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Free-boundary Electrophoresis of Acidic Polysaccharides from the Marine Alga *Ascophyllum nodosum* (L.) Le Jol.

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Different preparations of acidic polysaccharides from the marine alga *Ascophyllum nodosum* have been investigated by means of free-boundary electrophoresis. By taking advantage of the different dissociation constants for the carboxyl and sulphate half-ester groups the electrophoretic separation is substantially improved. This technique also indicates whether the molecule contains carboxyl or sulphate half-ester groups, or both. The application of this method demonstrated the presence of a new polysaccharide, for which the name ascophyllan is suggested, in the alkali soluble portion of the alga.

The use of free-boundary electrophoresis as a method of investigating the homogeneity of a polysaccharide preparation has received little attention in polysaccharide chemistry. With neutral polysaccharide, *i.e.* polysaccharides containing no charged groups, the method suffers the disadvantage of requiring the formation of a charged complex. Many neutral polysaccharides form such complexes with borate ions at high pH values, a fact that has been extensively utilized in paper electrophoresis. The separation of different components then depends on their ability to form complexes with different charge density. The acidic polysaccharides, on the other hand, carry charged groups in the molecule and thus possess the ability of moving in an electric field without the addition of a complexing agent. Except for polysaccharides recently isolated from microorganisms and shown to be significantly phosphorylated the naturally occurring acidic polysaccharides contain either uronic acid or sulphate half-ester as the charged group. The present communication describes an investigation of different polysaccharide preparations from the marine alga *Ascophyllum nodosum* (L.) Le Jol. Both uronic acid residues and sulphate half-ester groups were present, and in order to obtain electrophoretic separation of the different polysaccharides, advantage was taken of the different dissociation constants of the two types of charged groups.

EXPERIMENTAL

A sample of *Ascophyllum nodosum* harvested on July 5, 1957, at Garten was used as raw material in all experiments except those involving enzymic degradation or fractionation at pH 2. For the latter experiments a sample of the same species harvested in August

1959 at Være was used. Both samples were dried and milled prior to extraction of the polysaccharides.

Fucoidin. 20 g of the dried and milled algae were extracted overnight in a mechanical shaker with 500 ml of 0.2 N hydrochloric acid. The residue was removed by filtration and extracted once more with a new batch of acid for about 4 h. The two extracts were combined, neutralized with sodium hydroxide solution and evaporated *in vacuo* to approximately 100 ml. The solution was then dialysed against distilled water for 48 h with stirring and frequent changes of water. After filtration 10 ml of a 0.03 M calcium acetate solution were added to the non-dialysable portion and the polysaccharide precipitated by adding 2 volumes of ethanol. If the solution is sufficiently concentrated the addition of calcium ions may be omitted. About 0.3 % sodium chloride should then be added before precipitating with ethanol. The precipitate was filtered off, washed with alcohol and ether and dried in a vacuum desiccator. Before use the amount required for each experiment was dissolved in water and transformed to the sodium form by passing the solution through a column of Dowex 50 X 4 (20—50 mesh) and then neutralized with sodium hydroxide solution.

The alginate fraction. After the extraction of fucoidin the residue was suspended in 1000 ml of distilled water and sufficient 1 N sodium hydroxide solution added to render the mixture slightly alkaline. About 25 ml were required. The mixture was left overnight in the shaking machine and then filtered through Schleicher & Schüll 520 B filter paper. The residue was extracted once more with 1000 ml water and the two extracts combined. The solution was evaporated *in vacuo* to approximately 750 ml, 1.5 g sodium chloride was added and the alginate precipitated by adding 750 ml ethanol. The precipitate was washed with ethanol and ether and dried.

Preparation A. 5 g of the precipitate were extracted overnight with 500 ml of 0.06 N hydrochloric acid in the mechanical shaker. After filtration the residue was extracted once more with 500 ml of 0.01 N acid and the two extracts combined. The solution was neutralized with sodium hydroxide solution, evaporated *in vacuo* to approximately 100 ml and the polysaccharide precipitated by adding 200 ml of ethanol. The precipitate was washed and dried as described above. Yield approximately 0.6 g.

