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.

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Metabolism of Derivatives of Toluene

4. CRESOLS

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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.

Estimation of metabolites in cresol urines

Ether-soluble acid, reducing material and ethereal sulphate were estimated as previously described in the studies of the hydroxybenzoic acids and amides (Bray *et al.* 1947, 1948). The cresylglucuronides are considerably more stable than the glucuronides previously studied: the maximum liberation of reducing material was achieved by heating urine with 10N-H₂SO₄ (0.5 vol.) in a boiling water bath for 1.5 hr.

Estimation of cresols

The following method, using Folin & Ciocalteu's (1927) phenol reagent was devised for the determination of free and conjugated cresol in urine.

(a) *Free cresol.* Urine (20 ml.) as collected (pH 7.8–8.0) was continuously extracted with ether for 6 hr. Water (10 ml.) was then added to the 'boiler' and the ether carefully evaporated. The aqueous residue was then diluted to 25 ml. and portions (1–10 ml., depending on the free cresol content) transferred to boiling tubes, and the volume adjusted to 10 ml. Folin reagent (1 ml.) was then added, followed by 20% (w/v) NaCO₃ (2 ml.). The tubes were heated in a boiling water bath for 1 min., cooled in water and the resulting blue solution diluted to 25 ml. and compared with a standard prepared similarly using, instead of the urine extract, 1 ml. of a solution of the corresponding cresol (2 mg./100 ml. made by dilution of a 50 mg./100 ml. aqueous stock solution).

(b) *Total cresol.* This estimation was carried out as above using urine hydrolysed by boiling under reflux for 1 hr. with an equal volume of 10N-H₂SO₄ and then adjusted to pH 7.8–8.0. Control experiments showed that this method gave quantitative recovery of the cresols added to normal rabbit urine, even in the presence of added hydroxybenzoic acid. Attempts to adapt the method to the simultaneous estimation of the hydroxybenzoic acids which might arise by metabolic oxidation of the cresols have so far been unsuccessful. Actually the extent of this oxidation is small and the estimation of the acid produced is rendered more difficult by the fact that the hydroxybenzoic acids give only 36–61% of the intensity of the colour given by equivalent amounts of the corresponding cresols. Further the base line for ether-soluble phenolic acids is comparatively high (about 150 mg./24 hr., expressed as *p*-cresol), so that the

actual increases to be expected would represent only a relatively insignificant fraction of the total.

Paper chromatography

This technique was applied, as described by Bray, Thorpe & White (1950), to ether extracts of unhydrolysed and hydrolysed cresol urines.

RESULTS

Quantitative studies

Excretion of normal metabolites. The average daily excretion of normal metabolites by individual rabbits used in this study was as follows: (1) Reducing material expressed as glucuronic acid, 127–226 mg., the day-to-day variation in each experiment usually being less than $\pm 14\%$ of the weekly mean (average 12%); the average increase in reducing material on hydrolysis was 69%. (2) Ethereal sulphate, 28–47 mg. SO₃, daily variation usually within $\pm 13\%$ of the weekly mean (average 13%). (3) Free non-acidic phenols (i.e. those extracted at pH 7.8–8.0) expressed as cresols, 6–24 mg., daily variation within $\pm 25\%$ (± 4 mg.) of the weekly mean (average 20%). (4) Total non-acidic phenols expressed as cresol 40–87 mg., daily variation within $\pm 15\%$ of the weekly mean (average 11%).

Excretion of free and total cresols, ether glucuronide and ethereal sulphate by the rabbit after administration of cresols. The results obtained are summarized in Table 1. No increase in ester glucuronide excretion was observed. A large number of determinations of ether-soluble acid excretion gave erratic results and are not recorded. As mentioned above, the small percentages of the cresols which appear to be oxidized to the corresponding acids are insignificant in comparison to the normal fluctuations of the ether-soluble acid base line. In early experiments, when the animals were not given their food until after dosage, anorexia almost invariably occurred. This would affect the base-line values and almost cer-

