SUMMARY

1. The fate of phenacetin has been studied in the rabbit and it has been found to be largely transformed into *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, these occurring in the urine in the ratio 6·9 : 1. The major metabolic change undergone by phenacetin is, therefore, de-ethylation, followed by conjugation.

2. Deacetylation of phenacetin takes place only to a very minor extent. This was shown by the detection of traces of free *p*-phenetidine in the urine, and the isolation of minute amounts of a crystalline complex of *p*-toluidine and ammonium glucuronate derived from a labile glucuronide which is a major metabolite of *p*-phenetidine in the rabbit.

3. Acetanilide is also slightly deacetylated, for acetanilide urine also contains traces of a labile glucuronide which is a major metabolite of aniline (see Smith & Williams, 1949*a*).

4. The results obtained have been correlated with the known therapeutic and toxic effects of phenacetin and acetanilide.

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REFERENCES

Baccarani, U. (1900). *Jber. Fortschr. Tierchem.* **30**, 610.
Gaddum, J. H. (1944). *Pharmacology*, p. 144, 2nd ed. London: Oxford University Press.
Hinsberg, O. & Kast, A. (1887). *Jber. Fortschr. Tierchem.* **17**, 60.
Hinsberg, O. & Treupel, G. (1894). *Arch. exp. Path. Pharmak.* **33**, 216.
Krebs, H. A., Sykes, W. O. & Bartley, W. C. (1947). *Biochem. J.* **41**, 622.
Mahnert, F. (1888). *Dtsch. med. Wschr.* **14**, 1027.
Mörner, K. A. H. (1889). *Jber. Fortschr. Tierchem.* **19**, 80.
Müller, F. (1888). *Jber. Fortschr. Tierchem.* **18**, 149.
Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 538.
Smith, J. N. & Williams, R. T. (1949*a*). *Biochem. J.* **44**, 242.
Smith, J. N. & Williams, R. T. (1949*b*). *Biochem. J.* **44**, 250.

Studies in Detoxication

23. THE FATE OF ANILINE IN THE RABBIT

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In the past numerous investigations (see summary by Gross, 1946) have been carried out on the fate of acetanilide in the body, but few on aniline. This is probably the result of the belief that aniline and acetanilide are interconvertible *in vivo*. We have now found that in the rabbit this is not wholly true. In this animal acetanilide is almost entirely converted into the glucuronide and ethereal sulphate of *p*-acetamidophenol, the excretion of compounds containing free diazotizable amino groups being about 6–7 % of the dose (Smith & Williams, 1948*a*). Furthermore, *p*-substituted acetanilides are either excreted completely unchanged or deacetylated only to a very small extent (Smith & Williams, 1948*b*, 1949*a*). In this paper we shall show that aniline gives rise to metabolites different from those of acetanilide.

The recent work of Krebs, Sykes & Bartley (1947) has shown that the extent of deacetylation of the aromatic acetamido group depends on animal species, deacetylation being extensive in the cat, dog and pigeon, but very small in man and the rabbit. From this it follows that aniline and acetanilide are likely to give rise to similar metabolites in animals such as the dog and cat, but to different ones in man and the rabbit.

Earlier work on the fate of aniline appears to be very scanty. Müller (1887) studied a human case of poisoning by 25 g. of aniline, and found that the urine reduced Fehling's solution and contained conjugated *p*-aminophenol. Schmiedeberg (1878) fed dogs with aniline acetate and identified *p*-aminophenol in the urine after acid hydrolysis. According to Elson, Goulden & Warren (1946) rats probably excrete aniline as the ethereal sulphate of *p*-aminophenol. It is clear from this earlier work that conjugated *p*-aminophenol is a metabolite of aniline, but we shall show that in the rabbit this phenol is not a major metabolite.

The study of the metabolic fate of aniline is not only of considerable theoretical interest, but it is also important because of the possible role of aniline as a bladder carcinogen (for discussion see Goldblatt, 1947).

I. QUANTITATIVE INVESTIGATION OF TYPES OF COMPOUNDS EXCRETED

EXPERIMENTAL

Glucuronic acid and ethereal sulphate in urine were determined as described in earlier papers from this laboratory (e.g. Smith & Williams, 1948*a*).

Diazo test. Throughout this paper this term means diazotization with 1% $NaNO_2$ and dilute HCl, then addition of 1% ammonium sulphamate followed by coupling with 1% ethanolic 1-naphthyldimethylamine.

Free aromatic amino groups were determined using a modification of the Bratton & Marshall (1939) procedure for sulphanilamide. Three compounds, namely, aniline hydrochloride and *o*- and *p*-aminophenylglucuronides (Williams, 1943) were used as standards. If a dilute solution of any of these three compounds is diazotized and coupled with *N*-(1-naphthyl)ethylenediamine as in the Bratton & Marshall procedure a red (aniline and *o*-aminophenylglucuronide) or red-purple (*p*-aminophenylglucuronide) colour develops gradually and reaches a stable intensity in 24 hr. A similar slow rate of colour development was also found when 1-naphthyldimethylamine and sulphatoethyltoluidine were used as coupling agents, but they were not as sensitive as naphthylethylenediamine. In order to obtain reproducible results diazotization had to be prolonged for 10 min. at 20° before adding the coupling agent, and furthermore it was found that the final colour intensity depended on the concentration of acid in the solution, a point which had to be considered in the construction of calibration curves for 'free' and 'total' amino groups.

The procedure finally adopted was as follows. The urine was diluted so as to contain an equivalent of 1–8 mg./l. of aniline: 10 ml. of diluted urine were mixed with 0·5 ml. 4N-HCl followed by 1 ml. 0·1% $NaNO_2$ and allowed to stand 10 min. at room temperature, then 1 ml. of 0·5% ammonium sulphamate was added followed 2 min. later by 1 ml. of 0·1% aqueous *N*-(1-naphthyl)ethylenediamine hydrochloride. After mixing, the solution was left for 24 hr., then made up to 25 ml. with water, and the colour measured with a Spekker photoelectric absorptiometer using an Ilford no. 605 yellow-green filter. The readings were then referred to calibration curves constructed in the same way, but using standard aqueous solutions of aniline hydrochloride, *o*- or *p*-aminophenylglucuronide instead of urine. The *o*-glucuronide gives the same red colour as does aniline the colour ratio *o*-glucuronide/aniline being 1·04. The *p*-glucuronide gives a slightly different colour (red-purple), and using the same filter (no. 605) the colour ratio *p*-glucuronide/aniline = 0·84. In this procedure the free *o*- and *p*-aminophenols do not yield interfering colours, and if present in urine are not estimated.

