Studies in Detoxication

19. THE METABOLISM OF BENZENE. I. (a) THE DETERMINATION OF PHENOL IN URINE WITH 2:6-DICHLOROQUINONECHLOROIMIDE. (b) THE EXCRETION OF PHENOL, GLUCURONIC ACID AND ETHEREAL SULPHATE BY RABBITS RECEIVING BENZENE AND PHENOL. (c) OBSERVATIONS ON THE DETERMINATION OF CATECHOL, QUINOL AND MUCONIC ACID IN URINE

By J. W. PORTEOUS and R. T. WILLIAMS
The Department of Biochemistry, University of Liverpool

(*Received* 5 *May* 1948)

The fate of benzene in the body has been the subject of numerous investigations since 1867 when Schultzen & Naunyn (1867) discovered that benzene was converted to phenol in the animal body. Phenol, catechol, quinol and their conjugates (Munk, 1876; Schmiedeberg, 1881; Nencki & Giacosa, 1880; Baernstein, 1945), muconic acid (Jaffe, 1909; Thierfelder & Klenk, 1924; Drummond & Finar, 1938; Bernhard & Gressley, 1941) and L-phenylmercapturic acid (Zbarsky & Young, 1943) have been detected in 'benzene urine' from various animals. Of these metabolites, phenol, catechol, muconic acid and phenylmercapturic acid have been isolated in a pure crystalline state. Quinol and conjugates such as glucuronides and ethereal sulphates have not been isolated. Dihydroxydihydrobenzene (dihydroxy*cyclo*hexadiene) is also a possible metabolite, for analogous compounds have been isolated as metabolites of naphthalene (Young, 1947; Booth & Boyland, 1947), anthracene (Boyland & Levi, 1935, 1936), and phenanthrene (Boyland & Wolf, 1948). It is, therefore, clear that the metabolism of benzene is a complex problem, and hence no balance sheet for the fate of benzene in the body has yet been drawn up. Furthermore, no mechanism has been put forward to account for the formation of the divers oxidation products.

Of the benzene administered to an animal a large proportion may be eliminated unchanged via the lungs, and at least a third to a half may leave the body in this way (Nencki & Sieber, 1883; Lehmann, Gundermann & Kleiner, 1910).

The object of the present investigation was to determine the amount of phenol and other metabolites excreted by rabbits receiving benzene orally. A new method for the determination of phenol in urine in the presence of catechol and quinol was developed. This method depended on the steam distillation of phenol from urine at pH 6, and the determination of phenol in the distillate by means of the blue colour formed with 2:6-dichloroquinonechloroimide at pH 10. This reagent has been studied qualitatively by Gibbs (1927), Baylis (1928) and Fearon (1944). Scudi (1941) has used it for the determination of pyridoxine.

2:6-Dichloroquinonechloroimide gives a pure blue colour with phenol but it also reacts with phenols which are not substituted in the para position. The reagent is unaffected by quinol, but with catechol it gives a violet colour. Catechol, however, is not volatile in steam, and phenol can be separated from it by a steam distillation.

METHODS

THE DETERMINATION OF PHENOL IN URINE

Phenol gives a blue colour in aqueous solutions with 2:6-dichloroquinonechloroimide at pH 8–10. Under rigidly controlled conditions the colour is proportional to the amount of phenol present and a few μg. of phenol/ml. of solution can be accurately estimated.

Reagents required

Standard solutions of phenol. (*a*) A 0·1% stock solution was prepared by dissolving 1 g. pure phenol in 1 l. water. Its exact phenol content was determined by the bromination method of Day & Taggard (1928). This solution is stable for 2 weeks. (*b*) Phenol solutions (0·01, 0·005 and 0·001%) were prepared from the stock solution as required.

2:6-*Dichloroquinonechloroimide reagent.* This reagent (subsequently called the phenol reagent) was prepared immediately before use. Dry finely powdered 2:6-dichloroquinonechloroimide (British Drug Houses Ltd., 0·1 g.) was shaken for 10 min. with distilled water (100 ml.). The solution was filtered from undissolved solid and 10 ml. of the filtrate used in each determination. If more or less than 0·1 g. of the dichloroquinonechloroimide was used in the preparation of the phenol reagent, an unsatisfactory reagent was obtained. The method of preparation of the phenol reagent is simple but critical.

Buffer solutions. (*a*) *Phosphate buffers* (*Sorensen*); 11·9425 g. $Na_2HPO_4.12H_2O$ (A.R.) were dissolved in 500 ml. distilled water and 4·539 g. KH_2PO_4 in 500 ml. distilled water. From these two solutions were prepared the acid buffers, pH 5·2–6·8 (see Clarke, 1928) used

when distilling free phenol from urine without hydrolyzing conjugated phenol. (*b*) *Alkaline buffer of Kolthoff & Vlesschouwer* (1927); the colour between phenol and the phenol reagent was developed at pH 10·15 which was obtained by mixing 75·4 ml. of a solution of Na_2CO_3 (2·65 g. anhydrous (A.R.) in 500 ml. water) with 24·6 ml. of a solution of $Na_2B_4O_7.10H_2O$ (9·55 g. in 500 ml. of water).

Acid solutions: (*a*) 10 N-H_2SO_4 for the hydrolysis of conjugated phenol; (*b*) 6 % H_3PO_4 for titrating rabbit urine to the pH required for the distillation of free phenol.

