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Metabolism of dichloromethane (methylene chloride) to formaldehyde in human erythrocytes: influence of polymorphism of glutathione transferase Theta (GST T1-1)

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Abstract. Human hemolysate was incubated in vitro with different concentrations of dichloromethane (methylene chloride). The resulting enzymatically mediated production of formaldehyde was determined by two independent analytical methods (Nash-reaction/colorimetry or HPLC). The formation of formaldehyde from dichloromethane is influenced by the polymorphism of glutathione-*S*-transferase (GST) Theta, in the same way as the metabolism of methyl bromide, methyl chloride, methyl iodide and ethylene oxide. Three quarters of the population ("conjugators") possess, whereas one quarter ("non-conjugators") lack this enzyme activity in human erythrocytes. The metabolism of dichloromethane in hemolysate in vitro can be described by Michaelis-Menten kinetics; for an individual with high GST T1-1 enzyme activity, the maximum velocity of formaldehyde production was calculated to be approximately 180 pmol/min per mg Hb, the k_M being approximately 60 mM dichloromethane. Carcinogenicity of dichloromethane in long-term inhalation exposure of rodents has been attributed to metabolism of the compound via the GST-dependent pathway. Extrapolation of the results to humans for risk assessment should consider the newly discovered polymorphic enzyme activity of GST Theta. Furthermore, the possible existence of a "high-risk" population among humans should be considered in epidemiological research.

Key words: Dichloromethane – Methylene chloride – Formaldehyde – Human erythrocytes – Enzyme polymorphism – Glutathione-*S*-transferase Theta – Risk assessment

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Introduction

Dichloromethane (methylene chloride, DCM) induces lung and liver tumors in $B_6C_3F_1$ mice under long term exposure to 2000 ppm and 4000 ppm (NTP 1986). Likewise, exposure to this substance leads to the formation of DNA-protein crosslinks in liver of the same species (Casanova et al. 1992; Vangala and Bolt 1993). Such lesions are considered a typical effect of formaldehyde, a metabolite of dichloromethane formed via the glutathione-dependent pathway. Upon exposure to radiolabelled [^{14}C] dichloromethane, two- to four-fold higher levels of radioactivity were found in DNA and protein from lungs and livers of $B_6C_3F_1$ mice than in F344 rats (Green et al. 1988). In humans, data from epidemiological studies are inconsistent, and the International Agency for Research on Cancer (IARC) has concluded that the evidence for carcinogenicity of the compound to humans is inadequate (IARC 1986, 1987).

In mammals, dichloromethane is metabolized by two pathways: oxidation by cytochrome P450-dependent mixed function oxidases ("high affinity, low capacity"), ultimately leading to formation of carbon monoxide, and conjugation by glutathione *S*-transferases (GST) ("low affinity, high capacity"), leading to the production of chloromethyl glutathione and subsequently formaldehyde (Ahmed and Anders 1976, 1978). An investigation of the data from the long-term mouse inhalation studies by physiologically based pharmacokinetic modelling (PB-PK) revealed that the tumor incidence was correlated with the amount of dichloromethane metabolized by the GST pathway. However, no correlation could be established with the amount of DCM metabolized by the oxidative pathway (Andersen et al. 1987).

Based on these results, an extrapolation of tumor risk from rodents to humans was attempted. In experiments performed in vitro with lung and liver tissue from mice, rats, Syrian golden hamsters and humans, enzyme activity for these two major pathways was compared between the species (Reitz et al. 1989). It was found that GST activity was highest in these tissues in mice, and lowest in humans.

Upon this basis, it was estimated that tumor risk induced by DCM should consequently be very high in mice and negligible in humans under the existing occupational exposure limits (ECETOC 1987, 1988, 1989).

Several C_1- and C_2-compounds are enzymatically conjugated to glutathione by a GST in human erythrocytes which displays polymorphism (Schröder et al. 1992). Approximately three-quarters (so-called "conjugators") of the more than 200 subjects investigated so far possess, and one quarter ("non-conjugators") lack this enzyme activity in erythrocytes. A minority with especially high GST activity ("high conjugators") can be distinguished among the conjugators (Hallier et al. 1993).

