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THE METABOLISM OF ^{36}Cl-LABELLED TRICHLOROETHYLENE AND TETRACHLOROETHYLENE IN THE RAT

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Abstract—^{36}Cl-labelled tri- and tetrachloroethylene were synthesized and fed to rats. The partition of radioactivity in urine, faeces and expired air was subsequently measured and the nature and amount of individual metabolites determined. The specific activities of metabolic trichloroacetic acid and trichloroethanol were shown to be the same as that of the administered trichloroethylene thus demonstrating an intramolecular re-arrangement of chloride. About 15 per cent of the dose of trichloroethylene was excreted in the urine. Tetrachloroethylene underwent little metabolism and only 2 per cent of the radioactivity was excreted in the urine. Both trichloroethylene and tetrachloroethylene are largely excreted through the lungs although there is an appreciable difference between the rates of excretion of the two compounds. Tetrachloroethylene has no effect on the liver–lipid content of rats exposed to high concentrations (1000 p.p.m.) of the vapour. A scheme is presented which would account for the formation of the known metabolites of both tri- and tetrachloroethylene.

THE chlorinated hydrocarbons trichloroethylene and tetrachloroethylene are important industrial solvents especially for dry-cleaning and degreasing. They possess the advantage of non-inflammability and low toxicity. The metabolism of trichloroethylene has been studied both in man and in a variety of experimental animals while the metabolism of tetrachloroethylene has received but little attention and published results are conflicting. It is well known that trichloroethylene is excreted in the urine as trichloroacetic acid and trichloroethanol in all species of experimental animals studied while, in addition to these compounds, monochloroacetic acid is also reported to be a urinary metabolite in man. This information has been adequately reviewed by Defalque.[1] Despite the large amount of published work no adequate balance sheet has been presented for the excretion of trichloroethylene and its metabolites in urine, faeces and expired air. A further point of major importance which has not been elucidated is the nature of the re-arrangement which results in the formation of 2,2,2-trichloroethanol and trichloroacetic acid from 1,1,2-trichloroethylene. This may involve an exchange of a chloride-ion with the body chloride-pool or may be an intra-molecular re-arrangement.

Laham,[2] using a combination of gas- and paper-chromatographic techniques, identified di- and tri- chloroacetic acids and the corresponding alcohols in the urine of rats dosed orally with tetrachloroethylene (1 g/kg). Yllner[3] exposed mice to the vapour of ^{14}C-tetrachloroethylene and subsequently recognised trichloroacetic acid and oxalic

acid in the urine. These conflicting observations may in part be due to the different methods of dosage employed.

The metabolism of trichloroethylene and tetrachloroethylene, both uniformly labelled with ^{36}Cl, have been studied in the rat in an attempt to resolve some of the outstanding problems. In addition the effect of exposure to the vapour of tetrachloroethylene on the lipids of rat liver has been investigated following a report[4] that exposure of mice to tetrachloroethylene vapour (400 p.p.m.) produced fatty-infiltration of the liver.

MATERIALS AND METHODS

Synthesis of ^{36}Cl- labelled trichloroethylene and tetrachloroethylene

^{36}Cl- Hydrochloric acid (100 μc) was converted into AgCl which was subsequently oxidized by heating with a sulphuric acid–phosphoric acid–CrO_3 mixture.[5] The ^{36}Cl- chlorine gas so produced was allowed to react with an excess of acetylene for 60 min at 20° at 0·5 atm and the tetrachloroethane collected by vacuum distillation. The tetrachloroethane (1·47 mmole) was dehydrochlorinated with alcoholic-KOH[6] and the product of 1,1,2-trichloroethylene was separated and purified by gas-chromatography at 50° on a 2-m column of Celite (60–80 mesh; Johns-Manville Ltd). containing 25% (w/w) silicone grease (Dow-Corning Ltd.) as liquid-phase. The product was diluted with inactive trichloroethlene to a specific activity of 0·27 μc/mmole.

^{36}Cl- trichloroethylene (15 mmole) was allowed to react for 36 hr at 20° with an excess of ^{36}Cl- chlorine gas in the presence of anhydrous $FeCl_3$ (0·2 mmole). The pentachloroethane was collected, dehydrochlorinated and the product of ^{36}Cl-tetrachloroethylene purified by gas-chromatography as described above [^{36}Cl-C_2Cl_4] requires C, 14·3; Cl, 85·7; found C, 14·4; Cl, 85·4]. It was diluted with inactive tetrachloroethylene to a specific activity of 1·3 μc/mmole.

