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## METHYLENE CHLORIDE (DICHLOROMETHANE) : AN OVERVIEW OF EXPERIMENTAL WORK INVESTIGATING SPECIES, DIFFERENCES IN CARCINOGENICITY AND THEIR RELEVANCE TO MAN

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## SUMMARY

Inhalation studies have been carried out using high concentrations of methylene chloride in mice, rats and hamsters. No carcinogenic effect was observed in either rats or hamsters but an increase in the incidence of lung and liver tumours were observed in both sexes of the B3C6F1 mouse. These findings in mice are of concern because people are exposed to methylene chloride both in the occupational and non-occupational setting. This report summarises a series of experiments undertaken to explain the difference in response between mice and other species and hence to understand the relevance to man of the results obtained in mice, rats and hamsters.

Methylene chloride was known to be mutagenic to Salmonella and E. Coli and to induce chromosome damage to mammalian cells in vitro. Mutagenicity studies carried out as part of this programme have demonstrated that methylene chloride is not genotoxic in vivo using doses equivalent to those used in the carcinogenicity studies.

Sub-acute studies at doses used in the carcinogenicity experiments showed that methylene chloride produces an increase in liver weight and damage to Clara cells in the lung in mice. These effects were not seen in the rat.

Methylene chloride is metabolised by two pathways - a microsomal cytochrome P450 pathway and a cytosolic glutathione S-transferase pathway. Based on studies on of the metabolism of methylene chloride in rats and mice, it has been concluded that the carcinogenic effect in mice is associated with high rates of metabolism through the glutathione-S-transferase pathway. The rate of metabolism of methylene chloride by this pathway is much higher in mice than in other species. There is also a substantial difference between the relative rate of metabolism by the two pathways at low and high doses. These observations have been extended to include observations made on human tissues in vitro. On the basis of these data it concluded that man is not susceptible to the carcinogenic effect of methylene chloride at the doses encountered in occupational and other settings.

A risk assessment taking into account these differences in metabolic rates between the species indicates that the computed risk of cancer in man is negligible under normal use conditions. The current hygiene standards which are based on the need to restrict the degree of formation of carboxyhaemoglobin after metabolism of methylene chloride, adequately protect man from the carcinogenic risk of exposure to methylene chloride.

# 1. INTRODUCTION

Methylene chloride is a volatile liquid, boiling point 40°C, used extensively in industry and in a variety of consumer products including paint strippers and aerosols. It has low acute toxicity. The need to limit carbon monoxide formation from its metabolism is the basis of current occupational exposure limits.

In 1986 the United States National Toxicology Program reported a significantly elevated incidence of lung and liver tumours in B6C3F1 mice exposed to 2000 or 4000ppm of methylene chloride for 104 weeks (NTP 1986). Increased incidences of lung and liver tumours were not seen in Fischer F344 rats in the same study nor in Sprague-Dawley rats nor Syrian hamsters exposed at similar dose levels in an earlier study (Burek et al, 1984). Lower doses of methylene chloride given to mice either in drinking water (Serota et al, 1986) or by gavage (Maltoni et al, 1986) did not cause an increase in either lung or liver tumours.

Increases in other tumour types have been reported in these studies. In one study (Burek et al, 1984), an increased incidence of sarcomas in the submandibullar region was found in male rats exposed to 3500ppm and consistent increases in benign mammary cancer in female rats have been reported from several studies. The significance of these tumours for human risk assessment has been discussed elsewhere (ECETOC, 1987).

The finding of high incidences of lung and liver cancer in mice exposed to high doses of methylene chloride caused considerable concern particularly because methylene chloride is available to the general public in consumer products. At the same time the marked species differences in response seen in the cancer bioassays left considerable doubt about the relevance of the mouse data to man. This report presents a summary of a series of studies undertaken to explain both the species differences in the carcinogenicity of methylene chloride, and the relevance of the results in mice to man. The work was funded by the European manufacturers of methylene chloride through CEFIC and was supervised by ECETOC. The experimental work was carried out at Imperial

Chemical Industries, Central Toxicology Laboratory under the leadership of Dr T Green. Full details of these studies are contained in a series of reports available from Imperial Chemical Industries plc. Reviews of the toxicology of methylene chloride relevant to its carcinogenicity and a quantitative human risk assessment are available from ECETOC. These reports and the publications derived from them are given in the references at the end of this report.

