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Periodate Oxidation of the Jelly Coat Substance of *Echinocardium cordatum*

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The jelly coat which surrounds sea-urchin eggs is composed of a polysaccharide ethereal sulphate in combination with protein, the former being the main component [1]. Analyses carried out with the aid of paper partition chromatography [2] revealed the presence of but one sugar, fucose, in the hydrolysate from *Echinocardium cordatum* jelly. Chemical studies on the egg jellies of other species of sea-urchin [1–4] suggest that the comparatively high degree of specificity which the jellies show in the sperm-agglutination test [5–7] may depend on the carbohydrate component [8] of the jelly substance. Furthermore, these studies showed that polyfucose sulphate is the major carbohydrate component, not only in *E. cordatum* jelly (where no other sugar was detected), but also in *Strongylocentrotus droebachiensis* and *Paracentrotus lividus* jellies. In the latter two species the minor carbohydrate component is built up from galactose and glucose residues respectively [2]. It is therefore of interest to investigate the structure of the polyfucose sulphates in order to see if they differ, and the present paper presents the results of some studies on the structure of the *E. cordatum* jelly substance.

EXPERIMENTAL

Material. The material used was collected in the summer of 1949 and prepared in the usual manner [1] by dissolution in acidified sea-water (pH approximately 5.0), cleaning by repeated centrifugation, and precipitation with ammonium sulphate. The resulting precipitate was stored in the cold.

Very concentrated solutions were obtained from this precipitate by dialysis, after the addition of some water, against running tap water until free from sulphate ions and then against cold distilled water until free from chloride ions.

Methods. The solid content of these solutions was determined by evaporating and drying at 105°. Total nitrogen was determined both on dry samples and on aliquots of

solution by the procedure described by Parnas [9], using the combustion fluid of Arnold and Wedemeyer (*cf.* Parnas [9]).

Total sulphur was determined by the Grote – Krekeler method [10]. The absorption liquid and the ash were treated separately, and the sulphate was weighed as $BaSO_4$. As the ash was more or less insoluble, most probably owing to the presence of dead-burned $CaSO_4$, the apparent sulphate figure for the ash was corrected on this assumption. In most cases the entire ash could be considered to be composed of anhydrous $CaSO_4$. The excellent agreement between the values for the total sulphur obtained in this way and the values for the acid-hydrolysable sulphate (Table 1) suggests that this assumption is

Table 1. Analysis of total sulphur (Grote and Krekeler [10]) and acid-hydrolysable sulphate of the jelly substance of Echinocardium cordatum.

Preparation	Total sulphur as sulphate (SO_4)				Acid-hydrolysable sulphate (SO_4)		
	weight of substance mg	in abs. liquid per cent	in ash ($CaSO_4$) per cent	total per cent	weight of substance mg	time at 127° hours	per cent
Ec 49 : 1					27.97	0 *	17.64
					27.93	3	18.41
Ec 49 : 2	27.5	10.92	9.74	20.66			
	69.1	10.91	9.69	20.60			
Ec 49 : 3	29.0	12.76	9.96	22.72	29.72	1	22.44
	34.1	11.82	10.56	22.38			

* *i. e.* the gas was turned off immediately after the temperature had reached 127°. Fifteen minutes was allowed for the steam to displace the air in all autoclaving experiments.

justified. Acid-hydrolysable sulphate was obtained as $BaSO_4$ after hydrolysing weighed samples of the jelly solution with 0.5 *N* HCl in an autoclave at 127° *Cf.* [1]. The total fucose in the jelly solution was determined by the procedure described by Dische and Shettles [11].

Quantitative analyses of the monosaccharide content of the *E. cordatum* jelly hydrolysate were carried out following the procedure described by Flood, Hirst, and Jones [12], and the colorimetric determination described by Blass, Macheboeuf, and Nunez [13].

The jelly was hydrolysed with 0.5 *N* H_2SO_4 for 5 hours in a flask equipped with a reflux condenser and immersed in a boiling water bath. The fucose content [11] after hydrolysis was 33.6 per cent, compared with 34.4 per cent before hydrolysis. The optical rotation of the hydrolysate was strongly negative, indicating that the fucose is in the L-form. No reference sugar [12] was added to the hydrolysate. Instead, the concentrations of the various sugars were related to the concentration of fucose in the jelly before hydrolysis, *i. e.* 34.4 per cent of the dry weight. The hydrolysate was neutralised with $BaCO_3$ and freed from amino acids on columns of ion exchange resins (Amberlites IR 100 and IR 4 B). n-Butanol: water (6 : 1) was used for developing the chromatograms which were run on Munktell OB filter paper sheets. The techniques described by Trevelyan, Procter, and Harrison [14], and by Hough, Jones, and Wadman [15] were employed for the detection and

identification of the sugars. The sugars from the sections of the chromatograms corresponding to galactose and glucose were estimated as galactose, since the amount of glucose was very small, probably less than one third of the galactose content. The values obtained for galactose (+ glucose) were 1.55, 1.53, 1.58, mean 1.56 per cent, and for mannose 0.54, 0.21, 0.65, mean 0.50 per cent, referred to fucose as 34.4 per cent.

