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Studies in Detoxication

54. THE METABOLISM OF BENZENE. (*a*) THE FORMATION OF PHENYLGLUCURONIDE AND PHENYLSULPHURIC ACID FROM [^{14}C]BENZENE. (*b*) THE METABOLISM OF [^{14}C]PHENOL

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Parke & Williams (1953) have shown, using [$^{14}C_1$]benzene, that phenol is a major metabolite of benzene in rabbits. This phenol, however, is not excreted as such but as the glucuronide and ethereal sulphate. Porteous & Williams (1949) have estimated the glucuronic acid and ethereal sulphate outputs of rabbits receiving benzene, but their results represent the conjugates of all the phenolic metabolites of benzene, that is, phenol, quinol, catechol and hydroxyquinol. The use of [^{14}C]-benzene has now enabled us to determine the amount of benzene excreted as phenylglucuronide and phenylsulphuric acid.

In the intact animal, benzene gives rise to *trans-trans*-muconic acid, and it is possible that phenol is an intermediate in the formation of this acid. To test this possibility ^{14}C-labelled phenol, obtained as a metabolite of benzene in our previous experiments (Parke & Williams, 1953), was used. This phenol, since it is obtained from [$^{14}C_1$]benzene, is presumably a mixture of four isomeric [^{14}C]phenols, namely [1-, 2-, 3- and 4-^{14}C]phenols. Using this phenol, it was also possible to find out how much administered phenol was excreted as glucuronide and ethereal sulphate and how much was oxidized to quinol and catechol. ^{14}C-Labelled phenylglucuronide was also prepared from the urine of rabbits receiving labelled phenol or benzene. With this compound the reliability of the usual systematic lead-acetate-precipitation procedure for the isolation of glucuronides from urine could be tested.

EXPERIMENTAL

Measurement of radioactivity. The methods used were essentially the same as described earlier (Parke & Williams, 1953).

Reference compound. In one experiment the diphenylmethyl ester of β-phenyl-D-glucuronide was used. The compound is new and was prepared by treating phenylglucuronide (0·5 g.) dissolved in methanol (5 ml.) with an ethereal solution (10 ml.) of diazodiphenylmethane (0·5 g.) (Elvidge, Linstead, Sims & Orkin, 1950). After standing overnight the crystals which separated were collected and recrystallized from ethanol (yield, 0·6 g.). The *diphenylmethyl ester* formed colourless needles, m.p. 172°, $[\alpha]_D^{20}$ − 72±1° in ethanol (*c*, 1). (Found: C, 68·8; H, 5·8. $C_{25}H_{24}O_7$ requires C, 68·8; H, 5·5%.) (Light absorption in ethanol; $\lambda_{max.}$ 207, 212, 214, 261–262, 267, 274 mμ. with $\epsilon_{max.} \times 10^{-3}$ 17·1, 18·3, 17·1, 1·27, 1·62, 1·08, respectively.)

Experiments with [^{14}C]*benzene*

Phenylglucuronide. Rabbits were fed with [$^{14}C_1$]benzene (cf. Parke & Williams, 1953) and the urine collected for 48 hr. To one-quarter of the urine was added 200 mg. of β-phenyl-D-glucuronide dihydrate (m.p. 162°, cf. Garton, Robinson & Williams, 1949) dissolved in a little water. The glucuronide was then isolated from the urine as the basic lead salt as described by Porteous & Williams (1949). After removal of the lead with H_2S and concentrating, the glucuronide was obtained as a pale-yellow gum. This was purified by dissolving in methanol, filtering and evaporating to dryness *in vacuo*. The residue was recrystallized twice from water and its specific activity determined. The glucuronide (m.p. 161°) was converted into the triacetyl methyl ester (m.p. 115°) and then to phenylglucuronid-

amide (m.p. 225° decomp.) as described by Parke & Williams (1951). The specific activity of each of these derivatives was determined. The glucuronide, ester and amide had the same activity on a molecular basis.

In a second experiment, the isolated glucuronide was dissolved in ethanol and treated with an ethereal solution of diazodiphenylmethane. The phenylglucuronide diphenylmethyl ester was isolated (m.p. and mixed m.p. 170°). The total activity of the glucuronide was unchanged on converting it into this ester. The results are given in Table 1.

