It would be unwise to conclude either from the present, or from the previous results (Lees, 1946), that copper is essential to the nitrifying organisms. The balance, as well as the amounts, of trace elements in a nitrifying culture may profoundly affect the speed of nitrification therein (Meiklejohn & Lees, in preparation). The inhibition of soil nitrification that ensues when copper is removed from a soil may therefore be due, not directly to a copper deficiency, but to a resultant imbalance of the remaining available trace elements. Such a theory would perhaps explain the partial reversal of diethyldithiocarbamate poisoning by manganese. Lucas (1945) has shown by plant growth experiments that there is some interaction between soil copper and soil manganese.

The results of the present work suggest a possible basis for a direct biological test for trace element deficiencies in soil. A diethyldithiocarbamate-treated soil is copper-deficient; as a result of this deficiency the nitrifying power of the soil is lowered, but may be raised again if copper is added to the soil. Copper deficiency in soil is therefore biologically detectable by the fact that addition of copper to the soil increases the nitrifying power. The possibility of measuring natural trace-element deficiencies by this sort of technique, which is an extension of the culture test elaborated by Mülder (1940), is obvious.

SUMMARY

1. It has been found possible quantitatively to study the effects of zinc and copper on soil nitrification by the percolation technique.

2. The toxic effect of either element is less on an organic than on a mineral soil.

3. Sodium diethyldithiocarbamate poisons soil nitrification. The poisoning is reversible by copper and partially reversible by manganese.

4. The possible importance of copper in nitrification is discussed.

5. It is suggested that the results might form the basis of a direct soil test for trace-element deficiencies.

I am indebted to Mr W. J. Redmond for accurate and skilled assistance throughout the work, and to Dr J. B. Hale for spectrophotometric determinations of zinc.

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Studies in Detoxication

16. THE METABOLISM OF ACETANILIDE IN THE RABBIT

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It is now sixty years since Cahn & Hepp (1887) introduced acetanilide (antifebrin) as an analgesic and antipyretic. Nevertheless, its fate in the body has not been studied quantitatively until recently (Greenberg & Lester, 1946). Furthermore, its metabolites are not exactly known. Early work (Müller, 1887; Kumagawa, 1888; Gregoire & Hendrick, 1904, etc.) established that acetanilide was oxidized *in vivo*, and that by suitable treatment of the urine *p*-aminophenol could be isolated. Jaffé & Hilbert (1888) found that the metabolites of acetanilide in the dog were different from those in the rabbit. From dog urine the main compound isolated was benzoxazolone together with small amounts of *p*-aminophenol, whereas from rabbit urine only *p*-aminophenol was isolated. It is likely that the benzoxazolone of Jaffé & Hilbert (1888) is an artefact derived from *o*-aminophenol and urea. It appears therefore that in dogs acetanilide is oxidized mainly in the *o*-position and in rabbits in the *p*-position. None of the above-mentioned work gives any clue whether acetanilide is deacetylated or not.

In 1889 Mörner, carefully avoiding hydrolytic procedures, worked up urine from a subject to whom acetanilide had been administered, and isolated potassium *p*-acetamidophenylsulphate as a double

salt with potassium ethyl oxalate. He also isolated a glucuronide which he was unable to characterize, but thought to be *p*-acetamidophenylglucuronide. Mörner's work suggests that the metabolites of acetanilide in man are acetylated.

From a study of the pharmacological properties of acetanilide and a number of related compounds, Hinsberg & Treupel (1894) concluded that the antipyretic and analgesic properties of the acetanilide group of drugs were related to their conversion in the organism to *p*-aminophenol or *p*-acetamidophenol. They found both these phenols to have a prompt antipyretic action in man (*p*-acetamidophenol is a more powerful antipyretic than phenacetin), but the action was not prolonged enough for successful use in therapy. It therefore appeared that the activity of acetanilide or phenacetin could be explained by its slow conversion *in vivo* into one or other of these phenols. Hinsberg & Treupel (1894) favoured *p*-acetamidophenol. More recently, Michel, Bernheim & Bernheim (1937) have shown that acetanilide is split into aniline and acetic acid by rat liver and kidney tissue, and that aniline can be oxidized by the same tissues to *p*-aminophenol. From this it appears possible that acetanilide might be deacetylated and then oxidized to *p*-aminophenol.

