78. THE ISOLATION OF SOME OXIDATION PRODUCTS OF SULPHANILAMIDE FROM URINE

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OXIDATION is a primary detoxicating mechanism of the organism; the first stage is often the formation of a hydroxyl derivative, which is then conjugated and excreted as an ethereal sulphate or glucuronate. Such oxidation products are formed after the administration of aromatic amines such as aniline, and it has become of interest to determine whether similar products are formed from sulphanilamide, and what part they play in the therapeutic activity of the compound.

Jaffé & Hilbert [1888] isolated *p*-aminophenol and *o*-hydroxycarbanil and its acetyl derivative from urine after acetanilide administration. Herrick & Irons [1906] found *p*-aminophenol in the urine after acetanilide poisoning, and further found an increase in the ethereal sulphates.

Young & Wilson [1926] confirmed this and also showed an increase in the blood phenols. They attributed the observed cyanosis to the production of a dyestuff which coloured the blood corpuscles.

Ellinger [1920] isolated acetylphenylhydroxylamine from the blood of cats, following single acetanilide injections.

Hinsberg & Treupel [1894] concluded that primary and secondary aminophenols could produce methaemoglobin, while their acetyl derivatives were less active and the ethers inactive.

Heubner [1913; 1923], investigating the action of *p*-aminophenols, suggested that unstable quinonimine was an intermediate in methaemoglobin formation.

Alsted [1937], Gley [1937] and Mayer [1937] have all suggested by analogy the possibility of the formation of *p*-hydroxylaminobenzenesulphonamide and *p*-aminophenol. Mayer, indeed, suggests that the whole anti-bacterial activity is due to the formation of oxidation products. This paper makes no attempt to investigate that phase of the problem, but confines itself to the description of compounds obtained at various stages of the chemical oxidation of sulphanilamide.

Sulphanilamide is an aromatic amine, but stabilized to some extent by the sulphonamide grouping in the para position. Sulphanilic acid, however, may be oxidized to *p*-quinone, the end-product of the oxidation of aniline. Like aniline, sulphanilamide causes methaemoglobinaemia, but with less frequency. It is possible that similar intermediates are formed in the oxidation of the two substances in the body, and the attempt was therefore made to isolate them from urine.

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Examination of urine for evidence of conjugation

The ratio of inorganic to ethereal sulphates was determined by the method of Rosenheim & Drummond [1914] before, during and after sulphanilamide therapy, when this was possible. Many patients, however, were not available before medication began, or for sufficiently long after it had ceased.

The results in Table 1 show that the sulphate ratio during medication falls below the normal value of 10 : 1 [Hawk & Bergeim, 1938], giving evidence of the production of hydroxy substances during administration of sulphanilamide.

Table 1. *Ratio of inorganic to ethereal sulphates*

The figures in parentheses are days over which the average was taken.

Patient	Before treatment	During treatment	After treatment
1	—	3.6 (5)	—
2	—	2.8 (2)	10.0 (4)
3	6.2 (3)	2.9 (7)	8.7 (2)
4	6.1 (1)	2.8 (7)	7.0 (1)
5	—	3.4 (10)	7.0 (4)
Average	6.2 (4)	3.1 (31)	8.2 (11)

Glycuronic acid was estimated in some of the urines by the method of Quick [1924]. It does not occur in normal urines and its presence is additional evidence of the formation of hydroxyl derivatives from sulphanilamide.

It was noticed that the urine of two patients with urinary infections contained less than the urine of a patient with a streptococcal infection. This may be due to decomposition of the glycuronic acid by organisms of the coliform group observed by Kay [1926] and by Quick & Kahn [1929]. The results of the estimations are shown in Table 2. As before, the figures in parentheses are the days over which the average was taken.

Table 2. *Excretion of glycuronic acid; mg. per 100 ml.*

Patient	Infection	Before treatment	During treatment	After treatment
2	<i>Bact. coli</i>	—	4.2 (2)	0.8 (4)
4	<i>Bact. coli</i>	0.0 (1)	2.8 (7)	3.1 (1)
5	<i>Strep. haem.</i>	—	10.5 (10)	5.9 (4)
Average		0.0 (1)	7.0 (19)	3.3 (9)

The presence of glycuronic acid and the altered sulphate ratio lead to the conclusion that conjugation is occurring and that hydrolysis will be necessary in order to liberate any oxidation products.

