SUMMARY

The fate of metanilamide in the rabbit has been studied.

1. This compound is converted *in vivo* into *m*-acetaminobenzenesulphonamide and the ethereal sulphate and glucuronide of 4-hydroxymetanilamide.

2. About 50 % is acetylated and more than 25 % is oxidized to 4-hydroxymetanilamide.

3. 20–25 % of the metanilamide is excreted as an ethereal sulphate.

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The Metabolism of Sulphonamides

2. THE ISOLATION AND IDENTIFICATION OF 3-HYDROXYSULPHANILAMIDE AS THE OXIDATION PRODUCT OF SULPHANILAMIDE IN THE RABBIT

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Although it is known that a number of the sulphonamide drugs used in therapy undergo oxidation *in vivo*, none of the oxidation products have hitherto been chemically identified. Scudi (1940) isolated from the urine of dogs receiving sulphapyridine a monohydroxysulphapyridine, m.p. 180–181°, but left its structure undetermined. Weber, Lalic & Major (1943) isolated from dog's urine a glucuronide of a hydroxysulphapyridine in which the hydroxyl group is attached to the pyridine ring. No other data of this type has been published on sulphonamide oxidation products.

The determination of the exact chemical nature and the biological properties of sulphonamide oxidation products is highly important since certain theories relative to the mode of action of sulphonamide drugs involve the formation of oxidation products. Mayer & Oechsli (1937) suggested that the activity of sulphanilamide was due to its conversion *in vivo* to an oxidation product which they suggested might be *p*-hydroxylaminobenzenesulphonamide (*N*⁴-hydroxysulphanilamide), this being

the effective therapeutic agent. Another theory involving the hydroxylamine derivative was put forward by Locke, Main & Mellon (1938), who assumed that since hydroxylamine (NH₂OH) and oximes inhibit catalase, *p*-hydroxylaminobenzene-sulphonamide would do the same (this is actually true; see Sevag, Shelburne & Ibsen (1942)). Hydrogen peroxide, normally resulting from the metabolic activities of bacteria such as streptococci and pneumococci, would not be removed by catalase if *p*-hydroxylaminobenzenesulphonamide were present and would therefore accumulate, and in turn destroy the bacteria. Some workers (e.g. Rosenthal & Bauer, 1939; James, 1940) claimed to have detected hydroxylamine derivatives in the urine of patients and of animals receiving sulphanilamide, but these claims were shown to be invalid by Thorpe, Williams & Shelswell (1941). In all, some four compounds have been suggested as oxidation products of sulphanilamide, i.e. *N*⁴-hydroxy- (I), 3-hydroxy- (II) and 2-hydroxysulphanilamide (III) and *p*-aminophenol (IV) (cf. James, 1940; Thorpe *et al.*

1941), but hitherto, unequivocal proof has not been provided concerning the precise chemical nature of the oxidation product or products.

It has also been suggested (e.g. Ottenberg & Fox, 1938) that cyanosis produced in patients by sulphanilamide may be due to oxidation products.

METHODS

Ethereal sulphate and free and total sulphanilamide excreted by rabbits were determined as described by Dobson & Williams (1946).

RESULTS

Quantitative determinations

Ethereal sulphate excretion. In an earlier paper (Shelswell & Williams, 1940) it was shown that rabbits excreted from 6 to 12 % of doses of sulphanilamide varying from 0.2 to 1.0 g./kg. as an ethereal sulphate. In the present work, determinations confirmed these results. Two rabbits were given 500 mg./kg. orally; one excreted in 2 days 9.5 and the other 7.2 % of the dose as ethereal sulphate.

Free and total sulphanilamide. The results are given in Table 1.

