ALKYLATION OF DNA AND PROTEINS IN MICE EXPOSED TO VINYL CHLORIDE

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SUMMARY: Experiments with mice show that the pre-carcinogen vinyl chloride is metabolically converted to a short-lived alkylating intermediate which introduces the 2-oxoethyl group onto nucleophilic sites in DNA and proteins. The absolute and relative amounts of alkylated products support the hypothesis that the main reactive metabolite is chloroethylene oxide.

INTRODUCTION

The degree of alkylation of specific amino acids in Hb* or erythrocytes may be used as a measure of the *in vivo* dose both of electrophilic agents and of compounds which are metabolically converted to electrophilic agents (1). The present study on vinyl chloride is the basis of work in progress at the Wallenberg Laboratory on a) factors modifying the formation *in vivo* of electrophilic intermediates from vinyl chloride and b) the development of a gas-chromatographic method to alkylated amino acids in Hb from man occupationally exposed to vinyl chloride.

Exposure of a model nucleophile, 3,4-dichlorobenzenethiol to vinyl chloride in a microsomal system *in vitro* yields an alkylated product, identified as 3,4-dichlorophenylmercaptoacetaldehyde (2). This could result from chloroethylene oxide or chloroacetaldehyde as reactive metabolites of vinyl chloride. In their reactions with nucleophiles, both these compounds yield alkylated products containing the 2-oxoethyl group.

Reduction of the 2-oxoethyl groups with sodium borohydride gives the possibility of determining the degree of alkylation of macromolecules from the amounts of S-(2-hydroxyethyl)cysteine and (N-1-and N-3-)hydroxyethylhistidine or N-7-

*Abbreviations: Hb, globin precipitate of haemoglobin; HOEtCys, S-(2-hydroxyethyl)cysteine; HOEtHis, (N-1-and N-3-)hydroxyethylhistidine; HOEtGua, N-7--hydroxyethylguanine.

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-hydroxyethylguanine in hydrolysates from proteins and nucleic acids, respectively. The present investigation comprises quantitative measurement of these alkylated products in Hb, testes proteins and liver DNA after exposure of mice to ^{14}C-labelled vinyl chloride.

The fact that vinyl chloride binds covalently to proteins and RNA in the presence of fortified microsomal preparations has been demonstrated earlier (3). No characterization of the mode of binding to macromolecules *in vivo* has been published.

MATERIALS AND METHODS

Animals. Male mice from three strains, CBA, BALB and ATL, were used. The mice were 2-3 months old (average weight 20 g). They were fed on a standard pellet diet and water without restriction.

Chemicals. Vinyl-[1,2-^{14}C] chloride, specific activity 0.48 mCi/mmol, radiochemical purity 99%, was purchased from New England Nuclear, Boston, Mass. The vinyl chloride was dissolved in m-xylene to obtain 0.1 mCi/ml xylene. Chloroethylene oxide and chloroacetaldehyde were prepared as described earlier (4). Hydroxyethylcysteine was synthesized according to Zilkha and Rappoport (5) and the two isomeric hydroxyethylhistidines were synthesized by reacting ethylene oxide with N-acetylhistidine methyl ester in metanol-HCl, followed by hydrolysis of the product (6). Hydroxyethylguanine was synthesized from guanosine and ethylene oxide in acetic acid (7, 8).

Exposure of the animals to vinyl chloride. Ten to twelve mice at a time were exposed to labelled vinyl chloride in an 11 l all-glass system. The vinyl chloride was distilled from the solvent at 0°C into an evacuated 150 ml vessel and was then transferred to the treatment chamber by flushing with air (600 ml).

The air in the chamber was circulated by a propeller and the expired CO_2 was absorbed on an Ascarite layer. The pressure was kept constant by passing a slow stream of O_2 through a capillary inlet. The atmosphere was analyzed for ^{14}C at intervals by taking 2 ml samples by a gas syringe and rapidly injecting the gas through a small membrane on the cover of a scintillation flask, prefilled with 10 ml of scintillation liquid (5 g PPO and 0.05 g POPOP/l toluene).

In a control experiment the O_2 concentration in the chamber was measured as follows: A slow stream of CO_2 was led through 1 ml of water in a plastic cell (kept at 37°C) with a Clark-type oxygen electrode connected with a recorder. Air samples were rapidly injected into the CO_2 stream. The O_2 level was constant over a period of 11 hours with 10 mice in the chamber.

