We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and advice, Mr A. Renshaw for his assistance in building a gas chromatograph, Dr Lovelock and Dr James for prepublication advice on the techniques of gas chromatography, and Miss B. M. Notton for technical assistance. The work was aided by grants from the Rockefeller Foundation and the United States Department of Public Health. One of us, G.S.G., held a Nuffield Demonstratorship during the period of the research, and wishes to thank the Nuffield Dominions Trust for this financial support.

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Determination of Inorganic Sulphate in Studies on the Enzymic and Non-Enzymic Hydrolysis of Carbohydrate and Other Sulphate Esters

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(*Received* 21 *July* 1960)

During studies on the hydrolysis of potassium glucose 6-*O*-sulphate by hydrazine (see Dodgson & Lloyd, 1961) it became necessary to develop a new method for determining small amounts of inorganic sulphate. Previous methods developed in these laboratories have involved the use of benzidine (Dodgson & Spencer, 1953; Spencer, 1960) or barium chloranilate (Lloyd, 1959) but cannot be used in the presence of hydrazine. In a previous study of the non-enzymic hydrolysis of crude samples of glucose monosulphate (Dodgson & Spencer, 1954), liberated sulphate was estimated gravimetrically as barium sulphate, but the method was not satisfactory because of the relatively large amounts of substrate which had to be used.

The author's attention was directed (Dr B. Sörbo, personal communication) to the possible use of a turbidimetric method for the determination of inorganic sulphate, gelatin being used as a cloud stabilizer. A method of this type, based on that of Gassner & Friedel (1956), has been developed by Berglund & Sörbo (1959) for the determination of inorganic sulphate in blood and urine. The present paper shows how the method was adapted to the study of the non-enzymic hydrolysis of simple carbohydrate sulphates. Other modifications are described which enable the method to be used, under certain circumstances, for the assay of glycosulphatase and for the microanalysis of ester sulphates.

MATERIALS

Trichloroacetic acid. This was British Drug Houses Ltd. AnalaR reagent.

Barium chloride–gelatin reagent. Micromethods which depend on precipitation of SO_4^{2-} ions in one form or another generally require a certain minimum amount of

SO_4^{2-} ions to be present before precipitation becomes quantitative (see Dodgson & Spencer, 1957; Spencer, 1960). The presence of SO_4^{2-} ions in commercial gelatin preparations ensures that this requirement is fulfilled in the present case. Some gelatins which were tested contained excessive amounts of SO_4^{2-} ions; however, Difco Bacto Gelatin (Difco Laboratories Inc., Detroit, Mich., U.S.A.) proved to be satisfactory in all respects.

The reagent had to be prepared with some care. Gelatin (2 g.) was dissolved in 400 ml. of hot water (60–70°) and allowed to stand at 4° for at least 6 hr. and preferably overnight. Barium chloride (2 g.) was dissolved in the semi-gelatinous fluid and the resultant cloudy solution was allowed to stand for 2–3 hr. before use. Strict adherence to this procedure gave a stabilized suspension of $BaSO_4$ in the presence of excess of $BaCl_2$, which, when mixed with 4% trichloroacetic acid in the proportions 1:4 (v/v), gave an extinction (E) of approx. 0·15 when measured spectrophotometrically in 2 cm. quartz cells at 360 mμ against a water blank. The reagent was stored at 4° and could be used for about 1 week.

Glassware. Glassware was cleaned with HNO_3; mixtures containing H_2SO_4 were avoided. Micropipettes were of the Lang–Levvy constriction type (Lowry, Roberts, Leiner, Wu & Farr, 1954).

Spectroscopic measurements. These were made with the model H 700.308 Uvispek spectrophotometer. A suitable metal slit-mask was constructed to enable measurements to be made in 2 cm. quartz cells with 2 ml. samples (see method B), and the Uvispek quartz 1 cm. microcells and adaptor were used in method C.

