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Studies in Detoxication

76. THE METABOLISM OF HALOGENOBENZENES. 1:2:3:4-, 1:2:3:5- AND 1:2:4:5-TETRACHLOROBENZENES*

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Previous papers in this series have described the metabolic fate of the mono-, di- and tri-chlorobenzenes (Spencer & Williams, 1950; Smith, Spencer & Williams, 1950; Azouz, Parke & Williams, 1952, 1953, 1955; Parke & Williams, 1955; Jondorf, Parke & Williams, 1955). These studies have now been extended to the tetrachlorobenzenes. Cameron *et al.* (1937) have observed that tetrachlorobenzenes, unlike chlorobenzene or *o*-dichlorobenzene, do not cause liver injury in rats. We shall show that in the rabbit these compounds are in part slowly metabolized to tetrachlorophenols, and some evidence has been obtained which suggests that they may be partly dechlorinated in the gut to di- and tri-chlorobenzenes.

EXPERIMENTAL

Reference compounds. The following compounds were prepared or purchased, and purified: 1:2:3:4- and 1:2:3:5-tetrachlorobenzene, m.p. 45° and 51° respectively (Holleman, 1920); 1:2:4:5-tetrachlorobenzene, m.p. 140° (L. Light and Co.); these tetrachlorobenzenes were free from di- and tri-chlorobenzenes as judged by absorption spectra and m.p.; 2:3:4:5-tetrachlorophenol, m.p. 116°, and its benzoate, m.p. 110° (Tiessens, 1931); tetrachlorocatechol, m.p. 193°, and its diacetate, m.p. 190° (Huntress, 1948); 2:3:4:6-tetrachlorophenol, m.p. 70° (Kodak Ltd.), and its benzoate, m.p. 115°; 2:3:5:6-tetrachlorophenol, m.p. 115°, and its benzoate, m.p. 136° (Tiessens, 1931); tetrachloroquinol, m.p. 236° (Kodak Ltd.), and its benzoate, m.p. 233°.

Animals. Chinchilla doe rabbits, kept throughout on a diet of 80 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water/day, were used. The tetrachlorobenzenes were administered by stomach tube or subcutaneously as 10% (w/v) solutions in arachis oil, and urine was collected daily. In some experiments the animals were fitted with plywood collars to prevent coprophagy.

Analytical methods. Glucuronic acid, ethereal sulphate and mercapturic acid in urine were determined each day according to the methods of Paul (1951), Mead, Smith & Williams (1958), Sperber (1948), and Stekol (1936) respectively. Spectrophotometric determinations were made with a Unicam spectrophotometer (SP. 500).

Estimations of tetrachlorophenols. The total tetrachlorophenols in urine were estimated spectrophotometrically by determination of the difference between the light absorption of acid and alkaline solutions of steam-distillates of the urines previously hydrolysed in 5N-HCl, as described by

* Part 75: El Masri, Smith & Williams (1958).

Azouz *et al.* (1955) for dichlorophenols. The spectra of the phenols were recorded between the wavelengths 220 and 400 mμ, and the wavelengths chosen for the estimations were 315 mμ for 2:3:4:5- and 2:3:4:6-tetrachlorophenols and 308 mμ for 2:3:5:6-tetrachlorophenol. The free tetrachlorophenols were similarly estimated with steam-distillates of the urines adjusted to pH 3 with N-HCl. The spectra in acid and alkali of 2:3:4:5-tetrachlorophenol (ε at 315 mμ is 5600 in 0·1N-NaOH and 400 in 0·1N-HCl), 2:3:4:6-tetrachlorophenol (ε at 315 mμ is 5120 in 0·1N-NaOH and 0 in 0·1N-HCl) and 2:3:5:6-tetrachlorophenol (ε at 308 mμ is 6800 in 0·1N-NaOH and 0 in 0·1N-HCl) are shown in Fig. 1. Recoveries of 2:3:4:5-, 2:3:4:6- and 2:3:5:6-tetrachlorophenols from normal rabbit urine at concentrations of 5 mg./100 ml. were 97, 93 and 95±5% respectively.

Estimations of tetrachlorobenzenes. (i) In faeces: The tetrachlorobenzenes excreted unchanged were recovered by steam-distillation of the homogenized material. They were then extracted from the distillates into *n*-hexane and estimated spectrophotometrically. The spectra were recorded between 220 and 400 mμ (cf. Conrad-Billroth, 1932) and the wavelengths chosen for the estimations were 292, 293 and 296 mμ for 1:2:3:4-, 1:2:3:5- and 1:2:4:5-tetrachlorobenzenes respectively (see Table 1). With 1:2:3:5-tetrachlorobenzene in faeces the greatest maxima sometimes occurred at wavelengths lower than those shown by the authentic 1:2:3:5-tetrachlorobenzene (see below).

(ii) In tissues: The animals were killed 6 days after dosing, and the gut contents, liver, brain and portions of the skin, mixed depot fat and the remainder of the body were homogenized or minced and then steam-distilled to recover the unchanged tetrachlorobenzenes. The steam-distillates were extracted with ether, and the extracts were washed with 2N-NaOH followed by 2N-HCl, dried over anhydrous Na_2SO_4 and evaporated to dryness at 10°; the residue was weighed. The yields of unchanged tetrachlorobenzenes thus recovered were confirmed by spectrophotometric estimations of *n*-hexane extracts of the steam-distillates or of ethanolic solutions of the weighed residues.

