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The Ultramicro Determination of Inorganic Sulphate

By B. SPENCER
Department of Biochemistry, University of Wales, Newport Road, Cardiff

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In a previous paper (Dodgson & Spencer, 1953) a method was described for the determination of inorganic sulphate with particular reference to the study of sulphatases. The determination was a variant of the well-known benzidine method and involved treating the solution containing inorganic sulphate with an ethanolic solution of benzidine at acid pH, washing the precipitated benzidine sulphate with ethanol and estimating the benzidine moiety colorimetrically after diazotization and coupling with thymol. Stringent precautions were necessary to avoid contamination of the reaction tubes with benzidine and extreme care during manipulation was demanded. The method was therefore somewhat exacting and rather long but it has proved satisfactory, within certain limits, for various studies in this (Dodgson & Spencer, 1954; Dodgson, Lloyd & Spencer, 1957) and other laboratories (Isselbacher & McCarthy, 1959), and modifications have been introduced to meet different circumstances (Nechaeva, 1958; Slack, 1957).

To conserve laboriously prepared enzymes and other materials an attempt has been made in this Laboratory to reduce the scale of the commonly used assay techniques by a factor of usually 100 times. Many spectrophotometric determinations, such as those of arylsulphatases A and B, arylsulphatase C, β-glucuronidase, ribonucleases and non-specific phosphatases (Spencer, 1959), are readily adapted to the assay of 10 μl. samples simply by scaling down well-established methods, by the use of small reaction tubes, constriction micropipettes and microabsorption cells (cf. Lowry, 1955). However, in adapting the Dodgson & Spencer (1953) method for inorganic sulphate for use with 10 or 12 μl. samples certain changes in technique were found to be necessary. The ultramicro modification finally developed possesses considerable advantages over the original method since it is shorter, less tedious and twice as sensitive.

Bertolacini & Barney (1957) introduced a method for determining sulphate based on a new principle. It depends on the reaction in aqueous 50% ethanol between sulphate and slightly soluble barium chloranilate, leading to the formation of barium sulphate and the release of soluble chloranilic acid, which can be measured colorimetrically. The method has been modified by Lloyd (1959) for use in the presence of materials of biochemical interest, but although the procedure is simpler than that of the benzidine method of Dodgson & Spencer (1953) the sensitivity was only about one-fifth. The possibility of increasing the sensitivity of the barium chloranilate procedure and adapting it for use with small samples has now been examined.

EXPERIMENTAL

Materials. Commercial purified benzidine (May and Baker Ltd.) was recrystallized twice from 95% ethanol with the use of charcoal. Barium chloranilate was prepared and aged according to the directions of Bertolacini & Barney (1957). Powdered glass was prepared by grinding glass wool in a mortar, suspending the ground material in water and using only that part that did not settle in 10 min. under the influence of gravity. This material was washed on the centrifuge with N-HCl, water, ethanol and ether and then dried. The particle size of the powdered glass was such that in suspension it did not block the constriction micropipettes.

Apparatus. All glassware was cleaned with N-HCl; sulphuric acid mixtures must not be used for this purpose.

Benzidine sulphate precipitations were carried out in rimless centrifuge tubes of 5 cm. × 5 mm. internal diam. and tapering over the last 12 mm. to a tip of approx. 1 mm. internal diam. For the ethanolic precipitation of protein and mucopolysaccharides in both the benzidine and barium chloranilate methods rimless tubes of 5 cm. × 3 mm. internal diam. and tapering only over the last 5 mm. were preferred. These tubes were also suitable for the reaction between barium chloranilate and sulphate-containing solutions. All tubes were readily made from glass tubing.

Solutions were delivered with Lang–Levvy constriction micropipettes (Lowry, Roberts, Leiner, Wu & Farr, 1954). Owing to the low surface tension of ethanol, pipettes used for ethanolic solutions require rather smaller orifices and constrictions than are normal for aqueous solutions and should be calibrated (Lowry *et al.* 1954) with ethanol. Contents of the small tubes were mixed by 'buzzing' against a flattened nail rotating at speeds from 300 to 1000 rev./min., depending on the degree of agitation required. Tubes were sealed with Parafilm (A. Gallenkamp and Co. Ltd.) and centrifuging was carried out with multiplace bored-out aluminium blocks. Spectrophotometric readings were made in cuvette-type microcells (Lowry & Bessey, 1946) in a Hilger Uvispek spectrophotometer with the microcell adaptor. Practical details for the construction, use and cleaning of the apparatus of the type mentioned above have been reviewed by Lowry (1955).

