The Hypobranchial Mucin of the Whelk *Buccinum undatum* L.

THE POLYSACCHARIDE SULPHATE COMPONENT

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1. A polysaccharide sulphate has been isolated from the hypobranchial mucin of the whelk *Buccinum undatum*. 2. The molecular weight of this polysaccharide, which is a glucan carrying one ester sulphate group per monosaccharide residue, is 1.7×10^5 . 3. Some investigations bearing on the location of the ester sulphate groups are reported. 4. The viscosity of the whole mucin has been shown to depend mainly on the glucan sulphate.

The hypobranchial mucin of the whelk *Buccinum undatum* L. consists of a glycoprotein in loose association with a polysaccharide sulphate (Hunt & Jevons, 1963, 1965a). The polysaccharide, which has been shown previously to contain glucose as its only monosaccharide constituent, has now been isolated in a pure form and its structure investigated.

Polysaccharide sulphates composed of ester-sulphated neutral monosaccharide units are unusual in higher animals, in which sulphate-carrying polysaccharides commonly contain hexosamine and hexuronic acid (e.g. the chondroitin sulphates or heparin). Sulphated neutral polysaccharides occur commonly among the marine algae (Dillon, 1954; Ross, 1953) as structural polysaccharides and are sporadically distributed among the marine fauna either as epithelial secretions or as structural polysaccharides occupying the place of the chondroitin sulphates, hyaluronic acid or keratosulphate (Lash & Whitehouse, 1960; Bettelheim-Jevons, 1958; Stacey & Barker, 1962).

MATERIALS AND METHODS

Preparation of the polysaccharide sulphate. A solution (1%, w/v) of salt-free mucin, in water, was brought to 60° and an equal volume of 90% (w/v) phenol in water was added slowly with stirring. The mixture was stirred at 60° for 30 min. and then cooled to 4° and allowed to stand at this temperature overnight in the cold-room. During this period the mixture separated into three layers: a lower phenol layer, an upper aqueous layer (containing the polysaccharide sulphate) and a layer of denatured protein at the interface. Most of the lower phenol layer was withdrawn, by using a large hypodermic syringe with a long needle. The remaining aqueous phase and denatured protein were centrifuged at 20000g at 4° for 30 min. The residual phenol phase was again withdrawn and the aqueous phase then decanted from the protein precipitate. The aqueous phase was freeze-dried for 16 hr. to ensure complete removal of phenol and to yield a pure white product.

It was found that the separated polysaccharide sulphate material could be selectively precipitated by the addition of KCl. Precipitation as a final purification step was carried out from aqueous 1% (w/v) solutions of the polysaccharide sulphate by addition of solid KCl (to a final concentration 0.2M) and then allowing the solution to stand at 4° for 6 hr. The precipitate was collected by centrifugation at 20000g, redissolved by addition of water at room temperature, dialysed against repeated changes of distilled water for 36 hr. and freeze-dried. It was considered a measure of the homogeneity of the material that the precipitation took place completely within a fairly narrow concentration range (i.e. 0.2 ± 0.03 M-KCl). The supernatant solution after removal of the precipitate did not contain organic material.

Physical techniques. Analytical ultracentrifugation was carried out with a Spinco model E ultracentrifuge in the Department of Biochemistry at the Manchester College of Science and Technology, by kind permission of Professor A. A. Eddy and with the aid of Mr G. McBride.

Infrared spectra were measured with either a Unicam SP.200 recording spectrophotometer or a Perkin-Elmer model 28 recording spectrophotometer. Samples were examined, either as mulls in Nujol (liquid paraffin) or as freeze-dried films, between polished NaCl plates.

Molecular weights were determined either by the Archibald approach to sedimentation equilibrium method (Archibald, 1947; Klainer & Kegeles, 1955) or by a combination of sedimentation and diffusion coefficients (Schachman, 1957). Sedimentation coefficients at infinite dilution were determined by extrapolation to zero concentration after determination of the sedimentation coefficients at 0.1, 0.25, 0.5 and 1.0% (w/v) concentration in 0.02M-phosphate buffer, pH 7.9 (10.15 with respect to NaCl), and 59780 rev./min. (260000g). Diffusion coefficient determinations were carried out at 0.5% (w/v) concentration in the same buffer, with a Perkin-Elmer model 38A apparatus fitted with a schlieren optical system. Results were evaluated by the 'height-area' method of Lamm (1937).