(iii) In expired air: The unchanged tetrachlorobenzenes exhaled in the expired air were estimated spectrophotometrically (cf. Azouz *et al.* 1952) with the extinctions for these compounds in ethanol given in Table 1. The spectra of the ethanolic solutions of the expired air were recorded between the wavelengths 220 and 400 mμ at intervals of 6 or 12 hr. over a period of 3–5 days. It was found that

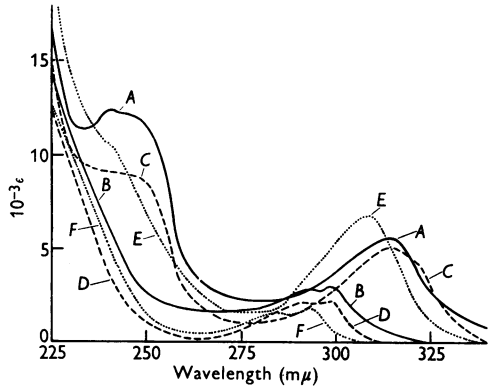


Fig. 1. Ultraviolet-absorption spectra of 2:3:4:5-, 2:3:4:6- and 2:3:5:6-tetrachlorophenols. *A*, 2:3:4:5-Tetrachlorophenol in 0·1N-NaOH ($\lambda_{max.}$ 240, 314–315 mμ: $\epsilon_{max.}$ 12 500, 5600) and, *B*, in 0·1N-HCl ($\lambda_{max.}$ 293, 298 mμ: $\epsilon_{max.}$ 2900, 3000); *C*, 2:3:4:6-tetrachlorophenol in 0·1N-NaOH ($\lambda_{max.}$ 240, 315 mμ; $\epsilon_{max.}$ 9200, 5100) and, *D*, in 0·1N-HCl ($\lambda_{max.}$ 290, 298 mμ; $\epsilon_{max.}$ 2100, 2250); *E*, 2:3:5:6-tetrachlorophenol in 0·1N-NaOH ($\lambda_{max.}$ 308 mμ; $\epsilon_{max.}$ 6800) and, *F*, in 0·1N-HCl ($\lambda_{max.}$ 286, 293 mμ, $\epsilon_{max.}$ 1650, 1800).

Table 1. *Absorption spectra of chlorinated benzenes in ethanol*

$\lambda_{max.}$ (mμ) with $\epsilon_{max.}$ italicized in parentheses.

Wavelength range ... Compound	240–249	250–259	260–269	270–279	280–289	290–299
C_6H_5Cl	245 (*90*)	258 (*220*) 251 (*135*)	264 (*280*)	272 (*210*)	—	—
1:2-$C_6H_4Cl_2$	—	256 (*135*) 250 (*95*)	263 (*230*)	277 (*235*) 270 (*275*)	—	—
1:3-$C_6H_4Cl_2$	—	256 (*140*) 250 (*80*)	263 (*250*)	278 (*270*) 270 (*330*)	—	—
1:4-$C_6H_4Cl_2$	—	258 (*175*)	266 (*290*)	273 (*400*)	281 (*320*)	—
1:2:3-$C_6H_3Cl_3$	—	—	265 (*125*)	273 (*160*)	280 (*125*)	—
1:2:4-$C_6H_3Cl_3$	—	—	—	278 (*565*) 270 (*350*)	287 (*525*)	—
1:3:5-$C_6H_3Cl_3$	—	—	266 (*180*)	273 (*235*)	281 (*170*)	—
1:2:3:4-$C_6H_2Cl_4$	—	—	—	274 (*260*) 274 (*245*)*	280 (*330*) 283 (*350*)*	291 (*290*) 292 (*305*)*
1:2:3:5-$C_6H_2Cl_4$	—	—	—	274 (*210*) 274 (*225*)*	281 (315) 284 (*420*)*	291 (*275*) 293 (*515*)*
1:2:4:5-$C_6H_2Cl_4$	—	—	—	276 (*640*) 278 (*620*)* 270 (*300*)*	285 (*1050*) 287 (*1150*)* 282 (*680*)*	294 (*1050*) 296 (*1400*)*

* In *n*-hexane.

absorption spectra of these solutions did not exactly coincide with those of the authentic tetrachlorobenzenes, and the greatest maxima sometimes occurred at wavelengths lower than those shown by the tetrachlorobenzenes (see Discussion). The maxima chosen for the present estimations were 291 mμ for 1:2:3:4- and 1:2:3:5-tetrachlorobenzenes and 294 mμ for the 1:2:4:5-isomer. Although water vapour was removed by passing the expired air through Drechsel bottles containing anhydrous magnesium perchlorate (Anhydrone), some water vapour (about 5%, v/v) was entrained in the ethanol absorption vessels. The absorption spectra of these compounds in ethanol were, however, unaffected by the addition of <5% (v/v) of water, and even 10% of water only slightly reduces the values of ϵ without affecting the wavelengths of the maxima.

Chromatography. Only one monophenol can be derived directly from each of the tetrachlorobenzenes. 1:2:3:4-Tetrachlorobenzene could, however, be metabolized to tetrachlorocatechol, and 1:2:4:5-tetrachlorobenzene to tetrachloroquinol. Both these dihydric phenols were readily separable from the monophenols by paper chromatography in solvent *A*, and were further distinguished by colour reactions (see Table 2). The tetrachlorophenols were separable from the tri- and di-chlorophenols by paper chromatography in solvent *B*; the colour reactions, R_F values and absorption spectra of di- and tri-chlorophenols are given by Jondorf *et al.* (1955), Azouz *et al.* (1955) and Parke & Williams (1955). Convenient amounts of the phenols for chromatography were of the order of 50 μg.

