Studies in Detoxication

40. THE METABOLISM OF NITROBENZENE IN THE RABBIT. *o*-, *m*- AND *p*-NITROPHENOLS, *o*-, *m*- AND *p*-AMINOPHENOLS AND 4-NITROCATECHOL AS METABOLITES OF NITROBENZENE

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Despite its relatively highly toxic nature, nitrobenzene has been used under the name of 'oil of mirbane' or 'artificial oil of bitter almonds' for flavouring and perfuming purposes. Its main industrial use is in the manufacture of aniline and benzidine. In spite of the importance of nitrobenzene, little is known about its metabolism. Reports on the nature of the urine from men or animals which had received nitrobenzene always refer to the dark colour of the urine. This was attributed to the presence of *p*-aminophenol (Meyer, 1905) which was, up to the present, the only proved metabolite of nitrobenzene. Small amounts of nitrobenzene are also excreted unchanged (e.g. Frossard, 1925; Meyer, 1905). The mechanism of the conversion of nitrobenzene to *p*-aminophenol is still unknown, although much has been written about this and its relation to methaemoglobin formation (e.g. Dresbach & Chandler, 1918; Heubner & Meier, 1923; Lipschitz, 1920). In yeast, nitrobenzene is reduced to aniline, probably via nitrosobenzene and *N*-phenylhydroxylamine (Neuberg & Welde, 1914).

In the present work we shall show that in the rabbit, nitrobenzene gives rise to at least seven phenols as well as to small amounts of aniline. Although we have attempted a quantitative assessment of the metabolism of nitrobenzene, we feel that our results in this direction are not entirely satisfactory, and although the quantitative data enable us to make some interesting suggestions, further work is necessary.

METHODS

The effect of nitrobenzene on rabbits. Female giant chinchilla rabbits were used in this work and were kept on a diet of 70 g. rat cubes per day and water *ad lib.* The nitrobenzene was administered in 25 ml. water by stomach tube. The resistance of the rabbits to nitrobenzene varied greatly, since some resisted a dose of 370 mg./kg., whereas others were killed by 180 mg./kg. The highest level which could be fed with reasonable safety was 200 mg./kg. Above this level 50% of the animals died. The toxic effect was not immediately apparent, for with doses of the order of 250 mg./kg. death occurred between 2 and 6 days later. With doses above 300 mg./kg. death occurred within 24 hr.

Nitro groups. Nitro groups were estimated by the titanous chloride method described in the preceding paper (Robinson, Smith & Williams, 1951).

Total p-*aminophenol* was estimated by the indophenol method (Robinson *et al.* 1951).

p-*Aminophenylsulphate.* The *p*-aminophenol present as ethereal sulphate was estimated by making the urine 0·5 N with respect to HCl and heating at 100° for 10 min. This short hydrolysis decomposes the ethereal sulphate and the liberated *p*-aminophenol can then be estimated by the indo-

Table 1. *Nitro compounds and* p-*aminophenol derivatives in the urine of rabbits 2 days after receiving* 0·5 *g. nitrobenzene orally*

(Values bracketed represent: 1st day / 2nd day; Total of 1st and 2nd day.)

	Percentage of the dose excreted as						
					p-Aminophenylglucuronide		
Rabbit no.	Nitro compounds *N*	Total *p*-aminophenol *A*	*N* + *A*	*p*-Aminophenyl sulphate *B*	By difference (*A* − *B*)	By diazo reaction *C*	(*A* − *B*) − *C**
26	17·9 / 8·4 } 26·3	30·9 / 11·1 } 42	68	4·3 / 2·6 } 6·9	35	20·5 / 8·0 } 28·5	6·5
27	11·5 / 6·3 } 17·8	15·9 / 11·0 } 26·9	45	3·3 / 1·9 } 5·2	21·7	10·8 / 9·5 } 20·3	1·4
28	8·9 / 6·3 } 15·2	25·0 / 9·9 } 34·9	50	3·3 / 1·1 } 4·4	30·5	12·4 / 5·6 } 18·0	12·5

* This figure gives an estimate of *p*-acetamidophenylglucuronide.

phenol method. The difference between the total *p*-aminophenol and the *p*-aminophenylsulphate estimations should give approximately the amount of *p*-aminophenol combined with glucuronic acid. This procedure, however, does not allow for the presence of *p*-acetamidophenylsulphate which may or may not be present. Mild hydrolysis of this compound would probably remove the sulphate group but not necessarily the acetyl group.

p-*Aminophenylglucuronide*. The procedure used was the same as that used for the estimation of *o*-aminophenylglucuronide in the preceding paper (Robinson *et al.* 1951) except that the standard curve was constructed with *p*-aminophenylglucuronide instead of the *o*-isomer. The results of this estimation should roughly give the same values as the difference between the two preceding indophenol estimations. In general, however, the results should be, and are, a little lower since *p*-acetamidophenylglucuronide is not estimated by the diazo method and is almost certain to be present (see Table 1). The approximate agreement between the two methods of assessing *p*-aminophenylglucuronide also suggests that *o*- and *m*-aminophenylglucuronides, although almost certain to be present (since the phenols themselves have been isolated), are only present in small amounts compared with the *p*-isomer. This view is supported by the isolation experiments (see later, p. 231).

