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Carbohydrate Sulfates

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Richard G. Schweiger, EDITOR
Stauffer Chemical Co.

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Robert F. Gould, *Editor*

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the SERIES parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. As a further means of saving time, the papers are not edited or reviewed except by the symposium chairman, who becomes editor of the book. Papers published in the ACS SYMPOSIUM SERIES are original contributions not published elsewhere in whole or major part and include reports of research as well as reviews since symposia may embrace both types of presentation.

PREFACE

At first glance, the subject "Carbohydrate Sulfates" appears to be so specific and so limited that one may think of it as a small field which can be covered thoroughly in a few articles. The title, however, is misleading, and there is considerably more to it than the title implies.

For example, there are polysaccharide sulfates, some of which occur naturally, such as carrageenan, furcellaran, agar, etc., and others, such as the cellulose and starch sulfates, are obtainable only synthetically. Other classes, like the sulfated glycoproteins and sulfated glycolipids, have important biological functions. And there are typical low molecular weight carbohydrate sulfates, many of which occur naturally in plants while others are accessible by synthesis only.

Such a variety of compounds obviously requires more than straightforward carbohydrate chemistry. Polysaccharide sulfates are typical polyanions and have colloidal characteristics. Consequently, they involve polyelectrolyte and colloid chemistry. The researcher working with sulfated glycolipids and glycoproteins must be knowledgeable in the general areas of lipids and proteins. Studying biological functions either in the body or in the plant requires familiarity with biological and medical sciences. Many more examples of the complexity of the field of carbohydrate sulfates could be given.

This symposium, therefore, cannot possibly present all of today's research in this field, not even a major portion of it. However, it does represent a nice cross-section of present research activities. Together with the references cited, the articles provide a good up-to-date overview of the various areas discussed. Hopefully, the symposium will stimulate the search for additional indepth knowledge, for only basic information of this kind will ultimately create new products for our industry, permit us to cure and prevent diseases, and, some day perhaps, let us understand nature and life more fully.

I wish to thank all of the participants, scientists from abroad and from this country, for their efforts and their contributions to bring this symposium to a successful conclusion.

Stauffer Chemical Co.
San Jose, California
February, 1978

RICHARD G. SCHWEIGER

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Monosulfate Esters of L-Ascorbic Acid

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L-Ascorbate 2-Sulfate. In 1972 Halver and his colleagues (1) reported L-ascorbic acid 2-sulfate (AA2S, Fig. 1) was as effective as L-ascorbic acid (AA) in preventing scurvy in rainbow trout and coho salmon. Prior to that time, Ford and Ruoff (2) synthesized AA2S and showed the sulfate ester was more stable than AA to oxidative degradation. Those two reports stimulated the interest of C. W. Deyoe in AA2S because he was engaged in research on formulating catfish feed at Kansas State University. Fish feed is often produced in floating form by extrusion puffing, a process that destroys 70-80% of the L-ascorbic acid in the feed (3). In addition, L-ascorbate 2-sulfate appeared attractive as a potential form of vitamin C since AA2S occurs in Nature; it is found in the dormant embryo of brine shrimp (4,5), in human urine (6), and in rat liver, spleen, adrenal gland, and urine (7,8). Since there was no commercial supply of AA2S, Prof. Deyoe asked that our group produce a large amount of AA2S. A chronology of events concerning L-ascorbate 2-sulfate is given in Table I.

The biological significance of AA2S has not been established. Its antiscorbutic activity appears species-dependent. AA2S is inactive in the guinea pig (14,15), but is active in fish and marginally active in the rhesus monkey (23). It is also partially active in insect diets (24). No data are available on man.

It has been suggested that AA2S may serve as a storage form of AA, or as a direct sulfate donor in the biosynthesis of sulfate esters (20,22). Wholebody radioautographs of fish intubated with radioactive L-ascorbate 1-^{14}C or L-ascorbate 2-sulfate-S^{35} showed the radioactivity of both compounds was concentrated in the same organs, mainly the skin, fins, lower jaw-plate, kidney, liver, and heart (25). However, in rats and guinea pigs (8), the uptake of AA by organs was different than observed for AA2S.

To obtain large amounts of AA2S we first modified (16) the original procedure of Ford and Ruoff (2) and produced AA2S in 75% yield starting from 5,6-O-isopropylidene-L-ascorbic acid. But we also found (16) a much better method to prepare AA2S (Figure 2) with the following attractive features; (a) the starting material

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Table I. Chronology of L-Ascorbate 2-Sulfate

Date	Reference	Event
1965	Ford and Ruoff (2)	Synthesis of 5,6-*O*-isopropylidene-L-ascorbate-2-sulfate.
1968	Chu and Slaunwhite (9) and Mumma (10)	*In vitro* oxidative sulfation of *n*-octanol and androsterone during mild oxidation of L-ascorbate 2-sulfate (AA2S) in non-aqueous media.
1969	Mead and Finamore (4)	Identification of AA2S in Nature from undeveloped cysts of brine shrimp.
1971	Baker et al. (6,11)	Discovery of AA2S in human urine; daily excretion reported to be 30-60mg of the dipotassium salt.
1972-3	Halver et al. (1,12)	Prevention of scurvy in coho salmon and rainbow trout by equivalent amount of AA2S or L-ascorbate.
1972-3	Bond et al. (5) and McClelland (13)	Determination of X-ray crystallographic structure.
1973-4	Kuenzig et al. (14) and Campeau et al. (15)	AA2S gave no antiscorbutic activity in guinea pigs.
1974	Seib et al. (16)	Synthesis of AA2S improved.
1974	Carlson et al. (17), Hatanaka et al. (18) and Roy, et al. (19)	Isolation of AA2S sulfhydrolase from a marine organism and and mammalian liver.
1975-6	Hatanaka et al. (20) and Fluharty et al. (21)	Incorporation of sulfate from AA2S into choindroitin sulfate. Discovery of widespread distribution of AA2S sulfhydrolase.
1976	Mohamaram et al. (22)	Demonstration of L-ascorbic acid sulfotransferase activity in rat liver and colon homogenates which catalyzed formation of AA2S-^{35}S from L-ascorbic acid and 3'-phosphoadenyl-sulfate-^{35}S or sodium sulfate-^{35}S.

is L-ascorbic acid, (b) the reaction solvent is water, (c) the conversion of AA to AA2S is 98%, (d) the sulfation reaction is rapid (< 30 minutes), (e) the sulfating agent is the sulfur trioxide complex of the least expensive tertiary amine (trimethylamine), (f) the isolation of AA2S is simple, and (g) analytically pure crystals of barium L-ascorbate 2-sulfate are isolated in 80% yield. Obviously, the chemical synthesis of AA2S presents little difficulty (16).

L-Ascorbate 6-Sulfate (AA6S). L-Ascorbate 6-sulfate (AA6S) is an intermediate (not isolated) in the production (26) of L-ascorbyl 6-palmitate, a material that is used mainly to prevent oxidative rancidity (27) in fats, oils, and dehydrated foods. In addition, L-ascorbyl 6-palmitate is a useful additive in breads (28) and carotenoid colorants (29).

L-Ascorbyl 6-palmitate is synthesized by reacting L-ascorbic acid (AA) with palmitic acid in concentrated sulfuric acid. The esterification of AA in concentrated sulfuric acid is at first surprising, since L-ascorbic acid is rapidly dehydrated and decarboxylated in hot acid to give almost quantitative yields of furfural (30). But the ring-structure of AA is very stable in concentrated sulfuric acid at room temperature. We previously showed (26) that the absorbance (265nm) of a solution of AA ($\sim 3 \times 10^{-5}$M) in 95-98% sulfuric acid was unchanged over a 46-day period. We also found the absorption maximum for unionized L-ascorbic acid (λ_{max} 245nm at pH 2) shifted to longer wavelength (λ_{max} 265nm) when AA was dissolved in concentrated sulfuric acid, indicating that a delocalized cation had formed in the strongly acidic medium (Figure 3). We further speculated that the primary hydroxyl group of AA is fully sulfated in concentrated sulfuric acid.

Carbon-13 nmr measurements confirmed (26) the formation of the hydroxyallyl cation shown in Figure 3. The ^{13}C-spectra of AA in 99% sulfuric acid and of 4-deuterio-L-ascorbic acid in water at pH 2 are shown in Figure 4. The assignments of the resonance signals were made from the relaxation rates of the carbon atoms and from proton-coupled spectra. The assignments will not be discussed here, since they are the subject of another paper (31).

When AA is dissolved in 99% sulfuric acid, spectrum B in Figure 4 shows the reaction products all have a set of six ^{13}C-signals which closely resembles the set of signals of AA in water. Spectrum B clearly shows the reaction product contains three components which are all closely related to the structure of AA. Thus, AA does not undergo dehydration and polymerization (32) in concentrated sulfuric acid at room temperature.

In this report, we describe the isolation and characterization of the two principal components formed when AA is dissolved in concentrated sulfuric acid, namely, L-ascorbate 6-sulfate (AA6S) and L-ascorbate 5-sulfate (AA5S). Other references to work on AA6S are given in Table II; AA5S has not been reported

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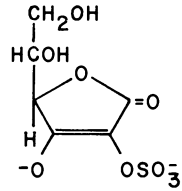

Figure 1. L-*Ascorbate 2-sulfate (AA2S)*

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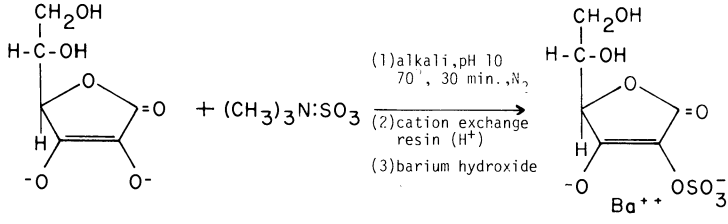

Figure 2. Synthesis of L-*ascorbate 2-sulfate*

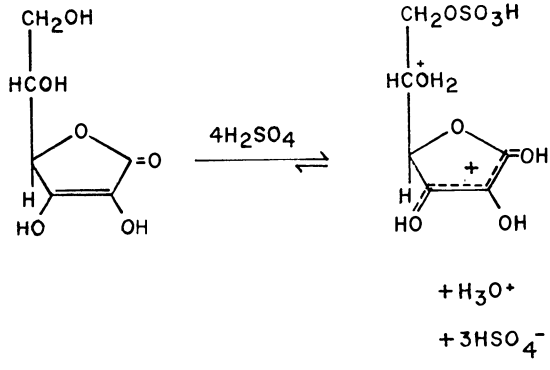

Figure 3. L-*Ascorbic acid in concentrated sulfuric acid*

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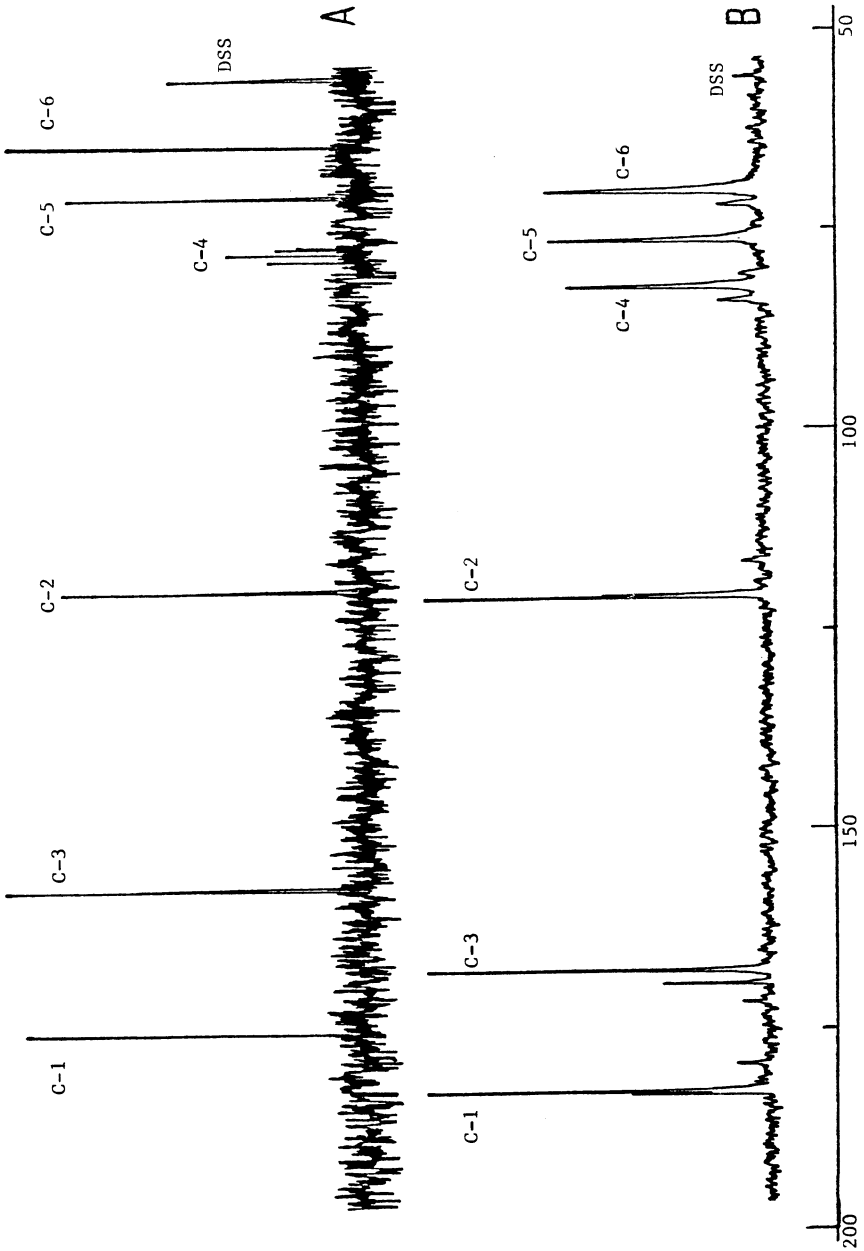

*Figure 4. Fourier transform, carbon-13 spectrum of: (A) 4-deuterio-*L*-ascorbic acid in deuterium oxide at 30°C, and (B)* L*-ascorbic acid in concentrated sulfuric acid at 30°C*

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Table II. Chronology of L-Ascorbate 6-Sulfate (AA6S)

Date	Reference	Event
1970	Allaudeen and Ramakrishnan (33)	Sulfated AA with pyridine-sulfur trioxide complex in pyridine; AA6S isolated by DEAE-cellulose chromatography. Yield not reported. Product chromatographically pure.
1974	Hatanaka, Ogawa, and Egami (34)	Sulfated AA with pyridine-sulfur trioxide complex in *N,N*-dimethylformamide (DMF). AA6S was characterized by p.m.r. and u.v. spectroscopy. Yield not reported
1975	Tolbert (35)	Sulfated AA with pyridine-sulfur trioxide complex in DMF. AA6S was purified by DEAE-cellulose chromatography in 20% yield. The compound had ε 12 x 10^3, λ_{max} 265nm (pH 7.0).

previously.

The isolation of L-ascorbate 6-sulfate was accomplished as follows. A 0.7M solution of L-ascorbic acid in 99% sulfuric acid was quenched by dropwise addition, with stirring, of the acidic mixture to ethyl ether at -65°. After neutralization with aqueous barium hydroxide and removal of ether and barium sulfate, iodimetric titration showed that 74% of the reducing power in the starting material was present in the aqueous phase.

The reaction products were separated (35)by ion-exchange column chromatography on DEAE cellulose using a gradient of very dilute sulfuric acid as eluting solvent (Figure 5). The percentage of each component in the reaction mixture was determined by iodine titration and u.v. absorbance at 265nm (Table III).

Component III, which accounted for 81-85% of the products (u.v. activity) applied to the ion-exchange column, was found to be L-ascorbate 6-sulfate. The barium salt of AA6S was isolated as an amorphous solid in an overall yield of 48% (u.v. on solids). Elemental analysis showed the solid contained 81% barium L-ascorbate 6-sulfate and 19% barium sulfate. No attempt was made to further purify the solid. We found when isolating AA6S, the yield of the product can be preserved by using a slurry of barium carbonate which had been previously de-aerated with nitrogen. Presumably oxygen is absorbed on the surface of the fine particles of barium carbonate, which leads to partial oxidation of the desired product.

L-Ascorbate 6-sulfate was characterized by u.v. and c.m.r. spectroscopy. The c.m.r. spectrum (Figure 6) shows that, relative to the shifts observed for AA, sulfation at C-6 shifted the signal of C-6 in water downfield by ∿6ppm while the signal of C-5 moved slightly upfield. Those shifts have been noted by others (36) in the spectra of sugar sulfates. The p.m.r. spectrum of AA6S showed that the signal of the H-6 protons are shifted downfield by ∿0.5 ppm in AA6S(δ H-6, H-6' 4.14-4.30 at pH 7.0) from those observed for L-ascorbate(δ H-6, H-6' 3.74 at pH 7).

Our method of preparing and purifying AA6S is somewhat better than those previously described. When we repeated the method of Allaudeen and Ramakrishnan (33) we found the crude sodium salt of L-ascorbate 6-sulfate could be isolated in only 26% yield. Tolbert (35) obtained AA6S in 20% yield, which is probably the same obtained by Hatanaka, et al. (34).

L-Ascorbate 5-Sulfate. We suspected that Component II (Figure 5 and Table III) was L-ascorbate 5-sulfate for several reasons: (1) primary hydroxyls are known to sulfate 3-10 times faster in concentrated sulfuric acid than are secondary hydroxyls, which explained the small percentage of Component II; (2) Component II gave one spot on paper chromatography in the monosulfate region, and the spot strongly reduced silver and ferric ions (Table IV); (3) when Component II was collected over 6% metaphosphoric acid and the acidic solution concentrated at 25° to a small

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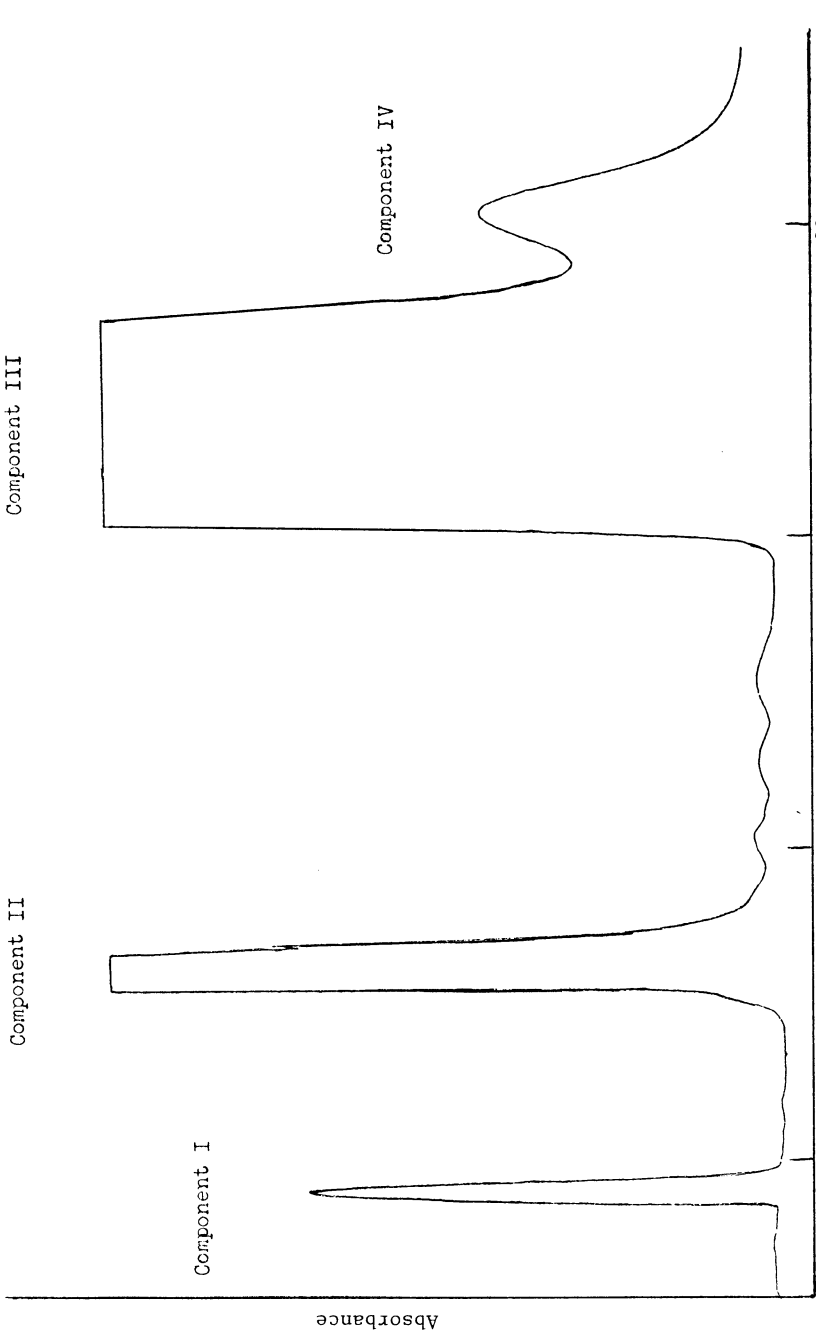

Figure 5. Separation of reaction products of L*-ascorbic acid in 99% sulfuric acid. Products were chromatographed on a column of DEAE-cellulose (35).*

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Table III. Ion-exchange column chromatography of reaction products formed by dissolving L-ascorbic acid in 99% sulfuric acid.

Component	Effluent pH	Total effluent, ml.	Percent of mixture[a]	
			uv	Iodine titration
I	6.0	351	0.3	0.5
II	5.0	570	4.0	5.0
III	1.7	1118	85.0	81.0
IV	1.7	1464	0.2	< 0.5
		Total Recovery	89.5	87.0

[a]Percentage determined by comparison of absorbance or iodine titer of a peak to the total absorbance or iodine titer of the mixture applied to the column.

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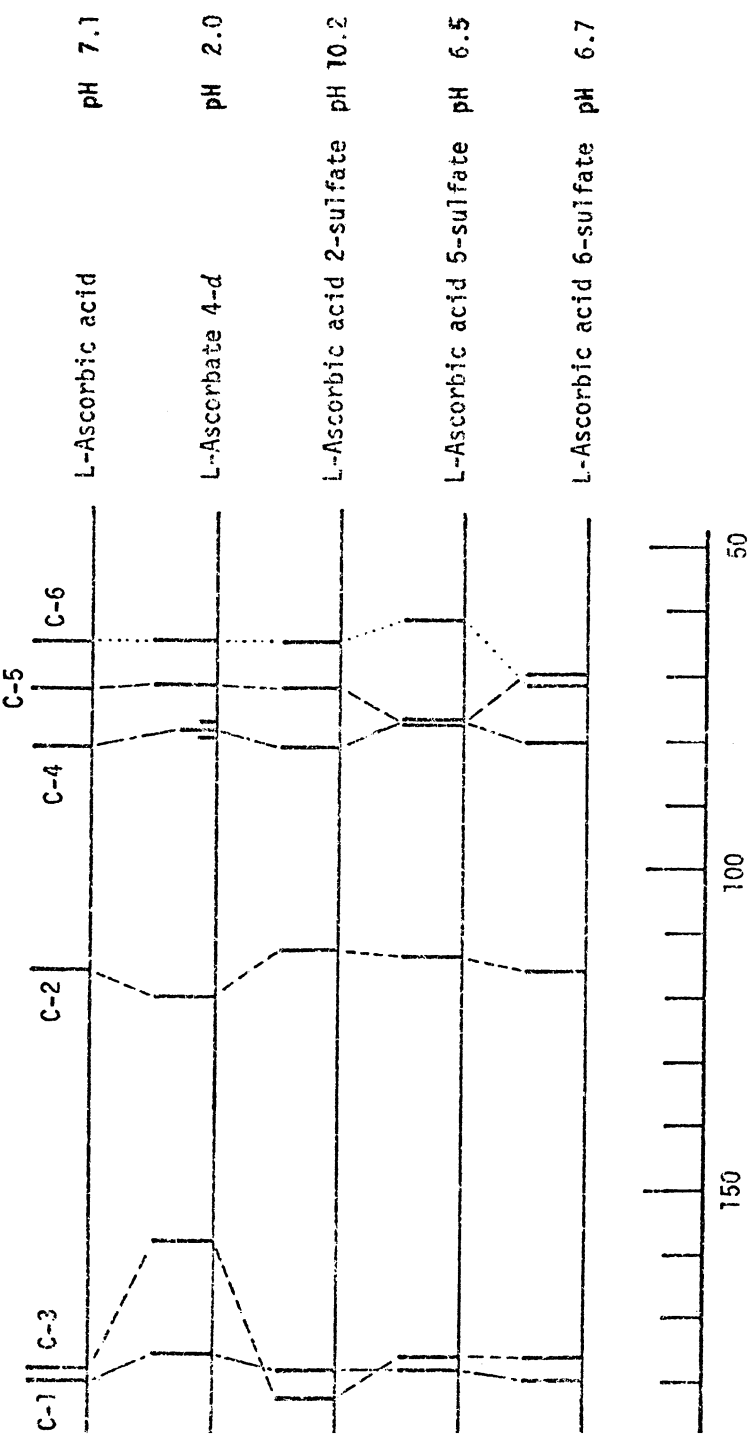

Figure 6. Comparison of the ^{13}C chemical shifts of L-ascorbic acid and its derivatives

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Table IV. Properties of the Monosulfate Esters of L-Ascorbate Acid

Derivative	Paper Chromatography[a] R_{ASC}[c]	Neutral Silver Nitrate Spray	Alcoholic Ferric Chloride Spray	u.v., λ_{max} in H_2O pH 2	pH 7	c.m.r. in Water, δ at pH 7[b] C-1	C-2	C-3	C-4	C-5	C-6	p.m.r. in D_2O, δ at pH 7[b] H-4	H-5	H-6, H-6'	pH
Unsubstituted	1.0	+	white	244	265	179.71	115.70	177.87	80.96	72.26	65.33	4.50	4.02	3.74	7.1
2-sulfate	0.5	-	red	232	255	178.08	113.01	182.34	81.31	72.28	65.12	4.57	4.05	3.73	7.0
5-sulfate	0.3	+	white	245	267	177.65	113.90	175.72	77.64	77.43	61.41	4.68	-[d]	3.86	6.5
6-sulfate	0.5	+	white	244	266	179.37	116.06	176.14	80.51	69.81	71.50	4.53	4.14-4.30	4.14-4.30	6.7

[a] See Experimental.

[b] Downfield from external (c.m.r.) or internal (p.m.r.) 2,2-dimethyl-2-silapentane-5-sulfonate.

[c] $R_{ASC.}$ = mobility relative to L-ascorbic acid.

[d] Sample not exchanged with D_2O. The H-5 signal is apparently under the HOD peak at 4.5-4.8.

volume, the resulting mixture contained only L-ascorbic acid as determined by paper chromatography; and (4) the 5- and 6-hydroxyls of AA would be expected to undergo very little disulfation since it is known (37) that vicinal diols do not form dinitrates upon reaction with the electrophilic nitronium ion. Presumably, reaction of diols with sulfur trioxide in concentrated sulfuric acid would give the same result.

Attempts to isolate Component II from the column effluent were unsuccessful, because as we discovered later, sulfate is eliminated from AA5S in the presence of barium ion. In fact, Component I was thought to be the 4,5-unsaturated product formed by the elimination reaction. The u.v. properties of Component I support the extended resonance system of three conjugated double bonds. Compared to L-ascorbic acid, the absorption maximum of Component I was shifted 15-20nm to longer wavelength (λ_{max} 260nm and 285nm at pH 2 and 7, respectively, *versus* λ_{max} 245nm and 265nm for AA at the same pH). In addition, Component I was very unstable above pH 7.

We therefore decided to prepare AA5S by an unequivocal route, which is shown in Figure 7. Reaction of L-ascorbic acid in pyridine with two equivalents of *t*-butyldimethylsilyl chloride followed by sulfation with pyridine-sulfur trioxide gave 2,6-*O*-*bis*-(*t*-butyldimethylsilyl)-L-ascorbate 5-sulfate (AA5S). Hydrolytic removal of the 2,6-blocking group with 80% acetic acid followed by ion-exchange chromatography gave the desired AA5S.

We found Component II was indeed identical to AA5S. The two materials co-migrated on paper chromatograms and on column chromatograms packed with *O*-(diethylaminoethyl)-cellulose. The structure of AA5S was readily confirmed by u.v., c.m.r., and p.m.r. The carbon-13 spectrum (Figure 6, Table IV) showed that C-5 shifted downfield by ∿6 ppm while C-4 and C-6 shifted slightly upfield. In the p.m.r. spectrum the signals of H-4 and H-6 were similar to those of L-ascorbic acid, whereas the signal of H-5 was apparently shifted downfield by 0.5-0.8 p.p.m. under the HOD peak (Table IV).

The isolation of barium L-ascorbate 5-sulfate in pure solid form is difficult because of the tendency of the molecule to undergo elimination of the sulfate group. In one attempt, a solid was isolated that contained mostly AA5S as shown by c.m.r. spectroscopy. When an aqueous solution of the barium salt of AA5S was allowed to stand at room temperature, barium sulfate precipitated in increasing amounts with time.

Experimental

General. All evaporations were done under reduced pressure below 50°. Melting points were determined with a Thomas-Hoover apparatus. Optical rotations were obtained using a Swiss-made Kern polarimeter. L-Ascorbic acid was obtained from ICN

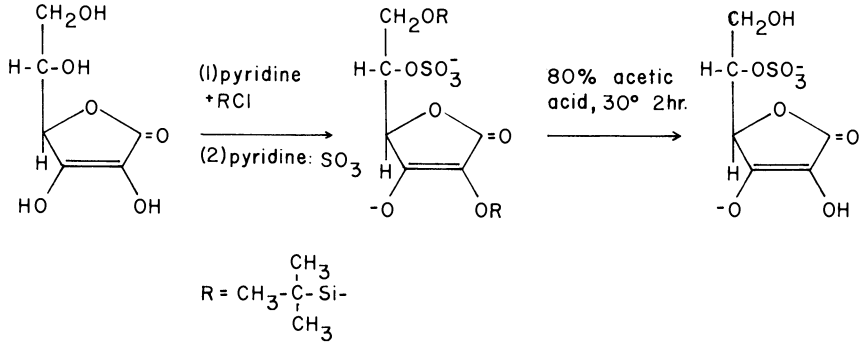

Figure 7. Synthesis of L-*ascorbate 5-sulfate*

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Pharmaceutical, Inc., Cleveland, Ohio. Concentrated sulfuric acid (99%, 37.0±0.15N) was obtained from Fisher Scientific, Fairlawn, NJ. Thin-layer chromatography was performed on microscope plates coated with silica gel G (Brinkman Instruments, Inc., Westburg, New York). Compounds were heated by spraying with 50% aqueous sulfuric acid followed by charring on a hot plate. Descending paper chromatography was done using ethyl acetate, acetic acid, and water (6:3:2, V:V). Components were located using one of three dip reagents. *Reagent A.* Strongly reducing components were detected by dipping a chromatogram in a solution of 0.7% (by weight) of silver nitrate in a mixture of acetone, concentrated ammonium hydroxide, and water (200:1:1). *Reagent B.* Moderate to weakly reducing components were detected by dipping in Reagent A followed by dipping in alcoholic sodium Hydroxide (39). *Reagent C.* Components with enolic hydroxyls were detected by dipping in a 1% ferric chloride in 95% methanol (40). The relative mobilities and color reactions of L-ascorbic acid and its monosulfate esters are given in Table IV.

U.v. spectra were obtained in aqueous solutions using a Beckman Model DB-G spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Varian Model XL-100 spectrometer. For carbon-13 spectra, the n.m.r. instrument was coupled to a Nicolet 1084 pulse Fourier transform system. Chemical shifts are given in ppm downfield from external (^{13}C) or internal (^{2}H) sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

L-Ascorbate 6-Sulfate (AA6S). To obtain good yields of AA6S, molecular oxygen should be eliminated from reagents and solvents by boiling and/or purging with nitrogen. (Pre-purified grade, Matheson, E., Rutherford, New Jersey). L-Ascorbic acid (3g) was dissolved with stirring at 25° in 99% sulfuric acid (10mL), and the solution held 4 h at 25°. The viscous reaction mixture was added dropwise with rapid stirring to ethyl ether (300mL) at -65°, which caused the sulfate esters to precipitate as a viscous gum. The ether was maintained at -65° by occasional, direct addition of solid carbon dioxide. While the ether mixture was still at -65°, aqueous saturated barium hydroxide was added (200mL) and the resulting mixture was allowed to warm to -5°. The ether layer was drawn off and washed twice with water (2 x 150mL). The combined aqueous layers were kept at 0-5° and neutralized by addition of solid barium hydroxide. Barium sulfate was removed by filtration, and the filtrate made to volume (1000mL). An aliquot (10.0mL) of the reaction mixture reduced 3.65mL of 0.1N iodine solution, whereas an aliquot (5.0mL) of a known amount (1.015g) of L-ascorbic acid in 6% aqueous metaphosphoric acid (100.0mL) reduced 11.6mL of 0.1N iodine. The titers showed the aqueous extract contained 74% of the ene-diol groups put into the concentrated sulfuric acid.

The remaining filtrate (990mL) was evaporated to a small

volume, and made to 25.0mL with water. An aliquot (10.0mL) was subjected to ion-exchange chromatography on a column (2.6 x 40cm) of *O*-(diethylaminoethyl)-cellulose (Whatman DE-52, H. Reeve Angel, Inc., Clifton, New Jersey), which had been previously converted to the hydrogen sulfate form by washing with 0.1M sulfuric acid. The column was developed at a flow rate of 45ml h^{-1} using a linear gradient (0 → 0.18M of sulfuric acid. The absorbance of the effluent was monitored at 254nm, and the components (Figure 5) were collected in a volumetric flask over 6% metaphosphoric acid. The amount of each component was determined by its absorbance (245nm) and by iodimetric titration (Table III).

Component III, which accounted for 81% of the reaction products, was found to give a single spot (paper chromatography, R_{AA} 0.59), which co-migrated with a sample of L-ascorbate 6-sulfate prepared as described by Allaudeen and Ramakrishnan (33). The spot rapidly reduced silver and ferric ions. Both samples had u.v. properties identical to those of L-ascorbic acid (λ_{max} 245nm at pH 2 and λ_{max} 265nm at pH 7).

In a separate experiment Component III was isolated as its barium salt after separation of the products obtained from a reaction starting with 2.85g of L-ascorbic acid. The column (2.6 x 40cm) was developed as previously described, except a total of 400mL of 0.18M sulfuric acid was used in the linear, gradient elution step, followed by 1800mL of 0.18M sulfuric acid. Component III was collected in a slowly stirred mixture of water (50ml) and barium carbonate that had previously been purged with nitrogen. After removal of barium sulfate, the filtrate was concentrated to dryness to give barium L-ascorbate 6-sulfate (5.0g) as an amorphous solid which was 75% barium AA6S as determined by u.v. analysis.

Anal. Calculated for 0.73 moles $C_6H_6O_9SBa$ + 0.27 moles $BaSO_4$: C, 13.44; H, 1.13; O, 34.35; Ba, 41.50. Found: C, 13.33; H, 1.13; O, 35.56; Ba, 47.54.

The p.m.r. spectrum of barium AA6S at pH 7 (Table IV) agreed with that reported by Hatanaka, et al. (34).

L-Ascorbate 5-Sulfate. To a pyridine (10mL) solution of L-ascorbic acid (1.5g) cooled in an ice bath was added 2.1 equivalents of *t*-butyldimethylsilyl chloride (3.0g) (Aldrich Chemical Company, Milwaukee, Wisconsin). After 3 hr stirring at 25°, t.l.c. (benzene, ethyl acetate 9/1, V/V) showed the reaction mixture contained one principal (R_f 0.7) and one minor component (R_f 0.8). Pyridine-sulfur trioxide (1.35g) was added, and the sulfation reaction allowed to proceed overnight at 25°. After sulfation t.l.c. (chloroform, acetic acid 3/2, V/V) showed one major spot at R_f 0.7.

The reaction mixture was evaporated to remove pyridine, and water (20mL) was added twice and the mixture re-evaporated to a thick syrup. The syrup was dissolved in glacial acetic acid (16mL), water (4mL) added, and the resulting mixture was held at 25°. After 12 hours t.l.c. (chloroform, acetic acid 3/2, V/V)

showed the reaction mixture contained one principal product at R_f 0.2. Evaporation of the hydrolysis reaction mixture to a small volume (10mL) removed water, acetic acid, and *t*-butyldimethylsilanol. Aqueous barium hydroxide was added to pH 7.0, and the mixture was concentrated to ∿25mL and applied to a DEAE-cellulose (H^+, SO_4^-) column. Developing the column with a gradient of sulfuric acid (0 → 0.18M) eluted a material whose mobility was the same as that of Component II (Figure 5). The column effluent was collected over barium carbonate as previously described to give 2.03 g of an amorphous solid. The solid barium salt of L-ascorbic 5-sulfuric acid had identical paper and chromatographic properties as those of Component II. The solid product did not give an acceptable elemental analysis. The u.v., p.m.r., and c.m.r. signals of AA5S are given in Table IV.

L-Ascorbic Acid 4-d. The title compound was prepared by a modification of the procedures of Tolbert (41) and Brenner (42). L-Ascorbic acid (0.05 moles) was dissolved and evaporated from deaerated deuterium oxide (10mL) three times. To the dry material was added 4M sodium methoxide in methanol-d (50mL), and the mixture was refluxed 24 hours. The cooled alcoholic solution was added to water (100mL) containing 45g of strongly acidic, cation exchange resin (H^+). The resin was removed, the filtrate freeze-dried, and the residue dissolved in warm acetonitrile. Cooling gave white crystals (2.8g, 32%) which had m.p. and a mixed m.p. of 192-3° with authentic L-ascorbic acid.

Acknowledgments

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Abstract

Methods to prepare L-ascorbate 2-,5-, and 6-sulfates are given. The compounds were characterized by ^1H-and ^{13}C- nuclear magnetic resonance spectroscopy and by ultraviolet spectroscopy. The biological properties of L-ascorbate 2-sulfate are reviewed.

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2

Glucosinolates and Other Naturally Occurring *O*-Sulfates

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The following discussion will be restricted to sulphates of natural provenance. Carbohydrate sulphates, the legitimate topic of this symposium, quantitatively dominate the class of naturally occurring, organic sulphates but by no means represent it fully. To be sure, the typical C-O-S ester linkage is also encountered in a vast and rapidly increasing number of other known sulphates of animal and plant origin. Moreover, chemical entities exhibiting other structural features, such as P-O-S, N-O-S, and N-S-linkages, are also known as natural products. The ensuing discussion will centre on the glucosinolates 1, a uniquely constituted and well-defined class of anions occurring as plant products that are, at the same time, organic sulphates and glucose derivatives, yet assembled in a fashion so unorthodox that the raison d'être for their presentation at this symposium may appear tenuous. The chemical character and biological degradation of glucosinolates, however, exhibit features of potential interest to carbohydrate sulphate chemistry; these aspects will be emphasized. The discussion will also include a brief, up-to-date survey of some other organic sulphates, selected to indicate the scope of structural variation encountered within the living world.

Glucosinolates

The collection of glucosinolates, thus far comprising about seventy-five individual anions, exclusively encountered in higher

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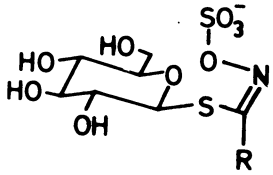
plants, possesses a remarkable structural uniformity. Thus, the β-D-thioglucopyranosidic linkage, the hydroxylamine-O-sulphate moiety, and the (Z)-thiohydroximate arrangement present in 1 are chemical features to which no known exception exists. Hence, the side-chain R of 1 constitutes the sole structural parameter. This remarkable chemical uniformity reflects, almost by necessity, a corresponding similarity in biosynthetic derivation. Few anabolic pathways in higher plants have been clarified in greater detail than those leading from α-amino acids, $R\cdot CH(NH_3^+)CO_2^-$, to glucosinolates 1, through a sequence of steps outlined in Scheme 1.



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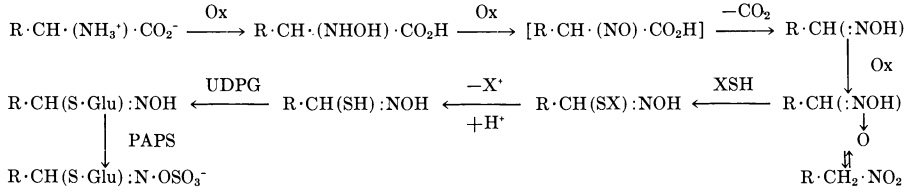
Scheme 1



1

The glucosinolates have been a subject of several reviews. Two major summaries (1, 2), chronologically followed by additional accounts (3, 4) and yearly reports (5), provide a broad background and detailed up-to-date status of our knowledge about this class of natural products. On this occasion, however, certain facets of the general theme, regarded as being of particular interest, have been selected for discussion.

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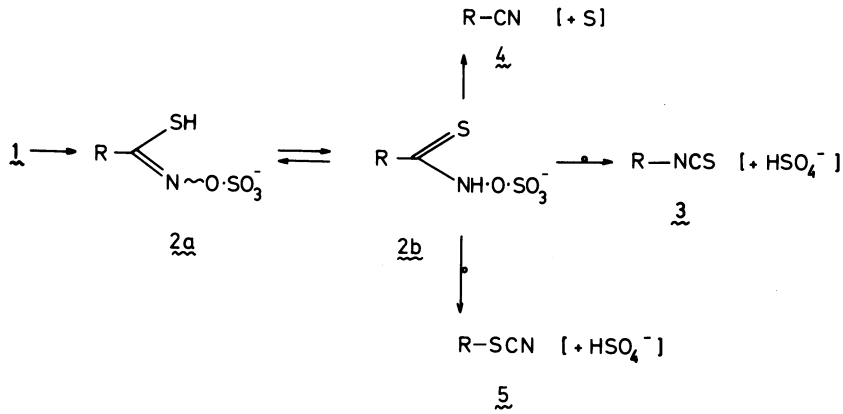
Despite reported sporadic occurrences in taxonomically remote families (2, 3), the glucosinolates are obviously concentrated in the large economically important botanical families Capparaceae and Cruciferae, but are present also in certain smaller families, such as Resedaceae, Moringaceae and Tovariaceae, all of established phylogenetic affinity to the crucifers and capers. Virtually every taxon within the order composed of the above families contains one or more glucosinolates in their fruits or vegetative parts. In fact, the absence or presence of glucosinolates ranks among the most clear-cut chemical characters within the class of higher plants. Invariably, the glucosinolates are accompanied in the natural sources by specific enzymes, the myrosinases, catalyzing the hydrolysis of the thioglucosidic linkage to produce glucose and the labile aglucone, 2a or 2b. Degradation of the latter may proceed in multiple ways, depending on circumstances such as the plant source and the pH-value, to give either isothiocyanates (mustard oils) 3, nitriles 4, or, rarely, thiocyanates 5; in many cases mixtures of these arise and in some instances the isothiocyanates undergo further reaction, such as intramolecular cyclization, to give more stable end-products.

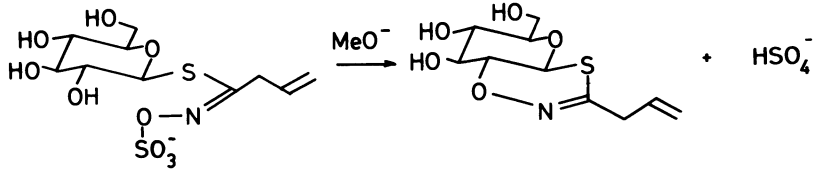


All observed transformations of the aglucone involve fission of the sulphate 'ester-bond'. Much controversy arose some years

ago concerning the enzymic, or non-enzymic, character of this cleavage. On the basis of simple chemical reasoning, however, the spontaneous conversion of 2a (or 2b) into 3, a reaction representing the sulphur equivalent of the well-known Lossen-rearrangement yielding isothiocyanates from hydroxamic acids, equipped with a suitable leaving group, is an altogether acceptable event. In fact, nature's device:to utilize sulphate as the leaving group, serves well in the ordinary Lossen-rearrangement (6).

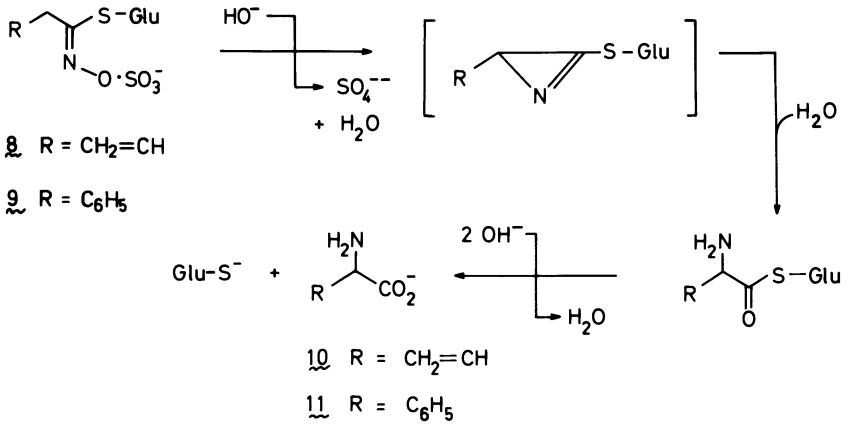
The enzymically initiated production of isothiocyanates 3 provides the rationale for the cherished pungency and flavour of numerous foodstuffs and condiments of cruciferous character such as mustard, radish, watercress, horse radish, turnips and rape. Enormous economic and considerable dietary problems are associated with the glucosinolate contents in cruciferous crops employed as fodder and for human consumption. In Japan alone, the amount of fresh or processed radish consumed by the local population has been estimated at five million tons per year (7).

