105. THE DETERMINATION OF SULPHANILAMIDE AND ITS DERIVATIVES

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THE extensive use of sulphanilamide and its derivatives in medicine during the past few years has naturally led to the investigation of methods for the estimation of this group of drugs in blood, especially as it seems that a knowledge of the blood level of the compound used is necessary for correct treatment. The work of Marshall *et al.* [1937] has shown that sulphanilamide is acetylated in the animal organism, and, since the therapeutic value is very considerably altered by this conjugation, a knowledge of the amount of this bound sulphanilamide as well as of the free compound is necessary.

The methods for the estimation of sulphanilamide in blood may be divided into two groups. In the first of these the sulphanilamide is diazotized, and the diazonium salt coupled with a suitable aromatic amine or phenol to give an intensely coloured azo dyestuff which may be determined colorimetrically. The coupling may be carried out in alkaline solution [Fuller, 1937], or in acid solution [Marshall, 1937-8; Scudi, 1937-8; Bratton & Marshall, 1939]. In the second group the sulphanilamide is combined directly with a suitable compound to give a coloured complex which is determined colorimetrically. Examples of this latter group are the sodium β -naphthaquinone-4-sulphonate method [Schmidt, 1937-8], and the *p*-dimethylaminobenzaldehyde methods described later.

The diazotization methods, especially that of Bratton & Marshall [1939], are extremely sensitive, and have been widely used. They have, however, several disadvantages, such as impermanence of colour, the use of unstable reagents, and interference by other substances such as traces of metals [Marshall, 1937-8], thiocyanates [Gregerson & Painter, 1938] etc.

Quantitative methods, based on the yellow colour given by sulphanilamide with *p*-dimethylaminobenzaldehyde in acid solution, have been worked out by Kühnau [1938], Kimmig [1938] and Werner [1939], while the latter's work has been partly substantiated by Andrews & Strauss [1941]. The advantages of the method as summarized by Andrews & Strauss are simplicity and rapidity of manipulation, and permanence of colour.

Work in this laboratory required a method for the determination of sulphanilamide in blood which would give accurate results in the presence of relatively high concentrations of thiocyanate, and accordingly Werner's [1939] method was studied. While, in agreement with Andrews & Strauss [1941], reasonably good results were obtained for free sulphanilamide, the values for acetylsulphanilamide were so erratic as to be worthless.

Closer study has revealed the following sources of error in Werner's [1939] method.

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(1) No provision is made for laking the blood before precipitation of the blood proteins. Marshall [1937-8] has indicated that this leads to low results, presumably due to incomplete equilibrium between the sulphanilamide in plasma and erythrocytes.

(2) The precipitation of the blood proteins is carried out at an insufficiently great dilution. Marshall [1937-8] has shown that while recovery of added sulphanilamide from blood is reasonably complete after precipitation in 1 : 10 dilution, recovery of added acetylsulphanilamide is not quantitative even in 1 : 20 dilution. Werner has used 1 : 5 dilutions, although his own results show that recovery of acetylsulphanilamide is incomplete in 1 : 10 dilutions under the conditions used by him. Work in this laboratory described later indicates that quantitative recovery of acetylsulphanilamide from blood may be obtained only in 1 : 20 dilutions.

(3) The use of trichloroacetic acid as hydrolysing agent for acetylsulphanilamide is unsatisfactory. Marshall [1937-8] has suggested that this is due to the decomposition of the trichloroacetic acid during the heating necessary for complete hydrolysis of the acetylsulphanilamide. This is borne out by experiments in this laboratory, which have shown that a 4% solution of trichloroacetic acid as used by Werner is approximately 50% decomposed by 30 min. heating at 100°. The extent of decomposition varies with the conditions of heating, such as shape of tubes used etc., and is not exactly reproducible. Apart from changes in the rate of hydrolysis, the variations in acid concentration will lead to corresponding variations in *pH* after partial neutralization, and as will be seen later the *pH* is of critical importance for reproducibility of colour.