Apparatus

For the steam distillation of phenol from a given solution the apparatus shown in Fig. 1 was used. *A* is the steam generator, i.e. a 2 l. conical flask. Zinc dust is added to the water

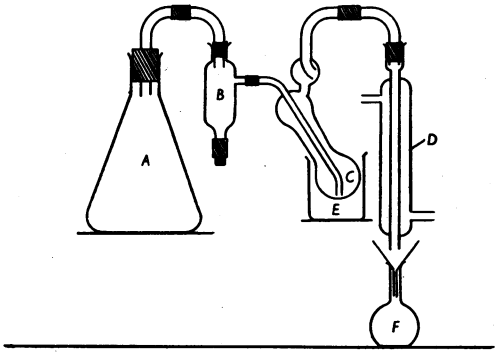

Fig. 1. Apparatus for the distillation of phenol from urine (see text for description).

for smooth boiling. *B* is a water trap. *C* is a micro-Kjeldahl distillation flask fitted with a bulb trap at its exit. *D* is a Liebig's condenser. *E* is a water bath (beaker) completely surrounding the end of *C* and the water in it is kept boiling during distillation. *F* is a volumetric flask used as a receiver. During the hydrolysis of conjugated phenol the condenser *D* is fitted vertically above *C*. No rubber should be exposed to the steam distillate, and the rubber bung at the upper end of the condenser *D* is covered with tin foil. Exposure of the distillate to rubber connexions results in a low recovery of phenol (an all glass apparatus would be ideal). Before use and between experiments the apparatus is thoroughly steamed out.

Construction of the phenol calibration curve

To a series of ten 25 ml. volumetric flasks were added 1–10 ml. of a standard 0·001 % phenol solution (10 μg./ml.). The Kolthoff-Vlesschouwer alkaline buffer pH 10·15 (5 ml.) and then the phenol reagent (10 ml.) were added to each flask. Finally, the volume in each flask was made up to 25 ml. with distilled water and the contents mixed and allowed to stand for 1 hr. when the blue colour reached a maximum. Colour measurement in a 1 cm. cell was made with a Spekker photoelectric absorptiometer with an Ilford no. 607 spectrum orange filter. The curve obtained is given in Fig. 2.

The effect of pH on the development of the colour was shown by repeating the above experiment using alkaline buffers of pH 9·2, 9·7 and 10·5 instead of that of pH 10·15. At pH 9·2 maximum colour development required 3 hr.; at pH 9·7 the result was similar to that at pH 10·15; at pH 10·5 the maximum colour developed in 1 hr., but it

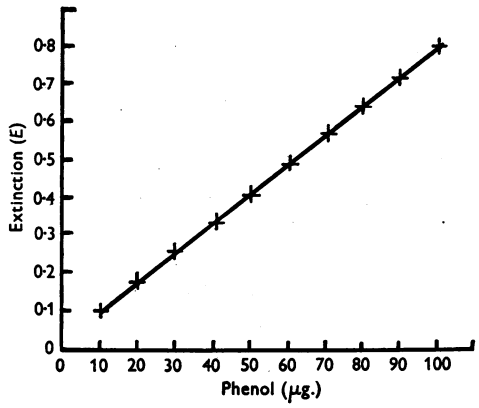

Fig. 2. Standard curve for the estimation of phenol using 2:6-dichloroquinonechloroimide at pH 10·15.

rapidly deteriorated; at pH 10·15 the blue colour was stable, reaching a maximum in 1 hr., and this pH was adopted in all subsequent estimations.

The recovery of phenol from pure solution by steam distillation

Mixtures of standard phenol solutions (1–5 ml.) and Sorensen buffer pH 6 (5 ml.) were steam distilled in the apparatus already described. Distillation was carried out at such a rate that 100 ml. of liquid were distilled in 10–15 min. A suitable sample (1–10 ml.) of the distillate was then buffered with the Kolthoff-Vlesschouwer alkaline buffer and the phenol reagent added as already described. The colour was measured as before. The results are given in Table 1 which shows that as little as 10 μg. of phenol/ml. solution can be recovered almost quantitatively by steam distillation.

Table 1. *The recovery of phenol from pure solution by steam distillation*

(The numbers in parentheses give the number of determinations of which the percentage recovery quoted is the average.)

Amount of phenol added	Vol. of distillate (ml.)	Phenol recovered (%)
1 mg. in 1 ml. solution	25	95·5 (8)
	50	96 (16)
	100	99 (11)
1 mg. in 5 ml. solution	50	96 (16)
	100	97 (16)
0·1 mg. in 1 ml. solution	25	92 (8)
	50	95 (4)
0·1 mg. in 5 ml. solution	25	91 (8)
	50	100 (4)
50 μg. in 1 ml. solution	25	98 (4)
50 μg. in 5 ml. solution	25	94 (4)

The determination of conjugated phenol in pure solution

The urines of animals receiving benzene or phenol are likely to contain phenylglucuronide and ethereal sulphate. The ethereal sulphates are readily hydrolyzed by dilute acid, but the stability of glucuronides to acid is variable (see Hanson, Mills & Williams, 1944). Menthylglucuronide, for example, is readily hydrolyzed by dilute acid, whereas *o*-aminophenylglucuronide is only hydrolyzed with difficulty. Phenylglucuronide appears to fall somewhere in between these two extremes. The following experiment was, therefore, designed to find out what length of time and strength of acid are required to hydrolyze phenylglucuronide completely, and whether the phenol thus set free can be recovered quantitatively by steam distillation.

A standard aqueous solution of phenyl-β-D-glucuronide (m.p. 160–162°) was prepared by dissolving 287·2 mg. of the glucuronide in 100 ml. water. This solution on complete hydrolysis should yield 1 mg. phenol/ml. When freshly prepared these solutions give no colour with the phenol reagent at pH 10·15. However, aqueous solutions of phenylglucuronide do not keep more than a few days and should be made up fresh on each occasion.

In earlier experiments HCl was used in the hydrolysis, but since this acid is volatile in steam it was subsequently replaced by H_2SO_4. Phenylglucuronide solution (1 ml.) with 5 ml. 2N- or 10N-H_2SO_4 were refluxed at 100° in the micro-Kjeldahl flask (*C*, Fig. 1) for varying times. The water in *E* (Fig. 1) was then replaced by iced water and the reflux condenser washed down with a few ml. of water. The condenser was then put in position for distillation and the solution steam distilled. The phenol in the distillate was then determined as before. Table 2 shows that optimum recovery (95%) of phenol occurs when the glucuronide solution has been hydrolyzed for 1 hr. with 10N-H_2SO_4 and these conditions were adopted for determination of conjugated phenol.

