SUMMARY

1. Sulphapyridine containing ^{35}S has been synthesized.

2. Metabolic experiments with this compound in the rabbit have shown that there is no complete catabolism to inorganic sulphate and no significant retention of the drug in the tissues after 3 days.

3. Differences between the values obtained by assays of ^{35}S and diazotizable material suggested the formation of a metabolite which does not diazotize or couple normally but this has not yet been isolated.

We wish to express our sincere gratitude to Prof. A. Wormall for his advice and encouragement throughout this work as well as for providing facilities in his laboratory for the radioactivity counts. We had the benefit of discussing preliminary plans with Dr J. C. Bournsnel. We also wish to thank our Birmingham colleagues Prof. M. L. Oliphant, F.R.S., Prof. P. B. Moon, F.R.S., and the late Dr F. E. Whitmore for their interest and advice. The ^{35}S was obtained from the U.S. Atomic Energy Control through the kind co-operation of Dr A. S. McFarlane and the Medical Research Council. The diazo estimations were carried out by Mr P. B. Wood. Part of the expenses of the work were defrayed by a Government Grant of the Royal Society to whom grateful acknowledgement is made.

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The Fate of Certain Organic Acids and Amides in the Rabbit

10. THE APPLICATION OF PAPER CHROMATOGRAPHY TO METABOLIC STUDIES OF HYDROXYBENZOIC ACIDS AND AMIDES

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(Received 24 October 1949)

Metabolic processes resulting in hydroxylation of aromatic compounds are well known. The proportion of a given compound so changed varies however with the structure and the consequent metabolic alternatives. If the compound already contains conjugable groups or readily convertible 'potential centres for conjugation' (Bray, Ryman & Thorpe, 1948) the phenols obtainable by hydroxylation may only be formed in small amount. In the past it has often not been possible to identify and determine very small quantities of such phenolic compounds in urines containing normal amounts of other phenols. We have now shown, however, that the method of paper chromatography originally developed for identification of small amounts of amino-acids by Consden, Gordon & Martin (1944) can usefully be adapted to the identification of phenolic compounds. This paper shows how the method has enabled us to confirm and to extend some earlier observations on the hydroxylation and glycine conjugation of the hydroxybenzoic acids and their amides.

METHODS

Paper

Whatman filter paper no. 4 was used.

Solvents

(All percentages are v/v.) The two main series of developing solvents used consisted of benzene-acetic acid-water and CHCl_3 -acetic acid-water. By varying the relative proportions of the constituents, R_F values could be varied within wide limits. By increasing the amount of acetic acid (formic acid was equally effective) the R_F value of a compound was increased. By these means R_F values could be adjusted so as to give maximum separation and bring compounds into suitable positions on the chromatogram. The benzene series, especially benzene (40%), acetic acid (40%), water (20%), was suitable for the majority of the compounds. The CHCl_3 series, e.g. CHCl_3 (50%), acetic acid (25%), water (25%), was used for separating compounds having low R_F values in the benzene series. *n*-Butanol-acetic acid-water mixtures gave similar results to the CHCl_3 series. The acetic or formic acid in these mixtures greatly reduced 'trailing'. These solvents have proved suitable for detection of aromatic monohydroxy acids and amides and

dihydroxy compounds, but we have not yet found a solvent mixture suitable for phenols, cresols and xylenols.

Apparatus and procedure

An ascending method essentially similar to that of Williams & Kirby (1948) was used. Cylinders of the paper, usually about 33 cm. high, were made from sheets by stapling so that the edges did not overlap. Spots of the required solutions containing 1–5 μ g. of the substance were applied to the paper so as to lie about 2.5 cm. from the lower edge of the cylinder and dried. The cylinder was placed in a Petri dish containing the non-aqueous phase of the solvent mixture, the whole standing in a filtrate jar, the bottom of which was covered with the aqueous phase of the mixture. The jar was covered with a glass food cover. A run of 3–5 hr. was sufficient for hydroxybenzoic acids, but for slower moving compounds, e.g. dihydroxy compounds and *m*- and *p*-hydroxyhippuric acids, a longer time, up to 15 hr., was required, evaporation at the solvent front taking place. The positions occupied by the compounds after development were revealed by spraying, after drying, with an appropriate detecting reagent.

Detecting reagents

(1) *Diazotized p-nitraniline*. (a) *p*-Nitraniline, 0.3% in 8% (w/v) HCl (25 ml.) and NaNO₂ (5% w/v) (1.5 ml.) mixed immediately before spraying. (b) Na₂CO₃ (20% w/v). The two solutions were used successively.