Isolation and detection of metabolites of 1:2:3:4-tetrachlorobenzene

From urine. A total of 4·5 g. of 1:2:3:4-tetrachlorobenzene was fed to three rabbits and their urine collected for 6 days. The neutral, non-reducing urine gave a strong naphtharesorcinol reaction and a blue colour with 2:6-dichloroquinonechloroimide. The urines left over from the analyses were pooled, adjusted to pH 3 with 2N-HCl, and continuously extracted with ether for 8 hr. The extract was concentrated and then chromatographed on paper in solvent system *A*. The zone of paper adjacent to the solvent front (R_F 0·80–1·00) was cut from the chromatogram and extracted with ether in a Soxhlet apparatus. After removal of the ether at room temperature, the residue (29 mg., 1·6% of the dose) was identified as 2:3:4:5-tetrachlorophenol, m.p. and mixed m.p. 116° after recrystallization from ethanol. This chromatographic procedure for the isolation of the free tetrachlorophenols was shown to be reasonably quantitative compared with spectrophotometric estimation.

The residual urine was then refluxed for 3 hr. in 5N-HCl to hydrolyse the conjugated phenols, and cooled and continuously extracted with ether for 8 hr. Paper chromatography of the extract in solvent *A* revealed the presence of 2:3:4:5-tetrachlorophenol and tetrachlorocatechol (about 0·2% of the dose). The ethereal solution was then extracted with N-NaOH (3 × 30 ml.) and the extract was acidified with 2N-H_2SO_4 and steam-distilled. The tetrachlorophenol removed by filtration had m.p. and mixed m.p. 116° with authentic 2:3:4:5-tetrachlorophenol, and was characterized as the benzoate, m.p. and mixed m.p. 110°, after recrystallization from ethanol. A further amount of tetrachlorophenol (total was 305 mg., 15·8% of the dose) was recovered by ether extraction of the steam-distillate. Paper chromatography of this ether extract in solvent *B* revealed the presence of 2:3:4:5-tetrachlorophenol and a minor spot (R_F 0·94) which gave colour reactions corresponding to 2:5- or 3:5-dichlorophenol (see Azouz *et al.* 1955; Parke & Williams, 1955) and amounted to ≯1% of the dose. The spectra of the acid and alkaline solutions of steam-distillates of the urine showed no peaks other than those due to 2:3:4:5-tetrachlorophenol.

The aqueous residues remaining from the steam-distillation were extracted continuously with ether and the extract was chromatographed on paper in solvent *A*. The tetrachlorocatechol band was located on the chromatogram by the green colour obtained with $FeCl_3$ solution and by its quenching of the background fluorescence of the paper in u.v. light. The band was cut out and eluted with ether. In this way a few milligrams of the catechol were freed from about 0·4 g. of normal excretion products. Further chromatography on paper in the same solvent mixture gave a

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

R_F values are for descending chromatography on Whatman no. 1 paper; *A*, in benzene–acetic acid–water (1:1:2, by vol.), run for 6 hr.; *B*, in ethanol–butan-1-ol–aq. 3N-NH_3 soln./3N-ammonium carbonate buffer (11:40:19, by vol.) (cf. Fewster & Hall, 1951), run for 12 hr. Sprays used were: Gibbs's reagent consisting of 2% ethanolic solution of 2:6-dichloroquinonechloroimide, followed by saturated $NaHCO_3$ for pH 8, saturated borax for pH 9 and 2N-Na_2CO_3 for pH>10 (pH values are in parentheses); diazotized *p*-nitraniline, followed by 2N-Na_2CO_3; diazotized sulphanilic acid, followed by 2N-Na_2CO_3; 0·1% aq. solution of Brentamine Fast Red B salt, followed by aq. 2N-NH_3 soln.; 0·5% aq. $FeCl_3$ followed by saturated $NaHCO_3$.

Phenol	R_F values		Colour reactions				
	A	*B*	Gibbs's reagent	Diazotized *p*-nitraniline	Diazotized sulphanilic acid	Brentamine Fast Red B salt	$FeCl_3$
2:3:4:5-Tetrachlorophenol	0·88	0·78	Pale blue (9)	None	None	Red	None
2:3:4:6-Tetrachlorophenol	0·91	0·70	Blue (9)	None	None	None	None
2:3:5:6-Tetrachlorophenol	0·92	0·72	Blue (9)	Red	Red	Red	None
Tetrachlorocatechol	0·55	—	Blue (8) / Violet (10)	Green	Orange	Yellow	Green (7) / Violet (9)
Tetrachloroquinol	0·66	—	Green (8) / Black (10)	Green (8)	Green (8)	Yellow	None (7) / Green (9)

band R_F 0·55 which gave a green colour with $FeCl_3$ solution and showed all the other colour reactions of tetrachlorocatechol. The eluate from the second chromatogram gave 12 mg. of non-crystalline material, which from its light absorption ($\lambda_{max.}$ 220, 225, 276 mμ in 0·1N-NaOH; $\lambda_{max.}$ 229 and 297 mμ in 0·1N-HCl) was shown to contain approx. 3 mg. of tetrachlorocatechol (see Fig. 2).

From faeces and tissues. 1:2:3:4-Tetrachlorobenzene, m.p. and mixed m.p. 45°, was isolated from the combined faeces of three rabbits receiving tetrachlorobenzene orally. Only traces of non-crystalline material were isolated from the tissues. Steam-distillation of the urine before and after acid hydrolysis did not yield any unchanged tetrachlorobenzene.

From expired air. The absorption spectra of the ethanolic solutions of the expired air showed maxima at 290, 287, 282, 278, 275 and 273 mμ. The greatest maxima occurred at 282, 278, 275 and 273 mμ, which suggests the presence of some trichlorobenzenes and perhaps even dichlorobenzenes (see Table 1). An approximate estimate of the trichlorobenzenes, calculated from the absorption at 278 and 273 mμ after correction for 1:2:3:4-tetrachlorobenzene, indicated that some 2–3% of the dose could be present as these dechlorination products.

