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Studies in Detoxication

66. THE METABOLISM OF HALOGENOBENZENES. 1:2:3-, 1:2:4- AND 1:3:5-TRICHLOROBENZENES*

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The trichlorobenzenes are decomposition products of the insecticide benzene hexachloride (gammexane). On treatment with alkali, the α-, β-, γ- and δ-isomers of benzene hexachloride are dehydrochlorinated to yield a mixture of the three isomeric trichlorobenzenes in which the 1:2:4-isomer predominates and amounts to about 80% of the total (cf. Hughes, Ingold & Pasternak, 1953). Benzene hexachloride also decomposes under field conditions, and it has been shown that the deleterious effects of crude benzene hexachloride on wheat seedlings and coniferous seedlings may be due to trichlorobenzenes, the 1:2:4-isomer being the most active towards wheat and the 1:3:5-isomer towards coniferous seedlings (Hocking, 1950; Simkover & Shenefelt, 1952). Benzene hexachloride is also decomposed in animals and insects, but nothing is known about the nature of the metabolites. However, it is known that the different isomers of benzene hexachloride are metabolized at different rates in mice and in flies (Asperen, 1954; Oppenoorth, 1954, 1955). When the common isomers of benzene hexachloride are fed to rats over a long period, the foreign material which accumulates in the tissues, particularly the fat, does not contain 1:2:4-trichlorobenzene, but consists of unchanged benzene hexachloride (Davidow & Frawley, 1951).

The present work was undertaken with the view in mind that benzene hexachloride may be converted into trichlorobenzenes *in vivo* and that these may be further metabolized to phenolic substances. A knowledge of the metabolism of the trichlorobenzenes could thus be useful in elucidating that of benzene hexachloride. The trichlorobenzenes themselves have been used as pesticides and they are also employed in industry for various purposes such as the manufacture of electrical insulating material. From a toxicological point of view, the trichlorobenzenes are less toxic to small laboratory animals than chlorobenzene and *o*-dichlorobenzene (Cameron *et al.* 1937), and this may be correlated with the extent of mercapturic acid formation, which diminishes as the number of chlorine atoms in these compounds increases.

The metabolism of the isomeric dichlorobenzenes in rabbits has already been described (Azouz, Parke & Williams, 1955; Parke & Williams, 1955).

* Part 65: Smith & Williams (1955).

EXPERIMENTAL

Materials and reference compounds

1:2:3-Trichlorobenzene, m.p. 53°, was purchased (L. Light and Co., Colnbrook, Bucks.); 1:2:4-trichlorobenzene, m.p. 17°, and 1:3:5-trichlorobenzene, m.p. 61°, were prepared according to Holleman (1918).

The following compounds were prepared by standard methods (m.p.'s are corrected; references to the known compounds are given by Huntress (1948)).

Compounds related to 1:2:3-*trichlorobenzene.* 2:3:4-Trichlorophenol, m.p. 81°, and benzoate, m.p. 142°; and 3:4:5-trichlorophenol, m.p. 98°, and benzoate, m.p. 119°, were prepared.

3:4:5-Trichlorocatechol was synthesized as follows. 2:3:4-Trichlorophenol was converted into 3:4:5-*trichlorosalicylaldehyde* as described by Duff (1941) for the preparation of *o*-hydroxyaldehydes. The aldehyde (yield 20%), m.p. 87°, formed yellow needles from aqueous ethanol. (Found: C, 37·4; H, 1·6; Cl, 47·0. $C_7H_3O_2Cl_3$ requires C, 37·3; H, 1·3; Cl, 47·2%.) The *semicarbazone*, m.p. 236°, formed pale-yellow needles from aqueous ethanol. (Found: C, 34·1; H, 2·4; Cl, 37·7. $C_8H_6O_2N_3Cl_3$ requires C, 34·0; H, 2·1; Cl, 37·7%.) The aldehyde in N-NaOH was treated with H_2O_2, as described by Dakin (1909) for the oxidation of *o*-hydroxyaldehydes, to yield 3:4:5-trichlorocatechol (yield

Table 1. *Synthetic hexachlorodiphenyl disulphides*

Trichloroaniline* used	Hexachlorodiphenyl disulphide isolated: Isomer	M.p.	Analysis (found): C	H	Cl
2:3:4-	2:3:4:2′:3′:4′-	185°	34·0	0·9	50·4
3:4:5-	3:4:5:3′:4′:5′-	152°	34·1	1·1	50·2
2:3:5-	2:3:5:2′:3′:5′-	168°	34·2	1·0	50·0
2:4:5-	2:4:5:2′:4′:5′-	136–137°	34·0	1·1	50·3
2:4:6-	2:4:6:2′:4′:6′-†	173°	33·8	0·9	49·9
	($C_{12}H_4Cl_6S_2$ requires		33·9	0·95	50·1)

* See text.

† This compound has previously been described by Baddeley & Bennett (1933), who record m.p. 165°.

80%), m.p. 105° [Huntress (1948) gives m.p. 106–109° and the compound is described as the hemihydrate], which was chromatographically pure. Its *dibenzoate*, m.p. 114–115°, formed colourless needles from aqueous ethanol. (Found: C, 57·1; H, 2·65; Cl, 25·3. $C_{20}H_{11}O_4Cl_3$ requires C, 57·0; H, 2·6; Cl, 25·2%.)

Compounds related to 1:2:4-*trichlorobenzene.* 2:3:5-Trichlorophenol, m.p. 62°, and its benzoate, m.p. 103°; 2:3:6-trichlorophenol, m.p. 58°, and its benzoate, m.p. 90°; 2:4:5-trichlorophenol, m.p. 68°, and its benzoate, m.p. 92°; and 2:3:5-trichloroquinol, m.p. 136°, were prepared.

3:4:6-Trichlorocatechol was synthesized. 2:4:5-Trichlorophenol was converted into 3:4:6-*trichlorosalicylaldehyde* as described above. The aldehyde formed yellow needles, m.p. 116°, from aqueous ethanol; yield 20%. (Found: C, 37·3; H, 1·5; Cl, 46·8%.) The *semicarbazone* was obtained as yellow needles, m.p. 244°, from aqueous ethanol. (Found C, 33·8; H, 1·9; Cl, 37·4%.) This aldehyde was oxidized to 3:4:6-*trichlorocatechol* (yield 80%) as above. It formed white needles of the hemihydrate, m.p. 92°, from water. (Found: C, 32·7; H, 2·1; Cl, 47·7. $C_6H_3O_2Cl_3$,0·5H_2O requires C, 32·3; H, 1·8; Cl, 47·8%.)