Viscosity measurements were carried out in Ostwald viscometers of 3 ml. capacity.

Analytical techniques. The techniques of qualitative and quantitative carbohydrate analysis were exactly as de-

scribed in Hunt & Jevons (1965a). D-Glucose was identified with D-glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Estimation of ester sulphate was carried out according to the procedure of Dodgson & Price (1962), the same technique being applied to the measurement of the rate of release of sulphate on dilute acid hydrolysis.

Materials. Monosaccharides, cellobiose and chondroitin sulphate were obtained from British Drug Houses Ltd., Poole, Dorset. Laminarin was obtained from H. Gurr Ltd., London. λ - and κ -Carrageenins were gifts from Dr C. H. Wynn of the Department of Chemistry, University of Manchester.

RESULTS

Physical characterization. The sedimentation pattern produced by centrifugation of the polysaccharide sulphate at 59780 rev./min. (260 000g) at 1% (w/v) concentration in phosphate buffer, pH 7.9 (10.15 with respect to sodium chloride), at 20° is shown in Fig. 1. Only one sedimenting component is apparent, the peak being symmetrical albeit hypersharp. The self-sharpening property of the material on sedimentation is apparently a function of the high viscosity. The material had $S_{20}^{1\%} 5.25 \times 10^{-13}$ and $S_{20}^0 9.75 \times 10^{-13}$, partial specific volume $\bar{v}_{20} 0.6$ and the diffusion coefficient $D_{20}^{0.5\%} 3.28 \times 10^{-7}$. The mol.wt. calculated from S_{20}^0 and $D_{20}^{0.5\%}$ was 1.72×10^5 and from the approach to sedimentation equilibrium method 1.70×10^5 and 1.10×10^5 at the meniscus and base respectively. The reduced viscosity at aqueous 0.25% (w/v) concn., at 25°, was 21320 ml./g. and the intrinsic

viscosity at the same temperature in 0.5M-sodium chloride was 500 ml./g.

The infrared spectrum of the material is shown in Fig. 2. Apart from the absorption frequencies due to carbohydrate and sulphate, discussed below, the following bands were noted. The intense absorption at 3390 cm^{-1} represents the hydrogen-stretching frequency arising from the polysaccharide hydroxyl groups. Normally this frequency would be in the range $3700\text{--}3510 \text{ cm}^{-1}$; its presence at 3390 cm^{-1} implies a considerable degree of hydrogen bonding involving the polysaccharide hydroxyl groups.

The absorption frequency at 1645 cm^{-1} arises not from carboxyl groups, which are almost totally absent, but from absorbed water which here is an integral part of the crystal of the polysaccharide molecules. This frequency has been shown by Forgiath & Rowen (1951) to be present in the spectrum of bacterial cellulose and was shown here to be present in the spectra of starch, cellulose, yeast mannan and laminarin.

Chemical characterization. Paper chromatography of acid hydrolysates of the material indicated that the only monosaccharide present was glucose and qualitative examination with glucose oxidase (Glucostat), according to the procedure described in the Nutritional Biochemicals Corp. method sheet for this reagent, showed this to be D-glucose. The polysaccharide can therefore be designated as a glucan.

Fig. 1. Sedimentation pattern of the glucan sulphate after 128 min. at 59780 rev./min. (260 000g) in 0.02M-sodium phosphate, 0.15M-sodium chloride buffer, pH 8.0. Concentration of glucan sulphate, 1% (w/v); temperature, 20°.

Fig. 2. Infrared spectra of the glucan sulphate: (a) as a freeze-dried film; (b) (inset) as a Nujol mull. Scale expansion $\times 2$ of the region $700\text{--}1250 \text{ cm}^{-1}$.

Analysis of the materials for hexose as glucose (modified orcinol reaction; Hunt & Jevons, 1965a) gave 54% (w/w), and for sulphate as SO_4^{2-} 30% (w/w). The moisture content of the freeze-dried material was 13% (w/w) and the peptide content, determined by quantitative amino acid analysis, was 2% (w/w).