Another facet of glucosinolate chemistry involving the sulphate grouping is apparent in the formation of, inter alia, merosinigrin upon treatment of allyl-glucosinolate 6 (the potassium salt of which is the classical 'sinigrin') with sodium methoxide, observed more than 60 years ago (8). Based on the now accepted general glucosinolate structure 1, merosinigrin most likely possesses the structure 7, arising from a base-induced, intramolecular displacement of the sulphate function by the 2-OH-grouping of the glucose moiety (9).



6 7

Subjecting allyl-(8) or benzylglucosinolate (9) to strong aqueous base results in the formation of vinylglycine (10) and phenylglycine (11), respectively, in addition to thioglucose and other products. The following pathway, involving a Neber-type reaction, has been invoked (9):



8 R = CH_2=CH

9 R = C_6H_5

10 R = CH_2=CH

11 R = C_6H_5

Intriguingly, the amino acids produced contain excess of the L-isomers, reflecting some steric control from the glucose ring, the only chiral moiety involved in the reaction.

Several glucosinolates have been synthesized. A characteristic feature of all the approaches is the introduction of the sulphate-grouping at the final stage of the synthetic sequence, normally with the SO_3-pyridine complex as a reagent. In this respect the approach simulates the biosynthetic assemblage; here, in the very last step (Scheme 1), the sulphate residue is transferred from 3'-phosphoadenosine 5'-phosphosulphate to the oxygen atom of the hydroxylamine grouping in a reaction probably catalyzed by the same enzyme for the whole range of glucosinolates (5a).

Pure glucosinolate salts are non-volatile, high-melting compounds, readily soluble in water by virtue of their combined glucoside and salt character; their readiness to crystallize is capricious and not always very pronounced. In no case have sugars other than glucose been observed in the thioglycosidic linkage of

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naturally occurring glucosinolates. Equally constant is the sulphate residue; the analogous phosphate esters, a priori not unreasonable alternatives, have never been encountered. It is tempting to assume that the raison d'être for the sulphate grouping in the glucosinolate is its character of an excellent leaving group. Pursuing this line of speculation, the true biological function of the glucosinolates may hence be that of serving as depositories of volatile reaction products, possessing specific biological properties and arising from the enzymically initiated degration reactions discussed above. In fact, much knowledge has accumulated over the years to indicate that isothiocyanates, and perhaps other degradation products as well, play a decisive rôle in several well-established and specific parasite-host relations within important plants of the crucifer family. Obviously, such functions will per se attribute to the glucosinolates a phylogenetic significance that would make their discontinuous natural distribution within the plant kingdom less surprising.

Other Organic Sulphates of Plant Origin

A few representatives of other groups of organic sulphates, occurring in higher plants selected rather arbitrarily by an organic chemist primarily concerned with structural aspects, may serve as an illustration of the versatility and scope of organic sulphates encountered even within the limited group of living species made up by higher plants.

Choline sulphate 12 accumulates, to the extent of more than one-third of the total amount of sulphate fed, in the roots of sulphur-deficient plants of corn, barley, and sunflower, even under sterile conditions. In non-deficient roots, and in all leaves, it accounts for 5-15 % of the total, soluble sulphur compounds. An important rôle of 12 as an effective transport agent for passage through the cell membrane has been suggested (11).

A dramatic development in our knowledge of flavonoid sulphates in higher plants should not pass unnoticed here. More than

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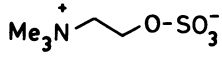
40 years ago, persicarin (isorhamnetin 3-sulphate) 13 was reported as a constituent of water-pepper (Polygonum hydropiper) (12); the isolation of only few additional and scattered flavonoid sulphates made these structures intriguing enough to prompt the statement, in 1971, that 'flavonoid bisulphates are amongst the rarest of naturally occurring flavonoids' (13). In the same year, however, a veritable landslide of discovery set in, triggered by Dr. J. B. Harborne and his associates and resulting in a profound revision of our view of these compounds. Flavonoid sulphates now must be regarded, not as phytochemical oddities, but rather as an important class of natural compounds. Thus, in a recent review (14) individual flavonoid sulphates 'detected variously in over 200 species from 20 plant families' are listed, and undoubtedly many more are likely to appear. This example illustrates how readily a large group of natural products may be overlooked because of neglect on the part of the chemist, in the present case undoubtedly caused by the salt character and over-all inconspicuousness of the compounds. Flavonols and flavones, as such or in glycosidic form, with one or more sulphate residues attached, mostly at the 3-OH and 7-OH, but occasionally also linked to the sugar moiety, are prevalent among the flavonoid sulphates known thus far. The possibility of flavonoid sulphates having an important function in salt uptake and metabolism, in addition to that of conferring water solubility to otherwise insoluble flavonoids, has been suggested in view of their preferred occurrence within plants with aquatic, often saline habitats.



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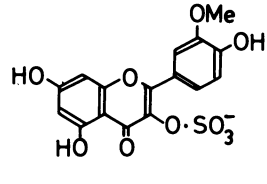

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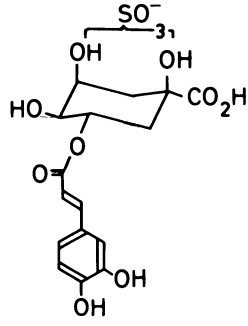
Other plants phenolics, recognized as sulphate conjugates, include the isomeric chlorogenic acid O-sulphates 14 (14); O-sulphates deriving from 1-caffeylglucose 15 and p-coumaryl-glucose 16 (14), as well as the 3'-(17) (15) and 6'-O-sulphate 18 (16) of betanin, a pigment characteristic of members of the taxonomically restricted order Centrospermae.



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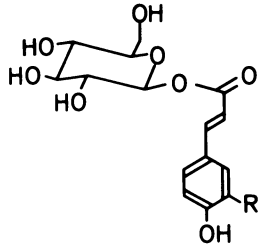
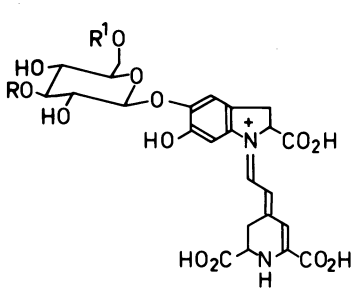
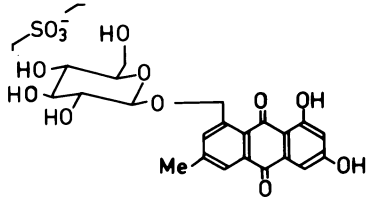


15 R = OH
16 R = H

Application of paper electrophoresis technique to extracts of several species of the family Polygonaceae recently led to the recognition of covalently bound sulphate in half of 27 species studied, belonging to the genus Rumex. From one of these an O-sulphate of emodin 1 (or 8)-glucoside 19 and a diglucosidesulphate of the corresponding emodin dianthrone were characterized, both containing the sulphate residue in the glucose moieties (17).





19

17 R = SO_3^- , R^1 = H
18 R = H , R^1 = SO_3^-

Conclusion

Our knowledge about covalently bound sulphates is obviously in its infancy. We know that sulfohydrolases, enzymes catalyzing the hydrolysis of organic sulphates, are wide-spread throughout the worlds of microorganisms, plants and animals. Although our understanding of these and related enzymes has increased remarkably recently we are still left with the rather paradoxical situation: many enzymes lack well-recognized natural substrates and, at the same time, natural sources provide organic sulphates for which no enzymic apparatus seems to exist. Obviously, continued efforts to clarify the distribution, chemistry and enzymic transformation of naturally occurring sulphates cannot fail to be rewarding to those possessing the imagination, skill and perseverance needed to help us understand the importance and biological function of these compounds.

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Abstract

The chemistry of the glucosinolates, a class of naturally occurring thioglucosides containing an O-sulphate grouping, is briefly discussed. Their enzymic degradation to isothiocyanates, and nitriles, as well as other reactions in which the sulphate grouping participates, are reviewed. Other classes of O-sulphates, occurring in higher plants, are listed, including flavonoid sulphates. The possible rôle of organic sulphates is briefly discussed and future research aspects adumbrated.

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Sulfate Ester Groups as Potential Informational Regulators in Glycoproteins

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The presence of ester sulphates in carbohydrate macromolecules is a feature widespread both in plants and animals. Considerable speculation exists about the biological functions played by the acidic ester groups, whether these groups solely serve to augment the anionic character of the macromolecule concerned or whether they exert more specific and perhaps more subtle biological effects.

The purpose of this paper is to consider these questions, in particular in terms of sulphated macromolecules present in animal tissues. The parallel aspects in plants are no less important, bearing in mind the location of sulphated polysaccharidic material in cell wall structures, their potential role in relation to water-retaining mechanisms and their capacity to interact with other macromolecules.

Sulphated Glycosaminoglycans

In animals, relevant macromolecules examined in greatest detail have been the sulphated glycosaminoglycans (mucopolysaccharides) associated with connective tissues eg. chondroitin sulphates, keratosulphate, heparan sulphate, derman sulphate. In principle, the sulphate ester groups are located in defined positions on oligosaccharide chains of regular repeating sugar sequences and these in turn are attached by alkali-label linkages to a protein (Table I). Thus in chondroitin 4-sulphate, sulphate ester groups are envisaged in the ideal situation as being attached to C-4 of every N-acetylgalactosaminyl residue and to C-6 of the same aminosugars in chondroitin 6-sulphate. Nevertheless considerable variations in practice have been noted in the actual extent of sulphate between chondroitin sulphate types of different biological origin. Chondroitin sulphate from shark cartilage, for example, exhibits the abnormally high sulphate content of 33.7% while that from bovine cortical bone has 10.3% sulphate.

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Table I. Composition of glycosaminoglycans

	Disaccharide repeating unit		Sulphate	Other sugar residues, including those in the linkage region
	Hexuronic acid	Hexosamine		
Hyaluronic acid	D-glucuronic acid	D-glucosamine	-	?
Chondroitin 4-sulphate	D-glucuronic acid	D-galactosamine	O-sulphate	D-xylose, D-galactose
Chondroitin 6-sulphate	D-glucuronic acid	D-galactosamine	O-sulphate	D-xylose, D-galactose
Dermatan sulphate	L-iduronic acid or D-glucuronic acid	D-galactosamine	O-sulphate	D-xylose, D-galactose
Keratan sulphate	D-galactose	D-glucosamine	O-sulphate	D-mannose, D-fucose, sialic acid, D-galactosamine
Heparan sulphate	D-glucuronic acid or L-iduronic acid	D-glucosamine	O-sulphate and N-sulphate	D-xylose, D-galactose
Heparin	D-glucuronic acid or L-iduronic acid	D-glucosamine	O-sulphate and N-sulphate	D-xylose, D-galactose

Incontrovertibly, such ester sulphate can contribute to the electrophoretic anionic character, and under experimental conditions at least, endow the macromolecule with dye-binding properties. Studies on the induced cotton effects of anionic dye-mucopolysaccharide complexes in the adsorption band of the dye eg. methylene blue (Stone, 1964, 1965) have been interpreted on the basis of polymer conformations, indicative of both random and helical forms (Hirano and Onodera, 1967). Whereas chondroitin (sulphate-free) exhibited random conformation, chondroitin 4-sulphate (like derman sulphate and hyaluronate) had high degrees of helical structures of the Left Screw sense. By contrast, chondroitin 6-sulphate, heparin and heparitin sulphate were helical conformations of the Right Screw sense. Interestingly, chondroitin polysulphate (chondroitin 4,6-sulphate) had the dye-binding characteristics of chondroitin 4-sulphate. Additional sulphation of chondroitin 6-sulphate (to OH-4 of the aminosugar residues) on the other hand leads to conformational inversion. In the heparin series, de N-sulphation of that material or of heparitin sulphate did not bring about change of conformation, though complete desulphation caused the helical structure to disappear. The primary structure plays a decisive part in conformational determination, the linked position of each ester sulphate being important in relation to hydrogen-bonding capability. Elegant X-ray analytical studies by Rees (1969) and by Atkins and Laurent (1973) have established fine details of ordered conformation of these mucopolysaccharides. Detailed reviews of these aspects have been presented by Kirkwood (1974) and Rees (1975).

The presence of ester sulphate groups in these defined and reiterated positions in sequence nevertheless does not necessarily impair the action of degradative enzymes. Hydrolytic endohexosaminidases eg. hyaluronidase act readily on chondroitin 4- and 6-sulphates, as on hyaluronate, giving corresponding disaccharide and tetrasaccharide products. In general, it would be concluded that in these instances the in-building of ester sulphates in a regular periodicity does not seemingly influence the metabolic stability nor their immunological properties, but rather the macromolecular shape and, by inference, their inter-molecular associations.

Sulphated Glycoproteins

Ester sulphates are however widely found in other situations, especially in a wide variety of sulphated glycoproteins (Table II). These were first shown to exist in gastro-intestinal epithelial mucins and corneal glycoproteins. The degree of sulphate here, too, is variable, though a value

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Table IIa Composition of Epithelial Sulphated Glycoproteins

(g/100g dry wt)

Source	Sulphate	Sialic acid	Fucose	Hexosamine	GlcN/GalN	Galactose	Ref.
A. G-i tract	2.1	5.8	18.2	36.2	2.0	26.3	
	3.8	3.2	17.3	35.8	1.9	25.4	
Human Gastric	6.5	3.1	15.4	32.8	1.9	25.0	Hakkinen et al (1965)
juice	7.1	3.8	16.2	33.2	2.3	22.0	
	5.1	2.5	17.3	22.6	-	34.1	Martin et al (1967)
	4.3	2.9	13.6	28.9	-	13.6	
Human Gastric	4.4	1.3	10.2	30.2	-	10.2	Martin et al (1968)
tissue	4.9	2.3	10.2	28.5	-	10.2	
Human Gastric carcinoma	1.8	8.5	7.4	21.1	1.4	32.2	Kimoto et al (1968)

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Table IIb. Composition of Epithelial Sulphated Glycoproteins (Pooled preparations)

Source	Sulphate	Sialic acid	Fucose	Hexosamine	GlcN / GalN	Galactose	Peptide	Ref
							(g/100g dry wt)	
Pig submaxillary gland		11.8	6.2	18.0	-	9.4	55.4	(1)
		24.5	5.0	24.5	-	19.6	40.0	
G-i tract								
Pig gastric mucosa	3.5	1.3	12.5	32.1	-	45		(2)
Pig Duodenal tissue	±0	3.0	5.6	52.3	0.9	13.9		(3)
	0.06	6.5	3.2	51.2	0.97	14.6		
	1.84	7.4	5.2	48.1	0.75	13.0		
Pig Colonic mucosa	3.2	7.4	11.2	21.3	3	21.7	8.0	(4)
	3.8	8.8	8.8	22.4	2	25.6	6.4	
	3.8	7.0	21.4	21.4	2	23.2	8.0	
	3.4	5.5	5.5	29.3	3	20.4	8.7	

References : (1) Katzman and Eylor (1966)
(2) Slomiary and Meyer (1972)
(3) Coles and Kent (1977)
(4) Inoue and Yosizawa (1966)

of about 2% is common i.e. about 20 sulphate residues per 100,000 daltons. Unlike the mucopolysaccharides, glycoproteins show little evidence of ordered sugar-repeating sequences and the task of allocating exact positions to such sulphate residues is considerable. In a study of glycoproteins (sulphate content between 2.8 and 5.9%) of pig gastric mucosa, Slomiany and Meyer (1972) showed that at least one N-acetyl-glucosamine residue was sulphate, at position 6, and located in close proximity to the peptide attachment (Fig.1a). This material is free from uronic and sialic acids. A similar arrangement has been indicated in the sheep epithelial glycoprotein in which sialic acid residues are also present (Kent, 1970).(Fig.1b).

The pig gastric glycoprotein exhibits strong (A + H) blood group activity and it is apparent that a single sulphate ester group remote from the immuno-determinant groups has no demonstrable effect on the blood group activity. The large majority of sulphated glycoproteins have not yet been investigated in sufficient detail to assign the structural position of the sulphate residues. In this respect, the chemical study of ^{35}S-labelled glycoproteins obtained by biochemical incorporation techniques, and well characterised by customary physical means, offers substantial advantages. It remains a matter for speculation whether in certain of these cases the presence of the small number of ester sulphate groups nevertheless act as 'informational blocks' either of immunologically important sites or of substrate specificity in enzymic degradation.

Semi-synthetic Studies

Means of gathering information about these important possibilities can be envisaged in other ways. In particular, the effects are open to investigations of introducing sulphate groups, ^{35}S-labelled or otherwise, into a well-characterized glycoprotein by chemical means. While it must be inevitably expected that multiple sites will be esterified even with low degrees of sulphation, nevertheless sufficiently selective chemical and enzymic techniques are now available to make exploration worthwhile. A particular study has been made of the glycoproteins obtained from pooled pig duodenal tissue by papain digestion and fractionation initially by ion-exchange (Bertillini *et al.* 1971).

The initial mixed glycopeptides had a sulphate content between 1.04% and 1.11% for a range of successive preparations, while the sialic acid content varied between 1.9% and 2.6%. Fractionation by anionic exchange chromatography (Watman DE52) was eluted with sodium acetate buffer followed by a sodium chloride gradient (Fig.2). This enabled six components to be

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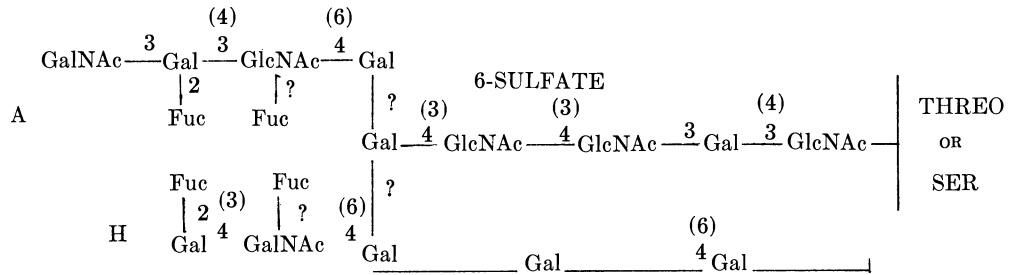

Figure 1a. Suggested structure of the carbohydrate chains of hog stomach blood group (A + H) sulfated glycoproteins

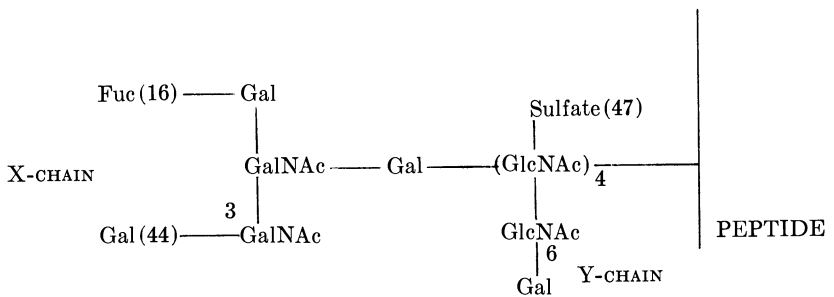

Figure 1b. Proposed oligosaccharide structure of desialated colonic goblet cell glycoprotein H_a (sheep). Numbers in parentheses are number of residues per average molecule. For each polypeptide, 30 main X-chain oligosaccharides are present, each bearing two or three side Y-chains (taken from Kent (1970)).

Abbreviations: Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine.

separated (Table III). The principal fraction (A) was a near-neutral PAS+ material accounting for 45% of the original material, followed by two lesser, but more acidic glycoproteins (C and D). In addition, a chondroitin sulphate fraction (F) and hyaluronic acid (E) were present.

Of the glycoprotein components, all three showed some (A and H) blood group activity and the third component (D) had in addition an ester sulphate content of 1.84% (Table IV). In a structural examination of the principal glycopeptide A, alkaline borohydride cleaved the oligosaccharide residues, with concomitant destruction of seryl and threonyl residues of the peptide chain and quantitative formation of N-acetylgalactosaminol. The oligosaccharide side chains were of at least three types, terminating in sialic acid, fucose and galactose, attached to the peptide chain by O-glycosyl residues.

The second glycopeptide (C) differed from A notably by its higher content of sialic acids, whilst glycopeptide (D) had both sialic acid and sulphate groups within it. Though the sites of sulphation (227 groups per molecule, m.wt. 3.5×10^5) have not yet been identified precisely, it is known from the work of Smith that the groups are not in the terminal regions of the oligosaccharide side branches but in those regions proximal to the carbohydrate-peptide junctions, as in the other epithelial glycoproteins mentioned earlier.

Complete sulphation (i.e. $>96\%$) of mixed duodenal glycopeptide preparations (or of the contributing purified GLP-A or GLP-C) has been achieved (Prino, Lietti and Paglialunga, 1971). Though the product is reported to possess some anti-coagulant activity *in vitro*, and its biological potential as an anti-ulcer agent is considerable, the latter property is common to a number of chemically sulphated macromolecules eg. dextran sulphate (Ricketts 1952, Ricketts and Walton 1952), amylopectin sulphate (for example, San and Ryan 1970), hyaluronic acid sulphate (Pantlitschko *et al.* 1951), but with the added advantage that the parent pig glycopeptide derives from an epithelial system related to the ABO blood group system.

Microtechniques have been devised for the ^{35}S-sulphation of the mixed duodenal glycopeptides, as well as for the component fractions, using $H^{35}SO_3Cl$ in pyridine. Near quantitative sulphation ($>96\%$ of available hydroxyl sites) produced a highly anionic derivative (GLPS) in which the sugar composition was in other respects undisturbed. This material (and also sulphated GLP-A) was a modest inhibitor of purified human and pig pepsins *in vitro* (Table V). It also was a powerful inhibitor of cell growth of Swiss 3T3 fibroblasts and of cell adhesion. It is doubtful however whether this property is specific to the derivative and other related

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Table III. Composition of Pig Duodenal Glycopeptides

Component	A	C	D	E_1 E_2	F
% of initial material	45%	16%	31%	3%	1%
identity	neutral glycopeptide	neutral glycopeptide	sulphated glycopeptide	hyaluronic acid (E_1)	chondroitin sulphate etc.
PAS	++	++	++	-	-
Alcian Blue	-	-	-	+	++

(Component B is of low molecular weight and accounts for about 1% of the initial material)

Table IV. Composition of Pig Duodenal Glycopeptides

Component	A	C	D
		(μmoles/g. dry wt)	
NANA NGNA	106	230	263
Fucose	342	199	316
Gal	772	812	721
GalNAc	1260	1170	1240
GluNAc	1140	1140	926
		(Xyl, Mann, Glucuronic acid not detected)	
Asp	55	55	49
Thre	567	533	711
Ser	310	304	290
Glu	85	87	66
Pro	369	331	447
Gly	63	66	67
Ala	151	138	72
Val	93	88	53
I-Leu	17	14	34
Leu	66	56	41
His	64	59	35
Lys	16	15	12
Arg	39	33	17
Tyr	ND	ND	ND
Phe	ND	ND	ND
		(Cys and Met not detected)	
Sulphate	less than 0.3	7.4	227

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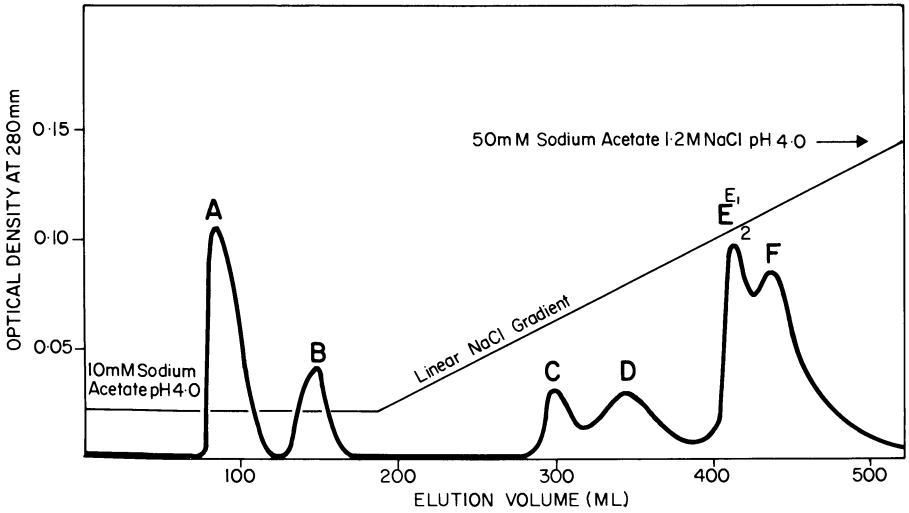

Figure 2. Fractionation of duodenal glycopeptides by ion exchange. 200 mg of GLP in 5mL starting buffer was applied to a column of Whatman DE 52 anion exchanger (120 × 1.25) equilibrated with 10mM sodium acetate, pH 4.0 (starting buffer). A linear ionic gradient terminating with 50mM sodium acetate, 1.2M sodium chloride, pH 4.0 was applied. Flow rate, 10mL/hr.

Table V. Effect of sulphated duodenal glycopeptides on purified pepsins

(in collaboration with Dr. W. H. Taylor)

Highly purified pig or human pepsins were incubated at 37° with glycopeptide (2.5mg/ml).

30 min, pH 4.0 (acetate); haemoglobin substrate.

Values expressed at % of glycopeptide-free control.

glycopeptide	human *	Pig	
native	118.6	112	substrate
glycopeptide sulphate (full)	91	72	inhibition
glycopeptide sulphate (33%)	-	90	inhibition

* (average of 3 experiments)

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polysulphates eg. dextran sulphate behaved similarly, both qualitatively and quantitatively (Fig.3) towards fibroblasts in culture.

Mild acidic hydrolysis results in cleavage of two sialic acid derivatives, separated by t.l.c. on cellulose and identified as the tetra-O-sulphates of N-acetylneuraminic acid and as the penta-O-sulphate of N-glycollylneuraminic acid. Sulphated forms of fucose have not yet been identified though presumably are cleaved simultaneously. Neither of these products are oxidised by sodium metaperiodate though they may be detected by the Svennerholm procedure. Not surprising, the fully sulphated glycopeptide (GLPS 96%) is not a substrate for V. cholerae neuraminidase and there is some evidence that it may be a non-competitive inhibitor of this enzyme towards other sialoproteins. The inhibition is not considered to be specific but rather a consequence of the polyanionic character of the substance.

It is of interest to examine the situation arising from much lower degrees of sulphation of the glycopeptide and empirically two derivatives representing 34% and 11% sulphation of available hydroxyl sites have been prepared. The low sulphation derivative (GLP-S 11%) is hydrolysable by dilute sulphuric acid, with release of sialic acids (NANA and NGNA) in which predominantly one hydroxyl group is replaced by sulphate. (The derivatives are oxidised by IO_4^-, suggesting that the sulphate ester in NANA is at C_7 or C_4).

Thin-layer chromatography of the hydrolysate showed the presence of at least three sulphates and it appears likely that a sulphated fucose is also released. It is estimated that 16% of the total sulphate content is present in sialic acid and fucose terminal residues. Unlike the fully sulphated derivative (GLP-S 96%), derivatives with lower sulphation had little effect on fibroblast cell growth or on pepsin activity.

The sulphated derivative (GLP-S 11%) also is not a substrate for V. cholerae neuraminidase and indeed shows some inhibition of the enzymic hydrolysis with a known susceptible substrate (Table VI). It is believed that this is a competitive inhibition and attributable to the presence of O-sulphate residue(s) in terminal sialic acids, since the sulphated glycopeptide residue after mild acidic hydrolysis was not such an inhibitor. It is of interest to consider whether the inhibition can be relieved by metabolic intervention of appropriate enzymes, and this would suggest a particular role for the sulphatases, lysosomal systems known to be capable of action both on glycoprotein and glycolipid carbohydrate sulphate esters. In appropriate condition this step could represent a further control mechanism regulating the metabolic turnover of important terminal sugar residues. A parallel situation exists with O-acetyl derivatives of NANA and NGNA residues in bovine, porcine and equine submaxillary gland glycoproteins (reviewed

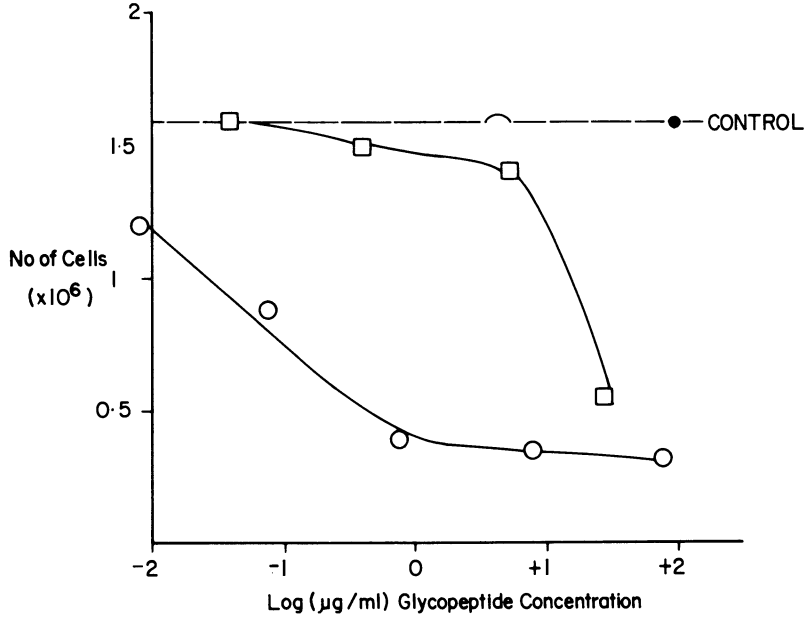

Figure 3. Effect of sulfated glycopeptides on 3T3 fibroblast growth

Table VI Sulphated Derivatives of Pig Duodenal Glycopeptide (sodium salts)

	glycopeptide	fully sulphated	partly sulphated S1	partly sulphated S2
				(g/100g dry wt)
% sulphate	-	49	14.2	4.7
degree of sulphation	-	>96%	ca.34%	ca.11%
NANA) NGNA)	5.0	2.3		
Gal	24.8	13.4		
Fuc	11.0	5.1		
Hexosamines	37.5	20.1		

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by Schauer, Buscher and Casals-Stenzel, 1974). In the native material those terminal sialic acids carrying a 4-O-acetyl substituent are reported to be insensitive to V. cholerae neuraminidase and here also an interesting regulatory role may be exercised by de-acetylases.

The intact nature of terminal sialic acids is now established as an important informational signal regulating certain forms of biological recognition of glycoproteins. In particular, this new concept of the role of carbohydrates is involved in regulating the serum survival time of plasma glycoproteins (reviewed by Ashwell and Morell 1974). Hydrolysis of terminal sialic acids by the action of neuraminidase, even in part, exposes subterminal sugar, galactose, and the consequent alteration in molecular identity leads to the removal of the glycoprotein by specific hepatic binding sites (Lunney and Ashwell, 1976; Kawasaki and Ashwell 1976). Thus any further modification of terminal sugars by additional substitution clearly has important implications for this recognition process.

It is appreciated that as yet, amongst the glycoproteins, additional substitution by sulphation rarely occurs on exterior sugar sites. Presence of these ester groups in the interior parts of the carbohydrate side-branches nevertheless can be envisaged as having informational significance of two kinds. The first aspect of this hypothesis akin to the above situation can be considered as influencing the susceptibility of the monosaccharide concerned towards exoglycosidases in the course of metabolic turnover.

The second aspect involves the changed acceptor status of a sulphated monosaccharide residue towards sugar nuleotide transferases. The possibility exists that these specific enzymes may well discriminate between the non-sulphated and sulphated residues of a given sugar within a single glycoprotein molecule with significant alteration in the patterns of biosynthesis or resynthesis of oligosaccharide chains.

Accumulated evidence suggests that in vivo sugar residues in the more exterior parts of the glycoprotein structure are more readily exchangeable than the deeper internal regions of the molecule. In this, O-sulphation may be one contributing feature.

The hypothesis that O-sulphation may have information consequences for synthesis and biosynthesis of glycoproteins is not solely restricted to those processes. The extensive change in anionic charge and the conformational effect of introducing a single sulphate ester into a carbohydrate residue involved in an immunological determinant site may be expected also to lead to alteration of antigen-antibody interactions.

The overall evidence suggests further roles for the part

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played by low levels of sulphation in a variety of processes. It points to the importance of identifying precisely the positions occupied by O-sulphates in glycoproteins and of finding new and specific means of introducing such esters at defined positions of oligosaccharides. The sulphatases would appear to be enzyme systems of considerable potential importance in respect to control of informational content of glycoproteins.

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Studies on the Synthesis of Sulfur-Containing Glycolipids ("Sulfogylcolipids")

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A sulphur-containing lipid was first detected in brain by Thudichum (1) about one hundred years ago and in 1910 Koch (2) postulated that this lipid was a complex between a phospholipid and a sulphated cerebroside. Later Levene (3-5) obtained a sulphur-containing brain lipid free from phosphate but it was not until 1933 that Blix (6) obtained a pure sample for which the analytical data indicated that it was a sulphate ester of a cerebroside (I). The presence of the sulphate group on the 3-position of the galactose residue was established in 1962 by Yamakawa (7-8) and Stoffyn (9) by methylation studies and by showing that the compound was stable to periodate oxidation. The β-configuration of the galactosidic-linkage was established by degradative studies (10) and thus the complete structure (II) of the sulphate ester of cerebroside ("sulphatide") was established. (For reviews see references 11-14).

Interest in the sulphatides has increased considerably since the demonstration (15-16) that these compounds accumulate in tissues in the inherited disease known as metachromatic leukodystrophy. This accumulation is due to the deficiency of a sulphatase which normally degrades the sulphatide (II) to a cerebroside (I).

In 1963, Mårtensson (17) isolated a new sulphated lipid, from human kidney, which contained both glucose and galactose and this was later (18,19) characterised as the sulphate ester (IV) of the lactosyl ceramide (III). Compound (IV) has also been detected in testicular tissue (20).

Another ceramide-containing sulphoglycolipid was detected in hog gastric mucosa in 1974 (21), and this was identified as a triglycosyl ceramide sulphate with the probable structure (VI). The triglycosylceramide (V) is a normal tissue constituent and accumulates in tissues in Fabry's disease due to the deficiency of an α-galactosidase.

In 1972 a new sulphoglycolipid was reported to be a major component of the glycolipids of mammalian testes and spermatozoa (22-24). This lipid ("seminolipid") differed from the known

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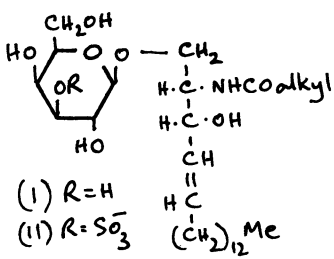
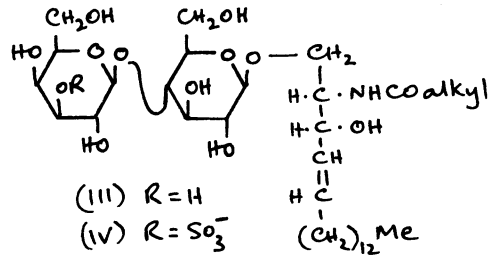
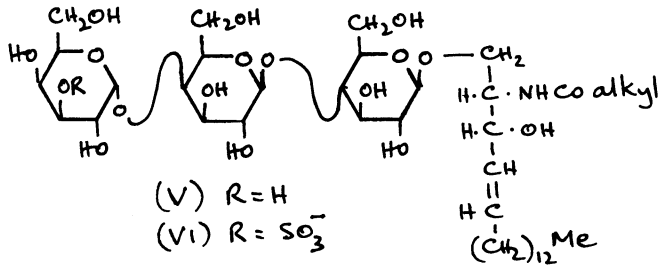


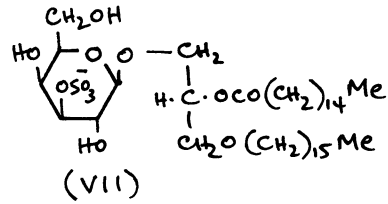
(I) R=H
(II) R=SO_3^-



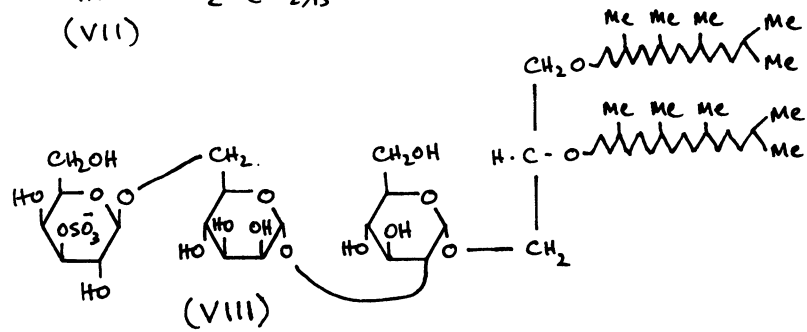
(III) R=H
(IV) R=SO_3^-



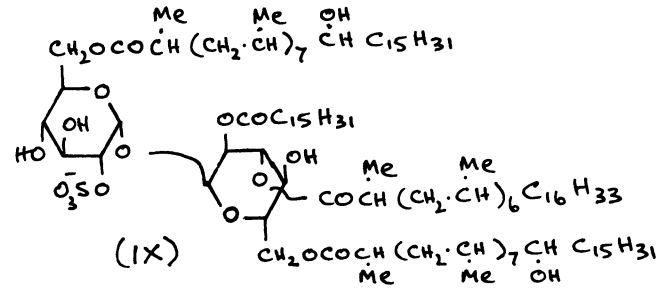
(V) R=H
(VI) R=SO_3^-



(VII)



(VIII)



(IX)

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mammalian sulphoglycolipids in that it contained 1-O-alkyl-2-O-acyl-L-glycerol instead of ceramide in the lipid moiety. The complete structure (VII) was established (25,26) for seminolipid and the compound was later detected in rat brain (27,28) but was found to be absent from bird and fish testes (20). In brain an analogous diacyl derivative also occurs (28-30).

Kates (31) reported the presence of a sulphated glycolipid in the membrane of the extremely halophilic bacterium Halobacterium cutirubrum. Like the previously described lipids from this microorganism, the sulphoglycolipid contained 2,3-di-O-phytanyl- L-glycerol in the lipid moiety as opposed to 1,2-di-O-acyl-L-glycerol or 1-O-alkyl-2-O-acyl-L-glycerol which is present in most of the known phospholipids and glycolipids based on glycerol. Mannose, glucose and galactose 3-sulphate were also identified and the complete structure (VIII) was later established (32) by methylation studies and by enzymic hydrolysis.

In 1959 (33), a sulpholipid was detected in a virulent species of Mycobacterium tuberculosis. A family of acylated derivatives of trehalose 2-sulphate was later identified by Goren (34,35) and the structures of some of these were established. The structure (IX), which represents a 2,3,6,6'-tetra-O-acyl-α:α'-D-trehalose 2'-sulphate was proposed (36) for the principal glycolipid. These compounds may prevent membrane fusion of lysosomes with phagosomes and thus hinder the host's normal method of destruction of invading microorganisms (37).

Slomiany (38) has recently reported the presence of sulphated glyceroglycolipids in human gastric secretion. The major compound was characterised as a glycoside (X) of a triglucosyl sulphate with an alkyl-acyl-glycerol.

The presence of sulphate esters of glucosyl diglycerides in seaweeds has also been reported (39).

In 1959, Benson (40,41) reported the occurrence of a sulphur containing lipid in plants. The sulphur was not released as sulphate on acidic hydrolysis and the structure was subsequently established (42) as a glycoside of 6-deoxy-6-sulpho glucose with a 1,2-di-O-acyl-L-glycerol (XI). This structure was confirmed (43) by X-ray crystallographic studies on the rubidium salt of the deacylated sulphonoglycolipid. This lipid is a major component of plant glycolipids and occurs in the chloroplast membrane (for a review see reference 13).

Other sulpho- and sulphonoglycolipids have been isolated from seaweeds (39).

Previous Synthetic Studies on Sulphoglycolipids

Flowers (44) reported the synthesis of a glycoside (XVI) of β-galactopyranosyl 3-sulphate with racemic dihydroceramide in 1966. He prepared the acetylated galactosyl bromide (XIII) containing a free 3-hydroxy-group, from 4,6-O-ethylidene-1,2-O-

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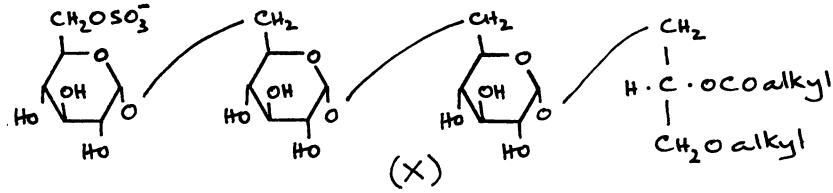
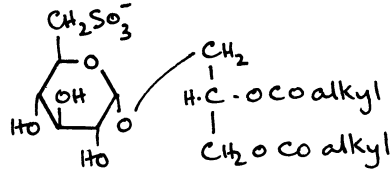
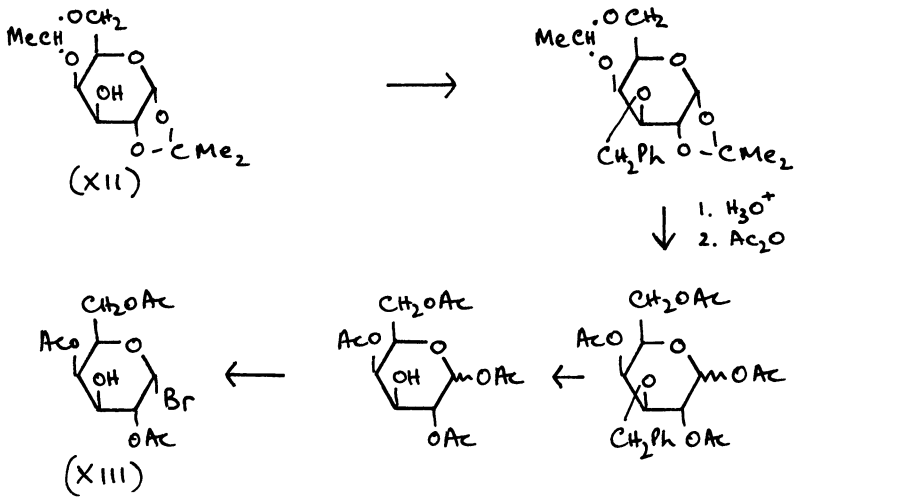


(X)

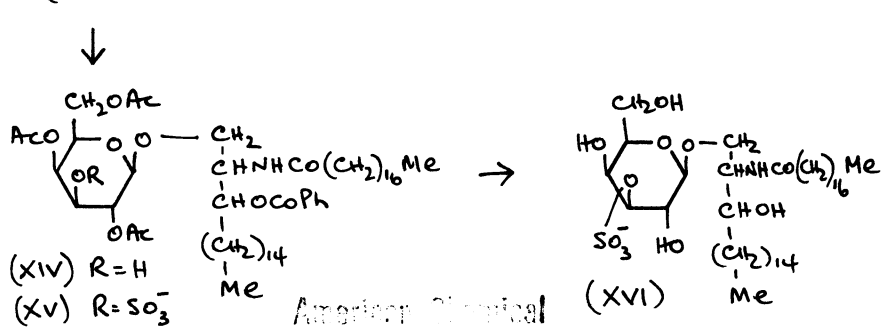


(XI)



(XII)

(XIII)



(XIV) R = H
(XV) R = SO_3^-

(XVI)

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isopropylidene-α-D-galactopyranose (XII) by the route shown. Compound (XIII) was condensed with racemic O-benzoyl-N-octadecanoyl dihydrosphingosine to give the β-galactopyranosyl derivative (XIV). Sulphation of compound (XIV) with the pyridine-sulphur trioxide complex and subsequent basic hydrolysis gave the dihydrosulphatide (XVI). In view of the known ease of migration of acetyl groups, it is surprising that this preparation was achieved without problems due to this type of rearrangement. Shapiro (45) has reported that attempts to apply this synthesis to the preparation of sphingosine containing sulphatides were unsuccessful.

Jatzkewitz and Nowoczek (46) and Yamakawa and his coworkers (8) have described the preparation of cerebrosides sulphated on the 6-position of galactose by the action of chlorosulphonic acid on cerebroside (1).

Miyano and Benson (47) synthesised the deacylated derivative (XXI) of the plant sulphonolipid but attempted acylation (42) of this did not give the sulphonolipid. The 6-deoxy-6-sulpho-D-glucose derivative (XVIII) was prepared by replacement of the tosyl group of compound (XVII). Compound (XVIII) was converted into the α-allyl glycoside (XIX) and the allyl group was hydroxylated with permanganate to give the crude diol (XX). Crystallisation of the crude compound (XX) gave the pure isomer (XXI) identical with the material prepared by deacylation of the natural lipid. Other attempts to acylate derivatives of the allyl glycoside (XIX) (47) were also unsuccessful.

