Studies in Detoxication

49. THE METABOLISM OF BENZENE CONTAINING [^{14}C]₁BENZENE

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In earlier papers in this series (Porteous & Williams, 1949*a*, *b*; Garton & Williams, 1949; Parke & Williams, 1950, 1951*a*, *b*; 1952) attempts were made to account for the whole of an oral dose of benzene in rabbits. About 75% could be accounted for in gross terms of oxidized and unchanged benzene, and only a few of the metabolites excreted could be fairly accurately assessed. No reliable estimate of metabolites other than catechol, phenylmercapturic acid and muconic acid could be made. Using benzene labelled with one ^{14}C -atom, we have been able to make a reasonable balance sheet for the fate of a single dose of benzene and also to make accurate estimates of most of its metabolites.

EXPERIMENTAL

The ^{14}C -labelled benzene used was prepared by Dr H. S. Turner (Turner, 1951) and contained about 100 μC /ml. of radioactivity. It was administered to rabbits by stomach tube and diluted where necessary with unlabelled benzene which had been purified through the nickel cyanide ammonia clathrate compound (Evans, Ormrod, Goalby & Stavely, 1950).

After dosing, each rabbit was placed in a metabolism chamber (cf. Parke & Williams, 1950) which was swept with a continuous stream of air that was then bubbled through ethanol at about -50° to trap unchanged benzene (Azouz, Parke & Williams, 1952*a*). In three out of four experiments arrangements were also made to collect CO_2 , and in these cases the absorption train consisted of 2 Drechsel bottles (250 ml.) containing 50 g. of anhydrous magnesium perchlorate (Anhydron) followed by 2 Drechsel bottles (50 ml.) immersed in a freezing mixture of acetone and solid CO_2 and containing 40 ml. of glass beads and 20 ml. ethanol to collect benzene. These were followed by the CO_2 absorbers consisting of 3 Drechsel bottles (250 ml.) fitted with sintered-glass dispersers and each containing 150 ml. of carbonate-free 5*N*-NaOH. CO_2 could be quantitatively recovered from the animal chamber by this train; thus the recovery of CO_2 liberated in the chamber from 25 g. of Na_2CO_3 by acid was 101% in 6 hr. During an experiment the ethanol and alkali in the absorption train were renewed every 6 hr. In the four experiments carried out, the absorption of unchanged benzene and CO_2 was continued for 30, 48, 48 and 70 hr., respectively, after dosing, after which times the animals were removed from the chamber.

In Exps. nos. 1 and 3 the animals were killed after 48 hr. and tissues analysed. In Exps. 2 and 4, the animals, after removal from the metabolism chamber, were placed in open

cages so that their urine could be collected up to 7 days after dosing. The animals were then killed and the tissues of no. 4 analysed.

Measurement of radioactivity. All measurements were carried out on solid samples using an end-window counter tube, the background of which was approximately 9–11 counts/min. The specific activities were determined by comparison with known amounts of labelled benzene as BaCO_3 and sometimes as the nickel cyanide ammonia complex (see below) using the 'infinite thickness' technique (see Calvin, Heideberger, Reid, Tolbert & Yankwich, 1949; Popják, 1950) with samples of 25 mg./sq.cm. of identical geometry prepared on aluminium planchettes (General Electric Co. Ltd.). A sample of about 2 sq.cm. containing 10^{-2} μC of ^{14}C /mg. of substance gave approximately 7000 counts/min. Sufficient counts to give a standard error of $\pm 2\%$ were taken. The variation in disk size gave an error of $\pm 5\%$. In the determination of the specific activity of the nickel cyanide ammonia complex the counting was carried out within 5 min. of the preparation of the sample on the planchette. This complex slowly loses its activity by loss of benzene from the surface layer and in an hour the counts/min. of an 'infinitely thick' sample dropped by about 2% at room temperature. This compound appears to be relatively stable in bulk. Permanent standards which showed no loss of activity over several months were prepared from [^{14}C]phenyl toluene-*p*-sulphonate. This ester was made from metabolic phenol isolated from the urine of rabbits receiving labelled benzene and standardized against BaCO_3 prepared from labelled benzene.

Urine and tissues. The total radioactivity eliminated in the urine was measured by evaporating to dryness, over P_2O_5 *in vacuo* at room temperature, 5 ml. urine mixed with 0.2 ml. *N*-NaOH. The residue was powdered and a weighed amount was submitted to wet combustion and the CO_2 evolved counted as BaCO_3 (see Calvin *et al.* 1949).

For the radioactivity of the tissues, 5 g. tissue were dispersed with 25 ml. 0.5*N*-NaOH in a Waring Blender. The procedure for urine was then followed.