Table 2. *Recovery of phenol from phenylglucuronide solutions*

(1 ml. of phenylglucuronide solution (phenol equiv. = 1 mg.) + 5 ml. H_2SO_4 hydrolyzed and steam distilled.)

Duration of hydrolysis (hr.)	Strength of acid (H_2SO_4) (N)	Phenol recovered (%)
1·5	2	30
0·25	10	81·5
0·5	10	88, 89, 99, 88
1·0	10	92, 99, 91·5, 93·5, 97·5
1·5	10	90, 93·5

During the action of hot mineral acids on glucuronic acid, furfural is formed. This aldehyde can be detected (by aniline acetate) in the distillates from the hydrolysis of phenylglucuronide. Furfural is said to give a green colour with 2:6-dichloroquinonechloroimide (Scudi, 1941). There is, therefore, the possibility that furfural in the distillates may interfere with the development of colour. It was found, however, that furfural in amounts equivalent to the phenol distilled had no effect on the development of the phenol colour. Calibration curves constructed by distilling phenol (10–100 μg.) alone and in the presence of equivalent amounts of twice-distilled furfural were coincident.

The recovery of phenol added to normal rabbit urine

Normal rabbit urine is usually alkaline and has to be brought to pH 6 before added phenol can be recovered by distillation. This can be achieved by titrating to pH 6 with 6% H_3PO_4 and then adding 5 ml. of a Sorensen buffer pH 6 to 5 ml. of the acidified urine. It was then found that on steam distilling such a buffered urine containing added phenol the distillate had a pH of 8–9. Such distillates did not give a pure blue colour with the phenol reagent at pH 10·15; the colours obtained were dirty blue or green. The interfering substance, especially with slightly stale urines, was eventually found to be largely NH_3, particularly in rabbit urine. The removal of ammonia, where it occurred above a critical concentration, was achieved by treating every 5 ml. of urine (brought to pH 6 with 6% H_3PO_4) with 2 g. of permutit (60 mesh), and filtering. A separate experiment showed that permutit did not remove added phenol from urine.

Urine containing added phenol was therefore treated as above and steam distilled; the recoveries are given in Table 3 which shows that 50–1000 μg. of phenol can be recovered by distillation almost quantitatively when added to urine.

Table 3. *Recovery of phenol added to normal rabbit urine*

Vol. of urine treated (ml.)	Phenol added (mg.)	Vol. of distillate (ml.)	Phenol recovered (%)
1	1	100	98, 100
1	1	50	100, 100
5	1	100	100, 100
5	1	50	99, 101
1	0·05	25	95
5	0·05	25	93, 97, 96·5

The phenol content of normal human and rabbit urine

Rabbit urine. The recovery of added phenol from normal urine raises the question of whether normal urine contains significant amounts of phenol. Normal rabbit urine was brought to pH 6 and treated with permutit as already described above. The treated urine (5 ml.) was then steam distilled. For total phenol, 5 ml. of urine was hydrolyzed by heating under reflux with 5 ml. 10N-H_2SO_4 and steam distilling.

Table 4 shows that normal rabbit urine may contain 10–13 μg. total phenol/ml., an amount of little significance when compared with the phenol which occurs in urine after feeding benzene or phenol. The only other figures which have been given for free and conjugated phenol in rabbit urine are those of Deichmann (1943). This worker gives, free phenol, 0–0·39 mg. and conjugated phenol, 1·15–10·0 mg./24 hr.

Table 4. *Free and combined phenol in normal rabbit urine*

Rabbit no.	Volume of urine (ml./24 hr.)	Phenol (mg./24 hr.) Free	Phenol (mg./24 hr.) Total	Phenol (mg./100 ml.) Free	Phenol (mg./100 ml.) Total
72	39	0·09	0·50	0·23	1·27
73	67	0·42	0·65	0·63	0·97
74	62	0·35	0·775	0·56	1·25
75	80	0·50	0·972	0·63	1·22
Sample					
1*	—	—	—	0·32	—
2*	—	—	—	0·22	—
3*	—	—	—	0·30	—
4*	—	—	—	0·26	—
5*	—	—	—	0·37	—
6*	—	—	—	0·53	—

* Mixed urines.

Table 5. *Free and combined phenol in normal human urine*

Subject	Vol. of fresh random sample (ml.)	Phenol Free (mg.)	Phenol Conjugated (mg.)	Phenol Conjugated (mg./100 ml.)
A. G.	120	0	1·23	1·0
F. W.	230	0	1·15	0·5
J. P.	204	0	1·83	0·9

Human urine. Since human urine is slightly acid, there is no need to titrate it with 6 % H_3PO_4 as in the case of rabbit urine. To 5 ml. human urine, 5 ml. of Sorensen buffer pH 6 were added and the resulting mixture had a pH very nearly 6. The urine was then treated with permutit and the filtrate distilled. Total phenol was determined as for rabbit urine. The results are given in Table 5.

General methods

Glucuronic acid was determined by the method of Hanson *et al.* (1944), using pure phenylglucuronide (m.p. 161–162°) as the standard.

Ethereal sulphate and neutral sulphur were determined gravimetrically by the Folin method.

RESULTS

Experiments with Phenol

Free and conjugated phenol. Rabbits were maintained on a diet of 50 g. Lever's cubes and 100 g. cabbage *per diem*. Phenol was administered by stomach tube at a dose level of 125 mg./kg. in 10 ml. of water in most experiments. In two experiments phenol dissolved in 0·9 % sodium chloride was injected intraperitoneally at a dose level of 60 mg./kg. Urine for analysis was centrifuged and then filtered. All determinations were carried out within 1–2 hr. of collecting the urine sample. The urine was analyzed for free and conjugated phenol by the method described above.