Periodate oxidation: Periodate oxidation of the jelly substance was carried out at 50° in a solution prepared as above which was 0.533 millimolar in $NaIO_4$ and buffered to pH 3.5 with a phthalate buffer [16]. About two thirds of the periodate had reacted after twelve hours.

The pH 3.5 was chosen since model studies on the periodate oxidation of maltose, melibiose, a dextrin containing 1,3-linkages, dextran, and other sugars [17] have shown that at this pH the amount of periodate consumed during the initial rapid phase of the oxidation is proportional to the number of α-glycol groupings in the carbohydrate. The rate of "overoxidation" [17–22] is much less at this pH than in more acidic solutions [18] or, in particular, at pH 7 [19]. At known times five-ml samples, equivalent to 10.0 mg of untreated jelly substance, were withdrawn with a pipet and run into 10 ml of ice-cold phosphate buffer of pH 7 [19, 23]. Solid potassium iodide was added and after a few minutes the mixture was titrated with 0.01 *N* thiosulphate. A blank, without jelly substance, was run simultaneously. Fucose was determined in these titrated samples after dialysis, and in the untreated sample, by the method of Dische and Shettles [11], and nitrogen according to Parnas [9]. The effect of dialysis alone on the fucose value was determined on an aliquot to which periodate was not added. This sample was treated with the same amount of phosphate buffer, potassium iodide, and starch as was added to the periodate-treated samples, and a little thiosulphate was added before the dialysis. The results of the oxidations carried out at pH 3.5 are summarised in Table 2. The values for the nitrogen content of the supernatants are not reliable, because it was found out that the cellophane of the dialysis bags under these conditions slowly went into solution, thus contaminating the samples and increasing their nitrogen content. It seems fairly certain that the entire nitrogen content ot the supernatant of all the periodate-treated samples, possibly except of the very first, comes from this source. After the last sample for periodate, etc. determinations had been withdrawn, the remainder of the reaction mixture, equivalent to 50.0 mg of untreated jelly substance, was dialysed. The precipitate and the supernatant were thereafter treated separately. They were hydrolysed with 0.5 *N* HCl in an autoclave for 3 hours at 127°, and the sulphate was precipitated and weighed as $BaSO_4$. The supernatant contained 17.45 per cent of sulphate, and the precipitate gave 1.8 mg (= 1.48 per cent as SO_4) of a brown precipitate, which probably contained some $BaSO_4$ but which was not analysed further.

In another experiment, at pH 1.7, a much stronger periodate solution was used so that there was more than a fivefold excess of periodate even after ten hours. The fucose content was affected in the same way as in the before-mentioned experiment, but the periodate consumption increased rapidly even after the polysaccharide was broken down.

DISCUSSION

The carbohydrate analyses by the quantitative micromethod of Flood, Hirst, and Jones [12] as modified by Blass, Macheboeuf, and Nunez [13] showed the presence of very small amounts of galactose, mannose, and glucose (total

about 2 per cent) as well as fucose (34.4 per cent). The former three sugars were present in this jelly preparation in such low amounts that it appears very doubtful if they really form part of the jelly molecule. It seems more probable that they represent a slight impurity derived from the eggs. This is all the more likely in view of the fact that *E. cordatum* eggs are known to be very fragile.

The analyses of total sulphur and acid-hydrolysable sulphate (Table 1) confirm the results obtained previously with *S. droebachiensis* jelly [24] and *Echinus esculentus* jelly [1] that practically all of the sulphur is present as easily acid-hydrolysable sulphate. The figures for the fucose content and for the content of acid-hydrolysable sulphate (and total sulphur) agree very well with the concept [1] that the polysaccharide part of the jelly is built up of fucose sulphate units (Table 3).

The periodate oxidation gives some very interesting information on the structure of the polysaccharide part of the *E. cordatum* jelly. During the first half hour of reaction at pH 3.5, periodate is consumed very rapidly; thereafter the rate decreases. During the initial rapid reaction half of the fucose residues are destroyed (Table 2), but the remainder are intact even after twelve hours of periodate treatment.

