Metabolism of *o*-, *m*- and *p*-Fluoro-, -Bromo- and -Iodo-nitrobenzenes in the Rabbit

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In a study of the metabolism of the 19 members of the chloromononitrobenzene series (for summary and references see Table 4 of Bray, James & Thorpe, 1957*b*) it was observed that in the rabbit several compounds were converted into mercapturic acids by replacement of a relatively labile chlorine atom. The formation of mercapturic acid by acetyl-cysteyldehalogenation of 4-bromo-3:5-dichloronitrobenzene (Betts, Bray, James & Thorpe, 1957) and of 2-bromo-4-chloro-, 2-bromo-5-chloro- and 4-bromo-3-chloro-nitrobenzene (Bray, James & Thorpe, 1957*a*) has also been noted. It was therefore of interest to examine the metabolic fate of some other halogenonitrobenzenes with particular reference to the formation of mercapturic acids. For this purpose the monohalogenonitrobenzenes were chosen since they were more readily available than polyhalogenonitrobenzenes. Some preliminary experiments have been reported by Bray & James (1957).

Betts *et al.* (1957) found that in the rabbit 2:4:5- and 2:4:6-trichloronitrobenzene yielded metabolites in which a chlorine atom was replaced by an hydroxyl group. The present investigation has revealed further examples of hydroxyldehalogenation.

MATERIALS

All melting points recorded are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn.

Most of the halogenonitrobenzenes were purchased. *o*-Fluoronitrobenzene was prepared by decomposition of *o*-nitrobenzenediazonium borofluoride (cf. Schiemann & Pillarsky, 1929; Dippy & Williams, 1934). The solid product of the reaction was extracted with ether, the solvent was removed from the extract and the residue was steam-distilled. The distillate was extracted with ether. After removal of ether the liquid residue from the extract was purified by fractional distillation. The product had b.p. 95° at 12 mm.; n_D^{20} 1.5452 [Schiemann & Pillarsky (1929) give $n_D^{17.2}$ 1.5331]. *o*- and *m*-Iodonitrobenzene, m.p. 49° and 32° respectively, were prepared by the method of Ullmann (1896).

o-Fluoro- and *o*- and *m*-iodo-aniline were prepared by reduction of the corresponding halogenonitrobenzenes with Sn and HCl in ethanol. The other halogenoanilines were purchased. All halogenoanilines were characterized as the corresponding acetanilides, which were prepared by treatment with acetic anhydride at room temperature. The

melting points of the acetanilides were: *o*-fluoro, 75°; *m*-fluoro, 86°; *p*-fluoro, 152°; *o*-bromo, 99°; *m*-bromo, 89°; *p*-bromo, 168°; *o*-iodo, 108° and *m*-iodo, 121°. *p*-Iodo-acetanilide, m.p. 184°, was purchased.

N-Acetyl-*S*-(2-nitrophenyl)- and *N*-acetyl-*S*-(4-nitrophenyl)-L-cysteine were prepared as described by Bray, James & Thorpe (1956). In that paper the m.p. of the former was described erroneously as 156–158° instead of 173–174°. 3- and 4-Nitrocatechols were prepared according to Weselsky & Benedikt (1882).

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 0.1 g./kg. body weight for *o*-fluoronitrobenzene, 0.15 g./kg. for *o*-bromo- and *o*- and *m*-iodo-nitrobenzene and 0.2 g./kg. for the other compounds. All the fluoro and all the *ortho* and *meta* compounds caused some anorexia. Occasionally the dose of *m*-fluoronitrobenzene was fatal.

Determination of metabolites. The methods were essentially as described by Bray *et al.* (1956, 1957*a*). Recoveries (% ± 5%) of the anilines added to urines were: *o*-fluoro, 93; *m*-fluoro, 108; *p*-fluoro, 108; *o*-bromo, 98; *m*-bromo, 103; *p*-bromo, 103; *o*-iodo, 103; *m*-iodo, 107; *p*-iodo, 105.

Qualitative examination of urines. This was carried out as described by Bray *et al.* (1956). Ether extracts B were prepared by continuous extraction with ether for 24 hr. of urine adjusted to pH 1 with 2*N*-H₂SO₄. Extraction of the residual urine after hydrolysis with an equal volume of 10*N*-H₂SO₄ gave extracts C. Extracts D were obtained from the 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 and loss of nitrophenols papers were dried without heating. The solvents and detecting reagents used and the *R_F* values of the compounds are given in Tables 3 and 4.

Indophenol reaction. Synthetic *p*-fluoroaniline reacts with the indophenol reagents to give a blue colour slowly. (Normally a blue colour is obtained with these reagents only from *p*-aminophenols.) This caused little difficulty in interpretation of paper chromatograms, since *p*-fluoroaniline runs faster than *p*-aminophenol with which it might otherwise have been confused (see Table 3). The *p*-fluoroaniline was shown to be free from *p*-aminophenol by paper chromatograms treated with other detecting reagents. The slow blue colour always appeared in the zone which other detecting agents had shown to be that occupied by *p*-fluoroaniline. It seems likely that a small proportion of

p-fluoroaniline is defluorinated by the reagents used in the indophenol reaction.