For the measurement of radioactivity present as benzene, the blood, urine or milk was mixed with an equal volume of *N*-NaOH, but tissues were dispersed as above. To 2.5 ml. of each mixture there were added 250 mg. of pure benzene as carrier and the mixture extracted with 5 ml. of light petroleum (b.p. 40 – 60°). The extract (2 ml.) was then shaken for 1 hr. with 15 ml. of an aqueous solution of ammonia nickel cyanide (prepared according to Evans *et al.* 1950). After keeping the mixture at 0° for a further 1 hr. the solid complex ($\text{Ni}(\text{CN})_4 \cdot \text{NH}_3 \cdot \text{C}_6\text{H}_6$) was collected in a Gooch crucible, washed with water, ethanol and ether and dried at room temperature. The radioactivity of the solid complex was then determined as above within 5 min. of its

Table 1. *The occurrence of radioactivity in the tissues of rabbits receiving [^{14}C]benzene*

Exp. no.	1* (2 days)†		3 (2 days)†		4 (7 days)†	
	Wt. of tissue (g.)	% of dose present in tissue	Wt. of tissue (g.)	% of dose present in tissue	Wt. of tissue (g.)	% of dose present in tissue
Spinal cord	—	—	3	0 (0)‡	3.5	0
Brain	—	—	1.5	0 (0.01)	—	—
Blood	—	0.8 (3-4)‡	—	0 (0)	—	—
Intestine and contents	—	—	205	0.55 (0.1)	—	—
Stomach contents	—	—	55	0.15 (0.03)	—	—
Stomach	—	—	26	0.05	—	—
Spleen	—	—	1.1	0	1.2	0
Bone-marrow	—	—	Approx. 8	0	5	0.01
Heart	7	0 (0)	9	0	—	—
Lungs	38	0.2 (0)	5	0	—	—
Kidney	11	0.06 (0)	14.5	0.015	—	—
Liver	63	0.5 (0)	79	0.07	94	0.05
Testes	—	—	10	0.02	—	—
Voluntary muscle	800	9.3 (0)	700	1.6	1200	1.2
Involuntary muscle	200	1.5 (0)	—	—	—	—
Fat	200	2.6 (0)	180	1.5	—	—
Bile	—	—	1.5	0.01	—	—
Uterus	45*	0.9 (0)	—	—	—	—
Ovary	1.2*	0.03 (0)	—	—	—	—
Faeces	2.2	0.3 (0)	22	0.86	—	—
Total accounted for	16.19		4.8			

* The animal in this experiment was found to be diseased; the uterus was much enlarged and contained a chorion epithelioma. The specific activity of the uterus and ovary was twice that of the most radioactive normal tissue.

† Number of days after dosing with benzene when animal was killed.

‡ Figures in brackets give the amount found as free benzene.

preparation. The results for various tissues are given in Table 1, and Fig. 1 shows the elimination of radioactivity in the urine.

Fig. 1. The excretion of radioactivity in the urine of rabbits receiving ^{14}C -labelled benzene orally. A, Exp. 2 (dose of benzene, 0.34 g./kg.). B, Exp. 4 (dose of benzene, 0.4 g./kg.).

Carbon dioxide. Each 6 hr. fraction of the CO_2 absorbed in the absorption train (see above) was converted into BaCO_3 and the activity measured, as described by Calvin *et al.*

(1949) (see Fig. 2 and Table 2). In Exp. 4 the radioactivity of the carbonate of the urine was determined. Sodium carbonate (0.5 g.) was added to 1/10 of the urine and this was acidified with 2N- H_2SO_4 and aerated. The CO_2 evolved was collected in N-NaOH and the carbonate converted to BaCO_3 .

Benzene. The benzene eliminated in the expired air was determined spectrophotometrically (cf. Azouz *et al.* 1952a) using a Unicam spectrophotometer. Of the several absorption maxima of benzene in ethanol, that chosen for the present work was at 255 m μ , where ϵ was 240 (slit width of 0.4 mm.). The benzene was also estimated in one experiment by measuring its radioactivity. Unlabelled benzene (1 g.) was added to 5 ml. of the ethanolic solution of expired benzene and the nickel cyanide clathrate complex was prepared as above. The solid complex was centrifuged and repeatedly washed with 20% (w/w) ammonia solution until the supernatant was almost colourless and then with water, ethanol and finally ether. The solid was counted immediately. By this method, 46.5% of the dose was found, whereas the spectrophotometric method gave 48.4%. In the other experiments the spectrophotometric method only was used since it was inherently much more accurate than the counting method.

The figures of elimination of unchanged benzene and CO_2 in the expired air are given in Table 2. Fig. 2 shows graphically the elimination of $^{14}\text{CO}_2$, which begins at about 20 hr. after dosing. It is clear from the curves that the elimination of $^{14}\text{CO}_2$ had not ceased at the end of the experiment. In Exp. 1 the expired air was only examined for 30 hr. and the elimination of $^{14}\text{CO}_2$ had probably not begun.

Table 2. *The elimination of unchanged benzene and CO_2 in the expired air of rabbits receiving ^{14}C -labelled benzene orally*

Exp. no.	Dose of benzene (g./kg.)	Dose of ^{14}C $\mu\text{c.}/\text{rabbit}$	Duration of exp. (hr.)	Unchanged benzene in expired air		Respiratory CO_2 (% of dose)
				Estimated spectrophotometrically (%)	Estimated by radioactivity (%)	
1	0.34	123	30	45.9	—	0*
2	0.34	4.6	48	34.0	—	—
3	0.5	128	48	45.2	—	1.75
4	0.4	171	70	48.4	46.5	1.0

* The duration of the experiment was probably not long enough to detect radioactive CO_2 ; furthermore this animal was diseased (see footnote to Table 1).

