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Determination of *p*-Aminosalicylic Acid as *m*-Aminophenol

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Several methods have been described for the estimation of *p*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid) in blood (Lehmann, 1946; Ragaz, 1948; Tennent & Leland, 1948, 1949; Klyne & Newhouse, 1948). None of the procedures, developed in connexion with therapeutic trials of this drug in tuberculosis, were micromethods; our aim was to develop a method suitable for use with capillary blood. Bratton & Marshall's (1939) estimation of sulphonamides in blood seemed an obvious choice. Such a method was used (but not described) by McClosky, Smith & Frias (1948). These workers recorded erratic results with *p*-aminosalicylic acid, but obtained quantitative linear curves by allowing

solutions of the drug to stand for several days at room temperature, or by heating them at 100° for 30–60 min.

Aqueous solutions of *p*-aminosalicylic acid are known to be decarboxylated on standing or heating (Venkataraman, Venkataraman & Lewis, 1948; Rosdahl, 1948; Oberweger, Seymour & Simmonite, 1948). The findings of McClosky *et al.* (1948) could be explained as the decarboxylation of *p*-aminosalicylate followed by diazotization of *m*-aminophenol. We thought that such a technique could be used for the micro-estimation of *p*-aminosalicylic acid and set about establishing conditions for complete decarboxylation.

While this work was in progress four diazotization methods were described. One is an adaptation by Marshall (1948) of the original Bratton & Marshall (1939) procedure, on which the two other methods (Way, Smith, Howie, Weiss & Swanson, 1948; Newhouse & Klyne, 1949) are also based. In the fourth method (Desbordes & Henry, 1948) diazotized *p*-aminosalicylic acid is coupled with thymol.

EXPERIMENTAL

Bratton & Marshall's procedure for estimating total sulphamide in blood was progressively modified, and two alternative methods, *A* and *B*, were finally adopted.

Reagents. Trichloroacetic acid (20% w/v in water). H_2SO_4 (21.5 $\pm$ 0.5 N; made by adding 60 ml. conc. H_2SO_4 to 40 ml. water, titrating and readjusting the volume). NaOH (8.1 N). NaNO_2 (1% w/v in water). Ammonium sulphamate (5% w/v in water). *N*-1-Naphthylethylenediamine dihydrochloride (0.1% in water). Ethanol (95%) or industrial methylated spirit.

Standard solutions. (1) Stock standard equivalent to 100 mg. *p*-aminosalicylic acid/100 ml.: 137.9 mg. pure sodium *p*-aminosalicylate dihydrate is dissolved in 100 ml. water; or 71.2 mg. pure *m*-aminophenol is dissolved in 100 ml. 0.01 N-HCl. These can be kept in colourless bottles at room temperature for at least 8 weeks. (2) Dilute standards representing 5, 10 and 20 mg. *p*-aminosalicylic acid/100 ml. blood: 0.25, 0.50 and 1.0 ml. of solution (1) and 15 ml. 20% (w/v) trichloroacetic acid are diluted to 100 ml. with water. These are referred to as '5, 10 and 20 mg. solutions'. With standard solutions containing *p*-aminosalicylate, 2.0 ml. samples are treated as supernatant liquids of the test solutions; using *m*-aminophenol standards, heating may be omitted.

Method A

Oxalated or freshly drawn capillary blood (0.2 ml.) is added to water (3.2 ml.), and the tube is allowed to stand for 30 min. Trichloroacetic acid (0.6 ml.) is added, and the mixture is shaken and centrifuged. Supernatant liquid (2.0 ml.), transferred to a centrifuge tube graduated at 4 ml., is heated with H_2SO_4 (0.5 ml.) in a boiling water bath for 1 hr. The solution is cooled in tap water (approx. 15°), partially neutralized with NaOH (1.0 ml.) and again cooled. Water is added to adjust the volume to 4.0 ml.

NaNO_2 solution (0.2 ml.) is added, and the solution is shaken. After 5 min. ammonium sulphamate solution is added, followed 20 $\pm$ 1 sec. later by naphthylethylenediamine (1.0 ml.), the tube being shaken after each addition.

