2. The average concentration of sodium in calf or cattle lens was 15–21 m-molal, and of potassium 119–122 m-molal.

3. The sodium concentration in calf or cattle lens increased from about 12 m-molal in the nucleus to about 80 m-molal in the capsule plus adhering tissue. The potassium concentration was maximal in the cortical fibres just below the capsule.

4. The excess of concentration of sodium in the outermost zone was reduced by blotting the lens, and was abolished by washing the lens in water or 0.25 M-sucrose.

5. Storage of the excised lens at 4° caused the sodium to become more evenly distributed throughout the tissue water.

6. Storage of the lens within the excised eye at 18° for periods up to 18 hr. caused an inward penetration of sodium from the aqueous humour, and a loss of potassium from the lens. The penetration started after storage for about 3 hr.

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Studies on Sulphatases

24. THE USE OF BARIUM CHLORANILATE IN THE DETERMINATION OF ENZYMICALLY LIBERATED SULPHATE*

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The benzidine micro-method (Dodgson & Spencer, 1953) has been used extensively in these Laboratories for the determination of enzymically liberated sulphate. However, as indicated by Dodgson & Spencer (1957), the procedure is both exacting and time-consuming. Consequently, the need has arisen for the development of a simpler method which can more readily be applied to the routine determination of a large number of samples. Such a method, based on the use of barium chloranilate (see Bertolacini & Barney, 1957), has now been devised.

EXPERIMENTAL AND RESULTS

Materials

Barium chloranilate. This reagent was prepared from chloranilic acid (2:5-dichloro-3:6-dihydroxy-*p*-benzoquinone; L. Light and Co.) and barium chloride, by the pro-

cedure of Bertolacini & Barney (1957). Commercial preparations of the reagent were also used, but these tended to give high blank values. These values could be minimized by washing the barium chloranilate several times by suspension in water, followed by filtering, washing with acetone and drying *in vacuo* at 50°. All preparations of barium chloranilate were finely powdered before use.

Tissue and enzyme preparations. Tissue suspensions were prepared according to Dodgson, Spencer & Thomas (1953). *Proteus vulgaris* chondrosulphatase was prepared by the method of Dodgson & Lloyd (1957), and glycosulphatase was obtained from extracts of the digestive gland of *Littorina littorea* by chromatography on diethylaminoethylcellulose (A. G. Lloyd, unpublished work).

Carbohydrate sulphates. Potassium glucose 3-sulphate was prepared by a modification of the method of Duff (1949); the potassium salts of glucose 6-sulphate, *N*-acetylglucosamine 6-sulphate and *N*-acetylgalactosamine monosulphate were prepared by the method of Lloyd (1959). Potassium chondroitin sulphate was obtained from ox tracheal cartilage by the method of Dodgson, Lloyd & Spencer (1957); heparin was a commercial preparation (British Drug Houses Ltd.).

* Part 23: Dodgson, Rose & Tudball (1958).

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All stages of the procedure were performed in 3 ml. Pyrex test tubes chosen for uniformity of bore. These tubes were suitable for the centrifuging steps when used in conjunction with an MSE 32-place angle-head centrifuge (catalogue no. 6885; Measuring and Scientific Equipment Ltd.). All glassware was cleaned with nitric acid; the use of sulphuric acid-containing mixtures was avoided. Reaction mixtures were agitated by holding the tube against a rapidly rotating flattened rod. A $\frac{1}{2}$ h.p. electric motor, with a flattened spindle, operating at 500–1000 rev./min., was suitable for this purpose.

Methods

Determination of sulphate in aqueous solution. In a 3 ml. test tube, 0.2 ml. of an aqueous solution of K_2SO_4 (5–80 μ g. of SO_4^{2-} ions/0.2 ml.) was mixed with 0.8 ml. of ethanol. A portion (0.8 ml.) of the ethanolic sulphate solution was withdrawn and mixed with 0.2 ml. of aqueous 0.05 M-potassium hydrogen phthalate solution, pH 4.0. To this was added 0.5 ml. of a suspension of barium chloranilate (approx. 400 μ g./0.5 ml.) in 0.25 M-sodium acetate-acetic acid solution, pH 4.0. Since the barium chloranilate is only slightly soluble and is always present in excess, the addition of an exact weight is unnecessary. The tube was capped with Parafilm (V. A. Howe and Co.) and the mixture was briefly agitated at 1 min. intervals for a total of 20 min. Excess of barium chloranilate and precipitated barium sulphate were removed by centrifuging at 4000 g and 0° for 10 min. The amount of chloranilate ion released into solution was measured against a reagent blank in the Uvispek spectrophotometer at 350 m μ in 1 cm. cells. The cell carrier of the instrument was modified to permit the use of 1 cm. cells with the 1.5 ml. test volume obtained in the above procedure. A straight-line relationship was obtained between the concentration of sulphate and the spectrophotometric reading.