(2) *Diazotized sulphanilic acid*. This was prepared and used as above, sulphanilic acid (0.3% in 8% w/v HCl), replacing *p*-nitraniline.

(3) *2:6-Dichloroquinone chloroimide*. (a) 2:6-Dichloroquinone chloroimide (0.1% w/v in absolute ethanol) freshly prepared; (b) veronal or phosphate buffer, pH 10. The two solutions were sprayed successively.

(4) *Ammoniacal silver nitrate*. 0.1 N-AgNO₃ (25 ml.) and NH₄OH (8 ml., sp.gr. 0.880) made up to 50 ml. with water.

(5) *Ferric chloride*. 0.2% (w/v) in water.

The diazo reagents are generally suitable for detection of the hydroxy acids and the AgNO₃ reagent for the dihydroxy compounds. The quinone reagent tends to be hypersensitive making identification in urine extracts difficult. The FeCl₃ reaction is not very sensitive when applied on paper and the colours are not always the same as those in the test tube. The responses of the phenolic compounds studied to some of these reagents are given in Table 1.

R_F values

The R_F values of a number of hydroxy acids, amides and dihydroxy compounds are given in Tables 1 and 2. It will be seen that by selecting appropriate solvents and detecting reagents, most of these compounds can be identified without ambiguity. It should be noted, however, that the solvent mixtures are liable to change in composition, on exposure to the atmospheric conditions in the apparatus, sufficiently to cause appreciable variation in R_F values. Changes in atmospheric temperature had a similar effect, but we did not determine whether this was related to accompanying changes in humidity. For our purpose, however, these variations were of little importance since reference compounds were always run on the chromatograms side by side with the unknowns.

Table 1. Colour reactions and R_F values of some phenolic compounds

(2:6-Dichloroquinone chloroimide gives a blue colour with all the compounds except salicylic acid, with which it gives an intense blue-green colour. Solvent mixture: A, benzene 40, glacial acetic acid 50, water 10% (all % v/v); B, benzene 40, glacial acetic acid 40, water 20%; C, benzene 40, glacial acetic acid 30, water 30%.)

Compound	Colour with diazotized		Reaction with ammoniacal AgNO ₃	<i>R_F</i> value in solvent mixture				
	<i>p</i> -Nitraniline	Sulphanilic acid		<i>A</i> (15 hr.)	<i>B</i>			<i>C</i> (15 hr.)
					(15 hr.)	(6 hr.)	(3 hr.)	
Salicylic acid	Pale yellow	Yellow	—	.	1.0	1.0	1.0	.
Saligenin	Mauve	.	—	.	0.95	0.8	.	.
<i>p</i> -Hydroxycoumaric acid	Blue	.	—	.	0.95	.	.	.
Salicylamide	Orange	.	—	.	.	.	0.9	.
<i>m</i> -Hydroxybenzoic acid	Red	Yellow	—	.	0.9	0.6	0.5	.
<i>p</i> -Hydroxybenzoic acid	Red	Yellow	—	.	0.9	0.6	0.5	.
<i>p</i> -Hydroxyphenylacetic acid	Blue	Red	—	.	0.85	0.55	0.5	.
Salicyluric acid	Orange	Yellow	—	.	0.75	0.5	0.5	.
2:4-Dihydroxybenzoic acid	Brown	.	—	.	0.7	.	.	.
<i>m</i> -Hydroxybenzamide	Red	.	—	.	.	.	0.15	.
<i>p</i> -Hydroxybenzamide	Red	.	—	.	.	.	0.1	.
<i>m</i> -Hydroxyhippuric acid	Red	Yellow	—	.	0.05	0.0	0.0	.
<i>p</i> -Hydroxyhippuric acid	Pale red	Yellow	—	.	0.05	0.0	0.0	.
2:3-Dihydroxytoluene	Pale brown	.	+	0.8–1.0	0.9–1.0	.	.	.
Catechol	Pale brown	.	+	.	0.9–1.0	.	0.75	.
2:3-Dihydroxybenzoic acid	Pale brown	.	+	0.8–1.0	0.9–1.0	.	0.75	0.7
2:3-Dihydroxybenzamide	Pale brown	.	+	0.8–1.0	0.9–1.0	.	0.65	0.4
2:5-Dihydroxytoluene	Pale brown	.	+	0.8–1.0	0.7	.	.	.
2:5-Dihydroxybenzoic acid	Pale brown	Pale yellow	+	0.8	0.5	.	0.35	0.15
Quinol	Pale brown	.	+	.	0.3	.	0.25	.
2:5-Dihydroxybenzamide	Pale brown	.	+	0.65	0.25	.	0.2	0.0–0.15
3:4-Dihydroxytoluene	Pale brown	.	+	.	0.2	.	0.0–0.2	0.0–0.15
3:4-Dihydroxybenzoic acid	Pale brown	.	+	0.45	0.2	.	0.0–0.2	0.0–0.15
3:4-Dihydroxybenzamide	Pale brown	.	+	0.25	0.05	.	0.0–0.2	0.0–0.15