Estimation of phenols

The urine from each rabbit was heated for 3 hr. under reflux with an equal volume of conc. HCl . This procedure hydrolysed glucuronides and ethereal sulphates. The various phenols were then isolated according to the following procedures.

Fig. 2. The excretion of radioactive CO_2 in the expired air of rabbits receiving ^{14}C -labelled benzene orally. A, Exp. 3 (benzene, 0.5 g./kg.). B, Exp. 4 (benzene, 0.4 g./kg.).

Phenol. To 20 ml. of the hydrolysed urine 50 mg. of phenol were added. The solution was then steam distilled until 10 ml. of distillate had collected. To this was added 2 ml. of 40% (w/v) NaOH and the solution was then shaken for 20 min. with 0.25 g. of toluene-*p*-sulphonyl chloride in 10 ml. acetone. After 1 hr. the mixture was poured into water and the precipitated phenyl toluene-*p*-sulphonate collected and recrystallized from ethanol. The radioactivity of the toluenesulphonate was measured by the infinite thickness technique. It was recrystallized from ethanol until its specific activity became constant (m.p. and mixed m.p. 95°).

Catechol. Catechol (100 mg.) in a little water was added to 25 ml. of the hydrolysed urine and the solution continuously extracted with peroxide-free ether for 8 hr. The ether was evaporated (phenol is largely removed during this process) and the residue dissolved in a little water and the solution filtered. The catechol was then precipitated at pH 8 as the basic lead salt by the addition of aqueous basic lead acetate. The precipitate was collected and washed, and then suspended in water. H_2S was passed through the suspension to

remove lead. After filtration, the catechol was extracted from the solution by continuous extraction with ether for 6 hr. After removal of the ether, the residue was dissolved in 5 ml. 2*N*- NaOH and shaken with 0.5 g. toluene-*p*-sulphonyl chloride in acetone (5 ml.). Catechol ditoluene-*p*-sulphonate was isolated by dilution with water and was recrystallized to constant specific activity from ethanol (m.p. and mixed m.p. 162°).

Quinol. In Exps. 1 and 2, quinol (200 mg.) was added to 20 ml. of the hydrolysed urine which was then continuously extracted with ether for 8 hr. After evaporation of the ether, the residue was dissolved in 10 ml. 2*N*- NaOH and quinol ditoluene-*p*-sulphonate prepared as for the catechol derivative. The ditoluenesulphonate was recrystallized first from ethyl acetate-ethanol and finally from ethanol until its specific activity was constant (m.p. and mixed m.p. 161°). In Exps. 3 and 4, the quinol was precipitated as the basic lead salt at pH 9.5 and the procedure for catechol followed.

Hydroxyquinol. Hydroxyquinol (200 mg.) was added to 20 ml. of the hydrolysed urine and the procedure of extraction and purification as the basic lead salt was carried out as for catechol. The final residue was then converted to the tritoluene-*p*-sulphonate. This ester was now further purified by dissolution in ethyl acetate and chromatography on a short alumina column. The ethyl acetate eluate was evaporated and the toluenesulphonate recrystallized from ethyl acetate-ethanol to constant specific activity (m.p. and mixed m.p. 106°).

Pyrogallol. This phenol (100 mg.) was added to 15 ml. of the hydrolysed urine and the procedure for hydroxyquinol followed exactly. On continued recrystallization from ethyl acetate-ethanol mixtures, then ethanol and finally aqueous ethanol the pyrogallol tritoluene-*p*-sulphonate (m.p. and mixed m.p. 139°) finally showed no radioactivity.

Resorcinol. Resorcinol (200 mg.) dissolved in a little water was added to 10 ml. of hydrolysed urine and recovered by continuous extraction with ether. The extracted resorcinol was purified through the basic lead salt and then converted to the dibenzoate (m.p. and mixed m.p. 117°). On repeated recrystallization from aqueous ethanol the dibenzoate never became completely free of radioactivity.

Since resorcinol has not been demonstrated colorimetrically or chromatographically in benzene urine, it was thought that the small amount of activity in the above dibenzoate might be due to adsorbed traces of other phenols. The phenols of benzene urine were therefore fractionated as follows.

To 1/10 of the total sample of hydrolysed urine, 1 g. of resorcinol in 10 ml. of water was added. The resorcinol was then extracted with ether in a continuous extractor for 8 hr. After evaporation of the ether, the residue was dissolved in 20 ml. water and filtered. Phenol (200 mg.), catechol (50 mg.) and quinol (50 mg.) were then added to the filtrate to effect the removal of the corresponding radioactive phenols by entrainment. The solution was steam-distilled to remove phenol. Catechol was removed from the solution as the insoluble lead salt by the addition of 20 ml. of saturated normal lead acetate solution at pH 6. After centrifuging to remove lead catecholate, the solution was adjusted to pH 9 with 2N-ammonia solution whereby the lead salt of resorcinol was precipitated. At pH 9, quinol, in the concentration used, just remains in solution. The lead salt of resorcinol was filtered off, well washed with water and dissolved in 2N-acetic acid and the precipitation of lead salts repeated. The purified salt of resorcinol was finally suspended in water and the lead removed with H_2S . Continuous ether extraction of the lead-free filtrate gave 0.6 g. of resorcinol, half of which was converted to the bis-3:5-dinitrobenzoate and the other half to the dibenzoate. After repeated recrystallization from acetone-ethanol the dinitrobenzoate (m.p. 200°) had a specific activity of 4 counts/min., whereas similar recrystallization of the dibenzoate (m.p. 116°) from ethanol also gave a constant count of 4/min. This specific activity corresponds to less than 0.1% of the dose but we never succeeded in obtaining an inactive resorcinol derivative. A qualitative examination of hydrolysed benzene urine by paper chromatography revealed the presence of phenol, catechol, quinol and hydroxyquinol but not of resorcinol, pyrogallol or phloroglucinol.