Table I gives the layout of the experiments with regard to the number of animals treated, duration of exposure, doses expressed in ppm·h and time from end of exposure to killing. Table I also gives the half-life to the radioactivity in the air and the total radioactivity in Hb.

Isolation of Hb, other tissue proteins and DNA. After anesthetizing the animals with ether, the blood was collected and suspended in 0.9% NaCl solution containing heparin. The erythrocytes were gently spun down and thoroughly washed with 0.9% NaCl. The cells were lyzed with water and cell membranes and debris were sedimented at 20,000 g for 10 min. The supernatant was dialyzed over night against 1 mM phosphate buffer pH 7 and was then brought to pH 4 by addition of acetic acid. After 3 h the pH was cautiously brought to pH 6 and half of the material was reduced with sodium borohydride (50 mg/10 ml). The globin was precipitated by the addition of 10 volumes of 1% HCl in acetone (9) and was then washed successively with HCl-acetone, 5% aqueous TCA, ethanol and ethyl ether.

Table I. Details of the layout of the experiments A, B and C.

Expt.	Number of mice and strain	Duration of exposure (h)	Dose (ppm·h)	Half-life of act. in the air (h)	Time from end of exposure to killing (h)	Tot. act. in Hb (dpm/mg)
A	11 CBA	10	160	3.3	12	-
B	4 CBA	10[a)]	302	1.2	12	16
	4 BALB					38
	4 ATL					14
C	3 CBA	2.33[a)]	98	0.9	1	7.7
	7 BALB				1	14.2
					3	14.0
					25	15.6

a) ^{14}C-labelled vinyl chloride was added at intervals to maintain a sufficently high vinyl chloride concentration in the treatment chamber (in expt. B every 2 h, in expt. C every 50 min).

Proteins and nucleic acids from livers and testes were separated by the phenol m-cresol 8-hydroxyquinoline method (10). The DNA was precipitated from the aqueous phase by 2 volumes of 2-ethoxyethanol, dissolved in 0.1 M phosphate buffer pH 7, reduced with sodium borohydride and reprecipitated with 2-ethoxyethanol.

Hydrolysis and ion-exchange chromatography of proteins and DNA. Samples of 100 - 300 mg of protein were hydrolyzed with 6 N HCl Suprapur (1 mg/0.1 ml) in evacuated tubes at 120°C for 15 h. After evaporation to dryness the product was dissolved in 2 - 5 ml of 0.1 N HCl and incubated at 37°C for 1 h to hydrolyze 2-chloroethylcysteine which forms from part of the hydroxyethylcysteine during protein hydrolysis. Hydroxyethylcysteine and the two hydroxyethylhistidines were added as carriers. The amino acids were then separated on a Dowex 50W-X4 column (80 x 1 cm) which was eluted with 400 ml of 1 N HCl followed by 2 N HCl. The effluent was collected in 4.5 - 5 ml fractions. Hydroxyethylcysteine eluted after 170 ml 1 N HCl, and the hydroxyethylhistidines after 160 ml and 190 ml 2 N HCl, respectively. To achieve a better separation of the histidine products, the corresponding fractions were re-chromatographed on an Aminex A-5 column (0.9 x 20). The column was eluted with 160 ml 0.12 M citrate buffer pH 4.26, followed by 0.12 M buffer pH 5.30. The two hydroxyethylhistidines eluted after 43 and 53 ml, respectively, of the last-mentioned buffer. The presence of amino acids was detected by thin layer chromatography (1).

Samples of about 20 mg DNA were hydrolyzed in 1 N HCl for 1 h at 100°C. After addition of hydroxyethylguanine, the hydrolysates were chromatographed on a Dowex 50W-X12 column (18 x 1.1 cm) with 1 N HCl. The fractions containing hydroxyethylguanine (eluted after ca. 700 ml) were evaporated to dryness and measured for radioactivity.

Radioactivity determinations were made using an Intertechnique SL 30 liquid scintillation spectrometer. Protein samples were dissolved in 1 ml 0.01 N NaOH and counted after addition of 10 ml of Instagel (Packard). Fractions from the ion-exchange columns were counted after addition of equal amounts of Instagel. The counting efficiency was determined using automatic external standardization.

