3. Earlier observations on the occurrence of a D-amino acid oxidase in *Helix aspersa* are confirmed.

We are grateful to the Director and Staff of the Marine Biological Laboratory for their help, and to the Osler Memorial Fund for a grant. One of us (D.B.H.) holds a Medical Research Council Scholarship.

REFERENCES

Bender, A. E. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
Blaschko, H. & Hawkins, J. (1951). *Biochem. J.* **49**, xliv.
Blaschko, H. & Hawkins, J. (1952). *Biochem. J.* **52**, 306.
Blaschko, H. & Hellmann, K. (1953). *J. Physiol.* **122**, 419.
Blaschko, H. & Himms, J. M. (1955). *J. Physiol.* **128**, 7*P*.
Blaschko, H. & Hope, D. B. (1955). *J. Physiol.* **129**, 11*P*.
Elks, J., Hems, B. A. & Ryman, B. E. (1948). *J. chem. Soc.* p. 1386.
Fearon, W. R. & Bell, E. A. (1955). *Biochem. J.* **59**, 221.
Florkin, M. (1952). *Annu. Rev. Biochem.* **21**, 459.
Gaudry, R. (1949). *Canad. J. Res.* B, **27**, 21.
Pugh, C. E. M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 2306.
Roche, J., Thoai, N-v. & Glahn, P. E. (1952). *Experientia*, **8**, 428.
Thayer, P. S. & Horowitz, N. H. (1951). *J. biol. Chem.* **192**, 755.
Work, E. (1955). *Biochim. biophys. Acta*, **17**, 410.

Studies in Detoxication

68. THE METABOLISM OF [^{14}C]NITROBENZENE IN THE RABBIT AND GUINEA PIG*

BY D. V. PARKE
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received* 27 *July* 1955)

In the previous paper on the metabolism of nitrobenzene in this series (Robinson, Smith & Williams, 1951), about 50% of an oral dose of nitrobenzene was accounted for as urinary metabolites. However, only the *p*-aminophenol and total nitro compounds excreted could be accurately determined, the amounts of the other metabolites formed being approximately assessed by paper chromatography and isolation procedures. Using nitrobenzene randomly labelled with ^{14}C in one carbon atom, it has now become possible to estimate accurately all the previously known metabolites, to demonstrate the existence of and identify the mercapturic acid suspected by Robinson *et al.* (1951) as a metabolite of nitrobenzene, and to show the formation of two new minor metabolites, namely carbon dioxide and nitroquinol. It has also been possible to measure the elimination of metabolites such as carbon dioxide, aniline and unchanged nitrobenzene in the expired air of the animal and to determine the amount eliminated in the faeces or retained in the tissues. It will be shown that some 85–90% of a single dose of nitrobenzene can now be accounted for.

MATERIALS AND METHODS

Melting points are corrected.

Preparation of [^{14}C]*nitrobenzene.* [^{14}C]Benzene (5 g.; Radiochemical Centre, Amersham) was added in small amounts to a mixture of HNO_3 (10 g.) and H_2SO_4 (15 g.), keeping the temperature below 45°. After keeping the mixture for 0·5 hr. at 0° the nitrobenzene layer was separated, and the remaining acid layer was diluted to 100 ml. with water and extracted with ether (2 × 10 ml.). The extract was added to the nitrobenzene and the mixture was washed with 10 ml. of water and then twice with 10 ml. of 10% (w/v) aqueous NH_4Cl. After drying over anhydrous $CaCl_2$, the ether was evaporated and the nitrobenzene distilled, b.p. 208–211° (yield 6·4 g., 69%). By isotopic dilution experiments it was shown that the non-volatile residue contained 24·5% of the radioactive benzene as *m*-dinitrobenzene, 2·4% as *o*-dinitrobenzene and 0·25% as *p*-dinitrobenzene.

Nitroquinol, m.p. 132° (Elbs, 1893); 3-nitrocatechol, m.p. 85°, and 4-nitrocatechol, m.p. 174° (Dakin, 1909); *o*-nitrothiophenol, m.p. 57° (Lecher & Simon, 1922); *m*-nitrothiophenol, m.p. 26°, and *p*-nitrothiophenol, m.p. 77° (Bennett & Berry, 1927); L-phenylmercapturic acid, m.p. 142° (Parke & Williams, 1951); and *trans-trans*-muconic acid, m.p. 300° (decomp.) (Ingold, 1921), were also prepared. Other known compounds required for isotope dilution experiments were purchased or prepared by standard methods and purified to constant m.p.

Animals. The [^{14}C]nitrobenzene contained about 100 μc/ml. of radioactivity, and was administered, alone or suitably diluted with inactive nitrobenzene (A.R.), b.p. 209–211°, to rabbits by stomach tube, and in one case to a guinea pig by intraperitoneal injection. After dosing, each rabbit was placed in a metabolism chamber through which a current of air was drawn into ethanol at −50° to trap any unchanged nitrobenzene or aniline, and thence into CO_2 absorbers (Parke & Williams, 1953). The expired CO_2 was collected only in Expts. 4 and 5 (see Table 1). In all the experiments the animals were removed from the chamber not later than 30 hr. after dosing.

* Part 67: Mead, Smith & Williams (1955).