Phenol at the dose level used is excreted almost entirely in a conjugated form, traces only of free phenol being excreted (Table 6). The excretion of free phenol is more significant after injection than after oral administration. On an average 77 % of the fed phenol and 62 % of the injected phenol are excreted as total phenol. Furthermore, the phenol was completely excreted within 24 hr. of its administration.

Table 6. *The excretion of free and conjugated phenol by rabbits receiving phenol orally and by injection*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	Method of administration	Phenol excreted: Free (mg.)	Free (% of dose)	Total (mg.)	Total (% of dose)
6	2·85	125	Oral	0	0	281·6	79
9	2·65	125	,,	0	0	232·5	70
10	2·95	125	,,	Trace	0	302·0	81·9
16	2·1	125	,,	Trace	0	170·6	65
31	2·45	125	,,	Trace	0	236·1	77·1
73	2·5	60	Injection	5	3·3	84·3	56·2
75	2·6	60	,,	4·2	2·4	98·0	65·3

Glucuronic acid excretion. The results (Table 7) show that at doses of 125 and 250 mg./kg. about 70 % of the administered phenol is excreted as a glucuronide (see Fig. 3). The figures for the dose of 50 mg./kg. are unreliable, for at these low doses, the variation in normal glucuronic acid excretion may cause significant errors.

Table 7. *The excretion of conjugated glucuronic acid in the rabbit after orally administered phenol*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	'Extra' glucuronic acid excreted (mg.)	Phenol equivalent to 'extra' glucuronic acid (% of dose)
66	2·82	50	314·0	107·9
68	2·8	50	271·4	93·2
72	2·75	50	325·6	114·7
75	2·62	50	264·6	96·7
78	2·5	125	548·5	85·0
82	2·45	125	494·2	78·3
83	2·65	125	398·0	58·2
66	2·77	250	1094·5	77·1
68	2·8	250	1216·7	83·6
72	2·7	250	875·0	62·8
75	2·6	250	706·8	52·6

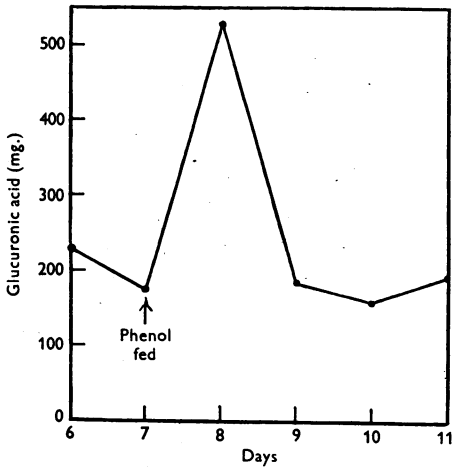

Fig. 3. Glucuronic acid excretion by rabbit no. 66 which had received 141 mg. of phenol orally on the 7th day of the experiment.

Isolation of phenylglucuronide. Five large rabbits were each given 0·9 g. of phenol (total fed, 4·5 g.) with water. Their urine, collected during 18 hr., was acidified with a little glacial acetic acid and treated with 0·2 vol. of saturated normal lead acetate solution. The solution was filtered and the filtrate neutralized with NH_4OH. An excess of saturated basic lead acetate solution was now added and the precipitate was filtered at the pump and washed with water. It was suspended in water and its lead removed with H_2S. After removal of PbS the filtrate was concentrated *in vacuo* at 40–50° until it began to crystallize. The whole was allowed to crystallize at 0° and the phenylglucuronide (4–6 g.) filtered off. It was recrystallized from benzene containing a little ethanol and it formed needles m.p. 161–162° sintering slightly at 110°.

Experiments with benzene

Free and conjugated phenol. Rabbits were maintained on a constant diet of 50 g. Lever's cubes and 100 g. cabbage *per diem.* Benzene was administered by stomach tube at a dose level of 500 mg./kg. with 10 ml. water. The urine excreted was analyzed daily for free and conjugated phenol by the method already described (see Table 8). Most of the phenol formed was excreted within 24 hr. of feeding the benzene (see Fig. 4) and furthermore very little occurred in the free state.

Excretion of conjugated glucuronic acid. The output of glucuronic acid after feeding benzene is given in Table 9. Fig. 5 illustrates a single experiment. These results show that on an average about 11 % of the dose of benzene is excreted as a glucuronide, assuming that one molecule of benzene gives rise to one of 'extra' glucuronic acid.

Excretion of ethereal sulphate and neutral sulphur. The results for these experiments are given in Table 10. In two of these experiments the benzene was fed dissolved in a vegetable oil, and in the others with 10 ml. of water. An average of 9·5 % of the benzene fed is excreted as an ethereal sulphate. The main bulk of the 'extra' ethereal sulphate is excreted during the first 24 hr. after feeding (see Fig. 5), although in one or two cases appreciable amounts were excreted in the second 24 hr.

In the case of the neutral-sulphur output, the daily variation of the normal value in the rabbit was such that it was difficult to reach a definite conclusion. We are, however, of the opinion that if any benzene is excreted by the rabbit as a mercapturic acid then the amount is small, and cannot be detected by neutral-sulphur determinations. Witter (1945) has concluded that the rabbit probably does not excrete phenylmercapturic acid, but Zbarsky & Young (1943) have isolated this conjugate from rat urines in yields up to 0·37 % of the dose of benzene. Such amounts could not be detected by neutral-sulphur determinations.