Table 2. R_F values of some phenolic compounds

(Solvent mixture: *D*, *n*-butanol 50, glacial acetic acid 5, water 45% (all %, v/v); *E*, aqueous benzene containing 0.1% acetic acid; *F*, CHCl_3 50, glacial acetic acid 25, water 25%; *G*, benzene 50, glacial acetic acid 5, water 45%.)

Compound	R_F value in solvent mixture			
	<i>D</i> (6 hr.)	<i>E</i> (6 hr.)	<i>F</i> (3 hr.)	<i>G</i> (15 hr.)
Salicylic acid	1.0	0.0-0.4	.	.
Vanillic acid	.	.	.	0.65
<i>m</i> -Hydroxybenzoic acid	0.9	.	0.7	0.07
<i>p</i> -Hydroxybenzoic acid	0.9	.	0.7	0.07
<i>m</i> -Hydroxyhippuric acid	0.7	.	0.3	.
<i>p</i> -Hydroxyhippuric acid	0.7	.	.	.

Preparation of urine extracts

Known fractions of 24 hr. urines (about one-tenth was usually convenient, corresponding to about 50 mg. of administered compound) were acidified (with 0.2 vol. of 2*N*- H_2SO_4) and continuously extracted with ether for 6 hr. to extract unconjugated phenols. The acid content of the urine was then adjusted to 2*N* and the urine was hydrolysed by boiling under reflux for 1 hr. and again continuously extracted with ether for 20 hr. to remove phenols originally conjugated.

The residues after removal of solvent from the two extracts were separately taken up in 5 ml. of acetone. Extracts of normal urine were used in conjunction with reference compounds to provide standards for identification or estimations.

Quantitative estimation

The method can be rendered roughly quantitative by comparing the size and intensity of the spots given by a known volume of unknown and standard solutions. This was most conveniently achieved by using a suitably graded series of standard spots of the reference compound alternating with those of the unknown. Reasonably accurate estimates, within 15% of the actual amount estimated, were obtained if the amount of compound used was between 1 and 5 μg . Measurement of the area of the spots as suggested by Fisher also gave satisfactory results (Fisher, Parsons & Morrison, 1948; Fisher & Holmes, 1949).

Animals, diet and dosage

Rabbits were fed and dosed as in previous investigations (Bray, Ryman & Thorpe, 1947).

RESULTS

Phenolic constituents of normal rabbit urine

Several phenolic compounds are known to occur in normal urine and may be revealed on the chromatograms. These normal constituents must be allowed for in the interpretation of chromatograms from metabolic urines. Most conspicuous in urines from rabbits on our standard pellet diet are *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids which occur free, and hydroquinone which is conjugated. So far we have not been able to distinguish between

m- and *p*-hydroxybenzoic acids on paper chromatograms of urine extracts. It is therefore possible that *m*-hydroxybenzoic acid may also be a normal constituent of urine (cf. Lederer & Polonsky, 1948). We have assumed that the spot observed on the chromatograms is due mainly to the para isomer since this is a well known constituent of vertebrate urines (Lederer & Polonsky, 1948). Normal rabbit urine was also found to contain small amounts of catechol and 3:4-dihydroxybenzoic acid (both conjugated) and up to the present we have identified traces of 2:3- and 2:5-dihydroxybenzoic acids (both conjugated), vanillic acid and *p*-coumaric acid (both free; cf. Lederer, 1949). We did not detect 3:4-dihydroxytoluene, which we have shown to be a metabolite of *p*-cresol (Bray, Thorpe & White, 1950). It has already been mentioned that we have not yet found a solvent mixture suitable for the detection of phenol and cresols.

