LIVER-MICROSOME-MEDIATED FORMATION OF ALKYLATING AGENTS FROM VINYL BROMIDE AND VINYL CHLORIDE*

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Summary: When a mixture of vinyl chloride/oxygen or vinyl bromide/air was passed through a mouse-liver microsomal system, volatile alkylating metabolites were trapped by reaction with excess 4-(4-nitrobenzyl)pyridine. The absorption spectra of the adducts, either from vinyl bromide or vinyl chloride, were identical with that obtained by reaction of chloroethylene oxide with 4-(4-nitrobenzyl)pyridine. Chloroethylene oxide decomposes in aqueous solution with a half-life of 1.6 minutes. After reaction of chloroethylene oxide and 2-chloroacetaldehyde with adenosine and Sephadex chromatography the binding products were compared with those formed in the presence of vinyl chloride, mouse-liver microsomes and adenosine. A common product of these reactions was tentatively characterized as 3-β-ribofuranosyl-imidazo-[2,1-*i*]purine.

The various adverse biological effects of VCM, its carcinogenicity in experimental animals[1,2] and in man[3-5] and its mutagenic action in microbial systems[6-9], appear to be largely dependent upon its metabolic activation[10], since hepatic microsomal mixed-function oxidases from rats, mice and humans have been shown *in vitro*[6-8] to be equally effective in generating metabolites which bind to bacterial or exogenous DNA[11]. Chloroethylene oxide, which has been proposed as a primary reactive metabolite of vinyl chloride[12], rearranges

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Abbreviations: VCM = vinyl chloride monomer; 4-NBP = 4-(4-nitrobenzyl)pyridine.

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spontaneously to 2-chloroacetaldehyde[13]; both compounds have been shown to be strong mutagens in *S. typhimurium* strains[7,8,14] and in Chinese hamster V79 cells[15]. We report here on mouse-liver-microsome-mediated formation of alkylating agents from vinyl bromide and VCM and the partial characterization of their binding products with adenosine.

MATERIALS AND METHODS

Chemicals

VCM (purity 99.9%) was generously provided by Rhône-Progil, Lyon, France; vinyl bromide (98%) was obtained from Aldrich Chemical Co.; 2-chloroacetaldehyde (50% aqueous solution) from Merck, Darmstadt, FRG and alcohol dehydrogenase (200 U/mg) from Boehringer, Mannheim, FRG. Chloroethylene oxide (b.p. 65-67°C; purity >95%) was prepared and stored as previously described[8].

Assays

I Rate of decomposition of chloroethylene oxide in aqueous solution: 40 µmol chloroethylene oxide in a total volume of 30 ml 0.1M Tris-HCl buffer pH 7.4) : acetone (2:1 v/v) were incubated at 37°C. Aliquots of 1.5 ml were removed at various intervals, and 2 ml ethylene glycol containing 0.04 g 4-NBP were added. After 30 min of incubation at 37°C, 2.5 ml of a mixture of triethylamine : acetone (1:1 v/v) were added, and absorbance at 560 nm was determined after two minutes[16].

II Reaction of 2-chloroacetaldehyde and chloroethylene oxide with 4-NBP: 2.4 µmol chloroethylene oxide or 2-chloroacetaldehyde were incubated in a mixture of 3 ml acetone, 6 ml 0.1M Tris-HCl buffer (pH 7.4) and 12 ml ethylene glycol containing 0.4 g 4-NBP. Aliquots of 3 ml were removed at various time intervals, 2.5 ml of a mixture of triethylamine : acetone (1:1 v/v) were added and absorbance at 560 nm was read against a blank[16].

III Absorption spectra of reaction products of chloroethylene oxide and 2-chloroacetaldehyde with 4-NBP: 1.21 µmol of chloroethylene oxide or 955 µmol chloroacetaldehyde were incubated in a mixture of 0.5 ml acetone, 1 ml 0.1M Tris-HCl buffer (pH 7.4) and 2 ml ethylene glycol containing 186 µmol 4-NBP for 30 min at 37°C. After cooling the reaction mixture to 0°C, 2.9 ml of a mixture of triethylamine : acetone (1:1 v/v) were added. The absorption spectra were recorded against an appropriate blank two or 20 min after addition of the amine[16].