Phenylsulphuric acid. Phenol (0·5 g.) was added to half of the urine (see above) which was then made N with respect to HCl. The acidified urine was then heated at 95° for 15 min. to hydrolyse ethereal sulphates. About 5% of the phenylglucuronide present is hydrolysed under these conditions (Garton *et al.* 1949). The mixture was continuously extracted with ether, the ether extract steam distilled and the phenol in the distillate converted into the toluene-*p*-sulphonate (cf. Parke & Williams, 1953) and its specific activity determined. This procedure gives phenol combined as ethereal sulphate after making a small correction of 5% for the phenylglucuronide hydrolysed.

Total phenol. This was estimated after adding 0·5 g. of carrier phenol to one-quarter of the urine by the method described by Parke & Williams (1953). In one experiment catechol and quinol were also determined isotopically (see Table 1). The difference between the total phenol and the phenol obtained by mild acid hydrolysis should be equal to the phenol present as glucuronide determined in the preceding section. Table 1 shows that this is so. Little if any phenylglucuronide could have been derived from other sources such as a hypothetical glucuronide of a 1:2-dihydrobenzene-1:2-diol.

Experiments with [^{14}C]*phenol*

Preparation of [^{14}C]*phenol.* The urines of rabbits which had received [$^{14}C_1$]benzene were pooled, made approx. 10 N with respect to H_2SO_4 (1 vol. conc. H_2SO_4 to 3 vol. urine) and heated under reflux for 1 hr. The liberated phenols were extracted with ether and then transferred to 2 N-NaOH. The alkaline solution was acidified and the phenol recovered by steam distillation. The phenol was converted into the toluene-*p*-sulphonate which was then purified by repeated crystallization from aqueous ethanol. The pure ester (m.p. 96°) was hydrolysed by heating under reflux for several days with 15 N-H_2SO_4. The liberated phenol was steam distilled and then extracted from the distillate with ether. The ethereal solution was passed through a short column of alumina, dried over anhydrous Na_2SO_4 and finally evaporated at 0° to yield [^{14}C]phenol as a colourless crystalline solid, m.p. 38°.

Preparation of phenylglucuronide labelled with ^{14}C *in the benzene ring.* This glucuronide (m.p. 161°) was prepared biologically from the [^{14}C]phenol according to the method of Porteous & Williams (1949).

The recovery of phenylglucuronide from urine. Systematic lead acetate precipitation has long been used as a general method for the isolation of glucuronides from urine, the glucuronides usually precipitating as basic lead salts. ^{14}C-Labelled phenylglucuronide enabled us to test the completeness of this precipitation.

Phenylglucuronide (165 mg.), labelled with ^{14}C, was added to 25 ml. normal rabbit urine. The solution was adjusted to pH 4·5 with 2 N-acetic acid and 5 ml. of saturated normal lead acetate added. The precipitate was collected on the centrifuge, dried and its specific activity determined. The filtrate was adjusted to pH 8 with 2 N-ammonia and 5 ml. of basic lead acetate solution added (the basic lead acetate solution was made by dissolving 56 g. PbO in a solution of 95 g. of $Pb(OCOCH_3)_2$, $3H_2O$ in 800 ml. water with heating, then cooling and diluting to 1 l.). The basic lead precipitate was collected, washed twice with water, dried and its activity determined. Excessive washing with water was avoided since the progressive removal of electrolyte produced a sol of basic lead salt. The basic lead salt was suspended in 30 ml. water and the lead removed with H_2S.

Table 1. *The excretion of phenylglucuronide and phenylsulphuric acid by rabbits receiving* [$^{14}C_1$]*benzene orally*

Dose of benzene (mg./kg.)	Isotope dose (μc)	Percentage of dose excreted in 48 hr. as: Total phenol	Phenyl-glucuronide	Phenyl-sulphuric acid*	Catechol	Quinol
150	2·5	21·6	8·1 (7·8†)	13·8	—	—
500	11·0	17·6	6·3 (6·5†)	11·1	—	—
350	9·5	18·2	—	—	4·4	7·5

* Calculated from phenol liberated by mild hydrolysis.

† Calculated from total phenol minus phenylsulphuric acid (i.e. phenol by strong hydrolysis minus phenol by mild hydrolysis).

