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A Note on the Characterization of Carbohydrate Sulphates by Acid Hydrolysis

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The recent observation (Barry & McCormick, 1957) that glucose 3-sulphate is hydrolysed significantly faster than galactose 6-sulphate has called in question the tentative conclusion drawn by Percival (1949), that differences in rates are generally too small to be useful for characterization purposes. The rates of hydrolysis of a number of carbohydrate sulphates are now reported, and it is shown that such rates can be useful for the characterization of single sulphates, and also for 'fingerprinting' compounds containing several different ester groups. The technique has helped to establish that λ -carrageenan contains galactose 2,6-disulphate units in addition to the galactose 4-sulphate units known to be present (Rees, 1963).

MATERIALS

Carbohydrate sulphates. The monosaccharide sulphates were synthesized by definitive routes (Peat, Turvey, Clancy & Williams, 1960; Turvey & Williams, 1962) and generously given by Dr J. R. Turvey. Evidence for the types of sulphate present in porphyran and the carrageenan polysaccharides is summarized elsewhere (Peat & Rees, 1961; Rees & Conway, 1962; Rees, 1961, 1963). Fucoidin and the *Enteromorpha* polysaccharide were given by Dr Elizabeth Percival. The dextran sulphate was Dr Ricketts's preparation E (Ricketts, 1956) and was known to contain a high proportion of equatorial ester. Laminarin sulphate was given by Dr D. Walker of Boots Pure Drug Co. Ltd. The mucopolysaccharides were given by Dr K. S. Dodgson (Lloyd, Dodgson, Price & Rose, 1961), the shark preparation being prepared from the head cartilage of the blue shark (*Charcharinus glaucus*) and the chondroitin sulphate A from ox cartilage by the usual methods. The presence of a small amount of 6-sulphate (probably contaminating chondroitin sulphate C) in the latter polysaccharide was confirmed by infrared spectroscopy.

METHODS

Acid hydrolysis of carbohydrate sulphates. Samples of a solution of the compound in 0.250 N-hydrochloric acid (0.250 ml., containing about 50 μ g. of esterified sulphate) were sealed in Pyrex test tubes (3 in. $\times$ $\frac{1}{8}$ in.) and heated in a boiling-water bath for varying periods. After cooling, centrifuging and opening, the contents of each tube were exactly neutralized with sodium hydroxide (2.50 N; 0.025 ml.) and a trace of Cetavlon and chloroaminodiphenyl reagent (0.250 ml.) added. The latter reagent was prepared as described by Jones & Letham (1954). After mixing, keeping for several hours, and clarification on the centrifuge, a portion of the supernatant solution was diluted and the extinction measured as described by Jones & Letham.

To avoid interference in the determination by non-hydrolysed polysaccharide sulphate, it was normally satisfactory to allow the reaction to proceed for about 30 min. to allow extensive glycosidic hydrolysis to occur, before making the first measurement. No interference through precipitation of chloroaminodiphenyl by polysaccharide was then observed. In certain cases (e.g. dextran sulphate) where the glycosidic linkages were very stable, or the sulphate very acid-labile, this was not practicable and an alternative procedure was followed (Foster, Martlew, Stacey, Taylor & Webber, 1961; K. O. Lloyd & P. F. Lloyd, unpublished work). After neutralization as above, a solution of Cetavlon (sufficiently concentrated to give an excess; 0.250 ml.) was added and the precipitate removed on the centrifuge. The supernatant solution (0.250 ml.) was then treated with chloroaminodiphenyl reagent, and the experiment completed as usual.

In certain cases, it was necessary to introduce corrections for the ultraviolet absorption of the degradation products of 3,6-anhydrogalactose (this sugar being a component of some of the polysaccharides that were examined). Larger samples were then heated in the sealed tubes and, after cooling and opening, portions (0.250 ml.) were removed and treated as usual. Further portions of each solution were diluted in the same ratio, but without the

addition of chloroaminodiphenyl reagent. The extinction of each member of the first series of solutions was then corrected for the presence of ultraviolet absorbing artifacts by subtracting from it the extinction of the corresponding member of the second series.

