3. Consideration of the physical and chemical properties of compounds I of bacterial catalase and of peroxidase has led us to suppose that these compounds are mixtures of two components. One of the components is a simple ferric porphyrin complex with the hydroperoxide. The other is the result of an attack of the hydroperoxide upon the porphyrin ring.

The authors wish to thank Professor Sir Hans Krebs, F.R.S., for providing laboratory facilities for preparing the solutions and for the use of the Cary spectrophotometer.

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Studies in Detoxication

85. THE METABOLISM OF *m*-DINITRO[¹⁴C]BENZENE IN THE RABBIT*

By D. V. PARKE

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 25 July 1960)

m-Dinitrobenzene is used industrially as a dye-stuff intermediate and as a constituent of mining explosives. Animals poisoned with *m*-dinitrobenzene develop methaemoglobinaemia, anaemia, liver damage, convulsions and cerebral paralysis (Kunz, 1942; Kiese, 1949). The tissues of rabbits dosed with *m*-dinitrobenzene have been shown to contain *m*-nitroaniline (Belaborodova, 1945) and *m*-nitrophenylhydroxylamine (Lipschitz, 1920), and reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine has been demonstrated *in vitro* in rabbit muscle (Comel, 1931) and blood (Lipschitz, 1948). In the presence of fermenting yeast, *m*-dinitrobenzene is converted into *m*-nitroaniline and 3:3'-dinitroazoxybenzene (Neuberg & Reinfurth, 1923) which is probably an artifact of *m*-nitrophenylhydroxylamine formed by atmospheric oxidation. The formation of dinitroazoxybenzene is evidence of the probable stepwise reduction of *m*-

dinitrobenzene to *m*-nitrosonitrobenzene, *m*-nitrophenylhydroxylamine and *m*-nitroaniline.

The present work with *m*-dinitro[¹⁴C]benzene has made it possible to account for the greater part of an oral dose, most of which is quickly excreted in the urine as *m*-nitroaniline, *m*-phenylenediamine, 2-amino-4-nitrophenol and 2:4-diaminophenol, together with traces of 2:4-dinitrophenol, 4-amino-2-nitrophenol, *m*-nitrophenylhydroxylamine and artifacts derived from this, and unchanged *m*-dinitrobenzene.

EXPERIMENTAL

Melting points are corrected.

Preparation of m-dinitro[¹⁴C]benzene. *m*-Dinitro[¹⁴C]benzene, m.p. 91°, randomly labelled with ¹⁴C in one carbon atom, was obtained as a by-product of the preparation of nitro[¹⁴C]benzene (Parke, 1956). Unlabelled *m*-dinitrobenzene (2 g.) was added to the solid residue which remained after distillation of the nitro[¹⁴C]benzene, and the material was recrystallized from ethanol until it was shown

* Part 84: Parke (1960).

to be free (< 0.1%) from *o*-dinitrobenzene, the principal radioactive impurity, by the colour test described by Fearon & Kawerau (1943). *m*-Dinitrobenzene (10 mg.) in 0.5 ml. of ethanol was heated with 1 ml. of aq. ascorbic acid solution (0.1%, w/v) and 0.2 ml. of 2*N*-NaOH in a boiling-water bath for 30 sec. The mixture was diluted to 10 ml. with water and the light-absorption at 560 μ was measured within 5 min. The violet colour that *o*-dinitrobenzene yields under these conditions has $\lambda_{\max}$ 560 μ (ϵ , 2.4×10^6). By this means 10 μ g. of *o*-dinitrobenzene can be detected in 10 mg. of *m*-dinitrobenzene.

Materials. The following were used: *m*-nitroaniline, m.p. 114°, and 3-nitrobenzanilide, m.p. 159°; *m*-phenylenediamine, m.p. 64°, and its *NN'*-dibenzoyl derivative, m.p. 240°; 2,4-dinitrophenol, m.p. 113°, and its toluene-*p*-sulphonate, m.p. 122°; 3,5-dinitrophenol, m.p. 126° (Sidgwick & Taylor, 1922); 2,6-dinitrophenol, m.p. 61°; 2,4-diaminophenol, m.p. 80°, and its *NN'*-tribenzoyl derivative, m.p. 242° (Meldola & Hollely, 1912); 3,5-diaminophenol, m.p. 180°, and its *NN'*-diacetyl derivative, m.p. 195° (Pollak, 1893); 2,6-diaminophenol sulphate, and its *NN'*-tribenzoyl derivative, m.p. 220° (Fromm & Ebert, 1924); 2-amino-4-nitrophenol, m.p. 142°, and its *O*-toluene-*p*-sulphonate, m.p. 122° (Misslin & Bau, 1919); 4-amino-2-nitrophenol, m.p. 131°, and 4-acetamido-2-nitrophenol, m.p. 158° (Friedländer & Zeitlin, 1894); 3-amino-5-nitrophenol, m.p. 169°, and 3-acetamido-5-nitrophenol, m.p. 270° (decomp.) (Heller & Kamman, 1909); 2-amino-6-nitrophenol, m.p. 112°, and 2-acetamido-6-nitrophenol, m.p. 122° (Newberry & Phillips, 1928); *m*-nitrophenylhydroxylamine, m.p. 119° (Brand, 1905); *m*-nitrosonitrobenzene, m.p. 90° (Brand, 1905) (light-absorption in ethanol, max. 750 μ , log ϵ , 1.8) (Nakamoto, 1952), and 4-bromo-3'-nitroazobenzene, m.p. 121° (Leandri & Risaliti, 1954); 3,3'-dinitroazoxybenzene, m.p. 146° (Lobry de Bruyn, 1893).

Animals. The *m*-dinitro[¹⁴C]benzene contained about 25 μ g. and was administered to rabbits by stomach tube as an aqueous suspension or by subdermal injection as a 10% (w/v) solution in arachis oil. The doses given were from 50 to 100 mg./kg. body wt. At the higher dose level (100 mg./kg.) the animals died on the third day. The rabbits were maintained on a daily diet of 100 g. of Diet 41B (E. Dixon and Sons, Ware, Herts) and 100 ml. of water.

Measurement of radioactivity. Measurements were usually carried out on solid and liquid samples of 'infinite thickness' with an end-window counter tube. The specific activities were determined by comparison with a stable polymer reference (The Radiochemical Centre, Amersham, Bucks.) which was standardized against the *m*-dinitro[¹⁴C]benzene. The radioactivity of the urines and tissues was determined as described by Parke (1956).

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined as described by Mead, Smith & Williams (1958).