Preparation B. After extraction of preparation A the residue was suspended in *ca.* 500 ml distilled water and dissolved by neutralizing with sodium hydroxide solution. After adding 1 g of sodium chloride the polysaccharide was precipitated by adding an equal volume of ethanol. The precipitate was then washed and dried as usual.

Enzymic degradation and fractionation at pH 2. A detailed description of the experimental procedure is given in the subsequent paper. This procedure was only applied when a partial degradation was desired, and the soluble, non-dialysable fraction was used for electrophoresis. When extensive enzymic hydrolysis was required, the sample, dissolved in the phosphate buffer to give a 1 % solution, was placed in a dialysis bag and a sufficient amount of enzyme concentrate added. The enzymic hydrolysis was allowed to proceed at 30°C, the solution inside the bag being stirred continuously. The solution outside the bag, consisting of the same phosphate buffer as used for dissolving the sample, was changed every 12 h. This technique provided a means of controlling the enzymic breakdown by removing the degradation products as soon as they reached a certain molecular size. Only the dialysable part was used for electrophoresis.

Desalting of enzymic hydrolysates. As ordinary dialysis could not be applied, samples subjected to extensive enzymic degradation had to be desalted by gel filtration with Sephadex after concentrating the dialysate *in vacuo*. Aliquots of 15 ml of the concentrated enzyme digest were put on a column (25 × 200 mm) of Sephadex G 25 (void volume 31 ml). The column was connected to a fraction collector and eluted with distilled water. The fractions, each having a volume of about 2 ml, were analysed for carbohydrate by the phenol sulphuric acid reaction [1] and for chloride by titration with 0.1 N silver nitrate. A qualitative assay for phosphate ions was carried out by spotting a drop of each fraction on a filter paper and spraying with ammonium heptamolybdate solution according to Hanes and Isherwood [2]. All fractions containing only carbohydrate material were pooled. Usually, two runs on the Sephadex column were necessary in order to obtain sufficient material for electrophoresis. The solution was then concentrated to 9 ml.

Electrophoresis. Preparation B was dissolved in the buffer to give a 0.8 % solution, the other substances to give a 1 % solution when sufficient material was available. The solutions (10 ml) were dialysed for at least 18 h against two batches of 300 ml each of the

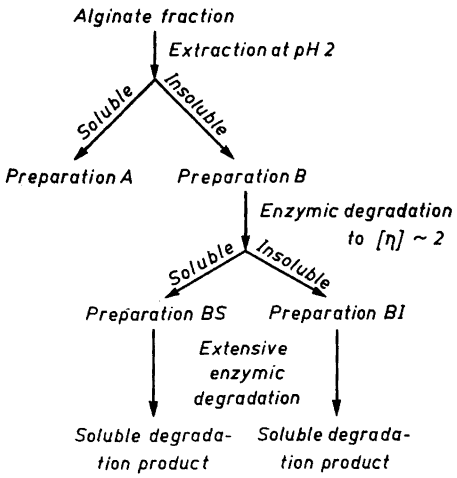

Fig. 1. Preparation of fractions.

same buffer. The desalted enzymic hydrolysates were adjusted to the proper pH and conductivity by addition of 1 N hydrochloric acid and 1 M sodium chloride solution. The electrophoresis was carried out in an Aminco Portable Electrophoresis Apparatus equipped with a standard analytical cell for Schlieren patterns. A current of 15 mA was used at pH values between 2.0 and 2.6, 10 mA with the other buffer systems. The specific conductivity of the dialysed solution was determined in the same apparatus by means of a Phillips conductivity bridge type GM 4249. As the separation was usually better at the ascending boundary and only relative values were needed, the apparent ascending mobilities were calculated.