Application of a General Method of Oligosaccharide Synthesis to the Synthesis of Sulphoglycolipids

We have recently developed and reviewed (48) a general method of oligosaccharide synthesis in which allyl ethers are used for 'temporary' protection and benzyl ethers are used for 'persistent' protection of hydroxy-groups and have found this method of particular value for the synthesis of glycolipids e.g. the digalactosyl diglyceride (XXII) (49), the trigalactosyl diglyceride (XXIII) (50) and the glucosyl diglyceride (XXIV) (51).

In this method a particular hydroxy-group, temporarily protected as an allyl ether, can be liberated by removal of the allyl group whilst the remaining hydroxy-groups of the oligosaccharide remain protected as benzyl ethers thus allowing a further glycosidation to be carried out specifically at the free hydroxy-group. It was apparent that other substitutions could also be carried out specifically at the liberated hydroxy-group and sulphation appeared to be an appropriate reaction to investigate so that specifically sulphated oligosaccharides or glycolipids could be prepared.

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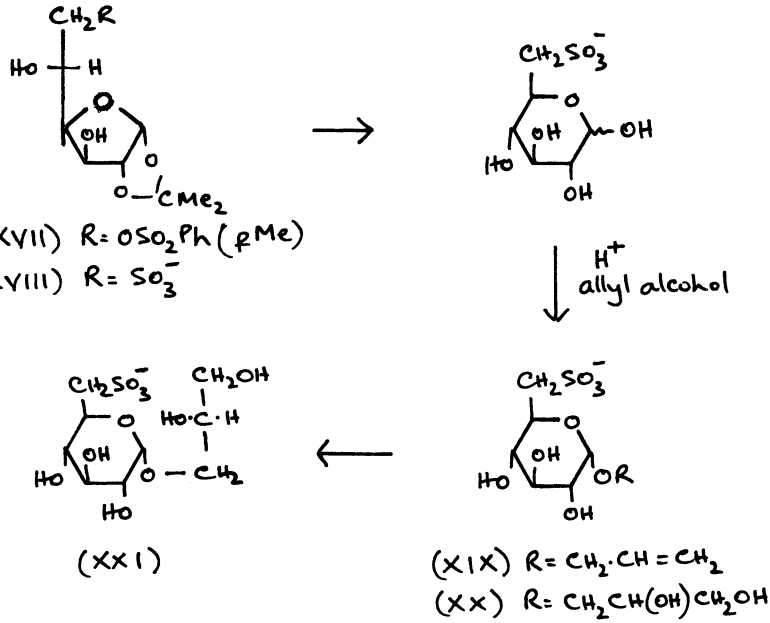
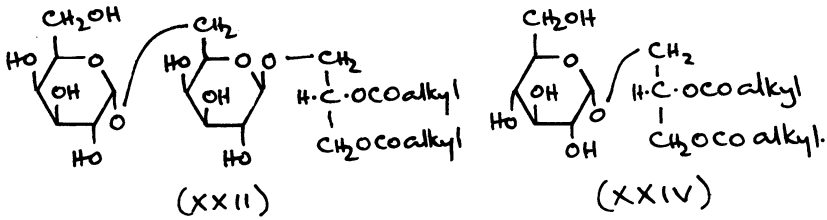
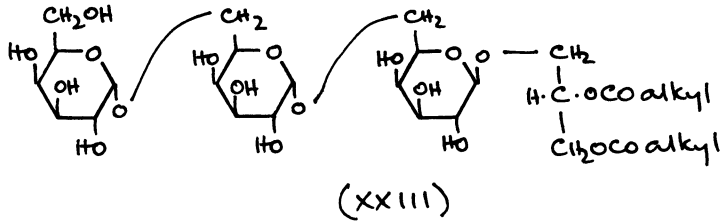




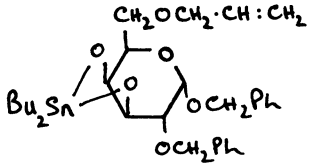


Since the success of this method depends largely on the ease of preparation of specifically benzylated and allylated derivatives of carbohydrates, new methods for achieving this are important. We have reviewed (48) our development of the allyl ethers (prop-2-enyl, prop-1-enyl, but-2-enyl and 2-methylprop-2-enyl ethers) as protecting groups and shown their value in the preparation of benzylated carbohydrate derivatives and recently some other developments have occurred which are useful in this respect. Augé, David and Veyrières (52) have shown that the axial-equatorial O,O-dibutylstannylidene derivative (XXV), (which is readily prepared by azeotropic distillation of water from a mixture of the diol and dibutyl tin oxide in benzene) was benzylated on the equatorial 3-hydroxy-group, to give the derivative (XXVI) with complete regiospecificity, when it was treated with benzyl bromide in dimethylformamide at 100°.

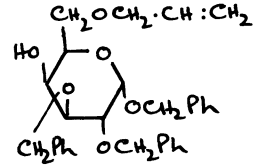
This high regiospecificity, in the galactose series, was confirmed by Nashed and Anderson (53) when they showed that a similar reaction occurred with allyl iodide. Thus the O,O-dibutylstannylidene derivative (XXVII) gave the 3-O-allyl derivative (XXVIII) with only trace amounts of the isomeric 4-O-allyl derivative being formed. A similar alkylation of the axial-equatorial system of the myo-inositol derivative (XXIX) again gave the equatorial allyl ether (XXX) with high regiospecificity (53.see also 54,55). We have also applied (56) this reaction to the O,O-dibutylstannylidene derivative (XXXI) of 1,6-anhydro-2-O-benzyl-β-D-galactopyranose and have prepared the 4-O-benzyl (XXXII) and 4-O-allyl (XXXIII) derivatives with high regiospecificity. We have previously (57) prepared the derivatives (XXXII and XXXIII) by more involved procedures.

Recently also a valuable new technique has been developed for the preparation of 4-O-benzyl ethers of carbohydrates by Nánási and Lipták (58-62). They have shown that 4,6-O-benzylidene derivatives of carbohydrates, which contain a bulky protecting group on the 3 hydroxy-group (e.g. benzyl), are hydrogenolysed by lithium aluminium hydride-aluminium chloride to give 4-O-benzyl derivatives with high regiospecificity. Thus the 4,6-O-benzylidene derivative (XXXIV) gave the 4-O-benzyl derivative (XXXV) in 92% yield under these conditions. It has been shown also that this reaction can be carried out satisfactorily in the presence of allyl ethers (63,64).

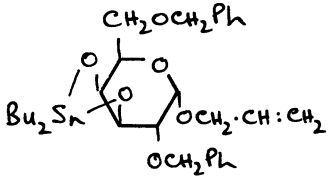
We have envisaged that a similar reaction should occur on the hydrogenolysis of acrolein acetals to give allyl (or prop-1-enyl) ethers. The acrolein acetal (XXXVII) was readily prepared from the diol (XXXVI) by the action of acrolein diethyl acetal and an acid catalyst in benzene at room temperature. Hydrogenolysis of compound (XXXVII) with lithium aluminium hydride-aluminium chloride gave (56) in 20% yield, a mixture of the 4-O-allyl derivative (XXXVIII) and the 4-O-(prop-1-enyl) derivative (XXXIX) (in a ratio of ca 3:2) which were characterised by com-



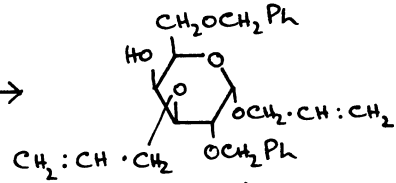
(XXV)



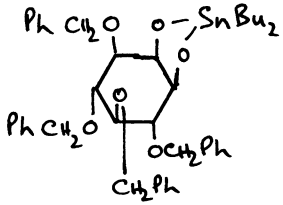
(XXVI)



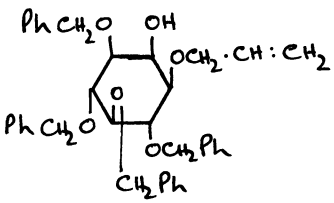
(XXVII)



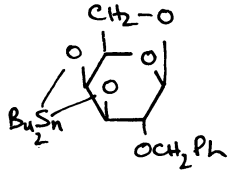
(XXVIII)



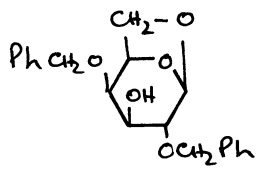
(XXIX)



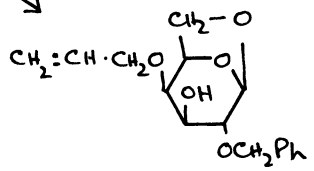
(XXX)



(XXXI)



(XXXII)



(XXXIII)

parison with authentic materials prepared by standard procedures [(XXXVIII), m.p. 76-79°, $[\alpha]_D^{26}$ + 39.2° (C 1 in $CHCl_3$); (XXXIX), m.p. 76-77°, $[\alpha]_D^{26}$ - 22.8° (C 1 in $CHCl_3$)]. Only traces of the corresponding 6-O-substituted derivatives were observed showing that the reaction occurs with the same regiospecificity as with the benzylidene derivative. Different conditions for the hydrogenolysis are being investigated in attempts to improve the yields of compounds (XXXVIII and XXXIX).

Since our previous review (48) on the use of allyl ethers as protecting groups, Boss and Scheffold (65) have shown that allyl ethers can be isomerised to prop-1-enyl ethers by the action of palladium on charcoal and it has also been shown (66, 67) that some hydrogenation of the allyl group to a propyl group may occur when tristriphenyl-phosphine rhodium (I) chloride is used for the isomerisation. The transfer of hydrogen from certain solvents to unsaturated compounds is a well known property of the rhodium catalyst (68,69). Several other reports on the use of allyl ethers as protecting groups in carbohydrate and other branches of chemistry have also appeared (52-55, 63, 64,66,67,70-81) since our previous review. See also (107,108).

In considering the application of this general method to the synthesis of carbohydrate sulphates it was necessary to show that benzyl ethers could be removed satisfactorily from the corresponding sulphated benzyl derivatives. It has been reported (82) that the benzyl and benzylidene groups of compounds (XL) and (XLI) could not be removed by catalytic hydrogenolysis although no details of the experimental conditions used were reported. Turvey (83,84) has also reported problems in the hydrogenolysis of benzyl ethers in the presence of sulphate groups and has used prolonged hydrogenation times in aqueous ethanol at elevated pressures. We decided therefore to study the hydrogenolysis of the benzyl groups from some model compounds before this general approach to sulphoglycolipid synthesis was further considered.

The crystalline benzylated derivatives (XLII) and (XLV) of benzyl α-D-galactopyranoside were available since they had been prepared previously (85) for investigations of the general oligosaccharide synthesis. These compounds were readily converted into the corresponding sulphates (XLIII) and (XLVI) by the pyridine-sulphur trioxide complex and hydrogenolysis of the barium salts over palladium-charcoal at atmospheric pressure in glacial acetic acid during three days gave products (XLIV) and (XLVII) which co-chromatographed with authentic standards (56). Thus this general approach appeared to be feasible and a synthetic route to a typical sulphoglycolipid ('seminolipid') containing a galactose 3-sulphate residue was initiated.

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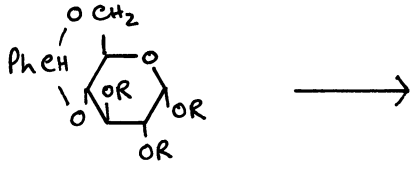
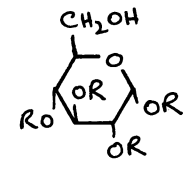
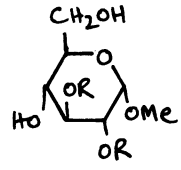


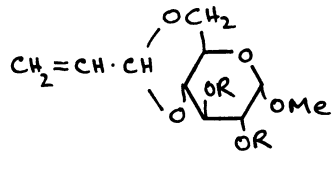
(XXXIV) R = CH_2Ph



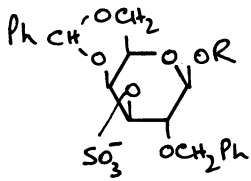
(XXXV) R = CH_2Ph



(XXXVI) R = CH_2Ph

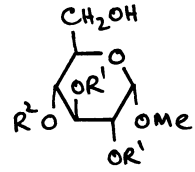


(XXXVII) R = CH_2Ph



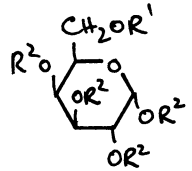
(XL) R = CH_2Ph

(XLI) R = Me



(XXXVIII) $R^1 = CH_2Ph$; $R^2 = CH_2 \cdot CH : CH_2$

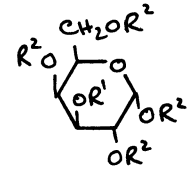
(XXXIX) $R^1 = CH_2Ph$; $R^2 = CH : CH \cdot Me$



(XLII) $R^1 = H$; $R^2 = CH_2Ph$

(XLIII) $R^1 = SO_3^-$; $R^2 = CH_2Ph$

(XLIV) $R^1 = SO_3^-$; $R^2 = H$



(XLV) $R^1 = H$; $R^2 = CH_2Ph$

(XLVI) $R^1 = SO_3^-$; $R^2 = CH_2Ph$

(XLVII) $R^1 = SO_3^-$; $R^2 = H$

Synthesis of Seminolipid

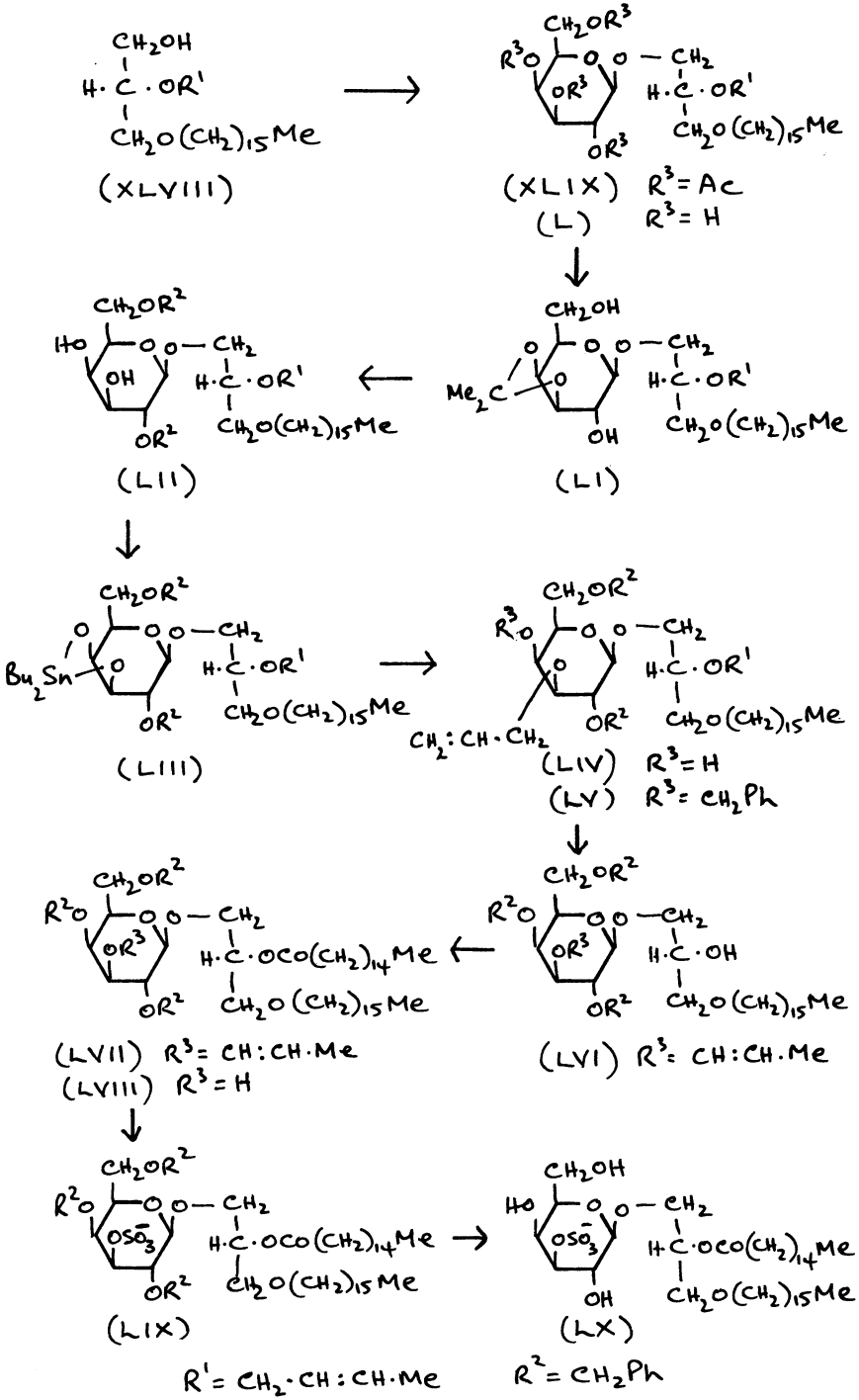
With all naturally occurring lipids a spectrum of long-chain acyl or alkyl groups (often unsaturated) exists in a particular species but it has been shown (25,26) that in seminolipid, from adult testes, the acyl group is predominantly hexadecanoyl and the alkyl group is predominantly hexadecyl as shown in formula (LX). Thus the almost complete absence of unsaturated alkyl chains from this lipid indicated that a route using benzyl ethers (which are removed by hydrogenolysis) would be useful for preparing material identical in all respects with the natural material.

The route used by Flowers (44) for the synthesis of the dihydrosulphatide (XVI) which involved, in the final stage, an alkaline hydrolysis to remove the acetyl protecting groups is also less useful in the case of an esterified glycerol derivative since the long-chain acyl group would also be removed at this stage. There are, however, examples in the literature (86-89) where acetyl groups have been cleaved preferentially from acetylated glycosyl diglycerides by hydrazinolysis. However, the possibility of acetyl migrations on the galactose residue during manipulations of the intermediates and the possibility of hydrazinolysis (90) of the sulphate group suggested that an approach using the stable allyl and benzyl ethers would be more definitive in this case.

The ideal intermediate for this approach appeared to be the benzylated desulpho-seminolipid derivative (LVIII) which on sulphation and hydrogenolysis would give seminolipid (LX). The route shown, starting from the glycerol derivative (XLVIII), was developed (56) for the synthesis of (LVIII) using the 'temporary' protecting properties of allyl, prop-1-enyl and but-2-enyl groups.

The starting alcohol (LXVIII≡XLVIII)) could be prepared readily from commercially available chimyl alcohol (LXIX) by tritylation, crotylation and subsequent acidic hydrolysis. The commercial chimyl alcohol is fairly crude (containing only ca 80% of hexadecyl chains) and therefore a purely synthetic route was developed (56) to compound (LXVIII), using again the 'temporary' protecting properties of the allyl ethers, so that a pure starting material was available.

1,2-O-Isopropylidene-L-glycerol (LXI), which is the standard starting material for synthetic glycerolipid chemistry, and is readily prepared from 1,2:5,6-di-O-isopropylidene-D-mannitol (91), was converted into the allyl ether and this was hydrolysed with acid to give 3-O-allyl-L-glycerol (LXII) (92). Compound (LXII) was tritylated and the product was converted into the but-2-enyl ether (LXIII) which was hydrolysed with acid to give 3-O-allyl-2-O-(but-2-enyl)-L-glycerol (LXIV). Compound (LXIV) was a liquid with properties similar (but with opposite optical rotation) to those of the 1,2-di-O-(but-2-

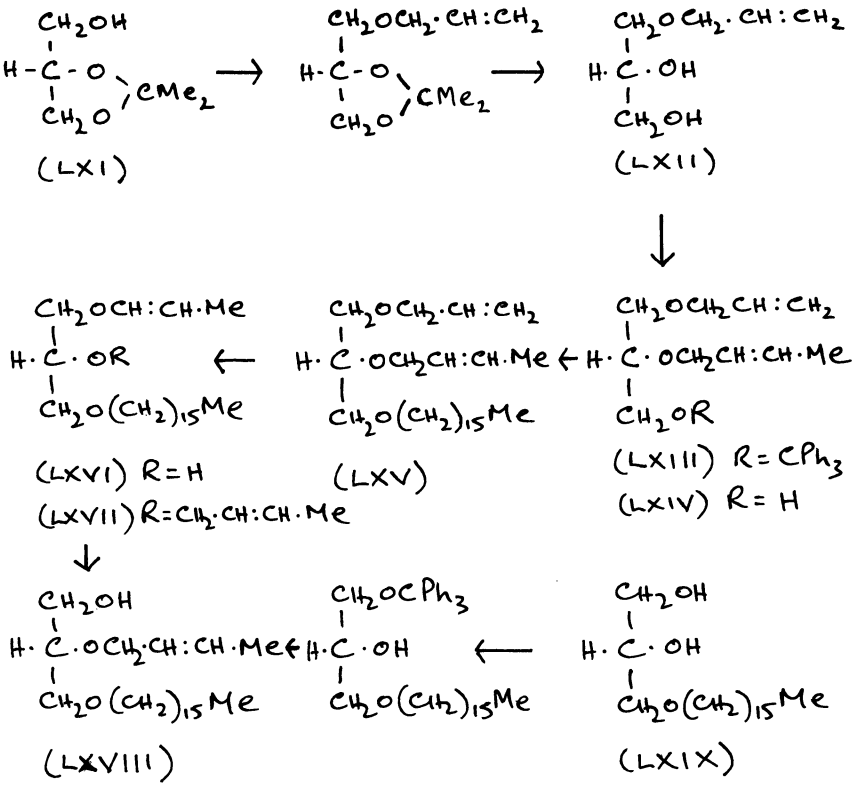


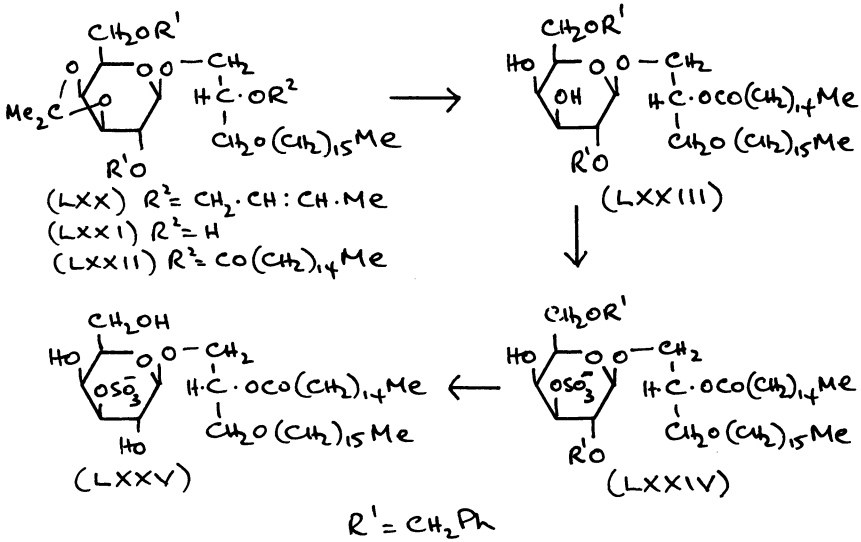
enyl)-L-glycerol which we have described previously (93). Alkylation of compound (LXIV) with pure hexadecyl bromide gave the hexadecyl ether (LXV) and this was treated with potassium t-butoxide in dimethyl sulphoxide which cleaved (94) the but-2-enyl group and isomerised (95) the allyl group, to a prop-1-enyl group, to give the alcohol (LXVI). Compound (LXVI) was converted into the but-2-enyl ether (LXVII) which was treated with dilute acid to remove the prop-1-enyl group giving the required alcohol (LXVIII). The alcohol (LXVIII) was characterised by removal of the but-2-enyl group by potassium t-butoxide in dimethyl sulphoxide to give pure 1-O-hexadecyl-L-glycerol (LXIX) whose bis-p-nitrobenzoate had the expected properties [m.p. 60-62°, $[\alpha]_D^{22}$ - 33.3° (C 1 in $CHCl_3$)].

The alcohol (XLVIII) was condensed with acetobromogalactose using the Helferich modification (96) of the Koenigs-Knorr reaction, to give the β-galactoside (XLIX). This reaction uses mercury (II) cyanide as a catalyst and we have shown (97) that mercury (II) chloride reacts slowly with the double bond of allyl ethers. We were therefore concerned previously (92) that this addition might also occur with the mercury salts in the Helferich condensation. However, Garegg and Norberg (74) had used the Helferich method in a glycosidation reaction with allyl 2,4-di-O-benzyl-α-L-fucopyranoside at 40° during twenty hours and had isolated a disaccharide in 70% yield and Sinaÿ and Jacquinet (98) had also carried out a Helferich condensation in the presence of allyl ethers, without problems and we were therefore encouraged to try the reaction in the presence of a but-2-enyl group. We have shown (56) that the but-2-enyl group reacts at approximately the same rate as the allyl group with mercury (II) chloride in aqueous-acetone whereas the 2-methyl-allyl group reacts at a very much higher rate.

The crude acetylated β-galactoside (XLIX) was treated with base which removed the acetyl groups to give the crude alcohol (L). This was treated directly with acidic acetone to give the crude isopropylidene derivative (LI). The pure product (LI) [m.p. 63-65°, $[\alpha]_D^{25}$ + 12.2° (C 1.9 in $CHCl_3$)] crystallised readily from light petroleum thus making this a convenient stage for purification from any by-products formed during the glycosidation reaction (96) and from any 4,6-O-isopropylidene derivative which can be formed in the acetalisation reaction (57,99). Compound (LI) was readily converted into the diol (LII) by benzylation and subsequent acidic hydrolysis. The diol (LII) was cleaved by periodate thus indicating that compound (LI) was indeed a 3,4-O-isopropylidene derivative.

The diol (LII) was converted into the O,O-dibutylstannylidene derivative (LIII) which was treated with allyl bromide in dimethylformamide at 100° during 5 hours to give the 3-O-allyl derivative (LIV). Thin layer chromatography showed the presence of another product (ca 5% of the major product), which is probably the 4-O-allyl derivative, but this was separated from





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the major product by chromatography on alumina. Nashed and Anderson (53) reported that allyl bromide reacted sluggishly with an O,O-dibutyl-stannylidene derivative and therefore used allyl iodide: we prefer the greater stability of the allyl bromide although the reaction may take longer.

The purified allyl ether (LIV) was benzylated to give the fully protected derivative (LV) which was treated with potassium t-butoxide in dimethyl sulphoxide to cleave the but-2-enyl group (94) and isomerise the allyl group (95) giving the prop-1-enyl ether (LVI). Hydrolysis of a portion of the product (LVI) with dilute acid gave crystalline 2,4,6-tri-O-benzyl-D-galactopyranose, identical with the material prepared previously (94), thus proving the substitution pattern of the galactose moiety.

Acylation of the alcohol (LVI) with hexadecanoyl chloride in pyridine gave the palmitoyl ester (LVII) which on treatment with mercury (II) chloride (97) gave the alcohol (LVIII). Sulphation of compound (LVIII) with the pyridine-sulphur trioxide complex (90,100) gave the tri-O-benzyl seminolipid derivative (LIX). This was hydrogenolysed in the presence of palladium on charcoal in acetic acid to give seminolipid (LX) which cochromatographed with an authentic sample kindly provided by Professor Yamakawa.

It has been shown (101) that, in the direct sulphation of D-galactose, preferential sulphation of the 6- and 3- positions occurs. This would indicate a more rapid route to seminolipid from the intermediate (LXX) which is readily available from the key intermediate (LI). Cleavage of the but-2-enyl group from (LXX) should give the alcohol (LXXI) which on acylation would give the ester (LXXII). Acidic hydrolysis of the isopropylidene group from (LXXII) should give the diol (LXXIII) and this should be sulphated preferentially on the 3 hydroxy-group to give the sulphate (LXXIV). Alternatively sulphation [like acylation (53)] of the O,O-dibutylstannylidene derivative of the diol (LXXIII) should again give (LXXIV) preferentially. Hydrogenolysis of (LXXIV) would then give seminolipid (LXXV). Any of the isomer of seminolipid, with the sulphate group on the 4-position, that was formed in these reactions could be preferentially destroyed by the action of periodate to give a pure sample of seminolipid. These alternative routes are being investigated since they would also be of value in the synthesis of other naturally occurring sulphoglycolipids which contain galactose 3-sulphate residues. The 1-O-hexadecyl-2-O-(but-2-enyl)- L-glycerol (LXVIII) should also serve as an intermediate for the synthesis of the glucose containing sulphoglycolipid of human gastric secretion (38).

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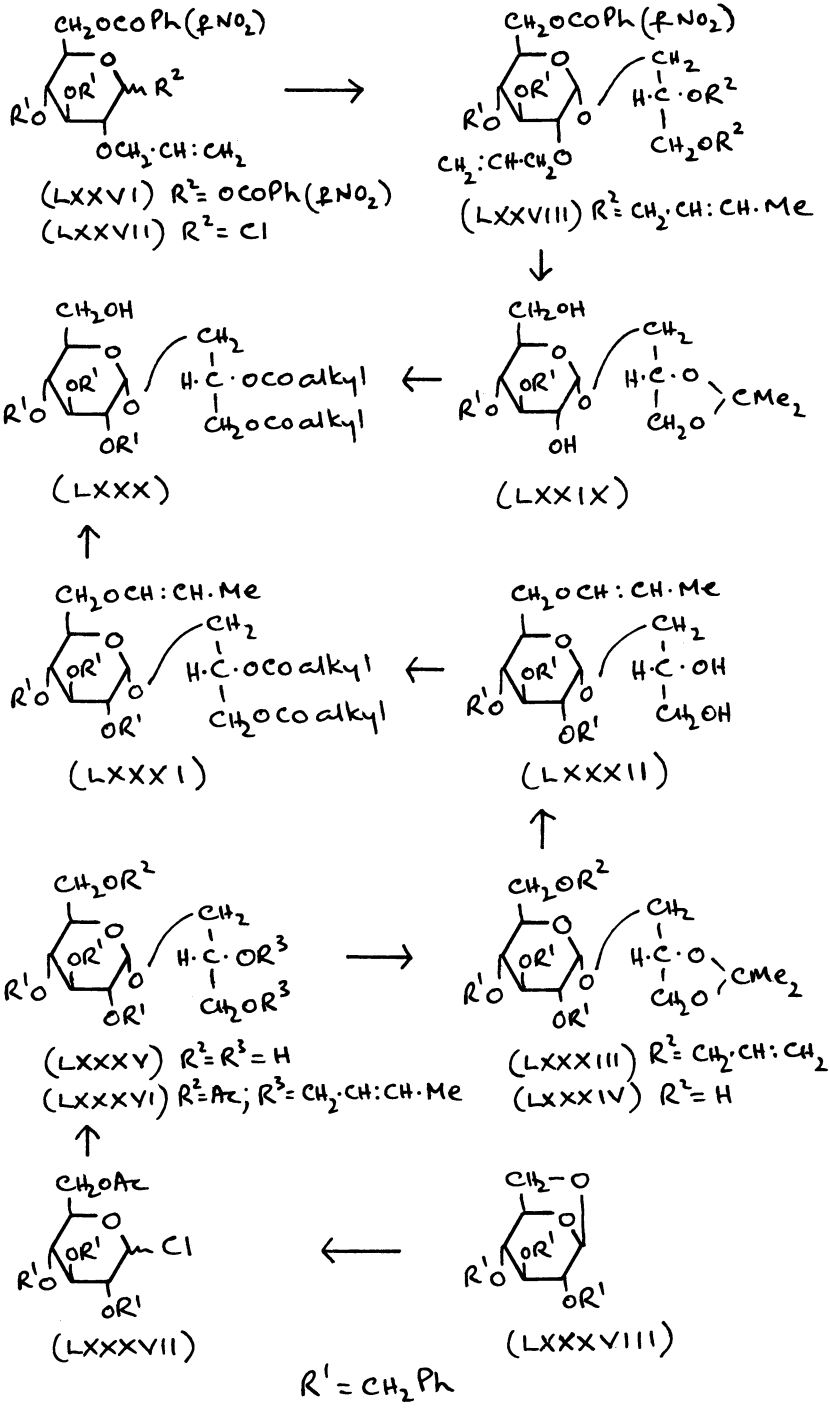
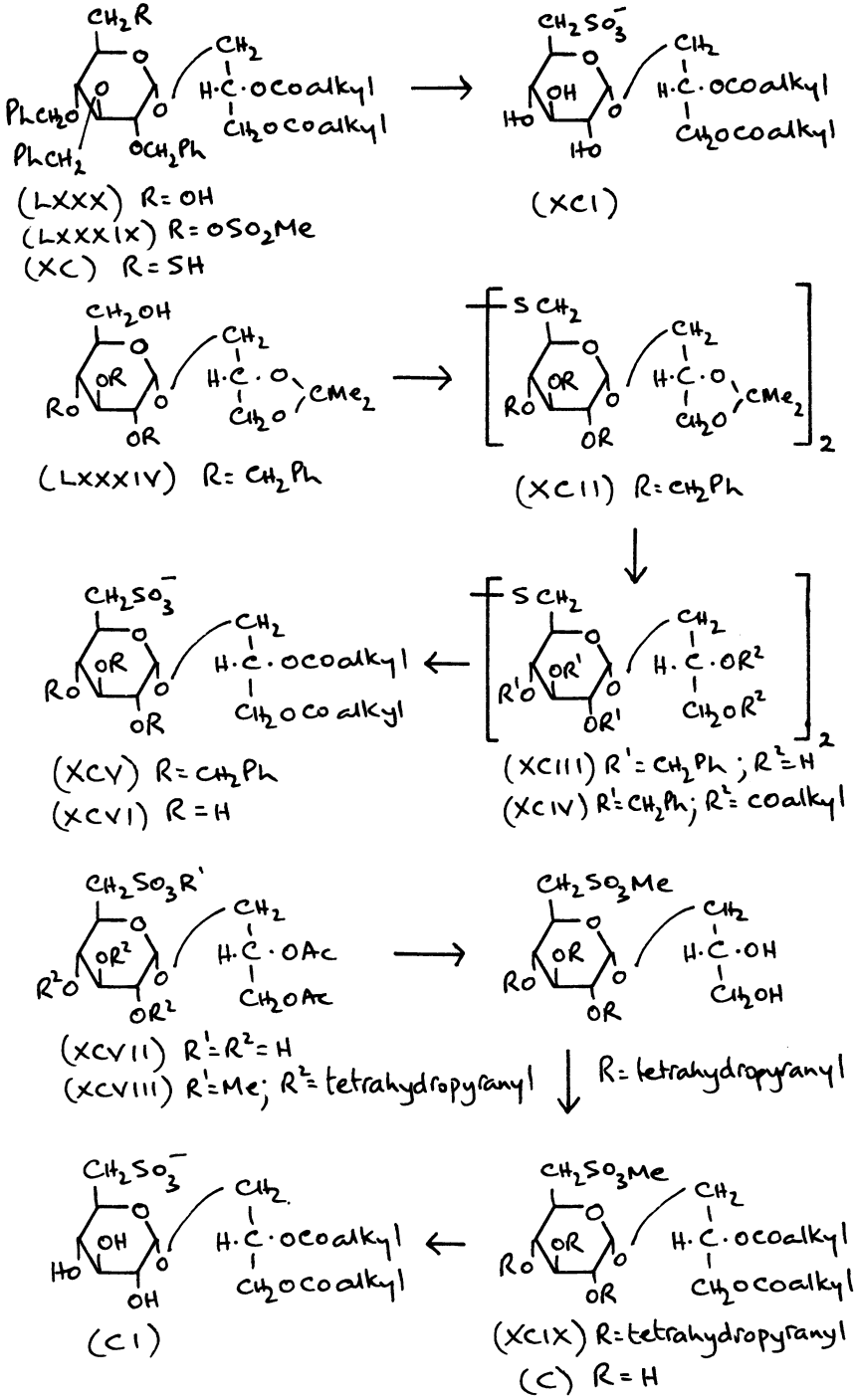


Studies Towards the Synthesis of the Plant Sulphonolipid

In studies aimed (48) at the synthesis of the immunologically interesting glycolipids of Streptococci (102) and the 'glucuronosyl diglyceride' of Pseudomonas diminuta (103) we prepared (93) the crystalline 3-O-(3,4-di-O-benzyl-α-D-glucopyranosyl)-1,2-O-isopropylidene-L-glycerol (LXXIX). This was obtained by converting the bis-p-nitrobenzoate (LXXVI) (104) into the chloride (LXXVII) which was condensed with 1,2-di-O-(but-2-enyl)-L-glycerol, under conditions shown previously (105) to give predominantly 1,2-cis-glycosidic linkages, to form the crude glycoside (LXXVIII). Compound (LXXVIII) was converted into the crystalline glycoside (LXXIX) and this was subsequently (51) converted into 3-O-(α-D-glucopyranosyl)-1,2-di-O-octadecanoyl-L-glycerol, a 'glucosyl diglyceride'. A route from compound (LXXIX) to the protected glucosyl diglyceride (LXXX) was also planned since the latter compound should serve as an intermediate for the synthesis of 3-O-(α-D-glucuronopyranosyl)-1,2-di-O-acyl-L-glycerol (a 'glucuronosyl diglyceride') and of a glucosyl diglyceride phosphorylated on the 6-position of glucose such as occurs in Streptococci (102). Moreover compound (LXXX) should also serve as an intermediate for the synthesis of the plant sulphonolipid since conversion into the sulphonate ester (LXXXIX) and replacement with sulphide should give the corresponding 6-thio derivative (XC). Oxidation of the thiol and hydrogenolysis of the benzyl groups should then give the plant sulphonolipid (XCI).

We subsequently found (57) that treatment of derivatives of 1,6-anhydro-sugars with acetyl chloride-hydrogen chloride gave the corresponding 6-O-acetyl glycosyl chlorides (a reaction which we have termed 'chloracetolysis') and this indicated a more convenient route to the derivative (LXXX) (51). 1,6-Anhydro-2,3,4-tri-O-benzyl-β-D-glucopyranoside (LXXXVIII) was treated with acetyl chloride-hydrogen chloride to give the crude 6-O-acetyl-2,3,4-tri-O-benzyl-D-glucopyranosyl chloride (LXXXVII) and this was condensed with 1,2-di-O-(but-2-enyl)-L-glycerol to give the crude α-glycoside (LXXXVI). Compound (LXXXVI) was treated with potassium t-butoxide in dimethyl sulphoxide which removed the acetyl and but-2-enyl groups to give the crystalline triol (LXXXV). The structure of compound (LXXXV) was confirmed by a further synthesis from compound (LXXIX) whose structure had been established previously (93).

The crystalline triol (LXXXV) was converted via the isopropylidene derivative (LXXXIV) into the allyl ether (LXXXIII). Acidic hydrolysis of the isopropylidene group and subsequent isomerisation of the allyl group gave the prop-1-enyl ether (LXXXII) which was acylated to give the bis-octadecanoyl ester (LXXXI). Hydrolysis of the prop-1-enyl group with mercury (II) chloride gave the protected glucosyl diglyceride (LXXX). Experiments on the conversion of compound (LXXX) into the plant sul-



phonolipid (XCI), by the method described above, are in progress. The chloride (LXXXVII) together with the alkyl glycerol derivative (LXVIII) should serve as intermediates for the synthesis of the sulphoglycolipid of human gastric secretion (38).

The availability of this route also suggested a more direct approach to the plant sulphonolipid via the intermediate (LXXXIV). Conversion of the alcohol (LXXXIV) into a sulphonate ester and replacement of this with potassium thiolacetate in dimethylformamide should give a thioacetate derivative. Alkaline hydrolysis of the thioacetate and oxidation of the thiol produced with iodine should give the oxidised thiol (XCII) [cf. reference (106)]. Acidic hydrolysis of compound (XCII) to the alcohol (XCIII) and acylation should give the ester (XCIV). Oxidation of the ester (XCIV) to the sulphonic acid (XCV) and subsequent hydrogenolysis of the benzyl groups should give the plant sulphonolipid (XCVI).

The naturally occurring plant sulphonolipid contains unsaturated acyl groups (42) but the routes proposed above will give only material containing saturated acyl groups since the benzyl groups are removed by hydrogenolysis. However, since sulphonic acids form methyl esters which can be cleaved by sodium iodide it should be possible to convert a synthetic diacetyl derivative (XCVII) via the methyl ester protected with tetrahydropyranyl groups (XCVIII) into a derivative (XCIX) containing unsaturated acyl groups. Mild acid hydrolysis should then give the sulphonate ester (C) which with sodium iodide should give the unsaturated sulpholipid (CI).

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Spectrofluorimetric Methods for Estimating and Studying the Interactions of Polysaccharides in Biological Systems

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The analysis of polyanions using metachromasia which accompanies dye binding has been successfully applied to plant sulphated polysaccharides using absorption[1] and fluorescence emission[2] spectroscopy. The latter is more useful because of its greater sensitivity at low polyanion concentrations, and because in fluorescence the spectral properties of cationic dyes such as acridine orange simplify into two well separated peaks corresponding to free dye (λmax 525 nm) and metachromatically bound dye (λmax 640 nm) (Fig. 1). By measuring the monomeric dye fluorescence at 540 nm (when excited at 400 nm) at successively increasing concentrations of a polyanion (carrageenan), a titration profile (Fig. 2) is obtained. The intercept with the polymer concentration axis gives the overall stoichiometry of the interaction with one dye molecule binding to each anionic site. Successive experiments with known polyanion concentrations can relate the intercept with the reciprocal of the anionic sites concentration (Fig. 3) and so allowing the rapid determination of the anionic site concentrations of unknown solutions.

Additives such as simple electrolytes, proteins and lipids etc. interfere with the dye binding properties of polyanions. Added salt 3, 4, 5 can release dye from the complex.

We have found that polymeric cations remove dye quantitatively[6] and more efficiently than salts, forming polyanion-polycation complexes which are stoichiometric in basic to acidic sites, and which are not influenced by added salts. This observation is the basis of the procedures we describe here to study interactions of polyanions with a range of synthetic and biological polymers. From our experience of such interactions, procedures have been devised for the quantitative estimation of polyanions, particularly sulphated polysaccharides in biological systems and so eliminating the interfering effects of associated materials such as salts, proteins and lipids.

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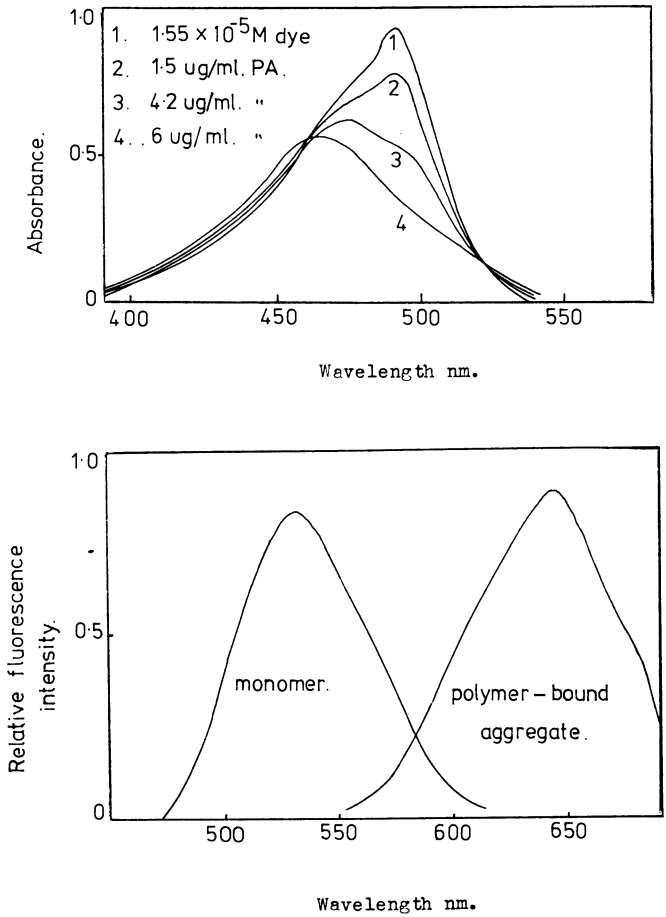

Figure 1. (Top) Absorption spectral shifts of acridine orange with aggregation of the dye on a polyanion, κ-carrageenan. (Bottom) Fluorescence shifts of acridine orange with aggregation (λ_{ex} = 400 nm).

