The Metabolism of 2:4-, 2:5- and 3:4-Dichloronitrobenzene in the Rabbit

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Previous studies of the metabolism of members of the chloronitrobenzene series (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Bray, James & Thorpe, 1955*b*, 1956) revealed that an appreciable proportion of a dose of most of these compounds was converted into mercapturic acid in the rabbit. These mercapturic acids had been formed by a process resulting in the substitution of either a nitro or a chloro group by the acetylcysteyl group (acetylcysteyldenitration or acetylcysteyldechlorination) instead of replacement of a nuclear hydrogen as had been observed in all previous instances of the formation of nuclear mercapturic acids (for references see Bray *et al.* 1956). Since penta- and two tetra-chloronitrobenzenes yielded mercapturic acids by acetylcysteyldenitration and the monochloronitrobenzenes by acetylcysteyldechlorination it was of interest to examine the intermediate members of the series and to attempt to trace the factors which determined the type of mercapturic acid formed.

The present paper describes experiments on the effect of the administration of three dichloronitrobenzenes on the excretion by the rabbit of mercapturic acid, glucuronic acid, ethereal sulphate and dichloroanilines. In each case the mercapturic acids have been isolated and several phenolic metabolites have been identified. A tetrachloroazoxybenzene has been found in the urine of rabbits given 3:4-dichloronitrobenzene. A preliminary account of some aspects of this work has been given (Bray, James & Thorpe, 1955*a*).

MATERIALS

All melting points recorded are uncorrected.

2:4-, 2:5- and 3:4-Dichloronitrobenzene, m.p. 30°, 56° and 42° respectively, were purchased. 2-Bromo-4-chloro-, 2-bromo-5-chloro- and 4-bromo-3-chloro-nitrobenzene, m.p. 49°, 70° and 61° respectively, were prepared by the action of potassium bromide and copper powder on diazotized 5-chloro-2-nitro-, 4-chloro-2-nitro- and 2-chloro-4-nitro-aniline respectively (cf. Gattermann, 1890). 4-Chloro-2-nitro- and 2-chloro-4-nitro-aniline were purchased and 5-chloro-2-nitroaniline was prepared as described by Hodgson & Kershaw (1929). 2:4-Dichloroacetanilide, m.p. 142°, and 2:5-dichloroacetanilide, m.p. 132–134°, were obtained by acetylation of the corresponding bases.

The following dichloronitrophenols were prepared: 2:4-dichloro-5-nitrophenol, m.p. 105°, as described by Groves, Turner & Sharp (1929); 3:5-dichloro-2-nitrophenol, m.p. 48°, by the method of Hodgson & Wignall (1927) from 3:5-dichloroaniline prepared according to Holleman (1904); 3:6-dichloro-2-nitrophenol, m.p. 67–68°, by the method of Hodgson & Kershaw (1929) from 2:5-dichlorophenol prepared as described by Noelting & Kopp (1905); 2:5-dichloro-4-nitrophenol, m.p. 115°, from 2:5-dichloro-4-nitroaniline (Holleman & Haeften, 1921) by decomposition of the diazo compound according to Noelting & Kopp (1905).

The dichloronitrophenols were reduced to the corresponding aminophenols with alkaline dithionite. The properties of 5-amino-2:4-dichlorophenol and 2-amino-3:5-dichlorophenol were as described by Hodgson & Wignall (1927) and Jacobs, Heidelberger & Rolf (1919) respectively. 2-*Amino*-3:6-*dichlorophenol* formed buff plates, m.p. 137°. (Found N, 7·95. $C_6H_5ONCl_2$ requires N, 7·9%.) 4-Amino-2:5-dichlorophenol, m.p. 174°, was characterized as 4-*acetamido*-2:5-*dichlorophenol*, colourless rosettes of needles from aqueous ethanol, m.p. 208–210°. (Found: N, 6·4. $C_8H_7O_2NCl_2$ requires N, 6·4%.) R_F values in solvents *B* and *C* of Table 2 were 0·59 and 0·64 respectively. The spots were detected by spraying with the Folin & Ciocalteu reagent.