Isolation of metabolites. Halogenoanilines were isolated as the acetanilides as described by Bray *et al.* (1956). Mercapturic acids and aminophenols were isolated as described by Bray *et al.* (1957*a*). The isolated acetanilides and mercapturic acids were shown to be identical with authentic synthetic specimens by mixed m.p. and by paper chromatography. The absorption spectra of the mercapturic acids were identical with those obtained from the corresponding synthetic acids. All the aminophenols isolated had m.p.'s in agreement with those given in the literature except the *o*-aminobromophenol (Found: N, 7.3. Calc. for C_6H_4ONBr : N, 7.4%) from *m*-bromonitrobenzene (see Table 2).

RESULTS

The average daily excretions of normal metabolites by the rabbits used were of the same order as those found previously (e.g. Bray, Hybs, James & Thorpe, 1953). The results obtained by quantitative analysis of the urines of dosed animals are summarized in Table 1. Except with *p*-bromo- and *p*-iodo-nitrobenzene, a relatively small proportion of the dose was excreted as the corresponding aniline. Except with *p*-fluronitrobenzene, a large part of the dose was excreted as phenolic metabolites. As several of the compounds caused anorexia, the values for glucosiduronic acid formed from these compounds are probably low, since the values for excretion before and after the experiment, which were used as base lines, would be higher than the true control values for the experimental period. Furthermore, with some of the compounds, significant amounts of phenolic metabolites seem to be excreted unconjugated (see Tables 3 and 4). For these reasons 'the totals accounted for' given in Table 1 for all compounds except *p*-bromo- and *p*-iodo-nitrobenzene are probably too low. The small values [by the modified iodometric method of Stekol (1936)] for mercapturic acid from the *meta* compounds, together with failure to detect any mercapturic acid in the urines, makes it probable that there is no significant excretion of mercapturic acid from these compounds. The *ortho* compounds and *p*-fluronitrobenzene form relatively large amounts of mercapturic acid.

Metabolites isolated or detected

The metabolites isolated and the yields obtained are given in Table 2. The metabolites detected by paper chromatography in extracts B, C and D of the urines are given in Tables 3 and 4. In addition to the nitro compounds listed there were faint traces of other yellow spots but these were not regularly reproducible and the amounts were too small for satisfactory identification after reduction. (The toxicity of the compounds administered precluded the use of larger doses.) All spots from

Table 1. *Excretion of metabolites of the halogenonitrobenzenes by the rabbit*

Compound	Isomer	Dose (g./rabbit)	Metabolite	Fluoronitrobenzene			Bromonitrobenzene			Iodonitrobenzene		
				<i>Ortho</i>	<i>Meta</i>	<i>Para</i>	<i>Ortho</i>	<i>Meta</i>	<i>Para</i>	<i>Ortho</i>	<i>Meta</i>	<i>Para</i>
Mercapturic acid	...	...	...	40 (36-48) [†]	2 (0-10) [†]	33 [†] (20-46) [†]	35 (20-54) [†]	0 [†]	5 [†] (1-9) [†]	15 (9-22) [†]	5 (0-19) [†]	3 [†] (1-4) [†]
Glucosiduronic acid†	...	...	...	26 (12-35) [†]	7 (0-13) [†]	2 (0-17) [†]	19 (3-38) [†]	25 (9-43) [†]	25 (10-49) [†]	12 (0-35) [†]	31 (15-39) [†]	43 (40-47) [†]
Ethereal sulphate	...	...	...	13 (0-24) [†]	36 (27-46) [†]	6 (0-12) [†]	10 (6-18) [†]	16 (12-22) [†]	28 (24-36) [†]	16 (10-21) [†]	22 (0-31) [†]	20 (16-26) [†]
Halogenoaniline, free	...	...	...	<1 [†]	4 (2-5) [†]	3 (2-4) [†]	8 (7-10) [†]	6 (5-10) [†]	19 (17-23) [†]	4 (3-7) [†]	5 (4-8) [†]	19 (15-22) [†]
Halogenoaniline, combined	...	...	...	0 [†]	1 (0-2) [†]	1 (0-1) [†]	1 (0-1) [†]	2 (2-3) [†]	8 (7-10) [†]	4 (1-6) [†]	1 (0-2) [†]	11 (9-12) [†]
Unchanged halogenonitrobenzene	...	...	...	0 [†]	0 [†]	0 [†]	0 (0-1) [†]	0 [†]	0 [†]	2 (1-2) [†]	0 [†]	0 [†]
Total accounted for	...	...	...	80	50	45	73	49	85	53	64	96

* By colorimetric method of Bray *et al.* (1956).

† The values for glucosiduronic acid are probably not reliable: anorexia often resulted from dosage with the halogenonitrobenzene and analytical results were variable and often difficult to interpret. Results are probably in general low and the total accounted for will be correspondingly low (see text).

Table 2. *Metabolites isolated from urine of rabbits given halogenonitrobenzenes*

Melting points given in parentheses are for the derivative isolated. All compounds isolated were recrystallized from aqueous ethanol.