(4) The *pH* of the solution in which the colour is developed is only partially controlled. Evidence is presented in the experimental section which shows that there is an optimal *pH* zone within which alone reproducible results can be obtained. Outside the limits of this zone a very marked decrease in colour intensity is observed. Werner [1939] observed that the presence of free trichloroacetic acid interfered with the maximal production of colour. The work of Kühnau [1938] has shown that this effect is not a specific property of trichloroacetic acid, since he estimated uliron (*N*⁴-sulphanilyl-*N*¹*N*¹-dimethylsulphanilamide) in blood by addition of a trichloroacetic acid solution of *p*-dimethylaminobenzaldehyde to a trichloroacetic acid blood filtrate without previous neutralization of the blood filtrate. Work in this laboratory has shown that the effect can be observed with a variety of acids including hydrochloric, sulphuric, *p*-toluenesulphonic and salicylsulphonic acids. The effect thus appears to be entirely a matter of *pH*. Werner attempted to overcome the difficulty by partial neutralization of the trichloroacetic acid filtrate with NaOH. The system trichloroacetic acid-sodium trichloroacetate is, however, a comparatively badly poised buffer since trichloroacetic acid is a relatively strong acid, and small pipetting errors might lead to a large shift in *pH*. A much greater source of error is the decomposition of trichloroacetic acid during heating in the determination of acetylsulphanilamide, and as will be seen from the last section, this is very difficult to control.

(5) The amount of *p*-dimethylaminobenzaldehyde used is insufficient for maximal colour development. While it is true that by the addition of exactly the same amount of *p*-dimethylaminobenzaldehyde reagent in every case, reproducible results will be obtained, such reproducibility will obviously depend on the accuracy of measurement of the reagent, and on the stability of the reagent solution. This is evidently undesirable for a routine clinical method. It will be seen from Fig. 2 (experimental section), that there is an optimal zone within

which small variations in the amount of *p*-dimethylaminobenzaldehyde reagent added produce practically no difference in colour.

The sources of error recapitulated above have been avoided in the method to be described by the following precautions.

- (1) Complete laking of the blood before precipitation of proteins.
- (2) Protein precipitation in 1 : 20 dilution.
- (3) The use of a *p*-toluenesulphonic acid-HCl mixture for precipitation of the blood proteins, and subsequent hydrolysis of the acetylsulphanilamide. This mixture is unaffected by much longer heating than is necessary to effect quantitative hydrolysis.
- (4) The use of a citrate-HCl buffer to maintain the pH within the optimal zone.
- (5) The amount of *p*-dimethylaminobenzaldehyde is regulated to fall within the optimal zone.

EXPERIMENTAL

The determination of sulphanilamide in blood

Reagents.

- (1) 20 g. *p*-toluenesulphonic acid in 100 ml. 0.2*N* HCl.
- (2) 0.75*M* disodium hydrogen citrate solution. 39.4 g. 'Analar' citric acid are dissolved in 188 ml. 2*N* NaOH, and diluted to 250 ml.
- (3) 2% alcoholic *p*-dimethylaminobenzaldehyde solution. 2.00 g. 'Analar' *p*-dimethylaminobenzaldehyde are dissolved in 100 ml. 95% alcohol. In estimations with the Pulfrich photometer or similar instruments in which the colour of the blank is compensated, the purity of the *p*-dimethylaminobenzaldehyde is not of very great importance since identical values were given by a 3 years old yellow specimen, and by a colourless preparation obtained by the method of Adams & Coleman [1922]. In estimations with the ordinary colorimeter, however, as Andrews & Strauss [1941] have noted, the question of purity is of importance, since certain preparations have a greenish colour which leads to high blank values. Such specimens can be purified by fractional precipitation from acid solution by the method of Adams & Coleman [1922].

All the above reagents appear to be stable indefinitely at 0°.

Method

1.0 ml. of whole blood is measured into 13.0 ml. of distilled water, the mixture shaken vigorously, and then kept for about 3 min., when laking is complete. 6.0 ml. of the 20% *p*-toluenesulphonic acid reagent are added slowly with vigorous shaking. The mixture is kept at least 5 min., and filtered through a Whatman No. 40 filter paper.

For the determination of free sulphanilamide, 5 ml. of the filtrate are treated with 1 ml. of the disodium hydrogen citrate solution, and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The yellow colour develops immediately to its maximum value, and is stable for at least a week, although if specimens are to be kept for more than 12 hr. it is advisable to stopper the tubes, as otherwise evaporation of the alcohol may lead to separation of crystals of *p*-dimethylaminobenzaldehyde.

For the determination of total sulphanilamide (free and acetylsulphanilamide), 5 ml. of the filtrate are transferred to a graduated tube, and heated for 60 min. in a boiling water bath. The tube is then cooled, the contents diluted to 5 ml. with water, and the colour developed as in the determination of free sulphanilamide.