'Total' aromatic amino groups were estimated by the same general procedure. In this case 10 ml. of diluted urine were mixed with 1·5 ml. 4N-HCl and heated for 1 hr. in a boiling water bath. After cooling the solution, the procedure was as indicated above, except that the absorptiometer readings were referred to calibration curves constructed using 1·5 ml. 4N-HCl instead of 0·5 ml. By this procedure acetanilide in pure solution could be determined almost quantitatively. Under the hydrolysis conditions used, *o*-aminophenylglucuronide is only hydrolyzed to a slight extent, for the colour ratio *o*-aminophenylglucuronide/aniline changes only from 1·04 to 0·91. With *p*-aminophenylglucuronide hydrolysis of the glycuronidic link proceeds further for the colour ratio *p*-aminophenylglucuronide/aniline changes from 0·84 to 0·59.

Owing to the variable extents of hydrolysis of the conjugated metabolites encountered in the urines examined, the estimations of 'total' aromatic amino groups are not easily interpreted, and, therefore, are quoted (in Table 1) with reserve. The estimations of 'free' aromatic amino groups, however, are more capable of interpretation.

RESULTS

The excretion of conjugated glucuronic acid and ethereal sulphates and of diazotizable amino groups by rabbits receiving oral doses of aniline, phenetidine, *o*- and *p*-aminophenols and their *N*-acetyl derivatives are given in Table 1.

The results for the acetylated compounds are more easily interpreted than those for the free amino compounds because the qualitative results (Smith & Williams, 1948*a*, 1949*a*) show that the metabolism of the acetyl derivatives is the less complicated; they do not undergo deacetylation to any great extent.

For the acetyl compounds the percentage of total diazotizable amino groups (Table 1, column 7) is expected to be only slightly less than the percentage of glucuronide (column 3), for the following reason.

Table 1. *The excretion of conjugated glucuronic acid and sulphate and of diazotizable amino groups by rabbits receiving aniline and its derivatives orally (averaged results)*

		Percentage excreted				
					Containing diazotizable amino groups	
Compound fed	Dose (mg./kg.)	As glucuronide (*G*)	As ethereal sulphate (*E*)	*G*/*E*	Free	After acid hydrolysis
Aniline	200	70	28	2·5	40*	60*
p-Ethoxyaniline (phenetidine)	300	120	30	4·0	3†	25†
p-Aminophenol	250	43	18	2·5	28†	45†
o-Aminophenol	210	(28)‡	25	1·1	33§	42§
Acetanilide	250	70	12	5·8	6,* 7†	37,* 61†
p-Ethoxyacetanilide (phenacetin)	350	48	7	6·9	0†	49†
p-Acetamidophenol	280	63	10	6·3	0†	49†
o-Acetamidophenol	280	39	20	2·0	3·5§	33§

* Aniline as standard. † *p*-Aminophenylglucuronide as standard.
‡ This figure will be too low because *o*-aminophenylglucuronide cannot be estimated by the naphthoresorcinol method.
§ *o*-Aminophenylglucuronide as standard.

Qualitative results show that the main glucuronide excreted after administration of acetanilide, phenacetin and *p*-acetamidophenol is *p*-acetamidophenylglucuronide and the ethereal sulphate is *p*-acetamidophenylsulphuric acid. Under the conditions of hydrolysis for the estimation of total amino groups the ethereal sulphate is completely hydrolyzed to *p*-aminophenol which gives no colour in the estimation procedure; the glucuronide, however, is deacetylated to *p*-aminophenylglucuronide which is only slightly hydrolyzed to *p*-aminophenol. Thus the amount of *p*-aminophenylglucuronide produced by hydrolysis, and estimated by diazotization and coupling should be only slightly less than the amount of *p*-acetamidophenylglucuronide estimated by the naphthoresorcinol reaction in the unhydrolyzed urine.

An examination of the figures for *p*-aminophenol suggests that both *p*-acetamido- and *p*-aminophenylglucuronide are its metabolites, because the figure for the total diazotizable amino groups (45 %) is almost identical with the figure for glucuronide output (43 %), and there is an increase in diazotizable amino groups on acid hydrolysis from 28 to 45 %. This increase must be due to hydrolysis of the *p*-acetamido- to the *p*-amino-glucuronide, because hydrolysis of the ethereal sulphate will give *p*-aminophenol which has no effect on the diazo reaction.

In the case of *o*-aminophenol, the total glucuronide formed is probably more accurately given by the figure for the total amino groups (42 %); the figure of 28 % obtained by the naphthoresorcinol method is definitely too low because Hanson, Mills & Williams (1944) have shown that *o*-aminophenylglucuronide is not completely hydrolyzed under the conditions of their method for estimating glucuronic acid.

II. THE ISOLATION OF ANILINE METABOLITES

Experimental

1. *The effects of aniline on rabbits*

Each dose (*c.* 0·5 g./kg. in most cases) was dissolved in 20 ml. water containing just enough HCl to give a clear solution. The amount of HCl used was less than that required to form the hydrochloride and this solution was better tolerated than aniline hydrochloride. Single doses of 1·3 g./kg. were not fatal in this form, but doses of 0·3 g./kg. repeated daily for 4 days were fatal. Aniline had no narcotic effect similar to that of acetanilide (Smith & Williams, 1948*a*).

2. *The nature of aniline urine*

The urine had a dark colour but a normal pH of 8. This colour did not appear to be due to porphyrins, for the urine under ultraviolet light showed not a red but a pale green fluorescence. The urine readily reduced Benedict's solution and the reducing substance was not glucose (osazone test). No reducing substance could be extracted by ether, butanol or amyl alcohol at different values of pH.