Mercapturic acid. The iodine consumption of nitrobenzene urine after alkaline hydrolysis was investigated as described by Parke & Williams (1951) for benzene urine. It was assumed that if nitrophenylmercapturic acid occurred in the urine it would be hydrolysed by alkali to nitrothiophenol. *o*- and *p*-Nitrothiophenols were prepared and shown to be stable to the alkali and the temperature used in the iodometric estimation of mercapturic acids. By iodine titration the percentage of nitrobenzene (at 150 mg./kg.) which may have been excreted as mercapturic acid was in three experiments 3·5, 2·8 and 4·8 %. These results are so low as to be just within the margin of error of the method and may be of no significance.

Detection and isolation of metabolites

Nature of nitrobenzene urine. Urine voided during the first 4 hr. after nitrobenzene administration was a clear golden-brown and slightly acid in reaction (pH 6·5). It gave a positive naphthoresorcinol reaction and turned a deeper yellow on adding alkali, showing the presence of nitro compounds. This urine gave only a feeble diazo reaction (nitrous acid and dimethyl-α-naphthylamine) before and after hydrolysis. It very slightly reduced Fehling's and Benedict's reagents. Ammoniacal silver nitrate was slowly reduced.

Urine voided 1 or 2 days after dosing was dark brown in colour and alkaline (pH 8). It gave an intense blue diazo reaction which became more intense after mild acid hydrolysis after which treatment it gave an intense indophenol reaction. After 2 days nitrophenols were not detectable, these being mainly excreted on the first day after dosing, with a little on the second. Most of the aminophenols had also been excreted on the first 2 days, although about 1 % of the dose still appeared as *p*-aminophenol on the third day. The urine, however, gave a weak diazo test for at least 7 days after dosing, suggesting that a part of the dose was being eliminated slowly. This is presumably the nitrobenzene which had been deposited in fatty tissues.

Steam-volatile metabolites of nitrobenzene

o-*Nitrophenol*. A total of 1·5 g. of nitrobenzene (A.R., b.p. 209°) was fed to three rabbits and urine was collected for 2 days. The urine was made 2 N with respect to H_2SO_4, heated under reflux for 2 hr. on a boiling-water bath and then steam distilled. The first few ml. were bright yellow and contained *o*-nitrophenol. The yellow colour of the distillate was intensified on making alkaline, the colour being discharged on acidification.

The *o*-nitrophenol in the distillate was identified by paper chromatography and spectroscopy as follows. The absorption spectrum of the distillate was determined at acid and alkaline pH, and whilst all the maxima of *o*-nitrophenol (at 283 and 415 mμ. in 0·05 N-NaOH and 280 and 351 mμ. in 0·5 N-HCl) were present their extinctions were not in the correct ratios due to irrelevant absorption. The impurities present were shown to be, by colour tests, volatile phenols of normal urine.

In another experiment it was shown that phenol and *o*-nitrophenol could be separated by paper partition chromatography using benzene, *n*-butanol and NH_4OH (sp.gr. 0·880) in the ratio 5 : 2 : 2, as solvent. In this mixture, *o*-nitrophenol moves very slowly and if the chromatogram is run for 24 hr. no trace of phenol could be found on the paper, whereas *o*-nitrophenol had only moved a short distance.

Purification of the *o*-nitrophenol distillate for spectroscopy was carried out by partition chromatography on a silica column. The column (4 × 50 cm.), was packed dry with silica (Harrington's 'pure precipitated') containing an equal weight of the aqueous phase of the solvent mixture described above. The *o*-nitrophenol distillate (containing about 0·5 mg. of phenols) from nitrobenzene urine was extracted with ether, and the extract mixed with a little dry silica which was then placed on the top of the column of silica. The column was then developed with the benzene-butanol-ammonia mixture. After 4 hr. the column was allowed to drain, thus removing interfering phenols. The column was extruded and the yellow *o*-nitrophenol band cut out and suspended in 0·1 N-NaOH and then steam distilled to remove ammonia and excess solvents. The suspension was then made acid and the *o*-nitrophenol present steam distilled. The steam distillate now showed the correct spectrum of *o*-nitrophenol in acid and in alkali with respect to the wavelengths of the maxima and their extinction coefficients (see Table 2). In this way, *o*-nitrophenol was satisfactorily identified in the urine.

For the estimation of *o*-nitrophenol, the urine of rabbits which had been fed 200–250 mg./kg. of nitrobenzene was hydrolysed as above and steam distilled until the distillate no longer had a yellow colour. The distillate was made up to 50 ml. with 0·1 N-NaOH and the extinction of the solution at 415 mμ. measured in the Unicam spectrophotometer. Results are given in Table 3.

