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Dear Mr Wheeler

VCM METABOLISM

You may care to have the enclosed publication by Green and Hathway in Chem-Biol Interactions (1977) pages 137 - 150. I also enclose a copy of the paper Dr Hathway gave at Bethesda, Maryland, on 2 - 4 May, 1977 - this is due to be published in Environmental Perspectives in the near future.

Yours sincerely

J Stafford
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ENC

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THE CHEMISTRY AND BIOGENESIS OF THE S-CONTAINING METABOLITES OF VINYL CHLORIDE IN RATS

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SUMMARY

In order to determine whether vinyl chloride yields chloroethylene oxide *in vivo*, the biogenesis of the various urinary S-containing metabolites in rats has been investigated.

N-Acetyl-S-(2-hydroxyethyl)cysteine is a major vinyl chloride metabolite in rats, but according to the method of protective esterification that is used, so either *N*-acetyl-S-(2-chloroethyl)cysteine or *N*-acetyl-S-(2-hydroxyethyl)cysteine may be isolated from the body fluids. *N*-Acetyl-S-vinylcysteine is a second related metabolite. These S-containing vinyl chloride metabolites are not mutagenic in *S. typhimurium*. Neutral methanol methylates *N*-acetyl-S-(2-hydroxyethyl)cysteine. *N*-Acetyl-S-(2-methoxyethyl)cysteine plus *N*-acetyl-S-vinylcysteine degrade to give the volatile S-(2-methoxyethyl)(prop-1 or 2-enyl)sulphide.

Administration of several vinyl chloride metabolites and closely related compounds to rats shows that chloroacetaldehyde and S-(carboxymethyl)cysteine, but not chloroacetic acid, lie on a pathway or pathways connecting vinyl chloride with thiodiglycollic acid. The fact (a) that chloroacetaldehyde affords both thiodiglycollic acid and *N*-acetyl-S-(2-hydroxyethyl)cysteine in the animal and (b) that S-(carboxymethyl)cysteine has been identified amongst the hydrolytic products from an hepatic extract prepared from vinyl chloride-treated animals is consistent with the formation of chloroacetaldehyde, and with the reaction of chloroethylene oxide or chloroacetaldehyde with glutathione in the presence of a glutathione S-epoxide transferase to give the identified S-containing metabolites.

Abbreviations: GC, gas chromatograph.

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INTRODUCTION

The way in which various S-containing metabolites of vinyl chloride are formed *in vivo* ought to provide evidence for the epoxidation which has been suggested by *in vitro* studies [1-4]. This supposition has caused us to investigate again the derivation of these substances, because in a previous paper [5], where the Fischer-Speier esterification with butan-1-ol had been used for stabilising the S-containing vinyl chloride metabolites, it became apparent (see Ref. 6) that such derivative formation may be less suitable for protecting these substances in comparison with amino acids, and, e.g. 2-chloroethyl sulphides would be expected to be highly reactive in water [7].

The present paper describes the results obtained and their possible interpretation. Poster material (no. 04-7-433) about some preliminary results was exhibited at the 10th International Congress of Biochemistry that was held in Hamburg during July 25-31, 1976.

MATERIALS AND METHODS

Chemicals

Bromoethanol and L-cysteine were condensed with Na and liquid NH_3 to give S-(2hydroxyethyl)-L-cysteine [8], which crystallized from 92% ethanol with a m.p. of 202°C (decomp.). (Carson and Wong [8] gave m.p. of $189-189.5^\circ\text{C}$). (Found: C, 36.5; H, 6.8; N, 8.4; S, 19.4%; $\text{C}_5\text{H}_{11}\text{O}_3\text{NS}$ requires C, 36.4; H, 6.7; N, 8.5; S, 19.4%). S-(2-Hydroxyethyl)-L-cysteine on heating in reagent hydrochloric acid (38%) gave S-(2-chloroethyl)-L-cysteine hydrochloride (m.p. 182°C), and from these 2 compounds, N-acetyl-S-(2-hydroxyethyl)-L-cysteine and N-acetyl-S-(2-chloroethyl)-L-cysteine were prepared [8]. The mass spectra of all 4 substances were in agreement with the proposed structures, and that of the O-methyl ester derivative of N-acetyl-S-(2-hydroxyethyl)-L-cysteine is shown in Fig. 1, but that of the O-butyl ester of N-acetyl-S-(2-chloroethyl)-L-cysteine was recorded previously [5].

S-vinyl-L-cysteine was prepared from trichloroethylene by 2-stage synthesis: trichloroethylene and L-cysteine were condensed with Na and liquid NH_3 to give S-(1,2-dichlorovinyl)cysteine, which was dechlorinated with Al-amalgam to give S-vinyl-L-cysteine [9]. Without further purification, the crude S-vinyl-L-cysteine was acetylated in boiling acetic anhydride. The structure of N-acetyl-S-vinyl-L-cysteine was confirmed by the mass spectrum, and that of the O-methyl ester derivative is shown in Fig. 1.

S-Carboxymethyl-L-cysteine was prepared [10] from chloroacetic acid plus L-cysteine hydrochloride, and the structure of the crystalline product (m.p. $175-176^\circ\text{C}$, decomp.) was confirmed by mass spectrometry.

Chloroethylene oxide was prepared pure by the method of Rannug et al. [11] *. Purity was assessed by gas chromatography, by which chloroethylene

* Under chemical conditions, chloroethylene oxide has been found to react with cysteine to give 4-carboxy-2-chloromethylthiazolidine, thus involving intermediate formation of S-(2-chloro-1-hydroxyethyl)cysteine but it is very unlikely that the analogous intermediate would have been formed from glutathione *in vivo* in the presence of glutathione S-epoxide transferase, namely S-(2-hydroxyalkyl)glutathione alkyl-epoxide-lyase [E.C. 4.4.1.7].

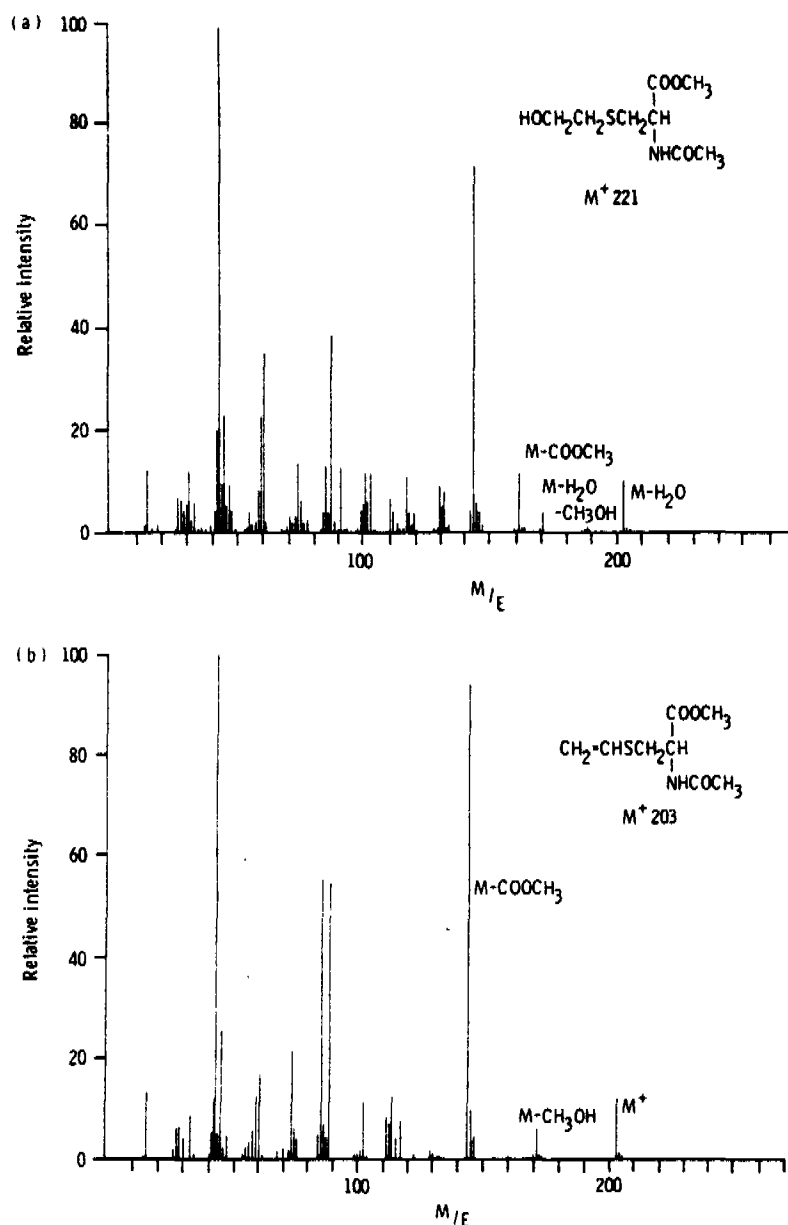


Fig. 1. Mass spectrum of: (a) the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl) cysteine; (b) the O-methyl ester of *N*-acetyl-S-vinylcysteine.

oxide was readily separable from its chloroacetaldehyde transformation product and from the ethylene oxide starting material. In the mass spectrometer, chloroethylene oxide is converted into chloroacetaldehyde.

