7-hydroxyl group of flavonols; for the present, it is suggested that a separate enzyme is required for catalysing this step in the synthesis.

SUMMARY

1. Nine flavonol glycosides have been found in potato flowers. They are the 3-glucosides, 3-rhamnosylglucosides, 3-diglucosides and 3-glucosylrhamnosylglucosides of kampferol and quercetin and the 3-rhamnosylglucoside of myricetin.

2. Kampferol 3-diglucoside 7-rhamnoside and kampferol 3-triglucoside 7-rhamnoside have been found in potato seeds.

3. Luteolin 7-glucoside has been found specifically in the flowers of a wild species, *Solanum stoloniferum*. The distribution of the flavonol glycosides in wild and cultivated potato species is recorded.

4. A gene controlling the transfer of glucose to quercetin 3-rhamnosylglucoside has been found.

5. A scheme is proposed outlining the pathway of biosynthesis of the flavonol glycosides and anthocyanins of the potato.

The author is grateful to Dr G. J. Paxman for valuable discussions and help with the genetic aspects of this work.

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Biochem. J. (1962) **84**, 106

A Note on the Determination of the Ester Sulphate Content of Sulphated Polysaccharides

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(*Received* 31 *January* 1962)

Estimation of the ester sulphate content of polysaccharide sulphates involves acid hydrolysis followed by determination of liberated inorganic sulphate. Methods which have been used for the determination of this sulphate include gravimetric estimation as barium sulphate (e.g. Hall, 1955), colorimetric or spectrophotometric estimation as benzidine sulphate (e.g. Gross, Mathews & Dorfman, 1960), titration with barium chloride with potassium rhodizonate as indicator (e.g. Polatnick, La Tessa & Katzin, 1957), and spectrophotometric estimation by the barium chromate (Egami & Takahashi, 1957) or 4-amino-4′-dichlorobiphenyl methods (Rees, 1961). None of these methods is

completely satisfactory, some being tedious or insensitive, others demanding a high degree of manipulative skill.

Dodgson (1961) described a method, based on those of Gassner & Friedel (1956) and Berglund & Sörbo (1959), in which sulphate is estimated turbidimetrically as barium sulphate, light-absorption at 360 mμ being measured and gelatin being used as a cloud-stabilizer. When this method was first applied to the analysis of chondroitin sulphate hydrolysates, the values obtained were significantly higher than when sulphate was determined gravimetrically as barium sulphate. These high results were shown to be due to the formation of ultraviolet-absorbing products during the hydrolytic procedure. The present work describes how the method was adapted to compensate for and to reduce the concentration of these products.

MATERIALS

Barium chloride–gelatin reagent. This was prepared as described by Dodgson (1961). The reagent was stored overnight at 4° before use and each new batch was tested by checking the recovery of a known amount of inorganic sulphate (usually 50 μg. of SO_4^{2-} ion).

Gelatin solution. This was prepared exactly as described for $BaCl_2$–gelatin reagent except that $BaCl_2$ was omitted.

Glassware and other reagents. These were as described by Dodgson (1961).

Polysaccharide sulphates. Unfractionated preparations of the potassium salts of the chondroitin sulphates of the cartilage of ox, blue shark (*Charcharinus glaucus*), dogfish (*Scyllium canicula*), skate (*Raia batis*) and fin-whale (*Baelaenopter physalus*), were obtained by the method of Dodgson, Lloyd & Spencer (1957) after papain digestion (Muir, 1958). Potassium charonin sulphate [S-rich fraction, prepared from the mucous gland of the mollusc, *Charonia lampas* (see Egami *et al.* 1955)] was given by Professor F. Egami; porphyran [the galactan sulphate from the seaweed, *Porphyra umbilicalis* (see Peat, Turvey & Rees, 1961)] by Dr D. Rees; the seaweed polysaccharide sulphate from *Chondrus ocellatus* (Mori, 1953) by Professor T. Mori; other seaweed polysaccharide sulphates, heparins, potassium chondroitin sulphate from pig nasal septum, ox sodium chondroitin sulphates and chemically sulphated hyaluronic acid by Dr W. J. C. Dyke.