Among the substrates for this polymorphic enzyme activity in human erythrocytes are the methyl halides methyl chloride (Peter et al. 1989), methyl bromide, methyl iodide (Hallier et al. 1990) and dichloromethane (Pemble et al. 1993). Incubation of ^{14}C-radiolabelled dichloromethane with whole blood of previously identified conjugators in head space vials led to a time dependent formation in vitro of soluble and protein-bound metabolites in the blood of conjugators but not in that of non-conjugators (Thier et al. 1991). Furthermore, the enzyme polymorphism influences the induction of sister chromatid exchanges (SCE) in human lymphocytes in vitro by dichloromethane and other compounds (Hallier et al. 1993).

In view of the theory that GST-dependent metabolism is responsible for the carcinogenic effects of dichloromethane in rodents, experiments were performed to investigate the production of formaldehyde from dichloromethane in human erythrocytes in vitro.

Materials and methods

Methyl bromide, 99% pure, was obtained from Linde, Essen, Germany; nitrogen, hydrogen and synthetic air for GC from Messer Griesheim, Dortmund, Germany; glutathione from Sigma, Munich, Germany; dichloromethane and other chemicals from Merck-Schuchardt, Darmstadt, Germany, in the highest purity available.

Preparation of hemolysate. Individual whole blood samples were drawn from human volunteers into heparinized vials by venipuncture. After centrifugation at 800 *g* for 10 min, the supernatant was discarded. The erythrocytes were washed in saline until no leukocytes were visible by light microscopy. The erythrocytes were lysed with an equal amount of distilled water (1 h at 4° C). The hemolysate was then dialysed overnight against 20 mM phosphate buffer, pH 7.4, with 2 mM EDTA. The hemoglobin content of the samples was determined by use of a standard reaction kit (Merck, Darmstadt, Germany).

Determination of conjugator status. Individual blood samples were subjected to the standard gas chromatography test procedure described earlier (Hallier et al. 1993). Conjugators (n = 7) displayed enzymatic conjugation of the test substrate methyl bromide to glutathione in erythrocyte cytoplasm, whereas non-conjugators (n = 6) showed no such enzyme activity for methyl bromide. The individual rate of methyl bromide disappearance was determined.

Incubation of blood samples with dichloromethane. Glass vials (9 ml) were filled with 1.8 ml of individual hemolysate, 4 mM glutathione (GSH) and 20 mM TRIS/HCl buffer, pH 7.4, to a total volume of 7.8 ml. Different amounts of dichloromethane were added to this mixture, so that practically no gas phase was left in the incubations.

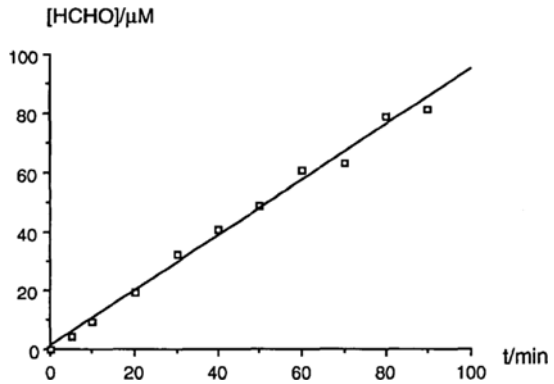

Fig. 1. Production of formaldehyde (HCHO) in an individual hemolysate sample incubated in vitro with 48 mM dichloromethane. Linear regression; r = 0.996

The vials were sealed with a Teflon septum and stored at 37° C for 1 h. Control samples were prepared likewise without addition of hemolysate.

Determination of formaldehyde production. To determine formaldehyde production, 1 ml of the liquid phase of each sample was drawn through the septum with a syringe every 20 min. This was treated with 333 µl 20% TCA and subsequent centrifugation for denaturation and removal of protein. Formaldehyde was determined in the supernatant by a photometric method according to Nash (1953); 1 ml of the supernatant obtained was added to 1 ml of Nash's reagent, and the yield of formaldehyde was measured in a spectrophotometer at 412 nm. Since the sensitivity given by Nash (1953) was sufficient for the practical purpose of the investigation, the method was not modified.