3:4:3':4'-*Tetrachloroazobenzene* was prepared by keeping 3:4-dichloronitrosobenzene (cf. Vanino, 1923) with an equivalent amount of 3:4-dichloroaniline in acetic acid overnight. It formed orange plates, m.p. 150°. (Found: C, 44·8; H, 1·6; N, 8·7; Cl, 44·6. $C_{12}H_6N_2Cl_4$ requires C, 45·0; H, 1·9; N, 8·8; Cl, 44·3%.) 3:4:3':4'-*Tetrachloroazoxybenzene*, m.p. 139°, was prepared (*a*) by oxidation of 3:4:3':4'-tetrachloroazobenzene in ethanol–acetic acid (1:1, v/v) by the daily addition of 2 g. of urea hydrogen peroxide (British Drug Houses Ltd.) for 7 days, or (*b*) by boiling 3:4-dichlorophenylhydroxylamine in 96% (w/v) ethanol for 15 min. (For analysis see below.) 3:4-Dichlorophenylhydroxylamine was prepared from 3:4-dichloronitrobenzene as described by Haworth & Lapworth (1921).

N-*Acetyl*-S-(5-*chloro*-2-*nitrophenyl*)-L-*cysteine*, m.p. 192°, N-*acetyl*-S-(4-*chloro*-2-*nitrophenyl*)-L-*cysteine*, m.p. 161°, and N-*acetyl*-S-(2-*chloro*-4-*nitrophenyl*)-L-*cysteine*, m.p. 193°, were prepared by the method of Parke & Williams (1951) from 5-chloro-2-nitroaniline, 4-chloro-2-nitroaniline and 2-chloro-4-nitroaniline respectively. The bases were diazotized in conc. H_2SO_4 as described for the preparation of *N*-acetyl-*S*-(2-nitrophenyl)-L-cysteine (Bray *et al.* 1956). Properties and elementary analyses are given in Table 1. The three mercapturic acids were also prepared by the method of Suter (1895) with ethylene glycol monoethyl ether as solvent. This is a more convenient method but it does not provide proof of the constitution of the acid formed.

Table 1. *Properties of three chloronitrophenylmercapturic acids*

	N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	$C_{11}H_{11}O_5N_2ClS$ requires
Cryst. form	Rosettes of yellow needles	Yellow needles	Cream needles	
Melting point	192°	161–162°	192–194°	—
$[\alpha]_D^{20}$ in ethanol (*c*, 0·5)	+63±3°	+104±7°	−4±4°	—
Found:				
C (%)	41·5*	41·3	41·6	41·5
H (%)	3·6	3·3	3·5	3·5
N (%)	8·3	8·8	9·2	8·8
Cl (%)	11·3	11·2	10·7	11·1
S (%)	9·8	9·9	9·9	10·1
Equiv.	310	314	314	319
Absorption spectra in 0·1N-KOH				
$\lambda_{max.}$ (mμ.)	253, 375	249, 390	345	—
$\epsilon_{max.}$ (synthetic)	13 540, 3156	17 380, 2815	10 025	—
$\epsilon_{max.}$ (biosynthetic)	14 420, 3406	16 903, 2806	10 010	—
$\lambda_{min.}$ (mμ.)	230, 328	226, 315	276	—
Inflexion (mμ.)	285	270	—	—

* Mean of triplicate analysis.

METHODS

Animals, diet and dosage. Doe rabbits (2–3 kg. body wt.) were maintained as previously described (Bray, Ryman & Thorpe, 1947). All compounds were administered by stomach tube as suspensions in water. The dose levels were: 2:4-isomer, 0·2, 0·3 and 0·4 g./kg.; 2:5- and 3:4-isomer, 0·4 g./kg. There was pronounced anorexia after giving 0·4 g. of the 2:4-isomer/kg.

Determination of metabolites

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

Glucuronic acid. A modification (Bray, Humphris, Thorpe, White & Wood, 1952) of the naphthoresorcinol method of Hanson, Mills & Williams (1944) was used. Reducing material was determined as described by Bray, Neale & Thorpe (1946).

Ether-soluble acid. The method of Bray *et al.* (1946) was used. The recovery of *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine added to urine was 109±16%.

Mercapturic acid. All the mercapturic acids formed were determined by the modified Stekol (1936) method described by Betts *et al.* (1955). The concentration of NaOH used for hydrolysis and time of boiling were 0·5N and 0·5 hr. for *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine and 2N and 1 hr. for *N*-acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine and *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine. The recoveries from urine were 46, 49 and 31% (all±5) respectively. *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine was also determined by the colorimetric method described for *N*-acetyl-*S*-(*p*-nitrophenyl)-L-cysteine (Bray *et al.* 1956). This gave 88±6% recovery of the acid added to urine.