Structure of the polysaccharide. (i) Desulphation; the infrared spectrum. In order that the specific infrared absorptions of the carbohydrate structure might be investigated without interference from the ester sulphate frequencies, desulphation of the glucan sulphate was carried out according to Kantor & Schubert (1956), by using methanol and acetyl chloride. Yields were not estimated because of difficulty experienced in recovering material from the reaction vessels completely. After two desulphation treatments no sulphate could be detected in the polysaccharide material. Fig. 3 shows the infrared spectrum of the material after desulphation (cf. the sulphated material, Fig. 2).

(ii) Colour reactions. Neither the polysaccharide sulphate nor the desulphated polysaccharide gave any colour reaction with a solution of iodine in potassium iodide (10%, w/v, iodine in aq. 20%, w/v, potassium iodide), thus rendering it unlikely that the linkages between the glucose residues are of the α -(1 $\rightarrow$ 4)- type. When the desulphated glucan was treated with chlor-zinc-iodine reagent (obtained from British Drug Houses Ltd., Poole, Dorset, under the name Schultze's solution; Egami *et al.* 1955) the particles of material, which is insoluble in water, showed a deep-blue colour (transmitted-light viewing) exactly comparable with that produced by the same reagent with particles of Whatman cellulose powder.

(iii) Degradation by cellulase. The micro-

organism *Myrothecium verrucaria* (strain 45541) produces an enzyme with a specificity for the hydrolysis of the β -(1 $\rightarrow$ 4)-linkages of cellulose. This enzyme may be obtained in culture filtrates of the organism (Selby, Maitland & Thompson, 1963). A culture filtrate was kindly provided by Dr K. Selby of the Shirley Institute, Didsbury, Manchester. The solution was free of low-molecular-weight material, 0.1M with respect to sodium chloride, 0.01M with respect to sodium phosphate at pH 6.4 and saturated with chloroform as preservative. The procedure, according to K. Selby (personal communication), for incubation with the enzyme was as follows. To 1.5mg. of the desulphated glucan was added 5.0ml. of the enzyme solution and the mixture incubated at 30° for 24hr. Samples of cellulose [β -(1 $\rightarrow$ 4)-], amylose [α -(1 $\rightarrow$ 4)-] and laminarin [β -(1 $\rightarrow$ 3)-] were also incubated with the enzyme. After incubation the enzyme action was halted, by heating to 90° for 10min., and the solutions were centrifuged at 9000g for 5min. Measurement of the increase of reducing power according to Somogyi (1952) indicated that degradation by the enzyme had occurred in both the glucan and cellulose but not in either the amylose or the laminarin. Of the available reducing groups calculated to be present in the glucan, 66% had been released. The glucose content of the desulphated glucan is 96% (w/w). The supernatant from the glucan and the cellulose digests were desalted, concentrated and examined by paper chromatography (Hunt & Jevons, 1965a) with glucose and cellobiose as standards. Staining with aniline hydrogen phthalate showed that degradation of both the glucan and the cellulose to glucose and cellobiose had occurred.

(iv) Partial acid hydrolysis. The glucan was subjected to partial acid hydrolysis. Glucan (1.0mg.) was dissolved in 10ml. of 0.1N-hydrochloric acid by heating under reflux at 100°. Refluxing was continued for 4hr. and, after removal of the acid *in vacuo*, the hydrolysate was redissolved in water (0.1ml.) and examined by paper chromatography. Partial acid hydrolysis yielded sugars with the R_F values of glucose, cellobiose and a smaller proportion of higher oligosaccharides.

Position of the sulphate residues. Barium sulphate was not precipitated by the addition of barium chloride solution to solutions of the glucan sulphate. Mild acid hydrolysis (1N-hydrochloric acid for 1 hr. at 100°), however, of the glucan sulphate released sulphate precipitable as barium sulphate. Thus the sulphate residues were apparently covalently bound to the polysaccharide.