Radioactive chemicals

[^{14}C]vinyl chloride with a specific activity of 0.46 mCi/mmol, and with a radiochemical purity exceeding 99.0% was synthesized as previously outlined [5].

L-[U- ^{14}C]cysteine hydrochloride with a specific activity of 24.5 mCi/mmol was obtained from the Radiochemical Centre, Amersham, Bucks. (Great Britain).

Experiments with animals

Adult male rats (approx. 2 months old, 200 g body weight) were used [Alderley Park strain (Wistar-derived), specific pathogen-free], and kept on a standard pellet diet.

For the purpose of identifying urinary metabolites [5], groups of 4 rats were administered intragastrically.

- (a) [^{14}C]vinyl chloride (100 mg/kg; 10 μCi) in corn-oil solution.
- (b) Chloroacetaldehyde (50 mg/kg) as an aq. soln.
- (c) S-(2-hydroxyethyl)-L-cysteine (500 mg/kg) as an aq. soln.
- (d) S-(carboxymethyl)-L-cysteine (250 mg/kg) in corn-oil suspension, and a 24-h collection of urine was made. Control animals were used in experiments (b)–(d).
- (e) Two rats were each given repeated daily i.p. injections of an aq. soln. of L-[U- ^{14}C]cysteine hydrochloride for 5 days (total dose, approx. 50 μCi). 30 min after the final injection, each animal was given a single dose of vinyl chloride (100 mg/kg, in corn-oil solution by stomach tube, and the 24-h urine was collected.
- (f) Two rats were each given a single intragastric dose of [^{14}C]vinyl chloride (450 mg/kg; 15 μCi) as a corn-oil solution. The animals, which were killed by cervical dislocation after 45 min, were dissected rapidly and their livers removed.

Measurement of radioactivity

An automated and computerized Intertechnique Model SL30 Liquid Scintillation Spectrometer was used for measurement of ^{14}C , making use of standard channels-ratio quench-correction curves. Liquid samples were mixed with standard scintillator and radio-assayed direct.

Systematic separation of the urinary metabolites into fractions of chemically similar substances

Urine from each group of rats, (a)–(e), was separated into 3 fractions by anion-exchange chromatography in a manner analogous to that described by Green and Hathway [5]. The 3 N acetic acid fractions and the 3 N HCl fractions were evaporated separately to dryness under reduced pressure, and the different residues were methylated with an ethereal solution of diazomethane. Methanolic solutions of the resulting O-methyl esters were analysed in the gas chromatograph–mass spectrometer system and by mass fragmentom-

etry. The following objectives were pursued with the various residues from the different groups of rats (a)–(e).

(a) Both the 3 N acetic acid and 3 N HCl fractions were investigated for [^{14}C]vinyl chloride metabolites.

(b) The 3 N acetic acid fraction was analysed by GC–mass spectrometry, as well as by mass fragmentometry, for the *N*-acetyl-S-(2-hydroxyethyl)cysteine metabolite (of vinyl chloride), and the 3 N HCl fraction was investigated for the presence of thiodiglycollic acid and chloroacetic acid by GC–mass spectrometry.

(c) and (d) The 3 N HCl fractions were analysed quantitatively for thiodiglycollic acid by GC–mass spectrometry.

(e) In the experiment in which unlabelled vinyl chloride was dosed to rats in which the cysteine–cystine pools had been labelled adequately with ^{14}C , the 3 N HCl fraction was examined in the GC–mass spectrometer for the presence of the thiodiglycollic acid metabolite and in the gas chromatograph for a peak of [^{14}C]thiodiglycollic acid.

Systematic separation of the hepatic metabolites into fractions of chemically similar substances

The combined livers from the 2 animals (f) (see above) were homogenized in 10 times the volume of extracting solution, which was prepared fresh by mixing 3 vols. of ice-cold ethanol with 1 vol. of ice-cold KH_2PO_4 buffer (24 mmole; pH 5.5) containing EDTA (0.1 mmole) [12]. The resulting homogenate was centrifuged at 10 000 g in a refrigerated head for 15 min, and the supernatant was removed and evaporated under reduced pressure to 5 ml. This concentrate, which contains glutathione and glutathione-related substances, was adjusted to pH 10–11 and applied to a column of IRA-410 anion-exchange resin in the CH_3CO_2^- cycle, which was successively washed with deionized water (500 ml) and eluted with 3 N acetic acid [5]. Bulk fractions containing significant amounts of ^{14}C were evaporated to dryness under reduced pressure, and a solution of the residue, which was formed by heating in 0.4 ml of formic acid (90%) for 5 min at 100°C, was mixed with 4 ml of 2 N HCl, and heated for 2 h at 100°C [13]. An evaporate, prepared from the resulting hydrolysate, was successively esterified with diazomethane and acylated with trifluoroacetic anhydride in methylene chloride solution. The derivatized sample was examined both by means of GC–mass spectrometry and by mass fragmentometry.

Gas chromatography

Fractions containing radioactive metabolites were examined with a Pye Model 104 instrument that was equipped with flame-ionization detection and that was coupled to an E.S.I. Nuclear 504 Radiogas detector. The column effluent was split in the ratio of 10 : 1 between the Radiogas detector and the flame-ionization detector. This gas chromatograph was fitted with glass columns (1.5 m long $\times$ 4 mm internal diameter), which were packed either with 6% (w/w) of OV-101 on Gas Chrom Q (80–100 mesh size) or

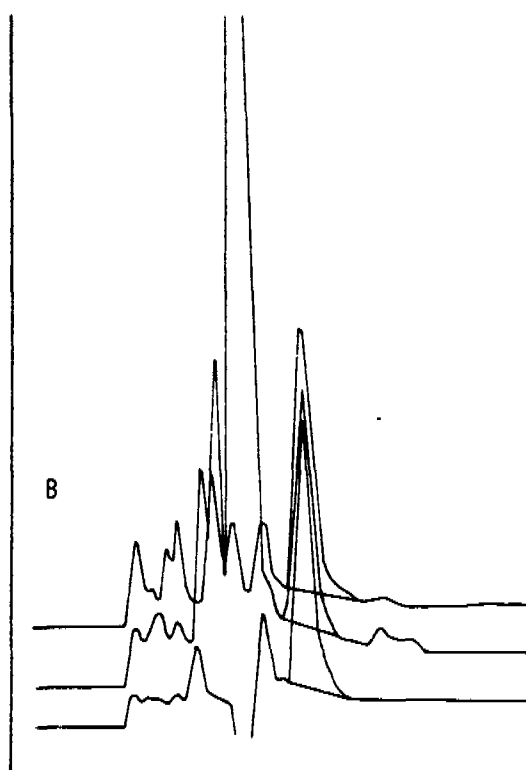
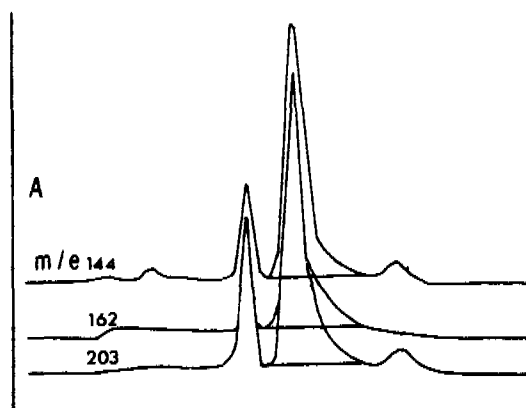


Fig. 2. Mass fragmentogram of the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine from the urine of chloroacetaldehyde-treated rats (the gains from m/e 144, m/e 162 and m/e 203 are in the ratio 1 : 10 : 10). A represents the injection point for a methanolic solution of the standard sample and B that for the 3 N acetic acid fraction (for details, see Materials and Methods, derived from urinary material. The latter shows all 3 ions with identical relative intensities and retention time as the standard.