Sulphate esters. Monosaccharide mono- and di-sulphate esters, shark chondroitin sulphate and ox chondroitin sulphates A and C were prepared by methods described by Dodgson & Lloyd (1961). The *O*-sulphate esters of serine, threonine and hydroxyproline, and a number of alkyl-sulphates, were prepared according to the methods of K. S. Dodgson, A. G. Lloyd & N. Tudball (unpublished work). Seaweed polysaccharide sulphates were purified and fractionated as described by Lloyd, Dodgson, Price & Rose (1961). Arylsulphate esters were prepared by the general methods outlined by Dodgson & Spencer (1957). Other sulphate esters were gifts.

EXPERIMENTAL AND RESULTS

Determination of sulphate in aqueous solutions

Three different modifications of what was essentially the same method were used. Method A, covering the range 0–200 μg. of SO_4^{2-} ions, was subsequently used to follow the rates of hydrolysis of carbohydrate sulphates by hydrazine (see Dodgson & Lloyd, 1961); method B (range, 0–40 μg. of SO_4^{2-} ions) was later used in enzyme studies on glycosulphatase (see Dodgson, 1961); method C (range, 0–12 μg. of SO_4^{2-} ions) was used in the analysis of various sulphate esters.

Method A. To the sulphate-containing solution (0·2 ml., contained in a tapered 10 ml. centrifuge tube) was added 3·8 ml. of 4% trichloroacetic acid followed by 1 ml. of the $BaCl_2$–gelatin reagent. After mixing, the whole was allowed to stand for 10–20 min. at room temperature. A reagent blank was prepared in the same way except that 0·2 ml. of water was substituted for the sulphate-containing solution. The extinction of the test solution was measured (against the reagent blank) in 2 cm. quartz cells at 360 mμ and a slit width of 0·2 mm. The cloud was stable for at least 1 hr., after which there was some tendency for $BaSO_4$ to settle. A calibration curve was prepared with solutions of K_2SO_4 containing between 20 and 200 μg. of SO_4^{2-} ions (Fig. 1). A second calibration curve was obtained by making spectrophotometric readings at 500 mμ with a slit width of 0·1 mm. (Fig. 1). Greater sensitivity was achieved by reading at 360 mμ, but the 500 mμ calibration curve can be used when estimating SO_4^{2-} ions in the presence of materials tending to absorb ultraviolet light.

Method B. Method A was modified to enable smaller volumes of starting material to be used with increased sensitivity. The sulphate-containing solution (0·1 ml.), contained in a 8 cm. × 1 cm. Pyrex test tube, was mixed

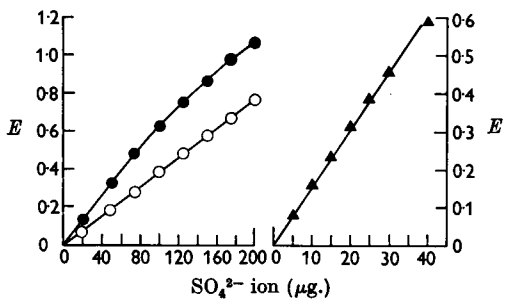

Fig. 1. Calibration curves for the determination of inorganic sulphate by methods A and B (see text). ●, Method A, measurements made in 2 cm. quartz cells at 360 mμ; ○, method A, measurements made in 2 cm. quartz cells at 500 mμ; ▲, method B, measurements made in 2 cm. quartz cells at 360 mμ with the aid of a slit mask.

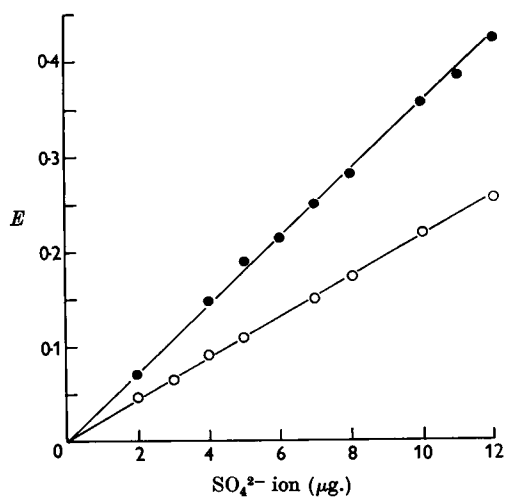

Fig. 2. Calibration curves for the determination of inorganic sulphate by method C (see text). ●, Measurements made in 1 cm. microcells at 360 mμ; ○, measurements made in 1 cm. microcells at 500 mμ.

