THE BIOLOGICAL FATE IN RATS OF VINYL CHLORIDE IN RELATION TO ITS ONCOGENICITY

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Running Title

BIOLOGICAL FATE OF VINYL CHLORIDE IN RATS

SUMMARY

The main eliminative route for [^{14}C] vinyl chloride after oral, i.v. or i.p. administration to rats is pulmonary; both unchanged vinyl chloride and vinyl chloride-related CO_2 are excreted by that route and the other [^{14}C] metabolites via the kidneys. After intragastric administration, pulmonary output of unchanged vinyl chloride is proportional to the logarithm of reciprocal dose. Excretion patterns after i.v. and i.p. injections are predictable from the characteristics of excretion following oral administration. Pulmonary excretion of unchanged vinyl chloride after oral dosing is complete within 3-4 h, but pulmonary elimination of CO_2 and renal excretion of metabolites occupies 3 days. In comparison, 99% of a small i.v. dose is excreted unchanged within 1 h of injection; 80% within 2 min.

The rate of elimination of single oral doses of [^{14}C]vinyl chloride is uninfluenced by up to 60 days' chronic dosing with the unlabelled substance.

The distribution volume of vinyl chloride as displayed by whole-animal autoradiography agrees with deductions from excretion data. Small localization of ^{14}C in the para-auricular region of appropriate sections occurs in sectioned tubules, belonging possibly to the Zymbal glands.

Biotransformation of vinyl chloride into S-(2-chloroethyl) cysteine and N-acetyl-S-(2-chloroethyl) cysteine occurs through free radical addition of cysteine, and biotransformation into

(i) chloroacetic acid, thiodiglycollic acid and glutamic acid, and (ii) into formaldehyde (methionine, serine), CO_2 and urea takes place through an associative reaction with molecular O_2 involving a singlet oxygen bonded transition state in dynamic equilibrium with a cyclic peroxide ground state. There is no evidence for chloroethylene oxide formation.

Thiodiglycollic acid is the major metabolite of chloroacetic acid in rats; more than 60% of the dose.

The interaction of vinyl chloride and of its primary metabolites with the intermediates of mammalian metabolism is discussed in relation to the oncogenicity of that substance.

INTRODUCTION

The discovery that one year's intermittent inhalational exposure of Wistar strain rats to 3% (v/v) of vinyl chloride elicited epidermoid and mucoepidermoid carcinomas in the para-auricular region of surviving animals[1,2] was confirmed in more detailed and extensive investigations[3-5], which revealed, in addition to the Zymbal gland carcinomas, liver angiosarcomas and nephroblastomas in Sprague-Dawley strain rats that had been exposed to atmospheric concentrations as low as 250 ppm. Vinyl chloride also induced liver angiosarcomas in Swiss mice at 500 ppm[4,5]. Moreover, a direct relationship was established by these workers between the dose level and length of treatment with vinyl chloride and the neoplastic response[5]. These findings as well as world-wide epidemiological recording by June 1974 of some twenty-four cases of liver angiosarcoma amongst workers, who had been engaged on vinyl chloride/polyvinyl chloride manufacture, make a better understanding of mammalian vinyl chloride pharmacodynamics desirable. Furthermore, the induction of angiosarcomas in rats by intraperitoneal (vinyl chloride) injection[6] implies that vinyl chloride, and not a product of its photolysis, is the causative agent.

At the outset of the present work, we envisaged that by analogy, with chloroform[7] the main eliminative route from mammals for this highly volatile (b.p.-14°, f.p.-160°C) and lipid-soluble, water-insoluble substance would be pulmonary, and that, since the halogen atom of vinyl chloride is stable to nucleophilic reagents, autoxidation

mechanisms and free-radical addition of intermediary metabolites may be important to its metabolism. The present paper describes the results of studying in rats the biological fate of vinyl chloride and their implications in respect of the oncogenicity of this substance. A report on some preliminary results was made to the XIth International Cancer Congress that was held in Florence during October 20-25, 1974.

MATERIALS AND METHODS

Chemicals

Vinyl chloride was supplied by Imperial Chemical Industries Limited, Mond Division, Runcorn, Cheshire.

Glutamic acid, methionine, serine and thiodiglycollic acid reference compounds were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and were all of grade 1 quality with a purity exceeding 99.5%.

All reagents and solvents were of AnalaR grade or of the next highest quality available.

Radioactive Chemical

[^{14}C] Vinyl chloride with a specific activity of 0.46 mCi (m mole)$^{-1}$, and with a chemical and radiochemical purity exceeding 99.0%, was synthesized from [U-^{14}C] ethylene via dehydrochlorination of [U-^{14}C] 1,2-dichloroethane by our colleague, Dr. J. A. Heslop of Imperial Chemical Industries Limited, Petrochemicals Division, Billingham, Teeside. On account of the risk of polymerization, it was convenient to store [^{14}C] vinyl chloride (60 mg) as a solution, for example, in peroxide-free corn oil (10 ml) at -20°C.

Experiments with Animals

Adult male rats (approximately 2 months old, 200 g body weight) were used (Wistar strain maintained as a specific pathogen-free colony at Alderley Park), and kept on a standard pellet diet.

(a) Animals were given single doses of [^{14}C] vinyl chloride as a solution in corn oil or β-hydroxyethyllactamide by the routes

of administration and at the dose levels that are shown in Table 1. Irrespective of the size of the dose, each dose contained approximately 2 μCi of [^{14}C] vinyl chloride. The animals were kept singly for 3 days in glass metabolism cages (Jencons of Hemel Hempstead, Herts.), which were designed for the study of the elimination pattern of ^{14}C by the urinary, faecal and pulmonary routes[8]. Unrestricted food and water were supplied, and the urine and faeces were collected in the dark and frozen at -70°C. The exhaled air was drawn through a gas train, comprising successively three Dreschel bottles, one containing 150 ml and two containing 50 ml of trichloroethylene at -70°C to remove unchanged [^{14}C] vinyl chloride, and two carbon dioxide absorber Nilox columns (Jencons of Hemel Hempstead), each containing 500 ml of 2N-NaOH.

(b) Groups of rats were chronically administered vinyl chloride by stomach tube at dose levels respectively of 3, 30 and 300 mg kg^{-1} day^{-1} for 60 days. Three animals from each group were each administered a single dose of [^{14}C] vinyl chloride (0.6 mg kg^{-1} containing 2 μCi) by stomach tube on day 1 and on day 60, and the urine and exhaled air were monitored [see (a)] for 24 h after administration.

(c) To each young rat (approximately 85 g body weight), there was administered by stomach tube a single dose of 30 μCi of [^{14}C] vinyl chloride. Fifteen, 30, 60, 120 and 240 min after ingestion, the animals were deeply anaesthetized, and were rapidly frozen by immersion in acetone, cooled to about -70°C. The frozen rats were transferred to a cryostat (Bright Instrument Company Limited, Huntingdon) at -20°C. Longitudinal sagittal sections, 20μ in thickness,

were cut with a mechanically operated Leitz microtome; some through the vertebrae and others through a kidney of each animal. Apposition autoradiograms were made by pressing the freeze-dried sections against Structurix D7 x-ray film (Agfa Gevaert) for 6 weeks in light-tight cassettes.

(d) For the identification of urinary metabolites, a group of 4 rats were dosed intragastrically 3 times at 3-h intervals with [^{14}C] vinyl chloride (50 mg kg^{-1}, containing 10 μCi) in corn-oil solution. The urine was collected throughout the period in repeated dosing and for 24 h after the last administration. Bulked urines were stored at -29°C.

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 admixed with standard scintillator and radio-assayed direct and samples of faeces were burnt in an Intertechnique 'Oxymat', solid-sample oxidizer.

Systematic separation of the urinary [^{14}C] vinyl chloride metabolites into fractions of chemically similar substances

The combined urine from a group of treated rats was filtered, and a sample taken for ^{14}C measurement. The remainder was evaporated to dryness under reduced pressure, <45°C. A solution of the residue in 5 ml of 0.1 N-KOH was percolated (20 drops min^{-1}) through a column (bed-volume, 100 ml) of Amberlite IRA 410 anion-exchange resin (14-52 mesh size, 1.40 mg equiv ml^{-1})

in the $CH_3CO_2^-$ cycle, and the column was then washed (300 ml h^{-1}) with 250 ml of de-ionized water. The total eluate was retained.

When 3N-acetic acid was percolated (20 drops min^{-1}) through this column, nine 50-ml fractions were collected. After the column had been washed with 50 ml of de-ionized water, it was stripped of the remaining [$^{14}C^-$] anions by the percolation of 3N-HCl; six 50-ml fractions were collected.

Radioactivity was measured in all of the separate 50-ml fractions. The ones, which contained the ^{14}C that was displaced with 3N-acetic acid, were combined and evaporated to dryness under reduced pressure, and similarly the ones, which contained the ^{14}C that was displaced with 3N-HCl. Successive O-n-butylation and N-trifluoroacetylation of the two residues were effected by established methods[10].

Meanwhile, evaporation under reduced pressure of the total eluate from the column in the $CH_3CO_2^-$ cycle left a residue, which was N-trifluoroacetylated. When the reaction mixture, which had been partially evaporated to 5 ml, was allowed to stand, the resulting semi-crystalline mass was recrystallized from chloroform-methanol mixture to a constant specific activity. The mass spectrum of the purified compound was examined in the LKB 9000 GC-MS system, using the direct insertion probe.

Thin-layer chromatography

The derivativized substances from both the 3N-acetic acid and the 3N-HCl fractions were applied separately as bands on 500u SiO_2-gel GF thin-layer plates, which were developed with chloroform.

Zones of ^{14}C were located with a Panax t.l.c. scanner, excised and eluted with methanol.

Gas chromatography

Radioactive substances from zones on thin-layer plates were analysed with a Pye Model 104 instrument that was equipped with flame ionisation detection and glass columns; some of which (7 ft long x ¼ in external diameter) were packed with 1% (w/w) of OV-1 on Gas Chrom Q (80-100 mesh size), and others (5 ft long x ¼ in external diameter) were packed with 5% (w/w) of DEGS on Gas Chrom Q. Where the stationary phase was OV-1, the column temperature was programmed to run from 150 to 220°C at 5°C min^{-1}, but where the stationary phase was DEGS, column temperatures of 140 and 180°C were used successively. All of the columns were operated at a 60 ml min^{-1} flow-rate of N_2.

