The Metabolism of 2:3:5:6- and 2:3:4:5-Tetrachloronitrobenzenes in the Rabbit and the Reduction of Aromatic Nitro compounds in the Intestine

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The metabolism of chloronitrobenzenes does not appear to have been studied. The fate of 2:3:5:6-tetrachloronitrobenzene (TCNB) in the animal body is of interest on account of its use in agriculture as an inhibitor of rotting and sprouting of potatoes. The toxicology of this compound has been studied by Buttle & Dyer (1950), and in this paper we report upon its metabolism in the rabbit, together with that of one of its isomers, 2:3:4:5-tetrachloronitrobenzene. Changes which tetrachloronitrobenzenes might be expected to undergo in the rabbit are (a) reduction of the nitro group and acetylation of the resultant tetrachloroaniline, (b) hydroxylation and conjugation of the nitro-, amino- or acetamidophenol with glucuronic or sulphuric acids, or (c) formation of a mercapturic acid. The absorption of the two tetrachloronitrobenzenes and their effect upon the excretion by rabbits of ethereal sulphate, glucuronic acid, mercapturic acid and phenols have been studied. Some of the major metabolites have been isolated. Since it was found that these compounds were reduced in the intestine the reduction of some other nitro compounds in this way has been examined.

EXPERIMENTS AND RESULTS

Materials. 2:3:5:6- and 2:3:4:5-Tetrachloronitrobenzenes (m.p. 99° and 65° respectively) were supplied by Bayer Agriculture Ltd. The corresponding tetrachloroanilines were prepared from the nitro compounds by treatment with Sn and HCl in ethanol at 100° for 2 and 6 hr. respectively. 2:3:5:6-Tetrachloroaniline (m.p. 107–108°, as recorded by Peters, Rowe & Stead, 1943) on diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride gives a rather feeble orange colour fading to brown. 2:3:4:5-Tetrachloroaniline, colourless needles m.p. 118–120°, gives a red colour on diazotization and coupling. (Found: Cl, 61.4. Calc. for $C_6H_2NCl_4$: Cl, 61.5%.)

Diet and dosage. The rabbits used were does weighing 2–3 kg. and were maintained throughout on the standard diet of rabbit pellets and water described previously (Bray, Ryman & Thorpe, 1947). Both compounds were administered by stomach tube as suspensions in water. No toxic effects were observed after giving repeated doses (3 g.) of 2:3:5:6-tetrachloronitrobenzene, but 2:3:4:5-tetrachloronitrobenzene in doses of 0.7 g. caused slight anorexia, some-

times persisting for 2 days. 2:3:5:6-Tetrachloroaniline was given in doses of 1 g. without toxic effects.

Estimation of ethereal sulphate. The method of Folin (1905–6) was used.

Estimation of glucuronic acid. A modification of the naphthoresorcinol method of Hanson, Mills & Williams (1944) was used (Bray, Humphris, Thorpe, White & Wood, 1952a).

Estimation of aromatic amino compounds. These were determined by the Bratton & Marshall (1939) method, using the appropriate metabolites as standards, and a Spekker photoelectric absorptiometer with a Chance glass filter OG 1.

Estimation of mercapturic acid. Stekol's (1936) iodometric method was modified by omission of ethanol in the titration because the pink colour of the end point in the original method was obscured by the colour developed in tetrachloronitrobenzene urines during the hydrolysis. Although the method gave good results with *p*-chlorophenyl mercapturic acid only 47 ± 6% of the mercapturic acid formed from 2:3:5:6-tetrachloronitrobenzene could be accounted for when it was added to normal urine. Allowance for this was made in calculating the results from metabolic urines. It should perhaps be mentioned that the values obtained in this way were supported by the amount of the mercapturic acid actually isolated from urine.

Estimation of 2:3:5:6- and 2:3:4:5-tetrachloronitrobenzenes. Since these compounds were not readily absorbed, it was necessary to determine the amounts excreted unchanged in faeces. 2:3:5:6-Tetrachloronitrobenzene is not readily reduced. This and the insolubility in water of the resulting amine made its quantitative estimation by the diazo method impracticable. A polarographic method based upon that of Dennis, Powell & Astle (1949) was, therefore, used. The polarograph was an automatically recording instrument (Cambridge Instrument Co. Ltd.) by means of which current-voltage changes are recorded photographically. A 2 ml. cell was used. The rate of dropping was 1 drop/2–2.5 sec. and the sensitivity was 1/7. The organic solvent was isopropanol and the buffer acetic acid (1 vol. 0.1N) and sodium acetate (1 vol. 0.1N). Estimations were carried out in solutions consisting of equal volumes of buffer and isopropanol solution saturated with N_2 . 'Day' faeces, collected over 24 hr. and uncontaminated by urine, were finely ground and extracted with isopropanol for 6 hr. in a Soxhlet extractor. The dark green viscous extract was diluted with further isopropanol to give a concentration of 2:3:5:6-tetrachloronitrobenzene within the range 10–100 µg./ml. The calibration curve, constructed by measuring the height of the step (half-wave potential = 0.8 V.), given by amounts of the compound within this range, was a straight line, 1 cm. step corre-