Dichloroanilines. These were determined, on the steam-distillates of urine samples adjusted to pH 10 or of whole 72 hr. faeces, by the method of Bratton & Marshall (1939) with the appropriate bases as standards and a Spekker photoelectric absorptiometer with a Chance glass filter OG. 1. Recoveries of 2:4-, 2:5- and 3:4-dichloroaniline added to urine were 88, 100 and 100% respectively.

Catechols. The method of Azouz, Parke & Williams (1953) was used with 4-chlorocatechol as standard.

Qualitative examination of urines

Ether extracts A, B, C, D and E were prepared from urines as described by Bray *et al.* (1956). Briefly, the extracts 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-H_2SO_4; D, from residual urine from C adjusted to pH 7; E, from hydrolysed urine adjusted to pH 7, was prepared only from urines of rabbits given dichloroanilines.

Paper chromatography. The procedure was as described by Bray, Thorpe & White (1950) except that papers were dried without heating to minimize decomposition of aminophenols. The solvents and detecting reagents used and the R_F values of reference compounds are given in Table 2.

Isolation of metabolites

Unless stated otherwise all metabolites identified were shown to be identical with synthetic specimens by mixed m.p. and paper chromatography.

Dichloroanilines. The urine was adjusted to pH 10 and steam-distilled. The distillate was continuously extracted with ether. The residue left after removal of ether from the extract either yielded the crystalline dichloroaniline or was treated with acetic anhydride to give the dichloroacetanilide which was recrystallized from aqueous ethanol.

Mercapturic acids. The urine was adjusted to pH 1 and continuously extracted with ether. Crystals of the mercapturic acid were isolated from the extract and recrystallized from aqueous ethanol. Results for elementary analyses, optical rotation and absorption spectra are given in Table 1.

Preparation of 3:3′-*dichloro*-4:4′-*dinitrodiphenyl disulphide.* *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (0·5 g.) was boiled for 1 hr. in N_2 with 2N-NaOH (40 ml.). The solution was cooled and acidified, when crystals m.p. 68–71° separated. These were immediately oxidized with ethanolic iodine to give yellow needles of 3:3′-*dichloro*-4:4′-

Table 2. R_F *values and detection of some possible metabolites of* 2:4-, 2:5- *and* 3:4-*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*, *n*-butanol–3N-$(NH_4)_2CO_3$–3N-NH_3 soln. (4:3:3, by vol.; Corner & Young, 1954) (2 hr.). Detecting reagents: *a*, 2N-HCl, 0·1% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); *b*, diazotized *p*-nitroaniline followed by 20% (w/v) Na_2CO_3 (Bray *et al.* 1950); *c*, 20% (w/v) Na_2CO_3. Colours of spots: B, blue; G, green; M, magenta; Oc, ochre; P, purple; R, red; Y, yellow; p, pale. The compounds in parentheses were not available as reference compounds and were observed only as metabolites in urine. The arguments for the suggested constitutions are given in the Results section.

Compound	R_F values in solvent mixture			Colour with detecting reagents		
	A	*B*	*C*	*a*	*b*	*c*
Possible metabolites of 2:4-dichloronitrobenzene						
5-Amino-2:4-dichlorophenol	0·76	—	0·70	M	Oc	—
2-Amino-3:5-dichlorophenol	0·89	—	0·83	Y*	P	—
(3-Amino-2:6-dichlorophenol)	0·50	—	—	pR	—	—
2:4-Dichloro-5-nitrophenol	—	0·64	—	—	—	pY
3:5-Dichloro-2-nitrophenol	—	1·0	—	—	—	pY
2:4-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	0·73	0·0	0·58	—	—	pY
Possible metabolites of 2:5-dichloronitrobenzene						
2-Amino-3:6-dichlorophenol	1·0	—	0·69	Y*	P	—
(3-Amino-2:5-dichlorophenol)	0·95	—	0·83	pR	—	—
4-Amino-2:5-dichlorophenol	0·72	—	0·77	B	pG	—
2:5-Dichloro-4-nitrophenol	—	0·55	—	—	—	pY
3:5-Dichloro-2-nitrophenol	—	1·0	—	—	—	Y
2:5-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	0·78	0·0	0·59	—	—	pY
Possible metabolites of 3:4-dichloronitrobenzene						
3:4-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	0·55	0·0	0·77	—	—	Y
(2-Aminodichlorophenol X)†	0·66	—	0·86	B*	—	—
(2-Aminodichlorophenol Y)†	0·72	—	0·75	Y*	—	—
(5-Amino-2:3-dichlorophenol)	0·75	—	0·86	pR	—	—

* Gave bright yellow colour with nitrous acid. † See text under 'Isolation of metabolites'.

dinitrodiphenyl disulphide, m.p. 163–164° after recrystallization from acetic acid. A further yield was obtained from the mother liquor. Total yield, 180 mg. (Found: C, 38·1; H, 1·3; N, 7·5; Cl, 18·6; S, 16·5. $C_{12}H_6O_4N_2Cl_2S_2$ requires C, 38·2; H, 1·6; N, 7·4; Cl, 18·8; S, 17·0%.)