Paper chromatography. The possible metabolites of *m*-dinitrobenzene were mostly separable by chromatography in solvents *A* and *B* (see Table 1). The only exception was the separation of *m*-dinitrobenzene and *m*-nitrosonitrobenzene, which was not achieved with these or any other solvent system. However, these two substances could be distinguished by the colour reaction with *p*-dimethylaminocinnamaldehyde (see Table 1). *m*-Nitrophenylhydroxylamine was unstable and appeared to be atmospherically oxidized to *m*-nitrosonitrobenzene or 3,3'-dinitroazoxybenzene during chromatography.

Estimation of *m*-nitroaniline and *m*-phenylenediamine. *m*-Nitroaniline was estimated volumetrically with TiCl_3 by the method described by Robinson, Smith & Williams (1951), and *m*-phenylenediamine was estimated colorimetrically after diazotization. To 5 ml. of a solution con-

Table 1. R_f values and colour reactions on paper of possible metabolites of *m*-dinitrobenzene

R_f values are for descending chromatography on Whatman no. 1 paper, *A*, in benzene-acetic acid-water (1:1:2, by vol.) run for 6 hr., *B* in diisopropyl ether saturated with water (see Parke, 1960). Detecting reagents were: 0.1*N*-NaOH; 1% aq. FeCl_3 ; Gibbs's reagent consisting of 0.2% ethanolic soln. of 2,6-dichloroquinone-chloroimide followed by 2*N*- Na_2CO_3 ; 0.02% soln. of *p*-dimethylaminocinnamaldehyde in ethanol containing 10% (v/v) of 2*N*- H_2SO_4 ; and reducing reagent consisting of the *p*-dimethylaminocinnamaldehyde soln. followed by a 1.5% (w/v) soln. of $\text{Ti}_4(\text{SO}_4)_3$ in *N*- H_2SO_4 , which gave a violet colour with all the compounds listed below.

Compound	R_f values		Colour reactions			
	<i>A</i>	<i>B</i>	NaOH	FeCl_3	Gibbs's reagent	Dimethylaminocinnamaldehyde
<i>m</i> -Dinitrobenzene	0.95	0.93	—	—	—	None
<i>m</i> -Nitrosonitrobenzene	0.96	0.93	—	—	—	Pale violet
<i>m</i> -Nitrophenylhydroxylamine	0.97	0.92	—	—	—	Pale violet
3,3'-Dinitroazoxybenzene	0.97	0.92	—	—	—	None
<i>m</i> -Nitroaniline	0.89	0.91	Orange	—	—	Violet
<i>m</i> -Phenylenediamine	0.05	0.20	—	—	—	Violet
2,4-Dinitrophenol	0.93	0.73	Yellow	None	Yellow	None
2,6-Dinitrophenol	0.91	0.71	Yellow	None	Yellow	None
3,5-Dinitrophenol	0.58	0.85	Yellow	None	Yellow	None
2-Amino-4-nitrophenol	0.08	0.75	Orange	Brown	Orange	Violet
2-Amino-6-nitrophenol	0.86	0.83	Orange	Brown	Violet	Violet
3-Amino-5-nitrophenol	0.07	0.74	Yellow	Buff	Violet	Violet
4-Amino-2-nitrophenol	0.56	0.82	Red	Brown	Violet	Violet
2,4-Diaminophenol	0.00	0.01	Blue	Red	—	Violet
2,6-Diaminophenol	0.00	0.03	Brown	Brown	—	Violet
3,5-Diaminophenol	0.00	0.00	Brown	Brown	—	Violet

taining 0.1–0.8 mg. of *m*-phenylenediamine in 0.5-HCl was added 1 ml. of a 0.1% (w/v) aq. solution of NaNO₂ followed by 2 ml. of 2*N*-Na₂CO₃; the mixture was diluted to 10 ml. and the light-absorption measured at 362 m μ . The standard curve is shown in Fig. 1. Recoveries of *m*-nitroaniline and *m*-phenylenediamine separately from aqueous solution were 95 $\pm$ 5% and 96 $\pm$ 5% respectively.

The total *m*-nitroaniline and *m*-phenylenediamine in solution in urines were estimated simultaneously by refluxing 25 ml. of urine with 12.5 ml. of conc. HCl for 3 hr. to hydrolyse the conjugates. The hydrolysed solutions were cooled, adjusted to pH 12.0 with 10*N*-NaOH and then extracted with three 20 ml. portions of ethyl acetate. The ethyl acetate extracts were bulked and extracted with three 10 ml. portions of 2*N*-HCl, and the acid extracts were bulked and diluted to 50 ml.; 5.0 ml. of this solution was used for the colorimetric determination of *m*-phenylenediamine, and 40 ml. was boiled with 5.0 ml. of TiCl₃ solution for the volumetric estimation of *m*-nitroaniline. Blank estimations were made on normal urines for 3 days before dosing with *m*-dinitrobenzene. Recoveries of mixtures of *m*-nitroaniline and *m*-phenylenediamine (25 mg./100 ml. each) from solutions in normal rabbit urine were 93 $\pm$ 5% and 83 $\pm$ 10% respectively. *m*-Nitroaniline also produces a colour on diazotization and the light-absorption at 362 m μ is shown in Fig. 1. Corrections were therefore made for the amount of *m*-nitroaniline present. The aminonitrophenols and 2:4-diaminophenol also produce a colour on diazotization and the aminonitrophenols consume TiCl₃, but since they are not extracted from aqueous solution at pH 12.0 they do not interfere in the estimations.

Estimation of metabolites

m-Dinitrobenzene. *m*-Dinitrobenzene (250 mg.) dissolved in 5 ml. of ethanol was added to a portion of the urine (10–20 ml.), and the solution was extracted with three portions of ether (10 ml.). The combined ethereal extracts were washed with *N*-HCl, *N*-NaOH, then water, dried over

anhydrous Na₂SO₄ and evaporated to dryness. The residue of *m*-dinitrobenzene was recrystallized from ethanol and toluene to constant specific activity (m.p. and mixed m.p. 91°).

m-Nitrosonitrobenzene. *m*-Nitrosonitrobenzene (250 mg.) was added to a portion of the freshly voided urine (10 ml.) and the mixture steam-distilled. The nitrosonitrobenzene in the distillate was filtered off, washed with *N*-HCl to remove traces of *m*-nitroaniline, and recrystallized from ethanol and aqueous ethanol to constant specific radioactivity (m.p. and mixed m.p. 91°). The material was then converted into the 4-bromoazo compound (Ingold, 1925) by warming with an equimolecular amount of *p*-bromoaniline dissolved in 2 ml. of acetic acid. The mixture was poured into water and the orange 4-bromo-3'-nitroazobenzene filtered off and recrystallized from dilute acetic acid to constant specific radioactivity (m.p. and mixed m.p. 121°).

m-Nitrophenylhydroxylamine. *m*-Nitrophenylhydroxylamine (500 mg.) in 5 ml. of ethanol was added to a portion of the freshly voided urine and the mixture extracted with three portions of ether. The ethereal extracts were bulked, the ether was evaporated, and the residual *m*-nitrophenylhydroxylamine was recrystallized from benzene to constant specific radioactivity (m.p. and mixed m.p. 118°). The phenylhydroxylamine was then treated with 50 ml. of 10% aq. FeCl₃ soln., and steam-distilled to convert it into *m*-nitrosonitrobenzene, which was removed from the distillate by filtration, recrystallized from ethanol and aqueous ethanol (m.p. and mixed m.p. 91°) and its radioactivity determined.