RESULTS

Benzidine method

Absorption spectrum of benzidine. In the benzidine method used previously (Dodgson & Spencer, 1953) the precipitated benzidine sulphate was measured colorimetrically after diazotization and subsequent coupling with thymol. The azo dye formed shows a broad peak at 510 mμ with ϵ_{510} 56 000 (Fig. 1). However, the factor which limits the sensitivity of the benzidine method is not the final colorimetric measurement but the inability to precipitate small amounts of benzidine sulphate. Some decrease in sensitivity in the measurement of the precipitated benzidine sulphate can therefore be accepted. Benzidine in N-HCl shows maximum absorption at 248 mμ with ϵ_{248} 19 200 (Fig. 1). Absorption at $\lambda_{max.}$ obeys the Lambert–Beer law up to the highest value tested, E 1·5, and measurements at 248 mμ in 1 cm. cells have proved satisfactory for the estimation of precipitated benzidine sulphate.

Determination of sulphate in aqueous solution. To 10 μl. of the sulphate-containing solution in a 5 mm. internal diam. tube were added 10 μl. of 20% (w/v) trichloroacetic acid (containing 10 mg. of powdered glass/ml. and 10 μg. of K_2SO_4/ml.) and 50 μl. of freshly prepared 1% (w/v) benzidine in ethanol. The tube was capped and the contents were mixed by 'buzzing'. After standing in the refrigerator (2°) for not less than 1 hr. the tube was centrifuged at 3000 g for 10 min. at 2°. The supernatant was removed by suction with a fine tube with a bent tip. With care, all the visible fluid can be removed without disturbing the precipitate. About 100 μl. of ethanol from a 120 μl. pipette was added into the top of the tube, which was turned during the addition to wash down its walls. The tube was 'buzzed' until the precipitated powdered glass was completely and evenly suspended and then the 20 μl. of ethanol remaining in the pipette was added to wash down the walls of the tube. The tube was capped, centrifuged and the supernatant removed as before. The precipitated benzidine sulphate was dissolved by 'buzzing' with 100 μl. of N-HCl. After a quick centrifuging to throw down the powdered glass, the absorption of the benzidine at 248 mμ was measured in 1 cm. microcells. Blank determinations, in which water was substituted for the sulphate-containing solution, were carried out. Both blank and test determinations were made in duplicate or triplicate. The amount of sulphate in the original sample was calculated from the difference in test and control absorption readings, the volume of the final solution and ϵ_{248} 19 200 for benzidine.

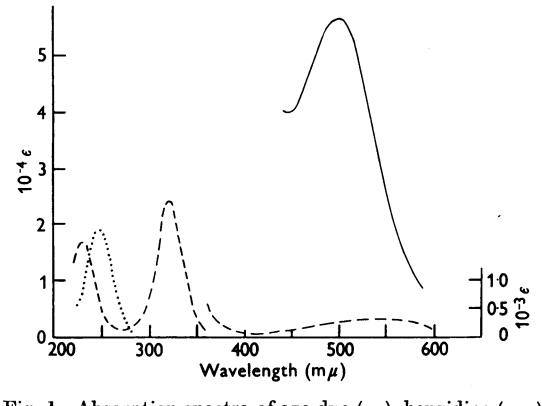

Fig. 1. Absorption spectra of azo dye (—), benzidine (. . .) and chloranilic acid (- - -). Azo dye was prepared by diazotization of benzidine followed by coupling with thymol under the conditions described in the text. The benzidine was dissolved in N-HCl. For the spectrum in the region 350–600 mμ, chloranilic acid was dissolved in 66·7% (v/v) ethanol containing 0·117 M-acetate, pH 4·0; these are the conditions used in the barium chloranilate method for large concentrations of SO_4^{2-} ion. For the region 200–350 mμ the chloranilic acid was dissolved in 6·1% (v/v) ethanol containing 0·46 M-acetate, pH 4·0; these are the conditions used in barium chloranilate method with low concentrations of SO_4^{2-} ion. The right-hand ordinate gives 10^{-3} ϵ values for chloranilic acid between 350 and 360 mμ; the left-hand ordinate gives 10^{-4} ϵ values for the remaining three curves.