with 1·4 ml. of 4% trichloroacetic acid followed by 0·5 ml. of $BaCl_2$–gelatin reagent. After the solution was mixed and allowed to stand as before, the extinction was measured (against an appropriate reagent blank) in 2 cm. cells at 360 mμ. Metal blocks (1 cm. high) were inserted in the cell-carrier in order to raise the position of the cells in relation to the light path. A metal plate (16 cm. × 5 cm. × 0·1 cm.), containing a hole (0·5 cm. × 0·5 cm.) suitably positioned to permit the passage of the beam of ultraviolet light, was placed between the cell carrier and the exit slit. The side flanges and two protruding screws which are already present in the cell compartment of the Uvispek provided a convenient means of securing the slit mask in position. All metal surfaces were painted black. A calibration curve was prepared as before in the range 0–40 μg. of SO_4^{2-} ions (Fig. 1).

Method C. This was essentially method B decreased in scale to one-fifth, the determinations being made in 50 mm. × 6 mm. Pyrex tubes. Spectrophotometric readings (at 360 mμ or 500 mμ) were made with the Uvispek 1 cm. microcells, the slit height of the microcell adaptor being adjusted to 4 mm. No 2 cm. microcells were available, consequently the readings were only half as sensitive as those in method B. However, up to 12 μg. of SO_4^{2-} ions could be measured with reasonable sensitivity (Fig. 2).

Effect of varying experimental conditions

Except where otherwise stated, these studies were carried out with method A. Control determinations, in which water was substituted for the solution of K_2SO_4, were made in each case, both test and control solutions being measured against a reagent blank. Generally speaking, control and reagent-blank readings were identical within the limits of experimental error.

Trichloroacetic acid concentration. Calibration curves obtained in presence of 2, 3 and 4% of trichloroacetic acid were identical. At concentrations below and above these limits (1, 5 and 6%) the results tended to be variable and less reliable. A concentration of 4% trichloroacetic acid was selected for general use because of the need to counteract the effect of buffers which were present in later experiments.

Concentration of barium chloride–gelatin reagent. The concentration of this reagent was varied by adding different volumes of the reagent to the trichloroacetic acid–K_2SO_4 mixture and adding appropriate volumes of water to give a final volume of 5 ml. In the one case where the volume of the reagent added exceeded 1 ml. (1·2 ml.), volume compensation was achieved by adding 3·6 ml. of the 4% trichloroacetic acid. Decrease of the volume of added reagent below 0·8 ml. resulted in increasingly poor recoveries of sulphate (Table 1).

Effects of various compounds on the recovery of sulphate

Buffers. Calibration curves for recovery of added K_2SO_4 in the presence of sodium acetate–acetic acid (M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid (0·5M), Na_2HPO_4–NaH_2PO_4 (0·2M) and sodium citrate–citric acid (0·5M) buffers of varying pH, were identical with that obtained in the absence of buffers.

Hydrazine and imidazole. Solutions of hydrazine hydrate in 0·5M-sodium acetate–acetic acid buffer at varying pH, and solutions of imidazole in the same buffer, did not affect recoveries of added K_2SO_4 (Table 2).

Carbohydrate sulphate esters. Recoveries of K_2SO_4 from buffered and aqueous solutions of various monosaccharide sulphate esters were good (Table 3). With the exception of tripotassium galactose trisulphate, all the esters were stable at room temperature in the presence of trichloroacetic acid for several hours. Galactose trisulphate (prepared by direct sulphation of galactose with excess of chlorosulphonic acid at 70°; Dr A. G. Lloyd, unpublished results) was rapidly hydrolysed by trichloroacetic acid at room temperature. Polysaccharide sulphates formed a complex with the gelatin in the reagent (irrespective of whether $BaCl_2$ was present or not) to give dense cloudy solutions. Berglund & Sörbo (1959) have previously noted that heparin interferes with this type of turbidimetric determination.

