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Properties of Highly Purified Fucan*

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Fucan was first isolated by Kylin from brown seaweed and named fucoidin by him (1). More recently fucan was isolated by Percival and Ross (2) from several Phaeophyceae. They considered it to be a polymeric salt of fucose monosulfate. However, their most highly purified preparation still contained approximately 5.6% sugars other than L-fucose and 3.3% uronic acid. A branched structure was suggested (3) for fucan with 1,2 links predominating (3, 4). The molecular weight of fucan determined by osmometric means was 133,000 ± 20,000 (4).

Anticoagulant active fractions were isolated from crude fucan in a yield of about 25% (5). Other fractions, although less active as anticoagulants, were potent lipemia clearers (6). All of these fractions contained a still unidentified sugar with chromatographic properties similar to but not identical with 3-*O*-methylfucose (5, 7).

In the present work, highly purified fractions were prepared from crude fucan and their physical, chemical, and biological properties investigated. It is hoped that the study of physical behavior and the chemistry of these fucan fractions will contribute to a better understanding of the relation of biological activity to physical and chemical characteristics of fucan and other sulfated high polymers.

EXPERIMENTAL PROCEDURE

Preparation of Highly Active Fractions—Crude fucan, prepared from *Fucus vesiculosus* as previously described (5), was made to 2% in water and fractionated with ethanol containing 0.3% sodium acetate. Material precipitating between 47.5 and 59% ethanol concentration showed the highest anticoagulant activity. After repeated ethanol subfractionating, the fraction precipitating between 50 to 59% ethanol (16.77 g) was collected and reprecipitated with acetone as described; 7 g of the material settling out at 55 to 60% acetone concentration were dissolved in 1.5% concentration in water. The invert soap (8) cetyldimethylbenzylammonium chloride (Winthrop-Stearns, Inc.) was added dropwise at room temperature (23–25°) to a final concentration of 0.5%. The precipitate was collected by centrifugation and suspended (2% dry weight) in 2 M aqueous $CaCl_2$. After mechanical shaking at room temperature for 6 hours, the mixture was centrifuged for 20 to 30 minutes at 2500 r.p.m. The clear middle layer was siphoned off and the residues re-extracted with the same volume of 4 M $CaCl_2$ and the soluble middle layer again removed. These solutions were then treated at room temperature with ethanol (final concentration, 77%). The precipitates were redissolved and dialyzed (Visking tubing, average pore size 48 A) under toluene and chloroform against 100 volumes of distilled water for 72 hours at 2–4°, the water being changed every 24 hours. Small amounts of sediment were then removed from the dialyzed solution which was subsequently precipitated with 10 volumes of 100% ethanol. The 4 M $CaCl_2$ extract showed highest activity and 140 mg of it were ultracentrifuged in a preparative Beckman-Spinco SL ultracentrifuge at various speeds. Virtually all anticoagulant activity resided in the supernatant remaining after centrifugation at 105,400 × *g*. About 70% of the total that was ultracentrifuged could be recovered in this fraction. This is approximately 2.5% of *purified* fucan before treatment with quaternary ammonium salt. This fraction was designated as Ca 408.

Fractions Ca 409 and Ca 410 differ in their way of preparation from Ca 408 in that crude fucan was first precipitated with invert soap. After dissolution of the fucan-invert soap complex with 4 M $CaCl_2$, the soluble fucan was precipitated with ethanol. Fractions obtained between 60 and 70% ethanol concentration under standard conditions were most active. Seven grams of this material were reprecipitated with invert soap as described for Fraction Ca 408. The most active materials obtained were those which precipitated at 0.5% and 1% final quaternary ammonium salt concentration and which were dissolved with 2 M (Ca 409) and 4 M (Ca 410) $CaCl_2$, respectively. These preparations were ultracentrifuged. Distribution of activity and yield was similar to that described for Fraction Ca 408.

