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The Metabolism of 2:3-, 2:6- and 3:5-Dichloronitrobenzene and the Formation of a Mercapturic Acid from 2:3:4:5-Tetrachloronitrobenzene in the Rabbit

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Previous papers (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Bray, James & Thorpe, 1956, 1957; Betts, Bray, James & Thorpe, 1957) have described the metabolism in the rabbit of all members of the chloromononitrobenzene series except 2:3-, 2:6- and 3:5-dichloronitrobenzene. One aspect of the metabolism of these compounds of particular interest is the readiness with which mercapturic acids are formed by most members of the series. Furthermore, these mercapturic acids are all formed, in effect, either by acetylcysteylidenitration or by acetylcysteyldechlorination, and not by acetylcysteyldeprotonation as had been observed in all previous instances of the formation of nuclear mercapturic acids (for references see Bray *et al.* 1956). The present paper describes the metabolic fate of 2:3-, 2:6- and 3:5-dichloronitrobenzene. No conclusive evidence has been obtained for the formation of mercapturic acid by these three compounds.

The early work on tetrachloronitrobenzenes (Bray *et al.* 1953) was published before an example of nuclear acetylcysteyldechlorination had been discovered. When this type of mercapturic acid formation was observed (Bray *et al.* 1956, 1957; Betts *et al.* 1957) it became necessary to re-examine the tetrachloronitrobenzenes to see whether a product of acetylcysteyldechlorination had been overlooked, especially with 2:3:4:5-tetrachloronitrobenzene, from which no mercapturic acid had been obtained. The search for a mercapturic acid formed by elimination of chlorine was facilitated by the fact that the resulting mercapturic acid retained the nitro group and, therefore, could be more readily detected by its colour in alkaline solution if only small amounts were present. It has, in fact,

now been shown that 2:3:4:5-tetrachloronitrobenzene does form a small amount of mercapturic acid by acetylcysteyldechlorination. The previous finding that no *N*-acetyl-*S*-(2:3:4:5-tetrachlorophenyl)-*L*-cysteine is formed has been confirmed.

MATERIALS

All melting points recorded are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn. 2:3- and 3:5-Dichloronitrobenzene were the gift of Bayer Products Ltd. Some 3:5-dichloronitrobenzene as well as 2:6-dichloronitrobenzene was obtained by the method of Holleman (1904).

2:3-Dichloroaniline was purchased; 2:6- and 3:5-dichloroaniline, m.p. 49° and 39° respectively, were prepared by reduction of the nitro compounds. The three bases were converted into the corresponding dichloroacetanilides, m.p. 159°, 174° and 187° respectively. The *picrate* of 2:6-dichloroaniline formed orange needles from water, m.p. 89° (Found: 2:6-dichloroaniline, 42.5. $C_{12}H_8O_7N_4Cl_2$ requires 2:6-dichloroaniline, 41.2%).

3:5:3':5'-Tetrachloroazoxybenzene was prepared by boiling 3:5-dichlorophenylhydroxylamine in 96% (w/v) ethanol for 15 min. according to the method of Haworth & Lapworth (1921). It formed fine colourless threads, m.p. 168°, from aqueous ethanol (Found: C, 42.7; H, 1.7; N, 8.3; Cl, 42.7. $C_{12}H_6ON_2Cl_4$ requires C, 42.9; H, 1.8; N, 8.3; Cl, 42.2%).

N-Acetyl-*S*-(2-chloro-6-nitrophenyl)-*L*-cysteine. This was prepared from 2-chloro-6-nitroaniline by the method of Parke & Williams (1951) as modified by Bray *et al.* (1956). 2-Chloro-6-nitroaniline was obtained by hydrolysis of 2-chloro-6-nitroacetanilide prepared as described by Franzen & Engel (1921). The mercapturic acid was also made by acetylation (cf. Zbarsky & Young, 1943) of *S*-(2-chloro-6-nitrophenyl)-*L*-cysteine obtained by interaction of 2:3-dichloronitrobenzene and cysteine in alkaline solution (Suter, 1895). *N*-Acetyl-*S*-(2-chloro-6-nitrophenyl)-*L*-cysteine formed pale-yellow rectangular plates from water, m.p. 185°

(decomp.), $[\alpha]_D^{25}$ in ethanol +72 $\pm$ 5° (c, 0.31); light-absorption max. in 0.1N-KOH, 260 m μ (ϵ , 4776), min., 241 m μ (Found: C, 41.6; H, 3.7; N, 8.9; Cl, 11.3; S, 9.7%; equiv. 310. C₁₁H₁₁O₅N₂ClS requires C, 41.5; H, 3.5; N, 8.8; Cl, 11.1; S, 10.1%; equiv. 319).

3:4-Dichloro-2-nitrophenol, m.p. 76°, was prepared by the method of Hodgson & Kershaw (1929), 3:5-dichloro-4-nitrophenol, m.p. 146–148°, by that of Hodgson & Wignall (1927), 2:4-dichloro-3-nitrophenol, m.p. 70°, by that of Groves, Turner & Sharp (1929) and 2:4-dichloro-6-nitrophenol, m.p. 121–122°, by the action of KClO₃ and HCl on *o*-nitrophenol (cf. Holleman, 1904). 2:6-Dichloro-4-nitrophenol, m.p. 123°, was purchased. The melting points of these compounds were substantially in agreement with those given in the literature.