Isolation and detection of metabolites of 1:2:3:5-tetrachlorobenzene

From urine. A total of 4·5 g. of 1:2:3:5-tetrachlorobenzene was fed to three rabbits, and the urine, collected for 6 days, was neutral, non-reducing and gave a moderate naphtharesorcinol reaction. By paper chromatography of an ethereal extract of the free phenols, 2:3:4:6-tetrachlorophenol (48 mg., 2·5% of the dose), m.p. and mixed m.p. 70°, was obtained. Ether extraction of the acid-hydrolysed urine also gave 2:3:4:6-tetrachlorophenol (31 mg., 1·6% of the dose), m.p. 70° (benzoate, m.p. and mixed m.p. 114–115°).

Daily examination of urine. The steam-distillates of the urines which were used for the daily spectrophotometric estimations of tetrachlorophenol were extracted continuously with ether, and the extracts on paper chromatography in solvent *B* revealed the presence of other chlorophenols. On the first day after dosing, two phenolic spots were detected, the major one (R_F 0·86) gave the colour reactions of 2:4-dichlorophenol, and the minor one (R_F 0·90) those of 3:4:5-trichlorophenol. 2:3:4:6-Tetrachlorophenol was not present. The spectra of the extract of the urine of this day showed a single maximum at 303 mμ in 0·1N-NaOH, and two maxima at 277 and 283 mμ in 0·1N-HCl, which would correspond with the presence of 2:4-dichlorophenol or 2:3:6-trichlorophenol. From the second to the twelfth day chromatography in solvent *B* revealed 2:3:4:6-tetrachlorophenol as the major spot with a series of spots of R_F 0·76–0·91 giving colour reactions corresponding to several di- and tri-chlorophenols. The spectra of the extracts of these urines showed a principal maximum at 315 mμ in 0·1N-NaOH (2:3:4:6-tetrachlorophenol) with one or more maxima at lower wavelengths corresponding to less chlorinated phenols. These di- and tri-chlorophenols collectively could account for about 5% of the dose.

After subcutaneous injection of 1:2:3:5-tetrachlorobenzene (dose, 200 mg./kg.), no chlorophenols were detected in the urine of the first day. During the second and third days, spectra and chromatography revealed the presence of 2:3:4:6-tetrachlorophenol only, but from the fourth to eighth day other phenols corresponding to various di- and tri-chlorophenols appeared in increasing amounts and on the seventh and eighth days were equal to the amount of tetrachlorophenol.

2:3:4:6-Tetrachlorophenol (100 mg.) added to 100 ml. of normal rabbit urine and then subjected to acid hydrolysis and steam-distillation as described above gave no trace of di- or tri-chlorophenols detectable by paper chromatography, and the spectrum showed only the absorption maxima characteristic of 2:3:4:6-tetrachlorophenol.

From faeces and tissues. 1:2:3:5-Tetrachlorobenzene, m.p. and mixed m.p. 51°, was isolated from the faeces, gut contents, body fat and the rest of the bodies of animals receiving the tetrachlorobenzene orally. After subcutaneous injection of 1:2:3:5-tetrachlorobenzene (dose, 200 mg./kg.), the unchanged tetrachlorobenzene was excreted in the faeces to the extent of about one-third of the dose in 6 days; the spectrum of the steam-distillate of the faeces also showed high absorption maxima at 266, 276 and 280 mμ, which suggests the presence of dechlorination products, i.e. di- and tri-chlorobenzenes. No unchanged tetrachlorobenzene was detected in the urine either before or after acid hydrolysis.

From expired air. The absorption spectra of the ethanolic solutions of the expired air showed maxima at 290, 282, 280, 278, 276, 274 and 273 mμ, and during the first 2 days the greatest maxima occurred at 276, 274 and 273 mμ, which suggests the presence of trichlorobenzenes and possibly also dichlorobenzenes. During the third to fifth days the greatest maxima occurred at 291 and 282 mμ, the principal maxima for 1:2:3:5-tetrachlorobenzene, but absorption at the other maxima at lower wavelengths was still too great to be due to tetrachlorobenzene alone. An approximate estimation of trichlorobenzenes calculated from the absorption at 278 and 273 mμ, after correction for the tetrachlorobenzene present, indicated that about 9% of the dose might be present as dechlorination products, most of which were excreted during the first 2 days. The absorption spectra of the ethanolic solutions of the expired air were unaffected by addition of acid or alkali, i.e. no chlorinated phenols were present.

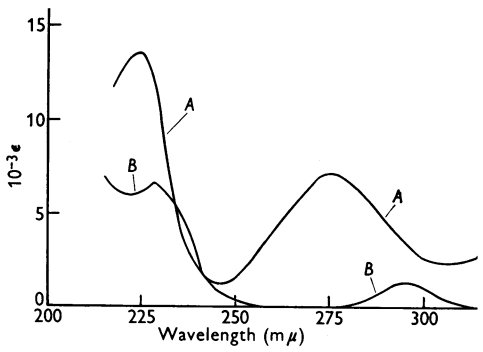


Fig. 2. Ultraviolet-absorption spectra of tetrachlorocatechol: *A*, in 0·1N-NaOH ($\lambda_{max.}$ 225, 275–276 mμ; $\epsilon_{max.}$ 13 500, 7100) and, *B*, in 0·1N-HCl ($\lambda_{max.}$ 229, 295–297 mμ; $\epsilon_{max.}$ 6600, 1400).