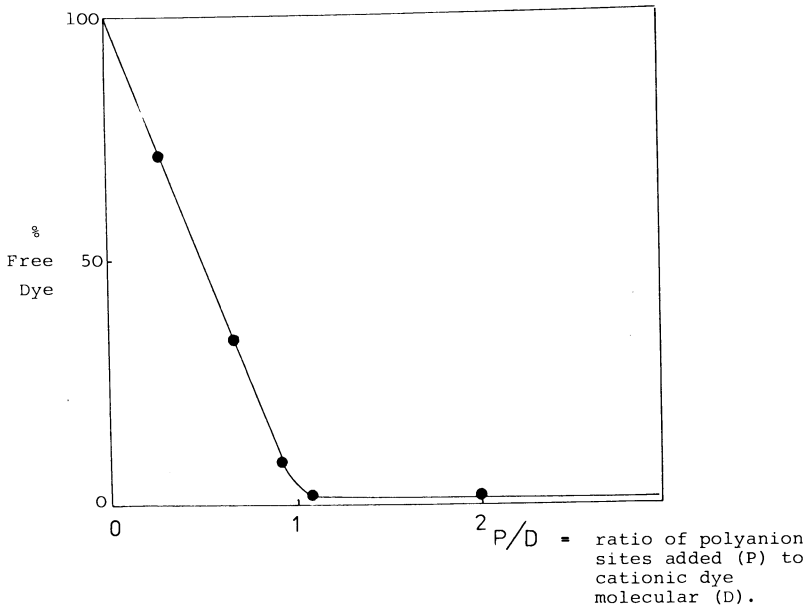

Figure 2. Titration of carrageenan vs. 10^{-5}M acridine orange in salt-free solution

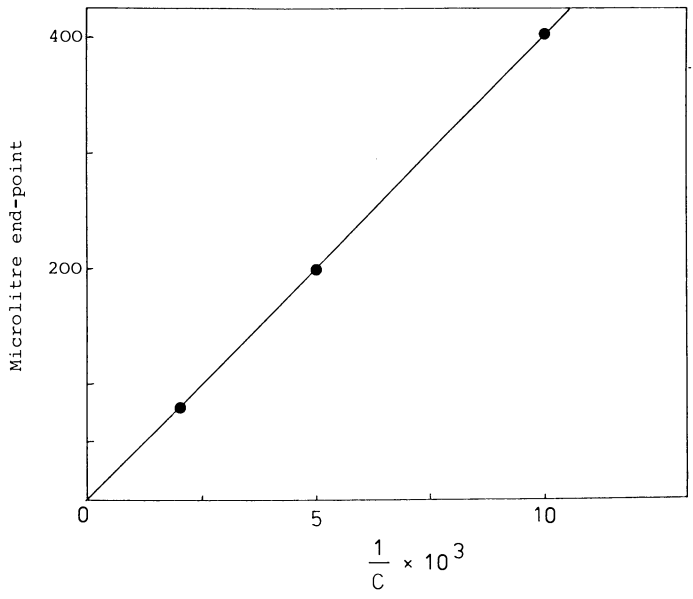

Figure 3. Calibration curve relating microlitre endpoints and polyanion concentrations

Analysis of polysulphates in the presence of polycarboxylates.

A stabiliser mixture extensively used in dairy products consists of one or more of various carrageenans, carboxymethyl celluloses, pectins, locust bean, guar and other gums, neutral polysaccharides and emulsifiers. The particular materials and relative proportions are chosen according to the specific properties desired for the particular product. Analysis of such complex mixtures is difficult and time consuming, but can be achieved if suitable fractionation procedures are utilised[11-13]. Here we describe a more rapid procedure for such mixtures.

The effect of pH on the stoichiometry of polyanion-acridine orange complexes is shown in (Fig. 5) after correction for variations of dye-fluorescence with pH. For polycarboxylates dye release is evident near the carboxyl pKa (~ pH 4). When only carboxyl groups are present (CMC and HA) the dye release is complete below pH 2.0, but the polysulphates (CAR and PSS) show considerably less dye release at this pH. Hence we can quantitatively distinguish between them.

By titrating stabiliser mixtures at pH values acid and alkaline to the carboxyl pK_A it is possible to determine separately the sulphate groups and total ionic site concentration, hence giving also a measurement of carboxyl sites concentrations. For a prepared 1 : 1 mixture of CAR and CMC, based on anionic sites, estimated site concentrations were within 2% of the predicted values. Complex ice-cream stabiliser mixtures could be similarly analysed satisfactorily (Table 2). The presence of emulsifiers and neutral polysaccharides had no effect on the analysis of commercial carrageenan (Stabiliser A). Fig. 6 shows that the dye binding of Stabiliser B and its component carrageenan varies with pH, indicating the high percentage of CMC present. Carrageenans used commercially present a particular difficulty in analysis because they may contain differing amounts of a particular isomer. We previously showed that these isomers differ significantly in the amount of dye bound per unit weight of polymer. Therefore, unless standards are available, or the ratios of isomers known, a fractionation step must be undertaken for complete accuracy. However, when κ - carrageenan only is present as in Stabiliser B, its proportion can be determined from the ratio of the titration end points in acid. The results shown in Table 2 correspond to 13.3% κ - carrageenan in Stabiliser B. The percentage of CMC in the sample can be determined from the difference between the anionic site concentrations of neutral and acid pH. Although some variation in dye binding properties occurs with changing degree of substitution (DS) the effect is relatively small. Of course, the DS of the CMC must be known to convert the determined site concentration into weight of polymer. Conversely the DS of any CMC, or other polymer of variable DS, can be determined by titration using a known weight of sample.

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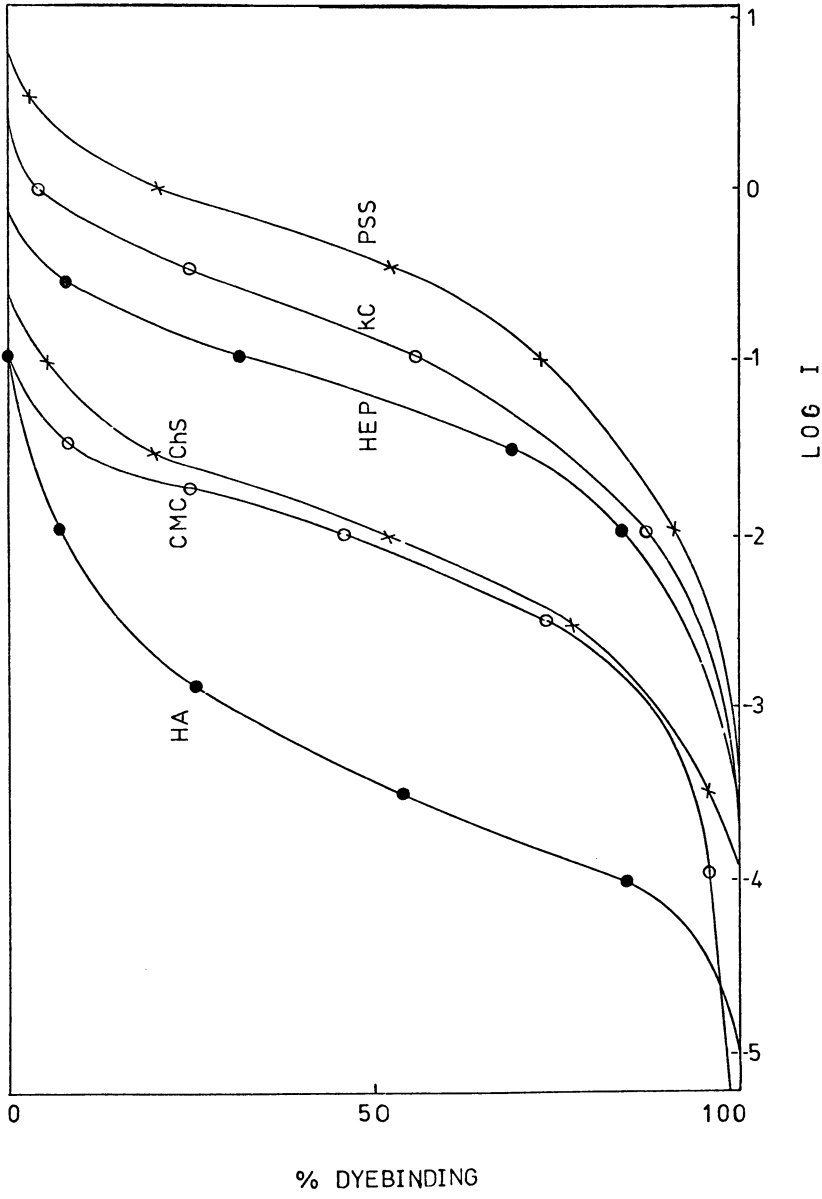

Figure 4a. Effect of simple electrolytes on polyanion–AO binding; I NaCl

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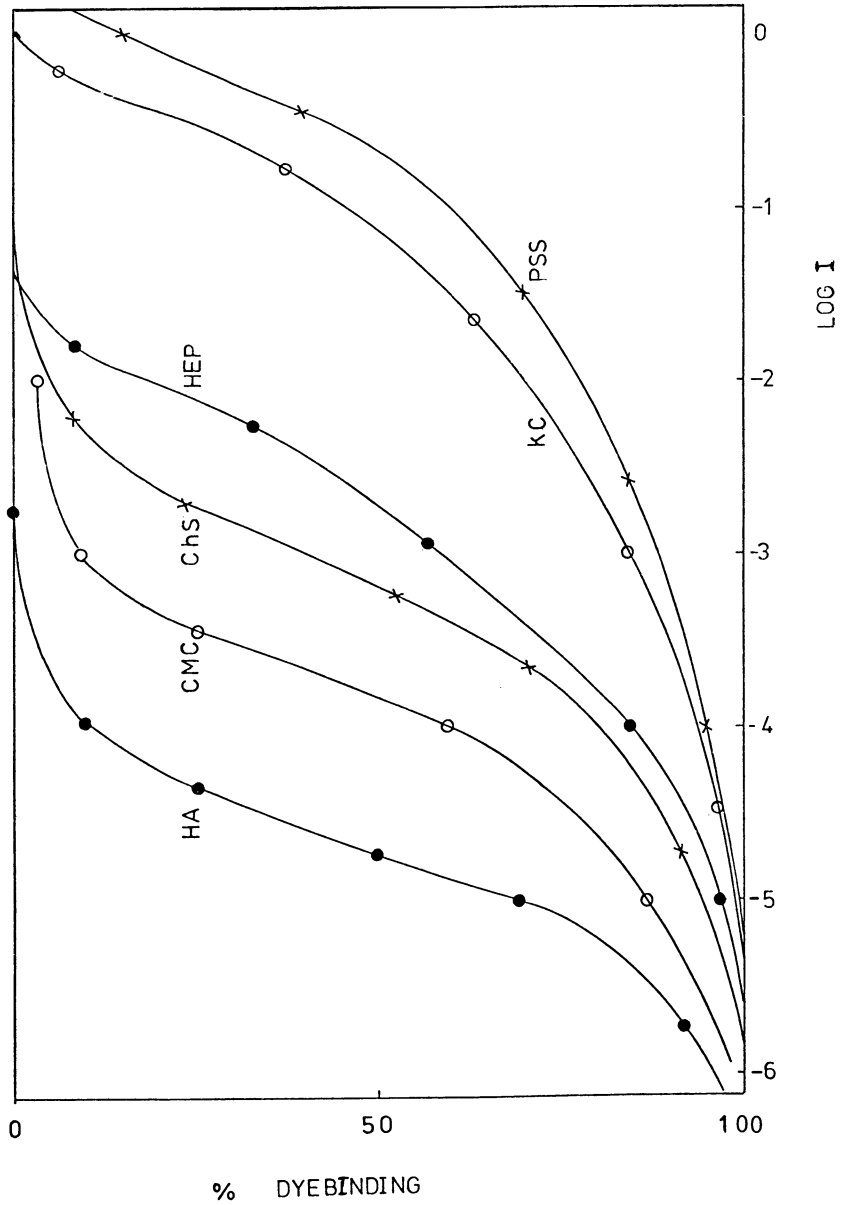

Figure 4b. Effect of simple electrolytes on PA–AO binding; 2 $CaCl_2$

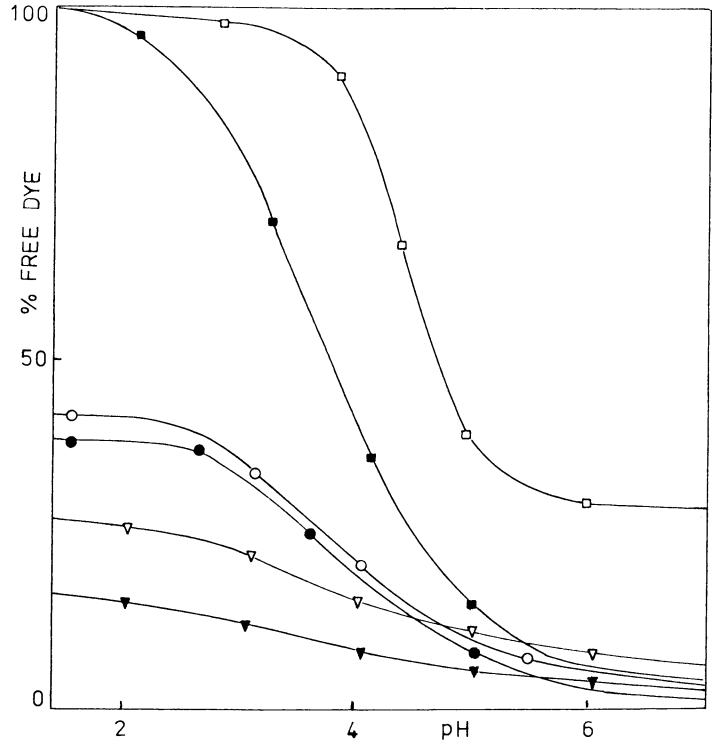

Figure 5. Variation of polyanion–AO binding with pH. (□) Hyaluronic acid; (●) chondroitin sulfate; (■) CMC; (▽) κ-carrageenan; (○) heparin; (▼) PSS.

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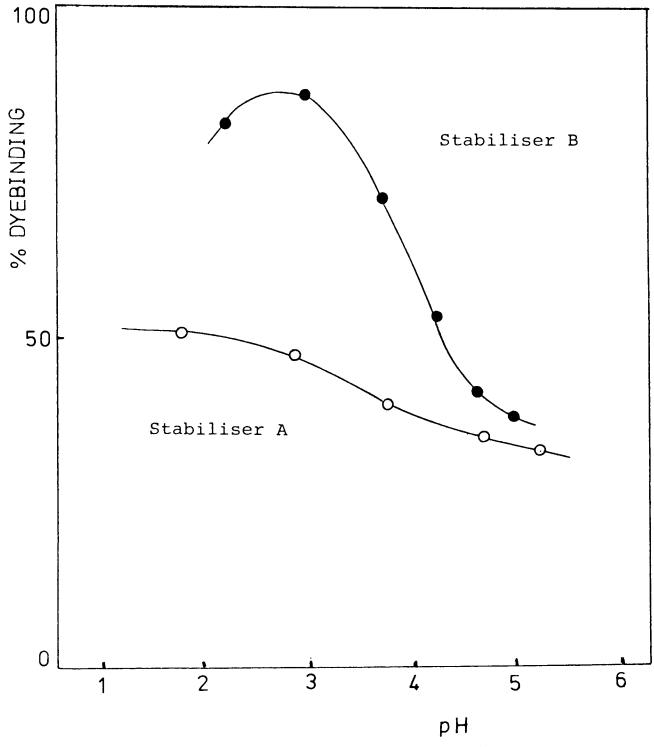

Figure 6. pH Dependence of dyebinding to two commercial ice-cream stabilizer mixes

Materials and Methods

Acridine Orange was dye-laser grade purchased from Kodak-Eastman, and was further repeatedly recrystallised from ethanol. A stock dye solution of 10^{-2}M was stable for up to 1 year in a blackened flask, and dye was withdrawn when required using a microlitre syringe and subsequently diluted to the required concentration. Unless stated, all dye solutions used were 10^{-5}M.

Hyaluronic acid (HA) and chondroitin sulphate (CS) were purchased from Sigma Biochemicals (London). Kappa-carrageenan (CAR) was provided by the Copenhagen Pectin Co. and sodium heparin (HEP) by Abbott Labs, North Chicago, with anticoagulant activity of 150 units per mg. Carboxymethyl cellulose 7 MP (CMC) was a medium viscosity grade of degree of substitution (DS) 9.7 to 0.8 from Hercules Inc. and sodium poly(styrene sulphonate) (PSS) was the isotactic conformer from Dow Chemical Co. Inc. Poly(p-xylyl viologen)[7] was prepared by Dr. G. Ashwell at Nottingham University, England, and was polycationic over the entire pH range. Poly-L-lysine hydrobromide was of molecular weight 15,000-30,000, and was purchased from Sigma (London). Samples of characterised Kappa caseins were prepared by Dr. R. G. Morley[8]. Calf-skin, acid soluble collagen was obtained from Sigma (London).

The ice-cream stabiliser mixture (Stabiliser B) and commercial carrageenan (Stabiliser A) were provided by Dari Tech Corporation, Atlanta. Fluorescence intensities were recorded on a fully compensated spectrofluorimeter[9]. In the dye binding profiles of standard solutions, the x-axis is represented by P/D, the ratio of polymer sites to the total concentration of dye molecules.

Results and Discussion

Binding Strengths of Polyanions. For a P/D = 1.0 complex of polyanion and 10^{-5}M dye (Fig. 4) shows the relationship of free dye with log. of salt concentration for a series of polyanions and two electrolytes (NaCl and $CaCl_2$). Intensities were corrected for salt-induced aggregation of the free-dye, and for quenching of the dye fluorescence by the chloride ion.

The results are in agreement with those obtained by pulse radiolysis for heparin and methylene blue[10]. Most of the dye release occurs within a decade of log. salt concentration. The critical electrolyte concentrations[3, 4] can be found by extrapolation to complete dye release, and values are given in Table 1. The order of increasing dye binding strength is:

$$HA < CMC < CS < HEP < CAR < PSS$$

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Table 1. Critical Electrolyte Concentrations for Polyanion—Acridine Orange Complexes from Fluorescent Intensity Data

POLYANION	Critical Electrolyte Concentration (M).	
	NaCl	$CaCl_2$
Hyaluronic Acid	3.9×10^{-3}	8.5×10^{-5}
CMC	3.7×10^{-2}	1×10^{-3}
Chondroitin Sulphate	7×10^{-2}	6×10^{-3}
Heparin	0.26	2.2×10^{-2}
K-Carrageenan	1.2	0.53
NaPSS	1.6	1.0

Table 2. Titrations at Varying pHs of Solutions Containing Polymer-Bound COO and SO_4 Groups

A. Ice Cream Stabilisers

Stabiliser	pH	End Point µl	% Carrageenan
A	3.07	69	)
A	2.57	69	) 98%
A	6.0	41.2	)
B	6.0	160	-
B	2.57	520	)
B	3.07	520	) 13.3%

B. Heparin

pH	Theoretical End Point	Experimental End Point (Average of 3)
7.0	40	39
3.0	56	57

Estimation of Carboxyl to Sulphate groups present in the same polyanion. Certain glycosaminoglycans contain both carboxyl and sulphate ester groups in a regular repeating structure along the polysaccharide backbone. The variation with pH of the stoichiometry of polyanion-acridine orange binding is shown in Fig. 5 for two such polysaccharides, heparin and chondroitin sulphate. The behaviour is intermediate between that of the polysulphates and polycarboxylates with incomplete dye release near the carboxyl pK_A. Recent workers[14, 15] have noted the variability of heparin structure. To interpret our results we have adopted the structural units proposed by Kennedy[16]. The carboxyl to sulphate ratio is approximately 2 to 5. Heparin (10^{-3}M in total anionic sites), was titrated against 10^{-5}M dye at pH 7.0 and pH 3.0. The results shown in Table 2B confirm the carboxyl to sulphate ratio 2 : 5 with total sites giving an overall accuracy of 2% in individual measurements.

Estimation of carrageenan in milk products. Bovine milk, carrageenan-free baby food and chocolate milk were doped with 1 mg per ml of κ - carrageenan. We have adopted various procedures to establish the standard titration curve for a 1 mg per ml carrageenan solution from each sample. Fig. 7 shows the direct titration of the standard carrageenan, doped baby food and undoped baby food, and indicates that the carrageenan cannot be assayed directly. Using model systems, the effect of lipids (lard) and protein (BSA) on the titration were investigated, and, as shown in Fig. 8, the presence of moderate concentrations of each virtually destroyed the quantitative nature of the dye binding. Using mixtures of BSA and κ - carrageenan it was found that interference from the protein could be removed by digestion with a mixture of trypsin, α- chymotrypsin and Carlsberg subtilisin at alkaline pH. Prior to digestion it was necessary to remove all lipid material from the dairy product. This could best be achieved by vigorous shaking with chloroform and methanol. The mixture after centrifugation had three phases, aqueous, organic, and between them the denatured protein layer. Analysis of the various layers confirmed that all the polyanion was sedimented along with the denatured protein. After freeze drying this layer and digestion with the 3-enzyme system, products were water-soluble. Fig. 9 shows the optimal conditions for recovery of polyanion. Dialysis of the digestion products gave 98% recovery of carrageenan from both bovine milk and baby food.

Chocolate pigments have previously been found to interfere with analytical procedures developed for non-pigmented milks[11-13], and the scheme we developed (Fig. 9) was no exception since the chocolate pigments inactivated the proteolytic enzymes. This difficulty was overcome using the procedures shown in Fig. 10. The carrageenan was extracted from the denatured protein layer, after de-fatting, using saline alkaline buffer, since the chocolate pigmented proteins were insoluble compared to those

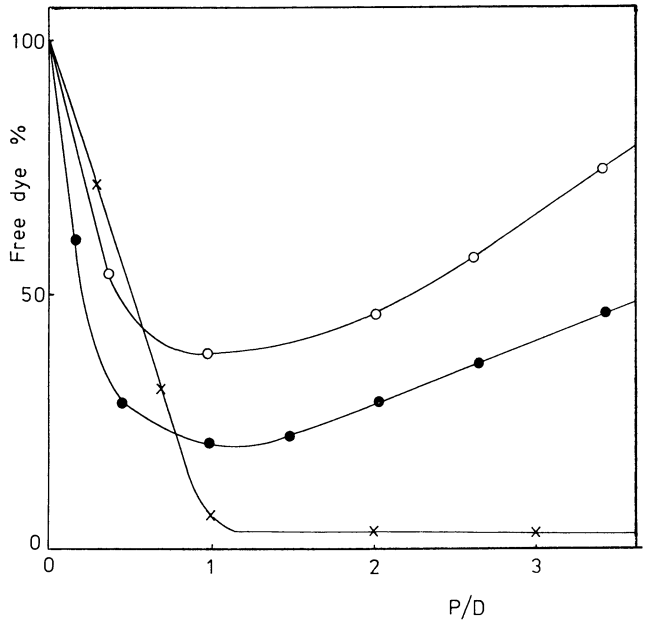

Figure 7. Doping of babyfood with carrageenan. Dyebinding titrations of doped and undoped sample and standard carrageenan. (○) Undoped SMA babyfood; (×) standard 10^{-3}M carrageenan; (●) doped sample.

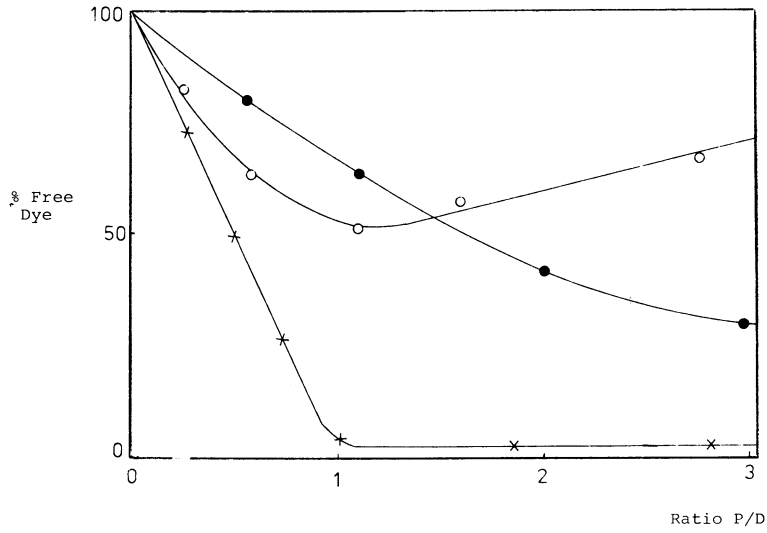

Figure 8. Effect of protein and lipid on the titration of acridine orange with 10^{-3}M carrageenan. (×) Standard carrageenan; (○) carrageenan with emulsified lard, 1 mg/mL; (●) carrageenan with BSA, 0.5 mg/mL.

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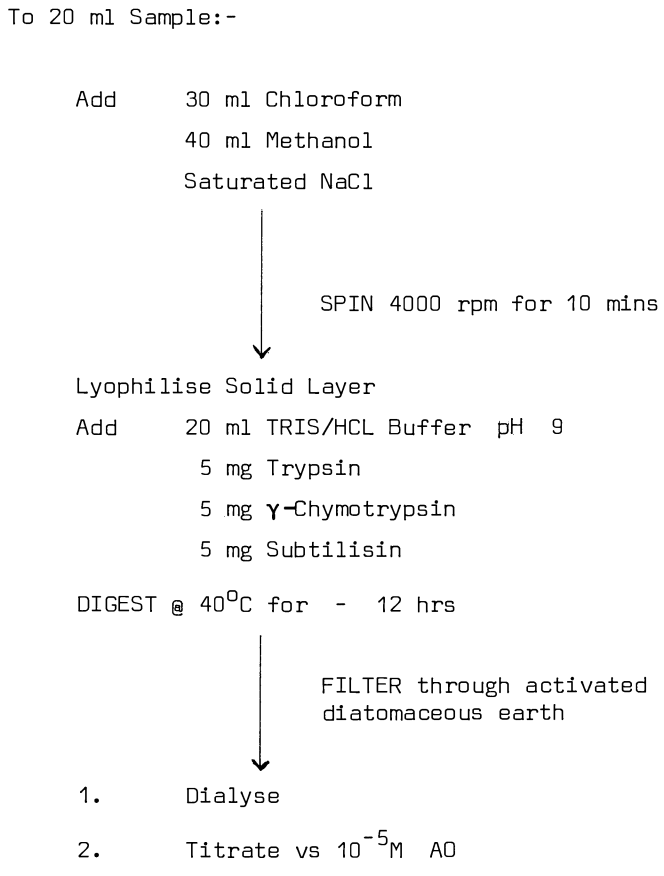

Figure 9. Procedure for the analysis of polyanion-containing solutions

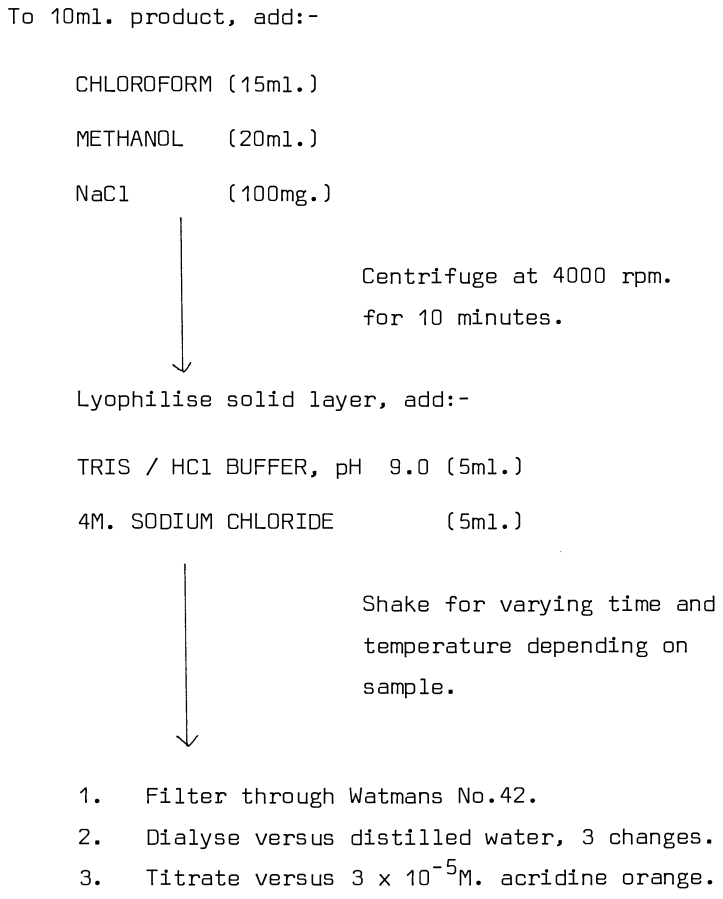

Figure 10. Procedure for the analysis of pigmented polyanion-stabilized dairy products

present in baby food. The extract after filtration remained slightly pigmented, and interfered slightly with the titration, giving reduced sharpness of the end point with 1 x 10^{-5}M dye. However, using a dye concentration of between 3 to 5 x 10^{-5}M increased the accuracy since dye binding to carrageenan is stronger at this higher concentration[17]. Polyanion recovery varied with the temperature of the extraction step, to a maximum of ~96%. The optimum temperature varies with the sample.

Carrageenan Standards. Table 5 shows the numerical values of the equivalent weights of several samples of carrageenan isomers, determined by Cundall et al[2]. In the situation when we are assaying for carrageenan on a weight per volume basis, the standard carrageenan sample must be of the same ratio of isomers as the unknown sample. Failure to account for this could lead to errors up to 30% in the extreme case of pure lambda and kappa isomers on a weight basis, although the concentration expressed in equivalents of anionic sites per litre would be unaffected.

A similar problem occurs with standards for CMC, as already noted. The difficulty here arises from the variable degree of substitution (DS) of commercial CMC samples. Here also conversion of concentration in terms of ionic sites, as determined by dye binding titration, into a weight of stabiliser, necessitates a knowledge of the DS or calibration with standard CMC samples.

Interaction of protein and polyanions.

The observation that cationic polypeptides quantitatively displace acridine orange from NaPSS, in these laboratories[6] is here utilised to study interactions which may occur in connective tissues and to investigate the mechanism of the stabilisation of dairy products by anionic gums. The normal titration of dye by polyanion is shown in Fig.11 (solid line). The system can be quantitatively reversed by titration with poly-L-lysine. The concentration x-axis is given as the ratio PC/PA, (polycation sites added to total polyanion sites), with PA constant at 2 x 10^{-5}M i.e. P/D = 2. The dye release curve closely parallels the dye binding curve with almost quantitative dye release occurring between PC/PA 0 to 1. Initial stoichiometric interaction occurs, therefore, between polycation and polyanion not associated with dye, followed by interaction of the polycation with dye polyanion complex, to give a complex with equivalence of acid and base sites and displacement of the dye.

With proteins the interaction is more complex since acidic and basic amino acid residues can be present. The acidic residues of the protein will modify the extent of interaction with polyanion, and the secondary and higher structure of the protein will affect the availability of the binding sites. The net charge of the protein can be varied by changing the pH, and at pH 3 most proteins have a net positive charge. The interaction between three proteins and one model polymer, polyvinyl

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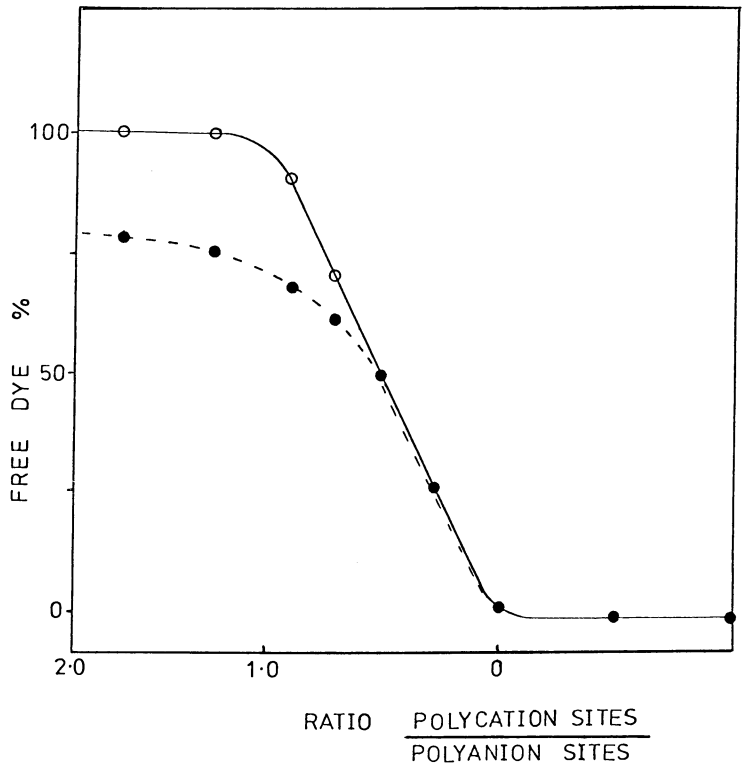

Figure 11. General effect of the time dependence of polyanion–polyanion interaction on binding profiles. (●) Points recorded instantaneously; (○) points recorded after 1.5 hr.

pyrrolidone, with a P/D = 2.0 complex of PSS and acridine orange at pH 3.0 is shown in Fig.12. The behaviour is not uniform. PVP does not displace dye at any pH indicating that the amide-linkage group does not participate in the binding. With κ - casein an initial dye release was observed, but the dye was re-bound with increasing protein concentration. All proteins used were 10^{-3}M in basic sites, the basic equivalent weight being defined as the weight of protein containing one basic amino acid residue, i.e. either lysine, arginine, histidine or hydroxylysine.

Bovine Serum Albumin (BSA) displaced dye linearly, indicating a final stoichiometry of about 6 to 1, BSA to PSS sites. By using structure breaking solvents such as urea[18] it is possible to increase the extent of BSA interaction. At pH 3 and an urea concentration of 4M, which is the maximum urea level at which reproducible dye binding occurred, the stoichiometry increases to 2 : 1, BSA to PSS. These findings demonstrate the importance of secondary structure of such globular proteins in regulating interactions.

The interaction of PSS and collagen is again different. Almost quantitative dye release is apparent at pH 3 and in the absence of a structure breaking solvent. This could be due to the proposed[19] heterogeneous charge distribution of collagen in which groups of amino acid residues of largely hydrophilic character are separated by regions of predominantly hydrophobic character. This effect would be allied to the more 'open' nature of the collagen triple helix, where charged groups are likely to be external for steric reasons, when compared with the globular proteins, where residues are buried deep within the folded structure as shown by BSA. The method could, therefore, offer a means of investigating distribution of charge within the protein tertiary sturcture.

Changes in stoichiometry of collagen - glycosaminoglycan (GAG) complexes with pH were studied by initially forming 1 : 1 complexes at acid pH, and observing the uptake of dye by the polyanion as the complex is dissociated with the addition of base. The interactions of hyaluronic acid and chondroitin sulphate are shown in Fig. 12 in comparison with those for model polyanions, CMC and PSS. All intensities were corrected for the acid dissociation of the dye-polyanion complex using data from Fig.5, and for the variation of dye fluorescence intensity with pH. At acid pH the dissociation closely follows the hydrogen-ion titration curve for collagen[20]. The interaction is virtually absent at neutral pH for the sulphated polymers. Interactions with the polycarboxylates are slightly more resistant to alkaline dissociation.

The results given in Fig. 13 were obtained using 10^{-5}M polymer concentrations. Increasing the concentrations of each polymer increased the stability of the complex to base dissociation, as also did the addition of 10^{-2}M calcium chloride for chondroitin sulphate.

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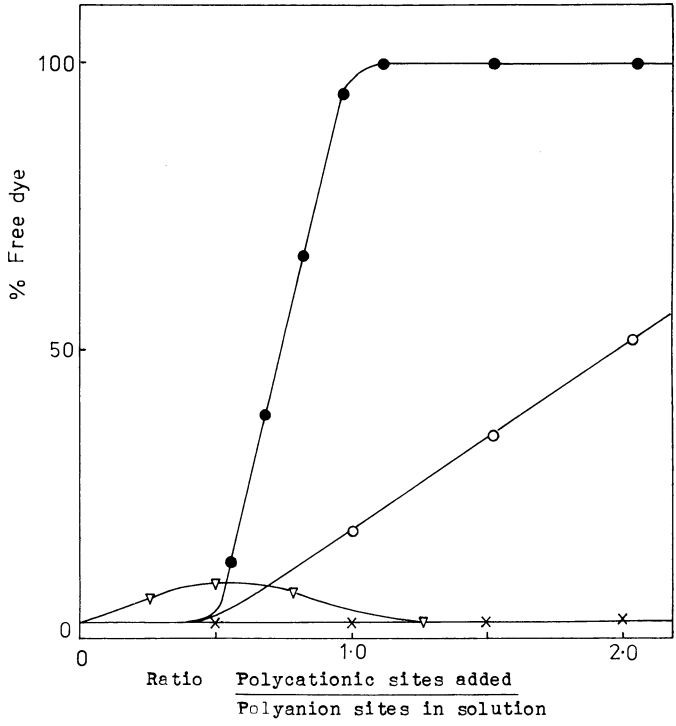

Figure 12. Dye release profiles for the interaction of three proteins and poly(vinyl pyrrolidone) with a P/D 2.0 complex of NaPSS-acridine orange at pH = 3.0 in salt-free solution. (×) Poly(vinyl pyrrolidone); (▽) kappa casein; (○) bovine serum albumin; (●) collagen.

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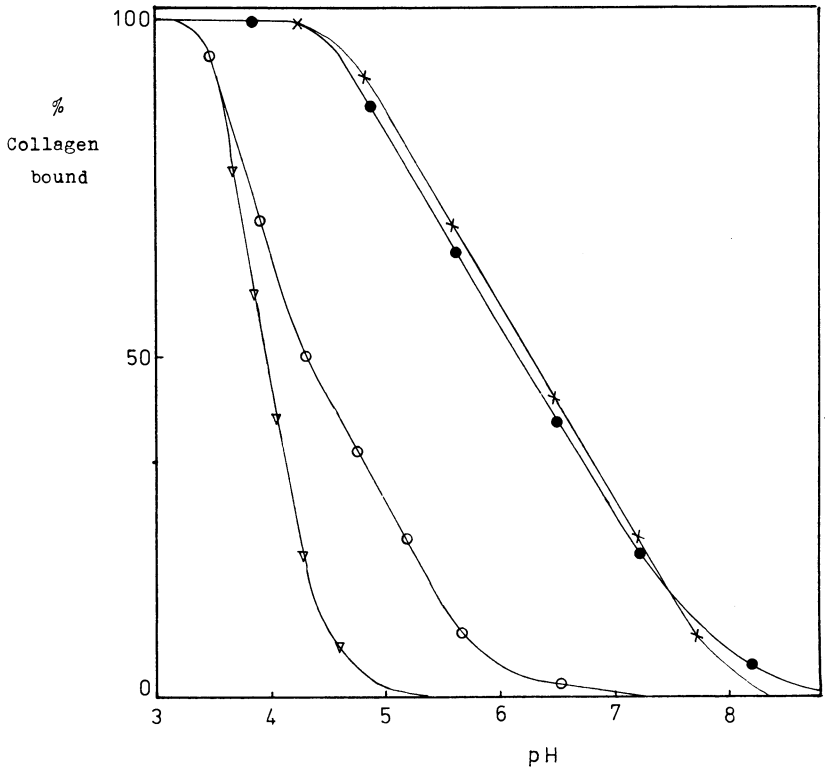

Figure 13. Variation of polyanion–collagen binding with pH. (▽) Choindroitin sulfate; (○) poly(styrene sulfonate); (×) CMC; (●) hyaluronic acid.

The relative strengths of the collagen - polyanion complexes were assessed by the 'critical electrolyte concentration' method of Scott[3, 4]. Table 3 shows the salt concentrations which were necessary to dissociate stoichiometric 10^{-3}M complexes of collagen polyanion. Values for PSS and heparin could not be obtained since the high salt concentrations for dissociation precipitated the collagen. Those for hyaluronic acid and chondroitin sulphate indicate that all the complexes exist in physiological conditions of ionic strength, and above.

Investigation of the structure of model polyelectrolyte complexes.

Using the technique we have described of dye displacement from a dye polyanion complex by the addition of polycations[6], we have studied the stoichiometry of the complexes formed between three polyanions (PSS, CMC and CAR) with two polycations (PPXV and PLL). A wide range of conditions of pH and salt concentrations were employed. Complexes were found to exhibit a 1 to 1 pairing of positive and negative ionic sites between pH 4 and 10, and in solutions of high salt concentration, up to 2M sodium chloride for complexes involving polystyrene sulphonate. The salt-induced dye release limited the salt concentration which could be employed.

A strong time-dependence of the binding was observed at polymer concentrations approaching the stoichiometric ratio, as shown in Fig.11,with binding usually being complete after about 1 hour. At this point complete electroneutrality of the complex is achieved. It is probable that the initial polycation added is bound instantaneously, since large numbers of polyanion sites are available. It has been observed[21] that when the polyelectrolyte counterions are H^+ and OH^- the binding is irreversible, and the contact configuration is retained, since the counterions react to give H_2O. However, the presence of other counterions such as Na^+ and Cl^- screens the polyelectrolyte sites to an extent which enables the re-orientation of the complex to give a lower free-energy state, as the counterions diffuse out. This is consistent with the slow dye-release at around the stoichiometric ratio observed here. The presence of some cross-linking must be predicted, in view of the molecular weight differences and polydispersity of the polymers.

Viscosity measurements (Fig. 14) on the CAR - PLL complex, and measurement of the Stern-Volmer quenching constant (Table 5, Figs. 15 and 16) of dye released by complex formation are consistent with the complete destruction of the electric field of the polyelectrolytes. In the former instance, the solution viscosity of the polyanion is dramatically reduced by the addition of polycation. Polyanion viscosity is intimately related to the degree of extension of the polymer chain, which is in turn a function of the degree of ionisation. The reduction of viscosity would, therefore, appear to be associated with the neutralisation

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Table 3. Critical Electrolyte Concentrations of Some Polyanion–Collagen Complexes Obtained by the Method of Scott (7)

Polyanion	pH	Critical electrolyte concentration (M)	
		NaCl	$CaCl_2$
Hyaluronic Acid	3.0	0.15	0.06
Chrondroitin Sulphate	3.0	(~0.5)	0.125

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Table 4. Stern–Volmer Quenching Constants for $10^{-5}M$ Acridine Orange Solutions Containing Stoichiometric Polyelectrolyte Complexes

Polyanion	Polycation	K_{sv}
-	-	70
k-Carrageenan	PLL	64
Chondroitin sulphate	PLL	54
NaPSS	PLL	54
CMC	PPXV	67
Hyaluronic Acid	PPXV	69

Table 5. Equivalent Weights of Repeating Units of Several Carrageenan Samples (2)

Carrageenan sample.	Equivalent weight per sulphate group.	
	From sulphate content.	By acridine orange titration.
Lambda (I).	286	298
Lambda (II).	258	266
Kappa	370	390
Iota	296	290

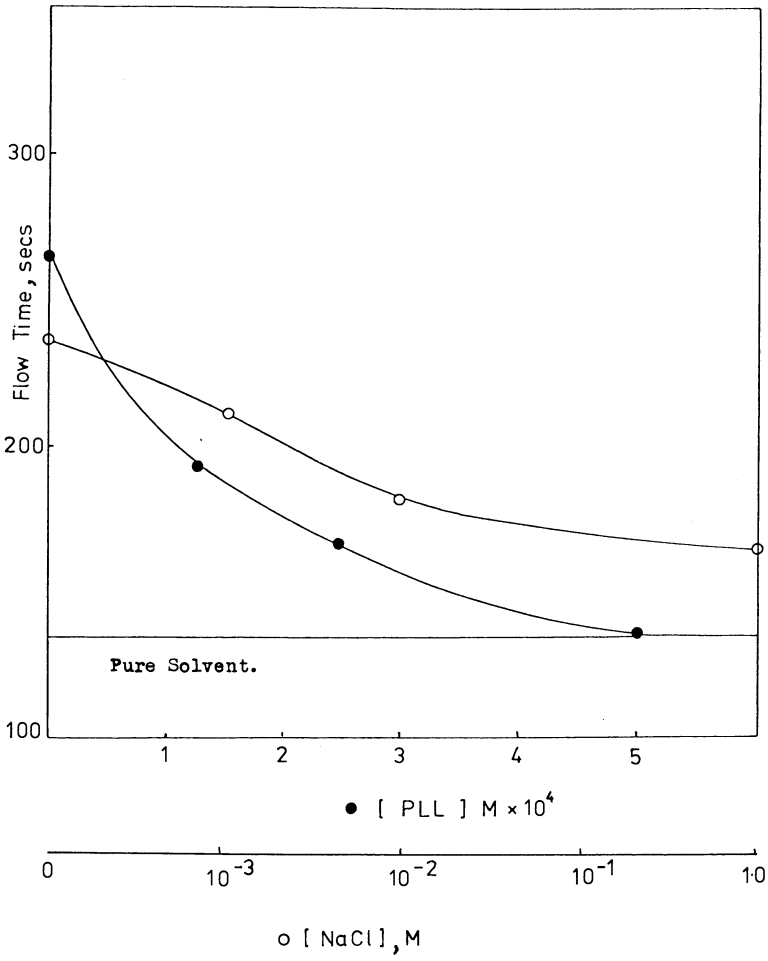

Figure 14. Ostwald viscometer flow times for a viscous polyanion, κ-carrageenan, at varying concentrations of simple (○) and polycationic (●) electrolytes

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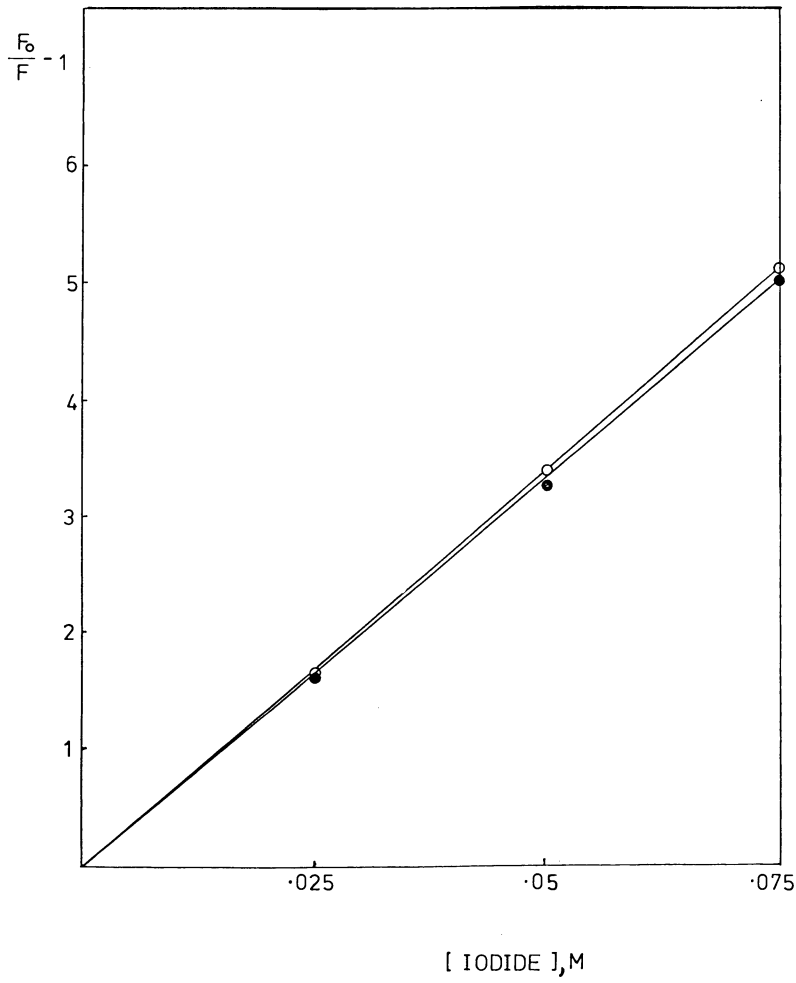

Figure 15. Stern–Volmer plots for the iodide quenching of dye released by polyanion—PPXV interaction. (●) Carboxymethyl cellulose; (○) hyaluronic acid.