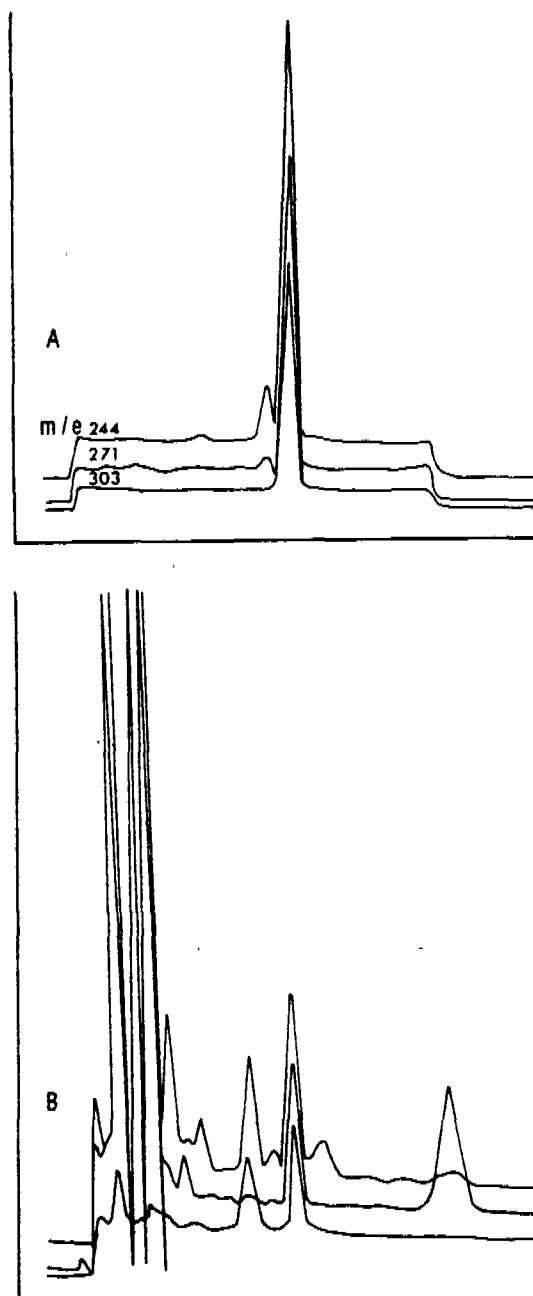


Fig. 3. Mass fragmentogram of the O-dimethyl ester of the *N*-trifluoroacetyl derivative of *S*-(carboxymethyl)cysteine from the urine of chloroacetaldehyde-treated rats (the gains for *m/e* 303, *m/e* 271 and *m/e* 244 are in the ratio 1 : 1 : 1). A represents the injection point for a solution of the standard sample and B that for the 3 N acetic acid fraction (for details, see Materials and Methods) derived from urinary material. The latter shows all 3 ions with identical relative intensities and retention time as the standard.

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with 10% (w/w) of SP2330 on Supelcoport (100–120 mesh size). All of the columns were operated at a 30 ml/min flow rate of a (95 : 5 v/v) A–CO₂ mixture.

Radioactive peaks were located, and the corresponding samples were analysed by GC–mass spectrometry, using the same columns under identical operating conditions. Fractions containing unlabelled metabolites were examined directly by GC–mass spectrometry, using the previously-described columns.

Fractions of chloroethylene oxide were tested in the gas chromatograph by using a column (2.7 m long × 2 mm internal diameter) which had been packed with 20% (w/w) of OV-275 on Chromosorb W-AW and which was run at 50°C at 30 ml/min flow rate of He. Under these conditions, chloroethylene oxide had a retention time of 2.2 min, chloroacetaldehyde of 3.6 min and ethylene oxide of 0.5 min.

GC–mass spectrometry

An LKB2091 GC–mass spectrometer system was used for the E.I. spectra and a Dupont 21-491B GC–mass spectrometer system for C.I. spectra.

Mass fragmentometry

In the case of the methyl ester of *N*-acetyl-*S*-(2-hydroxyethyl)cysteine (Fig. 2), 3 selected ions of known *m/e* value were *m/e* 203 corresponding to *M*–H₂O, *m/e* 162 corresponding to *M*–CO₂CH₃, and *m/e* 144 corresponding to *M*–H₂O–CO₂CH₃. The 6% OV-101 column was used and run at 195°C. Under these conditions, the methyl ester of *N*-acetyl-*S*-(2-hydroxyethyl)cysteine had a retention time of 5.6 min.

In the case of the dimethyl ester of the *N*-trifluoroacetyl derivative of *S*-(carboxymethyl)cysteine (Fig. 3), the selected ions were *m/e* 303 corresponding to the molecular ion, *m/e* 271 corresponding to *M*–CH₃OH and *m/e* 244 corresponding to *M*–CO₂CH₃. The column, used in the gas chromatograph, was one (2.7 m long × 2 mm internal diameter) which was packed with 20% (w/w) of OV-275 on Chromosorb W-AW and which was run at 250°C. Under these conditions, the dimethyl ester of the *N*-trifluoroacetyl derivative of *S*-(carboxymethyl)cysteine has a retention time of 5.4 min (Fig. 3).

Mutagenicity test

The mutagenic potential of *S*-(2-hydroxyethyl)cysteine and of *S*-(2-chloroethyl)cysteine was assessed by the method of Ames et al. [14] using *S. typhimurium* strains TA1535, TA1538, TA98 and TA100.

RESULTS AND DISCUSSION

N-Acetyl-*S*-(2-hydroxyethyl)cysteine is a major vinyl chloride metabolite in rats, but dependent upon the method of derivative formation that was used so either *N*-acetyl-*S*-(2-chloroethyl)cysteine (b) (Fig. 4) or *N*-acetyl-*S*-

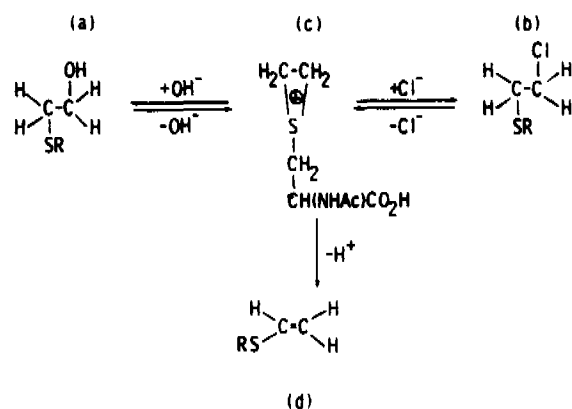


Fig. 4. Scheme for the interrelationship of some S-containing vinyl chloride metabolites.

(2-hydroxyethyl)cysteine (a) was isolated from the body fluids of vinyl chloride-treated animals. Thus, e.g. the O-methyl ester of *N*-acetyl-S-(2-chloroethyl)cysteine (b) was obtained if the Fischer-Speier reaction with methanol was employed for the purpose of esterification, whereas the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine (a) was produced if diazomethane was used instead. The mass spectrum of the O-methyl ester of *N*-acetyl-S-(2-chloroethyl)cysteine (b) was identical with that of the authentic substance [8]. It is noteworthy that the molecular ion ($M^+ = 239$) was absent from the conventional mass spectrum. Similarly, the mass spectrum (Fig. 1a) of the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine (a) (Fig. 4) was identical with that of authentic material [8], and again, the molecular ion ($M^+ = 221$) was absent from the conventional mass spectrum, but the $(M + 1)^+$ ion could be recognized in the mass spectrum, when the chemical ionization source was used in the GC-mass spectrometer system. Furthermore, treatment of the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine (a) with the methanol-HCl reagent furnished a mixture of the O-methyl esters of *N*-acetyl-S-(2-chloroethyl)cysteine (b) and S-(2-chloroethyl)cysteine of which the mass spectra were identical with those of the authentic substances [5]. Conversely, the O-methyl ester of *N*-acetyl-S-(2-chloroethyl)cysteine (b) was hydrolysed rapidly by water to the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine (a).