## 2. EXPERIMENTAL STUDIES

The objectives of the programme were as follows:-

1. To investigate the mechanism of action of methylene chloride as a carcinogen in the mouse.

2. To determine the basis of the species differences in response in laboratory animals.

3. To determine the relevance of the mouse carcinogenicity bioassay to man.

Within these objectives several areas of investigation were considered relevant.

Methylene chloride has been shown to be mutagenic in some test systems using microorganisms. The significance of these results for the development of tumours in mice has been determined.

The B6C3F1 strain of mouse has a high natural incidence of both liver and lung tumours. The increased incidence of these tumours following exposure to methylene chloride may merely be an increased expression of these naturally occuring tumours, stimulated possibly by cytotoxicity or increased cell division. The effects of methylene chloride on lung and liver cells were therefore investigated *in vivo*.

Mice generally metabolise chlorinated solvents at higher rates than other species. Differences in metabolism and pharmacokinetics were therefore

investigated since these might explain the species differences in carcinogenicity. Data on metabolism and pharmacokinetics were acquired for man for comparison with the experimental animal results and finally, the results of the experimental studies were incorporated into a human risk assessment for methylene chloride.

## 2.1 Mechanism of Action in Mice

### 2.1.1 Mutagenicity Studies

Methylene chloride is mutagenic in prokaryotic (Salmonella and E. Coli) and some eukaryotic (yeast-D7) microorganisms. In mammaliam cells the only positive findings are the induction of chromosomal aberrations in a variety of cells (human, hamster, mouse) at exceptionally high concentrations of methylene chloride. The significance of these studies has been reviewed previously (ECETOC, 1987). In contrast the results of studies on gene mutation and unscheduled DNA synthesis (UDS) in mammaliam cells are uniformly negative.

The apparant lack of correlation between findings in microorganisms and gene mutation and UDS in mammalian cells stimulated a further series of genotoxicity studies seeking evidence of genotoxicity in mammalian systems. These studies included examination of UDS, both in vivo and in vitro, and in vivo DNA binding. In an attempt to clarify the signficance of the chromosomal effects, the mouse micronucleus assay was performed. The ability of methylene chloride to induce mitosis (S-phase) was determined during the UDS studies. In most cases the B6C3F1 mouse and the F344 rat, or tissues from these animals, were used for these studies.

The potential for inducing unscheduled DNA synthesis was measured in the livers of B6C3F1 mice and F344 rats 4 and 12hr after in vivo exposure to 2000 or 4000ppm methylene chloride for up to 6hr (Trueman et al, 1986, Trueman and Ashby, 1987). Male AP:Alpk rats were also administered methylene chloride in corn oil by gavage at dose levels of up to 1g/kg. In all of these experiments methylene chloride failed to induce UDS in the livers of treated animals. However, some evidence for an increase in

mitosis (S-phase) was seen in mouse liver 36hr after exposure (Lefevre and Ashby, 1986). In further experiments, hepatocytes prepared from F344 rats and from B6C3F1 mice were exposed to methylene chloride at concentrations up to 4000ppm for a period of 8hr. These experiments again failed to detect an increase in UDS (Trueman et al, 1986, Trueman and Ashby, 1987).