IV Absorption spectra of reaction products of vinyl chloride or vinyl bromide with 4-NBP in the presence of a mouse-liver microsome system: A mixture of VCM or vinyl bromide in oxygen (50% by vol.) was sucked through a medium (final volume, 5 ml) containing 1.5 ml of a liver microsome fraction prepared[8] from phenobarbitone pre-treated male OF-1 mice (1% phenobarbitone in drinking-water for seven days), 40 µmol $MgCl_2$, 25 µmol glucose 6-phosphate, 20 µmol $NADP^+$, 10 U glucose 6-phosphate dehydrogenase and 92 µmol Sørenson phosphate buffer, pH 7.4. The average flow rate of the pre-warmed gas mixtures was 350 ml/min. The gas mixture and the volatile compounds formed were passed through a siphon containing a solution of 467 µmol 4-NBP in 5 ml ethylene glycol. After 30 min the gas flow was stopped, and 2 ml of the glycol solution were combined with 1.0 ml 0.1M Tris-HCl buffer (pH 7.4) and 0.5 ml acetone. After 30 min of incubation at 37°C, 2.9 ml of a mixture of triethylamine : acetone (1:1 v/v) were added.

The absorption spectra were recorded as described in assay III. Control assays were performed under identical conditions, except that $NADP^+$ and glucose 6-phosphate were omitted.

V Reaction of chloroethylene oxide or 2-chloroacetaldehyde with adenosine: 225 µmoles 2-chloroacetaldehyde were added to a solution of 45 µmol adenosine in 4 ml of ethanol : 9.5 mM citrate buffer, pH 7 (2:5 v/v). The reaction mixture was stirred under N_2 for two hrs at 37°C. After lyophilization, samples of the residue were redissolved and applied to Sephadex G-10 columns.

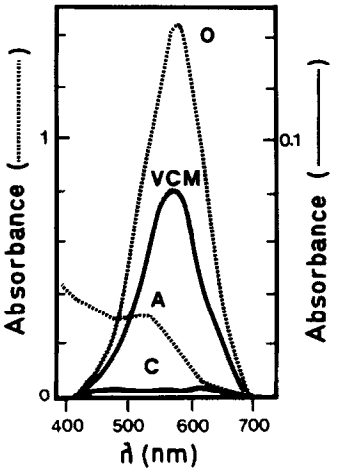
6.67 mmol chloroethylene oxide were added to a solution of 1.31 mmol adenosine dissolved in 40 ml of ethanol : 2 mM citrate buffer, pH 7 (2:5 v/v). The reaction mixture was stirred under N_2 for five min at 37°C and then another 17.2 mmol chloroethylene oxide were added. After a total of 15 min incubation at 37°C the reaction mixture was evaporated; the residue was lyophilized for 12 hrs then treated as above.

VI Reaction of VCM with adenosine in the presence of a liver microsome fraction: 5 ml of a mouse-liver microsome suspension, prepared as described in assay IV, were added to an aqueous solution of adenosine (15.1 mg/ml). A mixture of VCM : air (1:1 v/v), with an average flow rate of 385 ml/min, was bubbled through the solution at 37°C for 30 min. The reaction mixture was dried under vacuum and extracted with 0.05 M ammonium formate, pH 6.8. Insoluble material was removed by centrifugation and aliquots of the supernatant were applied to Sephadex G-10 columns as described in assay VII. Control assays were performed under identical conditions, except that $NADP^+$ and glucose 6-phosphate were omitted.

VII Chromatography of reaction products of VCM metabolites with adenosine on Sephadex G-10 columns: Dry residues from assays V and VI were dissolved in 1.5-4 ml 0.05M ammonium formate solution, pH 6.8, and applied to a Sephadex G-10 column (1.6 x 90 cm) previously equilibriated with 0.05 M ammonium formate. The column was eluted with the same buffer at a flow rate of 40 ml/hr; 10 ml fractions were collected and monitored at 254 nm with an LKB Uvicord III attached to an Ultrarac 7000. Spectra of the fractions with maximal absorbance were determined with a Pye Unicam SP 1800 spectrophotometer.