Compounds related to 1:3:5-*trichlorobenzene.* 2:4:6-Trichlorophenol, m.p. 67°, and its benzoate, m.p. 74°; 2:4:6-trichlororesorcinol, m.p. 83°; and 2:4:6-trichlorophloroglucinol, m.p. 134°, were prepared.

Synthesis of hexachlorodiphenyl disulphides. A cold mixture of sulphuric acid (8 ml.) and water (8 ml.) was added to a solution of the appropriate trichloroaniline (2 g.) in glacial acetic acid (4 ml.) and the whole cooled to 0°. The aniline was diazotized by gradual addition of solid sodium nitrite (1·4 g.). Ethyl potassium xanthate (2 g.) was slowly added in small quantities. The mixture turned yellow and a solid separated. On keeping in the ice bath, which was allowed to reach room temperature gradually, the solid decomposed with evolution of nitrogen. The oil which formed was separated, dissolved in ether and freed from phenol by washing with 2N-NaOH. The ethereal solution was then washed with 2N-H_2SO_4 and finally with water. The ether was evaporated and the oily residue dissolved in ethanol (20 ml.) and refluxed with solid KOH (0·7 g.) for 10–20 min. The ethanol was distilled off and the residue acidified with 2N-H_2SO_4, to liberate the trichlorothiophenol, which was steam-distilled and obtained as a waxy solid from the distillate by filtration. The thiophenol was dissolved in ethanol (10–15 ml.) and titrated with a 5% solution of iodine in ethanol. The *hexachlorodiphenyl disulphides* (0·4 g.) separated as white crystalline solids which were purified by recrystallization from aqueous ethanol or ethanol–ethyl acetate mixtures. Five of these disulphides were synthesized (see Table 1) and they crystallized as colourless needles, almost insoluble in water, sparingly soluble in cold ethanol but more soluble in hot ethanol.

Methods

Animals. Chinchilla rabbits, kept on a diet of 60 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water per day, were used throughout this work. The trichlorobenzenes were administered by stomach tube as 25% (w/v) solutions in arachis oil. Urine was collected daily.

Analytical methods. The urine was analysed daily for glucuronic acid by the modified naphthoresorcinol method of Paul (1951), ethereal sulphate by the turbidimetric method of Sperber (1948), and mercapturic acid by the iodine-titration method of Stekol (1936).

Determination of trichlorophenols. The total trichlorophenol was estimated spectrophotometrically by determination of the difference between the acid and alkaline spectra of steam distillates of the acid-hydrolysed urines as described by Azouz *et al.* (1955) for dichlorophenols. Chromatography of the phenolic metabolites of 1:2:3-trichlorobenzene showed that, compared with 2:3:4-trichlorophenol, the 3:4:5-isomer was a relatively minor constituent, and therefore the total phenol was estimated at 246 mμ. and calculated as 2:3:4-trichlorophenol. The spectra in acid and alkali of 2:3:4- and 3:4:5-trichlorophenols are shown in Fig. 1 (for 2:3:4-trichlorophenol ϵ at 246 mμ. is 7100 in 0·1N-NaOH and 1100 in 0·1N-HCl and for 3:4:5-trichlorophenol it is 10 700 in 0·1N-NaOH and 1050 in 0·1N-HCl). Recoveries of 2:3:4- and 3:4:5-trichlorophenols from normal rabbit urine at concentrations of 25 mg./100 ml. were 93±5% and 97±5% respectively.

Chromatography of the phenolic metabolites of 1:2:4-trichlorobenzene showed that only 2:3:5- and 2:4:5-trichlorophenol were present, in approximately equal amounts (see below), and the total phenol was estimated at 244 mμ. and calculated as 2:4:5-trichlorophenol. The spectra in acid and alkali of 2:3:5- and 2:4:5-trichlorophenols are shown in Fig. 2 (for 2:3:5-trichlorophenol ϵ at 244 mμ. is 7250 in 0·1N-NaOH and 1100 in 0·1N-HCl and for 2:4:5-trichlorophenol it is 8900 in 0·1N-NaOH and 1100 in 0·1N-HCl). Recoveries of 2:3:5- and 2:4:5-trichlorophenols from normal rabbit urine at concentrations of 25 mg./100 ml. were 95±5% and 100±5% respectively.

2:4:6-Trichlorophenol, the only monophenol obtained from 1:3:5-trichlorobenzene, was estimated at 244 mμ. The spectra of this phenol in acid and alkali are shown in Fig. 3 (ε at 244 mμ. is 8950 in 0·1 N-NaOH and 1050 in 0·1 N-HCl). Recovery of 2:4:6-trichlorophenol from normal rabbit urine at a concentration of 25 mg./100 ml. was 95±5%. The recoveries of the trichlorophenols (25 mg./100 ml.) from normal rabbit urine were completely unaffected by the addition of 25–75 mg. of glucurone/100 ml. of urine.

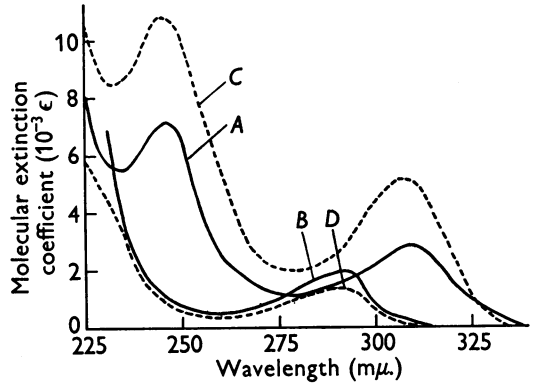

Fig. 1. Spectra of 2:3:4- and 3:4:5-trichlorophenols. *A*, 2:3:4-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 246, 309 mμ.; $\epsilon_{max.}$ 7100, 2800) and, *B*, in 0·1 N-HCl ($\lambda_{max.}$ 293 mμ.; $\epsilon_{max.}$ 1950); *C*, 3:4:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 243, 307 mμ.; $\epsilon_{max.}$ 10 800, 5050) and, *D*, in 0·1 N-HCl ($\lambda_{max.}$ 290 mμ.; $\epsilon_{max.}$ 1300).