Nitrobenzene and aniline. A rabbit was fed 500 mg. of nitrobenzene by stomach tube and a 48 hr. urine was made alkaline (pH 9–10) with Na_2CO_3 and steam distilled. Under these conditions *o*-nitrophenol was not distilled and the

Table 2. *Spectroscopic data for o-nitrophenol, aniline and nitrobenzene*

Compound	Solvent	Authentic material $\lambda_{max.}$	Authentic material $\epsilon_{max.}$	Authentic material $\epsilon_{max.\ short}/\epsilon_{max.\ long}$	Urinary material $\lambda_{max.}$	Urinary material E_{short}/E_{long}
o-Nitrophenol	0·05 N-HCl	280	6100	—	280	—
		351	3000	2·03	351	2·13
	0·05 N-NaOH	283	3850	—	283	—
		415	4500	0·86	415	1·04
Nitrobenzene	Water	270	7700	—	270	—
Aniline	0·1 N-NaOH	280	1500	—	280	—
	0·1 N-HCl	254	160	—	Maximum too small to be accurately measured	—

distillate contained nitrobenzene and aniline. That both these substances were present was suggested by the diazo test which was positive on the distillate and was increased in intensity when the distillate was reduced with Zn dust and HCl. To separate nitrobenzene, the distillate was made acid to congo red and steam distilled. This second distillate gave no diazo test until reduced with Zn dust and HCl. The absorption spectrum in water was determined, and it showed a large single band with a peak at 270 mμ. similar to that found for authentic nitrobenzene (cf. Doub & Vandenbelt, 1947). From the height of the peak and the known $\epsilon_{max.}$ (which was found to be 7700) for nitrobenzene, it was calculated that in 2 days after dosing 0·029 % of the nitrobenzene was excreted unchanged.

Table 3. *Excretion of o-nitrophenol by rabbits during 2 days after receiving nitrobenzene*

Rabbit no.	Weight (kg.)	Dose of nitrobenzene (mg.)	*o*-Nitrophenol excreted (% of dose)*
31	2·5	500	0·054
35	2·6	500	0·037
36	3·0	750	0·057
36†	3·0	750	0·165

* Estimated spectrophotometrically at 415 mμ. in 0·05 N-NaOH (see Table 2).

† Second dose fed to rabbit no. 36, 3 days after first dose.

The residue from the second steam distillation contained the aniline, and this was confirmed by distillation from alkaline solution and spectroscopic examination. In 0·1 N-NaOH a peak was found at 280 mμ. which disappeared on making acid. Aniline shows a peak at 280 mμ. ($\epsilon_{max.}$ 1500) in NaOH which disappears on acidification (see Table 2).

From the height of the peaks of the absorption spectrum it was calculated that in 2 days 0·5 % of the nitrobenzene fed appeared in the urine as aniline. This was confirmed in another experiment by colorimetric estimation of the aniline according to Smith & Williams (1949). By this method, the aniline excreted in 2 days was found to be 0·47 % of the dose of nitrobenzene.

The steam-volatile metabolites of nitrobenzene (0·5 g./rabbit) account for about 0·6–0·7 % of the dose in 2 days and consist largely of aniline (0·5 %) and traces of *o*-nitrophenol (0·05 %) and unchanged nitrobenzene (0·03 %).

Nitrobenzene in rabbit fat. A 3 kg. rabbit was given 0·75 g. of nitrobenzene by stomach tube with water. The animal died 2 days later. The fat (33 g.) around the kidneys and along the dorsal wall of the abdominal cavity was cut out and homogenized with 50 ml. water. The neutral homogenate was then steam distilled until 100 ml. of distillate had been obtained. This distillate gave no diazo reaction until reduced with Zn and HCl. On spectroscopic examination a peak at 270 mμ. was detected corresponding to nitrobenzene. From the extinction of the peak it was calculated that the above fat contained 0·54 % of the dose as unchanged nitrobenzene 2 days after dosing.

Nitrophenols

m- *and* p-*Nitrophenols and* 4-*nitrocatechol. Detection by paper chromatography.* The urine of a rabbit which had been fed 0·5 g. of nitrobenzene was collected for 36 hr. after feeding and made 4 N with respect to H_2SO_4. The mixture was heated under reflux for 4 hr. at 100°. After cooling, the solution was continuously extracted with ether for 6 hr. The extract (100 ml.) was washed once with dilute HCl and the nitrophenols transferred to 100 ml. 10 % NaOH. The alkaline solution was now acidified and the nitrophenols re-extracted with 100 ml. ether. This purified ether extract was now dried over anhydrous $CaCl_2$ and then used for paper chromatography.

For the paper chromatography of this solution, the paper and solvents used were those described in the preceding paper on nitrophenols (Robinson *et al.* 1951). Identification and elution for spectroscopic examination was also carried out as described before. The nitrophenols identified were *m*-nitrophenol (4 % of the dose), *p*-nitrophenol (5 %) and 4-nitrocatechol (0·5 %). *o*-Nitrophenol, is, of course, volatile and would not be detected by this procedure (Robinson *et al.* 1951).