Table 1. Excretion of free and total sulphanilamide by the rabbit

Rabbit no.	Dose (mg./kg.)	Time of excretion (days)	Sulphanilamide excreted, % of dose		
			Free	Total	Acetylated
2	100	2	54	94	40
5	100	2	8	90	81
8	100	2	32	89	57
2	250	2	45	57	12
2	250	1	52	63	11
4	250	1	62	81	19
5	250	1	15	44	29

It has been reported by Marshall, Emerson & Cutting (1937) and Gilligan (1945) among others, that acetylation of the sulphonamide drugs is an extremely variable process both in man and animals. The results in Table 1 add further confirmation to these reports.

Identification of metabolic products

The isolation of 3-hydroxysulphanilamide. Several unsuccessful attempts were made to isolate 3-hydroxysulphanilamide in a pure crystalline state from urines in which its presence was detected by colour reactions. The following is a description of an experiment in which the phenol was isolated in a crystalline state.

Twenty rabbits (2–3 kg. wt.) were each given by stomach tube 2 g. of sulphanilamide suspended in water. The dose was repeated on the two following days until a total of 120 g. of sulphanilamide had been fed. The urine was collected for 5 days after the first dose, the total volume (including funnel washings) being 10 l. It was alkaline to litmus.

(a) **Examination of the urinary deposits for azoxybenzene-4:4'-disulphonamide.** It is known that *p*-hydroxylaminobenzenesulphonamide is rapidly transformed in neutral or alkaline solution into azoxybenzene-4:4'-disulphonamide (Bratton, White & Marshall, 1939). If any of the hydroxylamine compound were excreted it would be rapidly transformed in the alkaline urine of the rabbit into the azoxy compound. The latter is very insoluble and would, therefore, be found in the urinary deposit together with acetylsulphanilamide. A separate experiment had shown that pure *p*-hydroxylaminobenzenesulphonamide, dissolved in normal rabbit urine (alkaline to litmus), was rapidly precipitated as insoluble azoxybenzenedisulphonamide. The latter compound can easily be detected in urinary deposits by means of its characteristic ultra-violet absorption spectrum, which in ethanol or in KOH shows a large band with maximum absorption at 333 m μ . (see Fig. 1).

Fig. 1. Spectral absorption of azoxybenzene-4:4'-disulphonamide. A. In ethanol: $\lambda_{\max}$ 333 m μ , $\epsilon_{\max}$ 20,665. B. In 0.01 N-KOH: $\lambda_{\max}$ 333 m μ , $\epsilon_{\max}$ 18,666.

The urine collected during the first 2 days of the experiment was centrifuged and the deposit obtained was extracted with 350 ml. 10 % (w/v) NaOH for 3 days. The mixture was filtered and the filtrate examined spectrographically for azoxybenzene-4:4'-disulphonamide. The result was negative. On acidifying a little of the alkaline extracted with strong HCl and allowing it to stand, long needles of acetylsulphanilamide, m.p. 216°, separated.

The urine collected on the third day was similarly treated, but spectroscopic examination failed to reveal any trace of the azoxy compound in the deposit.

The centrifuged urine of the first 2 days was now allowed to stand at 0° for 6 days and the deposit which formed

during this time was collected on the centrifuge. Spectroscopic examination of this deposit again gave a negative result for the azoxy compound.

(b) *Hydrolysis and extraction of the urine.* The centrifuged urine (c. 10 l.) was concentrated *in vacuo* to 675 ml. To this 400 ml. conc. HCl were added and the mixture was boiled for 2 hr. To the cooled mixture 40% (w/v) NaOH was added until it was just acid to litmus. The mixture was filtered from some black material (this material was examined for the azoxy compound with negative results), and kept overnight at 0°. A deposit of crude sulphanilamide (30 g.), which contained no azoxybenzenedisulphonamide, was removed by filtration.

The urine was now extracted with diethyl ether in a continuous extractor for 40 hr. Evaporation of the ether left 54.3 g. of pale brown crystalline material. This was dissolved in 400 ml. hot water and charcoal added. The solution was filtered and allowed to crystallize at 0°. 30 g. of sulphanilamide, m.p. 163–164° (free from azoxybenzenedisulphonamide by spectroscopic test), were filtered off. The filtrate gave a strong FeCl₃ colour reaction for 3-hydroxysulphanilamide, i.e. purplish red to red to brown. It was taken to dryness *in vacuo* leaving a semi-solid pale brown mass (D).