Production of methaemoglobin by urine

Neither normal urine nor that from patients receiving sulphanilamide, when incubated with defibrinated human blood for 18 hr. gave spectroscopic evidence of the presence of methaemoglobin.

When the urines were hydrolysed by boiling with conc. HCl for 15 min. and neutralized before incubating with the blood, the normal urine was still inactive, but the sulphanilamide urine caused very strong methaemoglobin bands to appear. The hydrolysis produces free hydroxylamine derivatives and also liberates *p*-aminophenol from its conjugation products.

Isolation of oxidation products

The increased ethereal sulphate and glycuronic acid in sulphanilamide urine and the failure to produce methaemoglobin except after hydrolysis all showed the advisability of hydrolysis prior to fractionation of the urine.

The urine was evaporated at 40° under reduced pressure, and the residues were then brought to pH 1.0 by the addition of HCl; a further addition of 5 ml. conc. HCl per 100 ml. residue was made to compensate for the loss caused by the decomposition of the urea present. The resulting liquid was then autoclaved at 15 lb. pressure per square inch (120°) for 2 hr. in a pressure bottle.

The pH of the final product varied from 3.0 to 5.0 and was adjusted to 5.0. Extraction with ether was then carried out several times. The aqueous residue still produced methaemoglobin when neutralized and incubated with blood, hence further ether extractions were made at pH 7.0 and 9.0, until finally the aqueous residue no longer produced methaemoglobin.

At this stage it was observed that the aqueous residue after extraction at pH 7 no longer produced methaemoglobin if the urine were from uncyanosed patients whilst if it were from cyanosed ones, then extraction at pH 9.0 was necessary for complete removal.

As a control specimens of urine from 8 normal puerperal patients who were not receiving sulphanilamide or its derivatives were processed, but none of the extracts produced methaemoglobin.

The ethereal extracts were separately evaporated and the residues so obtained were purified by recrystallization from water after treatment with activated charcoal. The partially purified products were again treated with activated charcoal and boiled for $\frac{1}{2}$ hr., filtered, recrystallized and the crystals dried at 110°. Pale yellow or white solids were obtained which were submitted to further examination.

*Examination of urine extracts**Substance A, extracted at pH 5.0.*

The purified residue was strongly acid in reaction and contained C, H, N, O and S. It gave a diazo reaction after reduction. The equivalent weight by titration with NaOH was 97 and it contained 7.2% N and 17.2% S; m.p. >300° with decomposition; it gave a violet colour with ferric chloride and benzoyl chloride (i.e. contained a hydroxylamine group).

These properties correspond to a substance of the formula $\text{NHOH} \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_3\text{H}$, mol. wt. 189, equiv. wt. 94.5 since the NHOH group is titratable with NaOH, it contains 7.4% N and 17.0% S.

Substance B, extracted at pH 7.0.

The substance was faintly acid; it gave no diazo reaction either directly or after reduction; equiv. wt. by titration, 228.6; it contained 12.6% N and 14.7% S; m.p. >300° with decomposition; it gave no hydroxylamine reaction but after boiling with dilute HCl and reducing it was possible to couple the compound. These properties correspond with those of the substance $\text{COCH}_3 \cdot \text{NH} \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2\text{NH}_2$ which is monobasic and has a mol. wt. of 230 and contains 11.6% N and 13.2% S.

This compound was prepared by acetylation of *p*-hydroxylaminobenzene-sulphonamide; the m.p. of the latter was >300° with decomposition and its properties were similar to those of the compound isolated. This substance is analogous with the acetylphenylhydroxylamine isolated by Ellinger [1920].

Substance C and pigment, extracted at pH 9.0.

(a) This substance was taken out of ethereal solution by dilute HCl, decolorized with charcoal and evaporated to crystallizing point when long needles separated which on analysis gave the following results: 9.0 % N, and 23.0 % Cl; m.p. 306° with decomposition. It gave a violet colour with ferric chloride and a blue indophenol reaction. The chlorine was ionized in solution which was neutral and the substance on treatment with chloroform and NaOH gave a carbylamine reaction for primary amines. The monoacetyl compound melted at 167°. These properties correspond to *p*-aminophenol, the hydrochloride of which melts at 304° (decomposition) and contains 9.65 % N and 24.4 % Cl and the monoacetyl derivative of which melts at 168°.