The urine from two rabbits, each of which had received 3 g. of aniline, was found to contain 4·4 g. extra glucuronic acid (estimated by the method of Hanson *et al.* 1944). By titration with Benedict's quantitative reagent the urine was found to contain 3·8 g. of reducing material, calculated as glucuronic acid. From this it can be deduced that aniline urine contains at least two glucuronides, one reducing and present in large amount, and the other non-reducing. The urine gave a strong red colour in the diazo test thus showing the presence of free aromatic amino groups. The Tollens test for glucuronic acid was given very readily, for a deep blue precipitate was formed even before the mixture had reached boiling point. These tests suggested that aniline urine may contain a very labile, reducing glucuronide. Neither the urine itself nor ether extracts of it gave any colour with $FeCl_3$ and from this it could be concluded that free aminophenols were not present. The urine was slightly laevorotatory; filtered and clarified with a few drops of HCl it gave α_D −0·18° in a 1 dm. tube. From a knowledge of the extra glucuronic acid content (0·8 g./100 ml.) of this urine and assuming that the glucuronide present is *p*-aminophenylglucuronide α_D for 1 dm. should be −0·64° (see Williams, 1943). Thus the conclusion can be drawn that the main glucuronide is not that of *p*-aminophenol.

3. *Extraction of aniline urine with ether. Isolation of unchanged aniline*

(*a*) *At acid reaction.* Continuous ether extraction of acidified aniline urine yielded no material derived from aniline.

(*b*) *At alkaline reaction.* A 24 hr. urine (1200 ml.), collected after feeding 12 g. aniline, was treated with a few ml. of 2N-KOH and extracted continuously with ether for 1 hr. The extract, dried over Na_2SO_4, was evaporated, leaving 0·42 g. of a dark oil which was identified as aniline by preparing from it tribromoaniline (m.p. and mixed m.p. 117–118°) and benzanilide (0·76 g.; m.p. and mixed m.p. 160°). In other experiments the dried ether extract was treated with a saturated ethereal solution of oxalic acid and the crystalline precipitate of aniline hydrogen oxalate (m.p. 158°; Anselmino (1903) gives m.p. 163°) collected, dried and weighed. The recoveries of free aniline in five experiments were 3, 5, 6, 9 and 9·5 % (average 6·5 %) of the dose. Neither acetanilide nor aminophenol was found.

(*c*) *The origin of the free aniline.* The aniline isolated could either be free, unchanged aniline or it could arise by decomposition of a labile precursor. That the second possibility was unlikely was shown as follows: 12 g. of aniline were fed to six rabbits and the urine (950 ml.) collected for 24 hr. To 350 ml. of the urine were added 10 g. of Na_2CO_3 and the whole was extracted with ether for 4 hr. On addition of ethereal oxalic acid to this extract there was obtained 0·8 g. of aniline hydrogen oxalate. Another 350 ml. of the urine was treated in a similar manner except that, before ether extraction, the alkaline urine was boiled vigorously for 10 min. The yield of aniline hydrogen oxalate was again 0·8 g. In another experiment 400 ml. of aniline urine were made alkaline and exhaustively extracted with ether to remove free aniline. To the urine were now added 40 ml. of conc. HCl and the whole was boiled for 20 min. The hydrolyzed urine was cooled, neutralized with 40 % NaOH,

made alkaline with solid Na_2CO_3 and extracted exhaustively with ether. Although this extract contained aminophenols (see below) it contained no trace of aniline.

These experiments show that the aniline present in the urine probably occurs as such for there is no increase in the amount present when the urine is heated with acid or alkali.

4. *The ethereal sulphate fraction of aniline urine. Isolation of* o- *and* p-*aminophenols and detection of* 4-*aminoresorcinol*

(*a*) *Mild hydrolysis of aniline urine.* Aniline urine (400 ml. from 5·8 g. aniline) was made alkaline with a little 2N-NaOH and the free aniline removed with ether as described above. It was then acidified with 40 ml. conc. HCl and boiled for 20 min. After cooling, it was neutralized with NaOH, made alkaline with 1 g. solid Na_2CO_3 and continuously extracted with ether for 1·5 hr. The extract was reduced to 10 ml. and the crystals (60 mg.) which separated collected: they had m.p. 180°, gave a purple colour with $FeCl_3$ and were identified as *p*-aminophenol by conversion to *ON*-dibenzoyl-*p*-aminophenol, m.p. and mixed m.p. 230° (yield, 0·9% of the dose). The filtrate was reduced to 0·5 ml. on the water bath and on cooling 50 mg. of yellow plates separated. These were collected and found to give a red colour with $FeCl_3$ and an intense yellow colour with nitrous acid, but no immediate red colour in the diazo test. They were identified as *o*-aminophenol by conversion into *ON*-dibenzoyl-*o*-aminophenol, m.p. and mixed m.p. 178–179° (yield, 0·7% of the dose). The residue, which remained after separation of the *o*- and *p*-aminophenols, gave colour reactions for 4-aminoresorcinol, for on shaking in air in the presence of NaOH it gave an intense blue colour which on standing faded to green and finally (1 hr.) to brown (Henrich & Wagner, 1902).

We prepared 4-aminoresorcinol hydrochloride by the method of Henrich & Wagner (1902) and carried out trial separations of the three phenols, but we failed to isolate the labile 4-aminoresorcinol from aniline urine in pure crystalline form. When fed to a rabbit, 4-aminoresorcinol (0·5 g.) causes the excretion of a urine which is almost black in colour.

(*b*) *Acetone-ammonium sulphate fractionation of aniline urine.* A total of 8 g. aniline was fed to four rabbits. The 24 hr. urine (800 ml.) was evaporated *in vacuo* at 40–45° to 100 ml. The concentrate was acidified with a little HCl, saturated with $(NH_4)_2SO_4$ and then shaken with 250 ml. acetone. The ethereal sulphates present, together with some glucuronides, passed into the acetone layer. The main bulk of the labile glucuronide (see p. 247) and other material reacting with naphthoresorcinol remained in the aqueous layer. The acetone extract was made alkaline with solid K_2CO_3 and concentrated to a small volume *in vacuo* at 40–50°. Dry acetone was now added and a small flocculent glucuronide-containing precipitate which rapidly turned into gum was deposited on standing. After removal of this gum, the acetone solution gave only a weak naphthoresorcinol reaction and contained no inorganic sulphate, but gave strong tests for ethereal sulphate. It was taken to small bulk *in vacuo*. Attempts to crystallize the organic sulphate or prepare crystalline derivatives such as benzylamine salts were unsuccessful. The concentrate gave strong tests for free amino groups and ethereal sulphates, but did not reduce ammoniacal $AgNO_3$. It was, therefore, acidified with 0·1 vol. conc. HCl and boiled for 10 min. to hydrolyze the ethereal sulphates and then diluted to 50 ml. with water. The solution now reduced ammoniacal $AgNO_3$ and gave an intense blue colour on shaking in air with NaOH solution, indicating the presence of 4-aminoresorcinol. The main bulk of the hydrolyzed solution was extracted with 2×100 ml. ether. Evaporation of the ether left a small amount of dark tar which gave no diazo or $FeCl_3$ test. The residual solution was, therefore, made alkaline with solid Na_2CO_3 and extracted with 2×100 ml. ether. From this extract *p*-aminophenol (3·1% of the dose) and *o*-aminophenol (0·3% of the dose) were isolated and identified as before.