3:4:3′:4′-Tetrachloroazoxybenzene. The ether extract from urine (pH 1) of rabbits dosed with 3:4-dichloronitrobenzene contained tetrachloroazoxybenzene in addition to the mercapturic acid. Ether was removed from the extract and the residue was extracted with dilute NaOH to remove the mercapturic acid. The insoluble material left was washed with water and recrystallized from ethanol to give pale-yellow needles of *3:4:3′:4′-tetrachloroazoxybenzene*, m.p. 139° (not examined by paper chromatography). (Found: C, 42·8; H, 1·8; N, 8·5; Cl, 42·2. $C_{12}H_6ON_2Cl_4$ requires C, 42·9; H, 1·8; N, 8·3; Cl, 42·2%.)

4-Amino-2:5-dichlorophenol. The urine was refluxed for 1 hr. with an equal volume of 10N-H_2SO_4 and was then made alkaline and steam-distilled to remove the dichloroaniline. The residual urine was adjusted to pH 6 and continuously extracted with ether. On evaporation of the ether, crude aminophenol was obtained, m.p. 160–170° after recrystallization from ethanol–light petroleum (b.p. 40–60°). This gave a positive indophenol test for a *p*-aminophenol and a blue colour with the Bratton & Marshall reagents. Acetylation with acetic anhydride at room temperature gave 2:5-dichloro-4-hydroxyacetanilide, m.p. 208°.

2-Aminodichlorophenol X. The urine was continuously extracted with ether at pH 1. The mercapturic acid was removed from the extract by extraction with N-$NaHCO_3$ and the remaining ethereal solution was extracted with 0·5N-NaOH. The extract was adjusted to pH 6 and re-extracted with ether. Evaporation of the ether gave a syrup, which rapidly darkened if kept. It formed with acetic anhydride a crystalline acetyl derivative, m.p. 220° (softening at 214°) after recrystallization from aqueous ethanol. Yield, 30 mg. (Found: C, 44·0; H, 3·5; N, 6·3. $C_8H_7O_2NCl_2$ requires C, 43·7; H, 3·2; N, 6·4%.) This aminophenol (prepared by hydrolysis of the acetyl derivative) when treated with the Bratton & Marshall (1939) reagents gave a bright yellow colour at the nitrous acid stage. When the coupling reagent was added the colour quickly changed to blue, and the blue dye was soon deposited from the solution.

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

Table 3. *Excretion of metabolites of* 2:4-, 2:5- *and* 3:4-*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. A superior number followed by p indicates a result for pooled urine from that number of rabbits. Urine was collected for 72 hr. except in experiments (indicated by m after the superior number) where separate consecutive samples, which were collected from rabbits given water at intervals (see Bray, Thorpe & White, 1951), were analysed until the excretion of metabolites was no longer detected. From 67 to 80% of the unabsorbed material in faeces was reduced to the dichloroaniline. The reducing value of the urine after feeding the 2:4-isomer increased to an extent corresponding to 62% of the glucosiduronic acid excreted and for the 2:5- and 3:4-isomers the increase was equivalent to the glucosiduronic acid excreted (3 expts. with each isomer).