Our results on phenols are summarized in Table 3.

Diphenyl derivatives

When benzene is oxidized by free hydroxyl radicals, in addition to phenols, diphenyl is produced as a result of the combination of two phenyl radicals (Merz & Waters, 1949; Stein & Weiss, 1948, 1950). Since the phenols produced *in vivo* are

similar to those produced by free-radical hydroxylation of benzene, the urine of rabbits receiving benzene was tested by the isotope dilution technique to see if it also contained diphenyl derivatives, namely diphenyl itself and *p*-diphenylglucuronide. Tests were made for the glucuronide because diphenyl is known to be oxidized *in vivo* to 4-hydroxydiphenyl (Stroud, 1940) which is excreted as the glucuronide (Dodgson, Garton, Stubbs & Williams, 1948).

Diphenyl. A solution of diphenyl (250 mg.) in ether (10 ml.) was shaken for 0.5 hr. with 10 ml. of unhydrolysed [^{14}C]benzene urine to which 1 ml. 2N-NaOH had been added to keep any free phenols present in the aqueous phase. The ether layer was separated from the urine, which was then further extracted with 10 ml. ether. The mixed ether extracts were washed with 2N-NaOH then three times with water and finally dried over anhydrous Na_2SO_4 . On evaporation of the ether, the residue of diphenyl, m.p. 60°, had a specific activity of 4 counts/min. After recrystallization from aqueous ethanol, the diphenyl had m.p. 66° (not depressing that of pure diphenyl, m.p. 69°) and no measurable radioactivity.

Diphenylglucuronide. The glucuronide (100 mg.) dissolved in 1 ml. of saturated aqueous $NaHCO_3$ was added to 10 ml. of the urine and the mixture filtered. On acidification with 2N-HCl, the glucuronide was precipitated: it was purified by dissolution in aqueous $NaHCO_3$, treatment with charcoal and reprecipitation with acid, and twice recrystallized from water. It had no radioactivity.

Phenylmercapturic acid. Synthetic L-phenylmercapturic acid (0.4 g.) in a little 2N- Na_2CO_3 was added to 20 ml. urine. The solution was acidified with conc. HCl and kept at 0° overnight. The brown deposit was collected, washed with water and dissolved in 10 ml. N- $NaHCO_3$. The solution was extracted with ether to remove phenols, treated with charcoal, acidified with conc. HCl and kept overnight at 0°. This process of dissolution in bicarbonate and acidification was repeated twice and the product had m.p. 142°. Its

Table 3. *The elimination of phenols and other aromatic compounds in the urine of rabbits receiving ^{14}C -labelled benzene**

Exp. no.	1†	2	3	4		
Duration of experiment (days)	2	2	2	3	5	7
	% of dose					
Phenol	16.7	23.7	30.8	22.8	22.9	22.9
Catechol	1.2	2.1	2.6	2.8	2.9	2.9
Quinol	6.2	4.8	4.9	3.5	3.6	3.6
Hydroxyquinol	0.35	<0.1	0.4	0.3	0.32	0.32
Resorcinol	± 0.3	± 0.2	± 0.2	—	—	—
Pyrogallol	0	—	—	0	0	0
Phenylmercapturic acid	0.7	—	0.4	0.4	0.4	0.4
Diphenyl	0	—	—	—	—	—
<i>p</i> -Diphenylglucuronide	0	—	—	—	—	—
Sum of aromatic compounds plus <i>trans-trans</i> -muconic acid	27.25	31.6	40.4	30.9	31.2	31.2
Total radioactivity of urine	35.0	34.8 (41.7)‡	39.7	28.5	28.7	28.8

* Doses as for Table 3.

† The animal in this experiment was found to be diseased (see footnote to Table 2).

‡ Urine collected for 7 days after dosing.