Reaction kinetics of conceivable vinyl chloride metabolites. The rate constants for the reactions of chloroethylene oxide, chloroacetaldehyde and chloroethanol with nucleophiles in water were determined by methods described earlier (10, 12). The data are partly published in another context (13).

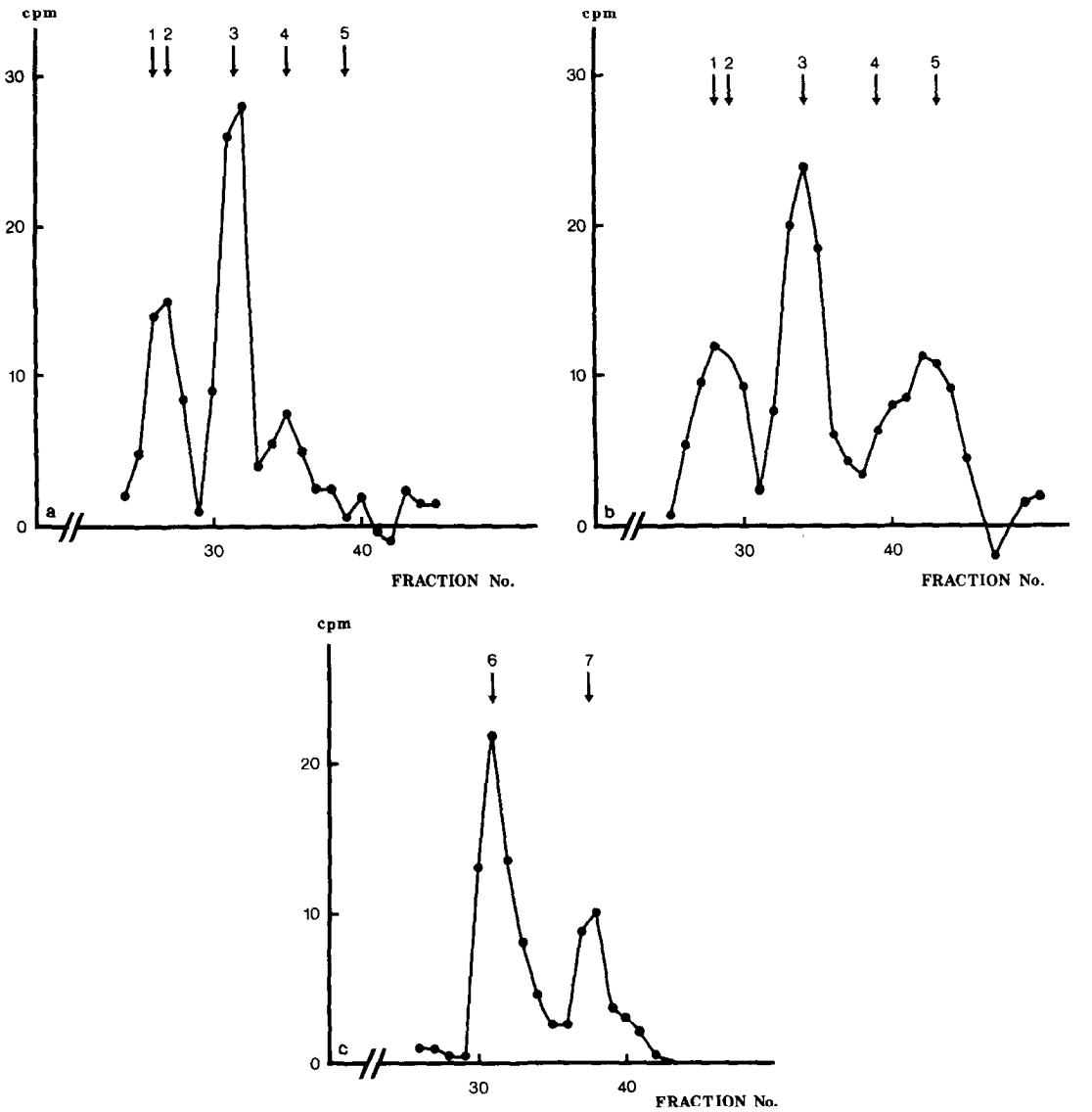

Fig. 1a. Part of the ion-exchange separation of acidic amino acids from a hydrolysate of Hb without preceding reduction of Hb, experiment A. The positions of some known amino acids are indicated 1) serine 2) aspartic acid 3) glycine 4) alanine 5) HOEtCys.
1b. The same fractions as in 1a but after reduction of the Hb (383 mg)
1c. Part of the ion-exchange separation on Aminex A-5 of basic amino acids from a hydrolysate of reduced Hb (383 mg), experiment A. The positions of the two isomeric HOEtHis, 6 and 7, are indicated.