The quantitative work of Greenberg & Lester (1946) on the fate of acetanilide in man has shown that 70–90 % of the dose is excreted as conjugated *p*-aminophenol, 96 % as *O* conjugates (i.e. glucuronide and ethereal sulphates) and 4 % as *N* conjugates (probably *p*-acetamidophenol). Other metabolites of acetanilide have been postulated by various workers, e.g. *N*-acetyl-*N*-phenylhydroxylamine (Ellinger, 1920) and certain azo compounds (Greenberg & Lester, 1946), but there appears to be no real basis for the existence of these metabolites.

From the work discussed it is therefore clear that the exact nature of the metabolites of acetanilide is not known. In the present paper evidence will be presented to show that, in the rabbit, the main metabolites are the glucuronide and ethereal sulphate of *p*-acetamidophenol. Other work in progress in this laboratory indicates that acetanilide and aniline are not metabolically equivalent as is sometimes thought, for the metabolites of aniline are not the same as those of acetanilide.

METHODS

Animals. Rabbits fed on 50 g. Lever's cubes daily were used throughout the work. Compounds were administered by stomach tube with water.

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944).

Ethereal sulphate was determined by Folin's gravimetric method.

EXPERIMENTAL AND RESULTS

Table 1 indicates that the ratio glucuronide/ethereal sulphate (*G*/*E*) for acetanilide (5·8) is similar to that for *p*-acetamidophenol (6·3) and different from that for *p*-aminophenol (2·5). These figures could be interpreted as meaning that acetanilide is first oxidized to *p*-acetamidophenol which then conjugates with glucuronic and sulphuric acids in the same ratio as though the phenol itself were fed. The *G*/*E* ratio for *p*-aminophenol is so different from that of acetanilide that it could hardly be expected that acetanilide is transformed to *p*-aminophenol, i.e. via aniline. That this interpretation is correct is supported by the isolation experiments described below. The sum of the glucuronic acid and ethereal sulphate outputs (*G*+*E*) shows that 82 % of the acetanilide can be accounted for. *G*+*E* is somewhat less for *p*-acetamidophenol (73 %) and for *p*-aminophenol (60·5 %). This lesser recovery of the phenols can be accounted for by the fact that they are rapidly absorbed and excreted and some appear in the free state in the urine. In the case of acetanilide there is a slow conversion to the phenol, which therefore becomes more completely conjugated. Here we have an illustration of the time factor in detoxication.

Table 1. *The output of glucuronic acid and ethereal sulphate in rabbits receiving orally acetanilide and related compounds*

Compound	Dose (g./kg.)	% of dose excreted as: Glucuronide* (*G*)	Ethereal* sulphate (*E*)	*G*+*E*	*G*/*E*
Acetanilide	0·25	70	12	82	5·8
p-Acetamidophenol	0·28	63	10	73	6·3
p-Aminophenol	0·25	43	17·5	60·5	2·5

* Average results.

The effect of acetanilide on rabbits. In all experiments with rabbits receiving oral doses of acetanilide of 0·5 g./kg. it was observed that the animals fell into a deep narcosis for 4–5 hr. The animals all recovered without any apparent deleterious effect. This narcosis was not observed when the animals received *p*-acetamidophenol or *p*-aminophenol.

ISOLATION OF METABOLITES FROM ACETANILIDE URINE

(*a*) *Nature of the urine.* Urine from rabbits receiving oral doses of 0·5 g./kg. of acetanilide does not reduce Benedict's reagent except on prolonged boiling. It does not reduce cold ammoniacal $AgNO_3$. The urine does not give a colour with $FeCl_3$, nor does an ether extract of the untreated urine. The urine gives an intense naphthoresorcinol reaction for glucuronic acid and is laevorotatory.

(*b*) *Extraction at alkaline* pH. 2 g. of acetanilide were fed to each of 5 rabbits and the urine (1 l.) collected during the next 24 hr. A few ml. of 2N-KOH were added to the urine, which was then extracted continuously with ether for 1 hr. The extract on evaporation left a small oily residue which partly crystallized. A few drops of 2N-HCl were added to the residue and the whole was filtered, leaving a crystalline mass (40 mg.), which on recrystallization from hot water, was identified as acetanilide (m.p. and mixed m.p. 112–113°). The filtrate from these crystals was now made alkaline and extracted with ether. The ether was evaporated and the residue benzoylated with benzoyl chloride and NaOH. From the mixture there were isolated 20 mg. of benzanilide (m.p. and mixed m.p. 160°). The yields of aniline and acetanilide in four experiments are given in Table 2. Aminophenols were not found in this extract.