Table 4. *Purification of phenylmercapturic acid from the urine of rabbits dosed with ^{14}C -labelled benzene*

Derivative	Purification procedure	^{14}C content	
		Counts/min.	Counts/min. as phenylmercapturic acid
Phenylmercapturic acid	Three precipitations by acid	175	175
Phenylmercapturic acid	1st recrystallization from water	135	135
Phenylmercapturic acid	2nd recrystallization from water	131	131
Phenylmercuric mercaptide	Conversion to mercaptide	144	126
Thiophenyl <i>p</i> -nitrobenzoate	Crystallization from ethanol-chloroform	122	132

activity was then measured. It was then recrystallized to constant activity from water. As a criterion of purity, two derivatives were prepared. The acid (120 mg.) was hydrolysed to thiophenol by heating in 10 ml. N-NaOH for 1 hr. To 2 ml. of the solution was added 1 ml. ethanol and 0.6 ml. 10% HgCl_2 . The precipitated phenylmercuric mercaptide (45 mg.) was filtered and washed with water. After drying its activity was determined. The thiophenol in the other 5 ml. of solution was continuously extracted with ether after acidification with 1 ml. conc. HCl . The ether extract was treated with 2 ml. pyridine and the ether allowed to evaporate at room temperature. Then 50 mg. *p*-nitrobenzoyl chloride was added and the mixture heated. The product was poured into water (10 ml.) and the precipitated thiophenol *p*-nitrobenzoate (25 mg.) washed with N-NaHCO_3 . After recrystallization from ethanol-chloroform, it had m.p. and mixed m.p. 157° (Grillot, Levin, Green & Bashford, 1950). The results of a typical experiment are given in Table 4. The results are summarized in Table 3.

Open-chain acids of benzene urine

The problem of the formation of *trans-trans*-muconic acid from benzene *in vivo* has been recently discussed by us (Parke & Williams, 1952). Since radioactive benzene was available the opportunity arose to prove without doubt that *trans-trans*-muconic acid was a true metabolite of benzene. On injection of the muconic acids into rabbits, only 50–60% is excreted unchanged in the urine (Parke & Williams, 1952). Muconic acids therefore appear to be further metabolized and they could be intermediate in the conversion of benzene to carbon dioxide. If a muconic acid is an intermediate, it could by the process of β -oxidation, etc., be converted into fumaric, succinic and acetic acids and, since these acids are components of the tricarboxylic acid cycle, the final oxidation of benzene to carbon dioxide would appear to go via this cycle. On these grounds the urine of rabbits receiving labelled benzene was examined for these three acids. The urine was also examined for adipic acid, since muconic acid could be hydrogenated to this acid which could be an intermediate between the various isomers of muconic acid. Adipic acid fed to animals is partly excreted unchanged in the urine (Mori, 1918; Enders, 1941; Weitzel, 1947). Both oxalic and mesotartaric acids were found by Cook & Schoental (1950) to be the products of the oxidation of benzene with osmium tetroxide and hydrogen

peroxide. The urine was therefore examined for these acids since osmium tetroxide oxidations seem to produce products analogous but not exactly similar to those produced in metabolic oxidations (cf. Schoental, 1950).

The muconic acids. (a) *The trans-trans-acid.* The acid (50 mg.) in 0.5 ml. $2\text{N-Na}_2\text{CO}_3$ was added to 10 ml. urine, and then precipitated by acidification with 2N-HCl . The dark brown precipitated material was dissolved in 2N-NaHCO_3 and the solution treated with charcoal and filtered. The muconic acid was precipitated with acid and the process repeated. The final product (m.p. 300°) was washed with water, ethanol and ether and its radioactivity measured. It was then converted into its benzhydryl ester (Parke & Williams, 1952) which was recrystallized to constant activity from ethyl acetate (m.p. and mixed m.p. 190°). In Exps. 1–4 the urinary elimination of *trans-trans*-muconic acid represented 1.8, 1.0, 1.3 and 1.1% of the dose of benzene respectively.

(b) *The cis-cis-acid.* The acid (200 mg.) in 2 ml. $2\text{N-Na}_2\text{CO}_3$ was added to 20 ml. urine and then precipitated with 2N-HCl . It was then purified by precipitation as for *trans-trans*-muconic acid. At this stage the acid had m.p. $188\text{--}189^\circ$ and was markedly radioactive (e.g. 400 counts/min.). It was then converted into the benzhydryl ester which after two recrystallizations from ethyl acetate was inactive (m.p. and mixed m.p. 156°).

(c) *The cis-trans-acid.* The acid (100 mg.) in 1 ml. $2\text{N-Na}_2\text{CO}_3$ was added to 20 ml. urine. After acidification to pH 2 with conc. HCl , the urine was continuously extracted with ether for 8 hr. The muconic acid was transferred from the ether to $3 \times 10\text{ ml. } 2\text{N-NaHCO}_3$. The bicarbonate extract was acidified and the muconic acid again extracted with ether. The ether was evaporated and the residual muconic acid converted into the benzhydryl ester. After a single recrystallization from ethanol, the ester had m.p. and mixed m.p. 142° and was inactive.

Acetic acid. Sodium acetate (250 mg.) was added to 10 ml. urine which was then acidified with 4 ml. 2N-HCl and continuously extracted with ether for 16 hr. The extracted acetic acid was taken into 5 ml. N-NaOH and the solution heated to remove ether, made just acid with 2N-HCl and then refluxed for 1 hr. with 0.65 g. of *p*-nitrobenzyl bromide. *p*-Nitrobenzyl acetate was precipitated by addition of water and cooling to 0° . After repeated recrystallization from ethanolethyl acetate, ethanol and aqueous ethanol the ester had m.p. 77° and had no appreciable radioactivity.