Nitrobenzene derivative administered	Dose/rabbit (g.)	No. of rabbits	Metabolite isolated	M.p.	Yield (% of dose)
<i>o</i> -Fluoro-	0.33	1	<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	176°	10
<i>m</i> -Fluoro-	0.52	2	<i>m</i> -Fluoroaniline*	(85°)	1
		6	4-Amino-2-fluorophenol	161†	2
<i>p</i> -Fluoro-	0.53	2	<i>p</i> -Fluoroaniline*	(151)	2
		3	<i>N</i> -Acetyl- <i>S</i> -(4-nitrophenyl)-L-cysteine	157	10
<i>o</i> -Bromo-	0.4	2	<i>o</i> -Bromoaniline*	(99)	2
		6	<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	172	12
		6	4-Amino-3-bromophenol	151‡	2
<i>m</i> -Bromo-	0.6	2	<i>m</i> -Bromoaniline*	(87)	1
		6	4-Amino-2-bromophenol	165§	1
		6	2-Amino-4- or -6-bromophenol	135	1
<i>p</i> -Bromo-	0.6	2	<i>p</i> -Bromoaniline*	(168)	4
		6	<i>N</i> -Acetyl- <i>S</i> -(4-nitrophenyl)-L-cysteine	156	0.1
		6	2-Amino-5-bromophenol	149‡	3
<i>o</i> -Iodo-	0.4	2	<i>o</i> -Iodoaniline*	(107)	1
		6	<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	170	4
		6	4-Amino-3-iodophenol	146‡	1
<i>m</i> -Iodo-	0.4	2	<i>m</i> -Iodoaniline*	(121)	3
<i>p</i> -Iodo-	0.6	2	<i>p</i> -Iodoaniline*	(183)	4
		6	2-Amino-5-iodophenol	139‡	2

* As monoacetyl derivative.

† Berg & Newbery (1949) give 162°.

‡ In near agreement with Hodgson & Kershaw (1928).

§ Gattermann (1894) gave 163° and Hodurek (1897) 155°.

|| Schlieper (1893) and Hunter & Barnes (1928) gave m.p. 88° and Schütt (1885) gave m.p. 128° for 2-amino-4-bromophenol. The acetyl derivative prepared from the phenol isolated had m.p. 184°. Phillips (1930) reported 177° for 2-acet-amido-4-bromophenol. 2-Amino-6-bromophenol has not been prepared.

diazotizable compounds have been listed in Tables 3 and 4 except two from slow-running derivatives of *o*-fluoronitrobenzene (see below).

Authentic synthetic specimens of the halogenonitrophenols and aminohalogenophenols were not available. As there are 60 possible metabolites of this type and at least 26 would be required for identification by direct comparison, the labour of attempting the synthesis of the appropriate reference phenols did not seem justified. The constitution of the phenols detected can, however, to some extent be deduced on the basis of chemical tests for *o*-, *m*- and *p*-aminophenols as described by Bray *et al.* (1957*b*). Six phenols which were isolated had properties in agreement with those given in the literature. With each halogenonitrobenzene the products which would result from hydroxydehalogenation were also looked for and each urine was examined for 3- and 4-nitrocatechol. To save space, the dehalogenated phenols are entered only once in Table 3, unless positive results were obtained. There is the possibility that some of the halogenophenols, which were detected in the (unhydrolysed) B extracts and tentatively identified as *m*-nitro- or *m*-amino-phenols, were, in fact, ether-soluble glucosiduronic acids of either these or

other phenols (cf. Bray *et al.* 1957*b*). If the phenols were so conjugated, tests for *o*- and *p*-aminophenols would no longer provide evidence of structure since the conjugates of *o*- and *p*-aminophenols would react more like *m*-aminophenols. For eight of the halogenonitrobenzenes, however, the possibility of the presence of such conjugates can be dismissed, since six of the B extracts gave no naphthoresorcinol tests for glucuronic acid and two, which gave a positive reaction (those from *p*-fluoro- and *p*-iodo-nitrobenzene), did not contain either nitro- or amino-halogenophenols. Doubt therefore remains only about the *m*-nitro- and *m*-amino-phenols obtained from *o*-fluoronitrobenzene, the B extract of which gave a positive naphthoresorcinol test. As spots corresponding to these two phenols were also obtained from paper chromatograms of the (hydrolysed) C or D extract, it is unlikely that they were glucosiduronic acids. This is supported by the finding of these spots on a paper chromatogram of hydrolysed B extract. This chromatogram also bore a spot corresponding to 4-amino-3-fluorophenol and one corresponding to the aminophenol formed by reduction of the 2- or 4-fluoro-3-nitrophenol which had been found in B extracts. These spots probably represented the

hydrolysis products of material, presumably glucosiduronic acids, which gave spots of R_F about 0 and 0.13 (solvent mixture *C*) on the chromatogram of unhydrolysed B extract. The following are the arguments for the constitution of the phenolic metabolites listed in Tables 3 and 4.

