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Molecular Mechanism of 1,1-Dichloroethylene Toxicity: Excreted Metabolites Reveal Different Pathways of Reactive Intermediates

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Abstract. The excretion and biotransformation of [^{14}C] 1,1-dichloroethylene (vinylidene chloride, VDC) after administration of a single oral dose has been investigated in female rats. Seventy-two hours after a dose of 0.5, 5.0, and 50.0 mg/kg, 1.26, 9.70, 16.47%, respectively, are exhaled as unchanged VDC, and 13.64, 11.35, 6.13% as $^{14}CO_2$. The main pathway of elimination is through renal excretion with 43.55, 53.88, 42.11% of the administered radioactivity. Through the biliary system, 15.74, 14.54, 7.65% of the activity are eliminated.

The isolation of the main metabolites of VDC from 24 h urine is accomplished through the combined application of solvent extraction, ion exchange chromatography and thin layer chromatography. Then gas chromatography and mass spectrometry are used for their identification. Three metabolites have been identified: thiodiglycolic acid, N-acetyl-S-(2-carboxymethyl)cysteine and methylthio-acetylaminoethanol. In addition, three smaller unidentified radioactive peaks have been found. Thiodiglycolic acid is the main metabolite in VDC metabolism. The simultaneous formation of an ethanolamine- and a cysteine-conjugation product points to different reaction pathways of the postulated intermediate reactive epoxide; ethanolamine probably originates from membrane lipids, which react with VDC-epoxide and/or its derivatives. This pathway could explain, in part, the parenchyma damaging effect of VDC.

Key words: 1,1-Dichloroethylene (Vinylidene chloride) – Pharmacokinetics – Biotransformation – Mercapturic acid – Molecular toxicity.

Introduction

1,1-Dichloroethylene (vinylidene chloride, VDC) has been employed for decades as a monomere in the manufacture of plastic materials. Not until 1977 was the carcinogenic effect of this substance demonstrated (Viola and Caputo, 1977). The initial suspicion of such a toxic property has been expressed with the recognition of the structurally closely related vinyl chloride as a carcinogen in humans (Creech and Johnson, 1974; Lee and Harry, 1974) and animals (Maltoni and Lefemine, 1975).

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Since then, numerous reports have appeared concerning the possible carcinogenic potential of all chlorinated ethylenes.

In bacterial test systems, after metabolic activation, a mutagenic effect has been proven for vinyl chloride, VDC and trichloroethylene. However, tetrachloroethylene and 1.2-dichloroethylenes (cis and trans) are not mutagenic (Bartsch et al., 1975; Greim et al., 1975). Previously unknown reactive metabolites are responsible for the mutagenic and carcinogenic effect of VDC (Bonse et al., 1975). The rate of metabolism of this substance is dose-dependent and saturable, as shown in experiments with isolated perfused rat livers (Reichert and Henschler, 1978) and whole animals (Jones and Hathway, 1978). However, it is suppressed by inhibitors of microsomal mixed-function-oxidases and through a lowered glutathione concentration in the liver (Reichert and Henschler, 1978; Reichert et al., 1978).

Studies concerning the fate of VDC in rats have recently been reported (Jones and Hathway, 1978; McKenna et al., 1978). Although we largely confirm the results of McKenna et al. (1978) with regard to pharmacokinetic data obtained after oral application (Reichert and Werner, 1978), differences continue to exist in the qualitative and quantitative characterization of the metabolites appearing in the urine. McKenna et al. (1978) have isolated four main metabolites from rat urine, two of which they have identified: thiodiglycolic acid and the mercapturic acid N-acetyl-S-(2-hydroxyethyl)cysteine. Jones and Hathway (1978) have found two main metabolites: besides thiodiglycolic acid, they postulate a cyclic product as an additional main metabolite. In addition, they have shown chloroacetic acid, dithioglycolic acid and thioglycolic acid in lesser concentrations.