Aminodichlorophenols were prepared by the action of alkaline dithionite on the corresponding dichloronitrophenols except 3:4-dichloro-2-nitrophenol, which did not yield the expected 2-amino-3:4-dichlorophenol. 3-Amino-2:4-dichlorophenol formed colourless needles from water, m.p. 83° (Found: N, 7.8; Cl, 39.7. C₆H₅ONCl₂ requires N, 7.9; Cl, 40.1%). This compound reacted abnormally with the Bratton & Marshall (1939) reagents (see Results section). At the nitrous acid stage it gave a very intense yellow colour, although the addition of the coupling reagent produced an immediate magenta colour. The yellow colour could be readily distinguished from that of the diazo oxides formed by *o*-aminophenols not only by the greater intensity of the colour but also by the rapidity with which the red colour is formed when the coupling reagent is added. With *o*-aminophenols the formation of the azo dye is relatively slow and the colour intensity is relatively weak. The melting points of 4-amino-3:5-dichlorophenol and 4-amino-2:6-dichlorophenol agreed with those previously recorded, but 2-amino-4:6-dichlorophenol (Found: N, 8.4. C₆H₅ONCl₂ requires N, 7.9%) had m.p. 92°. Hunter & Barnes (1928) give 109° (decomp.).

METHODS

Animals, diet and dosage. Doe rabbits (2–3 kg. body wt.) were maintained as described by Bray, Ryman & Thorpe (1947). Compounds were administered by stomach tube as suspensions in water. The dose levels were: 2:3-isomer, 0.1 g./kg.; 2:6-isomer, 0.3 g./kg.; 3:5-isomer, 0.2 g./kg. Dose levels above these were usually fatal; even the stated dose level for the 3:5-isomer was occasionally fatal.

Determination of metabolites

The methods used for determination of ethereal sulphate, glucuronic acid, reducing material, mercapturic acid, dichloroanilines and catechols were as given by Bray *et al.* 1957. The concentration of NaOH used for hydrolysis of mercapturic acid was 2N and the time of boiling was 1 hr. Recoveries of dichloroanilines added to urine were 97, 69, and 100% from the 2:3-, 2:6- and 3:5-isomers respectively.

Qualitative examination

Faeces. Steam-distillates of the 72 hr. faeces were examined for unabsorbed dichloronitrobenzene and dichloroaniline by determination of dichloroanilines before and after reduction.

Urine. Ether extracts A, B, C and D were prepared from urines as described by Bray *et al.* (1956). Briefly these were:

A, from urine at pH 7–8; B, from the residual urine from A adjusted to pH 1; C, from the residual urine from B hydrolysed with an equal volume of 10N-H₂SO₄; D, from residual urine from C adjusted to pH 7.

Paper chromatography. The procedure was as described by Bray, Thorpe & White (1950) except that to minimize decomposition of aminophenols papers were dried without heating. The solvents and detecting reagents used and the *R_F* values of reference compounds are given in Table 3. For the application of the indophenol test (Thorpe, Williams & Shelswell, 1941) papers were sprayed with 0.5N-Na₂CO₃, to which bromine had been added until just yellow, followed by 5% (w/v) aqueous phenol. The sodium hypobromite was freshly prepared and used within 1 hr. *p*-Aminophenols give a deep-blue colour changed to red by spraying with *n*-acetic acid.

In addition to the direct examination for mercapturic acids on paper chromatograms, extracts B of the urines were hydrolysed by refluxing with N-NaOH for 1 hr. and steam-distilled. The distillates were examined for thiophenols by paper chromatography as described by Parke & Williams (1955).

Isolation of metabolites

Unless stated otherwise all metabolites isolated were shown to be identical with synthetic specimens, by mixed m.p. and, except 3:5:3':5'-tetrachloroazoxybenzene, by paper chromatography. The methods were essentially as described by Bray *et al.* (1957).

Mercapturic acid from 2:3:4:5-tetrachloronitrobenzene

The 24 hr. urine of five rabbits, which had each received 0.7 g. of 2:3:4:5-tetrachloronitrobenzene, was acidified (pH 1) and continuously extracted with ether. The acids were separated from the ethereal solution by extraction with 0.5N-NaHCO₃. The aqueous extract was acidified and extracted with ether. The syrup obtained on evaporation of the solvent from the ether extract was dissolved in ethanol, placed on a column of Whatman paper powder (34 cm. long, 3 cm. diameter) and eluted with the upper layer of solvent mixture C (Table 3). That fraction of the eluate which was yellow was washed with water and evaporated to dryness under reduced pressure. The residue was dissolved in acetone and water was added until a turbidity developed. A small amount of charcoal was added and the solution was filtered. The crystals which separated from the filtrate were recrystallized from aqueous acetone and gave 27 mg. (0.5% of dose) of sheaves of yellow needles, m.p. 178° (decomp.), light-absorption max. 267 m μ (ϵ , 6103), min., 257 m μ (Found: N, 7.2; Cl, 26.7. C₁₁H₅O₅N₂Cl₄S requires N, 7.2; Cl, 27.5%).

RESULTS

The average daily excretions of normal metabolites by the rabbits used were of the same order as those found previously (e.g. Bray *et al.* 1953). The results obtained from quantitative analysis of the urines of rabbits dosed with dichloronitrobenzenes are summarized in Table 1 which also shows that about 5% of a dose of 2:3- and 3:5-dichloronitrobenzene and 1% of a dose of 2:6-dichloronitrobenzene is unabsorbed, the greater part of the unabsorbed material being reduced to the aniline. None of the

Table 1. *Excretion of metabolites of 2:3-, 2:6- and 3:5-dichloronitrobenzene by the rabbit*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. Urine was collected for 72 hr. except in experiments (indicated by superior m) where separate consecutive samples of urine, which were collected from rabbits given water at intervals (see Bray, Thorpe & White, 1951), were analysed until excretion of metabolites could no longer be detected. — Indicates not determined.