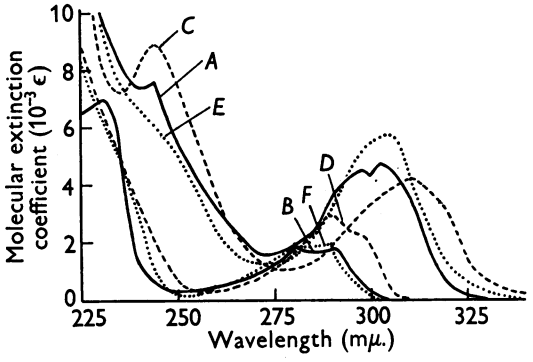

Fig. 2. Spectra of 2:3:5-, 2:4:5- and 2:3:6-trichlorophenols. *A*, 2:3:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 298, 302 mμ.; $\epsilon_{max.}$ 7250, 4450, 4700) and, *B*, in 0·1 N-HCl ($\lambda_{max.}$ 280, 290 mμ.; $\epsilon_{max.}$ 1900, 1900); *C*, 2:4:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 310 mμ.; $\epsilon_{max.}$ 8900, 4200) and, *D*, in 0·1 N-HCl ($\lambda_{max.}$ 290 mμ.; $\epsilon_{max.}$ 2900); *E*, 2:3:6-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 304 mμ.; $\epsilon_{max.}$ 5650) and, *F*, in 0·1 N-HCl ($\lambda_{max.}$ 280, 289 mμ.; $\epsilon_{max.}$ 1900, 1950).

Chromatography

1:2:3-*Trichlorobenzene metabolites.* The possible phenolic metabolites of 1:2:3-trichlorobenzene are 2:3:4- and 3:4:5-trichlorophenol, and 3:4:5-trichlorocatechol. The monophenols could be separated from the catechol by steam distillation. Two solvent systems were used: A, benzene–acetic acid–water (1:1:2, v/v) and B, the *n*-butanol–ethanol–ammonia–ammonium carbonate buffer system of Fewster & Hall (1951). The two monophenols were not completely separated in these solvents, but could readily be distinguished by their colour reactions with diazotized *p*-nitraniline and sulphanilic acid (see Table 2). Convenient amounts of phenols for chromatography were of the order of 50 μg.

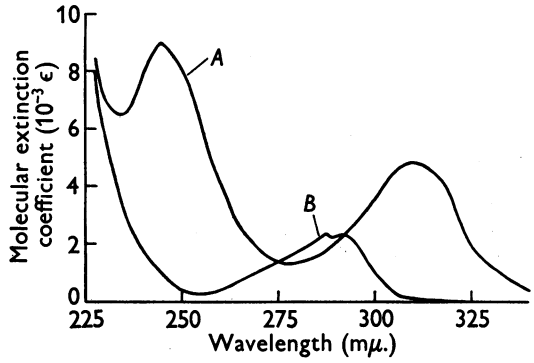

Fig. 3. Spectra of 2:4:6-trichlorophenol. *A*, in 0·1 N-NaOH ($\lambda_{max.}$ 244, 311 mμ.; $\epsilon_{max.}$ 8950, 4900) and *B*, in 0·1 N-HCl ($\lambda_{max.}$ 287, 292 mμ.; $\epsilon_{max.}$ 2350, 2300).

1:2:4-*Trichlorobenzene metabolites.* The possible phenolic metabolites are: 2:3:5-, 2:4:5- and 2:3:6-trichlorophenol; 3:4:6-trichlorocatechol and 2:3:5-trichloroquinol. The monophenols could be separated from the polyphenols by steam distillation and then on paper in solvent system B (Table 2). The 2:3:6-trichlorophenol could be separated from the other two monophenols, which, though not themselves separable in either solvent system, could be distinguished by colour reactions (see Table 2). The polyphenols were readily separable in solvent system A.

1:3:5-*Trichlorobenzene metabolites.* The possible phenolic metabolites are 2:4:6-trichlorophenol, 2:4:6-trichlororesorcinol and trichlorophloroglucinol. The monophenol was readily separated from the polyphenols by steam distillation, and the polyphenols were readily separable from each other in solvent system A (see Table 2).

Isolation and detection of metabolites

From 1:2:3-*trichlorobenzene*

Monophenols. A total of 4·5 g. of 1:2:3-trichlorobenzene was fed to three rabbits and their urine collected for 5 days. The neutral, non-reducing urine gave an intense naphthoresorcinol reaction and a blue colour with 2:6-dichloroquinonechloroimide. The urine was brought to pH 3 with HCl and extracted with ether for 5 hr. The phenols in the extract were transferred to N-NaOH (2 × 25 ml.), which was then acidified and steam-distilled. The colourless oil (50 mg.) in the distillate crystallized, m.p. 80°, and was benzoylated. The crystalline benzoate was identified after recrystallization as 2:3:4-trichlorophenyl benzoate, m.p. and mixed m.p. 142°. The residual urine was now made N with respect to HCl and heated on a water bath for 0·5 hr.

Table 2. *The R_F values and colour reactions on paper of trichlorophenols*

Figures in parentheses are pH values.

Phenol	R_F values in* A	R_F values in* B	Colour reactions: Gibbs reagent†	Diazotized‡ *p*-nitraniline	Diazotized sulphanilic acid§	Brentamine Fast Red B salt‖	$FeCl_3$¶
2:3:4-Trichlorophenol	0·92	0·83	Blue green (8)	Violet	Yellow	Violet	—
3:4:5-Trichlorophenol	0·88	0·89	Pale blue (10)	Orange	Orange	Orange	—
3:4:5-Trichlorocatechol	0·55	0·76	Green (8)	Buff	—	—	Green (7) Violet (9)
2:3:5-Trichlorophenol	0·90	0·84	Blue (8)	Orange	Orange	Orange	—
2:3:6-Trichlorophenol	0·92	0·73	Blue (8)	Magenta	Yellow	Magenta	—
2:4:5-Trichlorophenol	0·92	0·83	Blue (8)	Violet	Orange	Violet	—
3:4:6-Trichlorocatechol	0·67	0·72	Blue (8)	Buff	—	—	Green (7) Violet (9)
2:3:5-Trichloroquinol**	0·70	—	Violet (7)	Yellow	—	—	—
2:4:6-Trichlorophenol	0·92	0·74	Blue (8)	No colour	No colour	No colour	—
2:4:6-Trichlororesorcinol	0·64	0·94	Violet (8)	Green	Violet	Yellow	—
Trichlorophloroglucinol	0·10	—	Violet (8)	Brown	Orange	Orange	Violet

* Descending chromatography on Whatman no. 1 paper. In solvent A (benzene–acetic acid–water, 1:1:2, v/v), values are after 6 hr. and in solvent B (ethanol–*n*-butanol–3 N-ammonia–ammonium carbonate buffer, 11:40:19, v/v) after 16 hr.