3:3'-Dinitroazoxybenzene. 3:3'-Dinitroazoxybenzene (250 mg.) in 10 ml. of benzene was added to a portion of the freshly voided urine and the mixture shaken for 0.5 hr. to equilibrate. The mixture was then extracted with three portions of benzene, the benzene extracts were bulked, washed with *N*-NaOH, *N*-HCl and water, dried over anhydrous Na₂SO₄, and the benzene was removed. The residue was recrystallized from a mixture of ethanol and ethyl acetate, and then from formic acid to constant specific activity (m.p. and mixed m.p. 146°).

m-Nitroaniline. *m*-Nitroaniline (500 mg.) was added to a portion of the urine, which was then refluxed with an equal vol. of conc. HCl for 3 hr. The hydrolysed solution was adjusted to pH 8.0 with Na₂CO₃ and extracted with three portions of ether. The combined ethereal extracts were washed with water and evaporated to dryness, and the residue was steam-distilled. The *m*-nitroaniline in the steam-distillate was filtered off and recrystallized from water (m.p. and mixed m.p. 114°). It was finally dissolved in acetone (5 ml.) and treated with 1 equiv. of benzoyl chloride and excess of 2*N*-NaOH to give 3-nitrobenzanilide (m.p. and mixed m.p. 159°); this was recrystallized from pentanol to constant specific activity.

m-Phenylenediamine. *m*-Phenylenediamine (500 mg.) was added to a portion of the urine, which was then refluxed with an equal vol. of conc. HCl for 3 hr. The hydrolysed solution was adjusted to pH 8.0 with Na₂CO₃ and extracted with three portions of ethyl acetate. The combined extracts were washed with a little 0.2*N*-NaOH, and the *m*-phenylenediamine was transferred into 2*N*-HCl. The acid solution was then adjusted to pH 8.0 with Na₂CO₃ and extracted with three portions of ethyl acetate. The combined extracts were washed with a little 0.2*N*-NaOH, dried over anhydrous Na₂SO₄ and evaporated to dryness. The

Fig. 1. The light-absorption at 362 m μ of alkaline solutions of diazotized *m*-phenylenediamine (●) and *m*-nitroaniline (○).

residue was dissolved in water, filtered and treated with 2 equiv. of benzoyl chloride and excess of 2*N*-NaOH to yield the *NN'*-dibenzoyl derivative, which was recrystallized from acetic acid, methyl ethyl ketone and cyclohexanone to constant specific activity (m.p. and mixed m.p. 240°).

Dinitrophenols. 2:4-, 2:6- and 3:5-Dinitrophenol (500 mg.) were separately added to portions of the urine, which were then refluxed with an equal vol. of conc. HCl for 3 hr. The solution of 2:4-dinitrophenol was cooled, the 2:4-dinitrophenol filtered off and dissolved in ether (20 ml.); the ethereal extract was washed with 2*N*-HCl, dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was steam-distilled and the 2:4-dinitrophenol removed from the distillate by filtration and recrystallized from ethanol (m.p. 113°). The 2:4-dinitrophenol was then dissolved in acetone and treated with 1 equiv. of toluene-*p*-sulphonyl chloride and excess of 2*N*-NaOH to yield 2:4-dinitrophenyl toluene-*p*-sulphonate (m.p. and mixed m.p. 122°), which was recrystallized from ethanol to constant specific activity.

The solution containing 2:6-dinitrophenol was extracted with three portions of ether, and the combined ethereal extracts were washed with 2*N*-HCl, dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was steam-distilled, the 2:6-dinitrophenol was extracted from the distillate with three portions of ether, and the ether evaporated. The residue of 2:6-dinitrophenol was recrystallized from ethanol, aq. ethanol and 0.1*N*-HCl until free from radioactivity (m.p. and mixed m.p. 61°).

The solution containing 3:5-dinitrophenol was extracted with three portions of ether and the combined ethereal extracts were washed with 2*N*-HCl. The dinitrophenol was then transferred into *N*-NaOH which was acidified with 2*N*-HCl and cooled. The precipitated dinitrophenol was filtered off, redissolved in *N*-NaOH, reprecipitated with 2*N*-HCl and collected by filtration. The 3:5-dinitrophenol was then recrystallized from 0.1*N*-HCl until free from radioactivity (m.p. and mixed m.p. 126°).

Aminonitrophenols. 2-Amino-4-nitrophenol, 2-amino-6-nitrophenol, 3-amino-5-nitrophenol and 4-amino-2-nitrophenol (500 mg.) were separately added to portions of the urine which were then refluxed with an equal volume of conc. HCl for 3 hr. The hydrolysed solutions were made alkaline with 10*N*-NaOH and extracted once with ether, and the ether was discarded. The solutions were then adjusted to pH 7.0 with 2*N*-HCl and extracted with three portions of ether. The three ethereal extracts were bulked and washed once with water, and the aminonitrophenols were transferred into 2*N*-NaOH. Acidification of these solutions with 2*N*-HCl precipitates the aminonitrophenols, which are removed by filtration. The 2-amino-4-nitrophenol, m.p. 138°, was recrystallized from water and converted into its *O*-toluene-*p*-sulphonate, by treatment with one equivalent of toluene-*p*-sulphonyl chloride and excess of 2*N*-NaOH; it was then recrystallized from ethanol to constant activity (m.p. and mixed m.p. 122°). The three other aminonitrophenols were recrystallized from water, then dissolved in 2*N*-NaOH and treated with 1 equiv. of acetic anhydride. The solutions were acidified and the products filtered off and recrystallized from ethanol to constant specific activity (2-acetamido-6-nitrophenol, m.p. and mixed m.p. 122°; 3-acetamido-5-nitrophenol, m.p. 270° (decomp.); and 4-acetamido-2-nitrophenol, m.p. 157°).