Since this method is not specific for formaldehyde, parallel incubations were subjected to an HPLC determination of formaldehyde according to Grömping and Cammann (1983). This procedure involves reaction of formaldehyde with 2,4-dinitrophenylhydrazine (DNPH) to obtain a hydrazone derivative, which can be quantified by UV detection. The conditions for chromatography were: Spectroflow Monitor SF 770 UV-detector; Spherisorb ODS 2 5 µm 250×4.6 mm column; flow 1 ml/min; UV detection at 360 nm, AU 0.04; injection volume 20 µl, retention time 7.9 min.

The results obtained by both methods were statistically compared by use of the Kolmogorov-Smirnov test.

Results

A background of formaldehyde without exposure to methylene chloride was detectable in all samples of hemolysate prepared as described in the Methods section. This remained unchanged when the samples were stored for 1 h at room temperature or at 4° C. The background concentration of formaldehyde ranged from 0.46 µM to 2.81 µM in the samples with hemolysate of conjugators and from 1.7 µM to 2.73 µM in those with hemolysate of non-conjugators. A difference between the two groups could not be found using the Wilcoxon test (two-sided, $p > 0.05$).

Under incubation of the blood hemolysate samples with dichloromethane, no generation of formaldehyde above the background level could be detected in the case of non-conjugators (n = 6). In the hemolysate of conjugators (n = 7), a time dependent linear increase of the formaldehyde concentration was observed. An example of an incubation with 48 mM dichloromethane is shown in Fig. 1. When the

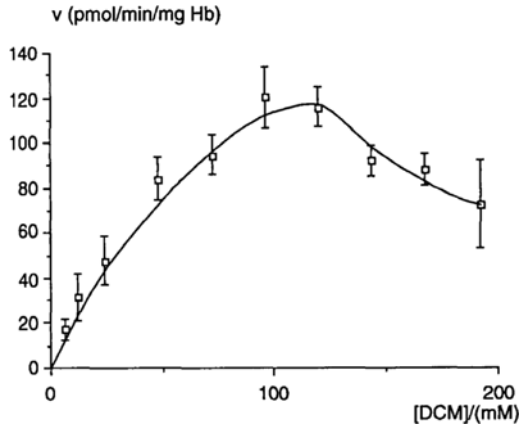

Fig. 2. Velocity of formaldehyde production in hemolysate from a conjugator. The hemolysate was incubated in vitro with different concentrations of dichloromethane (DCM). Mean values and standard deviations are given for each DCM concentration ($n = 4$–5 determinations each)

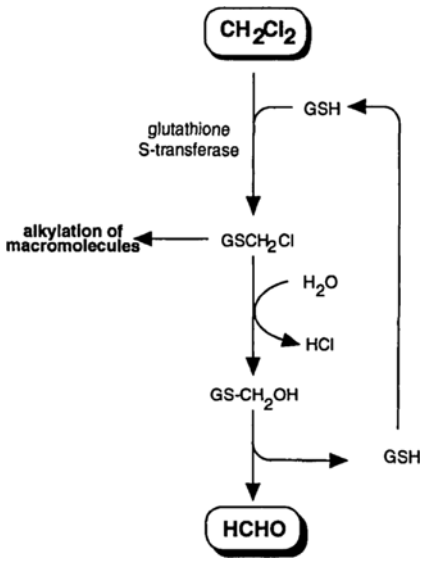

Fig. 3. Glutathione-dependent metabolism of dichloromethane to formaldehyde

hemolysate samples of conjugators had been heated to 100° C for 1 h prior to incubation with dichloromethane, no production of formaldehyde could be detected.

