Studies in Detoxication

22. THE METABOLISM OF PHENACETIN (*p*-ETHOXYACETANILIDE) IN THE RABBIT AND A FURTHER OBSERVATION ON ACETANILIDE METABOLISM

BY J. N. SMITH AND R. T. WILLIAMS
Department of Biochemistry, University of Liverpool

(*Received* 21 *July* 1948)

We have shown that acetanilide is converted in the rabbit mainly into the glucuronide and ethereal sulphate of *p*-acetamidophenol (Smith & Williams, 1948), not more than 6–7% being excreted as compounds containing free aromatic amino groups. Phenacetin has antipyretic and analgesic properties similar to those of acetanilide, but is less toxic. The similar pharmacological properties suggest that phenacetin may give rise to the same metabolites as acetanilide, and in this paper we shall show that this suggestion is true.

Earlier workers showed that phenacetin caused an increased output of ethereal sulphate in man, the dog and the rabbit (Müller, 1888; Mörner, 1889; Baccarani, 1900; Hinsberg & Kast, 1887; Mahnert, 1888) and Mörner isolated potassium *p*-acetamidophenylsulphate from the urine of patients receiving phenacetin orally. The presence of a glucuronide and of compounds of *p*-aminophenol in phenacetin urine from man, the dog and the rabbit was also suggested by this early work. According to Müller (1888) no unchanged phenacetin is excreted, but Mörner (1889) found small amounts of phenetidine in human urine. Hinsberg & Kast (1887) observed that a dog which had received 3 g. of phenacetin orally excreted urine which strongly reduced alkaline copper solutions; the significance of this observation will be discussed later.

EXPERIMENTAL

The quantitative analyses carried out on phenacetin urine are quoted in the succeeding paper (Smith & Williams, 1949*a*), in which analytical figures for a number of related aromatic amines are discussed together. At this point it may be mentioned that phenacetin undergoes only a very slight deacetylation in the rabbit and it is to be expected, therefore, that the major metabolites will be aromatic acetamido compounds.

The nature of phenacetin urine. The urine of rabbits receiving 0·3–0·7 g./kg. phenacetin orally had pH *c.* 8 and was normal in appearance. It did not reduce Benedict's reagent immediately, but a slight reduction was apparent on allowing the test mixture to stand. It gave no colour with $FeCl_3$ and only a very slight red colour in the diazo test (diazotization and coupling with 1-naphthyldimethylamine). The urine yielded no ether-soluble material on shaking with ether in a separating funnel.

1. *The ethereal sulphate fraction of phenacetin urine*

(*a*) *Detection of free* p-*phenetidine*

A 24 hr. urine (1 l.), collected after the feeding of 15 g. phenacetin, was evaporated *in vacuo* at 40–50° to 200 ml. Phenetidine was detected in the distillate by the permanganate-like colour it gives with $FeCl_3$, but it could not be isolated in sufficient amount for identification. The concentrated urine was saturated with $(NH_4)_2SO_4$ and then extracted with 2 × 200 ml. portions of acetone. The acetone extract was separated (leaving an aqueous layer *G* containing glucuronide, see section 2 (*b*) (i)), neutralized with solid K_2CO_3, filtered and reduced to 50 ml. *in vacuo*. The residue *B* was shaken with 500 ml. dry acetone and the supernatant layer separated and reduced to 50 ml. (*C*) *in vacuo*. *C* was now extracted with ether and the extract on evaporation yielded a small oily residue (*D*) which appeared by colour tests to be phenetidine. *D* was dissolved in 1 ml. 2N-HCl, filtered, neutralized with dilute Na_2CO_3 solution and extracted with ether. The extract was evaporated, and the residue treated with a little acetic anhydride and dilute Na_2CO_3 solution. Phenacetin separated and, recrystallized from a little hot water, had m.p. and mixed m.p. 132° (yield, 10 mg., or 0·07% of dose).