In some experiments 4-amino-2-nitrophenol was determined after hydrolysis of the urine with a crude preparation of β-glucuronidase and arylsulphatase. The urine (10 ml.) was adjusted to pH 4.6 with acetic acid and then incubated at 30° for 16 hr. with 1 ml. of crop fluid from *Helix pomatia* (see Leon, Bulbrook & Corner, 1960). Carrier 4-amino-2-nitrophenol (5–10 mg.) was then added to the mixture, which was again adjusted to pH 4.6, and incubated for a further 24 hr. after addition of another 1 ml. of the glucuronidase preparation. The mixture was then adjusted to pH 8.0 and extracted twice with 10 ml. portions of ether. The ethereal extracts were concentrated and chromatographed on Whatman no. 3MM paper in solvent mixture *B*. The band corresponding to 4-amino-2-nitrophenol was cut out and eluted with acetone, and the eluate rechromatographed on Whatman no. 1 paper in solvent *A*. The 4-amino-2-nitrophenol band was again cut out and eluted with acetone, and the eluate evaporated on a planchet; the residue was weighed and the radioactivity determined with an infinitely thin sample. The specific radioactivity remained constant after further chromatography in solvent *A*.

Diaminophenols. 3:5-Diaminophenol (500 mg.), 2:4-diaminophenol dihydrochloride (1 g.) and 2:6-diaminophenol sulphate (1 g.) were separately added to portions of the urine which were then refluxed for 3 hr. with an equal vol. of conc. HCl. The hydrolysed solutions were adjusted to pH 8.5 with Na₂CO₃ and extracted with three portions of butan-1-ol. The butanol extracts were bulked, and the diaminophenols were transferred into 2*N*-HCl. The acid solutions were adjusted to pH 8.5 with Na₂CO₃ and again extracted with butan-1-ol, and the diaminophenols transferred to 2*N*-HCl. The acid solutions were washed with ether and the ethereal extracts discarded. The solutions of 2:4- and 2:6-diaminophenol were then treated with excess of benzoyl chloride, solid Na₂SO₃ and 2*N*-NaOH to give the *ONN'*-tribenzoyl derivatives; the solution of 3:5-diaminophenol was treated with excess of acetic anhydride and 2*N*-NaOH to give the *NN'*-diacetamidophenol. 2:4-Diaminophenol tribenzoate was recrystallized from ethyl acetate to constant specific activity (m.p. and mixed m.p. 241°). 2:6-Diaminophenol tribenzoate (m.p. and mixed m.p. 220°) was recrystallized from ethanol and then ethyl acetate and 3:5-diacetamidophenol (m.p. and mixed m.p. 195°) was recrystallized from ethanol, until free from radioactivity.

Chromatographic detection of metabolites

The urine from a rabbit dosed with *m*-dinitro[¹⁴C]benzene (Expt. 3), which remained after isotope dilution estimations, was hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed urine was then continuously extracted with ether and twice again extracted with ether after adjusting to pH 7.0 and then 9.0 with Na₂CO₃. The three extracts were chromatographed on Whatman no. 1 paper in solvents *A* and *B*, and radioautographs were subsequently obtained.

The extract from acid solution showed radioactive bands with colour reactions and *R_F* values corresponding to *m*-dinitrobenzene and 3:3'-dinitroazoxybenzene, each present to about 1% of the dose, and 2:4-dinitrophenol (0.1% of dose). In addition a dark-brown band (15–20% of the dose) was present at the origin of the chromatograms in both solvents, and probably consisted of polymeric

oxidation products of *m*-phenylenediamine and diamino-phenol. 2:6- and 3:5-Dinitrophenol were not present in amounts $> 0.02\%$ of the dose. The extract at pH 7.0 showed radioactive bands with colour reactions and R_f values corresponding to *m*-phenylenediamine (10% of dose), 2-amino-4-nitrophenol (10% of dose), *m*-nitroaniline (5% of dose), 4-amino-2-nitrophenol (1% of dose), and a band at the origin in both solvents (10% of dose) consisting of oxidation products of *m*-phenylenediamine and diamino-phenol. 2-Amino-6-nitrophenol and 3-amino-5-nitrophenol were not present in amounts $> 0.05\%$ of the dose. The extract at pH 9.0 showed only two radioactive bands, one at the origin (25% of dose) and one corresponding to *m*-nitroaniline (3% of dose).

Isolation of metabolites from unhydrolysed urine

***m*-Nitroaniline.** *m*-Dinitrobenzene (0.25 g.) in arachis oil (5 ml.) was administered orally to each of two rabbits. The urines, which were collected for 2 days, were dark brown, had pH 7.4, did not reduce Benedict's reagent but gave a strong positive naphtharesorcinol reaction for glucuronides. They frequently contained albumin, erythrocytes, epithelial cells and cellular casts. The combined urines were adjusted to pH 2 with 2*N*-HCl and then continuously extracted with ether for 6 hr.; the ethereal extract was concentrated to 20 ml. and extracted three times with 10 ml. portions of 2*N*-HCl. The acid extracts were bulked, washed with 20 ml. of ether, adjusted to pH 10 with 10*N*-NaOH and then extracted three times with 10 ml. portions of ether. The bulked ethereal extracts were washed with water (10 ml.) and then extracted twice with 5 ml. portions of 5*N*-HCl. The acid extracts were adjusted to pH 10 with 10*N*-NaOH, and, on cooling, crystals of *m*-nitroaniline were deposited (yield, 43 mg. equivalent to 9% of dose) which after recrystallization from aqueous ethanol had m.p. 113°, mixed m.p. 114°. Benzoylation of this material with benzoyl chloride and 2*N*-NaOH gave 3-nitrobenzanilide, m.p. and mixed m.p. 159°.

3:3'-Dinitroazoxybenzene and *m*-dinitrobenzene. The ethereal extracts of the urines remaining after extraction with 2*N*-HCl and the ether washings of the acid extracts were bulked, washed with 2*N*-NaOH and water and evaporated to dryness. The residue (125 mg.) was digested with benzene, and the benzene solution was filtered and evaporated to dryness. The residue (113 mg.) gave a deep-violet colour with acetone and NaOH, indicative of *m*-dinitrobenzene or *m*-nitrosonitrobenzene, and was dissolved in 5 ml. of hot ethanol. On cooling, the solution deposited a few dark-brown crystals, m.p. 138° (1.5 mg., equivalent to 0.3% of dose) which on recrystallization from formic acid gave 3:3'-dinitroazoxybenzene (m.p. 142°, mixed m.p. 144°). The ethanolic solution was diluted with water and the precipitate (12 mg., 2.5% of the dose) which was deposited was filtered off and recrystallized repeatedly from aqueous ethanol to yield colourless crystals of *m*-dinitrobenzene, m.p. 90° (admixture of *m*-nitrosonitrobenzene with *m*-dinitrobenzene in the proportions of 0.5, 1 and 2 to 1 does not significantly depress the m.p. from 89–90°). The eutectic of *m*-nitrosonitrobenzene (25%) and *m*-dinitrobenzene (75%) is distinctly green (Nakamoto, 1952).