† 2% ethanolic solution of 2:6-dichloroquinonechloroimide, followed by saturated $NaHCO_3$ for pH 8, saturated borax for pH 9, and 2 N-Na_2CO_3 for pH > 10.

‡ Diazotized *p*-nitraniline, followed by 2 N-Na_2CO_3.

§ Diazotized sulphanilic acid, followed by 2 N-Na_2CO_3.

‖ 0·1% aqueous solution of Brentamine Fast Red B salt, followed by 2 N-ammonia.

¶ 1% aqueous $FeCl_3$, followed by saturated $NaHCO_3$.

** This compound turns green, then violet, in dilute alkali.

to hydrolyse ethereal sulphates. It was then continuously extracted with ether for 10 hr. From this extract 2:3:4-trichlorophenol (50 mg.) was isolated as described above and identified as the benzoate, m.p. and mixed m.p. 142°. The residual urine was now made 3 N with respect to HCl and boiled under reflux for 3 hr. to hydrolyse glucuronides. The phenols were extracted and distilled as before and 750 mg. of 2:3:4-trichlorophenol, m.p. 81°, were isolated and characterized as the benzoate, m.p. and mixed m.p. 142°. The total trichlorophenol isolated accounted for 17% of the dose.

The steam distillates, after the separation of 2:3:4-trichlorophenol from them, were extracted with ether to remove residual phenols. The extract was subjected to paper chromatography, the solvent system B being used. On spraying the paper with diazotized *p*-nitraniline or diazotized sulphanilic acid two spots were revealed near the solvent front and close together (R_F, 0·83 and 0·89). The faster-moving spot was light orange with diazotized *p*-nitraniline and orange with diazotized sulphanilic acid and corresponded to 3:4:5-trichlorophenol (R_F, 0·89, see Table 2). The slower spot was violet with diazotized *p*-nitraniline and light yellow with diazotized sulphanilic acid and corresponded to 2:3:4-trichlorophenol (R_F, 0·83). Thus it appeared that, although the 2:3:4-phenol is the major metabolite, the 3:4:5-phenol, which is readily distinguishable from the 2:3:4-isomer by colour reactions, is also formed in small amounts.

3:4:5-*Trichlorocatechol.* The residue from the steam distillates of the extracts described above contained non-volatile phenols. The residues from several experiments were combined, and on testing for catechols with $FeCl_3$ a positive green colour was obtained. The combined residues were extracted with ether and the extract was chromatographed in two solvents. When $FeCl_3$ was used for spraying, a catechol was detected, with R_F 0·55 in solvent A and R_F 0·76 in solvent B. These R_F values corresponded to 3:4:5-trichlorocatechol (see Table 2), and from the spot dimensions the amount present was equivalent to approx. 0·5% of the dose. The catechol fraction was therefore separated from the extract as the lead salt (Porteous & Williams, 1949) and the lead salt treated with H_2S to remove lead. The filtrate from the lead sulphide was extracted with ether to remove the catechol and the whole of the extract chromatographed on a large scale on no. 1 Whatman paper with benzene–acetic acid–water (1:1:2, v/v) as solvent. The position of the catechol on the paper was located and the catechol-containing portion of the paper excised. On elution of the excised strip with ethanol and evaporation of the eluate a small amount of the crystalline catechol was obtained. After recrystallization from light petroleum b.p. 60–80°, and then water, the catechol (5 mg. from 8 g. of trichlorobenzene) had m.p. and mixed m.p. 103° with authentic 3:4:5-trichlorocatechol.

The mercapturic acid fraction. The urine of three rabbits which had collectively received 4·5 g. of 1:2:3-trichlorobenzene was collected for 3 days, and adjusted to pH 2 with 2 N-H_2SO_4. It was then continuously extracted with ether for 24 hr. The carboxylic acids in the ether extract were then transferred into 2 N-$NaHCO_3$, and the bicarbonate extract was twice washed with ether to remove any contaminating phenols. This extract was then made N with respect to NaOH and refluxed for 0·5 hr. to hydrolyse the mercapturic acids to the corresponding thiophenols. The hydrolysed solution was acidified with 2 N-H_2SO_4 and steam-distilled to yield trichlorothiophenol as a colourless oil

(60 mg.) which crystallized with difficulty. On oxidation of the thiophenol with ethanolic iodine solution, 3:4:5:3′:4′:5′-hexachlorodiphenyl disulphide was obtained as a colourless solid, m.p. and mixed m.p. 152° after repeated recrystallization from aqueous ethanol. The m.p. of 2:3:4:2′:3′:4′-hexachlorodiphenyl disulphide (185°) was depressed by the material from the urine. This suggests that the acid formed from 1:2:3-trichlorobenzene is 3:4:5-trichlorophenylmercapturic acid.

From 1:2:4-*trichlorobenzene*

Monophenols. Steam distillation of the ether extract of the acid (3N)-hydrolysed urine collected for 4 days from four rabbits which had collectively received 6 g. of 1:2:4-trichlorobenzene gave a liquid mixture of phenols (0·9 g.), which on benzoylation yielded 2:4:5-trichlorophenyl benzoate, m.p. and mixed m.p. 92° after repeated recrystallization from aqueous ethanol. Paper chromatography of the ether extract in solvent B and spraying with diazotized *p*-nitraniline revealed a violet spot, R_F 0·83, and an orange spot, R_F 0·84, which corresponded to synthetic 2:4:5- and 2:3:5-trichlorophenol respectively. Attempts to isolate 2:3:5-trichlorophenol in the pure state were unsuccessful, although it was present in the mixture in appreciable amounts. From a melting point–composition curve of mixtures of pure 2:4:5- and 2:3:5-trichlorophenols, the minimum m.p. (39–43°) was found at about 40% of the 2:3:5- and 60% of the 2:4:5-isomer. The solidification point of this mixture was 18°. The phenol mixture from the urine was liquid at room temperature (22°). From this evidence and from paper chromatography we conclude that the urinary phenol contained slightly more of the 2:4:5- than of the 2:3:5-isomer. The presence of 2:3:6-trichlorophenol in the extracts could not be shown.