Table 1. *Effect of variation of the concentration of barium chloride–gelatin reagent on recoveries of potassium sulphate from aqueous solutions*

Vol. of $BaCl_2$–gelatin reagent present in final vol. of 5 ml. (ml.)	SO_4^{2-} ion (μg.) Added	Recovered
1·2	120	119
	40	39
1·0	200	200
	100	100
	20	20
0·8	200	194
	100	96
	20	19
0·6	200	188
	100	93
	20	18
0·4	200	171
	100	88
	20	15

Table 2. *Effect of hydrazine and imidazole on the recovery of potassium sulphate from solutions buffered with 0·5M-sodium acetate–acetic acid*

	pH	SO_4^{2-} ion (μg.) Added	Recovered
Hydrazine hydrate (0·33M)	4·2	50	50
		100	102
	5·3	50	51
		100	101
	6·8	50	50
		100	102
Imidazole (0·20M)	5·0	25	25
		50	52
		200	199
	6·0	25	25
		50	51
		200	198
	7·5	25	25
		50	51
		200	194

Other sulphate esters. Method B was used for these experiments, the test solutions being measured directly against controls in which water was substituted for the K_2SO_4 solution. Good recoveries were obtained in most cases (Table 4), exceptions being holothurin (a sulphated steroid saponin from sea cucumbers; Nigrelli, Chanley, Kohn & Sobotka, 1955), sodium cortisone 21-sulphate, sodium dehydro*iso*androsterone sulphate (DHAS) and sodium 2-(2:4-dichlorophenoxy)ethyl sulphate (Crag herbicide, see Vlitos, 1953). Holothurin formed an insoluble complex with gelatin, and in the remaining cases recoveries of SO_4^{2-} ions were high. All the sulphate esters tested were stable at room temperature in the presence of trichloroacetic acid during the time of the experiment.

Table 3. *Effect of various carbohydrate sulphate esters on the recoveries of potassium sulphate from aqueous solutions or solutions buffered with sodium acetate–acetic acid (acetate) or tris–acetic acid*

Chondroitin sulphates A and C, shark chondroitin sulphate, charonin sulphate, fucoidin, *Chondrus ocellatus* mucilage and κ- and λ-carrageenins from *C. crispus*, *Gigartina stellata* and *G. radula*, at concentrations of 0·1% in water, all interfered with the method by forming insoluble complexes with gelatin.

Sulphate ester (K^+ or Na^+ salt)	Concn. (mM)	Buffer	Concn. of buffer (M)	pH	SO_4^{2-} ion (μg.) Added	Recovered
Glucose 3-*O*-sulphate	80	Tris–acetate	0·5	5·7	20	20
	50	(Water)	—	—	20	20
Glucose 6-*O*-sulphate	80	Tris–acetate	0·5	5·7	20	21
	80	Acetate	0·5	5·8	20	20
	80	(Water)	—	—	20	20
Glucose disulphate	50	(Water)	—	—	40	42
	50	(Water)	—	—	100	104
Galactose 6-*O*-sulphate	80	Tris–acetate	0·5	5·2	20	20
	25	Acetate	0·25	5·3	40	41
Galactose disulphate	50	Acetate	0·25	5·3	40	39
Galactose trisulphate	50	Acetate	0·25	5·3	40	*

* Rapidly hydrolysed in 4% trichloroacetic acid.

Table 4. *Effect of various sulphate esters on the recoveries of potassium sulphate from solutions buffered with* 0·5M-*tris–acetic acid*

Sulphate ester (K^+ salt)	Concn. (mM)	pH	SO_4^{2-} ion (μg.) Added	Recovered
Methyl sulphate	40	5·5	20	20
Ethyl sulphate	40	5·5	20	20
Ethanolamine sulphate	40	5·5	15	16
n-Propyl sulphate	40	5·5	20	20
*iso*Propyl sulphate	40	5·5	20	20
*iso*Propanolamine *O*-sulphate	40	5·5	20	20
n-Butyl sulphate	40	5·5	20	20
sec.-Butyl sulphate	40	5·5	20	20
Propane-1:3-diol disulphate	20	5·1	15	16
n-Pentyl sulphate	40	4·5	20	19
n-Hexyl sulphate	40	5·5	20	20
*cyclo*Pentyl sulphate	40	5·5	20	20
*cyclo*Hexyl sulphate	40	5·1	25	26
Tetrahydrofuran 2-methyl sulphate	40	5·5	20	20
Tetrahydropyran 2-methyl sulphate	40	5·5	20	19
Serine *O*-sulphate	20	5·7	20	20
	20	8·5	20	20
Threonine *O*-sulphate	20	5·7	20	20
	20	8·5	20	20
Hydroxyproline *O*-sulphate	20	5·7	10	11
	20	8·5	10	11
Holothurin	20	5·5	20	*
Dehydro*iso*androsterone sulphate†	6	5·1	25	28
Cortisone 21-sulphate†	20	5·6	15	24
2-(2:4-Dichlorophenoxy)ethyl sulphate†	40	5·1	25	30