Radioactive peaks, which had been identified by the trapping of individual peaks in 10 ml of Packard 'Instagel' scintillator solution and the measuring of ^{14}C, were isolated by preparative gas chromatography on glass columns (5 ft long x ⅛ in external diameter), which were packed either with 5% (w/w) of OV-1 or with 5% (w/w) of DEGS on Gas Chrom Q. Effluent gas from these columns was split in the ratio of 25:1.

Mass spectrometry

Mass spectra of the purified metabolites were obtained by using an LKB 9000 gaschromatograph-mass spectrometer system. The gas chromatograph was fitted with a glass column (7 ft long x ¼ in external diameter), which was packed with 1% of OV-1 on Gas Chrom Q.

Mass spectra were measured at 70eV and 20eV.

Accurate mass measurements were made on an Associated Electrical Industries (Trafford Park, Manchester, England) M.S.9. double focusing mass spectrometer.

NMR spectra were obtained with a Bruker Fourier-transform spectrometer, using a chloroform-d solution of the metabolite.

RESULTS

Excretion of radioactivity

Total excretion data obtained from rats after 250-μg and 450-mg intragastric, intravenous and intraperitoneal doses of [^{14}C] vinyl chloride per kg are detailed in table 1. In all cases, almost all of the radioactivity was recovered during the first 72 h after administration, but very small amounts of ^{14}C were still being excreted during 72-96 h after intragastric dosing. The change in excretion pattern after intragastric administration at the different dose levels (Table 1) is striking. Whereas with the higher dose, more than 90% as unchanged vinyl chloride and less than 1% of CO_2 are excreted via the lungs, with the lower dose, urinary excretion accounts for nearly three-quarters, and a few per cent of unchanged vinyl chloride together with 12-15% of CO_2 are eliminated by the pulmonary route. However, about one-hundred times more vinyl chloride was metabolized at the higher dose level than at the lower one. The conclusion is drawn that this change in excretion pattern is due to a saturable drug metabolism and to an highly efficient arterial-alveolar transfer of unchanged vinyl chloride from systemic blood that leaves a relatively low concentration of material available for biotransformation in successive passes through the liver. Thus, 99% of a small intravenous dose of vinyl chloride is excreted unchanged from systemic blood within an hour of injection;

TABLE 1 NEAR HERE

80% within 2 min. Furthermore, the excretion pattern after a small intraperitoneal injection is intermediate between that resulting from intravenous injection and that from intragastric administration; some of the vinyl chloride is taken up into systemic blood and is excreted unchanged via the lungs and some is absorbed into the hepatic-portal system and is metabolized by the liver. In this investigation, the rate of pulmonary excretion of unchanged vinyl chloride after oral dosing is rapid, and is complete within a few hours of administration, whereas urinary (vinyl chloride) metabolites and the vinyl chloride-related CO_2 are excreted respectively from the kidneys and lungs throughout 3 days (Fig. 1). The conclusion is drawn that the matching formation of CO_2 probably belongs to the same metabolic pathway as that for the urinary metabolites.

FIG 1 NEAR HERE

Sixty days' chronic dosing with unlabelled vinyl chloride at dose levels of 3, 30 and 300 mg kg^{-1} do not affect the rate of elimination of a single dose of [^{14}C] vinyl chloride from the body. This observation indicates that vinyl chloride is not an inducer of the rate of its own metabolism, and that excretion data for a single dose also applies to the chronic situation.

Whole-animal Autoradiography

Autoradiograms of longitudinal sagittal sections that were taken sequentially through whole animals, which had been dosed orally with ^{14}C material (Fig. 2), show (i) conspicuously less radioactivity in the gut than would have been the case had faecal excretion been a major eliminative route for vinyl chloride, and (ii) the passage

FIG 2 NEAR HERE

of ^{14}C through the lungs, liver, kidneys and gastro-intestinal tract. At 4 h, except for the presence of a little radioactivity in the large intestine, the gut is markedly free from ^{14}C. The visual record of the drug-distribution volume for vinyl chloride agrees with deductions derived from extensive excretion data. Appropriate sections at 2 h reveal a discrete localization of ^{14}C in the para-auricular region within sectioned tubules, which belong possibly to the Zymbal glands.

Separation and identification of urinary (vinyl chloride) metabolites

Out of the [^{14}C]vinyl chloride metabolites in the urine of treated animals, solvent extraction with ethyl acetate removed only chloroacetic acid, which was a minor metabolite. Identification involved paper chromatography of the substance itself and of glycine derived from it [9]. Attempted separations from the urine by ion-exchange chromatography showed that much of the ^{14}C could be removed by these means. The fact that a large proportion of the radioactivity was displaced successively from strong cation - and anion - exchange resins in the usual way showed that some of the major constituents were zwitterions. Accordingly, N-trifluoroacetyl-O-n-butyl esters were prepared[10] from the evaporates of the effluent from linked cation - and anion - exchangers in order to facilitate metabolite identification through the recognition of mass fragmentation patterns. Separation of these [^{14}C]N-trifluoroacetyl-O-n-butyl ester derivatives by GC on 1% of OV-1[11] showed the presence of the corresponding derivatives of [^{14}C] glutamic acid, [^{14}C]methionine and

[^{14}C] serine with retention times in the ratio of 100:85:44. The mass fragmentation patterns of the glutamic acid, methionine and serine derivatives in the mass spectrometer were identical in all respects with those of the appropriate derivatives that were prepared from the authentic substances, and these mass spectra have been recorded in detail elsewhere[11]. Thus, two minor vinyl chloride metabolites, chloroacetic acid and glutamic acid, and two trace (vinyl chloride) metabolites, methionine and serine, were identified in preliminary separatory processes.

These exploratory investigations provided clues for the systematic separation of major [^{14}C] vinyl chloride metabolites from the bulked urines of treated animals. Thus, percolation of urinary constituents through a column of strong (Amberlite IRA-410) anion-exchange resin in the acetate form effected an uptake of more than 90% of the urinary ^{14}C. Residual ^{14}C in the eluate was shown by t.l.c. to be due to [^{14}C] urea. This observation was confirmed by isotope-dilution analysis; the N-trifluoroacetyl derivative was crystallized (with the naturally occurring unlabelled urea) to constant specific activity, and the purified product was shown by mass spectrometry to be identical in all respects with the N-trifluoroacetyl derivative of authentic material. Displacement from Amberlite IRA-410 anion-exchange resin was implemented successively with 3N-acetic acid and 3N-HCl, and N-trifluoroacetyl and O-n-butyl ester derivativizations were carried out on each fraction.

T.l.c. of those derivatives of material that was eluted with 3N-acetic acid separated two major radioactive metabolites of R_F 0.24 and R_F 0.59 (Table 2), and t.l.c. of the O-n-butyl ester of material eluted with 3N-HCl resolved a single major radioactive metabolite of R_F 0.74. Each zone from the thin-layer plates, when analysed by GC on columns of 1% OV-1 or 5% DEGS, afforded 15-20 peaks on the recorder trace. The peak, which contained the radioactivity, was isolated by preparative GC methods. (Accurate GC retention times are given in Table 2.)

TABLE 2 NEAR HERE

The three derivatives that had been purified successively by t.l.c. and preparative GC were examined by gas chromatography-mass spectrometry (Tables 3-5), and accurate mass measurements were made on two of those substances (Tables 3,4) with a double focusing spectrometer. The purified ^{14}C substance, which was present in the thin-layer band of R_F 0.74, was identified as the O-n-butyl ester of thiodiglycollic acid (Table 3) by the mass fragmentation pattern and by accurate mass measurements of the molecular and $(M-C_4H_9OH)$ ions. The mass spectrum of this derivativized metabolite was identical in all respects with that of the authentic O-n-butyl ester of thiodiglycollic acid. The purified ^{14}C substance, which was present in the thin-layer band of R_F 0.59, was identified as the O-n-butyl ester, N-trifluoroacetyl derivative of S-(2-chloroethyl) cysteine (Table 4) by the mass fragmentation pattern and by accurate mass measurements of the (M-HCl), the $(M-HCl-NH_2COCF_3)$ and the $(M-CH[NH_2COCF_3]CO_2C_4H_9)$ ions. Confirmatory evidence for the structure of this

TABLE 3 NEAR HERE

TABLE 4 NEAR HERE

Fig. 3 NEAR HERE

compound was derived from the NMR spectrum (Fig. 3). The purified ^{14}C substance, which was present in the thin-layer band of R_F 0.24, was identified as the O-n-butyl ester of N-acetyl-S(2-chloroethyl) cysteine (Table 5) by the mass fragmentation pattern, which is precisely analagous to that (Table 4) of the O-n-butyl ester, N-trifluoroacetyl derivative of S-(2-chloroethyl) cysteine, and accordingly no accurate mass measurements were necessary in this case (Table 5).

TABLE 5 NEAR HERE

TABLE 6 NEAR HERE

The relative proportions of $[^{14}C]$ vinyl chloride metabolites in the urine of intragastrically treated rats are detailed in Table 6.

The identifications of urinary vinyl chloride metabolites were made excessively difficult by the very small amounts of ^{14}C-labelled material that were available, because of the highly efficient pulmonary excretion of unchanged material, and of the difficulty of retaining sufficient (vinyl chloride) in the body for long enough for biotransformation. In addition, the difficulty of individual compound identification, for example of the O-n-butyl ester, N-trifluoroacetyl derivative of S-(2-chloroethyl) cysteine, was exacerbated by the absence of the molecular ion from the mass spectrum, and by the rather complex mass fragmentation pattern.

Remember there are tremendous advantages to approval via inhalation which will allow more metabolites to be found!

DISCUSSION

Three points arising out of this investigation merit discussion.

The first one concerns the fate of a dose of vinyl chloride in the mammalian body. A marked change in excretion pattern that accompanies a two thousand-fold difference in intragastric dose level has been attributed to a saturable drug metabolism and to an highly efficient pulmonary excretion of original substance from the body that leaves a relatively low concentration of material remaining in the circulating blood for hepatic biotransformation (see Results section). For groups of rats that had been administered various dose levels within that dose range, the plot of the pulmonary excretion of unchanged vinyl chloride (expressed in terms of percentage of the dose) against the logarithm of reciprocal doses is a straight line (Fig. 4). It seems that this observation reflects a more general phenomenon, since the same sort of logarithmic relationship holds for the biliary/faecal excretion of unchanged Ionox 220 against reciprocal doses[12], and whilst the antioxidant, Ionox 220, is lipid-soluble and water-insoluble, it is involatile. The fact that the main eliminative route for vinyl chloride is pulmonary agrees with Schaumann's observations of over forty years ago[13] that in mammals the pulmonary excretion of unchanged vinyl chloride follows its inhalational administration.