Table 1. *Excretion products of cresols in the rabbit*

Compound administered	Type	Excretion products		
		No. of experiments	Percentage of dose excreted	
			Range	Average
<i>o</i> -Cresol (500 mg.)	Ethereal sulphate	4	13–19	15
	Ether glucuronide	6	55–91	72
	Free cresol	4	0–2	1
	Total cresol	4	75–83	80
<i>m</i> -Cresol (500 mg.)	Ethereal sulphate	5	4–15	10
	Ether glucuronide	6	53–70	60
	Free cresol	6	0–4	1
	Total cresol	6	81–86	84
<i>p</i> -Cresol (250–500 mg.)	Ethereal sulphate	4	12–21	15
	Ether glucuronide	9	32–85	61
	Free cresol	5	0–12	2
	Total cresol	5	52–85	65

tainly reduce the net excretion of all metabolites during the 24 hr. after dosage. This effect was diminished by allowing the animals to satisfy their hunger before dosage, but was not entirely eliminated since even then they did not always consume the whole of their daily ration. It is thus possible that the results obtained are all somewhat low, especially with *p*-cresol, the most toxic isomer. It will be observed from the ranges quoted in Table 1 that *p*-cresol did in fact give more variable results than the other isomers. The excretion of the metabolites under investigation during the second 24 hr. period after dosage was however in general within the normal range.

It can be seen from Table 1 that the extra total non-acidic phenol (i.e. cresol) excretion is approximately equal to the sum of that of ethereal sulphate and ether glucuronide, i.e. that conjugation of the hydroxyl group accounts for the major part of the dose. The discrepancies with the *o*- and *m*-isomers are presumably due mainly to experimental error, since those minor metabolites which have been detected (Table 2) would be included in the total phenol values. The discrepancy with the *p*-isomer must, in part, be due to *p*-hydroxybenzoic acid which is not estimated by the phenol method used, but if conjugated with glucuronic or sulphuric acids would be included in the values for ethereal sulphate and ether glucuronide. Unconjugated *p*-hydroxybenzoic acid is not estimated by any of the chemical methods used: paper chromatography suggested that about 7% of the dose was excreted as free *p*-hydroxybenzoic acid and 3% conjugated.

The total percentages of the cresols for which we accounted are much higher than those found by Jonescu (1906) in the dog. It is possible that his low values were the result of failure to decompose the ether glucuronides during estimation, since we found that values for total phenol little greater than the ethereal sulphate were obtained if we used the method of Volterra (1942), in which the urine is hydrolysed by 2*N*-H₂SO₄ and the liberated cresol simultaneously removed by distillation.

Excretion of minor metabolites. By means of paper

chromatography it was possible to detect and approximately to estimate metabolites which gave phenolic reactions. By comparing the ether extracts of urine before and after hydrolysis, the free and conjugated forms could be distinguished. Experimental details have been given elsewhere (Bray *et al.* 1950). Table 2 lists the possible metabolites and summarizes the results obtained.

Qualitative experiments

The standard lead precipitation method was applied to cresol urines. The *o*- and *m*-isomers yielded syrups which could not be crystallized, but, being sparingly soluble in ether, could be partially purified by continuous extraction with that solvent. The syrups gave a strong naphthoresorcinol reaction, but, on hydrolysis, gave reducing material and cresols identified as cresyl carbanilates. *p*-Cresol urine, however, yielded a crystalline product, m.p. 147° (decomp.), which gave a positive naphthoresorcinol test and, after hydrolysis, a positive Folin & Ciocalteu reaction and reduction with Benedict reagent. (Found: C, 50.6; H, 5.8; glucuronic acid 56.5%. Equiv. (by titration) 299, $[\alpha]_D^{25} - 71.8^\circ$ (water; *c*, 0.4). C₁₃H₁₈O₈ requires: C, 51.7; H, 6.0; glucuronic acid 64.2%. Equiv. 302.) From the products of hydrolysis (boiling under reflux with 2*N*-HCl for 2 hr.), *p*-cresol was isolated as its benzoyl derivative, m.p. 67–68°. Mixed melting point with authentic specimen (m.p. 70–71°) 68–68°. It was concluded that the compound isolated was the *monohydrate* of *p*-cresylglucuronide.

Isolation of 2:5-dihydroxytoluene. This compound was isolated from both *o*- and *m*-cresol urines. The process of isolation was greatly facilitated by examining the fractions at each stage by paper chromatography (Bray *et al.* 1950).