Chemical Determinations—Phosphorus was determined according to the method of Zilversmit and Davis (9), calcium according to Clark and Collip (10), sodium and potassium by means of a Baird flame photometer, and nitrogen was determined by a micro-Kjeldahl method (11). Methylpentose was measured spectrophotometrically (12) with a Beckman DU spectrophotometer. L-Fucose (Mann Biochemicals) recrystallized from methanol was used as the standard.

Content of easily removable water was determined after drying in an Abderhalden pistol at 80° over P_2O_5. Drying to constant weight at 80° in a vacuum was also done at Huffman Microanalytical Laboratories, Wheatridge, Colorado. As constant weight was reached rather fast, no Fischer (13) water determination was carried out, but the following additional analyses were made at the Huffman Laboratories: carbon, hydrogen (both Pregl method), sulfur (Schoniger method), sulfated ash, and alkoxyl.

Fractions Ca 409 and 410 (2.5 mg per ml) were hydrolyzed in 1 N H_2SO_4 in sealed tubes at 100° for 7 hours and then dialyzed (Visking tubing) in the cold (toluene added) against 2 times 150

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TABLE I

*Analytical data on highly purified fucan fractions**

Component analyzed	Fraction		
	Ca 408	Ca 409†	Ca 410‡
	%	%	%
Sulfur	10.36	9.15	
Carbon	24.68	27.72	30.02
Hydrogen	4.24	4.25	4.83
Residue (C—H)	28.8 (not alkaline)	23.8 (alkaline)	
Nitrogen	None demonstrable	None demonstrable	None demonstrable
Alkoxyl		0.06	
Ash (with H_2SO_4)	28.4	29.24	
Ca^{++}		6.76	7.04
Na^+		2.94	1.05
K^+		0.02	0.05
Methylpentose		63	65

* All data obtained on vacuum-dried material (80°, 0.1 mm, P_2O_5).

† $[\alpha]_{D\,1\,dec}^{27}$ −137° (c 1.0 in water).

‡ $[\alpha]_{D\,1\,dec}^{27}$ −140° (c 0.8 in water).

volumes of distilled water. The dialysates were concentrated under reduced pressure, whereas the dialyzed solutions (referred to as "cores") were rehydrolyzed another 6 hours with 2 N H_2SO_4 (final concentration). All hydrolysates were neutralized to pH 5.5 with stoichiometric amounts of $Ba(OH)_2$ in the presence of a few drops of glacial acetic acid. The samples were then treated with small amounts of Duolite C-20 (H^+) and IR-45 (OH^-) resins. Paper chromatographic analysis was performed by the descending technique on Whatman No. 1 paper. The following three solvents were used: *n*-butanol-ethanol-water (5:1:4) (14), *n*-butanol-pyridine-water (6:4:3)[1], and phenol-water (14); spraying reagent was aniline-oxalate (15).

Physical Determinations—1. Sedimentation. A Spinco model E analytical ultracentrifuge with a temperature control unit was used. Sedimentation velocity experiments were carried out at 59,780 r.p.m. in a conventional 12-mm cell. The solvent was sodium phosphate buffer, pH = 6.8, μ = 0.6 (containing 0.2 M NaCl) and polysaccharide concentrations were between 0.2 and 0.7%. Concentrations were determined after exhaustive dialysis by drying measured volumes of both solvent and solution to constant weight at 105° and atmospheric pressure. Ultracentrifuge patterns were traced on 6× (linear) enlargements and the boundary displacement was measured. Sedimentation constants were then computed from plots of log x (x being the boundary displacement) *versus* t (time). These were corrected to standard conditions and extrapolated to zero concentration.

2. Diffusion. Owing to the limited amount of material available, diffusion measurements were carried out in the Spinco ultracentrifuge at 7928 r.p.m., with the use of a synthetic boundary cell (capillary type). The polysaccharide concentration was between 0.4 and 0.5% in the same solvent as that used for the sedimentation experiments. Fifteen photographs were taken at regular intervals during 4 hours. Schlieren patterns were enlarged, areas were measured with a mechanical integrator, and the diffusion constant was computed from plots of $(A/H)^2/4\pi$ *versus* t (A being the area under the peak, H the height of the peak). This was then corrected to standard conditions.