***m*-Nitrosonitrobenzene.** The urines from six rabbits fed a total of 1.5 g. of *m*-dinitrobenzene were collected for 2 days, bulked, adjusted to pH 6.0 and steam-distilled. The steam-distillate was extracted with ether, and the extract dried

over anhydrous Na_2SO_4 and evaporated to give a non-crystalline residue, which by paper chromatography was shown to contain *m*-nitroaniline and a trace of *m*-dinitrobenzene. The urine was again steam-distilled after addition of 20 g. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and the steam-distillate extracted with ether; the extract was dried over anhydrous Na_2SO_4 and evaporated. The residual trace of material was recrystallized from a little aqueous ethanol to give a few crystals (< 1 mg.) (m.p. 89°, mixed m.p. with *m*-nitrosonitrobenzene 90°) which were green in liquid state and on paper gave a violet colour with *p*-dimethylaminocinnamaldehyde alone. This material by virtue of its m.p., colour and colour reaction was most probably *m*-nitrosonitrobenzene, formed from *m*-nitrophenylhydroxylamine by the oxidation with FeCl_3 .

Isolation of metabolites from hydrolysed urine

2:4-Dinitrophenol. The combined urines from two rabbits which had been fed a total of 0.5 g. of *m*-dinitrobenzene were hydrolysed by refluxing for 3 hr. with an equal volume of conc. HCl. The urines were then cooled and continuously extracted with ether, and the ethereal extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (220 mg.) on chromatography on paper in solvents *A* and *B* revealed the presence of trace amounts (equivalent to about 0.1% of dose) of 2:4-dinitrophenol and *m*-nitroaniline. 2:6- and 3:5-Dinitrophenol were not present in amounts greater than 0.02% of the dose. The residue was redissolved in ether, washed with 2*N*-HCl, and the ether solution dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was digested with hot water and on cooling the solution deposited a few crystals (1 mg.), m.p. 114°, not depressed on admixture with 2:4-dinitrophenol. Paper chromatography of an ethereal extract of the HCl washings revealed only *m*-nitroaniline.

The absence of significant amounts of dinitrophenols was confirmed by u.v.-absorption spectra. The urines obtained from two rabbits dosed orally with a total of 0.5 g. of *m*-dinitrobenzene, collected over 2 days, were bulked and hydrolysed by refluxing for 3 hr. with 0.5 vol. of conc. HCl. The hydrolysed urine was cooled and continuously extracted with ether for 8 hr. The ethereal extract (50 ml.) was extracted with two 20 ml. portions of 2*N*-NaOH, and the alkaline extracts were bulked, washed with ether, acidified with conc. HCl and extracted with two 20 ml. portions of ether. The ethereal extract was washed with water, dried over anhydrous Na_2SO_4 , and evaporated to dryness at room temperature. The residue (about 10 mg.) was dissolved in 10 ml. of 0.1*N*-NaOH, filtered and diluted 1 to 10 with 0.1*N*-NaOH and 0.1*N*-HCl. The absorption spectra of these solutions were determined with a Unicam SP. 500 spectrophotometer and compared with the absorption spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0.1*N*-HCl and 0.1*N*-NaOH (see Figs. 2, 3). From the absorption spectra it is seen that no significant amounts of dinitrophenols were present, and 2:4-dinitrophenol was estimated to be present in an amount not greater than 0.1% of the dose. Flexser, Hammett & Dingwall (1935) showed that 2:4-dinitrophenol has light-absorption max. 261 μ (ϵ 11 200) in 0.1*N*-HCl; and 364 μ (ϵ 14 800) in 0.1*N*-NaOH.

2-Amino-4-nitrophenol. The hydrolysed urine after ether extraction of 2:4-dinitrophenol was then adjusted to

pH 8.0 with solid Na_2CO_3 and continuously extracted with ether. The ethereal extract (50 ml.) was extracted twice with 20 ml. portions of 2*N*-HCl; the acid extracts were combined, washed with ether, adjusted to pH 8.0 with Na_2CO_3 and extracted with three 20 ml. portions of ether. The ethereal extracts were bulked, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (215 mg.) was digested with hot water and filtered, and, on cooling the solution, red crystals, m.p. 70° (28 mg., equivalent to 5% of the dose) were deposited. Chromatography of this material on paper in solvents *A* and *B* revealed three spots with R_f values and colour reactions corresponding to 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and *m*-nitroaniline. The bulk of the crystalline material was chromatographed on Whatman no. 3 MM paper in solvent *B* and a broad orange band containing the two aminonitrophenols (R_f 0.7–0.85) and a yellow band (R_f 0.9–0.95) containing *m*-nitroaniline were cut out and eluted with ether. The material from the first band was rechromatographed on Whatman no. 1 paper in solvent *A* and gave a major band with R_f 0.08 and a minor one with R_f 0.56. The major band was cut out and eluted with ether; the ether was evaporated and the residue recrystallized from water to give 2-amino-4-nitrophenol, m.p. and mixed m.p. 143° (16 mg., equivalent to 3% of the dose). Treatment with 1 equiv. of toluene-*p*-sulphonyl chloride and excess of 2*N*-NaOH gave the *O*-toluene-*p*-sulphonate, m.p. and mixed m.p. 122°. Elution of the minor band gave a trace of red material which did not crystallize and did not yield a crystalline derivative on treatment with acetic anhydride

and excess of 2*N*-NaOH; light-absorption max., 239 and 495 $\text{m}\mu$ in *N*-NaOH and 267 and 340 $\text{m}\mu$ in *N*-HCl, indicative of 4-amino-2-nitrophenol [light-absorption max. 239, ~295 and 500 $\text{m}\mu$ (ϵ 16 300, 4100 and 3800 respectively) in *N*-NaOH and 267 and 340–345 $\text{m}\mu$ (ϵ 5400 and 2800) in *N*-HCl, cf. Parker (1952)].

***m*-Phenylenediamine.** The combined urines collected over 2 days from two rabbits fed a total of 0.5 g. of *m*-dinitrobenzene were hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed urines were adjusted to pH 8.0 with solid Na_2CO_3 and extracted three times with 0.5 vol. of ethyl acetate. The extracts were combined and in turn extracted twice with 25 ml. of 2*N*-HCl. The acid extracts were adjusted to pH 8.0 with solid Na_2CO_3 and extracted twice with 25 ml. of ethyl acetate. The ethyl acetate extracts were dried over anhydrous Na_2SO_4 , concentrated *in vacuo*, and the residue was chromatographed on Whatman no. 3 MM paper in solvent *B*. The band with R_f 0.20, corresponding to *m*-phenylenediamine, was cut out, eluted with *N*-HCl, and the eluate treated with an excess of benzoyl chloride and 2*N*-NaOH to yield *NN'*-dibenzoyl-*m*-phenylenediamine (yield 70 mg., equivalent to 6% of the dose), m.p. and mixed m.p. 240° after recrystallization from ethanol and acetic acid.