With regard to these discrepancies, further studies concerning the metabolism of VDC appear to be necessary. In this paper, we report on the metabolism of VDC after oral administration in rats, which together with the discovery of previously undescribed metabolites, expands knowledge of the behavior of this substance in organisms and demonstrates new possibilities of reaction mechanism(s).

Materials and Methods

Chemicals. 1.2-[^{14}C] VDC was purchased from New England Nuclear Corp. and had a specific radioactivity of 0.475 mCi/mmole. Its radiochemical purity exceeded 98%, as shown by radiogas-chromatography. The ^{14}C-labeled VDC was solubilized in tricapriline.

Thiodiglycolic acid was purchased from E. Merck, Darmstadt, FRG, S-(2-carboxymethyl)-L-cysteine from EGA-Chemie, Steinheim, FRG. N-acetyl-S-(2-carboxymethyl)-L-cysteine was prepared by acetylation of S-(2-carboxymethyl)-L-cysteine with acetic anhydride according to Ozawa (1963). Its identity was confirmed by GC/MS. Methylthio-acetylaminoethanol (MAAE) was prepared as follows: mercaptoacetic acid (EGA-Chemie, Steinheim, FRG) (3 mmol, 276.3 mg) was methylated with an ethereal solution of diazomethane. The reaction mixture was then treated with 2-aminoethanol (3 mmol, 183 mg) in a water bath (80° C) for 20 min. The reaction product was characterized by GC/MS. Further derivatisations of MAAE were achieved by reaction with propionyl chloride and with trifluoroacetic anhydride: 50 µl of dry MAAE was reacted with an excess of propionyl chloride in a water bath at 80° C for 15 min and subsequently evaporated to dryness in a stream of dry nitrogen. This synthesis was in analogy to a similar reaction between colamine and propionyl chloride, described by Jenden et al. (1972). Trifluoroacetylation was performed by established methods (Kaiser et al., 1974). For esterification of carboxylic acids, an excess of freshly prepared n-butanol-3N HCl was added to the sample. Butylation was then performed in a water bath (80° C/15 min) as described by Kaiser et al. (1974).

Animals and Treatments. Female Wistar rats (180—220 g), Institut für Versuchstierzucht, Hannover, FRG were used in all studies. Pairs of rats were administered single doses of [^{14}C] VDC (as a solution in tricapriline, 3 ml/kg) by stomach tube between 9 and 10 a.m. The animals were transferred into an all-glass metabolism cage immediately after dosing. A constant rate of dry air (500 ml/min) was drawn through the cage. Standard diet (Altromin®) and water were supplied ad libitum.

Sampling and Measurement of Radioactivity. Excreted [^{14}C] activity was followed for 72 h. Urine and feces were collected at 6 h intervals, the cage being rinsed with a total volume of 100 ml water in several portions. [^{14}C] VDC and $^{14}CO_2$ were collected in 0.5 h and 3 h intervals by drawing the air from the metabolism cage through a series of traps. Before entering the first trap, the moisture of the air was removed with Drierite (W. H. Hammond, Drierite Co.). Following the procedure described by Watanabe et al. (1976), we separated the [^{14}C] VDC and $^{14}CO_2$ in four consecutive traps. The first two contained 50 ml toluene/2-methoxyethanol solution (8 : 2, v/v) each, and were cooled in a dry ice bath. The remaining two traps contained 100 ml of diethanolamine/2-methoxyethanol (1 : 1, v/v) each. The traps for $^{14}CO_2$ were maintained at room temperature.

Samples from the traps and urine samples were transferred directly in Rotiszint 22® liquid scintillation medium (C. Roth, Karlsruhe, FRG) for counting the radioactivity. The carcass was weighed and digested in 20% KOH. Portions of the digests were added to scintillation counting vials. The activity in each vial was measured in a Packard liquid scintillation counter.