Dose (g.)	2:3-Dichloronitrobenzene		2:6-Dichloronitrobenzene	3:5-Dichloronitrobenzene	
	0.3	0.3	1.0	0.6	0.5
Metabolite					
Unabsorbed					
Dichloronitrobenzene	—	1.5 (0, 3) ²	0.5	—	0.4 (0.2, 0.6) ²
Dichloroaniline	—	3.5 (2, 5) ²	0.35 (0.4, 0.3) ²	—	4.0 (3, 5) ²
Mercapturic acid	13 ^m (12–14) ³	—	0 (0) ³	0 (0) ³	0 (0) ³
Glucosiduronic acid	33 ^m (30, 36) ²	45 (11–90) ⁵	45 (27–64) ⁴	59 (29–106) ⁵	76 (62–80) ³
Copper-reducing material (as glucuronic acid)	—	24 (12–35) ⁵	22 (11–27) ⁴	—	48 (44–56) ³
Ethereal sulphate	16 ^m (6–24) ³	—	10 (7–15) ³	8 (4–15) ⁵	—
Dichloroaniline, free	—	14 (7–17) ⁹	4 (2–7) ⁵	32 (22–45) ¹⁵	24 (16–32) ³
Dichloroaniline, combined	—	5 (3–8) ³	0.1†	0	—
'Catechols'	—	10† (7–15) ³	—	—	—
Azoxy compound	—	0	0	>2	>2

* It is very doubtful whether this represents mercapturic acid (see text).

† On pooled urine from two rabbits.

‡ For interpretation see text.

three dichloronitrobenzenes was found in urine in the form of mercapturic acid. The urine from animals given 2:3-dichloronitrobenzene had an uptake of iodine by the Stekol (1936) method equivalent to a conversion of about 13 % of the dose into mercapturic acid, but no evidence for the presence of a mercapturic acid could be obtained even by paper chromatography (see below). If such an amount had been present it should have been possible to isolate some of it, especially as a synthetic specimen of the expected mercapturic acid was available as a guide to its properties. The explanation of the iodine uptake is unknown. With 3:5-dichloronitrobenzene the urine had a marked uptake of iodine before, but not after, alkaline hydrolysis. The excretion of a large proportion of the dose as glucosiduronic acid and ethereal sulphate supports the expectation that some of each isomer would be hydroxylated. Approximately half of the glucuronic acid was excreted in a form which reduced alkaline cupric solutions, but there is no evidence to show whether this is due to an *N*-glucosiduronic acid (cf. Boyland, Manson & Orr, 1957) or a labile ether-type glucosiduronic acid. With the 2:6-isomer large quantities of an *N*-

glucosiduronic acid are ruled out because only 4 % of the dose was excreted as dichloroaniline. The ethereal sulphate is presumably formed from amino- or nitro-dichlorophenols. With the 2:3- and 3:5-isomers considerable reduction occurred and most of the base was excreted as such. 2:3-Dichloronitrobenzene was the only isomer from which significant amounts of the aniline were excreted combined. The 'catechol' determination, with 4-chlorocatechol as standard, gave results corresponding to an excretion of 10 % of the dose of 2:3-dichloronitrobenzene as 'catechol'. No catechol derivative, however, could be detected in the urine by paper chromatography. The probable explanation is that the blue colour obtained with the reagents of Azouz, Parke & Williams (1953) is caused by 4-amino-2:3-dichlorophenol, since an extract D, in which this aminophenol but no catechol was detected, gave a blue colour when treated with alkali (see Table 3) or with the reagents of Azouz *et al.* (1953) under the conditions for determination of catechol. With 2:6-dichloronitrobenzene, from which a catechol derivative was identified as a metabolite, the quantitative method could not be applied since 4-amino-3:5-dichlorophenol gave a calibration curve

with a slope half of that obtained with 4-chlorocatechol. 4-Amino-2:3:5-trichlorophenol also gives a blue colour with the reagents so that results obtained by the method of Azouz *et al.* (1953) should be accepted with caution when aminophenols may be present.

Metabolites isolated or detected

The metabolites isolated and the yields obtained are listed in Table 2 and the metabolites detected by paper chromatography in extracts A, B, C and D of the urines are given in Table 3. Authentic specimens

Table 2. *Metabolites isolated from urine of rabbits given 2:3-, 2:6- and 3:5-dichloronitrobenzene*

Solvents for recrystallization: E, ethanol; W, water. Those given in parentheses are for the derivative isolated.