RESULTS AND DISCUSSION

We have compared the kinetics of the alkylation of 4-NBP by chloroethylene oxide and by 2-chloroacetaldehyde. The colorometric method[16] used is based on the formation of 4-NBP derivatives with absorption maxima of between 530 and 570 nm (see Fig. 1). With a molar ratio of 775 : 1 of 4-NBP: chloroethylene oxide, absorbance at 560 nm (A560) increased from 0.01 to 0.9 after 90 seconds of reaction at 37°C; no further increase was noted up to 60 min (Materials and Methods, Assay II). Under the same conditions, the A560 for 2-chloroacetaldehyde showed no time-dependent increase up to 60 min. Since it appears unlikely that these differences in the rate of alkylation are due to large variations in the extinction coefficients of the adducts formed, the data indicate that chloroethylene



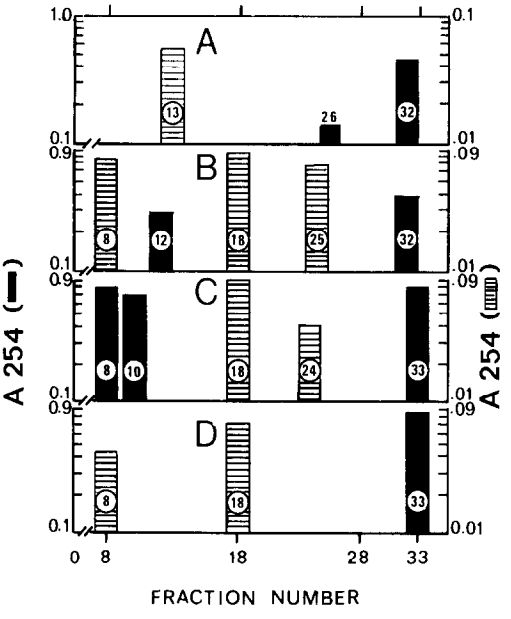
Legend to Fig. 1

Absorption spectra were recorded after reaction of 4-NBP with chloroethylene oxide (------, O) or with 2-chloroacetaldehyde (------, A). The spectrum of VCM (———, VCM) was recorded by passing a mixture of VCM/oxygen through a medium consisting of liver microsomes from phenobarbitone-pre-treated mice and an NADPH-generating system and reacting the volatile metabolite with 4-NBP in ethylene glycol. For the control (———, C), $NADP^+$ and glucose 6-phosphate were omitted (Materials and Methods, Assays III and IV).

oxide is a highly reactive electrophile.

The rate of decomposition of chloroethylene oxide in acetone buffer at pH 7.4 by hydrolysis or by rearrangement was determined using the same colorimetric method[16] after different lenths of pre-incubation at 37°C up to 10 min (Materials and Methods, Assay I). The time-dependent disappearance of chloroethylene oxide, as monitored at A560, is a first-order kinetic reaction: $A560 = A560(0) \times e^{-kt}$; [A560(0) (no pre-incubation) = 5.8; k = 0.43 (min^{-1}); $t_{\frac{1}{2}}$ = 1.6 min]. The high alkylating potency of chloroethylene oxide and its short half-life in aqueous solution link this compound to the group of highly reactive α-chlorinated aliphatic ethers, among which bis(chloromethyl)ether ($t_{\frac{1}{2}}$ for hydrolysis, <2 min[17]) is carcinogenic in animals and in man[18].