In Expts. 4 and 5 the animals were killed and their tissues analysed for radioactivity. In the other experiments the animals were removed from the metabolism chamber after 30 hr. and placed in open cages so that their urine could be collected up to 10 days after dosing. The daily volume of urine was approximately 100 ml., and portions of 1–10 ml./day, according to the doses of ^{14}C, were taken for the isotopic dilution technique.

Measurement of radioactivity. Measurements were carried out on solid samples of 'infinite thickness' on nickel-plated iron planchets (General Electric Co. Ltd.) *in vacuo* (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). In some experiments determinations of the total radioactivity were made by counting the solid residue obtained by evaporation of the urine under infrared lamps. These latter results did not differ significantly from those obtained by the combustion procedure.

The radioactivity of the tissues was measured as $BaCO_3$ or $CaCO_3$ obtained by wet combustion of the tissue dried on filter paper. The results for the various tissues are shown in Table 2, and Fig. 2 shows the rate of elimination of the radioactivity in the urine.

Table 1. *Elimination of unchanged nitrobenzene, aniline and* CO_2 *in the expired air of rabbits receiving* [^{14}C]*nitrobenzene orally*

Expt.	Dose of nitrobenzene (mg./kg.)	Dose of ^{14}C (μc/rabbit)	Duration of expt. (hr.)	Percentage of dose eliminated as: Nitrobenzene	Aniline	CO_2
3	250	0·6	24	0·7	0·04	—
5*	200	25	30	0·6	0	0·5
4	250	35	30	0·6	0	1·2

* This animal died after 30 hr. and was found to have large fat depots.

Table 2. *Occurrence of radioactivity in the tissues of rabbits receiving* [^{14}C]*nitrobenzene*

	Expt. 4 (8 days after dosing)*: Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	% of dose present in tissue	Expt. 5 (1·5 days after dosing)*: Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	% of dose present in tissue
Lungs	16	0·001	<0·1	18	0·003	0·2
Liver	46	0·001	0·1	52	0·004	0·8
Kidney	17	0·001	<0·1	18	0·050	3·6†
Heart	—	—	—	8	0·003	0·1
Spleen	—	—	—	2	0·004	0·05
Voluntary muscle	600	0·001	1·8	750	0·004	12·0
Brain	—	—	—	30	0·006	0·7
Fat (intestinal)	250	0·004	3·0	290	0·001	11·6
Fat (kidney)	210	0·004	2·4	230	0·017	15·4
Stomach contents	58	0·003	0·5	75	0·010	3·0
Intestinal contents (caecum)	36	0·004	0·4	42	0·018	3·1
Intestinal contents (small intestine)	—	—	—	72	0·010	2·9
Faeces	—	—	11·3	22	0·030	2·7
Total in tissues and faeces	—	—	19·6	—	—	56·2
Total in urine and expired air	—	—	58·8	—	—	24·1
Total accounted for	—	—	78	—	—	80

* No. of days after dosing with nitrobenzene when the animal was killed.
† This high figure is due to the high level of urinary excretion at the time of death.

using an end-window counter tube, the background of which was 9–12 counts/min. The specific activities were determined by comparison with a stable polymer reference (Radiochemical Centre, Amersham), which was standardized against ^{14}C-labelled *m*-dinitrobenzene, prepared from the labelled nitrobenzene. A sample of 1·75 sq.cm. containing 1 μc of ^{14}C/g. of substance gave approximately 1150 counts/min. The variation in disk size and preparation of sample gave an error of $\pm 5\%$.

Urine and tissues. The total radioactivity in the urine was measured as $BaCO_3$ obtained by wet combustion of the residue formed by evaporation of the urine over P_2O_5

ESTIMATION OF METABOLITES

Carbon dioxide. Each 4 hr. fraction of the CO_2 absorbed in the Drechsel bottles containing NaOH was converted into $BaCO_3$ and the radioactivity measured as described by Calvin *et al.* (1949).

Nitrobenzene and aniline in the expired air. The nitrobenzene and aniline eliminated in the expired air were determined spectrophotometrically in three experiments (cf. Azouz, Parke & Williams, 1952), using a Unicam spectrophotometer SP. 500. For the estimations the selected u.v. absorption maximum of nitrobenzene in

ethanol was at 260 mμ. where ε was 10 400 (slit width of 0·2 mm.), and that of aniline in ethanol was at 233 mμ. where ε was 11 500 (slit width of 0·5 mm.). It is unlikely that any metabolite, other than nitrobenzene, aniline or CO_2, was eliminated in the expired air, since in all experiments the u.v. absorption spectra of the ethanolic solutions from the absorption train showed no maxima other than those corresponding to nitrobenzene and aniline. Nitrobenzene was excreted in the expired air in all the experiments, to the extent of about 0·6% of the dose, but aniline was detected in only one experiment and amounted to 0·04% of the dose.