Table 2. *Recovery of phenylglucuronide from urine by systematic lead-acetate precipitation*

Fraction	Wt. (mg.)	^{14}C concn. (10^{-5} μg./mg.)	Total ^{14}C content (10^{-5} μg.)	Recovery (%)
Phenylglucuronide added	165	62·0	10 230	—
Normal lead acetate precipitate	290	1·3	377	4 } 98
Basic lead acetate precipitate	1505	6·4	9 632	94 }
Lead sulphide	880	< 0·1	< 88	< 1
Glucuronide gum	235	40·0	9 400	92
Phenylglucuronide diphenylmethyl ester isolated	254	34·5	8 760	85

The PbS was collected, washed with water and found to contain no radioactivity. The filtrate was concentrated *in vacuo* below 40°. The glucuronide gum thus obtained was purified by dissolving in ethanol, filtering and evaporating to dryness *in vacuo*. The specific activity of the dried material was determined; the glucuronide was then converted into the crystalline diphenylmethyl ester, the activity of which was also determined.

The results are given in Table 2, which shows that 94% of the glucuronide appears in the basic lead acetate precipitate, thus justifying this procedure. Loss of glucuronide in this process obviously occurs in the stages after the precipitation by basic lead acetate.

The metabolism of phenyl acetate. The possibility was considered that phenol might be so rapidly conjugated that it would not readily reach sites in the body where it could be oxidized. An attempt was made to circumvent this by the administration of phenyl acetate. As a criterion of oxidation the formation of catechol was measured.

Two rabbits were given phenyl acetate (350 mg./kg.), one by mouth and the other by intraperitoneal injection. The output of catechol was determined by the method of Azouz, Parke & Williams (1953). The yield of catechol in the urine in 48 hr. was 0·7 and 1·4% of the dose, respectively. Thus phenyl acetate produced essentially the same amount of catechol as did phenol (see Table 3) and is presumably rapidly hydrolysed by esterases to phenol.

Table 3. *The metabolites of orally administered* ^{14}C*-labelled phenol in rabbits*

	Expt. 1	Expt. 2	Expt. 3
Dose of phenol:			
Total (mg.)	160	140	550
(mg./kg.)	60	50	200
Total dose of radioactivity (μc ^{14}C)	0·5	0·5	2
Metabolite excreted (in 2 days after dosing)	As percentage of dose		
Total phenol	95	84	—
Phenylglucuronide*	50	37	—
Phenylsulphuric acid†	45	47	—
Catechol	1·0	0·5	—
Quinol	9·5	10·0	—
Hydroxyquinol	<0·5	—	—
Phenylmercapturic acid	0	—	—
Muconic acid:			
cis-cis-	0	—	0
cis-trans-	—	0	0
trans-trans-	0	0	0
Total urinary activity	105	88	—
Sum of phenols	105·5	94·5	—

* Calculated from the difference between total phenol and phenol liberated by mild hydrolysis.

† Calculated from the phenol liberated by mild hydrolysis.

The metabolism of [^{14}C]*phenol.* The phenol, dissolved in olive oil, was administered orally to rabbits. The urine was collected for 48 hr. The total urinary radioactivity was determined as $BaCO_3$ after combustion of a dried urine sample. Phenol, catechol, quinol, hydroxyquinol, phenylmercapturic acid and *cis-cis-*, *cis-trans-* and *trans-trans-*muconic acids were determined by isotope dilution as described by Parke & Williams (1953). Phenylsulphuric acid and phenylglucuronide were determined as above. The results are given in Table 3.

DISCUSSION

Porteous & Williams (1949) showed that when 0·5 g./kg. of benzene was fed to rabbits, about 11% of the dose was excreted as glucuronides and 10% as ethereal sulphates (total, 21%) of phenol, catechol, quinol and hydroxyquinol. More recently Azouz, Parke & Williams (1952) gave the values 11% glucuronides and 25% ethereal sulphates (total, 36%) which are more in keeping with the value (31%) for total phenols, determined isotopically by Parke & Williams (1953). In the present work we have been able to find out how much of the glucuronide excreted is actually phenylglucuronide and how much of the ethereal sulphate is phenylsulphuric acid (see Table 1), and it appears that most of the former conjugate is phenylglucuronide, whereas only about a half of the latter is phenylsulphuric acid. This confirms the suggestion of Parke & Williams (1951) that the polyphenols of benzene urine are excreted mainly as ethereal sulphates, whereas the glucuronide fraction is almost entirely phenylglucuronide. The ratios of phenylglucuronide to phenylsulphate in both experiments were 0·59 and 0·57. The phenol obtained by acid hydrolysis of benzene urine could arise from phenylsulphuric acid, phenylglucuronide and the hypothetical 1:2-dihydrobenzene-1:2-diol and its glucuronide (cf. naphthalene and anthracene). On gentle hydrolysis the latter compound could give rise to phenylglucuronide (cf. the conversion of 1:2-dihydroanthracene-1:2-diol glucuronide into 1-anthrylglucuronide (Boyland & Levi, 1936)). Table 1 shows that the amount of phenylglucuronide found by direct isolation is almost identical with that calculated from the difference between total phenol and phenol obtained by mild hydrolysis. This suggests that little if any glucuronide of the dihydrodiol of benzene is present in the urine.