The pink colour is allowed to develop for at least 2.5 hr. and is then compared with the appropriate standard solution. An Evans Electroselenium Ltd. portable photoelectric colorimeter with an Ilford 625 filter gives readings proportional to concentration if set to zero against a full reagent blank (2 ml. 3% w/v trichloroacetic acid taking the place of the supernatant liquid). An Ilford 404 green filter, though not giving fully linear results, has also been found satisfactory if a calibration curve is prepared using all three standards (the 5, 10 and 20 mg. solutions).

Method B

Method *A* is carried out to the coupling stage. 20 $\pm$ 1 sec. after coupling with naphthylethylenediamine solution,

ethanol (1.0 ml.) is added, the tubes are shaken, and the colours compared after 1 hr. These are proportional to concentration when using an Ilford 625 filter and a modified reagent blank (2.0 ml. water taking the place of the supernatant liquid). The Ilford 404 filter is unsuitable for this method.

RESULTS

Decarboxylation and hydrolysis. Our first experiments showed that solutions of *p*-aminosalicylate heated with concentrated hydrochloric acid gave deeper colours than solutions treated with hydrochloric acid in the cold or heated with trichloroacetic acid. Sulphuric acid was substituted for hydrochloric to prevent the formation of hydroxyazo compounds during the diazotization stage. The strength of acid and the heating time finally adopted give complete decarboxylation, as is shown by carrying out the method on *p*-aminosalicylate and *m*-aminophenol solutions with and without heating (Table 1). Determinations carried out on 20 mg.

Table 1. *The conversion of p-aminosalicylate into m-aminophenol*

(20 mg. standards used in each case.)

Exp. no.	Compound and treatment	Final concentration of <i>m</i> -aminophenol expressed as	
		Colorimeter reading (100 $\times$ $\bar{E}$)	Percentage of result in Exp. 1
1	<i>m</i> -Aminophenol, not heated	54	100
2	<i>m</i> -Aminophenol, heated	55.5	103
3	<i>p</i> -Aminosalicylate, not heated	17	31.5
4	<i>p</i> -Aminosalicylate, heated	53	98

solutions of *m*-aminophenol and *p*-acetamidosalicylic acid show that the *N*-acetyl derivative of *p*-aminosalicylic acid is completely hydrolysed by this treatment. Thus the present estimation is one of 'total *p*-aminosalicylate'. When heating is omitted, solutions of the *N*-acetyl compound give no colour reaction.

Diazotization. Partial neutralization with sodium hydroxide gives the moderately acid medium usual for diazo coupling. Observations which led to this step being introduced are summarized in Table 2. Diazotization for 5 min. was chosen as a convenient time, since the colour intensity/diazotization time curve showed only a very gradual decrease between 3 and 10 min. (Colorimeter reading at 3 min., 60; 5 min., 59.5; 10 min., 59; 15 min., 57.) Minor changes in the temperature of diazotization introduce appreciable errors; all test and standard solutions should be placed in the same cooling bath.

Table 2. *Effect of pH on colour intensity in the estimation of p-aminosalicylate as m-aminophenol*

(20 mg. standards used in each case.)

Exp. no.	Decarboxylation medium	Diazotizing and coupling medium	Colour intensity (percentage of value in Exp. 2)
1	Strongly acid (21.5N-H ₂ SO ₄)	Strongly acid (unchanged)	51.5
2	Strongly acid (21.5N-H ₂ SO ₄)	Less acid (partly neutralized with 8.1N-NaOH)	100
3	Less acid (9N-H ₂ SO ₄)	Less acid (unchanged)	81
4	Less acid (9N-H ₂ SO ₄)	Strongly acid (9N-H ₂ SO ₄ added until pH approx. that of Exp. 1)	12

Newhouse & Klyne (1949) allowed 10 sec. between adding ammonium sulphamate and coupling. Our sulphamate time of 20 sec. was adopted directly from their method. It gives colours about 20 % deeper than we had formerly obtained using longer times (cf. Table 3).

Table 3. *The effect of varying diazotizing and sulphamate times on the colour intensities obtained from p-aminosalicylate and m-aminophenol solutions*

(20 mg. standards used in each case.)