3:4:6-*Trichlorocatechol.* The residue from the steam distillation of the extract described above, which contained the non-volatile phenols, gave a green colour with $FeCl_3$. Paper chromatography of an ether extract of this residue and spraying with $FeCl_3$ followed by 2N-$NaHCO_3$ revealed a single violet spot, R_F 0·67 in solvent A and 0·72 in B, which corresponded to 3:4:6-trichlorocatechol. From the dimensions of the spot the amount present was estimated to be about 0·2% of the dose, but the crystalline catechol was not isolated.

The mercapturic acid fraction. Steam distillation of the alkali-hydrolysed mercapturic acid fraction of the urine from three rabbits which had collectively received 4·5 g. of 1:2:4-trichlorobenzene yielded 100 mg. of trichlorothiophenol, which partly crystallized. The crystals were separated mechanically from the liquid. On oxidation with ethanolic iodine, the crystalline thiophenol yielded 2:4:5:2′:4′:5′-hexachlorodiphenyl disulphide, m.p. and mixed m.p. 136–7° after recrystallization from aqueous ethanol, and the liquid fraction gave 2:3:5:2′:3′:5′-hexachlorodiphenyl disulphide, m.p. and mixed m.p. 168° after recrystallization. The isolation of these disulphides suggests that two mercapturic acids, namely 2:4:5- and 2:3:5-trichlorophenylmercapturic acids, are metabolites of 1:2:4-trichlorobenzene.

From 1:3:5-*trichlorobenzene*

Phenols. Steam distillation of the ether extract of the acid-hydrolysed urine from a rabbit which had received 1:3:5-trichlorobenzene (1·5 g.) yielded 2:4:6-trichlorophenol (50 mg.), m.p. 67° after recrystallization from light petroleum b.p. 60–80°; the benzoate had m.p. and mixed m.p. 74°. Chromatography of the ether extract in solvent B and spraying with Gibb's reagent revealed a blue spot, R_F 0·74, corresponding to synthetic 2:4:6-trichlorophenol, which appeared to be the only metabolite of 1:3:5-trichlorobenzene. 2:4:6-Trichlororesorcinol and trichlorophloroglucinol were not detected.

The mercapturic acid fraction. Alkaline hydrolysis of the mercapturic acid fraction of the urine from three rabbits which had collectively received 4·5 g. of 1:3:5-trichlorobenzene yielded what appeared to be a trace of a thiophenol (detected by odour) on steam distillation. Oxidation with ethanolic iodine, however, gave no crystalline disulphide.

Unchanged trichlorobenzene. Six rabbits were each fed with 1·5 g. of 1:3:5-trichlorobenzene. The faeces were collected daily, homogenized with an equal volume of water, and steam-distilled. Unchanged 1:3:5-trichlorobenzene (a total of 800 mg. or 9% of the dose), m.p. and mixed m.p. 61°, was recovered from the distillate on the first 2 days after dosage. No further unchanged material was obtained on subsequent days. The urines were collected over 5 days, adjusted to pH 3 with HCl, and continuously extracted with ether for 8 hr. The ethereal extract was evaporated to dryness, suspended in N-NaOH and steam-distilled. The steam distillate contained no unchanged trichlorobenzene. Traces, however, were found in the steam distillate from acid-hydrolysed urine. Similar experiments were carried out on the faeces of rabbits receiving 1:2:3- and 1:2:4-trichlorobenzene, but in both cases none of the unchanged material was found in the faeces.

RESULTS

Table 3 shows that the major metabolites of the trichlorobenzenes are oxygen conjugates containing glucuronic acid and sulphuric acid. In the case of the 1:2:3-isomer, which appears to be the most rapidly metabolized of the three isomers, these amount to about 62% of the dose in 5 days, and after this time metabolites are not detectable in the urine. The glucuronide and ethereal sulphate excretions were found to rise to a maximum usually on the first day after dosing. None of this isomer was found unchanged in the faeces. With the 1:2:4-isomer, the oxygen conjugates amount in 5 days to about 38% of the dose, which is less than with the 1:2:3-isomer. Furthermore, the maximum excretion of conjugates was found to be reached usually on the second day after dosing, and the excretion of metabolites was still detectable after 5 days. None of the unchanged 1:2:4-isomer was found in the faeces. With the 1:3:5-isomer, the total conjugation in 5 days was much less than with the other isomers and amounted to only about 23% of the dose. The glucuronide and ethereal sulphate excretion was found to rise after the first day and remain at about the same level for the first 3 days after dosing and then progressively diminish. However, metabolites were still being excreted after 5 days and unchanged 1:3:5-

Table 3. *Quantitative excretion of metabolites of the isomeric trichlorobenzenes*

Dose fed, 0·5 g./kg. wt. of rabbit. Figures refer to percentage of dose excreted during 5 days after dosing. Figures in parentheses are mean values. Superior figures indicate the number of experiments.

Trichlorobenzene fed	Percentage of dose excreted in the urine as					
					Trichlorophenols	
	Glucuronide	Ethereal sulphate	Mercapturic acid	Total conjugation	Free	Total
1:2:3-	46–55 (50)[3]	9–13 (12)[3]	0·2–0·5 (0·3)[3]	55–69 (62)[3]	3–5 (4)[3]	64–89 (78)[4]
1:2:4-	18–33 (27)[3]	10–12 (11)[3]	0·2–0·5 (0·3)[3]	30–45 (38)[3]	1–2 (1·5)[3]	33–51 (42)[3]
1:3:5-	16–23 (20)[5]	1–5 (3)[5]	0 (0)[2]	17–28 (23)[5]	0·4–0·5 (0·5)[3]	7–13 (9)[5]

trichlorobenzene was found in the faeces. The ethereal sulphate excretion after this isomer was small and probably not significant. This is perhaps to be expected, since the only phenolic metabolite detected was 2:4:6-trichlorophenol, and this phenol is known not to form an ethereal sulphate in rabbits (Dodgson, Smith & Williams, 1950).