From the sedimentation and diffusion constants, the molecular weight was calculated using Svedberg's formula $M = sRT/D(1 - \bar{v}\rho)$, where s is the sedimentation constant, R the universal gas constant, T the absolute temperature, D the diffusion constant, $\bar{v}$ the partial specific volume (calculated to be 0.53 from the analytical data and the densities of chemical components of fucan (*cf.* reference 16); electrostriction effects were not taken into account), ρ the solvent density.

3. Infrared spectroscopy. A Perkin-Elmer model 21 double beam spectrograph with a NaCl prism was used. Aqueous solutions of fucan were freeze-dried and the pressed KBr disk technique was used to prepare specimens.

4. Electrophoresis. Electrophoretic experiments were performed at 1° in a Strubin apparatus, equipped with both schlieren and interferential optics. Fucan was used as a 0.7% solution in sodium acetate buffer pH = 5.0, μ = 0.1. The current density was 13.35 ma per cm². Boundary displacements were measured on 7× enlargements of the photographic plates and the mobilities were computed from these values and the conductivity of the solvent at 1°.

Biological Determinations—Clotting time and recalcification time were determined with standard procedures (17). Antithrombin activity was measured by adding fucan and heparin to 0.1 ml of a 1% bovine fibrinogen solution (Armour Laboratories) and determination of inhibition of transformation of fibrinogen to fibrin after the addition of 10 units (0.1 ml) of topical bovine thrombin (Parke-Davis Laboratories). All biological activities are expressed on the basis of weight of vacuum-dried (over P_2O_5) material and are compared to those of a 1% solution of Na-heparinate (Abbott Laboratories 1000 U.S.P. units per ml). Blood used for these studies *in vitro* was obtained rom healthy human donors selected at random.

RESULTS

General and Chemical—The fucan fractions rapidly lost between 18 and 20% of weight upon drying at 80° (0.1 mm over P_2O_5). Weight constancy was reached in less than 20 hours. The material dissolved readily at concentrations of 3% (weight per volume) in water, in physiological saline (0.85%), and all buffers tested. It gave a clear, slightly yellowish solution. Phosphorus and nitrogen were not demonstrable.

The fractions (0.1% in water) showed no specific absorption bands between the wave lengths of 240 and 320 mμ. The optical rotation of the different fractions was strongly *levo* and is listed together with the analytical data in Table I.

Paper chromatograms of hydrolysates of Fractions Ca 409 and 410 showed that Ca 409 consisted almost entirely of fucose.[2] There were traces of galactose and a small amount of an unidentified "fast" component migrating at a rate nearly equal to that of 3-*O*-methylfucose. A trace of xylose was also detected, when large quantities of hydrolysate (600 to 800 μg) were spotted.

[1] R. L. Whistler, personal communication.

[2] The fucose in fucan has been characterized as the L-enantiomorph (*cf.* references 1–4). In the present study, the fucose of the hydrolysates was eluted from chromatograms and tested for its activity as blood group H(O) hapten (18–20). The eluate was found to be as active as authentic L-fucose, indicating that it was predominantly or exclusively the L-enantiomorph. D-Fucose is inactive in this test.

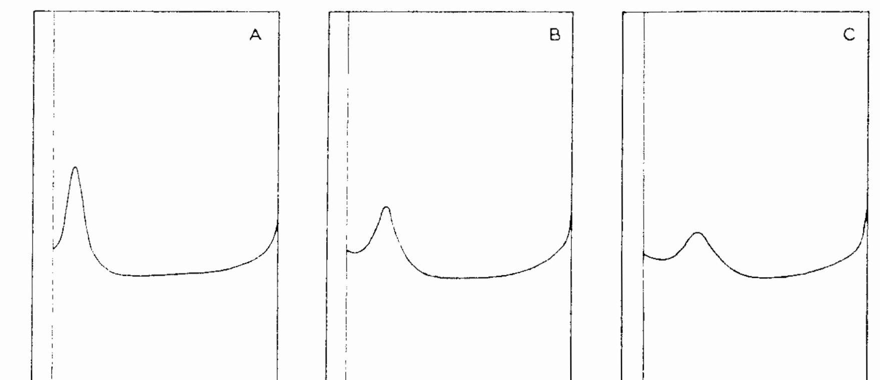

FIG. 1. Sedimentation pattern of fucan (Fraction Ca 410), c = 0.66%, in sodium phosphate buffer, pH 6.8, ionic strength 0.6 (0.2 M NaCl); 30 (*A*), 46 (*B*), and 62 minutes (*C*) after start; 59,780 r.p.m.