sponding to 14 $\mu\text{g.}/\text{ml.}$ 2:3:5:6-tetrachloronitrobenzene. Known amounts of this compound added to normal faeces gave results corresponding to recoveries of 93–100%. At the dilutions used (e.g. 24 hr. faeces in 10000 ml.) interference by normal constituents of faeces is negligible. (Dilution should not be less than 100 ml. for 24 hr. faeces.) As little as 1 mg. 2:3:5:6-tetrachloronitrobenzene in 24 hr. faeces may be determined in this way. The method can also be applied to the ether-extractable material of urine. Although interfering substances are present their effect is usually negligible if the extract from 24 hr. urine is diluted to not less than 50 ml.

The polarographic method can be applied to pure 2:3:4:5-tetrachloronitrobenzene but not to faecal extracts containing it. Addition of *isopropanol* extracts of normal faeces suppresses reduction at the cathode and inconsistent results are obtained. A sufficiently accurate estimate of this isomer in faeces may, however, be made by exhaustive steam distillation of an aqueous suspension of faeces. The solid (a mixture of the tetrachloronitrobenzene with some tetrachloroaniline) from the distillate is collected either by filtration or by ether extraction and weighed. The former method gives a recovery of 2:3:4:5-tetrachloronitrobenzene added to faeces of about 80% and the latter 90%. (The error in calculating this as the nitro compound is about 2% since about 20% of amine is formed, see p. 269.) Alternatively, the distillate may be used for polarographic estimation of the tetrachloronitrobenzene alone. This method is applicable also to 2:3:5:6-tetrachloronitrobenzene.

Paper chromatography. The general method was described by Bray, Thorpe & White (1950b).

RESULTS

The average daily excretions of ethereal sulphate by the rabbits used in this investigation ranged from 24 to 54 mg. SO_3 . The average percentage by which the normal individual daily values differed from the corresponding weekly averages used as 'baseline' for purposes of calculation was ± 6.8 (± 2 mg. SO_3). The corresponding values for glucuronic acid were 90–211 mg. and $\pm 5.7\%$ (± 8.5 mg. glucuronic acid) for the rabbits dosed with 2:3:5:6-tetrachloronitrobenzene and 85–284 mg. and $\pm 13.6\%$ (± 26 mg. glucuronic acid) for those dosed with 2:3:4:5-tetrachloronitrobenzene. The wider range with the latter compound is probably due to its toxicity.

Metabolism of 2:3:5:6-tetrachloronitrobenzene

Excretion in faeces. Table 1 summarizes the results obtained. In no case was any of the compound detected in the faeces passed more than 3 days after dosage. It will be seen that over a wide range of dose (0.1–3.0 g.) 59–78% is unabsorbed. Even a dose as small as 10 mg. is absorbed to an extent of only 70–78%. Evidence was obtained that some reduction of the nitro group takes place in the gut (see below).

Table 1. *The excretion of 2:3:5:6-tetrachloronitrobenzene in faeces after its administration to rabbits*

(Data for 1.5 g. dose are given in detail, other doses as totals only.)

Dose (g.)	Percentage of dose excreted in faeces			
	1st day	2nd day	3rd day	Total
0.01	—	—	—	22, 30
0.1	—	—	—	60, 60
0.5	—	—	—	67, 72
1.5	58.5	4.9	0.5	64
	47.4	11.0	0.7	59
	52.0	7.0	0	59
3.0	—	—	—	77, 78
Average for doses 0.1–3.0 g.				66

Urinary excretion products. Table 2 summarizes the quantitative results obtained. The values give the total excretion in 48 hr. after dosage. In large-scale quantitative experiments (dose 3.0 g.) it was found that very small amounts of tetrachloroaniline and mercapturic acid were excreted on the third day. If any glucuronide was excreted on the third day the amount was too small to be distinguished from baseline values. It can be seen that if the amount of the compound unabsorbed is taken into account 96% of a dose of 1.5 g. can be accounted for. It will be seen later that at least 9% of the dose is excreted as 2:3:5:6-tetrachloroaniline and 2% as free 4-amino-2:3:5:6-tetrachlorophenol. Attempts were made to determine (by the Folin and Ciocalteu method as used by Bray, Thorpe & White, 1950c) the amounts of phenolic metabolites excreted in free and conjugated forms, but the results obtained were invariably low, only about 3–5% of the dose being accounted

Table 2. *The amounts of metabolites excreted in urine in 48 hr. by rabbits after administration of 2:3:5:6-tetrachloronitrobenzene*

(Results are expressed as percentage of the dose.)