Aminophenols

o-, m- *and* p-*Aminophenols. Detection by paper chromatography.* In our first experiments, benzene-*n*-butanol-water mixtures were tried, but it was found that in this solvent mixture *o*- and *m*-aminophenols had the same R_F values. Good separation was eventually obtained using wet *iso*propyl ether and Barcham-Green no. 401 paper. The solvent was prepared by shaking 2 l. of *iso*propyl ether with 200 ml. water, allowing the mixture to stand overnight and using the top layer. Owing to the volatility of the solvent, the tank had to be made carefully air-tight but it had the advantage

of rapid equilibration. This solvent travels rapidly and a good separation of the aminophenols can be obtained in as little as 6 hr. The R_F values obtained are given in Table 4. All the aminophenols can be located by spraying with ammoniacal silver nitrate which is reduced by these phenols which then show up as dark spots. A better method of spotting, however, was found in the use of a 1 % solution of salicylaldehyde in ethanol containing 5 % glacial acetic acid as spraying agent. The paper was sprayed with the reagent and, after drying at room temperature, the aminophenols show up as yellow spots which fluoresce a vivid yellow or green in ultraviolet light.

Table 4. *R_F values of aminophenols in wet isopropyl ether*

(Chromatograms run for 6 hr.)

Phenol	R_F
o-Aminophenol	0·77
m-Aminophenol	0·60
p-Aminophenol	0·40
4-Aminocatechol	0·065
4-Aminoresorcinol	0·10
2-Aminoresorcinol	0·41

The urine was hydrolysed and the nitrophenols extracted as already described (p. 230). The residual urine was then brought to pH 7–7·5 with solid $NaHCO_3$, a little sodium bisulphite added and the solution extracted continuously with peroxide-free ether for 24 hr. Large amounts of *p*-aminophenol (20–30 % of the dose) separated in the extract at this stage and were filtered off. The extracted aminophenols were then transferred to dilute HCl. The acid solution was neutralized with $NaHCO_3$ and the aminophenols transferred to peroxide-free ether, and this solution used for paper chromatography. In this way, the *o*-, *m*- and *p*-aminophenols were detected and their presence confirmed by spectroscopy. *o*-Aminophenol was eluted from its spot with ethanol and its spectrum in ethanol, acid and alkali determined. The maxima found ($\lambda_{max.}$ in ethanol, 288 and 232 mμ; in 0·1 N-HCl, 270 mμ.; and in 0·1 N-NaOH, 241 and 298 mμ.) were identical with those of authentic *o*-aminophenol. From the extinctions of the maxima, it was found that 0·54 % of the nitrobenzene fed was excreted as *o*-aminophenol. *m*-Aminophenol was eluted from its spot with 0·1 N-HCl and its amount was estimated both spectroscopically and with the Bratton and Marshall reagents as described above for aniline. By spectra in ethanol, the yield was 0·58 % of the dose and by the colorimetric procedure using diazotization followed by coupling with naphthylethylene diamine (Robinson *et al.* 1951), 0·3 %.

Isolation of the phenols of nitrobenzene metabolism

Nitrophenols. The 48 hr. urine from ten rabbits which had collectively received 6 g. of nitrobenzene was made 4 N with respect to H_2SO_4 and heated under reflux for 4 hr. at 100°. The hydrolysate was continuously extracted with ether for 18 hr. The ether was removed leaving a residue of mixed nitrophenols (0·25 g.). A large column (200 × 5 cm.) was packed with silica with which had been intimately mixed an equal weight of the aqueous phase of a solvent mixture of benzene-acetic acid-water (1:1:2). The phenols were intimately mixed with a little silica and this was placed at the top of the column as a layer 1–2 cm. thick. The column was then developed for 24 hr. with the non-aqueous layer of the solvent mixture. As soon as the eluates turned yellow on addition of alkali, collection of fractions was commenced. A single fraction (*A*) was then collected until the eluate, after reduction with Zn dust and HCl, showed the indophenol reaction. This fraction contained mainly *m*-nitrophenol. A second fraction (*B*) was then collected by allowing the solvent to run through until no further yellow colour was obtained on adding alkali.

Isolation of m-*nitrophenol.* Paper chromatography of fraction *A* showed that it contained mainly *m*-nitrophenol with small amounts of *p*-nitrophenol. The nitrophenols in *A* were extracted with 10 % NaOH, the extract acidified and the phenols taken into ether (3 × 50 ml.). The ether was evaporated. The solid residue was treated with 2–3 ml. of 10 % NaOH drop by drop. A small amount of the sparingly soluble Na salt of *p*-nitrophenol was filtered off and the filtrate acidified and extracted with ether. Evaporation of the ether left 65 mg. of *m*-nitrophenol which on benzoylation yielded 120 mg. of *m*-nitrophenyl benzoate, m.p. 94° and mixed m.p. 95° with authentic material, m.p. 95°. (Found: C, 64·1; H, 3·7. Calc. for $C_{13}H_9O_4N$: C, 64·2; H, 3·7 %.)