Preparation of intermediates

2,2,2-Trichloroethyl-β-D-glucosiduronic acid (Na^+ salt) was isolated from the urine of dogs dosed with 2,2,2-trichloroethanol (700 mg/kg) (Imperial Chemical Industries Ltd.; General Chemicals Division, Widnes) as described by Külz.[7] Methyl 2,2,2-trichloro-(tri-*o*-acetyl-β-D-glucosid)-uronate (m.p. 154·5°) was prepared according to the method of Smith and Williams.[8]

Animal studies

Wistar rats from a closed colony, randomly mated, (180–220 g) were dosed with trichloroethylene and tetrachloroethylene respectively by stomach-tube. Animals were then transferred to individual glass metabolism cages which permitted collection of urine, faeces and expired air.[9] In order to collect the radioactivity in the expired air the metabolism cage was vented by a current of air (500 ml/min). The air then passed through a vertical glass column (30 × 300 mm) which was loosely packed with stainless-steel rings through which toluene percolated from a reservoir at 30 ml/hr. Chlorinated material was readily extracted into the toluene which was collected in fractions each of 1 hr until no further radioactivity was detected. While this was a convenient procedure for extracting the radioactivity from the expired air it was not possible to concentrate the fractions so as to allow analysis by gas chromatography. To achieve this purpose the expired air from dosed animals was drawn through two

absorbers containing anhydrous $CaCl_2$ and soda-lime respectively. It was then drawn through two traps each containing $\frac{3}{16}$ in. Lessing-rings kept at the temperature of liquid-air. The chlorinated metabolites were retained in the traps under these conditions and were subsequently recovered from the traps by vacuum distillation. The distillate was then examined by gas chromatography at 50° on 1-m column of Celite (60–80 mesh) containing 28·4 per cent (w/w) Reoplex 400 as liquid-phase and a Katharometer as detector.

Quantitative analysis

Radioactivity was measured in a liquid Geiger–Müller tube (Mullard MX 124) at 420 V. Urine and expired air fractions were counted directly. A 30% (w/v) homogenate of faeces was prepared in water and the homogenate counted directly.

Estimation of trichloroacetic acid

Trichloroacetic acid was determined in the urine using a modification of the Fujiwara procedure. Urine (1 ml), containing not more than 200 μg trichloroacetic acid was added to 25% NaOH (16 ml) in a test-tube fitted with a ground-glass stopper. The contents of the tube were heated, with frequent shaking, on a water-bath at 60° ± 1° for 15 min. When cool the contents of the tube were transferred to a separating funnel and the lower aqueous layer discarded. The red-coloured pyridine layer was collected, diluted to 10 ml with ethanol and the extinction measured in either 10 mm or 40 mm glass cells at 520 mμ in a suitable spectrophometer. Under these conditions 2,2,2-trichloroethanol gives a yellow colour (λ_{max} 412 mμ; ϵ_{max} 8000). This colour does not interfere with the procedure described for the estimation of trichloroacetic acid. A standard of trichloroacetic acid was included with each estimation since the colour developed varies appreciably with the temperature of the water-bath.

Trichloroethylglucosiduronic acid. This was estimated as trichloroethanol after hydrolysis of the urine either with mineral acid or with β-glucronidase. Optimum conditions for acid hydrolysis were 15 min at 100° with 3 N HCl. Since prolonged heating with acid results in destruction of the liberated trichloroethanol enzymic hydrolysis is to be preferred for quantitative work. Urine (1 ml) was incubated for 18 hr at 37° with a β-glucuronidase preparation[10] in 0·2 M acetate buffer (1 ml; pH 4·5). The liberated trichloroethanol was separated by steam-distillation until a 10 ml-vol. of distillate had been collected. The distillate (2 ml) was heated in a stoppered-tube at 100° for 2 min with NaOH (0·33 N: 1 ml) and the liberated formaldehyde estimated using the acetylacetone reagent described by Nash.[11]

Mercapturic acid excretion. Estimated using the iodometric procedure of Stekol.[12]

Qualitative analysis

Trichloroethylene. The combined urines (48 hr) of 6 rats given ^{36}Cl-trichloroethylene (4·6 μc) were pooled, acidified with HCl and extracted continuously with ether for 168 hr. During the first 4 hr Fujiwara-positive reacting material was extracted from the urine (Fraction A). At this stage therefore the solvent was separated, fresh solvent was added and the extraction continued until the bulk of the radioactivity in the urine had been removed (168 hr). This second fraction (Fraction B) was free of Fujiwara reacting material. A portion (20 ml) of the residual urine was treated with N $AgNO_3$ and the precipitate collected, washed with water and dissolved in ammonia