(i) Infrared spectra. The infrared spectra of the glucan sulphate (Fig. 2) show the presence of intense absorptions at 1240 cm^{-1} and over the

Fig. 3. Infrared spectrum of the desulphated glucan sulphate, as a Nujol mull, in the range 700–1300 cm^{-1} .

range 800–840 cm^{-1} . On desulphation these two bands disappear entirely (Fig. 3) indicating ester sulphate (Hoffman, Linker & Meyer, 1958). Apart from these spectral changes it is to be noted that desulphation also brings about the disappearance of absorptions at 940 cm^{-1} , 920 cm^{-1} and 1000 cm^{-1} . Examination of the infrared spectra of λ -carrageenin, κ -carrageenin and chondroitin sulphate A before and after desulphation showed similar changes. The possibility that spectral changes were arising in part from structural changes in the polysaccharide itself, brought about by the desulphation reaction and resulting in the formation or destruction of 3,6-anhydroglucose units, was obviated by testing for 3,6-anhydro-sugars (O'Neill, 1955) in both the sulphated and desulphated glucans. The tests on both materials were negative.

(ii) Rate of hydrolysis of the sulphate groups. With the procedure of Rees (1963) it was found that release of ester sulphate residues from the glucan sulphate, in 0.25N-hydrochloric acid at 100°, proceeded linearly according to a plot of $\log(a/a-x)$ versus t (where a is the total sulphate present and x the amount released after time t). The half-time of this reaction was calculated to be of the order 0.41 hr.

(iii) Potassium chloride precipitation. As already stated the glucan sulphate was readily precipitable below 4° by addition of potassium chloride to a concentration of 0.2M. The solubility properties of the freshly formed precipitate and of the centrifuged consolidated precipitate differ, the former redissolving in the 0.2M-potassium chloride immediately on allowing it to come to room temperature whereas the latter remained completely insoluble

in the 0.2M-potassium chloride at room temperature and could moreover be redissolved in distilled water, at room temperature, only with difficulty. The property of precipitation by potassium chloride may be related to the position and configuration of the sulphate groups on the glucan.

Fig. 4. Absorption spectra of (a) free toluidine blue in aqueous solution at 20 μM concentration and (b) the metachromatic complex, in aqueous solution, of toluidine blue (20 μM) and the glucan sulphate (60 μM with respect to sulphate).

Fig. 5. (a) Proportion of metachromatic complex present, at constant dye concentration (20 μM -toluidine blue), in aqueous solutions of glucan sulphate at various concentrations with respect to sulphate. (b), (c) and (d) Stability, in aqueous solution, of the metachromatic complex of toluidine blue (20 μM) and the glucan sulphate (60 μM with respect to sulphate) to increasing temperature ($\blacktriangle$), to decreasing pH ($\circ$) and to increasing ionic strength ($\bullet$).

(iv) *Metachromasia*. The dependence of metachromasia upon concentration of the polysaccharide, upon pH, temperature and ionic strength of the solvent were investigated for the glucan sulphate. The material readily formed a complex with toluidine blue, the absorption spectrum of which showed one maximum at $512\text{m}\mu$, in contrast with the spectrum of the free dye which had maxima at 598 and $653\text{m}\mu$ (Fig. 4). An isosbestic point, for the spectra of the complex and free dye, occurs at $545\text{m}\mu$, indicating an equilibrium between the metachromatic and unbound forms of the dye.

The concentration of metachromatic complex present, at constant dye concentration in solutions of varying glucan sulphate concentration, is expressed in Fig. 5 as the E_{512}/E_{598} ratio, as is also the stability of the complex (at the optimum dye and glucan sulphate concentration) to temperature, pH and ionic strength.

Glucan sulphate as the main viscous component of the mucin. A solution of the mucin (0.1%, w/v) in water was adjusted to pH 7.0 with sodium hydrogen carbonate. An appropriate quantity of the proteolytic enzyme Pronase P (Kaken Chemical Co., Tokyo, Japan) was added (1mg. of Pronase P/50mg. of substrate), the solution rapidly mixed and a sample placed in a viscometer maintained at 37° . The remainder of the solution was maintained also at 37° and samples were withdrawn, for degree of proteolysis assay by ninhydrin (Moore & Stein, 1948), at regular intervals over a 24 hr. period. The viscosity of the solution was also measured at

intervals over the same period (Fig. 6). It can be seen that during removal of the protein by proteolysis the viscosity fell by a negligible amount only.