There is a strong supposition that the reversible reaction processes connecting the substances, *N*-acetyl-S-(2-hydroxyethyl)cysteine (a) and *N*-acetyl-S-(2-chloroethyl)cysteine (b), are modulated through the intermediacy of episulphonium ion (c) (Fig. 4), and on this basis, e.g. the formation of (c) would be rate-determining in respect of the hydrolysis of (b). Moreover, nucleophilic attack of hydroxyl ion on the episulphonium ion (c) would be expected to afford olefine through β -elimination [15]. In fact, a small yield of a second product, *N*-acetyl-S-vinylcysteine (d), was recovered from the urine of [^{14}C]vinyl chloride-treated animals, whenever diazomethane esterifi-

cation was used for protecting the S-containing metabolites. The mass spectrum of the O-methyl ester of *N*-acetyl-S-vinylcysteine was identical with that of authentic material [9]. In this case, the molecular ion ($M^+ = 203$) was produced by electron impact (Fig. 1b).

Manipulation of the S-containing metabolites of vinyl chloride revealed them to be highly reactive substances with a specialized chemistry. Thus, the reaction of the O-methyl ester of *N*-acetyl-S-(2-hydroxyethyl)cysteine with methanol at neutral pH is noteworthy. While the molecular ion ($M^+ = 235$) was absent from the conventional mass spectrum, the $(M+1)^+$ ion could be recognized in the mass spectrum, when chemical ionization was used as the ion source. In another situation, (i) the loss of label from thin-layer plates containing a mixture of the O-methyl esters of *N*-acetyl-S-(2-methoxy[^{14}C]-ethyl)cysteine and *N*-acetyl-S-[^{14}C]vinylcysteine, and (ii) the presence of ^{14}C in the methanol solvent peak of the GC trace of the 2 labelled compounds, indicate their ready transformation under mild conditions of reaction. A volatile product has been identified by mass spectrometry as either [^{14}C]S-(2-methoxyethyl)(prop-1 or 2-enyl)sulphide. Whilst the mechanism of formation has not been investigated, it is felt that acetaldehyde, a known dissociation product of S-vinylcysteine-derived S-vinylcysteine-S-oxide [9] might be involved in a concerted condensation reaction with *N*-acetyl-S-(2-methoxy[^{14}C]ethyl)-cysteine, (leading to possible elimination of glyoxylate).

Since the S-containing vinyl chloride metabolites that generate the episulphonium ion (c) (Fig. 4) do not behave as mutagens in *S. typhimurium* strains, their further investigation was considered to be unrewarding.

At this stage in the work, it seemed important to investigate the biogenesis in the mammal of the S-containing vinyl chloride metabolites. Initially, we sought to determine whether S-(2-hydroxyethyl)cysteine would serve as a source of thiodiglycollic acid, and we were able to isolate the authentic substance [5] in 0.5% theoretical yield from the urine of rats dosed with S-(2-hydroxyethyl)cysteine. This result seemed to be highly significant, because of the previously-described instability of that compound under exceedingly mild conditions of reactions. Furthermore, the metabolic pathway (Fig. 5) concerned, which appears to include end-group oxidation, amino acid transamination and oxidative decarboxylation, admits of other sources for thiodi-

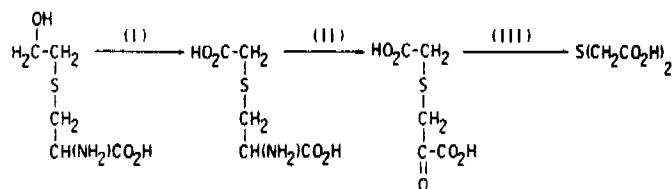


Fig. 5. Scheme suggesting the biotransformation of S-(2-hydroxyethyl)cysteine into thiodiglycollic acid. (I) End-group oxidation; (II) transamination; (III) oxidative decarboxylation.

glycollic acid formation. The fact that high yields (Table I) of thiodiglycollic acid (j) (Fig. 6) were obtained in rats, which were dosed separately with chloroacetaldehyde (g), chloroacetic acid (h) and *S*-(carboxymethyl)cysteine (i) (Fig. 6) suggested that these compounds might lie on a common metabolic pathway connecting vinyl chloride (e) with thiodiglycollic acid (j) and possibly with the other *S*-containing metabolites, namely *N*-acetyl-*S*-(2-hydroxyethyl)cysteine and *N*-acetyl-*S*-vinylcysteine.

Nevertheless, further experimental evidence suggests that the inclusion of chloroacetic acid (h) (Fig. 6) on this metabolic pathway may be incorrigible. Thus, (1) very little chloroacetic acid, <0.1% of the dose, has ever been detected in the body fluids of any of our vinyl chloride-treated rats, and this fact implies either a high rate of turnover for (h) or the possibility that this substance is not a major vinyl chloride metabolite. The latter supposition now seems the more likely, particularly since relatively large amounts of chloroacetic acid have been found [16] in the body fluids of rats, treated with vinylidene chloride, and in those animals, thiodiglycollic acid accounted for an even higher proportion of the dose than in parallel experiments with vinyl chloride. (2) A feasible metabolic pathway of thiodiglycollic acid from chloroacetic acid and involving cysteine desulphydrase [E.C. 4.4.1.1] has now been shown to be unacceptable in experiments with unlabelled vinyl chloride in rats, in which the cysteine-cystine pools had been labelled adequately with [^{14}C]. The formation of labelled thiodiglycollic acid showed that a part of the C-skeleton must be derived in fact from cysteine. (3) Moreover in rats treated with chloroacetaldehyde, the presence of thiodiglycollic acid (j) (Fig. 6) and of *N*-acetyl-*S*-(2-hydroxyethyl)cysteine, but not of chloroacetic acid (h), amongst the urinary metabolites has been established by mass fragmentometry (Fig. 2).

Hence, there is a strong supposition that in rats chloroethylene oxide (f) (Fig. 6) is formed from vinyl chloride [17] and transformed spontaneously into chloroacetaldehyde (g) [18]; there is supporting evidence [1-3] for vinyl chloride epoxidation *in vitro*. The biological interrelationship of glutathione *S*-epoxide transferase [EC 4.4.1.7] and substrate epoxide is well

TABLE I

RELATIVE PROPORTIONS OF THIODIGLYCOLLIC ACID IN THE URINE OF RATS DOSED INTRAGASTRICALLY WITH VARIOUS METABOLITES OF VINYL CHLORIDE

Vinyl chloride metabolites	Yield of thiodiglycollic acid (% of the dose)
<i>S</i> -(2-hydroxyethyl)cysteine	0.5
Chloroacetaldehyde	9.2
Chloroacetic acid	31.0
<i>S</i> -(carboxymethyl)cysteine	20.0

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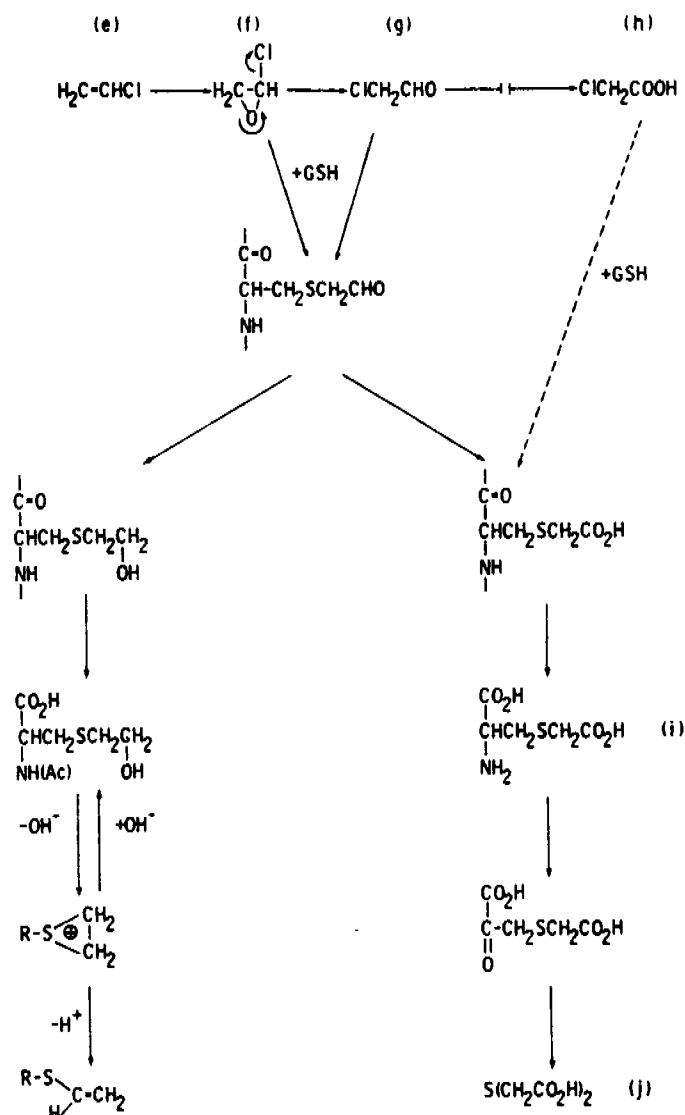


Fig. 6. Scheme for the biogenesis of S-containing vinyl chloride metabolites.

known, and the fact that chloroacetaldehyde affords *in vivo* both thiodiglycollic acid and *N*-acetyl-*S*-(2-hydroxyethyl)cysteine infers that vinyl chloride metabolism involves a reaction between glutathione and chloroethylene oxide or chloroacetaldehyde (Fig. 6). This supposition is supported by the identification, by mass fragmentometry, of *S*-(carboxymethyl)cysteine (i) (Fig. 6) amongst the hydrolytic products prepared from an hepatic extract from vinyl chloride-treated animals. Since chloroacetaldehyde and chloroethylene oxide are mutagens in *S. typhimurium* strains [19-21] and in

Chinese hamster V79 cells [22], both of these substances may contribute substantially to vinyl chloride carcinogenicity in mammals.

