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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.

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°C). (Found: C, 36.5; H, 6.8; N, 8.4; S, 19.4%; $C_5H_{11}O_3NS$ 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°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].

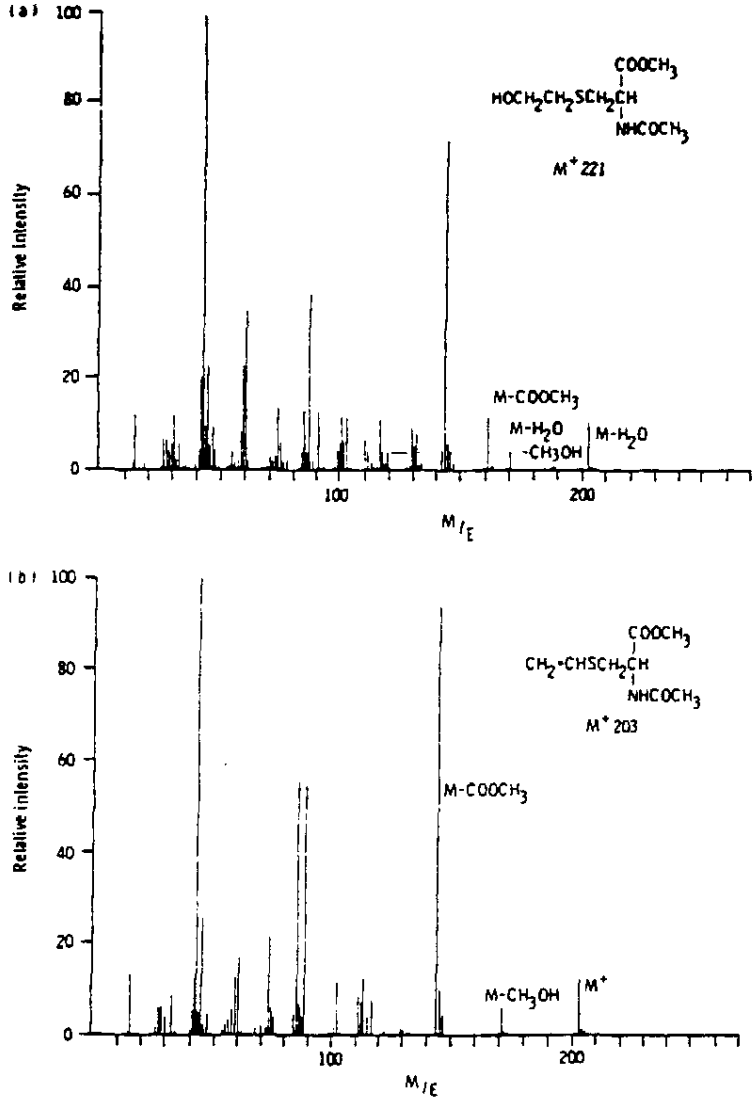


Fig. 1. Mass spectrum of: (a) the O-methyl ester of *N*-acetyl-*S*-(2-hydroxyethyl) cysteine; (b) the O-methyl ester of *N*-acetyl-*S*-vinylcysteine.

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/mmole, 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/mmole 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.

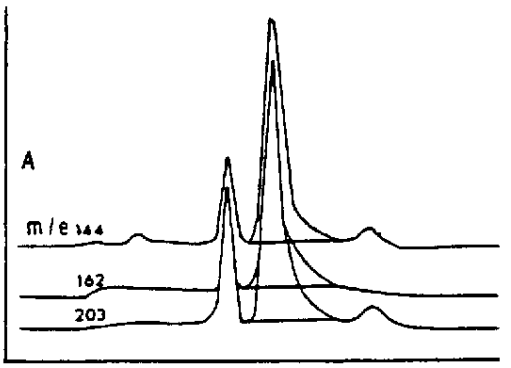
(e) In the experiment in which unlabelled vinyl chloride was dosed to rats in which the cysteine—cystine pools had been labelled adequately with ^{14}C, the 3 N HCl fraction was examined in the GC—mass spectrometer for the presence of the thiodiglycollic acid metabolite and in the gas chromatograph for 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]. Bulked 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 derivativized 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 × 4 mm internal diameter), which were packed either with 6% (w/w) of OV-101 on Gas Chrom Q (80—100 mesh size) or