Exp. no.	Compound	Diazotizing time (min.)	Sulphamate time	Colorimeter reading (100 × E)
1	<i>p</i> -Aminosalicylate	5	20 sec.	57
2	"	5	30 min.	38
3	"	20	20 sec.	49
4	"	20	10 min.	44.5
5	<i>m</i> -Aminophenol	5	20 sec.	58
6	"	5	20 min.	50
7	"	20	20 sec.	53
8	"	20	20 min.	43

Properties of the dye solutions. The colours given by methods A and B follow Beer's law between 2.5 and 20 mg./100 ml. levels using an Ilford 625 filter. The similar development (Fig. 1) of the colours obtained from equimolar solutions of *p*-aminosalicylate after heating and of *m*-aminophenol confirms the identity of the two dyes. Unheated solutions of *p*-aminosalicylate develop colour more rapidly than either of these two solutions. This indicates a structural difference between the dyes rather than a partial decarboxylation of the acid in the cold which would then give less of the same dye.

Both conclusions are borne out by the absorption spectra of the dyes (Fig. 2). These were recorded by

Dr I. D. P. Wootton, using a Beckmann spectrophotometer. The solutions used were those obtained by carrying through 20 mg. solutions of *p*-aminosalicylate and *m*-aminophenol. The heated *p*-aminosalicylate and the unheated *m*-aminophenol solutions have identical spectra. The unheated *p*-aminosalicylate solution gives a dye with the expected lower extinction values and an absorption maximum at a somewhat shorter wavelength.

Fig. 1. Rate of colour development in the estimation of *p*-aminosalicylate and *m*-aminophenol (20 mg. standards, using method A): - - - - -, *m*-aminophenol, unheated; —, *p*-aminosalicylate, heated; — x — x — x, *p*-aminosalicylate, unheated. Fig. 2. Absorption spectra of dyes (20 mg. standards, using method A): - - - - -, *m*-aminophenol, unheated; —, *p*-aminosalicylate, heated; — x — x — x, *p*-aminosalicylate, unheated.

The colours produced in method A are stable to light and are unchanged after 90 hr. at room temperature. The solution of the dye obtained from unheated *p*-aminosalicylate is stable only for a few hours. The dye slowly separates as a flocculent precipitate, leaving a colourless solution. On shaking

the mixture the precipitate is dispersed; the solution appears clear and has the same colour intensity as it had before flocculation occurred. The precipitate can be separated easily by filtration. Since in the aqueous acid medium containing a high salt concentration the more acid dye produced from the unheated *p*-aminosalicylate would probably be less soluble than its *m*-aminophenol analogue, the precipitate was filtered off and the filtrate kept for some time. No further colour developed; thus the low colour intensity of this solution is not due to a solubility effect leading to incomplete dye formation.

Table 4. Recoveries of *p*-aminosalicylate added to blood

(Aqueous *p*-aminosalicylate solution (100 mg./100 ml.) added to oxalated blood.)

Blood <i>p</i> -aminosalicylate level (mg./100 ml.)	No. of exp.	Recovery (%)	
		Range	Mean
10	17	89-102	96
20	8	91-102	98

Attempts were also made to follow the diazotization of *p*-aminosalicylic acid and *m*-aminophenol by means of free nitrous acid estimations. The *m*-phenylenediamine method (see Welcher, 1947) used cannot be described as a strictly quantitative method; it does indicate, however, that the amount of nitrous acid used in the reaction is in excess of that required by a simple diazotization.

Recoveries. The results of these are given in Table 4. The determinations were carried out on fresh oxalated blood to which the requisite amount of *p*-aminosalicylate (stock standard solution) was added.

Penicillin did not interfere with the estimation. Sulphonamides and sulphones (Sulphetrone) do, of course, interfere. An unsuccessful attempt was made to overcome this difficulty by converting the diazotized *m*-aminophenol to resorcinol and estimating the product by an adaptation of Seliwanoff's reaction.

SUMMARY

1. A method for the estimation of 'total *p*-aminosalicylate' in capillary blood is described.

2. *p*-Aminosalicylic acid and its *N*-acetyl derivative are quantitatively converted to *m*-aminophenol; this compound gives deeper colours than *p*-aminosalicylate when diazotized and coupled with *N*-1-naphthylethylenediamine.

3. The main aspects of the method have been surveyed and the behaviour of *p*-aminosalicylic acid and *m*-aminophenol compared.

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