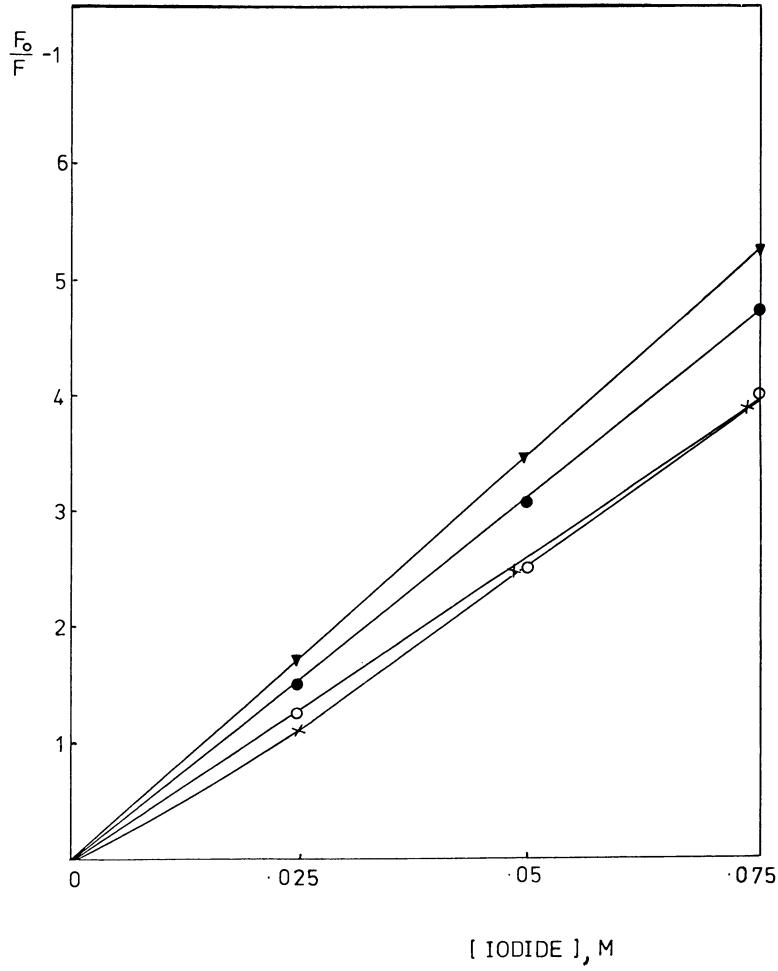

Figure 16. Stern–Volmer plots of dye released by polyanion/poly-L-lysine interaction. (▼) Acridine orange; (●) κ-carrageenan; (○) chondroitin sulfate; (×) polystyrene sulfonate.

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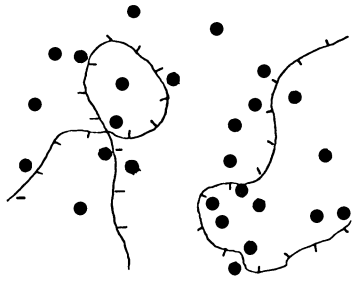
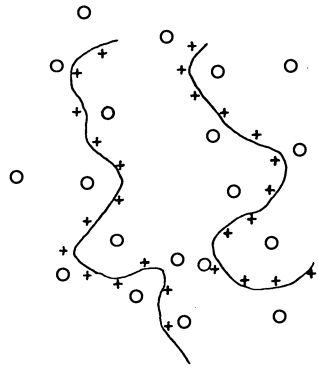


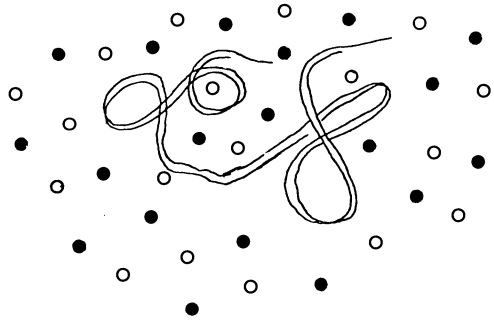
Poly-L
- lysine
hydrobromide

● Bromide ions



Sodium polystyrene
sulphonate

○ Sodium ions



Polyelectrolyte complex and free counterions

Figure 17. Interaction of polyanions and polycations—a simplified picture of polymer conformations and counter-ion distribution

of charged groups. Similarly measuring the Stern-Volmer quenching constants of the released dye indicates that the dye molecules are truly 'free' and under no constraint from the polyelectrolyte complex, since binding of monomeric dye to polyanions greatly reduces the value of the quenching constant[6].

A simplified picture consistent with such observations is indicated in Fig. 17. More direct evidence for this more compact structure of the complex comes from ORD/CD spectroscopy[22]. The presence of acid glycosaminoglycans had an α-helical or random coil-directing effect on cationic polypeptides which are normally in the charged-coil form at neutral pH.

ABSTRACT

The fluorescence characteristics of Acridine Orange bound to naturally occurring and synthetic anionic polysaccharides can be distinguished from free Acridine Orange, and is the basis for the quantitative estimation of these materials. By suppressing the ionization of individual anionic groups, sulphated polysaccharides can be estimated in the presence of carboxylated polysaccharides, and the proportions of the O- and N- sulphate groups relative to carboxylic groups within the same polyanion (e.g. in heparin) determined. Proteins, lipids, fats, and salts, which can occur in biological systems and food products, interfere with the basic procedure, but modifications have been introduced to allow the analysis to be undertaken in the presence of these additivies. The method has permitted the quantitative study of interactions between proteins and glycosaminoglycans. The extent to which such interactions occur under physiological conditions is assessed.

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Sulfated Glycosaminoglycans Obtained by Chemical Modification of Polysaccharides

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As part of the general theme of the symposium devoted to sulfated carbohydrates, this report presents some of the work from our laboratory concerned with the natural, sulfated glycosaminoglycan, heparin, both from the standpoint of structural elucidation and more especially from that of synthetic production of similarly constituted, sulfated polysaccharides that have biological properties resembling those of heparin.

There is not time to present a detailed historical development of the entire story of the biological role and efforts to establish the chemical structure of heparin, but it has been known for many years that this biopolymer contains residues of a uronic acid and 2-amino-2-deoxy-D-glucose in approximately equal proportion, and the material is highly sulfated, both at the amino positions and also at certain of the hydroxyl positions (1). The compound is widely distributed in many connective tissues, where it appears to be produced as an intracellular component in mast cells. It is widely used in therapy as an anticoagulant in cardiovascular disorders resulting from thrombosis, and it also displays antilipemic (clearing factor) properties.

Much current evidence indicates that heparin as used therapeutically is the carbohydrate portion alone of a proteoglycan that is the native tissue component; a long, single, protein chain may be envisaged as being substituted by a large number of lateral chains of the sulfated polysaccharide, attached by a bridge region containing two D-galactose and a D-xylose residue, attached via L-serine to the peptide chain (2). Not only has the total constitution of this native proteoglycan not been elucidated, but controversies still remain concerning the exact constitution of the glycan chains that are detached during the processes used for extraction of heparin on the commercial scale for therapeutic use as an anticoagulant.

Details of the exact procedure used in the commercial isolation of heparin from such rich sources as hog gastric mucuosa and beef lung are not readily available, but in general terms, the process involves autolysis of the tissue with concomitant

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degradation of the non-heparin components, followed by alkaline treatment to remove as much as possible of the protein component, and subsequent purification by use of cationic detergents. The glycan material thus isolated is of much lower molecular weight than the parent proteoglycan, and the product most widely used in therapy has a molecular weight in the region of 12,000, with a distribution of molecular weights above and below this figure. The molecular weight is difficult to estimate accurately unless careful attention is paid to the effects of the polyanionic character of the compound, but the accompanying graph (Figure 1) from a recent article (3) shows the results of molecular weight determinations on various heparin preparations by ultracentrifugation methods in comparison with viscosity measurements; it may be seen that the more readily applied viscosity technique may be used at least as a rough approximation in estimating the molecular weight of a heparin sample.

The anticoagulant activity of such heparin preparations is critically dependent on the level of sulfation; commercial products assayed by a conventional technique using sheep plasma typically show activities in the range of 110 to 180 International Units per milligram for material containing approximately 5 sulfate residues per nominal tetrasaccharide unit of the polymer. Partial desulfation leads to lowering of the biological activity, without necessarily causing changes of more-profound significance in the polymer; milder isolation techniques leading to products having still higher degrees of sulfation have displayed activities at higher levels than those encountered normally in commercial material.

Elucidation of the chemical constitution of the heparin chain has proved remarkably difficult; the high degree of sulfation renders the material intractable in many of the conventional methods for polysaccharide structure-determination. It has been established with little doubt that the uronic acid component involves D-glucuronic acid and L-iduronic acid, but the exact proportions of these, and their ratio in the native proteoglycan, still remain controversial. It is by no means certain that the ratio of these uronic acids present in commercial heparin reflects that present in the original proteoglycan, as these two uronic acids could interconvert by C-5 epimerization under the alkaline conditions used in the isolation procedure. In the biosynthesis of the polymer chain, it is very probable that this C-5 inversion occurs at the polymer level (4), and in the native state there may exist an entire spectrum of glycan chain-compositions ranging from fractions rich in D-glucuronic acid on the one hand to fractions rich in L-iduronic acid on the other.

Working on the premise of a linear, alternating chain of amino sugar and uronic acid components, work from this laboratory (5) involving N- and O-desulfation of commercial heparin, followed by carboxyl reduction with diborane and then selective fragmentation of the resultant, reduced, desulfated heparin led,

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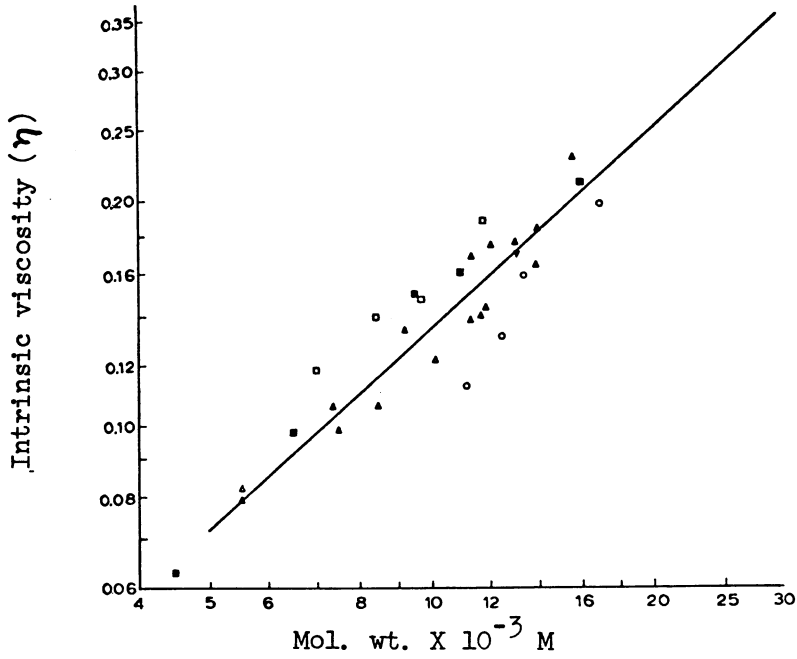

Figure 1. Relation between intrinsic viscosity and molecular weight (sedimentation) from $[\eta] = 1.35 \times 10^{-5}M^{0.9}$ *in aqueous sodium chloride medium. Data from Ref. 2.*

according to the hydrolytic procedure used, to two disaccharides, as illustrated in Figure 2.

One of these disaccharides, characterized on a crystalline basis, had a structure that indicated an α-D-(1→4) linkage between 2-amino-2-deoxy-D-glucose and D-glucuronic acid in the original polymer. The second disaccharide "maltosamine", for which a reference sample was synthesized from maltose, indicates from its structure the occurence of an α-D-(1→4) linkage between glucuronic acid and 2-amino-2-deoxy-D-glucose in the original polymer. This information led to the formulation of a partial backbone structure as shown in Figure 2, and subsequent unequivocal identification of L-iduronic acid residues in some preparations of heparin (6) led to the type of general formulation shown in Figure 3, which depicts a hypothetical tetrasaccharide repeating-unit for heparin based on the occurence of L-iduronic acid residues and D-glucuronic acid residues, complete sulfation of the amino groups, complete sulfation of the primary hydroxyl groups, and additional sulfate residues at one of the secondary positions, probably position 2, in the L-iduronic acid component; the preferential susceptibility of the D-glucuronic acid residues toward degradation by periodate provided evidence that the D-glucuronic acid residues were largely non-sulfated at the 2,3 positions.

Since the time of these formulations, other workers have proposed on the basis of enzymic studies with degradation fragments from heparin that some or all of the linkages of the glycuronic acid components have the β-D or α-L configuration (7), and support for these proposals (Figure 4) has been provided from inspection of n.m.r. spectra of the polymer (8) and also from X-ray diffraction studies (9) on the oriented polymer. The X-ray diffraction studies do accord with the general idea of fairly short, linear chains for the macromolecule. However, unequivocal structural proof by degradation and synthesis of fragments at a level beyond that of disaccharide components is still lacking, as is such evidence obtained from native proteoglycan material that has not been subjected to an unspecified level of possible alkaline treatment during a commercial isolation process.

Our continuing studies on heparin have been directed along two fronts, first of all the systematic structural elucidation of components of the polymer chain at a level beyond that of the disaccharide, together with procedures for determining uronic acid ratios and positions of sulfation in the chain. In parallel investigations, we have examined the chemical synthesis of modified polymers whose structure simulates that of heparin, to afford potential replacements for the expensive natural material as a therapeutic anticoagulant, and to provide a product for attachment to the surface of synthetic objects for implantation in the circulatory system, with the objective of conferring a biologically acceptable, nonthrombogenic surface-characteristic for such devices. It is the latter aspect of chemical synthesis

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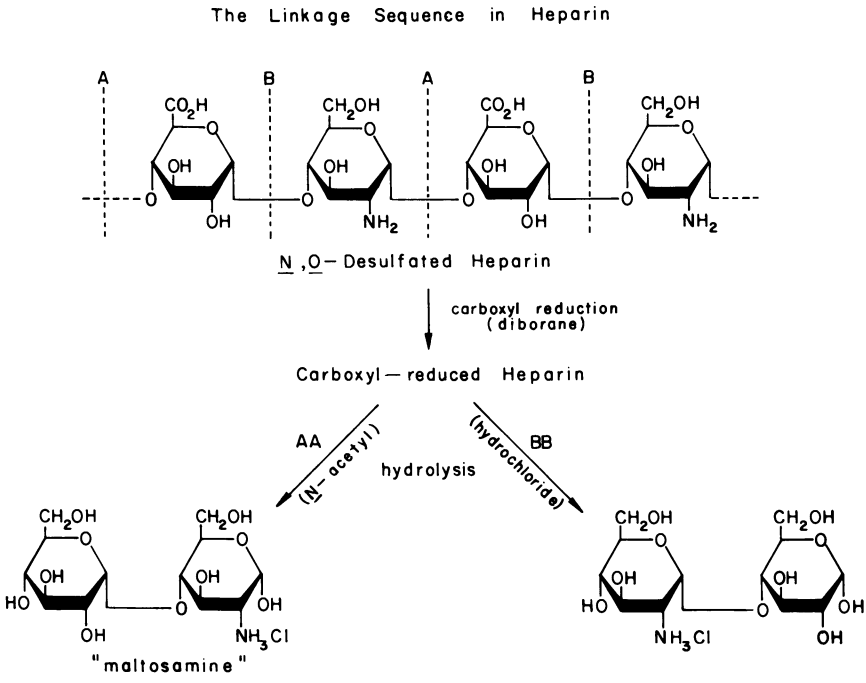

Figure 2. Disaccharides from carboxyl-reduced heparin

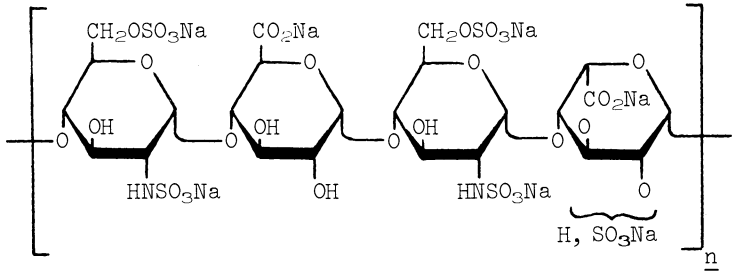

Figure 3. Component residues present in heparin

of heparin-like polysaccharides that constitutes the principal theme of this presentation, but the following discussion presents a brief survey of some of the more recent efforts to degrade commercial heparin into oligosaccharides above the disaccharide level that might be useful in structure-determination studies.

The next scheme (Figure 5) illustrates a degradative method based on the recognized susceptibility of D-glucuronic acid residues in heparin to attack by periodate. Exposure of heparin to 1 molar equivalent of periodate per tetrasaccharide unit gives a product in which the D-glucuronic acid components are selectively degraded; subsequent borohydride reduction of this material, followed by mild hydrolysis with a polystyrenesulfonic acid of high molecular weight confined within a dialysis membrane, shows that degradation to a low molecular weight, trisaccharide-tetronic acid component does take place by this procedure. The recovery of the degraded, dialyzable components is relatively low in proportion to the total product that still retains polymeric character (10). This result tends to argue against the idea that the periodate-labile uronic acid residues are uniformly distributed along the chain. The dialyzate, after purification by preparative paper-chromatography, yields a material that appears to be a homogeneous tetrasaccharide whose constitution may be tentatively depicted as in Figure 6.

If the periodate-oxidized, borohydride-reduced heparin is further subjected to carboxyl-group reduction, and the product is then subjected to acid hydrolysis under conditions where the highly acid-resistant 2-amino-2-deoxy-D-glucosyl residues would be expected to remain attached to their aglycons, a mixture of products is obtained from which the aglycons may be detached by deamination with nitrous acid. This procedure gives erythritol arising from the original D-glucuronic acid residues that had been cleaved by periodate, and L-threitol arising from any L-iduronic acid residues that had undergone C-2—C-3 cleavage by periodate (Figure 7). Although the observed ratio (10) of these two products (2:1) is strongly in favor of erythritol, the fact that some threitol is also formed indicates that some of the L-iduronic acid residues in the original polymer were not sulfated at positions 2 and 3. The quantitative results obtained by this sequence of experiments depend to a large extent on the method of the treatment of heparin with periodate; there is no clear-cut, limiting consumption of periodate at the level of 1 mole per tetrasaccharide, and the use of an excess of periodate leads to a progressive increase in the total periodate uptake.

The possibility that other sulfated amino sugar polysaccharides might have anticoagulant properties has attracted a good deal of attention. In an early application, Wolfrom and Shen Han (11) utilized N-deacetylated chitin (chitosan) and sulfated it with pyridine–chlorosulfonic acid to produce an N-sulfated, partially O-sulfated product (Figure 8). This compound had relatively high anticoagulant activity, although this was not so

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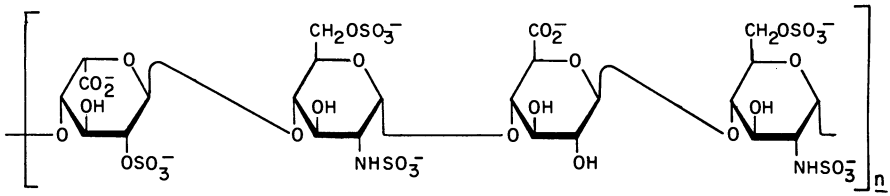
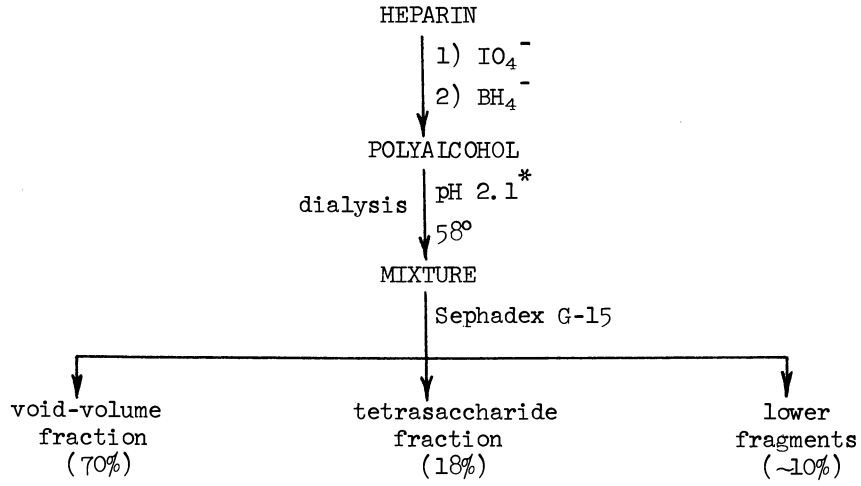


Figure 4. Heparin: a suggested tetrasaccharide repeating-unit



*with polystyrenesulfonic acid

Figure 5. Degradative scheme for heparin based on selective periodate cleavage of D-*glucuronic acid residues*

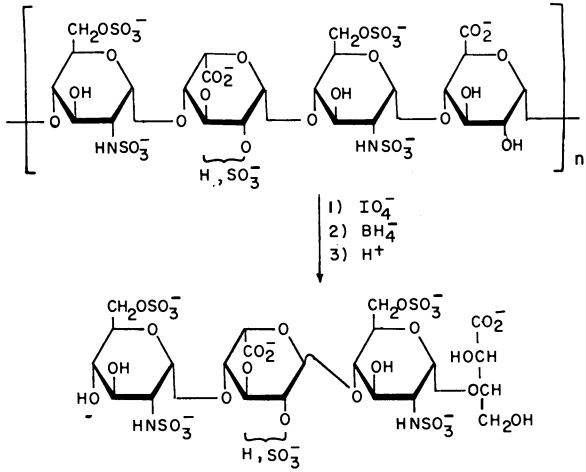


Figure 6. Tetrasaccharide fragment from periodate-oxidized, borohydride-reduced heparin

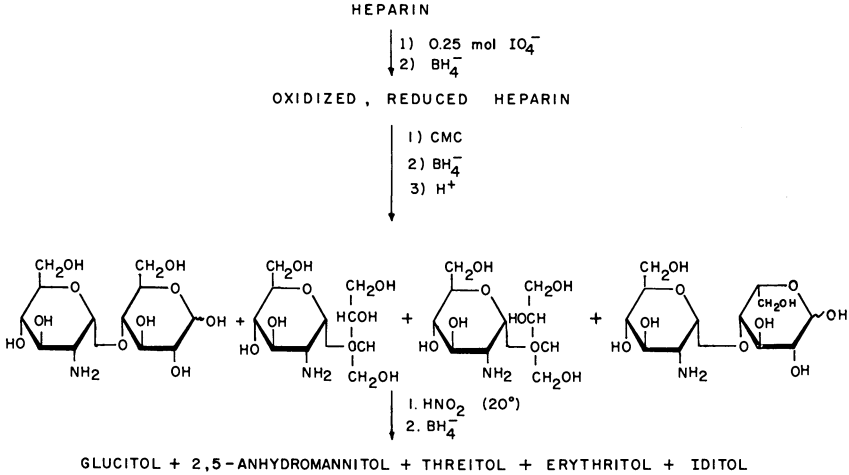


Figure 7. Alditol components from heparin after sequential periodate oxidation, borohydride reduction, carboxyl reduction, acid hydrolysis, nitrous acid deamination, and borohydride reduction

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high as that of heparin itself. Part of the reason for this difference may probably be traced to the structural dissimilarity between the chitosan backbone and the alternating backbone of heparin itself, but another important factor is undoubtedly the fact that the sulfated chitosan had a molecular weight some 20 to 40 times higher than the range considered optimal for high activity in heparin. The material showed acute toxicity in the mouse very similar to that of heparin, although there is evidence for a disadvantageous, delayed toxicity with this material. In subsequent studies by Whistler and Kosick (12), a similar approach was used, but in addition, the procedure included an oxidation step by use of dinitrogen tetraoxide or oxygen—platinum to introduce some carboxyl groups into the polymer (Figure 9). This material likewise showed activity of the same order of magnitude as that obtained by Wolfrom and Shen Han (11), and it was shown that sulfated and oxidized products were somewhat more active than materials that were sulfated only; in all instances the activity increased with increasing levels of sulfation, and products of the highest activity were obtained when the sequence was allowed to give sufficient degradation to shorten the polymer chain to molecular-weight levels (osmometry) of the same order of magnitude as that of heparin.

In view of the somewhat nonspecific and degradative characteristic of dinitrogen tetraoxide as an oxidant, an effort was made in our laboratory (Figure 10) to achieve oxidation of chitosan at C-6 with a high degree of specificity (13). To accomplish this objective, chitosan was converted into its perchlorate salt, following the hypothesis that the strongly cationic group at C-2 should protect the hydroxyl group at C-3 against oxidation and allow specific and complete oxidation at C-6. The latter step was indeed achieved by use of chromium trioxide, and a product was obtained that was completely carboxylated; it presumably exists as an inner salt. Sulfation of the latter product gave the fully N-sulfated, C-6 carboxylated chitosan analog. The properties of this product are displayed in Figure 11, where it may be observed that the product does have anticoagulant properties, although the procedure used led in large measure to retention of the high-polymeric nature of the original polysaccharide, and so the molecular-weight range of the product thus obtained lay above that considered optimal for heparin-like activity. Controlled degradation prior to sulfation of this product would offer promise as a potential method for obtaining a synthetic heparinoid of higher activity.

The former approaches, based as they are upon the chitosan structure, do suffer from the fundamental drawback that the polymer chain manifestly differs from that in heparin itself. In view of this disadvantage, we initiated a new series of investigations based on an abundant (1→4)-linked α-D-glucan, namely amylose. In the initial series of studies (14), amylose was converted by oxidation of its 6-trityl ether with methyl

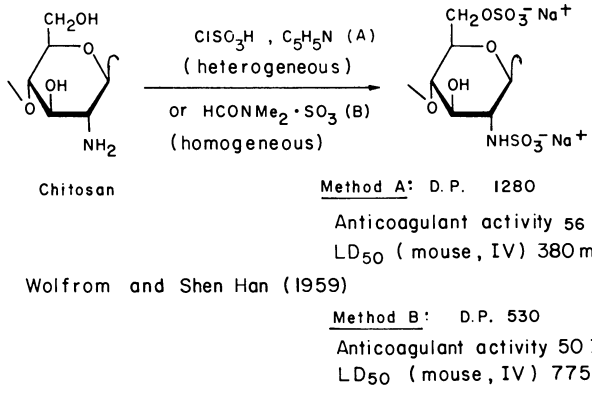

Figure 8. Sulfation of chitosan

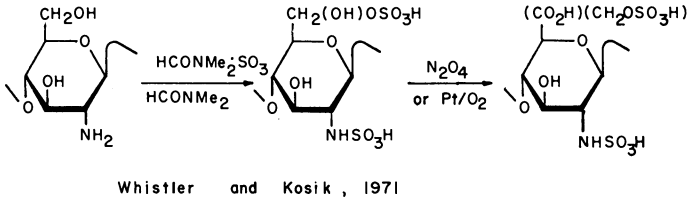

Figure 9. Sulfation and oxidation of chitosan

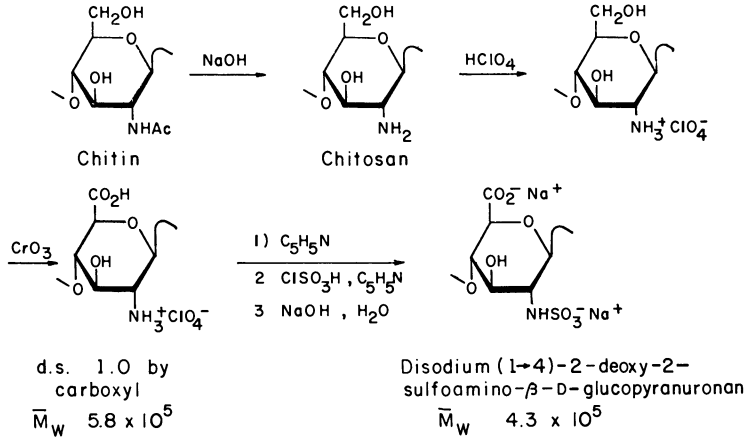

Figure 10. Specific C-6 oxidation and N-sulfation of chitosan

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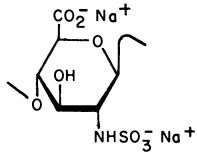
sulfoxide—acetic anhydride to give a product largely oxidized to the 2-keto derivative at certain of the residues (Figure 12). Conditions affording a degree of substitution of approximately 0.5 were selected. This product was successively oximated and then reduced with lithium aluminum hydride to give, after tritylation, an aminated amylose in which the amino groups had been largely incorporated at the 2-position in the D-gluco configuration, as demonstrated by acid hydrolysis. The oxidation procedure used also introduced (methylthio)methyl ether groups at O-3, at least in certain of the residues. Trifluoroacetylation of the free amino groups and the residual hydroxyl groups in the reduction product, followed by detritylation, exposed the hydroxyl groups at C-6, and these were subjected to oxidation by the action of oxygen—platinum. In view of the fact that this oxidation proceeds much more rapidly in D-glucose residues than in those of 2-amino-2-deoxy-D-glucose, it was hoped that the procedure would lead to a polymer in which residues of 2-amino-2-deoxy-D-glucose would be largely unoxidized at the C-6 position, whereas those of the non-aminated, D-glucose residues would be oxidized to the corresponding D-glucuronic acid residues. Removal of residual protecting groups, followed by sulfation of this polymer, gave a compound containing the requisite functional groups for a heparin-like structure, although, as shown in Figure 12, the anticoagulant activity of this material was low. This factor may arise from the fact that the product was of lower molecular weight than the values considered optimal for heparin-like activity. This degradation occurred principally at the stage where rather severe conditions were required to remove the O-(methylthio)methyl groups that had become introduced during the oxidation step with dimethyl sulfoxide—acetic anhydride.

In view of the drawbacks of the use of this particular oxidant, Dr. Usui performed a new series of experiments in which 6-O-tritylamylose was oxidized by mixtures of N,N-dicyclohexylcarbodiamide (DCC) and dimethyl sulfoxide, in various proportions. A range of oxidized products was obtained, as shown in Figure 13, and these were free of sulfur. Reduction of these polymers by borohydride, followed by analysis of the sugar composition of the products by means of the alditol acetates, revealed that oxidation had taken place selectively at the 2-position at low levels of oxidation. At higher levels of oxidation, the 3 positions were also affected, as shown by the nature of the alditols produced in the analytical sequence of reactions.

Oximation of the 6-O-tritylamyloses that had been oxidized to a fairly low degree of substitution gave the corresponding oximes, whose nitrogen content corresponded to the level of oxidation determined previously by reduction of the oxidized products with sodium borodeuteride, followed by analysis for the deuterium-containing sugar component (Figure 14). The oximated product was in turn acetylated to acylate the free hydroxyl groups in the polymer chain and also the hydroxyl group of the oxime function.



LD_{50} (intraperitoneal, mouse), 237 mg/kg

Anticoagulant activity, 26 I.U./mg

Leukemia L-1210 *in vitro* inhibition, 50% at 0.2 μM (100 μg/ml)

Figure 11. Biological properties of C-6 oxidized, N-sulfation chitosan

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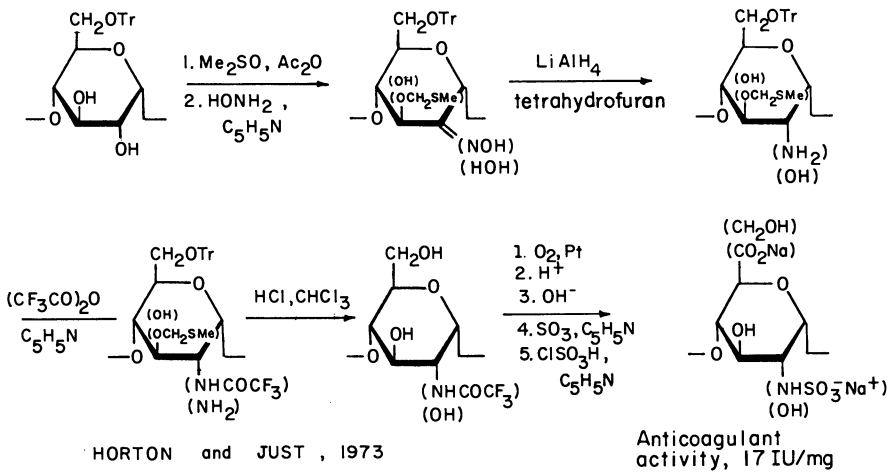

Figure 12. Conversion of amylose into a 6-carboxyl, 2-sulfoamino analog

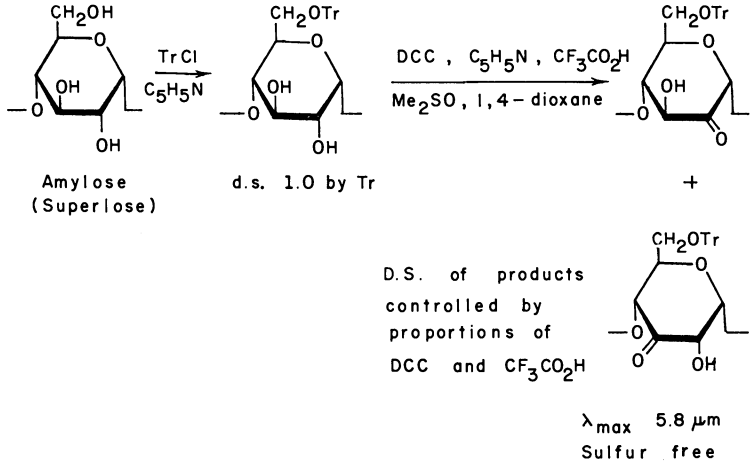

Figure 13. Alternative oxidation procedure for 6-O-tritylamylose

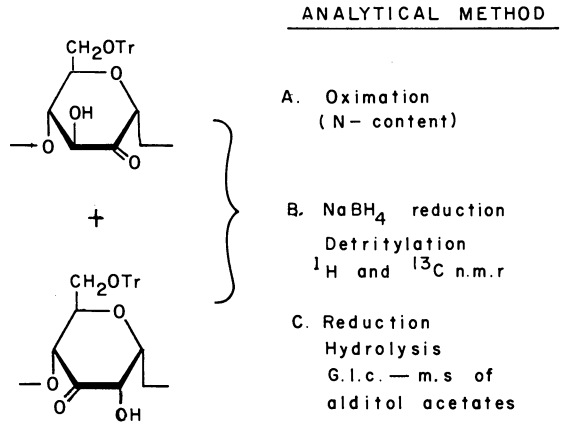

Figure 14. Procedures for analysis of oxidized 6-O-tritylamyloses

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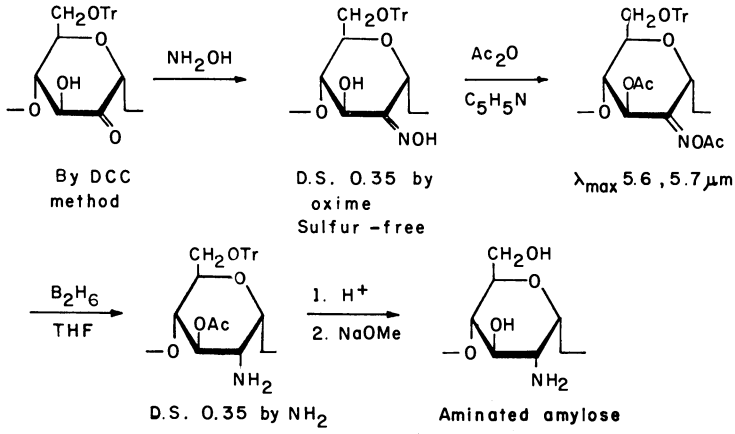

Figure 15. Preparation of 2-amino-2-deoxyamylose of d.s. 0.35

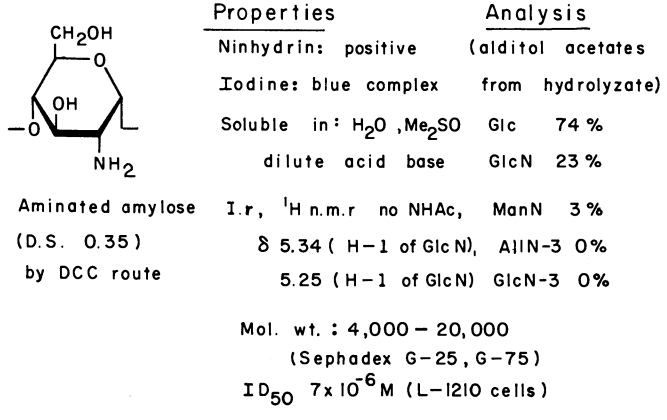

Figure 16. Characterization and properties of aminated amylose

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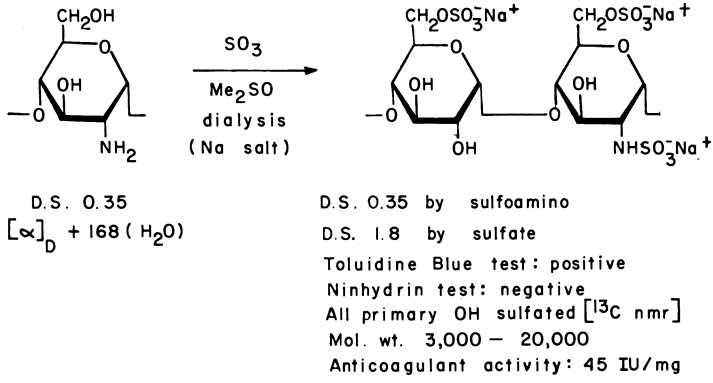

Figure 17. Sulfation of aminated amylose

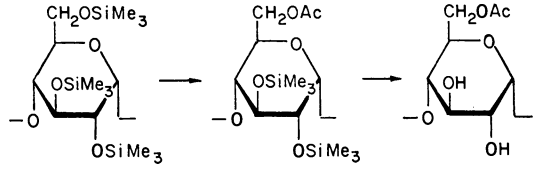

Figure 18. Preparation of 6-O-acetylamylose

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Table I. Analysis of DCC—CF_3CO_2H-Oxidized 6-O-Tritylamylose

Expt. no.	DCC/CF_3CO_2H per mol.	D.S. by oximation	Results of g.l.c. analysis[a]			Mol. wt.[b]	Iodine
			Glc	Man	All		
1[c]	5:1	70%	75%	4%	21%	8,000	-
2[d]	4:1	80%	93%	3%	4%	~ 20,000	-
3[d]	2:0.5	35%	95%	1%	4%	~ 20,000	+
4[d]	1:0.25	20%	99%	trace	trace	~ 35,000	+

[a] Supported by ^{13}C and ^{1}H n.m.r. analysis of reduced, detritylated product.

[b] By gel-permeation chromatography of reduced, detritylated product.

[c] Superlose 149974.

[d] Superlose 394554.

Reduction of the latter product by diborane proved to be a much more satisfactory method for introducing the amino group than the sequence previously used that involved direct reduction of the oxime with lithium aluminum hydride.

The sequence (Figure 15) gave, after detritylation, an aminated amylose whose degree of substitution corresponded to the original oxime content of the precursor, and the properties of the keto products at different degrees of substitution and as obtained by different means of preparation are shown in Table I. Acid hydrolysis of the aminated amylose showed that the net amination had been achieved with high regio- and stereo-selectivity. At a d.s. level of 0.35, essentially all of the amino groups had become incorporated at position 2 in the D-gluco configuration; no significant amino sugar components arising from oxidation at C-3 were detected in the product at this degree of substitution. Detritylation of the product was readily achieved by use of methanolic hydrogen chloride followed by treatment with sodium methoxide in methanol to remove the residual O-acetyl groups. The product (Figure 16) was readily soluble in water, in dilute acid, and also in dilute alkali, still showed the iodine-staining ability characteristic of amylose, but also showed the ninhydrin reaction characteristic of the presence of amino groups; the infrared spectrum, showing absence of carbonyl absorption, established that no migration of acetyl groups from oxygen to nitrogen had occurred during the saponification step.

Figure 17 shows the results of sulfation of this aminated amylose by use of dimethyl sulfoxide–sulfur trioxide. This product, obtained in 74% yield after dialysis, was soluble in water, gave a negative ninhydrin test for free amino groups, and a positive Toluidine Blue test for sulfoamino groups. Its anticoagulant activity, as assayed with sheep plasma, was 45 International Units per milligram, namely about 40% of that of commercial heparin. The ^{13}C n.m.r. spectrum of the product showed that the primary alcohol groups were essentially completely sulfated, because the CH_2 resonance normally occurring near 61.5 p.p.m. was shifted downfield by about 6 p.p.m. by the substituent effect of the sulfate groups.

The product was quite polydisperse, and its molecular-weight range (3,000–20,000) indicated that some measure of degradation had occurred during the total sequence utilized. Approximately one half of the material in this product had a molecular weight below the range considered optimum for heparin activity.

Continuing work in our laboratory is concerned with the fractionation of the material just described and assay of the anticoagulant activity of products containing their major component in the 10 to 12,000 molecular weight range, with various levels of amination at the C-2 position. In a further development of potential utility (Figure 18) a procedure has been established (15) for specific 6-substitution of amylose by an acetyl group;

this 6-O-acetylamylose and analogous primary monoesters of polysaccharides may prove more useful than the trityl ethers as starting points for synthesis of heparin analogs.

Acknowledgments

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Heparin Derivatives of High Molecular Weight

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Many attempts have been made in the last two decades (1-6) to modify the structural features of the heparin molecule (7,8) in order to produce changes in its biological activities (9,12), but only very few, to modify the size of the molecule (10,11,13). High molecular weight heparin preparations are now obtained through methacrylation of heparin and polymerization of the heparin methacrylate monomer.

Methacrylation and polymerization

Heparin (0.1 mMol) is dissolved in 0.5 N sodium hydroxide solution and methacryl chloride (4 mMols) is added with stirring at room temperature and then heated to 80°C for 2 hours. The solution is neutralized with acetic acid and evaporated under reduced pressure.

The heparin methacrylate ester (Figure 1) is polymerized by heating with azodiisobutyronitrile as catalyst in 1,4-dioxane solution at pH = 5. The polymerization is stopped by adding hydroquinone and the solution is evaporated under reduced pressure.

Operating in this way, the main product is a water soluble heparin methacrylate polymer having a molecular weight of about 40.000, as shown by ultracentrifugation. The molecular weight of the initial heparin being 18.000, the polymer is a dimer of heparin. The polymerized heparin is isolated from unchanged heparin by passage through a Sephadex G-200 column, equilibrated with an aqueous ammonia solution to pH = 8. The heparin-rich fractions are detected by their methacromatic effect with toluene blue. The fractions containing the water soluble heparin polymer are lyophilized and investigated by ultracentrifugation.