2:4-Diaminophenol. The combined urines collected over 24 hr. from two rabbits fed a total of 0.5 g. *m*-dinitrobenzene were heated on a boiling-water bath for 0.5 hr. after addition of 0.1 vol. of conc. HCl. The partially hydrolysed urine was cooled and continuously extracted with ether for 8-hr. periods. The ethereal extract of the first 8 hr. period was evaporated to dryness, and the residue was

Fig. 2. The absorption spectrum in 0.1*N*-HCl (100 ml.) of an extract of urine from rabbits after an oral dose (500 mg.) of *m*-dinitrobenzene (---), compared with the spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0.1*N*-HCl. *A*, 2:4-Dinitrophenol (λ_{max} 261, ~300 $\text{m}\mu$; ϵ_{max} 13 600, 9400); *B*, 2:6-dinitrophenol (λ_{max} 248, 345 $\text{m}\mu$; ϵ_{max} 10 800, 5400); and *C*, 3:5-dinitrophenol (λ_{max} 252, 340 $\text{m}\mu$; ϵ_{max} 15 300, 3100).

Fig. 3. The absorption spectrum in 0.1*N*-NaOH (100 ml.) of an extract of urine from rabbits after an oral dose (500 mg.) of *m*-dinitrobenzene (---), compared with the spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0.1*N*-NaOH. *A*, 2:4-Dinitrophenol (λ_{max} 226, 258, 358 $\text{m}\mu$; ϵ_{max} 10 300, 7200, 14 800); *B*, 2:6-dinitrophenol (λ_{max} ~258, 428 $\text{m}\mu$; ϵ_{max} 8000, 8200); *C*, 3:5-dinitrophenol (λ_{max} 268, 400 $\text{m}\mu$; ϵ_{max} 13 000, 2800).

digested with water (20 ml.) and acidified by addition of a few drops of 2*N*-acetic acid to which a crystal or two of Na_2SO_3 was added. The aqueous solution was filtered and treated with an excess of a saturated solution of lead acetate. The ppt. which formed (mostly the lead salt of 2-amino-4-nitrophenol) was filtered off, and the filtrate was adjusted to pH 8.0 with aq. 2*N*- NH_3 soln. and treated with an excess of a saturated solution of basic lead acetate (2:4-diaminophenol forms an insoluble salt with basic lead acetate but not with lead acetate). The ppt. formed was deposited by centrifuging, washed with water, suspended in water, and the suspension saturated with H_2S . The precipitated PbS was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue (about 20 mg.) was treated with 0.1 ml. of benzoyl chloride and an excess of solid Na_2SO_3 , and finally made alkaline with solid Na_2CO_3 , but no crystalline material was obtained. The ethereal extract of the second 8 hr. period was treated similarly. No ppt. was obtained with lead acetate solution, but a substantial ppt. was formed after addition of basic lead acetate. The residue finally obtained after removal of the lead and evaporation was dissolved in 5 ml. of water. The solution gave a red colour with Br_2 water or 10% (w/v) aq. FeCl_3 soln., and a blue colour with 2*N*- NaOH , which are colour reactions given by 2:4-diaminophenol. The solution was treated with 0.1 ml. of benzoyl chloride and an excess of solid Na_2SO_3 and finally made alkaline with 2*N*- NaOH , to yield a small amount of crystalline material (2 mg., equivalent to 0.1% of dose) which was washed with water and recrystallized from a mixture of ethanol and ethyl acetate to give the *ONN'*-tribenzoate of 2:4-diaminophenol (m.p. and mixed m.p. 240°). The very small yield of 2:4-diaminophenol isolated is probably due to its facile oxidation; Ogino & Yasukura (1957) who isolated 2:4-diaminophenol from the urine of rabbits dosed with 2:4-dinitrophenol were able to obtain only small amounts of material, equivalent to 0.2% of the dose. Attempts at the isolation of 2:4-diaminophenol from urine hydrolysed by refluxing for 3 hr. with an equal volume of conc. HCl yielded no crystalline material.

RESULTS AND DISCUSSION

After an oral dose of *m*-dinitro[^{14}C]benzene some 65–93% is excreted in the urine in 2 days (see Table 2), and subsequently only trace amounts of radioactivity are found in the urine. The faeces contain only small amounts of radioactivity (1–5% of dose), and the rest of the radioactivity is presumably retained in the tissues, from which it is slowly excreted in the urine. The radioactivity of the tissues could not be accurately determined, owing to the low specific activity of the administered *m*-dinitrobenzene. The mean values for urine conjugates for three rabbits dosed orally with *m*-dinitrobenzene (80 mg./kg. body wt.) were: glucuronides, 30% of dose (range 21–41%); and ethereal sulphates, 6% of dose (range 0–11%).

The major metabolites of *m*-dinitrobenzene found in the urine were *m*-nitroaniline and *m*-phenylenediamine, which together amount to 35% of the dose, 2:4-diaminophenol and 2-amino-4-nitrophenol. The minor metabolites present were unchanged *m*-dinitrobenzene, 2:4-dinitrophenol, 4-amino-2-nitrophenol, *m*-nitrophenylhydroxylamine and artifacts derived from this, 3:3'-dinitroazoxybenzene and *m*-nitrosodinitrobenzene. 2:6- and 3:5-Dinitrophenol, 2:6- and 3:5-diaminophenol, 2-amino-6-nitrophenol and 3-amino-5-nitrophenol were not present in amounts greater than 0.05% of the dose. All the metabolites estimated by isotope dilution procedures, with the exception of 4-amino-2-nitrophenol and *m*-nitrophenylhydroxylamine, have been isolated. The presence of 4-amino-2-nitrophenol has been confirmed by paper chromatography and u.v.-absorption spectra; the presence of *m*-nitrophenylhydroxylamine, which is