	2:4-Dichloronitrobenzene			2:5-Dichloronitrobenzene	3:4-Dichloronitrobenzene
Dose (g.)	0·5	0·7	1·0	1·0	1·0
Metabolite					
Unabsorbed	—	—	0·3 (0·3, 0·3)^{2}	1·1 (0·5, 1·7)^{2}	0·2 (0·1, 0·4)^{2}
Mercapturic acid	84 (61–98)^{6}	67 (24–91)^{6}	53 (38–67)^{5}	27 (9–33)^{7}	39 (16–70)^{9}
		49 (20–72)^{6}*	—	—	45 (30–56)^{6}†
Glucosiduronic acid	22 (17, 26)^{2m}	—	5 (0–13)^{10}	29 (8–56)^{14}	13 (7–23)^{7}
Ethereal sulphate	5 (0–14)^{3m}	—	6 (0–12)^{8}	14 (3–21)^{5}	12 (9–13)^{3}
Dichloroaniline, free	6 (6, 6)^{2m}	—	8^{3p}	15 (10–19)^{13}	17 (8–23)^{12}
Dichloroaniline, combined	—	—	3^{3p}	1^{10p}	5 (0–11)^{9}
'Catechols'	—	—	5 (4, 6)^{2}	6 (1–9)^{3}	2 (1–2)^{3}
Azoxy compound	—	—	—	—	2^{6p}‡
Total accounted for, (excluding 'catechols')	—	—	78	87	94

* Determined as ether-soluble acid. (Not the same animals as for Stekol method.)
† Colorimetric method. ‡ By isolation.

obtained from the quantitative analysis of urines are summarized in Table 3, which shows that with each compound a satisfactory proportion of the dose has been accounted for. In each case the greater part of the dose was excreted as mercapturic acid and phenolic metabolites. Relatively little was excreted as the dichloroaniline. The totals do not include the small amounts of phenolic metabolites found unconjugated in urine (Table 4, extracts A and B). These may have been formed by hydrolysis of glucosiduronic acids, which are presumably labile since there was good agreement between the results of glucuronic acid and reducing-value determinations. The values for mercapturic acids by the Stekol method have been corrected according to recoveries obtained when the pure acids were added to urine. With the 3:4-isomer the validity of the correction is supported by the results of analyses by the colorimetric method. The colour given by the other mercapturic acids with NaOH was too feeble for satisfactory quantitative determination by this method. The presence of the small amounts of catechol derivatives indicated by quantitative determination has not been confirmed by either isolation or detection of catechol derivatives. These amounts have not, therefore, been included in the totals accounted for. Unchanged dichloronitrobenzenes were apparently absent from the urines, since the amounts of chloroanilines found were not increased after treating the urine with zinc and hydrochloric acid.

Metabolites identified

The metabolites identified by paper chromatography are listed in Table 4. No nitro- or amino-monochlorophenols and no acetamido compounds other than the mercapturic acids were detected. No nitrophenols were detected after administration of 3:4-dichloronitrobenzene. Table 4 includes the metabolites detected by paper chromatography in urines from rabbits dosed with 2:4-, 2:5- and 3:4-dichloroaniline.

Metabolites isolated

The yields of the metabolites isolated are given in Table 5. A tetrachloroazoxybenzene was found only in the urines of animals dosed with 3:4-dichloronitrobenzene. The constitution of the 2-aminodichlorophenol X isolated from such urines was not fully established. The intense yellow colour given on treatment with nitrous acid points to its being an *o*-aminophenol, i.e. either 2-amino-4:5-dichlorophenol or 6-amino-2:3-dichlorophenol. (When 3:4-dichloroaniline was given, the other *o*-aminophenol, 2-aminodichlorophenol Y, was excreted.) 5-Amino-2:3-dichlorophenol is suggested as the probable constitution of the other phenolic metabolite (R_F 0·75 in solvent *A*) since this gave no yellow colour with nitrous acid and it is the only other possible aminodichlorophenol from 3:4-dichloronitrobenzene. The aminomonochlorophenol which would be expected if a chlorine atom were

Table 4. *Metabolites of dichloronitrobenzenes and dichloroanilines detected by paper chromatography*

Metabolites of dichloronitrobenzenes were sought in extracts A, B, C and D, and of dichloroanilines in extract E. For preparation of extracts see Methods. Compounds found in extracts of hydrolysed urine (C and D) are probably excreted in combined form. +, Present; (+), present in traces only; −, absent.

	Detected in extract				
	A	B	C	D	E
Metabolites of 2:4-isomer					
2:4-Dichloroaniline	+	+	+	+	+
2:4-Dichloro-5-nitrophenol	+	+	(+)	(+)	−
3:5-Dichloro-2-nitrophenol	+	+	(+)	−	−
2-Amino-3:5-dichlorophenol	(+)	(+)	(+)	+	+
3-Amino-2:6-dichlorophenol*	(+)	−	−	−	−
5-Amino-2:4-dichlorophenol	(+)	(+)	(+)	(+)	−
N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	−	+	−	−	−
Metabolites of 2:5-isomer					
2:5-Dichloroaniline	+	+	(+)	−	+
2:5-Dichloro-4-nitrophenol	−	(+)	−	−	−
3-Amino-2:5-dichlorophenol*	−	(+)	−	−	−
4-Amino-2:5-dichlorophenol	(+)	(+)	−	+	+
N-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	−	+	−	−	−
Metabolites of 3:4-isomer					
3:4-Dichloroaniline	+	+	+	+	+
2-Aminodichlorophenol X*†	(+)	(+)	(+)	+	+
2-Aminodichlorophenol Y*†	−	−	−	−	−
5-Amino-2:3-dichlorophenol*	−	+	−	+	−
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	−	+	−	−	−