Table 2. *Recovery of acetanilide and aniline from the urine of rabbits receiving acetanilide orally*

	% of dose recovered as	
Exp.	Acetanilide	Aniline
1	0	0·5
2	0·4	0·1
3	0·2	0·1
4	0·2	0

(*c*) *Extraction at acid* pH. The above urine was made acid to congo red with HCl and extracted with ether for 1 hr. A small tarry residue remained on evaporation of the ether, but this contained nothing of significance.

(*d*) *Mild hydrolysis of acetanilide urine.* A total of 6 g. of acetanilide was fed to three rabbits and the urine (500 ml.) was collected during the following 24 hr. 350 ml. of the urine were made alkaline with a few ml. of 2N-KOH and then extracted continuously for 5 hr. with ether. Evaporation of the ether left 10 mg. of acetanilide; no aniline was found. The extracted urine was now made normal with respect to HCl by the addition of conc. HCl and then boiled gently for 20 min. A separate experiment showed that *p*-aminophenylglucuronide was only slightly hydrolyzed under these conditions. The urine was now cooled and made slightly alkaline with solid Na_2CO_3 and then extracted for 1 hr. with ether. On cooling the ether extract, 200 mg. of *p*-aminophenol separated (m.p. 184° after recrystallization). The ether was now taken to 5 ml. and a further 350 mg. of *p*-aminophenol were recovered. The filtrate from the second crop of crystals was now evaporated to a paste and the solid matter separated and benzoylated. It yielded 20 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. 229–230°). The mother liquor of the above paste was now stirred with 2N-NaOH and then extracted with ether. The ether yielded nothing. The alkaline liquor was made less alkaline by acidifying and then adding Na_2CO_3. Extraction with ether yielded a small residue, which on benzoylation yielded 1 mg. of dibenzoyl-*p*-aminophenol (m.p. 225–230°). No trace of *o*-aminophenol was found (*ON*-dibenzoyl-*o*-aminophenol has m.p. 180°). Further extraction of the urine with ether for 8 hr. yielded a small amount of *p*-aminophenol.

The total amount of *p*-aminophenol obtained by this mild hydrolysis was equivalent to 11% of the acetanilide fed. Quantitative determinations of the ethereal-sulphate output showed that 12% of acetanilide was excreted as a sulphate (see Table 1). It is therefore concluded that the ethereal-sulphate fraction of acetanilide urine contains no other phenol than *p*-aminophenol. Since very little of free amino compounds is excreted in acetanilide urine the actual phenol excreted as a sulphate is *p*-acetamidophenol (cf. Mörner, 1889).

(*e*) *The glucuronide fraction.* (1) *Detection of p-aminophenol.* Two rabbits were each fed 2 g. of acetanilide and the urine collected for 24 hr. The urine was made faintly acid with acetic acid and treated with saturated normal lead acetate. The precipitate was removed and discarded. The filtrate was made faintly alkaline and basic lead acetate added until precipitation was complete. The basic lead precipitate was filtered and washed with water. It was then suspended in water and the lead removed with H_2S. After removal of the PbS, the filtrate was evaporated with addition of ethanol *in vacuo* at 40–50° to a thin syrup. A small amount of inorganic material was removed from this syrup by diluting with ethanol and filtering. On addition of ether or chloroform to the ethanolic solution, the glucuronide was thrown down as amorphous hygroscopic flocks. In four separate experiments this material could not be induced to crystallize. The glucuronide gum gave only a very feeble diazo reaction indicating the almost total absence of free aromatic amino groups. On keeping a small portion of the gum with conc. HCl at room temperature there was no change in the diazo reaction.

The gum obtained after the feeding of 4 g. of acetanilide was now boiled for 1 hr. with 100 ml. 5N-HCl. The resulting solution gave an intense *p*-aminophenol type of diazo reaction (i.e. on diazotizing and coupling with dimethyl-α-naphthylamine there is obtained at first a fleeting dirty blue colour and then, on standing, the solution becomes a deep blue colour). The solution was made just alkaline and extracted with ether in a separatory funnel. The ether extract was evaporated and the residue benzoylated. There were obtained 800 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. and mixed m.p. 234°) after recrystallization from ethanol. The yield was 5% of the dose of acetanilide.