(*b*) *Isolation of* p-*aminophenol*

The ether-extracted concentrate *C* (above) was a syrupy liquid. It was practically free of glucuronides and gave no precipitate with $BaCl_2$ until boiled with dilute HCl. It did not reduce ammoniacal $AgNO_3$ and gave no colour in the diazo test, but both these tests became positive after hydrolysis. This fraction, therefore, contained the ethereal sulphate of an acetylated aminophenol.

A 30 ml. portion of *C* was boiled for 10 min. with 3 ml. of conc. HCl. The mixture was cooled and extracted with ether. The extract was evaporated to 5 ml. and stirred with dilute Na_2CO_3 solution and a little acetic anhydride. The solid which separated was recrystallized from benzene and the crystals (plates) obtained were identified as *ON*-diacetyl-*p*-aminophenol, m.p. and mixed m.p. 150°. (Found: N, 7·3. Calc. for $C_{10}H_{11}O_3N$: N, 7·25%.) The yield was 120 mg., or 1·3% of dose. The ethereal sulphate fraction, therefore, contained *p*-acetamidophenylsulphuric acid.

2. *The glucuronide fraction of phenacetin urine*

(*a*) *Isolation of* p-*acetamidophenylglucuronide*

The 24 hr. urine (400 ml.) from two rabbits, which had each received 2 g. (0·7 g./kg.) of phenacetin, was acidified

with a little glacial acetic acid and treated with 100 ml. saturated lead acetate solution. The precipitate was discarded. The filtrate was made just alkaline with ammonia (sp.gr. 0·88) and the precipitate which formed was collected, washed well with water, then suspended in water and treated with a current of H_2S to remove Pb. After filtering from PbS, the filtrate was reduced *in vacuo* at 45° to 5 ml. Addition of ethanol threw down a ropy precipitate which dissolved on warming and came down again on cooling as a gum containing some crystals. The whole was, therefore, reduced *in vacuo* to 5 ml. and then mixed with 4 ml. of benzylamine. This mixture was now diluted to 1 l. by alternate additions of ethyl acetate and ethanol so that the last addition of ethyl acetate produced a slight cloudiness. After keeping at 0° for 3 hr. the crystalline precipitate (1·65 g.) was filtered off and the mother liquor diluted to 2 l. with ethyl acetate. On keeping overnight at 0° a further 0·65 g. of crystals was obtained. Concentration and redilution of the mother liquor yielded a further 0·6 g. These crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–197° after recrystallization from aqueous ethanol, $[\alpha]_D^{15°}$ −60° (*c*, 1·4 in water). (Found: N, 6·2. Calc. for $C_{21}H_{26}O_8N_2.H_2O$: N, 6·2%.) (See Smith & Williams, 1948.) The yield corresponded to 32% of the dose of phenacetin; quantitative estimations showed that 47% of phenacetin is excreted as a glucuronide (Smith & Williams, 1949*a*).

(*b*) *The detection of a labile glucuronide*

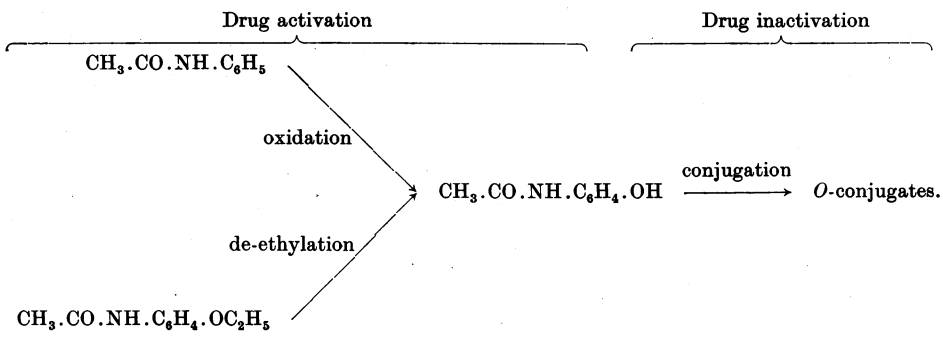
In the succeeding papers (Smith & Williams, 1949*a*, *b*) it is suggested that the main metabolites of aniline and phenetidine in the rabbit are labile glucuronides. Now both acetanilide (Smith & Williams, 1948) and phenacetin undergo a very slight deacetylation in the rabbit and should, therefore, give rise to small amounts of these labile glucuronides. These substances can be detected in urine because they yield, in the presence of *p*-toluidine and NH_4^+ ions, a crystalline complex of *p*-toluidine and ammonium glucuronate. The nature of this complex is discussed by Smith & Williams (1949*b*).