Nitrobenzene derivative administered	Dose/rabbit (g.)	No. of rabbits	Compound isolated	Solvent	Yield (% of dose)
2:3-Dichloro-	0.3	6	2:3-Dichloroaniline (as acetyl deriv.)	(WE)	5
2:6-Dichloro-	1.0	3	2:6-Dichloroaniline (as picrate)	(W)	2
	1.0	3	4-Amino-3:5-dichlorophenol	W	7
3:5-Dichloro-	0.6	3	3:5-Dichloroaniline (as acetyl deriv.)	(WE)	3
	0.5	6	3:5:3':5'-Tetrachloroazoxybenzene	E	2
	0.6	6	2-Amino-4:6-dichlorophenol	W	0.6
	0.8	10	4-Amino-2:6-dichlorophenol	WE	0.1

Table 3. *R_F values and detection of some possible metabolites of 2:3-, 2:6- and 3:5-dichloronitrobenzene*

Solvent mixtures and times of run on Whatman no. 4 paper: A, benzene-acetic acid-water (2:2:1, by vol.) (1.5 hr.); B, light petroleum (b.p. 90°)-benzene-98% formic acid (3:1:2, by vol.) (1 hr.); C, butanol-3N-(NH₄)₂CO₃-aq. 3N-NH₃ soln. (4:3:3, by vol.; Corner & Young, 1954) (2 hr.). Detecting reagents: a, 2N-HCl, 0.1% NaNO₂, 0.5% ammonium sulphamate and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); b, diazotized p-nitroaniline followed by 20% (w/v) Na₂CO₃ (Bray *et al.* 1950); c, 20% (w/v) Na₂CO₃; d, indophenol reaction (see Methods). Colours of spots: B, blue; Bn, brown; G, green; Gy, grey; M, magenta; O, orange; P, purple; R, red; Y, yellow; d, deep; p, pale; vp, very pale. Synthetic specimens of the compounds in parentheses were not available for comparison; the arguments for the suggested constitutions are given in the Results section.

Metabolites were sought in extracts A, B, C and D (see Methods). Compounds found in extracts of hydrolysed urine (C and D) are probably excreted in combined form. +, Present; (+), present in traces; -, absent.

	<i>R_F</i> values in solvent mixture			Colour with detecting reagent				Detected in extract			
	A	B	C	a	b	c	d	A	B	C	D
Derivatives of 2:3-dichloronitrobenzene											
(2-Amino-3:4-dichlorophenol)	0.90	0.15*	0.90	vpR†	—	—	—	—	(+)	(+)	(+)
(3-Amino-4:5-dichlorophenol)	0.71	0.25*	0.82	M	—	—	—	(+)	(+)	—	—
(4-Amino-2:3-dichlorophenol)	0.77	0.14*	0.72	B	pG	B	B	(+)	—	(+)	+
3:4-Dichloro-2-nitrophenol	1.0	1.0	0.83	—	—	Y	—	—	—	—	—
(4:5-Dichloro-3-nitrophenol)	1.0	0.50	0.68	—	—	Y	—	+	(+)	(+)	—
N-Acetyl-S-(2-chloro-6-nitrophenyl)-L-cysteine	—	0.0	0.46	—	—	vpY†	—	—	—	—	—
2:3-Dichloroaniline	1.0	1.0	0.94	M	pY	—	—	+	+	(+)	—
Derivatives of 2:6-dichloronitrobenzene											
3-Amino-2:4-dichlorophenol	1.0	0.80*	0.72	M§	RBn	—	GyBn	—	—	—	—
4-Amino-3:5-dichlorophenol	0.88	0.15*	0.90	M	pG	GyB	B	+	+	+	+
2:4-Dichloro-3-nitrophenol	—	0.78*	0.81	—	—	Y	—	+	—	—	—
3:5-Dichloro-4-nitrophenol	—	0.43*	0.75	—	—	Y	—	—	—	—	—
(3:5-Dichloro-4-nitrocatechol)	0.52	0.0	0.53	—	—	pO	—	—	(+)	—	—
2:6-Dichloroaniline	1.0	1.0	0.90	pOR	—	—	—	+	—	—	—
Derivatives of 3:5-dichloronitrobenzene											
2-Amino-4:6-dichlorophenol	1.0	0.0	0.72	vpR†	BnG	Bn	Bn	—	+	+	+
4-Amino-2:6-dichlorophenol	0.55	0.15	0.50	B	pG	Gy	B	(+)	—	(+)	+
2:4-Dichloro-6-nitrophenol	1.0	1.0	0.72	—	—	dY	—	—	—	—	—
2:6-Dichloro-4-nitrophenol	0.96	0.60	0.59	—	—	Y	—	—	—	—	—
3:5-Dichloroaniline	1.0	1.0	0.86	M	—	—	—	+	+	(+)	—

* Approximate value; elongated spots.

† Black in ultraviolet light.

§ Intense yellow with nitrous acid (see Materials section).

|| Detected by 0.2% FeCl₃, which gave typical catechol green turned red by NH₃.

of the possible aminophenols from 2:3-dichloronitrobenzene were not available but the structure of the aminophenols can be attributed with fair certainty on the basis of the chemical tests which distinguish between *o*-, *m*- and *p*-aminophenols. *o*-Aminophenols give a bright-yellow diazo oxide colour at the nitrous acid stage of the Bratton & Marshall (1939) test and the final colour appears slowly, *m*-aminophenols give usually a magenta colour immediately with the Bratton & Marshall reagents and *p*-aminophenols give usually a bluish colour slowly. *p*-Aminophenols are readily identified by the indophenol test (see Methods). Only the following metabolites require comment.