Reasonable care is required during these operations if contamination of the tubes with benzidine is to be avoided. Only the tip of the pipette used for benzidine addition should come into contact with the stock benzidine solution and this pipette should be cleanly inserted to near the bottom of the tube before delivery of the solution. 'Buzzing', at all stages, should be vigorous enough to suspend the glass powder evenly but not so strong as to throw the solution more than about half-way up the tube.

The above directions were suitable for 10 μl. samples containing 20–400 μmg. of SO_4^{2-} ion, giving E 0·040–0·800. For samples containing over 400 μmg. of SO_4^{2-} ion the washed benzidine sulphate should be dissolved in a proportionately greater volume of N-HCl. The procedure can be adapted to the estimation of SO_4^{2-} ion in 20 μl. samples by doubling the volume of all the reagents used, and this scale has been preferred by some users of the method. The size of tube remains the same. The method has also been used for 100 μl. samples of sulphate-containing solution by working in 15 ml. tapered centrifuge tubes. The method and precautions are as described before but all volumes must be increased ten times. On this scale the final solution for light-absorption measurement is 1 ml. and readings can be made in normal 1 cm. cells by masking the appropriate spectrophotometer slit with a piece of opaque paper to restrict the beam of light so that it passes under the meniscus of the liquid in the cell.

An alternative procedure for the measurement of the precipitated benzidine sulphate, recommended for use only when a u.v. spectrophotometer is not available, is carried out as follows. The benzidine sulphate was dissolved in HCl–$NaNO_2$ mixture (3 vol. of N-HCl and 1 vol. of 0·1% $NaNO_2$, added together immediately before use). The diazotized benzidine was coupled with thymol by the addition, with immediate and rapid mixing, of 0·5% thymol in 7·5% (w/v) NaOH containing 10% (w/v) of Na_2SO_3. The absorption of the coloured product was measured at 510 mμ. The HCl–$NaNO_2$ mixture and the thymol solution should be added in the ratio 4:5, in volumes calculated from the value ϵ_{510} 56 000 for the coloured product and the approximate sulphate concentration of the original sample. For example, when the coloured product was contained in a final volume of 360 μl., i.e. 160 μl. of HCl–$NaNO_2$ and 200 μl. of thymol solution, the readings

were E 0·081–0·810 for the range 50–500 μmg. of SO_4^{2-} ion/10 μl. of original sample. This volume of liquid was large enough for the absorption at 510 mμ to be read in normal 1 cm. cells by using a suitably masked spectrophotometer as previously described. The Na_2SO_3 was added to the thymol solution to prevent the formation of a brown oxidation product which was formed, in its absence, at the interface between the HCl–$NaNO_2$ solution and the thymol before the two solutions could be mixed. This trouble was not encountered when 15 ml. centrifuge tubes and larger volumes were used as in the original method (Dodgson & Spencer, 1953), since considerable mixing occurred in these wider tubes during the addition of the thymol solution.

Determination of sulphate in biological materials. To 12 μl. of the tissue suspension or other material contained in a 3 mm. internal diam. tapered tube was added 48 μl. of ethanol and the tube was capped, 'buzzed' and centrifuged at approx. 3000 g for 10 min. A 50 μl. portion of the supernatant was pipetted into a 5 mm. internal diam. tapered tube and 10 μl. of 20% (w/v) trichloroacetic acid (containing 10 mg. of powdered glass and 10 μg. of K_2SO_4/ml.) and 10 μl. of a freshly prepared ethanolic 5% (w/v) benzidine were added. The tube was kept at 0° for at least 1 hr. and the benzidine sulphate precipitated, washed and determined as before. Control determinations, in which the tissue suspension was replaced by the same amount of suspension medium, were carried out. In the calculation, allowance must be made for a 2% contraction in volume when 48 μl. of ethanol is added to 12 μl. of aqueous solution.