FIG 5 NEAR HERE

The second point relates to the primary metabolic changes, which vinyl chloride undergoes in mammals. In the first place, glutathione reacts directly with the vinyl chloride in a completely anti-Markownikov manner to give S-(2-chloroethyl) cysteine (a)(Fig. 5), which is further metabolized into the mercapturic acid, N-acetyl-S-(2-chloroethyl) cysteine (b). The anti-Markownikov addition is in fact a free radical reaction (v. inter alia ref. 14), which would be induced by traces of peroxide in vitro[15]. It is important that the retention of chlorine in metabolites (a) and (b) would have been realized only through the reaction processes under discussion. A second reaction, an associative reaction with molecular oxygen, typically involves the bonding of both olefinic carbon atoms at the transition state. We envisage a singlet oxygen bonded form[16] in dynamic equilibrium with a cyclic peroxide form. We consider that chloroacetic acid (c) would be formed through rearrangement of a singlet oxygen bonded transition state, and that (c) is metabolized further into thiodiglycollic acid (d) and glutamic acid (e), whereas formaldehyde (f), and through it CO_2 (g) and urea (h), is formed by dismutation of a cyclic peroxide transition state. Evidence for formaldehyde formation rests on the production of CO_2 and of urea, and on the detection of the trace (vinyl chloride) metabolites, methionine (i) and serine (j), themselves established metabolic products of formaldehyde[17]. Formation of formaldehyde and CO_2 by this metabolic pathway finds confirmation in their production in vitro amongst the reaction products of a cyclic peroxide of vinyl chloride[18,19].

Another reaction that seems feasible involves anti-Markownikov addition of a thiol to vinyl chloride and a second anti-Markownikov addition of the resulting 2-chlorothioethane to another molecule of vinyl chloride. The resulting sulphur mustard, di-(2-chloroethyl) sulphide, which would hydrolyse to thiodiglycol, might be expected to yield thiodiglycollic acid (d) by subsequent oxidation. Whilst present work does not exclude formation of di-(2-chloroethyl)sulphide, it is unlikely that this intermediate is a major source of thiodiglycollic acid. In fact, at least one-half of a dose of di-(2-chloroethyl) sulphide administered to rats was excreted in the urine as di-β-alanylthioethylsulphone in conjugated form, 15-20% of the metabolites are the same as those derived from thiodiglycol including about 1% of thiodiglycollic acid, and a further 10-15% the same as those metabolites formed from 2-chloroethyl-2'-hydroxyethylsulphide[20,21]. In comparison, our finding that thiodiglycollic acid is the hitherto undiscovered major metabolite (more than 60% of the dose) of chloroacetic acid in rats strongly supports its genesis from vinyl chloride by that metabolic pathway.

Although present work does not exclude the occurence *in vivo* of chloroethylene oxide, it provides no evidence for its formation, despite the fact that rearrangement of this substance to chloroacetaldehyde[22] would account for chloroacetic acid production. However, if the epoxide were formed even as a bonded transition state, then ensuing reaction of the epoxide or its rearrangement product with glutathione would afford products[23], which would differ structurally from the ones that have been established

rigorously for metabolites (a) and (b). Moreover, chloroethylene oxide is not prepared direct from vinyl chloride in vitro[22].

Our third point delineates a possible relationship between the metabolism of vinyl chloride and its oncogenicity. Formation of the three major urinary metabolites, thiodiglycollic acid, S-(2-chloroethyl) cysteine and N-acetyl-S-(2-chloroethyl) cysteine, involves glutathione utilization. Under certain circumstances, exposure to vinyl chloride would deplete the glutatione pool seriously, despite compensating mechanisms. In fact, there has been found some lowering in the availability of hepatic non-protein sulphydryl groups in exposed rats[24], besides significant blocking of non-protein sulphydryl groups in the blood of vinyl chloride operatives[25]. The latter effects were less pronounced in workers whose contact was discontinuous. Since it has been proposed that a fundamental role for glutatione in the body may be to protect tissues against electrophilic attack by drug metabolites and other alkylating agents[26-30], it follows that chronic exposure to high concentrations of vinyl chloride would lower the body's protection against attack by reactive metabolites.

FIG 6 NEAR HERE

Biotransformation of [^{14}C] vinyl chloride involves interaction with mammalian metabolism, and formation of the minor metabolite [^{14}C] glutamate implies C_2-alkylation of an oxaloacetate intermediate (Fig. 6), whilst production of the major metabolite, [^{14}C] thiodiglycollate involves double C_2-alkylation of cysteine-derived

hydrogen sulphide (see ref. 31). Furthermore, well-established metabolic pathways for formaldehyde afford very small quantities of [^{14}C]methionine and [^{14}C]serine[17]. The fact that this study provides ample evidence for C_1 and C_2 alkylation of intermediary metabolites suggests that this sort of chemical reaction may lead to the transcriptional changes of the RNA, DNA or other macromolecular cell constituents, which would appear to be responsible for vinyl chloride-induced liver neoplasm. In an extension of the present work, the way in which vinyl chloride, vinylidene chloride and the reactive molecular species to which they give rise, alkylate selected nucleotides, poly-nucleotide and microsomal RNA is the subject of rigorous investigation. However, an alternative metabolic pathway to those that have been discussed may contribute to the physico-chemical mechanism of carcinogenesis, and in that context, formation of, for example, di-(2-chloroethyl) sulphide ought to be considered, in view of the tragic incidence of respiratory neoplasia in man following mustard gas inhalation and poisoning[32-36].

It may be significant that in the present study, vinyl chloride has been shown to react *in vivo* with certain nucleophilic protein constituents, with which N-hydroxy-2-fluorenylacetamide sulphate (the ultimate carcinogen about which most is known) reacts both *in vitro* and *in vivo*[37]. Those reactions are considered to be especially important to the oncogenicity of 2-fluorenylacetamide through its interaction with nucleic acid[38].

Finally, the carcinogenic potential of vinyl chloride would be expected to increase with increasing intensity and frequency of exposure, when the concerted interaction of the parameters that have been discussed under the third point would be expected to be most effective.

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TABLE 1

Excretion of radioactivity in rats, given a single dose of [^{14}C] vinyl chloride

Four rats were each dosed i.g. with 250 µg of [^{14}C] vinyl chloride per kg in corn oil solution, and another 4 rats were each dosed similarly with 450 mg of [^{14}C] vinyl chloride per kg. Four rats were each injected in the femoral vein with 250 µg of [^{14}C] vinyl chloride per kg in N-(β-hydroxyethyl) lactamide. Four rats were each injected i.p. with 250 µg of [^{14}C] vinyl chloride per kg in N-(β-hydroxyethyl) lactamide, and another 4 animals were each injected similarly with 450 mg of [^{14}C] vinyl chloride.

Size of dose	Time (h)	Radioactivity Excreted (% of dose)[a]											
		Intragastric				Intravenous				Intraperitoneal			
		Exhaled air		Urine	Faeces	Exhaled air		Urine	Faeces	Exhaled air		Urine	Faeces
		Vinyl chloride	CO_2			Vinyl chloride	CO_2			Vinyl chloride	CO_2		
250 µg kg^{-1}	0-24	3.7±1.2	12.6±1.1	71.5±5.0	2.8±2.5	99.0±0.8	0.1	0.5	0.1	43.2±4.6	10.3±2.2	41.5±4.8	1.6
	24-48		0.9	3.3	1.6						0.7	1.6	0.2
	48-72			0.3	0.2								
	Total	3.7±1.2	13.5±1.3	75.1±4.2	4.6±3.0	99.0±0.8	0.1	0.5	0.1	43.2±4.6	11.0±1.2	43.1±5.7	1.8
450 mg kg^{-1}	0-24	91.9±2.5	0.6	4.5±2.3	0.4					96.2±4.1	0.7	2.5±0.9	0.1
	24-48		0.1	0.8	0.3							0.1	
	48-72			0.1									
	Total	91.9±2.5	0.7	5.4±2.2	0.7					96.2±4.1	0.7	2.6±0.9	0.1

[a] Values shown are the means ± S.D. of those means.

TABLE 2

Thin-layer chromatographic and gas chromatographic characteristics of the O-n-butyl, N-TFA derivatives of the principal urinary metabolites of vinyl chloride

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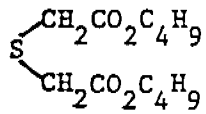
Derivativized vinyl chloride metabolites	R_F values on SiO_2-gel plates developed with chloroform	GC-retention times (min)	
		Column coated with OV-1 and run at 175°	Column coated with DEGS and run at 140° and 180°
O-n-Butyl ester of N-acetyl-S-(2-chloroethyl) cysteine	0.24	5.00	
O-n-Butyl ester, N-TFA derivative of S-(2-chloroethyl) cysteine	0.59	2.20	14.0*
O-n-Butyl ester of thiodiglycollic acid	0.74	2.50	4.6*

*O-n-Butyl thiodiglycollate was stripped from the second column at 140° and the O-n-butyl ester, N-TFA derivative of S-(2-chloroethyl) cysteine at 180°.