Blanks for photometric determinations are made by mixing 1.5 ml. of the *p*-toluenesulphonic acid reagent, 1 ml. of the citrate reagent and 2 ml. of the *p*-dimethylaminobenzaldehyde reagent, and diluting to 8 ml. with water.

It must be emphasized that the coloured solutions may not be diluted, since the alteration in the ionic strength of the buffer apparently produces a change of *pH*, and a marked relative decrease in colour is observed.

Measurement of colour

The majority of the determinations have been carried out with the Pulfrich photometer using filter S 47, and 5 mm. and 10 mm. cells according to the intensity of colour. Then

$$\text{mg. sulphanilamide/100 ml. blood} = E \times 11.7,$$

where *E* is the extinction for 10 mm. solution thickness using filter S 47. The value of the constant should of course be determined independently in the user's laboratory, since small variations may occur, owing to variations in filters or reagents.

Some determinations have also been made with the Hilger Spekker photoelectric absorptiometer. In this case the most suitable filter is the Ilford Spectrum Blue, which has maximum transmission at 4700 Å.

Since Beer's law is valid for the concentration range studied (0.5–12 mg. sulphanilamide/100 ml. blood), the method should be applicable to the ordinary colorimeter, provided that *p*-dimethylaminobenzaldehyde of adequate purity is available. The difficulty of matching yellow solutions can be obviated by the use of a suitable blue filter. The question of suitable standard solutions is discussed in the paper of Andrews & Strauss [1941]. The use of permanent standards prepared from potassium chromate solutions as suggested by Werner [1939] is not recommended for accurate work, as such solutions have a different absorption spectrum from the solutions to be measured.

RESULTS

Recoveries of sulphanilamide and acetylsulphanilamide from blood in 1:10 and 1:20 dilutions are shown below.

Table 1

Dilution 1:10

Substance	Added mg./100 ml.	Found mg./100 ml.	% recovery
Sulphanilamide	3.2	3.2	100
	6.4	6.35	99
	9.6	9.45	98
Acetylsulphanilamide	4.0	4.0	100
	8.0	7.85	98
	12.0	11.2	93

Table 2

Dilution 1:20

Substance	Added mg./100 ml.	Found mg./100 ml.	% recovery
Acetylsulphanilamide	4.0	4.0	100
	8.0	8.0	100
	12.0	11.9	99

Each of the above values is the mean of three estimations.

Derivatives of sulphanilamide

In addition to sulphanilamide itself, three derivatives in common use in medicine have been studied, albucid (*p*-aminobenzenesulphonacetamide), sulphapyridine (dagenan, M & B 693, 2-sulphanilamidopyridine) and sulphathiazole (2-sulphanilamidothiazole). All three compounds give yellow colours with *p*-dimethylaminobenzaldehyde in acid solution, which may be used for their estimation. The method used is identical with that used for sulphanilamide. The molecular extinction coefficients for sulphanilamide, sulphapyridine and sulphathiazole are identical, so that the latter substances may be estimated by applying a molecular weight correction to the sulphanilamide factor. Thus:

$$\text{mg. sulphapyridine/100 ml. blood} = E_1 \times 16.0,$$

$$\text{and mg. sulphathiazole/100 ml. blood} = E_2 \times 17.35,$$

where E_1 and E_2 are the respective extinctions for 10 mm. solution thickness using filter S 47 of the Pulfrich photometer.

In cases where low blood levels of sulphapyridine and sulphathiazole are anticipated, 20 mm. cells should be used with the Pulfrich photometer.

In the case of albucid, the theoretical extinction coefficient is not obtained unless the albucid solution is heated for 60 min. at 100° as in the case of the acetylsulphanilamide estimation. In direct determinations slightly low but consistent values are obtained, possibly owing to the presence of *p*-acetylaminobenzenesulphonacetamide in the preparation used. In such cases a separate calibration curve must be made.

The *p*-dimethylaminobenzaldehyde method appears to be applicable to all derivatives of sulphanilamide which have a free primary amino group directly attached to an aromatic residue. Its range of utility is thus similar to that of the diazotization methods.

The variation of colour with hydrogen ion concentration

Buffer solutions were made up with varying ratios of 0.75*M* disodium hydrogen citrate and 0.75*N* HCl. The pH values of these were determined

Fig. 1. The relationship between pH and colour intensity.

electrometrically using the quinhydrone electrode. 5 ml. portions of the buffers were treated with 0.30 ml. of a sulphanilamide solution containing 8.0 mg./100 ml., and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution added. The results are shown in Fig. 1.