Recovery of added sulphate. Previous experience with the benzidine method and 1 ml. samples had shown that under certain conditions the recovery of added sulphate was not quantitative (Dodgson & Spencer, 1953). The main sources of error were the incomplete precipitation of small amounts of sulphate and interference at the precipitation stage by various anions and cations. The ultramicro modification was found to be subject to the same errors. In pure aqueous solution, recovery from samples containing less than 75 μmg. of SO_4^{2-} ion as K_2SO_4/10 μl. was low. Good recoveries (±4%) were obtained by adding 100 μmg. of K_2SO_4 to both the tests and controls and this procedure was incorporated into the standard method. Recovery of 100 μmg. of SO_4^{2-} ion from 12 μl. of 0·5 M-sodium acetate–acetic acid containing various sulphatase substrates or tissue suspensions was satisfactory (Table 1). Variations of the pH of the acetate buffer between pH 3·5 and 7·0 did not affect the recoveries. In acetate buffer, pH 5·0, NaCl, KNO_3, $MgCl_2$, adenosine triphosphate, glucose at a concentration of 0·01 M and $FeCl_3$ and $CaCl_2$ at a concentration of 0·001 M did not affect the recovery of 122 μg. of SO_4^{2-} ion/12 μl. of sample. The cations Fe^{3+} and Ca^{2+} at a concentration of 0·01 M considerably depressed the precipitation of benzidine sulphate and recovery of added sulphate was less than 50%. The method was inoperable in the presence of traces of barium. Low recoveries of sulphate were observed in the presence of KH_2PO_4 at concentrations exceeding 0·03 M.

Barium chloranilate method

Absorption spectrum of chloranilic acid. The sensitivity of the barium chloranilate method is governed by the low absorption maximum of chloranilic acid in the visible region, although recently Lloyd (1959) has increased the sensitivity by making measurements at 350 mμ. The spectrum of chloranilic acid at pH 4·0 shows absorption maxima at 530, 327·5 and 235 mμ with ϵ 220, 24 300 and 17 400 respectively (Fig. 1). A 100-fold increase in sensitivity in estimating chloranilic acid can therefore be obtained by making absorption measurements at 327·5 rather than 530 mμ. The Lambert–Beer law is obeyed at 327·5 mμ up to E 1·5.

Determination of sulphate in aqueous solution. To 10 μl. of the sulphate-containing solution in a 3 mm. internal diam. tapered tube was added 40 μl. of ethanol. After mixing by 'buzzing', 5 μl. of 0·5 M-acetate buffer, pH 4·0, containing approx. 72·5 μg. of K_2SO_4/ml., was added and the solutions were mixed. A further 5 μl. of 0·5 M-acetate buffer, pH 4·0, containing 20 mg. of barium chloranilate/ml., was then added, the tube was capped with Parafilm and 'buzzed' vigorously at minute intervals over 10 min. The mixture was centrifuged at 3000 g for 10 min. and a 20 μl. portion of the supernatant was diluted with 200 μl. of 0·5 M-acetate buffer, pH 4·0. The absorption of the liberated chloranilic acid in the diluted solution was measured at 327·5 mμ in 1 cm. cells. Determinations were made in duplicate with duplicate controls in which water was substituted for the sulphate-containing sample. These directions were suitable for use with 10 μl. samples containing 0·25–2·5 μg. of

Table 1. *Recovery of added potassium sulphate from biological materials by the benzidine method*

Material	Concn. in the sample (%, w/v)	SO_4^{2-} ion added (μmg./12 μl.)	SO_4^{2-} ion recovered (μmg.)
Acetone-dried human liver	0·5	100·7	98·3
Fresh rat liver (wet wt.)	1·0	100·7	97·7
Fresh rat kidney (wet wt.)	2·0	100·7	98·0
Acetone-dried *Proteus vulgaris*	0·5	100·7	98·9
Acetone-dried *Alcaligenes metalcaligenes*	0·5	100·7	102·3
Acetone-dried *Charonia lampas*	0·5	100·7	99·5
Taka-diastase in 0·5 M-acetate buffer, pH 3·5	0·5	100·7	98·3
Taka-diastase in 0·5 M-acetate buffer, pH 5·0	0·5	100·7	97·9
Taka-diastase in 0·5 M-acetate buffer, pH 6·0	0·5	100·7	99·6
Chondroitin sulphate C (shark cartilage)	0·15	122·3	118·8
Chondroitin sulphate A (ox cartilage)	0·15	122·3	118·7
Potassium *p*-nitrophenyl sulphate	0·05 M	122·3	120·0
Potassium glucose 6-sulphate	0·02 M	122·3	119·4
Choline sulphate	0·05 M	122·3	118·4
Sinigrin	0·05 M	122·3	121·1

SO_4^{2-} ion giving E 0·096–0·960. With higher sulphate concentrations, 5·0–25·0 μg. of SO_4^{2-} ion/10 μl. of sample, 10 μl. of 0·5 M-acetate containing 10 mg. of barium chloranilate/ml. was added to the sample plus ethanol mixture instead of the two 5 μl. additions as detailed above. Measurements were then made on the undiluted final supernatant at 530 mμ. The sulphate contents of the samples were calculated from the dilutions made by using the appropriate molecular extinction coefficient for chloranilic acid (see above). The procedures were readily adapted to either a 10- or 50-fold increase in scale by using 15 ml. tapered centrifuge tubes.