* Too cloudy to measure. † Sodium salt.

Sodium cortisone 21-sulphate. Table 5 show the effect of increasing concentrations of this ester on recoveries of SO_4^{2-} ions in the presence of 0·5M-tris–acetic acid buffer. Similar results were obtained in the presence of 0·25M-tris–acetic acid and 0·25M-sodium acetate–acetic acid buffers. The results can be explained by assuming that the minimum concentration of SO_4^{2-} ions which must be present before $BaSO_4$ is precipitated quantitatively is comparatively high when cortisone 21-sulphate is present in high concentrations. At lower concentrations of the ester there are sufficient SO_4^{2-} ions present in the test solutions to cause complete precipitation for $BaSO_4$, but the SO_4^{2-} ion content of the controls is insufficient for this purpose. Support for this came from experiments in which extra SO_4^{2-} ion was added to both test and control solutions. This extra SO_4^{2-} ion was incorporated (as K_2SO_4) in the trichloroacetic acid solutions at a concentration of 12 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid. Provided that the initial concentration of cortisone 21-sulphate did not exceed 0·01M, satisfactory recoveries of SO_4^{2-} ion could be obtained (Table 5).

Table 5. *Effect of various concentrations of sodium cortisone 21-sulphate on recoveries of potassium sulphate from solutions buffered at* pH 5·1 *with* 0·5M-*tris–acetic acid*

Concn. of cortisone 21-sulphate (mM)	SO_4^{2-} ion (μg.) Added	Recovered
80	20	3
80	10	2
40	10	3
20	10	14
20	15	24
10	10	14
5	15	18
2·5	15	17
10*	25	25
10*	15	15
10*	10	10
10*	5	5

* Extra SO_4^{2-} ion added to tests and blanks at a concentration of 12 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid.

Table 6. *Effect of various compounds on the recoveries of potassium sulphate from aqueous solution*

Compound	Concn. (M)	SO_4^{2-} ion (μg.) Added	Recovered
Glucose	0·25	20	20
NaCl	0·10	20	20
$BaCl_2$	0·10	20	7
$MgCl_2$	0·10	20	20
Na_2SO_3	0·10	20	*
$MnCl_2$	0·10	20	20
$ZnCl_2$	0·10	20	20
NaF	0·10	20	20
KCN	0·10	20	20
$Na_4P_2O_7$	0·10	20	15
	0·02	20	19
	0·01	20	20
Hydroxylamine–HCl	0·10	20	20
Semicarbazide–HCl	0·10	20	20
Ethylenediaminetetra-acetic acid	0·10	20	20

* Too cloudy to measure.

Dehydroisoandrosterone sulphate and Crag herbicide. An initial concentration of 3 mM-DHAS in 0·5M-tris–acetic acid buffer resulted in slightly high recoveries of SO_4^{2-} ion (approx. 10%). Recoveries were good when extra SO_4^{2-} ion was added with the trichloroacetic acid to test and control solutions at a concentration of 10 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid. In the presence of 0·5M-sodium acetate–acetic acid buffer, recoveries were good without the need to add extra SO_4^{2-} ion. Recoveries of SO_4^{2-} ion from buffered solutions of Crag herbicide also tended to be high. However, good recoveries were obtained when extra SO_4^{2-} ion was added as before, provided that the concentration of the ester did not exceed 0·01M.

Some common enzyme inhibitors and activators. Method B was used for these experiments. Recoveries of SO_4^{2-} ion from unbuffered solutions containing $BaCl_2$ or $Na_4P_2O_7$ (at concentrations greater than 0·01M) were low, and solutions containing Na_2SO_3 were too cloudy to read (Table 6).