The residue after separation of these two phenols left on evaporation a small tarry residue giving a faint red colour in the diazo test. On shaking the tar in air with NaOH solution an intense blue colour was obtained which faded to brown in about an hour. 4-Aminoresorcinol was thus present, but attempts to obtain crystalline derivatives were unsuccessful.

5. *The glucuronide fraction of aniline urine*

(*a*) *Preparation of the glucuronide gum.* 12 g. of aniline were fed to eight rabbits and the urine was collected for 24 hr. Free aniline (3% of the dose) was removed with ether as described in section 3 (*b*). The alkaline urine was now made faintly acid and lead acetate added until no further precipitation occurred. The precipitate was removed and the filtrate was neutralized with ammonia and an excess of saturated basic lead acetate added. The precipitate was filtered, washed with water, and then suspended in water and Pb removed with H_2S. The Pb-free filtrate was dried *in vacuo* at 40–50° to a reddish gum (24 g.) which was purified by dissolving in the minimum of water and pouring into ethanol. A precipitate of inorganic material separated and after filtration the solution was concentrated *in vacuo* at 40–50° to a gum which reduced Benedict's solution on warming, gave a weak red colour in the diazo test, and reacted very rapidly in the Tollens test for glucuronic acid. The gum could not be induced to crystallize and its potassium, brucine, *o*-toluidine and benzylamine salts separated from ethanol as flocculent precipitates which readily formed gums. It contained no ether-soluble material.

(*b*) *Isolation of* p-*aminophenol from the gum.* The purified glucuronide gum obtained after feeding 5 g. of aniline was dissolved in 150 ml. 5N-H_2SO_4, filtered and then boiled for 2·5 hr. The dark hydrolysate, after cooling and making alkaline, was extracted for 3 hr. with ether which on evaporation left a crystalline residue from which 150 mg. (5% of the dose) of *p*-aminophenol, m.p. 182–184°, were obtained. No other phenol was identified in the hydrolysate.

(*c*) *Methylation and acetylation of the gum. Isolation of* p-*acetamidophenyltriacetylglucuronide methyl ester.* Methylation of the gum with dimethyl sulphate and alkali followed by Ag_2O and methyl iodide yielded no crystalline product. A similar result was obtained on acetylation of the gum with pyridine and acetic anhydride. A crystalline derivative was, however, obtained by the following procedure: 2 g. of the gum dissolved in 10 ml. absolute ethanol were treated with an excess of diazomethane in 100 ml. dry ether; a small precipitate appeared. After standing for 24 hr. at room temperature the precipitate had partly dissolved again

and the whole was evaporated *in vacuo*. The product, a brown gum which would not crystallize, was dissolved in 30 ml. of a pyridine-acetic anhydride mixture (1:1) and kept overnight. It was then poured into 200 ml. water and the solution was extracted with chloroform. The extract, washed free of acetic acid with water, gave on evaporation a gum which crystallized from acetone-water mixtures as long needles, m.p. 100° (resolidifying and then melting again indefinitely at 170–180°). This compound appears to be a dimorphic *p*-acetamidophenyltriacetylglucuronide methyl ester (yield 70 mg.; 1% of the dose). On recrystallization from absolute ethanol it yielded needles, m.p. 205°, not depressed by an authentic sample (Smith & Williams, 1948*a*). It showed $[\alpha]_D^{20°}$ −22·4° (*c*, 4 in chloroform). (Found: C, 54·3; H, 5·5. Calc. for $C_{21}H_{25}O_{11}N$: C, 54·0; H, 5·4%.) In another experiment 0·6% of the dose was isolated as this ester.

The two methyl esters, m.p.'s 100 and 205°, have been described by us in an earlier paper (Smith & Williams, 1948*a*) and we suggested that the former was a hydrate of the latter, but we only quoted analyses for the latter compound. We now find that both give analyses correct for the anhydrous compound and show identical specific optical rotations in chloroform. The ester of m.p. 100° gave the following analysis: Found: C, 53·6; H, 5·65; N, 3·2. Calc. for $C_{21}H_{25}O_{11}N$: C, 54·0; H, 5·4; N, 3·0%. $[\alpha]_D$ −22·1° (*c*, 7 in chloroform). We conclude, therefore, that we have here a case of dimorphism. Neither of the two forms obtained from the aniline gum depressed the m.p.'s of the corresponding authentic forms prepared synthetically from *p*-aminophenylglucuronide.

Our results so far indicate that the gum contains either *p*-aminophenylglucuronide or *p*-acetamidophenylglucuronide, or both, but the small yields of the above derivatives suggest that these glucuronides are not major metabolites.

(*d*) *Isolation of* p-*aminophenylglucuronide*. The evidence presented on the nature of the glucuronide gum suggests that it contains at least two glucuronides, viz. large amounts of a labile, reducing glucuronide and the non-reducing *p*-aminophenylglucuronide. Since the ethereal sulphate fraction contains *o*-aminophenol, it is also possible that the gum may contain *o*-aminophenylglucuronide. Attempts were, therefore, made to fractionate the gum.

At first advantage was taken of the fact that the aminophenylglucuronides have isoelectric points where they show minimum solubility. It was found that the *o*-compound had a minimum solubility at *c*. pH 3·0–3·5 and the *p*-compound at *c*. pH 4·0–4·5. Thus the *p*-compound should crystallize best at about pH 4·0–4·5.

Aniline urine (140 ml.) clarified with 50 ml. colloidal iron was evaporated *in vacuo* to 70 ml. then buffered to pH 4·3 and seeded with *p*-aminophenylglucuronide. No precipitate appeared after 5 days at 0°.