Quantitative recoveries of sulphate were obtained when the sulphate standards were prepared in the presence of sodium acetate-acetic acid solution up to a concentration of 0.25 M at pH values within the range 3.0–7.5. To estimate sulphate in the presence of buffered tissue and enzyme preparations, a portion (0.1 ml.) of a solution of K_2SO_4 (20–30 μ g. of SO_4^{2-} ions/0.1 ml.) in 0.2 M-sodium acetate-acetic acid solution, pH 5.5, was mixed with 0.1 ml. of a tissue or enzyme preparation in the same medium. A suitably diluted tissue or enzyme preparation was used as a control. After incubation for 1 hr. at 37.5°, 0.8 ml. of ethanol was added and, after mixing, the whole was allowed to stand at 0° for 10 min. Precipitated protein, and polysaccharide where present, was removed by centrifuging at 3500 g and 0° for 15 min. A portion (0.8 ml.) of the clear supernatant was withdrawn and the inorganic sulphate determined as above. Sulphate could be recovered quantitatively from all the tissue and enzyme preparations tested (see Table 1). Na^+ , K^+ , NH_4^+ , Mg^{2+} , Cl^- , F^- , CN^- , PO_4^{3-} , HCO_3^- and oxalate ions when present in concentrations up to 0.02 M did not interfere with the recovery of sulphate from acetate-buffered tissue suspensions. However, Cu^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} , Fe^{3+} , Pb^{2+} and Ca^{2+} ions at the same concentration all interfered strongly (Table 2). The presence of the potassium salts of glucose 6-sulphate (0.02 M), glucose 3-sulphate (0.02 M), *N*-acetylglucosamine 6-sulphate (0.015 M), *N*-acetylgalactosamine monosulphate (0.015 M), chondroitin sulphate (0.25 %, w/v) and heparin

(0.23 %, w/v) did not affect the recoveries of sulphate. However, increase of the concentration of both chondroitin sulphate and heparin above the values quoted decreased the recovery of added inorganic sulphate.

Assay of glyco- and chondro-sulphatase. As a check of the method the enzymic release of sulphate from potassium glucose 6-sulphate and potassium chondroitin sulphate by glyco- and chondro-sulphatase respectively was examined. Experimental details are given in Table 3. The results obtained by the chloranilate procedure were compared with a similar series of determinations made simultaneously with the benzidine micro-method (Dodgson & Spencer, 1953). In each experiment four sets of six test determinations and six control determinations were made. After incubating each set for the time interval indicated and precipitating protein and polysaccharide by the addition of ethanol, the inorganic sulphate in three tests and three controls was determined by the chloranilate procedure, and inorganic sulphate in the remaining tests and controls was determined by the benzidine method. Table 3 shows that good agreement was obtained between the two methods.

Table 1. Recovery of potassium sulphate from tissue and enzyme preparations after incubation for 1 hr. at 37°

Preparation	Final concn. of suspension (g. dry wt./100 ml.)	SO_4^{2-} ions added (μ g.)	Recovery (%)
Rat			
Liver	1.8	25.8	98.2
Kidney	2.1	25.8	100.8
Spleen	2.0	25.8	99.4
Spleen	1.9	30.4	104.1
Lung	2.1	25.8	98.6
Cartilage	2.0	30.4	99.8
Pancreas	1.8	30.4	97.6
Human			
Liver	2.0	22.6	99.1
Kidney	2.0	22.6	99.6
Proteus vulgaris			
Chondrosulphatase	0.1	22.6	99.6
Littorina littorea			
Glycosulphatase	0.5	22.6	101.5

Table 2. Recovery of potassium sulphate in the presence of inorganic ions

Compound added	Concn. (M)	SO_4^{2-} ions added (μ g.)	Recovery (%)
NaCl	0.02	22.0	98.0
KCl	0.02	22.0	97.2
MgCl ₂	0.02	22.0	96.8
NH ₄ Cl	0.02	35.8	14.5
CaCl ₂	0.02	35.8	9.8
CoCl ₂	0.019	35.8	11.5
MnCl ₂	0.02	35.8	0
FeCl ₃	0.02	35.8	0
PbCl ₂	0.02	35.8	7.4
CuCl ₂	0.18	35.8	8.2
NaF	0.02	30.4	101.4
NaH ₂ PO ₄	0.02	30.4	98.6
NaHCO ₃	0.02	30.4	104.8
Sodium oxalate	0.02	30.4	101.9