The ability of methylene chloride or its metabolites to bind covalently to DNA in the livers and lungs of exposed F344 rats and B6C3F1 mice was determined following a 3hr exposure to 4000ppm of $^{14}C$ radiolabelled methylene chloride. High purity DNA was isolated from the livers and lungs 6, 12 and 24hr after the start of exposure and the associated radioactivity determined. The nature of this radioactivity was investigated by further analysis of the DNA. One route of metabolism of methylene chloride is to formaldehyde, formic acid and carbon dioxide in vivo, and as a result radiolabel becomes incorporated into DNA through the C-1 pool. In order to distinguish between $^{14}C$ covalently bound to DNA and that incorporated into DNA through the C-1 pool, the DNA isolated from exposed animals was hydrolysed to the constituent deoxynucleosides and analysed by high pressure liquid chromatography. All of the radioactivity associated with the DNA was shown to be present as a result of incorporation through the C-1 pool. There was no evidence of alkylated deoxynucleosides in the liver or the lungs of either rats or mice, the study had the power to detect 1 alkylation in $10^6$ nucleosides (Green et al, 1986a, 1988).

Chromosomal effects following methylene chloride exposure have been reported in a number of studies using cells in vitro, (Thilager and Kumaroo, 1983; Thilager et al, 1984) but not in vivo, (Gocke et al, 1981; Burek et al, 1984). The significance of the in vitro results to the tumours seen in mice exposed in vivo was further investigated using the mouse micronucleus assay (Sheldon et al, 1986, 1988). In these studies with C57BL/6 mice, methylene chloride did not induce any specific increase in polychromatic erythrocytes containing micronuclei when tested orally in corn oil at dose levels up to 4g/kg (80% of the median lethal dose).

These new studies (Table 1) confirmed those of the previously published short term tests; methylene chloride is mutagenic in some tests using

microorganisms, but genotoxicity is not detectable in mammalian cells either in vivo or in vitro.

### 2.1.2 Cytotoxicity Studies

The effect of methylene chloride on lung and liver tissues was determined in F344 rats and B6C3F1 mice exposured to 2000 or 4000ppm methylene chloride, 6hr/day for either 1 day or 10 days. The animals were killed 24hr after the start of the exposure and the lungs and livers taken for histopathological examination by light and electron microscopy (Hext et al, 1986).

Methylene chloride was not cytotoxic to the livers of either rats or mice at the dose levels used at either time point. Although there were no morphological changes in the livers of mice, a significant increase in relative weight was seen after 10 days of exposure. This effect was not seen in the rat. Species specific effects were also found in the mouse lung in the form of a marked lesion in the Clara cells seen after a single exposure to either dose level. The lesion comprised a highly selective vacuolation and pyknosis of the Clara cells of the bronchiolar epithelium. After 10 days exposure the vacuolation of the cells was no longer evident.

There is therefore clear evidence that both target organs respond differently to methylene chloride in the mouse than 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.

### 2.1.3 Conclusions

No clear explanation has emerged for the mechanism of action of methylene chloride as a carcinogen in the B6C3F1 mouse. Neither the genotoxicity studies carried out as part of this programme nor those reported in the literature have detected evidence for genotoxicity in mammalian systems. Within the limitations of the currently available short term tests it must be concluded therefore that methylene chloride is non- genotoxic although

the activity in microorganisms may suggest a certain potential not detected in higher organisms. The full significance of the effects of methylene chloride on the lungs and livers of mice cannot be determined without significantly more work, but it does appear that these species-specific effects may influence the development of cancer in a strain of mouse (B6C3F1) which has significant background tumour rates in the two target organs.

## 2.2 The Basis for the Species Differences

Methylene chloride is metabolised by two pathways (Kubic and Anders, 1975, 1978; Ahmed and Anders, 1976, 1978; Gargas et al, 1986). One is catalysed by microsomal monooxygenases (cytochrome P-450 enzymes) and yields carbon monoxide, resulting in the formation of carboxyhaemoglobin, and carbon dioxide. The other pathway is catalysed by a cytosolic glutathione-S-transferase enzyme and yields carbon dioxide in vivo (Figure 1). In vitro the two pathways may be conveniently separated by liver fractionation into cytosolic microsomal components and the rate of metabolism by each pathway assayed by the formation of carbon monoxide from the cytochrome P-450 pathway (P-450) or formaldehyde from the glutathione-S- transferase pathway (GST).

