coffee Association study (Serota et al, 1986b). There is some question as to whether the MTD has been achieved as there was no decrease in bodyweight gain or increase in mortality in this study. However, histopathological changes were observed in the livers of animals at the highest concentration and it is doubtful if they would have survived a substantially higher concentration. In the NTP study, using the same B6C3F1 mouse strain, clear evidence of a liver and lung neoplastic response was obtained in both males and females.

In hamsters there was no increase in the incidence of neoplasms at concentrations up to 3500ppm in a life-time inhalation study (Burek et al, 1984). In this study there was no adverse effect on bodyweight gain or survival but taking all parameters into account 3500ppm can still be considered an acceptable upper concentration.

There have been a number of other carcinogenicity studies carried out and reported in the literature on methylene chloride. These include a dermal study in NMRI mice (Muller, 1968), an intraperitoneal study in strain A mice (Theiss et al, 1977) and an NTP oral gavage study in Fischer 344 rats and B6C3F1 mice which was not published due to irregularities in the conduct of the study. In all three of these studies the design or conduct of the experiments were such that no conclusions as to the carcinogenicity of methylene chloride can be made.

6. THE RELEVANCE OF RAT MAMMARY CARCINOGENESIS TO HUMAN HAZARD ASSESSMENT

Methylene chloride administered by inhalation increased the incidence and/or the number of benign mammary neoplasms (fibroadenomas) in F344 and Sprague-Dawley rats (Table 2). In the Sprague-Dawley rats lifetime administration of doses of 500ppm and above increased the number of benign neoplasms per rat, but no increase was seen at 50 and 250ppm. In the F344 rats increases in the number of fibroadenomas per animal and the incidence of rats with fibroadenomas occurred at all dose levels (1000, 2000 and 4000ppm lifetime exposure). It is noteworthy that there was no increase in the number of malignant neoplasms in either of these studies.

Methylene chloride administered for a lifetime in drinking water at levels of up to 250mg/kg/day did not increase mammary neoplasms in F344 rats. No increase in mammary neoplasms were seen in mice or hamsters (Table 2).

The dependence of rat mammary tumours upon pituitary hormones has been established unequivocally (Welsch and Nagasawa, 1977; Welsch, 1985). In the rat, prolactin acts as both an "initiator" and "promoter" of mammary carcinogenesis. There is good evidence that increased prolactin levels increase the incidence of mammary cancer (eg grafting of multiple pituitaries into Sprague-Dawley rats increases the incidence of mammary cancer (Welsch et al, 1970) and there is a positive correlation between elevated blood prolactin levels and mammary tumours in aged R-Amsterdam female rats (Kwa et al, 1974)). Treatments that induce hyperprolactinaemia in female rats which have received carcinogens induce a dramatic increase in tumour incidence. These treatments include adrenalectomy, pituitary homografts and high dietary fat (Welsch and Nagasawa, 1977).

The mechanism by which methylene chloride induces mammary adenoma is important for human hazard assessment. Female Sprague-Dawley rats administered methylene chloride have a high blood level of prolactin (Breslin and Landry, 1986). In common with the response to other agents which act via hyperprolactinaemia, the methylene chloride-induced response is of benign neoplasms only. There is no evidence for binding of methylene chloride to the DNA of other tissues and hence it seems unlikely that it will bind to mammary tissue when the primary site of metabolism is in the liver. It seems most likely, therefore, that the increased incidence of mammary adenomas is the result of an indirect mechanism operating via hyperprolactinaemia.

This mechanism apparently does not operate in hamsters or mice at comparable dose levels as there is no increase in mammary adenoma.

The relevance of these findings to human hazard assessment is unclear. In women there is conflicting evidence on whether or not mammary neoplasms are as responsive to prolactin as is the case in the rat (Sinha, 1981). The rat has elevated levels of prolactin when fed ad libitum in comparison to a restricted dietary regimen and this may explain why the mammary tumour incidence is so easily responsive to a variety of environmental and other effects.

The mechanism of production of mammary tumours involving hyper-prolactinaemia will only occur at doses of methylene chloride which affect prolactin levels.

There is no direct information on prolactin levels at low doses, but no increase in mammary adenomas was observed at low doses in the inhalation or drinking water studies. This is compatible with the absence of an effect of methylene chloride on prolactin levels at lower doses (ie below 250ppm).

The evidence on mechanism of action, although not conclusive, taken together with the absence of a carcinogenic effect in mice and hamsters suggests that the findings in rats are not of relevance to low dose exposure in humans.

TABLE 2
SUMMARY OF AVAILABLE ANIMAL CARCINOGENICITY STUDIES ON METHYLENE CHLORIDE

Reference	Species/ Strain/Sex	Route of Administration/ Protocol/Group Size	Doses	Observations
Burek et al (1984)	Rat: Sprague-Dawley Male and female	Inhalation : 6hr/day, 5 days/week for 2 years: 95/group.	Control, 500, 1500, 3500ppm	'Salivary gland' tumours - see Table 3 Benign mammary tumours - no increase in number of female rats with benign mammary tumours but increase in total number of benign mammary tumours in female rats exposed to methylene chloride therefore increase in number of benign mammary tumours/ tumour bearing female rat. Effect is concentration related.
	Hamster: Male and female	Inhalation : 6hr/day, 5 days/week for 2 years: 95/group.	Control, 500, 1500, 3500ppm	No effect on tumour incidence.
Nitschke et al (1982)	Rat: Sprague-Dawley Male and female	Inhalation : 6hr/day, 5 day/week for 20 months (male) or 24 months (female) 90 animals/sex/ exposure concentration 5/group killed at 6, 12, 15 or 18 months	Control, 50, 250, and 500ppm (Further group of 30 females exposed to 500ppm for 12 months, with recovery for 12 months - further group of 30 females exposed to 500ppm during second 12 months of lifetime).	Increase in number of benign mammary tumours/ tumour bearing female rat exposed to 500ppm. No effect on 'salivary gland' tumours in males.