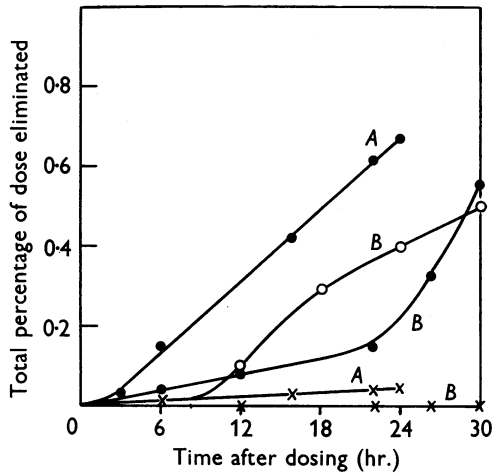

Fig. 1. Excretion of nitrobenzene (●), aniline (×), and CO_2 (○), in the expired air of rabbits receiving [^{14}C]-nitrobenzene orally. *A*, Expt. 3 (nitrobenzene, 0·25 g./kg.); *B*, Expt. 5 (nitrobenzene, 0·2 g./kg.).

The figures for the elimination of unchanged nitrobenzene, aniline, and CO_2 in the expired air are given in Table 1. Fig. 1 shows graphically the rate of elimination of nitrobenzene and aniline in Expts. 3 and 5. In none of the experiments was the expired air examined for longer than 30 hr. because of distress of the experimental animals. From the curves it is clear that the exhalation of unchanged nitrobenzene at 30 hr. after dosing was still at its maximal rate, so that the total nitrobenzene eliminated in the expired air might amount to 1–2% of the dose. The elimination of CO_2 in the expired air, which begins about 12–20 hr. after dosing, was also at its maximal rate at the end of the experiments.

Nitrobenzene and aniline in the urine. Nitrobenzene (100 mg.) and aniline (100 mg.) were added to one-half of the urine immediately it was passed, and were recovered diluted with the labelled materials by steam distillation of the urine made alkaline with Na_2CO_3 (pH 9). The aqueous distillate was acidified with 2N-H_2SO_4 and distilled to remove the nitrobenzene. Then, after addition of an excess of 2N-NaOH, the non-volatile residue was distilled to remove the aniline. The aqueous distillate containing the nitrobenzene was treated with an excess of $Ti_2(SO_4)_3$ (15%, w/v, in 23%, w/v, H_2SO_4) and the aniline so formed was converted into 2:4:6-tribromoaniline by addition of an excess of a saturated aqueous solution of bromine. The product was filtered, and recrystallized from ethanol to constant specific activity (m.p. and mixed m.p. 120°). The aqueous distillate containing the aniline was likewise treated with bromine water and the 2:4:6-tribromoaniline was recrystallized from ethanol to constant specific activity (m.p. 121°).

Phenols

Nitrophenols. *o*-, *m*- and *p*-Nitrophenols (100 mg.) were added to separate portions of the urine, which were then refluxed with 0·5 vol. of conc. HCl for 3 hr. to hydrolyse the phenolic conjugates. The solution containing added *o*-nitrophenol was then steam-distilled and the *o*-nitrophenol in the distillate was collected by filtration and dried over P_2O_5. The radioactivity of the *o*-nitrophenol (m.p. 45°) was determined, and the material was then dissolved in 5 ml. of 2N-NaOH and treated with 0·2 ml. of benzoyl chloride. The product was poured into water and the precipitated benzoate collected and recrystallized from aqueous ethanol. The *o*-nitrophenyl benzoate was further recrystallized from ethanol and methanol until its specific activity was constant (m.p. and mixed m.p. 59°).

The solutions containing the added *m*- and *p*-nitrophenols were separately extracted with ether continuously for 3 hr. The ether extracts were washed repeatedly with N-H_2SO_4 to remove aminophenols, and finally washed with water and evaporated to dryness. The residues were dissolved in NaOH and treated with benzoyl chloride, as above, to yield *m*-nitrophenyl benzoate, m.p. and mixed m.p. 95°, and *p*-nitrophenyl benzoate, m.p. and mixed m.p. 143° respectively, after recrystallization from ethanol and were further recrystallized from ethanol, methanol and light petroleum (b.p. 60–80°) to constant specific activity.

Aminophenols. *o*- and *m*-Aminophenols (200 mg.) and *p*-aminophenol sulphate (300 mg.) were added to separate portions of the urine, which were then refluxed with an equal vol. of conc. HCl for 5 hr. The solutions were cooled, adjusted to pH 8 with solid $NaHCO_3$, and continuously extracted with ether for 3 hr. The ethereal extracts were washed with water, and the aminophenols were transferred into 50 ml. of 2N-H_2SO_4. The acid solutions were washed with ether to remove nitrophenols, and then after adjusting to pH 8 with solid $NaHCO_3$ were extracted twice with 20 ml. portions of ether. The ethereal extracts were evaporated to dryness, and the residual aminophenols were benzoylated to yield the *ON*-dibenzoyl derivatives of *o*-, *m*-, and *p*-aminophenols (m.p.'s and mixed m.p.'s 183, 153 and 237° respectively), after recrystallization from ethanol. These benzoyl derivatives were then further recrystallized successively from ethanol, ethyl acetate, light petroleum (b.p. 60–80°), and a mixture of $CHCl_3$ and CCl_4, to constant specific activity. In some experiments, in addition to the preparation of the dibenzoyl derivatives, the aminophenols were converted into the corresponding acetamidophenols by warming with acetic anhydride. The products were poured into a little water, and recrystallized from hot water to give *o*-, *m*-, and *p*-acetamidophenols (m.p. and mixed m.p. 207°, 149° and 169° respectively), which were further recrystallized from water and aqueous ethanol to constant specific activity.