However, it ought to be explained that the proposed enzymic reaction between glutathione and chloroethylene oxide, catalysed by glutathione S-epoxide transferase, would give a product, which is unlikely to be stable in the isolation procedure. In fact, the reaction product of the terminal cysteinyl residue undergoes ring-closure (on hydrolysis) to afford 1,4-thiaz-1-ene-6-carboxylic acid.

The foregoing evidence suggests a scheme (Fig. 6) which is economical in respect of primary biotransformations, for the biogenesis of vinyl chloride S-containing metabolites. It now (cf. ref. 5) seems unlikely that glutathione reacts directly with vinyl chloride (e) (Fig. 6) as well as with chloroethylene oxide (f) or its chloroacetaldehyde (g) transformation product, and present work provides convincing evidence for a metabolic pathway, involving chloroethylene oxide and chloroacetaldehyde, for vinyl chloride in the living mammal.

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COMPARATIVE MAMMALIAN METABOLISM OF VINYL
AND VINYLIDENE CHLORIDES IN RELATION TO
ONCOGENIC POTENTIAL⁺

by David E Hathway *

Elucidation of the role of vinyl chloride metabolites in the various reaction sequences which comprise the metabolic pathway, including the interaction of reactive metabolites with some purine and pyrimidine residues of target-organ DNA, provides some explanation for the (oncogenic) properties associated with the original substance. Comparative investigation of the biological fate of vinylidene chloride reveals (a) an agent of low oncogenic potential which is likely to be damaging only under special circumstances, and (b) species differences which suggest that the mouse is more susceptible than the rat towards vinylidene chloride oncogenicity.

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The research work with which this communication is concerned is based on the idea that knowledge of the biology of the reactive metabolites of chemical carcinogens in the mammal, including the precise nature of the chemical changes to the DNA of the nucleus, ought to give an insight into the (oncogenic) properties of the parent compounds.

In tracer studies, N-acetyl-S-(2-hydroxymethyl)cysteine was shown to be a major vinyl chloride metabolite in rats, but according to the method of protective esterification that was used so a derivative either of N-acetyl-S- (2-chloroethyl)cysteine or of N-acetyl-S-(2-hydroxyethyl)cysteine was isolated from body fluids (1, 2). Thus, by Fischer-Speier methylation, N-acetyl-S-(2-chloroethyl)cysteine was obtained, and with diazomethane, N-acetyl-S-(2-hydroxyethyl)cysteine. It might be stated *en passant* that throughout the investigations described, mass spectrometry, involving E.I. and chemical ionisation sources and multiple-ion detection and all combinations of these facilities, was used extensively both for product identification and analysis and for the purposes of detection. Treatment of the O-methyl ester of N-acetyl-S-(2-hydroxyethyl)cysteine (a) (Fig. 1) with the methanol-HCL reagent gave a mixture of N-acetyl-S-(2-chloroethyl)cysteine (b), and S-(2-chloroethyl)cysteine, and conversely, the O-methyl ester of N-acetyl-S-(2-chloroethyl)cysteine (b) was hydrolysed rapidly by water to that of N-acetyl-S-(2-hydroxyethyl)cysteine (c) (2). Hence, the reversible reaction processes connecting the two substances would seem to be modulated through the intermediacy of episulphonium ion (c) and formation of this ion would in fact be rate-limiting in respect of the hydrolysis of N-acetyl-S-(2-chloroethyl)cysteine. Nucleophilic attack of OH⁻ on the episulphonium ion would be expected to give olefin (3), and in fact, N-acetyl-S-vinylcysteine (d) was recovered from the urine of vinyl

Fig. 1

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chloride-treated animals whenever diazomethane esterification was used to protect S-containing metabolites.

Surprisingly, N-acetyl-S-(2-hydroxyethyl)cysteine O-methyl ester was methylated with neutral methanol, and the O-methyl esters of N-acetyl-S-(2-methoxy[¹⁴C]ethyl)cysteine plus N-acetyl-S-[¹⁴C]vinyl-cysteine degrade to give the volatile [¹⁴C]S-(2-methoxyethyl)(prop-1 or 2-enyl) sulphide. Although the mechanism of formation was not investigated, we felt that acetaldehyde, a known dissociation product of S-vinylcysteine-derived S-vinylcysteine-S-oxide (4) might undergo concerted condensation with N-acetyl-S-(2-methoxyethyl)cysteine leading to elimination of thermodynamically stable glyoxylate. [There is an analogy for such a concerted condensation reaction in the work of Dabritz & Virtanen (4) on the tear-producing volatile components of the onion.]

The half-mustard S-containing metabolites of vinyl chloride did not behave as mutagens in the Ames test (2).

Thiodiglycollic acid is another major vinyl chloride metabolite (1).

In order to determine whether vinyl chloride yielded chloroethylene oxide in vivo, the biogenesis of several vinyl chloride metabolites and related compounds were investigated in rats (2). S-(2-Hydroxyethyl)cysteine gave 0.5% of the authentic thiodiglycollic acid, and this result was seen to be highly significant, because of the instability (v. supra) of the starting material under exceedingly mild conditions of reaction. The metabolic pathway concerned (Fig. 2) appears to include end-group oxidation, amino-acid transamination and oxidative decarboxylation, and the results of the animal feeding experiments suggest that chloroacetaldehyde (g) (Fig. 3),

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Fig. 3 chloroacetic acid (h), and S-(2-carboxymethyl)cysteine (i) might lie on a common pathway connecting vinyl chloride (e) with thiodiglycollic acid (j). However, other evidence implies that chloroacetic acid (h) does not belong to this metabolic pathway (e-j) (Fig.3). Thus, (i) <0.1% has even been detected in the body fluids of any of our vinyl chloride-treated animals. Either there is a high rate of turn-over or this compound is not a major vinyl chloride metabolite. The latter possibility seems more likely, since relatively large amounts are produced in vinylidene chloride metabolism, and in those animals, thiodiglycollic acid accounts for an even greater proportion of the dose than in parallel experiments with vinyl chloride. (ii) A feasible metabolic pathway for thiodiglycollic acid from chloroacetic acid and involving cysteine desulphhydrase is unacceptable. Experiments with unlabelled vinyl chloride in rats in which the cysteine-cystine pools had been labelled adequately with ^{14}C gave [^{14}C]thiodiglycollic acid, showing that a part of the C-skeleton must be derived in fact from cysteine. (iii) In rats treated with chloroacetaldehyde, the presence of thiodiglycollic acid and N-acetyl-S-(2-hydroxyethyl)cysteine, but not of chloroacetic acid, amongst the urinary metabolites was established by mass fragmentometry.

Thus, it is probable that in vivo chloroethylene oxide (f) (Fig.3) was formed (5) from vinyl chloride (e) and transformed spontaneously (6) into chloroacetaldehyde (g); there is supporting evidence (7-10) for vinyl chloride epoxidation in vitro. This supposition is supported by the fact (A) that chloroacetaldehyde affords both N-acetyl-S-(2-hydroxyethyl)cysteine and thiodiglycollic acid in vivo and (B) that S-(2-carboxymethyl)cysteine has been identified by mass fragmentometry amongst the hydrolytic products of an hepatic extract prepared from vinyl chloride-treated animals.

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Since chloroacetaldehyde and chloroethylene oxide are mutagenic in the Ames test (11-13) and in Chinese hamster V79 cells (14), they may be relevant to vinyl chloride carcinogenicity.