Metabolite	No. of exp.	Dose (g.)	Range	Average
Ether glucuronide	2	1.5	11–13	12
	2	3.0	11–13	12
Ethereal sulphate	2	1.0	0–1	1
	1	1.5	—	1
Mercapturic acid	6	1.5	9–15	11
Unconjugated 4-amino-2:3:5:6-tetrachlorophenol	1*	1.5	—	2
2:3:5:6-Tetrachloroaniline	1*	1.5	—	9
	1*	3.0	—	12

* By isolation from pooled urines of several rabbits.

for as total phenol as against 12% as glucuronide alone estimated by the naphthoresorcinol method. The standard used was 4-amino-2:3:5:6-tetrachlorophenol isolated from metabolic urines, and it is possible that the mixture of various phenols in the phenol fraction interfered with the intensity of colour given by 4-amino-2:3:5:6-tetrachlorophenol with the Folin and Ciocalteu reagent.

Isolation of 2:3:5:6-tetrachloronitrobenzene from faeces. Faeces of rabbits which had received 2:3:5:6-tetrachloronitrobenzene were ground, suspended in water, and steam-distilled. The solid which separated from the distillate was collected and recrystallized from aqueous ethanol. This was identified as the unchanged compound by comparison with an authentic specimen. The crude solid gave a feeble orange colour on diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine similar to that given by 2:3:5:6-tetrachloroaniline. Analysis of the crude solid gave Cl, 53.8%. (Calc. for $C_6H_2O_2NCl_4$: Cl, 54.4% and for $C_6H_3NCl_4$: Cl, 61.5%.) This suggests that only very small amounts of the reduced compound were present.

Absence of 2:3:5:6-tetrachloronitrobenzene from urine. The urine from a rabbit which had received 2:3:5:6-tetrachloronitrobenzene was extracted with ether for 24 hr. at pH 8. The solvent was removed from the extract, and the residue dried *in vacuo*. This gave no colour with tetraethylammonium hydroxide (Auerbach, 1950). It thus appears that the amount of unchanged 2:3:5:6-tetrachloronitrobenzene excreted in urine is negligible.

Isolation of 2:3:5:6-tetrachloroaniline from urine. The urine of rabbits which had been dosed with 2:3:5:6-tetrachloronitrobenzene (3 g. each) was collected for 3 days after dosage. Each day's urine was adjusted with HCl to pH 3 and the flocculent precipitate separated and dried. (The third day's urine usually gave a small precipitate but the fourth day's urine none.) The precipitates were extracted with successive small volumes of cold acetone. The addition of a small amount of water to the extracts caused the ready separation of fine colourless needles of 2:3:5:6-tetrachloroaniline, m.p. 107°, not depressed by admixture with an authentic specimen. The residue from the acetone extraction consisted mainly of a mercapturic acid, m.p. 212°, with small amounts of tetrachloroaniline (for the treatment of this residue see below). The total yield of 2:3:5:6-tetrachloroaniline was 1.35 g. from 18 g. tetrachloronitrobenzene (8.5% of dose). A better yield (0.63 g. from 6 g., or 12% of dose) was obtained by suspending the acid precipitate from urine in 2*N*-NaOH and separating the tetrachloroaniline by exhaustive steam distillation. Practically pure 2:3:5:6-tetrachloroaniline, m.p. 106°, separated from the distillate. This method has the disadvantage of destroying the mercapturic acid, but provides a convenient method of assessing the total excretion of tetrachloroaniline. (No tetrachloroaniline was obtained when the pure mercapturic acid was steam-distilled under these conditions.) The yield obtained by the acetone method and the absence of any indication of the presence of tetrachloroacetanilide makes it reasonable to assume that the steam-distillation method gives a reliable measure of the total excretion of free tetrachloroaniline.

Isolation of a mercapturic acid from urine. This compound was first obtained by continuous extraction with ether of the urine at pH 4, but is more conveniently prepared from the precipitate formed on adjusting the urine to pH 3. The residue left after extraction of this precipitate with small volumes of cold acetone, whereby much tetrachloroaniline was removed (see above), was dissolved in a large volume of

a mixture of acetone (1 vol.) and 96% (v/v) ethanol (3 vol.), leaving a small brown granular residue. The extract was treated with 2 vol. water and left to stand overnight at 0°. The crystals which separated were purified by solution in acetone-ethanol, decolorizing by filtration through charcoal if necessary, and crystallizing by addition of water as colourless elongated needles, m.p. 212°. By further addition of water, crops of crystals were obtained from the mother liquors which were either tetrachloroaniline or a mixture of tetrachloroaniline and the mercapturic acid, from which the two pure compounds could be separated by repetition of the acetone treatment. Total yield of mercapturic acid, 1.75 g. from 18 g. tetrachloronitrobenzene (7% of dose).