Buffers.
pH 2.0. 0.05 M sodium chloride adjusted to pH 2.0 with hydrochloric acid. Ionic strength $\mu = 0.06$.
pH 2.6. As above but adjusted to pH 2.6. $\mu = 0.05$.
pH 3.0. 0.05 M sodium citrate-hydrochloric acid. $\mu = 0.05$.
pH 3.6. Same buffer system. $\mu = 0.05$.
pH 4.2. Same buffer system. $\mu = 0.06$.
pH 6.0. M/30 phosphate buffer. $\mu = 0.04$.

RESULTS

Paper chromatography of acid hydrolysates of the alginate fraction from *Ascophyllum nodosum* revealed the presence of a considerable proportion of

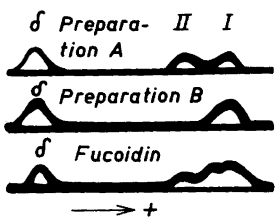

Fig. 2. Ascending boundaries obtained with 1 % solutions in 0.05 M citrate buffer pH 4.2.

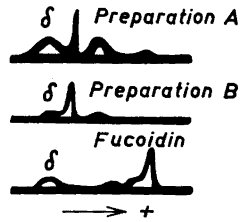

Fig. 3. Ascending boundaries obtained with 1 % solutions at pH 2.

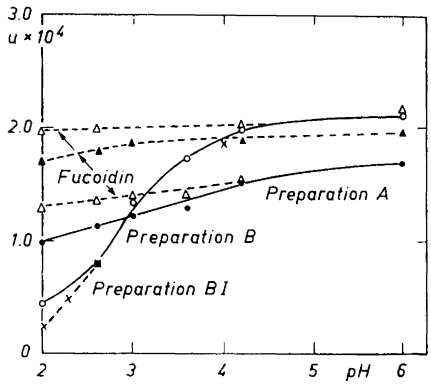

Fig. 4. The variation of mobilities with pH.

fucose and xylose, thus suggesting that this material was heterogeneous. It was also observed that a fractionation could be obtained by extracting the alginate fraction with a buffer of pH 2. The soluble fraction obtained with this procedure will be referred to as preparation A, the insoluble part as preparation B (*cf.* Fig. 1). A detailed report on the low-pH fractionation is given in the following paper. Preparations A and B, together with fucoidin, were then investigated by free-boundary electrophoresis to obtain further information on the suspected heterogeneity.

The ascending boundaries obtained with fucoidin, and preparation A and B in the citrate buffer of pH 4.2 are shown in Fig. 2. Concentration boundaries are marked with the letter δ. Of the three preparations only B appeared to be homogeneous. Fucoidin apparently consisted of a mixture of at least three components, the major one having a mobility equal to that of preparation B at this pH. Under similar conditions O'Neill [3] observed 2 boundaries in fucoidin with mobilities of 2.3 and 2.5 $\times$ 10^{-4} cm^2 per sec. volt. Preparation A gave two completely separated peaks, the fast one (I) having the same mobility as preparation B, the slower one the same mobility as the minor component in fucoidin. These are the only conclusions that can be drawn from experiments carried out at a single pH value.

The ascending boundaries obtained at pH 2.0 are shown in Fig. 3. Fucoidin again showed three components, the separation being a little better at this pH. From the method of preparation it follows that preparation B is insoluble at this pH. According to the results given in the subsequent paper, it can, however, be partially brought into solution by enzymic degradation to a value of intrinsic viscosity of 2 (*cf.* p. 1654). By dialysing the enzyme digest against the buffer of pH 2, a soluble, non-dialysable fraction is obtained, originating from preparation B. This will be referred to as preparation BS (*cf.* Fig. 1) and has been used for the electrophoretic experiments at pH values below 3. At pH 2, this preparation gave a sharp boundary of very low mobility (0.45 $\times$ 10^{-4} cm^2/sec.volt) and traces of a compound corresponding to the major component of preparation A. Preparation A contained traces of fucoidin and of the component with low mobility observed in BS, but the major component had a mobility definitely different from both of these substances.