Determination of sulphate in biological materials. A 12 μl. sample of the tissue suspension or other material was deproteinized with 48 μl. of ethanol as described for the benzidine method. To 50 μl. of the deproteinized solution were added 5 μl. of the acetate–K_2SO_4 solution plus 5 μl. of acetate–barium chloranilate suspension or 10 μl. of the acetate–barium chloranilate suspension, depending on the approximate sulphate content of the original sample. The further treatment was in the manner described for the determination of sulphate in aqueous solutions.

Recovery of added sulphate. When the reaction mixture was composed of 10 μl. of the sulphate-containing mixture, 40 μl. of ethanol and 10 μl. of 0·5 M-acetate buffer, pH 4·0, containing 10 mg. of barium chloranilate/ml., the recovery of 5·0–25·0 μg. of SO_4^{2-} ion/10 μl. of sample was 100 ± 2 %, but in the range 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample the recovery was low. Fig. 2 shows that at least 0·1 μg. of SO_4^{2-} ion/10 μl. of sample must be present before release of chloranilic acid from barium chloranilate starts. This limiting effect was readily corrected, as detailed in the method, by adding extra SO_4^{2-} ions to the sample plus ethanol, in both test and controls, before addition of the barium chloranilate. With this procedure there was a straight-line relationship between concentration of SO_4^{2-} ion, in the range 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample, and the difference between test and control readings at 327·5 mμ (Fig. 2). With the higher sulphate concentrations the error introduced by the limiting-concentration effect was not significant and there was no need to add extra sulphate.

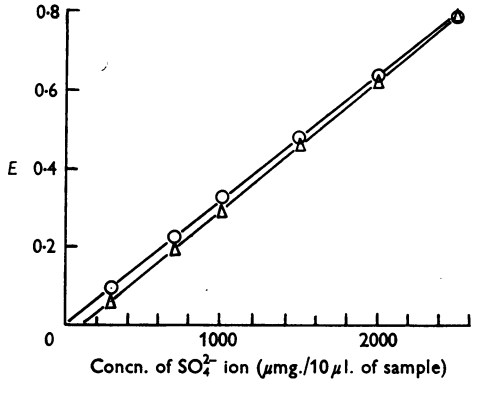

Fig. 2. Relationship between chloranilic acid liberated and sulphate concentration. The chloranilic acid released from barium chloranilate by 10 μl. samples of known amounts of SO_4^{2-} ion under the conditions described in the text was measured at 327·5 mμ. △, Normal 10 μl. samples; ○, samples containing an extra 200 μmg. of SO_4^{2-} ion.

Unsuccessful attempts were made to estimate 25–251 μmg. of SO_4^{2-} ion/10 μl. of sample by making absorption readings at 327·5 mμ on the undiluted supernatant. With this procedure it was found that the 200 μmg. of SO_4^{2-} ion/10 μl. of sample, added to avoid the limiting-concentration effect, resulted in the control readings being very high compared with the difference between the test and control. Furthermore, the exchange of barium between chloranilic acid and SO_4^{2-} ion did not appear to proceed to completion and with amounts of SO_4^{2-} ion of the order of 100 μmg./10 μl. low recoveries of approx. 70 % were obtained. An increase in recovery of SO_4^{2-} ion to 85 % was made by using aqueous ethanol in the procedure so as to obtain an ethanol concentration of 35 % (v/v) in the reaction mixture (instead of the usual 66·5 %). Unfortunately there was also a large increase in control reading under this circumstance and further lowering of the ethanol concentration was not feasible.

The recovery of 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample was tested in the presence of a number of anions and cations, sulphatase substrates and tissue and enzyme preparations. In general, the findings of Lloyd (1959) were repeated except that recovery of low sulphate concentrations was 5–15 % too high in the presence of 0·02 M-PO_4^{3-} ions. At concentrations of PO_4^{3-} ion below 0·01 M no interference was observed.