The nature of the mercapturic acid. The acid obtained above was very sparingly soluble in water and in acetone, contained S, and reacted with I_2 under the conditions of mercapturic acid estimation. $[\alpha]_D^{25} + 33^\circ \pm 2^\circ$ in ethanol (*c*, 0.24). No nitro group was detected by the polarograph, and there was no colour when treated with the Bratton & Marshall (1939) or Folin and Ciocalteu reagents. More than half of the compound was recovered unchanged after hydrolysis for 5 hr. with 2*N*-HCl in a sealed tube in boiling water. It was established that this compound was *N*-acetyl-S-(2:3:5:6-tetrachlorophenyl)-L-cysteine. (Found: C, 35.1; H, 2.3; N, 4.0 (Dumas and Kjeldahl); Cl, 37.4; S, 8.2; equiv. 372. $C_{12}H_9O_3NSCl_4$ requires C, 35.0; H, 2.4; N, 3.7; Cl, 37.7; S, 8.5%; equiv. 377.) On treatment with diazomethane it formed a methyl ester, as colourless needles, m.p. 174°. (Found: C, 37.1; H, 2.9; Cl, 36.4; S, 8.3. $C_{12}H_{11}O_3NSCl_4$ requires C, 36.8; H, 2.8; Cl, 36.3; S, 8.2%.) The mercapturic acid was hydrolysed by boiling under reflux with *N*-NaOH for 1 hr. On acidification of the cooled reaction mixture a white precipitate was formed which, after recrystallization from aqueous acetone, gave colourless needles, m.p. 104°. (Found: C, 29.3; H, 0.9; Cl, 57.7; S, 12.5. 2:3:5:6-Tetrachlorothiophenol, $C_6H_2SCl_4$, requires C, 29.0; H, 0.8; Cl, 57.3; S, 12.9%.) This compound gave a deep blue colour with Folin and Ciocalteu reagent. In another experiment the mercapturic acid (0.48 g.) was hydrolysed with NaOH as above, and the cooled reaction mixture extracted with ether. The aqueous layer was then acidified and again extracted with ether. The first extract yielded 75 mg. of a product which crystallized with some difficulty from ethanol, had m.p. 128–130° and contained S and Cl but gave no blue colour with Folin and Ciocalteu reagent. The second extract yielded 180 mg. 2:3:5:6-tetrachlorothiophenol. Assuming the compound in the first extract to be 2:3:5:6-tetrachlorophenyldisulphide, the yield of thiophenol and disulphide was 80% of the theoretical. The residue from the ether extractions was evaporated to dryness and extracted with ethanol. The extract (36 mg.) was examined by paper chromatography for amino-acids. Several ninhydrin-reacting spots were observed but could not be identified with certainty.

Isolation of 4-amino-2:3:5:6-tetrachlorophenol from urine. This metabolite was isolated both from metabolic urines as collected and from a hydrolysed glucuronide syrup. The 48 hr. urine from seven rabbits, each of which had received 2:3:5:6-tetrachloronitrobenzene (1.5 g.) was continuously extracted with ether at pH 8.0. The residue, after removal of ether from the extract, was mainly solid which on recrystallization from aqueous ethanol (charcoal) gave colourless elongated platelets m.p. 186°, identified as 4-amino-2:3:5:6-tetrachlorophenol. This gave a blue colour with Folin and Ciocalteu reagent, a feeble orange colour in the diazo

reaction resembling that given by 2:3:5:6-tetrachloroaniline, and a strongly positive indophenol test. (Found: C, 29.6; H, 1.3; N, 6.0; Cl, 56.6. $C_6H_3ONCl_4$ requires C, 29.2; H, 1.2; N, 5.7; Cl, 57.5%.) Yield 200 mg. or 2% of dose. The same compound was isolated in small yield from the crude glucuronide syrup (see below) after hydrolysis by boiling with HCl (0.1 vol. 12N) under reflux for 1 hr. Continuous extraction of the hydrolysate with ether for 24 hr. yielded brown, viscous, ether-soluble material which partially crystallized on chilling. On recrystallization from aqueous ethanol (charcoal) slightly coloured elongated platelets, m.p. 186°, were obtained. These were identical (mixed m.p.) with those obtained by direct extraction of urine.

Attempted isolation of a glucuronide from urine. Isolation of *m*-hydroxybenzoic acid. The glucuronide syrup obtained by the usual lead procedure (Bray *et al.* 1947) did not crystallize. As mentioned in the previous section, 4-amino-2:3:5:6-tetrachlorophenol was isolated after hydrolysis. During attempts to purify the syrup it was continuously extracted with ether and the extract contained, besides hippuric acid, a small amount of a chlorine-free phenolic acid, m.p. 198°. (Yield 30 mg. from 48 hr. urine of ten rabbits.) Comparison by means of paper chromatography with phenolic acids known to occur in normal rabbit urine (see Bray *et al.* 1950*b*) suggested that it was *m*-hydroxybenzoic acid (m.p. 201°). This was confirmed by the method of Bray, Lake, Thorpe & White (1950*a*), which clearly differentiates between *m*- and *p*-hydroxybenzoic acid. Mixed m.p. with an authentic specimen of *m*-hydroxybenzoic acid (m.p. 201°) was 200°.