From o-fluoronitrobenzene. Hydroxylation can give one *o*-, two *m*- and one *p*-nitrofluorophenol and the corresponding aminophenols. The main phenolic metabolites were a *p*-aminofluorophenol and its corresponding nitrophenol (shown by reduction to the aminophenol). No *o*-amino- or

o-nitro-fluorophenol was detected. Small amounts of a *m*-amino- and *m*-nitro-phenol were found in the B extracts but the *m*-aminophenol was not that which was formed by reduction of the *m*-nitro-phenol. The *m*-aminophenol (R_F 0.5, solvent mixture *C*) corresponding to the nitrophenol was obtained after hydrolysis of the B extract and was therefore probably present only as an ether-soluble conjugate (see above).

From m-fluoronitrobenzene. Two *o*-, one *m*- and one *p*-nitrofluorophenol and the corresponding aminophenols are possible. The main phenolic

Table 3. R_F values and detection of metabolites of fluoronitrobenzenes

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-3*N*-(NH₄)₂CO₃-aq. 3*N*-NH₃ soln. (4:3:3, by vol.; Corner & Young, 1954) (3 hr.); *D*, benzene-acetic acid-water (1:1:2, by vol.) (1.5 hr.). Detecting reagents: *a*, 2*N*-HCl, 0.1% NaNO₂, 0.5% ammonium sulphamate and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); *b*, indophenol reaction (Bray *et al.* 1957*b*); *c*, 20% (w/v) Na₂CO₃; *d*, 0.5% FeCl₃. Colours of spots: B, blue; Bn, brown; G, green; M, magenta; O, orange; P, purple; Y, yellow; p, pale; d, deep. Authentic specimens of the compounds in parentheses were not available for comparison; the arguments for the suggested constitutions are given in the Results section. Under R_F values, × indicates that the spots obtained were not satisfactory for identification, e.g. due to streaking. Metabolites were sought in extracts 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.

	R_F values in solvent mixture			Colour with detecting reagent				Detected in extract		
	<i>A</i>	<i>B</i>	<i>C</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>B</i>	<i>C</i>	<i>D</i>
Derivatives of <i>o</i> -fluoronitrobenzene										
<i>o</i> -Fluoroaniline	1.0	×	0.09	M	.	.	.	Steam-distillate		
<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	0.65	0.0	0.46	.	.	Y	.	+	-	-
(3-Fluoro-4-nitrophenol)	0.68	0.08	0.40	.	.	Y	.	+	+	-
(2- or 4-Fluoro-3-nitrophenol)*	×	×	0.50	.	.	Y	.	(+)	(+)	-
(4-Amino-3-fluorophenol)	0.0	0.0	0.71	B	B	.	.	-	-	+
(3-Amino-2- or -4-fluorophenol)*†	×	×	0.50	M	.	.	.	-	-	-
(3-Amino-4- or -2-fluorophenol)*†	0.0	0.0	0.79	M	.	.	.	(+)	-	(+)
2-Nitrophenol	1.0	1.0	×	.	.	dY	.	(+)	(+)	-
2-Aminophenol	0.02	0.0	0.81	B‡	.	.	Bn	-	-	-
Derivatives of <i>m</i> -fluoronitrobenzene										
<i>m</i> -Fluoroaniline	1.0	0.15	0.89	M	.	.	.	+	-	+
(2-Fluoro-4-nitrophenol)	0.7	0.06	0.57	.	.	Y	.	+	+	-
4-Amino-2-fluorophenol§	0.17	0.0	0.61	B	B	.	.	-	-	+
(2-Amino-4- or -6-fluorophenol)	0.52	×	0.25	B‡	.	.	.	-	-	(+)
3-Nitrophenol	1.0	0.11	0.81	.	.	dY	pP	(+)	(+)	-
3-Nitrocatechol	×	×	0.25	.	.	OBn	G	-	-	-
4-Nitrocatechol	0.17	0.0	0.28	.	.	O	G	(+)	(+)	-
3-Aminophenol	0.05	0.0	0.77	BP	.	.	PBn	-	-	(+)
Derivatives of <i>p</i> -fluoronitrobenzene										
<i>p</i> -Fluoroaniline	0.70	0.0	0.90	M	B	.	.	-	-	+
<i>N</i> -Acetyl- <i>S</i> -(4-nitrophenyl)-L-cysteine	0.87	0.0	0.60	.	.	dY	.	+	-	-
(2-Amino-5-fluorophenol)	0.20	×	×	B‡	.	.	.	-	-	(+)
(5-Amino-2-fluorophenol)	0.75	0.05	0.70	M	.	.	.	-	-	(+)
4-Nitrophenol	0.83	0.05	0.58	.	.	Y	pP	-	(+)	-
4-Nitrocatechol	0.17	0.0	0.28	.	.	O	G	(+)	(+)	-
4-Aminophenol	0.01	0.0	0.72	B	B	.	P	-	-	+

* Reduction of the nitrophenol gave the aminophenol of R_F 0.5 which was not found in extracts B and D but was obtained when extract B was hydrolysed (see Results section).

† Lack of a reference compound made it impossible to decide which R_F values pertained to the given aminophenol.

‡ Bright-yellow colour with nitrous acid. Blue develops slowly after addition of the coupling reagent.