Application of the benzidine method

Chondrosulphatase of Charonia lampas. Extracts of the tropical marine-mollusc *C. lampas* have been reported to contain both a chondrosulphatase and a chondroitinase (Soda & Egami, 1938). Before attempting to extend this observation, samples of acetone-dried *Charonia* liver, obtained from Professor F. Egami, were tested for their ability to degrade chondroitin sulphate by incubation of the preparations with chondroitin sulphate and measurement of the release of inorganic sulphate and reducing activity by the benzidine method and an ultramicro modification (B. Spencer, unpublished work) of the Somogyi (1952) method respectively.

To 50 μl. of a 4 % (w/v) suspension of acetone-dried *Charonia* liver in 0·5 M-acetate buffer, pH 5·0, was added 50 μl. of water or 50 μl. of a solution containing 2 mg. of sodium chondroitin sulphate/ml. and the mixture incubated at 38°. Samples (12 μl.) of the incubation mixture were removed at zero time and after incubation for 3 hr., and SO_4^{2-} ion and reducing activity were determined. The experiment was repeated with the centrifuged supernatant of the *Charonia* suspension.

Unmeasurably large amounts of reducing activity were liberated during 3 hr. incubation of the *Charonia* suspension in the absence of added substrate. This was attributed to the enzymic degradation of endogenous insoluble polysaccharides, for when the supernatant of the centrifuged *Charonia*

preparation was used the release of reducing material was much smaller and readily measured. No extra release of reducing activity was observed in the presence of chondroitin sulphate from ox or shark cartilage. There was a slight liberation of SO_4^{2-} ions from endogenous substrates in the *Charonia* suspension amounting to 5 μg./g. of *Charonia* powder/3 hr., but no extra SO_4^{2-} ion was released from the chondroitin sulphate preparations during the incubation with the whole suspension of *Charonia* powder or the centrifuged supernatant. The experiment was repeated at pH 4·0 and 6·0 but no significant liberation of sulphate or reducing activity from the chondroitin sulphates was observed during 3 hr. incubation. The lack of chondrosulphatase activity in three different *Charonia lampas* preparations has been confirmed by other workers in this Laboratory.

Acid hydrolysis of choline sulphate. During an investigation of the role of choline sulphate in the sulphur metabolism of fungi (Spencer & Harada, 1959) it became necessary to know the conditions necessary for complete hydrolysis of choline sulphate by acid.

The acid solution of choline sulphate (0·5 mg./ml.) was heated under a finger reflux condenser in a boiling-water bath and 1 ml. samples were removed at various times. To each sample was added 2 μl. of bromocresol green-indicator solution; the mixture was neutralized with NaOH and made up to 2 ml. The SO_4^{2-} ion in 10 μl. of the diluted samples was estimated by the benzidine method.

Complete hydrolysis of choline sulphate took place in 25 min. in 2N-HCl at 100°. In 0·3N-HCl for 30 min. at 100°, the conditions used by Raistrick & Vincent (1948) for the hydrolysis of ester sulphates in fungi, only 23% hydrolysis had occurred.

DISCUSSION

The small-scale modification of the benzidine method for sulphate estimation offers certain advantages over the original method (Dodgson & Spencer, 1953). In addition to the saving of materials and the application to small sample volumes the modification is twice as sensitive as, and very much more rapid than, the larger-scale method. The number of manipulations is halved and in practice it has proved relatively simple to gain proficiency in its use. Apart from the applications already given, the modified method is being used in this Laboratory in the study of the glycosulphatase of *Littorina littorea* (K. S. Dodgson, unpublished work), for the analysis of sulphate esters isolated in small amounts by paper chromatography and for the determination of SO_4^{2-} ions and ester sulphates in fungal mycelia.

The success of benzidine methods for sulphate estimation depends largely on the efficient removal of unprecipitated benzidine. In the small-scale method all benzidine-containing mixtures were restricted to the bottom half of the reaction tube and no serious contamination of the upper parts of the tube occurred. In the larger-scale method (Dodgson & Spencer, 1953) the benzidine-containing solutions were poured from the tube and much of the subsequent manipulation was concerned with the removal of the contaminating benzidine from the mouth and walls of the reaction tube. Of course, some benzidine was left behind in the tubes in the small-scale method but with a little practice in the technique the differences between the amounts of benzidine remaining in the individual tubes, as shown by the differences between the absorption readings of duplicate determinations, were not significant. Although no advantages were gained (cf. Nechaeva, 1958), it was noted that some of the remaining benzidine can be removed and the blank values lowered a little by a second washing of the precipitated benzidine sulphate with 120 μl. of ethanol. The recovery of SO_4^{2-} ion by this procedure was only 96 ± 4% (cf. Dodgson & Spencer 1953).