Respective formation of 3 β -D-ribofuranosylimidazo-[2,1-i]purine or 6 β -D-ribofuranosyl-5-oxo-5,6-dihydroimidazo-[1,2-c]pyrimidine from adenosine or cytidine by reaction with chloroacetaldehyde (15) or chloroethylene oxide was readily confirmed. The presence of these two imidazo-nucleoside derivatives has now been established by mass fragmentometry (16) in the enzymic hydrolysate of modified rat-liver DNA, prepared from rats, which had been exposed chronically to vinyl chloride

Fig. 4

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(250 ppm in their drinking water) for 1 year (Fig. 4). A smaller proportion of the 3 β -D-ribofuranosylimidazo-[2,1-i]purine, than would have been expected to have been formed, was found both in the animal experiments with vinyl chloride and in model reactions between chloroacetaldehyde and calf-thymus DNA (16). This observation is consistent with some degree of DNA depurination brought about by the reaction of vinyl chloride, and in our model experiments, we have found evidence for the presence of the detached purine, Viz. imidazo-[2,1-i]purine. Hence, the alkylation that produces imidazo-derivative formation (with DNA) labilizes the N₉ purine β -glycoside linkage, which leads to depurination. The gap so produced might then be filled by various bases, resulting in 'mispairing' during DNA replication. These results are very important, because in general, there is excellent agreement between the severe damaging effect of depurination to DNA and mutagenicity (17-19).

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Thus, in retrospect, one would suspect vinyl chloride of being mutagenic/carcinogenic.

On the other hand, vinylidene chloride (a) (Fig. 5) metabolism in rats gave thiodiglycollic acid (g) and an N-acetyl-S-cysteinyl-acetyl derivative (e) as major urinary metabolites, plus substantial amounts of chloroacetic acid (b), dithioglycollic acid (j) and thioglycollic acid (h) (20). It is probable that chloroacetic acid (b), which is a vinylidene chloride metabolite per se, lies on a major metabolic pathway for vinylidene chloride (Fig. 5), since it affords several metabolites in common with vinylidene chloride (20).

There is a strong supposition that detoxification of chloroacetic acid (b) is effected through a glutathione S-acyl transferase catalysed reaction process and ensuing degradative sequence for the resulting carboxymethylglutathione (d), and that this represents the principal metabolic pathway for chloroacetic acid and a major one for vinylidene chloride. Thiodiglycollic acid is the ultimate detoxification product, and previous work (2) established the biotransformation of S-(2-carboxymethyl) cysteine (f) into that substance. A feasible metabolic pathway to thiodiglycollic acid from chloroacetic acid and involving cysteine desulphhydrase is unacceptable. In experiments (rats) with unlabelled vinylidene chloride in which the cysteine-cystine pools had been labelled with ^{14}C , labelled thiodiglycollic acid resulted, and a part of the C-skeleton of that substance must be derived in fact from cysteine (20). Formation of a small amount of [^{14}C]dithioglycollic acid (j) (and hence of the intermediate [^{14}C]thioglycollic acid) (h) is reconcilable with the action of Michaelis's (21) unspecific β -thionase, which would lyse a small proportion of the preponderating [^{14}C]thioglycollic acid (v. infra)

Moreover, Kolbe electrolysis (22) of one molecular proportion of the [^{14}C]thiodiglycollic acid metabolite from [$1\text{-}^{14}\text{C}$]1,1-dichloroethylene or [$1\text{-}^{14}\text{C}$]chloroacetic acid gave 1 equivalent of $^{14}\text{CO}_2$ (23), and this evidence is consistent with the transformation of vinylidene chloride into chloroacetic acid by a mechanism involving migration of one Cl atom and the loss of the other one (20, 23). Hence, the metabolic pathway (Fig. 5), which was tentatively proposed for the biotransformation of vinylidene chloride into thiodiglycollic acid does in fact operate in rats.

It is equivocal whether the very small amounts of CO_2 and urea are produced by the action of epoxide hydratase on 1,1-dichloroethylene oxide or by a minor oxidative pathway for chloroacetic acid.

There is a strong supposition that the N-acetyl-S-cysteinylactyl derivative (e) (Fig.5), which is a metabolite of vinylidene chloride, but not of chloroacetic acid, may be formed in fact from 1,1-dichloroethylene oxide through the agency of glutathione S-epoxide transferase to afford S-glutathione acetyl chloride (c) and its subsequent reactions (20). This supposition is important, since the reactivity displayed by 1,1-dichloroethylene oxide (v. supra) appears to be relevant to the possible interaction of reactive vinylidene chloride metabolites with mouse-kidney DNA (Fig. 6), which is a prerequisite of tumour initiation (24). Such interaction would be analogous to that of vinyl chloride with rat-liver DNA in vivo, which forms imidazo derivatives with some nucleoside residues (16). Further work in progress to investigate this hypothesis.

Fig. 6

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Comparative studies (25) provide clues of differences between rats and mice in the processing of vinylidene chloride (Fig. 7). Thus, in mice, the production of thiodiglycollic acid is considerably reduced and the formation of the N-acetyl-S-cysteinylacetyl metabolite is increased. The higher β -thionase activity in mice than in rats accounts for the greater conversion of thiodiglycollic acid into dithioglycollic acid via thioglycollic acid in the former species of animal. Yllner's (26) mice excreted a proportion of a dose of chloroacetic acid as unchanged starting acid. Thus, in mice, the metabolic pathway from chloroacetic acid to thiodiglycollic acid seems to be readily saturable, possibly on account of an inadequacy in the reaction catalysed by glutathione S-acyl transferase. Under these circumstances, detoxification of 1,1-dichloroethylene oxide by glutathione S-epoxide transferase and the modification of DNA by 1,1-dichloroethylene oxide or chloroacetyl chloride would be expected to be more significant in mice than in rats. This diagnosis of species susceptibility seems to accord with Maltoni's (24) discovery of vinylidene chloride oncogenicity in (the kidneys of) mice.

Vinylidene chloride emerges as an agent of low, perhaps very low, oncogenic potential, which can be damaging only in a special set of biological circumstances, which we have partially defined (v. supra) and on which work is continuing.

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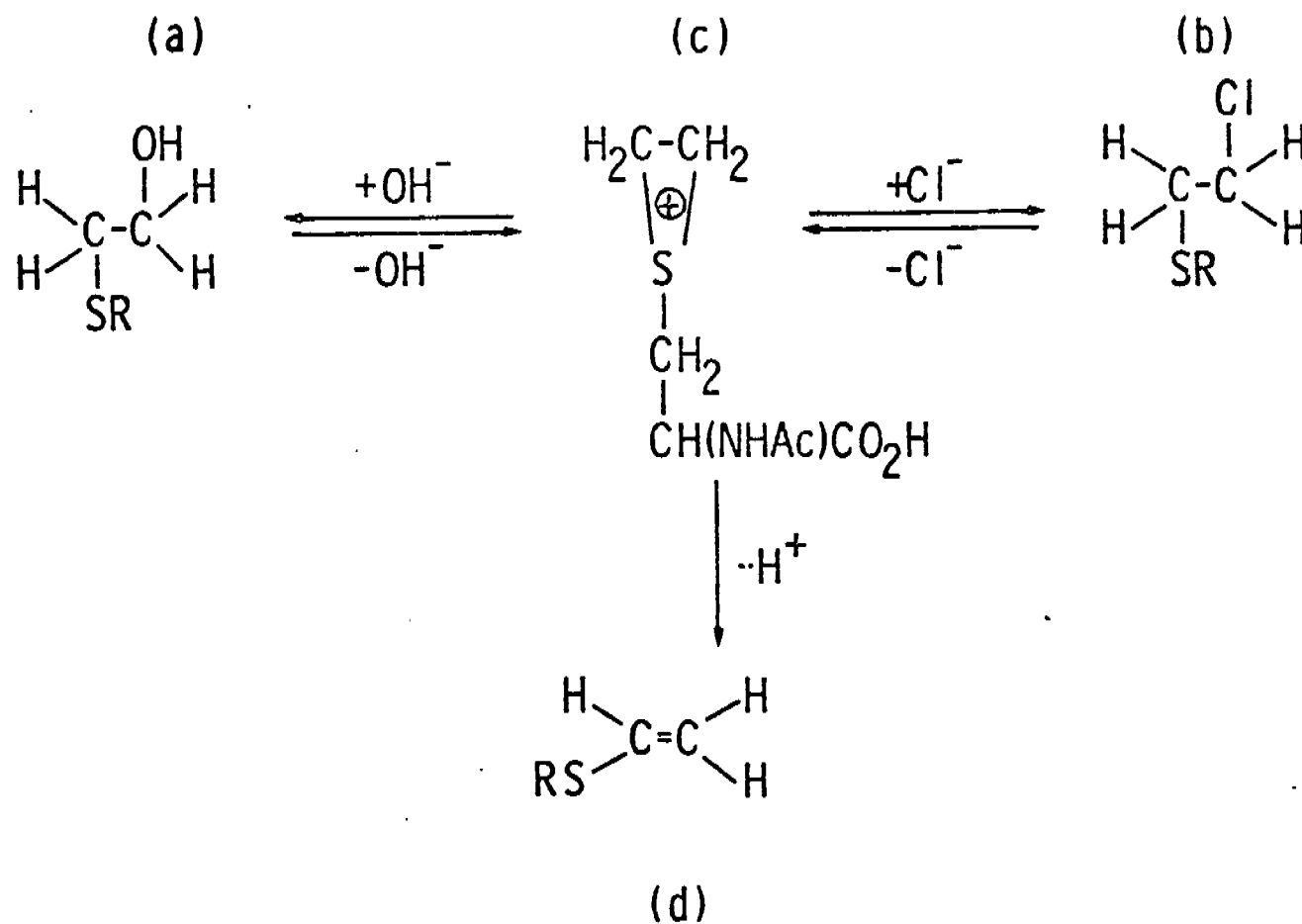