Metabolism of 2:3:5:6-tetrachloroaniline

Excretion of faeces. The 24 hr. faeces from two rabbits which had each been given 1 g. of 2:3:5:6-tetrachloroaniline yielded on steam distillation 360 mg. (18% of dose) of unchanged compound which separated from the distillate as a flocculent precipitate.

Urinary excretion products. Unchanged 2:3:5:6-tetrachloroaniline separated when the urine was acidified (pH 3). Yield 0.3 g. or 15% of the dose. Estimation of glucuronic acid and ethereal sulphate in 48 hr. urines indicated that 60% of a dose of 2:3:5:6-tetrachloroaniline was excreted as glucuronide and 10% as ethereal sulphate. Thus 70% of the dose, or 85% of absorbed 2:3:5:6-tetrachloroaniline, is hydroxylated. Mercapturic acid is apparently not formed since there was no significant increase in iodine titration after dosage, and no mercapturic acid was detected in the acid precipitate of the urine.

Metabolism of 2:3:4:5-tetrachloronitrobenzene

Excretion of faeces. Owing to its toxicity this compound was not usually administered for quantitative experiments at dose levels greater than 0.3 g./kg. From 27 to 36% (average 33%) of a dose of 2:3:4:5-tetrachloronitrobenzene was excreted as a mixture of unchanged compound and tetrachloroaniline in the faeces passed during 48 hr. after dosage. This was mainly excreted during the first 24 hr. and none was found after 48 hr. The solid material isolated from the faeces by steam distillation gave a strongly positive diazo reaction similar to that given by 2:3:4:5-tetrachloroaniline. Both 2:3:4:5-tetrachloronitrobenzene and 2:3:4:5-tetrachloroaniline were isolated from this material. Estimation by the diazo method indicated that 20% was in the reduced form. This extensive reduction of 2:3:4:5-tetrachloronitrobenzene in the gut suggests that at least a part of the tetrachloroaniline appearing in urine (see below) may be formed before absorption.

Urinary excretion products. The quantitative results obtained are summarized in Table 3. The values give the total excretion in 48 hr. No metabolites were detected in the third day's urine. Allowing for the failure to absorb 33%, an average of 91% of a dose of 0.7 g. can be accounted for. In one experiment (not included in Table 3) a value of 17% was obtained for glucuronide. This was almost certainly due to anorexia since the baseline on the 2nd day after dosage was considerably lower than before dosage. Determinations of free or conjugated phenols were not made since 2-amino-3:4:5:6-tetrachlorophenol was not available as a standard. Estimation of amino compounds by the Bratton & Marshall (1939) method was unsatisfactory since the diazo colour produced in urines (and in ether extracts of the urines) was orange whereas the colour given by 2:3:4:5-tetrachloroaniline was the usual red. This may have been due to the presence of 2-amino-3:4:5:6-tetrachlorophenol which would be expected to give the yellow colour typical of diazo oxides when treated with nitrous acid.

Isolation of 2:3:4:5-tetrachloroaniline from urine. Tetrachloroaniline was isolated from the 24 hr. urine of rabbits dosed with 2:3:4:5-tetrachloronitrobenzene either by continuous extraction with ether from the urine adjusted to pH 4, by steam distillation of the urine, or by precipitating from the urine by addition of acid. For example, the urine from four rabbits which had each received 0.7 g. 2:3:4:5-tetrachloronitrobenzene was adjusted to pH 1 by addition of conc. HCl. The precipitate was collected and recrystallized from aqueous acetone as colourless needles, m.p. 118°

Table 3. *The amounts of metabolites excreted in urine in 48 hr. by rabbits after administration of 2:3:4:5-tetrachloronitrobenzene*

(Results are expressed as percentage of the dose.)

Metabolite	No. of exp.	Dose (g.)	Range	Average
Ether glucuronide	8	0.7	29-57	41
	1*	1.0	—	54
Ethereal sulphate	4	0.7	3-10	6
	1*	1.0	—	5
Mercapturic acid	2	0.7	0	0
2:3:4:5-Tetrachloroaniline	1†	0.7	—	11
	1†	1.0	—	16

* Pooled urine of five rabbits each given dose of 1 g.