Isolation of p-*nitrophenol.* Fraction *B* was shown by paper chromatography to contain mainly *p*-nitrophenol with a little *m*-nitrophenol. The nitrophenols were isolated as in the preceding paragraph. The sparingly soluble Na salt of *p*-nitrophenol was filtered off and dissolved in acid and the acid solution ether extracted. Evaporation of the ether gave *p*-nitrophenol (30 mg. or 0·44 % of the dose), m.p. and mixed m.p. 114° after recrystallization from light petroleum. (Found: C, 51·5; H, 3·5. Calc. for $C_6H_5NO_3$: C, 51·8; H, 3·6 %.) The benzoate has m.p. and mixed m.p. 142°. (Found: C, 63·7; H, 3·6 %. Calc. for $C_{13}H_9O_4N$: C, 64·2; H, 3·7 %.)

Isolation of 4-*nitrocatechol.* The silica was extruded from the column and a greenish-yellow band near the top was cut out. The material in this band was extracted quickly with 10 % NaOH and the red extract centrifuged. It was then acidified and extracted with ether. The ether extract was dried with anhydrous $CaCl_2$ and evaporated to yield 20 mg. (0·26 % of the dose) of 4-nitrocatechol. Benzoylation by benzoyl chloride and alkali yielded 4-nitrocatechol dibenzoate, m.p. and mixed m.p. 156°, after two recrystallizations from aqueous ethanol. (Found: C, 65·9; H, 3·8. Calc. for $C_{20}H_{13}O_6N$: C, 66·1; H, 3·6 %.) In another experiment in which 4 g. of nitrobenzene were fed, the yields of benzoates were *m*-nitrophenol 70 mg., *p*-nitrophenol 70 mg. and 4-nitrocatechol 15 mg. In yet another experiment in which the nitrophenol fraction was not chromatographed and in which 6 g. of nitrobenzene were fed, only *m*-nitrophenol was isolated, in this case as the toluene-*p*-sulphonate (28 mg.) m.p. 113° and mixed m.p. 114°. (Found: C, 53·4; H, 3·8; Calc. for $C_{13}H_{11}O_5NS$: C, 53·2; H, 3·8 %.)

Aminophenols

p-*Aminophenol.* The urine from six rabbits which had collectively received 6 g. of nitrobenzene was hydrolysed and the nitrophenols removed as above. The urine was brought to pH 7·5–8·0 with solid $NaHCO_3$ and continuously extracted with ether for 6 hr. Evaporation of the ether yielded 0·54 g. (10 % of the dose) of almost pure *p*-aminophenol, m.p. 180° and mixed m.p. 181° (authentic m.p. 184°). The *ON*-dibenzoyl derivative was prepared and had m.p. and mixed m.p. 235°.

o-Aminophenol. The ether residues after crystallizing out the *p*-aminophenol as above was chromatographed on a column of silica (75 × 4 cm.) containing an equal volume of water. The column was developed with wet peroxide-free ether and 50 ml. fractions were collected. Each fraction was chromatographed on paper to establish its composition. The third fraction collected appeared to contain only *o*-aminophenol. It was evaporated and the light-brown residue was benzoylated with benzoyl chloride and pyridine. This yielded 100 mg. (0·64 % of the dose) of *ON*-dibenzoyl *o*-aminophenol. On recrystallizing twice from ethanol (charcoal) and once from ligroin (b.p. 120°), 20 mg. of the pure dibenzoyl derivative, m.p. and mixed m.p. 182° were obtained. (Found: C, 75·0; H, 4·9. Calc. for $C_{20}H_{15}O_3N$: C, 75·7; H, 4·8 %.) We were not successful in obtaining *m*-aminophenol or a derivative in a pure crystalline state.

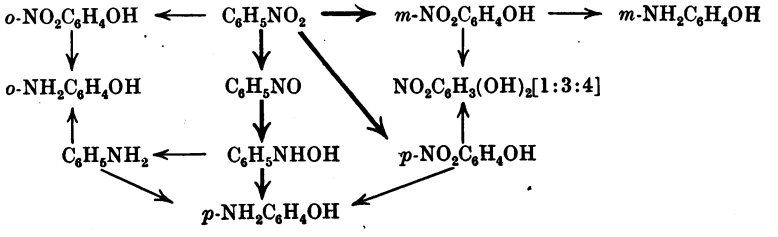
DISCUSSION

The present work has shown that the metabolism of nitrobenzene is complicated like that of aniline (Smith & Williams, 1949). At best we have only accounted for just over 50 % of the dose as the metabolites excreted during the 2 days subsequent to dosing. It appears that nitrobenzene is to some extent retained in the body of the rabbit for some time, for its metabolites can be detected in the urine a week after dosing. Our quantitative findings are briefly summarized in Table 5. It is clear that unlike the nitrophenols, nitrobenzene is quite extensively reduced *in vivo*, roughly two-thirds of the material excreted being in the reduced form and only one-third as nitro compounds. Furthermore, the amount of nitrobenzene excreted unchanged is almost negligible (0·03 %), so that the whole of the dose is either oxidized or reduced. The major metabolic product is *p*-aminophenol, which is not excreted in the free state but combined with glucuronic and sulphuric acids. The exact nature of these conjugates has not yet been investigated.