(i) *Detection of a labile glucuronide in phenacetin urine.* The aqueous residue *G* (see Section 1 (*a*)) left after removal of the sulphate fraction from phenacetin urine by acetone consisted of 50 ml. of urine saturated with $(NH_4)_2SO_4$. Into this was stirred 1 g. of *p*-toluidine dissolved in a little acetone. After several hours the crystalline precipitate which had separated was filtered off and washed with acetone to remove excess *p*-toluidine. The crystals (50 mg.) were recrystallized from water and had m.p. 125–128°, not depressed on admixture with the *p*-toluidine-ammonium glucuronate complex from aniline or phenetidine urine. The compound rapidly reduced Benedict's reagent and gave the Tollens naphthoresorcinol reaction very readily.

(ii) *From acetanilide urine.* A 24 hr. urine (800 ml.) from rabbits which had collectively received 10 g. acetanilide was reduced *in vacuo* at 40–50° to 160 ml. The concentrate was acidified with a little 2N-HCl, saturated with $(NH_4)_2SO_4$ and extracted with acetone (2 × 150 ml.). The aqueous layer was separated and treated with an ethanolic solution of 5 g. *p*-toluidine followed by 50 ml. ethanol. On keeping this mixture, solid $(NH_4)_2SO_4$ separated and settled on the bottom of the vessel whereas crystals of the *p-toluidine-ammonium glucuronate complex* collected at the ethanol-water interface. The complex was collected and recrystallized from water (yield, 30 mg. or 0·1% of dose). It had m.p. and mixed m.p. 125–128° with the corresponding product from aniline urine. (Found: N, 9·6. $C_{20}H_{31}O_7N_3$ requires N, 9·9%.) It gave a characteristically rapid Tollens reaction and reduced Benedict's solution readily on warming.

DISCUSSION

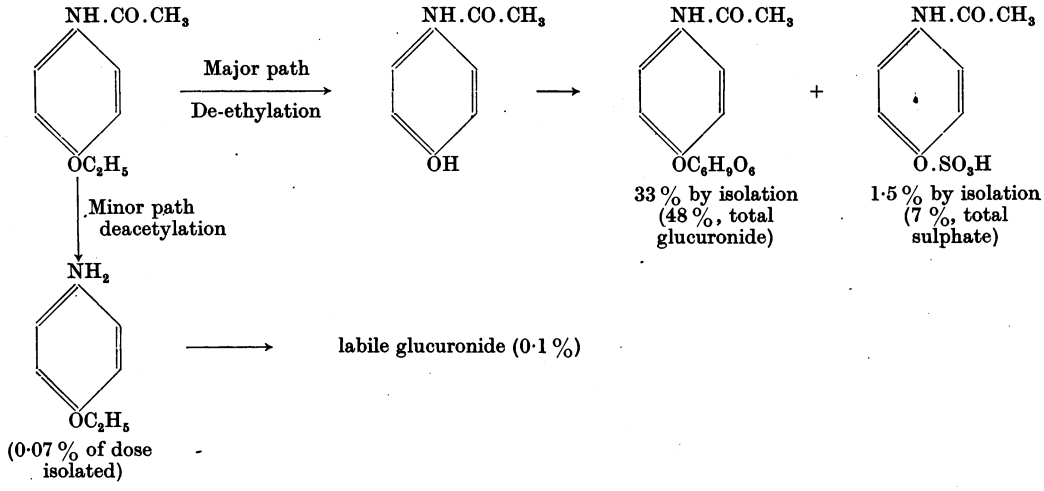
The present work shows that the main metabolites of phenacetin in the rabbit are the same as those of acetanilide, i.e. *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, and quantitatively these account for 54% (47% as glucuronide and 7% as ethereal sulphate) of the phenacetin fed. These metabolites are produced in roughly the same ratio as found in *p*-acetamidophenol or acetanilide metabolism, for the glucuronide/ethereal sulphate ratio for phenacetin is 6·9, for *p*-acetamidophenol, 6·3 and for acetanilide, 5·8 (see Smith & Williams, 1948). From our present results and those on acetanilide it appears that phenacetin and acetanilide owe their therapeutic activity to their metabolic conversion to *p*-acetamidophenol (cf. Hinsberg & Treupel, 1894). This phenol is probably then inactivated by conjugation with glucuronic and sulphuric acids. In the case of acetanilide the process of activation involves oxidation, whereas with phenacetin it involves de-ethylation thus:



Phenacetin is more satisfactory as an analgesic and antipyretic drug than acetanilide. It has been suggested that the rate at which a drug of this group

is metabolized (presumably to *p*-acetamidophenol) depends on its molecular size (Hinsberg & Treupel, 1894). Thus molecules smaller than phenacetin are rapidly metabolized and are consequently more toxic, whereas with larger molecules such as the propyl and amyl analogues of phenacetin, the change is probably slower and, while toxic effects are less marked, there is also a loss of therapeutic effect (see Gaddum, 1944).

we have not yet elucidated. These glucuronides are relatively easy to detect for they readily decompose yielding free glucuronic acid which forms a characteristic crystalline complex in the presence of *p*-toluidine and ammonium ions. This complex was isolated from both phenacetin and acetanilide urines, the yields corresponding to 0·1 % of the dose in each case. It was suggested in an earlier paper (Smith & Williams, 1948) that the metabolism of

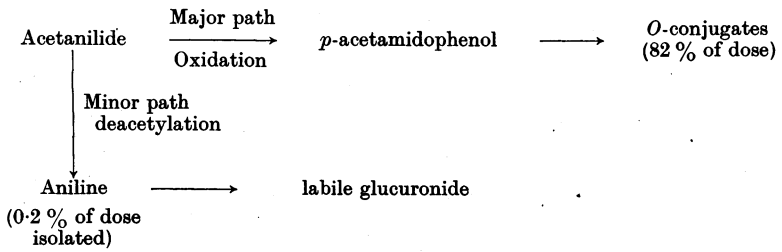


If it is conceded that both the therapeutic and toxic effects of acetanilide and phenacetin are due to the metabolic formation of *p*-acetamidophenol, then the greater efficiency and lesser toxicity of phenacetin are due to its de-ethylation being a slower process than the oxidation of acetanilide. The toxic effects of phenacetin are also less than those of acetanilide because the slower rate of production of *p*-acetamidophenol permits a more efficient detoxication by conjugation.

The extent of deacetylation of phenacetin in the rabbit is very small, but we were able to detect traces of phenetidine in the urine. This small amount of phenetidine should give rise to phenetidine metabolites. Similarly, acetanilide is slightly deacetylated by the rabbit, and should, therefore, give rise to small amounts of aniline metabolites. Now the major metabolites of both aniline and phenetidine in the rabbit are labile glucuronides whose structure acetanilide would be similar to that of aniline in animals capable of extensively deacetylating the aromatic acetamido group. Such animals are the dog, cat, and pigeon (Krebs, Sykes & Bartley, 1947). As mentioned earlier Hinsberg & Kast (1887) observed that a dog receiving phenacetin excreted a strongly reducing urine. This observation can now be explained, for the dog probably converts phenacetin to a considerable extent to phenetidine which may then give rise to the labile glucuronide which we have found to be the major metabolite of phenetidine in the rabbit.

The main features of the metabolisms of phenacetin in the rabbit are as shown above. These findings are probably applicable to man but not to the pigeon, dog and cat.

In the case of acetanilide, a minor pathway of metabolism can now be added to the results given earlier (Smith & Williams, 1948):



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.

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

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

BY J. N. SMITH AND R. T. WILLIAMS
Department of Biochemistry, University of Liverpool

(*Received* 21 *July* 1948)

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*).