Table 8. *The excretion of phenol by the rabbit after oral administration of benzene (dose 500 mg./kg.)*

Rabbit no.	Wt. (kg.)	Dose (g.)	Phenol excreted: Free (mg.)	Total (mg.)	Free (% of dose)	Total (% of dose)
7	2·65	1·325	0	271·0	0	17·0
36	2·15	1·075	0	118·2	0	9·1
41	2·0	1·000	0	117·7	0	9·7
44	1·85	0·925	0	65·3	0	5·9
72	2·65	1·300	24·6	112·9	1·6	7·2
74	2·6	1·300	6·2	98·0	0·4	6·3

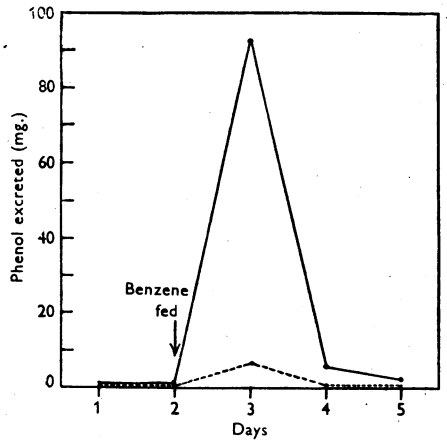

Fig. 4. The excretion of free (broken line) and total (continuous line) phenol by rabbit no. 74 after an oral dose of 1·3 g. benzene.

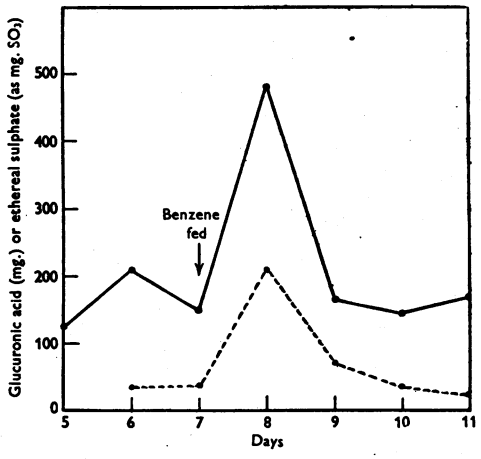

Fig. 5. Glucuronic acid excretion by rabbit no. 84 (continuous line) and ethereal sulphate excretion by rabbit no. 9 (broken line), after oral doses of benzene. Doses: rabbit no. 84, 1·3 g. (500 mg./kg.); rabbit no. 9, 2·85 g. (1·0 g./kg.).

Table 9. *Excretion of conjugated glucuronic acid by rabbits receiving orally 500 mg. of benzene/kg.*

Rabbit no.	Wt. (kg.)	Dose (g.)	Extra glucuronic acid (mg.)	Extra glucuronic acid (% of dose)
73	2·65	1·325	139·5	4·2
79	2·45	1·235	149·0	4·8
74	2·5	1·25	182·6	5·9
79	2·55	1·275	260·5	7·9
84	2·65	1·325	268·8	8·15
83	2·57	1·3	279·8	8·6
84	2·6	1·3	309·7	9·5
79	2·45	1·225	418·5	13·7
82	2·5	1·275	556·6	17·5
84	2·57	1·31	681·4	20·9
83	2·55	1·31	739·7	22·7

Table 10. *The excretion of ethereal sulphate and neutral sulphur by rabbits receiving benzene orally (dose 500 mg./kg.)*

Rabbit no.	Wt. (kg.)	Dose (g.)	Extra ethereal sulphate: (mg. SO_3)	(% of dose)	Neutral sulphur (% of dose)
7	2·5	1·3*	78·2	5·85	0
6	2·8	1·4*	113·8	7·9	0
6	2·7	1·35*	110·9	8·0	0
9†	2·85	2·694*	231·3	8·4	0
10†	2·9	2·893*	250·4	8·4	0
6	2·65	1·31*	140·2	10·4	0
6	2·6	1·30‡	142·8	10·7	0
7	2·6	1·30‡	224·2	16·8	0

* Benzene fed with water. † Dose of 1 g./kg. ‡ Benzene fed dissolved in oil.

Observations on catechol and quinol

The only previous attempt to estimate catechol and quinol in urine appears to be that of Baernstein (1945). In this, the phenols were extracted from neutralized acid-hydrolyzed benzene urines with ether, and the catechol was separated from phenol and quinol as a lead salt. We attempted unsuccessfully to find specific reagents for catechol and quinol in order to avoid lead separation. Although we devised methods for the determination of catechol and quinol added to normal urine, these methods gave erratic results when applied to benzene urines owing to the presence of interfering substances, one of which we identified as hydroxyquinol. It is, therefore, proposed to mention our experiments only briefly.

Determination of catechol in pure solution. Catechol can be readily estimated in pure solution with 2:6-dichloroquinonechloroimide. With this reagent catechol gives at an optimum pH of 6·6–6·8 a blue-purple colour which is proportional to the catechol present. Quinol does not interfere, but phenol and *o*- and *m*-cresols do. A calibration curve was prepared by mixing, in 20 ml. flasks, acetate buffer pH 6·7 (5 ml.) and a solution of 2:6-dichloroquinonechloroimide (3 ml.) prepared as for phenol estimations, with a series of 1–10 ml. of a fresh 0·002% catechol solution and making up to 20 ml. with water. The colours produced 1 hr. after mixing were read in a Spekker absorptiometer with an Ilford no. 606 spectrum yellow filter. A reproducible straight line calibration curve was obtained.

Catechol in quantities of 0·1–1·0 mg./ml. can be recovered from aqueous solution by ether extraction for 4 hr. to the extent of 80% of that added.

Determination of quinol in pure solution. The estimation of quinol was based on the method of Oglesby, Sterner & Anderson (1947) for the determination of *p*-benzoquinone in air. The standard curve was obtained by mixing in 20 ml. flasks a series of 1–10 ml. of a 0·002% quinol solution with 1 ml. of 0·5% phloroglucinol and then adding water to within 3 ml. of the mark. 0·1 N-KOH (1 ml.) was added and then water to 20 ml. The mixed solutions were kept for 1 hr. and then the red colour was measured in the absorptiometer using an Ilford no. 604 spectrum green filter. A reproducible straight line calibration curve was obtained.

By this method quinol can be estimated in the presence of phenol, *o*-, *m*- and *p*-cresols and furfural, but catechol interferes. Quinol (0·05% solutions) can be recovered by ether extraction for 4 hr. from aqueous solution to the extent of 90%.

Neither of these methods for catechol and quinol is as sensitive as the method used for phenol.