In order to verify that the generated substance determined by the photometric method according to Nash (1953) was formaldehyde, parallel incubations of three individual hemolysate samples were analyzed by HPLC according to Grömping and Cammann (1983). A comparison of the formaldehyde concentrations obtained by the two analytical methods (Table 1) showed that the results were in good agreement. Using the Kolmogorov-Smirnov test, the hypothesis that both methods yield the same results could not be rejected at $p = 0.05$

As shown in Fig. 1, the production of formaldehyde was linear over time within the first 60 min of incubation of blood samples of conjugators with dichloromethane. By fitting a linear regression, the individual metabolic rate constants for incubations with hemolysates of seven conjugators were obtained. These are shown in comparison to the individual rate constants of methyl bromide conjugation in vitro according to Hallier et al. (1993) of the same subjects (Table 2). It is obvious that three of the seven conjugators display a higher velocity of metabolism of both

Table 1. Concentration of formaldehyde (given in μM) in hemolysate of three individual conjugators in parallel incubations with 48 mM dichloromethane in 9 ml vials. Formaldehyde was determined according to Nash (1953) or Grömping and Cammann (1989)

Time (min)	Subject 1		Subject 2		Subject 5	
	Nash	Grömping	Nash	Grömping	Nash	Grömping
10	11.22	13.25	14.82	12.74	8.42	7.81
15	15.56	16.66	17.78	16.55	10.94	9.74
20	22.66	22.11	22.24	19.81	12.08	14.35
25	29.14	27.17	33.66	21.39	13.94	16.10
30	35.92	33.36	17.92	29.69	17.92	19.09

Table 2. Individual kinetic rate constants of seven conjugators for the conjugation of methyl bromide by hemolysate in vitro and the production of formaldehyde from dichloromethane in vitro by the erythrocytic GST T1-1

Subject	Sex	Age (years)	Smoking habit	Conjugation of methyl bromide (nmol/min per mg Hb)	Production of formaldehyde from dichloromethane (pmol/min per mg Hb)
1	m	27	–	1.75	17.7
2	f	29	–	3.32	15.4
3	m	32	+	0.56	6.0
4	m	34	–	0.41	7.6
5	m	28	+	0.36	7.2
6	f	28	–	2.22	17.8
7	f	22	–	0.56	4.3

Table 3. Pharmacokinetic parameters of formaldehyde production from dichloromethane in human hemolysate calculated by different models, k_M given as mM, v_{max} as pmol/min per mg Hb. The linear correlation coefficients (r) are listed

Method of calculation	k_M	V_{max}	r
Non-linear regression	62.6	181.2	
Lineweaver-Burk	59.9	180.0	0.945
Eadie-Hofstee	54.3	168.0	–0.945
Hanes-Woolf	59.8	176.8	0.981

methyl bromide and dichloromethane and may therefore be classified as high conjugators.

Blood samples of one of the high conjugators (subject no. 2) were incubated with different concentrations of dichloromethane ranging between 6 and 192 mM, and the initial velocity of metabolism was calculated. A minimum of three incubations was performed for each concentration. The rates of formaldehyde production are presented in Fig. 2 as a function of the dichloromethane concentration. The velocity of metabolism increased with the substrate concentration, up to a concentration of approximately 120 mM dichloromethane; at higher concentrations a decrease (substrate inhibition) was observed. Using the data obtained up to a concentration of 120 µM dichloromethane, the kinetic parameters were calculated using the models of Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf. The maximum velocity of formaldehyde production was calculated to be approximately 180 pmol/min per mg Hb, the k_M being approximately 60 mM dichloromethane. The results are summarized in Table3.

Discussion

Dichloromethane (methylene chloride) is enzymatically metabolized in human blood lysate to formaldehyde. This metabolism is subjected to the same polymorphism as methyl chloride (Peter et al. 1989), methyl bromide, methyl iodide (Hallier et al. 1990), and ethylene oxide (Föst et al. 1991). Conjugators, approximately three quarters of the population, possess, and non-conjugators lack this particular enzyme activity in erythrocytes.

The enzyme responsible for this polymorphic activity has been characterized by Schröder et al. (1992). It is a glutathione *S*-transferase belonging to class Theta, which was first described to occur in humans by Meyer et al. (1991). In contrast to the other human GST families, this GST can neither be separated by affinity chromatography, nor does it show activity towards the standard test substrate 1-chloro-2,4-dinitrobenzene (CDNB). The genetic background of the polymorphic enzyme activity in human hemolysate has recently been disclosed by PCR (Pemble et al. 1993). The GSTT1 cDNA cloned from human lymphocytes was highly related to the known GSTsu5 of rats, the homology being over 82%. Non-conjugators have a homozygous null genotype, whereas conjugators are either homozygous or heterozygous bearers of the gene.

