Studies on Sulphatases

5. THE DETERMINATION OF INORGANIC SULPHATE IN THE STUDY OF SULPHATASES

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(Received 18 April 1953)

The various enzymes which are collectively termed sulphatases are able to hydrolyse certain organic sulphate esters. Four different sulphatases are known: arylsulphatase (Derrien, 1911), glucosulphatase (Soda, 1936), chondrosulphatase (Neuberg & Hofmann, 1931) and myrosulphatase (Neuberg & Wagner, 1927). These enzymes have been reviewed by Fromageot (1950).

Sulphatase studies have been limited by the difficulty of estimating the end products of hydrolysis. Arylsulphatase can be studied by estimating the phenolic end product of hydrolysis and many procedures are available (cf. Dodgson & Spencer, 1953*a*). However, it is more difficult to estimate the organic end products produced by other sulphatases and in the case of certain natural substrates the chemical structure of the organic end product is in doubt. Soda (1936) studied glucosulphatase by determining glucose liberated from glucose 6-sulphate, but this method depended on yeast fermenting small amounts of glucose in the presence of large amounts of glucose 6-sulphate and was complex and time consuming.

Attempts were made to determine liberated sulphuric acid by gravimetric (Soda & Hattori, 1931), nephelometric (Soda, 1936) and manometric (Lindahl, 1935) methods, and by potentiometric titration (Soda, 1936), but the methods were insensitive and necessitated incubation periods of several days. Under such conditions the validity of quantitative enzyme experiment is questionable; furthermore, the suitability of these methods was not fully investigated. Greater sensitivity was achieved by Tanaka (1938) who modified the colorimetric benzidine method, but insufficient experimental evidence was given to justify using the method under widely varying conditions. Recently Lowenstein & Young (1952) adapted the manometric method of Lindahl (1935) to the Warburg apparatus, but the method was available over a limited pH range only.

For studying sulphatases it is desirable to have a method of measuring inorganic sulphate which is sensitive enough for 1 or 2 hr. incubations to be used and which permits many determinations to be made simultaneously. Furthermore, it must be

capable of use under varying conditions of pH, molarity of buffer and in the presence of various inorganic ions. A sensitive method of determining blood sulphate has recently been described by Nalefski & Takano (1950), but the method depends on measurement of barium sulphate by photoelectric nephelometer. Cope (1931) and Guillaumin (1940) have defined the errors inherent in precipitating barium sulphate in the presence of biological material and for this reason the method was considered unsuitable. Methods involving precipitation of sulphate as benzidine sulphate and estimation of benzidine in one of several ways (British Drug Houses Ltd., 1948) appeared to be most suitable for the present purpose. Many such methods are described in the literature, but of those examined (e.g. Das Gupta, 1938; Eichelberger, Brower & Roma, 1951; Cuthbertson & Tompsett, 1931) none was directly applicable to enzyme studies. The present paper describes a method which has proved satisfactory, within certain limits for the study and sulphatases.

EXPERIMENTAL AND RESULTS

Materials

Benzidine (Hopkin and Williams, 'Pure' or B.D.H., A.R.). This was twice recrystallized from 95% ethanol (charcoal). For determining sulphate in tissue suspensions a 5% (w/v) solution of benzidine in ethanol was used. The solution was stored in a warm room (otherwise the benzidine tended to crystallize) and was stable for 5-6 weeks. When determining sulphate in aqueous solution a 1% (w/v) ethanolic solution of benzidine was used. All ethanol was filtered or redistilled immediately before use. Powdered glass was washed with distilled water, ethanol and ether and then dried.

Centrifuge tubes. For precipitating benzidine sulphate, 15 ml. centrifuge tubes of even taper with a tip of int. diam. 4-5 mm. were used. All glassware was cleaned with nitric acid; sulphuric acid-containing mixtures were avoided.

Tissue suspensions. These were prepared according to Dodgson, Spencer & Thomas (1953).