Separation of Urinary Metabolites. The collected 24 h urine of two rats was freeze-dried, the residue was extracted with methanol at room temperature until it contained less than 1% of the radioactivity. The extract was evaporated to a small volume at 40° C under reduced pressure. One part of this solution was analysed by thin-layer chromatography. Aliquots were spotted on silica gel plates (0.25 mm layers of silica gel, F 254, E. Merck and developed in n-butanol : acetic acid : water solution (40 : 15 : 15, v/v/v). Compounds were detected by spraying with ninhydrin or with a reagent for bivalent sulfur compounds (Knight and Young, 1958). The radioactive zones were located with a Berthold radiochromatogram scanner, and removed by scraping off and extraction of the silica gel with methanol (50 ml). These fractions were analysed by GC/MS. Another part of the methanolic solution was evaporated to dryness, dissolved in pyridine-formiate buffer (0.05 M, pH 3) and passed through a 200 × 6 mm column of Aminex A 5 (Bio Rad Lab.). Operating pressure was 10—15 bar at 50° C. The eluate was collected in 1 ml fractions. Single fractions were evaporated and derivatized as described below.

Detection and Identification of Urinary Metabolites. Radioactive substances from zones on the thin-layer plates and fractions eluted from the cation exchange column were butylated following the procedure of Kaiser et al. (1974). GC/MS was performed with a Varian CH 7 mass spectrometer combined with a Varian 2700 gas chromatograph, and Varian SS 100 MS data system. Mass spectra were taken at 70 eV. Fractions containing radioactive metabolites were analyzed by radio gas chromatography using a Varian 2700 GC equipped with a flame ionization detector and a Berthold RGC 170 radiogas detector. The separation was carried out on a 6 ft glass column packed with 3% OV 225 on Gas Chrom Q, 100/120 mesh. Carrier gas He 30 ml/min. Temperatures: column oven 80—270° with 4° C/min, injector 250° C, detector 270° C.

Results

Pharmacokinetics of [^{14}C] VDC after Oral Administration

In Table 1, the elimination pathways of [^{14}C] activity after a single oral application are compiled. The main portion of the [^{14}C] (42—53%), in a dose-range from 0.5 to 50.0 mg/kg, is recovered from the urine. Both, in urine and in feces, only the non-volatile radioactivity has been determined.

Table 1. Excretion of radioactivity following oral single-dose administration of [^{14}C] VDC to rats

Specimen	Time (h)	Dose		
		0.5 mg/kg	5.0 mg/kg	50.0 mg/kg
		% of administered activity		
Exhaled air				
VDC	0–72	1.26 ± 0.39	9.70 ± 1.26	16.47 ± 4.24
CO_2	0–72	13.64 ± 4.72	11.35 ± 2.65	6.13 ± 0.33
Total		14.90 ± 4.76	21.05 ± 3.69	22.60 ± 4.37
Urine	0– 6	18.40 ± 3.93	16.39 ± 2.20	4.76 ± 1.39
	6–12	14.59 ± 1.73	22.79 ± 1.87	12.83 ± 2.20
	12–24	7.97 ± 0.55	11.14 ± 2.25	19.65 ± 2.41
	24–48	1.99 ± 0.69	3.00 ± 1.52	4.06 ± 0.83
	48–72	0.60 ± 0.02	0.56 ± 0.36	0.81 ± 0.25
Total		43.55 ± 4.59	53.88 ± 4.14	42.11 ± 3.53
Feces	0–12	0.11 ± 0.07	1.68 ± 1.23	0.75 ± 0.48
	12–24	8.52 ± 2.68	6.24 ± 3.02	2.71 ± 1.17
	24–48	5.86 ± 0.52	6.24 ± 1.89	3.69 ± 0.47
	48–72	1.25 ± 0.47	0.38 ± 0.06	0.50 ± 0.18
Total		15.74 ± 3.32	14.54 ± 1.18	7.65 ± 1.47
Carcass and tissues		5.57 ± 0.60	2.83 ± 0.42	2.77 ± 0.34
Cage rinse		9.80 ± 3.13	7.51 ± 0.31	5.91 ± 0.79
Total		89.56 ± 3.74	99.81 ± 1.21	81.04 ± 3.90