DISCUSSION

Homogeneity. The results obtained from analytical ultracentrifugation and potassium chloride precipitation suggest that the polysaccharide sulphate obtained from the *B. undatum* mucin is a homogeneous preparation.

General composition. The infrared spectra of the material (Fig. 2) indicate that it consists of the major carbohydrate component of the whole mucin and that it is the carrier of the ester sulphate groups detected in the whole mucin (Hunt & Jevons, 1963, 1965a), these conclusions being derived from observation of the characteristic carbohydrate bands at $900\text{--}1100\text{cm}^{-1}$ and ester sulphate bands at about 820 and 1250cm^{-1} (Barker, Bourne & Whiffen, 1956). Peptide bond frequencies were absent, in keeping with the low percentage of peptide detected chemically. The small proportion of peptide material remaining attached to the polysaccharide sulphate is apparently an integral, covalently bound, component and is discussed by Hunt & Jevons (1965b). The value 30% for the sulphate (SO_4^{2-}) composition of the material when corrected for peptide and moisture content becomes 35%, which, assuming that the polysaccharide is a glucan, suggests that the ester sulphate residues may be distributed one per monosaccharide unit.

Structure of the polysaccharide. From its monosaccharide composition the polysaccharide can only be a glucan.

Examination of the infrared spectrum of the glucan indicates a strong absorption at 895cm^{-1} and the absence of absorptions at 844 and 766cm^{-1} , suggesting, on the basis of the criteria of Barker, Bourne, Stacey & Whiffen (1953, 1954) and Barker, Bourne, Weigel & Whiffen (1956), that the linkages might be β rather than α .

The enzymic degradation and partial-acid-hydrolysis data require care in interpretation. Both results tend to suggest the presence of β -(1 $\rightarrow$ 4)-linkages, indicating that the glucan has a structure similar to that of cellulose. Moreover the solubility and iodine-staining properties of the glucan are again both similar to those of cellulose. However, with the enzymic evidence a similar effect might have been produced if a second enzyme, specific for β -(1 $\rightarrow$ 3)-linkages, were present and if the glucan had a similar structure to lichenin, which contains 30% of β -(1 $\rightarrow$ 3)-glucosidic linkages in addition to β -(1 $\rightarrow$ 4)-linkages. Dr K. Selby has informed us that the presence of such an enzyme, in the particular culture filtrate used here, cannot be completely discounted. It should, however, be

Fig. 6. Course of proteolytic degradation of the whole mucin in aqueous solution. ●, Proteolysis measured by reaction with ninhydrin and expressed as percentage of the total degradation obtained by hydrolysis of an equal quantity of mucin, in 5.7N-hydrochloric acid at 100° for 16 hr. ○, Viscosity of the mucin solution relative to water at the same temperature (37°).

noted when considering this possibility that the culture filtrate was without action against laminarin, which also has β -(1 $\rightarrow$ 3)-linkages. Thus while the results point to some if not all β -(1 $\rightarrow$ 4)-structure the possibility of other types of linkage and of a low degree of branching cannot be ruled out.

Position of the sulphate groups. (i) Infrared spectra. The results obtained by infrared spectroscopy are inconclusive. Since all the hydroxyl groups of β -D-glucose are known to be equatorial (Reeves, 1951) it is to be expected that the infrared spectrum should show the characteristic C—O—S frequency (Thompson & Torkington, 1945; Colthup, 1950; Orr, Harris & Sylven, 1952; Orr, 1954) at about 820 cm.⁻¹ rather than at 855 cm.⁻¹. The broadness of the absorption band (Fig. 2), however, renders difficult any differentiation between primary and secondary substituted equatorial hydroxyl groups although the position of the maximum at 820 cm.⁻¹ tends to favour the primary (i.e. the 6-position) (Lloyd & Dodgson, 1961). The inference might be drawn that a proportion of both types is substituted were it not for the information derived from other sources.