TABLE 2 - continued

SUMMARY OF AVAILABLE ANIMAL CARCINOGENICITY STUDIES ON METHYLENE CHLORIDE

Reference	Species/ Strain/Sex	Route of Administration/ Protocol/Group Size	Doses	Observations
Serota et al, 1986a	Rat: F344 Male and female	Drinking water ad lib for 104 weeks 85 animals/sex/dose scheduled kills 5 at 26 weeks 10 at 52 weeks 20 at 78 weeks Also additional groups of controls and 250mg/kg (50/sex) which received methylene chloride for 78 weeks only.	0, 5, 50, 125 or 250 mg/kg/day	No increase in incidence of neoplasms. Survival and other findings not affected by methylene chloride. Significant decreases in bodyweight gain at 125 and 250mg/kg/day and evidence of liver damage at doses above 50mg/kg/day.
Serota et al, 1986b	Mouse: B6C3F1	Drinking water ad lib for 104 weeks Group size, 125 m 100 f 200 m 100 f 100 m 50 f 100 m 50 f 125 m 50 f	mg/kg/day 0 60 125 185 250	No increase in incidence of neoplasms. Evidence of slight liver damage at 250mg/kg/day.

TABLE 2 - continued.

SUMMARY OF AVAILABLE ANIMAL CARCINOGENICITY STUDIES ON METHYLENE CHLORIDE

Reference	Species/ Strain/Sex	Route of Administration/ Protocol/Group Size	Doses	Observations
National Toxicology Program (TR306)	Rat: F344 Male and female	Inhalation : 6hr/day, 5 days/week for 102 weeks 50/group	Control 1000, 2000, 4000ppm	Dose-dependent increase in benign mammary neoplasms (male 0/50, 0/50, 2/50, 5/50 female 7/50,13/50,14/50,23/50).
National Toxicology Program (TR306)	Mouse: B6C3F1 Male and female	Inhalation : 6hr/day, 5 days/week for 102 weeks 50 group	Control 2000 and 4000ppm	Dose-dependent increase in alveolar/bronchiolar adenomas (males 3/50, 19/50, 24/50 females 2/50, 23/48, 28/48) alveolar/bronchiolar carcinomas (males 2/50, 10/50, 28/50 females 1/50, 13/48, 29/48) hepatocellular adenoma and carcinoma combined (males 22/50, 24/49, 33/49 females 3/56, 16/48, 40/48)

TABLE 3

THE FREQUENCY OF OCCURRENCE OF TUMOURS IN THE REGION OF THE SALIVARY GLAND IN MALE RATS EXPOSED TO METHYLENE CHLORIDE

Tumour Type	Controls (n = 95)	1500ppm methylene chloride (n = 95)	3500ppm methylene chloride (n = 95)
(a) Subcutaneous neoplasms			
Undifferentiated sarcoma	1	0	1
Pleomorphic sarcoma	0	0	3
Round cell sarcoma	0	2	1
Fibrosarcoma	0	0	2
Neurofibrosarcoma	0	0	2
(b) Salivary gland tumours			
Malignant schwannoma	0	2	0
Undifferentiated schwannoma	0	1	0
Carcinosarcoma	0	0	1
Fibrosarcoma	0	0	1

Taken from Burek et al, 1984.

7. FACTORS INFLUENCING THE INDUCTION OF NEOPLASMS IN MICE

Of the three species used to evaluate the carcinogenicity of methylene chloride, the B6C3F1 mouse, with the highest basal metabolic rate and known susceptibility to liver and lung tumours, is predictably the most sensitive species. However the degree of specificity in the development of lung and liver tumours after exposure to methylene chloride is remarkable and suggests a causal event, or more likely, a combination of circumstances which are unique to this species. Similar species specificity has been seen with several other chlorinated solvents indicating that this mouse strain is uniquely sensitive to this class of chemical. Although methylene chloride is genotoxic in some microorganisms the lack of measurable genotoxicity in mammalian cells both *in vitro* and *in vivo* suggest other contributing factors which either enhance an undetectable level of DNA alkylation or cause cancer by an epigenetic mechanism. The increased incidence of lung and liver tumours which are morphologically identical to those in control animals, suggests a promotional mechanism. Promotion is often associated with cytotoxicity, cellular regeneration or cell division as the stimulus for an increased expression of the high background tumour incidences found in B6C3F1 mice.