In the exhaled portion, unchanged [^{14}C] VDC and $^{14}CO_2$ are identified. After application of 0.5 mg/kg, only 1.2% of the total radioactivity is exhaled as unchanged [^{14}C] VDC, 13.6% as $^{14}CO_2$. A reversal in the proportion of VDC to CO_2 has occurred after a dose of 50.0 mg/kg. In this case, about three times more VDC than CO_2 is excreted. The elimination of [^{14}C] VDC as a function of time after 0.5, 5.0, and 50.0 mg/kg is shown in Fig. 1. Figure 2 represents the biphasic pattern of $^{14}CO_2$ exhalation after a dose of 0.5 and 50.0 mg/kg over a period of 72 h. In Table 2 are the calculated elimination half-lives of radioactivity listed, excreted as [^{14}C] VDC, $^{14}CO_2$, and urinary activity. From both, figures (1 and 2) and tables (1 and 2), the biphasic elimination is evident at all dose levels by pulmonary and urinary excretion.

Isolation and Identification of Urinary Metabolites

The isolation of the main metabolites of VDC in the urine of rats is accomplished through the successive procedures of solvent extraction, ion exchange chromatography and thin layer chromatography. Finally the metabolites are separated and identified by means of combined gas chromatography and mass spectrometry. Thin layer chromatography has revealed 5 radioactive, clearly resolved zones (A, B, C, D, and E). The least polar fraction E (Rf 0.54, about 12% of the activity) on the

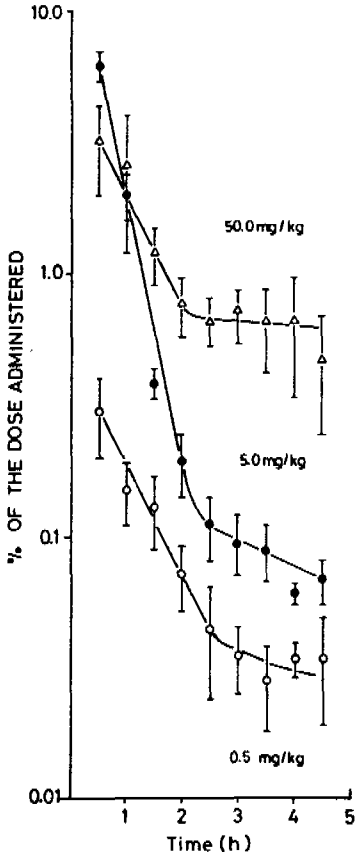

Fig. 1

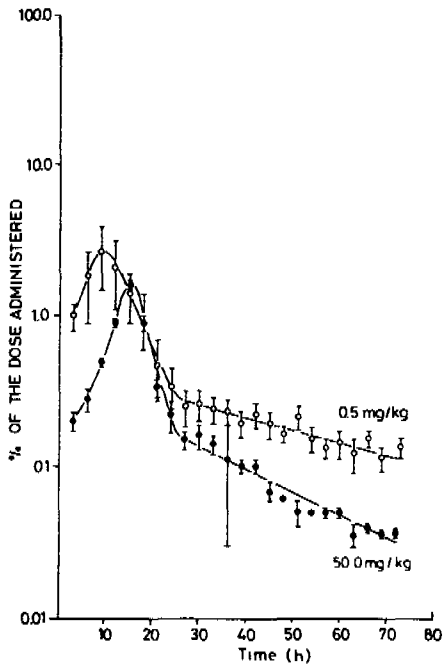

Fig. 2

Fig. 1. Expired [^{14}C] VDC expressed as percentage of the administered dose (0.5, 5.0, and 50.0 mg/kg) versus time (h). In this figure and Fig. 2 each value is the mean ± S.D. from three experiments

Fig. 2. Semilogarithmic concentration-time curves of $^{14}CO_2$ in the expired air following doses of 0.5 and 50.0 mg/kg [^{14}C] VDC orally