From 2:3-dichloronitrobenzene. There are three possible aminodichlorophenols and an *o*-, an *m*- and a *p*-aminophenol were detected. It is probable that these aminophenols have the constitutions shown in Table 3 since the only other phenols which could be confused with these would be those formed by hydroxyldechlorination (cf. Betts *et al.* 1957) of 2:3-dichloroaniline, i.e. either 2-amino-6-chlorophenol or 3-amino-2-chlorophenol. R_f values (Bray *et al.* 1956) distinguish the former (0.8 in solvent *A*) from 2-amino-3:4-dichlorophenol (0.9). 3-Amino-2-chlorophenol has almost the same R_f value (0.7) in solvent *A* as has 3-amino-4:5-dichlorophenol (0.71) but in chloroform-acetic acid-water (2:1:1, by vol.; 2.2 hr.) the R_f values are 0.91 and 1.0 respectively. Furthermore, it is unlikely that 3-amino-2-chlorophenol would be formed since this would involve the replacement of chlorine in the *meta* position which is well known to be stable.

The structure attributed to 4:5-dichloro-3-nitrophenol is based upon the formation of a phenol identical with the 3-amino-4:5-dichlorophenol when spots of the nitrophenol were eluted from the chromatograms with aqueous ethanol and reduced with tin and hydrochloric acid. This nitrophenol (R_f 0.50 and 0.68 in solvents *B* and *C* respectively) was not 2-chloro-3-nitrophenol, which has R_f values of 0.75 and 0.69 in these solvents (Bray *et al.* 1956).

The abnormal iodine uptake of the hydrolysed urine of animals given 2:3-dichloronitrobenzene (see above) prompted a particularly careful search for mercapturic acid. *N*-Acetyl-*S*-(2-chloro-6-nitrophenyl)-L-cysteine was not detected on any paper chromatograms. A chromatogram was run from extract *B* with solvent *C*; strips were cut and eluted with ethanol but no sulphur was detected in any of the eluates. It was concluded, therefore, that the increased iodine uptake was not caused by mercapturic acid.

From 2:6-dichloronitrobenzene. No mercapturic acid was detected by the methods used above. Synthetic specimens were available for comparison with all the metabolites listed except the catechol.

The products of hydroxyldechlorination would be 3-chloro-2-nitrophenol and 2-amino-3-chlorophenol. The R_f values of the former (1.0 and 0.69 in solvents *B* and *C* respectively, Bray *et al.* 1956) are different from those of 2:4-dichloro-3-nitrophenol (0.81 and 0.75) and 2-amino-3-chlorophenol is an *o*-aminophenol whereas only a *p*-aminophenol was detected. Since the catechol gave an orange colour with alkali and no colour with the Bratton & Marshall reagents it was probably a nitrocatechol. The only nitrocatechol which could be formed from 2:6-dichloronitrobenzene is 3:5-dichloro-4-nitrocatechol.

Paper chromatograms of extracts *B* detected with the Bratton & Marshall reagents showed a magenta spot, R_f 0.22 in solvent *C*. (There was no movement with solvents *A* or *B*.) The aminophenols which could be formed from 2:6-dichloronitrobenzene have R_f values much greater than 0.22. By elution with water of strips cut from a paper chromatogram it was shown that the zone around R_f 0.22 contained material which gave a naphthoresorcinol reaction corresponding in intensity with that of the colour obtained with the Bratton & Marshall test. After an eluate had been hydrolysed with 5*N*-H₂SO₄ at 100° for 1 hr. examination by paper chromatography revealed no spot at R_f 0.22 but one at R_f 0.90 which reacted in the same way as 4-amino-3:5-dichlorophenol. It was concluded that the material causing the original spot at R_f 0.22 was probably 4-amino-3:5-dichlorophenyl glucosiduronic acid and that this acid was sufficiently soluble in ether to be extracted in extracts *B*.

From 3:5-dichloronitrobenzene. No mercapturic acid was detected and all the metabolites found were identified by comparison with synthetic specimens. The 2-amino-4:6-dichlorophenol had m.p. 92° (see Materials). The products of hydroxyldechlorination would be 3-chloro-5-nitrophenol and 3-amino-5-chlorophenol. No nitrophenol was detected and 3-amino-5-chlorophenol is an *m*-aminophenol whereas only an *o*- and a *p*-aminophenol were detected.

From 2:3:4:5-tetrachloronitrobenzene. The properties and elementary analysis of the mercapturic acid isolated were in accord with those of an *N*-acetyl-*S*-(trichloronitrophenyl)-L-cysteine formed by acetylcysteyldechlorination. Since chlorine in a *meta* position to the nitro group is the most stable (see Bunnett & Zahler, 1951) and no labile *meta* chlorine atom has been found in any member of the chloromononitrobenzene series (see Betts *et al.* 1957), it is reasonable to conclude that the acetylcysteyl group would replace the chlorine in either position 2 or position 4. Two observations make the position 4 unlikely. The mercapturic acid does not give, when heated with alkali, the intense yellow colour which is typical of all known mercapturic acids with a nitro group *para* to the acetylcysteyl group (see Betts *et al.* 1957). These mercapturic acids

also all have a light-absorption maximum in the region of $340\text{ m}\mu$ (see Betts *et al.* 1957). The absorption spectrum of the mercapturic acid isolated from 2:3:4:5-tetrachloronitrobenzene has no maximum in this region and resembles the spectra of mercapturic acids with a nitro group in the *ortho* position. It is, therefore, reasonable to conclude that the acid isolated was *N*-acetyl-*S*-(2:3:4-trichloro-6-nitrophenyl)-L-cysteine.

From penta- and 2:3:4:6- and 2:3:5:6-tetra-chloronitrobenzene (cf. Bray *et al.* 1953; Betts *et al.* 1955). A re-examination by paper chromatography of urines from rabbits given these compounds failed to reveal the presence of mercapturic acids containing a nitro group.