Determination of added catechol and quinol in urine. Normal rabbit urine (5 ml.) containing 10 mg. each of catechol and quinol were acidified with 5 drops of 2 N-H_2SO_4 and then extracted continuously for 4 hr. with ether. Acetate buffer pH 6·7 (10 ml.) was added to the extract and the ether was removed from the mixture by suction at room temperature. The resulting solution was then neutralized with solid $NaHCO_3$, filtered and made up to 25 ml. with the acetate buffer pH 6·7. A 5 ml. sample of the solution was then placed in a fine sintered-glass filter (Pyrex SF2A/4) and mixed with 5 ml. buffer pH 6·7 and 5 ml. 0·1 M-lead acetate (normal) solution. Catechol is thus precipitated as a lead salt (precipitate A). The glass filter was then subjected to suction, the filtrate being sucked into a test tube containing 5 ml. of 2% sodium oxalate which precipitated the excess lead in the filtrate. The excess lead was removed by filtration through asbestos in a Gooch crucible and the filtrate made up to 50 ml. with water. The quinol in a sample of this solution was then determined by the phloroglucinol-KOH method described above. Glacial acetic acid (3 ml.) was now added to the lead catecholate (precipitate A) in the sintered-glass filter. When the lead salt had dissolved, the resulting solution was sucked into a test tube containing 5 ml. of 2% sodium oxalate. The glass filter was carefully washed with 10 ml. water which was also sucked into the test tube. The mixture was then filtered through the same glass filter to remove lead oxalate, and the final clear filtrate, after neutralization with solid $NaHCO_3$, was made up to 50 ml. with water. Catechol was determined in 5 ml. samples of this solution as described above for catechol in pure solution. The recoveries of both catechol and quinol by this method were 80–90% of the amounts present. Such recoveries were also obtained when phenol was present, for phenol appears in the quinol fraction during the lead separation.

The recovery of catechol and quinol was also tested under hydrolysis conditions. Urine (5 ml.) containing the added phenols was heated on the water bath for 1 hr. under reflux with 10 N-H_2SO_4 (5 ml.). The mixture was cooled and extracted with ether for 4 hr. Catechol and quinol in the extract were then determined as in the preceeding paragraph. Under these conditions 80% of the added phenols were recovered.

The above method was now applied to urines of rabbits receiving benzene or phenol orally. With benzene urines erratic results were obtained due apparently to the other metabolites in such urines. The results were more erratic for catechol than for quinol, for we found that benzene urine contained hydroxyquinol which was precipitated with catechol during the lead separation. Our results with benzene are therefore not worth quoting.

Some results with administered phenol are given in Tables 11 and 12. It should be noted that no free catechol or quinol was excreted. These tables show that the main oxidation product of phenol is quinol. After a single dose of phenol, quinol is excreted, but not catechol. With continuous feeding of phenol about 5% of the dose appears as conjugated quinol, with traces only of catechol. These results confirm the earlier observations of Baumann & Preusse (1879), who isolated quinol from the hydrolyzed urine of a dog whose skin had been painted with phenol twice a day for 6 days, and from the urine of a dog poisoned with phenol; catechol was only detected in small amounts. Baumann & Preusse also detected quinol in the urine of dogs receiving

Table 11. *The excretion of combined catechol and quinol by a rabbit receiving daily doses (250 mg./kg.) of phenol*

Day	Dose (g.)	Volume of urine (ml.)	Catechol (% of the day's dose)	Catechol (% of the total fed)	Quinol (% of the day's dose)	Quinol (% of the total fed)
1	0·727	60	0	0	2·3	2·3
2	0·721	138	0	0	7·6	4·95
3	0·717	84	Traces	0	Present*	?*
4	0·710	115	0	0	5·1	5·0†
5	0·712	140	0	0	4·8	4·95†

* Result unreliable. † Excluding day 3.

Table 12. *The excretion of catechol and quinol by a rabbit receiving a single oral dose (200 mg./kg.) of phenol*

Day	Dose (g.)	Percentage of the dose excreted as: Catechol Free	Catechol Combined	Quinol Free	Quinol Combined
1	0·62	0	0	0	1·0
2	Nil	0	0	0	0
3	Nil	0	0	0	Trace

doses of potassium phenylsulphate, but in lesser amounts than after phenol, the phenylsulphate being more rapidly excreted than phenol. It should also be noted (see Tables 11 and 12) that continuous dosing with phenol gives rise to the excretion of larger amounts of quinol than does a single dose.

Muconic acid

If the ring of benzene undergoes fission in the body to muconic acid it should give rise to the *cis-cis* form, but the isomer which has been isolated from benzene urines in the past has always been the *trans-trans* form (see Williams, 1947). The amount of muconic acid formed from benzene is small and uncertain so we attempted to find a method for estimating this acid in urine. The occurrence of a conjugated double bond system in the acid immediately suggested that it might be estimated spectroscopically. We have, therefore, examined the ultraviolet absorption spectra of the two isomers (see Fig. 6).

cis-cis-Muconic acid, m.p. 175°, was prepared according to Böeseken & Kerkhoven (1932). Its absorption spectrum has not been previously recorded. In ethanol it shows one absorption band with $\lambda_{max.}$ 258·5 mμ. and $\epsilon_{max.}$ 19,880.

trans-trans-Muconic acid, m.p. 296°, was prepared according to Ingold (1921). Its ultraviolet absorption spectrum in ethanol has been previously recorded by Hausser, Kuhn, Smakula & Deutsch (1935) who give $\lambda_{max.}$ at 261–263 mμ., but $\epsilon_{max.}$ was not quoted. In ethanol we found one band with $\lambda_{max.}$ 259 mμ. and $\epsilon_{max.}$ 27,100 and in 2% NaOH the band was at 261 mμ. and $\epsilon_{max.}$ 24,600. Thus both forms of muconic acid show maximum absorption at the same wavelength, but the *trans-trans* isomer absorbs more strongly than the *cis-cis* isomer (cf. *cis-* and *trans-*cinnamic acids; Smakula & Wassermann, 1938). It is possible that during isolation from urine the *cis-cis* acid may be converted to the *trans-trans* acid (cf. Drummond & Finar, 1938). We have investigated the possibility that this change may be brought about by heat, our criterion of change being alteration in the extinction at 259 mμ. We found, however, that the *cis-cis* acid in ethanolic solution (0·025%) was not converted to the *trans-trans* acid by heat alone.