In contrast to earlier reports (*cf.* reference 2), uronic acid or mannose were not demonstrable.

The hydrolysate of the "core" of Fraction Ca 409 yielded, in addition to small amounts of fucose, an unidentified component, probably an oligosaccharide which migrated slightly slower than lactose in all three solvents. The "core" of Fraction Ca 410 showed the same pattern, whereas the material obtained by the first hydrolysis of Fraction Ca 410 contained somewhat more galactose than the corresponding sample of Fraction Ca 409.

Physical—1. Sedimentation. The fucan fractions showed only one sedimenting boundary in the ultracentrifuge. A slight degree of asymmetrical spreading of the boundary during the sedimentation (Fig. 1) suggested the existence of some polydispersity of the preparations. Fraction Ca 410 showed a sedimentation constant $s^0_{20,w}$ = 4.5 S. Sedimentation was concentration dependent, the relationship between s (sedimentation coefficient) and c (concentration) being linear, with ds/dc = −1.2 S. Fraction Ca 409 exhibited substantially the same sedimentation constant as Fraction Ca 410. For Fraction Ca 408, a lower sedimentation constant of $s^0_{20,w}$ = 2.9 S was found at one finite concentration (c = 0.66%), the corresponding value for Fraction Ca 410 being $s^0_{20,w}$ = 3.7 S.

2. Diffusion. Fraction Ca 410 had a diffusion constant $D_{20,w}$ of 3.0 F (c = 0.50%). The boundary was not remarkably skewed (Fig. 2) and no significant decrease of the area under the peak was observed to take place during the experiment.

By introducing the sedimentation and diffusion constants obtained for Fraction Ca 410 into Svedberg's formula, a molecular weight $M = 7.8 \times 10^4$ was obtained.

From the above data a value of f/f_0 = 2.8 could be computed for the frictional ratio of the macromolecule.

3. Electrophoresis. Fraction Ca 409 showed one migrating boundary on the descending limb; its mobility was -27×10^{-5} cm²/V/sec. In the ascending limb, beside the main peak showing a mobility of -28×10^{-5} cm²/V/sec, a minor component was also detectable with mobility of -17×10^{-5} cm²/V/sec.

The physical data mentioned above are summarized in Table II.

4. Infrared spectroscopy. The infrared spectrum of fucan (Fraction Ca 410) is shown in Fig. 3. Its main features are: (*a*) the strong absorption at 1000 to 1200 cm⁻¹, due to C═O stretching and C—O—H bending, which is characteristic of all sugar derivatives; (*b*) the very strong band at 1250 cm⁻¹ caused by S═O stretching vibrations; (*c*) the band at 840 cm⁻¹ and the shoulder at 820 cm⁻¹ to be ascribed to the sulfate group; (*d*)

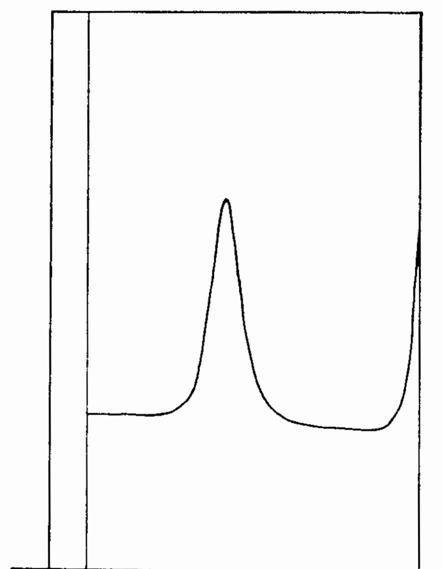

FIG. 2. Diffusion pattern of fucan (Fraction Ca 410), c = 0.50%, in sodium phosphate buffer, pH = 6.8, ionic strength 0.6 (0.2 M NaCl) at 62 minutes after start; 7928 r.p.m.