Table 5. *A summary of some of the quantitative aspects of the metabolism of nitrobenzene in the rabbit*

	Compounds found	Amounts excreted in 2 days (% of dose)	
Nitro compounds	Nitrobenzene	0·03	
	o-Nitrophenol	0·05	
	m-Nitrophenol	4·0	9·6
	p-Nitrophenol	5·0	
	4-Nitrocatechol	0·5	
	Total nitro compounds (by $TiCl_4$ titration)		20
Amino compounds	Aniline	0·50	
	o-Aminophenol	0·54	
	m-Aminophenol	0·58	36·2
	p-Aminophenol	35·0	

The *o*- and *m*-aminophenols are also present in small but appreciable amounts, and the *p*:*m*:*o* ratio is about 70:1:1. The fact that the *m*- and *p*-nitrophenols occur in roughly equal amounts, whereas *p*-aminophenol occurs in very much greater amounts than *m*-aminophenol, suggests that the major portion of the *p*-aminophenol is not derived from *p*-nitrophenol. Furthermore, since *p*-aminophenol is also present in much greater amounts than *o*-aminophenol, it suggests that *p*-aminophenol is not mainly derived from aniline, for although aniline does yield in the rabbit somewhat more *p*- than *o*-aminophenol, it does not do so in very great excess (Smith & Williams, 1949). Aniline, when fed to rabbits, gives rise to a highly reducing urine (Smith & Williams, 1949), the reducing substance being glucuronic acid which may be present



Quantitatively the metabolites of next importance are the *m*- and *p*-nitrophenols, which probably account for the major part of the nitro-compounds excreted. *o*-Nitrophenol is a relatively minor metabolite and using the figures in Table 5, the *p*:*m*:*o* ratio for the nitrophenols is 100:80:1. The numerical value of this ratio is given with reserve, but the *m*-and *p*-nitrophenols are certainly excreted in approximately equal amounts and in very much as such or as a labile glucuronide. Nitrobenzene urine, however, is not highly reducing, and this suggests that aniline is not formed in large amounts. It seems, therefore, that *p*-aminophenol does not arise via aniline or *p*-nitrophenol. That a hydroxylamine is an intermediate in the biological reduction of nitro compounds seems to be established (Channon, Mills & Williams, 1944) and our present results suggest that most of the *p*-aminophenol may

be derived from nitrobenzene via phenylhydroxylamine. There is as yet no rigid proof (cf. Sieberg, 1914) that phenylhydroxylamine can be converted *in vivo* directly to *p*-aminophenol, although this change readily occurs *in vitro* in acid solution.

Minor sources of *p*-aminophenol are aniline, which is derived via phenylhydroxylamine, and *p*-nitrophenol. The formation of aniline has been proved by its spectroscopic detection, and *p*-nitrophenol is slightly reduced (14%) *in vivo* to *p*-aminophenol (Robinson *et al.* 1951). The probable main source of *o*-aminophenol is aniline although a very minute amount could arise from *o*-nitrophenol. The main source of *m*-aminophenol is probably *m*-nitrophenol, for aniline does not give rise to *m*-aminophenol *in vivo* (Smith & Williams, 1951, unpublished observations). The above scheme is suggested by our results (major metabolic paths are in thick arrows).

The metabolism of nitrobenzene and the orientation of biological hydroxylation

That *meta*-hydroxylation takes place *in vivo* was shown by Smith & Williams (1950) when they isolated *m*-cyanophenol as a metabolite of cyanobenzene. The isolation of *m*-nitrophenol as a metabolite of nitrobenzene suggests that *meta*-hydroxylation may be a general metabolic reaction for monosubstituted benzenes containing a *meta*-directing (ring deactivating) group.

Since nitrobenzene has now been shown to be hydroxylated in the rabbit in all three positions, *o*-, *m*- and *p*-, the process is therefore analogous with the hydroxylation of nitrobenzene by free hydroxyl radicals (Loebl, Stein & Weiss, 1950; Stein & Weiss, 1950). Similar results have been obtained with phenol which *in vivo* yields catechol and quinol but no resorcinol (Garton & Williams, 1949). The same two dihydric phenols are produced *in vitro* by the action of free hydroxyl radicals on phenol (Stein & Weiss, 1950) and, furthermore, the relative amounts of the two phenols produced are dependent upon pH. These results strongly suggest that the biological process may be free-radical in nature. There are, however, differences between the *in vivo* and *in vitro* results which, nevertheless, need not be contrary to the free-radical concept. In the first place, nitrobenzene is converted *in vivo* to 4-nitrocatechol, whereas *in vitro* the formation of this compound was not reported by Loebl *et al.* (1950). Loebl, Stein & Weiss (1951) have, in fact, commented upon the absence of dihydric phenols during the free-radical hydroxylation of benzoic acid which yields *o*-, *m*- and *p*-hydroxybenzoic acids. In the second place, *in vitro* free-radical hydroxylation of nitrobenzene produces *o*-, *m*- and *p*-nitrophenols in roughly equal amounts (Loebl *et al.* 1950), whereas *in vivo* the *m*- and *p*-isomers are produced in roughly equal amounts but in very much greater amounts than the *o*-isomer. This may mean that *in vivo* if a free radical is involved it may be very large and cannot readily approach the *ortho*-position.