The basic lead acetate precipitate from aniline urine after feeding 10 g. of aniline was prepared in the usual manner and Pb removed with H_2S. The filtrate (350 ml.) from the PbS had pH 2·3. A 50 ml. portion of this filtrate was brought to pH 4·3 with acetate buffer, but no precipitate was obtained after 2 days at 0°. The rest of the filtrate (300 ml.) was made faintly alkaline with ammonia and then treated with cold saturated mercuric acetate solution. A small pinkish precipitate separated and was filtered off. The labile glucuronide appeared in the filtrate together with the main bulk of the naphthoresorcinol-reacting material of the urine. The mercuric acetate precipitate did, however, contain a glucuronide. It was suspended in water and Hg removed with H_2S. The filtrate from the HgS was evaporated *in vacuo* at 40–50° to 15 ml. and this was brought to pH 4·3 with 0·5 M-Na acetate. The mixture was kept at 0° overnight. The crystalline precipitate (180 mg., or 0·6% of the dose) was filtered off, washed with water, ethanol and then ether. It was recrystallized from hot water, from which it formed felted needles, m.p. 215–216°, not depressed by authentic *p*-aminophenylglucuronide (Williams, 1943). It showed $[\alpha]_D^{20°}$ −83·4° (*c*, 4·2, in 0·5 N-H_2SO_4). (Found: C, 48·2; H, 5·6; N, 4·6. Calc. for $C_{12}H_{15}O_7N.H_2O$: C, 47·5; H, 5·6; N, 4·6%.) In another experiment, using 12 g. aniline, 295 mg. (0·8% of the dose) of the glucuronide were isolated.

This experiment was repeated in exactly the same manner, except that the glucuronide gum solution was saturated with SO_2 prior to the Hg precipitation. The yield of *p*-aminophenylglucuronide was not increased, nor was it increased by boiling the urine with hot alkali prior to preparation of the gum. These results indicate that *p*-aminophenylglucuronide occurs in the urine as such and is not derived from a labile glucuronide.

(*e*) *Isolation of* p-*acetamidophenylglucuronide*. The glucuronide fraction from the urine (350 ml.) of four rabbits which had collectively received 8 g. of aniline was prepared as before. The fraction was concentrated to 30 ml. of syrupy liquid to which was added, with shaking, 200 ml. of absolute ethanol. A reducing gum was precipitated which yielded 7 g. (15% of the dose) of the crystalline *p*-toluidine-ammonium glucuronate complex (see p. 247). The ethanolic solution after removing the gum was now reduced *in vacuo* to 10 ml., and again precipitated with ethanol to remove further traces of the labile glucuronide. The concentration and precipitation with ethanol was repeated once more. In this way most of the labile glucuronide was removed and the final ethanolic solution was concentrated at 40–50° to a non-reducing gum (2 g.) which contained conjugated glucuronic acid, but only gave a diazo test after hydrolysis by acid. The non-reducing gum was now dissolved in 50 ml. 95% ethanol and the solution treated with 1 g. of benzylamine followed by 400 ml. ethyl acetate. After 1 hr. at 0° the crystalline precipitate (200 mg., 0·5% of the dose) was collected and recrystallized twice from ethanol and ethanol-ethyl acetate. The crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–198°, $[\alpha]_D^{15°}$ −59° (*c*, 0·7 in water) (see Smith & Williams, 1948*a*). A further quantity of this salt was obtained from the mother liquor, but it could not be satisfactorily purified.

We have attempted to assess by various means the amount of *p*-acetamido- and *p*-amino-phenylglucuronides in aniline urine. By actual isolation we found 0·8% of the dose as *p*-amino- and at least 0·5% as *p*-acetamidophenylglucuronide, and by isolation of the acetylated methyl ester, 1% of the dose. These values are obviously minimal. A study of the ultraviolet absorption spectrum of the gum (see p. 247) gave an estimate of 10%. The difference between the 'extra' glucuronic acid content of the acid content of the urine and its reducing power towards Benedict's reagent gave a value of 15%. We can, therefore, conclude that about 10–15% of the aniline fed is excreted as free and acetylated *p*-aminophenylglucuronide.

(*f*) *The isolation of free* D-*glucuronic acid from the gum.* It was mentioned in § 2 that aniline urine had strong reducing properties, Benedict's and Fehling's solution being very readily reduced on warming. Furthermore, the reducing substance was not glucose. The reducing substance appeared in the basic lead acetate precipitate of the urine, and constituted the major fraction of the glucuronide gum prepared from this precipitate. On examination of the gum it was found possible to isolate, without the use of hydrolytic methods, considerable amounts of free glucuronic acid. The significance of this free glucuronic acid is not yet clear to us.

The isolation of glucuronic acid was achieved through the use of *p*-toluidine. Glucuronic acid forms in the presence of *p*-toluidine and NH_4^+ ions a sparingly soluble, crystalline compound whose components are two molecules of *p*-toluidine, and one of ammonium glucuronate. It appears to be a convenient compound for isolating free glucuronic acid, but it is not formed from the lactone, glucurone. The preparation of authentic samples of this compound, and of the corresponding compound from D-galacturonate together with a discussion of their nature are described in the succeeding paper (Smith & Williams, 1949*b*).

The isolation of the compound from aniline urine was achieved as follows. A solution of 8 g. of the purified glucuronide gum (see § 5 (*a*)) dissolved in 5 ml. cold water was mixed with a suspension of 4 g. *p*-toluidine in 1 ml. ethanol and 4 ml. warm water. The toluidine dissolved on stirring, and, on cooling, the mixture crystallized. After keeping at 0° for a few hours the crystals were filtered at the pump, washed with a little ethanol followed by a large volume of ether (yield, 7 g.: this corresponds to 21% of the dose of aniline, assuming that one molecule of aniline gives rise to one of glucuronic acid). The *p-toluidine-ammonium glucuronate complex*, recrystallized from hot dilute ethanol, formed colourless rectangular plates, m.p. 125–128° (decomp.), $[\alpha]_D^{20°}$ +20°→ +11° (constant value in *c.* 3 hr.) (*c*, 5 in 0·4 N-HCl). Ultraviolet absorption spectrum: in water, $\lambda_{max.}$ 234 mμ., $\epsilon_{max.}$ 16,900, $\lambda_{max.}$ 285 mμ., $\epsilon_{max.}$ 2700; in N-HCl, $\lambda_{max.}$ 264 mμ., $\epsilon_{max.}$ 400. Elementary analysis suggested that two hydrates of this complex existed (cf. the hydrates of glucose toluidide; Irvine & Gilmour, 1909). The one less frequently obtained contained 0·5 H_2O more than the other. (Found: (1) C, 55·5; H, 7·2; N, 9·6. $C_{20}H_{29}N_3O_6.1{\cdot}5H_2O$ requires: C, 55·3; H, 7·4; N, 9·7%. (2) C, 56·7; H, 7·2; N, 9·25; glucuronic acid, 47·6. $C_{20}H_{29}N_3O_6.H_2O$ requires C, 56·45; H, 7·3; N, 9·9; glucuronic acid, 45·7%.) The compound quickly reduced Benedict's reagent on warming and gave the Tollens test for glucuronic acid very rapidly. With Nessler's reagent it gave a brown precipitate, a reaction not given by *p*-toluidine.