(sp. gr. 0·90). No radioactivity was detected in this ammoniacal solution. Fraction B was concentrated *in vacuo* and the residue redissolved in 0·5 N acetate buffer (pH 4·5). A saturated solution of normal lead acetate (0·5 vol.) was added and the precipitate separated on the centrifuge. The residue was washed twice with water on the centrifuge and the washings added to the supernatant which was then adjusted to pH 8 by the drop-wise addition of ammonia (sp. gr. 0·90). The precipitate that formed was collected on the centrifuge, washed three times with water, suspended in water and gassed with H_2S. The PbS was removed by filtration and the filtrate concentrated *in vacuo*. The residue was dissolved in methanol and filtered free of insoluble material. The filtrate was then treated with an excess of diazomethane in ether and allowed to stand overnight at room temperature. The solvent was distilled and the process of methylation repeated. The gummy residue of the methyl-ester was dissolved in pyridine (5 ml) and acetic anhydride (5 ml) added. After standing for 18 hr at room temperature the solution was poured into water and the solid which separated was collected and recrystallized, to constant specific activity, from aqueous ethanol. The material was identified as methyl 2,2,2-trichloroethyl-(tri-*o*-acetyl-*β*-D-glucosid)-uronate (m.p. and mixed m.p. 154–155°; $[\alpha]_D$ − 38·6° (c. 1·1 in EtOH) [Smith and Williams,[8] quote m.p. 158° $[\alpha]_D$ − 37° (c. 1·0 in $CHCl_3$): Seto and Schultze[13] quote m.p. 157–158° $[\alpha]_D$ − 40·7° (c. 1·1 in $CHCl_3$)] $C_{15}H_9O_{10}{}^{36}Cl_3$ requires ^{36}Cl 23·11; found ^{36}Cl 22·7. The specific activity of this material was 0·31 μc/mmole.

Fraction A contained both trichloroacetic acid and the normal urinary ether-soluble acids. It was shaken with Na_2CO_3 (2 N) and the ether layer discarded. The aqueous solution was adjusted to pH 4 with 70% H_3PO_4 and extracted for 18 hr with ether. This procedure removes a considerable amount of non-radioactive ether-soluble material which would otherwise interefere with subsequent procedures. No significant amount of radioactivity was extracted. The aqueous solution was then acidified with conc. HCl and extracted for 4 hr with ether. The ethereal extract was concentrated to a small volume (5 ml) and treated with excess of an ethereal solution of diazomethane. Gas chromatography of the mixture of the methyl-esters thus obtained was carried out on a 2-m column of Celite (60–80 mesh) containing 30% by weight of di-n-decylphthalate as liquid phase operating at 105° with nitrogen (70 ml/min) as carrier gas. Two peaks were obtained which were separately collected in traps cooled in liquid-N_2. The contents of both traps were weighed, dissolved in 10 ml toluene and counted. Only one fraction was radioactive and corresponded to that peak given to methyl trichloroacetate. The material isolated (7 mg) had a specific activity of 0·29 μc/mmole.

^{36}Cl-Trichloroethylene (0·6 μc) was administered to a rat and urine was collected for 72 hr. To the urine (10 ml) was added trichloroacetic acid (533 mg) and conc. HCl (5 ml). The solution was refluxed for 30 min and the urine was extracted with ether. The residue, after evaporation of the solvent, was treated with *p*-chlorobenzyl-pseudothiuronium chloride.[14] The resulting solid, *p*-chlorobenzylpseudothiuronium trichloroacetate (m.p. 150°) was recrystallized from dioxane to constant specific activity and corresponded to 1·7 per cent of the dose of administered trichloroethylene. No activity was found when mono- and di-chloroacetic acids were separately added to urine (10 ml) and the above procedure carried out.

^{36}Cl-Tetrachloroethylene. Trichloroacetic acid (530 mg) was added to urine (20 ml) of rats which had received ^{36}Cl-tetrachloroethylene (2 μc) by stomach tube. The urine

was then acidified with conc. HCl and extracted continuously with ether for 4 hr. The ether was separated and distilled in a current of nitrogen. The residue (1·02 g) was dissolved in ethanol (5 ml) and treated with a solution of *p*-chlorobenzylpseudothiuronium chloride (1 g) in ethanol (5 ml). The solid which separated was collected and recrystallized from aqueous ethanol to constant specific activity and melting point (150°), and corresponded to 0·6 per cent of the dose of the administered tetrachloroethylene.