Table 2. *Metabolites of m-dinitro[^{14}C]benzene excreted in urine by rabbits*

<i>m</i> -Dinitrobenzene was administered orally as aqueous suspensions. Urines were collected for 2 days.								
Expt. no.	1*	2	3	4	5	6	7	
Dose of <i>m</i> -dinitrobenzene (mg./kg.)	100	70	70	60	60	50	50	
Dose of ^{14}C ($\mu\text{C}/\text{animal}$)	5	4	5	4	4	2	3	Average
Percentage of dose								
<i>m</i> -Dinitrobenzene	<0.1	0.2	—	2.4	—	0.4	0.7	0.7
<i>m</i> -Nitrophenylhydroxylamine	—	—	—	—	0.7	1.1	0.75	0.8
<i>m</i> -Nitrosodinitrobenzene	—	—	—	0.5	0.2	0.1	0.25	0.25
3:3'-Dinitroazoxybenzene	—	—	—	0.3	0.2	0.3	—	0.3
<i>m</i> -Nitroaniline (total)	28	35	10	18	—	—	—	} 35
<i>m</i> -Phenylenediamine (total)	<0.2	<0.2	23	25	—	—	—	
2:4-Dinitrophenol (total)	0.1	0.1	<0.1	—	—	—	—	0.1
2-Amino-4-nitrophenol (total)	12.5	15	12	16	—	—	—	14
4-Amino-2-nitrophenol (total)	1.4	1.6	2.1	2.6	—	1.9	2.4	2.0
						(0.6)†	(1.0)†	(0.8)†
2:4-Diaminophenol (total)	19	37	38	28	—	—	—	31
Total metabolites	61	89	85	93	—	—	—	83
Total radioactivity in urine	65	89	82	93	71	89	75	81
Total radioactivity in faeces	—	0.3	1.0	—	5.2	—	—	—

* Animal died on 3rd day.

† Estimated after enzymic hydrolysis.

readily oxidized to 3:3'-dinitroazoxybenzene in alkaline solution and to *m*-nitrosonitrobenzene in neutral and acid media, has been indirectly confirmed by the isolation of both of these, the latter after oxidation with FeCl₃. *m*-Nitrophenylhydroxylamine and 3:3'-dinitroazoxybenzene were both estimated in freshly voided urine, but the possibility of their being artifacts arising from oxidation of *m*-nitrophenylhydroxylamine during the estimation procedures cannot be eliminated.

The excretion of traces of unchanged *m*-dinitrobenzene is surprising, for at similar doses neither benzene (Parke & Williams, 1950) nor nitrobenzene (Parke, 1956), both of which are more soluble than *m*-dinitrobenzene in water, is excreted unchanged in the urine. Possibly the excretion of traces of the unchanged material is due to the kidney damage which was indicated in many of the animals.

Reduction products. The excretion of some 1% of the dose as *m*-nitrophenylhydroxylamine or its artifacts confirms the findings of Lipschitz (1920) and Comel (1931) that *m*-nitrophenylhydroxylamine is formed from *m*-dinitrobenzene in animal tissues. A considerable amount of dinitrohydroxylaminotoluene was isolated from the urine of rabbits dosed with trinitrotoluene (Channon, Mills & Williams, 1944), but in the metabolism of nitrobenzene no trace of phenylhydroxylamine nor any artifact of it was found (Parke, 1956). Jackson & Waters (1958) have demonstrated in non-biological systems that free radicals may catalyse hydrogen transfer with *m*-dinitrobenzene or *s*-trinitrobenzene as oxidizing agents. In such reductions, Jackson & Waters (1960), using benzyl radicals, have shown that the free radicals react readily with trinitrobenzene to give a dinitro-

phenylhydroxylamine derivative, less readily with *m*-dinitrobenzene, to give small amounts of the *m*-nitrophenylhydroxylamine derivative together with *m*-nitroaniline, and with nitrobenzene to give only aniline. This is qualitatively and quantitatively similar to the metabolic reductions of the aromatic nitro compounds, and it is possible that the biological reduction of these compounds may be effected by a similar free radical mechanism involving flavins. The nitro-reductases described by Fouts & Brodie (1957), Bueding & Jolliffe (1946) and Müller (1958) are considered to be flavoproteins, which may be replaced by flavin mononucleotide, flavinadenine dinucleotide and even riboflavin; and free radical flavin semiquinones have been shown to arise as intermediates of the flavoprotein acyl dehydrogenases (Beinert, 1957; Beinert & Page, 1957; Ke, 1957).

The two other reduction products of *m*-dinitrobenzene found in the urine, *m*-nitroaniline and *m*-phenylenediamine, together account for about 35% of the dose. The former usually amounts to 14% and the latter to 21%, but in the first two experiments with *m*-dinitro[¹⁴C]benzene no significant amount of *m*-phenylenediamine could be detected, and the amounts of *m*-nitroaniline were approximately equal to the total of the two reduction products of subsequent experiments. The reduction of only one of the nitro groups of *m*-dinitrobenzene has been observed in polarographic studies of the blood of dogs fed *m*-dinitrobenzene, reduction to *m*-nitroaniline occurring within the erythrocytes (Roubal, Tuný & Pokorný, 1946). In two different breeds of rabbit dosed with unlabelled *m*-dinitrobenzene (Table 3) both *m*-nitroaniline and *m*-phenylenediamine were formed in

Table 3. *Excretion of m-nitroaniline and m-phenylenediamine by rabbits after dosage with m-dinitrobenzene*

The dose of *m*-dinitrobenzene was 80 mg./kg. Urines were collected for 2 days. The mean values and standard errors of the mean are given in parentheses.

Breed of rabbit	Route of administration	Percentage of dose excreted as	
		<i>m</i> -Nitroaniline	<i>m</i> -Phenylenediamine
English	Oral	14	16
English	Oral	10 (14 ± 3)	12 (17 ± 3)
English	Subcutaneous	19	22
Chinchilla	Oral	9	18
Chinchilla	Oral	16 (14 ± 2)	23 (21 ± 2)
Chinchilla	Subcutaneous	16	23
Chinchilla	Oral*	9	12
Chinchilla	Oral*	15 (12 ± 2)	16 (13 ± 2)
Chinchilla	Subcutaneous*	11	10
Chinchilla	Oral*†	14	11
Chinchilla‡	Subcutaneous*†	14 (12 ± 2)	9 (8 ± 2)
Chinchilla‡	Subcutaneous*†	8	5

* Aureomycin (30 mg.) administered orally 12 hr. before dosage with *m*-dinitrobenzene and then again at 12 hr. intervals.

† *m*-Dinitrobenzene (80 mg./kg.) without aureomycin was administered orally 14 days previously.