(*g*) *Spectroscopic observations on the glucuronide gum.* The isolation of free glucuronic acid from the gum raises the question of its source. It could arise from a labile glucuronide of aniline metabolism or aniline may stimulate the excretion of free glucuronic acid. We thought that spectroscopic examination of the gum might give us information on the first of these alternatives. A possible glucuronide would be the unknown β-phenylhydroxylamine glucuronide. This compound would probably be labile and break up to phenylhydroxylamine which would rapidly form azoxybenzene. The latter can be detected by its characteristic ultraviolet absorption. When fed to rabbits, β-phenylhydroxylamine (200 mg.) does not cause the excretion of a reducing urine.

The glucuronide gum for spectroscopic examination was obtained by working up the 24 hr. urine of a rabbit which had received 2 g. aniline (see § 5 (*a*)). It was dissolved in 2·5 l. 50% aqueous ethanol and the solution examined with a Hilger E 3 spectroscope. The absorption spectra of the gum solution, *p*-aminophenylglucuronide, *p*-acetamidophenylglucuronide and azoxybenzene are reproduced in Fig. 1.

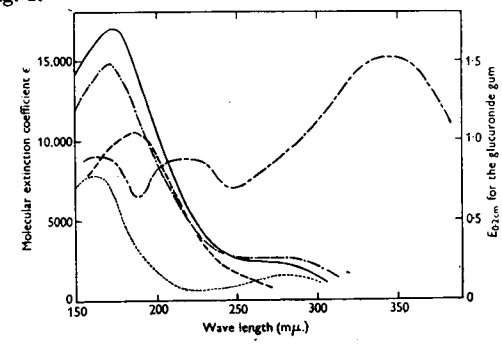


Fig. 1. The ultraviolet absorption spectrum of the glucuronide gum from aniline urine compared with the spectra of possible components. –·–·–·, the glucuronide gum (after feeding 2 g. aniline) dissolved in 2·5 l. 50% aqueous ethanol: $\lambda_{max.}$ 236 mμ., $E_{0\cdot2\,cm.} = 1{\cdot}5$; ———, *p*-acetamidophenylglucuronide in water: $\lambda_{max.}$ 244 mμ., $\epsilon_{max.}$ 10,800; ·····, *p*-aminophenylglucuronide in 0·1 N-KOH: $\lambda_{max.}$ 231 mμ., $\epsilon_{max.}$ 7700 and $\lambda_{max.}$ 291 mμ., $\epsilon_{max.}$ 1600; ————, sum of the absorptions of *p*-acetamido- and *p*-aminophenylglucuronides; — · — ·, azoxybenzene in ethanol: $\lambda_{max.}$ 235 mμ., $\epsilon_{max.}$ 9000; $\lambda_{max.}$ 249 mμ., $\epsilon_{max.}$ 8900; $\lambda_{max.}$ 324 mμ., $\epsilon_{max.}$ 15,200.

The gum solution showed a band at $\lambda_{max.}$ 236 mμ. and an inflexion at 285 mμ. There was no significant absorption at 324 mμ., in which region azoxybenzene absorbs strongly. It can, therefore, be concluded that the gum contains no azoxybenzene, and consequently that phenylhydroxylamine is unlikely to be present (cf. the cases of sulphanilamide and 4:4′-disulphonamidoazoxybenzene studied by Williams (1946) and of 4-hydroxylamino-2:6-dinitrotoluene and 2:2′:6:6′-tetranitro-4:4′-azoxytoluene in TNT metabolism studied by Channon, Mills & Williams (1944)). The peak at 236 mμ. may be due to *p*-amino- and *p*-acetamido-phenylglucuronide, for if the curves for these compounds are summated, the resultant curve has the same shape as that of the gum. This argument is only valid if irrelevant absorption at the shorter wavelengths is assumed to be negligible. The glucuronide gum may contain, on these grounds, *p*-amino- and *p*-acetamido-phenylglucuronide in roughly equal amounts.

Results

The results of the work on isolation of aniline metabolites are summarized in Table 2.

DISCUSSION

The present work shows that the metabolism of aniline in the rabbit is a more complicated process than one would have imagined at first sight and, although a number of the metabolites have been identified, what we think is a major metabolite has,

Table 2. *Derivatives isolated from the urine of rabbits receiving aniline orally*

Derivative	M.p.	$[\alpha]_D$	Yield as % of dose
(*a*) Unconjugated:			
Aniline hydrogen oxalate	158°	—	6–9
Benzanilide	160°	—	3–9
(*b*) From glucuronide fraction:			
p-Toluidine complex of ammonium glucuronate	125–128°	+21° (in 0·4 N-HCl)	21
p-Aminophenylglucuronide	215–216°	−83·4° (in 0·5 N-H_2SO_4)	0·8
Benzylamine salt of *p*-acetamidophenylglucuronide	195–198°	−59° (in water)	0·5
p-Acetamidophenyltriacetylglucuronide methyl ester	205° and 100°	−22·4° (in chloroform)	1
p-Aminophenol (by hydrolysis)	182–184°	—	5
(*c*) From ethereal sulphate by hydrolysis:			
p-Aminophenol	183°	—	3
o-Aminophenol dibenzoate	176–179°	—	0·3
4-Aminoresorcinol (detected)	—	—	?

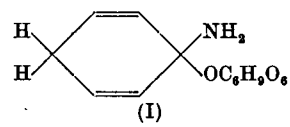
so far, eluded our efforts to isolate and identify it. In what follows we shall attempt to assess, in various ways, the amounts of each metabolite excreted.