Our main interest in the metabolism of phenol was to find out whether it gave rise to *trans-trans-*muconic acid as does benzene. Both phenol and catechol can be oxidized chemically to *cis-cis-*muconic acid by peracetic acid (Böeseken, 1932). However, Table 3 shows that none of the three isomers of this acid is found in the urine of rabbits fed with phenol at levels from 50 to 200 mg./kg., and if any had been formed we could have detected it down to 0·05% of the dose of phenol. From this we can conclude that either phenol is not a precursor of muconic acid *in vivo* or that the conjugation

reactions are too rapid to allow the phenol to reach the site of muconic acid formation. Phenol, however, is appreciably oxidized (10%) to quinol and catechol and if catechol were the precursor of muconic acid, one would have expected the latter to be formed in these experiments. This suggests that neither phenol nor catechol are precursors of muconic acid in the intact animal and we are therefore inclined to the hypothesis that a reduced benzene derivative such as 1:2-dihydrobenzene-1:2-diol may be the precursor of muconic acid. In fact, the formation of this acid might well be an indication of the production of such reduced compounds *in vivo*. Our attempts to synthesize this diol in order to test this hypothesis have, so far, failed.

Most of the phenol fed was excreted in conjugated form and at the level fed (50–60 mg./kg.) the glucuronide and ethereal sulphate were excreted in approximately equal amounts. The values for ethereal sulphate (45 and 47%; see Table 3) confirm the earlier values (36–45%) of Williams (1938) for low levels of phenol (25–50 mg./kg.) (cf. Bray, Thorpe & White, 1952).

SUMMARY

1. Using ^{14}C-labelled benzene it has been shown in rabbits that 6–8% of an oral dose of benzene (150–500 mg./kg.) is excreted as phenylglucuronide and 11–14% as phenylsulphuric acid.

2. [^{14}C]Phenol and [^{14}C]phenylglucuronide have been prepared biosynthetically by feeding rabbits with [^{14}C]benzene and [^{14}C]phenol, respectively.

3. At a dose level of 50–60 mg./kg., orally administered phenol can be almost entirely accounted for as urinary metabolites in 2 days after dosing. These metabolites are phenylglucuronide (40–50%), phenylsulphuric acid (about 45%), quinol (about 10%) and catechol (0·5–1·0%).

4. Orally administered phenol does not give rise to any of the isomers of muconic acid or to phenylmercapturic acid.

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The Precipitation of 3β-Hydroxysteroids by Digitonin

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Most 3β-hydroxysteroids are precipitated by digitonin in ethanolic or methanolic solution, whilst most 3α-hydroxysteroids and steroids not carrying a hydroxyl group at C-3 are not precipitated by digitonin. This rule, originally suggested by Fernholz (1935), is subject to certain exceptions about which no simple generalizations can be made; a variety of 3β-hydroxysteroids are not precipitated in the usual conditions, and a few other steroids are precipitated. The literature up to 1949 is summarized by Fieser & Fieser (1949) (cf. also Strain, 1943). Some further exceptions to the general rule are mentioned in the discussion.

Recent work in this laboratory on the steroids of pregnant mares' urine (Brooks, Klyne, Miller & Paterson, 1952) and on the determination of the '3α' and '3β' ketosteroid fractions of human urine (Haslam & Klyne, 1952), led us to investigate the precipitation of 3β-hydroxysteroids by digitonin in a roughly quantitative manner by the method of Haslewood (1947*a*).

The results show that the configuration at C-5, and the presence or absence of substituents in the *D*-ring area, affect considerably the precipitability of 3β-hydroxysteroids by digitonin. A few miscellaneous experiments with 3β-acetoxysteroids and