The 24 hr. urine from ten rabbits, each of which had received 0.5 g. of *m*-cresol, was hydrolysed by heating to 90° with 0.1 vol. of conc. HCl for 30 min., and continuously extracted with ether for 24 hr. The ether-soluble material was dissolved in acetone and evaporated under reduced pressure to 20 ml. On chilling, hippuric acid (1 g.) separated and was

Table 2. *Metabolites of cresols in the rabbit detected by paper chromatography*

(+, about 7% of dose; +, about 3% of dose, or less; Tr., trace; –, absent.)

Compound administered	Metabolites						
	Hydroxybenzoic acid		Position of hydroxyls in dihydroxy compounds	Dihydroxytoluene		Dihydroxybenzoic acid	
	Free	Conjugated		Free	Conjugated	Free	Conjugated
<i>o</i> -Cresol	–	–	2:5 2:3	–	+	–	–
<i>m</i> -Cresol	–	–	2:5 2:3 3:4	–	+	–	–
<i>p</i> -Cresol	++	+	3:4	–	Tr.	–	–

removed. The filtrate was treated with charcoal and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum necessary amount of boiling water and the solution made just alkaline with ammonia. The precipitate formed by addition of excess of saturated basic lead acetate was collected, suspended in water (100 ml.) and treated with hydrogen sulphide to remove lead. After addition of excess $\text{Na}_2\text{S}_2\text{O}_4$, the lead-free filtrate was continuously extracted with ether. The ethereal extract was concentrated *in vacuo* to give a syrup which, on treatment with small volumes of water and chilling, gave a crude product, m.p. 105° , consisting mainly of 2:5-dihydroxytoluene. Recrystallization from water gave colourless needles, m.p. 124° , mixed m.p. with authentic 2:5-dihydroxytoluene (m.p. 125°), 125° . Yield 50 mg. (1% of dose). (Found: C, 67.5; H, 6.6. Calc. for $\text{C}_7\text{H}_8\text{O}_2$: C, 67.7; H, 6.5%.) The identity of the isolated metabolite with 2:5-dihydroxytoluene was further confirmed by paper chromatography and by the preparation of the dibenzoyl derivative, m.p. $118\text{--}119^\circ$, alone or mixed with an authentic specimen. 2:5-Dihydroxytoluene was similarly isolated from *o*-cresol urine. Yield 10 mg. from a total dose of 5 g.

DISCUSSION

The cresols are excreted principally as conjugates through the hydroxyl group, 60–72% as ether glu-

curonide and 10–15% as ethereal sulphate. Negligible amounts are excreted unconjugated and, as would be expected in compounds possessing a readily conjugable group, little conversion of the potential centre for conjugation or hydroxylation occurs. With regard to the extent to which such processes occur there exists a reciprocal relationship: in *o*- and *m*-cresols, where no conversion of the potential centre could be detected, hydroxylation occurs and in *p*-cresol, of which *p*-hydroxybenzoic acid was shown to be a metabolite, no significant hydroxylation could be detected.

SUMMARY

1. The cresols are excreted by the rabbit mainly as oxygen-conjugates; 60–72% as ether glucuronides and 10–15% as ethereal sulphates.
2. It has been shown by paper chromatography that *o*- and *m*-cresols are hydroxylated to a small extent and that *p*-cresol gives rise to some *p*-hydroxybenzoic acid.
3. *p*-Cresylglucuronide was isolated as a crystalline monohydrate from *p*-cresol urine and 2:5-dihydroxytoluene from *o*- and *m*-cresol urines.

The authors are indebted to the Royal Society for a Government Grant which defrayed part of the expenses of this investigation and to Miss B. G. Humphris for assistance with some of the analyses. The microanalyses were carried out by Drs Weiler and Strauss, Oxford.

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Further Observations on Changes in β -Glucuronidase Activity in the Mouse

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Increased β -glucuronidase activity in mouse liver or kidney was shown to reflect an increase in the proliferative activity of the tissue (Levy, Kerr & Campbell, 1948). A similar relationship in uterus has been found to explain changes in the glucuronidase activity of this organ produced by certain sex

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hormones (Fishman & Fishman, 1944; Fishman, 1947; Kerr, Campbell & Levy, 1949). Investigation of unexpected changes in glucuronidase activity in mouse tissues led to the discovery of new factors controlling growth in liver and uterus (Kerr *et al.* 1949). Further studies of these factors are described in the present communication. Additional examples of parallelism between the β -glucuronidase activity