Recoveries of sulphate from preparations of glycosulphatase

Several methods for the estimation of enzymically liberated SO_4^{2-} ion have been used in the study of glycosulphatase (see Dodgson & Spencer, 1957). All these methods suffer from limitations of one sort or another but the benzidine micromethod developed by Dodgson & Spencer (1953) can probably be regarded as the most sensitive and generally applicable of these methods. However, the method is laborious and time-consuming and demands a high degree of manipulative skill. It also suffers from the disadvantage of being inapplicable in the presence of concentrations of potassium glucose 6-*O*-sulphate (the substrate for glycosulphatase) greater than 0·02M. Attempts were therefore made to use the present turbidimetric method for the assay of glycosulphatase. Although the sensitivity of this method compares unfavourably with that of the benzidine procedure, this disadvantage would be outweighed by the simplicity and rapidity of the method and its applicability over a wide range of experimental conditions.

Crude extracts of the visceral regions of *Littorina littorea* (one of the best sources of glycosulphatase) gave cloudy suspensions when mixed with trichloroacetic acid. These suspensions could not be centrifuged under routine centrifuging conditions. However, with partially purified preparations of the enzyme (Dodgson, 1961) no cloud or precipitate appeared on addition of trichloroacetic acid. Seven separate preparations of the enzyme behaved similarly. The following procedure was used to check recoveries of SO_4^{2-} ion from these preparations. The enzyme solution (0·05 ml.) was incubated at 38° with 0·05 ml. of a solution of K_2SO_4 in water, sodium acetate or tris. After 1 hr., 1·4 ml. of 4% trichloroacetic acid was added and thereafter the procedure was exactly as described for method B. Control determinations were made in which water or buffer was substituted for the K_2SO_4 solution. Test solutions were measured against controls. Good recoveries were obtained in all cases (Table 7).

Good recoveries of SO_4^{2-} ion were also obtained from diluted preparations of the digestive juice of the snail

(*Helix pomatia*) (see Dodgson, 1961) and from a purified arylsulphatase preparation obtained from snail digestive gland. The method was subsequently used to study the hydrolysis of sodium cortisone 21-sulphate by these preparations.

Recoveries of sulphate from rat-tissue homogenates

In these experiments 0·2 ml. of a 4% (w/v) suspension of the tissue in M-tris–acetic acid buffer, pH 6·5, was incubated for 1 hr. with an equal volume of an aqueous solution of K_2SO_4. Trichloroacetic acid (4·6 ml. of 4%) was added and, after standing for 60 min. at −5°, precipitated protein was centrifuged down. A portion (4 ml.) of the clear supernatant was added to 1 ml. of the $BaCl_2$–gelatin reagent and thereafter method A was followed. Appropriate control determinations were made. Recoveries of SO_4^{2-} ion from liver preparations were poor (Table 8) unless extra SO_4^{2-} ion was added with the trichloroacetic acid (13 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid).

Analysis of ester sulphates

The turbidimetric method was readily adapted to the analysis of ester sulphates. The ester sulphate (usually about 1 mg.) was dissolved in 1 ml. of water. A portion (0·02 ml.)

Table 7. *Recoveries of potassium sulphate in the presence of enzyme preparations*

Reaction mixtures were incubated for 1 hr. at 37·5° before analysis.

Enzyme preparation	Incubation medium	Concn. (M)	SO_4^{2-} ion (μg.) Added	Recovered
Littorina littorea concentrate	Water	—	30	30
			25	26
			20	20
			15	16
			10	10
	Sodium acetate	0·5	30	29
			20	20
			10	9
	Tris	0·25	30	29
			20	20
			10	10
		0·5	20	20
Snail digestive juice (diluted 10-fold)	Tris	0·5	20	19
			10	10
Snail digestive juice (diluted 30-fold)	Tris	0·5	10	10
Snail digestive-gland arylsulphatase	Tris	0·5	10	10

Table 8. *Recoveries of potassium sulphate from various rat-tissue suspensions*

Tissue suspensions (at a final concentration of 2%, w/v) were incubated with K_2SO_4 in the presence of 0·5 M-tris–acetic acid, pH 6·5, for 1 hr. at 37·5° before determination of SO_4^{2-} ion.