Determination of sulphate in aqueous solution

In a 15 ml. centrifuge tube was placed 1 ml. of a solution of K_2SO_4 (containing from 10 to 70 $\mu\text{g./ml. SO}_4^{2-}$), 1 ml. of 20% (w/v) trichloroacetic acid and 5 ml. of a 1% (w/v)

ethanolic solution of benzidine. Mixing was by means of a jet of air passing through a fine capillary which was subsequently washed with a few drops of ethanol. The tube was stoppered and allowed to stand for at least 2 hr. at 0°. A small pinch (3–5 mg.) of powdered glass was then added and benzidine sulphate separated by centrifuging at 2250 g for 15 min. The supernatant was discarded. In the absence of powdered glass, small particles constantly broke away from the precipitate at this stage, but when powdered glass was present the precipitate packed down tightly on top of the glass and no such losses were experienced. The tube was inverted on filter paper and allowed to drain for 5–10 min. The mouth of the tube was wiped with filter paper and the outside washed with acetone. After standing for 1 min. on filter paper the mouth of the tube was washed with ethanol whilst the tube was held in an inverted position. After draining for a further 5 min. on filter paper, the mouth of the tube was again wiped with filter paper and about 10 ml. of ethanol was added, care being taken not to disturb the precipitate otherwise large fragments broke away which could not effectively be washed. After breaking up the precipitate with a glass rod the whole was mixed and the glass rod washed down with a little ethanol. The benzidine sulphate was separated by centrifuging, and after pouring off the supernatant solution the tube and contents were treated exactly as before except that instead of adding 10 ml. ethanol, the benzidine sulphate was dissolved in 3 ml. of *n*-HCl. After a few minutes 1 ml. of a 0.1% (w/v) solution of NaNO₂ was added. After mixing, 5 ml. of a 0.5% (w/v) solution of thymol in 7.5% (w/v) NaOH was added and the tube centrifuged to remove powdered glass. The intensity of the red colour (λ_{max} , 500 m μ .) was measured against a reagent blank with the Hilger Spekker absorptiometer using blue glass filters (Chance O.B. 2) and 2.5 mm. cells. The amount of sulphate was read from a calibration curve prepared by diazotizing and coupling known amounts of benzidine in similar volumes. Between the range 10–70 μ g. SO₄²⁻ the calibration curve prepared in this way did not differ from that prepared by actual precipitation and determination of known amounts of K₂SO₄. When present in amounts less than 10 μ g., sulphate was not precipitated quantitatively.

Variation of experimental conditions

The effects of varying certain experimental conditions was tried before applying the method to enzyme studies.

Table 1. *The effect of the initial concentration of trichloroacetic acid on the recovery of K₂SO₄ from aqueous solutions*

Initial trichloroacetic acid concentration (g./100 ml.)	SO ₄ ²⁻ added (μ g.)	Recovery (%)
30	21.8	86.7
26	15.6	93.6
25	31.2	97.2
24	15.6	100.6
20	15.6	98.1
15	15.6	101.9

Concentration of trichloroacetic acid. Trichloroacetic acid concentration was varied between 15 and 25% (w/v) without affecting sulphate recoveries. Below 15% high recoveries

were obtained in the presence of 0.01 M-chloride and 0.001 M-phosphate. When the concentration of acid was greater than 25% recoveries of sulphate were low (Table 1).

Concentration of benzidine solution. When the concentration of the added trichloroacetic acid was 20%, low sulphate recoveries were obtained with concentrations of benzidine below 0.75% (w/v).

Time of standing. Good recoveries of sulphate were obtained when the time of standing of the solution, after addition of benzidine, varied between 2 and 48 hr. Recoveries were low when solutions were allowed to stand for shorter periods.

Estimation of sulphate in the presence of buffered tissue suspensions

Recovery of sulphate from pure solution in amounts less than 10 μ g. was not quantitative. At concentrations suitable for enzyme experiment, the amount of SO₄²⁻ present in tissue suspensions was usually less than 10 μ g. and enzyme control determinations were often low. It was therefore necessary to add sulphate to both tests and controls before precipitating with benzidine. Since sulphate inhibits certain sulphatases (Dodgson & Spencer, 1952) the addition of sulphate was made after completing the incubation. It was convenient to add the sulphate in the trichloroacetic acid solutions. In practice a control value of between 15 and 25 μ g. SO₄²⁻ was aimed at.