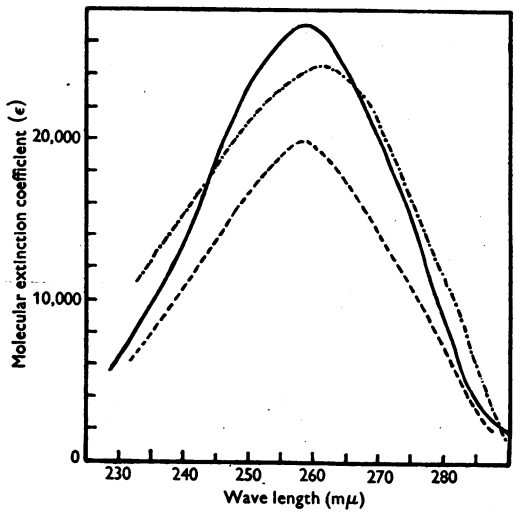

Fig. 6. Spectral absorption of *cis-cis-* and *trans-trans-*muconic acids. —— *trans-trans*-Muconic acid in ethanol; $\lambda_{max.}$, 259 mμ., $\epsilon_{max.}$ 27,100; ·—· *trans-trans*-muconic acid in 2% NaOH; $\lambda_{max.}$ 261 mμ., $\epsilon_{max.}$ 24,600; - - - - *cis-cis*-muconic acid in ethanol; $\lambda_{max.}$ 258·5 mμ., ϵ_{max} 19,880.

The determination of trans-trans-*muconic acid in pure solution and in urine. trans-trans*-Muconic acid is adsorbed by an alumina column from aqueous solution and it can be eluted by 2% NaOH. The amount of the acid in the eluate

can be determined spectrographically. For example, 2·5 mg. muconic acid in 200 ml. water were completely adsorbed by a column of alumina (Savory and Moore Ltd.) 20 × 1·25 cm. After washing the column with 100 ml. water, the muconic acid was completely eluted by 90 ml. 2% NaOH, and the muconic acid content of the eluate was determined spectrographically. In 8 experiments using 2·5 mg. of the acid in 200 ml. water the following recoveries were obtained; 100, 86, 96, 96, 100, 109, 92 and 100%. With shorter columns the muconic acid was not completely adsorbed. The same experiment was repeated with muconic acid added to normal rabbit urine at varying pH, but on eluting the column with 2% NaOH it was found that some constituent of the urine, which absorbed all light of wave lengths less than 300 mμ., was also eluted. Although much time was spent on this problem, it finally had to be abandoned.

DISCUSSION

In Table 13 the results quoted in preceding tables have been averaged and summarized. With benzene there is a considerable scatter in the glucuronide figures which vary from 4–22%. The figures for ethereal sulphate (5·8–10·7%, with one extreme of 16·8%) and phenol (6·3–9·7%, with one extreme of 17%) are more consistent. This scatter may in part be due to the volatility of benzene, for there is little doubt that a large and variable amount of the benzene fed escapes unchanged through the lungs. Although we have not yet investigated the elimination of unchanged benzene we suspect that the amount thus eliminated may be as high as 80% of the dose. The scatter in the glucuronide figures is also partly due to the quantitative method being less accurate than the methods for ethereal sulphate and phenol. We can, however, use the averaged figures of Table 13 for the purposes of discussion.

total phenols that 25% of injected benzene was oxidized to phenols in the rabbit. We have also found that the phenols excreted are almost entirely conjugated. Small amounts of free phenol but no free catechol, quinol and hydroxyquinol were found. Concerning the amounts of the latter phenols excreted it appears that together they amount to about 11% of the dose of benzene, but we were unable to estimate each phenol separately for reasons already mentioned. The qualitative results of the following paper (Porteous & Williams, 1949), however, indicate that catechol and quinol are probably excreted in roughly equal amounts, whereas hydroxyquinol excretion is about a quarter that of catechol. A very rough assessment of the amounts of each phenol excreted would be phenol 10, catechol 4–5, quinol 4–5 and hydroxyquinol about 1% of the dose of benzene.

With reference to phenol itself it is clear that its main metabolites are phenylglucuronide and phenylsulphuric acid which together account for 75% of the phenol fed. Furthermore, we found that only traces of free phenol were excreted, a result which is contrary to that found by Deichmann (1944), who reports that half of the phenol excreted in the urine of rabbits receiving phenol orally is in the free state. That our results are correct is supported by the fact that the total conjugation is 89% which in experiments of this type represents an almost quantitative recovery of the phenol fed. The difference between the total conjugation and total phenol amounts to 14% (see Table 13). This figure, which is necessarily only approximate, indicates that phenol is converted to a small extent into other phenols which, according to the early work of Baumann & Preusse (1879), are

Table 13. *The excretion of metabolites by rabbits receiving benzene or phenol orally (averaged results)*

Compound fed	Dose (mg./kg.)	Percentage of dose excreted as: Ethereal sulphate	Glucuronide	Total phenol	Total conjugates	Other phenols (i.e. total conjugates – total phenols)
Benzene	500	9·5	11·2	9·2	20·7	11·5*
Phenol	125–250	19†	70	75	89	14‡

* Catechol, quinol and hydroxyquinol. † Value quoted from Williams (1938). ‡ Mainly quinol.