(2) *Acetylation and methylation of the glucuronide gum.* The gum from the urine of rabbits after the feeding of 10 g. of acetanilide was divided into two equal parts. The first part was methylated with methyl sulphate and alkali followed by methyl iodide and Ag_2O. The product was an intractable chloroform-soluble gum which was not further studied.

The second part was dissolved in 100 ml. of a 50 : 50 pyridine-acetic anhydride mixture and left overnight at room temperature. The mixture was then poured into 1 l. of dilute HCl, and the solution extracted with chloroform.

The chloroform extract was dried with anhyd. $CaCl_2$ and evaporated on a water bath. From the residue there were obtained 600 mg. (3·5% of the dose) of a white amorphous solid. This solid recrystallized from acetone water as needles, m.p. 224° (decomp.). It was acid to litmus and gave the naphthoresorcinol reaction on prolonged boiling. The diazo reaction was negative, but was positive after hydrolysis. It was laevorotatory, $[\alpha]_D^{20°} = -15·1°$ (*c*, 2·8 in acetone). This compound appears to be *p-acetamidophenyltriacetylglucuronic acid*. (Found: C, 52·7; H, 5·2; N, 3·7%. $C_{20}H_{23}O_{11}N$ requires C, 53·0; H, 5·1; N, 3·1%.) (Ultraviolet absorption spectrum: $\lambda_{max.}$ 245 mμ.; $\epsilon_{max.}$ 18,000 in ethanol (see Fig. 1).)

300 mg. of the above acid dissolved in acetone were methylated with methyl iodide and Ag_2O at room temperature overnight. Removal of the solvent yielded an *ester* (210 mg.) which crystallized from ethanol in small sheaves of rods with m.p. 210° and showing $[\alpha]_D^{25°} = -22·5°$ (*c*, 3·3 in chloroform). (Found: C, 54·05; H, 5·55; N, 3·4%. $C_{21}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Its ultraviolet absorption spectrum in ethanol (see Fig. 1) was determined, and was found to contain a band with $\lambda_{max.}$ at 246 mμ.; $\epsilon_{max.}$ 15,600. It was neutral in reaction, sparingly soluble in water, but easily soluble in acetone and ethanol. It was identified as the *methyl ester* of *p-acetamidophenyltriacetyl-β-D-glucuronic acid*, for it did not depress the melting point of an authentic sample of this ester and was identical with it in all respects (see below).

(3) *Isolation of the benzylamine salt of p-acetamidophenylglucuronide.* Identification of the glucuronide of acetanilide urine was finally established through the use of benzylamine. It was found that under certain conditions aminophenylglucuronides could be isolated from solutions in almost quantitative yields as crystalline benzylamine salts. This observation was made after testing a large number of bases including brucine, *o*-toluidine, benzidine, aniline, benzylthiourea and guanidine.

A rabbit was given 2 g. of acetanilide orally and its urine (225 ml.) was collected during the next 24 hr. This urine was estimated to contain about 1 g. of glucuronic acid more than normal. The glucuronide gum was prepared by systematic lead precipitation as already described. The gum was freed from small amounts of inorganic material by dissolution in ethanol and filtering. The purified gum was now dissolved in sufficient aqueous ethanol to produce a 5% solution of the gum. To this solution was added slightly more than one equivalent of benzylamine. Then ethyl acetate and ethanol were added alternately so that the gum concentration was 0·1–0·5% and enough ethyl acetate had been added to make the solution cloudy. The mixture was then allowed to stand at 0° for a few days. The crystalline precipitate (1·03 g., m.p. 196–197°) was filtered off and washed with ethanol. The filtrate was taken down and then diluted with ethanol-ethyl acetate as before. On standing a further 0·35 g. of crystals (m.p. 195–196°) was obtained. Repetition of the process yielded another 0·21 g. The total material recovered was 1·59 g. or 71% of the extra glucuronic acid in the urine. The crude salt gave a very faint diazo reaction which disappeared after one recrystallization from 95% ethanol. The salt showed $[\alpha]_D^{25°} = -63·8°$ (*c*, 4·7 in water), m.p. 195–197° and mixed m.p. 195–197° with authentic benzylamine *p*-acetamidophenylglucuronate (see below). (Found: C, 55·3; H, 6·2; N, 6·5%. $C_{21}H_{26}O_8N_2.H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.)