TABLE 3

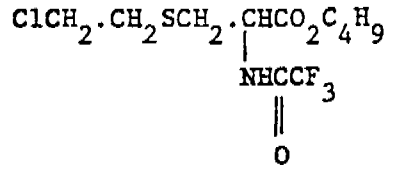
Mass spectral data for O-n-butyl thiodiglycollate



Ion	m/e	%	Measured Mass	Corresponding Formula	Calculated Mass
Molecular	262	18	262.1236	$C_{12}H_{22}O_4S$	262.1238
$M-C_4H_9OH$	188	33	188.0509	$C_8H_{12}O_3S$	188.0507
$M-CO_2C_4H_9$	161	6			
$M-C_4H_9OH-C_4H_9$	132	100			

TABLE 4

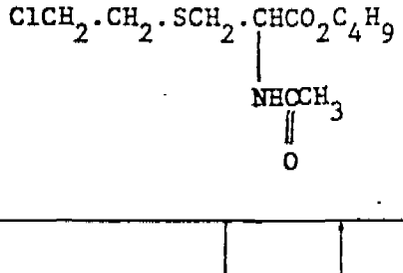
Mass spectral data for the O-n-butyl ester, N-trifluoroacetyl derivative of S-(2-chloroethyl) cysteine



Ion	m/e	%	Measured Mass	Corresponding Formula	Calculated Mass
Molecular	335				
M-HCl	299	10	299.0814	$C_{11}H_{16}NO_3SF_3$	299.0803
$M-CO_2C_4H_9$	234	15			
$M-NH_2COCF_3$	222	19			
$M-HCl-NH_2COCF_3$	186	86	186.0721	$C_9H_{14}O_2S$	186.0715
$M-HCl-NH_2COCF_3-C_4H_8$	130	39			
$M-CH(NH_2COCF_3)CO_2C_4H_9$	109	100	108.9873	C_3H_6SCl	108.9878
C_4H_9	57	36			

TABLE 5

Mass spectral data for the O-n-butyl ester of N-acetyl-S-(2-chloroethyl) cysteine



Ion	m/e	%
Molecular	281	0.5
M-HCl	245	13
$M-NH_2COCH_3$	222	6
$M-HCl-NH_2COCH_3$	186	60
$M-HCl-NH_2COCH_3-C_4H_8$	130	49
$CH_2.CH_2.SCH_2$	74	40
$COCH_3$	43	100

TABLE 6

Relative proportions of vinyl chloride metabolites in the urine of rats, dosed p.o. with [^{14}C] vinyl chloride

Vinyl chloride Metabolites	% of urinary radioactivity
Thiodiglycollic acid	47.0
S-(2-Chloroethyl) cysteine	23.0
N-Acetyl-S-(2-chloroethyl) cysteine	23.0
Urea	6.0
Glutamic acid	0.5
Chloroacetic acid	0.5
Methionine	tr
Serine	

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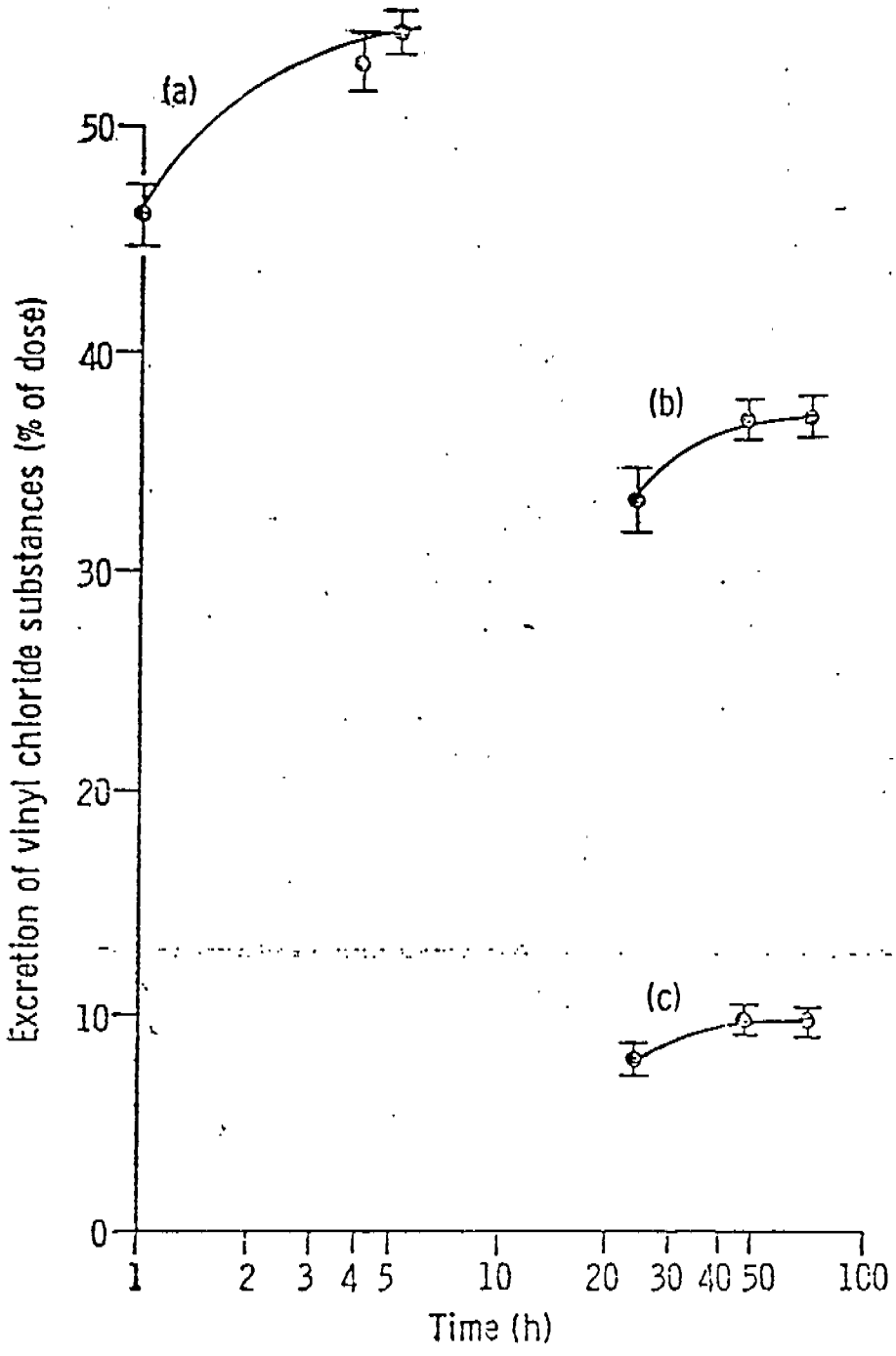
(a)
(b)
(c)
50
40
30
20
10
0
Excretion of vinyl chloride substances (% of dose)
1
2
3
4
5
10
20
30
40
50
100
Time (h)

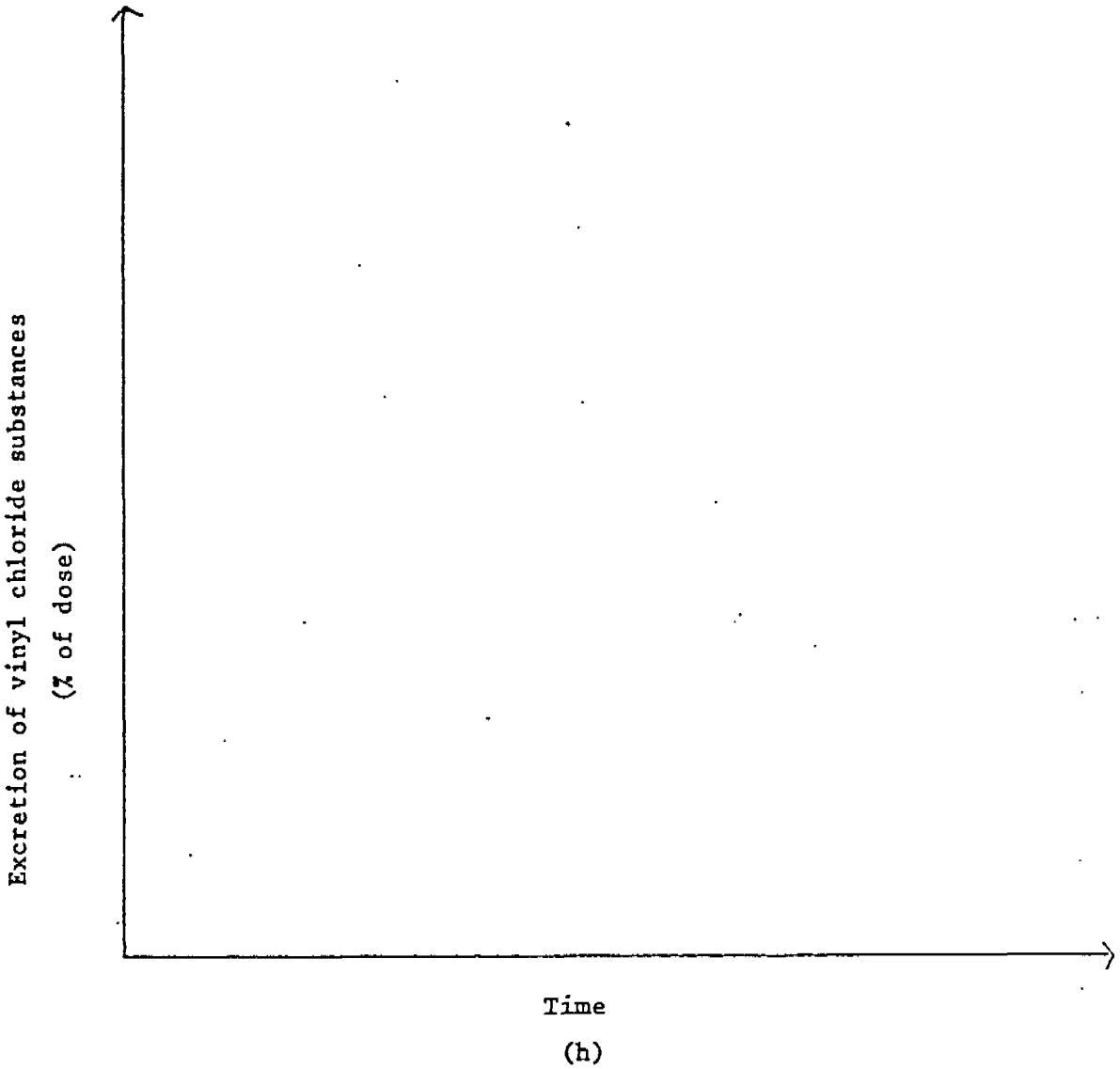

Fig. 1 Linear-log plots of the cumulative excretion of vinyl chloride-related materials from rats after administration of single doses of [^{14}C] vinyl chloride

[^{14}C]Vinyl chloride (30 mgkg^{-1}) was administered i.g. to rats in corn oil solution. Three animals were dosed, and mean values were used to construct the curves. Plot (a) represents pulmonary excretion of unchanged vinyl chlori (b) the urinary excretion of [^{14}C] metabolites, and (c) the matching pulmonary excretion of $^{14}CO_2$.