Nitrocatechols. 3- and 4-Nitrocatechols (100 mg.) were added to separate portions of the urine which were then refluxed with 0·5 vol. of conc. HCl for 3 hr., cooled, and continuously extracted with ether for 4 hr. The ethereal extracts were washed with 2N-H_2SO_4 and then with water,

and finally were evaporated to dryness. The residue of 4-nitrocatechol was dissolved in 5 ml. of water and treated with 5 ml. of 10% (w/v) $BaCl_2$ solution to precipitate the barium salt. This material was purified by dissolving in 10 ml. N-HCl, treating with 5 ml. of 10% (w/v) $BaCl_2$ solution and reprecipitating the barium salt by adjusting to pH 7 with 2N ammonia. The barium salt was removed by filtration, dried *in vacuo* and its activity determined. The barium 4-nitrocatecholate was then dissolved in N-HCl, and the 4-nitrocatechol was extracted into ether, transferred into 2N-NaOH and treated with benzoyl chloride to yield 4-nitrocatechol dibenzoate (m.p. and mixed m.p. 156°) after recrystallization from aqueous ethanol. The activity of this material was then measured.

light petroleum (b.p. 60–80°) to give 3-nitrocatechol (m.p. and mixed m.p. 85°), which showed no radioactivity.

Nitroquinol. Nitroquinol (100 mg.) was added to a portion of the urine which was then refluxed with 0·5 vol. of conc. HCl for 3 hr., cooled, and twice extracted with ether. The ether extracts were washed with 2N-H_2SO_4 and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue on recrystallization from CCl_4 gave nitroquinol (m.p. 131°), and its specific radioactivity was determined. This material was then benzoylated to give nitroquinol dibenzoate (m.p. and mixed m.p. 142°), which was recrystallized from ethanol to constant specific radioactivity.

The results on phenols and other aromatic metabolites are given in Table 3.

Table 3. *Elimination of phenols and other aromatic compounds in the urine of animals receiving [^{14}C]nitrobenzene*

The nitrobenzene was given orally to rabbits, except in Expt. 7, when it was administered to a guinea pig by intraperitoneal injection.

	Rabbits						Guinea pig
Expt.	1	2*	3	4	5†	6‡	7
Dose of nitrobenzene (mg./kg.) ...	250	400	250	250	200	200	500
Dose of ^{14}C (μc/animal)	0·6	1·0	0·6	35	25	25	18
Duration of expt. (days)	10	2	7	8	1·5	5	4
	Percentage of dose						
Nitrobenzene	<0·05	<0·05	0·05	<0·05	—	—	—
Aniline	0·2	0·3	0·2	0·6	—	—	—
o-Nitrophenol	0·1	<0·1	0·2	0·05	0·1	0·1	0
m-Nitrophenol	10·5	4·6	8·0	5·7	2·3	10·0	1·2
p-Nitrophenol	11·2	4·8	6·4	7·0	3·4	10·5	10·0
o-Aminophenol	1·5	3·5	3·8	3·1	3·4	3·1	3·6
m-Aminophenol	—	7·7	4·0	2·2	2·1	2·9	7·5
p-Aminophenol	33	26	23	33	8·4	33	26
3-Nitrocatechol	—	—	—	0	—	0	—
4-Nitrocatechol	0·9	0·4	0·2	0·5	0·4	1·0	0·5
Nitroquinol	—	—	—	0·1	0·05	0·05	0·1
o-Nitrophenylmercapturic acid	—	—	—	0	0	0	—
m-Nitrophenylmercapturic acid	—	—	—	0	0	0	—
p-Nitrophenylmercapturic acid	—	—	—	0·1	0·1	0·5	0·1
Nitrosobenzene	—	—	—	0	—	—	—
Azoxybenzene	—	—	—	0	0	—	0
Azobenzene	—	—	—	0	0	—	0
Benzidine	—	—	—	—	0	0	—
Phenol	—	—	—	0	0	—	0
Catechol	—	—	—	<0·1	<0·05	0	<0·05
Phenylmercapturic acid	—	—	—	0	—	0	0
trans-trans-Muconic acid	—	—	—	0	—	0	0
Sum of aromatic compounds	58	48	46	53	20	61	50
Total radioactivity of urine	68	41	45	57	23	63	58

* This animal died after 2 days. † This animal died after 30 hr., and was found to have abnormally large fat depots.
‡ This animal littered on the third day after dosing.

The residue of 3-nitrocatechol was dissolved in 5 ml. of water and treated with 10 ml. of lead acetate solution (3%, w/v) to precipitate the nitrocatechol as its lead salt. This was filtered off, suspended in 20 ml. of water and saturated with H_2S to remove the lead. After filtration, the 3-nitrocatechol was extracted from the solution by continuous extraction with ether for 3 hr. The ethereal extract was then evaporated to dryness, and the residue extracted with light petroleum (b.p. 60–80°) in which 4-nitrocatechol is insoluble. The soluble material was recrystallized from

Mercapturic acids

The mercapturic acid excretion of rabbits dosed with nitrobenzene (150 mg./kg.) was estimated by Robinson *et al.* (1951) by I_2 titration, and was found to amount to about 3% of the dose, which was within the margin of error of the method. This iodometric estimation was repeated on the whole urine from two rabbits which had been fed with nitrobenzene (200 mg./kg.), and amounted in 2 days to 0·4 and 0·6±0·2% of the dose. The existence and identity

of the mercapturic acid were therefore investigated by the isotopic dilution technique, using *o*-, *m*-, and *p*-nitrothiophenols, since it was assumed that if any nitrophenylmercapturic acid were present it could be hydrolysed by alkali to the corresponding nitrothiophenol.