Table 2. Half lives of elimination of [^{14}C] VDC, $^{14}CO_2$ and activity in urine after oral administration of [^{14}C] VDC

Phase of elimination	Dose					
	0.5 mg/kg		5.0 mg/kg		50.0 mg/kg	
	Rapid	Slow	Rapid	Slow	Rapid	Slow
VDC	43 min	–	18 min	2 h 54 min	27 min	6 h 21 min
CO_2	4 h 30 min	35 h	4 h 45 min	24 h	2 h 45 min	20 h 45 min
Urine	4 h 30 min	18 h 30 min	3 h 45 min	14 h	5 h 15 min	25 h

plate has been scraped off and eluted with methanol. An initial analysis with GC/MS has indicated that the active material could be methylthio-acetylaminoethanol (MAAE). Therefore, we have synthesized this substance. The synthetic product and metabolic fraction E have shown identical retention times on columns with different stationary phases (SE 30 and OV 225). The mass spectra are in agreement with respect to the molecular ion and the typical fragments (Fig. 3). For further characterization, the metabolite and synthetic product were then converted into two differ-

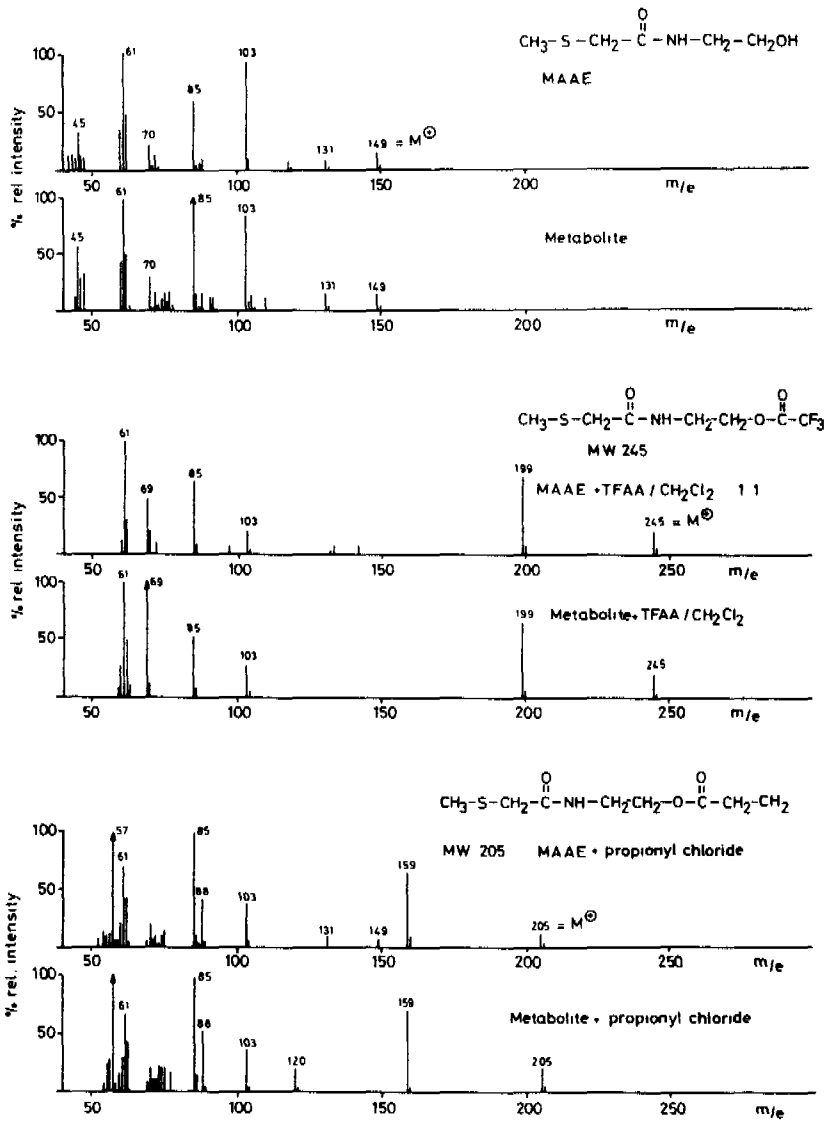

Fig. 3. Mass spectra of the metabolite in TLC zone E and synthesized methylthio-acetylaminoethanol. Trifluoroacetyl- and propionyl-derivatives of metabolite and authentic substance are compared