§ Isolated specimen of this compound had m.p. agreeing with that given in the literature.

|| 3- and 4-Nitrocatechol have R_F values 1.0 and 0.2 respectively in solvent mixture *D* and give purple and red colours respectively with *N*-NaOH.

metabolites were the *p*-nitro- and the *p*-amino-phenol. Traces of an *o*-aminophenol were found but it was not possible to distinguish between the two possible *o*-aminophenols. A *m*-nitro- and *m*-amino-phenol were detected and are recorded as 3-nitro- and 3-amino-phenol in Table 3, since it is unlikely that 3-fluoro-5-nitro- and 3-amino-5-fluoro-phenol would both have the same R_F values as have 3-nitro- and 3-amino-phenol respectively. The presence of 4-nitrocatechol is also more readily accounted for as a product of further hydroxylation of 3-nitrophenol.

From p-fluoronitrobenzene. One *o*- and one *m*-nitrofluorophenol and the corresponding aminophenols are possible. Both the aminophenols were detected but the main phenolic metabolite was the defluorinated product, 4-aminophenol.

From the bromo- and iodo-nitrobenzenes. Similar arguments to those used for the fluoro compounds apply. The absence of dehalogenated phenols simplified the identification. The compound from *m*-bromonitrobenzene described in Table 4 as 2-amino-4- or -6-bromophenol was isolated, but its properties were not in satisfactory agreement with

Table 4. R_F values and detection of metabolites of bromo- and iodo-nitrobenzenes

Solvent mixtures, times of run, paper, detecting reagents and colour abbreviations are as in Table 3. Authentic specimens of the compounds in parentheses were not available for comparison; the arguments for the suggested constitutions are given in the Results section. Under R_F values, × indicates that the spots obtained were not satisfactory for identification, e.g. due to streaking. Metabolites were sought in extracts 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.

	R_F values in solvent mixture			Colour with detecting reagent			Detected in extract		
	A	B	C	a	b	c	B	C	D
Derivatives of <i>o</i> -bromonitrobenzene									
<i>o</i> -Bromoaniline	1.0	1.0	1.0	M	.	.	−	−	+
<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	0.65	0.0	0.46	.	.	Y	+	−	−
(3-Bromo-4-nitrophenol)	0.90	0.0	0.63	.	.	Y	+	+	−
4-Amino-3-bromophenol*	0.62	0.0	0.90	B	B	.	+	−	+
(3-Amino-2- or -4-bromophenol)	0.50	0.25†	0.10	M	.	.	−	−	(+)
Derivatives of <i>m</i> -bromonitrobenzene									
<i>m</i> -Bromoaniline	1.0	1.0	1.0	M	.	.	+	−	+
(2-Bromo-4-nitrophenol)	0.31	0.22	0.50	.	.	Y	+	+	−
4-Amino-2-bromophenol*	×	0.0	0.70	B	B	.	−	−	+
(2-Amino-4- or -6-bromophenol)	×	×	0.87	B†	.	.	−	−	+
(3-Amino-5-bromophenol)	×	0.25†	0.80	M	.	.	(+)	−	(+)
Derivatives of <i>p</i> -bromonitrobenzene									
<i>p</i> -Bromoaniline	1.0	×	1.0	M	.	.	+	−	+
<i>N</i> -Acetyl- <i>S</i> -(4-nitrophenyl)-L-cysteine	0.85	0.0	0.60	.	.	dY	+	−	−
2-Amino-5-bromophenol*	0.63	0.0	0.92	B†	.	.	−	−	+
(5-Amino-2-bromophenol)	×	×	0.05	M	.	.	−	−	(+)
Derivatives of <i>o</i> -iodonitrobenzene									
<i>o</i> -Iodoaniline	1.0	×	1.0	M	.	.	+	−	−
<i>N</i> -Acetyl- <i>S</i> -(2-nitrophenyl)-L-cysteine	0.65	0.0	0.46	.	.	Y	+	−	−
(3-Iodo-4-nitrophenol)	×	0.02	0.66	.	.	Y	+	+	−
4-Amino-3-iodophenol*	0.30	0.0	0.69	B	B	.	−	−	+
(3-Amino-2- or -4-iodophenol)§	0.0	×	0.06	M	.	.	−	−	(+)
(3-Amino-4- or -2-iodophenol)§	0.95	×	0.28	M	.	.	−	−	(+)
Derivatives of <i>m</i> -iodonitrobenzene									
<i>m</i> -Iodoaniline	1.0	×	0.90	M	.	.	+	−	+
(2-Iodo-4-nitrophenol)	0.76	0.10	0.67	.	.	Y	+	+	−
(2-Amino-4- or -6-iodophenol)	0.28	0.0	0.83	B†	.	.	−	−	+
Derivatives of <i>p</i> -iodonitrobenzene									
<i>p</i> -Iodoaniline	1.0	×	0.90	P	.	.	+	−	+
<i>N</i> -Acetyl- <i>S</i> -(4-nitrophenyl)-L-cysteine	0.85	0.0	0.60	.	.	dY	+	−	−
2-Amino-5-iodophenol*	0.0	×	0.80	B†	.	.	−	−	+

* Isolated specimens of these compounds had m.p. agreeing with those given in the literature.

† Approximate; spot very elongated.

‡ Bright-yellow colour with nitrous acid. Blue develops slowly after addition of the coupling reagent.