RESULTS AND DISCUSSION

The concentration of vinyl chloride in the inhalation chamber decreased exponentially with time. From Table I it is clear that an increased proportion of BALB

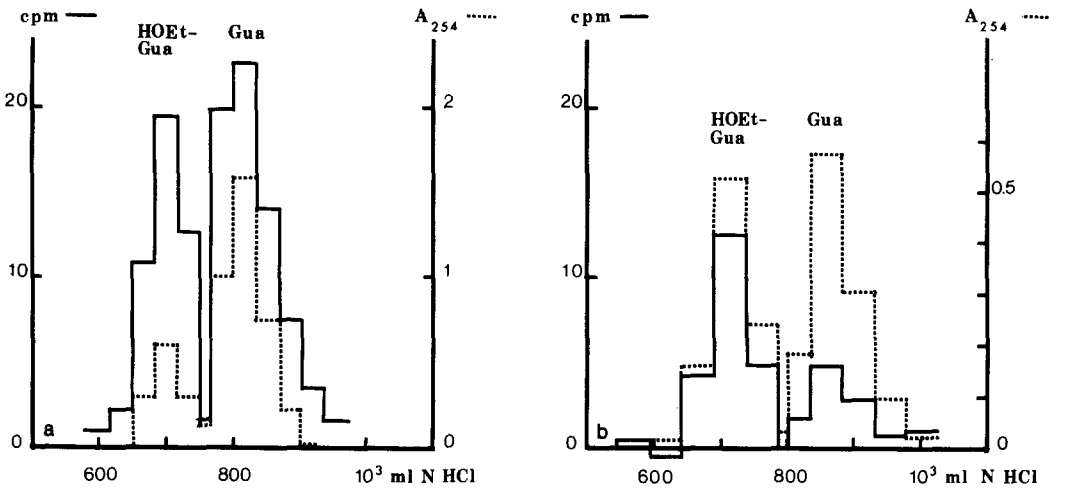

Fig. 2. Ion-exchange separation of reduced liver DNA, experiment B. 2a. 24 mg DNA from BALB mice, 2b. 8.7 mg DNA from CBA mice.

mice in the treatment led to a faster clearance of vinyl chloride from the system. The total incorporation of radioactivity found in Hb from BALB mice is about twice as high as that of the strains CBA and ATL. These observations are consistent with the finding that the rate of uptake of vinyl chloride reflects the rate of metabolic conversion of vinyl chloride (14, 15).

The elution patterns of chromatographed hydrolysates of Hb and liver DNA are shown in Figs. 1 and 2, respectively. Fig. 1a shows part of the chromatogram after hydrolysis of Hb without preceding borohydride reduction, and Fig. 1b shows the same fractions after such reduction. Only in the latter case is a radioactive peak corresponding to hydroxyethylcysteine obtained. The basic fractions, Fig. 1c, show the appearance of the two isomeric hydroxyethylhistidines in the hydrolysate of reduced Hb. The hydrolysate of unreduced material contained no radioactivity in the corresponding fractions. The hydroxyethylguanine recovered after hydrolysis of reduced DNA presents a peak which is well separated from that of guanine (Fig. 2a, b). The labelling of the latter has to be ascribed to metabolic incorporation of ^{14}C.

These results show that vinyl chloride through metabolic activation gives

Table II. Rate constants for reactions of chloroethylene oxide, chloroacetaldehyde, chloroethanol and, for comparison, ethylene oxide (cf. 17) with different nucleophiles in water; k_{H_2O} in h^{-1}, other constants in $l \cdot mole^{-1} \cdot h^{-1}$.