* Reference compounds not available. See Results section for arguments for the suggested constitutions.
† Probably either 2-amino-4:5- or 2-amino-5:6-dichlorophenol (see text).

Table 5. *Metabolites isolated from urine of rabbits given dichloronitrobenzenes and bromochloronitrobenzenes*

Nitrobenzene derivative administered	Dose/rabbit (g.)	No. of rabbits	Compound isolated	Yield (% of dose)
2:4-Dichloro-	0·7	6	2:4-Dichloroaniline (as acetyl deriv.)	1
		6	*N*-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	23
2:5-Dichloro-	1	10	2:5-Dichloroaniline	13
		10	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	2
		6	4-Amino-2:5-dichlorophenol	1
3:4-Dichloro-	1	10	3:4-Dichloroaniline	5
		12	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	17
		6	3:4:3′:4′-Tetrachloroazoxybenzene	2
		12	2-Aminodichlorophenol X (as acetyl deriv.)	1
2-Bromo-4-chloro-	0·5	2	*N*-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	10
2-Bromo-5-chloro-	0·5	2	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	1
4-Bromo-3-chloro-	0·5	2	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	7

replaced by a hydroxyl group would be 4-amino-3-chlorophenol, since the chlorine on C-4 of 3:4-dichloronitrobenzene is the more labile one (Holleman, Mooy & Weel, 1915). This aminochlorophenol has in solvent *A* an R_F value of 0·23 (Bray *et al.* 1956) which is much lower than that of the metabolite detected (R_F 0·75). Similar arguments apply for the attribution of the probable constitutions of 3-amino-2:6-dichlorophenol and 3-amino-2:5-dichlorophenol to the aminophenols detected in urine after administration of 2:4- and 2:5-dichloronitrobenzene respectively. Table 5 includes the mercapturic acids isolated from urines of rabbits dosed with bromochloronitrobenzenes, which were given in order to provide additional evidence for the constitutions of the mercapturic acids formed from the dichloronitrobenzenes.

DISCUSSION

The most conspicuous quantitative difference between the metabolic fates of the three dichloronitrobenzenes and those of the monochloronitrobenzenes (Bray *et al.* 1956) is that in the former mercapturic acid formation provides the major metabolic pathway, whereas with the latter only

small amounts of mercapturic acid are excreted and hydroxylation is the principal metabolic process. The excretion of chloroanilines in the two groups is quantitatively similar. In both cases a relatively small proportion was excreted in combined form but acetylation could not be detected.

It is difficult to predict the position of hydroxylation in the three dichloronitrobenzenes, since in the same nucleus there are two *ortho–para*-directing, but deactivating, chloro groups together with a nitro group. The aminodichlorophenols detected in urines of rabbits dosed with the dichloroanilines are those which would be expected from a dominating *ortho–para*-directing amino group. The hydroxylation products detected from the dichloronitrobenzenes and dichloroanilines have been summarized in Fig. 1, which shows, on the basis of the existing evidence, the probable stage at which hydroxylation occurs. It seems likely, for example, that 5-amino-2:4-dichlorophenol is a product of the reduction of 2:4-dichloro-5-nitrophenol rather than a product of the hydroxylation of 2:4-dichloroaniline, since the aminophenol is a *m*-aminophenol which was not detected as a metabolite of the dichloroaniline. 2-Amino-3:5-dichlorophenol, on the other hand, could have been formed either by reduction of 3:5-dichloro-2-nitrophenol or by hydroxylation of 2:4-dichloroaniline.