PREPARATION OF REFERENCE COMPOUNDS

Benzylamine salt of p-acetamidophenylglucuronide. Four rabbits were each fed 1·5 g. of *p*-acetamidophenol by stomach tube. This phenol is rapidly conjugated and excreted and therefore the urine was collected for 3–4 hr. after feeding. The basic lead acetate precipitate was obtained in the usual manner and the Pb removed by H_2S. The filtrate from the PbS gave, on concentration, *p*-acetamidophenylglucuronide as a gum which could not be crystallized. (Crystalline *p*-acetamidophenylglucuronide was obtained by acetylation of *p*-aminophenylglucuronide (see below).) The gum was dissolved in 3 vol. of ethanol and benzylamine and a mixture of ethyl acetate and ethanol was added to give a cloudy solution with a concentration of 0·1% as regards the gum. On standing at 0° the *benzylamine salt* crystallized out as needles (yield, 0·5 g.). The salt was recrystallized from water-ethanol-ethyl acetate mixtures, and had m.p. 195–197° and $[\alpha]_D^{25°}, = -64·1°$ (*c*, 4·4 in water). (Found: C 55·9; H, 6·3; N, 6·4%. $C_{21}H_{26}O_8N_2.H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.) It was very soluble in water but insoluble in dry organic solvents.

Benzylamine salt of p-aminophenylglucuronide. *p*-Aminophenylglucuronide (m.p. 215–216°; $[\alpha]_D = -82·7°$ in dilute HCl) was prepared according to Williams (1943). From the urine collected during the first 3 hr. after feeding 10 g. of *p*-aminophenol, 3·3 g. of the glucuronide were isolated. For the 24 hr. urine the yield of crystalline glucuronide was 3·95 g. or 13% of the dose. To prepare the benzylamine salt, the glucuronide was suspended in a small amount of water and slight excess of benzylamine added. This was followed by 5 vol. ethanol and then ethyl acetate until the solution became cloudy. On standing at 0°, the *benzylamine salt* of *p-aminophenylglucuronide* separated as square plates. This was recrystallized from the same solvents, and had m.p. 211–212° and $[\alpha]_D^{20°} = -62·7°$ (*c*, 6·5 in 0·2 N-HCl). (Found: C, 53·1; H, 6·6; N, 6·4%. $C_{19}H_{24}O_7N_2.2H_2O$ requires C, 53·3; H, 6·6; N, 6·5%.)

p-Acetamidophenylglucuronide. 2 g. of *p*-aminophenylglucuronide were dissolved in excess dilute Na_2CO_3 solution and shaken with an excess of acetic anhydride until tests for free aromatic NH_2 groups were negative. The *N*-acetylated glucuronide was obtained from the solution as the insoluble lead salt by addition of basic lead acetate solution. The lead salt, suspended in water, was freed from Pb by H_2S. The filtrate from the PbS was concentrated *in vacuo* to a clear syrupy solution (5–10 ml.). To this were added 100 ml. ethanol and the mixture was concentrated *in vacuo* at 40° to 5 ml. 50 ml. ethanol were now added followed by enough ether to give a slight precipitate. The whole was now kept at 0° for 48 hr. whereby *p-acetamidophenylglucuronide* (0·7 g.) crystallized as long, narrow plates. It was recrystallized from hot 95% ethanol (m.p. 193°, decomp. after sintering at 100°); $[\alpha]_D^{22°} = -63·9°$ (*c*, 7·5 in water). (Found: C, 45·3; H, 6·2; N, 3·95%. $C_{14}H_{17}O_8N.2\frac{1}{2}H_2O$ requires C, 45·2; H, 6·0; N, 3·8%.) It was very soluble in water, soluble in ethanol and insoluble in ether. It did not convert haemoglobin to methaemoglobin *in vitro* at 37° (cf. Williams, 1943).

p-Acetamidophenyltriacetylglucuronic acid methyl ester. 300 mg. of *p*-acetamidophenylglucuronide were dissolved in 50 ml. of absolute ethanol and an ethereal solution of