This table shows that about 20–21% of the benzene fed is excreted as conjugated sulphates and glucuronides, the ratio glucuronide/ethereal sulphate being roughly unity. About one-half of these conjugates contains phenol and the other half contains catechol, quinol and hydroxyquinol (Porteous & Williams, 1949). Thus about 20–21% of the administered benzene is oxidized to phenols, a figure which agrees with the results of Braunstein, Parschin & Chalisowa (1931), who found by direct estimation of mainly quinol with small amounts of catechol. In fact Baumann & Preusse isolated quinol from the urine of a dog which had had phenol rubbed into its skin. Our results on the determination of catechol and quinol in phenol urine indicate that at least 5% of the phenol fed is excreted as quinol conjugates. Whether or not phenol is oxidized to catechol in the higher animals has yet to be proved. In certain phenol-utilizing micro-organisms, however, phenol is oxidized to catechol (Evans, 1947).

SUMMARY

1. A quantitative study has been made of the excretion of phenol, glucuronic acid and ethereal sulphate by rabbits receiving oral doses of benzene or of phenol.

2. A new colorimetric method for the estimation of free and combined phenol in urine has been studied. This method is based on steam distillation of phenol from urine and determination of phenol in the distillate by means of the blue colour with 2:6-dichloroquinonechloroimide under specified conditions.

3. Normal human urine contains about 5–10 mg. of total phenol/l. in a conjugated form, whereas rabbit urine contains 2–6 mg. free and 10–13 mg. total phenol/l.

4. The results indicate that about 21 % of administered benzene is excreted as phenols, 9·2 % as phenol and 11·5 % as other phenols; 9·5 % of the benzene fed is excreted as ethereal sulphates and 11·2 % as glucuronides. Only traces of free phenol are excreted.

5. When phenol is fed to rabbits it is largely excreted as phenylglucuronide and phenylsulphuric acid, but some is oxidized to quinol which is excreted conjugated. Only small amounts of free phenol are excreted.

6. Attempts to estimate catechol and quinol in benzene urine were unsuccessful because of interference by hydroxyquinol, a metabolite of benzene.

7. The absorption spectra of *cis-cis-* and *trans-trans-*muconic acid are recorded. These acids can be estimated in pure solution spectrographically, but the method fails with urine.

The expenses of this work were in part defrayed by a grant from the Medical Research Council.

REFERENCES

Baernstein, H. D. (1945). *J. biol. Chem.* **161**, 685.
Baumann, E. & Preusse, C. (1879). *Hoppe-Seyl. Z.* **3**, 156.
Baylis, J. R. (1928). *J. Amer. Wat. Wks Ass.* **19**, 597.
Bernhard, K. & Gressley, E. (1941). *Helv. chim. Acta*, **24**, 83.
Böeseken, J. & Kerkhoven, C. L. M. (1932). *Rec. Trav. chim. Pays-Bas*, **51**, 964.
Booth, J. & Boyland, E. (1947). *Biochem. J.* **41**, xxix.
Boyland, E. & Levi, A. A. (1935). *Biochem. J.* **29**, 2678.
Boyland, E. & Levi, A. A. (1936). *Biochem. J.* **30**, 728, 1225.
Boyland, E. & Wolf, G. (1948). *Biochem. J.* **42**, xxxii.
Braunstein, A. E., Parschin, A. N. & Chalisowa, O. D. (1931). *Biochem. Z.* **235**, 311.
Clarke, W. M. (1928). *Determination of Hydrogen Ions*, p. 215. London: Baillière, Tindall and Cox.
Day, A. R. & Taggard, W. T. (1928). *Industr. Engng Chem.* **20**, 545.
Deichmann, W. B. (1943). *Fed. Proc.* **2**, 77.
Deichmann, W. B. (1944). *Arch. Biochem.* **3**, 345.
Drummond, J. C. & Finar, I. L. (1938). *Biochem. J.* **32**, 79.
Evans, W. C. (1947). *Biochem. J.* **41**, 373.
Fearon, W. R. (1944). *Biochem. J.* **38**, 399.
Gibbs, H. D. (1927). *J. biol. chem.* **72**, 649.
Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.
Hausser, K. W., Kuhn, R., Smakula, A. & Deutsch, A. (1935). *Z. phys. Chem.* **29** B, 383.
Ingold, C. K. (1921). *J. chem. Soc.* **119**, 951.
Jaffe, M. (1909). *Hoppe-Seyl. Z.* **62**, 58.
Kolthoff, I. M. & Vlesschouwer, J. J. (1927). *Biochem. Z.* **189**, 191.
Lehmann, K. B., Gundermann, S. O. & Kleiner, R. (1910). *Arch. Hyg., Berl.*, **72**, 307.
Munk, I. (1876). *Pflüg. Arch. ges. Physiol.* **12**, 146.
Nencki, M. & Giacosa, P. (1880). *Hoppe-Seyl. Z.* **4**, 325.
Nencki, M. & Sieber, N. (1883). *Pflüg. Arch. ges. Physiol.* **31**, 319.
Oglesby, F. L., Sterner, J. H. & Anderson, B. (1947). *J. industr. Hyg.* **29**, 60.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 56.
Schmiedeberg, O. (1881). *Arch. exp. Path. Pharmak.* **14**, 288.
Schultzen, O. & Naunyn, B. (1867). *Arch. Anat. Physiol., Lpz.*, p. 349.
Scudi, J. V. (1941). *J. biol. chem.* **139**, 707.
Smakula, A. & Wassermann, A. (1938). Cited by R. A. Morton in *Practical Aspects of Absorption Spectrophotometry*, p. 41. London: Institute of Chemistry.
Thierfelder, H. & Klenk, E. (1924). *Hoppe-Seyl. Z.* **141**, 29.
Williams, R. T. (1938). *Biochem. J.* **32**, 878.
Williams, R. T. (1947). *Detoxication Mechanisms*, p. 38. London: Chapman and Hall Ltd.
Witter, R. F. (1945). *Univ. Microfilms Ann Arbor*, **6**, no. 1, p. 21.
Young, L. (1947). *Biochem. J.* **41**, 417.
Zbarsky, S. H. & Young, L. (1943). *J. biol. Chem.* **151**, 487.