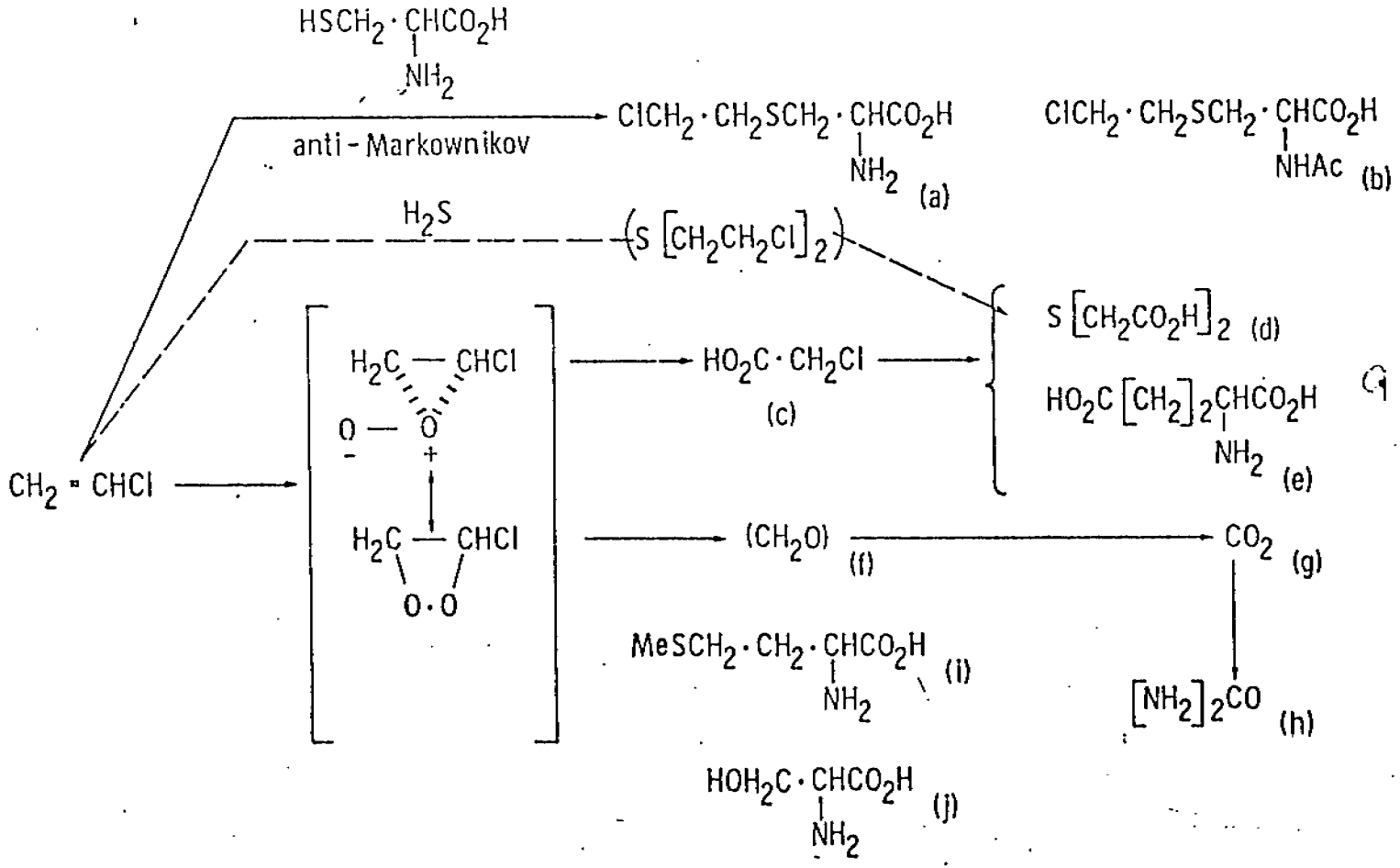
HSCH2·CHCO2H
NH2
anti-Markownikov
ClCH2·CH2SCH2·CHCO2H
NH2 (a)
ClCH2·CH2SCH2·CHCO2H
NHAc (b)
H2S
S[CH2CH2Cl]2
S[CH2CO2H]2 (d)
HO2C[CH2]2CHCO2H
NH2 (e)
CH2 = CHCl
H2C — CHCl
O — O
H2C — CHCl
O·O
HO2C·CH2Cl
(c)
(CH2O)
(f)
CO2 (g)
[NH2]2CO (h)
MeSCH2·CH2·CHCO2H
NH2 (i)
HOH2C·CHCO2H
NH2 (j)

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Fig. 6 Scheme for the derivation of thiodiglycollic and glutamic acids

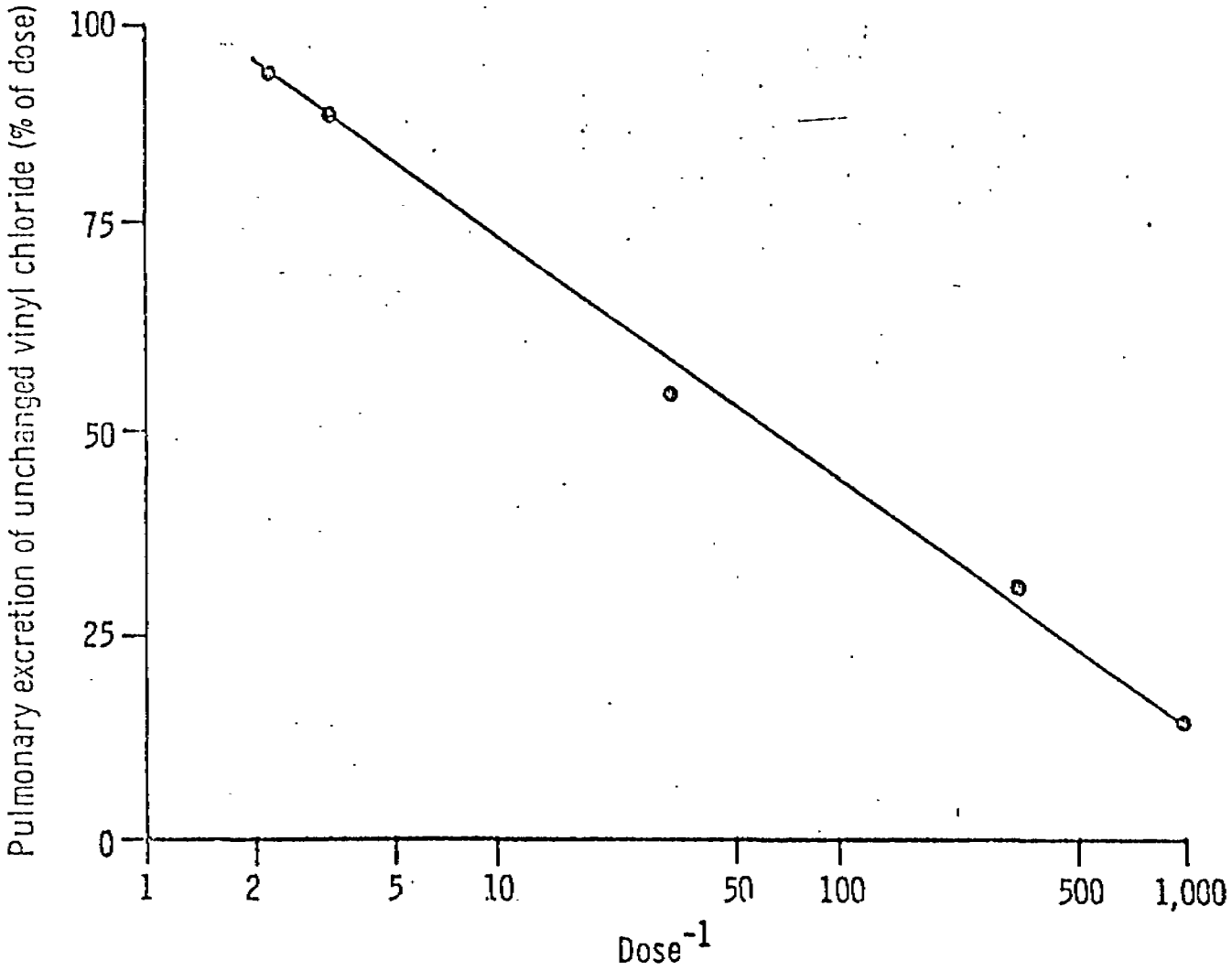
Pulmonary excretion of unchanged vinyl chloride (% of dose)
100
75
50
25
0
1
2
5
10
50
100
500
1,000
Dose^{-1}

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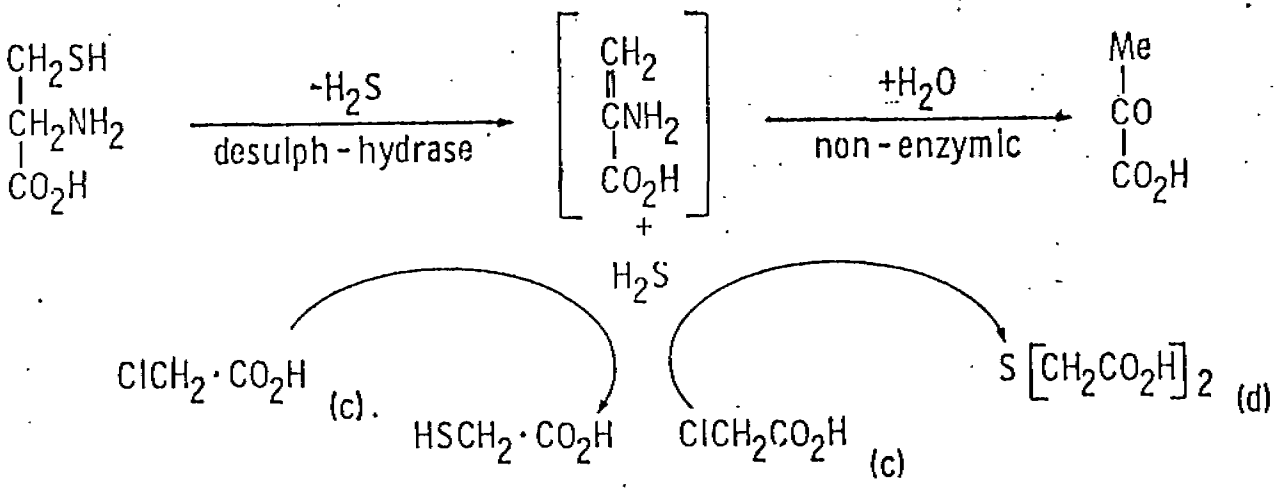
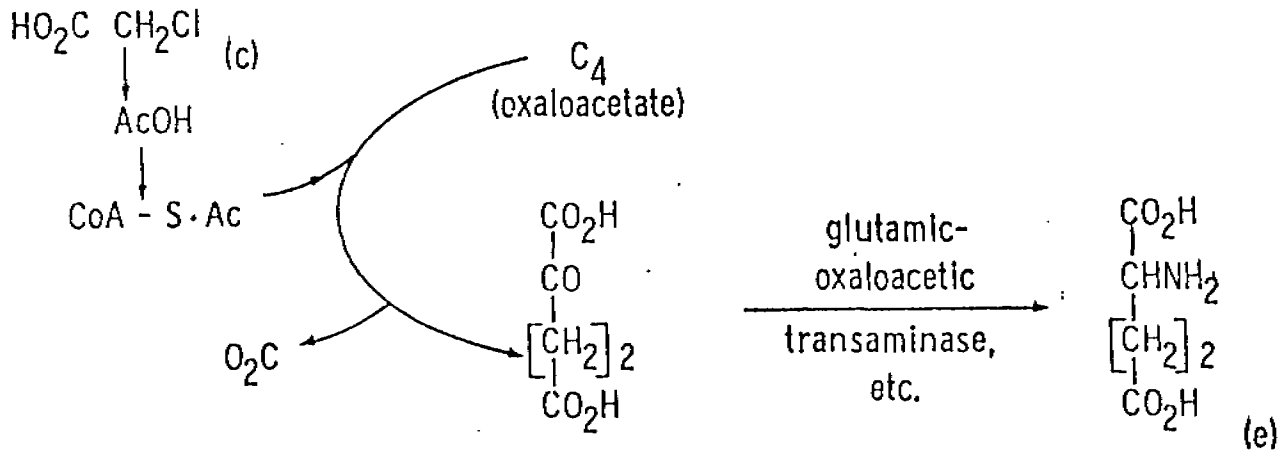
Fig. 5 Scheme for the metabolism of vinyl chloride in rats