With regard to the other frequencies correlated with the presence of sulphate groups (other than at 1240 cm.⁻¹), these do not as yet provide information about the position of the groups but are of interest since such absorptions have not previously been widely reported. That the frequencies (940, 920 and 1000 cm.⁻¹) are in fact manifestations of the ester sulphate groups, seems to be definitely established by their disappearance on desulphation. A number of workers have reported the presence of absorption frequencies from 920 to 955 cm.⁻¹ in the spectra of polysaccharide sulphates but without positively identifying them with the sulphate groups. For example, absorptions at 928 cm.⁻¹ in the spectrum of chondroitin sulphate A (Mathews, 1958), at 955 cm.⁻¹ in the spectrum of a new chondroitin sulphate (Bettelheim & Philpott, 1960) and at 925 cm.⁻¹ for both λ - and κ -carrageenins (Bayley, 1955) have been noted but their disappearance on desulphation has not, however, been recorded. Mathews (1958) has also reported a 1000 cm.⁻¹ absorption in chondroitin sulphate and its disappearance on desulphation was reported by Hoffman, Linker & Meyer (1958).

(ii) Rate of hydrolysis of sulphate. The figure of 0.41 hr. for the half-time of the sulphate-release reaction on dilute acid hydrolysis is comparable with that obtained by Rees (1963) for the release of sulphate from β -D-glucose 3-sulphate (i.e. a secondary equatorial hydroxyl position). The release of sulphate from the primary equatorial hydroxyl group at position 6 should, according to Rees (1963), have a half-time of the order 1.5–2.4 hr. The observation that the rate curve was

linear suggests strongly that one class of ester sulphate group only is present.

(iii) K⁺ ion packing. The property of the material to form an insoluble potassium 'salt', in the presence of potassium chloride, suggests an arrangement of the sulphate on the polysaccharide chain which allows approach and packing together of adjacent chains to produce a highly ordered stack of polymer units in which the 'cement' is provided by hydrated K⁺ ions. The diameter of the hydrated K⁺ ion is of the order 2.5–4.0 Å (Moelwyn-Hughes, 1940) and, according to Bayley (1955), the optimum distance between alternate sulphate groups attached to adjacent polysaccharide chains, such that entry of a hydrated K⁺ ion might be permitted and packing produced, would be of the order 6.0 Å. Examination of molecular models has shown here that with sulphated β -(1 $\rightarrow$ 4)-glucans, irrespective of whether the chain is substituted entirely in the 2-, the 3- or the 6-position, the ester sulphate groups will lie at intervals of approximately 12.0 Å and hence when adjacent chains are packed an interval of the order 6.0 Å recurs and K⁺ ion stabilization and precipitation could result. Unless all groups are similarly placed, entirely on the 6-, the 3- or the 2-position, this situation would not be permitted. It seems likely therefore that only one type and position of sulphate group on the glucan is possible. This is in keeping with the kinetics of release of sulphate by hydrolysis. Exactly why the precipitation should require a low temperature is not clear.

(iv) Metachromasia. It is apparent from the results that even where the concentration of the polysaccharide is in excess (60 μ M with respect to sulphate) over toluidine blue, the bound toluidine blue is all in the metachromatic state, implying that the nearest neighbour binding site must be preferred and that random interactions are at a minimum (Schoenberg & Moore, 1964). The results show clearly that the metachromatic complex has considerable stability even at low pH, high temperature and high ionic strength.

The formation of these highly stable metachromatic complexes by the glucan sulphate would seem to indicate that the polysaccharide molecule possesses a linear configuration upon which the ester sulphate residues are arranged in a precise and regular, rather than random, manner (the considerable stability of the complex might stand as evidence against any significant degree of branching in the polysaccharide chain). This arrangement would agree with the potassium packing property. According to Schoenberg & Moore (1964), for the formation of a metachromatic complex with toluidine blue the acidic residues of a polysaccharide should be capable of approach to about 7 Å separation. Examination of molecular models has

indicated that with a β -(1 $\rightarrow$ 4)-glucan chain occupying the normal unstrained configuration, substitution of the 2-, 3- or 6-position by ester sulphate will not fulfil the requirements. If, however, a certain degree of rotation about the glycosidic bond is postulated (the possibility of free rotation about the glycosidic oxygen bond in cellulose is recognized; Woods, 1959), then it becomes possible for ester sulphate residues upon positions 2 or 3, but not on position 6 of the glucose molecules, to approach within the required limits without too great a degree of rotation and strain.