Hydroxylation of the hydroxybenzoic acids and amides

Accounts of investigations of these compounds by ordinary chemical methods have been published (Bray *et al.* 1947; Bray, Ryman & Thorpe, 1948), but although the main metabolic pathways of these six compounds were traced some details, relating to the hydroxylation products, could not be satisfactorily elucidated. The hydroxylation products which have since been detected in ether extracts of the urines by means of paper chromatograms are shown in Table 3.

Both the dihydroxy compounds which can be formed from salicylic acid and salicylamide by *o*- or *p*-hydroxylation (Bray, Lake, Neale, Thorpe & Wood, 1948) have been detected in rabbit urine after administration of the *o*-hydroxy compounds. All three possible dihydroxy compounds have been found in *m*-hydroxybenzamide urine, although the 2:3 compound was not detected in *m*-hydroxybenzoic acid urine. Previously, using ordinary chemical methods, we had been able to isolate only 2:5 compounds, gentisic acid or its amide, as metabolites of the *o*-hydroxy compounds. There was indirect evidence that gentisic acid, or amide, was formed from *m*-hydroxybenzamide in that a nitrogen-containing complex was separated from the urine which yielded hydroquinone on dry distillation (Bray, Ryman & Thorpe, 1948, pp. 564-5). Reinvestigation of this complex on the paper chromatogram has shown that it contained gentisic acid, its amide, 2:3-dihydroxybenzoic acid and its amide.

The present investigation has also revealed the true extent of hydroxylation of salicylic acid and salicylamide. Since a hydroxybenzoic acid contains two conjugable groups whereas its amide contains one and one potential centre for conjugation, which is not readily convertible, it would be expected that the amide would be hydroxylated to a greater extent

Table 3. *Hydroxylation products of hydroxybenzoic acids and amides in the rabbit detected by paper chromatography*

(The symbols ++ indicate up to 3% of the dose, + up to 1%, - not identified.)

	Metabolites identified											
	Dihydroxybenzoic acids						Dihydroxybenzamides					
	2:3		2:5		3:4		2:3		2:5		3:4	
	Free	Con- jugated	Free	Con- jugated	Free	Con- jugated	Free	Con- jugated	Free	Con- jugated	Free	Con- jugated
<i>o</i> -Hydroxybenzoic acid	+	-	++	+	.	.	.	.	.	.	.	.
<i>o</i> -Hydroxybenzamide	-	+	-	+	.	.	-	+	+	++	.	.
<i>m</i> -Hydroxybenzoic acid	-	-	++	-	++	+	.	.	+	.	.	.
<i>m</i> -Hydroxybenzamide	-	-	-	+	-	+	-	+	+	++	-	+
<i>p</i> -Hydroxybenzoic acid	.	.	.	.	++	+	.	.	.	.	.	.
<i>p</i> -Hydroxybenzamide	.	.	.	.	-	+	.	.	.	.	+	++

than the acid. In the earlier investigations this appeared to be true for the *m*- and *p*-isomers, but the isolation of gentisic acid in greater amount from salicylate urine than from salicylamide urine suggested that the generalization did not apply to the *o*-isomers. It is now clear, however, from paper chromatograms that the total extent of hydroxylation of the amide is greater than that of the acid so that the *o*-isomers do conform with the generalization. The original difficulty in isolating hydroxylation products from salicylamide would seem to be due to that of separating four compounds having very similar properties.

Glycine conjugation of o- and m-hydroxybenzoic acids

It is well established that salicyluric acid is a metabolite of salicylic acid in man (Quick, 1933; Kapp & Coburn, 1942). In our earlier investigation we were unable to isolate it from rabbit salicylate urine, although we had no difficulty in obtaining it from human salicylate urine (Bray, Ryman & Thorpe, 1948). We have now shown by paper chromatogram that salicyluric acid is excreted by the rabbit to an extent of about 5% of the dose.

Owing to failure to isolate *m*-hydroxyhippuric acid we had also been led to conclude that conjugation of *m*-hydroxybenzoic acid with glycine did not occur in the rabbit. When, however, we examined paper chromatograms of ether extracts of *m*-hydroxybenzoic acid urines we observed a spot in a position which, by analogy with the *p*-isomer, *m*-hydroxyhippuric acid would be expected to occupy. (At that time we had no *m*-hydroxyhippuric acid to use as a reference compound.) Guided by chromatograms we were able to isolate the substance responsible, separating it from *m*-hydroxybenzoic acid by means of cold ether in which the latter is more readily soluble. This was found more satisfactory

than the extraction with boiling toluene used previously.