The primary physiological disturbance in all animals and humans exposed to methylene chloride is the formation of carboxyhaemoglobin (COHb). Levels of carboxyhaemoglobin are however limited to around 12% in rats and mice by saturation of the enzyme that metabolises methylene chloride to carbon monoxide. This was found to be the case in rats and mice exposed to methylene chloride at the dose levels used in the NTP study (Green *et al*, 1987b). Similar studies in the hamster (Burek *et al*, 1984) have shown COHb levels of approximately 30%. The physiological consequences of these levels of carboxyhaemoglobin on the liver and lungs of mice and the lack of any major species differences in the levels between rats and mice together with the higher levels found in the hamster, indicate that carbon monoxide formation is unlikely to be related to the development of tumours in mice. Although methylene

chloride is cytotoxic to the livers of mice at high dose levels (Weinstein et al, 1972) it was not cytotoxic to the livers of either rats or mice exposed for 10 days at the dose levels used in the NTP 2-year study (Hext et al, 1986). However at these dose levels and under conditions of continuous exposure at lower dose levels (Kjellstrand et al, 1986), significant increases in relative liver weight were seen in exposed mice but not rats. Species specific effects were also seen in the lungs of mice exposed to methylene chloride (Hext et al, 1986). A lesion was seen in the Clara cell of the mouse lung after a single exposure to either 2000 or 4000ppm methylene chloride which was not seen in the rat under the same conditions. There is therefore clear evidence that both target organs respond differently to methylene chloride in the mouse than they do in the rat. Although the relevance of these observations to the subsequent development of lung and liver tumours is uncertain, the possible contribution of liver growth and pulmonary cytotoxicity to the development of cancer in the B6C3F1 mouse cannot be ignored.

In addition to the specific effects on mouse lung and liver, the mouse, as the smallest and most metabolically active of the species used in the cancer studies might be expected to metabolise inhaled methylene chloride to a significantly greater extent than the other species. This has been found to be so with several other volatile chlorinated solvents and in each case the mouse was, for its size, exposed to significantly higher concentrations of the metabolites of these chemicals than other species (Stott et al, 1982; Schumann et al, 1980; Prout et al, 1985; Brown et al, 1974). As a result the mouse will be exposed to a higher body burden of reactive metabolites and thus will be at increased risk from those chemicals which require metabolic activation to exert their harmful effects.

Until recently little was known about the relative rates of metabolism of methylene chloride in the mouse and other species, particularly at the high exposure levels used in the carcinogenicity bioassays. Comparative studies in the species and at the dose levels used in carcinogenicity studies would allow a direct correlation to be drawn between the fate of a chemical and the development of cancer. Such

studies are particularly useful when species differ in the way they respond to a chemical. Identification of the key interactions with the organism, whether they arise from the parent chemical or a particular metabolite, allows selection of the most appropriate animal model and also facilitates the use of the internal dose rather than external dose, in the risk assessment process. Confidence in risk extrapolation between species is directly related to the available database in animals and in man. Unfortunately adequate data in relevant animals and at relevant doses rarely exists. Methylene chloride is perhaps unique in the depth of information now available from animals and from man that can directly be used in risk assessment. Comprehensive animal studies (Green et al, 1987b,c) have been completed in the mouse and the rat. Comparative human and hamster data is also available from in vitro studies (Green et al, 1987c).

8. COMPARATIVE METABOLISM AND PHARMACOKINETICS

Recent reviews (eg EPA 1985) on the metabolism of methylene chloride have concluded that the data available at the time of the review was inadequate for a variety of reasons, but principally that there were "no pharmacokinetic/metabolism data that:

- indicate species similarities or differences at high doses.
- relate the carcinogenic response at doses used in this NTP bioassay to metabolic saturation.
- allow for the determination of the relationship between administered dose and the effective dose, and
- allow for determining the concentration or biological half-life of the reactive intermediates responsible for the carcinogenic response."

The data available at that time did allow a number of conclusions about the metabolism and pharmacokinetics of methylene chloride. Among the most important were:

(a) Methylene chloride is metabolized via two pathways. One pathway is catalysed by microsomal monooxygenases (cytochrome P-450 enzymes), requires oxygen and yields carbon monoxide as an end product. The other pathway is initiated by conjugation with glutathione, catalysed by cytosolic glutathione-S-transferase, does not require molecular oxygen and yields formaldehyde and carbon dioxide as end products. The pathways were reviewed in more detail in Section 2.

(b) Both pathways probably have highly reactive short-lived metabolites (formyl chloride for the cytochrome P-450 pathway and S-chloromethyl-glutathione for the glutathione pathway). However the chemical reactivity and short-lived nature of these metabolites precludes confirmation by chemical analysis of their presence in biological tissue.

(c) The liver is the primary site of metabolism.

(d) There was evidence for metabolic saturation at high doses of methylene chloride; a non-linear relationship was shown between administered dose of methylene chloride and total carbon monoxide and carbon dioxide exhaled and a less than proportional increase in tissue levels of radioactivity. There was insufficient data to deduce the precise levels at which saturation occurs.

(e) The microsomal cytochrome P-450 pathway appears to be a high affinity low capacity pathway while the cytosolic glutathione conjugation pathway is a low affinity, high capacity pathway. These data, based on metabolic inhibitor studies, could not be compared directly with other experimental data on metabolism and pharmacokinetics.

(f) The interpretation of the metabolic studies on methylene chloride was based on the assumption that the activity of the cytochrome P-450 pathway could be monitored accurately by estimation of carbon monoxide (or carboxyhaemoglobin levels) and that the glutathione pathway could be monitored by estimation of exhaled carbon dioxide. Recent data shows that significant amounts of carbon dioxide are also derived from the cytochrome P-450 pathway (Gargas et al, 1986; Reitz et al, 1986), so that conclusions based on the belief that the relative molar amounts of CO and CO_2 exhaled provide an index of the activity of the two pathways may be in error.