(b) The residual ethereal solution was coloured purple although some pigment had been removed by the acid extraction. The solution was evaporated, leaving a tarry residue which could not be crystallized, although some purification was effected by adsorption on to charcoal and elution by absolute alcohol.

The pigment appeared to belong to the indophenol group since it was decolorized by stannous chloride to give a leuco base, and was regenerated by oxidation with ferric chloride [Rota, 1898]. The ethereal solution did not give up its colour to 5 % acetic acid and the colour changed to red after gentle warming with dilute HCl. Dr C. Rimington kindly examined a sample of this pigment and stated that it was not a porphyrin.

Extraction of p-aminophenol from unhydrolysed urine

Phenylhydroxylamine is converted into *p*-aminophenol by heating with HCl and an unsuccessful attempt was made to convert *p*-hydroxylaminobenzene-sulphonamide into *p*-aminophenol under the conditions of hydrolysis. It was nevertheless desirable to isolate *p*-aminophenol from unhydrolysed urine.

p-Aminophenol was administered to mice and the urine collected to discover the form in which it is excreted and the best method of isolating it. The following method was adopted.

The urine was evaporated to a syrup which was extracted with 80 % alcohol, the extract filtered and the filtrate evaporated to a syrup and allowed to stand in a refrigerator. Crystals separated; the liquid was poured off and the crystals were rapidly washed with alcohol and dissolved in water. They gave positive diazo and indophenol reactions. After hydrolysis with dilute acid there were strongly positive sulphate and indophenol reactions but only a weak diazo reaction. The crystals were probably $\text{NH}_2\cdot\text{C}_6\text{H}_4\cdot\text{OSO}_3\text{H}$ showing that the substance was excreted principally as the acid sulphate.

This substance was also obtained from concentrated urines from patients receiving sulphanilamide who had shown cyanosis.

It was found that both the hydroxylamine compound and sulphanilamide formed compounds with xanthyrol by the method of Wood [1937], the hydroxylamine derivative melting at 260° with decomposition, whilst that of sulphanilamide melted at 208° (Wood found 209° for this compound).

When an alcoholic solution of xanthyrol was added to urine made acid with acetic acid and allowed to stand, crystals were obtained which were fractionated from 50 % acetic acid. The least soluble fraction on further crystallization and drying had a melting point of 258° while the more soluble fraction on similar purification melted at 208°. This showed the presence of free hydroxylamine compound and also of free sulphanilamide in the urine.

Xanthyrol did not form a condensation product with *p*-aminophenol.

Effect of cyanosis

The urine of a cyanosed patient was collected in two periods, firstly, during and immediately after cyanosis, secondly up to the cessation of medication. The urines were fractionated and the crude fractions weighed.

Table 3. *Yields of derivatives from the urine of a cyanosed patient*

Period	Days	Vol. urine/day	Substance A		Substance B		Substance C	
			mg./day	mg./100 ml.	mg./day	mg./100 ml.	mg./day	mg./100 ml.
1	10	663	120	18	45	7	29	4
2	2	112	200	18	82	7	12	1

Table 3 shows that while much less of substances A and B was passed per day during cyanosis, much more of substance C was excreted. This connexion between *p*-aminophenol and cyanosis has been repeatedly noticed.

Bactericidal properties of the substances

The bactericidal activity of the compounds was examined by Mr W. R. Maxted who found that substances A and B were bactericidal to group A haemolytic streptococci in whole blood, whilst substance C and *p*-aminophenol were not but produced methaemoglobin. In serum both C and *p*-aminophenol were bactericidal and produced a purple indophenol pigment soluble in serum but extracted by absolute alcohol. The failure of these substances to be bactericidal in whole blood is due to their interaction with the haemoglobin.

Estimation of the substances

It was found possible to estimate the *p*-aminophenol by the indophenol reaction and the hydroxylamine compound by the method of Pucher & Day [1926]. Making use of these estimations it was found that the sulphanilamide excreted in various forms approximated to 100 % of the amount given. Marshall *et al.* [1937, 1] found 70–87 % recovery of the drug in patients with normal renal function but only 12–57 % in patients with impaired renal function. The renal condition of the following cases was not investigated but it will be seen that the total recovery is often much greater than that indicated by the amount of sulphanilamide.