ent derivatives by esterification of the primary alcohol group with trifluoroacetic anhydride and propionyl chloride. The conversion has proceeded smoothly in both cases. The trifluoroacetyl and propionyl esters from the synthetic product and metabolic fraction E show the same retention times, and their mass spectra are identical (Fig. 3). This indicates that the fraction E metabolite is identical with MAAE.

The main activity (approx. 40%) on the thin layer plate is found in zone D (Rf 0.37). The column chromatographically purified acid fraction is converted to the butyl ester with n-butanol-3N HCl. Radio GC of this derivatized sample reveals a major peak cochromatographing with the dibutylester of authentic thiodiglycolic acid. Moreover, the mass spectrum of the butylated metabolite is identical with that of the reference compound, thus proving that the metabolite in fraction D is thiodiglycolic acid.

In addition to MAAE and to thiodiglycolic acid, three additional radioactive compounds were demonstrated by radio GC in the eluate of the ion exchange column after butylation. There is reason to suggest that one of the components might be a mercapturic acid. The mass spectra of one of the metabolites proved to be identical with that of the butyl ester of N-acetyl-S-(2-carboxymethyl)cysteine (Fig. 4). Cochromatography of the synthetic products with radioactive biological material has verified the identity of both substances. At this point it has been certain that N-acetyl-S-(2-carboxymethyl)cysteine is a metabolite of TLC zone A, B, or C. A comparison of the RF-value of the synthetic product and radioactive material from the thin layer plate is interpreted to mean that fraction B contains this mercapturic acid. A lack of agreement in retention time and mass spectrum has been found in a further comparison to other mercapturic acids (hydroxyethyl- and methyl-mercapturic acid).

The mass spectra of the remaining two urinary metabolites are shown in Fig. 5. The spectra imply that the metabolites may have closely related structures, because the type of fragmentation appears to be similar. The ion at m/e 57 and the difference of 100 between the ions with the highest mass (presumably the molecular ion) may indicate that the metabolites are mono- and dicarboxylic acids. However, no unequivocal structure could be assigned to these metabolites so far.

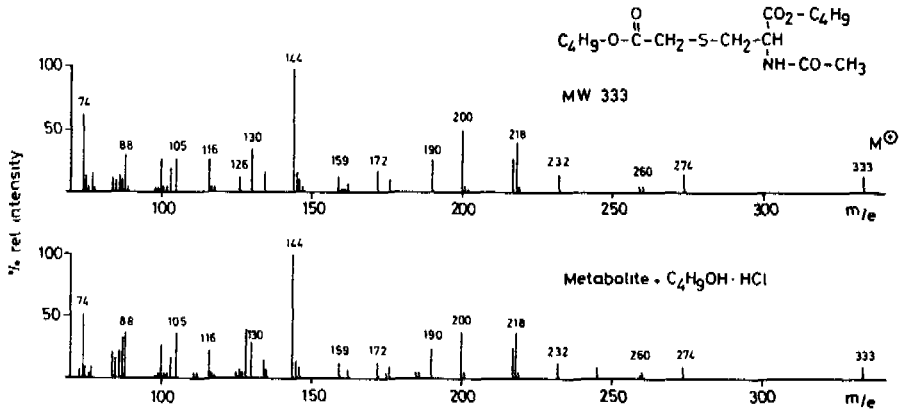

Fig. 4. Mass spectra of the butyl esters of N-acetyl-S-(2-carboxymethyl)cysteine and metabolite