The substance formed colourless needles, m.p. 185°. On hydrolysis with 5*N*-HCl for 6 hr. *m*-hydroxybenzoic acid and glycine, both identified by paper chromatogram, were formed. It was noted that the compound did not depress the melting point of hippuric acid, m.p. 187°. (Found: C, 55.4; H, 4.8; N, 7.5%. Equiv. by titration 193. Calc. for *m*-hydroxyhippuric acid, C₉H₇O₄N: C, 55.4; H, 4.6; N, 7.2%. Equiv., 195. Calc. for hippuric acid, C₉H₇O₃N: C, 60.2; H, 5.0; N, 7.8%. Equiv., 179.) It seems probable that our original failure to isolate *m*-hydroxyhippuric acid was largely due to confusion with hippuric acid which has practically the same melting point which is not depressed by admixture; in the absence of a chromatogram, melting point had been the chief guide in isolation. Recently, through the kind co-operation of Prof. F. Smith and Prof. Homer Smith, we received a specimen of *m*-hydroxyhippuric acid which was in every respect identical with our specimen.

Hydroxylation by rabbit-liver slices

It was noted in an earlier paper (Bray, James, Ryman & Thorpe, 1948) that rabbit-liver extracts and slices could bring about hydroxylation of hydroxybenzoic acids and amides as evidenced by the appearance of substances giving colours with ferric chloride. By means of a paper chromatogram of ether extracts of strongly acidified digests both gentisamide and 2:3-dihydroxybenzamide have now been identified as hydroxylation products of salicylamide by liver slices. In the absence of 2:3-dihydroxybenzamide as a reference compound, this amide was identified after hydrolysis to 2:3-dihydroxybenzoic acid which was run alongside an authentic specimen.

SUMMARY

1. A procedure is described for the identification of phenolic compounds by paper chromatography.

2. Examples are given of the use of the method in metabolic studies.

3. All the theoretically possible *o*- and *p*-hydroxylation products have now been identified in rabbit urine after administration of the *o*-, *m*- and *p*-hydroxybenzoic acids and amides.

4. It has been shown that both salicylic acid and *m*-hydroxybenzoic acid are conjugated with glycine in the rabbit.

The authors' thanks are due to Dr R. Consden for his advice in preliminary discussions on the technique of paper chromatography. We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. Some micro-analyses were carried out by Drs Weiler and Strauss, Oxford.

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Metabolism of Derivatives of Toluene

4. CRESOLS

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(Received 24 October 1949)

The fate of the cresols in the animal body has been studied by various workers. Baumann & Herter (1877-8) observed that commercial cresol gave rise to ethereal sulphate formation in the horse and dog. Williams (1938) found that the percentages of doses of the three isomers excreted conjugated as ethereal sulphate by the rabbit were 22 (ortho and meta), and 16 (para). Neuberg & Kretschmer (1911) demonstrated conjugation of the hydroxyl group in *p*-cresol with glucuronic acid by the isolation, from dog *p*-cresol urine, of a complex of the barium salts of *p*-cresylglucuronide and *p*-cresylsulphuric acid; they were able to separate potassium *p*-cresylsulphate, but not the glucuronide. Jonescu (1906) also showed that the cresols were conjugated with both sulphuric and glucuronic acids in the dog, but could account for only 30-50% of *o*-, 46.5-50% of *m*- and 23-27% of *p*-cresol administered. Preusse (1881) found that in the dog *p*-cresol gave rise to some *p*-hydroxybenzoic acid and *o*-cresol to 2:5-dihydroxytoluene.

The cresols are compounds with one conjugable group and one potential centre for conjugation (Bray, Ryman & Thorpe, 1948), and it was from this point of view that the present investigation was carried out. An attempt has been made to draw up complete balance sheets for the three isomers and to determine whether the same metabolites were formed by the rabbit as by the dog.

Diet and dosage

METHODS

Rabbits (2-3 kg.) were maintained throughout the investigation on the diet of rabbit pellets and water previously described (Bray, Ryman & Thorpe, 1947). The cresols were administered by stomach tube as solutions in NaHCO₃. The *o*- and *m*-isomers could be given safely at dose levels of 0.2 g./kg. (dose 500-600 mg.) without further precautions, but the *p*-isomer was more toxic, 200-300 mg. being the maximum safe dose under fasting conditions. If, however, the rabbits were given their daily food 1-2 hr. before administration of the dose, the symptoms of toxicity were much less noticeable and *p*-cresol could be administered at the same level as the other isomers.