A mixture of 0.6 ml. of acetate-buffered K₂SO₄ solution and 0.6 ml. of similarly buffered tissue suspension, suitably diluted, was placed in a 15 ml. stoppered centrifuge tube and mixed thoroughly. A buffered suspension without K₂SO₄ was used as a control. After incubation 4.8 ml. of ethanol were added to the tube and the contents were mixed. Protein and, in certain cases, polysaccharide material was precipitated and, after standing for a few minutes to allow flocculation, the tube was capped and the precipitate separated by centrifugation. A 5 ml. portion of the supernatant was transferred to a 15 ml. centrifuge tube and 1 ml. of a 25% (w/v) solution of trichloroacetic acid (containing about 15 μ g. SO₄²⁻ as K₂SO₄) followed by 1 ml. of a 5% (w/v) solution of benzidine in ethanol was added to the tube. The contents were mixed by a stream of air as previously described. The tube was stoppered, allowed to stand overnight at 0° and the benzidine sulphate separated and determined as before. The amount of sulphate in the test determination was read from the calibration curve and the corresponding control value was then subtracted. Allowance was made for the contraction in volume which occurred when 1.2 ml. of an aqueous solution was mixed with 4.8 ml. ethanol (cf. Dodgson & Spencer, 1953a).

Sulphate could be recovered from a wide variety of tissue suspensions after incubating at 37° for 1 hr. (Table 2). Longer incubation periods did not affect the results, but unsatisfactory recoveries were obtained when the concentration of the tissue suspension in the incubation mixture exceeded 3% (w/v).

Effect of varying buffer concentration and pH. Recoveries of sulphate from incubated tissue suspensions in acetate buffers of varying molarity (0.1–0.75 M) and pH (4.2–7.2) were satisfactory (Table 3).

Effect of inorganic ions on sulphate recoveries. K, Na, Mg, CN, Cl and F at concentrations of 0.01 M, and Fe, Ca and PO₄ at concentrations of 0.001 M did not interfere with the recovery of sulphate from acetate-buffered suspensions

Table 2. *The recovery of K_2SO_4 from various tissue suspensions after incubation for 1 hr. at 37.5°*

Organism	Organ	Final concn. of suspension (g./100 ml.)	SO_4^{2-} -added (μg.)	Recovery (%)
Rat	Liver	2.0	15.8	104.3
	Kidney	2.0	15.8	99.4
	Brain	2.0	15.8	99.4
	Intestine	2.0	15.8	103.8
	Spleen	2.0	15.8	96.8
	Lung	2.0	15.8	101.9
	Pancreas	2.0	15.8	104.4
	Ovary	2.0	15.8	100.0
	Testis	2.0	15.8	101.9
	Heart	2.0	15.8	104.4
Cat	Liver	1.0	35.9	103.3
	Kidney	1.8	31.2	96.8
<i>Littorina littorea</i>	Visceral hump	3.0	23.7	99.6
<i>Patella vulgata</i>	Visceral hump	3.0	23.7	101.7
	Muscular foot	2.0	18.9	100.4
<i>Buccinum undatum</i>	Digestive gland	2.0	23.7	100.0
<i>Cardium edule</i>	Visceral hump	2.0	23.7	101.3
<i>Helix aspersia</i>	Visceral hump	2.0	19.0	98.5
<i>Aspergillus oryzae</i> (Parke-Davis Co. Taka diastase diluted with lactose)		1.5	37.0	101.2

Table 3. *Recoveries of K_2SO_4 from rat-tissue suspensions after incubation for 1 hr. at 37.5° in acetate buffers of varying molarity and pH*