The results clearly demonstrated that a better separation is obtained at low pH values, and we therefore decided to investigate the influence of pH on the mobility of these three preparations. Fig. 4 shows the apparent ascending mobilities of the different components as a function of pH. When the pH of the solution was between 2.6 and 3.6 some of the boundaries were less well-defined than at other pH values, and a reliable mobility determination could therefore not always be obtained. As expected, the mobility of the main component of fucoidin varied only slightly with pH. The mobility of the two minor fractions decreased slightly with decreasing pH. With preparation B, and BS, the mobility decreased from 1.99×10^{-4} at pH 4.2 to 0.45×10^{-4} at pH 2.0 following a curve resembling the acid dissociation curve of the carboxyl group (*cf.* Ref.[9]). The mobility of the main component of preparation A varied from 1.0×10^{-4} at pH 2 to 1.7×10^{-4} at pH 6.

DISCUSSION

Not considering the bacterial polysaccharides containing phosphate, the acidic polysaccharides may be divided into three groups:

(1) Those containing uronic acid as the only group carrying a negative charge, *e.g.* alginic acid, pectin, hyaluronic acid, hemicelluloses and various plant and seed gums.
(2) Those containing only sulphate half-ester groups, *e.g.* carrageenin and fucoidin.
(3) Those containing both these groups in the same molecule, *e.g.* chondroitin sulphates and polysaccharides from marine green algae [4].

At any value above 5 an electrophoretic separation of two polysaccharides belonging to different groups but having the same charge density is unlikely to occur. Below pH 5, however, a separation can in many cases be obtained due to the different dissociation contants for the carboxyl and sulphate half-ester groups. The sulphate half-ester group has a very low pK value, and the mobility of a polysaccharide containing only such groups should therefore remain almost constant at least down to pH 2. This was clearly demonstrated by Goring [5,6] for carrageenin. With a polysaccharide containing only uronic acids a decrease in pH below 5 will lead to a lower degree of ionization until, at a pH of about 2, the charge should become negligible. Consequently, the mobility will decrease and, provided the undissociated molecule is soluble, will approach zero at a pH of approximately 2. This has been shown to occur with pectin and gum arabic by Säverborn [7]. A polysaccharide contaning both uronic acid and sulphate in the same molecule would occupy an intermediate position. The results obtained with chondroitin sulphate A and B by Mathews [8] verify this assumption.

From Fig. 4 it is evident that the separation of the acidic polysaccharides from *Ascophyllum nodosum* is very much improved at pH values below 4. Between pH 4 and 6 only three components would be observed. A single run at pH 2 allows the identification of all five components.

Preparation B would be expected to consist mainly, if not exclusively, of alginic acid. Owing to the insolubility of alginic acid, and of preparation B, below pH 3, preparation BS had to be used between pH 2 and 3. This prepara-

tion does, however, only represent a fraction of preparation B (*cf.* following paper). The electrophoretic pattern of preparation BS at pH 2 also demonstrated the presence of a certain proportion of the main component of preparation A, thus showing that B is not a pure alginic acid. The mobility-pH curve obtained may therefore not be representative of alginic acid.

To further investigate this point extensive enzymic degradation was applied to three samples: the alginate fraction, preparation BS and the insoluble fraction obtained when preparing BS. This insoluble fraction will be referred to as preparation BI (*cf.* Fig. 1). The dialysis technique used for the extensive degradation involves a fractionation in dialysable and non-dialysable material. For these experiments only the dialysable fraction was used. According to the results given in the subsequent paper, the main component of preparation A is then retained in the non-dialysable fraction and will thus not appear during electrophoresis of these samples. The products obtained were completely soluble at pH 2.