DISCUSSION

2:3-, 2:6- and 3:5-Dichloronitrobenzene

The most conspicuous difference between the metabolic fates of the three dichloronitrobenzenes now reported and those of the three previously examined (Bray *et al.* 1957) is that none of the former appears to yield mercapturic acid. This may be correlated with the stability of the chlorine atoms in these isomers. In the 3:5-isomers the two *meta* chlorine atoms are stable and the values for the mobilities of the labile chlorine atoms in the 2:3- and 2:6-isomers are lower than those for the other three dichloronitrobenzenes (see Table 4). Presumably the nitro groups are as stable as the chlorine atoms. In the absence of mercapturic acid formation, the main metabolic pathways of 2:3-, 2:6- and 3:5-dinitrochloronitrobenzene are reduction and hydroxylation. Less than 5% of a dose remains unabsorbed.

Reduction. All the isomers are reduced to the corresponding dichloroanilines. The extent of this reduction with the 2:6-isomer (4% of dose) is less than for any member of the chloromononitrobenzene series. Only 2:3-dichloroaniline was excreted in significant amounts in combined form. 3:5-Dichloronitrobenzene provides another example of a nitro compound which is partly excreted as the corresponding azoxy compound. The origin of the corresponding compound from 3:4-dichloronitrobenzene has been discussed by Bray *et al.* (1957).

Hydroxylation. The hydroxylation products detected are summarized in Fig. 1. The metabolic pathways shown are based on the assumption that hydroxylation of a dichloronitrobenzene will occur in a *meta* position to the nitro group and hydroxylation of a dichloroaniline will occur either *ortho* or *para* to the amino group. This assumption has justification on general chemical grounds and from previous findings in the chloronitrobenzene series (e.g. Bray *et al.* 1957; Betts *et al.* 1957) as well as from the compounds identified in urine. It has been shown (Betts *et al.* 1957) that 2:4:5- and

2:4:6-trichloronitrobenzene both yield aminophenols which have been formed by substitution of one chlorine atom by a hydroxyl group in addition to aminophenols formed by the usual (direct) hydroxylation of an unsubstituted position. No dichloronitrobenzene, however, forms an aminophenol by hydroxyldechlorination. 2:3-Dichloronitrobenzene forms the two aminophenols which would be expected by hydroxylation of 2:3-dichloroaniline as well as that which would be expected by reduction of the nitrophenol formed by direct hydroxylation of the dichloronitrobenzene. This nitrophenol was detected in the urine.

With 2:6-dichloronitrobenzene two aminophenols would be expected, one from the aniline and one from the *m*-nitrophenol. The former was detected but not the latter, although the corresponding nitrophenol was shown to be present in small amount. 2:6-Dichloronitrobenzene is the only member of the series which yielded evidence for the excretion of a catechol derivative. With 3:5-dichloronitrobenzene no *m*-nitrophenol is possible and the two aminophenols expected from 3:5-dichloroaniline were identified. Thus, with the exception of 3-amino-2:4-dichlorophenol, all the aminodichlorophenols expected from the three dichloronitrobenzenes have been identified in urine.

The chloromononitrobenzene series

The main features of the metabolism of the 19 members of the chloromononitrobenzene series have been summarized in Table 4. The most interesting is that 15 members form mercapturic acids in a way not previously observed, that is, in effect, either by acetylcysteyldenitration or nuclear acetylcysteyldechlorination. [Benzyl chloride (Stekol, 1939) undergoes acetylcysteyldechlorination in the side chain.] There is a fairly close correlation between the type of acid formed and the lability of the displaced chloro or nitro groups to nucleophilic substitution reagents as measured by the extent of the hydrolysis of the groups by sodium methoxide or ethanolic sodium hydroxide. Qualitatively the only exception appears to be 2:4:5-trichloronitrobenzene, in which the chlorine in position 2 is replaced, whereas sodium methoxide replaces the chlorine in position 4. A chlorine atom in an *ortho* position in halogenonitrobenzenes is, however, usually more readily displaced by amine reagents than is a chlorine atom in the *para* position (Bunnett & Zahler, 1951), and Beilstein & Kurbatov (1879) found that ethanolic ammonia at 200° displaced the chlorine in position 2 from 2:4:5-trichloronitrobenzene. In the rabbit, chlorine is not displaced from the *para* position of any chloronitrobenzene in which there is an *ortho* chlorine atom (see Table 4). The displacement of chlorine in the rabbit may, therefore, resemble that by amine reagents more

closely than that by sodium methoxide. Quantitative data obtained with the former reagents are not, however, available. The positions of the chlorine atoms displaced by sodium methoxide from tetra- and penta-chloronitrobenzenes are not known.

All the ten mercapturic acids formed by acetylcysteyldechlorination are excreted as nitro compounds. No *N*-acetyl-*S*-(aminochlorophenyl)-L-cysteine was ever detected. Several chloroanilines were fed to rabbits but none caused the formation of mercapturic acids.