Calculation of half-lives. No problem exists in the calculation of half-lives from straight-line graphs (e.g. porphyran and κ -carrageenan in Fig. 1), but it is necessary to assume, in calculating the results for polysaccharides containing more than one type of sulphate, that the curve (e.g. laminarin sulphate in Fig. 1) represents several simultaneous apparent first-order reactions, each reaction corresponding to the hydrolysis of one of the isomers. This may be justified because: (i) all known carbohydrate sulphates that contain substantially one ester (whether the carbohydrate moiety is a monosaccharide, a glycoside or a polysaccharide) show an apparent first-order hydrolysis of ester (Turvey & Clancy, 1961; Guiseley & Ruoff, 1961; Percival & Soutar, 1940; see also the curves for porphyran and κ -carrageenan in Fig. 1); and (ii) interpretations of complex curves according to this principle gave results (Table 1) that are consistent with what is known from other evidence of the types of ester present, and their proportions (see Discussion). Lee & Kolthoff (1951) showed that, if the rate coefficients are known for the separate reactions in such a system, then the mixture of esters can be fairly accurately analysed. In the present study such information has not generally been available, and the graphical method of Brown & Fletcher (1949) was used. The assumption is made in this method that the later linear part of the curve represents the slower process only, the faster process having gone to completion. This is justified if there is a great difference between the rates of the two processes, or if the total reaction is studied over a period that is long relative to the half-life of the faster process. In some of the experiments reported here the half-lives differed only by a factor of about 5 (Table 1), and it was not practicable to follow the reaction beyond 90% hydrolysis because any experimental error then had considerable effect on the quantity $\log a/(a-x)$. The reliability of the method when applied to the data obtained in our experiments was therefore tested by applying it to a series of hypothetical situations. In this way it was shown that some error is indeed always introduced by the assumption made in the calculations. The error is substantial (about 20%) when the more labile component is present in greater proportion. Some of the half-lives are also in error for experimental reasons (see Table 1). It therefore seems best to regard the figures in Table 1 that are calculated from non-linear curves as approximate values only. This does not invalidate the grouping of the esters into three categories differing in acid-stability, because the direction of any error would be such as to mask rather than exaggerate the differences.

RESULTS AND DISCUSSION

Some typical sulphate-release curves are given in Fig. 1, and these show that different esters are often hydrolysed at very different rates. The results may be rationalized and summarized (Table 1) in terms of a generalization analogous to one well established in the cyclohexane series (Barton, 1953), namely that esters of equatorial secondary

Fig. 1. Curves showing the release of free sulphate on acid hydrolysis of dextran sulphate (○), laminarin sulphate (□), κ -carrageenan from *Chondrus crispus* (■) and porphyran (●). Experiments were carried out according to the standard method described in the text.

hydroxyl groups are more rapidly hydrolysed than those of axial alcohols. It would further seem that the sulphates of primary alcohols are more acid-stable than those derived from either type of secondary alcohol (Table 1).

A number of the polysaccharides examined contained more than one type of ester, and in each case the relative amounts indicated by the hydrolysis curve were in accord with previous evidence, although the approximations necessary in the analysis of the kinetics did not allow any precise estimations of proportions. Thus fucoidin (Conchie & Percival, 1950; Côté, 1959) and the *Enteromorpha* polysaccharide (McKinnell & Percival, 1961; E. E. Percival & J. K. Wold, unpublished work) each appeared from its hydrolysis curve to contain axial sulphate as the major component, with a smaller proportion of equatorial ester also present. The hydrolysis curve for laminarin sulphate suggested similar proportions of 6-sulphate and equatorial sulphate, consistently with the results obtained by Whistler & Spencer (1961) for the distribution of ester groups in several polysaccharide sulphates prepared by direct sulphonation. As well as the 6-sulphate, which was the main ester of the shark mucopolysaccharide (cf. the infrared evidence, Lloyd *et al.* 1961), the hydrolysis curve suggested that a small amount of a more labile component was present (cf. Suzuki, 1960).

SUMMARY

1. The rates of acid hydrolysis of a number of carbohydrate sulphates have been examined.

2. It is concluded that the rates of acid hydrolysis differ sufficiently to be useful for the characterization of single sulphates.

Table 1. *Acid hydrolysis of some carbohydrate sulphates in 0.25N-hydrochloric acid at 100°*

The half-lives were reproducible to within $\pm 5\%$ unless stated otherwise in the footnotes.

Compounds	Half-lives (hr.) of esters derived from		
	Equatorial hydroxyl	Axial hydroxyl	Primary hydroxyl
Galactose 4-sulphate	—	1.45	—
Glucose-3-sulphate	0.42	—	—
Galactose 6-sulphate	—	—	1.55
Glucose 6-sulphate	—	—	2.40*
Porphyran	—	—	2.40
Laminarin sulphate	0.13†	—	2.40†
Mucopolysaccharide from shark cartilage	0.13††	—	2.13†
Dextran sulphate	0.10	—	—
Chondroitin sulphate A	—	0.40†	1.88†
κ -Carrageenan (<i>Chondrus crispus</i>)	—	1.00	—
κ -Carrageenan (<i>Hypnea musciformis</i>)	—	1.03	—
λ -Carrageenan	0.23†	1.02†	
<i>Enteromorpha</i> polysaccharide	0.20††	1.01†	—
Fucoidin	0.20††	1.38†	—

* Calculated from Guiseley & Ruoff's (1961) data.

† From a non-linear curve (see text).

†† This ester was present in only small amount and an accurate figure could therefore not be obtained. The value given is the maximum consistent with the experimental data.

3. For compounds containing several different sulphate groups, the hydrolysis curve can often be used as a guide to the types of ester present, and their approximate proportions.

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