Isolation and detection of metabolites of 1:2:4:5-tetrachlorobenzene

From urine. The urine collected in 6 days from three rabbits, which had collectively received 4·5 g. of 1:2:4:5-tetrachlorobenzene orally, was neutral, non-reducing and gave a moderate naphtharesorcinol reaction. The ethereal extract of the free phenols after paper chromatography gave 2:3:5:6-tetrachlorophenol (30 mg., 1·6% of the dose), m.p. and mixed m.p. 115°. Ether extraction of the acid-hydrolysed urine also gave 2:3:5:6-tetrachlorophenol, m.p. 115° (30 mg., 1·6% of the dose), (benzoate m.p. 136° and mixed m.p. 134–5°). Paper chromatography of the ethereal extract in solvent *A* revealed 2:3:5:6-tetrachlorophenol, but 2:3:5:6-tetrachloroquinol was not present in amounts greater than 0·2% of the dose, the limits of detection.

Daily examination of urine. Paper chromatography in solvent *B* of ethereal extracts of the urine steam-distillates used in the quantitative estimation of tetrachlorophenol revealed other chlorophenols. On the first day a single spot (R_F 0·89) giving the colour reactions of 2:5-dichlorophenol or 2:3:5-trichlorophenol was revealed; 2:3:5:6-tetrachlorophenol was not present. Spectra of the steam-distillate gave a single maximum at 298 mμ in 0·1N-NaOH and at 278 mμ in 0·1N-HCl, which could indicate the presence of either 2:5-dichlorophenol or 2:3:5-trichlorophenol. The second to the seventh days showed a principal spot corresponding with 2:3:5:6-tetrachlorophenol, and a series of other spots (R_F 0·75–0·89), whose colour reactions and R_F values correspond with 2:3-, 2:5- and 2:6-dichlorophenols and 2:3:5-trichlorophenol. The spectra showed maximum absorption at 308 mμ in 0·1N-NaOH and 293 mμ in 0·1N-HCl (2:3:5:6-tetrachlorophenol) but also exhibited several minor peaks and inflexions at lower wavelengths indicative of di- and tri-chlorophenols. The amounts of di- and tri-chlorophenols increased with time, until at the eighth to the twelfth days the tetrachlorophenol spot (R_F 0·72) was no longer the major one and finally disappeared. Instead the major spot had R_F 0·80 with colour reactions indicative of 2:5-dichlorophenol or 2:3:5-trichlorophenol, and the spots R_F 0·86–0·89, corresponding to 2:3- and 2:6-dichlorophenols, were still present. The spectra of the eighth to twelfth days showed maxima at wavelengths 298–308 mμ in 0·1N-NaOH and 275–295 mμ in 0·1N-HCl, but the highest maxima no longer corresponded with those of 2:3:5:6-tetrachlorophenol (308 mμ in 0·1N-NaOH and 293 mμ in 0·1N-HCl), but occurred at lower wavelengths. These di- and tri-chlorophenols together could account for about 5% of the dose.

After subcutaneous injection of 1:2:4:5-tetrachlorobenzene (400 mg. to one rabbit), a slight trace of 2:3:5:6-tetrachlorophenol only was found in the urine of the first day. During the second and third days, chromatography and spectra revealed only the tetrachlorophenol, but on the fourth to the sixth days traces of other chlorophenols, including probably 2:5-dichloro- or 2:3:5-trichloro-phenol, were detected. During the seventh and eighth days these lower chlorophenols and tetrachlorophenol were present in about equal amounts.

2:3:5:6-Tetrachlorophenol (100 mg.) in 100 ml. of normal rabbit urine after acid hydrolysis gave a steam-distillate which contained no di- or tri-chlorophenols detectable by paper chromatography, and the spectrum showed only the absorption maxima characteristic of 2:3:5:6-tetrachlorophenol.

From faeces and tissues. 1:2:4:5-Tetrachlorobenzene, m.p. and mixed m.p. 140°, was isolated from the faeces, gut contents, skin, body fat and the rest of the bodies of animals receiving the tetrachlorobenzene orally. After subcutaneous injection of 1:2:4:5-tetrachlorobenzene only some 2% of the dose was excreted unchanged in the faeces.

From expired air. The absorption spectra of the ethanolic solutions of the expired air showed maxima at 277–278, 270, 265 and 255 mμ with only a slight inflexion at 294 mμ. The amount of 1:2:4:5-tetrachlorobenzene exhaled unchanged was therefore very small and the principal material exhaled by the rabbit probably consisted of dechlorination products of the tetrachlorobenzene, which from the position and extinctions of the absorption maxima appeared to be dichlorobenzenes. A rough calculation from the absorption at 270 and 265 mμ suggests that about 10% of the dose may be exhaled as dechlorination products over 3 days. No phenols were excreted in the expired air.

RESULTS

The quantitative aspects of the metabolism of the tetrachlorobenzenes are given in Tables 5 and 6. These tables show that some 70–80% of the dose can be accounted for, and we regard this recovery as reasonably satisfactory in view of the difficulties involved. There appears to be a difference between 1:2:3:4-tetrachlorobenzene on the one hand and its two isomers on the other, since it appears to be more rapidly metabolized and more extensively oxidized to a tetrachlorophenol than its isomers. All the isomers appear to be slowly but fairly well absorbed in 6 days at the dose level (0·5 g./kg.) administered, since even with 1:2:4:5-tetrachlorobenzene only 16% appears in the faeces. Only about 5% of the dose of 1:2:3:4-tetrachlorobenzene appears in the faeces.

1:2:3:4-Tetrachlorobenzene appears to be mainly metabolized to 2:3:4:5-tetrachlorophenol (43%) which is partly excreted in the urine free (8%), but is largely conjugated (Table 5). With this compound the main excretion of the conjugated tetrachlorophenol occurred during the first 3 days after dosing. Apart from the small amount of the compound excreted unchanged in the faeces, some 10% of the dose remained after 6 days in the tissues, mainly the fat (Table 4), and some 8% had been eliminated in the expired air (Table 3). There seems to be a small conversion (about 2%) into less chlorinated benzenes which were detected in the breath of the animals.