METHODS AND RESULTS

Determination of ester sulphate

Gravimetric method. Polysaccharide sulphates (200–400 mg., depending on sulphate content) were hydrolysed by refluxing for 20 hr. with 20 ml. of 3·5N-hydrochloric acid. After the condenser had been washed down, the hydrolysate was filtered through a sintered-glass Gooch filter (porosity × 4) to remove suspended carbon and the filtrate and washings were made up to a volume of 100 ml. with water. Liberated inorganic sulphate was then determined gravimetrically as barium sulphate on 25 ml. portions of the solution.

Turbidimetric method. The method given is the one finally developed. Reasons for the selection of particular experimental conditions are given below.

The polysaccharide sulphate, usually 2–4 mg., accurately weighed, was dissolved in sufficient N-hydrochloric acid to give a final concentration of SO_4^{2-} ion of between 40 and 90 μg./0·2 ml. A portion (approx. 0·5 ml.) was transferred to a glass tube (approx. 0·5 cm. internal diam. × 10 cm. long) which was sealed in an oxy-gas flame and kept in an oven at a temperature of 105–110° for 5 hr. After being cooled, the contents of the tube were mixed before opening. A portion (0·2 ml.) was transferred, by tapered constriction-pipette, to a suitable 10 ml. tube containing 3·8 ml. of 3% (w/v) trichloroacetic acid. Barium chloride–gelatin reagent (1 ml.) was added and, after mixing, the whole was kept at room temperature for 15–20 min. The extinction of this 'test' solution was then measured at 360 mμ against an appropriate reagent blank as described by Dodgson (1961) for 'method A'.

A second 0·2 ml. portion of the hydrolysate was mixed with 3·8 ml. of trichloroacetic acid, as described above, before adding 1 ml. of gelatin solution (i.e. containing no barium chloride). The extinction of this 'control' solution was then measured at 360 mμ against a reagent blank consisting of 0·2 ml. of N-hydrochloric acid, 3·8 ml. of trichloroacetic acid and 1 ml. of gelatin solution. This control gave a measure of the ultraviolet-absorbing materials produced during hydrolysis and the reading obtained was subtracted from that of the test solution, the resultant value being applied to the 'method A' calibration curve (see Dodgson, 1961). Under the hydrolytic conditions described above the contribution of ultraviolet-absorbing materials was very small or was absent. In no case did it exceed an extinction of 0·04.

Selection of hydrolysis conditions

In preliminary investigations the polysaccharide sulphates were hydrolysed overnight in sealed tubes at 120–125° with either 8% (w/v) trichloroacetic acid or 2N-hydrochloric acid. The contribution of ultraviolet-absorbing products was high under these circumstances, particularly with esters containing uronic acid residues when hydrochloric acid was used. In many cases hydrolysates were coloured and contained suspended carbon particles which had to be separated by centrifuging. Lowering the temperature to 105–110° decreased these effects without markedly interfering with the efficiency of the hydrolysis, and these temperature conditions were used in subsequent work. A further complication with trichloroacetic acid

hydrolysates was the degradation of the acid to give droplets of chloroform. These could be separated by centrifuging but micro-pipetting of the resultant supernatant solution was difficult, the pipettes behaving as though they were greasy.

Further investigations with hydrochloric acid as hydrolysis agent showed that acid concentration and hydrolysis time affected the production of ultraviolet-absorbing materials.