Fig. 1 Scheme for the interrelationship of some S-containing vinyl chloride metabolites.

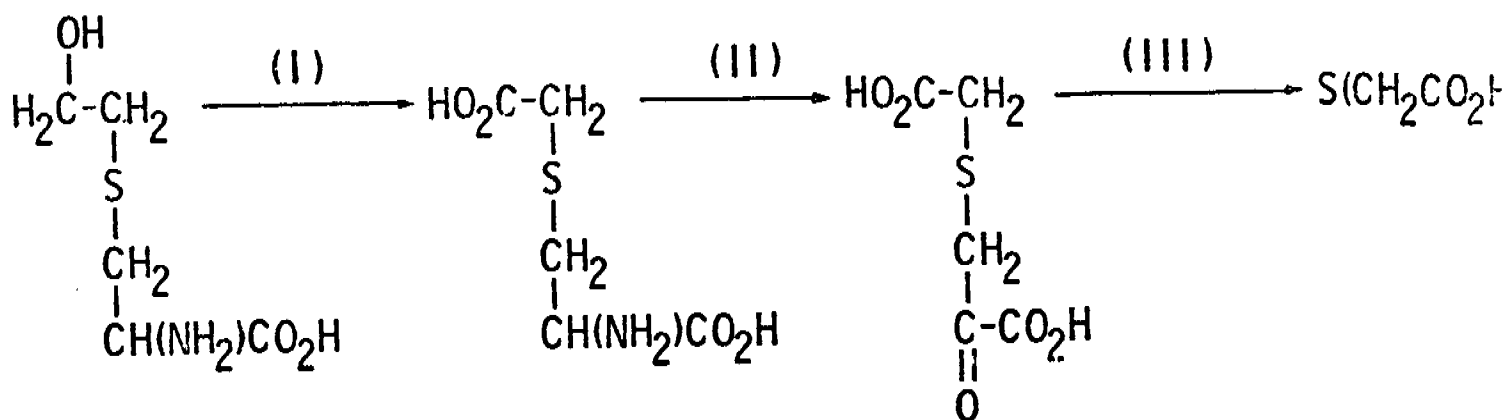


Fig. 2. Scheme suggesting the biotransformation of S-(2-hydroxyethyl) cysteine into thiodiglycollic acid. (I) End-group oxidation; (II) transamination; (III) oxidative decarboxylation.

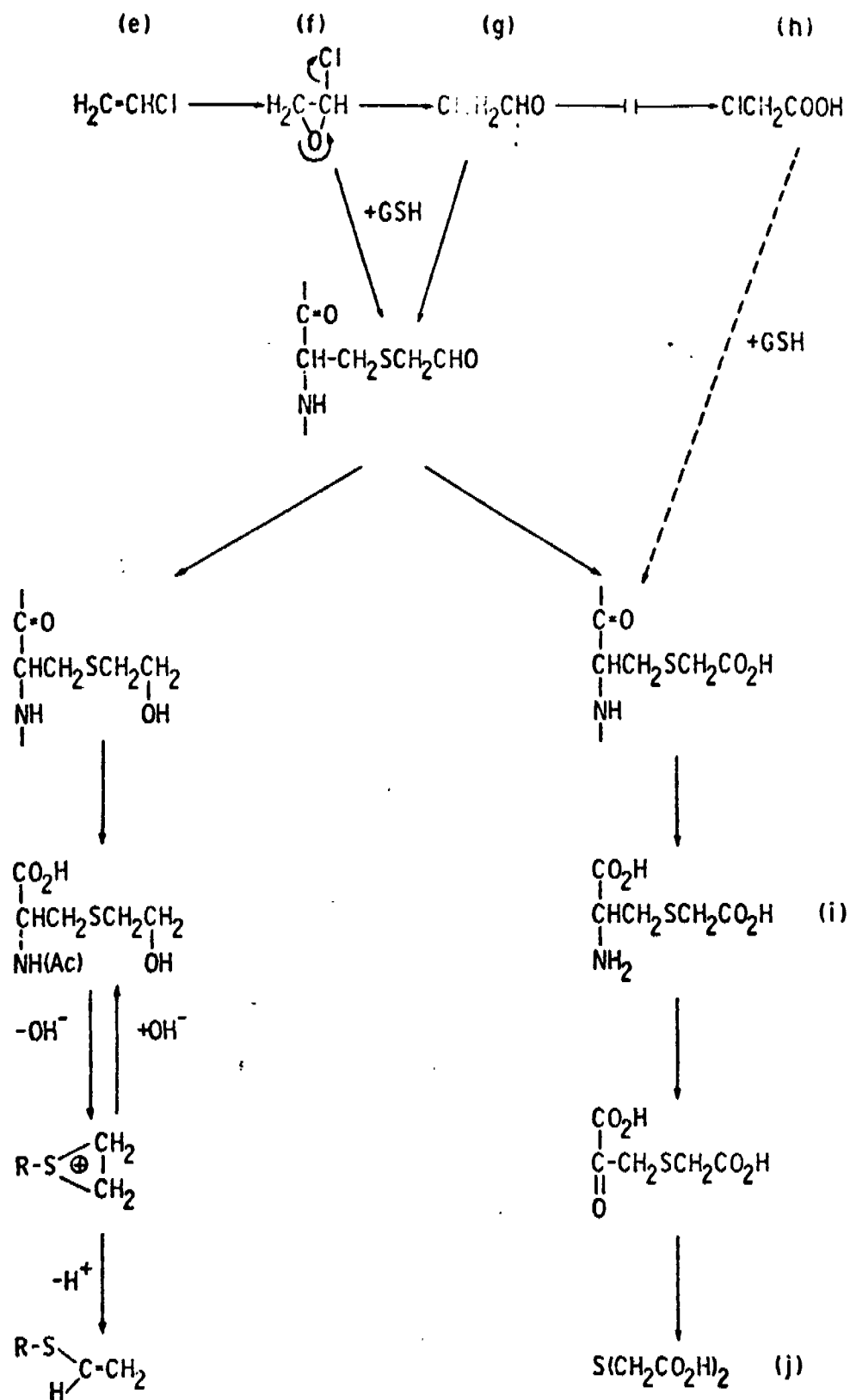
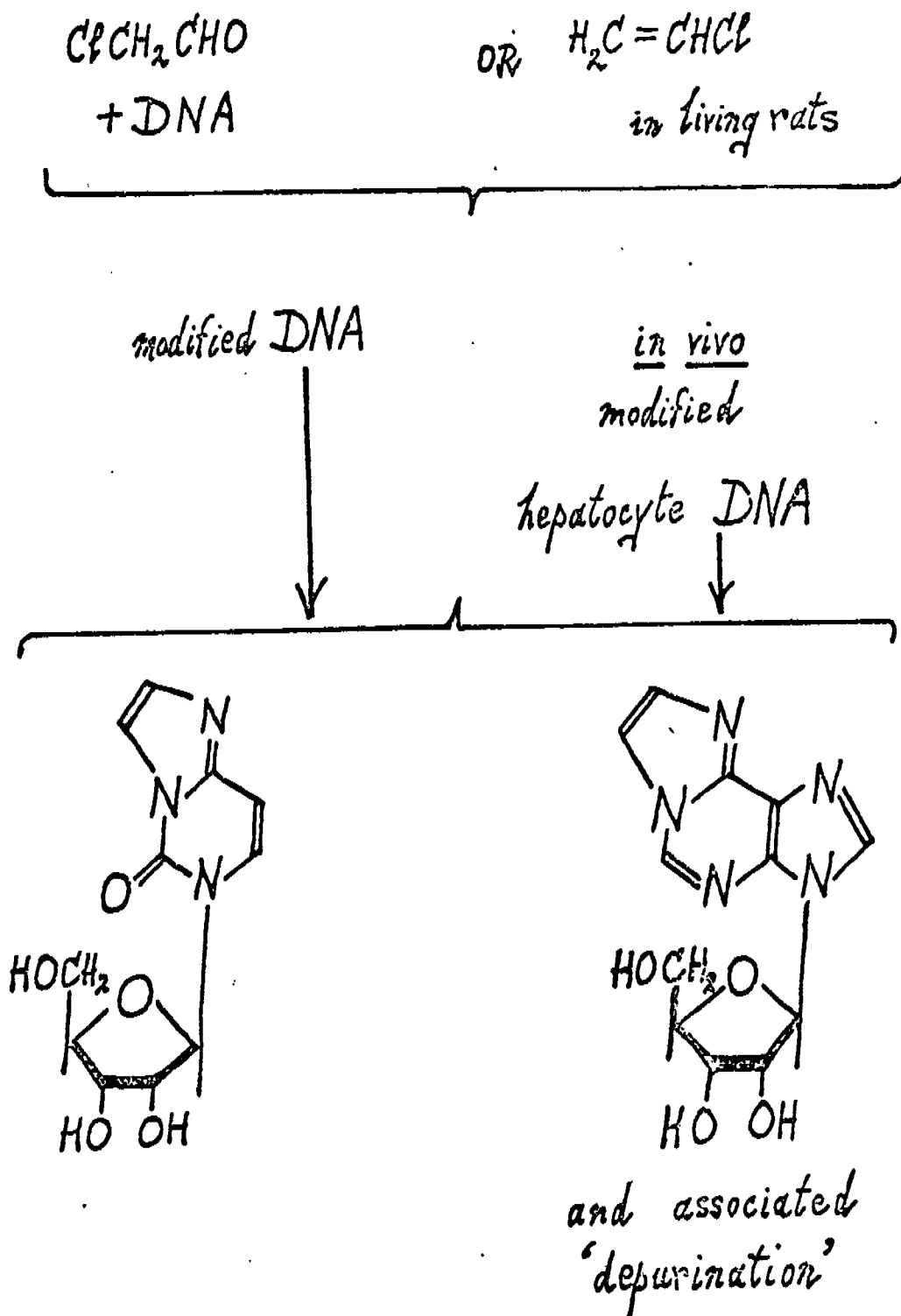