In all cases small amounts of free phenols were excreted, and there is an approximate agreement between the total *O*-conjugates and the conjugated phenols excreted in the case of the 1:2:3- and 1:2:4-isomers. The higher values of the total phenol as compared with the total conjugates are in part due to the fact that a mixture was estimated and the results were calculated on the basis of the extinction value of the major component phenol. With the phenols from 1:2:3-trichlorobenzene, for example, the major component, 2:3:4-trichlorophenol, has a smaller ϵ value at 246 mμ. (7100 in 0·1 N-NaOH) than the minor component, 3:4:5-trichlorophenol (ϵ, 10700 in 0·1 N-NaOH). With the 1:3:5-isomer, the total conjugates amounted to 17–28 %, but the amount of conjugated 2:4:6-trichlorophenol excreted was 7–13 %. At first it was thought that this discrepancy might have been due to the excretion of the other possible phenolic metabolites of 1:3:5-trichlorobenzene, namely 2:4:6-trichlororesorcinol or trichlorophloroglucinol. However, neither of these compounds could be detected in the urine, and the reason for the discrepancy has to be left undecided.

DISCUSSION

The trichlorobenzenes are metabolized slowly in rabbits by oxidation to monophenols, which are mainly excreted in combination with glucuronic acid and to a lesser extent with sulphuric acid. The rate at which these compounds are metabolized appears to be in the descending order 1:2:3-, 1:2:4-, 1:3:5-. In contrast with monochlorobenzene and the *o*- and *m*-dichlorobenzenes, the amounts of mercapturic acid and catechols formed are very small. With monochlorobenzene, the mercapturic acid and catechols formed account for about 20 and 27 % of the dose respectively; with the *o*- and *m*-dichlorobenzenes, the values are 5–11 and 3–4 % respectively; but with the 1:2:3- and 1:2:4-trichlorobenzenes the values are less than 0·5 % of the dose in each case. As pointed out by Azouz, Parke & Williams (1953) catechol and mercapturic acid formation appear to be related in some way. In the case of 1:3:5-trichlorobenzene the formation of a catechol derivative is not possible; mercapturic acid formation was doubtful and the amount was certainly less than 0·1 % of the dose. The orientations of the metabolites formed are summarized in Fig. 4.

Phenols

The major metabolites of the trichlorobenzenes are trichlorophenols. With the 1:2:3-isomer, the major phenol is 2:3:4-trichlorophenol (I). The other possible isomer, 3:4:5-trichlorophenol (III), is also formed, but only in small amounts. The only possible catechol, 3:4:5-trichlorocatechol, is also formed in very small quantities. The orientations of these phenols could be explained (cf. Parke & Williams, 1955) by postulating the formation of a 3:4:5-trichloro-1:2-dihydro-1:2-dihydroxybenzene (II), which could be dehydrated to form the monophenols found and dehydrogenated to form the catechol (Fig. 5). The dihydro-diol (II) on dehydration would be expected to yield mainly (I), i.e. the hydroxyl group remaining after dehydrating the diol should be located mainly at the carbon atom of 1:2:3-trichlorobenzene that is most activated in electrophilic substitutions (Badger, 1949; cf. Robinson, Smith & Williams, 1951). The major product of nitration of 1:2:3-trichlorobenzene is 2:3:4-trichloronitrobenzene (cf. Huntress, 1948).

In the case of 1:2:4-trichlorobenzene, three trichlorophenols, namely 2:4:5- (IV), 2:3:5- (VI) and 2:3:6-, are possible. 2:3:6-Trichlorophenol,

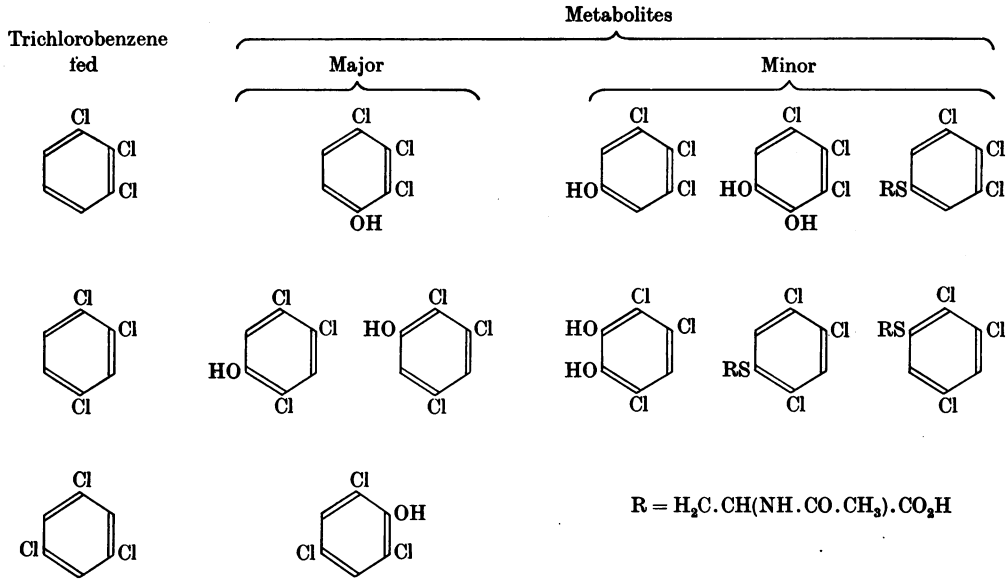

Fig. 4. Orientation of the metabolites of trichlorobenzene.