Acid concentration. Fig. 1 (*A*) shows the ultraviolet-absorption spectra obtained with some of the polysaccharide sulphates. The esters (at a concentration of 2–4 mg./ml. of N-hydrochloric acid) were hydrolysed for 17 hr. at 105–110° in sealed tubes, and 0·2 ml. portions were mixed with 3·8 ml. of 3 % (w/v) trichloroacetic acid and 1 ml. of water. Spectra were measured against a reagent blank in which 0·2 ml. of N-hydrochloric acid was substituted for the hydrolysate. Spectra of the esters before hydrolysis were also measured but with one exception, a chemically treated heparin, absorption in the region 250–400 mμ was negligible and these spectra are therefore omitted from Fig. 1. All the esters gave hydrolysates which absorbed to a greater or lesser extent in the ultraviolet region, maximum absorption being in the range 270–290 mμ. Although not shown in Fig. 1, free glucuronic acid gave a similar absorption spectrum ($\lambda_{max.}$ approx. 270 mμ) when treated in an identical manner.

An example of the effect of increasing acid concentration on the production of ultraviolet-absorbing materials is given in Fig. 1 (*B*).

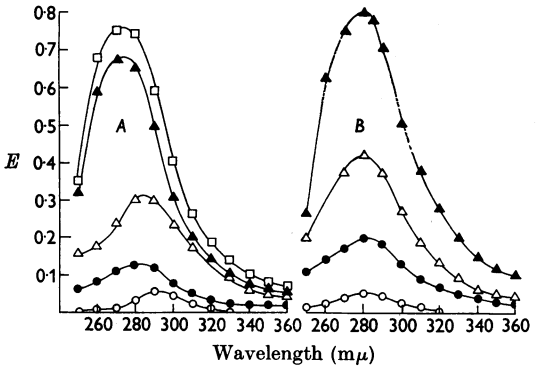

Fig. 1. (*A*) Ultraviolet-absorption spectra of some polysaccharide sulphate preparations after hydrolysis with N-hydrochloric acid for 17 hr. in sealed tubes at 105–110°. Experimental details are given in the text. ○, Charonin sulphate; ●, *Chondrus ocellatus* polysaccharide; △, heparin; ▲, skate potassium chondroitin sulphate; □, ox potassium chondroitin sulphate. (*B*) Spectra of whale potassium chondroitin sulphate (initial concentration 2·5 mg./ml., see text) after hydrolysis for 17 hr. at 105–110° with hydrochloric acid at concentrations of: ○, 0·25N; ●, 0·5N; △, 1·0N; ▲, 2·0N.

Time of hydrolysis. Polysaccharide sulphates could be classified into three groups on the basis of the effects of time of hydrolysis on the absorption spectra. Hydrolysates of esters known to contain uronic acid residues showed a four- to seven-fold increase in maximum absorption on increasing the period of hydrolysis from 4 to 17 hr. Fucoidin gave a twofold increase over the same period, but with the remaining esters the bulk of the absorption developed during the first 4 hr. (see Fig. 2 for examples).

Rate of liberation of sulphate. The collective findings illustrated the need to use relatively low concentrations of acid and short hydrolysis periods. However, there was a progressive decrease in the rate of liberation of inorganic sulphate with decrease in acid concentration (Table 1), and N-hydrochloric acid was therefore selected as the optimum acid concentration. It only remained to establish the minimum time of hydrolysis which was necessary to ensure complete liberation of sulphate. In all cases liberation was complete in

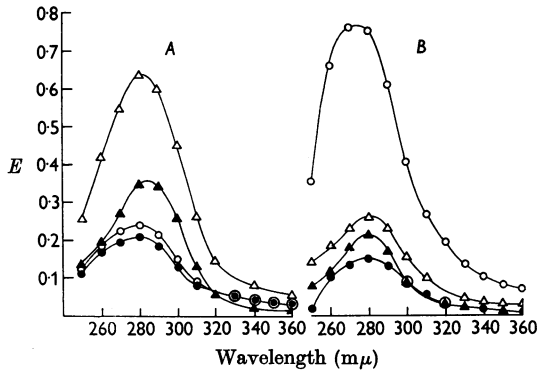

Fig. 2. Effect of time of hydrolysis on the ultraviolet-absorption spectra of some polysaccharide sulphates hydrolysed with N-hydrochloric acid at 105–110°. (*A*) Carrageenin: ●, 4 hr. hydrolysate, and ○, 17 hr. hydrolysate; fucoidin: ▲, 4 hr. hydrolysate, and △, 17 hr. hydrolysate. (*B*) Ox sodium chondroitin sulphate: ●, 4 hr. hydrolysate, and ○, 17 hr. hydrolysate; porphyran: ▲, 4 hr. hydrolysate, and △, 17 hr. hydrolysate.