1:2:3:5-Tetrachlorobenzene is not oxidized to a great extent in 6 days after dosing, since only about 10% could be accounted for as urinary chlorinated phenols and nearly half the dose (49%) was found unchanged in the faeces (14%), expired air (12%) and the tissues (23%; mainly in the fat, which contained 11%) (Tables 3, 4 and 6). With this

isomer there was evidence that it was partly dechlorinated in the gut to di- and tri-chlorobenzenes to the extent of 10–15 %, and we assume that this dechlorination was bacterial. Its main urinary metabolite was 2:3:4:6-tetrachlorophenol, which was partly excreted free and partly conjugated with glucuronic and sulphuric acids (Table 5). Small amounts of urinary metabolites were still being excreted 12 days after dosing.

1:2:4:5-Tetrachlorobenzene was the least metabolized of all three isomers. Only 2–3 % of the dose was accounted for as 2:3:5:6-tetrachlorophenol. Nearly 70 % of the dose was unchanged in 6 days after dosing (Table 6) and most of this (48 % of the dose) was found in the tissues, mainly the fat (Table 4). There was evidence that this isomer was also partly dechlorinated, perhaps to a slightly greater extent than 1:2:3:5-tetrachlorobenzene.

Table 3. *Elimination of unchanged tetrachlorobenzenes in the expired air of rabbits receiving these compounds orally*

Tetrachlorobenzene fed	Dose (g./kg.)	Percentage of dose in expired air: Days after dosing 1	2	3	4	5	Total
1:2:3:4-	0·5	1·9	2·2	1·6	0·2	—	5·9
	0·3	0·8	1·7	6·7	—	—	9·2
1:2:3:5-	0·5	2·1	2·1	1·2	2·9	2·6	10·9
	0·3	0·9	3·2	9·8	—	—	13·9
1:2:4:5-	0·5	1·2	0·2	0·2	—	—	1·6

Table 4. *Tetrachlorobenzenes in tissues*

Dose, 0·5 g./kg. orally. Rabbits were killed 6 days after dosing.

Tetrachlorobenzene fed	Percentage of dose found unchanged in: Liver	Brain	Skin	Depot fat	Gut contents	Rest of body	Total
1:2:3:4-	0·1	—	2	5	0·5	2·0	10
1:2:3:5-	<0·5	<0·2	5	11	1·4	5·2	23
1:2:4:5-	0·1	<0·1	10	25	6·2	6·4	48

Table 5. *Urinary excretion of the metabolites of tetrachlorobenzenes*

Dose, 0·5 g./kg. orally. Figures given are mean values with ranges in parentheses and the number of experiments indicated by superior figures.

Tetrachlorobenzene administered	Percentage of dose excreted as: Glucuronide	Ethereal sulphate	Mercapturic acid	Tetrachlorophenol: Free	Total
1:2:3:4-	30 (22–36)[5]	3 (1–8)[5]	<1[5]	8 (7, 9)[2]	43 (38, 48)[2]
1:2:3:5-	6* (2–10)[9]	2 (1–6)[9]	0[3]	1·9 (1·2, 2·5)[2]	5 (4, 6)[2]
1:2:4:5-	4† (1–8)[11]	1 (<1–2)[11]	0[3]	1·3 (0·9, 1·6)[2]	2·2 (1·1, 3·2)[2]

* Without collars, 5·5 (2–8)[6]; with collars, to prevent coprophagy, 6 (4–10)[3].
† Without collars, 2 (<1–8)[6]; with collars, 4 (3–6)[3]. Ethereal sulphate values were not significantly different with or without collars.

Table 6. *Summary of excretion of metabolites of the isomeric tetrachlorobenzenes*

Dose, 0·5 g./kg. orally. Figures are mean values of two or three experiments covering excretion during 6 days after dosing.

Tetrachlorobenzene fed	Percentage of dose eliminated as: Phenols: Tetrachlorophenols	Other phenols	Unchanged tetrachlorobenzene in: Faeces	Tissues	Breath	Other chlorobenzenes in breath	Total
1:2:3:4-	43	<1	5	10	8	2	68
1:2:3:5-	5	5	14	23	12	9	68
1:2:4:5-	2	5	16	48	2	10	83

During the period of the experiments, which extended up to 12 days in some cases, it appeared that the expired air was a more important channel of elimination of 1:2:3:5- and 1:2:4:5-tetrachlorobenzenes than the urine, and was almost equal in importance to the faeces (Table 6).

DISCUSSION

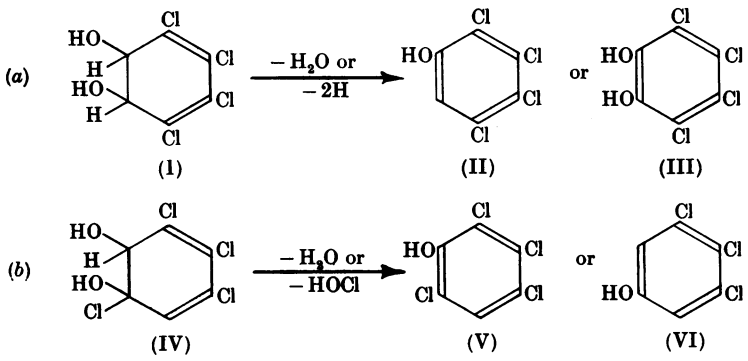
When tetrachlorobenzenes are administered orally to rabbits it appears that four things can happen to them apart from some remaining for some time unchanged in the tissues. They can be (i) eliminated in the faeces, (ii) eliminated in the expired air, (iii) oxidized in the tissues to tetrachlorophenols, which are excreted in the urine free and conjugated, and (iv) partly dechlorinated in the gut.