diazomethane (prepared from 1 g. of nitrosomethylurea) added. After 3 hr. the yellow solution was filtered and evaporated *in vacuo* leaving a white, amorphous, hygroscopic solid which was neutral to litmus. This was dissolved in 5 ml. of pyridine and then 5 ml. of acetic anhydride were added. After standing overnight, the mixture was diluted with 50 ml. of water and cooled to 0° for 2 hr. A mass of silky white needles (190 mg.) was deposited. A further 80 mg. were obtained by extracting the mother liquor with chloroform, and crystallizing from water. The product at this stage was apparently a *hydrate* for it melted at 100°, and then resolidified to a glass which melted indefinitely at 150–200°. Drying at 110° in air led to some decomposition because the material turned brown. The anhydrous product was obtained by dissolving the hydrate in absolute ethanol and scratching the beaker, whereby the *methyl ester* of *p-acetamidophenyltriacetylglucuronide* crystallized as sheaves of short needles, m.p. 200–205° and showing $[\alpha]_D^{25°} = -22{\cdot}1°$ (*c*, 7 in chloroform). (Found: C, 54·5; H, 5·6; N, 2·9%. $C_{31}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Ultraviolet absorption spectrum, $\lambda_{max.}$ 246 mμ., with ϵ_{max} 15,600. The compound is very soluble in acetone, ethanol and chloroform, but sparingly soluble in water and ether.

Benzylamine salt of o-aminophenylglucuronide. *o*-Aminophenylglucuronide (decomp. at 300°; $[\alpha]_D = -76{\cdot}1°$ in dilute HCl) was prepared by feeding rabbits with *o*-aminophenol according to Williams (1943). The *benzylamine salt* was prepared as for the *p*-isomer. The salt formed clumps of short needles, m.p. 204–205° and $[\alpha]_D^{20°} = -61{\cdot}3°$ (*c*, 6·1 in water). (Found: C, 58·0; H, 6·1; N, 6·9%. $C_{19}H_{24}O_7N_2$ requires C, 58·2; H, 6·1; N, 7·1%.)

SPECTROSCOPIC OBSERVATIONS

The ultraviolet absorption spectra of *p*-aminophenylglucuronide and its acetyl derivatives and *p*-acetamidophenol are recorded in Fig. 1. From this figure it can be seen that derivatives of *p*-aminophenol can be readily distinguished from those of *p*-acetamidophenol. Whereas the spectra of *p*-aminophenol (see Williams, 1947; Morton & Stubbs, 1940) and *p*-aminophenylglucuronide show two maxima in the region 220–320 mμ., with molecular extinction coefficients less than 10,000, all the derivatives of *p*-acetamidophenol exhibit only one band in this region with an extinction coefficient greater than 10,000. *p*-Acetamidophenol itself shows a band at $\lambda_{max.}$ 250 mμ., $\epsilon_{max.}$ 13,800 and an inflexion at 285 mμ., ϵ 3500, whereas *p*-aminophenol shows two bands at $\lambda_{max.}$ 234 and 395 mμ. with $\epsilon_{max.}$ 7950 and 2700 respectively. For the glucuronides, the figures are: *p*-acetamidophenylglucuronide, $\lambda_{max.}$ 243 mμ., $\epsilon_{max.}$ 10,800 and *p*-aminophenylglucuronide, $\lambda_{max.}$ 231 and 291 mμ., $\epsilon_{max.}$ 8700 and 1600.

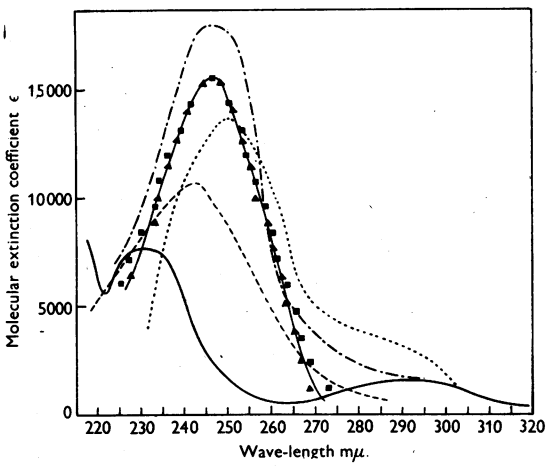
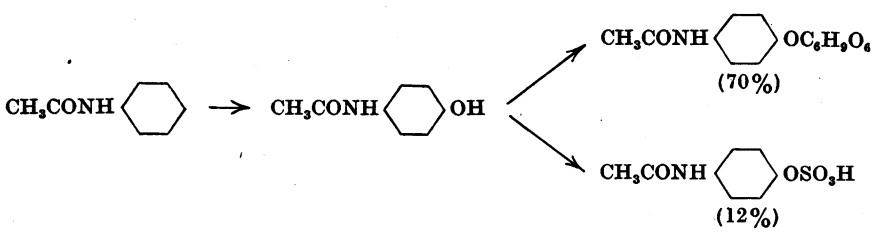