The measurement of the benzidine of the precipitated benzidine sulphate by direct spectrophotometric measurement involved only one pipetted addition, whereas three additions were required for the development of the azo dye in the larger-scale method. No interference in the absorption at 248 mμ was observed with the variety of biological materials listed in Table 1. Proteins were, of course, precipitated by ethanol and removed by centrifuging and other materials which absorb in the 248 mμ region and which were soluble in the aqueous 80% ethanol were effectively washed away in the subsequent manipulations.

The procedure adopted for the determination of SO_4^{2-} ion by the barium chloranilate method was slightly different from that of Lloyd (1959) and two extra steps were introduced: the addition of a small amount of extra SO_4^{2-} ion and the dilution of the final supernatant before making absorption readings at 327·5 mμ. In the method of Lloyd (1959) no absolute reference was made to the chloranilic acid liberated from barium chloranilate by SO_4^{2-} ion and values for concentrations of SO_4^{2-} ion were obtained by reference to a standard curve obtained with known amounts of SO_4^{2-} ion in the range 25–400 μg. of SO_4^{2-} ion/ml. of sample. The requirement for a limiting concentration of 10 μg. of SO_4^{2-} ion/ml. of sample before exchange of barium between chloranilic acid and SO_4^{2-} ion takes place was also found to apply to Lloyd's method, but with the use of the standard curve the

accuracy of the method was not affected. However, in both Lloyd's method and the present small-scale method the limiting concentration of SO_4^{2-} ion was found to vary slightly in the presence of biological materials and various ions, particularly PO_4^{3-} and Mg^{2+}. With concentration of 110 μg. of SO_4^{2-} ion/ml. of sample the effect of this variation influenced the recovery by less than 3 % (cf. Lloyd, 1959), but with lower sulphate values, such as 35 μg. of SO_4^{2-} ion, the error was sometimes greater than 10 %. These discrepancies were readily corrected by the addition of extra SO_4^{2-} ion, to both test and controls.

The dilution of the final supernatant before measuring the absorption at 327·5 mμ was necessary because of the high $\epsilon_{327\cdot5}$ of chloranilic acid. The pitfalls in making absorption readings at a wavelength where the absorption curve is steeply ascending are well recognized and the measurement of chloranilic acid at 350 mμ (Lloyd, 1959) is not recommended (cf. Fig. 1).

It is disappointing that the sensitivity of the barium chloranilate procedure has been increased only by a factor of 2 over the procedure of Lloyd (1959) and that it does not seem possible to determine less than 25 μg. of SO_4^{2-} ion/ml. by this method. The benzidine and barium chloranilate methods therefore remain complementary, the small-scale benzidine method being the choice for sensitivity and for use in the presence of small amounts of bivalent cations and the present barium chloranilate method or method of Lloyd (1959) being the choice for rapidity and convenience and for use in the presence of PO_4^{3-} ions.

SUMMARY

1. A scaled-down version of the benzidine method of Dodgson & Spencer (1953) has been developed for the estimation of 20–400 μmg. of SO_4^{2-} ion in 10 μl. samples. The precipitated benzidine sulphate is estimated by direct spectrophotometric measurement of benzidine at 248 mμ. Modifications of the method for use with higher concentrations of SO_4^{2-} ion and for 20 and 100 μl. samples are described.

2. The scaled-down version is twice as sensitive as, and very much more rapid than, the original method.

3. By means of the benzidine method potassium sulphate can be quantitatively recovered in the presence of various tissue suspensions, sulphatase substrates and small amounts of certain inorganic ions.

4. The barium chloranilate method has been scaled down for use with 10 μl. samples containing 0·25–2·5 or 2·5–25 μg. of SO_4^{2-} ion.

5. With the benzidine method it was observed that three different samples of acetone-dried liver of *Charonia lampas* failed to liberate SO_4^{2-} ion from chondroitin sulphate.

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