In the meantime a series of studies has been carried out at the dose levels used by the NTP and in the species of interest for risk assessment purposes (Green et al, 1987b,c). These new studies provide data directly relevant to risk assessment and help to provide a better understanding of methylene chloride pharmacokinetics, metabolism and toxicity.

9. KINETICS OF THE METABOLISM OF METHYLENE CHLORIDE BY THE MICROSOMAL P-450 PATHWAY

a) In Vivo Studies

Green et al (1987b) have reported on the pharmacokinetics and metabolism of methylene chloride in Fischer 344 rats and B6C3F1 mice, the strains used in the NTP studies. Rats and mice were exposed to methylene chloride at doses of 500, 1000, 2000 and 4000ppm for 6 hours. The NTP study used doses of 1000, 2000 and 4000ppm.

The rates of metabolism via the cytochrome P-450 pathway were monitored by determining the carboxyhaemoglobin levels and the exhalation of ^{14}CO after exposure to methylene chloride had ceased.

Methylene chloride is metabolised by this pathway to a similar extent in rats and mice as was shown by the similar levels of carboxyhaemoglobin (12%) in the two species and by the similar amount of carbon monoxide exhaled after cessation of exposure. Steady state levels of carboxyhaemoglobin were reached significantly faster in mice than rats suggesting a 3-fold greater rate of metabolism in mice.

There is clear evidence from these studies that the cytochrome P-450 monooxygenases are saturated at levels of 500ppm and above. Blood levels of methylene chloride were relatively low after 500ppm exposure but increased substantially at higher exposures (Table 4). Carboxyhaemoglobin levels were similar in both rats and mice at 500ppm and did not increase at higher doses. Indeed there was a considerable reduction in the level of carboxyhaemoglobin at 4000ppm when compared with 500ppm. The absence of further increases in carboxyhaemoglobin levels at exposures of methylene chloride above 500ppm, contrasts strongly with the much higher levels which can be produced from inhaled carbon monoxide providing clear evidence of saturation of the metabolic pathway. Saturation occurred at the same dose level in the hamster but the levels of carboxyhaemoglobin were found to be approximately 30% (Burek et al, 1984).

TABLE 4

METHYLENE CHLORIDE AND CARBOXYHAEMOGLOBIN LEVELS IN RATS AND MICE EXPOSED TO METHYLENE CHLORIDE

Exposure Level (ppm)	Rats			Mice		
	Blood Levels[1] (µg/ml)	AUC[2]	COHb[3] (%)	Blood Levels[1] (µg/ml)	AUC[2]	COHb[3] (%)
500	6	0.1	15	6	0.2	16
1000	62	1.0	14	31	1.0	13
2000	125	2.1	12	40	1.3	14
4000	230	3.7	10	54	2.1	8

1. Average blood levels between 3 and 6 hours after exposure commenced.

2. Area under the methylene chloride blood level curve, taking the 1000ppm value as 1.

3. Average carboxyhaemoglobin values between 3 and 6 hours after exposure commenced.

b) In Vitro Studies

Confirmation of these dose and species specific effects on methylene chloride metabolism were obained by studies in vitro. The affinity constants (Km) for the cytochrome P-450 pathway of rat and mouse liver were similar (Table 5) although the rate constant (Vmax) was higher (3x) in mouse than rat liver in agreement with the in vivo studies.

The in vitro metabolic studies were also carried out on hamster and human liver. Of the four species studied the hamster liver was the most active tissue in metabolising methylene chloride by

the cytochrome P-450 pathway, with Km values 3 times higher than rats and mice and Vmax values similar to those in mice (Table 5). The metabolism by human liver was of the same order as that of rat liver (Figure 2), although no rate constants were obtained.

Lung tissue is also capable of metabolising methylene chloride, the rate being greatest in mouse lung followed by hamster lung with rat lung at a much lower level (Fig 2).

A clear conclusion that derives from the study of relative metabolic rates in vivo and in vitro is that there is no correlation between the rate of metabolism via the cytochrome P-450 pathway and the susceptibility of the tissue or species to the development of cancer after methylene chloride exposure.

TABLE 5

KINETIC CONSTANTS FOR THE METABOLISM OF METHYLENE CHLORIDE BY THE CYTOCHROME P-450 PATHWAY

Species	Tissue	Km (mM)	Vmax (nmoles/min/mg protein)
Mouse	Liver	0.79	1.94
Rat	Liver	0.86	0.58
Hamster	Liver	2.83	1.85

Kinetic constants were not obtained for lung samples or for human liver samples.

