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

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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).

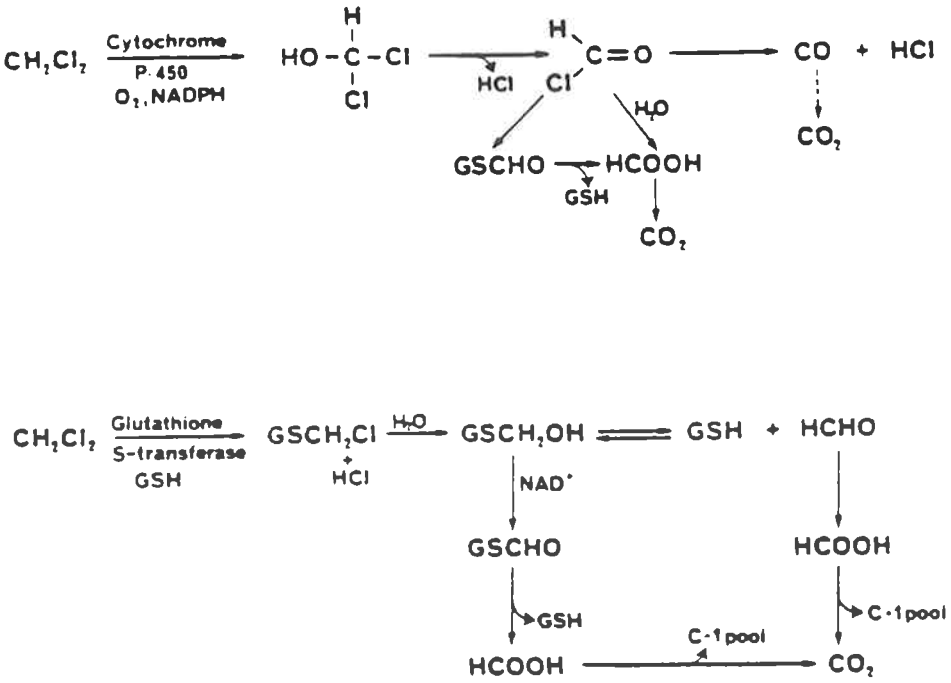

Fig 1. The proposed pathways of methylene chloride metabolism . (Adapted from Anders et al, 1977 and Gargas et al, 1986).

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