Nitrothiophenols. *o*-, *m*-, and *p*-Nitrothiophenols were separately added to portions of the urine and heated with 0·2 vol. of 10N-NaOH on a sand bath (120°) for 40 min. In the case of the *o*- and *p*-nitrothiophenols, the hydrolysed solutions were cooled, acidified with 2N-H_2SO_4 and continuously extracted with ether for 3 hr. The ethereal solutions were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residues were dissolved in ethanol and treated with an excess of 5% (w/v) ethanolic I_2 to convert the thiophenols into the corresponding diphenyl disulphides. The resulting mixtures were diluted with water and the precipitated disulphides were filtered off, washed with water and recrystallized from benzene to yield 2:2′- and 4:4′-dinitrodiphenyl disulphides (m.p. and mixed m.p. 198° and 181° respectively). These derivatives were then further recrystallized from mixtures of benzene–ethanol and ethanol–ethyl acetate to constant specific activity.

The hydrolysed urine containing the *m*-nitrothiophenol was acidified with 2N-H_2SO_4 and was then steam-distilled. The *m*-nitrothiophenol in the distillate was collected into ether and treated with an excess of ethanolic I_2 (5%, w/v), and the resulting mixture was diluted with water. The precipitated dinitrodiphenyl disulphide was filtered off, washed with water and recrystallized from benzene to give 3:3′-dinitrodiphenyl disulphide (m.p. and mixed m.p. 83°), which on further recrystallization from ethanol–ethyl acetate showed no radioactivity.

Of the added nitrothiophenols, only the *para*-isomer was found to entrain radioactivity. From these results it is deduced that *p*-nitrophenylmercapturic acid is a minor metabolite of nitrobenzene, equivalent to about 0·3% of the dose.

Reduction products

Robinson *et al.* (1951) suggested that most of the *p*-aminophenol which results from the metabolism of nitrobenzene is derived via phenylhydroxylamine. The urines were therefore examined for the presence of nitrosobenzene which is an atmospheric oxidation product of β-phenylhydroxylamine and a possible intermediate in the biological formation of phenylhydroxylamine from nitrobenzene. In the presence of nitrosobenzene, phenylhydroxylamine *in vitro* gives rise to azoxy, azo- and hydrazo-benzene. Hydrazobenzene is also a product of the metabolism of azobenzene in the rat and was isolated from the acid-treated urine as benzidine (Elson & Warren, 1944). The urines were therefore also examined for the presence of azoxybenzene, azobenzene and benzidine.

Nitrosobenzene. Nitrosobenzene (100 mg.) was added to a portion of the urine, which was refluxed with 0·5 vol. of conc. HCl for 3 hr. and then steam-distilled. The nitrosobenzene was collected from the steam distillate into ether, and the ethereal solution was washed with N-NaOH and then water to remove any *o*-nitrophenol. After drying over anhydrous Na_2SO_4, the ethereal solution was evaporated to dryness and the residue of nitrosobenzene was repeatedly recrystallized from aqueous ethanol. The final product (m.p. 68°) contained no radioactivity.

Azoxybenzene. Azoxybenzene (100 mg.) was added to a portion of the urine, which was then refluxed with 0·5 vol. of conc. HCl for 3 hr. The solution was cooled and continuously extracted with ether for 4 hr. The ether extract was washed several times with N-NaOH to remove phenolic materials, and was then evaporated to dryness. The residue of crude azoxybenzene was recrystallized repeatedly from aqueous ethanol and the final product (m.p. 36°) contained no radioactivity.

Azobenzene. A portion of the urine (10 ml.) was shaken for 2 hr. with 100 mg. of azobenzene dissolved in 10 ml. of ether. The ether layer was separated and was washed with 2N-NaOH to remove nitrophenols, with 2N-H_2SO_4 to remove aminophenols, and finally with water. The ethereal solution was then dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of azobenzene was recrystallized several times from ethanol and from light petroleum (b.p. 60–80°) and the final product (m.p. 68°) showed no radioactivity.

Benzidine. Benzidine (100 mg.) was added to a portion of urine which was refluxed for 3 hr. with 0·5 vol. of conc. HCl. The solution was cooled and treated with an excess of 2N-H_2SO_4 to precipitate the benzidine sulphate, which was removed by centrifuging, and washed with water, ethanol, and finally ether. The benzidine sulphate was then dissolved in warm N-NaOH, filtered, and reprecipitated by addition of excess of 2N-H_2SO_4. This process was repeated several times, and the product, although still radioactive, showed a continuously decreasing specific activity. The benzidine sulphate was then treated with a slight excess of N-NaOH and the solution was continuously extracted with ether for 3 hr. The ethereal solution was dried over anhydrous Na_2SO_4, evaporated to dryness, and the residue of benzidine was recrystallized from aqueous ethanol. The product (m.p. 125°) was still slightly radioactive, and was therefore treated in solution in 2N-NaOH with benzoyl chloride to yield the dibenzoyl derivative (m.p. 350° decomp.), which was free from radioactivity.