§ Lack of a reference compound made it impossible to decide which R_F values pertained to the given aminophenol.

those given in the literature for 2-amino-4-bromophenol (see Table 2). 2-Amino-6-bromophenol is unknown.

DISCUSSION

The results in the present investigation are, to a large extent, such as would have been expected from those obtained by Bray *et al.* (1956) with the corresponding chloro compounds. All the *o*- and *p*-halogenonitrobenzenes form mercapturic acids by acetylcysteyldehalogenation. The evidence now presented is stronger than that obtained with the chloro compounds, from which the formation of mercapturic acid was shown by paper chromatograms, since the mercapturic acids have been isolated from the urines of rabbits given *o*- and *p*-fluoro-, *o*- and *p*-bromo- and *o*-iodo-nitrobenzene. Halogens in a *meta* position in nitrobenzenes are particularly stable (see Bunnett & Zahler, 1951) and the lack of evidence for the formation of mercapturic acids from the *m*-halogenonitrobenzenes, apart from *m*-fluoronitrobenzene, is not surprising. *m*-Fluoronitrobenzene yields *m*-nitroanisole by treatment with sodium methoxide (Holleman & Beekman, 1903). Furthermore, the present work suggests that hydroxyldefluorination of *m*-fluoronitrobenzene can occur in the rabbit. Formation of mercapturic acid by acetylcysteyldefluorination might therefore have been expected. There is, however, no satisfactory evidence for the formation of mercapturic acid from *m*-fluoronitrobenzene.

Hydroxyldechlorination has been observed in two trichloronitrobenzenes (Betts *et al.* 1957), but not in the monochloronitrobenzenes. The monobromo- and moniodo-nitrobenzenes do not appear to undergo hydroxyldehalogenation but there is evidence that this occurs in the monofluoronitrobenzenes. It is unequivocal that defluorination of *p*-fluoronitrobenzene occurs since a *p*-aminophenol is the main phenolic metabolite excreted and only *o*- or *m*-aminophenols can be formed by hydroxylation without defluorination. At least some defluorination must occur in the nitro compound, since 4-nitrophenol and 4-nitrocatechol were detected in urine. The defluorination probably occurs within the animal since no nitrophenol could be detected after aqueous suspensions of *p*-fluoronitrobenzene had been kept at 37° for 4 hr. or at room temperature for 18 hr. *p*-Fluoroaniline is equally stable under these conditions.

The extent of hydroxyldefluorination of *o*- and *m*-fluoronitrobenzene is not so great, since only traces of defluorinated products were detected. With both these compounds, however, hydroxylation in a position *ortho* or *meta* to the nitrogen can occur without displacement of fluorine. As the properties of the phenols which can be so formed

are unknown, it cannot be stated unequivocally that they can be distinguished with certainty from the relevant phenols formed by defluorination, although the detection of 4-nitrocatechol strengthens the probability that defluorination of *m*-fluoronitrobenzene occurs. The absence of 3-nitrocatechol amongst the metabolites of *o*-fluoronitrobenzene is not unexpected since Robinson, Smith & Williams (1952) failed to detect 3-nitrocatechol as a metabolite of *o*-nitrophenol.

Although lack of reference compounds prevented a complete identification of the hydroxylated halogenated metabolites, sufficient evidence has been obtained to show a general similarity with the chlorophenol metabolites of the monochloronitrobenzenes (Bray *et al.* 1956). The results are summarized in Table 5. In general, fewer halogenophenols have been detected than with the chloro compounds, but this may have been due partly to the greater difficulty of interpreting paper chromatograms when reference compounds were not available. The chemical similarity of the phenols may also have offset the probability of revealing differences when mixtures of phenols were examined in even three different solvent mixtures. If the distinction between unreduced ($-\text{NO}_2$) and reduced ($-\text{NH}_2$) forms is neglected, only *m*-bromonitrobenzene yields a metabolite which does not correspond to one which was identified from *m*-chloronitrobenzene.

The position of the hydroxyl group in the nitrophenols is of interest. With the di- and the trichloronitrobenzenes (Bray *et al.* 1957*a, b*; Betts *et al.* 1957), where nitrophenols were detected as metabolites, they were those which would be formed if the nitro group directed hydroxylation into the *meta* position, except that 3:5-dichloro-2-nitrophenol and, in traces, 2:5-dichloro-4-nitrophenol were identified as metabolites of 2:4- and 2:5-dichloronitrobenzene respectively and traces of 2:3:5-trichloro-4-nitrophenol were obtained from 2:3:6-trichloronitrobenzene. In the monohalogenonitrobenzenes, however, *m*-nitrophenols were found only in traces and only as metabolites of *o*-fluoro-, *o*-chloro- and *p*-chloro-nitrobenzenes. The predominant nitrophenols from all the *o*- and *m*-halogenonitrobenzenes were *p*-nitrophenols. The major phenolic metabolites of the *p*-halogenonitrobenzenes were *o*-aminophenols and no *o*-nitrophenols were detected. The question whether hydroxylation precedes or follows reduction of the nitro group was studied for the monochloronitrobenzenes by Bray *et al.* (1956) by examination of the phenolic metabolites excreted after rabbits had been given the corresponding chloroanilines. These compounds yielded only *o*- and *p*-aminophenols and there was no evidence of *meta*-hydroxylation. It was therefore concluded that *meta*-hydroxylation