When a mixture of VCM : oxygen (1:1 v/v) was passed through a medium consisting of liver microsomes from phenobarbitone-pre-treated mice and an NADPH-generating system, a volatile metabolite was formed; this was trapped by its reaction with 4-NBP in ethylene glycol (Fig. 1). The absorption spectrum

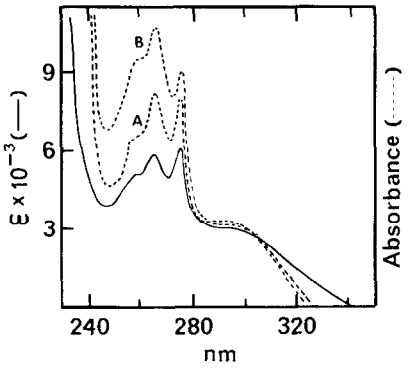


Legend to Fig. 2

Elution profiles after Sephadex G-10 chromatography of products obtained by reaction of adenosine with 2-chloroacetaldehyde (A), with chloroethylene oxide (B), with VCM/air in the presence of a mouse-liver microsomal system (C) or with VCM/air in the absence of cofactors for mixed-function oxidase (D). Aliquots of the lyophilized samples (Materials and Methods, Assays V and VI) corresponding to 5-17 mg adenosine were applied on the column as starting material. Adenosine was eluted as fractions 32-33. Two scales (0.1-0.9 and 0.01-0.09) for absorbance at 254 nm (A254) are given.

of the adduct (A570 = 0.07) proved to be identical to that obtained with chloroethylene oxide and 4-NBP; 2-chloroacetaldehyde showed a different spectrum (Fig. 1).

When a mixture of VCM/air was passed through a similar microsomal system, the absorption maximum of the adduct of the resulting metabolite with 4 NBP at 570 nm was reduced to 0.03. Using the same assay, vinyl bromide : air (1:1 v/v) in the presence of a mouse-liver microsomal fraction yielded a product which upon reaction with 4-NBP gave an identical spectrum with A570 = 0.05 (Fig. 1). In the absence of NADP+ and glucose 6-phosphate no such product was formed from either VCM or vinyl bromide (Fig. 1). These data strongly suggest that vinyl chloride and vinyl bromide are converted by microsomal enzymes into



Legend to Fig. 3

Comparison of the UV spectra of the reaction products of adenosine with 2-chloroacetaldehyde (A) or with chloroethylene oxide (B) in 0.05 M ammonium formate, pH 6.8. The products were obtained after chromatography on Sephadex G-10 as fractions A26 and B25, respectively (Fig. 2). The authentic spectrum of 3-β-D-ribofuranosyl-imidazo-[2,1-*i*]purine[19] is also plotted (———).

the corresponding epoxides[10,12], which react with 4-NBP to give similar adducts. Vinyl bromide and its presumed metabolites have been shown to be strong mutagens in *S. typhimurium* strains (Malaveille, Bartsch and Montesano, unpublished).

2-Chloroacetaldehyde reacts with nucleotides. Its reaction with adenosine forms a cyclic fluorescent product which has been characterized as 3-β-D-ribofuranosyl-imidazo-[2,1-*i*]purine[19]. The reaction products of 2-chloroacetaldehyde or chloroethylene oxide with adenosine (identified as fractions A26 and B25) have been separated on Sephadex G-10, and their elution positions have been compared with those of the products obtained from reaction of vinyl chloride with adenosine in the presence of a microsomal system (Fig. 2). With thin-layer chromatography on cellulose these fractions appeared as fluorescent spots with identical Rf-values (0.58 in isopropanol:ammonia:water = 6:3:1 by vol.). Their UV absorption spectra matched that reported for the cyclic fluorescent adenosine derivative[19] (Fig. 3). When adenosine was reacted with VCM/air in the presence of a liver-microsome system, the same product (Fig. 2, fraction C24) was isolated by column chromatography. Elucidation of the structures of compounds eluted in peaks A13, B8, 12, 18 and C8,10 (Fig. 2) is being attempted.

The identification of chloroethylene oxide as a primary reactive metabolite of VCM *in vitro*, its alkylating capacity and the fact that it and its rearrangement product, 2-chloroacetaldehyde, both bind covalently to nucleophilic centres[20] e.g., adenosine, suggest that similar base alterations in DNA may explain the mutagenic activity of 2-chloroacetaldehyde and of chloroethylene oxide in bacterial[7,8,14] and mammalian cell systems[15].

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