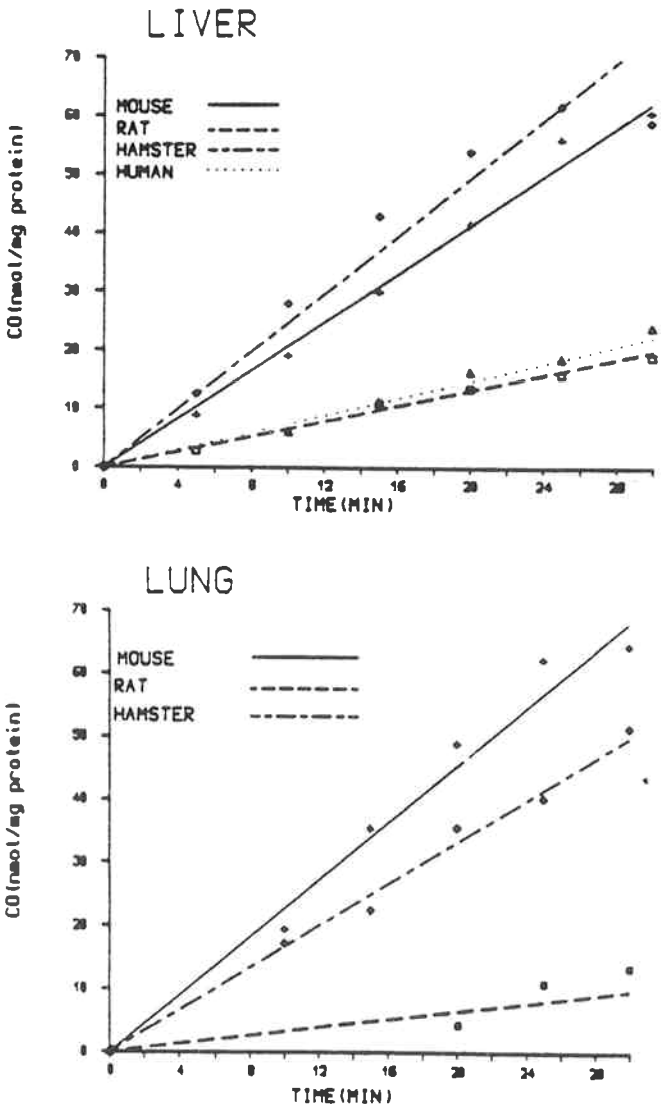

Fig 2. The metabolism of methylene chloride (15mM) by the cytochrome P-450 pathway in mouse, rat and hamster liver and lung tissue and in human liver tissue.
(Taken from Green et al, 1987c).

10. KINETICS OF THE METABOLISM OF METHYLENE CHLORIDE BY THE GLUTATHIONE PATHWAY

a) In Vivo Studies

The rates of metabolism of methylene chloride by the glutathione pathway were estimated from studies of the exhalation of $^{14}CO_2$ and from the levels of methylene chloride in blood in the same experiments described earlier (Green et al, 1987b).

The relative blood concentrations of methylene chloride increase more rapidly than the exposure concentrations in both rats and mice. The effect is particularly marked between 500ppm and 1000ppm when saturation of the cytochrome P-450 pathway has occurred. At the higher dose levels (1000-4000ppm) there was a linear increase in blood levels of methylene chloride, which leads to the conclusion that there is little glutathione metabolism occurring at dose levels over 500ppm. In contrast, for a 4-fold increase in dose from 1000-4000ppm in the mouse, there was only a doubling of the blood levels of methylene chloride indicating significant glutathione metabolism at high doses. Over the full dose range there was a 38-fold increase in blood levels in the rat compared to a 9-fold increase in mice, a clear illustration of the greater metabolic capacity of the mouse via the glutathione pathway.

Comparison of the expired carbon dioxide levels after exposure to 4000ppm methylene chloride had ceased showed that in the mouse the rate of metabolism to CO_2 was almost 10 times higher than in the rat. There was also a marked difference in the rate of clearance of methylene chloride from tissues, the mouse clearing methylene chloride more rapidly (in less than 2 hours) than the rat (up to 8 hours).

b) In Vitro Studies

The mouse liver has substantially higher kinetic constants for the metabolism of methylene chloride by glutathione conjugation than the rat; the affinity constant Km was 86mM for mouse and 21mM for rat and the rate constant Vmax was 36.4 nmoles/min/mg protein for mouse and 2.9 for rat (Table 6). It is of significance that no time or concentration dependent rate could be detected for hamster liver or for human liver for this pathway.

Comparison of the rate of metabolism in the different tissues (Fig 3) showed that the rate of metabolism by mouse liver was about 12 times that in rat liver. The rate in mouse lung was lower than that for rat liver. However, the site of metabolism in the mouse lung is likely to be the Clara cell, the only cell showing damage after methylene chloride exposure. The Clara cell forms only about 5% of the cells in the lung, and hence, if the metabolism of methylene chloride resides in the Clara cell, the rate of metabolism in that cell type must be about 20-fold higher than that measured for the whole lung. This would give a value similar to that in mouse liver.

In conclusion, methylene chloride has a much higher rate of metabolism via the glutathione pathway in the mouse at high dose levels than in any of the other species studied. Glutathione conjugation is only a minor pathway in the rat and appears to be absent in hamster and human tissues.