Succinic acid. Succinic acid (0.5 g.) in 2 ml. $2\text{N-Na}_2\text{CO}_3$ was added to 10 ml. urine and after acidification was extracted with ether continuously for 16 hr. The residue after removal of the ether was dissolved in 2N-NaHCO_3 and the solution extracted twice with ether to remove phenols,

acidified with 2*N*-HNO₃ and 10 ml. *N*-AgNO₃ added. Silver succinate was precipitated by dropwise addition of 2*N*-ammonia solution. The precipitation process was twice repeated. Finally the silver succinate was dissolved in 0.5*N*-HNO₃ and the succinic acid extracted with ether (continuous, 8 hr.). After evaporation of the ether the acid was converted into the benzhydryl ester which after two recrystallizations from ethanol had no appreciable radioactivity (m.p. and mixed m.p. 142°).

Benzhydryl succinate was prepared by mixing succinic acid (0.1 g.) in 5 ml. methanol with diazodiphenylmethane (0.75 g.) in 5 ml. ether. After 6 hr. at room temperature, the solvents were evaporated. The residue (0.7 g.) washed with a little ether and recrystallized from methanol gave colourless needles, m.p. 143°. (Found: C, 79.0; H, 5.6. C₃₀H₂₈O₄ requires C, 80.0; H, 5.8%.)

Fumaric acid. Fumaric acid (1 g. in 10 ml. water) was added to 5% of the urine collected, followed by 20 ml. 10% AgNO₃. The mixture was adjusted to pH 8 with 2*N*-ammonia solution and the precipitate collected, washed with water, and then dissolved in 20 ml. of *N*-HNO₃ and the solution filtered to remove AgCl. The filtrate was treated with charcoal, filtered, and the silver fumarate precipitated with ammonia solution. It was collected, washed, dissolved in *N*-HNO₃ and reprecipitated with ammonia solution. At this stage the fumarate showed appreciable activity. The salt was therefore dissolved in 20 ml. *N*-HNO₃ and extracted continuously with ether. Concentration of the ethereal solution yielded fumaric acid which was still slightly active. On conversion to dimethyl fumarate (m.p. 101°), recrystallized from ethyl acetate-methanol, the radioactivity disappeared.

Adipic acid. The acid (0.5 g.) in 2 ml. 2*N*-Na₂CO₃ was added to 10 ml. of urine. Repeated acidification, ether extraction and solution in 2*N*-NaHCO₃ was carried out as for succinic acid. The final aqueous bicarbonate solution was acidified with 2*N*-H₂SO₄ and 10 ml. *N*-CuSO₄ added. Cupric adipate was then precipitated by the dropwise addition of 2*N*-ammonia solution. It was collected, washed with water, ethanol and ether, dissolved in 0.5*N*-H₂SO₄ and reprecipitated with 2*N*-ammonia solution. This was twice repeated and the final product dissolved in 0.5*N*-H₂SO₄ and the adipic acid extracted continuously (8 hr.) with ether. The extracted acid was converted into the dibenzhydryl ester which after two recrystallizations from ethanol had m.p. 90° and no significant radioactivity.

Benzhydryl adipate was prepared in the same way as the succinate. It formed colourless rhombs, m.p. 92°. (Found: C, 80.8; H, 6.6. C₃₂H₃₀O₄ requires C, 80.3; H, 6.3%.)

Oxalic acid. To 10 ml. of urine, 100 mg. of oxalic acid was added. The solution was slightly acidified with 2*N*-HCl, 10 ml. 10% CaCl₂ added and calcium oxalate precipitated by the dropwise addition of 2*N*-ammonia solution to the boiling solution. After 1 hr., the precipitate was filtered off, washed well with water, ethanol and ether. It was then dissolved in 10 ml. 2*N*-HCl, treated with charcoal and reprecipitated in boiling solution with 2*N*-ammonia solution. This was repeated and then the activity measured. In all cases the radioactivity was insignificant.

mesoTartaric acid. *mesoTartaric acid* (1 g.) in 10 ml. water was added to about 5% of the urine collected. The solution was neutralized with 2*N*-ammonia solution and then made just acid with 2*N*-acetic acid. On addition of 20 ml. 10% CaCl₂, calcium *mesotartrate* was precipitated. After standing 24 hr. the precipitate was collected, washed with water and redissolved in 2*N*-HCl. After treatment with charcoal, the calcium salt was reprecipitated with ammonia solution. This process was repeated and finally the radioactivity disappeared.