‡ These animals died 20–24 hr. after dosing.

amounts similar to those found in Expts. 3 and 4 with *m*-dinitro[¹⁴C]benzene. The subcutaneous administration of *m*-dinitrobenzene did not lessen the amounts of the reduction products appearing in the urine, from which it may be inferred that neither compound arises to any extent from reduction of the *m*-dinitrobenzene by intestinal bacteria (cf. Bray, Hybs, James & Thorpe, 1953). The simultaneous administration of aureomycin has no effect on the reduction of the first nitro group of *m*-dinitrobenzene, which is in agreement with the observations of Fouts & Brodie (1957) that reduction of *m*-dinitrobenzene and other aromatic nitro compounds by rabbit-liver nitro-reductase is not inhibited by aureomycin; reduction of *p*-nitrobenzaldehyde by rat liver is, however, inhibited by the antibiotic (Green, Josimovich, Tsou & Seligman, 1956). The extent of the reduction of *m*-dinitrobenzene to *m*-phenylenediamine seems to be inhibited, however, especially when the animals had been dosed with *m*-dinitrobenzene 2 weeks earlier, and the toxic effects were much more severe, two of the animals dying at the end of the first day. Fouts & Brodie (1957) have shown that in rabbit liver there are at least two systems reducing aromatic nitro groups, one reducing nitrophenols and another reducing non-phenolic compounds. This would explain a second anomaly of Expts. 1 and 2, for despite the absence of *m*-phenylenediamine, 2:4-diaminophenol was present to about the same extent as in Expts. 3 and 4, where both reduction products were formed. The anomalous absence of *m*-phenylenediamine in two experiments, and the effect of aureomycin on the reduction of the second nitro group only, suggests that the reduction of the two nitro groups of *m*-dinitrobenzene involves at least two different systems, enzymic or otherwise, whereas the reduction of dinitrophenols involves at least one more.

Phenols. The only dinitrophenol detected in the urine of rabbits dosed with *m*-dinitrobenzene was 2:4-dinitrophenol, which was present in only trace amounts (0.1 % of dose). This is in marked contrast to nitrobenzene which is metabolized by the rabbit to give 18 % of the dose as nitrophenols (Parke, 1956). Since 2:4-dinitrophenol administered to rats is excreted partly unchanged (Parker, 1952) it would seem that the direct hydroxylation of *m*-dinitrobenzene is only a minor process. Alternatively, the 2:4-dinitrophenol formed by hydroxylation of *m*-dinitrobenzene *in situ* may be almost completely metabolized to aminonitrophenols and diaminophenols, at sites not readily accessible to dinitrophenol administered by injection. The aminonitrophenol metabolites of *m*-dinitrobenzene are 2-amino-4-nitrophenol (14 % of dose) and 4-amino-2-nitrophenol (0.8 % of dose). Parker (1952) found that although 2:4-dinitrophenol was reduced

by rat-liver homogenates to 2-amino-4-nitrophenol (10 %) and 4-amino-2-nitrophenol (90 %), the latter is further metabolized more rapidly, probably to 2:4-diaminophenol, so that only the 2-amino-4-nitrophenol is excreted in the urine of rats injected with 2:4-dinitrophenol. However, both aminonitrophenols were found in the urine and tissues of dogs injected with 2:4-dinitrophenol (Guerbet & Mayer, 1932). The 4-amino-2-nitrophenol detected and estimated after acid hydrolysis could have been an artifact of *m*-nitrophenylhydroxylamine, which is converted into the aminonitrophenol on treatment with strong mineral acids. Estimations of 4-amino-2-nitrophenol after hydrolysis of conjugates with snail crop-fluid as a source of arylsulphatase and β -glucuronidase show that it is a true metabolite, and the difference between the estimations after enzymic hydrolysis (0.8 % of dose) and acid hydrolysis (2.0 % of dose) is approximately equal to the amount of *m*-nitrophenylhydroxylamine present (0.9 %). This is additional evidence of the presence of *m*-nitrophenylhydroxylamine. 2:4-Diaminophenol is the only diaminophenol present and is the major phenolic metabolite of *m*-dinitrobenzene. It has been isolated from the urine of rabbits injected with 2:4-dinitrophenol and is itself converted in the animal into 2-amino-4-quinoneimine, which is bound to tissue protein and causes cataract (Ogino & Yasukura, 1957). Some of the radioactivity remaining in the tissues is probably present in this form.

SUMMARY

1. The metabolism of *m*-dinitro[¹⁴C]benzene has been studied in the rabbit at oral doses of 50–100 mg./kg.; 65–93 % of the dose is excreted in the urine during the first two days and 1–5 % in the faeces.

2. The principal metabolites in the urine are 2:4-diaminophenol (31 % of dose), *m*-phenylenediamine (21 %), *m*-nitroaniline (14 %), and 2-amino-4-nitrophenol (14 %). Traces of unchanged *m*-dinitrobenzene (0.7 %), 2:4-dinitrophenol (0.1 %), 4-amino-2-nitrophenol (0.8 %), *m*-nitrophenylhydroxylamine (0.8 %), *m*-nitrosonitrobenzene (0.25 %) and 3:3'-dinitroazoxybenzene (0.3 %) are also present. 2:6- and 3:5-Dinitrophenol, 2-amino-6-nitrophenol and 3-amino-5-nitrophenol, and 2:6- and 3:5-diaminophenol, are not present in amounts greater than 0.05 % of the dose.

3. With oral doses of non-radioactive *m*-dinitrobenzene, an average of 30 % of the dose is excreted as glucuronide conjugates and 6 % as ethereal sulphates.

4. 2:4-Diaminophenol, *m*-phenylenediamine, *m*-nitroaniline, 2-amino-4-nitrophenol, 2:4-dinitrophenol, 3:3'-dinitroazoxybenzene, *m*-nitrosonitro-

benzene, and unchanged *m*-dinitrobenzene, or derivatives of these, have been isolated from the urine of rabbits dosed with *m*-dinitrobenzene. The presence of 4-amino-2-nitrophenol has been confirmed by paper chromatography and ultraviolet-absorption spectra.

5. The ultraviolet-absorption spectra of 2:4- and 2:6- and 3:5-dinitrophenol in 0.1N-hydrochloric acid and 0.1N-sodium hydroxide are recorded, and the absence of these dinitrophenols except for traces of 2:4-dinitrophenol (0.1% of dose) is confirmed from ultraviolet spectra of extracts of the hydrolysed urine from rabbits dosed with *m*-dinitrobenzene.

6. 4-Amino-2-nitrophenol was estimated after acid hydrolysis (2.0% of dose), and after enzymic hydrolysis (0.8% of dose). The estimation after acid hydrolysis includes 4-amino-2-nitrophenol arising as an artifact of 3-nitrophenylhydroxylamine (0.8% of dose).

7. In two experiments with *m*-dinitro[¹⁴C]-benzene, no *m*-phenylenediamine was formed and the amounts of *m*-nitroaniline present in the urine were approximately equal to the sum of the two reduction products (35% of dose) formed in other experiments.

8. The reduction of *m*-dinitrobenzene to *m*-phenylenediamine is inhibited by aureomycin.

9. A mechanism for the reduction of aromatic nitro compounds involving flavin free radicals is postulated.

I am indebted to Professor R. T. Williams for his constant interest in this work.

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