TABLE II
*Summary of physical data for fucan**

$s^0_{20,w}$ (Svedberg units)	4.5
ds/dc (Svedberg units/1%)	−1.2
$D_{20,w}$ (Fick units)	3.0 (c = 0.5%)
$\bar{V}$ (ml/g)	0.53 (calculated)
Molecular weight	7.8×10^4
f/f_0	2.8
Electrophoretic mobility (-10^{-5} cm²/sec/V)	27

* Reported values are for Fraction Ca 410, except electrophoretic mobility, which was measured on Fraction Ca 409.

the absorption at 1640 cm⁻¹, which can almost certainly be attributed to the presence of moisture in the sample as shown by the decrease of this peak on samples extensively dried over phosphorous pentoxide.

Biological—The inhibition *in vitro* of human blood clotting and recalcification of human plasma are shown in Figs. 4 and 5. The three fucan preparations are of similar activities, Fraction Ca 408 being the most active one. These fractions possess 60 to

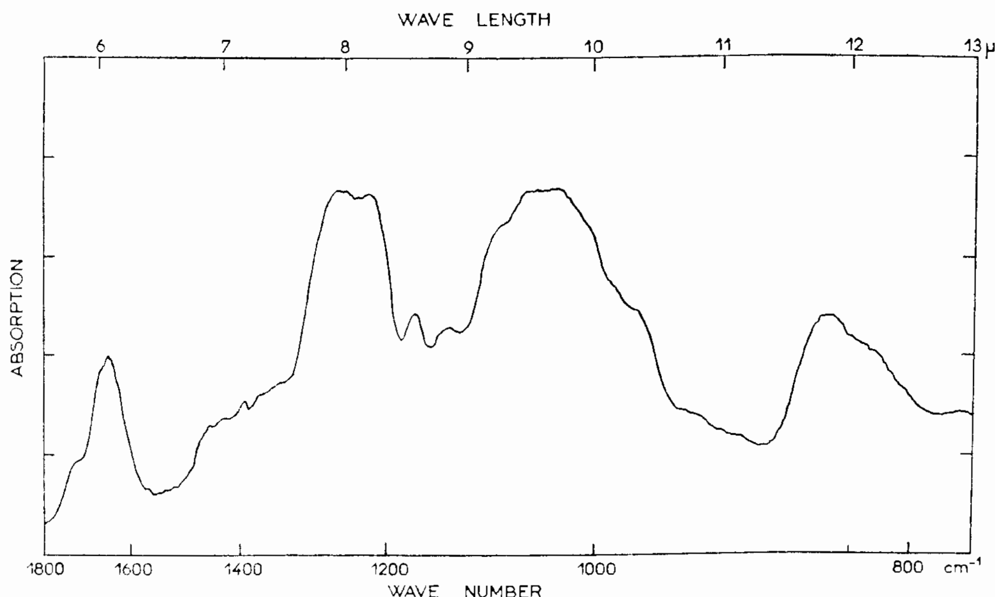

FIG. 3. Infrared spectrum of fucan (Fraction Ca 410)

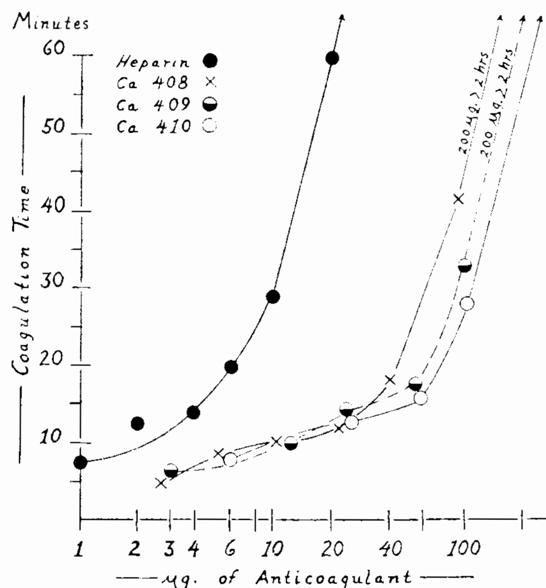

FIG. 4. Influence of fucan and heparin on coagulation of whole human blood.