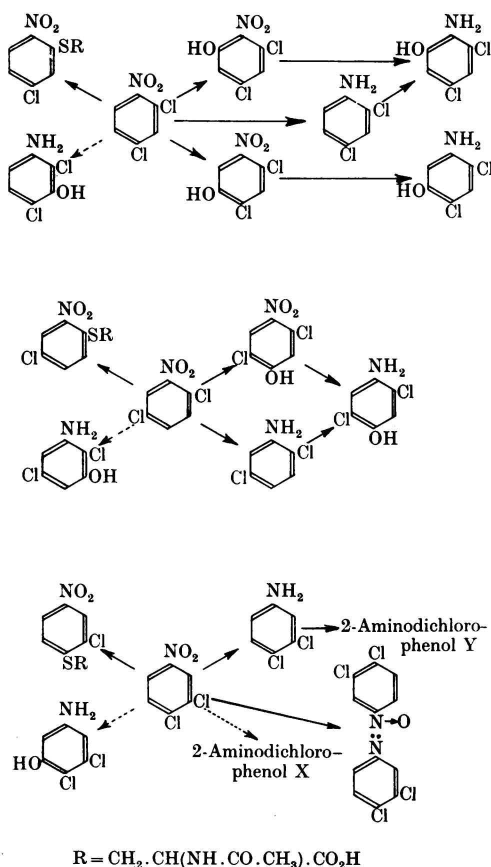

Fig. 1. Metabolites excreted in urine by rabbits dosed with 2:4-, 2:5- and 3:4-dichloronitrobenzene and 2:4-, 2:5- and 3:4-dichloroaniline. The broken arrows point to aminophenols, which were probably formed from nitrophenols not detected in the urines and which were not found as metabolites of the dichloroaniline. Aminodichlorophenol Y was not detected as a metabolite of 3:4-dichloronitrobenzene.

The most interesting feature of the metabolism of the three dichloronitrobenzenes is the nature of the mercapturic acids formed. All the mercapturic acids have retained the nitro group and one of the chloro groups has been substituted by an acetylcysteyl group. The process is, in effect, one of acetylcysteyldechlorination. In certain higher members of the chloromononitrobenzene series (penta- and some tetra-chloronitrobenzenes) it has been shown that mercapturic acid formation was, in effect, an acetylcysteyldenitration (Bray *et al.* 1953; Betts *et al.* 1955). A preliminary examination of some trichloronitrobenzenes has shown that these compounds appear to form mercapturic acid by acetylcysteyldechlorination (Bray *et al.* 1955*b*). The reason for the formation of mercapturic acids by substitution of chloro or nitro groups rather than by the more usual process of acetylcysteyldeprotonation observed in aromatic hydrocarbons and the halogenobenzenes (for references see Bray *et al.* 1956) seems to be related to the lability of the groups displaced by the acetylcysteyl residue. The correspondence between the ease of hydrolysis of the nitro groups by alkali in some polychloronitrobenzenes and the extent of mercapturic acid formation in the rabbit has already been reported (Betts *et al.* 1955). A similar relation for the displaced chloro group of the dichloronitrobenzenes is shown in Table 6, in which the rate coefficients for hydrolysis with sodium methoxide (determined by Holleman *et al.* 1915) are compared with the amounts of mercapturic acids excreted by the rabbit. Table 6 also shows that the mercapturic acids formed have the acetylcysteyl group in the position occupied by the labile chlorine in the original chloronitrobenzene. The structures of all three mercapturic acids have been established by comparison with the acids synthesized via the diazonium salts of the corresponding chloronitroanilines. This method of synthesis leaves no doubt as to their structure. Further evidence that the mercapturic acids are formed by substitution of the labile chlorine atom is provided by the experiments with bromochloronitrobenzenes. In these compounds bromine is more labile than chlorine and the bromine occupies the position of the labile chlorine in

Table 6. *Mercapturic acid formation and chloride mobility in some chloromononitrobenzenes*

The positions of the labile chlorine atoms and their mobilities (rate coefficients for hydrolysis with methanolic sodium methoxide at 25°) are those given by Holleman *et al.* (1915). In column 3, *m* represents mobility of labile chlorine.

			Mercapturic acid formed by rabbit		
			Amount (% of dose)		Position of acetylcysteyl group relative to nitro group
Nitrobenzene derivative	Position of labile halogen	10^5m (l. mole^{-1}min.$^{-1}$)	By quantitative analysis	By isolation	
2:4-Dichloro-	2	50	53	23	2
2-Bromo-4-chloro-	2	—	—	10	2
2:5-Dichloro-	2	10	27	2	2
2-Bromo-5-chloro-	2	—	—	1	2
3:4-Dichloro-	4	55	45	17	4
4-Bromo-3-chloro-	4	—	—	7	4
o-Chloro-	2	1*	7	—	2
m-Chloro-	Stable	0	0	—	—
p-Chloro-	4	4*	2	—	4

* Interpolated from values at 85° and 110°.

the corresponding dichloro compound. In each case the mercapturic acid isolated from urine after giving a bromochloronitrobenzene to rabbits was the same as that obtained after giving the corresponding dichloronitrobenzene. Although a considerable part of each dichloronitrobenzene was reduced in the rabbit to amino compounds, no *N*-acetyl-*S*-(aminochlorophenyl)-L-cysteine was ever detected.