This procedure was subsequently repeated after the addition of monochloroacetic and dichloroacetic acid respectively. The products were not radioactive.

NaCl (20 mg) was added to a portion of the urine (20 ml), the solution acidified with a few drops of conc. HNO_3 and treated with 0·1 N $AgNO_3$ (3 ml). The precipitate was collected on the centrifuge, washed three times with water, dried and weighed. It was then dissolved in ammonia (sp. gr. 0·90) and the radioactivity determined. The AgCl was reprecipitated by the addition of acid and the cycle repeated until the precipitated material was of constant activity. It had previously been shown that trichloroacetic acid is not precipitated from urine under these conditions.

In order to determine whether any trichloroethanol was present the urine (100 ml) was adjusted to pH 4·5 with acetate buffer (0·1 M) and incubated for 18 hr with β-glucuronidase. Volatile metabolites were separated by steam-distillation and the amount of radioactivity in the distillate determined. Only background activity was obtained indicating the absence of chlorinated alcohols.

The effect of tetrachloroethylene vapour on liver lipids. Animals were exposed to the vapour of tetrachloroethylene (1000 p.p.m.) for three successive periods each of 6 hr. Animals had ready access to food and water during pre- and post-exposure periods. Atmospheres were prepared as described by Gage.[15] The animals were killed, the livers removed and extracted for 6 hr with 25 ml of a mixture of alcohol–ether (3:1). The dried, defatted tissue was then separated and weighed. The solvent mixture was distilled, the residue weighed and the results expressed as mg lipid per 100 mg dry weight of liver.

RESULTS AND DISCUSSION

The distribution of radioactivity in the urine, faeces and expired air of rats dosed with ^{36}Cl-trichloroethylene and ^{36}Cl-tetrachloroethylene is shown in the accompanying tables (Tables 1 and 2). Both compounds are largely excreted through the lungs, the excretion following an approximately exponential course. Trichloroethylene (half-time of expiration 5 hr) is more rapidly eliminated than tetrachloroethylene (half-time of expiration 8 hr). There was no evidence from analysis of the expired air that any

TABLE 1. THE EXCRETION OF RADIOACTIVITY IN EXPIRED AIR, URINE AND FAECES AFTER THE ORAL ADMINISTRATION OF ^{36}Cl-TRICHLOROETHYLENE

Dose (μc)	Radioactivity (%) in			Total
	Expired air	Urine	Faeces	Radioactivity (%)
8·6	84·8	10·7	0·5	96·0
7·5	82·3	13·8	0	96·1
3·7	—	11·3	0	[11·3]
4·0	72·1	20·6	0	92·7

TABLE 2. THE EXCRETION OF RADIOACTIVITY IN EXPIRED AIR, URINE AND FAECES AFTER THE ADMINISTRATION OF ^{36}Cl-TETRACHLOROETHYLENE

Dose (μc)	No of animals	Radioactivity (%) in Expired air	Urine	Faeces
1·75	1	97·9*	2·1†	0
13	4	—	1·6†	0

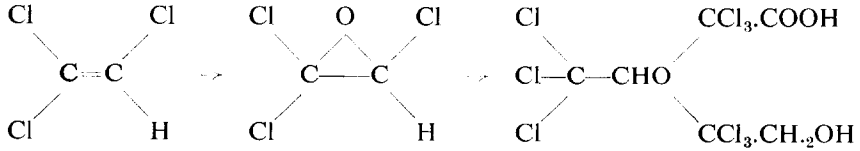
* 48 hr.
† 18 days.

metabolite of either compound was present, although the presence of chloroform in the expired air of men exposed to the vapour of trichloroethylene[16] has been reported. In a single experiment a small amount of (1–2%) of *trans* 1,2-dichloroethylene was identified in the expired air of a rat dosed with trichloroethylene. This observation could not be confirmed in subsequent experiments and the result was thought to be due to traces of impurities in the trichloroethylene. Quantitatively the urinary metabolites of trichloroethylene (10–20%) in the rat are due to trichloroacetic acid (1–5%) and trichloroethanol (10–15%). No monochloroacetic acid was detected in the urine.