TABLE 6

KINETIC CONSTANTS FOR THE METABOLISM OF METHYLENE CHLORIDE BY THE GLUTATHIONE-S-TRANSFERASE PATHWAY

Species	Tissue	Km (mM)	Vmax (nmoles/min/mg protein)
Mouse	Liver	86	36.4
Rat	Liver	21	2.9
Hamster	Liver	------- no detectable rate -------	
Man	Liver	------- no detectable rate -------	

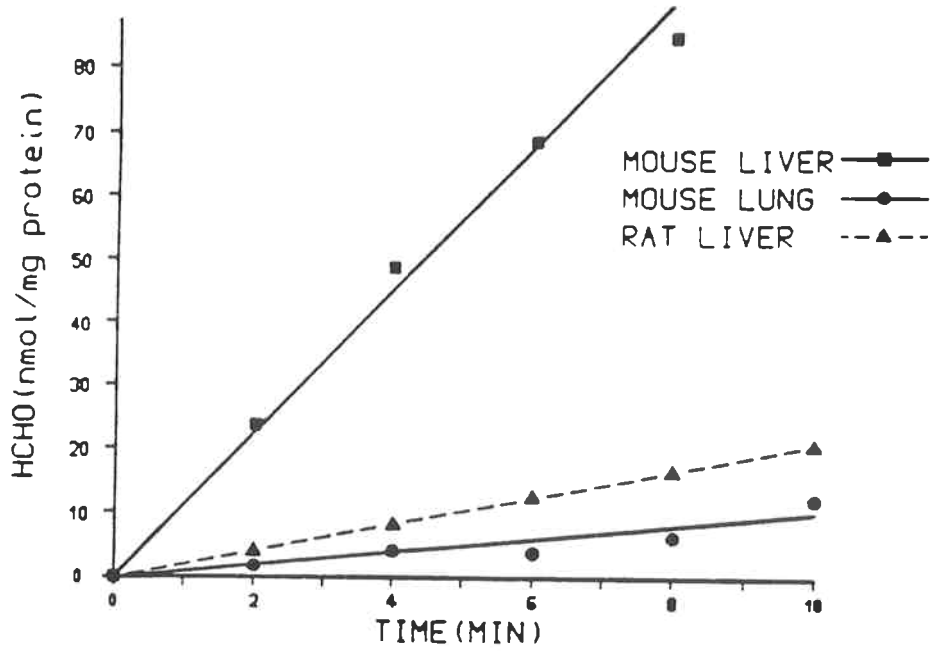

Fig 3. The metabolism of methylene chloride (35mM) by the glutathione-S-transferase pathway in mouse and rat liver and mouse lung. Metabolism by this pathway was not detected in hamster liver or lung nor in human liver.
(Taken from Green et al, 1987c).

11. ASSESSMENT OF HUMAN HAZARD FROM METHYLENE CHLORIDE

a) The Link Between Metabolism and Cancer

There is substantial evidence to suggest that methylene chloride requires metabolic activation to act as a carcinogen. Neither the structure nor the chemical reactivity of methylene chloride are those normally associated with direct acting carcinogens. Experimental evidence in rats and mice also supports this view. The specificity for a single cell type in the lung (Hext et al, 1987b) is not consistent with a direct acting chemical, particularly when that cell type, the Clara cell, is known to have high metabolic capacity (Boyd, 1980). The dose received by the Clara cell is the same in both rats and mice since the cells line the airways of the lung and are exposed to atmospheric concentrations of methylene chloride, yet only mouse Clara cells are damaged. Methylene chloride blood levels in the rat are 4 to 5-fold higher than those in mice (Green et al, 1987b). Consequently the rat liver, where tumours are not seen, is exposed to far more methylene chloride than the livers of mice where dose-related tumours are seen. Finally the marked species variability in 2 year studies, and in short term tests, is not consistent with the response normally seen with direct acting carcinogens.

The comparative animal studies (Green et al, 1987b,c) have provided evidence that metabolism by the glutathione pathway is linked to the carcinogenicity of methylene chloride in the mouse. This conclusion was reached after detailed comparisons of the relative utilisation of the two metabolic pathways by rats and mice (Green et al, 1987b,c) and similar comparisons between rat, mouse and hamster tissues in vitro (Green et al, 1987c). The oxidative pathway was shown, to be very similar both qualitatively and quantitatively in rats and mice. Furthermore hamster liver tissue was more active in vitro in metabolising methylene chloride to carbon monoxide than either mouse or rat

tissues. There is thus no correlation between metabolism to carbon monoxide and the tumour incidences seen in the three species. It seems likely that this pathway was saturated at the top dose level used in the NCA study (Kirschman, 1984) and therefore that the dose to the livers of mice of metabolites produced via this pathway was the same in the negative NCA study as in the positive NTP study. This further supports the view that the liver tumours seen in exposed mice are not related to metabolism by this pathway.

By contrast the glutathione pathway appears to be a major metabolic pathway for methylene chloride only in mice. The pathway was detectable in rat liver in vitro but the rate was 12 times lower than in the mouse; there was little evidence in vivo for metabolism by this route in the rat. This marked difference in glutathione conjugation between rats and mice and the fact that this pathway was not detectable in hamster liver in vitro is the basis of the conclusion that glutathione conjugation is a necessary step in the mechanism of carcinogenicity.

b) Mechanism of Action of Methylene Chloride

The positive results in prokaryotic microorganisms and the chromosomal effects seen in mammalian cells in vitro are sufficient evidence to consider methylene chloride a potential mutagen. The negative results in mammalian in vitro gene mutation assays and in the in vivo assays for chromosomal effects and for damage to DNA, including the DNA binding study, lead to the conclusion that methylene chloride is not a somatic cell mutagen and is therefore non-genotoxic in vivo. Thus the potential indicated by the in vitro mutagenicity tests is not expressed in vivo even in the species and strain susceptible to methylene chloride induced cancer.