(c) *Chromatographic analysis of D.* The material D was dissolved in 200 ml. absolute ethanol and then 200 ml. benzene were added. The solution was now chromatographed on an alumina column (aluminium oxide standardized according to Brockmann, Savory and Moore, Ltd., London). The column was washed with ethanol until the diazo test (nitrous acid and dimethyl- α -naphthylamine) carried out on the washings was very feeble. On removal of the solvent the washings yielded 6.15 g. of crystalline material, which was identified as mainly sulphanilamide.

The 3-hydroxysulphanilamide remained on the column which showed a broad yellow band (B), located near the bottom, the rest (T) of the column above this band having a pale brownish yellow colour. Colour tests indicated that the main bulk of the phenol was in the broad yellow band B with smaller quantities in T. The column was cut, separating the two parts. B was extracted with hot water until it no longer gave a colour with FeCl₃. The extract was filtered and taken to dryness *in vacuo*, affording 1.7 g. of a pale brown, sticky solid giving intense colour reactions for 3-hydroxysulphanilamide. This solid was recrystallized from a little hot water with addition of a little charcoal, and after several days there were obtained 200 mg. of crude 3-hydroxysulphanilamide, m.p. 148°. On recrystallization from hot water, it formed small prisms, m.p. 161°, mixed m.p. 161° with authentic 3-hydroxysulphanilamide, m.p. 164°. (Found: C, 38.7; H, 4.4; N, 15.2; S, 17.2%. Calc. for C₈H₉N₂SO₃: C, 38.3; H, 4.3; N, 14.9; S, 17.0%.) The crystals gave all the colour reactions of authentic 3-hydroxysulphanilamide, which are as follows: (a) On adding NaNO₂ and HCl to a solution of the phenol an intense yellow colour is obtained. If ethanolic dimethyl- α -naphthylamine is added to the diazotized solution (excess nitrous acid having been destroyed by ammonium sulphamate) coupling to an orange-red colour takes place slowly. (b) On adding 2% FeCl₃ to the phenol a purplish red colour which rapidly turns red and finally brown, is obtained. If this reaction is carried out in the presence of peroxide-free ether, the ether becomes coloured an intense ruby red. This ruby red ethereal solution shows an absorption band with a maximum

at 530 m μ . (this reaction will be described in greater detail in a later publication) and the colour is stable for about a day. (c) On heating an ethanolic solution of the phenol with 2:4-dinitrobenzaldehyde in the presence of a few drops of glacial acetic acid for about 15 min., a mixture is obtained which gives an intense red colour with alkali (10% KOH). The red colour is discharged by acid.

The crystals were also carefully compared with all the other possible oxidation products of sulphanilamide (see introduction). The above tests and analyses provide unequivocal proof of the identity of the phenol isolated.

The portion T of the column was treated in a similar manner to B and from it there was obtained 1.3 g. of a sticky solid which yielded, on repeated recrystallization from water, 55 mg. of 3-hydroxysulphanilamide, m.p. 160°, mixed m.p. 163° with authentic phenol, m.p. 164°.

The injection of p-hydroxylaminobenzenesulphonamide. Water was heated to boiling and allowed to cool to 40–45° with a stream of nitrogen passing through it. 250 mg. of p-hydroxylaminobenzenesulphonamide (m.p. 140°) were dissolved in 15 ml. of this water and 10 ml. of the solution were quickly injected subcutaneously into a rabbit. The animal became cyanosed after about an hour. Its urine (60 ml.) was collected for 19 hr., centrifuged and the deposit tested for azoxybenzene-4:4'-disulphonamide. The result was negative.