The alternative explanation for the *in vivo* orientations is to assume the occurrence of a known metabolic reaction, namely 'perhydroxylation', which has been proved to occur to chlorobenzene (Smith, Spencer & Williams, 1950) and the polycyclic hydrocarbons (see Boyland, 1950; Young, 1950). If it be assumed that nitrobenzene forms two dihydrodiols in unequal amounts, there being much more of the 3:4 than the 2:3-diol, our results (and those with phenol) can be explained as follows. The 3:4-dihydrodiol on dehydration (see Fig. 1) could yield

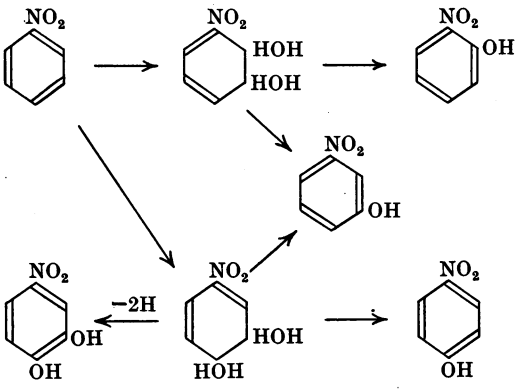


Fig. 1. Possible courses of perhydroxylation of nitrobenzene.

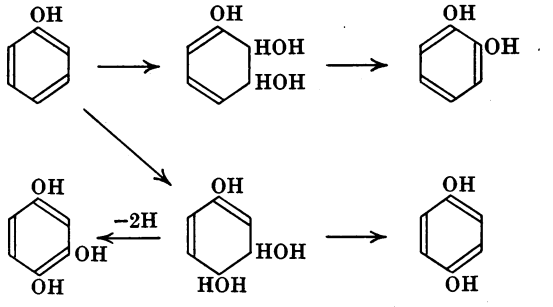


Fig. 2. Possible courses of 'perhydroxylation' of phenol.

m- and *p*-nitrophenol and on dehydrogenation, 4-nitrocatechol. The 2:3-dihydrodiol could yield *o*- and *m*-nitrophenol and 3-nitrocatechol. If it is assumed that only very small amounts of the 2:3-diol are formed, then the yield of *o*-nitrophenol would be very small and the 3-nitrocatechol almost undetectable. The *m*- and *p*-nitrophenols should be formed in roughly equal amounts (cf. cyanobenzene, Smith & Williams, 1950).

In the case of phenol the situation would be as shown in Fig. 2.

The dehydration of the dihydrodiols of phenol to give only one phenol and the dihydrodiols of nitrobenzene to two phenols can be explained, theoretically, by a consideration of the transition states in the dehydration. The dehydration is visualized as taking place as shown in Fig. 3. Whether or not the dihydrodiol (I) decomposes to give III or V will be determined by the speed of the two reactions which in turn will be dependent on the resonance energy of the two possible ionic transition states (II and IV in square brackets). When the group *X* is one of the classical *meta*-directing groups (e.g. NO_2 in nitrobenzene), no extra resonance stabilization is conferred on either of the ionic transition states (cf.

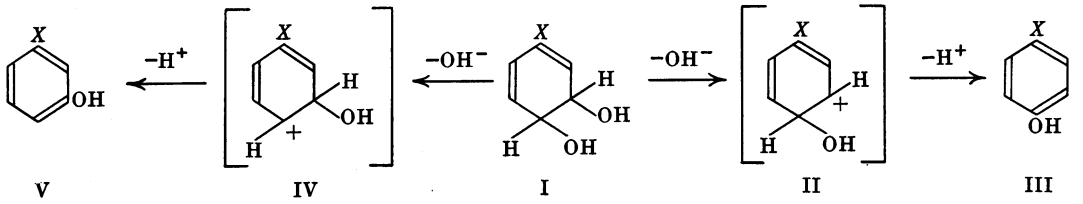


Fig. 3. Dehydration of dihydrodiols.

Dewar, 1949) and we may expect both reactions to proceed with comparable velocities to yield a mixture of the *m*- and *p*-phenols. On the other hand, if *X* is an *o*-*p*-directing group (e.g. OH in phenol) extra resonance stabilization is possible in the transition state (II) on the right and the dihydrodiol will yield the *p*-hydroxy compound III rather than the *m*-hydroxy compound V. An example of the latter case is 3:4-dihydro-chlorobenzene-3:4-diol which we have shown to dehydrate to *p*-chlorophenol only (Smith *et al.* 1950).

It appears to us that on our present evidence biological hydroxylation could be explained by direct hydroxylation by free radicals or by the 'perhydroxylation' hypothesis. The 'perhydroxylation' hypothesis is perhaps to be the more favoured at present. The whole process is, however, complicated by the fact that the phenols produced are conjugated and it is not absolutely certain whether conjugation is a secondary reaction to hydroxylation, although the conjugation of phenols is well known.