The lung and liver cancer of mice must result from exposure to the parent chemical or to a product or products of one of the two metabolic pathways, and hence mice must either respond differently from rats and hamsters to methylene chloride or its metabolites or be exposed to significantly different quantities of those metabolites. If this could be established then the differences between mice and other species could be used as the basis for a human risk assessment, even though the mechanism of action in mice is not known. Such information was sought through a series of studies investigating the rates of metabolism by the two pathways, and the circulating levels of the parent chemical in B6C3F1 mice and F344 rats. These studies were followed by a series of in vitro studies measuring metabolic rates in rat, mouse, hamster and human tissues. The in vivo experiments in rats and mice were repeated in in vitro experiments in order

to examine whether the in vitro techniques quantitatively reflected the species differences observed in vivo.

Prior to these studies the metabolism and pharmacokinetics of methylene chloride were unknown in the mouse, the glutathione-S-transferase pathway had not been demonstrated in man, nor were there any detailed comparisons of the fate of methylene chloride in the four species of interest.

2.2.1 In Vivo Studies

A full pharmacokinetic profile of methylene chloride and its metabolites was determined in B6C3F1 mice and F344 rats both during and after a 6hr exposure to atmospheres containing various concentrations from 100 to 4000ppm methylene chloride (Green et al, 1986b, 1987a). Blood levels of methylene chloride and carboxyhaemoglobin and the rates of elimination of methylene chloride, carbon monoxide and carbon dioxide in exhaled air were measured. Stable isotopes were used to quantify the amount of carbon dioxide from each pathway at dose levels of 100, 500 and 4000ppm only in the mouse (Green et al, 1987a).

The steady state blood levels of methylene chloride during exposure were up to 5-times higher in rats than in mice at the higher dose levels. A comparison of the carboxyhaemoglobin levels in blood (Figure 2) and carbon monoxide levels in expired air showed that rate of metabolism by the cytochrome P-450 pathway was similar in both rats and mice. The pathway was saturated in both species at exposures of less that 500ppm resulting in maximal carboxyhaemoglobin levels of 16%.

Saturation of the cytochrome P-450 pathway in mice was also clearly shown by a 5-10 fold increase in the blood levels of methylene chloride when the inhaled concentration was doubled from 500 to 1000 ppm.

The stable isotope studies demonstrated that the cytochrome P-450 pathway was the major source of carbon dioxide at low exposure levels (100ppm) whereas at high levels (4000ppm) the glutathione-S-transferase pathway was the principal source of carbon dioxide. A comparison of the rate of elimination of the carbon dioxide by rats and mice at the top dose level

found the glutathione-S-transferase pathway to be 10-12 times more active in mice than rats (Figure 3). This higher rate of metabolic conversion of methylene chloride by mice largely accounts for the low blood levels of parent chemical in this species. The dose-dependance of the two pathways in mice, determined by these studies, is shown in Figure 4.

In summary, the in vivo studies provided evidence for the following:-

1. The circulating levels of methylene chloride in blood are 5-times higher in rats than in mice at the dose levels used in the NTP 2-year study (NTP, 1986).

2. The cytochrome P-450 pathway is saturated at 500ppm and is quantitatively similar in rats and mice.

3. The glutathione-S-transferase pathway is a major pathway only in mice, its activity at the 4000ppm dose level being an order of magnitude greater than in rats.

4. The rate of metabolism is dose-dependant. At the high dose levels used in the long term carcinogenicity studies, the utilisation of the two pathways is markedly different from that at low dose levels.

2.2.2 In Vitro Studies

A comparison of the rates of metabolism of methylene chloride by each pathway in liver fractions from rats, mice, hamsters and man was carried out. These experiments demonstrated that the rates of metabolism in vitro had similar differences to those seen in vivo in rats and mice, and enabled a comparison to be made with those species (hamster and man) where in vivo data was not available (Green et al, 1986c, 1987b).