Tetrachloroethylene undergoes little metabolism and only about 2 per cent of the radioactivity is excreted in the urine. Trichloroacetic acid (0·6%) and inorganic chloride were the only metabolites detected. About 25 per cent of the total radioactivity in the urine was precipitated as chloride on the addition of silver nitrate solution. The radioactivity remaining in the urine could be accounted for as trichloroacetic acid. Little oxalic acid could, therefore, be present since there was a 1:1 ratio between trichloroacetic acid and chloride ion. These results are in partial accord with those described by Yllner for the metabolism of ^{14}C-tetrachloroethylene in mice.

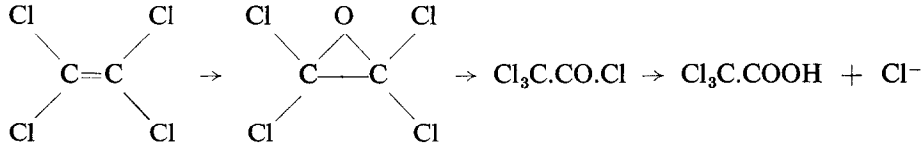
Radioactivity is excreted in the urine for upwards of 18 days after the administration of both trichloroethylene and tetrachloroethylene. It is well known that trichloroacetic acid is slowly eliminated from the body[17] after exposure to trichloroethylene. The excretion of radioactivity after dosing with trichloroethylene corresponds closely to the excretion of trichloroacetic acid in rats dosed orally with sodium trichloroacetate (unpublished experiments). It may be assumed that the radioactivity after dosing with tetrachloroethylene is due to the excretion of both trichloroacetic acid and of chloride ion since the latter has a biologic half-life of 29 days. It is certain that 70–80 per cent of the radioactivity excreted during the third week is precipitated as inorganic chloride.

The specific activities of both trichloroacetic acid (0·29 μc/mmole) and trichloroethanol (0·31 μc/mmole) isolated from the urine after dosing with trichloroethylene were approximately equal to that of the administered trichloroethylene (0·27 μc/mmole). This indicates that there is an intra-molecular rearrangement of trichloroethylene and no exchange of chloride with the body chloride pool. A reaction mechanism which may reasonably be expected to operate *in vivo* and which would account for the known metabolites of trichloroethylene would be,



The formation of an hypothetical oxide was originally postulated by Powell.[18] Trichloroethylene oxide is thought to be formed *in vitro* when trichloroethylene is oxygenated in the presence of actinic radiation. (British Patent 523, 55; 1940). Rearrangement of the oxide would give trichloroacetaldehyde the formation of which, in men exposed to trichloroethylene vapour, has been quantitatively determined.[19] Chloral appeared in the blood within 30 min of exposure but subsequently underwent rapid metabolism. The oxidation of chloral to trichloroacetic acid may be carried out by an enzyme present in the liver of a variety of experimental animals.[20] Reduction of chloral to trichloroethanol would involve alcohol dehydrogenase. That trichloroethanol is not the precursor of trichloroacetic acid follows from the observation that little trichloroacetic acid (0·3%) is formed when trichloroethanol (300 mg/kg) is administered orally to rats (unpublished results). The formation of trichloroethylene oxide *in vivo* is probably due to a non-specific enzyme system. Bartoniçek and Teisinger[21] have shown that the oxidation of trichloroethylene in man is inhibited by tetraethylthiuram disulphide (Disulfiram). This was demonstrated by a considerably increased excretion of trichloroethylene through the lungs. The oxidation is not however inhibited by SKF-acid (50 mg/kg) since the excretion of radioactivity and of metabolites of trichloroethylene in the urine is the same both in control animals and in those animals treated with SKF-acid 60 min before dosing.

The metabolism of tetrachloroethylene may involve the following series of reactions,



The acid chloride would be rapidly hydrolysed to trichloroacetic acid[22] and neither trichloroethanol or oxalic acid would be formed.

There was no increase in the urinary excretion of mercapturic acid following the administration of either trichloroethylene or tetrachloroethylene.

The absence of any effect of tetrachloroethylene on the liver-lipid content in rats (Table 3) is contrary to that reported in mice[4] but is consistent with the low mammalian toxicity of tetrachloroethylene.

TABLE 3. THE EFFECT OF EXPOSURE OF RATS TO THE VAPOUR OF TETRACHLOROETHYLENE (1000 p.p.m.) ON THE LIVER-LIPID CONTENT

No. of animals	Sex	Series	mg lipid/100 mg dry wt. liver
7	male	control	11·2 ± 1·4
7	male	test	11·3 ± 2·2
7	female	control	10·7 ± 2·2
7	female	test	8·0 ± 1·5

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