Table 5. *Phenolic metabolites of the monohalogenonitrobenzenes*

The major phenolic metabolites are indicated by ++, those present in small amounts or traces by + and compounds not detected by -. It should be emphasized that this grading is based mainly on the appearance of spots on paper chromatograms and not on quantitative determinations; particularly in the ++ grading, it should not be taken that the amounts of nitro- and amino-phenols excreted are approximately the same. The aminophenols were almost certainly present in greater amounts, e.g. only aminophenols were present in sufficient amount to permit isolation (see Table 2). An inferior figure indicates the position of the halogen relative to the hydroxyl group if more than one product is possible with the hydroxyl group in the given position. Results for the chloronitrobenzenes are from Bray *et al.* (1956).

Position of hydroxyl relative to the nitrogen ...	Phenols formed by hydroxylation						Phenols formed by hydroxyldehalogenation		
	<i>Ortho</i>		<i>Meta</i>		<i>Para</i>				4-Nitro-catechol
	Nitro	Amino	Nitro	Amino	Nitro	Amino	Nitro	Amino	
Nitrobenzene derivative									
<i>o</i> -Fluoro-	-	-	+ _{2 or 4}	+ ₂ , + ₄	++	++	+	-	-
<i>o</i> -Chloro-	+	+	+ ₂ , + ₄	+ ₂ , ++ ₄	++	++	-	-	-
<i>o</i> -Bromo-	-	-	-	+ _{2 or 4}	++	++	-	-	-
<i>o</i> -Iodo-	-	-	-	+ ₂ , + ₄	++	++	-	-	-
<i>m</i> -Fluoro-	-	+ _{2 or 4}	-	-	++	++	+	+	+
<i>m</i> -Chloro-	-	++ ₄	-	-	++	++	-	-	-
<i>m</i> -Bromo-	-	+ _{2 or 4}	-	+	++	++	-	-	-
<i>m</i> -Iodo-	-	++ _{2 or 4}	-	-	++	-	-	-	-
<i>p</i> -Fluoro-	-	+	-	+	} <i>Para</i> position occupied by halogen }		+	++	+
<i>p</i> -Chloro-	-	++	+	+			-	-	-
<i>p</i> -Bromo-	-	++	-	+			-	-	-
<i>p</i> -Iodo-	-	++	-	-			-	-	-

occurred only in the nitro compounds. All the four possible chloronitrophenols were excreted by rabbits given *o*-chloronitrobenzene so that in this compound *ortho*-, *meta*- and *para*-hydroxylation can occur before reduction. With the other *o*-halogenonitrobenzenes the products of *ortho*-hydroxylation were not detected (see Table 5), but this is not surprising for the reasons given above, in conjunction with the fact that only traces of the products of *ortho*-hydroxylation of *o*-chloronitrobenzene were found (Bray *et al.* 1956). As the phenolic metabolites of the fluoro- (apart from products of hydroxyldefluorination), bromo- and iodo-nitrobenzenes so closely resembled those from the corresponding chloro compounds (see Table 5) it seems reasonable to suppose that the origins of the various hydroxylation products are similar to those of the corresponding chloro compounds.

The most conspicuous general features of the hydroxylation products of the halogenonitrobenzenes are: (1) the main phenols from the *ortho* compounds are *p*-aminophenols and those from the *meta* compounds are *p*- and *o*-aminophenols (4-amino-2-iodophenol was the only phenol of this type not detected); (2) the main phenols from the *p*-halogenonitrobenzenes are *o*-aminophenols (with *p*-fluoronitrobenzene, however, only a small amount of this metabolite was excreted, probably because the major phenolic metabolite was one formed by hydroxyldefluorination); (3) only in the fluoronitrobenzenes is the halogen group sufficiently labile to be replaced by hydroxyl.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-fluoro-, *o*-, *m*- and *p*-bromo- and *o*-, *m*- and *p*-iodo-nitrobenzene has been studied in the rabbit.

2. The *ortho* and *para* compounds form mercapturic acids (over 30% of the dose with the fluoro- and *o*-bromo- compounds) but the *meta* compounds do not.

3. Except with *p*-fluoronitrobenzene (less than 10%) about 30-60% of the dose is excreted as phenols conjugated with glucuronic and sulphuric acids; except with *p*-bromo- and *p*-iodo-nitrobenzene (30%) less than 10% of the dose is excreted as the corresponding anilines.

4. The main phenolic metabolite of *p*-fluoronitrobenzene is *p*-aminophenol. *o*- and *m*-Fluoronitrobenzene also probably undergo hydroxyldefluorination.

5. Apart from hydroxyldehalogenation in the fluoro compounds the metabolic fate of the fluoro-, bromo- and iodo-nitrobenzenes is, in general, qualitatively similar to that of the corresponding chloro compounds.