DISCUSSION

Within the limits of error of the methods used, we feel that we can now account for most of an oral dose of benzene administered to rabbits. This is shown in the summarizing Table 5. Further details of the exact nature of the conjugates and the mechanism of the formation of muconic acid, however, have still to be worked out. Within 2-3 days of dosing, some 80% is eliminated from the body in the expired air, urine and faeces. The rest appears to remain in the body to be eliminated slowly in the urine, as shown in Exp. 4 where 28.5% of the dose had been eliminated in the urine in 3 days after dosing but only a further 0.3% was excreted during the succeeding 4 days, and in the expired air probably as carbon dioxide. The curves for the elimination of carbon dioxide (see Fig. 2) suggest that the elimination of benzene as carbon dioxide is far

Table 5. *The metabolic fate of a single oral dose of ¹⁴C-labelled benzene in the rabbit, 2-3 days after dosing*

Nature of metabolite	% of dose (average)	
Benzene exhaled unchanged	43	} 44.5 in expired air
Respiratory CO ₂	1.5	
Phenol*	23.5	} 32.6 in urine
Catechol*	2.2	
Quinol*	4.8	
Hydroxyquinol*	0.3	
<i>trans-trans</i> -Muconic acid	1.3	
L-Phenylmercapturic acid	0.5	
(Urinary radioactivity)	(34.5)	} 77 (79)†
Metabolized benzene in tissues and faeces†	About 5-10 in body	
Total accounted for	84-89%	

* These phenols are excreted conjugated.

† About 0.5% of dose in faeces.

‡ Benzene in expired air + urinary radioactivity.

from complete after 2 days. That portion of the dose which remains in the body after 2 days appears to be present largely as metabolites. Only traces of free benzene were found and these were mainly in the stomach and intestinal contents. The radioactivity left in the body seemed to be generally distributed in most tissues and it appeared in greatest concentration in the bile, stomach, intestine, muscle and fat. Most of the radioactivity was found in the muscle and fat owing to their bulk. Exp. 4 (see Table 1) shows that over 1 % of the radioactivity remained in the animal after 7 days. The accurate assessment of the radioactivity remaining in the body is difficult owing to dilution by the bulk of the tissues.

More than half of the material eliminated from the body appears in the expired air, largely as unchanged benzene and to a small extent as respiratory carbon dioxide. In fact, the only new metabolite of benzene that these experiments have revealed is carbon dioxide. The formation of carbon dioxide was to be expected since it was already known that the benzene ring was broken *in vivo* to yield muconic acid. However, no more than 1-2 % of the dose appeared as carbon dioxide in 2 days, and no radioactivity was detected in the urinary carbonate, although carbon dioxide elimination was probably not complete in this time.

About 30 % of the dose is excreted in the urine as compounds containing the intact aromatic ring. The L-phenylmercapturic acid output in three experiments was 0.7, 0.4 and 0.4 % of the dose, which is somewhat lower than that (about 1 %) found by Parke & Williams (1951b) using non-isotopic methods. The major urinary metabolite of benzene has now been proved to be phenol which accounts for nearly one-quarter of the dose or about 70 % of the benzene metabolized and excreted in the urine. This phenol and the others are excreted almost entirely conjugated (Porteous & Williams, 1949a, b). Quinol is quantitatively the next major metabolite accounting for nearly 5 % of the dose and catechol, just over 2 %, the next. Hydroxyquinol is a minor metabolite (0.3 %). The urine was also tested for resorcinol and pyrogallol and although we were unable to eliminate the radioactivity completely from the carrier resorcinol, we feel, from other observations, that it and other phenols are not formed from benzene *in vivo*. At this point it is interesting to compare the outputs of quinol and catechol after feeding benzene with those obtained after feeding phenol. With benzene the ratio quinol/catechol is about 2, but with phenol the ratio is about 10 (Porteous & Williams (1949a) give the quinol output after phenol as 5 % of the dose, and Azouz, Parke & Williams (1952b) the catechol output as 0.6 %). This may suggest, as pointed out by Garton & Williams (1949), two routes from

benzene to catechol, one via phenol and the other through an unknown intermediate.

The experiments with diphenyl and *p*-diphenylglucuronide were carried out because of the similarity of orientation found in the biological and free-radical hydroxylation of benzene. The present experiments, however, show that diphenyl derivatives are probably not formed in the biological process, although diphenyl is found during the free-radical process in the presence of oxygen (Stein & Weiss, 1948, 1950).

Our experiments with the isomeric muconic acids now prove definitely that *trans-trans*-muconic acid is a metabolite of benzene. The results with these acids were very clear-cut and leave no doubt that *cis-cis*- and *cis-trans*-muconic acids are not excreted in the urine of rabbits dosed with benzene. They do not, however, throw any further light on why the *trans-trans*- is excreted rather than the expected *cis-cis*-isomer. In an earlier paper (Parke & Williams, 1952) we suggested that *cis-cis*-muconic acid need not be a precursor of *trans-trans*- during its formation from benzene. A reconsideration of the results of Bernhard & Gressly (1941), however, has caused us to doubt the validity of this suggestion. Bernhard & Gressly fed benzene containing 10.7 atoms % deuterium and isolated *trans-trans*-muconic acid containing 7.21 atoms % deuterium. The calculated deuterium content of a muconic acid containing 4 deuterium atoms would be $4/6 \times 10.7 = 7.13$ atoms % which agrees with the value found experimentally. This agreement suggests that *cis-cis*-muconic acid is first formed and is isomerized to the *trans-trans*-acid without the removal of the deuterium atoms. The formation of hydroxylated intermediates as suggested by us would have resulted in the removal of deuterium atoms, and a lower deuterium content would be expected in the isolated muconic acid.