The isolation experiments account for nearly 40 % of the aniline fed (see Table 2). This figure is made up as follows: free and acetylated *p*-aminophenylglucuronide, 5 % (based on the isolation of *p*-aminophenol from the glucuronide gum); free aniline, up to 9 % (see § 3 (*b*)); *p*-aminophenylsulphuric acid, 3·1 %; *o*-aminophenylsulphuric acid, 0·7 % (from hydrolysis of the ethereal sulphate fraction, see §§ 4 (*a*) and (*b*)). Assuming that each molecule of glucuronic acid isolated as the *p*-toluidine complex is derived from a labile glucuronide generated from one molecule of aniline, then 21 % of the aniline fed is excreted as a labile glucuronide (§ 5 (*f*)).

The excreted free diazotizable amino groups correspond to 40 % of the aniline fed (Table 1). The compounds which could contribute to this figure are free aniline (6·5 %, see § 3 (*b*)), *p*-aminophenylglucuronide (5–8 %, § 5 (*d*) and (*g*)) and *p*-aminophenylsulphuric acid (*c.* 28 %). *o*-Aminophenylsulphuric acid probably does not contribute because Burkhardt & Wood (1929) have shown that when diazotized it loses its sulphate group yielding *o*-aminophenol.

If it is assumed that one molecule of aniline gives rise to one of glucuronide, then 70 % of the amine is excreted as conjugated glucuronic acids (Table 1). The isolation experiments indicate that aniline urine may contain three glucuronides, i.e. *p*-amino- and *p*-acetamido-phenylglucuronides and a 'labile glucuronide'. The amount of the first two was assessed at 10–15 % and, therefore, about 55–60 % of the aniline fed may be excreted as the labile glucuronide. We can do little more than speculate at present on the nature of the labile glucuronide.

Aniline urine is reducing and free glucuronic acid in large amounts can be isolated from it as the *p*-toluidine-ammonium glucuronate complex. Now this free glucuronic acid could occur in the urine as such or be produced by the breakdown of a labile glucuronide. The excretion of free glucuronic acid as such, appears to us, at the moment, to be unlikely though not impossible. The existence of a labile glucuronide, however, is feasible on the following grounds. Less than 50 % of the aniline fed can be accounted for as diazotizable compounds; the rest may be changed in the body to a non-diazotizable form which is the aglycone of a labile glucuronide. A dihydrohydroxyaniline glucuronide (I) could account for some of the facts. Such a structure as (I) contains an aliphatic amino group, a new asymmetric carbon atom—which could account for the low optical rotation of the urine—and an aldehyde-ammonia group—which would make it labile and release ammonia and glucuronic acid to form the *p*-toluidine-ammonium glucuronate complex. When this complex is formed from the acidic glucuronide gum no external ammonia is necessary, yet when it is formed from pure glucuronic acid ammonia must be added. Structure (I) is that of a hexa-1:4-diene



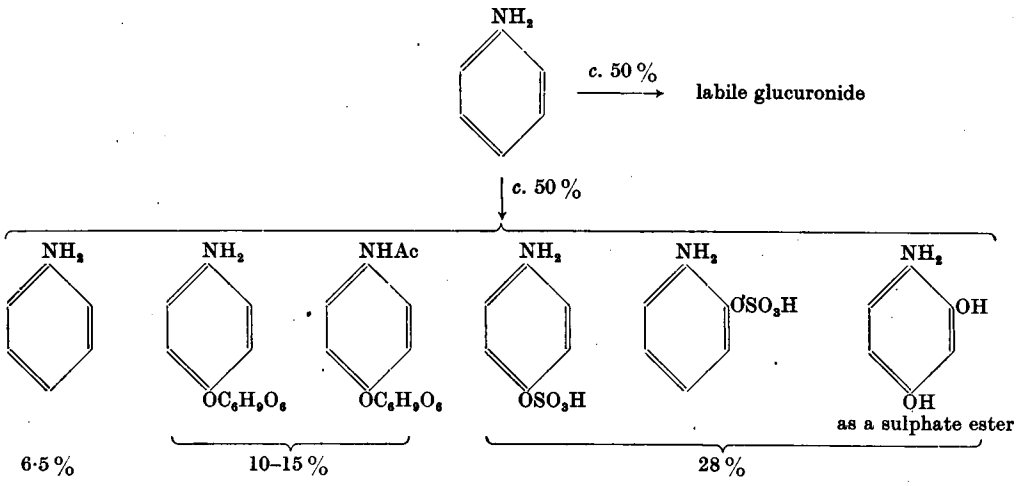
(I)

and therefore should show little or no light absorption. It will be recalled that ultraviolet absorption spectrum of the glucuronide gum appears to be that of glucuronides of *p*-acetamido- and *p*-amino-phenol only.

We have also proved that aniline is slightly acetylated *in vivo* by the isolation of *p*-acetamidophenylglucuronide, but no acetanilide was detected. It is possible that here aniline is first oxidized to

p-aminophenol which is then acetylated and *O*-conjugated; there may be no direct acetylation of aniline.

It is clear that the metabolism of aniline is very complex. On available evidence we suggest the following tentative scheme:



Three aminophenols were found in the urine. The qualitative experiments on the ethereal sulphate fraction suggest that *p*-aminophenol preponderates. *o*-Aminophenol and 4-aminoresorcinol were found only in the ethereal sulphate fraction. The only phenol isolated from the glucuronide fraction was *p*-aminophenol, but we cannot eliminate the possibility that the *o*-compound is also present in this fraction, for Hanson *et al.* (1944) have shown that *o*-aminophenylglucuronide is very resistant to acid hydrolysis. We feel, however, that the *o*-glucuronide is not present in appreciable amounts because although it is more easy to isolate from urine (Williams, 1943) than its *p*-isomer, we isolated only the latter from the glucuronide gum.

The detection of 4-aminoresorcinol shows that the *o*- and *p*-aminophenols derived from aniline undergo further oxidation to a trisubstituted benzene. Porteous & Williams (1949) have already shown that benzene gives rise to a trisubstituted compound, hydroxyquinol, in the rabbit.

We suggested in an earlier paper (Smith & Williams, 1948*a*) that biological oxidation of an aromatic ring takes place at those carbon atoms which possess a certain minimum of electronic activation. On this basis aniline should be oxidized *in vivo* in the *o*- and *p*-positions and this is in agreement with our findings (cf. acetanilide, Smith & Williams, 1948*a*).

SUMMARY

1. The metabolic fate of aniline in the rabbit has been studied.

2. About 28 % of the dose is excreted as the ethereal sulphates of *o*- and *p*-aminophenol and 4-aminoresorcinol. The *o*- and *p*-aminophenols were isolated.