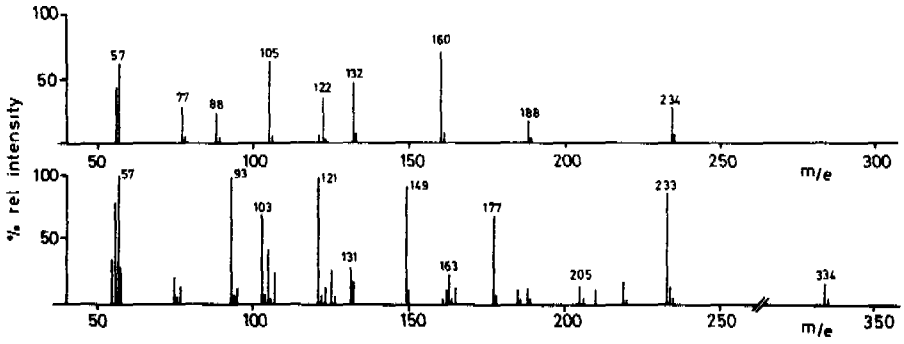

Fig. 5. Mass spectra of unidentified VDC metabolites after butylation

Discussion

The results of this investigation confirm the report by McKenna et al. (1978) concerning the pharmacokinetics of VDC after a single oral application in rats. In a dose range of 0.5—50.0 mg/kg the orally administered VDC is quickly and completely absorbed. This is substantiated on the one hand by the initial part of the pulmonary excretion of VDC and CO_2 and on the other hand through a rapid and extensive renal and biliary excretion of metabolic end products.

However, differences exist in the qualitative and quantitative interpretation of the metabolic end products of VDC metabolism. The reason for this disparity could stem from different analytical techniques. McKenna et al. (1978) have employed high pressure liquid chromatography, Jones and Hathway (1978) thin layer and gas chromatography.

Thiodiglycolic acid has been confirmed as the main metabolite in urine. Starting from chloroacetic acid as an intermediate in VDC metabolism, the formation of thiodiglycolic acid can be explained (Fig. 6): chloroacetic acid has been identified as a metabolite from VDC in liver tissue (Reichert and Bashti, 1976) and in urine (Jones and Hathway, 1978). Yllner (1970) has shown thiodiglycolic acid in the urine of mice after application of monochloroacetate.

Chloroacetic acid reacts with glutathione in an enzyme-catalyzed reaction, to form carboxymethyl glutathione (Fig. 6). Cleavage of the glycine and glutamine fragments yields carboxymethylcysteine. Apparently, this compound is preferentially deaminated and subsequently decarboxylated. The main metabolite thiodiglycolic acid is the end product of this reaction sequence. Up to now, the intermediate product, carboxymethylcysteine has not been shown in VDC metabolism. However, the identification of the N-acetyl derivative of this compound can be considered as indirect evidence. N-acetyl-S-(2-carboxymethyl)cysteine is the only mercapturic acid we have found in VDC metabolism. In our studies, there was no indication of the formation of the hydroxyethylmercapturic acid (McKenna et al., 1978). We cannot account for this pathway, which would involve a reductive dechlorination of VDC as a major step in metabolism.

The great importance of glutathione in the metabolization rate and the toxicity of VDC has been stressed previously (Reichert et al., 1978; Jaeger et al., 1974).