Denitration products

The action of X-rays on dilute solutions of nitrobenzene is similar to the action of the free hydroxyl radicals of Fenton's reagent, but, in addition to the production of *o*-, *m*- and *p*-nitrophenols, the nitro group is eliminated with the formation of phenol (Loebl, Stein & Weiss, 1949, 1950). Furthermore, Bray, Hybs, James & Thorpe (1953) have shown that 2:3:5:6-tetrachloronitrobenzene is converted in the rabbit into L-2:3:5:6-tetrachlorophenylmercapturic acid, the nitro group of the original substance being eliminated. The metabolism of 2:3:4:6-tetrachloronitrobenzene and pentachloronitrobenzene into L-2:3:4:6-tetrachlorophenyl- and L-pentachlorophenyl-mercapturic acids by the rabbit are two further examples of conjugation with acetylcysteine with the concomitant elimination of an aromatic nitro group (Betts, James & Thorpe, 1953). It was therefore necessary to ascertain whether denitration of nitrobenzene to benzene or its metabolites could occur in the animal body, and accordingly the rabbit urines were examined for phenol, and for catechol, *trans-trans*-muconic acid and L-phenylmercapturic acid, which are metabolites of benzene which may not arise via phenol (Parke & Williams, 1953).

Phenol and catechol. These phenols (100 mg. each) were added to separate portions of the urine which were subsequently hydrolysed by refluxing with 0·5 vol. of conc. HCl for 3 hr. The phenol and catechol were then isolated by the

procedures described by Parke & Williams (1953) and their toluene-*p*-sulphonates were found to contain no radioactivity.

Phenylmercapturic acid. Synthetic L-phenylmercapturic acid (200 mg.) was added to a portion of the urine, which was then heated with 0·2 vol. of 10N-NaOH on a sand bath (120°) for 40 min. The hydrolysed urine was acidified with 2N-H_2SO_4 and steam-distilled. The thiophenol in the distillate was collected into ether and treated with an excess of 5% (w/v) ethanolic I_2, and the product diluted with water. The precipitate of diphenyl disulphide was filtered off and after repeated recrystallization from ethanol (m.p. 61°) was found to contain no radioactivity.

trans-trans-*Muconic acid.* This carboxylic acid oxidation product of benzene was estimated by the isotopic dilution procedure described by Parke & Williams (1953), and was shown not to occur in nitrobenzene urine.

RESULTS AND DISCUSSION

The fate of an oral dose of nitrobenzene administered to rabbits has now been accounted for within the limits of the experimental methods, and is shown in summary in Table 4. During 4–5 days after dosing with [^{14}C]nitrobenzene some 70% of the radioactivity is eliminated from the body in the expired air, urine and faeces. The rest remains in the body, from which it is slowly excreted in the urine and probably also in the expired air as carbon dioxide. The urinary excretion of metabolites is almost complete within 5 days of dosing, but the urines are still weakly radioactive even after 10 days (see Fig. 2). The radioactivity remaining in the body in Expt. 4, when the animal was killed 8 days after dosing, was probably present entirely as metabolites, since no odour of nitrobenzene was detectable in the tissues. In Expt. 5, however, where the animal died 30 hr. after dosing, the odour of nitrobenzene in the tissues was pronounced, particularly in the fat depots, which were abnormally large in this animal. This confirms the findings of Robinson *et al.* (1951) that the fat of a rabbit killed 2 days after dosing contained small amounts of unchanged nitrobenzene. The radioactivity in the body was located principally in the fat, but apart from this it was equally distributed among the other tissues.

Only a very minor fraction of the material was eliminated from the body in the expired air. About 0·5% of the dose was exhaled as unchanged nitrobenzene, and in one case only (Expt. 3) a trace of aniline (0·04% of the dose) was also found. Respiratory carbon dioxide amounting to about 1% of the dose was excreted in 30 hr., but it was unlikely that elimination of this metabolite was complete in this time.

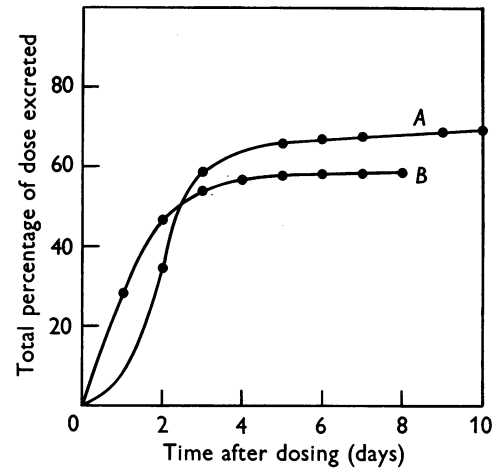

Fig. 2. Excretion of radioactivity in the urine of rabbits receiving [^{14}C]nitrobenzene orally (dose, 0·25 g./kg.). *A* is Expt. 1 and *B* is Expt. 4.