By electrophoresis at pH 2 the following mobilities were found: alginate fraction 0.30, preparation BI 0.27, preparation BS 0.36, all figures given as 10^{-4} cm^2/sec.volt. The difference in mobility between the degraded preparations BS and BI, 0.09×10^{-4}, can not be considered as proving that these preparations are different. This conclusion is also supported by the fact that the degraded alginate fraction, which contains both BS and BI, gave only one boundary upon electrophoresis.

For preparation BI the mobility was also determined at pH 2.3, 2.6, and 4.0. These results are shown in Fig. 4. The lower mobility obtained with this preparation between pH 2.0 and 2.6 would seem to indicate that it is different from preparation BS. It should be noted, however, that BS has an intrinsic viscosity of approximately 2, whereas BI has been extensively degraded. The results from the precipitation experiments given in the subsequent paper suggested that the main component of preparation A may be chemically bound to alginic acid, the complex thus possessing sulphate half-ester groups which would influence the mobility in the low pH-region. It was also demonstrated that the amount of preparation A was higher in the pH 2 soluble fraction (BS) than in the insoluble fraction (BI) (*cf.* Table 2, subsequent paper). The existence of such a complex, which would release the component A upon enzymic degradation, might explain the different curves obtained for BS and BI. It then also seems reasonable to assume that the curve given for B—BI is representative for alginic acid.

The three components observed in fucoidin may differ only with respect to charge density, the major component, *i.e.* the one with the highest mobility probably carrying one sulphate on each L-fucopyranose unit. The slight decrease in mobility with decreasing pH observed for the slow moving component of fucoidin might indicate the presence of carboxyl groups in this compound.

The results obtained with the main component of preparation A unequivocally demonstrates the presence of a new polysaccharide in the alkali soluble portion of *Ascophyllum nodosum*. We suggest the name ascophyllan for this polysaccharide. The marked increase in mobility with increasing pH obtained with ascophyllan strongly suggested the presence of carboxyl groups. To check the validity of this assumption we also determined the mobilities at

Table 1. Apparent ascending mobilities of polysaccharides containing uronic acid and sulphate half-ester groups.

	$u \times 10^4$ cm²/sec.volt			
	Ascophyllan	*Enteromorpha compressa*	*Ulva lactuca*	Chondroitin sulphate
pH 2	0.99	1.36	1.72	1.43
pH 4	1.52	1.95	2.34	2.06

pH 2 and 4 of a chondroitin sulphate and two algal polysaccharides known to contain both uronic acid and sulphate half-ester groups [4]. Chondroitin sulphates A and B have previously been examined by Mathews [8] who determined the electrophoretic mobilities at three different pH values, *i.e.* 7.0, 3.0, and 2.1. Owing to different experimental conditions with respect to ionic strength and temperature his results could not be used for comparison. The chondroitin sulphate used by us was a commercial preparation (L. Light & Co.). The algal polysaccharides were isolated from the green algae *Enteromorpha compressa* and *Ulva lactuca* by Dr. E. Percival of the University of Edinburg and kindly supplied by her. The apparent ascending mobilities of these polysaccharides are given in Table 1.

The increase in mobility is about the same for the four samples, thus justifying the conclusion that ascophyllan is of the same type as the others. This has also been verified by chemical investigation.

The comparatively low mobility observed at pH 4 may indicate the presence of neutral sugar units in the polysaccharide. A quantitative interpretation of the mobility-pH curve should, however, not be attempted. Owing to the binding of counter-ions the charge within the shear surface is not necessarily the same as the charge of the particle determined by, for instance, potentiometric titration (*cf.*, *e.g.*, Overbeek [10]). The counterion binding is probably different for different polysaccharides depending upon the buffer electrolyte and the ionic strength, and a quantitative comparison of mobilities may therefore be invalid.

Although a complete purification has not yet been accomplished the results obtained with paper chromatography of acid hydrolysates strongly suggested the presence of uronic acids together with fucose, xylose and sulphate groups. A detailed report on these investigations will be published later.

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