Table 2. Periodate oxidation of the jelly substance of Echinocardium cordatum at pH 3.5 and 50°.

Time of periodate treatment, min	Untreated		1	10	30	60	120	240	360	540	730
	non-dialysed	dialysed									
Periodate consumed, μmol IO_4^-/mg dry subst.			0.289	1.024	1.32	1.50	1.73	2.08	2.43	2.83	3.24
Total fucose, per cent	30.7	25.3	21.5	17.9	13.9	13.9	12.4	14.9	14.9	14.0	12.8
Fucose in supernatant, per cent	29.3	23.4	20.2	15.6	12.2	13.0	10.0	13.0	14.3	13.2	12.5
Nitrogen in precipitate, per cent	4.8	0.7	3.1	4.0	3.5		3.0	2.7		2.1	
Nitrogen in supernatant, per cent		4.5	2.1	1.6	1.7		1.0	1.3		1.3	
Nitrogen in supernatant corrected for contamination from dialysis bag (see text), per cent		ca. 3.3	<1.0	<0.3	<0.4		0	0		0	

Further at pH 1.7 half of the fucose residues are destroyed during the first half hour, but no more are oxidised in spite of the large excess of periodate present. However, the plot of periodate consumed against time shows no break after 30 minutes so that in this case the periodate consumption cannot be used to calculate the number of α-glycol groupings in the jelly substance.

On the other hand, the experiment carried out at pH 3.5 lends itself better to such calculation as there is a pronounced break in the rate of periodate consumption, which occurs simultaneously with the termination of the breakdown of the fucose residues in the jelly substance. If the reacting fucose residues each consume one molecule of periodate (see below), then the periodate consumption should be 0.934 μmol per mg of dry substance. The actual periodate consumption at this point (30 minutes) is however 1.32 μmol per mg and this fact indicates that structures other than the polyfucose sulphate consume periodate as well. The galactose, glucose, and mannose, present as impurities (see above), would consume not more than 0.23 μmol of periodate per mg of dry substance, probably considerably less, if they are in the form of neutral polysaccharides. Therefore, other components of the jelly must be considered to be oxidised by the periodate.

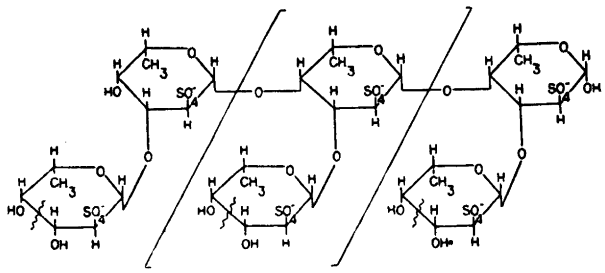
This assumption is substantiated by the observations that a precipitate is formed in the jelly solution during the course of periodate oxidation. This precipitate first becomes evident after one hour, and its formation seems to be essentially complete after four hours. When these samples were dialysed (for the fucose determination), a flocculent precipitate was formed in all the samples except the non-oxidised sample in which comparatively little precipitate was formed. Part of this precipitate seemed to consist of retrograded starch (from the periodate titration). However, the analyses of the clear supernatants and of the precipitates (Table 2) show that, whereas practically all of the fucose is in the clear solution, the major part of the nitrogen is contained in the precipitate. Moreover, the nitrogen values indicate a slow decrease in the nitrogen content of the precipitate during the periodate treatment. This indicates that the protein component is attacked by the periodate and precipitated in denatured form. Desnuelle, Antonin, and Casal [25] found that egg albumin, when treated with periodate at pH 5.5 and 27°, was partly oxidised and precipitated. The precipitated albumin did not contain any cysteine and cystine and also the tryptophane and tyrosine contents were reduced. On the other hand, the methionine, threonine, and serine contents remained practically constant.

Acid-hydrolysable sulphate analyses showed that about 95 per cent of the original sulphate was in the supernatant when the periodate oxidation was complete. This together with the fucose analyses again demonstrates that each

fucose residue carries one sulphate group. Furthermore, it demonstrates that the sulphate linkage is not split during periodate oxidation, even in the oxidised fucose residues.

The fucose content before and after oxidation with periodate indicates that the fucose sulphate residues in the polysaccharide cannot be linked in an unbranched chain since in such a structure only one or both (or possibly none) of the terminal residues have the unsubstituted α-glycol grouping which is a prerequisite for attack by the periodate ion [26].