Tissue	Departure from normal reaction mixture	SO_4^{2-} ion (μg.) Added	Recovered
Liver	None	11	5
		31	15
		62	69
		99	116
		124	141
	40 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	*
		31	29
		62	60
		99	94
	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	10
Spleen	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	14
Lung	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	12
Heart	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	12

* Results were inconsistent.

Table 9. *Analytical results for the* SO_4^{2-} *ion content of various sulphate esters*

Sulphate ester	SO_4^{2-} ion content (%) Found	Theory
Potassium *n*-amyl sulphate	46·0	46·6
Potassium *n*-butyl sulphate	50·3	49·2
Potassium *sec.*-butyl sulphate	50·3	52·3
Potassium *iso*propyl sulphate	53·9	53·9
Potassium tetrahydropyran 2-methyl sulphate	43·7	41·2
Potassium tetrahydrofuran 2-methyl sulphate	46·0	43·2
Dipotassium propane-1:2-diol disulphate	59·5	61·5
Potassium serine *O*-sulphate monohydrate	40·2	40·0
Potassium threonine *O*-sulphate monohydrate	38·2	37·6
Potassium hydroxyproline *O*-sulphate	39·4	38·5
Potassium *p*-nitrophenyl sulphate	36·3	37·4
Potassium *m*-cresyl sulphate	41·3	42·5
Dipotassium 2-hydroxy-5-nitrophenyl sulphate dihydrate	27·7	27·7
Potassium glucose 6-*O*-sulphate	32·8	32·3
Potassium methyl sulphate	65·0	64·1
Sodium dehydro*iso*androsterone sulphate	33·6	24·6
Sodium dehydro*iso*androsterone sulphate	26·2*	24·6

* After intermediate centrifuging (see text).

was transferred to a 100 mm. × 6 mm. stout-walled Pyrex tube containing 0·3 ml. of 4% trichloroacetic acid. The tube was sealed in an oxygen–coal gas flame and placed in an oven at 110°. Heating was continued for at least 4 hr., although it was generally convenient to leave the tubes overnight. After cooling, the tubes were shaken and allowed to stand for 5 min. in an upright position before opening. A portion (0·3 ml.) was withdrawn with a tapered constriction pipette and added to 0·1 ml. of the $BaCl_2$–gelatin reagent contained in a 50 mm. × 6 mm. test tube. After mixing, the extinction was measured in 1 cm. microcells against a reagent blank. Some sulphate esters, on hydrolysis, yielded parent compounds which absorbed in the 360 mμ region and in these cases spectrophotometric measurements were made at 500 mμ. In one case (DHAS) the trichloroacetic acid solution, after heating, was cloudy and the final SO_4^{2-} ion figure was high (Table 9). Good results were obtained by centrifuging the cloudy solution before removing the 0·3 ml. sample.

DISCUSSION

The difficulties associated with the measurement of microgram quantities of SO_4^{2-} ion under a wide variety of experimental conditions have long been recognized (see Dodgson & Spencer, 1953, 1957; Lloyd, 1959; Cope, 1931). In addition many of the methods which are available for the determination of SO_4^{2-} ion are time-consuming and exacting or, when used for studies on the enzymic and non-enzymic hydrolysis of sulphate esters, are very wasteful of starting material. The adaptations of the turbidimetric barium sulphate method which have been described above have the advantage of simplicity and rapidity and, on the whole, are less subject to interference from other materials which may be present. Interference is probably minimized by the considerable dilution which occurs when the relatively large volume of trichloroacetic acid is added to the reaction mixture.

The method is less sensitive than either the benzidine or the chloranilate procedure, but this fact is compensated for by the simplicity of the method. Enzyme experiments with purified glycosulphatase, which normally take 1 or 2 days to complete, can be accomplished in 2 or 3 hr. Moreover, it is a simple matter to check recoveries of SO_4^{2-} ion whenever new experimental conditions are to be used.