All the five chloronitrobenzenes which have chlorine atoms in position 2, 6 and at least one other position form mercapturic acids by acetylcysteyldechlorination. That chlorine substituted into the *meta* position has a greater influence on the mobility of the nitro group than has chlorine in a *para* position (Bunnett & Zahler, 1951) is well illustrated by the

extent of the formation of mercapturic acid by these five compounds. The four which have chlorine in the *meta* position readily lose the nitro group and yield mercapturic acids (10–37% of dose), whereas 2:4:6-trichloronitrobenzene, which has chlorine in the *para* but not in the *meta* position, is more resistant to ethanolic sodium hydroxide and forms only a very small amount of mercapturic acid (Table 4). 2:6-Dichloronitrobenzene, which has no chlorine in either *meta* or *para* position, does not yield a mercapturic acid.

The anilines formed by reduction of the chloronitrobenzenes are of interest in that, although many appeared to be excreted partly in conjugated form, in no instance could proof be obtained that acetyl derivatives were excreted. The anilines were excreted mainly in uncombined form and 2:4:6-trichloro-, 2:3:4:6-tetrachloro- and pentachloronitrobenzene were the only compounds from which

Fig. 1. Probable metabolic pathways of 2:3-, 2:6- and 3:5-dichloronitrobenzene in the rabbit.

Table 4. *Summary of the metabolism of the 19 chloromononitrobenzenes*

The values for the average amounts of metabolites excreted are expressed as percentage of the absorbed dose (for details of methods, dosage, and ranges see papers cited). The positions of labile chlorine atoms (indicated in bold type) and their mobilities, m_{Cl} (rate coefficient for hydrolysis with sodium methoxide at 25°), are based on those given by Holleman & van Haeften (1921) and Holleman, de Mooy & Weel (1915). The lability of nitro groups, m_{NO_2} , is based on liberation of NO_2 ions with ethanolic NaOH at 100° (Betts *et al.* 1955).

References: 1, Bray *et al.* 1953; 2, Betts *et al.* 1955; 3, Bray *et al.* 1956; 4, Bray *et al.* 1957; 5, Betts *et al.* 1957; 6, this paper.

Position of chlorine atoms	Lability of groups		Group replaced by acetylstyrene in rabbit, and position	Metabolites excreted in urine						Ref.
	$10^5 m_{Cl}$ (l. mole ⁻¹ min. ⁻¹)	m_{NO_2} (percentage of N liberated as NO ₂ ⁻)		Chloroaniline		Chlorazoxy-benzene (% of dose)	Glucosiduronic acid		Ethereal sulphate (% of dose)	
				Free (% of dose)	Combined (% of dose)		Naphtho-resorcinol method (A) (% of dose)	Copper-reducing method (% of A)		
2:3:4:5:6*	—	43	NO ₂	37	10	21	—	†	0	2
2:3:4:5*	—	0	Cl, 2	6†	16	Trace	—	61	9	1, 6
2:3:4:6	—	20	NO ₂	37	11	20	—	33	0	2
2:3:5:6*	—	82	NO ₂	36	23	0	—	35	3	1
2:3:4	191	0	Cl, 2	41	11	4	—	28	11	5
2:3:5	84	14	Cl, 2	21	13	3	—	36	11	5
2:3:6	5	8	NO ₂	10	19	0	—	67	8	5
2:4:5§	729	0	Cl, 2	30	21	8	>1	40	4	5
2:4:6§	9	4	NO ₂	<1	10	22	—	31	2	5
3:4:5	814	0	Cl, 4	24	24	2	>1	27	13	5
2:3	5	—	None	0	15	5	—	47	17	6
2:4	50	—	Cl, 2	53	8	3	—	5	6	4
2:5	10	—	Cl, 2	27	15	1	—	29	14	4
2:6¶	<1	—	None	0	4	Trace	—	45	10	6
3:4	55	—	Cl, 4	45	17	5	>2	13	12	4
3:5	—	—	None	0	29	0	>2	70	8	6
2	1	—	Cl, 2	7	9	0	—	42	24	3
3	—	—	None	0	11	0	—	33	18	3
4	4	—	Cl, 4	7	9	4	—	19	21	3

* From 33 to 62% of these compounds were unabsorbed.

† Rough value based on amount isolated.

¶ A small amount of a catechol derivative was detected by paper chromatography.

† Determination not reliable, see reference 2.

§ These compounds undergo hydroxy/dechlorination.

|| For chlorine atom in position 4.

more conjugated than free trichloroaniline was excreted.

From four members of the series, what are probably products of intermediates in the reduction were identified. 3:4- and 3:5-Dichloro- and 2:4:5- and 3:4:5-trichloro-nitrobenzene yielded the corresponding azoxy compounds. Experiments with the 3:4-dichloronitrobenzene did not provide any evidence that the azoxy compound was an artifact formed in the urine from excreted 3:4-dichlorophenylhydroxylamine.

Chloronitrobenzenes would be expected to form phenols by hydroxylation of either the chloronitrobenzene or the chloroaniline formed from it by reduction. The nitrophenol formed from the former could be reduced to an aminophenol. With the exception of two monochloronitrobenzenes, the phenols formed are those which would result from hydroxylation of the nitro compound in the *meta* position and of the aniline in *ortho* or *para* positions or both. With the *o*- and *m*-chloronitrobenzene, there is evidence of hydroxylation of the nitro-compound in the *ortho* and *para* as well as in the *meta* positions [cf. metabolism of nitrobenzene (Robinson, Smith & Williams, 1951)].

Several halogenobenzenes are excreted by the rabbit in the form of catechol derivatives; with some monohalogenobenzenes, in fact, 20–30% of a dose may be dihydroxylated in this way (Azouz *et al.* 1953; Azouz, Parke & Williams, 1955; Parke & Williams, 1955). Theoretically catechols could be formed by dihydroxylation of 2:3:4- and 2:3:6-trichloro- and of the dichloro- and the monochloro-nitrobenzenes. Urines from rabbits given these compounds were examined for catechols by paper chromatography, but only 2:6-dichloronitrobenzene provided evidence of dihydroxylation.