Elimination in the faeces. All three tetrachlorobenzenes, when given by mouth in doses of 0·5 g./kg. in arachis oil, are partly eliminated unchanged in the faeces. However, this process is not important with the vicinal 1:2:3:4-tetrachlorobenzene, but does account for some 15% of the dose of 1:2:3:5- and 1:2:4:5-tetrachlorobenzenes in 6 days. There is little doubt that part of the material excreted in the faeces, especially from the 1:2:3:5-isomer, is material that has been absorbed and then excreted via the bile into the intestine, since on subcutaneous injection of 1:2:3:5-tetrachlorobenzene (0·2 g./kg.) about a third of the dose is eliminated as such in the faeces in 6 days. Only 2% of an injected dose of 1:2:4:5-tetrachlorobenzene found its way into the faeces in 6 days.

Elimination in the expired air. Tetrachlorobenzenes are volatile in steam and tend to sublime. 1:2:3:4- and 1:2:3:5-Tetrachlorobenzenes are about equally volatile in steam, and the 1:2:4:5-isomer is less so (this was determined by steam-distillation of 50 mg. quantities of each and estimating the tetrachlorobenzenes spectrophotometrically in the distillates collected at intervals). The first two could therefore be expected to appear in the breath in greater amounts than the last. Table 3 suggests that this is true. Since 1:2:3:4-tetrachlorobenzene is more readily metabolized by oxidation than the 1:2:3:5-isomer, it could be expected that 1:2:3:5-tetrachlorobenzene would be the isomer appearing unchanged in greatest amounts in the breath. Table 3 suggests that this may also be true. Azouz *et al.* (1952) have already commented that some compounds may be eliminated in the expired air by a process analogous to steam-distillation.

Oxidation to tetrachlorophenols. The tetrachlorobenzenes are oxidized to tetrachlorophenols, but this process is of importance only with the vicinal 1:2:3:4-isomer, where it accounts for nearly half the dose. With the other two isomers it only accounts for 5% or less of the dose. This may not be entirely a matter of absorption in view of the faecal excretion of injected 1:2:3:5-tetrachlorobenzene already mentioned. There is a possibility that since 1:2:3:4-tetrachlorobenzene has two vicinal unsubstituted positions in the ring, it is more readily oxidized *in vivo* than are the 1:2:3:5- and 1:2:4:5-isomers, which have no vicinal unsubstituted positions. This recalls the case of 1:3:5-trichlorobenzene, which also possesses no vicinal unsubstituted positions and is not hydroxylated to any great extent *in vivo* compared with its 1:2:4- and 1:2:5-isomers (Jondorf *et al.* 1955). Other work in this Laboratory has shown that dichlorophenols are excretory products of 1:3:5-trichlorobenzene (unpublished work). 1:2:3:4-Tetrachlorobenzene could form a 1:2-dihydro-1:2-diol, i.e. 1:2:3:4-tetrachloro-5:6-dihydrobenzene-5:6-diol (I), but its isomers could not. In fact 2:3:4:5-tetrachlorophenol (II) and 3:4:5:6-tetrachlorocatechol (III), metabolites of 1:2:3:4-tetrachlorobenzene, could be formed via the diol *a* (see below).

Diol formation with the 1:2:3:5- and 1:2:4:5-isomers would require hydroxylation of a carbon atom already substituted by chlorine. If such a diol were formed then trichlorophenols could be metabolites. One possibility with 1:2:3:5-tetrachlorobenzene is shown in *b* (see below).



We have in fact some evidence, though not unequivocal, that 3:4:5-trichlorophenol (VI) is a minor metabolite of this isomer. That (V) is a metabolite has been proved by isolation. The formation of trichlorophenols, however, could be the result of the oxidation of partly dechlorinated tetrachlorobenzenes which are discussed below.

Dechlorination products. Table 1 shows that the absorption peaks in ethanol of monochlorobenzene are in the range 245–272 mμ, those of the dichlorobenzenes 250–281 mμ, those of the trichlorobenzenes 265–287 mμ and those of the tetrachlorobenzenes 274–294 mμ. Spectroscopic examination has suggested that rabbits given tetrachlorobenzenes eliminate di- and tri-chlorobenzenes in expired air (see Fig. 4). With 1:2:3:5-tetrachlorobenzene this is most marked on the first 2 days after dosage. This suggestion was supported, particularly for 1:2:3:5- and 1:2:4:5-tetrachlorobenzenes, by the finding by chromatography and spectroscopy of small amounts of di- and trichlorophenols in the urine, particularly on the first day after dosing (see Fig. 3). On subsequent days the major phenol found was the corresponding tetrachlorophenol. We believe that these less chlorinated benzenes were formed from the tetrachlorobenzene in the gut, since on injection of 1:2:3:5- or 1:2:4:5-tetrachlorobenzene, only the corresponding tetrachlorophenol was detected in the urine for the first 3 days after dosing, but on the fourth to the eighth days di- and tri-chlorophenols appeared in the urine in increasing amounts. This reversal of the appearance of di- and tri-chlorophenols in the urine after injection of the tetrachlorobenzene is presumably the result of slow excretion of tetrachlorobenzene via the bile into the gut, where it is then partly dechlorinated by bacteria. The partly dechlorinated benzenes are then absorbed and oxidized to phenols. We have no information on the mechanism of the dechlorination but our data suggest that it is not performed by the tissues of the rabbit.