Fig. 1. Ultraviolet absorption spectra of *p*-aminophenylglucuronide and related compounds. · · · · *p*-acetamidophenol in ethanol: $\lambda_{max.}$ 250 mμ., $\epsilon_{max.}$ 13,800; ~ 285 mμ., ϵ 3500. - - - - - *p*-acetamidophenylglucuronide in water: $\lambda_{max.}$ 243 mμ., $\epsilon_{max.}$ 10,800. -·-·- *p*-acetamidophenyltriacetylglucuronic acid in ethanol: $\lambda_{max.}$ 245 mμ., $\epsilon_{max.}$ 18,000. ■—■ *p*-acetamidophenyltriacetylglucuronide methyl ester (synthetic) in ethanol: $\lambda_{max.}$ 246 mμ., $\epsilon_{max.}$ 15,600. ▲—▲ *p*-acetamidophenyltriacetylglucuronide methyl ester (from acetanilide urine) in ethanol: $\lambda_{max.}$ 246 mμ., $\epsilon_{max.}$ 15,600. —— *p*-aminophenylglucuronide in 0·1 N-KOH: $\lambda_{max.}$ 231 and 291 mμ., $\epsilon_{max.}$ 8700 and 1600.

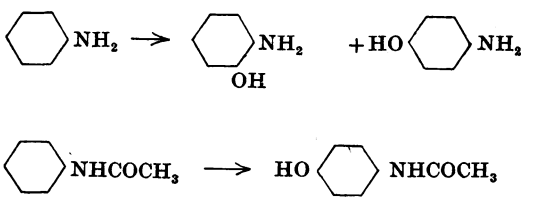
DISCUSSION

The present work proves that the metabolites of acetanilide in the rabbit are *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid which are excreted in the ratio of about 6 to 1 and account for over 80% of the acetanilide fed. Small traces of acetanilide (*c.* 0·2% of dose) and of aniline (*c.* 0·2%) are also found in the urine. The metabolism of acetanilide in the rabbit can therefore be represented as follows:



Acetanilide undergoes very little deacetylation (less than 5 % of the dose as determined by diazotization and coupling), an observation which agrees with our earlier results on *p*-substituted acetanilides (Smith & Williams, 1948). Michel *et al.* (1937) have shown that acetanilide is deacetylated by isolated rat tissues. Our results indicate that in the intact rabbit either such a deacetylating system does not occur or, if it does, it has no opportunity of functioning to any great extent. Some animals may, however, be able to deacetylate the aromatic acetamido group, for Sykes (1944) has shown that whereas N^4-acetylsulphamezathine is excreted almost entirely unchanged by man, 40 % is deacetylated in the pigeon. Whether or not an acetamido compound is deacetylated *in vivo* may have an important bearing on its fate in the body, as will be seen from what follows.

We have found (Smith & Williams, unpublished) that in rabbits aniline is oxidized but is not acetylated, and the products of oxidation are *o*- and *p*-aminophenols (together with small amounts of what we think is 2:4-dihydroxyaniline). No trace of *o*-aminophenol or its *N*-acetyl derivative was detected in acetanilide urine:



Present views (see Pauling, 1945) concerning the effect of substituents on the activity of aromatic rings in substitution reactions indicate that the amino group in aniline activates, mainly through resonance, the ortho and para positions. It is to be expected therefore that biological oxidation will take place in these positions. This actually occurs with aniline in the rabbit. In the case of acetanilide, biological oxidation occurs only in the para position. This result is similar to the behaviour of acetanilide on nitration which gives rise to the formation of 95 % *p*- and 5 % *o*-nitroacetanilide, whereas nitration of aniline gives 50 % *p*- and 40 % *o*-nitro compound (Cumming, Hopper & Wheeler, 1937). Now acetylation of the amino group of aniline will result in some deactivation of the ring. This deactivation is more pronounced in the *o*-position than in the *p*-position through the so-called inductive effect of the acetamido group working in opposition to the resonance effect. In addition to this, the acetamido group is larger than the amino group and will therefore exert a steric hindrance effect on ortho substitution. From these arguments it can be seen that ortho substitution of acetanilide may be hindered both by steric hindrance and by deactivation through induction. For biological oxidation to take place, it may be assumed that a minimum activation of the position to be oxidized is necessary. The absence of biological oxidation of acetanilide in the ortho position could therefore be explained on the grounds that (1) the ortho positions are not sufficiently activated for biological oxidation, and (2) steric hindrance due to the acetamino group prevents the approach of the biological oxidizing system to the ortho positions. Oxidation of acetanilide should therefore take place in the para position, for here there is no steric hindrance and the position is sufficiently activated, through resonance, for the enzyme system to attack it.