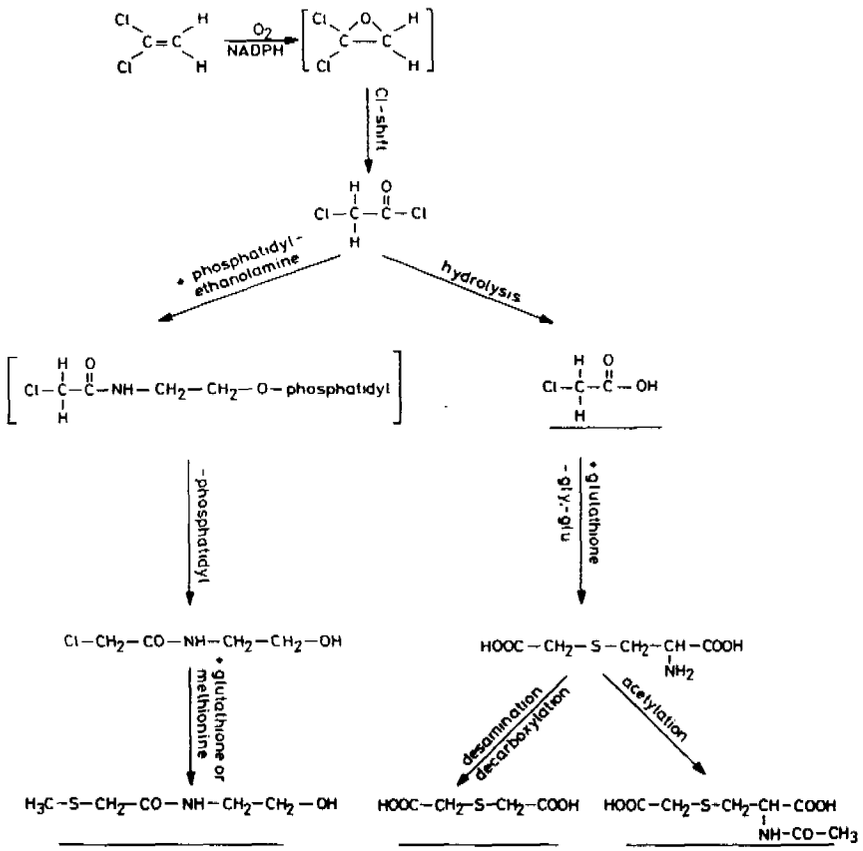

Fig. 6. Metabolic pathways of VDC. Identified metabolites underlined

However, in addition to the described reaction sequence involving glutathione conjugation and the elimination of S-containing end products, another totally different reaction mechanism must exist which leads to the formation of ethanolamine derivatives. The formation of this unusual metabolite is not yet clarified in detail. An initial step could be the formation of chloroacetic acid chloride (Fig. 6). This electrophilic intermediate product arises from the very unstable epoxide by an intramolecular chloride shift. The largest part is hydrolyzed to chloroacetic acid, which, as described above, conjugates with glutathione. A smaller portion, however, evidently remains bound to the membrane and can directly react there with phosphatidylethanolamine, an obligatory constituent of lipid membranes. The subsequent enzymatic cleavage of the phosphatidyl residue leads to the ethanolamine derivative of chloroacetic acid. Additional support for this metabolic pathway is the fact that chloroacetic acid chloride is electrophilic enough to react directly with nucleophiles, e.g., functional SH-groups (Reichert and Werner, unpublished). A similar mechanism has recently been postulated by Cohen et al. (1975) in halothane metabolism. They

presume that trifluoroacetic acid, a major halothane metabolite, can react directly with phosphatidylethanolamine. The enzymatic cleavage then results in N-trifluoroacetylethanolamine.

Now the question remains as to how the methylthio-group is introduced into the intermediary product chloroacetyl aminoethanol. Two ways are conceivable: direct nucleophilic attack of a methylthio-group (i.e., of methionine), or formation of a glutathione conjugate. To conclusively answer this question, further investigations are required. The increasing number of metabolites with the insertion of the methylthio-group on an electrophilic carbon atom have been compiled recently by Jenner and Testa (1978). They conclude from the present data that, in most cases, the direct nucleophilic attack is the probable pathway.

The results of this study show that the inactivation of VDC in vivo occurs by a large number of reactions, some of which could be demonstrated. The identification of the main metabolites from VDC in rats leads to an improved understanding of the toxic properties of this substance. Consequently, the covalent binding of chloroacetic acid chloride on membrane constituents (i.e., phosphatidylethanolamine) could explain, in part, the parenchyma damaging effect of VDC.

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