The production of formaldehyde from dichloromethane in human blood lysate is saturable and can be described by Michaelis-Menten kinetics (Fig. 2, Table 3). It is not present in heat-inactivated lysate. At high substrate concentrations (above 120 mM dichloromethane), a loss of enzyme activity is observed, probably due to toxic effects of the solvent. Among the seven conjugators investigated in this study, three subjects have higher enzyme activity for methyl bromide and dichloromethane than the others (Table 2). The same distinction between heterozygous conjugators and homozygous high conjugators has previously been shown for the substrate ethylene oxide (Hallier et al. 1993).

The glutathione dependent pathway for metabolism of dichloromethane is summarized in Fig. 3. In mice, this metabolism is considered to be responsible for the formation of lung and liver tumors (Andersen et al. 1987; Reitz et al. 1989). The mutagenic activity underlying this carcinogenic potential has recently been investigated in a series of animal experiments (Anderson and Maronpot 1993).

Risk assessment for carcinogenicity of dichloromethane to humans has been based on extrapolation of data from the NTP carcinogenicity studies (NTP 1986) by applying pharmacokinetic modelling and comparing the competitive metabolic pathways via cytochrome P_{450} dependent oxidases and glutathione *S*-transferases (Andersen et al. 1987; Reitz et al. 1989). A basic assumption underlying this extrapolation was that GST activity for dichloromethane is relatively high in liver and lungs of mice, lower in rats, and practically not detectable in humans (Green et al. 1988). This view has led to the evaluation that carcinogenic risk from this major organic solvent must be negligible in humans under the common exposure conditions and the current Occupational Exposure Limits (ECETOC 1987, 1988, 1989).

Until 1991, glutathione transferase activity had been regarded as practically synonymous with activity towards the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). Risk extrapolation had therefore not considered the existence of the new class Theta glutathione *S*-transferases, which cannot be distinguished by affinity chromatography and show no activity for CDNB. Recently, Thier et al. (1993) have incorporated the gene for GST 5-5 of rats, the ortholog of human GSTT1, into *S. typhimurium*, and used this construct in the Ames test, which led to intrabacterial production of a mutagen from dichloromethane. It should be noted that in rats, GST 5-5 is constitutionally expressed in liver but not in erythrocytes. A phenomenon analogous to a human enzyme polymorphism has so far not been observed for GST 5-5 in this species.

Under consideration of this role of GST Theta activity for mutagenicity and possible carcinogenicity of dichloromethane, the polymorphism of GSTT1-1 in humans may be of particular importance (Bolt 1994). Previous investigations have shown that it is a disposition factor for the induction of sister chromatid exchanges (SCE) in vitro by dichloromethane as well as by methyl bromide and ethylene oxide (Hallier et al. 1993). The polymorphism may not be restricted to erythrocytes alone, since non-conjugators have a homozygous null allele and will not express GSTT1 in other organs as well (Pemble et al. 1993). Evidence for this has been provided by Bogaards et al. (1993), who investigated metabolism of dichloromethane in vitro in liver tissue of 22 humans. The authors registered great interindividual differences in metabolism, which suggested the

existence of an enzyme polymorphism. The activity was independent of the classic GST families Alpha, Mu and Pi.

In a large epidemiologic cohort study on 1013 workers occupationally exposed to dichloromethane at the Eastman-Kodak plant in Rochester, N.Y., Hearne et al. (1987) found no significant increase of tumors of the lung or liver. The authors focussed their interest on these organs, since these tumor sites corresponded to the carcinogenicity results of the NTP study. Mirer et al. (1988), however, pointed out that the study showed an increased incidence of carcinomas of the pancreas (8 observed, 3.1 expected, SMR = 2.58). Since conjugators and especially high conjugators are able to metabolize dichloromethane via GST1-1 in blood and possibly other organs as well, these may represent a high risk group for genotoxic effects of the substance. The consideration of this polymorphism in future epidemiologic research may therefore lead to a higher sensitivity of the studies and may open new aspects in risk assessment of dichloromethane.

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