† By isolation from pooled urines of several rabbits.

not depressed by admixture with authentic specimen of 2:3:4:5-tetrachloroaniline, m.p. 118°. Yield 0.12 g. A further 0.14 g. was obtained by steam distillation of the residual urine. Total yield 10.5% of dose. The urine left after steam distillation was hydrolysed by boiling under reflux for 2.5 hr. with H_2SO_4 (equal vol. 10N). This was made just alkaline, and a small amount (11 mg., 0.5% of dose) of tetrachloroaniline was obtained by steam distillation. This suggests that the tetrachloroaniline is excreted almost entirely free and that the extent of acetylation, if any, must be very small. No mercapturic acid was detected either in the acid precipitate from urine or in ether extracts, and no 2:3:4:5-tetrachloroacetanilide was obtained from ether extracts. It may be noted that if 2:3:4:5-tetrachloroaniline is present in urine hydrolysed with H_2SO_4 it is extracted by ether as a *sulphate* forming platelets, m.p. 285° (decomp.) after darkening at 260°. (Found: equiv. 160. $\text{C}_6\text{H}_5\text{NCl}_4 \cdot \text{H}_2\text{SO}_4$ requires 164.) Recrystallization from ethanol in absence of H_2SO_4 gave 2:3:4:5-tetrachloroaniline. The sulphate could be prepared by extracting with ether the suspension formed by boiling 2:3:4:5-tetrachloroaniline in 5N- H_2SO_4 .

Attempted isolation of glucuronide and phenolic metabolites. The glucuronide syrup obtained by the usual lead procedure could not be crystallized. It was hydrolysed by heating on a boiling-water bath for 2 hr. with HCl (equal vol. 10N), and the hydrolysate was extracted with ether at pH 8. From the extract a yellow syrup was obtained which gave a strongly positive Folin and Ciocalteu reaction, but no crystalline compound could be isolated. Other attempts to isolate a phenol from hydrolysed 2:3:4:5-tetrachloro-nitrobenzene urines were also unsuccessful. The separation of black tarry material during hydrolysis suggests that the phenolic metabolite is unstable.

Reduction of aromatic compounds in the intestine

The stomach and intestines were removed from a freshly killed rabbit. Samples of the contents of (i) the stomach, (ii) the small intestine and (iii) the large intestine were suspended in water, incubated at 37° with 2:3:4:5-tetrachloro-nitrobenzene (0.2 g.) for 48 hr. and then filtered. The filtrates from (ii) and (iii) gave strong cherry-red diazo colours but that from (i) gave a negative reaction.

One-tenth of the mixed contents of the small and large intestine of a rabbit were made up to 50 ml. in water, and sodium *p*-nitrobenzoate (50 mg. in 10 ml.) added. The mixture was incubated at 37° and samples (5 ml.) were withdrawn at hourly intervals. The samples were mixed with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5 ml. 10% w/v) and NaOH (5 ml. 0.5N) and filtered. The amino compound present in the filtrates was determined by the Bratton & Marshall (1939) method using *p*-aminobenzoic acid as standard. Some results obtained are shown in Fig. 1. It can be seen that more than 7% of *p*-nitrobenzoic acid was reduced in 1 hr. and 24% in 3 hr. In 24 hr. over 60% was reduced. This suggests that slowly absorbed compounds which remain in the gut for a long time may be largely reduced before absorption. Reduction at similar rates was observed with 2:3:4:5-tetrachloronitrobenzene, nitrobenzene, *m*-nitrophenol and *o*-, *m*- and *p*-nitrobenzoic acids and their amides. *o*- and *p*-Nitrotoluenes and *p*-nitrophenol were reduced very slowly.

Little reduction was observed when *p*-nitrobenzoic acid was incubated for 3 hr. with the contents of the small

intestine of the rat and only 7% after 18 hr. When the contents of the large intestine were used, however, reduction was appreciable after 15 min. and 10, 20 and 74% had been reduced after 1, 2 and 18 hr. respectively.

Reduction of 2:3:4:5-tetrachloronitrobenzene and p-nitrobenzoic acid by bacteria from rabbit intestine

Agar slopes were inoculated with intestinal contents and incubated for 48 hr. at 37°. Three successive subcultures were made and a final one in three Petri dishes containing (a) medium with 2:3:4:5-tetrachloronitrobenzene, (b) medium with *p*-nitrobenzoic acid, and (c) medium alone. Round white colonies appeared in (a) after 72 hr. and in (b) after 6 weeks. The media were dissolved in water and the diazo reaction applied; in cultures (a) and (b) a positive reaction was obtained while (c) gave a negative reaction. This indicates that the reduction is bacterial in origin.

Fig. 1. Reduction of sodium *p*-nitrobenzoate by intestinal contents of the rabbit at 37°.

Reduction of 2:3:4:5-tetrachloronitrobenzene by liver extract

Incubation of 2:3:4:5-tetrachloronitrobenzene with rabbit-liver extract for 3 hr. caused some reduction to the corresponding aniline. The diazo reaction was intense after 24 hr. The reduction of the nitrobenzoic acids and amides by liver extracts has been reported previously (Bray, Thorpe & Wood, 1949).