The explanation for these differing results probably lies in the lack of mutagenic activity in methylene chloride itself and the fact that the highly reactive and probably mutagenic metabolic intermediates (formyl chloride and S-chloromethyl-glutathione) are extremely short-lived. In assay systems where the active metabolites can gain access to DNA in spite of their very short half lives (prokaryotic cells where the DNA is in intimate contact with the cytosol and mammalian cell cultures undergoing cell division) a mutagenic event is seen. In eukaryotic microorganisms a marginal response is seen only at toxic dose levels. In all other systems in mammalian cells or whole mammals negative results are observed presumably because the reactive metabolites cannot penetrate to the DNA.

The mechanism by which methylene chloride produces lung and liver cancer in mice is an enigma. The mouse metabolises methylene chloride at very high (carcinogenic) doses via the glutathione pathway which includes the reactive intermediate S-chloromethyl-glutathione. However there was no evidence of binding of methylene chloride or its metabolites to mouse liver or lung DNA in studies using ^{14}C-labelled material which gave a sensitivity of detection of 1 affected nucleotide in 10^6. Further improvements in sensitivity are precluded by the introduction of ^{14}C into the C-1 pool by the metabolism of methylene chloride. There was no evidence of the induction of UDS in mouse liver even at toxic doses of methylene chloride. Hence, using sensitive assays for the detection of the potential interaction of methylene chloride or its metabolites with the DNA of the target tissues, there was no evidence of DNA damage.

There are two possible explanations for these observations. The first is that the assay systems were not sufficiently sensitive to detect the genotoxicity of methylene chloride to mouse lung and liver. In the case of mouse lung, this may well be true as the target cell is the Clara cell and it constitutes only 5% of the lung. In the liver, the hepatocyte is the target cell.

Other genotoxic hepatocarcinogens bind to DNA and this can be detected by the techniques used here (Lutz, 1979). The high incidence of hepatocellular adenomas and carcinomas induced by methylene chloride would be expected to be associated with DNA damage if this were the mechanism of action.

The second explanation is that methylene chloride produces its effects by a non-genotoxic mechanism. This is supported by the absence of evidence of DNA damage in the target tissues but there is no further experimental evidence to indicate the precise mode of action. The most probable mechanism is that methylene chloride affects a later stage in the carcinogenic process and that it accelerates the development of neoplasms which have a spontaneous incidence in the mouse strain under study.

c) Suitability of the Mouse as a Model for Human Hazard Assessment

In the absence of appropriate mechanistic studies in humans the species which is most like humans, in the manner and rate at which its tissues and organs metabolise the chemical, is the best predictive model. This guiding principle of extrapolation between species for the purpose of risk assessment can now be applied with some confidence in the case of methylene chloride. Studies in humans are confined to epidemiology studies, which do not indicate an occupational risk from methylene chloride (Friedlander, 1986), and to measurements of the metabolism of methylene chloride to carbon monoxide. Equivalent studies designed to investigate the glutathione-dependent metabolism of methylene chloride *in vivo* have not been performed, indeed, there is no evidence to suggest that this pathway exists in man. In the absence of such data, species comparisons and the selection of the most appropriate species, must rely on data from *in vitro* studies; these are now available. Because these studies in rats and mice accurately reflect the species differences seen *in vivo* there is some basis for confidence that the *in vitro* human studies, carried out alongside the animal work, will reflect *in vivo* human metabolism of methylene chloride.

The data derived from studies of the metabolism and pharmacokinetics of methylene chloride show that the mouse differs substantially not only from man but also from the other species with respect to the rate of metabolism and the relative importance of the metabolic pathways. The microsomal cytochrome P-450 pathway is a high affinity low capacity pathway with similar kinetics in the rat and mouse in vivo and in all 4 species studied, including man, in vitro. It is saturated at exposure levels above 500ppm and does not correlate with the species differences in carcinogenicity seen in the various bioassays. Although not involved in the development of cancer the cytochrome P-450 pathway nevertheless plays an important role in protecting against the carcinogenic response. At low dose levels the high affinity of the enzyme for the substrate results in the majority of the methylene chloride which is metabolized being metabolized via the cytochrome P-450 pathway. Thus even in the mouse, which has the most active glutathione pathway, the bulk of the solvent is metabolised via the cytochrome P-450 pathway at low doses.

In contrast, at high doses glutathione metabolism of methylene chloride has been shown to correlate well with the cancer seen in mice. At low doses in mice there is insufficient metabolism by this pathway to induce a carcinogenic response, as is evidenced by the absence of effect in the NCA study. In the rat at high doses the glutathione pathway is at least 10-12 times less active than in the mouse, and possibly substantially less because part of the CO_2 exhaled (which forms the basis of the estimate) is derived from the cytochrome P-450 pathway. In vitro the rat liver is about 12 times less active in this respect. The hamster, which was not susceptible to the carcinogenic effects of methylene chloride at doses greater than the effective carcinogenic dose in mice, had no detectable activity in vitro. Similarly human liver tissue showed no detectable glutathione pathway activity in spite of the fact that other substrates were actively metabolised. A low rate of metabolism by this pathway was observed in vitro in mouse but not rat lung.

In addition to, or more probably as a consequence of, the differences in metabolism, the target tissues in the mouse respond differently from those in the rat during sub-acute exposure to methylene chloride. The liver growth and pulmonary cytotoxicity seen in the mouse may not alone be responsible for the observed carcinogenicity, but such effects may play an important role, particularly when the tumours are an increased expression of a pre-existing tumour type found in control populations.