Table 4. *Metabolic fate of a single oral dose of* [^{14}C]*nitrobenzene in the rabbit during 4–5 days after dosing*

Metabolite	Percentage of dose (average)		
Respiratory CO_2	1	2 in expired air	60 total
Nitrobenzene	0·6*		
Aniline	0·4†		
o-Nitrophenol	0·1	58 in urine	
m-Nitrophenol	9		
p-Nitrophenol	9		
o-Aminophenol	3		
m-Aminophenol	4		
p-Aminophenol	31		
4-Nitrocatechol	0·7		
Nitroquinol	0·1		
p-Nitrophenylmercapturic acid	0·3		
(Total urinary radioactivity)	(58)		
Metabolized nitrobenzene in faeces	9‡		
Metabolized nitrobenzene in tissues	15–20		
Total accounted for	85–90%		

* 0·5% in the expired air and <0·1% in the urine.
† 0·3% in the urine and <0·1% in the expired air.
‡ 6% of the dose was present in the faeces as *p*-aminophenol.

About 58% of the dose is excreted in the urine, all of which is present as aromatic compounds. The major urinary metabolite of nitrobenzene is *p*-aminophenol, which accounts for 31% of the dose, or more than half of the nitrobenzene metabolized. *m*- and *p*-Nitrophenols are the next major metabolites, each accounting for 9% of the dose, and then *m*- and *o*-aminophenols, which amount to 4 and 3% respectively. 4-Nitrocatechol (0·7%), aniline (0·3%), *o*-nitrophenol (0·1%), and nitroquinol (0·1%) are minor metabolites, nitroquinol being a hitherto-undescribed metabolite of nitrobenzene. 3-Nitrocatechol was not found to be a metabolite. Apart from aniline and these phenolic compounds, the presence of unchanged nitrobenzene in the urine in amounts of less than 0·1% of the dose (Robinson *et al.* 1951) was confirmed. These quantitative figures are in very good agreement with those obtained by Robinson *et al.* (1951) with unlabelled nitrobenzene (see Table 5).

Table 5. *Comparison of quantitative estimations of the urinary metabolites of nitrobenzene in the rabbit, using non-radioactive and radioactive nitrobenzene*

Metabolites	With non-radioactive nitrobenzene* (Robinson *et al.* 1951) (% of dose)	With radioactive nitrobenzene† (this paper) (% of dose)
Nitrobenzene	0·03	< 0·1
o-Nitrophenol	0·05	0·1
m-Nitrophenol	4·0	9
p-Nitrophenol	5·0	9
4-Nitrocatechol	0·5	0·7
Nitroquinol	—	0·1
Total nitro compounds	20‡	18·9
Aniline	0·50	0·3
o-Aminophenol	0·54	3
m-Aminophenol	0·58	4
p-Aminophenol	35·0	31
Total metabolites	56	57

* Urine collected for 2 days after dosing.
† Urine collected for 4–5 days after dosing.
‡ Estimated by $TiCl_3$ titration.

The mercapturic acid was identified as *p*-nitrophenylmercapturic acid, and was estimated to be equivalent to about 0·3% of the dose. This is considerably less than the estimate of 3% of Robinson *et al.* (1951), but more in agreement with the figure of 0·5% obtained in the present paper when the whole of the urine was used for the iodometric titration.

L-Phenylmercapturic acid, which could have arisen by conjugation with acetylcysteine with concomitant displacement of the nitro group (see Bray *et al.* 1953), was found not to be a metabolite of nitrobenzene. This result, together with the absence from the urine of phenol, catechol and *trans-trans*-muconic acid, shows that elimination of the nitro group does not occur while the aromatic ring is still intact. Since carbon dioxide is a metabolite of nitrobenzene, the elimination of the nitro group must occur either during the opening of the ring, when the product of fission is other than *trans-trans*-muconic acid, or at some subsequent stage of degradation of this fission product.

As nitrosobenzene and β-phenylhydroxylamine are probably intermediates in the formation of *p*-aminophenol, the major metabolite of nitrobenzene, and are undoubtedly also the precursors of aniline (Robinson *et al.* 1951), it seemed reasonable to expect to find evidence of the existence of these intermediate compounds, as traces of azoxy-, azo- or hydrazo-benzene, in the urine. Since azobenzene is metabolized by the rabbit into aniline and hydrazobenzene (Elson & Warren, 1944), and the latter is converted into benzidine by treatment with strong acids, the most likely evidence to be found in the urine of the intermediate stages of reduction would appear to be benzidine. Since none of these compounds were found in the urine, the stepwise reduction of nitrobenzene to nitrosobenzene and β-phenylhydroxylamine, and the rearrangement to *p*-aminophenol, *in vivo*, remain only conjectural.