The evidence therefore seems to be weighted slightly against position 6 as the site for the ester sulphate residues on the glucan chain.

Viscosity. From Fig. 6 it can be seen that a 70% degradation of the protein component of the whole mucin, brought about by proteolytic enzyme action, produces no significant change in the viscous properties of the mucin. It seems apparent therefore that the glucan sulphate alone is responsible for the viscosity of the mucin in solution. This situation is comparable with that of the complex of hyaluronic acid and protein occurring in synovial fluid. Here 65% degradation of the protein, by trypsin and chymotrypsin, was achieved without any reduction in the high and anomalous viscosity of the solution occurring (Ogston & Sherman, 1959). It should be borne in mind, however, that the viscosity measurements upon the *Buccinum undatum* mucin were made with dispersions (Hunt & Jevons, 1963) of the original secretion and that these solutions do not possess all of the original rheological properties, i.e. thixotropy and syneresis, in which the protein might well be implicated.

In contrast with a value of the order 5500 ml./g. for the intrinsic viscosity of native hyaluronic acid in 0.2 M-sodium chloride (Blumberg & Ogston, 1957), that of the glucan sulphate in 0.5 M-sodium chloride was of the order 500 ml./g. This value should be regarded only as an approximation, as the extrapolation to zero concentration, even in the presence of swamping electrolyte, becomes extremely difficult with the wide scatter of the reduced viscosity values obtained at very high dilutions of polyelectrolytes (see the review by Yang, 1961). The low value for the intrinsic viscosity of the glucan sulphate in comparison with that of hyaluronic acid is most probably a function of the chain length. Calculation of the chain length of the glucan sulphate from the molecular weight 1.7×10^5 and by the use of 10.3 Å for the length of the diglucose unit (cellobiose, according to Meyer & Misch, 1937) yields a value of the order of 3200–3300 Å. Hyaluronic acid has a chain length of 25 000 Å (Schubert, 1964) and is thus capable of spreading throughout a large volume of space such that considerable interactions between molecules are possible even in very dilute

solution and hence result in elevated viscosities. At equivalent concentrations solutions of the shorter, more compact, sulphate molecules would be expected to produce less extensive interactions than would occur in solutions of the larger, more diffuse, hyaluronic acid molecules.

Comparison with related substances. The value 1.7×10^5 (number average) obtained for the molecular weight of the glucan sulphate is apparently lower than the average for this type of molluscan material. Although no direct data for the molecular weight of the β -(1 $\rightarrow$ 4)-linked glucan sulphate in *Charonia lampas* are available, a quoted value of 15s (Iida, 1963), for the sedimentation coefficient, suggests that the molecular weight might be somewhat greater than that of *B. undatum* glucan sulphate (9.75s at infinite dilution). The molecular weight, based on sedimentation data, of the α -(1 $\rightarrow$ 6)-glucan sulphate from *C. lampas* mucin was estimated by Iida (1963) as being of the order of 10^6 (estimations of reducing power suggested a value of 10^4). The estimated molecular weight of the polyhexosamine sulphate of *Busycon canaliculatum* mucin, based on light-scattering data (weight average) was 2.5×10^6 (Kwart & Shashoua, 1958). In contrast, chondroitin sulphate, a polysaccharide sulphate of higher animal tissue, has been variously estimated as having chain weights of 2.8×10^4 to 5.0×10^4 (Partridge, 1961; Mathews & Lozaityte, 1956).

With regard to the chemical character, the observation might be made that whereas in higher animals viscous, epithelial secretions consist largely of proteins bearing oligosaccharide side chains with strongly acidic terminal residues in the shape of the sialic acids (Bettelheim-Jevons, 1958; Stacey & Barker, 1962), the corresponding secretions of these lower animals would appear to have achieved the same effect by substitution of linear high-molecular-weight polysaccharides with strongly acidic sulphate groups. The reasons underlying this divergence may reside in the ready availability of sulphate in the marine environment, and here the ubiquity of polysaccharide sulphates in the marine algae (Dillon, 1954) is worthy of note.

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