The variation of colour with p-dimethylaminobenzaldehyde concentration

A series of tubes were set up containing 0.3 ml. 8 mg./100 ml. sulphanilamide solution, 1.5 ml. *p*-toluenesulphonic acid reagent, 1 ml. disodium hydrogen citrate reagent and 3.2 ml. of water. Varying volumes of the 2% alcoholic *p*-dimethylaminobenzaldehyde reagent were added, and the contents of the

Fig. 2. The relationship between *p*-dimethylaminobenzaldehyde concentration and colour intensity.

tubes were diluted to 8.5 ml. with 95 % alcohol. A series of corresponding blanks were set up in which the 0.3 ml. of sulphanilamide was replaced by 0.3 ml. of water. Each tube was measured against the corresponding blank. The results are shown in Fig. 2.

Identification of the chromophoric complex as p-dimethylaminobenzylidene-p-aminobenzenesulphonamide

0.3 ml. of a solution of *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide [Gray *et al.* 1937] containing 14.10 mg. in 100 ml. *p*-toluenesulphonic acid reagent, was treated with 1.2 ml. of the *p*-toluenesulphonic acid reagent, 3.5 ml. of water, 1 ml. of the disodium hydrogen citrate reagent and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The extinction was measured using a 10 mm. cell and filter S 47.

0.3 ml. of an equivalent solution of sulphanilamide (8 mg./100 ml.) was treated with 1.5 ml. of the *p*-toluenesulphonic acid reagent, 3.2 ml. of water, 1 ml. of the disodium hydrogen citrate reagent and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The extinction was measured as above.

Table 3

Substance	Extinction
<i>p</i> -Dimethylaminobenzylidene- <i>p</i> -aminobenzenesulphonamide	0.885
Sulphanilamide	0.878

DISCUSSION

Any explanation of the *pH*-colour, and *p*-dimethylaminobenzaldehyde concentration-colour relationships shown in Figs. 1 and 2 necessitates an examination of the reaction responsible for colour formation. The compound responsible for the colour produced when sulphanilamide reacts in acid solution with *p*-dimethylaminobenzaldehyde is probably the Schiff base *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide

This base has been synthesized by Gray *et al.* [1937] by direct condensation of sulphanilamide with *p*-dimethylaminobenzaldehyde at 140°. Its melting point is given as 229°. Werner [1939], by condensation of sulphanilamide with *p*-dimethylaminobenzaldehyde in acid solution, prepared a substance of melting point 224°, for which he gives the anomalous formula $\text{C}_{15}\text{H}_{16}\text{O}_4\text{N}_3\text{S}$ (the Schiff base would be $\text{C}_{15}\text{H}_{17}\text{O}_3\text{N}_3\text{S}$). It is probable that the substance isolated by Werner was slightly impure *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide. It is shown in the experimental section that a preparation of this base synthesized according to the method of Gray *et al.* [1937] had, under appropriate conditions, the same colour intensity as that given by an equivalent amount of sulphanilamide in the colorimetric determination. It may therefore be concluded that the chromophoric complex is indeed *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide.

Dimroth & Zoeppnitz [1902], in the course of investigations into the mechanism of Schiff base formation, suggested the following reaction scheme:

They found that in those cases where the aromatic aldehyde had a *para*-substituted positive (electron-repelling) group, and the aromatic amine a *para*-substituted negative (electron-attracting) group, the amino-carbinol A was stable in strongly acid solution. Since these conditions are fulfilled in the reaction between sulphanilamide and *p*-dimethylaminobenzaldehyde, and since the amino-carbinol A would be expected to have a less intense colour than the Schiff base B, owing to the interruption of the series of conjugated double bonds, this may provide an explanation for the diminution in colour below *pH* 1 as shown in Fig. 1. The decreased colour above *pH* 2 may possibly be explained by the failure of reaction I to proceed to completion, or by the diminished ionization of the *p*-dimethylamino group.

The very large excess of *p*-dimethylaminobenzaldehyde necessary for maximum development of colour (Fig. 2) may also be explained by the reversible nature of the above mechanism.

SUMMARY

1. The conditions affecting the reaction between sulphanilamide and *p*-dimethylaminobenzaldehyde in acid solution have been studied.
2. A simple and rapid colorimetric estimation of sulphanilamide and its derivatives in blood based on this reaction has been developed.
3. The compound responsible for the colour has been identified as *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide.

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