DISCUSSION

The quantitative results obtained with the two tetrachloronitrobenzenes and 2:3:5:6-tetrachloroaniline are compared in Table 4, in which the values are expressed as a percentage of the amount of compound absorbed, i.e. the amount of unabsorbed material has been deducted from the actual dose administered. The most conspicuous differences between the results for 2:3:5:6 compounds are in the amounts of ethereal sulphate and mercapturic acid excreted. The nitro compound yields a relatively large amount of mercapturic acid but very little ethereal sulphate, whereas administration of the amine, which is formed from it *in vitro*, gives no

Table 4. *Excretion of metabolites of 2:3:5:6- and 2:3:4:5-tetrachloronitrobenzenes and of 2:3:5:6-tetrachloroaniline by the rabbit*

(Results are expressed as the average percentage of the absorbed dose. Absorbed dose = actual dose administered less material found in faeces after 48 hr.)

	2:3:5:6-Tetrachloro- nitrobenzene	2:3:5:6-Tetra- chloroaniline	2:3:4:5-Tetrachloro- nitrobenzene
Absorbed dose (g.)	0.58	0.82	0.47
Metabolite:			
Tetrachloroaniline	23	18	16
Tetrachloroaminophenol	5	—	—
Glucuronide	31	73	61
Ethereal sulphate	3	12	9
Mercapturic acid	28	0	0
Total accounted for	90	103	86

detectable mercapturic acid but considerable ethereal sulphate. The extent of hydroxylation is, in fact, very much greater with the amine, since not only is 12% of the absorbed dose excreted as ethereal sulphate but also the glucuronide output is more than doubled; 85% of absorbed 2:3:5:6-tetrachloroaniline is hydroxylated and conjugated compared with 34% of the nitro compound. Comparable amounts of free tetrachloroaniline are excreted after administration of the two compounds and all identified metabolites are amino compounds.

tetrachloroaminophenol formed from it will be lower than when preformed tetrachloroaniline is administered. This would account for the total extent of hydroxylation being so much less when tetrachloronitrobenzene is administered, but under these conditions it would be expected that the proportion of tetrachloroaminophenol conjugated with sulphuric acid would be greater, giving a glucuronide/sulphate ratio, G/S, lower than that obtained when a higher body level of the aminophenol is achieved by administration of tetrachloroaniline

It seems unlikely that the failure to excrete an appreciable amount of ethereal sulphate after a dose of 2:3:5:6-tetrachloronitrobenzene can be ascribed to a limitation of sulphur available for the conjugation since it has been shown that the rabbit can normally provide about 5 mg. sulphur/hr. for ethereal sulphate formation (Bray *et al.* 1952b). The total amount of sulphur required to produce the mercapturic acid, which is excreted over at least 48 hr., is only 20 mg. Tetrachloronitrobenzene is absorbed with great difficulty and its metabolites are excreted up to 3 days after dosage, whereas tetrachloroaniline is both more readily absorbed and more rapidly eliminated. Tetrachloronitrobenzene is, in effect, being given in repeated very small doses so that the body levels of tetrachloroaniline and

(see Bray, Thorpe & White, 1952c). It seems unlikely that a satisfactory explanation of the small ethereal sulphate excretion after giving tetrachloronitrobenzene can be given without knowledge of the kinetics of the reactions involved. It may be relevant that a significant part of a dose of tetrachloronitrobenzene is excreted as unconjugated tetrachloroaminophenol. The absence of the mercapturic acid formation after administration of tetrachloroaniline must be ascribed to mercapturic acid being formed only from the nitro compound or some intermediate in its reduction (see below). The experimental results can be summarized in the scheme above in which the percentage figures indicate the proportion of a dose of 1.5 g. converted. There is at present no clear evidence as to the extent

to which 2:3:5:6-tetrachloronitrobenzene may be reduced by bacterial reduction before absorption. The amount of 2:3:5:6-tetrachloroaniline formed in the gut would appear to be small, judging by the examination of faeces, in which only traces of the compound were found, and the fact that the tetrachloroaniline is itself not rapidly absorbed (18% of a dose of 2:3:5:6-tetrachloroaniline was found in faeces after 24 hr.). The formation of the mercapturic acid and the low sulphate conjugation would also support the view that 2:3:5:6-tetrachloronitrobenzene is absorbed mainly as such. No unequivocal evidence of acetylation of any of the amino compounds was obtained.