In the single experiment in which the [^{14}C]-nitrobenzene was administered to a guinea pig, the only marked differences between this animal and the rabbits were the complete absence of *o*-nitrophenol and the decrease of *m*-nitrophenol in the urine of the guinea pig. It is possible that the *o*- and *m*-nitrophenols are more readily reduced to the corresponding aminophenols in the guinea pig than in the rabbit, since the total *ortho* and *meta* compounds (i.e. nitro plus amino) are approximately the same in comparative cases of both animals. Thus the sum of *o*-nitro- and *o*-aminophenols in Expt. 4 is 3·15% and in Expt. 7 is 3·6%; and the figures for the corresponding *meta* compounds are: Expt. 4, 7·9%; Expt. 7, 8·7%. The total amount of reduction is approximately constant however, the sum of the aminophenols in Expt. 4 being 38·3%, and in Expt. 7, 37·1%.

SUMMARY

1. A study has been made of the fate in rabbits of nitrobenzene labelled randomly in one carbon atom with ^{14}C.

2. In 30 hr. the expired air contains about 1% of the dose as carbon dioxide and 0·6% as unchanged nitrobenzene. The elimination of carbon dioxide is not complete within this time.

3. In 4–5 days, 58% of the nitrobenzene is eliminated in the urine. This is present as the previously established metabolites: *p*-aminophenol

(31%), *m*-nitrophenol (9%), *p*-nitrophenol (9%), *m*-aminophenol (4%), *o*-aminophenol (3%), 4-nitrocatechol (0·7%), aniline (0·3%) and *o*-nitrophenol (0·1%). Two new metabolites, nitroquinol (0·1%) and *p*-nitrophenylmercapturic acid (0·3%), were found in the urine.

4. The denitration products, phenol, catechol, L-phenylmercapturic acid, and *trans-trans*-muconic acid, did not occur in the urine.

5. Nitrosobenzene, azoxybenzene, azobenzene and benzidine, the presence of which would have substantiated the occurrence of nitrosobenzene and phenylhydroxylamine as intermediates in the formation of *p*-aminophenol, were not detected in the urine.

6. The total excretion of radioactivity in the expired air, urine and faeces accounts for nearly 70% of the dose of nitrobenzene in 4–5 days after dosing. The remainder is present in the tissues and is eliminated slowly as metabolites in the urine, which is still radioactive 10 days after dosing, and probably also as carbon dioxide.

7. Two days after dosing, about 54% of the administered radioactivity was found in the tissues, particularly in the fat and the intestinal tract. Unchanged nitrobenzene was present in the tissues of this animal. Eight days after dosing, in another experiment, 8% of the radioactivity was found in the tissues, the fat being the most active. No free nitrobenzene was detected in this animal.

8. The faeces contain about 9% of the administered radioactivity, of which about 6% is present as *p*-aminophenol.

9. These experiments account for 85–90% of the administered nitrobenzene.

I am indebted to Professor R. T. Williams for his constant interest in this work. The expenses of this work were in part defrayed by a grant to Professor Williams from the Medical Research Council.

REFERENCES

Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.
Bennett, G. M. & Berry, W. A. (1927). *J. chem. Soc.* p. 1668.
Betts, J. J., James, S. P. & Thorpe, W. V. (1953). *Biochem. J.* **54**, xxxi.
Bray, H. G., Hybs, Z., James, S. P. & Thorpe, W. V. (1953). *Biochem. J.* **53**, 266.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. H. & Yankwich, P. F. (1949). *Isotopic Carbon.* New York: Wiley.
Dakin, H. D. (1909). *Amer. chem. J.* **42**, 490.
Elbs, K. (1893). *J. prakt. Chem.* **48**, (2) 179.
Elson, L. A. & Warren, F. L. (1944). *Biochem. J.* **38**, 217.
Ingold, C. K. (1921). *J. chem. Soc.* **119**, 951.
Lecher, H. & Simon, K. (1922). *Ber. dtsch. chem. Ges.* **55**, 2427.
Loebl, H., Stein, G. & Weiss, J. (1949). *J. chem. Soc.* p. 2074.
Loebl, H., Stein, G. & Weiss, J. (1950). *J. chem. Soc.* p. 2704.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **61**, 569.
Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 624.
Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.

Studies in Carotenogenesis

19. A SURVEY OF THE POLYENES IN A NUMBER OF RIPE BERRIES*

By T. W. GOODWIN
Department of Biochemistry, The University, Liverpool 3

(*Received* 19 *May* 1955)

During the past few years a number of new C_{40} polyenes have been found in plants (Karrer & Jucker, 1950; Goodwin, 1952*a*, 1955*a*). Perhaps the most important of these from the viewpoint of carotenogenesis is the series of hydrocarbons, chemically related to lycopene, consisting of tetrahydrophytoene, phytofluene, ζ-carotene, neurosporene and lycopene. (The first three members of this series are colourless; hence the term 'carotene' will be used only in discussing the coloured members of the series, and the term 'polyene' will be used as a general term including both coloured and colourless members.) As a result of a study of the distribution of these polyenes in the fruit of various tomato crosses, Porter & Lincoln (1950) concluded that the biosynthesis of lycopene probably occurred via this series by sequential dehydrogenation of the almost completely saturated tetrahydrophytoene.

One of the aims of the present survey was to discover, within the limits of the species available, how widespread was the distribution of the 'Porter–Lincoln' series in fruit and berries.

Goodwin (1952*a*, 1955*a*, *b*) lists the polyenes found in the fruit of some 100 plant species. Most of these species, however, were investigated from

* Part 18: Goodwin & Jamikorn (1956).