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REFERENCES

- Berg, S. S. & Newbery, G. (1949). *J. chem. Soc.* p. 642.
 Betts, J. J., Bray, H. G., James, S. P. & Thorpe, W. V. (1957). *Biochem. J.* **66**, 610.
 Bratton, A. C. & Marshall, E. K. jun. (1939). *J. biol. Chem.* **128**, 537.

- Bray, H. G., Hybs, Z., James, S. P. & Thorpe, W. V. (1953). *Biochem. J.* **53**, 266.
- Bray, H. G. & James, S. P. (1957). *Biochem. J.* **66**, 45P.
- Bray, H. G., James, S. P. & Thorpe, W. V. (1956). *Biochem. J.* **64**, 38.
- Bray, H. G., James, S. P. & Thorpe, W. V. (1957*a*). *Biochem. J.* **65**, 483.
- Bray, H. G., James, S. P. & Thorpe, W. V. (1957*b*). *Biochem. J.* **67**, 607.
- Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
- Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.
- Bunnett, J. F. & Zahler, R. E. (1951). *Chem. Rev.* **49**, 273.
- Corner, E. D. S. & Young, L. (1954). *Biochem. J.* **58**, 647.
- Dippy, J. F. J. & Williams, F. R. (1934). *J. chem. Soc.* p. 1466.
- Gattermann, L. (1894). *Ber. dtsh. chem. Ges.* **27**, 1927.
- Hodgson, H. H. & Kershaw, A. (1928). *J. chem. Soc.* p. 2703.
- Hodurek, O. (1897). *Ber. dtsh. chem. Ges.* **30**, 477.
- Holleman, A. F. & Beekman, J. W. (1903). *Proc. K. Akad. Wet. Amst.* **6**, 327. [Quoted from *J. chem. Soc. Abstr.* (1904), **86**, i; 232.]
- Hunter, L. & Barnes, R. S. (1928). *J. chem. Soc.* p. 2051.
- Phillips, M. A. (1930). *J. chem. Soc.* p. 2400.
- Robinson, D., Smith, J. N. & Williams, R. T. (1952). *Biochem. J.* **50**, 221.
- Schiemann, G. & Pillarsky, R. (1929). *Ber. dtsh. chem. Ges.* **62**, 3035.
- Schlieper, F. W. (1893). *Ber. dtsh. chem. Ges.* **26**, 2469.
- Schütt, F. (1885). *J. prakt. Chem.* [2], **32**, 61.
- Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
- Ullmann, F. (1896). *Ber. dtsh. chem. Ges.* **29**, 1878.
- Weselsky, P. & Benedikt, R. (1882). *Mh. Chem.* **3**, 386.

Displacement Fractionation of Deoxyribonucleoproteins by Heparin and Dextran Sulphate

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Recent investigations have shown that the total deoxyribonucleic acid, isolated from a single biological source, may be regarded as a group of closely related polymeric species having significantly different compositions.

The methods of fractionation commonly employed hitherto fall into two groups: (i) those dependent on the extraction of denatured deoxyribonucleoproteins with solutions of sodium chloride, either on a concentration gradient (Crampton, Lipshitz & Chargaff, 1954*a*, *b*; Chargaff, Crampton & Lipshitz, 1953; Lipshitz & Chargaff, 1956) or on a time gradient (Lucy & Butler, 1955), and (ii) methods involving the interaction of isolated deoxyribonucleic acid with proteins (Brown & Watson, 1953), polypeptides (Spitnik, Lipshitz & Chargaff, 1955) or ionic-exchange reagents (Bendick, Fresco, Rosenkranz & Beiser, 1955).

The present studies are concerned with an alternative means of fractionation of undenatured deoxyribonucleoproteins from calf-thymus gland and ox spleen, founded on the stepwise displacement by strong anionic polymers of the protein moiety of the nucleoproteins, and the accompanying liberation of deoxyribonucleic acid fractions. It was shown by Chargaff & Olson (1938), in

another connexion, that protamine combines with heparin yielding an insoluble complex conjugate. We have now shown that isolated calf-thymus histone behaves in a similar way and is precipitated in 0.14M-sodium chloride by titration with heparin or with dextran sulphate. Addition of sub-equivalent quantities of these polysulphates to deoxyribonucleoprotein in 0.14M-sodium chloride leads to the liberation of deoxyribonucleic acid in solution. Unchanged nucleoproteins and the insoluble conjugate formed were removed by centrifuging. Repetition of the process thus led to the preparation of a series of deoxyribonucleic acid fractions.

EXPERIMENTAL

Materials

Calf-thymus deoxyribonucleoprotein. The material was extracted from minced glands (obtained immediately after slaughter) by the method of Daly, Allfrey & Mirsky (1949). The product was reprecipitated four times from M-NaCl soln. and obtained as a gel by centrifuging at 81 000 g. Where necessary, this was stored at -18° .

Ox-spleen deoxyribonucleoprotein. Fresh tissue (150 g.) was homogenized in a Waring Blendor for 6 min. in 350 ml. of 0.14M-NaCl soln. containing 0.01M-trisodium citrate. The solid material was separated by centrifuging (4000 g for 30 min.) and washed twice with 3 vol. of NaCl-trisodium citrate soln. The washed solid was then mixed rapidly in a Waring Blendor with M-NaCl (3 vol.). After

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