The isolation of *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-*L*-cysteine is of interest, not only because this appears to be the first example of the formation of a mercapturic acid from a polysubstituted aromatic compound, but also because the attachment of the cysteic group has involved the elimination of the N attached to C in the benzene ring. In previously described halogeno-mercapturic acids the acetylcysteic group has replaced H attached to the C in the benzene ring which is in the *para* position to the halogen atom (see Williams, 1947). It is reasonable to conclude that *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-*L*-cysteine is formed either directly from tetrachloronitrobenzene or from some intermediate product of its reduction to tetrachloroaniline, but not from tetrachloroaniline itself since no mercapturic acid is formed when tetrachloroaniline is administered. It may be pertinent that in tetrachloronitrobenzene C₍₁₎ is surrounded by three electron-attracting groups, the chlorine atoms attached to C₍₂₎ and C₍₆₎ and the nitro group. No increase in excretion of nitrite, which might be expected if the N was liberated from some possible intermediates, e.g. tetrachlorophenylhydroxylamine, was detected in urines passed while 2:3:5:6-tetrachloronitrobenzene was being metabolized. It is hoped that a study of mercapturic acid formation in some related com-

pounds will help to elucidate the mechanism of this reaction.

The metabolism of 2:3:4:5-tetrachloronitrobenzene differs from that of its symmetrical isomer in several respects. More is absorbed; more glucuronide and appreciable amounts of ethereal sulphate are excreted. No mercapturic acid formation was detected. The general quantitative pattern, in fact, resembles that obtained after administration of 2:3:5:6-tetrachloroaniline, which appears to be absorbed and metabolized at much the same rate as 2:3:4:5-tetrachloronitrobenzene and more rapidly than the symmetrical isomer. The observed results can be summarized in the following scheme:

The percentage figures indicate the proportion of a dose of 0.7 g. converted. With this isomer it has been shown that 20% of the material in faeces is in the form of 2:3:4:5-tetrachloroaniline. This makes it probable that at least some of the dose is absorbed in the reduced form. Some of the material absorbed is excreted as free 2:3:4:5-tetrachloroaniline. 6-Amino-2:3:4:5-tetrachlorophenol was not isolated. For both isomers, the failure to detect nitro compounds amongst the metabolites, and isolation of tetrachloronitrobenzenes precedes hydroxylation.

The observation that several aromatic nitro compounds are readily reduced by bacteria in intestinal contents shows that this factor should be considered when the fate of aromatic nitro compounds is studied. If the compound is rapidly absorbed the extent of reduction may only be small, e.g. the nitrobenzoic acids and amides are only slightly reduced by the rabbit (Bray *et al.* 1949), but if compounds are absorbed slowly it is possible that virtually all the reduction observed may have taken place in the intestine before absorption. There is, however, evidence that reduction of nitro compounds can also take place within the body, since 2:3:4:5-tetrachloronitrobenzene injected intraperitoneally gave rise to the excretion of diazotizable compounds, and liver extracts have been shown to

reduce nitro compounds. The observation that nitro compounds may be reduced by intestinal contents has also been made by Glazko, Wolf, Dill & Bratton (1949) in a study of chloramphenicol (2-dichloro-acetamido-1-*p*-nitrophenyl-1:3-propanediol).

The isolation of *m*-hydroxybenzoic acid from urine after administration of 2:3:5:6-tetrachloronitrobenzene is of interest. This compound cannot reasonably be considered a metabolite of 2:3:5:6-tetrachloronitrobenzene and its isolation can be compared with that of the same compound by Bielig & Hayashida (1940) from the urines of rabbits dosed with β -ionone. Its isolation from normal rabbit urine has not yet been reported, although Lederer & Polonsky (1948) obtained it from the urine of pregnant mares. Bray *et al.* (1950*a*) obtained evidence on paper chromatograms that it was present in normal rabbit urine in greater amounts than the para isomer.

SUMMARY

1. The metabolism of 2:3:5:6- and 2:3:4:5-tetrachloronitrobenzenes in the rabbit has been studied.

2. The compounds are poorly absorbed, an average of 66 % of the 2:3:5:6- and 33 % of the 2:3:4:5-isomer being excreted in faeces.

3. The average percentages of a dose of 2:3:5:6-tetrachloronitrobenzene excreted in urine as 2:3:5:6-tetrachloroaniline, glucuronide, ethereal sulphate

and mercapturic acid were 10, 12, 1 and 11 respectively. Some 4-amino-2:3:5:6-tetrachlorophenol was excreted unconjugated. This metabolite, 2:3:5:6-tetrachloroaniline and *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine were isolated.

4. *m*-Hydroxybenzoic acid was isolated from urine after administration of 2:3:5:6-tetrachloronitrobenzene.

5. When 2:3:5:6-tetrachloroaniline was given to rabbits the average percentages of the dose excreted were 18 % unchanged in faeces, 15 % unchanged in urine, 60 % as glucuronide and 10 % as ethereal sulphate. No mercapturic acid formation was detected.

6. The average percentages of a dose of 2:3:4:5-tetrachloronitrobenzene excreted in urine as 2:3:4:5-tetrachloroaniline, glucuronide and ethereal sulphate were 13, 46 and 6 respectively. 2:3:4:5-Tetrachloroaniline was isolated. No mercapturic acid formation was detected.

7. Various aromatic nitro compounds are reduced by intestinal bacteria.

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