SUMMARY

1. A study has been made of the fate of nitrobenzene in the rabbit.

2. Nitrobenzene undergoes extensive reduction *in vivo* and at dose levels of 150–200 mg./kg. about 55% is excreted as metabolites in the urine during 2 days after feeding; 20% is in the form of nitro compounds and 35% as amino compounds mainly *p*-aminophenol derivatives. Small amounts of nitrobenzene metabolites are excreted for a week after dosing, and appreciable amounts of nitrobenzene can be detected in the fat of the animal 2 days after dosing.

3. The nitro compounds found in the urine were nitrobenzene and *o*-nitrophenol, which occurred in very small amounts, *m*- and *p*-nitrophenol, which occurred in relatively large amounts, and 4-nitrocatechol. The last three phenols were isolated by partition chromatography on silica.

4. The amino compounds found were aniline, *o*- and *m*-aminophenol each amounting to about 0·5% of the dose, and *p*-aminophenol which was the main metabolite (35%). The *o*- and *p*-aminophenols were isolated.

5. Paper chromatography of the aminophenols has been studied using wet *iso*propyl ether as solvent.

6. All the phenols excreted were conjugated.

7. The results suggest that the main path of nitrobenzene metabolism is through phenylhydroxylamine to *p*-aminophenol. This has been discussed.

8. The results have also been discussed in relation to the perhydroxylation and free radical hypotheses of aromatic hydroxylation *in vivo*.

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Studies in Detoxication

41. A STUDY OF THE OPTICAL ROTATIONS OF THE AMIDES AND TRIACETYL METHYL ESTERS OF SOME BIOSYNTHETIC SUBSTITUTED PHENYLGLUCURONIDES

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Smith (1949) observed that the triacetyl methyl ester of β-*o*-cyanophenylglucuronide had an optical rotation in chloroform which was appreciably more negative than the corresponding *m*- and *p*-isomers. We decided, therefore, to prepare, by feeding the necessary phenols to rabbits, a number of such substituted phenylglucuronides and their derivatives to obtain further information on this point. Our results suggest that in the case of the triacetyl methyl esters of the *ortho*-substituted phenylglucuronides we have instances of the phenomenon of restricted rotation which shows itself as an abnormal negative optical rotation. The abnormal positive optical rotations of triacetyl β-*o*-nitrophenyl-D-glucuronide and its methyl ester have already been commented upon (Robinson, Smith & Williams, 1951).

EXPERIMENTAL

General method of preparation of glucuronides

In most cases the glucuronides were isolated as basic Pb salts. Two rabbits (3 kg.) were each given by stomach tube 2 g. or less of the phenol suspended in water. The 24 hr. urine was brought to pH about 4 with a little glacial acetic acid and then treated with saturated aqueous normal lead acetate until precipitation was complete. The precipitate was removed by centrifuging and then discarded. The supernatant fluid was now brought to pH about 8 with a little NH_3 (sp.gr. 0·88) and saturated aqueous basic lead acetate added in excess. The basic lead precipitate was washed on the centrifuge, made into a fine suspension in water and the lead removed by saturation with H_2S. After removal of PbS by filtration, the aqueous solution of the glucuronide was concentrated to a small volume *in vacuo* at 45°. Some glucuronides crystallize out at this stage, whereas others are obtained as water-soluble gums often containing inorganic material. These gums can be purified by dissolution in water and precipitation of the inorganic material by adding ethanol. The precipitate of inorganic material may sometimes contain salts of the glucuronides from which the glucuronide can be freed by careful addition of dilute H_2SO_4 to an ethanolic suspension. The extent to which these salts are formed appears to depend to some extent on the purity of the basic lead acetate used.

Methyl esters of glucuronides were prepared by dissolving the glucuronide gum in methanol and methylating with diazomethane in ether until the gum obtained on evaporating the solvents was neutral. In most instances the methyl esters were not obtained crystalline even when pure crystalline glucuronides were methylated. The methyl esters of glucuronides were found to be somewhat unstable and tended to decompose on boiling in water or drying at 100°.

Crystalline derivatives were usually obtained by acetylating the methyl esters at room temperature overnight with equal volumes of pyridine and acetic anhydride. On pouring the mixture into water, the triacetyl methyl ester of the glucuronide usually separated in crystalline form. Since these triacetyl esters crystallize easily, they are eminently suitable for characterizing glucuronides.

Crystalline glucuronidamides were prepared by dissolving the triacetyl methyl esters in methanol and saturating the solution with dry ammonia. The amides separated from the solution either on keeping or on concentrating. Most of these amides were sparingly soluble compounds of high melting point at which they decompose. Many tend to retain half a molecule of water. In view of their unsatisfactory melting points and sparing solubility, they are not nearly as suitable as the triacetyl methyl esters for characterizing glucuronides.

Melting points are uncorrected; rotations were measured in 2 dm. tubes and when *c* was about 1%, the error in $[\alpha]_D$ was $\pm 1°$ or less. With the sparingly soluble glucuronidamides *c* was usually 0·1 and the error in $[\alpha]_D$ was $\pm 5°$. Compounds were dried at room temperature over $CaCl_2$, unless otherwise stated.