Table 4. *Recovery of sulphanilamide and its oxidation products, expressed as % of dose given*

Patient	Sulphanilamide %	Hydroxylamine <i>p</i> -Aminophenol as sulphanilamide		Total recovery %
		as sulphanilamide %	as sulphanilamide %	
1	55.7	24.3	2.3	82.3
2	30.0	16.0	1.0	47.0
3	33.8	24.8	0.3	58.9
4	82.5	4.5	0.0	87.0
5	74.9	10.5	0.1	85.5
6	42.0	11.2	0.3	53.5

DISCUSSION

The isolation of the oxidation products of sulphanilamide presents some difficulties, for they are very reactive substances and are excreted in the conjugated form with these reactive groups protected.

p-Acetylhydroxylaminobenzenesulphonamide (substance B) definitely occurs in the urine, and substance A, *p*-hydroxylaminobenzenesulphonic acid, is probably derived from it during the acid hydrolysis of the urine.

This view was confirmed, for substance A was obtained when either *p*-acetylhydroxylamino- or *p*-hydroxylamino-benzenesulphonic acid was hydrolysed with acid in the autoclave.

There is no certain evidence whether the hydroxylamine compound is excreted entirely in the acetylated form or whether some occurs free. At the time when this work was done, it was not known that simple diazotization also estimates the free hydroxylamine substance. Repeated failure had resulted from attempts to obtain increased sulphanilamide values from urine after reduction and this had been ascribed to the absence of the hydroxylamine compound.

The free compound cannot be present in large quantities, since unhydrolysed urine does not produce methaemoglobin. The isolation of the xanthidrol compound from untreated urine, on the other hand, suggests that it does occur in the free state, although even in this experiment the use of 50 % acetic acid may have produced some of it by hydrolysis.

It may not be as stable to oxidation as substance A (*p*-hydroxylaminobenzenesulphonic acid), and may have been oxidized further to the azoxy compound and beyond. Other oxidation products of the azoxy and quinone types are probably present in the urine, but these, if present, were destroyed during hydrolysis or removed by the extensive charcoal treatments. Polymerization and condensation of these products to give tarry substances probably occurs.

This investigation is only of a preliminary character and a more thorough fractionation might yield other substances.

The *p*-aminophenol is probably present in the urine in the conjugated form, for there was evidence of its ethereal sulphate in untreated urine. The failure of the urine to produce methaemoglobin or to give an indophenol reaction before hydrolysis shows that free *p*-aminophenol is not present. There is a possibility that some is derived from the other oxidation products during the acid hydrolysis of the urine. Phenylhydroxylamine yields *p*-aminophenol by heating with acid but it was not detected in control experiments after acid hydrolysis in the autoclave of hydroxylaminobenzenesulphonamide or its acetyl derivative. The conversion is probably hindered by the presence of the sulphonamide grouping in the para position.

Some of the *p*-aminophenol is excreted with the amino group free and the phenol group conjugated, for it gives the diazo reaction, but hydrolysis leads to a negative diazo test owing to oxidation of the free *p*-aminophenol by the nitrous acid.

The pigment of the indophenol group is interesting in view of the theory of Young & Wilson [1926] that a dyestuff was the cause of cyanosis in aniline poisoning and of the isolation of a pigment from the blood cells by Lundsten *et al.* [1938] after acetanilide poisoning. Marshall *et al.* [1937, 2], however, were unable to isolate a pigment from the blood of patients treated with sulphanilamide. The production of pigments by oxidation of sulphanilamide has been reported by many authors and illustrates the complexity of the substances that may be formed by the condensation of the primary oxidation products.

The stages of sulphanilamide oxidation and excretion may be represented as follows [cf. Rimington & Hemmings, 1939], although future knowledge may clarify some reactions which at the moment are obscure.

The oxidation products may be the cause of some of the toxic effects noticed by clinicians. Both the hydroxylamine compound and *p*-aminophenol in the unconjugated form can produce methaemoglobin. The hydroxylamine derivatives may be the cause of the drug rash, since phenylhydroxylamine causes eczema in susceptible persons [Gattermann & Wieland, 1937].

SUMMARY

p-*N*-Acetylhydroxylaminobenzenesulphonamide, *p*-hydroxylaminobenzenesulphonic acid and *p*-aminophenol have been isolated from the urine of patients treated with sulphanilamide. They appear to be excreted in conjugation with sulphates and glucuronates. The *p*-aminophenol undergoes further change to give rise to a pigment which is also excreted.

It is suggested that these compounds may be the cause of some of the toxic effects noted during therapy with sulphanilamide.

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