80% or more of the activity of heparin in the recalcification test and 15 to 18% heparin activity in the whole human blood coagulation inhibition assay. The fucan fractions delayed the action of thrombin on fibrinogen considerably more than heparin (Fig. 6).

DISCUSSION

The purification method described here has yielded products of high biological activity in which no significant inhomogeneity could be demonstrated physically and chemically. Chemically this is indicated by the high L-fucose and sulfur content and by the absence of more than trace amounts of sugars other than L-fucose. The high sulfur content of the most active Fraction Ca 408 amounts to over 90% of that of the best heparin (21). It is noteworthy that an unidentified carbohydrate of chromatographic characteristics similar to that of methylethers of L-fucose (22) has been regularly detected in hydrolysates of the most highly purified fucan fractions. The release of somewhat different carbohydrate components from the "core" upon prolonged hydrolysis points to structural differences of the remaining material. It is conceivable that this represents in part contaminants which may be more resistant to acid hydrolysis. No investigation has been made as to the degree of sulfation of the core after the first hydrolysis.

The results of hydrodynamic measurements indicate a molecular weight $M = 7.8 \times 10^4$ for fucan (Fraction Ca 410). This estimate cannot be accepted without some reserve because of the incertitude in the value of the partial specific volume. Furthermore, the diffusion constant was obtained from experiments carried out in the ultracentrifuge cell and was not extrapolated

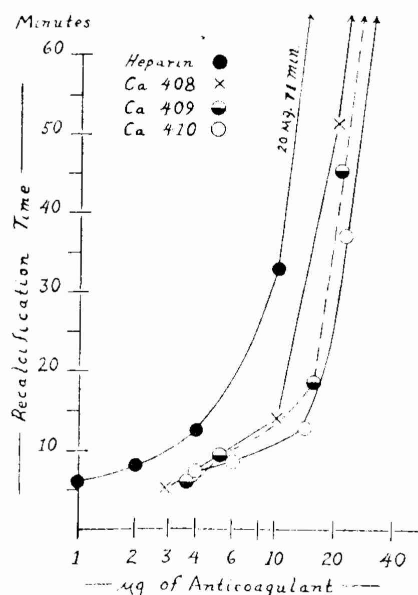

FIG. 5. Influence of fucan and heparin on human plasma recalcification.

to zero concentration. The correct value for this constant at infinite dilution is expected to be slightly lower; therefore the molecular weight should be slightly higher. A remarkably lower molecular weight is suggested for Fraction Ca 408 by its lower sedimentation constant.

Despite the uncertainties, the molecular weight obtained in the present investigation by sedimentation and diffusion for the fucan samples is definitely lower than the value reported by O'Neill (4) on the basis of osmometry (133,000 ± 20,000). No definite statement can be made at present about the molecular shape of the polysaccharide under study, as it is hazardous to rely on the frictional ratio for estimation of the molecular asymmetry and hydration in polydisperse systems. There is, however, little doubt that a branched coil is the model which best describes the fucan molecule. It is quite possible that branching contributes a certain degree of rigidity to the molecule.

It is also difficult to account for the very high electrophoretic mobility of fucan. The value found in the present work is somewhat higher than that reported by O'Neill (4). It is noteworthy that this value is much higher than that reported for carrageenans (23).