This leaves as the most probable structure a branched polysaccharide chain. If it is assumed that the fucose residues are in the pyranose form, and that the sulphate groups are attached to the same carbon atoms of the fucose residues in both the main chain and the branches, only a few structures will account for the analytical results.



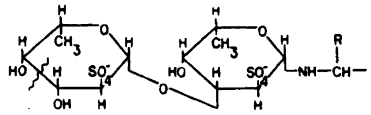
I

One of the possible structures is shown in I, in which the points of attack by the periodate ion are indicated with the wavy lines. The configuration of the glycosidic linkage is not known. Two other possibilities are (1) a main chain linked by 1 : 3-linkages with the branching point at C_2 and with the sulphate at C_4 both in the main chain and in the branches; (2) the same main chain but with the branching point at C_4 and the sulphate at C_2.

The structure of the polyfucose sulphate, fucoidin, extracted from the brown seaweed, *Fucus vesiculosus*, appears to be quite dissimilar [27]. Hydrolysis of methylated fucoidin gave L-fucose (ca. 1 part), 3-methyl L-fucose (ca. 3 parts), and 2 : 3-dimethyl L-fucose (ca. 1 part). The sulphate was considered to be linked to C_4. Only one fucose residue of five would thus be oxidised by periodate. The periodate consumption by the fucoidin prepared from *Himanthalia lorea* [28] corresponds to the breakdown of one fucose residue of between three and four monosaccharide units [27].

However, the results on the *E. cordatum* jelly may be interpreted in still another way. The fucose sulphate residues might be attached to the protein in

a similar fashion to the way in which, according to Aminoff and Morgan [29], the fucose residues of the blood group A substance are linked to hexosamine: (L-fucose)-(L-fucose)-(N-acetylhexosamine) —. In such a structure there would be one terminal fucose sulphate residue which is destroyed by periodate and another residue which cannot be attacked by periodate since it has no free α-glycol grouping (II).



II

This structure would be in line with the interpretation by Tyler [7] that the "protein and polysaccharide constituents do not exist as such in the molecule but rather that the various sugars and amino acids are interlinked". However, the formation upon periodate oxidation of a precipitate, which most probably consists of denatured protein, and of a clear supernatant, which contains the polysaccharide, confirms the hypothesis that the jelly substance is composed of a protein and a polysaccharide component.

It seems rather likely that the fucose sulphate residues form a polysaccharide with a structure similar to that depicted in I. This polysaccharide is then attached to the protein by comparatively strong linkages [1, 7]. One of these linkages may be of the kind shown in II. Isbell and Frush [30] have shown that the hydrolysis of arabinosylamine, which contains such a linkage, proceeds at a maximum rate at pH 5. It is a remarkable fact that the jelly coat of most sea-urchin species can be dissolved at about this pH.

Table 3. Fucose, sulphate, and nitrogen contents of the Echinocardium cordatum jelly preparations used in this study.

Preparation	Collected	Fucose		Sulphate		Poly-fucose sulphate per cent	Nitrogen per cent	Protein N × 6.25 per cent
		per cent	mmoles/g	per cent	mmoles/g			
Ec 49 : 1	July 5 to 20	30.7	1.87	18.4	1.92	46.5	4.8	30
Ec 49 : 2	July 19 to 28	34.4	2.10	20.6	2.15	52	3.4	21
Ec 49 : 3	July 29 to August 9	43.0	2.62	22.5	2.34	57—64	4.0	25

The data in Table 3 indicate the possibility of a seasonal variation in the composition of the jelly coat. In the beginning and middle of July, when the eggs are underripe, the content of polyfucose sulphate is low (Ec 49 : 1); it increases slightly during the latter part of the month (Ec 49 : 2) and attains its maximum value during the height of the spawning season (Ec 49 : 3). However, the data indicate also that about 23, 27, and 15 per cent, respectively, of the jelly substance are not accounted for in these preparations. Therefore, further investigations will be needed to prove the seasonal variation. On analogy with similar substances it seems probable that most of the unidentified material is bound water [cf. 28].

SUMMARY

The jelly coat substance of the *Echinocardium cordatum* egg consists of polyfucose sulphate in combination with protein. On periodate oxidation (at pH 3.5 and 1.7) half of the fucose residues are destroyed rapidly, but the remaining residues are not attacked even after prolonged periodate treatment.

This result suggests that the polysaccharide is built up in a comb-like fashion with one main chain of fucose sulphate residues each of which carries another fucose sulphate residue as a side chain.

The protein component is oxidised by the periodate as well and is precipitated during the reaction. The oxidised polysaccharide, on the other hand, remains in solution.

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