Table 1. *Effect of acid concentration on the rate of liberation of inorganic sulphate from whale chondroitin sulphate at* 105–110°

Experimental details are given in the text.

Time (hr.)	Sulphate liberated (% of total available) 0·25N-HCl	0·5N-HCl	1·0N-HCl
1	40·5	60·0	75·0
2	72·0	91·0	95·0
3	83·0	95·0	100·0

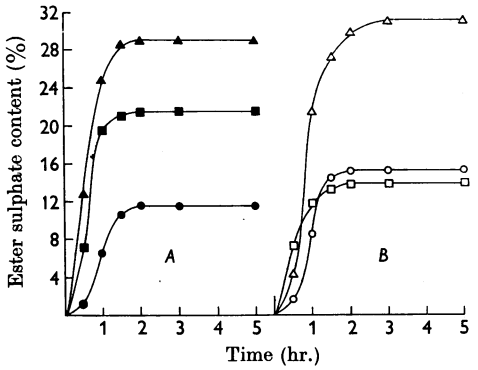

Fig. 3. Rates of liberation of inorganic sulphate from polysaccharide sulphates hydrolysed with N-hydrochloric acid in sealed tubes at 105–110°. To avoid undue overlapping of curves, results are expressed in terms of ester sulphate content, maxima on the curves representing the total ester sulphate content of the samples. No additional liberation of sulphate occurred when the hydrolysis period was extended to 17 hr., but these experimental points have been omitted. (*A*) ●, Porphyran; ■, fucoidin; ▲, carrageenin. (*B*) ○, Dogfish potassium chondroitin sulphate; □, ox sodium chondroitin sulphate; △, heparin.

5 hr. (see Fig. 3 for examples). Before hydrolysis, all the polysaccharide sulphates complexed with gelatin to a greater or lesser extent to give cloudy suspensions or precipitates [see also Dodgson (1961)] but such complexes were not obtained after the first 15–20 min. of the hydrolysis period. In many cases only relatively small amounts of sulphate had been liberated in this time. This suggests that polymeric size or shape or both may be more important than acidic groupings in the formation of these complexes.

Accuracy of the method

The ester sulphate contents of the available polysaccharide sulphates were determined by both turbidimetric and gravimetric procedures. The results obtained by both methods agreed closely (Table 2).

DISCUSSION

Compared with other available methods the present procedure has the advantage of speed and simplicity. Comparatively small amounts of starting material are required and this is of some importance: many polysaccharide sulphates are difficult to isolate in appreciable quantities. There appears to be no reason why the procedure could not be reduced still further in scale by substituting 'method B' or 'method C' (see Dodgson, 1961) for 'method A'. The principal limitation of the method would then be the accuracy with which the polysaccharide sulphate could be weighed out.

Table 2. *Analysis of ester sulphate content of some polysaccharide sulphates by gravimetric and turbidimetric methods*

Experimental details are given in the text.

Compound	Sulphate content (% of SO_4^{2-} ion) Gravimetric	Turbidimetric
Potassium chondroitin sulphates from:		
Ox	13·6	13·9
Ox*	14·1	13·9
Ox*	14·3	13·7
Pig	12·3	12·3
Whale	12·2	12·2
Shark	14·2	14·3
Skate	13·7	13·8
Dogfish	15·4	15·2
Heparin	31·2	31·6
Heparin†	26·6	26·1
Hyaluronic acid (chemically sulphated)	4·9	5·4
Charonin sulphate	42·1	41·5
Carrageenin (preparation 1)	25·3	25·1
Carrageenin (preparation 2)	28·9	29·0
Chondrus ocellatus polysaccharide	26·6	27·1
Fucoidin	21·2	21·5
Porphyran	11·7	11·5

* Sodium salts.