3. About 70 % of the aniline fed is excreted as glucuronides, two of which were isolated and identified as *p*-acetamido- and *p*-amino-phenylglucuronide. These two account for 10–15 % of the aniline fed.

4. It is suggested that the main metabolite of aniline is a labile glucuronide. This substance accounts for more than 50 % of the aniline fed and may be a reduced aniline derivative. It also accounts for the reducing properties of aniline urine.

5. The labile glucuronide readily breaks up to give free glucuronic acid, which has been isolated as a crystalline *p*-toluidine-ammonium glucuronate complex.

6. No evidence was found to support the view that aniline is converted to phenylhydroxylamine *in vivo*.

7. Aniline gives rise to excretion of small amounts of *N*-acetyl derivatives. Whether or not aniline is directly acetylated has not been proved.

8. Aniline is oxidized in the *o*- and *p*-positions and the significance of this finding is discussed.

9. The metabolites of aniline in the rabbit are not the same as those of acetanilide. The significance of this is discussed.

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REFERENCES

Anselmino, O. (1903). *Ber. dtsch. pharm. Ges.* **13**, 494.
Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
Burkhardt, G. N. & Wood, H. (1929). *J. chem. Soc.* p. 141.
Channon, H. J., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 70.
Elson, L. A., Goulden, F. & Warren, F. L. (1946). *Biochem. J.* **40**, xxix.
Goldblatt, M. W. (1947). *Brit. med. Bull.* **4**, 405.
Gross, M. (1946). *Acetanilide.* New Haven: Hillhouse Press.
Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.
Henrich, F. & Wagner, B. (1902). *Ber. dtsch. chem. Ges.* **35**, 4205.
Irvine, J. C. & Gilmour, R. (1909). *J. chem. Soc.* **95**, 1545.
Krebs, H. A., Sykes, W. O. & Bartley, W. C. (1947). *Biochem. J.* **41**, 622.
Müller, F. (1887). *Dtsch. med. Wschr.* **13**, 27.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 56.
Schmiedeberg, O. (1878). *Arch. exp. Path. Pharmak.* **8**, 10.
Smith, J. N. & Williams, R. T. (1948*a*). *Biochem. J.* **42**, 538.
Smith, J. N. & Williams, R. T. (1948*b*). *Biochem. J.* **42**, 351.
Smith, J. N. & Williams, R. T. (1949*a*). *Biochem. J.* **44**, 239.
Smith, J. N. & Williams, R. T. (1949*b*). *Biochem. J.* **44**, 250.
Williams, R. T. (1943). *Biochem. J.* **37**, 329.
Williams, R. T. (1946). *Biochem. J.* **40**, 219.

Studies in Detoxication

24. THE METABOLISM OF *p*-PHENETIDINE (*p*-ETHOXYANILINE) WITH SOME OBSERVATIONS ON THE ANISIDINES (METHOXYANILINES)

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This study of the fate of alkoxyanilines in the rabbit was pursued because we wanted to know the effect of alkoxyl groups on the biological acetylation of the amino group in compounds of the type $R.C_6H_4NH_2$. The study became of further interest when it was found that administration of *p*-phenetidine and the *o*-, *m*- and *p*-anisidines resulted in the excretion of reducing urines as in the case of aniline (Smith & Williams, 1949*b*).

Little previous work has been done on *p*-phenetidine; Edlefson (1900) showed that, in man, it gave rise to conjugated *p*-aminophenol and Elson, Goulden & Warren (1946) suggested that it behaved similarly in the rat.

EXPERIMENTAL

Methods. The quantitative estimations of the excretion of glucuronic acid, ethereal sulphate, and diazotizable amino groups in the urine of rabbits receiving *p*-phenetidine hydrochloride orally are quoted in the preceding paper (Smith & Williams, 1949*b*).

Phenetidine was fed as the hydrochloride, m.p. 234°.

The isolation of phenetidine metabolites

(1) *The nature of phenetidine urine*

After feeding phenetidine in doses of 0·2–0·5 g./kg., rabbits excreted a brown urine (pH *c.* 8) similar in appearance to aniline urine. It reduced Benedict's reagent and gave a very rapid naphthoresorcinol reaction. It gave a permanganate colour with $FeCl_3$ and a positive diazo reaction.

The urine was optically active. The urine from two rabbits, each of which had received 2 g. of phenetidine hydrochloride, was estimated to contain 4·7 g. of extra glucuronic acid. A 10 ml. portion of this urine was clarified with dialyzed iron and made up to 20 ml. In a 2 dm. tube α_D was −0·42°, whilst normal rabbit urine treated in the same way has no detectable rotation. If the glucuronide in the urine were that of either *p*-aminophenol or *p*-acetamidophenol, the expected α_D would be *c.* −1·3°. Thus it follows that the glucuronides from phenetidine cannot be entirely *p*-aminophenol derivatives.

(2) *Isolation of unchanged phenetidine*

The filtrate from the preparation of the basic lead acetate fraction of phenetidine urine (see p. 251) was made alkaline with KOH, filtered and continuously extracted with ether for 4 hr. The bases in the ether were extracted with 2 N-HCl. This solution was made alkaline and extracted in a funnel with ether. This ethereal solution was dried over NaOH for 24 hr., filtered, and saturated with dry HCl gas. The crystalline precipitate of phenetidine hydrochloride (m.p. and mixed m.p. 234°) was filtered off. The yields of unchanged phenetidine in these experiments were 1·5% of the dose at a level of 0·5 g./kg. and 2 and 5% at 0·8 g./kg.

(3) *The ethereal sulphate fraction*

(*a*) *Isolation of 2-hydroxy-4-ethoxyaniline (hydroxyphenetidine).* The 24 hr. urine (1250 ml.) after feeding 18 g. phenetidine hydrochloride was concentrated at 40° *in vacuo* to 250 ml., acidified with dilute HCl, saturated with $(NH_4)_2SO_4$ and extracted with 2 × 250 ml. acetone. The acetone extract was made alkaline with solid K_2CO_3, concentrated *in vacuo* to 100 ml., treated with 1 l. dry acetone, filtered and concentrated to 50 ml. The concentrate was now extracted five times with an equal volume of ether to remove free phenetidine (recovered as the hydrochloride, m.p. 230°). (Found: C, 55·1; H, 6·9; N, 7·6. Calc. for