The experiment was repeated with two rabbits each receiving 200 mg. of the hydroxylamine; both animals became cyanosed. The urine was collected separately on each of the following 2 days. The two deposits were examined for the azoxy compound but only uncertain traces were found spectroscopically.

The combined urines were extracted for 4 hr. with peroxide-free ether, which on evaporation left a brownish crystalline residue. Qualitative tests showed that this residue contained free sulphanilamide but no hydroxylamine derivative. On recrystallization from water the residue yielded 50 mg. of pure acetylsulphanilamide (m.p. 213°).

DISCUSSION

The present work proves that the oxidation product excreted in the urine of rabbits fed sulphanilamide is 3-hydroxy-4-aminobenzenesulphonamide (3-hydroxysulphanilamide). No evidence was obtained of the presence of other oxidation products such as p-hydroxylaminobenzenesulphonamide and its N-acetyl derivative, 2-hydroxysulphanilamide and p-aminophenol. Other work in progress in this laboratory has shown that 3-hydroxysulphanilamide also occurs in the urine of hospital patients treated with sulphanilamide. The phenol is not excreted as such but appears in the urine mainly as an ethereal sulphate. Whether or not a glucuronide is excreted after sulphanilamide administration appears to be doubtful. Gilligan (1945) has reported that sulphanilamide does not increase the glucuronic acid output in man and Scudi & Robinson (1941) found a similar result with rats. It appears likely that, since the amount of 3-hydroxysulphanilamide formed is small, not enough is produced to give rise to appreciable glucuronide formation, the sulphate

mechanism being able to cope with most of the phenol. When, however, 3-hydroxysulphanilamide is fed to rabbits, it is partly excreted in conjugation with glucuronic acid (Williams, 1943). It may be stated, therefore, that when sulphanilamide is fed to rabbits, the urine is found to contain free sulphanilamide, acetylsulphanilamide and the ethereal sulphate of 3-hydroxysulphanilamide,

Concerning the formation of *p*-hydroxylaminobenzenesulphonamide, the present work does not entirely eliminate the possibility that it may be formed *in vivo*, but it does prove that this hydroxylamine is not excreted in the urine. The possibility still exists that *p*-hydroxylaminobenzenesulphonamide may be formed and rearranged *in vivo* to 3-hydroxysulphanilamide. There is, however, circumstantial evidence against this possibility. First, Bratton *et al.* (1939) have shown that on injection of this hydroxylamine into dogs, it is largely converted into sulphanilamide (dogs do not acetylate the aromatic amino group). In the rabbit, the subcutaneously injected hydroxylamine is largely converted into sulphanilamide and acetylsulphanilamide. Secondly, in the analogous case of *p*-hydroxybenzenesulphonamide, Williams (1941) has proved that this compound is oxidized in the rabbit to catechol-4-sulphonamide, a result which shows that the benzene ring can be oxidized in the *meta* position to a sulphonamide group and *ortho* to a hydroxyl

group when both these groups occur together *para* to each other. Thirdly, the chemical transformations of *p*-hydroxylaminobenzenesulphonamide show that this compound is transformed in neutral or alkaline solution solely to azoxybenzene-4:4'-disulphonamide (Bratton *et al.* 1939; Burton & Walker, 1943),

whereas on heating in acid solution it forms the azoxy compound and *p*-aminophenol (Burton & Walker, 1943). *p*-Aminophenol was not detected and this confirms earlier work (Thorpe *et al.* 1941). It has not yet been shown that this hydroxylamine can be converted chemically into 3-hydroxysulphanilamide.

SUMMARY

1. The oxidation product of sulphanilamide excreted by rabbits has been isolated and proved to be 3-hydroxy-4-aminobenzenesulphonamide. This compound is excreted as an ethereal sulphate.
2. Injection of *p*-hydroxylaminobenzenesulphonamide into rabbits results in the excretion of sulphanilamide and acetylsulphanilamide.
3. The ultra-violet absorption spectrum of azoxybenzene-4:4'-disulphonamide has been recorded.

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