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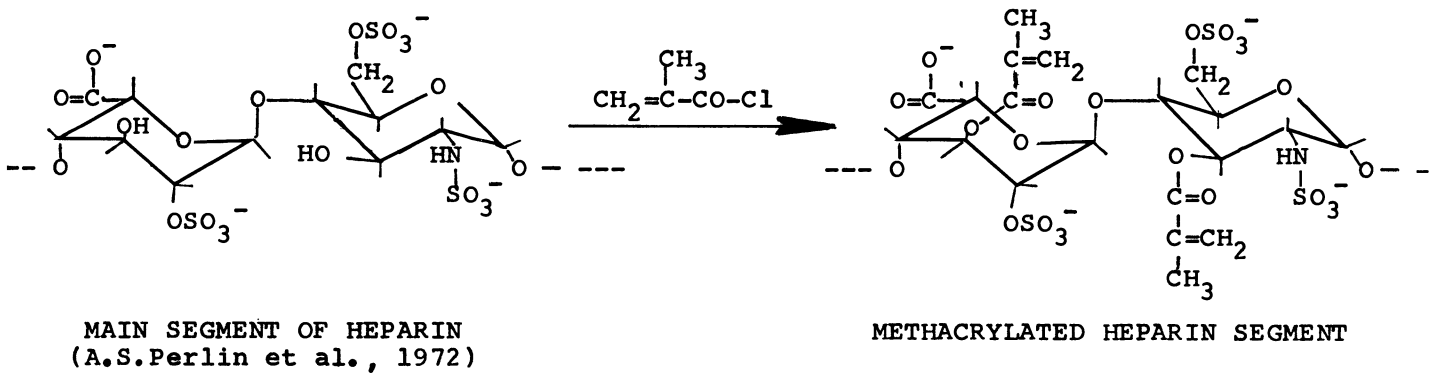

Figure 1. Methacrylated heparin segment

Water insoluble methacrylated heparin polymers are obtained when more methacryl chloride is used for esterification and when the polymerization is carried out at pH = 3. The insoluble heparin preparations are washed three times with ice cold water, centrifugated and lyophilized. The molecular weight of the insoluble heparin polymers is higher than 200.000. The water insoluble polymers may be used for coating metal surfaces with layers showing antithrombic activity.

Ultracentrifugation

Ultracentrifugation of heparin (I) and of methacrylated heparin polymer (II) was carried out in a sucrose gradient of 5 % to 20 %, in SPINCO Centrifug (41.000 RPM, 30 hours, 2°C), using serum albumin (SA) and cytochrom (CY) as internal references.

On Figure 2, fraction I is heparin with a constant of sedimentation S = 2 corresponding to a molecular weight of 18.000, fraction II is a polymer of methacrylated heparin, isolated on Sephadex G-200 column and showing a sedimentation constant S = 4, which corresponds to a molecular weight of about 40.000.

Copolymerization

Copolymerization of methacrylated heparin with vinyl or methacryl monomers is an unique method to change the geometry and physico-chemical properties of the heparin molecule. Figure 3 shows : A. a fragment of the methacrylated heparin polymer and B. a fragment of a copolymer of methacrylated heparin with butyl methacrylate. Copolymerization of methacrylated heparin with vinyl laurate gave liposoluble polymers.

Copolymerization with reticulants, like divinylbenzene or N,N'-methylene bis-acrylamide is especially effective to change the geometry of the polymerized heparin molecule.

Biological activities of the polymers

One of the most important current aims for chemical modification of the structure of heparin is the dissociation of its antithrombic and antilipemic activities. A decrease in the first and/or an increase in the second has been observed earlier by partial hydrolysis (4), periodate oxidation (6), irradiation (5) or modification of the N-sulfo groups (2,3).

A similar dissociation of the two activities is now reported through polymerization and copolymerization of methacrylated heparin.

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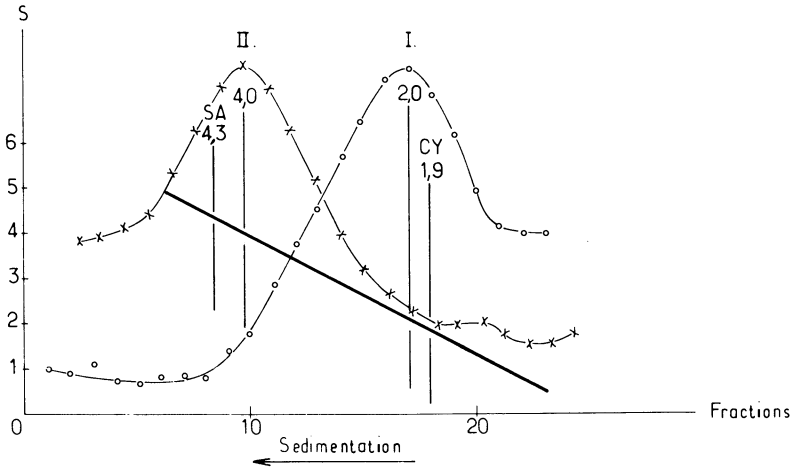

Figure 2. Ultracentrifugation in sucrose gradient 5–20% (Spinco, 41.000 RPM, 30 hr, 2°C); heparin (I) and methacrylated heparin polymer (II).

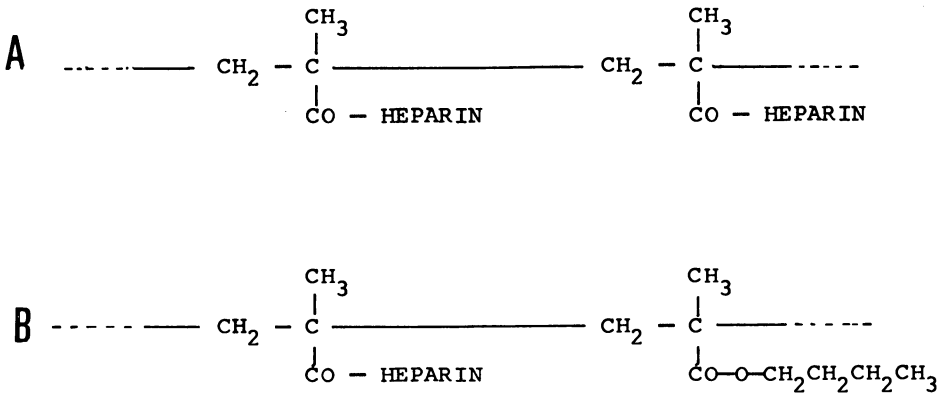

Figure 3. (A) Polymer of heparin methacrylate; and (B) co-polymer of heparin methacrylate with butyl methacrylate

Table I shows thrombin-time and antilipemic activity of the methacrylated heparin polymer (II) and of the copolymer of methacrylated heparin with butyl methacrylate (III), compared with the corresponding values obtained with heparin (I) in rats by intravenous injection of 1 mg/kg doses of the compounds. All samples were dissolved in physiological sodium chloride solution. Polymer (II) shows an increased antithrombic activity of short duration and a decreased antilipemic activity. The butyl methacrylate copolymer (III) distinguished itself through a considerably increased antilipemic activity with slightly decreased antithrombic activity.

Similar results were obtained through intravenous administration of 2 mg/kg doses of the compounds in rabbits, as shown in Table II.

In subcutaneous administration (5 mg/kg) most of the polymers and copolymers show a heparin-like activity, however, their antilipemic activity was decreased or considerably delayed.

Circular dichroism measurements

Circular dichroism data of heparin^{14}(I), of methacrylated heparin polymer (II) and of the copolymer of methacrylated heparin with butyl methacrylate (III) in water solution are shown on Figure 4, measured with a "Dichrograph-II" JOUAN, Paris.

In both polymers (II) and (III), the (+) and (-) Cotton-effects are increased, when compared with heparin. However, for the polymer (II) the increase of the (-) Cotton-effect is greater (110 %) than the increase of the (+) Cotton-effect (69%). The reversed phenomenon is observed for the copolymer (III). These changes in the Cotton-effects are due probably to a change in the geometry of the polymerized heparin molecule with methacrylate and/or with butyl methacrylate. This observation should also be taken in consideration to explain the dissociation of the antithrombic and antilipemic activities of the heparin polymers.

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Abstract

Heparin methacrylate is polymerized in 1,4-dioxane solution by heating with azodiisobutyronitrile as cata-

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TABLE I

ANTILIPEMIC ACTIVITY AND THROMBIN TIME AFTER INTRAVENOUS INJECTION OF 1 mg/kg HEPARIN OR HEPARIN POLYMER IN RATS

COMPOUNDS	THROMBIN TIME IN SECOND+			ANTILIPEMIC ACTIVITY		
	10 min.	60 min.	180 min.	10 min.	60 min.	180 min.
I. Heparin Roche	11.2	9.2	8.8	18	8	4
II. Heparin methacrylate polymer	14.5	7.5	-	12	2	1
III. Copolymer of heparin methacrylate with butyl methacrylate	10.5	7.5	7.3	30	17	6

TABLE II

ANTILIPEMIC ACTIVITY AND THROMBIN TIME AFTER INTRAVENOUS INJECTION OF 2 mg/kg HEPARIN OR HEPARIN POLYMER IN RABBITS

COMPOUNDS	THROMBIN TIME IN SECOND+			ANTILIPEMIC ACTIVITY		
	0 min.	20 min.	120 min.	0 min.	20 min.	120 min.
I. Heparin Roche	4.3	8.9	5.5	3	20	4
II. Heparin methacrylate polymer	4.6	14.0	4.9	2	12	2
III. Copolymer of heparin methacrylate with butyl methacrylate	4.3	6.4	4.7	3	20	5

+ Quick's method using 16 E/ml thrombin solution

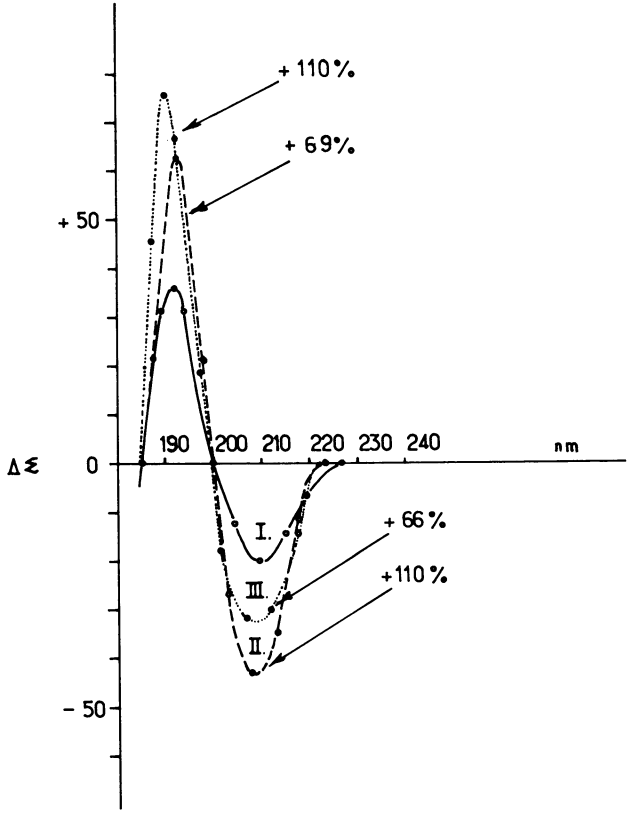

Figure 4. Circular dichroism data in water (0.75 mg/mL) of heparin (I), methacrylated heparin polymer (II), and co-polymer of methacrylated heparin with butyl methacrylate (III)

lyst. Depending on the degree of polymerization, soluble of gelatinous heparin preparations of high molecular weight are obtained, and separated on a column of Sephadex G-200 from unchanged heparin. A higher degree of polymerization of heparin methacrylate with alkyl methacrylates or vinyl derivatives resulted in liposoluble heparin preparations. Copolymerization with such reticulants as divinylbenzene or N,N'-methylene-bisacrylamide changed completely the geometry of the molecule. Circular dichroïsm measurements were used to follow the structural change of heparin methacrylate polymer and of its copolymer with butyl methacrylate. Some of the high molecular weight heparin preparations show a dissociation between the anticoagulant and antilipemic activities of heparin : the antithrombic activity decreased, while the antilipemic activity increased considerably or remained unchanged. Insoluble heparin preparations can be used for coating of surfaces.

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8

Enzymatic Formation and Hydrolysis of Polysaccharide Sulfates

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Naturally occuring polysaccharide sulfate esters are widely distributed. These high molecular weight anionic polymers possess rheological and complexing properties that have drawn the attention of industrial interests and lead to extensive commercialization of several of the polysaccharides. This, in turn, has influenced fundamental and application research to bring about a better understanding of the behavior of hydrocolloids. The next step is a rational design and development of polysaccharide sulfates to more perfectly serve practical needs.

The great bulk of natural occurring polysaccharide sulfates are found in seaweeds where they serve structural functions and possibly act as ion exchange agents and natural absorbents to hold large quantities of water for proper functioning of the sea plant. While normally water soluble, the polysaccharides are retarded from escape into the enveloping sea by the matrix character of the plant cell wall. Man has extracted these polymers and made use of their highly viscous nature and their gel forming properties. In addition, use is made of their unique characteristic of combining with protein to produce complexes such as the useful one between carrageenan and the protein in chocolate to maintain suspension uniformity in chocolate milk.

Other sulfated polysaccharides are widely distributed in animal tissues where they again serve a water holding use, provide emolliency, lubricity and complexing characteristics. Heparin serves a special function in the control of blood coagulation.

It can be expected that sulfated, and perhaps phosphorylated polysaccharides, will develop greater pharmaceutical and industrial applications. Applications would be facilitated by finding techniques by which sulfate-groups could be inserted into and removed from polysaccharides by expectedly low cost and surely the more specific means provided by enzymes. The present review is a summary of the existing knowledge of enzymes that transfer sulfate to ester positions in polysaccharides and of those

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enzymes that catalyze sulfate ester hydrolysis. Although little exact information is presently available, the excellent work so far done points to the likelihood of rapid further development. With proper application of evolving information, practical use may well be made of sulfate ester forming and hydrolyzing enzymes.

In the enzymatic formation of sulfate esters, the sulfate group is transferred from a donor substrate to a polysaccharide acceptor. Only three donor substrates have been identified. Ascorbate 2-O-sulfate and adenine 3'-O-phosphate-5'-O-phosphosulfate are two natural donors and p-nitrophenyl sulfate is a synthetic donor. Additional synthetic donors may soon be designated. Sulfate transfer from p-nitrophenyl sulfate and from ascorbate 2-O-sulfate require the presence of ATP as a cofactor and, hence, adenine 3'-O-phosphate-5'-O-phosphosulfate (PAPS) may be the universal designate intermediate and sole donor for all sulfate transferases. When other donors are present they presumably interact with ATP through the catalytic influence of two additional enzymes to provide PAPS.

Sulfatases capable of hydrolyzing the half-ester sulfate linkage to carbohydrates have been observed since 1931. The group of esterases that hydrolyze sulfate linkages in a variety of simple sugar sulfates are termed glycosulfatases (sugar sulfate sulfohydrolases E C. 3.1.6.3). Sulfatases capable of hydrolyzing sulfate linkages in polysaccharide sulfates are named after their substrate, as for example, chondrosulfatases hydrolyze chondroitin sulfates, and cellulose sulfatase hydrolyzes cellulose sulfates. The sulfatases may be completely substrate specific even as to location of the sulfate group, but some are not fully substrate specific since apparently a single enzyme can hydrolyze both charonin (charonan) sulfate and cellulose sulfate. All sulfatases are absolutely specific for the sulfate group. The enzymes are obtained from a variety of sources such as microbial, molluscan and higher animals. None have been observed in higher plants.

Enzymatic Sulfation

Enzymatic sulfation of polysaccharide is usually effected through the transfer of sulfate from adenine-3'-phospho-5'-phosphosulfate (3'-PAPS) in a reaction catalyzed by sulfotransferase [E C. 2.8.2] as shown originally by F. D'Abramo and F. Lipmann (1). The process of sulfation with inorganic sulfate proceeds in several steps. First is the activation of sulfate to form adenine-5'-phosphosulfate. This is immediately followed by phosphorylation at the 3'-position to produce adenine 3'-phospho-5'-phosphosulfate, followed by the transfer of sulfate from 3'-PAPS to the acceptor, catalyzed by sulfotransferase.

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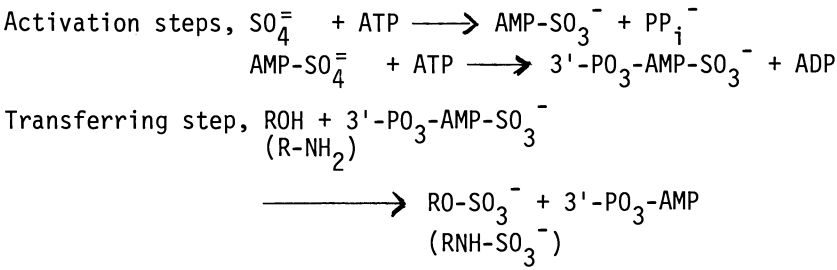

Sulfotransferases are of common occurance in animal tissues that contain aminoglycans and in plant tissues, especially algal cells, that are major producers of sulfated polysaccharides. However, evidence for the mechanism of the enzymatic reaction and characterization of the enzyme is incomplete due to difficulties in obtaining homogeneous enzyme preparations.

Enzyme preparations from chick embrionic cartilage (1-3), hen oviduct (4), hen uterus (5), rabbit uterus (6), beef lung (7), beef eyes (8), rat brain (9), mouse liver (10), serum (11), mast cell tumor (12-14), squid cartilage (15), molluscus (16), and marine gastropod (17) have been reported to catalyze sulfation of exogeneous and endogeneous acceptors.

Occurrence of sulfotransferase and 3'-PAPS as sulfate donors (18) are indicated in red algae Chondrus crispus (19), Porphyridium (20, 21) and in the brown algae, Fucus (22-25). Experiments with incorporation of radioactive sulfate into carrageenan of Chondrus (19) and into capsular polysaccharide of Porphyridium (20) demonstrate that the incorporation is a remarkably rapid process through 3'-PAPS as a sulfate pool. In Porphyridium 3'-PAPS was actually isolated as a water soluble intermediate (21). The rapidity with which sulfate incorporation takes place seems to favor the idea that the sulfation of a preformed polymer occurs. During early embryogenesis of Fucus, exogeneous radioactive sulfate can be incorporated into an acid-soluble fucose polymer within 10 hr. after fertilization. Experimental evidence indicates that enzymatic sulfation occurs in the region where fucan is formed and where it can immediately act as an acceptor (25). Although several attempts have been made to obtain from these algae, cell-free enzyme system which can transfer the sulfate from 3'-PAPS to free sugar, sugar nucleotide, or polysaccharide, no positive results have yet been seen.

Little is known of the specificity of sulfotransferase and it is not established whether there is a single non-specific sulfotransferase or a number of more specific enzymes. The latter case becomes more likely as the enzyme systems are investigated in greater detail.

Results of the earlier work on the specificity of the crude enzyme preparation are summarized in Table I.

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Table I

Acceptor specificity of some crude preparations of mucopolysaccharide sulphotransferases

Tissue	Potential Acceptors Utilized	Not Utilized	Reference
rabbit skin	dermatan sulphate		26
hen oviduct	heparitin sulphate		27
chick cartilage	chondroitin 4- and 6-sulphates	dermatan sulphate	28
human leimyosarcoma	dermatan sulphate	chondroitin 4- and 6-sulphates	29
human mammary carcinoma	chondroitin, chondroitin 4- and 6-sulphates, dermatan sulphate, heparitin sulphate		30

More detailed investigations on the specificity of sulfotransferase have been conducted in an enzyme system solubilized from hen oviduct (4). This cell-free system is capable of catalyzing the transfer of radioactive sulfate from 3'-PAPS to a number of aminoglycans. The relative rates of transfer are shown in Table II. Further experiments of the system show that simple oligosaccharides containing N-acetyl-D-galactosamine can also act as acceptors for the sulfotransferase although the oligosaccharides are rather less efficient than the polysaccharides. Both mono- and disulfated derivatives of N-acetyl-D-galactosamine residues in the oligosaccharides are formed in the reaction (31, 32). Measurements of reaction velocity at different concentrations show that the V_{max} and the Michaelis constant (K_m) differ among carbohydrate substrates. The higher V_{max} for chondroitin 6-sulfate as an acceptor compared to chondroitin 4-sulfate is in agreement with the data shown in Table II.

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Table II

Relative extents of sulphate transfer to different acceptors by the amino glycan sulphotransferases of hen oviduct and of chick embryo cartilage

Acceptor	Oviduct (4)	Cartilage (2)
chondroitin 4-sulphate	1.00	1.00
chondroitin 6-sulphate	1.8	0.46
dermatan sulphate	0.72	0.75
heparitin sulphate	0.45	1.3
chondroitin (natural)	1.8	-
chondroitin (chemical)	0.62	0.13
hyaluronic acid	0.00	0.08
heparin	0.00	0.26
keratosulphate	0.00	0.25

The sulfotransferase system in the 105000 x g supernatant fraction prepared from the homogenate of chick embryo cartilage (2) can use a greater number of polysaccharide acceptors than the oviduct enzyme (Table II). An endogeneous polysaccharide acceptor occurs in the same enzyme preparation (33). After desulfation of this acceptor with methanolic-hydrogen chloride, it no longer accepts sulfate. Similarly, chondroitin prepared by desulfation of chondroitin 4-sulfate does not act as an acceptor. The ability of sulfate acceptors is little influenced by their molecular size but is strongly dependent on the charge on the molecules. Treatment of protein-polysaccharide complex endogeneous acceptors with proteinase or with alkali increase their ability to accept sulfate. However, there is a striking change in the type of sulfation that results from removal of protein. When the aminoglycan is combined with protein sulfate, esters are formed predominantly at axial hydroxyl groups. For example, sulfate groups are introduced at position 4 of N-acetyl-D-galactosamine units. On removal of protein, sulfation occurs principally at equatorial or primary hydroxyl groups. One possible explanation for this is that although the protein portion of the endogeneous protein-polysaccharide complex is not necessary for sulfate incorporation, it aids in determining the sites for sulfation.

A sulfotransferase purified from mouse liver homogenate (10) transfers sulfate from 3'-PAPS only to position C-6 of an N-acetyl-D-galactosamine residue in chondroitin sulfate and its Michaelis constant can be closely correlated to the degree of sulfation.

In the soluble enzyme systems, only a small amount of sulfate is incorporated into polysaccharide. Most of the chondroitin sulfating activity is found to occur as microsomal enzyme rather than as soluble enzyme (34,35). A sulfotransferase

in a microsomal preparation from chick embryo cartilage transfers a greater amount of sulfate from 3'-PAPS to endogeneous glycosaminoglycan than does the soluble enzyme (3, 36). Incubation of the mcirosomal preparation at pH 6.5 with 3'-PAPS results in the incorporation of sulfate into endogeneous chondroitin 6-sulfate (60-70%) and into chondroitin 4-sulfate (30-40%) while incubation at pH 7.8 results in the incorporation of sulfate into chondroitin 6-sulfate exclusively (37).

Although the sulfotransferase activity of chick embryo cartilage is predominantly associated with membrane, a significant proportion of the enzyme activity is found in the 105000 x g supernatant fraction (Golgi preparation). This preparation contains activity for 4- and 6-sulfation of endogeneous polysaccharide. However, heat treatment of the preparation causes more rapid loss of ability to sulfate position C-4 of the polysaccharide than loss of ability to sulfate position C-6 (38) (Fig. 1). This suggests that a specific enzyme is active for sulfation at each position. Moreover, by chromatography on Sephadex G-200 the enzyme for 6-sulfation can be obtained essentially free from the activity for 4-sulfation. The enzyme effecting 4-sulfation is not recovered from chromatography perhaps because of inactivation.

Thus, within the limited work on the microsomal preparation and the Golgi preparation, the sulfation system of chick embryo cartilage seems to consist of at least two enzyme species that are highly specific with regard to the position sulfated.

An aminoglycan sulfotransferase utilizing 3'-PAPS as a sulfate donor and endogeneous protein-polysaccharide complex as an acceptor has been characterized (15). This enzyme, from squid cartilage, shows high specificity for position C-6 of N-acetyl-D-galactosamine moieties that already bear a sulfate group at position C-4. The sulfotransferase is separated from endogeneous protein polysaccharide complex and purified some nine-fold on DEAE-Sephadex A-50. The enzyme is active only with exogeneous acceptors. Although various mono-, di-, and polysaccharide are sulfate acceptors, it is essential that they have a sulfate at position C-4 of their N-acetyl-D-galactosamine residues. The purified enzyme specifically catalyzes the sulfation of N-acetyl-D-galactosamine 4-sulfate residues, and produces N-acetyl-D-galactosamine 4,6-disulfate, whether N-acetyl-D-galactosamine 4-sulfate is in a protein-polysaccharide complex or whether it is protein-free. Most likely, therefore, the specificity of this enzyme does not involve the recognition of a particular size or a particular monosaccharide sequence of an aminoglycan acceptor molecule.

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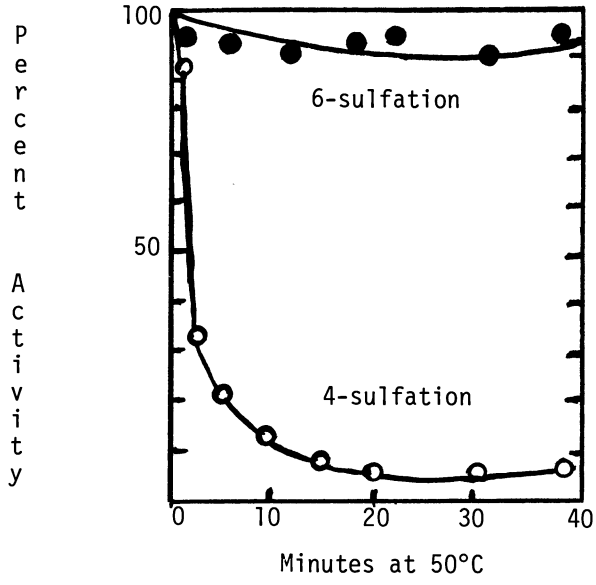

Figure 1. Heat labilities of two different sulfotransferase activities in the 105,000× g supernatant of cartilage homogenate (38)

Another enzyme has been purified from a homogenate of the albumin secreting region of hen oviduct (39-41). The enzyme catalyzes the transfer of sulfate from 3'-PAPS to position C-6 of the N-acetyl-D-galactosamine 4-sulfate moiety of uridine diphosphate-N-acetyl-D-galactosamine 4-sulfate. Although UDP-N-acetyl-D-galactosamine 4-sulfate is the most active carbohydrate substrate for the enzyme, N-acetyl-D-galactosamine 4-sulfate and its 1-phosphate act as a sulfate acceptor at a comparable rate and $\Delta^{4,5}$-glucuronido-N-acetyl-D-galactosamine 4-sulfate [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyl-uronic acid)-4-O-sulfo-D-galactose] and chondroitin accept sulfate slowly. A more completely purified enzyme has a pH optimum for sulfate transfer to UDP-N-acetyl-D-galactosamine 4-sulfate at pH 4.8. The kinetic data for sulfate transfer to several substrate are summarized in Table III.

Table III
Specificity of a sulphotransferase from hen oviduct which can utilize simple sugars or sugar derivatives as substrates (39)

Acceptor	K_m (mM)	V (relative)
UDP-N-acetylgalactosamine 4-sulfate	0.05	1.00
N-acetylgalactosamine 4-sulfate	1.4	0.72
N-acetylgalactosamine 1-phosphate 4-sulfate	0.13	0.39
$\Delta^{4,5}$-glucuronido-N-acetylgagactosamine 4-sulfate	2.0	0.05

The sulfotransferases considered so far have all formed sulfate esters. However, in the formation of heparin which contains O-sulfate and N-sulfate, a separate sulfotransferase is involved. This is demonstrated by the separate identification of N- and O-sulfotransferase activity in an enzyme preparation from mouse mastocytoma (14). Both sulfotransferases can be solubilized from mastocytoma microsomal fraction. The pH optimum for the enzymes is about 7.5. The Michaelis constant for 3'-PAPS is estimated to be 2×10^{-5}M for the N-sulfotransferase and 1×10^{-4} for the O-sulfotransferase. The O-sulfotransferase is more sensitive to heat-inactivation; 60% of the activity being lost after 1 min. at 50°. Under the same conditions only 15% of N-sulfotransferase is lost. The N-sulfotransferase is selectively inhibited by sodium chloride, whereas, the O-sulfotransferase is essentially unaffected. Work on the acceptor specificity of the O-sulfotransferase from mouse mastocytoma shows that N-sulfate groups in acceptor polysaccharides are essential for O-sulfation. The O-sulfate group is

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preferentially introduced into the N-sulfated (and thus previously N-deacetylated) rather than into N-acetylated regions at heparan sulfate (42,43).

Almost all work on sulfotransferase has been done on tissues in which sulfation of aminoglycan occurs. Little work has been done on the mechanism of sulfation of nitrogen-free glycans. However, a sulfotransferase from the mucous gland extracts of the marine gastropod, charonia lampus, catalyzes the transfer of sulfate from 3'-PAPS to the glucan polysulfate, charonan sulfuric acid (17). Charonan sulfuric acid consists of two fractions, one a sulfur-poor fraction with a glycogen-like structure and the other a sulfur-rich fraction with a cellulose-like structure (44,45). When ^{32}S is used in the donor, more than 95% of the activity is incorporated to the sulfur-rich fraction.

In addition to 3'-PAPS, other sulfate donors exist. One is p-nitrophenyl sulfate, an effective sulfate donor in the sulfotransferase preparation of beef cornea epithelical extract. Here, the sulfate group is transferred from p-nitrophenyl sulfate to polysaccharide in the presence of adenosine 3',5'-diphosphate (56). The sulfation of polysaccharide seems to occur by polysaccharide sulfotransferase coupling with phenol sulfotransferase or phenol sulfatase which is also present in the corneal extract. Another donor is ascorbate 2-sulfate (47). When radioactive sulfate is used, the sulfate is found incorporated into chondroitin sulfate in chick embryo cartilage epiphyses.

Enzymatic Desulfation

Glycosulfatasewas observed first in the digestive organs of the tropical marine mollusc Charonia lampas (48-52). Subsequently, the enzymewas observed (53-63) in extracts of the digestive organs of marine molluscs from British waters, in the large periwinkle Littorina littorea and the common limpet Patella vulgata. It is also present in the mould Trichoderma viride (64), and the bacteria Pseudomonas carrageenovora (65). A similar glycosulfatase is observed in the isthmus region of the hen oviduct (95).

From the liver of the marine gastropod Charonia lampas (Tritonalia sauliae) there can be obtained a cellulose polysulfatase (66-68) and a chondrosulfatase (79). A porphyran sulfatase can be prepared from the algae Porphyra umbilicalis (69-72).

Chondrosulfatase is observed also in putrifactive bacteria (73-75), Proteus vulgaris (76,77) and Flavobacterium heparinum (78). In addition to occurance in the liver of C. lampas, the enzyme is found in the liver of squid Ommastrephes sloani

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pacificus (81-83), in rat liver lysosomes (84), in bovine aorta (85) and the viscera of Patella vulgata (80).

Two kerato sulfatases are present in C. lampus (86). A heparin sulfatase has been isolated from Flavobacterium heparinum (87).

Cerebroside sulfatase can be isolated from the digestive gland of abalone, Haliotis (88) and the lysosomes of pig kidney and from other organs as liver and spleen (89-91).

Glycosulfatases

The glycosulfatases (sugar-sulfate sulfohydrolases, E.C.3.1.6.3) capable of hydrolyzing ester sulfate linkages in a variety of mono-, di-, and tri-sulfate substituted monosaccharides and disaccharides have been partially purified and isolated by a number of different groups from a variety of different sources. Most of the procedures are rather long and involved. Several are given as examples.

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Example 1. Washed and macerated viscereal organs of Littorina (57) are placed in volumes of acetone at 0°, filtered, macerated in fresh acetone and thoroughly washed in acetone. The residue is suspended in cold water, homogenized at pH 9 and adjusted to pH 7 for 15 minutes, centrifuged and the clear supernatant dialyzed and the dialyzate adjusted to pH 2.3 for 2 min and readjusted to 4.6, centrifuged, dialyzed, adjusted to pH 6.7, ribonucleic acid and salmon roe protamine sulfate added to complete precipitation. After centrifugation the supernatant is dialyzed, centrifuged and the supernatant adjusted to pH 8, centrifuged, dialyzed and at pH 5.7 treated with ammonium sulfate to 55% of saturation. After centrifugation the supernatant at pH 4.9 is 85% saturated with ammonium sulfate. The precipitate is removed by centrifugation and dialyzed. The final product has a high concentration of glycosulfatase as well as a high content of arylsulfatase.

The purification procedure results in the elimination of the chondroitinase and most of the β-N-acetylglucosaminidase but other sulfatase enzymes are still present in appreciable amounts. Nucleic acid concentration has been considerably decreased and the preparation retains activity when stored in the frozen state.

It is noticed that glycosulfatase activity in Littorina varies considerably with season. Relatively small amounts of the enzyme can be detected in the winter months, whereas, organisms collected in July provide the most active preparation.

Example 2. Trichoderma viride (64) is grown on a medium containing the 6-O-sulfate of D-galactose or D-glucose. The

washed mycelium is frozen and ground with alumina at -15° and extracted with cold Tris solution at pH 7.9, centrifuged and supernatant stored.

Example 3. Pseudomonas carrageenovora (65) cells are harvested, and centrifuged. The supernatant is treated with streptomycin sulfate to remove nucleic acids, centrifuged and the supernatant is treated with ammonium sulfate. The precipitate is dissolved in water and dialyzed.

Example 4. The liver of Charonia lampas (94) is homogenized with sodium chloride, centrifuged, and the supernatant dialyzed at pH 3.6 and centrifuged. The supernatant is then successively passed through columns of phosphocellulose, Sephadex G-150 and Concanavalin A-Sepharose when purified glycosulfatase I is isolated. Glycosulfatase II, which is still unseparated from aryl sulfatase at this stage, is purified by isoelectric focussing.

Example 5. Hen oviduct isthmus (95) is treated with Tris-HCl at pH 7.2, homogenized, and centrifuged. The supernatant is treated with ammonium sulfate to 40% saturation, centrifuged, and the supernatant is treated again with ammonium sulfate to 65% saturation, and centrifuged. The precipitate is dissolved in Tris-HCl at pH 7.2 and dialyzed. This is then applied to a column of DEAE-cellulose. The pooled fraction is concentrated, dialyzed at pH 4.5 and then applied to another column of CM-cellulose at pH 4.5. The pooled fractions are concentrated and dialyzed at same pH. The enzyme retained 95% and 90% of its activity for 2 and 9 months, respectively, when stored at -20°.

Assay of Glycosulfatases

Sulfatases catalyze the reaction:

$$R\text{-}O\text{-}SO_3^- + H_2O \longrightarrow R\text{-}O\text{-}H + SO_4^{2-} + H^+$$

Enzyme assay is based upon measurement of unchanged substrate, desulfated residue, inorganic sulfate or change of pH.

The earlier investigation of glycosulfatases has employed assay methods for indirect determination of unhydrolyzed substrate, by gravimetric estimations of inorganic sulfate liberated on acid hydrolysis and the measurement of liberated inorganic sulfate by potentiometric titration (50) or nephelometrically (50). These methods are not capable of a high degree of accuracy unless applied to large quantities. Methods for the assay of sulfatases based on the turbidimetric determination of barium sulfate have been described (96,97). Colorimetric methods of sulfatase assay also have been used (98), and a small-scale version of each of these methods has been devised (99).

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The method of Dodgson and Spencer (54), in which precipitated benzidine sulfate is estimated colorimetrically, although it is time-consuming and demands very careful manipulation, has proved useful for a number of sulfuric esters. A glucose-oxidase-peroxidase-O-dianisidine method (100) is applicable also.

Properties of glycosulfatases

Charonia lampas glycosulfatase: The earliest study of this glycosulfatase has been made during the years 1931 through 1948 and this work has been partly reviewed (101). Unfortunately, the glycosulfatase used is not pure. It shows greatest activity against D-glucose 6-O-sulfate and shows activity also against a variety of sulfate esters of monosaccharides and disaccharides as well as against adenosine 5'-sulfate.

Recently purified glycosulfatase I and II from *C. lampas* have been reported as active against D-glucose 6-O-sulfate, showing the same K_m of 25.0 mM, and the same optimum pH of 5.5.

Littorina littorea glycosulfatase: This glycosulfatase has been extensively investigated. The specificity of the enzyme is relatively low as it hydrolyzes D-glycose 6-O-sulfate, D-glucose 3-O-sulfate, and D-galactose 6-O-sulfate (Table IV). In common with most other sulfatase enzymes, glycosulfatase is strongly inhibited by phosphate and pyrophosphate, and to a less extent by fluoride ions. Some increase in enzyme activity is observed in the presence of magnesium and manganous chlorides.

Table IV

Substrate	Optimum pH	Optimum Substrate conc x 10^2 (M)	K_m x 10^2	Relative activity
Potassium D-glucose 3-O-sulfate	5.7-5.9	7.0	3.0	1.0
Potassium D-glucose 6-O-sulfate	5.5-5.6	4.0	1.7	10.0
Potassium D-galactose 6-O-sulfate	5.2	>8.0	7.2	2.0

None of a wide range of polysaccharides of plant or animal origin is attacked by the enzyme, nor are the oligosaccharides produced by the action of hyaluronidase on chondroitin 4-sulfate or chondroitin 6-sulfate (102).

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Patella vulgata glycosulfatase: This enzyme has a wide specificity for 6-O-sulfate esters of D-glucose, D-galactose (Table V), and D-mannose, and also for 2-O-, 3-O- and 4-O-sulfate esters of L-fucose.

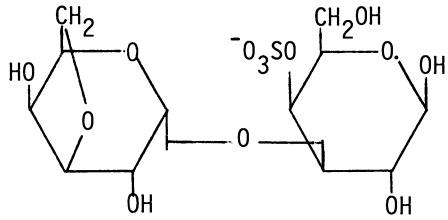
Table V

Substrate	Optimum pH	Optimum substrate conc (M)	Activity (units)	K_m (mM)
D-glucose 6-O-sulfate	6.0	0.04	3295	1.86
D-galactose 6-O-sulfate	6.0	0.04	4035	28.3

The enzyme activity is strongly inhibited by phosphate and borate ions, while the effects of cyanide, ferric and cupric vary with the substrate.

Trichonderma viride glycosulfatase: The enzyme is a simple glycosulfatase active towards the 6-O-sulfate esters of D-glucose and D-galactose but not towards D-glucose 3-O-sulfate. Activity decreases sharply at either side of the optimum temp. of 28°. The pH for maximum activity is 7.8-7.9. The glycosulfatase seems to be different from analogous molluscan enzymes that also can hydrolyze monosaccharide sulfate esters.

Pseudomonas carrageenovora glycosulfatase: The enzyme shows a remarkable degree of specificity for hydrolyzing neocarrabiose sulfate, 3-O-(3,6-anhydro-α-D-galactopyranose 4-O-sulfate, a degradation product of K-carrageenan, with hydrolysis leading to the corresponding disaccharide. The enzyme also acts on D-galactose 6-O-sulfate, but not on D-galactose 4-O-sulfate; a surprising result since D-galactose 4-O-sulfate is a unit in the neocarrabiose sulfate.



Neocarrabiose 4-O-sulfate

Enzyme activity is completely inhibited in 0.1 M acetate buffer, pH 4.0, and by 25 mM mercuric ion. Partial inhibition occurs with 0.1 M acetate buffer, pH 5.6, and 25 mM phosphate buffer, pH 7.5. There is no inhibition with 25 mM sulfate and 3 mM ethylene diamine tetraacetic acid (EDTA).

Hen oviduct sulfatase: This enzyme removes only the sulfate at position C4 and shows remarkable specificity towards UDP-N-acetyl-D-galactosamine-4-O-sulfate, UDP-N-acetyl-D-galactosamine-4,6-di-O-sulfate, and N-acetyl-D-galactosamine-4,6-di-O-sulfate, but not towards N-acetyl-D-galactosamine-4-O-sulfate. (Table VI).

Table VI

Substrate	Maximum velocity V_{max} (units/mg protein)	K_m (M)
1. UDP-Gal NAc-4-sulfate	4,200	4×10^{-4}
2. UDP-Gal NAc-4,6-disulfate	10,500	6×10^{-6}
3. N-Acetylgalactosamine 4-sulfate	0	0
4. N-Acetylgalactosamine 4,6-disulfate	560	3×10^{-5}

The enzyme is not active toward p-nitrophenyl sulfate, D-glucose 6-O-sulfate, D-galactose 3-O-sulfate, D-galactose 6-O-sulfate, or a number of sulfated polysaccharides of plant and animal origin. The enzyme is distinct from any of the known arylsulfatase and glycosulfatase.

Polysaccharide sulfatases

A number of natural polysaccharide sulfates of both plant and animal origins are known, for example: chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, shark chondroitin sulfate, keratan sulfate, heparan sulfate, heparin, porphyran, τ, κ, ι and μ-carrageenan, charonan sulfate and fucoidan. A number of enzymes and enzyme-systems have been isolated that desulfate some of these sulfated polysaccharides.

Cellulose polysulfatase: An enzyme hydrolyzing sulfuric ester bonds in cellulose polysulfate and charonan sulfate occurs in liver extract of the marine gastropod, Charonia lampas. Since

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chondroitin sulfate and amylose polysulfate are scarcely hydrolyzed by the enzyme preparation, this new sulfatase has been called "cellulose polysulfatase".

The liver of Charonia lampas (68) is macerated at pH 6.0, centrifuged, and to the supernatant is added acriflavine to 1% concentration. Precipitated material is separated by centrifugation, ethanol is added to the supernatant to 70% concentration, centrifuged, and the precipitate dialyzed in water and centrifuged. The supernatant, at pH 5.2, is purified in a column of CMC, changing the eluent pH gradually from 5.2 to 6.0. The appropriate fractions are pooled, and dialyzed.

The crude cellulose polysulfatase from C. lampas hydrolyzes a random sulfated cellulose removing most of the sulfate groups while presumably other enzymes present hydrolyze the cellulose to D-glucose and D-glucose sulfates from which sulfate groups are then hydrolyzed.

The purified cellulose polysulfatase hydrolyzes sulfate groups from the polysaccharide without degrading the chain. This enzyme shows remarkable specificity towards cellulose polysulfate and charonan sulfate by converting these from high sulfur content to low sulfur content polysaccharides. It slowly attacks dextran sulfate, but not amylose sulfate (Table VII). Regarding the effect of the position of sulfate bond, Takahashi suggests that cellulose polysulfatase preferentially attacks ester bonds at the C2 and C3 positions, while glucosulfatase attacks preferentially C6 position ester bonds.

Porphyran sulfatase: An enzyme present in Porphyra extracts is capable of hydrolyzing porphyran, a complex sulfated polysaccharide containing residues of D-galactose, L-galactose, 6-O-methyl-D-galactose, and 3,6-anhydro-L-galactose.

Porphyra seaweed (70) is washed with water, minced into aqueous sodium carbonate at pH 8.3, pH adjusted to 6.0-6.5 and centrifuged. To the supernatant is added a calcium phosphate gel, stirred and centrifuged. The gel is washed at pH 7.5, centrifuged, and the supernatant treated with solid ammonium sulfate to 0.8% saturation. The precipitate is isolated by centrifugation, dissolved in water, and dialyzed.

The enzyme desulfates porphyran but does not attack other sulfated polysaccharides. Complete inhibition of the enzyme occurs on addition of metal binding reagents, thus indicating that there is a bi- or tervalent cation essential to the enzyme. No inhibition is observed when a mixture of Zn^{2+} or Co^{2+} ions (1.5 mM) and ethylenediaminetetra acetic acid (EDTA) (0.5 mM) is present. A 50% inhibition occurs if Mn^{2+} ion is substituted for one of these cations, and complete inhibition occurs if

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Mg^{2+} ion is used. Borate is a powerful activator producing 60% activation at pH 7.6.

Since porphyran is itself a polyelectrolyte, it is conceivable that certain of the activators influences the reaction by interaction with the substrate rather than the enzyme. It is known that salts present in solution with polysaccharide polyanions can alter the configuration of the polymer, and it is possible that some of the cation activators operate by increasing the time spent by the substrate in a configuration favorable for the reaction.

Chondrosulfatase

Chondrosulfatase of *Proteus vulgaris*: A strain of *Proteus vulgaris* (N.C.T.C. 4636) appears to be a particularly potent source of chondrosulfatase (E.C.3.1.6.4). The enzyme is associated with a chondroitinase system which can degrade the chain of chondroitin sulfate producing sulfated oligosaccharides which are subsequently desulfated by chondrosulfatase.

Proteus vulgaris (76,77) is cultured, and harvested by centrifuging. The cells are macerated in acetone, filtered, homogenized at pH 7.0, incubated at 37°, and centrifuged. The supernatant is dialyzed, centrifuged, and to the supernatant added sodium salt of yeast nucleic acid at pH 7.4. The pH is adjusted to 4.0, the precipitate removed by centrifuging and dissolved in aqueous sodium hydroxide at pH 7.4. The solution is adjusted to pH 6.7 and an aqueous solution of protamine sulfate added, dialyzed and centrifuged. The supernatant is adjusted to pH 8.0, centrifuged, and the supernatant treated with calcium phosphate gel at pH 6.55. The gel-enzyme complex is separated by centrifugation, washed and treated repeatedly with sodium acetate solution at pH 8.0 until no further protein is eluted from the gel. The eluate containing sulfatase are combined and dialyzed.

Chondrosulfatase, in the absence of chondroitinase, is virtually inactive towards the polymerized form of chondroitin sulfate. However, chondroitin sulfate degraded with testicular hyaluronidase is readily hydrolyzed.