Tissue	Molarity of buffer	pH	SO_4^{2-} -added (μg.)	Recovery (%)
Liver	0.10	5.50	19.0	101.6
	0.10	5.20	14.3	101.3
Spleen	0.50	4.20	15.8	100.0
Kidney	0.50	5.20	19.0	103.1
	0.50	5.53	15.8	99.4
Liver	0.50	6.44	15.8	98.1
	0.50	7.20	16.8	98.2
Kidney	0.75	6.44	15.8	98.9

Table 4. *Recoveries of K_2SO_4 from acetate-buffered rat-liver suspensions in the presence of various inorganic ions after incubation for 1 hr. at 37.5°*

Molarity of buffer	pH	Compound added	Molarity	SO_4^{2-} -added (μg.)	Recovery (%)
0.1	5.5	KCN	0.010	19.0	95.3
0.1	5.5	NaF	0.010	19.0	101.6
0.1	5.5	$FeCl_3$	0.010	19.0	55.2
0.1	5.5	$Fe(CH_3COO)_3$	0.010	19.0	55.8
0.1	5.5	$MgCl_2$	0.010	19.0	95.8
0.1	5.5	$CaCl_2$	0.010	19.0	33.2
0.5	5.8	KH_2PO_4	0.010	19.0	*
0.5	5.8	KH_2PO_4	0.001	37.0	95.5
0.5	5.8	$FeCl_3$	0.001	24.7	95.2
0.5	5.8	$CaCl_2$	0.001	24.7	103.7
0.5	5.8	$BaCl_2$	0.001	24.7	0.0

* The final solution was too dense to measure on the Spekker absorptiometer.

(Table 4). However, Fe, Ca and PO_4 in higher concentrations caused considerable interference while Ba, even in traces, rendered the method invalid.

The effect of certain sulphatase substrates. Potassium *p*-acetylphenylsulphate (0.05 M), sodium chondroitinsulphate (1%, w/v), potassium myronate (0.01 M) and potassium glucose 6-sulphate (0.02 M) did not affect recoveries. Higher concentrations of glucose 6-sulphate resulted in high recoveries which were due to low blank values.

Check of the method

Dodgson *et al.* (1953) described a spectrophotometric method for the determination of *p*-hydroxyacetophenone liberated from potassium *p*-acetylphenylsulphate by rat arylsulphatase. The method outlined in the present paper was designed to follow the experimental procedure of the spectrophotometric method, particularly as regards volumes and the use of ethanol as protein precipitant. The two methods were checked against each other after incubating rat-tissue suspensions with potassium *p*-acetylphenylsulphate using the experimental conditions for rat arylsulphatase outlined by Dodgson *et al.* (1953). In each experiment six test and six control determinations were made. After incubating and precipitating protein with

were tried in this laboratory, there is need for extreme care during manipulation. The method described here is no exception. It has been noted consistently that the chief source of error is connected with separating and washing the benzidine sulphate. There is a tendency for the centrifuged precipitate to break away during decantation of the supernatant solution, and although many different types of centrifuge tubes have been tried the only really satisfactory solution to the problem is to use powdered glass as a rough surface on which the precipitate can pack down. Over-generous use of glass must be avoided, however, if the desired effect is to be obtained. More serious is the problem of freeing the precipitated benzidine sulphate from contaminating benzidine. The solution to this would seem to be a second washing with ethanol, but this leads to low results and successive washings of ethanol and acetone are no more satisfactory. However, if the experimental procedure is followed exactly the danger of contamination is reduced to a minimum and once the technique is mastered there is no trouble from this source.

The use of ethanol as a protein precipitant is a departure from the procedure normally followed in the benzidine method. Hitherto, trichloroacetic acid or uranium acetate has been used, but these were found to be generally unsuitable in the present work. Precipitation of protein with uranium acetate necessitates careful adjustment of pH and this is not always easy when solutions of varying pH are involved. Trichloroacetic acid was a satisfactory precipitant with tissue suspensions of rat and dog but, following the removal of protein from certain cat-liver suspensions and preparations from many marine organisms and seaweeds, there was a considerable precipitation of polysaccharide material when ethanolic solutions of benzidine were added. This material later interfered with efficient washing of the benzidine sulphate and agreement between duplicate determinations was poor. However, by altering the sequence of addition of the various reagents and thus using ethanol as protein precipitant, polysaccharide was removed at the same time.