SUMMARY

1. The metabolic fate of 1:2:3:4-, 1:2:3:5- and 1:2:4:5-tetrachlorobenzenes in rabbits has been investigated.

2. 1:2:3:4-Tetrachlorobenzene (dose 0·5 g./kg. orally) is slowly metabolized and about 43 % is oxidized in 6 days to 2:3:4:5-tetrachlorophenol, which is excreted in the urine, partly unchanged and partly conjugated. About 5 % is eliminated in the faeces, 8 % in the expired air and some 10 % is still in the tissues after 6 days. There is some evidence that a small proportion (2 %) of the dose is partly dechlorinated, probably in the gut.

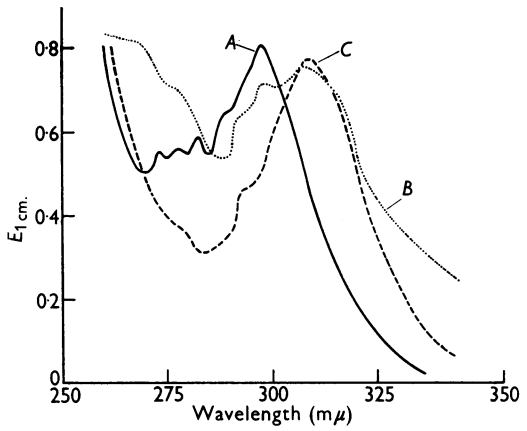


Fig. 3. Ultraviolet spectra in 0·1 N-NaOH of the steam-volatile products of acid-hydrolysed urines from a rabbit which had been dosed with 1:2:4:5-tetrachlorobenzene (1·5 g.). Curve *A* (—) is that from urine of the first day after dosing and the main peak is at 298 mμ, which corresponds to 2:5-di- or 2:3:5-tri-chlorophenol. Curve *B* (· · ·) is that of the second day after dosing and the main peaks are at about 298 and 308 mμ, and correspond to a mixture of the components of curves *A* and *C*. Curve *C* (– – – –) is that of the sixth day after dosing and the main peak is at 308 mμ and corresponds to 2:3:5:6-tetrachlorophenol.

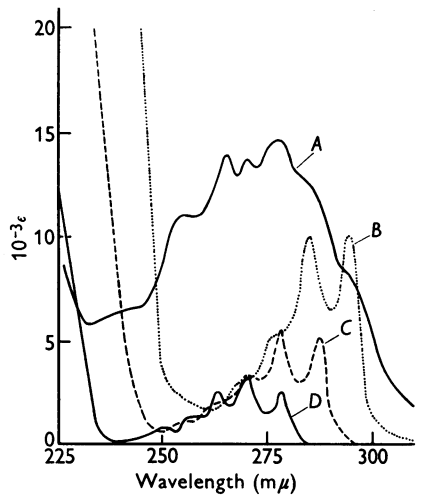


Fig. 4. Absorption spectrum in ethanol of the expired air of a rabbit collected for 12 hr. after an oral dose of 1·5 g. of 1:2:4:5-tetrachlorobenzene compared with the spectra of 1:3-di-, 1:2:4-tri- and 1:2:4:5-tetra-chlorobenzene in ethanol. Ordinates for curve *A* are arbitrary.

Expired air:	*A*	255, 265, 270, 277–278, 285, 294 mμ
Chlorobenzenes:		
1:2:4:5-tetra-	*B*	— — — 276, 285, 294
1:2:4-tri-	*C*	— — 270, 278, 287, —
1:3-di-	*D*	256, 263, 270, 278, — —

3. 1:2:3:5-Tetrachlorobenzene is very slowly metabolized. Only about 5 % is oxidized to and excreted as 2:3:4:6-tetrachlorophenol in 6 days. Some 14 % is eliminated unchanged in the faeces, 12 % in the breath and 23 % remains in the tissues after 6 days. There is evidence that some 9 % of the dose is dechlorinated and eliminated in the expired air as less chlorinated benzenes. Some 5 % of the dose may also be excreted in the urine as di- and tri-chlorophenols. Injected 1:2:3:5-tetrachlorobenzene is partly excreted as such in the faeces, probably via the bile.

4. 1:2:4:5-Tetrachlorobenzene appears to be the least readily metabolized of the three isomers. Only about 2 % is converted into 2:3:5:6-tetrachlorophenol in 6 days; 48 % of the dose was found in the tissues after 6 days, and 16 % was in the faeces and 2 % in the expired air. Dechlorination products could account for 15 % of the dose, about 10 % of the dose appearing in the expired air as less chlorinated benzenes and 5 % in the urine as di- and tri-chlorophenols. Dechlorination of the tetrachlorobenzenes is believed to occur in the gut, probably under the influence of bacteria.

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Glutamic–Alanine and Glutamic–Aspartic Transaminases of Wheat Germ

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Since the discovery of the transamination reaction by Braunstein & Kritzmann (1937), transaminase systems have been studied in extracts from a number of plant and animal tissues and micro-organisms. This work has been reviewed by Braunstein (1947) and Cohen (1951, 1954). Results of many of the early investigations were conflicting, as crude enzyme preparations and non-specific quantitative methods were used. Recently much information on the properties of animal and microbial transaminases has been obtained with purified enzyme preparations and specific quantitative methods. There is, however, little precise information on the plant transaminases.

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In the present investigation a study has been made of some of the properties of two partially purified transaminase enzymes from wheat germ. Wheat germ was chosen as the plant material because Leonard & Burris (1947) had previously demonstrated that it contained active transaminase systems. The two transaminase systems were as follows:

(1) L-Glutamic acid + pyruvic acid ⇌ α-oxoglutaric acid + L-alanine

(catalysed by glutamic–alanine transaminase)

(2) L-Glutamic acid + oxaloacetic acid ⇌ α-oxoglutaric acid + L-aspartic acid

(catalysed by glutamic–aspartic transaminase)