The crude *Proteus* concentrate, which is active towards chondroitin sulfate, is inactive towards the sulfated polysaccharides agar, fucoidan, carrageenan, *chondrus ocellatus* mucilage and sulfated laminaran.

Recently, two chondrosulfatases are isolated from the extracts of *Proteus vulgaris* (N.C.T.C. 4636). One of these enzymes, chondro 4-sulfatase, is active against 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyl-uronic acid)-4-*O*-sulfo-*D*-

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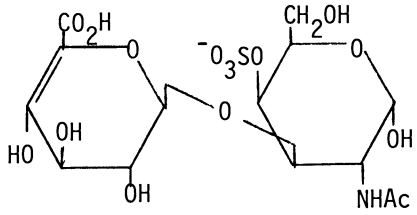
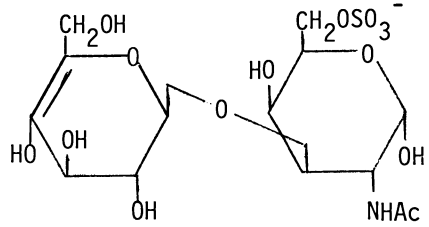
galactose, the product from the degradation of chondroitin sulfate A or B by chondroitinase, and its saturated analogue, acetylchondrosin 4-sulfate, but does not attack the corresponding 6-O-sulfate isomer, the product from the degradation of chondroitin sulfate C by chondroitinase, and its saturated analogue, acetylchondrosin 6-sulfate. In contrast, chondro 6-sulfatase desulfates disaccharide 6-sulfates andacetyl-D-galactosamine 4,6-disulfate at position 6, but does not attack the disaccharide 4-sulfate isomers.



2-Acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose



2-Acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose

The enzymes do not attack polymer chondroitin sulfates, hexa-, penta-, tetra-, or trisaccharides derived from chondroitin sulfates A and C by digestion with crude testicular hyaluronidase, or acetyl-galactosamine 4- and 6-sulfates.

Chondrosulfatase of *Patella vulgata*: The viscera of *Patella vulgata* (60) is reported to contain a glycosulfatase and also an enzyme that is active against chondroitin 4-sulfate. The chondrosulfatase has been purified by separating from the glycosulfatase by fractionation with ammonium sulfate.

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Chondrosulfatase from this source is reported to be capable of desulfating chondroitin sulfate without prior depolymerization. Sulfate is also liberated by the enzyme from a range of chondroitin sulfates from different sources, including a commercial preparation believed to be predominately chondroitin 6-sulfate.

Chondrosulfatase of Squid liver: The liver extracts of the squid (Ommastrephes sloani pacificus) (83) contains chondrosulfatase along with three other enzymes, hyaluronidase (E.C. 3.2.1.35), β-N-acetyl-hexosaminidase (E.C. 3.2.1.30), and β-glucuronidase (E.C. 3.2.1.31). Crude enzyme is prepared from squid liver by extraction with acetone and then purified by fractionation with ammonium sulfate and by using a column of Sephadex G-100. The purification achieved is about 15-fold from the crude enzyme. The partially purified chondrosulfatase is stored at -20°, without appreciable loss of activity for a month.

The squid chondrosulfatase attacks chondroitin 4-sulfate at greater rate than it does chondroitin 4,6-disulfate. Chondroitin 6-sulfate is also desulfated to a slight extent, but keratan sulfate, dermatan sulfate and heparin are not desulfated with this enzyme. A sulfated tetrasaccharide and a mixture of sulfated oligosaccharides, both prepared from chondroitin 4-sulfate by testicular hyaluronidase digestion, are respectively desulfated to the extent of about 32% and 56% of the sulfate released from chondroitin 4-sulfate polymer. Desulfation from the sulfated disaccharide, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-4-O-sulfo-D-galactose, is negligible. These studies show that the squid chondrosulfatase is capable of desulfating mainly 4-sulfate in chondroitin sulfates without prior depolymerization.

Chondrosulfatase activity is extremely inhibited by cupric sulfate, sodium phosphate, sodium fluoride, borax and heparin. Chondrosulfatase is activated by sodium chloride, cupric chloride, EDTA, and cysteine hydrochloride.

The apparent K_m of this chondrosulfatase is estimated as 0.49 mM for reactions at pH 5.0 and 37° for 3 h with chondroitin 4-sulfate as the substrate.

Chondrosulfatase of bovine aorta: It has been known (103, 104) that rats desulfate chondroitin sulfate in vivo and it has been claimed (105) that chondrosulfatase can be detected histochemically in human sweat glands, but attempts have been unsuccessful (56) to prepare it from mammalian tissue, extracts with chondrosulfatase activity. On the other hand, crude preparations from pig kidney are known to liberate sulfate from chondroitin sulfate.

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Bovine arterial tissue (aorta) contains a chondrosulfatase (85). The enzyme is purified by ammonium sulfate fractionation, precipitation at pH 4.6, and by gel filtration. An 85 fold purification is achieved.

The enzyme is active against chondroitin 4-sulfate, but it does not attack chondroitin 6-sulfate, dermatan sulfate, and keratan sulfate. In acetate buffer, the pH optimum is 4.4, and K_m is 0.735 m*M*.

Keratosulfatase: Two forms of keratosulfatase (86) (I and II) are isolated from the marine gastropod, *charonia lampas*. Both forms release all the sulfate from kerato sulfates and neither appears identical with glycosulfatase or chondrosulfatase, both of which are also present in *charonia lampas*.

The crude liver extract is purified by fractionation with ammonium sulfate and then by successive column chromatography of CM-Sephadex C-50 and DEAE-Sephadex A-50. Keratosulfatase preparations I and II are stored at -20° in the presence of 0.1 *M* sodium chloride without appreciable loss of activity for several weeks.

Keratosulfatases desulfate keratopolysulfate, keratosulfate and horatin sulfate, a sulfated polysaccharide composed of *L*-fucose, *D*-mannose, *D*-glucose, *D*-galactose, *D*-glucosamine and *D*-galactosamine, isolated from the liver of *C. lampas*. Keratosulfatase I does not liberate sulfate from horatin sulfate (Table VIII).

Table VIII

Enzyme preparation		Substrate	Incubation time (h)	SO_4^{2-} liberated (%)
1.	I	Keratopolysulfate	96	80
	II	Same	96	100
2.	I	Kerato[^{35}S]sulfate	96	95
	II	Same	76	100
3.	I	Horatin sulfate	48	< 3
	II	Same	48	100

It also appears that keratosulfatase first liberates sulfate from keratosulfates and the desulfated polymer is then degraded to *D*-galactose and *N*-acetyl-*D*-glucosamine by the action of β-*D*-galactosidase and β-*N*-acetyl-*D*-glucosaminidase, enzymes also present in the partially purified keratosulfatase.

Potassium dihydrogen phosphate, sodium fluoride and p-chloromercuribenzoate inhibit both keratosulfatases I and II, as they do most other known C. lampus sulfatases.

The optimum pH values of keratosulfatases I and II are 4.5 and 6.0, respectively.

Heparin degrading multienzyme system: No single enzyme has been isolated which can desulfate heparin or heparitin sulfates. However, five enzymes from Flavobacterium heparinum (87), capable of degrading heparin to its basic constituents, have been purified and characterized. The purified heparinase degrades heparin to trisulfated disaccharide. This is desulfated further by a disaccharide sulfatase yielding the disulfated disaccharide. This product is a substrate for glycuronidase giving glucosamine-2,6-disulfate and α,β-keto acid. D-glucosamine-2,6-disulfate is desulfated by a sulfatase and a sulfamidase yielding free glucosamine and inorganic sulfate.

The crude extract from F. heparinum is purified by successively using a column chromatography of Sephadex G-200 and then by agarose gel electrophoresis.

The heparinase has no activity upon chondroitin sulfates A, B and C or on heparitin sulfates A and B, but degrades heparitin sulfates C and D, yielding sulfated disaccharides with the same chromatographic mobility as the ones obtained from heparin.

The specificity of the enzyme, disaccharide sulfatase, is limited. The enzyme acts only upon the trisulfated disaccharide, forming disulfated disaccharide and inorganic sulfate. Heparin, sulfated hexa- and tetra-saccharides, disulfated disaccharide, and glucosamine-2,6-disulfate are not substrates for this enzyme.

Glycuronidase acts only upon the disulfated disaccharide, yielding glucosamine-2,6-disulfate. This enzyme does not act upon the trisulfated disaccharide.

Monosaccharide sulfatase and sulfamidase act only upon the suflated hexosamine monosaccharides. No activity upon heparin or the disaccharides has been detected.

The pathway of enzymatic degradation of heparin may proceed as shown.

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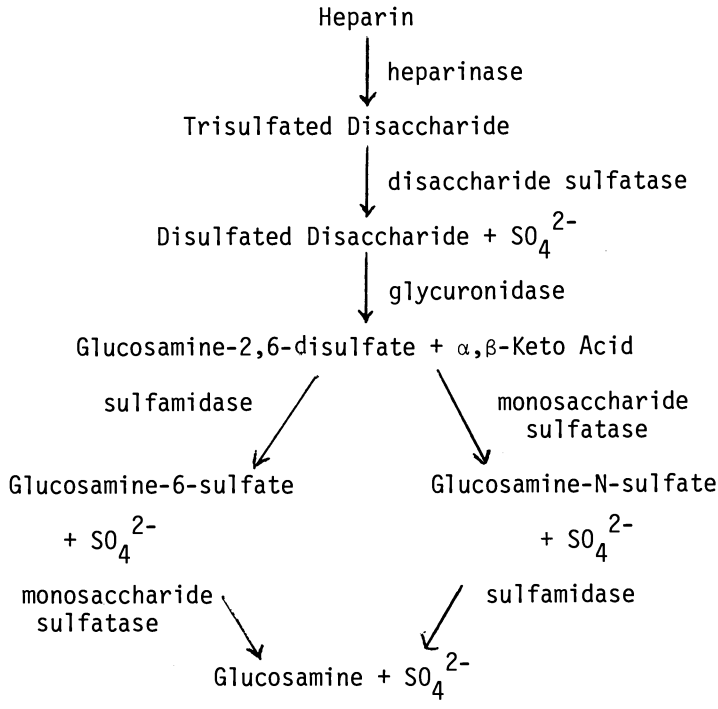

Cerebroside sulfatase: Presence of a cerebroside sulfatase has been first reported in the digestive gland of the abalone, Haliotis (88). This enzyme, in addition to hydrolyzing cerebroside, can also desulfate chondroitin sulfate but not phenylsulfate nor glucose 6-sulfate.

Cerebroside sulfatase has been purified from the lysosomes of pig kidney (90) and its presence has been shown in other organs, particularly liver and spleen. The purification is achieved by high voltage electrophoresis of the crude extract.

This enzyme desulfates only cerebroside 3-sulfates. Cerebroside 6-sulfates are not desulfated. D-Galactose 3-sulfate is hydrolyzed at about 20% the rate of cerebroside 3-sulfate but D-galactose 6-sulfate is not hydrolyzed. The enzyme also desulfates cerebron and kerasin sulfates. The optimum pH is 4.5, and K_m is 0.1 mM with cerebroside 3-sulfates. Enzyme activity is inhibited by sulfite, sulfate, phosphate, and fluoride while activated by hydroxylamine.

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The enzyme has striking resemblance to arylsulfatase A and the enzymes could be identical (107). Both cerebroside sulfatase and arylsulfatase A have been isolated from human liver (108) and perhaps their absence could cause metachromatic leucodystrophy in humans.

Hunter's Syndrome

Hunter's Syndrome is a genetic disorder associated with failure to degrade dermatan sulfate and heparan sulfate. Lysosomal storage of these polymers leads to numerous clinical problems. Fibroblasts cultured from the skin of Hunter's patients do not adequately degrade sulfated mucopolysaccharides because of a deficiency of a specific protein that is present in cell secretions, cells, and urine of individuals who do not have Hunter's Syndrome (113). Recent studies show (109) that a deficiency of a sulfatase, L-idurono-sulfate sulfatase, which specifically cleaves the ester sulfate of iduronic acid, to be responsible for the defect in Hunter's Syndrome. This sulfatase, when added exogenously to Hunter cells, accelerates the degradation of sulfated mucopolysaccharides.

Sanfilippo Syndrome

Sanfilippo Syndrome is a familial disorder of mucopolysaccharide metabolism, transmitted in autosomal recessive fashion. Excessive urinary excretion of heparan sulfate is the major diagnostic criterion. Fibroblasts from patients with Sanfilippo Syndrome fall into two subgroups, each manifesting a deficiency of a specific factor required for normal metabolism of sulfated mucopolysaccharide. The factor deficient in the A subgroup has been isolated from normal human urine. The factor accelerates degradation of stored mucopolysaccharide in Sanfilippo A fibroblasts. It has been suggested that Sanfilippo A factor is a heparan sulfate sulfatase (110). When the factor is administered exogenously to Sanfilippo fibroblasts, a correction of the abnormal metabolism occurs.

Metachromatic Leucodystrophy (MLD)

Metachromatic leucodystrophies are a group of human genetic disorders which are characterized by the deposition of cerebroside sulfate rich granules in the central and peripheral nervous systems with resultant progressive neurological degeneration. It has been suggested that the turnover of cerebroside sulfates to cerebrosides might be blocked as a result of deficiency of cerebroside sulfatase (111, 112). Cerebroside sulfatase activity is demonstrated in normal mammalian tissues such as kidney and brain, where in MLD patients the cerebroside sulfate accumulation is especially high.

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Biogenesis

Recent work has shown that prophyran contains residues of both 3,6-anhydro-L-galactose and L-galactose 6-sulfate (69, 71). When extracts of porphyran containing seaweed are incubated with the isolated polysaccharide, free sulfate is liberated and synthesis of 3,6-anhydro-L-galactosyl residues occurs. Equimolar quantities of the two products are present in the mixture. These results strongly indicate that L-galactose 6-sulfate is an immediate biological precursor of 3,6-anhydro-L-galactose, the residue present in commercial agar. This enzymic hydrolysis can provide a way of making commercial agar from 6-sulfated polysaccharides.

Structural work of polysaccharides

Enzymic hydrolysis of polysaccharides can provide interesting structural information. Thus, the substrate specificity of five enzymes leads to the formulation of a pathway for the sequential degradation of heparin (87). Likewise, the demonstration of the presence of chondroitinase-ABC, chondroitinase-AC, chondro-4-sulfatase, chondro-6-sulfatase, and unsaturated disaccharide glucuronidase provides an enzymatic mechanism for the degradation of chondroitin sulfates (78). Another obvious feature of these enzymes is the use to which they may be put as reagents in studying the structure of chondroitin sulfates as well as the composition of a given mixture of isomeric chondroitin sulfates.

Conclusion

Work on enzymatic sulfation and desulfation of polysaccharides is in its formative stages but has already reached a point where certain beneficial applications are apparent. Clinical control of several syndromes may be facilitated by appropriate manipulation of enzymes effecting sulfate groups. On a broader basis there is indication of the potential use of enzymes for sulfation of at least certain polysaccharides and even a more immediate use of enzymes for desulfation and perhaps desulfation with resultant introduction of anhydro rings. Consequently, further industrial examination of these enzyme systems can be expected.

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9

Some Novel Methods and Results in the Sulfation of Polysaccharides

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The methods used in the sulfation of polysaccharides are fairly limited, because the properties of sulfating agents and those of polysaccharides combine to put severe constraints on the synthetic chemist. Thus, in virtually all the literature, we find essentially the same techniques - the treatment of a polysaccharide with a Lewis base complex of sulfur trioxide, under anhydrous conditions, usually in the presence of a base such as pyridine. One of the commonest techniques involves the addition of chlorosulfonic acid to pyridine in the cold, followed by introduction of the polysaccharide, and subsequent heating (1-8). The product is usually recovered and purified by alcohol precipitation, dissolution in water, neutralization, dialysis, and alcohol precipitation.

The vigor of the sulfating agent is a function of the strength of the Lewis base with which it is combined. Pyridine, being a moderate base, effectively controls the potency of the sulfur trioxide, and the crystalline pyridine-sulfur trioxide complex is readily prepared (9). Triethylamine and trimethylamine form less reactive sulfating reagents (10a), which are so stable, that sulfations in aqueous systems have been reported (4, 11). At the opposite end of the activity scale are the complexes with dimethylformamide (DMF) and dioxane (10a). These, being much weaker bases, hold the sulfur-trioxide less tightly, permitting reactions at lower temperatures.

Virtually all polysaccharides have been the object of sulfation reactions, starting with cellulose in 1819 (12). A series of review articles in Industrial and Engineering Chemistry in the 1950's and 1960's, by Gilbert and Jones refer to most of these, and a concise, but thorough, coverage was given the subject in Gilbert's book, "Sulfonation and Related Reactions" (10b).

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Because of the physiological importance of heparin, many attempts have been made over the years to produce a synthetic heparin by sulfation of polysaccharides (5-7, 13, 14), and antiulcerative medications (pepsin inhibitors) have been similarly prepared (1, 15-17).

Direct sulfation of polysaccharides, as, for example, by the pyridine-chlorosulfonic acid method, generally results in degradation of the polymer, with resultant loss of colloidal properties. However, employment of gentler conditions often fails to effect enough sulfation to change the properties of the polysaccharide. Wolfrom and Juliano (3) recognized the need to render polysaccharides more amenable to sulfation through an activation process, and Schweiger furthered the concept in a series of patents describing the sulfation of algin (18), starch (19), xanthan (20), and cellulose (21). In these cases, the activation was accomplished by treatment of the polysaccharide in a series of solvent washes which dehydrated it, yet prevented its becoming inert to sulfation, as does oven-drying.

The principal subject of this paper is a generally applicable method of activating polysaccharides, which we developed at about the same time. Basically, it consists of a two-step process involving hydration of the gum, followed by distillative solvent drying. For its hydration, the gum may be totally dissolved, or merely swollen with a limited quantity of water. In the dehydration step, the water and any other solvent which may react irreversibly with a sulfating agent, are removed by distillation in the presence of a higher boiling, water-miscible solvent. Products of predictable degrees of substitution and high molecular weight can then be prepared by conventional methods of sulfation.

Our interest in sulfating polysaccharides was primarily one of producing a synthetic carrageenan (22). Carrageenans are galactan sulfates present in a number of red seaweeds, and which are extracted and used for their gelling, suspending, and viscosity-producing properties in foods, particularly dairy products, and in such other diverse products as the popular air freshener gel. A semi-synthetic gum with properties similar to carrageenan could prove to be of great economic value in the face of rising seaweed costs which put the price of carrageenan at roughly four times that of guar and locust bean gum, and twenty to thirty times that of starch.

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Results and Discussion

The effects of time, temperature, sulfating reagent, and precipitating solvent were determined on polysaccharides activated by the dissolution/reprecipitation method, after earlier experiments showed that other methods of drying inhibited reaction (Table 1).

Effect of Temperature. For guar and locust bean gum sulfated with DMF:SO_3, the extent of sulfation was quite comparable at a given temperature, whereas other gums varied somewhat in their tendency toward being sulfated. Reaction was found to be quite rapid initially, as indicated in Fig. 1, with about 80% of the possible substitution for a given temperature occurring in the first hour. Using this knowledge, it was possible to obtain a product with a desired DS, merely by employing the calculated amount of sulfating reagent and carrying out the reaction at a temperature somewhat above the temperature which would produce that DS when an excess of sulfating reagent was present. An added advantage in this technique was the fact that there was no residual sulfating reagent left following the reaction.

Effect of Precipitating Solvent. A factor which, rather surprisingly, affected the DS achievable under a given set of sulfation conditions, was the particular solvent used for the initial precipitation of the gum. This is illustrated in Table 2. With all three gums tested, methanol was generally the most efficient at activating, whereas DMF varied the most, being least efficient with starch and most with locust bean gum. Isopropyl alcohol worked well for starch but was unimpressive with the two galactomannans. Acetone was generally poor. A possible explanation is that the order is, in general, related to the dehydrating ability of the solvent, modified by an affinity of each gum for a particular solvent. In all these cases, the gum was activated by the dissolution/reprecipitation method and sulfated at 25° for 24 hours, using sufficient 1 M DMF:SO_3 to totally sulfate the gum, i.e., to produce a DS of 3.0. Pyridine was present in an amount equal to twice the number of moles of DMF:SO_3.

Effect of Sulfating Reagent. Using DMF as a solvent, and locust bean gum as the substrate, four sulfating reagents were tested for their activity at 70° (for 4 hours). Here, again, there were differences in DS. Generally, they followed the order predicted from

Table 1. Effect of drying method on degree of substitution (DS) and viscosity (1%, 25°, Brookfield LVF, 6 rpm) for locust bean gum + 1 mole DMF:SO_3/mole anhydro sugar, 24 hrs., room temperature.

	D.S.	$\eta^{25}_{1.0}$ (cps)
Oven Drying, 60°C. ATM.	0.006	2425
Benzene Distillation, ATM.	0.03	3450
Benzene Distillation, VAC.	0.07	1100
DMF Distillation, VAC.	0.47	1150

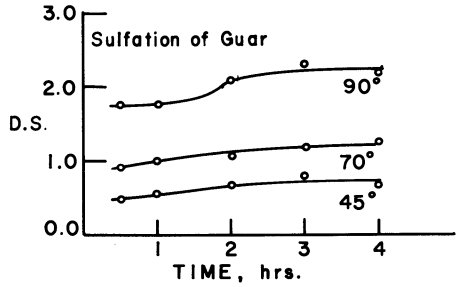

Figure 1. Effect of time at different temperatures in the sulfation of guar with DMF:SO_3

Table 2. Effect of precipitating solvent on degree of sulfation of starch, guar, and locust bean gum (DMF:SO_3, 25°/24 hrs.)

Starch		Guar		LBG	
MeOH	1.65	MeOH	1.51	DMF	1.67
i-PrOH	1.37	DMF	0.70	MeOH	1.08
Acetone	0.57	Acetone	0.65	Py	0.94
DMF	0.31	i-PrOH	0.60	Acetone	0.53
				i-PrOH	0.47

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the strength of the base used to complex the SO_3, with the exception of triethylamine, which produced the highest DS rather than the second lowest as anticipated:

Sulfating Reagent	DS
$Et_3N:SO_3$	1.35
$DMF:SO_3$	0.99
$Py:SO_3$	0.88
$Me_3N:SO_3$	0.39

From a practical point of view, there is no advantage in using any of the complexes other than that with DMF, since it can be prepared and used directly, and is very efficient. This aspect was investigated no further.

Analysis for Active Sulfating Reagent. Because of the desire to control DS and to minimize polymer degradation, it was necessary to analyze for the concentration of both active sulfating reagent and hydrolyzed $DMF:SO_3$ which may be present. Published methods for such an analysis usually direct the reader to add an excess of water and titrate the sulfuric acid produced (23). This method fails to make the necessary distinction between the active and the hydrolyzed reagent. If, however, one uses anhydrous methanol in place of water in a second titration, it is possible to determine, by difference, both the amount of active sulfating reagent and that of sulfuric acid.

Calling the reaction with water Titration A, sulfuric acid from all sources is determined:

$$SO_3 + H_2O \rightarrow H_2SO_4 \quad (2 \text{ meq. } H^+)$$
$$H_2SO_4 \rightarrow H_2SO_4 \quad (2 \text{ meq. } H^+)$$

Titration B distinguishes between the two species:

$$SO_3 + CH_3OH \rightarrow CH_3OSO_3H \quad (1 \text{ meq. } H^+)$$
$$H_2SO_4 \rightarrow H_2SO_4 \quad (2 \text{ meq. } H^+)$$

Therefore,

$$A = 2 \times \text{meq. } SO_3 + \text{meq. } H_2SO_4$$
$$B = \text{meq. } SO_3 + \text{meq. } H_2SO_4$$
$$A\text{-}B = \text{meq. } SO_3$$

and,

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$$2B = 2 \times \text{meq. } H_2SO_4 + 2 \times \text{meq. } SO_3$$
$$A = \text{meq. } H_2SO_4 + 2 \times \text{meq. } SO_3$$
$$2B-A = \text{meq. } H_2SO_4$$

To illustrate this, 10 ml. of DMF:SO_3 reagent added to 50 ml. of water required 22.65 ml. of 1 N NaOH. Another 10-ml. portion was added to 10 ml. of anhydrous DMF plus 2.5 ml. of anhydrous methanol and 5 ml. anhydrous pyridine. The mixture was allowed to stand overnight, then 50 ml. of water was added, and the solution titrated to a phenolphthalein endpoint with 1 N NaOH: 11.90 ml. was required.

$$A-B = 22.65 - 11.90 = 10.75 \text{ meq. } SO_3$$
$$2B-A = 23.80 - 22.65 = 1.15 \text{ meq. } H_2SO_4$$

Effect of Residual Water After Activation. This analytical scheme was put to use to determine how much residual water was present in an activated gum, by carrying out the analysis on the reaction mixture after the reaction was complete. Guar gum was activated by the dissolution/reprecipitation method, four samples being prepared identically except that each was taken to a different state of dryness with the DMF. Sulfation was carried out with two moles of DMF:SO_3 per mole of anhydrohexose, and two moles of pyridine for each mole of DMF:SO_3, employing conditions of time and temperature that would normally produce a DS of about 1.25. When the reaction was complete, the product was separated and worked up, and the reaction mixture analyzed for active DMF:SO_3 and sulfuric acid. The viscosity of the product and its DS were determined and compared with the level of sulfuric acid found. These results are shown in Table 3. It is quite clear that differences in viscosity are not due to variations in DS, since those are virtually identical, but it does appear significant that even in the presence of an excess of pyridine, degradation occurs in proportion to the amount of sulfuric acid produced. In view of the fact that the sulfate ester of the polysaccharide should be as acidic as the sulfuric acid (pyridinium ion being the counterion in both cases), a possible explanation is that oxidative degradation is occurring, rather than hydrolytic chain cleavage. (The titratable acidity would then be explained in terms of H_2SO_3 plus unreduced H_2SO_4).

Other Activation Methods. Alternative methods of

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hydrating the gums were investigated in the interest of economy - the dissolving and reisolation of a gum is very costly in view of the energy requirements for solvent recovery. In one, the gum was briefly soaked in aqueous isopropyl alcohol, then the mixture was boiled rapidly to evaporate the alcohol. The resulting wet, swollen powder was mixed with DMF, broken up, and allowed to stand overnight. The water and DMF were removed by vacuum distillation as in the precipitation method, and the resulting activated gum sulfated. For a given sulfation condition, the DS and molecular weight (as indicated by viscosity) were dependent on the concentration of alcohol in the original mixture (Table 4). At low titers of alcohol, low viscosities suggested incomplete dehydration with resulting degradation. At intermediate titers, viscosities increased markedly, actually exceeding the viscosity and DS of the typical DS 1.25 guar sulfates described above. Finally, at the higher titers, the DS again fell, probably a result of incomplete activation.

A variation of this process was the use of aqueous DMF to hydrate the gum. This did not work well when the original mixture placed on the gum was distilled off *in vacuo*, but if the mixture were heated, and the gum then filtered off and dehydrated with fresh DMF, sulfation without excessive degradation was possible. For example, heating guar with 30% aqueous DMF, filtering, dehydrating, and sulfating under conditions that gave a DS of 1.25 and a viscosity of about 2000 cps with precipitated guar, a DS of 1.09 and a viscosity of 4240 cps were obtained, again somewhat better than by the precipitation method.

A very unexpected result came out of a modification of these methods: guar was mixed with 60% aqueous isopropyl alcohol and refluxed 15 minutes, then filtered off and dehydrated and sulfated as described above. The product had a DS of only 0.16, but a viscosity of 29,000 cps at 1%. (The untreated guar had a viscosity of 6600 cps measured by the same procedure.) A repeat of the experiment resulted in a product with a DS of 0.20 and a 1% viscosity of 26,000 cps.

Effect of Bases. Because the primary goal of the work was to make a synthetic carrageenan, and since carrageenan is used so extensively in foods, an attempt was made to replace pyridine in the reaction mixture with a less noxious base. The difficulty was to find one which would not be so alkaline as to react preferentially with the DMF:SO_3, nor so weak as to fail to pick up protons as they were liberated (the possibility

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Table 3. Effect of sulfuric acid in reaction mixture on viscosity of sulfated guar. (8.1 g. guar → 13.1 g. Na guar SO_4).

Meq. H_2SO_4				
From DMF:SO_3 (92 ml. 1.106 M)	From H_2O in Guar	Total	$\eta^{25}_{1.0}$(cps)	DS
9.9	10.0	19.9	2375	1.27
9.9	14.3	24.2	2175	1.27
9.8	26.8	36.6	1725	1.22
9.9	38.5	48.4	1525	1.24

Table 4. Effect of alcohol titer on DS and viscosity of guar gum hydrated by alcohol boil-off (8.1 g. guar + 100 ml. aqueous isopropyl alcohol).

% i-PrOH	DS	$\eta^{25}_{1.0}$(cps)
10	0.70	840
20	0.33	605
30	1.25	850
40	1.26	1450
50	1.46	3400
60	0.88	5700
70	0.21	7300

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of oxidative degradation had not been considered at the time). Although none was found that worked as well as pyridine (likely because of the heterogeneity of the system), ones that showed promise were sodium and potassium acetates and disodium phosphate. In Table 5 are given some results. In these instances, the aqueous DMF method was used except for the example with potassium acetate; this was carried out with freshly precipitated guar.

Control of DS. An interesting sidelight to our work was demonstrating the apparent identity of funoran and sulfated agarose. Funoran is a seaweed polysaccharide used in great quantities in the Far East. Extracted from Gloiopeltis furcata and related species, it finds use as a thickener or glue. It was identified as a galactan sulfate in the early 1900's, and the presence of 3,6-anhydro-L-galactose was established in 1956 by Hirase, Araki, and Ito (24). This meant that instead of being a carrageenan, it was more like a sulfated agarose. When agarose was sulfated to the same extent as funoran, and their infrared spectra compared (Fig. 2), they were found to be essentially identical as proposed by Stancioff and Stanley in 1968 (25). Additional proof of funoran's structure was given by Penman and Rees in 1973 (26).

Conclusion - Properties of the Products. Sulfated polysaccharides prepared in this work were found to have one feature in common - they were non-gelling. As such, they were most like lambda-carrageenan, and when tested in a variety of applications, demonstrated an ability to replace lambda-, but not kappa- or iota-carrageenan. Of all the products made, the most commercially promising was a guar sulfate having a DS of 2.19 - it could be used to stabilize a cold-mixed milkshake at one-fifth the level of a carrageenan used for that purpose. Several of the products made satisfactory chocolate syrups which, when mixed with cold milk, would thicken it and prevent settling of the cocoa, as is characteristic of lambda-carrageenan. A few of the products were also satisfactory as toothpaste binders - another function normally performed by lambda-carrageenan.

A number of factors combined to lessen the commercial interest in these semi-synthetic gums, the principle ones being the increased concern over the safety of food additives (and the requiring of extensive and costly animal testing), and the availability of larger quantities of seaweeds through the successful develop-

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Table 5. Replacement of pyridine by salts of weak acids. (Guar gum heated with aqueous DMF, filtered, dehydrated, and sulfated with excess DMF:SO_3).

Base	SO_4'n. Conditions	DS	$\eta^{25}_{1.0}$ (cps)
Na_2HPO_4	70°/1 hr.	0.59	600
Na_2HPO_4	70°/2 hrs.	0.50	350
NaOAc	50°/1 hr.	0.63	1150
NaOAc	50°/2 hrs.	0.66	1100
NaOAc	50°/2 hrs.	0.60	3580
KOAc	70°/4 hrs.	0.77	1130

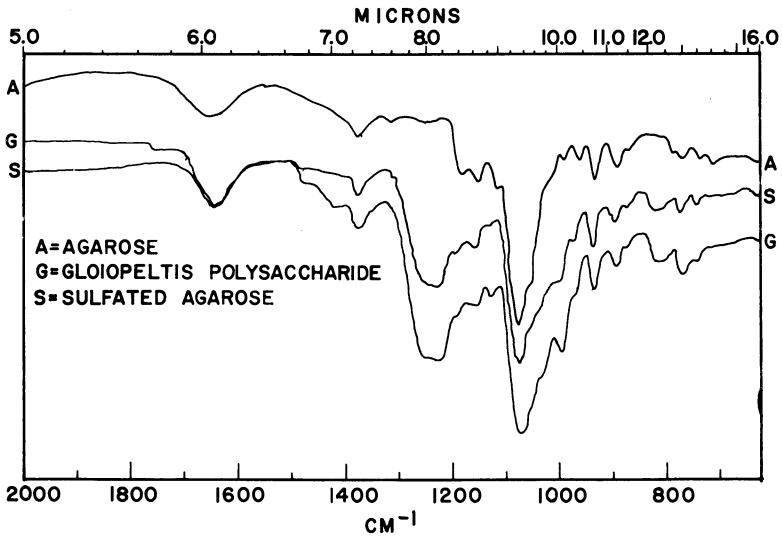

Figure 2. IR spectra of agarose, sulfated agarose (25.5% SO_4), and funoran, the polysaccharide from Gloiopeltis furcata *(24.7% SO_4)*

ment of seaweed farms in the Philippines (27, 28). In addition, since the major uses of carrageenan are dependent on its gelling properties, further developmental work with these non-gelling derivatives was not warranted. Nonetheless, a controllable means of producing polysaccharide sulfates was developed and because of its general applicability, it can readily be put into practice, utilizing virtually any polysaccharide available.

Experimental

Materials. Polysaccharides used were of standard commercial food grade. Sulfur trioxide was obtained from Allied Chemical Corp. as Sulfan B. Isopropyl alcohol was technical grade, 99%. Trimethylamine-sulfur trioxide was purchased from Hexagon Laboratories, Inc., Bronx, N.Y., and used as received. All other solvents and chemicals were reagent grade. DMF:SO_3 was prepared by the method of Garbrecht (23). Pyridine-sulfur trioxide was made by the method given in Inorganic Syntheses (9), except that Sulfan B was used in place of chlorosulfonic acid. In this way, the product was free from chloride ion. Triethylamine-sulfur trioxide was prepared as described by Whistler and Spencer (29).

Activation of Polysaccharides. 1. Dissolution/Reprecipitation. The polysaccharide was dispersed into rapidly agitated water at room temperature, generally at a level of 1%. If necessary, the mixture was heated to dissolve the polysaccharide. For guar, it was not necessary; locust bean gum, 85°C; starch and agarose were boiled. The sol was then poured, in a steady stream, into twice its volume of the solvent selected, with agitation provided by a glass stirring rod. The gum was separated by straining it through a piece of polyester cloth suspended on a screen or collander, and further dried by drawing up the corners of the cloth and squeezing out excess liquid. The polysaccharide was then shredded by hand into a large necked standard-taper round-bottom flask, and covered with anhydrous DMF to the extent of about ten times the initial weight of gum. The flask was then attached to a rotating evaporator and the solvents removed at 5-10 mm pressure. The temperature of the bath surrounding the flask was allowed to increase to 80°. Distillation was stopped when no visible liquid remained in the flask.

2. Aqueous alcohol boil-off. The polysaccharide was mixed with aqueous isopropyl alcohol in the ratio of 8.1 g. gum to 100 ml liquid, and the mixture quickly

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brought to a boil in a steam-jacketed stainless steel beaker. The alcohol and some of the water were quickly removed. The resulting damp, sticky gum was mixed with 150 ml. of DMF, allowed to stand overnight, and the vacuum distillation carried out as in 1.

3. Aqueous DMF. The polysaccharide was mixed with about 12 parts by weight of aqueous DMF and heated in a boiling water bath for 5-10 minutes. The mixture was separated by filtration, and the gum dehydrated by vacuum distillation as in 1.

4. Aqueous alcohol soak. The polysaccharide was mixed with aqueous isopropyl alcohol and refluxed for 15 minutes or allowed to stand at room temperature overnight, then separated by filtration and dehydrated by DMF distillation as in 1.

Sulfation of Polysaccharides. The activated polysaccharide was sulfated in the flask in which it had been dried down by distillation. An amount of approximately 1 M DMF:SO_3 was added, according to the desired result or experimental condition being investigated. For example, for 8.1 g. of starch, guar, or locust bean gum, each of which has an average of three hydroxyl groups per anhydro sugar, there is a total of 150 meq. of hydroxyl. To provide enough sulfating agent to completely sulfate the gum to a DS of 3.0, 150 ml. of 1 M DMF:SO_3 would be used; for a DS of 1.0, only 50 ml. of the reagent would be added. Pyridine was added at twice the milliequivalent level of the sulfating reagent. The flask was stoppered and placed on a shaker for the desired time. For work at elevated temperatures, a water bath was positioned beneath the arm of the shaker and the flask immersed in it.

When the reaction time had elapsed, the mixture was quickly poured through a sintered glass funnel on a filter flask and the gum washed with dry DMF and/or isopropyl alcohol to remove adhering unreacted sulfating reagent. It was then dispersed in water (about 100 parts, based on initial weight), and stirred. Dilute (1 N) sodium hydroxide was added to maintain a pH somewhat over 7 as the gum dissolved. Usually, it was heated toward the end of the dissolution process, then added to 2 volumes of isopropyl alcohol to reprecipitate it. It was washed 2-3 times with 85% isopropyl alcohol sufficient to cover it, in order to remove residual pyridine. Finally, it was squeezed out and dried in a circulating air oven at 60° and ground through a 40-mesh screen in a laboratory Wiley Mill.

Analysis of DMF:SO_3 Reagent. This is described in

the Results and Discussion section.

Analysis of Products. Moisture was determined by drying at 70°C and <10 mm. overnight.

Sulfate was determined by digesting a sample of the gum with boiling concentrated nitric acid until all organic matter was destroyed, then removing excess nitric acid by addition of sulfate-free formalin. Usual procedures were followed for precipitation with barium chloride and weighing as barium sulfate.

Since the products were isolated as sodium salts, DS was calculated directly from the sulfate analysis (anhydrous basis) using the formula

$$DS = \frac{162\,(\%SO_4)}{9600 - (102)\,(\%SO_4)}.$$

This holds true, without modification, only for sodium salts of sulfated gums initially having an average of three hydroxyls per anhydro sugar unit.

Viscosity Measurements. Viscosities were determined on 1% solutions of the gums at 25°C using a Brookfield model LVF or LVT rotational viscometer at 6 rpm, selecting the appropriate spindle for the viscosity being measured. Solutions were prepared by dispersing the gum into 99 times its weight of room temperature distilled water using good agitation. Although most of the sulfated gums were soluble at this temperature, the mixture was routinely heated for 15 minutes in a boiling water bath to assure dissolution of such materials as low DS locust bean gum sulfate, and to make all solution preparations similar. After heating, the sol was cooled, and water lost by evaporation replaced and thoroughly admixed prior to making the measurement.

Milkshake Test. A dry blend consisting of 20 g. of sugar, 2 g. of cocoa, 0.01 g. artificial vanilla flavor and 0.5 g. of carrageenan (or sulfated polysaccharide) passing a 270-mesh screen, was added to 8 fl. oz. of cold milk and the mixture shaken vigorously for 15 seconds, then poured into a 400-ml. Berzelius beaker. A shake of high quality filled the beaker to the brim, retaining the volume for over 5 minutes, and no settling of the cocoa occurred. Test materials were evaluated against this standard.

Cold Mix Chocolate Milk and Toothpaste Tests. These are rather specialized tests peculiar to the industry, and somewhat beyond the scope of the subject at hand. Consequently, they will not be included here.

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Abstract

In an attempt to prepare sulfated polysaccharides which might have the gelling, suspending, and stabilizing properties of carrageenan, techniques were developed which led to products having controlled degrees of substitution with retention of high molecular weight. Basic to the process was an activation step which involved the sequential hydration-dehydration of the polysaccharides. Several methods proved effective, with one resulting in a product having four times the viscosity of the starting material. An analytical method for distinguishing between active sulfating reagent and that which was hydrolyzed, was developed. Several of the products demonstrated carrageenan-like properties, most notably, those of the non-gelling fraction, lambda-carrageenan.

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Sodium Cellulose Sulfate via Cellulose Nitrite

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It was reported previously that polyhydroxy polymers including cellulose react under certain conditions with N_2O_4 to form nitrite esters (1). The reaction medium must contain a proton acceptor, which may be a highly polar aprotic solvent, such as N,N-dimethylformamide (DMF) or N,N-dimethylacetamide (DMAC). The reaction path is shown in Figure 1. The assymetric isomer of N_2O_4, nitrosyl nitrate, reacts quantitatively with the cellulosic hydroxyl groups to result in cellulose trinitrite ester and an equivalent amount of nitric acid.

The nitrite ester has been found to be quite labile and to decompose immediately with a protic solvent, such as water or alcohol, in the presence of an acidic catalyst. It results in regenerated cellulose and nitrous acid or alkyl nitrite depending on the protic solvent used as shown in Figure 2. Analytical data showed that no modification or significant depolymerization occur during this process unless the temperature is excessively high.

It was found that, due to the lability of the nitrite groups, cellulose nitrite can be used as a chemical intermediate for the preparation of cellulose derivatives, particularly cellulose sulfate (2). If, to the reaction mixture containing the nitrite ester, SO_3 is added, preferrably in the form of a complex with, for example, DMF to avoid an excessive heat of reaction and degradation, an equivalent number of nitrite groups will be replaced by sulfuric acid ester groups as shown in Figure 3. This results in the formation of a mixed cellulose nitrite sulfate ester and an equivalent amount of N_2O_4 by the reaction of nitrosyl ion with nitrate ion that was formed previously during nitrosation.

The mixed ester is brought into contact with a protic solvent, such as water or alcohol. In the presence of the nitric acid formed during nitrosation, residual nitrite groups are removed immediately as shown in Figure 4 with the formation of cellulose sulfate ester and nitrous acid or alkyl nitrite. The sulfate ester may be precipitated with

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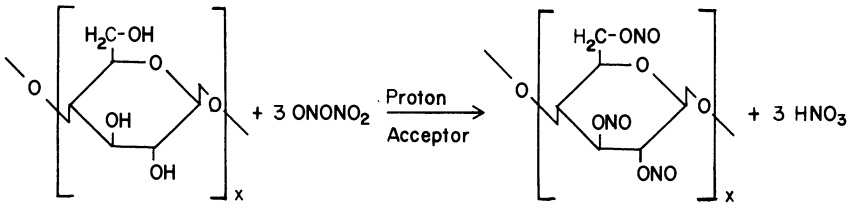

Figure 1. Formation of cellulose nitrite

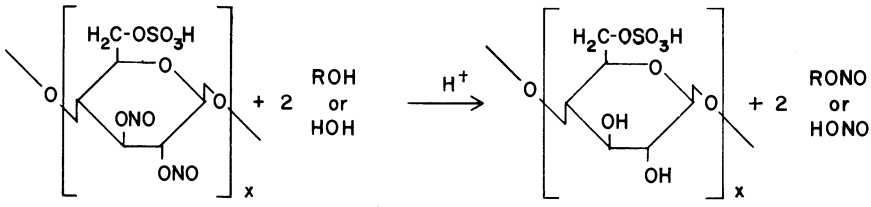

Figure 2. Hydrolysis of cellulose nitrite

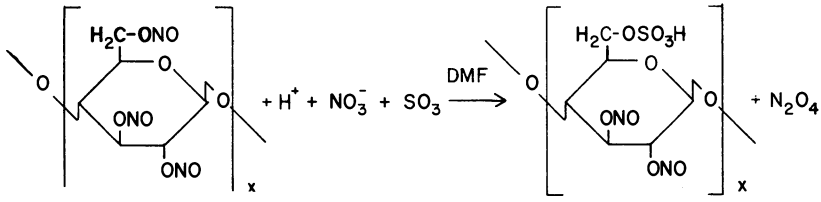

Figure 3. Sulfation of cellulose nitrite

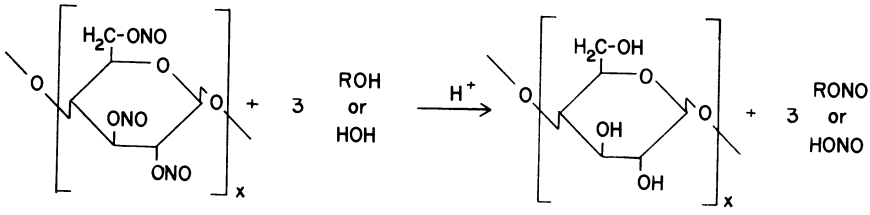

Figure 4. Hydrolysis of mixed cellulose nitrite sulfate ester

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acetone, separated, and neutralized. However, it is preferred to neutralize the complete mixture and precipitate the sodium cellulose sulfate with alcohol. By this method, any D.S. of up to about 1.1 is obtainable simply by calculating and using the stoichiometric amount of SO_3.

If D.S. values exceeding about 1.1 are to be obtained, lower D.S. nitrite esters have to be used as the intermediate, such that the total D.S., i.e., the sum of the degree of nitrosation plus the degree of sulfation, is about 3. The amount of N_2O_4 for producing the nitrite ester is again calculated stoichiometrically while that of SO_3 may be the stoichiometric amount or a slight excess. For example, the preparation of a sulfate ester having a D.S. of 1.4 requires nitrosation to a D.S. of about 1.6 followed by the addition of a stoichiometric quantity of DMF-SO_3 complex to produce a degree of sulfation of 1.4 or of a slight excess. At the higher degrees of sulfation, a slight excess of SO_3 is preferred since this assures a complete reaction. Thus, degrees of substitution of up to close to 2 can be obtained.