One drawback to the method, when it is used for enzyme studies, is that care must be taken to ensure that sufficient substrate is present to enable kinetic interpretations of results to be made. For example, with method B, complete hydrolysis of 0·05 ml. of 0·02 M-potassium glucose 6-*O*-sulphate by an equal volume of a glycosulphatase solution would yield a total of 96 μg. of SO_4^{2-} ion and hydrolysis of 10% of the substrate would yield a sufficient amount of SO_4^{2-} ion (9·6 μg.) to be measured accurately. On the other hand, 10% hydrolysis of 0·05 ml. of a 2 mM-solution of the substrate would yield only 0·96 μg. of SO_4^{2-} ion. Fortunately, the optimum substrate concentration for glycosulphatase activity is high (0·04 M-potassium glucose 6-*O*-sulphate; see Dodgson, 1961) and the method has been of great value in studies on this enzyme. On the other hand, the method is not entirely suitable for the assay of the 3β-steroid sulphatase, which is also present in glycosulphatase preparations, since the optimum substrate concentration of this enzyme is less than 1 mM. The activity of this enzyme must therefore be measured at substrate concentrations greater than optimum when some inhibition by excess of

substrate is obtained (see Dodgson, 1961). The method would also appear to be of doubtful value for use with crude mammalian-tissue preparations in view of the need to add relatively large amounts of extra SO_4^{2-} ion to tests and controls before good recoveries of SO_4^{2-} ion can be obtained.

The method has also been successfully used to follow the alkaline- and acid-hydrolysis rates of the *O*-sulphates of serine and threonine (K.S. Dodgson, A. G. Lloyd & N. Tudball, to be published) and to measure the ester sulphate content of chondroitin sulphate preparations after hydrolysis with hydrochloric acid (R. G. Price, unpublished results).

SUMMARY

1. Three variations of a turbidimetric method for the determination of small amounts of inorganic sulphate have been described.

2. One variation, covering the range 0–200 μg. of SO_4^{2-} ion, is suitable for following the non-enzymic hydrolysis of monosaccharide sulphates by hydrazine; a second variation, covering the range 0–40 μg. of SO_4^{2-} ion, has been developed for the assay of purified glycosulphatase preparations; the other variation, covering the range 0–12 μg. of SO_4^{2-} ion, has been successfully used for the microanalysis of various ester sulphates.

3. The method can be used in the presence of hydrazine, imidazole, various buffers, anions, cations, monosaccharide mono- and di-sulphate and other sulphate esters. SO_3^{2-}, Ba^{2+} and $P_2O_7^{4-}$ ions (in concentrations greater than 0·01 M), and a large number of polysaccharide sulphate esters which were tested, interfered with the method. Other limitations of the method are described.

This work has been supported by a research grant (A 1982) from the Arthritis and Metabolic Diseases Division of the U.S. Public Health Service. The author wishes to thank Mr E. Peterson for his technical assistance and to acknowledge gifts of various ester sulphates by Merck and Co. Inc., N.J., and the Union Carbide and Carbon Corp., New York.

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Potassium Glucose 6-*O*-Sulphate as a Substrate for Glycosulphatase

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(*Received* 21 *July* 1960)

During a study of the glycosulphatase of *Littorina littorea*, Dodgson & Spencer (1954) observed anomalies in the behaviour of the enzyme towards increasing concentrations of potassium glucose monosulphate (prepared by direct sulphation of glucose and presumed to be glucose 6-*O*-sulphate), which they suggested might be due to the heterogeneity of the substrate preparation. Evidence for the presence of more than one sulphate ester in the preparation was obtained by paper chromatography and from experiments in which it was shown that about 25 % of the material could be desulphated by treatment with 0·33 M-hydrazine at pH 5·3 and 38° (see also Egami, 1938, 1940, 1942). Further work on the enzyme was deferred until pure potassium glucose 6-*O*-sulphate could be obtained.

Recent work from these laboratories (Lloyd, 1960) has shown that glucose, potassium glucose 6-*O*-sulphate and dipotassium glucose disulphate are present in preparations of potassium glucose monosulphate obtained by direct sulphation of glucose. The present study shows that the partial hydrolysis of such preparations by hydrazine can be attributed to the hydrolysis of one of the sulphate groups present in potassium glucose