Another possible but unusual route for the formation of phenols was revealed with 2:4:5- and 2:4:6-trichloronitrobenzene. Both these compounds underwent hydroxyldechlorination with the formation of aminodichlorophenols. With 2:4:6-trichloronitrobenzene the aminodichlorophenol was, in fact, the major phenolic metabolite. The same phenol was the major phenolic metabolite of 2:4:6-trichloroaniline. Careful search failed to reveal evidence of this unusual reaction with the other chloronitrobenzenes.

With the 15 members of the series examined, the excretion of copper-reducing material appeared to be related to the excretion of glucuronic acid and in five compounds was quantitatively equivalent; in none did the copper-reducing value of the urine correspond to less than 50% of the glucuronic acid excreted. A detailed examination of 2:3:6-trichloronitrobenzene showed that the ratio of the glucuronic acid/copper-reducing material was constant in successive urine samples. On the basis of existing

evidence, however, it cannot be decided whether the reducing material excreted was labile *O*-glucosiduronic acid, some other form of glucuronic acid conjugate, e.g. an *N*-glucosiduronic acid, or both. Since in many instances the amount of aniline excreted was far less than the amount of glucuronic acid excreted, it is probable that at least some of the glucuronic acid is in the form of labile ether-type glucosiduronic acids. This is supported by the fact that many of the aminophenols were isolated from unhydrolysed urine.

The study of the chloronitrobenzene series has revealed new ways in which, indirectly, conjugation *in vivo* can take place through nuclear substituents. Bray, Ryman & Thorpe (1948) distinguished three types of conjugation reactions according to the nature of the substituent groups involved at the site of conjugation, namely those which could be conjugated directly without modification, e.g. $-\text{CO}_2\text{H}$, $-\text{OH}$, $-\text{NH}_2$ (centres for conjugation), those occurring at groups which could be converted *in vivo* into centres for conjugation, e.g.

(potential centres of conjugation), and those which were replaced by a new conjugable group before conjugation could occur (formation of new centres for conjugation). At that time the formation of a new centre was envisaged as the replacement of the hydrogen in an unsubstituted position in the ring either by hydroxyl or by cysteyle; the hydroxyl group would then be conjugated with glucuronic or sulphuric acid in the usual way and the cysteyle group with acetic acid to form mercapturic acids, e.g. 1-naphthylmercapturic acid from naphthalene (Bourne & Young, 1934). The chloronitrobenzenes have shown that groups other than hydrogen can be replaced to provide a new centre for conjugation. Such groups may be described as providing 'replacement centres for conjugation'. Both nitro and chloro groups may be replaced by a cysteyle group and the resulting compound can then be conjugated with acetic acid to give a mercapturic acid. A nitro group may thus act as either a potential

or, when labile, a replacement

centre for conjugation with acetic acid. Chloro groups may also be replaced by a hydroxyl group and the resulting phenol can be conjugated in the usual way. Thus chloro groups may act as replacement centres in two ways, although in the compounds examined there is no example of chlorine being replaced in the two ways at the same position in the ring, e.g. in 2:4:5-trichloronitrobenzene the mercapturic acid is formed by replacement of

chlorine in position 2 but the aminodichlorophenol by replacement in position 4. The latter metabolite may, however, have been formed from the trichloro-aniline rather than the trichloronitrobenzene (cf. 2:4:6-trichloronitrobenzene). Fluoro, bromo and iodo groups have also been shown to act as replacement centres for the formation of mercapturic acid (Bray *et al.* 1957; Betts *et al.* 1957; Bray & James, 1957).

One reason for the examination of all 19 members of the chloromononitrobenzene series was to search for compounds which would be suitable for a study of the mechanism of the formation of mercapturic acid. 2:3:4:6-Tetrachloro-, 3:4:5-trichloro- and 3:4-dichloro-nitrobenzene seem the most suitable for this purpose, and experiments are in progress with these compounds and with benzyl chloride and its nuclear-substituted derivatives in which the side-chain chlorine atom is much more labile than in nuclear chloronitrobenzenes. Preliminary results support the view that glutathione is involved in the process (Barnes & James, 1957; Bray & Franklin, 1957). The effect of the length of the side chain in ω -bromoalkylbenzenes on the formation of mercapturic acid is also being investigated.

SUMMARY

1. The metabolism of 2:3-, 2:6- and 3:5-dichloro-nitrobenzene has been studied in the rabbit.

2. The main products excreted are phenols, conjugated with glucuronic and sulphuric acids, and dichloroanilines. No mercapturic acids were detected in the urines.

3. Several nitro- and amino-dichlorophenols have been identified. A small amount of a catechol derivative is probably formed from 2:6-dichloro-nitrobenzene.

4. 3:5:3':5'-Tetrachloroazoxybenzene has been isolated from the urine of rabbits given 3:5-dichloro-nitrobenzene.

5. A mercapturic acid, probably *N*-acetyl-S-(2:3:4-trichloro-6-nitrophenyl)-L-cysteine, has been isolated from the urine of rabbits given 2:3:4:5-tetrachloronitrobenzene.

6. The chief features of the metabolism of the 19 chloromononitrobenzenes have been summarized and discussed.

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