While theoretically the reduction of a nitro group would be expected to involve the intermediate formation of nitroso and hydroxylamino compounds, the biological production of such compounds has been difficult to demonstrate. If these intermediates were produced *in vivo*, it would be expected that the corresponding azoxy or azo compounds might be formed. Channon, Mills & Williams (1944), however, showed that the tetranitroazoxytoluene, which was found in the urine of subjects with 2:4:6-trinitrotoluene poisoning, was an artifact formed from 4-hydroxylamino-2:6-dinitrotoluene when the urine was kept. The origin of the tetrachloroazoxybenzene found in the urine of rabbits after administration of 3:4-dinitrochlorobenzene is, therefore, of interest. So far it has not been possible to prove that it is an artifact and attempts to detect or measure the excretion of hydroxylamino compounds in freshly voided urine have not been successful, partly because 3:4-dichloroaniline does not react readily with acetic anhydride under the conditions of the Rosenthal & Bauer (1939) method of determination. The Pucher & Day (1926) reaction for hydroxylamine was also negative with 3:4-dichlorophenylhydroxylamine. The formation of the 3:4-3′4′-tetrachloroazoxybenzene could not be detected when 3:4-dinitrophenylhydroxylamine was added to urine under the conditions used by Channon *et al.* (1944), although the azoxybenzene was easily isolated from the urine of rabbits dosed with 3:4-dichloronitrobenzene. While it is probable that the azoxy compound is derived from an intermediate in the reduction of the nitrocompound there is as yet no evidence to show how or where it is formed. The available evidence suggests that the azoxy compound is not an artifact.

SUMMARY

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

2. The main products excreted in urine are mercapturic acids (corresponding to 30–50 % of the dose) and phenols conjugated with glucuronic and sulphuric acids. Smaller amounts of dichloroanilines are excreted.

3. The mercapturic acids have been isolated and their structure has been confirmed by synthesis. In each case they are formed by substitution of the labile chlorine atom.

4. 2-Bromo-4-chloro-, 2-bromo-5-chloro- and 4-bromo-3-chloro-nitrobenzene when fed to rabbits yield the same mercapturic acids as do the corresponding dichloronitrobenzenes.

5. Several of the nitro- and amino-dichlorophenols excreted have been identified.

6. 3:4:3′:4′-Tetrachloroazoxybenzene has been isolated from the urine of rabbits given 3:4-dichloronitrobenzene.

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Boundary Spreading in Sedimentation-Velocity Experiments

4. MEASUREMENT OF THE STANDARD DEVIATION OF A SEDIMENTATION-COEFFICIENT DISTRIBUTION: APPLICATION TO BOVINE ALBUMIN AND β-LACTOGLOBULIN*

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Methods of studying the heterogeneity of proteins have been essential to the modern development of protein chemistry. One of the most useful methods has been analysis by means of the velocity ultracentrifuge, in which molecular species of different size, shape or density are resolved. This article provides a method for measuring the standard deviation of a sample's distribution of sedimentation coefficient. One would prefer to measure the distribution itself, but for several systems this is not possible while measurement of the standard deviation, p, of the distribution is possible.

One reason for studying the heterogeneity of a protein preparation is to find out whether homogeneous proteins exist in Nature. Because of the possibility of changes produced during fractionation or upon storage, measurements of heterogeneity must be interpreted with great caution. This subject has been reviewed by Pirie (1940) and recently by Colvin, Smith & Cook (1954). Another reason for studying the heterogeneity of a protein sample is to aid in interpreting other physical measurements. For example, heterogeneity enters into the measurements of sedimentation coefficient, diffusion coefficient and intrinsic viscosity in different ways, so that, in interpreting these measurements by a theory such as that of Scheraga & Mandelkern (1953) for the hydrodynamic behaviour, one must have detailed knowledge of the heterogeneity. A brief review of the measurement of heterogeneity by diffusion, electrophoresis

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