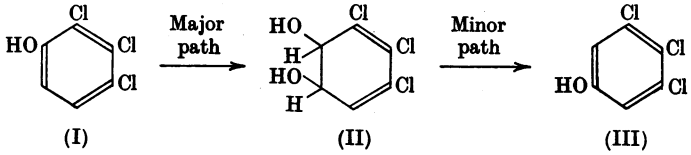

Fig. 5

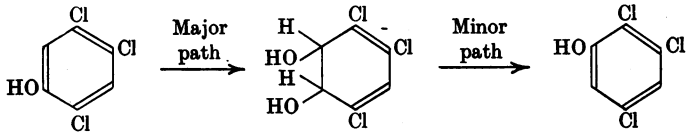

Fig. 6

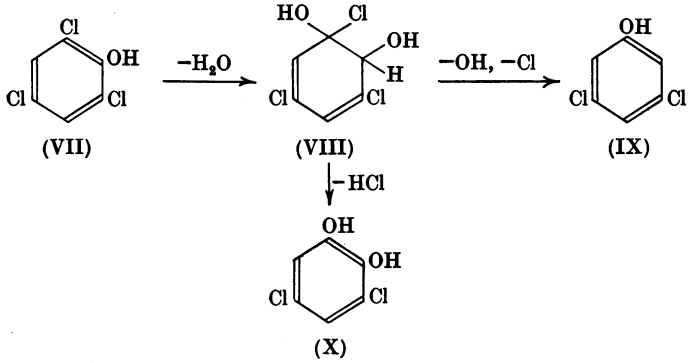

Fig. 7

however, was not found as a metabolite. The other two phenols were both found, and we believe that they were present approximately in the ratio 3:2, although only the major one (2:4:5-) was isolated in pure crystalline form. Again an intermediate 3:4:6-trichoro-1:2-dihydro-1:2-dihydroxybenzene (V) could be postulated as the precursor of these two phenols (Fig. 6). The most activated carbon atom in 1:2:4-trichlorobenzene is the one at position 5, since, on nitration with fuming nitric acid, 2:4:5-trichloronitrobenzene is formed exclusively (for references see Huntress, 1948). On these grounds one would expect (V) to yield mainly the phenol (IV), with small amounts of (VI). Thus qualitatively the orientations of the metabolic phenols and catechols of both 1:2:3- and 1:2:4-trichlorobenzenes could be explained by postulating the intermediary 1:2-dihydro-1:2-dihydroxy derivatives (II) and (V).

In the case of 1:3:5-trichlorobenzene, a 1:2-dihydrodiol at unsubstituted carbon atoms cannot be formulated. The alternative is to postulate a 1:3:5-trichloro-2-hydro-1:2-dihydroxybenzene (VIII) (Fig. 7). Dehydration of (VIII) would give (VII), the actual phenol found. Loss of HCl would give 3:5-dichlorocatechol (X), and of OH and Cl 3:5-dichlorophenol (IX), but neither of these compounds was found as metabolites of 1:3:5-trichlorobenzene. In fact, no other chlorinated phenol but (VII) was found.

1:3:5-Trichlorobenzene differs from its isomers in that the ethereal sulphate output after its administration is insignificant. In Table 4 the pK values of the monophenolic metabolites of the di- and trichlorobenzenes and the percentages of the dose of the chlorinated benzenes excreted as ethereal sulphates are given. It is to be noted that only in 2:4:6-trichlorophenol does the pK value fall below 7, and this phenol is at least ten times stronger than any other phenol given in the table. Anderton, Smith & Williams (1948) suggested that phenols with pK_a values less than 7 are not conjugated with sulphuric acid in rabbits, and Dodgson, Smith & Williams (1950) showed that 2:6-di-, 2:4:6-tri- and penta-chlorophenols (pK_a, 6·80, 6·42 and 5·26 respectively) were not excreted as ethereal sulphates, whereas chlorophenols with pK_a values higher than 7 were. On these grounds, 1:3:5-trichlorobenzene, since it is oxidized to 2:4:6-trichlorophenol, would not be expected to increase ethereal sulphate output. Since the ethereal sulphate output in this instance is practically zero, it supports the finding that 2:4:6-trichlorophenol is the only phenol formed.

Table 4. *Ethereal sulphate formation of chlorinated benzenes and the* pK_a *of the phenolic metabolites*

Chlorobenzene fed	Sulphate conjugation (% of dose)	Chlorophenol metabolites	pK_a of metabolites*
1:2-Di-	21†	3:4-Di-	8·39
		2:3-Di-	7·44
1:3-Di-	7†	2:4-Di-	7·74
		3:5-Di-	7·92
1:4-Di-	27†	2:5-Di-	7·35
1:2:3-Tri-	12	2:3:4-Tri-	7·58
		3:4:6-Tri-	7·33
1:2:4-Tri-	11	2:4:5-Tri-	7·74
		2:3:5-Tri-	7·27
1:3:5-Tri-	0	2:4:6-Tri-	6·42

* Quoted from Murray & Gordon (1935).
‡ From Parke & Williams (1955).

Mercapturic acids

The acetylcysteine conjugation of the trichlorobenzenes is very small, being less than 0·5 % of the dose with the 1:2:3- and 1:2:4-isomers and negligible with the 1:3:5-isomer. Previous work with 1:2- and 1:3-dichlorobenzenes had shown that the orientations of the mercapturic acids from these compounds were identical with those of the major phenolic metabolites. Thus the major phenol of 1:2-dichlorobenzene was 3:4-dichlorophenol and the mercapturic acid was 3:4-dichlorophenylmercapturic acid (Azouz *et al.* 1955; Parke & Williams, 1955). This is again true of 1:2:4-trichlorobenzene, but not of the 1:2:3-isomer. In the latter case the mercapturic acid appears to be 3:4:5-trichlorophenylmercapturic acid, whose orientation is similar to that of the minor metabolic phenol (3:4:5-trichlorophenol) of 1:2:3-trichlorobenzene. The case of 1:2:4-trichlorobenzene is interesting because it appears to give rise to two mercapturic acids in roughly equal amounts, namely 2:4:5- and 2:3:5-trichlorophenylmercapturic acids (cf. Fig. 4), and these are similar in orientation to the two major phenolic metabolites. In most instances hydroxylation and mercapturic acid formation occur in the chlorinated benzenes at the most electronegative or least electropositive carbon atom, as indicated by the position of *in vitro* nitration of these compounds. The agreement, however, is not general, as is shown in Table 5. At present no *in vitro* pattern has been found which agrees entirely with the *in vivo* one.