The variations in conditions described here are not exhaustive, and further data become available as the use of the method extends. It is important to test the method (by recovery of added potassium sulphate) whenever new conditions of enzyme experiment are used. However, it is possible to study the various sulphatases under a wide range of experimental conditions, and preliminary work on gluco- and chondro-sulphatases has been reported (Dodgson & Spencer, 1953b). No attempts have been made to study the effect on the method of buffers other than acetate since the majority of workers on sulphatases have preferred acetate

Table 5. *Comparison of the SO_4^{2-} liberated by rat arylsulphatase (benzidine method) with that calculated from the amount of *p*-hydroxyacetophenone liberated (spectroscopic method) under identical experimental conditions*

(A suitably diluted tissue suspension (0.6 ml.) was incubated with 0.6 ml. of potassium *p*-acetylphenylsulphate (0.007 M) in 0.5 M-acetate buffer (pH 7.2) for 1 hr. at 37.5°.)

Source of arylsulphatase	SO_4^{2-} liberated ($\mu\text{g.}$)	
	Measured by benzidine method	Calculated from spectroscopically determined <i>p</i> -hydroxyacetophenone
Liver	30.7	30.1
	26.6	26.9
	13.3	13.8
	20.2	20.7
Spleen	21.8	23.1
Kidney	33.9	35.9
Lung	15.6	16.3

ethanol, the inorganic sulphate present in three of the tests and three of the controls was determined by the benzidine method, while the *p*-hydroxyacetophenone present in the remaining tests and controls was measured spectrophotometrically. The results obtained by the two methods were related to each other by calculation and are recorded in Table 5.

DISCUSSION

It should be emphasized that in the benzidine methods previously described in the literature which

buffers which appear to be very suitable for these enzymes.

Determination of sulphate is not adversely affected by most of the common ions used in enzymology. Even when a particular ion interferes the difficulty may often be resolved by using smaller volumes of a more concentrated enzyme solution during the preliminary incubations and then diluting to the correct volumes immediately before addition of ethanol. The necessity for adding inorganic sulphate to both 'test' and 'control' determinations is regrettable but unavoidable. However, provided the sulphate is added after incubation in order to avoid possible enzyme inhibition by sulphate, no serious objection can be raised. The fact that sulphate cannot be recovered when present in amounts less than 10 μg . means that the method cannot be used directly for determining the absolute sulphate content of tissues. Using the volumes suggested earlier and suspensions of the order of 3% (wet, w/v), it was invariably found that the sulphate content of these suspensions was such that less than 10 μg . SO_4^{2-} was actually present in the final solution. It is possible that by adjusting the volumes and using a more concentrated suspension the method might be used to determine tissue sulphate, but since this was not the object of the work the problem was not pursued.

SUMMARY

1. An adaptation of the benzidine method for determining small amounts of enzymically liberated sulphuric acid in the presence of acetate-buffered biological material is described.

2. Using this method potassium sulphate can be recovered quantitatively from tissue suspensions of different organisms under varying conditions of pH and buffer molarity and after incubation for 1 hr. at 37.5°.

3. With the exception of barium, recoveries of sulphate are unaffected by the presence of small amounts of certain inorganic ions.

4. Within certain limits potassium *p*-acetylphenylsulphate, potassium glucose 6-sulphate, potassium myronate and chondroitinsulphuric acid are without effect on the method.

5. The method has been checked under conditions of enzyme experiment by measuring the sulphate liberated from potassium *p*-acetylphenylsulphate by rat arylsulphatase. The values obtained were in good agreement with those obtained by calculation after spectrophotometric measurement of the liberated *p*-hydroxyacetophenone.

We wish to thank Dr S. H. Edgar of Reckitt and Colman Ltd. for a generous gift of potassium myronate, and Dr R. C. Jordan for a gift of sodium chondroitinsulphate.

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