CH2SH
CH2NH2
CO2H
-H2S
desulph-hydrase
CH2
CNH2
CO2H
+
H2S
+H2O
non-enzymic
Me
CO
CO2H
ClCH2·CO2H
(c)
HSCH2·CO2H
ClCH2CO2H
(c)
S[CH2CO2H]2
(d)


HO2C CH2Cl
(c)
AcOH
CoA - S·Ac
C4
(oxaloacetate)
O2C
CO2H
CO
[CH2]2
CO2H
glutamic-
oxaloacetic
transaminase,
etc.
CO2H
CHNH2
[CH2]2
CO2H
(e)

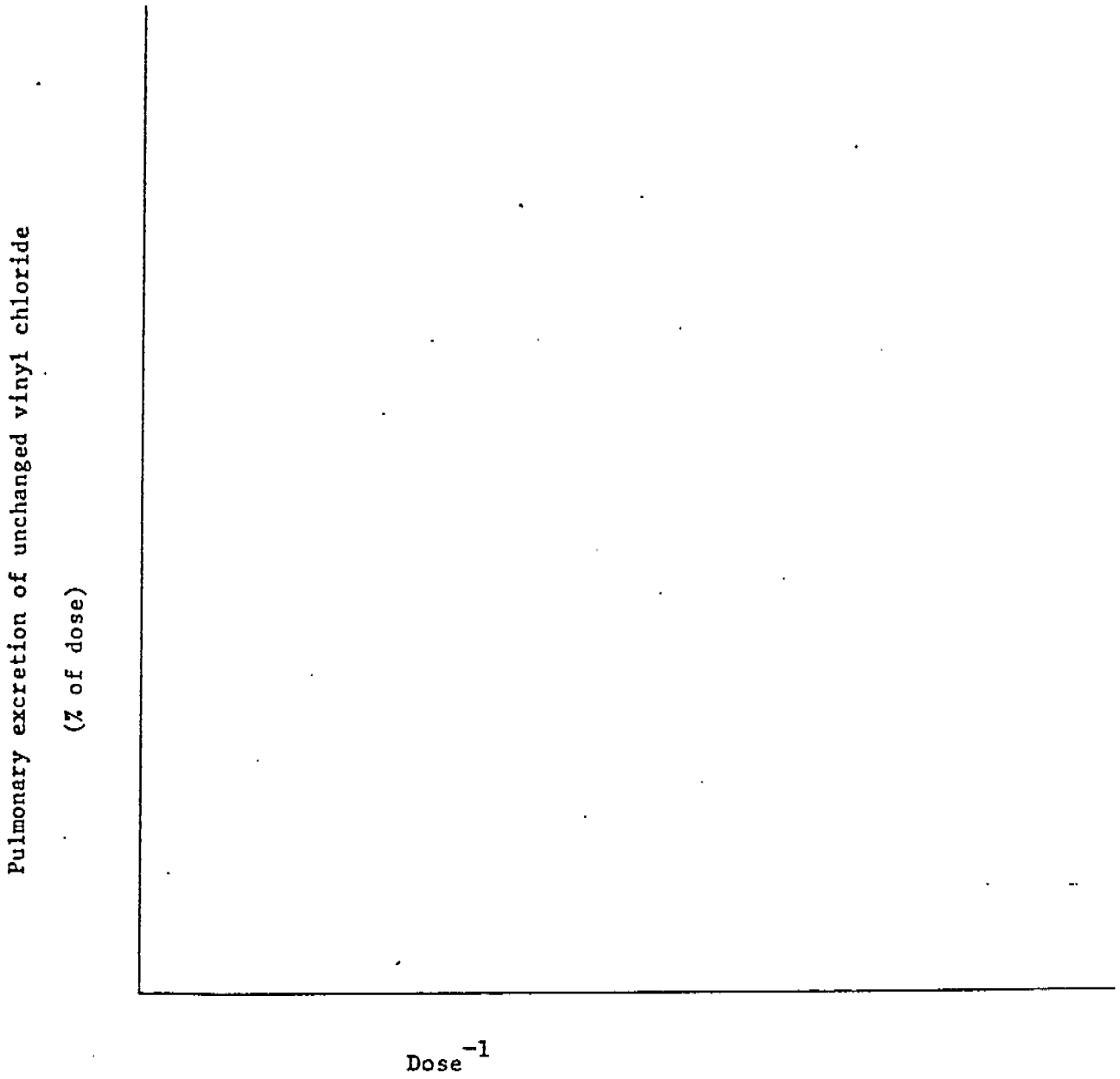


Fig. 4 Linear-log plot of the pulmonary excretion of unchanged vinyl chloride against the reciprocal doses

Five groups of 3 rats were dosed i.g. with vinyl chloride at dose levels in the range of 1 to 450 mgkg^{-1}; each point represents 3 independent values.

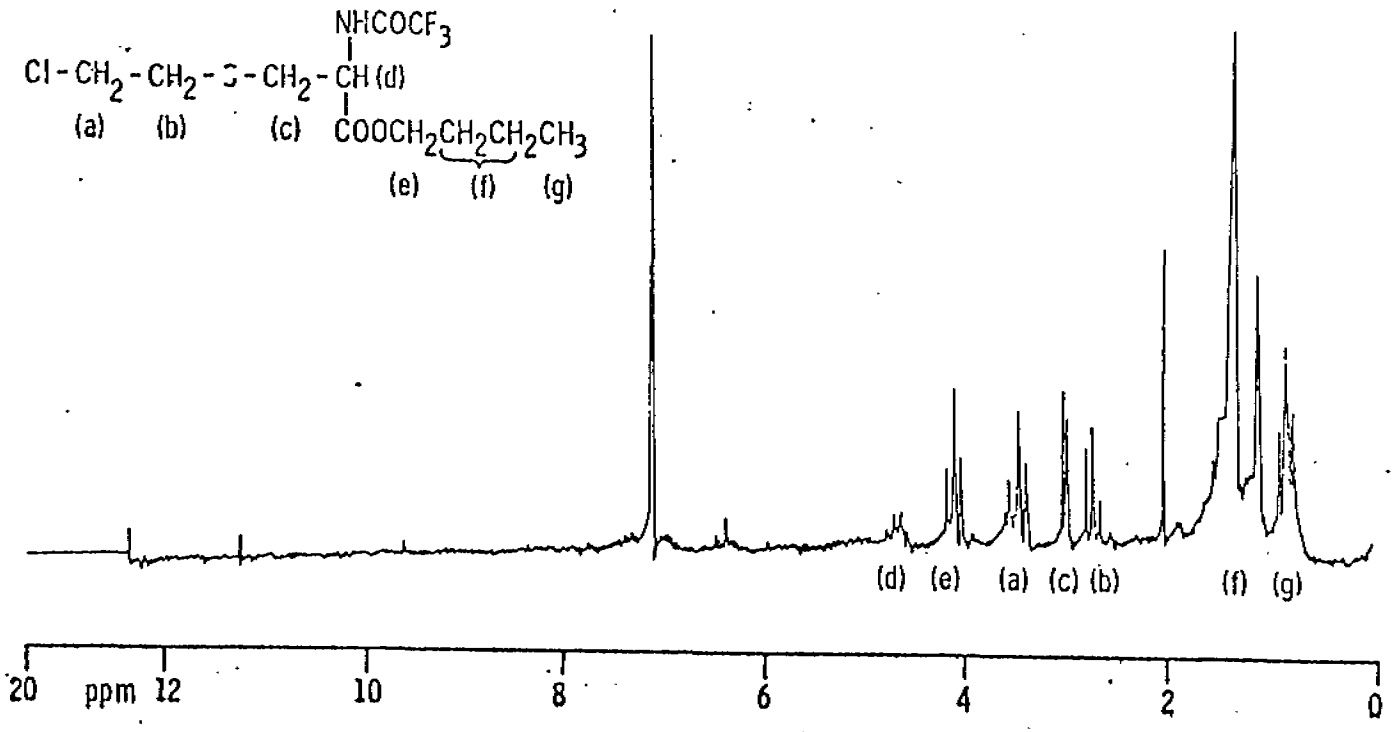
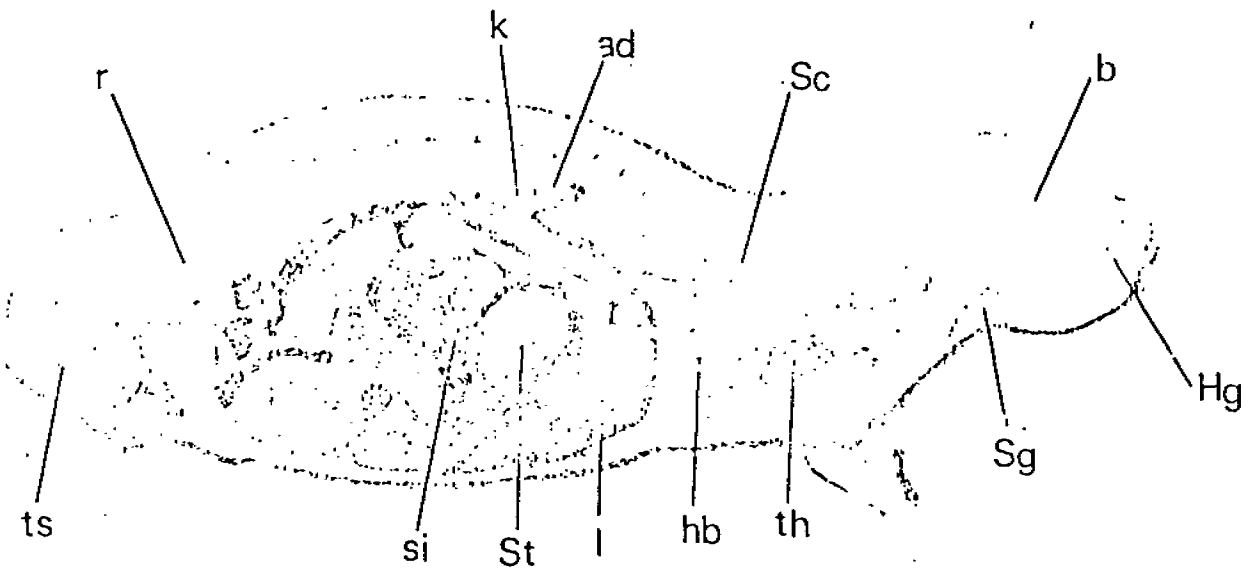
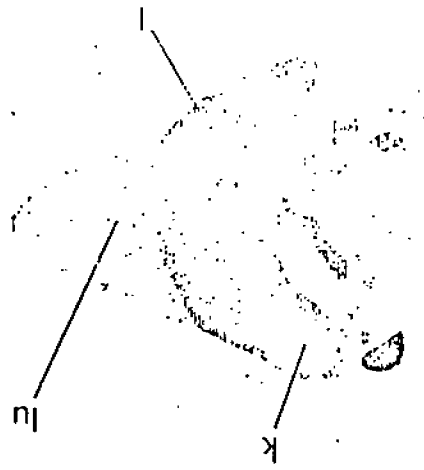