The metabolic rates for each pathway in liver fractions of the four species are shown in Figure 5.

The 10-fold difference in glutathione-S-transferase activity measured *in vivo* in mice and rats was also found *in vitro*. It is also clear from Figure 4 that there is an excellent correlation between glutathione-S-transferase metabolism and the outcome of the two year cancer studies in the three animal species. No such correlation exists for the cytochrome P-450 pathway where, for example, the metabolic rate in hamsters is very similar to that in the mouse.

Cytochrome P-450 catalysed metabolism of methylene chloride could be detected in lung tissue from all three animal species, the relative activities being similar to those in the livers. Glutathione-S-transferase activity was detectable only in mouse lung fractions.

The low rates of metabolism of methylene chloride by the glutathione-S-transferase pathway in human liver samples has been attributed to a deficiency in the transferase isoenzyme responsible. The same liver samples had similar activity to rat liver when assayed with an alternative substrate for these enzymes.

### 2.2.3 Pulmonary Metabolism in the Mouse

The type of lesion observed in the Clara cell after a single exposure to methylene chloride is known to result in a loss of cytochrome P-450 enzymes from damaged cells. This effect was also seen in a series of studies measuring the metabolic capacity of the lung after exposure to methylene chloride for up to 10 days (Green *et al*, 1987c). By measuring metabolic rates using mouse lung fractions and studying the distribution of isoenzymes in lung sections with polyclonal antibodies it was found that cytochrome P-450 isoenzymes were completely lost from damaged Clara cells after a single exposure to methylene chloride. In whole lung fractions the metabolism of methylene chloride to carbon monoxide was reduced by 50%, suggesting that half of the capacity of the lung to metabolise methylene chloride by this pathway is located in the Clara cells, which themselves only account for 5% of the total cells in the mouse lung. After 10 days of continued exposure the lesion recovered, as did some isoenzymes of cytochrome P-450. Isoenzymes responsible for the metabolism of methylene chloride did not, however, recover and therefore metabolism to carbon

monoxide by the whole lung still remained at 50% of control value. Throughout these experiments, at either 1 or 10 days, glutathione-S-transferase metabolism was not reduced.

As a result of these changes the Clara cells in the mouse lung were no longer able (at least up to 10 days) to metabolise methylene chloride by the cytochrome P-450 pathway, but the glutathione- S-transferase pathway, the one associated with cancer induction remained fully competant.

### 2.2.4 Conclusions from the Metabolism and Pharmacokinetics Studies

The studies of the comparative metabolism and pharmacokinetics of methylene chloride in rat, mouse, hamster and man provided a plausible explanation for the species differences in the carcinogenicity of this chemical. The differing metabolic rates by the glutathione-S-tranferase pathway are consistent with the outcome of the cancer studies whereas the blood levels of parent chemical and the metabolic rates by the cytochrome P-450 pathway clearly are not. These results are also consistent with the different responses seen in the three mouse cancer bioassays (NTP, 1986; Serota et al, 1986; Maltoni et al, 1986). At the high dose levels used in the NTP study the glutathione-S-transferase pathway would have been the major metabolic pathway and high tumour incidences were observed. At the lower dose levels used by Serota et al (1986) and Maltoni et al (1986), methylene chloride would have been metabolised mainly by cytochrome P-450 and glutathione-S-transferase metabolism would have been minimal; consequently there were no significant increases in either lung or liver tumours in these studies.

## 3. RISK ASSESSMENT

A guiding principle of extrapolation between species for the purpose of risk assessment is to use the species which is most like man both in the manner and rate of metabolism of the chemical in tissues. From these studies it can be concluded that the rat or hamster should be used and that the mouse is not an appropriate model. Cancer is not induced by methylene chloride in the rat or hamster. Therefore a cancer risk assessment based on mouse data is not the