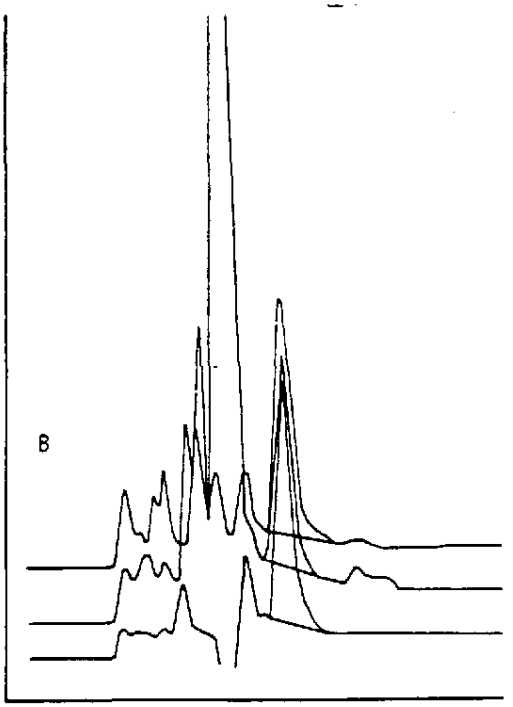


Fig. 2. Mass fragmentogram of the O-methyl ester of *N*-acetyl-*S*-(2-hydroxyethyl)cysteine from the urine of chloroacetaldehyde-treated rats (the gains from m/e 144, m/e 162 and m/e 203 are in the ratio 1 : 10 : 10). A represents the injection point for a methanolic solution of the standard sample and 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.

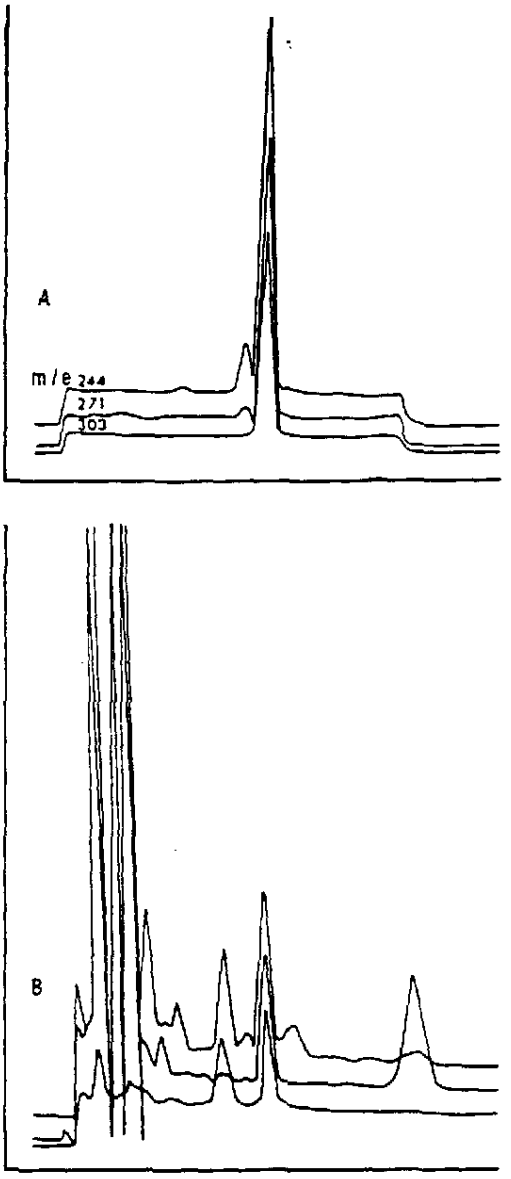


Fig. 3. Mass fragmentogram of the O-dimethyl ester of the *N*-trifluoroacetyl derivative of *S*-(carboxymethyl)cysteine from the urine of chloroacetaldehyde-treated rats (the gains for m/e 303, m/e 271 and m/e 244 are in the ratio 1 : 1 : 1). A represents the injection point for a solution of the standard 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.