NHCOCF3
Cl - CH2 - CH2 - C - CH2 - CH (d)
(a) (b) (c) COOCH2CH2CH2CH3
(e) (f) (g)
(d) (e) (a) (c) (b) (f) (g)
20 ppm 12 10 8 6 4 2 0

Fig. 3 NMR spectrum of the O-n-butyl ester, N-trifluoroacetyl derivative of S-(2-chloroethyl) cysteine from rat urine



P
k
Sp
lu
Zg
St
l
h

R&S 135246



15 min
after
dosing

2 h
after
dosing

4 h
after
dosing

Fig. 2 The distribution of radioactivity in the tissues of rats 15 min, 2 h and 4 h after intragastric dosing with [^{14}C] vinyl chloride (mgkg^{-1})

ad, adrenal; b, brain; Hg, Harder's gland; h, heart; k, kidney; l, liver; lu, lung; r, rectum; Sc, spinal cord; Sg, salivary gland, si, small intestine; Sp, spleen; St, stomach; th, thymus; ts, testis; Zg, Zymbal gland.

Biotransformation in rats of vinyl chloride vis-à-vis its oncogenicity

Early warning that one year's intermittent inhalational exposure of Wistar rats to 3% (v/v) of vinyl chloride elicited epidermoid and mucoepidermoid carcinomas in the para-auricular region of surviving animals[1] has been confirmed in more detailed and extensive investigations[2], which revealed, in addition to Zymbal gland carcinomas, liver angiosarcomas and nephroblastomas in Sprague-Dawley rats that had been exposed to atmospheric concentrations as low as 250 ppm. Vinyl chloride also induced liver angiosarcomas in Swiss mice at 500 ppm[2]. This latter work[2] established a direct relationship between the dose level and length of treatment with vinyl chloride and neoplastic response. Those observations and the world-wide record by June 1974 of 24 cases of liver angiosarcoma amongst workers, who had been engaged upon vinyl chloride/polyvinyl chloride manufacture, demand a better understanding of the biological fate of vinyl chloride in mammals in relation to its established oncogenicity. Moreover, angiosarcoma induction by chronic intraperitoneal (vinyl chloride) injections[3] implies that vinyl chloride, and not a product of its photolysis, is the causative agent.

Excretion data showed that the main eliminative route for [^{14}C] vinyl chloride after oral, intravenous or intraperitoneal administration to rats is pulmonary (Table 1) (see also ref 4); both unchanged vinyl chloride and vinyl chloride-related CO_2 are excreted through the lungs and other [^{14}C] metabolites via the kidneys. A marked change in

excretion pattern that accompanies a nearly two thousand-fold difference in intragastric dose level is due to a saturable drug metabolism and to an highly efficient pulmonary excretion of original substance that leaves the relatively low concentration of vinyl chloride material remaining in circulating blood for hepatic biotransformation. For groups of animals that had been orally dosed vinyl chloride within a dose-level range of 1-450 $mgkg^{-1}$, the pulmonary output of unchanged substance (expressed in terms of % of the dose) is proportional to the logarithm of the reciprocal dose (Fig 1). Excretion patterns after i.v. and i.p. injections are predictable from the characteristics of excretion after oral administration (Table 1). Whilst pulmonary excretion of unchanged vinyl chloride after oral dosing is complete within 3-5 h, pulmonary elimination of CO_2 and renal excretion of vinyl chloride metabolites occupies 3 days. In comparison, 99% of a small i.v. dose was excreted unchanged within 1 h of injection; 80% within 2 min. Since the rate of elimination of single oral doses of $[^{14}C]$ vinyl chloride is unaffected by up to 60 days' chronic dosing with the unlabelled substance at 3, 30 and 300 $mgkg^{-1}$ day^{-1}, excretion data for an acute dose apply also to the chronic situation.

Distribution studies by whole-animal authoradiography agree with deductions made from the excretion data, but small localizations of ^{14}C are visible in the para-auricular region of appropriate sections, possibly in the Zymbal glands.

Of the vinyl chloride in the circulating blood, which escapes the highly efficient pulmonary arterial-alveolar transfer and is metabolized, thiodiglycollic acid, S-(2-chloroethyl) cysteine, N-acetyl-S-(2-chloroethyl) cysteine and urea are major urinary metabolites, chloroacetic acid and glutamic acid comprise the minor metabolites, and methionine and serine are trace metabolites. Families of related [^{14}C] metabolites were separated from the urine of [^{14}C] vinyl chloride-treated rats by anion-exchange chromatography, and residues from successively displaced fractions were derivativized, purified by t.l.c. and preparative GC and examined by GC-mass spectrometry[5]. Accurate mass measurements of the principal ions and NMR spectroscopy aided allocations of structure, and where possible, mass spectra were compared with those of authentic substances.

The halogen atom of vinyl chloride is stable to nucleophilic reagents, and glutathione reacts directly <u>in vivo</u> with [^{14}C] vinyl chloride in an anti-Markownikov manner to give S-(2-chloroethyl) cysteine (a) (Fig 2), which is further metabolized into the mercapturic acid, N-acetyl-S-(2-chloroethyl) cysteine (b). Retention of chlorine in metabolites (a) and (b) would have been realized only through the reactions that have been mentioned. Associative reaction with molecular O_2 typically involves the bonding of both olefinic C atoms at the transition state. We envisage a singlet oxygen-bonded form[6] in dynamic equilibrium with a cyclic peroxide form. Chloroacetic acid (c) would be formed through rearrangement of a singlet oxygen-bonded transition state, and (c) is metabolized further into thiodiglycollic acid (d) and glutamic acid (e), whereas

formaldehyde (f), and through it CO_2 (g) and urea (h), is formed by dismutation of a cyclic peroxide transition state. Evidence for formaldehyde formation rests on the production of $^{14}CO_2$ and [^{14}C] urea, and on the detection of [^{14}C] methionine (i) and [^{14}C]serine (j), themselves established formaldehyde metabolites. Formation of formaldehyde and CO_2 along this metabolic pathway finds confirmation in their production in vitro amongst the reaction products of a known cyclic peroxide of vinyl chloride[7,8].

Another reaction that seemed feasible would involve addition of a thiol to vinyl chloride followed by addition of the resulting 2-chlorothioethane to another molecule of vinyl chloride to yield di-(2-chloroethyl) sulphide, which would hydrolyse to thiodiglycol, and which might be expected to yield thiodiglycollic acid by subsequent oxidation. Whilst present work does not exclude di-(2-chloroethyl) sulphide formation, it is unlikely that this intermediate is a major source of thiodiglycollic acid (see refs. 9,10). In comparison, our observation that thiodiglycollic acid is the hitherto undiscovered major metabolite (more than 60% of the dose) of chloroacetic acid in rats strongly supports its genesis from vinyl chloride by this metabolic pathway.

Although present work does not exclude the occurrence in vivo of chloroethylene oxide, it provides no evidence for its formation, despite the fact that rearrangement of this substance to chloroacetaldehyde[14] would account for chloroacetic acid production. However, if the epoxide were formed even as a bonded transition state, the ensuing reaction of the epoxide or its rearrangement product with glutathione would afford

products[12], which would differ structurally from the ones, which have been established rigorously for metabolites (a) and (b). Moreover, chloroethylene oxide is not prepared direct from vinyl chloride in vitro[11].

The metabolism of vinyl chloride is important to its oncogenicity. Thus, (i) formation of all three major urinary metabolites, thiodiglycollic acid S-(2-chloroethyl) cysteine and N-acetyl-S-(2-chloroethyl) cysteine, utilizes glutathione, and under certain circumstances, vinyl chloride exposure would deplete the glutathione pool seriously, despite compensating mechanisms. Besides significant blocking of non-protein sulphydryl groups in the blood of vinyl chloride operatives[13], some decrease in the availability of hepatic non-protein sulphydryl groups has been found in treated rats[14]. Since a fundamental role for glutathione in the body may be to protect against electrophilic attack by drug metabolites and other alkylating agents[15], chronic exposure to an high dose level of vinyl chloride would lower the body's protection against attack by reactive metabolites. (ii) Biotransformation of vinyl chloride involves intermediary metabolism, and formation of glutamic acid implies C_2-alkylation of an oxaloacetate intermediate, whilst thiodiglycollate production involves double C_2-alkylation of cysteine-derived hydrogen sulphide[16]. In addition,well-established metabolic pathways for formaldehyde afford very small amounts of methionine and serine[17]. The fact that this study provides ample evidence for C_1 and C_2 alkylation of intermediary metabolites suggests that this sort of chemical reaction may lead to the transcriptional changes of the RNA, DNA or other macromolecular cell constituents, which would appear to be responsible

for vinyl chloride-induced neoplasm. However, alternative reactive metabolites may contribute to the physico-chemical mechanism of carcinogenesis, and for example, di-(2-chloroethyl) sulphide may be important.