Alkylating agent	Nucleophile	k (20°C)	k (37°C)	s[a]
Chloroethylene oxide	H_2O	6.9	52	0.8
	aniline	820		
	$S_2O_3^{2-}$	$13 \cdot 10^3$		
Chloroacetaldehyde	H_2O		$2 \cdot 10^{-6}$	1.3
	$S_2O_3^{2-}$		13	
2-Chloroethanol	$S_2O_3^{2-}$		$4 \cdot 10^{-2}$	
Ethylene oxide	H_2O	$9.1 \cdot 10^{-3}$		0.96

[a] The s-value describes the sensitivity of an alkylating agent to changes in the nucleophilic strength, n, of a nucleophile. The s-value is calculated from the Swain-Scott equation (cf. e.g. refs. 11 and 17).

rise to an alkylating agent which introduces the 2-oxoethyl group onto nucleophilic sites. It may also be concluded that alkylation by chloroethanol is negligible.

The degree of alkylation, $[RY]/[Y]$, of a nucleophilic group Y *in vivo* is proportional to the rate constant, k_y, for the reaction of the alkylating agent RX with Y, to the initial concentration (or the total amount formed) of RX, $[RX]_0$, and to its mean-life, $1/\lambda$, in the immediate environment of Y, according to the equation

$$[RY]/[Y] = k_y \cdot [RX]_0 \cdot 1/\lambda \qquad [1]$$

where λ is the rate constant for the disappearance of RX (1, 8, 17).

The rate constants for the reactions of chloroacetaldehyde and chloroethylene oxide with amino acids in Hb and with guanine-N-7 in DNA are not known, but may be estimated from known rate constants for reactions with simple nucleophiles (Table II) and by comparison with corresponding values for ethylene oxide (1, 8, 17). Estimated values of the rate constants for the reaction of histidine in Hb with chloroacetaldehyde and chloroethylene oxide at 37°C are $3 \cdot 10^{-7}$ and 0.1 $l \cdot (g\ Hb)^{-1} \cdot h^{-1}$, respectively.

Table III. Degree of alkylation of cysteine and histidine of haemoglobin and guanine-N-7 in liver DNA.

Expt.	Strain	HOEtCys (nmole/g Hb)	The two isomeric HOEtHis (nmole/g Hb)		HOEtGua (nmole/g DNA)
A	CBA	0.17	0.21	0.07	0.6±0.4
B	CBA	1.5	1.9	0.8	4.3
	BALB	3.4	4.2	1.3	2.6

Insertion of this rate constant of chloroacetaldehyde into eqn [1] shows that, in order to account for the found degree of alkylation of Hb, it has to be assumed that practically all vinyl chloride contained in the alveolar ventilation (8) is converted to chloroacetaldehyde, and that this compound has a mean retention time ($1/\lambda$) of several hours. These are very unlikely assumptions. On the contrary, if vinyl chloride is converted to chloroethylene oxide, the mean-life of this compound could be estimated to be in the order of seconds, which seems very reasonable.

Considering the s-values of the proposed intermediates (Table II) and the fact that cysteine and histidine residues differ by about 2 units in the Swain-Scott nucleophilicity scale (cf. 17), the ratio of cysteine to histidine alkylation in Hb is expected to be 2 times lower for chloroethylene oxide and about 5 times higher for chloroacetaldehyde than for ethylene oxide. The experiments showed this ratio to be 0.56 (cf. Table III) compared to 0.75 for ethylene oxide (17). This further strengthens the conclusion that chloroethylene oxide is the main reactive metabolite. The degree of alkylation of guanine-N-7 in DNA is close to that expected on the basis of Hb data, assuming the dose to be equal in the liver and the blood (cf. 1, 8, 17). BALB mice show a higher ratio of Hb to DNA alkylation (Table III), and a higher ratio of metabolic incorporation to alkylation of guanine in DNA (Fig. 2) than CBA mice. This indicates that there are strain differences at several levels.

Small amounts of the two hydroxyethylhistidines were found in proteins from

pooled testes (12 and 6 dpm, respectively, in 134 mg of testis proteins from BALB mice, expts. B and C). Thus the male gonads are exposed to the alkylating intermediate and a risk of heritable damage, in addition to the cancer risk must be expected.

Further studies on the recovery of alkylation products by this method, and studies on the relative role of alternative pathways, e.g. formation of imidazo derivatives with adenosine and cytidine (18), are required for meaningful risk evaluations.

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