This comparison of the metabolism and pharmacokinetics of the rat, mouse, hamster and human liver in vitro together with the responsiveness of mouse tissue to the sub-acute effects of methylene chloride suggests that the mouse is a most inappropriate species from which to assess human hazard. In summary the reasons for this conclusion are:

i) The mouse is the most sensitive species to the sub-acute effects of methylene chloride which causes Clara cell damage in the lung and an increase in liver weight. These changes indicate a generally higher susceptibility of the mouse than other species tested to the toxic effects of methylene chloride. In the absence of a coherent explanation of the mode of carcinogenic action, these changes may be significant in relation to the subsequent development of tumours.

ii) At high doses, when the high affinity cytochrome P-450 pathway is saturated, the mouse is the only species which shows a capacity to metabolise methylene chloride by the glutathione pathway.

iii) The mouse has a high and/or variable natural incidence of lung and liver neoplasms. A variety of environmental factors, including for example cytotoxicity or even changes in dietary intake, can affect their incidence. Increased incidences of these tumours must therefore be considered with caution in risk assessment.

d) The Suitability of the Rat and Hamster for Human Hazard Assessment

On the basis of comparative metabolism and kinetics the rat and hamster are the two species which are the most suitable for human hazard assessment. The rates of metabolism *in vitro* suggest that human liver tissue is less active in metabolising methylene chloride via the glutathione pathway than rat liver and is similar, in having no detectable activity, to hamster liver. By the cytochrome P-450 pathway human liver is similar to rat liver and less active than hamster liver. Hence the use of these two species should provide a model which is qualitatively similar to man (hamster) or has higher rates of metabolism (rat) via the active glutathione pathway. The use of the rat as a model should therefore provide a margin of safety for human hazard assessment.

12. QUANTITATIVE DATA FOR RISK ASSESSMENT

In the knowledge of substantial quantitative differences between mice and other species in the kinetics of the metabolic pathways, careful consideration has to be given as to which data should be used for risk assessment. The hamster and rat show no evidence of carcinogenic responses to methylene chloride. However, an increased incidence of benign mammary cancer did occur in rats. This is an unusual response in that only benign neoplasms were increased in incidence. Recent studies (Breslin and Landry 1986) have shown a significant increase in prolactin levels in female rats exposed to methylene chloride suggesting that hormonal changes may be the cause or be associated with the cause, of this increase in benign tumours. Furthermore the hormonal control of these neoplasms in rats, particularly their responsiveness to prolactin levels and oestrogens, is sufficiently different from that in man as to suggest that it is biologically implausible to use these data to assess risk in man. It is concluded from the data in rats and the negative result in the test in hamsters, that methylene chloride is unlikely to present a carcinogenic hazard to man.

The tumour incidence data from mice is more amenable to conventional quantitative analysis because of the large increase in incidences of malignant tumours and at first sight the data might appear to provide a good basis for quantitative risk assessment. However, in addition to the mouse being an inappropriate model, conventional risk assessment is also inappropriate in the case of methylene chloride. This process normally utilises a multistage dose-response model which is incorporated in the Global 83 computer program developed by Howe (1983). The model assumes linear single pathway metabolism and uses a body surface area correction to extrapolate across species. From the preceding account of current understanding of the complex metabolic processes involved in the carcinogenicity of methylene chloride, it will be clear that such a model is both a poor representation of *in vivo* exposure to methylene chloride at different dose levels and a poor basis for extrapolation of risk. In reality, risk is dependent on the inter-relationship of two dose-dependent saturable pathways with markedly different enzyme-substrate affinities. These factors can only be incorporated into a risk assessment by use of the "internal dose" concept as described by Clewell and Anderson (1987) and Reitz *et al* (1986). This process not only takes into account species differences in metabolism and kinetics but also includes species specific physiological variables such as ventilation rates, cardiac output and tissue partition coefficients to enable the "internal dose" to be calculated in several species including man. This type of risk modelling is now feasible because of the extensive data *in vivo* in rats and mice and *in vitro* in 4 species including man. The availability of *in vivo* data in two species at 4 dose levels allows the model to be fully validated prior to calculation of human internal dose and risk. Two remaining key areas are the subject of current research; one is the relative utilisation of the two pathways at low dose levels (<500ppm), the other, a further investigation of the apparent absence of the glutathione pathway in human liver. Following completion of this research the data will be used in the calculation of the internal dose as the basis of a risk assessment.

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ECETOC PUBLICATIONS

Good Laboratory Practice.	Monograph no.1	October, 1979	(Out of Print)
Contribution to Strategy for Identification and Control of Occupational Carcinogens.	Monograph no.2	September, 1980	(Out of print)
Definition of a Mutagen, for 6th Amendment.	See Appendix in Monograph no.2	September, 1980	
Risk Assessment of Occupational Chemical Carcinogens.	Monograph no.3	January, 1982	
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Acute Toxicity Tests, LD_{50}(LC_{50}) Determinations and Alternatives	Monograph n° 6	May, 1985	
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Joint Assessment of Commodity Chemicals, 1-4 Dioxane	JACC Report n°2	February, 1983
Joint Assessment of Commodity Chemicals, Methyl Ethyl Ketone	JACC Report n°3	February, 1983
Joint Assessment of Commodity Chemicals, Methylene Chloride.	JACC Report n°4	January, 1984
Joint Assessment of Commodity Chemicals, Vinylidene Chloride	JACC Report n° 5	August, 1985
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