Bromobenzene on injection into rats causes central hepatic necrosis, which can be prevented by methionine or cysteine supplements (Koch-Weser, Huerga, Yesinick & Popper, 1953). Chlorobenzene, *o*-dichlorobenzene and *m*-bromochlorobenzene, but not *p*-dibromobenzene or *p*-bromochlorobenzene, also produce liver damage in small animals. The trichlorobenzenes have only a slight effect on the liver, whilst the tetra- and hexa-derivatives are

Table 5. *Orientation of* in vivo *hydroxylation and mercapturic acid formation with chlorinated benzenes, compared with that of* in vitro *nitration*

Compound	Position of: Hydroxylation *in vivo* — Mono	Hydroxylation *in vivo* — Di	Mercapturic acid formation *in vivo*	Nitration *in vitro*
Chlorobenzene	4 (trace at 2)	3:4-*	4	4 (70%), 2 (30%)
1:2-Dichlorobenzene	4 (a little at 3)	3:4-†	4	4
1:3-Dichlorobenzene	4 (a little at 5)	4:5-†	4	4 (a little at 2)
1:2:3-Trichlorobenzene	4 (a little at 5)	4:5-†	5	4 (a little at 5)
1:2:4-Trichlorobenzene	5 and 6	5:6-†	5 and 6	5

* 4-Chlorocatechol is the major metabolite of chlorobenzene in rabbits.
† These catechols are minor metabolites.

without effect even in high doses (Cameron *et al.* 1937; Koch-Weser *et al.* 1953). The mono- and *o*- and *m*-di-halogenobenzenes form appreciable amounts of mercapturic acids *in vivo*, whilst the *p*-di- and the tri-halogenobenzenes form little or none. It thus appears that the toxic effects of these compounds are due to withdrawal of methionine and cysteine from the liver for mercapturic acid formation. It seems likely that since the tetra-, penta- and hexa-chlorobenzenes are relatively non-toxic they do not form mercapturic acids to any appreciable degree. This has already been found to be true for some of the tetrachlorobenzenes (unpublished data).

SUMMARY

1. A study has been made of the fate in the rabbit of oral doses (0·5 g./kg.) of the three isomeric trichlorobenzenes.

2. The 1:2:3-isomer is the most rapidly metabolized isomer. It is nevertheless slowly metabolized, 62% of the dose appearing, during 5 days after dosing, as conjugates of glucuronic (50%) and sulphuric acids (12%). The maximum excretion of these conjugates occurred on the first day after dosing. The major metabolite was 2:3:4-trichlorophenol, but small amounts of 3:4:5-trichlorophenol and traces of 3:4:5-trichlorocatechol were also formed. Small quantities (0·3%) of mercapturic acid were formed and identified as 2:3:4-trichlorophenylmercapturic acid.

3. The 1:2:4-isomer was more slowly metabolized and oxygen conjugates accounted for 38% of the dose in 5 days, the maximum occurring on the second day after dosing. Two major phenols, 2:4:5- and 2:3:5-trichlorophenol, were excreted, together with small amounts of 3:4:6-trichlorocatechol. Small amounts (0·3%) of two mercapturic acids, 2:3:5- and 2:4:5-trichlorophenylmercapturic acid, were excreted.

4. 1:3:5-Trichlorobenzene was the least rapidly metabolized of the isomers, and only about 23% of the dose was excreted as oxygen conjugates in 5 days. Practically no ethereal sulphate or mercapturic acid was formed and the only phenol detected was 2:4:6-trichlorophenol. Unchanged 1:3:5-trichlorobenzene was found in the faeces.

5. The paper chromatographic behaviour and colour reactions of 11 trichlorophenols have been recorded, together with the ultraviolet spectra of some of them.

6. Five isomeric hexachlorodiphenyl disulphides have been synthesized as reference compounds for the identification of trichlorophenylmercapturic acids.

7. The orientations of the phenolic metabolites found have been discussed in the light of current theories of metabolic hydroxylation.

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Studies on Ali-esterases and other Lipid-hydrolysing Enzymes

3. INHIBITION OF THE ESTERASES OF PANCREAS*

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Extensive studies of the substrate specificity of crude preparations of pancreatic lipase have been carried out by many investigators (cf. Ammon & Jaarma, 1950), though they usually failed to consider the fact that the pancreas contains more than one enzyme with esterase activity. The results obtained by Loevenhart & Pierce (1907) with sodium fluoride and by Platt & Dawson (1925) with albumin and edestin as inhibitors suggest that different enzymes are responsible for the hydrolysis of ethyl esters and olive oil by pancreas. Although these authors did not draw this conclusion, later experiments by Wolvekamp & Griffoen (1934) and Fodor (1946, 1950*a*, *b*) showed that the suggestion is justified.

It is generally agreed that pancreatic lipase can hydrolyse tributyrin as well as triolein (cf. Willstätter & Memmen, 1923), and recent evidence presented by Fodor (1950*a*) and Aldridge (1954) supports the assumption that the activity of pancreas preparations towards tributyrin is due largely, if not entirely, to the lipase. Fodor (1950*b*) suggested further that the hydrolysis of esters such as methyl butyrate is due largely to cholesterol esterase, an enzyme which was shown by Kelsey (1939) to be distinct from the lipase of pancreas. The methods used by Fodor (1946, 1950*a*, *b*) to distinguish these two enzymes (effect of gum acacia, bile salts, trypsin digestion and exposure to high temperatures and to alkali) did not permit of a definition of their relative substrate specificities. However, Webb (1948) has suggested that organophosphorus inhibitors would be useful tools in a study of the substrate specificity of esterases and lipases, since the lipases are relatively resistant to inhibition by these compounds. The results obtained by Aldridge (1953*a*, 1954), Mounter & Whittaker (1953) and Myers *et al.* (1955) with serum and other tissues have confirmed the value of this technique. In the present investigation we have therefore reinvestigated the specificity of the pancreas esterases, using various organo-phosphorus derivatives as esterase inhibitors.

We found that the pancreas enzymes responsible for the hydrolysis of tributyrin and methyl butyrate could indeed be readily distinguished. Further attention was paid to the hydrolysis of salicyl butyrate since Hofstee (1952*a*, *b*) reported that pancreas preparations contain two esterases capable of hydrolysing salicyl esters, neither of which is identical with lipase. Our experiments confirm the conclusions of Hofstee (1952*a*, *b*), as well as those of Fodor (1950*a*, *b*), and show that it is possible to distinguish at least three types of enzymes with esterase activity in pancreas by the use of organo-phosphorus inhibitors.

MATERIALS AND METHODS

Substrates. Many of the substrates were prepared in this laboratory by Miss J. W. Tol according to the methods described in the literature; these esters include phenyl

* Part 2: Myers, Schotte & Mendel (1955).