1. McDonald, T. R. R. and Beevers, C. A. *Acta Cryst.* **3** (1950) 394; **5** (1952) 654.
2. Hudson, C. S. *J. Am. Chem. Soc.* **31** (1909)66.
3. Blom, J. *Acta Chem. Scand.* **13** (1959) 388; **15** (1961) 1667.
4. Böeseken, J. *Advances in Carbohydrate Chem.* **4** (1949) 189.
5. Blom, J. *Acta Chem. Scand.* **16** (1962) 922.
6. Hassel, O. and Ottar, B. *Acta Chem. Scand.* **1** (1947) 929.
7. Reeves, R. E. *Advances in Carbohydrate Chem.* **6** (1951) 107.
8. Sørensen, N. A. *Kgl. Norske Videnskab Selskabs Skrifter* **2** (1937) 104, 106.
9. Isbell, H. S. and Pigman, W. W. *J. Research Natl. Bur. Standards* **18** (1937) 141.

Received May 22, 1962.

A Modification for the Determination of Sulphate in Mucopolysaccharides by the Benzidine Method

CRISTOS A. ANTONOPOULOS

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The methods for determination of microgram quantities of sulphate in mucopolysaccharides have been studied. Gravimetric methods have been found to be unsuitable for small amounts of sulphate, while certain colorimetric methods have been found to be generally more suitable. One of the most frequently used techniques is to precipitate the sulphate with benzidine and then to determine the amount of benzidine-sulphate spectrophotometrically.

When using a modification of this procedure described by Kent and Whitehouse [1] difficulties were encountered, particularly in filtering the benzidine-sulphate precipitate through sintred glass to remove the excess benzidine. The process is slow and the results were found to be inconsistent.

A modification of this method in which the benzidine sulphate was separated by centrifugation was also tried [2] but the results obtained were unsatisfactory. With additional modifications of the above methods, more consistent results were obtained with standard solutions as well as with mucopolysaccharides after hydrolysis, and these modifications are described here.

Reagents.

98—100 % Formic acid.
95 % Ethanol.
Benzidine reagent: 0.5 % solution benzidine in 95 % ethanol. Kept in the dark in a refrigerator and renewed every 3 weeks.

Acetone-ethanol mixture, 1:1 (v/v).
1.0 N Hydrochloric acid.
0.5 % (w/v) Thymol in 2 N NaOH.
0.1 N sodium nitrite.

The mucopolysaccharides are hydrolyzed in sealed tubes with 25 % formic acid on a boiling water bath. The hydrolysis curve (Fig. 1) shows that 24 h are sufficient to liberate all the sulphate. Usually 0.75 ml of a mucopolysaccharide solution containing $10-80$ μg of sulphate is mixed with 0.25 ml of formic acid.

Standard solutions. 10, 20, 40 and 50 μg SO_4/ml in 25 % formic acid.

Procedure. 0.3 ml of the hydrolysate is placed in a conical centrifuge tube (15 ml) to which 0.5 ml 95 % ethanol, 0.2 ml benzidine reagent and 0.5 ml amyl alcohol are added. The standard solutions are treated in the same way using 0.3 ml aliquots. The tubes are shaken and allowed to stand 60 min at 0°C. They are then centrifuged for 6 min in an ordinary laboratory centrifuge with "swing out" head at 4000 r.p.m. and the supernatant is carefully decanted and discarded. 1 ml acetone-ethanol and 0.5 ml amyl alcohol are added to each tube, the tubes are shaken to disperse the precipitate and then recentrifuged for 6 min. The supernatant is decanted as before, after which 1 ml acetone-ethanol is added, the tubes are shaken and centrifuged again. The supernatant is again decanted.

The precipitate is dissolved in 3 ml of 1.0 N hydrochloric acid and 2 ml of distilled water. The benzidine solutions are diazotized at room temperature by the addition of 1 ml of 0.1 N sodium nitrite. After 3 min, 5 ml of the alkaline thymol solution is added and the contents of the tubes are mixed.

The optical density is measured at 505 mμ in 1 cm cells. The colour is stable for at least 4 h. The amount of sulphate is calculated from a standard curve.
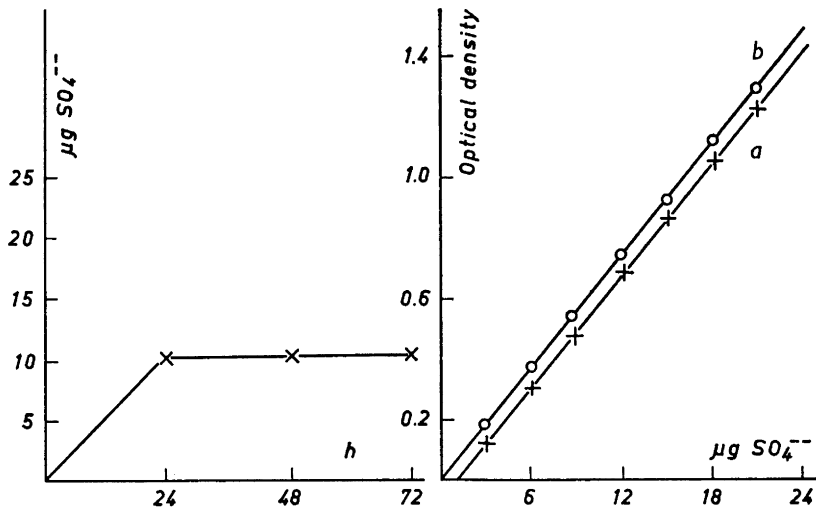
Fig. 1. Liberation of sulphate from chondroitin-sulphate hydrolysed by 25 % formic acid on a boiling waterbath.

Fig. 2. Curve *a*: Typical standard curve for different amounts of sulphate. Curve *b*: Curve obtained when benzidine corresponding to the amounts of sulphate indicated is diazotized directly.

Results and discussion. Curve *a* in Fig. 2 shows a typical standard curve obtained by the method described. This curve shows a linear relation between the amount of colour formed in the reaction and the amount of sulphate to as little as 3 μg. However, at lower sulphate concentrations deviation from linearity occurs and, more-over, the curve does not pass the origin.

When known amounts of benzidine corresponding to 3—21 μg of sulphate were diazotized a straight line passing through the origin is obtained (Fig. 2, *b*). The two curves are parallel.

The intersection of curve *a* with the ordinate occurs at a point which corresponds to the solubility of benzidine sul-phate in the system used. According to Kent and Whitehouse optimum results by this method are found in the pH-range 2.5 to 2.7; the pH under the described conditions was 2.5.

The author is greatly indebted to Dr Sven Gardell for invaluable advice and criticism during the performance of the work.

1. Kent, P. and Whitehouse, M. *Analyst* **80** (1955) 630.
2. Häkkinen, I. *Nature* **186** (1960) 232.