† A chemically treated preparation for which no details are available.

Although not all types of polysaccharide sulphate have been tested, it seems likely that the method is of general applicability. In the series of esters examined, sulphate is bound in different types of linkage on various monomer units. Thus, in the mammalian chondroitin sulphates, the secondary alcohol groupings at $C_{(4)}$ of the *N*-acetylgalactosamine residues are principally involved; in shark chondroitin sulphate the corresponding $C_{(6)}$ (primary alcohol) groupings are concerned. In heparin at least two different types of grouping are involved in the binding of sulphate, namely the $C_{(2)}$ (amino) and $C_{(4)}$ (hydroxyl) groups of the glucosamine residues. It is also possible that some secondary alcohol groups in the uronic acid residues may be sulphated (Wolfrom, Montgomery, Karabinos & Rathgeb, 1950). In other cases, primary or secondary alcohol groupings of glucose, galactose or fucose are involved. With all types of ester, liberation of sulphate is relatively rapid under the hydrolysis conditions used, although complete hydrolysis of the polysaccharide molecule is not necessarily achieved.

The production of ultraviolet-absorbing materials during hydrolysis is of some interest. The strongly absorbing hydrolysates of esters of animal origin

may be presumed to result from degradation of uronic acid residues, but the origin of the absorption of the hydrolysates of the remaining esters is less certain. The structures of these compounds are still not clearly understood and the available methods of preparation usually yield heterogeneous products (see Mori, 1953). However, the nature of the absorbing materials did not fall within the scope of the present investigation and the problem was not pursued further.

SUMMARY

1. A method is described for the analysis of the ester sulphate contents of polysaccharide sulphates of plant and animal origin.

2. The esters are hydrolysed with acid under conditions designed to reduce the rate of formation of ultraviolet-absorbing materials and liberated sulphate is then measured turbidimetrically by a modification of the procedure of Dodgson (1961).

3. Results obtained with the method agreed closely with those obtained by a conventional large-scale gravimetric procedure.

This work was supported by Grant A-1982 from the Arthritis and Metabolic Diseases Division of the United States Public Health Service. We are grateful to Dr A. Jonsgård and to Dr K. Enge for supplies of fin-whale trachea. One of us (R.G.P.) wishes to thank the Department of Scientific and Industrial Research for a research studentship.

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Biochem. J. (1962) **84**, 110

The Asymmetrical Stimulation of a Membrane Adenosine Triphosphatase in Relation to Active Cation Transport

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(*Received* 25 *January* 1962)

The concept that adenosine triphosphate is needed for the active transport of Na^+ and K^+ ions across cell membranes has led to several studies of the effects of these ions on the activity of adenosine triphosphatases in disrupted tissues and cells. A stimulation of enzymic activity has been described when both ions are present in such diverse preparations as suspensions of fragmented erythrocyte membranes (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961), a microsomal fraction obtained from crab nerve (Skou, 1957, 1960) and brain (Deul & McIlwain, 1961; Järnefelt, 1961), and in a nuclear fraction of a kidney homogenate (Whittam & Wheeler, 1961). The stimulation by Na^+ and K^+ ions is counteracted by digoxin and ouabain and related glycosides, which are known to inhibit the active transport of ions in intact cells. A partial inhibition by digoxin of the hydrolysis of adenosine triphosphate in intact human erythrocytes was shown by Whittam (1958), and this appeared to be the only effect of digoxin on the chemical reactions investigated.

The adenosine triphosphatase of erythrocytes is situated exclusively in the membranes (Clarkson & Maizels, 1952; Herbert, 1956), which, unlike those of other cells and tissues, can be readily obtained in a homogeneous suspension particularly suitable for study. Post *et al.* (1960) found with human erythrocyte membranes that the concentrations of Na^+ and K^+ ions required for half-maximal stimulation of the adenosine triphosphatase were the same as those for the ion-pumping system (Post & Jolly, 1957). Dunham & Glynn (1961)