The infrared spectrum of fucan gives strong indications as to the position of the sulfate group in the sugar ring. It has been suggested by Orr (24) that a sulfate band around 840 cm^{-1} is related to an axial position of the sulfate group, whereas an equatorial position would give rise to a band near 820 cm^{-1}. The correctness of this suggestion has been shown by examination of the infrared spectra of a number of sulfated polysaccharides (25). In fucan the sulfate band is close to 840 cm^{-1}, but a shoulder is evident at about 820 cm^{-1}. It is inferred that most of the sulfate groups in fucan are on the C-4 of fucose (the only C atom in the molecule with an axial hydroxyl), which is in agreement with chemical data (3). It is also suggested that a smaller number of sulfate groups are in an equatorial position. This conclusion is supported not only by the shoulder at 820 cm^{-1} but also by results obtained in investigations on carrageenan. The infrared spectrum of this polysaccharide (26) is very similar to that of fucan in the region 800 to 900 cm^{-1}, and existing chemical evidence indicates that a large proportion of the galactose residues are disulfated (27). Therefore, besides the axial sulfates on C-4, equatorial sulfates also are present.

In comparing the biological activities of the fucan fractions with those of commercial heparin the qualitative similarity is striking. Quantitatively, fucan was more potent than heparin in only one of the three test systems *in vitro*, namely, as inhibitor of the fibrinogen to fibrin transformation. The biological similarity is especially remarkable because fucan, in contradistinction to heparin, contains no sulfoamino groups, is nitrogen free, has 3 or 4 times the molecular size of heparin, and has an optical rotation opposite to that of heparin. Furthermore, the carbohydrate building stones for fucan appear to be exclusively or almost exclusively fucose units linked predominantly 1,2 (3, 4), whereas in heparin the basic structure is glucosamine-glucuronic acid units alternating in 1,4 and 1,3 linkages (28). Also there are somewhat more sulfate groups in the heparin molecule

Other naturally occurring sulfated nitrogen-free polysaccharides may show inhibition of blood coagulation and indeed sulfuric acid esters of galactan have been demonstrated to possess anticoagulant activity (29, 30). However, attempts in this laboratory to isolate anticoagulant active fractions from the red seaweed *Chondrus crispus* or from commercial carrageenan

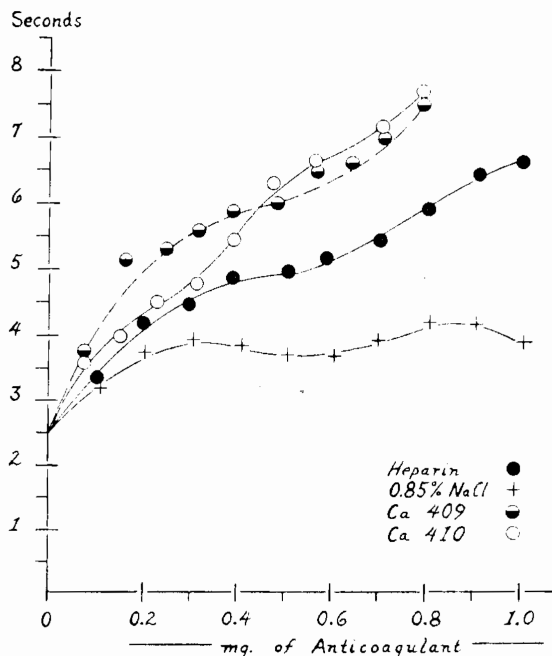

FIG. 6. Inhibition of thrombin-fibrinogen reaction

have yielded products, the most active of which had less than 10% of the anticoagulant activity of the fucan fractions described here.[3]

SUMMARY

The preparation of highly purified fucan fractions from *Fucus vesiculosus* has been described and some of their physical, chemical, and biological properties have been investigated. The material is essentially homogeneous, and contains approximately 10% sulfur, 65% L-fucose and one unidentified "fast sugar." From sedimentation and diffusion measurements, a molecular weight of about 7.8×10^4 has been calculated. In salt solution the macromolecule can best be depicted as a highly charged random coil. The electrophoretic mobility is very high (-27×10^{-5} $cm^2/sec/V$; pH 5). The infrared spectrum suggests that axial sulfate groups on C-4 predominate but a small number of equatorial sulfates are also present. The significant anticoagulant properties of fucan fractions have been compared with those of heparin.

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