Fig. 3 Scheme for the biogenesis of S-containing vinyl chloride metabolites



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Fig. 4 Scheme suggesting the model reaction of chloroacetaldehyde with (calf-thymus) DNA and the biotransformation of hepatocyte DNA by vinyl chloride in vivo. Both reaction processes afford 6β-D-ribofuranosyl-5-oxo-5,6-dihydroimidazo-[1,2-c]pyrimidine (left-hand side) and 3β-D-ribofuranosylimidazo-[2,1-i]purine (right-hand side)

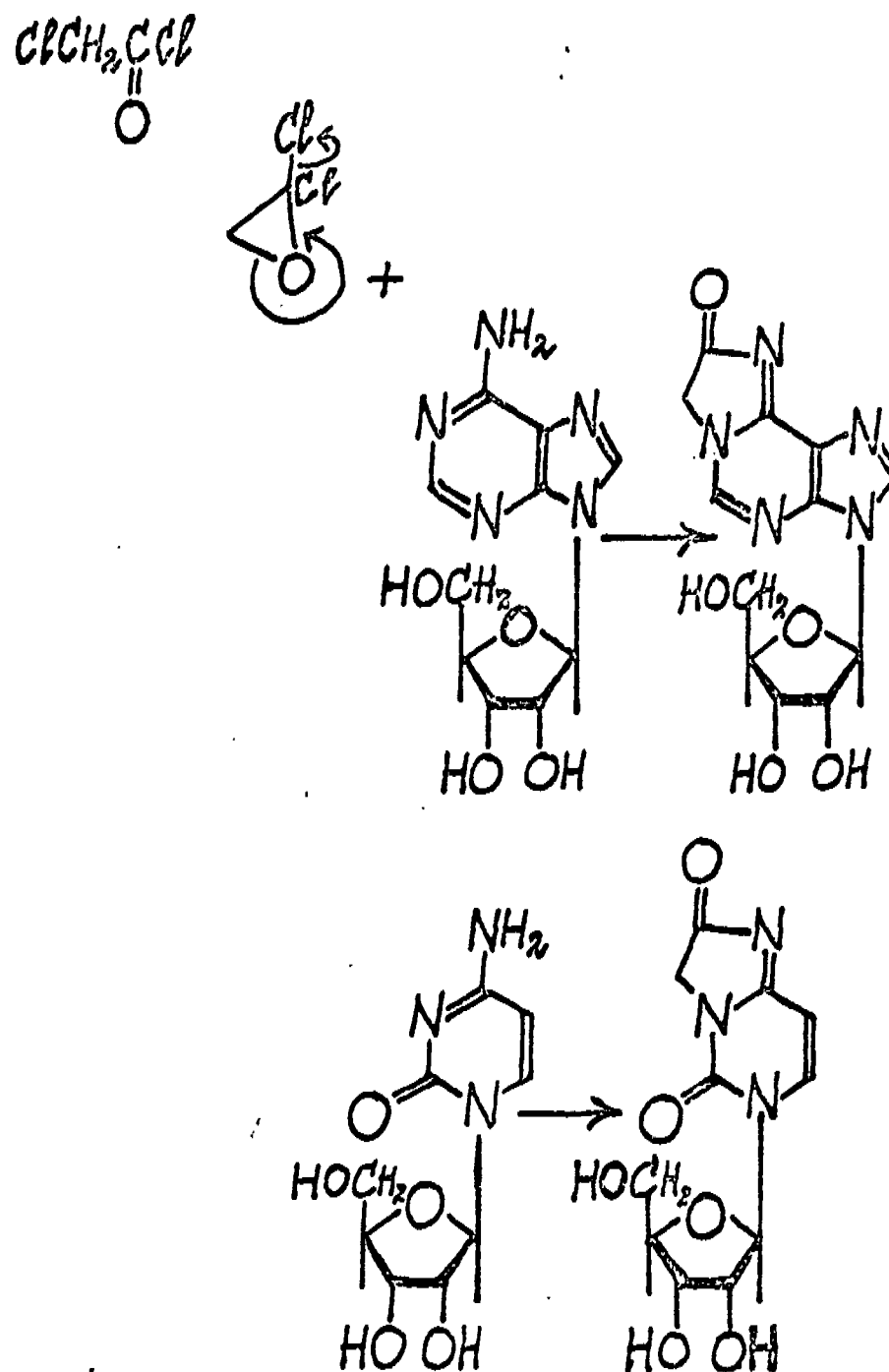


Fig. 6. Scheme suggesting the feasible interaction of reactive vinylidene chloride metabolites, 1,1-dichloroethylene oxide and chloroacetyl chloride, with adenosine and cytidine respectively.

METABOLISM(1) of Chloroacetic acid

<u>Metabolite</u>	<u>Rats</u>	<u>Mice</u>	
		<u>Yllner</u>	<u>BKJ-DEH</u>
Chloroacetic acid	-	6-22%	-
Thiodiglycollic acid	90	37	30-40
<u>N</u> -Acetyl- <u>S</u> -(2-carboxymethyl)cysteine	2	40	40

(2) of Vinylidene chloride

Chloroacetic acid	3	-
Thiodiglycollic acid	37	3
Thioglycollic acid	3	5
Dithioglycollic acid	5	20
<u>N</u> -Acetyl- <u>S</u> -cysteinylacetyl derivative	48	70

Fig. 7. The relative proportions of products resulting from the metabolism (1) of chloroacetic acid and (2) of vinylidene chloride in rats and mice.

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