It may be significant that vinyl chloride has been shown to react *in vivo* with the same sort of nucleophilic protein constituents, with which N-hydroxy-2-fluorenylacetamide sulphate (the ultimate carcinogen about which most is known) reacts *in vitro* and *in vivo*[18]. Those reactions are held to be especially important to the oncogenicity of 2-fluorenylacetamide through its reaction with nucleic acid.[19]

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TABLE 1

Excretion of radioactivity in rats, given a single dose of [^{14}C] vinyl chloride

Four rats were each dosed i.g. with 250 µg of [^{14}C] vinyl chloride per kg in corn oil solution, and another 4 rats were each dosed similarly with 450 mg [^{14}C] vinyl chloride per kg. Four rats were each injected in the femoral vein with 250 µg of [^{14}C] vinyl chloride per kg in N-(β-hydroxyethyl) lactamide Four rats were each injected i.p. with 250 µg of [^{14}C] vinyl chloride per kg in N-(β-hydroxyethyl) lactamide, and another 4 animals were each injected similarly with 450 mg of [^{14}C] vinyl chloride.

Size of dose	Time (h)	Radioactivity Excreted (% of dose)[a]											
		Intragastric				Intravenous				Intraperitoneal			
		Exhaled air		Urine	Faeces	Exhaled air		Urine	Faeces	Exhaled air		Urine	Faeces
		Vinyl chloride	CO_2			Vinyl chloride	CO_2			Vinyl chloride	CO_2		
250 µg kg^{-1}	0-24	3.7±1.2	12.6±1.1	71.5±5.0	2.8±2.5	99.0±0.8	0.1	0.5	0.1	43.2±4.6	10.3±2.2	41.5±4.8	1.6
	24-48		0.9	3.3	1.6						0.7	1.6	0.2
	48-72			0.3	0.2								
	Total	3.7±1.2	13.5±1.3	75.1±4.2	4.6±3.0	99.0±0.8	0.1	0.5	0.1	43.2±4.6	11.0±1.2	43.1±5.7	1.8
450 mg kg^{-1}	0-24	91.9±2.5	0.6	4.5±2.3	0.4					96.2±4.1	0.7	2.5±0.9	0.1
	24-48		0.1	0.8	0.3							0.1	
	48-72			0.1									
	Total	91.9±2.5	0.7	5.4±2.2	0.7					96.2±4.1	0.7	2.6±0.9	0.1

[a]Values shown are the means ± S.D. of those means.

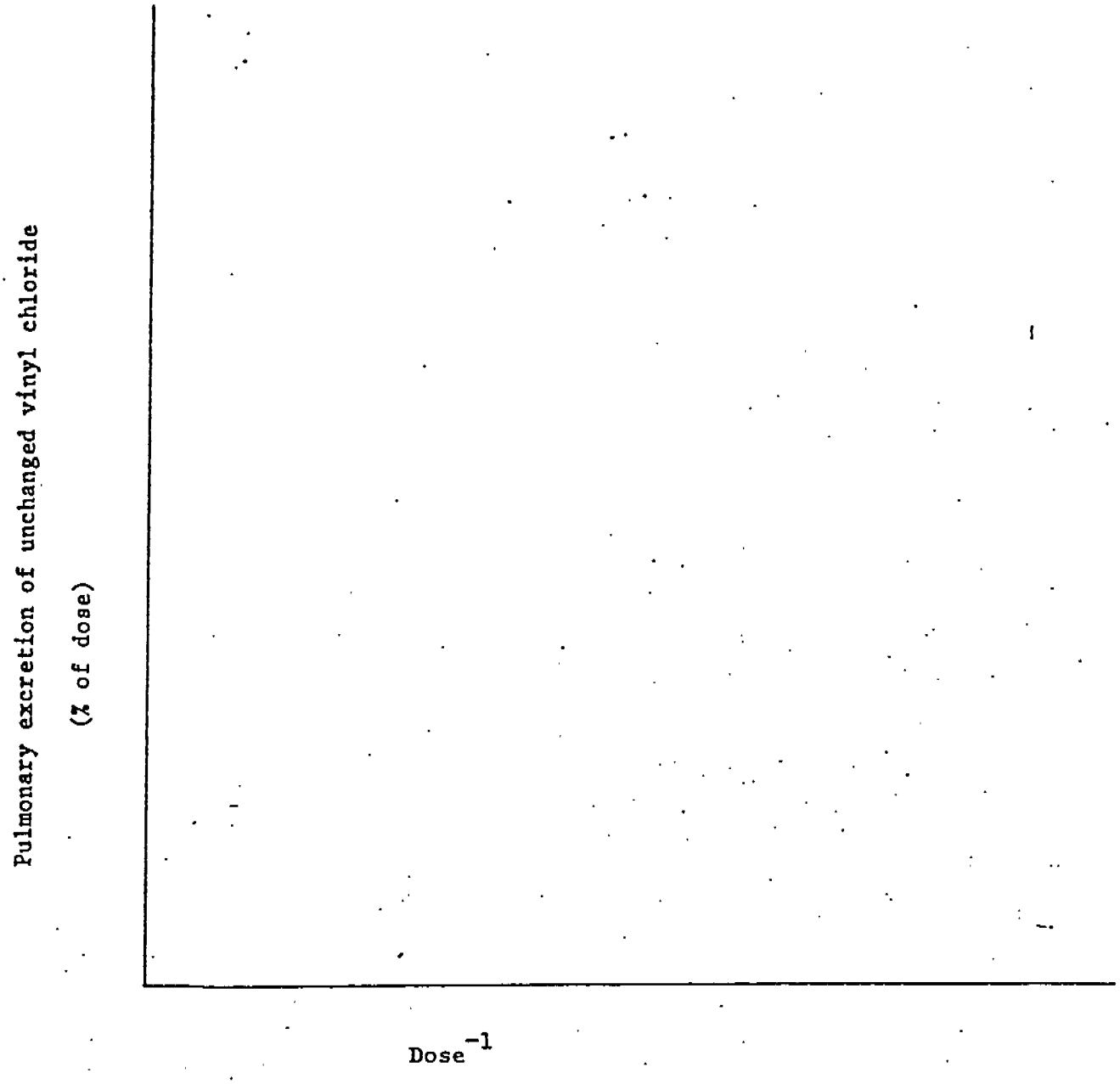

Fig. 1 Linear-log plot of the pulmonary excretion of unchanged vinyl chloride against the reciprocal doses

Five groups of 3 rats were dosed i.g. with vinyl chloride at dose levels in the range of 1 to 450 mgkg^{-1}; each point represents 3 independent values.

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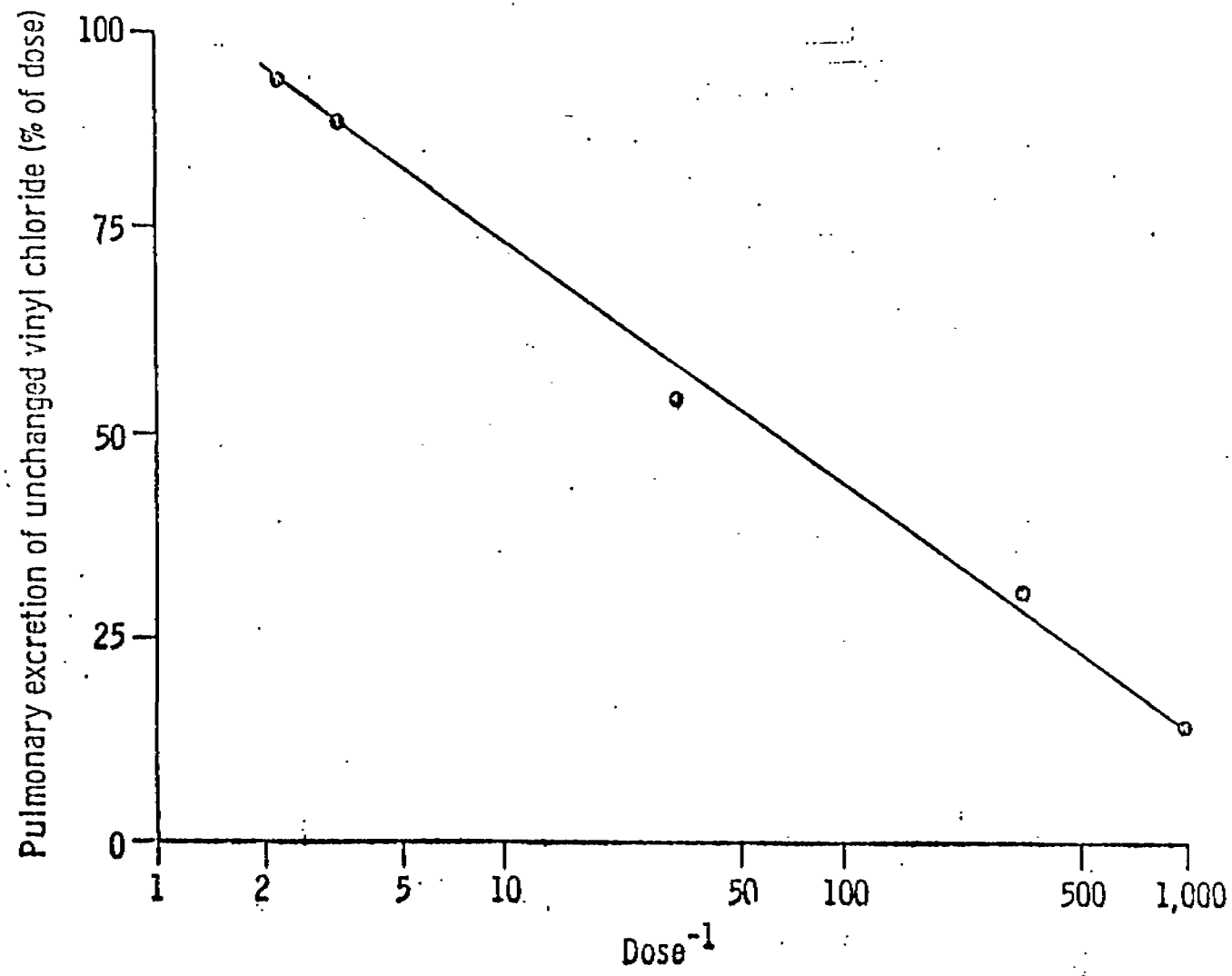
Pulmonary excretion of unchanged vinyl chloride (% of dose)
100
75
50
25
0
1
2
5
10
50
100
500
1,000
Dose^{-1}