Table 3. Comparison of the benzidine and barium chloranilate methods for the determination of SO_4^{2-} ions liberated by carbohydrate sulphatases

(a) A portion (0.1 ml.) of potassium chondroitin sulphate solution (0.25%, w/v, in 0.2M-sodium acetate-acetic acid solution, pH 7.0) was incubated at 37.5° with 0.1 ml. of *Proteus vulgaris* chondrosulphatase (0.1%, w/v, in 0.2M-sodium acetate-acetic acid solution, pH 7.0). At the end of the incubation period 0.8 ml. of ethanol was added and inorganic sulphate released by enzyme action was determined. Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only immediately before the addition of ethanol.

Incubation period (min.)	SO_4^{2-} ions liberated ($\mu\text{g.}$)	
	Chloranilate method	Benzidine method
10	15.6	16.4
20	28.8	27.4
30	40.4	40.8
40	48.8	51.2

(b) Potassium glucose 6-sulphate (0.1 ml. of 0.01M solution in acetate buffer, pH 5.5) was incubated at 37.5° with 0.1 ml. of *Littorina littorea* glycosulphatase (0.5%, w/v, in the same buffer). Control determinations were made as described above.

Incubation period (min.)	SO_4^{2-} ions liberated ($\mu\text{g.}$)	
	Chloranilate method	Benzidine method
10	8.4	9.8
20	17.6	18.2
30	26.3	25.8
40	35.7	35.3

DISCUSSION

The chloranilate procedure for the determination of sulphate compares favourably with the benzidine method for the assay of both glyco- and chondrosulphatase. The validity of the benzidine method as a means of estimating enzymically released sulphate has already been amply demonstrated (Dodgson & Spencer, 1953). The principal advantage of the present method is the considerable reduction in the time required to complete the assay of such carbohydrate sulphatases. The procedure is particularly useful therefore for the determination of a large number of samples as required, for example, in chromatographic studies on these sulphatases. However, at the present stage of development it offers no increase in sensitivity as compared with the benzidine method.

The introduction of the ethanol-precipitation step serves a double purpose. First, treatment of incubation mixtures with ethanol to 80% (v/v) results in the precipitation of both protein and polysaccharide which might otherwise interfere

with subsequent stages in the procedure. Secondly, as originally noted by Bertolacini & Barney (1957), the presence of ethanol in the test solution results in a decrease in the solubility of both barium sulphate and barium chloranilate. In aqueous solution the solubilities of barium sulphate and barium chloranilate are 9.6×10^{-6} and 2.2×10^{-4} mole/l. respectively. In aqueous 50% (v/v) ethanol these values are reduced to 2.5×10^{-7} and 5.2×10^{-6} mole/l. respectively.

The determination of sulphate by this method is not adversely affected by a number of common anions used in enzymology. However, it is unfortunate that, with the exception of certain univalent ions, cation interference is rather pronounced.

The variations in experimental conditions described here are not exhaustive, and further information about the performance of the technique will become available as the use of the method is extended. In general it has been found advisable to test the method (by recovery of added potassium sulphate) whenever new conditions are employed. The effect of buffer solutions other than acetate has not been examined here since the majority of workers on sulphatases have preferred this type of incubation medium. For similar reasons, no attempt has been made to examine the effect of values higher than pH 7.5 or lower than pH 3.0 for the incubation mixtures, since the range pH 3.0–7.5 embraces the pH optima of all the carbohydrate sulphatases at present known.

SUMMARY

1. A modification of the chloranilate procedure for determining small amounts (5–80 $\mu\text{g.}/0.2$ ml.) of enzymically liberated inorganic sulphate is described.

2. With the method potassium sulphate has been recovered quantitatively from a number of different enzyme and tissue preparations.

3. Recoveries of sulphate are unaffected by the presence of a number of common anions. On the other hand, interference by cations is rather pronounced. Within certain limits recoveries of sulphate are unaffected by the presence of the assay substrates for carbohydrate sulphatases.

4. The procedure has been compared with the benzidine method in the determination of the release of sulphate from glucose 6-sulphate and chondroitin sulphate by glycosulphatase and chondrosulphatase respectively.