It follows from the above arguments that if an animal can deacetylate acetanilide to aniline, then it is to be expected that acetanilide will give rise to *o*- and *p*-aminophenol in such an animal. When acetanilide is fed to dogs, both *o*- and *p*-aminophenol can be detected in the urine (Jaffé & Hilbert, 1888). It is probable that the dog possesses an active deacetylating enzyme for aromatic acetamido compounds.

The fate of acetanilide in man (cf. Mörner, 1889) is probably very similar to that in the rabbit. Lester & Greenberg (1947) have recently shown that the major metabolites of acetanilide in man are *p*-acetamidophenol and its *O* conjugates, although they did not isolate these compounds.

SUMMARY

1. It has been shown that, in the rabbit, acetanilide is oxidized almost entirely to *p*-acetamidophenol, which is excreted in the urine as *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, these occurring in the ratio 6 : 1.

2. Less than 5 % of the acetanilide fed is deacetylated and only traces of aniline and unchanged acetanilide were isolated.

3. *p*-Acetamidophenylglucuronide can be conveniently isolated as the benzylamine salt.

4. The following reference compounds have been synthesized: the benzylamine salts of *o*- and *p*-aminophenylglucuronides and of *p*-acetamidophenylglucuronide; *p*-acetamidophenylglucuronide and the triacetyl derivative of its methyl ester.

5. The ultraviolet absorption spectra of the compounds studied have been recorded and discussed.

6. The theoretical implications of the biological oxidation of acetanilide have also been discussed.

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The Molecular Weight of Insulin and its Dependence upon pH, Concentration and Temperature

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In a previous paper (Gutfreund, 1948) it was shown from the results of osmotic and diffusion-sedimentation measurements that the molecular weight of insulin is 47,000–48,000 in solutions of pH 7·0 containing 0·5–1·0% protein. Molecules of that size were found to dissociate into smaller units on dilution to protein concentrations of 0·2% or less. Sjögren & Svedberg (1931) have determined the sedimentation constant of insulin over a range of pH from 3·5 to 12·3 and found from their data that the molecular weight has a maximum at pH 6·8. They concluded that insulin dissociates both in acid and in alkaline solutions and also that this dissociation is reversible if the solution is not kept too long at a pH outside the stability region. Gutfreund & Ogston (1946) re-examined the sedimentation constant over a range of pH from 3·5 to 9·6 and found that the sedimentation velocity of the main boundary remained constant between pH 6·7 and 9·6. In some cases, however, some slower-sedimenting material was present.

Gutfreund & Ogston were interested in the sedimentation rate of the main boundary. A re-examination of the sedimentation diagrams showed the presence, in solutions more alkaline than pH 8, of some more slowly sedimenting material which did not form a clear boundary.

The recent work of Waugh (1944, 1945, 1946) showed that insulin undergoes drastic and reversible changes in acid solutions, and the studies of the influence of pH on the properties of insulin solutions have therefore been continued and extended by means of both the osmotic pressure and ultra centrifugal technique.

EXPERIMENTAL

The sedimentation constants were determined at Oxford in the oil-turbine ultracentrifuge (Svedberg type). The sedimenting boundaries were observed by the Philpot (1938) method. The speed of the centrifuge during all the experiments was about 1000 rev./sec. A detailed description of the accuracy of the measurements is given by Gutfreund & Ogston (1946). All values of sedimentation constants (S_{20}) recorded are corrected to sedimentation in water at 20°.

For the osmotic-pressure measurements an apparatus of the type described by Adair (1925) was used. The pressure was measured directly as the height of solution in the capillary tube. The protein concentration of the equilibrated solution was calculated from Δn (refractive index difference between buffer and solution) assuming a specific refractive increment of 180×10^{-5}. Δn was measured in a Zeiss interferometer, the usual type of refractometers not being accurate enough for measurements on the very dilute solutions used throughout this work.

Since the solutions were very dilute all the values for molecular weights, recorded in this paper, were calculated by means of the simple formula

$$M = \frac{RTC}{P},$$

where M = molecular weight; P = osmotic pressure; R = gas constant; C = concentration of protein in g./l. of solution; and T = absolute temperature. Except where explicitly