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—CO_2 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—H_2O, m/e 162 corresponding to M—CO_2CH_3 and m/e 144 corresponding to M—H_2O—CO_2CH_3. 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—CH_3OH and m/e 244 corresponding to M—CO_2CH_3. 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*-

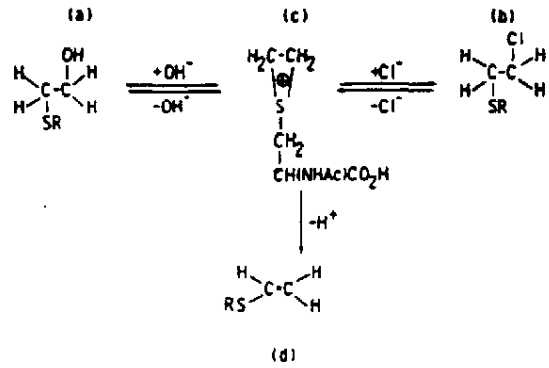


Fig. 4. Scheme for the interrelationship of some S-containing vinyl chloride metabolities.

(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)cystein (b) and *S*-(2-chloroethyl)cystein 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)cystein (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 ($M^{\cdot}$ = 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 ($M^{\cdot}$ = 235) was absent from the conventional mass spectrum, the $(M+1)^{\cdot}$ 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-

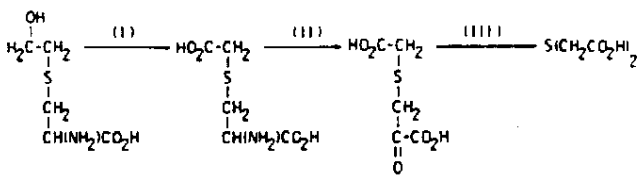


Fig. 5. Scheme suggesting the biotransformation of *S*-(2-hydroxyethyl)cysteine into thiodiglycollic acid. (I) End-group oxidation; (II) transamination; (III) oxidative decarboxylation.

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)
S-(2-hydroxyethyl)cysteine	0.5
Chloroacetaldehyde	9.2
Chloroacetic acid	31.0
S-(carboxymethyl)cysteine	20.0

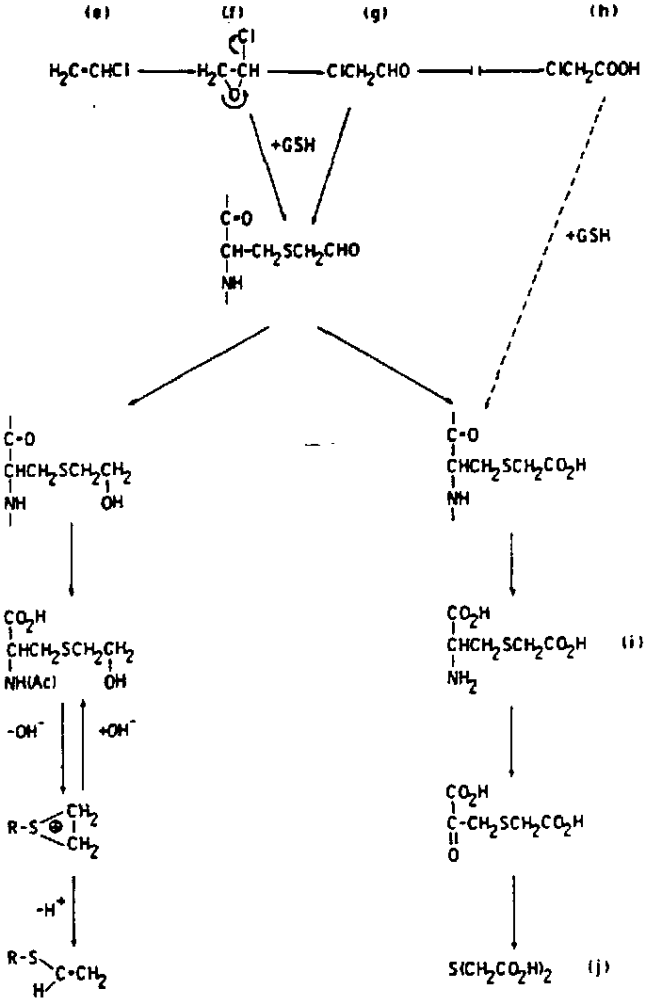


Fig. 6. Scheme for the biogenesis of S-containing vinyl chloride metabolites.

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 suppostion 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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