Possible β -oxidation products of muconic acid are acetic and fumaric acids. Neither these nor succinic, oxalic, adipic and mesotartaric acids were found in the urine to possess radioactivity after [^{14}C]benzene feeding.

Further details of the metabolism of benzene are now being pursued.

SUMMARY

1. A study has been made of the fate in rabbits of an oral dose of benzene labelled in one carbon atom with ^{14}C .

2. In 2 days about 45 % of the dose is eliminated in the expired air, of which 43 % is unchanged benzene and about 1.5 % is carbon dioxide. Whereas the elimination of unchanged benzene is nearly complete in this time, that of carbon dioxide is not.

3. In the same time nearly 35 % of the benzene is eliminated as metabolites in the urine. These consist mainly of conjugated phenols. Phenol is the major metabolite accounting for about 23 % of the dose. The other phenols excreted are quinol (4.8 %), catechol (2.2 %) and hydroxyquinol (0.3 %). *p*-Phenylmercapturic acid accounts for 0.5 % of the dose.

4. The only open-chain acid which is found in the urine and is derived from benzene, is *trans-trans*-muconic acid (1.3 % of the dose). Radioactive *cis-cis*- and *cis-trans*-muconic acids, acetic, oxalic, adipic, succinic, fumaric and *mesotartaric* acids were not found in the urine. The carbonate of the urine contained no radioactivity.

5. Diphenyl and *p*-diphenylglucuronide added to the radioactive urine were recovered devoid of activity, and hence these are not produced biologically from benzene.

6. The respiratory and urinary excretions account for nearly 80 % of the dose of benzene in 2-3 days after dosing, and the remainder appears to remain in the body to be eliminated slowly in the urine and probably in the expired air as carbon dioxide.

7. Two days after dosing, about 5 % of the administered radioactivity was found in the tissues generally. This occurred mainly as metabolites of benzene, and very little unchanged benzene was found after this time.

8. These experiments account for 80-90 % of the administered benzene.

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REFERENCES

- Azouz, W. M., Parke, D. V. & Williams, R. T. (1952a). *Biochem. J.* **50**, 702.
 Azouz, W. M., Parke, D. V. & Williams, R. T. (1952b). *2nd Int. Congr. Biochem. Abstr.* p. 345.
 Bernhard, K. & Gressly, E. (1941). *Helv. chim. acta*, **24**, 83.
 Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. H. & Yankwich, P. F. (1949). *Isotopic Carbon*. New York: Wiley.
 Cook, J. W. & Schoental, R. (1950). *J. chem. Soc.* p. 47.
 Dodgson, K. S., Garton, G. A., Stubbs, A. L. & Williams, R. T. (1948). *Biochem. J.* **42**, 357.
 Enders, A. (1941). *Arch. exp. Path. Pharmacol.* **197**, 706.
 Evans, R. F., Ormrod, O., Goalby, B. B. & Stavely, L. A. K. (1950). *J. chem. Soc.* p. 3346.
 Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **45**, 158.
 Grillot, G. F., Levin, P. M., Green, R. & Bashford, R. I. (1950). *J. Amer. chem. Soc.* **72**, 1863.
 Merz, J. H. & Waters, W. A. (1949). *J. chem. Soc.* p. 2427.
 Mori, J. (1918). *J. biol. Chem.* **35**, 341.
 Parke, D. V. & Williams, R. T. (1950). *Biochem. J.* **46**, 236.
 Parke, D. V. & Williams, R. T. (1951a). *Biochem. J.* **48**, 621.
 Parke, D. V. & Williams, R. T. (1951b). *Biochem. J.* **48**, 624.
 Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **51**, 339.
 Popják, G. (1950). *Biochem. J.* **46**, 560.
 Porteous, J. W. & Williams, R. T. (1949a). *Biochem. J.* **44**, 46.
 Porteous, J. W. & Williams, R. T. (1949b). *Biochem. J.* **44**, 56.
 Schoental, R. (1950). *Symp. biochem. Soc.* no. 5, p. 3.
 Stein, G. & Weiss, J. (1948). *Nature, Lond.*, **161**, 650.
 Stein, G. & Weiss, J. (1950). *Nature, Lond.*, **166**, 1104.
 Stroud, S. W. (1940). *J. Endocrin.* **2**, 55.
 Turner, H. S. (1951). *Nature, Lond.*, **168**, 73.
 Weitzel, G. (1947). *Hoppe-Seyl. Z.* **282**, 185.

Biosynthesis of Milk-fat in the Rabbit from Acetate and Glucose. The Mode of Conversion of Carbohydrate into Fat

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The main object of the investigation described in this article was to study the mode of conversion of carbohydrate into fat. It will be shown that glucose is converted very effectively not only to fatty acids but also to glycerol. With the aid of acetate, pyru-

vate and glucose labelled with ^{14}C it has been possible to demonstrate that the conversion of glucose into fatty acids takes place after the breakdown of glucose to acetate, probably via pyruvate. The lactating rabbit was used as the experimental animal and the biosynthesis of glyceride fat studied in the mammary gland, because recent experience has shown that the mamma of a lactating animal is particularly suited for such purpose (cf. reviews by Folley, 1952; Popják, 1952a, b).

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