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Location of ^{14}C in Protein from Isolated Rat Diaphragm Incubated *in vitro* with ^{14}C Amino Acids and with $^{14}\text{CO}_2$

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When isolated rat diaphragm is incubated *in vitro* with radioactively labelled amino acids, radioactive material is transferred to the protein (the trichloroacetic acid-insoluble portion) of the diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*). Manchester & Young (1958*b*) found that when rat diaphragm is incubated with ^{14}C glucose, ^{14}C pyruvic acid, ^{14}C oxoglutaric acid or with $^{14}\text{CO}_2$, ^{14}C is incorporated into protein. By the use of column chromatography we have now determined in which amino acids this incorporated ^{14}C is contained, and to what extent ^{14}C amino acids are converted by diaphragm into other ^{14}C amino acids before incorporation into protein.

MATERIALS AND METHODS

Radioactive materials. Labelled substances were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and sodium pyruvate were $[1\text{-}^{14}\text{C}]$ labelled; L-alanine, L-serine, L-glutamic acid, L-aspartic acid and D-glucose were all uniformly ^{14}C -labelled ($[\text{U-}^{14}\text{C}]$). Sodium α -oxoglutarate contained ^{14}C in the C-5 position. ^{14}C -Glucose had a specific activity of about $67\text{ }\mu\text{C}/\text{mg.}$, ^{14}C -sodium pyruvate about $43\text{ }\mu\text{C}/\text{mg.}$, ^{14}C -alanine $36\text{ }\mu\text{C}/\text{mg.}$, ^{14}C -glycine $37\text{ }\mu\text{C}/\text{mg.}$ and ^{14}C -serine $15\text{ }\mu\text{C}/\text{mg.}$ Each of these materials was used at a final concentration of $2\cdot5\text{ }\mu\text{C}/\text{ml.}$ ^{14}C -Glutamic acid (specific activity $62\text{ }\mu\text{C}/\text{mg.}$) and ^{14}C -aspartic acid (specific activity $32\text{ }\mu\text{C}/\text{mg.}$) were used at a concentration of $10\text{ }\mu\text{C}/\text{ml.}$ The protein from diaphragm incubated with ^{14}C glutamic acid and with ^{14}C oxoglutaric acid had been prepared in a previous experiment (Manchester & Young, 1958*b*).

The bicarbonate medium of Gey & Gey (1936), pH 7.4, gassed with $\text{O}_2 + \text{CO}_2$ (95:5, v/v), was used throughout. The incubation flasks each contained 2 ml. of medium. No unlabelled glucose was added to the medium in any experiment. In the experiment with $^{14}\text{CO}_2$, $16\text{ }\mu\text{C}$ of $\text{NaH}^{14}\text{CO}_3$ was

added to each flask, its final specific activity being $160\text{ }\mu\text{C}/\text{m-mole.}$

Preparation of diaphragm. Diaphragm was removed from female rats, of about 120 g. weight, which had starved for 20–24 hr. The two halves of the diaphragm were washed in freshly gassed buffer, then gently blotted and transferred to small conical flasks containing the incubation medium. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr. the diaphragm was removed, blotted and homogenized in a Potter-Elvehjem homogenizer in 10 ml. of 10% trichloroacetic acid. The precipitate was spun down, resuspended in 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90° . The insoluble material was extracted twice with 1.5 ml. of 0.4*N*-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% trichloroacetic acid. It was washed with 2 ml. of ethanol-ether (50:50, v/v), followed by 2 ml. of ether, and was dried in a vacuum desiccator.

Hydrolysis of protein. Protein (10 mg.) in 0.3 ml. of 6*N*-HCl was sealed in a thick-walled glass tube and heated to 105° for 20–24 hr. The hydrolysate was left to dry in a vacuum desiccator over CaCl_2 and redissolved in 1 ml. of 1.5*N*-HCl.

Chromatographic separation of amino acids. The amino acids in the protein hydrolysate were separated on a column (50 cm. $\times$ 1 cm.) of either Dowex-50 or Amberlite CG-120. The resin of both columns had been prepared for use by the method of Moore & Stein (1951), and was prepared for each experiment by washing with 2*N*-NaOH, followed by water and 1.5*N*-HCl. Washing with HCl was continued until the effluent left no deposit of NaCl when evaporated to dryness. The protein hydrolysate was introduced at the top of the column and solvent run through at room temperature at the rate of about 4 ml./hr. As solvents 1.5*N*-, 2.5*N*- and 4*N*-HCl were successively used (Stein & Moore, 1949). The eluate was collected in fractions of about 3.3 ml. by means of an automatic fraction collector.