Although the fact that lower D.S. nitrite esters have to be used to obtain degrees of sulfation of above 1.1 may suggest a direct substitution of hydroxyl groups, experimental evidence supports ester exchange similar to the sulfation of cellulose trinitrite. Cellulose trinitrite, for example, can be sulfated successively to D.S. values of 0.7 and 1.1 with about stoichiometric amounts of SO_3. If, however, after sulfation to D.S. 0.7, the residual nitrite groups were removed by the addition of a stoichiometric amount of methanol, no further sulfation occurred, even when an amount of SO_3 was added that was sufficient to raise the D.S. to about 2.

This new method of derivatizing cellulose is unique since it is the first method to utilize an active cellulose intermediate and, thus, to permit derivatization in a homogeneous reaction medium. All other methods are heterogeneous reactions using insoluble cellulose as the starting material and directly substituting free hydroxyl groups. As a result, the substituents are uniformly distributed over the molecule, i.e., a D.S. of 1 indicates substantially anhydroglucose monosulfate units and a D.S. of 2 anhydroglucose disulfate units. In contrast, the D.S. of products of all prior methods refers to an average value, and the D.S. of their individual units probably varies between 0 and 3. This leads to remarkable differences between products of prior methods and those of the present method.

1. Water soluble sulfate ester products are obtained at a D.S. of as low as about 0.3. Authors of comparable prior methods state that a D.S. of greater than 1.0 is required for water solubility (3,4).

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Table I. Compatibility with Polyvalent Metal Ions

Metal Ion	Sodium Cellulose Sulfate D.S. ≤ 1.3	D.S. ≥ 1.3
Mg^{2+}	+	+
Ca^{2+}	+	+
Sr^{2+}	+	+
Ba^{2+}	+	-
Zn^{2+}	+	+
Cu^{2+}	+	+
Co^{2+}	+	+
Ni^{2+}	+	+
Fe^{2+}	+	+
Cd^{2+}	+	+
Hg^{2+}	+	+
Pb^{2+}	+	+
Sn^{2+}	+	+
Al^{3+}	+	+
Ce^{3+}	+	-
Cr^{3+}	+	+
Fe^{3+}	+	-

+ Compatible

- Incompatible

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2. Aqueous solutions are perfectly clear and do not contain gelatinous transparent particles, such as those of most commercially available cellulose products having high viscosities. Dilute solutions, for example, can be filtered directly through fine millipore filters without plugging or substantial reduction of the flow rate.
3. All products, particularly those with a D.S. of below about 1.3, exhibit an unusual compatibility with polyvalent metal ions as shown in Table I. Only if the D.S. exceeds about 1.3 is some incompatibility observed with Ba^{2+}, Ce^{3+}, and Fe^{3+}. Solutions of products showing a plus do not form a precipitate, remain clear, and maintain viscosity even when the solution is saturated with the metal salts indicated. There is hardly any other water soluble polymer - particularly anionic polymer - that exhibits complete compatibility over such a wide range and in saturated salt solutions.

Another great advantage of this method is the fact that no significant depolymerization occurs during sulfation via nitrite ester intermediate. It has been shown previously that nitrite ester formation with N_2O_4 does not depolymerize cellulose unless the reaction mixture is kept at room temperature over an extended period of time (1,2). Similarly, no significant depolymerization occurs during the subsequent sulfation by ester exchange as indicated by the extremely high viscosities of the final products and by the fact that increasing the reaction time for sulfation from 1-2 hrs. to about 20 hrs. does not significantly reduce the viscosity of the products. Table II summarizes the viscosities of 1% solutions of products with varying D.S. prepared from high D.P. cotton linters.

Table II. Viscosity and i.r. Spectrum vs. Degree of Substitution

App. D.S.	Viscosity, cps	Frequency (-OH), CM^{-1}
0		3400 - 3420
0.3 - 0.5	5000 - 7000	∿3430
0.6 - 0.9	2000 - 4000	∿3440
1.0 - 1.3	1000 - 2000	∿3450
1.4 - 1.6	500 - 1000	3460 - 3470

Products having maximum viscosities of up to about 7000 cps are obtained. No cellulose derivative prepared by prior methods, irrespective of the type of substituent introduced, exhibits viscosities of such magnitude. This is of particular significance since sulfation of polysaccharides generally causes more serious degradation than most other reactions. Apparently, a fully substituted cellulose is considerably more resistent to depolymerization than a partially substituted or unsubstituted cellulose when subjected to chemical reactions.

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In comparing viscosity with D.S., a great viscosity increase is observed in the lower D.S. range as the D.S. decreases. A certain viscosity increase, of course, is expected since the product concentrations in the solutions are determined by weight and, therefore, the number of actual cellulose molecules in solution increases as the D.S. decreases. However, even when solutions of equal molar concentrations are compared to eliminate this effect remains the viscosity increase unexpectedly high. An explanation for this phenomenon is hydrogen bond activity. In cellulose, there is intensive inter-molecular hydrogen bond formation, in fact, to such an extent that the cellulose is water insoluble. It is, therefore, conceivable that substitution of hydroxyl groups to a low D.S. may reduce hydrogen bond formation sufficiently to solubilize the cellulose but not enough to eliminate a cross-linking, i.e., viscosity increasing, effect in solution. Such an effect would diminish and finally disappear as the D.S. increases. Support for this assumption is in the i.r. data in Table II. The frequency of the OH peak of products with the lowest degrees of substitution is close to that of the highly bonded hydroxyl groups in unsubstituted cellulose. There is a gradual shift towards higher frequencies as the D.S. increases indicating diminishing hydrogen bond activity of the hydroxyl groups with increasing D.S. Similar thoughts were expressed previously with regard to algin sulfates (5).

Similar to other polysaccharide sulfates (4-7), all the cellulose sulfate products prepared via nitrite ester intermediates are protein reactive assumingly by the formation of ionic bonds between the negative sulfate ester groups and the positive sites in the protein. This results in viscosity increases or precipitation if both the polysaccharide sulfate and the protein are in solution prior to combining them. Table III shows viscosities of 0.5% solutions of sodium cellulose sulfate at varying D.S. with and without 5% sodium caseinate.

Table III. Reaction with Protein

App. D.S.	Viscosity, cps With Caseinate	Without Caseinate
0.5 - 0.6	810	690
0.7 - 0.8	754	550
1.1 - 1.2	1420	303
1.4 - 1.5	2400	226

All products show a significant viscosity increase over that of the control. The viscosity increase becomes greater as the D.S. of the cellulose sulfate ester increases. Combined with the viscosity increase is a change of the flow characteristics in that a shorter flow is noticed and the solution

appears to become more coherent and exhibits a higher degree of pseudoplasticity. Similar results are obtained when, instead of caseinate, soy protein isolate is used.

A number of methods for the sulfation of cellulose have been published previously, among them reactions with SO_3 (8-10), chlorosulfonic acid (11,12), and sulfuric acid with (13-15) and without (16,17) an aliphatic alcohol in the reaction medium. Most of these methods, particularly those employing SO_3 or sulfuric acid, severely degrade the cellulose resulting in products of little commercial value.

Two other methods have been developed more recently, one by Whistler employing dimethylsulfoxide (DMSO)-SO_3 (4) and another by Schweiger using DMF-SO_3 complex (6). In both methods, cellulose is sulfated directly under heterogeneous conditions to result in products having relatively high viscosities. So far, they have been the only methods with potential commercial feasibility. Table IV summarizes some of the principal differences relating to the water soluble products obtainable by the two prior methods and the present method.

Both heterogeneous methods are similar in that they result in products within a narrow D.S. range. Relatively large excesses of sulfating agent are required, and the D.S. cannot be effectively controlled. The products have similar maximum viscosities of about 200 to 500 cps and poor compatibility with a number of metal ions particularly at high concentrations. In contrast, the homogeneous sulfation via nitrite ester permits easy controllability of the D.S. within a wide D.S. range - including previously unaccessible low D.S. ranges - by employing essentially stoichiometric quantities of reagent and results in products exhibiting much higher maximum viscosities and much greater compatibility with metal ions.

Another method has been reported most recently by B. Philipp et al. employing nitrosyl sulfuric acid in DMF (18,19). Like the present method, this procedure results in a mixed cellulose nitrite sulfate ester intermediate but, in contrast, is a direct substitution of cellulose in a heterogeneous system.

Experimental

Preparation of Sodium Cellulose Sulfates. Cotton linter pulp (50 g.) is suspended in 1.5 l DMF using a 3 l three neck round bottom flask equipped with a mechanical stirrer, $CaCl_2$ tube, and dropping funnel. About 75 g N_2O_4 dissolved in 100-150 ml DMF is slowly added over a period of 30-45 min. and stirring continued until a clear highly viscous solution is obtained. A saturated solution of DMF-SO_3 complex prepared as described previously (6) is then slowly added to the

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Table IV. Comparison of Different Methods

Method	D.S. Obtainable	Controllability of D.S.	App. Maximum Viscosity	Compatibility K^+	Compatibility Ca^{2+}	Compatibility Al^{3+}	Sulfating Agent Required
DMSO-SO_3 heterogeneous	≲1.0, not obtainable ∿1.0-1.5 most difficult ∿1.5-1.8, usual D.S.	not possible	200-500	-	-	-	large excess
DMF-SO_3 heterogeneous	≲1.2, not obtainable ∿1.2-2.0, difficult ≳2.0, usual D.S.	not possible	200-500	-	-	-	large excess
Via Nitrite Ester homogeneous	∿0.3-≲2.0, easily obtainable ≳2.0, not obtainable	easily controllable	up to ∿7000	+	+	+	stoichiometric to slight excess

nitrite ester solution over a similar period of time with continuous stirring and cooling in an ice bath to maintain the temperature at about 10-15°. The amount of SO_3 is calculated stoichiometrically to result in the desired degree of sulfation. The reaction mixture is then mixed with about 50 ml methanol or water, transferred into a large beaker, and neutralized with an aqueous solution of Na_2CO_3. During neutralization, strong agitation and cooling are required. The sodium cellulose sulfate is precipitated by the addition of methanol, washed with 70% aqueous methanol, and dried. The yield is quantitative.

Cellulose nitrite esters with a D.S. of below about 3 for the preparation of sulfate esters with a D.S. of above about 1.1 are obtained similarly by using correspondingly smaller, stoichiometrically calculated amounts of N_2O_4. The further procedure for sulfation and neutralization is the same as described above.

Analytical and Property Determinations. Viscosities were measured with a Brookfield Viscometer, LVT Model, at 20° and 60 rpm.

For the i.r. spectra, films of the products were used prepared by casting a 1-2% solution on a glass plate, drying at room temperature and, subsequently, at 80°, and removing the dry film with a razor blade.

The D.S. of the sodium cellulose sulfate was determined gravimetrically as described previously (6) by hydrolyzing the product in 10% HCl at 100° over night and precipitating the sulfuric acid formed as $BaSO_4$.

The compatibility with metal ions was determined by directly dissolving increasing amounts of the crystalline salt in a 1% aqueous solution of the sodium cellulose sulfate until saturated. Metal salts used were chlorides, sulfates, or acetates. Incompatibility was indicated by the formation of a precipitate combined with a loss of viscosity.

To demonstrate protein reactivity, the viscosity of a solution containing both 0.5% sodium cellulose sulfate and 5% sodium caseinate was compared with that of a 0.5% solution of the corresponding sodium cellulose sulfate.

Summary

Cellulose nitrite prepared as described previously was allowed to react with DMF-SO_3 complex. This resulted in the replacement of part of the nitrite ester groups by sulfate ester groups with the formation of mixed cellulose nitrite sulfate ester. Residual nitrite groups were removed by the reaction with a protic solvent, such as alcohol or water, and the cellulose sulfate ester was neutralized to form sodium cellulose sulfate. Both nitrosation and sulfation appear to be stoichiometric reactions, and the degree of sulfation can be calculated by the amount of SO_3 used. Water soluble

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products are thus obtained within a D.S. range of about 0.3 to 2. Chemical and physical properties of cellulose sulfate esters prepared via nitrite ester intermediates differ significantly from those of similar products prepared by prior methods.

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11

Reaction of Starch with the Chlorosulfonic Acid-Formamide Reagent

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Reactions of starch polysaccharides with sulfating agents have been carried out (1) for making heparinoids with blood anti coagulating properties (2-4), for obtaining products having anti ulcer action by inhibiting pepsin (5-7), to produce thickening agents for foods (8) and auxiliary substances in various techniques (9,10) and, generally to substitute for naturally occuring polysaccharide gums (11-13). The methods used for introducing sulfuric acid groups into the molecules of starch vary widely. There are (1) reactions in homogeneous nonaqeous or aqueous systems, (2) reactions in heterogeneous liquid systems, and (3) reaction in dry state. A summarizing survey on the most important methods and reagents used so far is given in Table 1. The properties of the resulting starch esters depend largely on these methods and reagents, and it is shown that reactions in homogeneous systems give products with low, medium and high degrees of substition (3,6,14-17). It is necessary, however, to pre-gelatinize the starch thus limiting the starch concentration by its viscosity. Reactions in heterogeneous systems on the other hand which may be performed at higher concentrations lead to low degrees of substitution (11,13,18-20). The lowest degrees of substitution are obtained by reactions in a dry state (8,21,22). The system SO_3-trialkylamine is frequently used and well studied as a reactant in aqueous alkaline (6,19, 17,20,23,24) and nonaqeous solvents (pyridine, DMF, formamide, dimethylsulfoxide) (6,9,11,15,16,25) using liquid or gaseous sulfur trioxide. Another reactive system, chlorosulfonic acid (4,6,14,26-28) and formamide (4,14,28,29), seems to be of partial

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interest with respect to costs and availability of chemicals in the case of upscaling to an industrial level for making starch sulfates as protein complexing agents (30). But there is poor knowledge on the influence of the reaction conditions on the properties of the end products.

The purpose of the present article is to present information on a correlation between principal reaction conditions and the fundamental properties of the resulting esters regarding degree of substitution (DS), degradation and contamination by decomposition reactants.

The chlorosulfonic acid-formamide system has been chosen because of its several advantages as compared with other reactive systems that have been investigated in numerous preliminary experiments. Formamide has various functions and acts as a swelling and solubilizing agent for starch granules, a complexing agent with chlorosulfonic acid, a buffering agent during the reaction, and as a solvent for the reaction products. Also, it may be destroyed in sufficient concentrations of alkali. The main benefit, however, is in the simplification of the initial reaction steps i.e., the complex may be formed in the reaction vessel by adding chlorosulfonic acid to formamide (calculated amounts for complex formation and for dissolving the starch)immediately followed by the addition of the dry starch. Under these conditions, the starch is suspended at ambient temperature up to concentrations of 30 % of the amount of formamide used for dissolving the starch. There is no problem with the fluidity of this system if it is heated slowly up to the reaction temperature desired. At a sufficiently high temperature, the starch granules begin to dissolve slowly in the reactive system. Consequently, the reaction may be carried out alternatively in heterogenous or homogeneous system (30).

Results

Coefficients of determination. For 16 effect variables out of 20 which have been investigated at a reaction temperature of 50 °C (level α), correlations by variance between effect variables and process variables have been established. Only the following items are not relevant in mathematical sense: $[\alpha]$, calculated on the basis of anhydroglucose unit (AGU) and of pure starch sulfate, R_G, calculated on the basis AGU, and inorganic sulfate (% S_A). At a reaction temperature of 70 °C (level β), 12 effect

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variables out of 20 are correlated with process variables. Similarly, properties without satisfactory correlation are $[\alpha]$, inorganic sulfur, RG, calculated on the basis of pure and crude starch sulfate, the optical density of solutions, and the yield of pure starch sulfate, the latter being evidently more dependent on purification than reaction conditions.

Fisher Test. The combination of four independent process variables by assuming a single effect and double and triple interactions results in 16 explanatory variables. Twelve of these variables show a significant influence on effect variables. They are summarized in Table 2 and are valid for the two reaction temperatures used. Independent from the general action of the temperature, which is of predominant influence on the quality of effect variables, Table 2 gives a sequence of validity for the explanatory variables involved in the process. The concentration of water (w), which is shown 67 times to be significant in single and multiple action and the concentration of SO_3 (r), significant in 64 cases of action are most essential from this point of view. The concentration of starch in formamide (s) with 19 significant actions and finally the time of reaction (t) with only 7 cases of significance are less important. The reaction time, however, may be of greater importance if the reaction is carried out at a temperature of below 50 °C.

Analysis and interpretation of eqations of regression. In the following only a few correlations between process variables and effect variables may be regarded, selecting those with the greatest importance for the process to be studied: Sulfur, bound in ester groups, S_E: Highly esterified products containing more than 16 % S_E, (DS > 1,4) may be obtained only at a temperature of 70 °C and a SO_3/AGU ratio of 4:1. The water content of starch, when increased from 5 % to 20 %, acts in favor of higher degrees of esterification (fig.1). At a SO_3/AGU ratio of 2:1, however, the degree of esterification is low and appears to decrease with increasing water content.

Very low degrees of esterification (<10 %, DS < 0,7) are also obtained at 50 °C. At both SO_3/AGU ratios, the degree of esterification increases with increasing water content, the increase being more pronounced at the 4:1 than the 2:1 ratio (fig.1).

Fig.2 shows that, at 70 °C, the degree of este-

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Tab. 1 Summarizing survey on preferred methods and reagents, used for preparing starch sulfates (taken from literature)

Characteristics of System	Solvent	Complexing Agent	SO_3 Donor	Temp. °C	Time	Degree of Esterification DS
Non aqueous, homogeneous heterogeneous	organ. N-containing bases	organ. N-containing bases	chlorosulfonic acid, oleum, SO_3	0 - 100	15 min - several days	0,1 - 2,6
Aqueous, alkaline, homogeneous, heterogeneous	dil. alkali soln. (+swelling inhibitors)	trialkyl-amines	liqu. SO_3	20 - 65	1h - 24 h	0,01 - 1,7
Nonfluid, dry,	—	— (urea amides)	acidic sulfate - or sulfite - salts	65 - 300	1h - several hours	max. 0,22

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TAB. 2 F I S H E R - T E S T FOR PROVING SIGNIFICANCE BETWEEN EXPLANATORY VARIABLES AND EFFECT VARIABLES IN THE REACTION OF THE CHLOROSULFONIC ACID - FORMAMIDE SYSTEM WITH STARCH

TYPE OF ACTION	$F > 10$	$F > 5$	$F > 3$	$\sum$ ACTIONS
SINGLE EFFECT				
w	17	7	1	25
r	12	3	4	19
s	6	4	2	12
t	-	-	1	1
DOUBLE INTERACTION				
w • r	10	7	4	21
s • r	4	4	1	9
s • w	-	3	3	6
t • w	-	1	1	2
t • r	-	1	1	2
t • s	-	-	1	1
TRIPLE INTERACTION				
s • w • r	2	5	5	12
t • s • r	-	-	1	1

(w WATER CONTENT OF THE STARCH, r MOL. RATIO AGU/SO_3, s STARCH CONCENTRATION IN FORMAMIDE
t TIME OF ACTION)

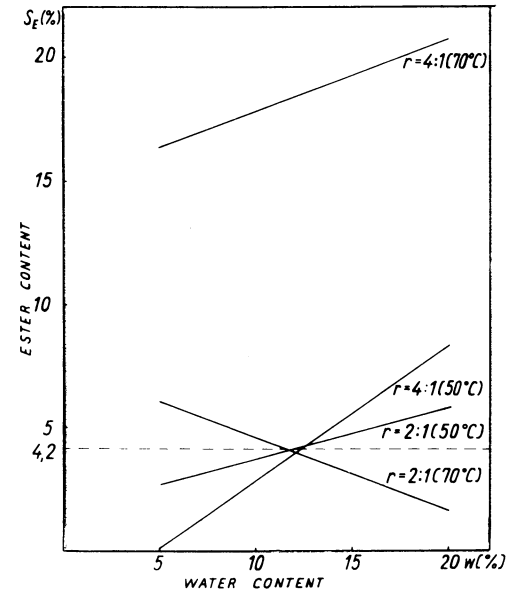

Figure 1. Lines of regression for the influence of the water content (w) of the starch on esterification (% S_E) (r = SO_3/AGU ratio)

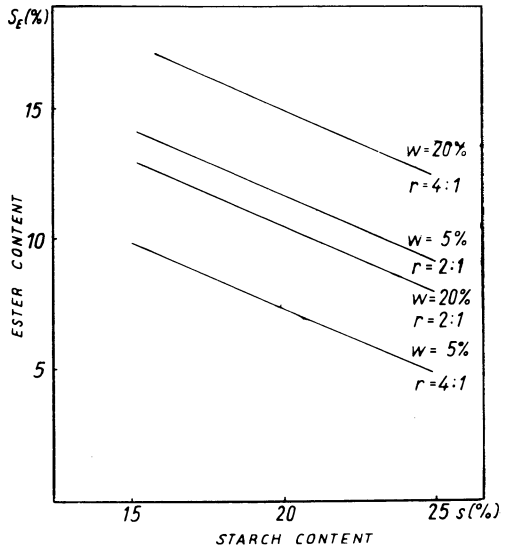

Figure 2. Lines of regression for the influence of the concentration of the starch in formamide (s) on esterification (% S_E) (w = water content, r = SO_3/AGU ratio)

rification decreases slightly with increasing concentration of starch in all cases. The limiting viscosity number may be considered as a general measure for molecular degradation without using exact values for the molecular weight. Generally, solutions with excess electrolyte are used to eliminate polyelectrolyte behavior. Viscosity measurements show a pronounced decrease of $[\eta]$ takes place with increasing water content (fig.3). A comparision of the values of $[\eta]$ indicates that degradation usually takes place under conditions which produce high degrees of esterification (high temperature and high SO_3/AGU ratio) and, inversely, that slightly degraded esters may be obtained under conditions favoring low degrees of esterification (lower temperature and lower SO_3/AGU ratio).

The high molecular weight portion as determined in the excluded volume through gel permeation chromatography on SEPHADEX G 200 may be interpreted similarly. There is a strong tendency to decrease with increasing water content at a SO_3/AGU ratio of 4:1, both with 15 % and 25 % s, and a tendency to increase with an SO_3/AGU ratio of 2:1 (fig.4). In this case, however, the different polyelectrolyte behavior of the variously substituted polysaccharide esters could not be overcome by the solvent and therefore, a few results are inconsistant with these tendencies. The apparent viscosity (η) of 5 % aqueous solutions is highly influenced by the water content of the starch at 70 °C and decreases at a high SO_3/AGU ratio (fig.5), The same tendencies have been observed at the higher viscosity level at 50 °C. It is surprising, that high ratios of SO_3/AGU as well as high concentrations of starch give rise to higher viscosities of the ester. It is possible that the hydrolysis which is accelerated by the higher water content of the starch may be reduced by higher amounts of formamide. It is, therefore, recommended to carry out the reaction at an SO_3/AGU ratio of 4:1 and a starch concentration of 25 % at 70 °C in order to obtain starch esters with higher viscosities.

With respect to the yield of starch sulfate, which is significantly correlated with process variables only at 50 °C, the same tendency is observed as in the dependency of S_E on process variables (see fig.1). The tendency of the yield value to increase with increasing water content and increasing SO_3/AGU ratio, is due to a more complete esterification. Thus, high degrees of esterification coincide

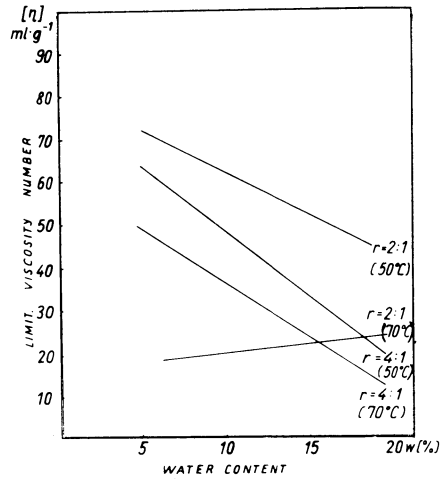

Figure 3. Lines of regression for the influence of the water content (w) *of the starch on limiting viscosity number* $[\eta]$ (r = SO_3/AGU *ratio*)

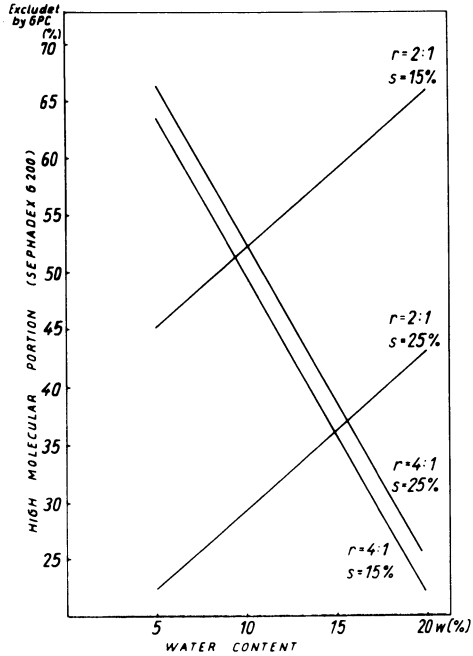

Figure 4. Lines of regression for the influence of the water content (w) *of the starch on the high molecular weight portion as excluded by gel permeation chromatography (SEPHADEX G 200)* (r = SO_3/AGU *ratio*, s = *starch concentration*)

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with high yields.

The amount of formamide necessary for producing 1 g starch sulfate is shown in fig.6 and appears to decrease with increasing starch concentration (s). An increasing water content, preferably with a SO_3/AGU ratio of 2:1, leads to a slight decrease of the formamide amount also.

The amount of chlorosulfonic acid necessary for 1 g ester decreases only slightly with increasing water content (fig.7); The reason probably is the increase of S_E as well as of the yield. Of course, the higher level refers to a SO_3/AGU ratio of 4:1.

The nitrogen content of the esters, the principal contamination, results from formamide bound by the sulfate group. Therefore, the nitrogen content exhibits the same tendencies in dependence from process variables as the degree of esterification, i.e., the higher the degree of esterification, the higher the contamination with formamide. Independent of this, the yield of ammonium sulfate as a byproduct resulting from decomposition of formamide is highest at 70 °C and an SO_3/AGU ratio of 4:1 and is increasing considerably with the water content.

Discussion

The mutual actions of the explanatory variables within the given level of numerical values chosen for this process are summarized in Table 3 with respect to high degrees of esterification, high yield values, low molecular destruction, and low specific amount of raw materials. Among the explanatory variables affecting the process, the temperature has the greatest influence on the effect variables. Therefore high degrees of esterification will result only at the higher temperature level. At this level the reaction proceeds completely in the homogeneous system, the starch being dissolved in the reagent. At the 50 °C level, on the other hand, the reaction takes place in a predominantly heterogeneous system, rendering some starch soluble and reacting to a higher degree and leaving another part of the starch undissolved and unreacted. Consequently these reaction products have a non-uniform distribution of substituents and a wide rang of molecular degradation. The water content of the starch has twofold effect, (a): it results in high degrees of esterification and a high efficiency regarding raw materials, and (b) it leads to a pronounced hydrolytic degradation of molecules. The increase in reactivity is due to a

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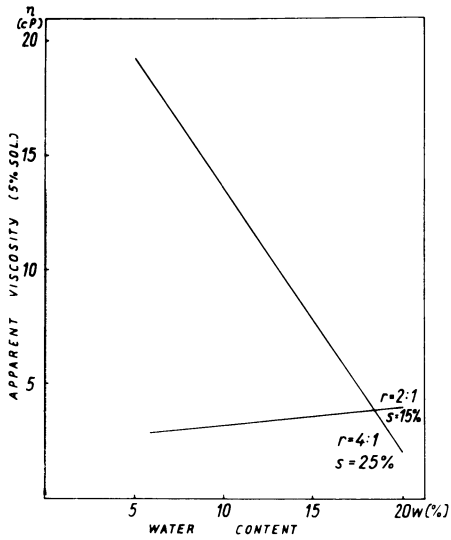

Figure 5. Lines of regression for the influence of the water content of the starch on apparent viscosity (5% sol) (r = SO_3/AGU ratio, s = starch concentration)

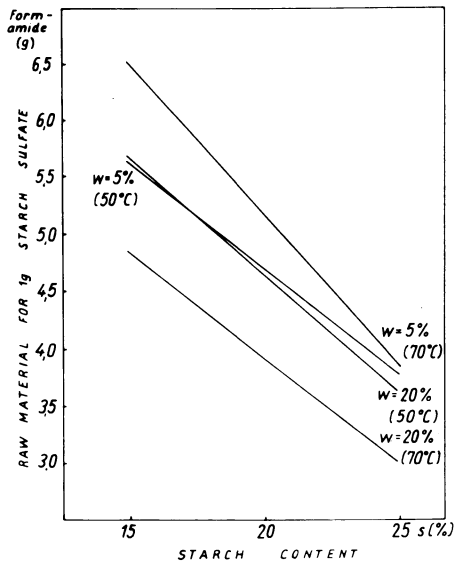

Figure 6. Lines of regression for the influence of the starch concentration on the amount of formamide needed for producing 1 g of starch sulfate (w = water content of the starch)

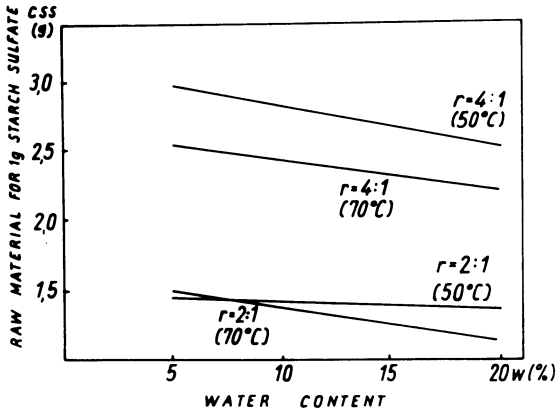

Figure 7. Lines of regression for the influence of the water content of the starch on the amount of chlorosulfonic acid needed for producing 1 g of starch sulfate (r = AGU/SO_3 ratio)

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TAB. 3 EFFECT OF PROCESS VARIABLES ON THE STARCH CHLOROSULFONIC ACID - FORMAMIDE SYSTEM WITH RESPECT TO HIGH DEGREES OF ESTERIFICATION (S_E) AND YIELD VALUES, SMALL MOLECULAR DESTRUCTION (HIGH $[\eta]$ VALUES) AND BY - PRODUCTS (N_G), AND LOW AMOUNTS OF RAW MATERIALS

EXPLANATORY VARIABLES	HIGH S_E	HIGH VIELD VALUE	HIGH $[\eta]$	LOW N_G	LOW AMOUNTS OF FORMAMIDE	LOW AMOUNTS OF CHLOROSULFONIC ACID
WATER CONTENT %	20	20	5	5	20	20
SO_3/AGU RATIO	4 : 1	4 : 1	2 : 1	4 : 1	2 : 1	2 : 1
STARCH CONCENTRATION %	25	-	-	25	-	25
TEMPERATURE °C	70	50	50	50	50,70	50,70

pronounced swelling of the starch that contains relatively large amounts of water, but the amount of water that causes swelling and increases reactivity will also cause an increased hydrolytic activity and result in cleavage of glycosidic bonds. When the sulfur trioxide content, i.e., the SO_3/AGU ratio, that is highly signifiant in all actions observed is increased, higher degrees of esterification, high yields and higher molecular destruction are observed. With respect to the efficiency, it must be kept in mind, however, that the amount of raw materials may be increased also. The concentration of starch in the formamide may be increased up to 30 %, but concentrations of starch between 15 % and 25 % are less significant than other explanatory variables. Increasing amounts of starch lead to a slight decrease in degree of esterification but also reduce the amount of formamide and by-products. As to the reaction time between 15 and 30 min, it has been pointed out already that the insignificant influence on effect variables may be due to the small difference between the temperatures used. It can therefore be stated that the reaction times are long enough for the esterifications at 70 °C, but not for those at 50 °C.

Conclusions

The mutual actions described above lead to some difficulties with respect to the selection of proper conditions for producing starch sulfates with predetermined properties. It must be realized, however, that the linear regression adopted in the study of this process generally allows the predetermination of tendencies qualitively, but does not produce quantitative results. A prediction may be allowable only if the steps in the system are within concrete limits and rather narrow. Within these restrictions, the selection of reaction conditions for the preperation of starch sulfates consists of a compromise between process variables being either positive or negative in their action. As an example for utilizing the tendencies elucidated in this study, Table 4 shows the recommended process variables for producing starch sulfates with a high degree of esterification.

Experimental

General procedure. The chlorosulfonic acid-formamide complex was formed by adding dropwise chloro-

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TAB. 4 PROPOSED PROCESS CONDITIONS FOR PREPARING STARCH SULFATES WITH A HIGH DEGREE OF ESTERIFICATION AS DERIVED FROM INTERPRETATION OF THE EQUATIONS OF REGRESSION

PROCESS CONDITIONS PROPOSED		RESULTING PROPERTIES OF RAW STARCH SULFATE	
WATER CONTENT OF STARCH	12 %	ESTER CONTENT	14 - 18 % S
SO_3/AGU RATIO	1 : 1	CONTAMINATION	5 - 6,5 % N
CONCENTRATION OF STARCH	15 %	[η]	30 ml g^{-1}
TEMPERATURE	70 °C	APPARENT VISCOSITY	2 - 11 cP (5 % SOL.)
TIME OF REACTION	15 MIN	SPECIFIC CONSUMPTION:	
		FORMAMIDE	5,3 g/1g STARCH SULFATE
		CHLOROSULFONIC ACID	2,4 g/1g STARCH SULFATE

sulfonic acid to formamide in a round bottom flask that was cooled to maintain the temperature at below 40 °C. The complex formed was diluted by addition of the amount of formamide required for dissolving the dry starch. Potato starch with a known moisture content was added to the complex solution at 20 °C with vigorous stirring, the reaction vessel being immersed in a temperature controlled bath. The temperature of the bath was raised to the desired value within 30 min and the mixture maintained at this temperature for the desired reaction time and immediately poured with vigorous agitation into excess methanol (1,5 l). The precipitate formed was filtered off by suction, washed with 100 ml methanol, and finally dried by infrared radiation in a stream of cold air.

Reaction variables. The general procedure described above was performed with the following variation of reaction conditions:

1. reaction temperature, 50 °C and 70 °C (α,β)
2. time of reaction at given temperature, 15 and 30 min (t)
3. concentration of dry starch, 15, 20 and 25 % (related to formamide) (s)
4. moisture content of potato starch, 5 and 20 % (w)
5. molar ratio of SO_3 to AGU, 2:1 and 4:1 (r) (AGU anhydro glucose unit)

The special conditions, applied in 48 resulting preparations are summarized in Table 5.

Analytical. The analytical procedure used for characterizing reaction products relate to molecular as well as to functional properties of the crude reaction products. Further purification as may be necessary for practical application of the esters has not been applied. It should be noted, however, that most of the ammonium sulfate, the main by-product, precipitates later than the starch sulfate. Therefore a relatively pure starch sulfate may be obtained by filtering it off immediately after precipitation. The following analytical methods have been applied during the course of the complete program of investigation.

(a) Total sulfur content, S_G %, by total hydrolysis with HCl, precipitation with $BaCl_2$ and gravimetric determination as $BaSO_4$; sulfur, bound in ester groups, S_E %, calculated by $S_E = S_G - S_A$; sulfur in anorganic compounds, S_A %, by complexo-

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TAB. 5 EXPERIMENTAL CONDITIONS FOR ESTERIFICATION OF POTATO STARCH BY MEANS OF THE CHLOROSULFONIC ACID - FORMAMIDE REAGENT

SO_3/AGU MOL. RATIO	CHLOROSULFONIC ACID g	FORMAMIDE g	CONCENTRATION OF STARCH %
2 : 1	43,1	186,7	15
2 : 1	43,1	136,7	20
2 : 1	43,1	106,7	25
4 : 1	86,2	203,4	15
4 : 1	86,2	153,4	20
4 : 1	86,2	123,4	25

SAMPLE AMOUNTS OF STARCH: 31,6 g (5 % H_2O), 37,5 g (20 % H_2O)

metric titration of the aqueous solution with CHELAPLEX.

(b) Limiting viscosity number $[\eta]$, in 0,33 M NaCl.
(c) Total nitrogen content, N_G %, by steam distillation of the alkaline solution and titration according to the KJELDAHL semi-micro method.
(d) Reduction value, calculated as glucose, R_G %, according to Willstaetter - Schudel.
(e) Optical rotation value, $[\alpha]$, of 1 % solutions.
(f) Dynamic viscosity, η (cP), of 5 % solutions in the HÖPPLER Viscosimeter.
(g) Coldwater solubility, derived from the refractive index n of a 10 % dispersion.
(h) pH-value of 10 % solution.
(i) Turbidity of 1 % solution, 8 weeks at 4 °C; cuvettes 1 cm, transmittance at 650 nm.
(j) Reaction with iodine, maximum aborbance λ_{max} with 10^{-3}N iodine.
(k) Gel permeation chromatography, GPC, on Sephadex G 200, measuring the high molecular weight portion (resulting from the excluded volume).

The results for $[\eta]$ and $[\alpha]$ refer to pure starch sulfate and to AGU, respectively and those for R_G to crude and pure starch sulfate and to AGU, respectively. The yield values were calculated with reference to pure starch sulfate and ammonium sulfate.

Evaluations. The above results derived from determinations and calculations lead to 20 effect variables at two levels each "α" at a temperature level of 50 °C and "β" at a temperature level of 70 °C. The calculation of results was carried out by means of statistical methods using orthogonal polynomials for description of correlation between independent variables (process variables) and resulting property variables (effect variables). In first approximation, a linear theory of adjustment between two variables each was chosen. Consequently the resulting equations of regression must be of first order and are correlated with 4 independent variables, time of reaction (t), concentration of starch in formamide (s), concentration of water in starch (w) and molar ratio of SO_3 to AGU (SO_3/AGU) (r). A number of 2^4=16 explanatory variables results for each of the 40 effect variables (y). For each y, the coefficient of determination (B) has been estimated, the B values of 0,80 being neglected since they are considered to be irrelevant in a mathematical sense. The Fisher test has been carried out to check the

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significance between independent and effect variables within the limits $F > 10$, $F > 5$, $F > 3$. $F \leq 3$ indicates that there is no proof for significance between variables. Equations of regression were calculated for the evident correlations between variables y and independent variables x according to

$$y = a + b_1\left[K_o(x_1 - \bar{x}_1)\right] + b_2\left[K_o(x_2 - \bar{x}_2)\right] + \ldots b_n\left[K_o(x_n - \bar{x}_n)\right]$$

(a absolute value, K_o orthogonal factor, b regression coefficient, $\bar{x}$ mean values between levels of the independent variables)

The correlations between process and effect variables (properties) as shown by the equation of regression were plotted and explained under the aspects of interaction between process variables and resulting properties as well as between reaction variables and yield values of the final products.

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Summary

Preparative and analytical studies on a laboratory scale have been carried out concerning the behavior of the starch-chlorosulfonic acid-formamide system. The results have been evaluated with respect to the influence of the explanatory (process) variables on effect variables. The calculation was performed by means of a theory of adjustment using orthogonal polynomials.

The explanatory variables exhibit a significant influence on effect variables in single effects and double and triple interactions. In addition to the temperature, the concentration of water in starch and the SO_3/AGU ratio are essential. Less important is the concentration of starch and the least important the time of reaction within the limit of 15 to 30 min.

High degrees of esterification (DS > 1) and yields are obtained by increasing amounts of water and at a high SO_3/AGU ratio, simultaneously leading to a higher extent of molecular destruction and formation of contaminants and by-products.

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Sulfonic Acid and Sulfomethyl-Containing Graft Co-Polymers of Xanthan Gum

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Xanthan gum is a high molecular weight polysaccharide produced in a pure culture fermentation process by the microorganism Xanthomonas campestris [1]. The structure of the polysaccharide is shown in Figure 1. As detailed in this figure, each repeating unit contains five sugar units consisting of two glucose units, two mannose units, and one glucuronic acid unit. The main chain of xanthan gum is built up of β-D-glucose units linked through the 1- and 4-positions; i.e., the chemical structure of the main chain is identical to the chemical structure of cellulose. The side chain comprises the two mannose units and the glucuronic acid unit. The terminal β-D-mannose unit is linked glycosidically to the 4-position of β-D-glucuronic acid, which in turn is linked glycosidically to the 2-position of α-D-mannose. This three-sugar side chain is linked to the 3-position of every other glucose residue in the main chain. Also, about half of the terminal D-mannose residues carry a pyruvic acid residue ketalically linked to the 4- and 6-positions. The nonterminal D-mannose unit contains an acetyl group at the 6-position [2].

Xanthan gum was commercialized by Kelco Company, now Kelco Division of Merck & Co., Inc., in 1964 and has found widespread utility in food and industrial applications because of its unique properties.

Attempts have been made to alter the properties of xanthan gum by chemical derivatization and modification. These derivatives and modifications include deacetylated xanthan gum [3], carboxymethyl ethers [4], cationic derivatives [5,6], propylene glycol esters [7], hydroxyalkyl ethers [8], sulfates [9], and graft co-polymers [10]. However, with the exception of deacetylated xanthan gum, none of these derivatives have been commercialized to date.

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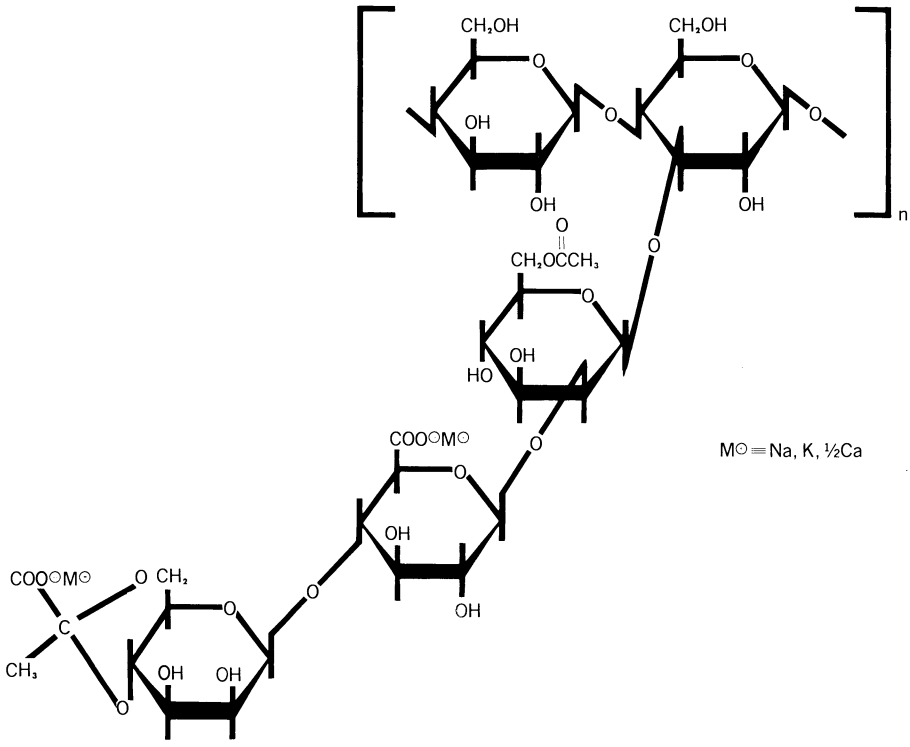

Figure 1. Structure of xanthan gum

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This paper describes the preparation of graft copolymers of xanthan gum containing sulfonic acid groups attached by either graft copolymerizing acrylamide and 2-acrylamido-2-methylpropane sulfonic acid (AMPSA) onto xanthan gum, or by sulfomethylating xanthan gum-polyacrylamide. These reactions are shown schematically in Figure 2.

Results and Discussion

The sulfonic acid and sulfomethyl-containing graft copolymer was prepared by grafting acrylamide (AM) and 2-acrylamido-2-methylpropane sulfonic acid (AMPSA) onto xanthan gum in an aqueous medium with a ceric salt present (Figure 2). The ratio of acrylamide to AMPSA was adjusted to give graft polymers with varying degrees of anionic character. Also, the ratio of total monomer to xanthan gum was varied to give polymers with a range of viscosities (Table I). Preliminary screening of the product polymer from example 5 in Table I (Polymer I) has revealed that it is pseudoplastic, is stable between pH 4∿12, and exhibits a synergistic viscosity increase with guar gum. Effect of pH and temperature on viscosity and the shear-stress/shear-rate relationship are shown in Figures 3, 4, and 5, respectively. These polymers also possess good salt stability, which makes them suitable for oil field uses, such as flooding and drilling. In addition, they can be used in other applications where thickened water is desirable, such as textile printing paste thickening, slurry explosive formulations, and coating technology.

The aforementioned polymer was also prepared by introducing sulfonic acid groups into xanthan gum by combining graft polymerization and chemical modification techniques. First, polyacrylamide was grafted onto xanthan gum at 1:1 ratio by weight, then the amide function was sulfomethylated with formaldehyde and sodium metabisulfite (Figure 2) [11]. Preliminary screening of this anionic polymer (Polymer II) has shown that it is pseudoplastic (Figure 5), has good high-temperature stability (Figure 4), and is stable between pH 6 and 11 (Figure 3). The polymer also exhibits a synergistic viscosity effect with guar gum. Possible applications of this polymer are use as a dispersant and stabilizer in slurries; in petroleum recovery (drilling and flooding), as an anti-static film former; and possibly in textiles.

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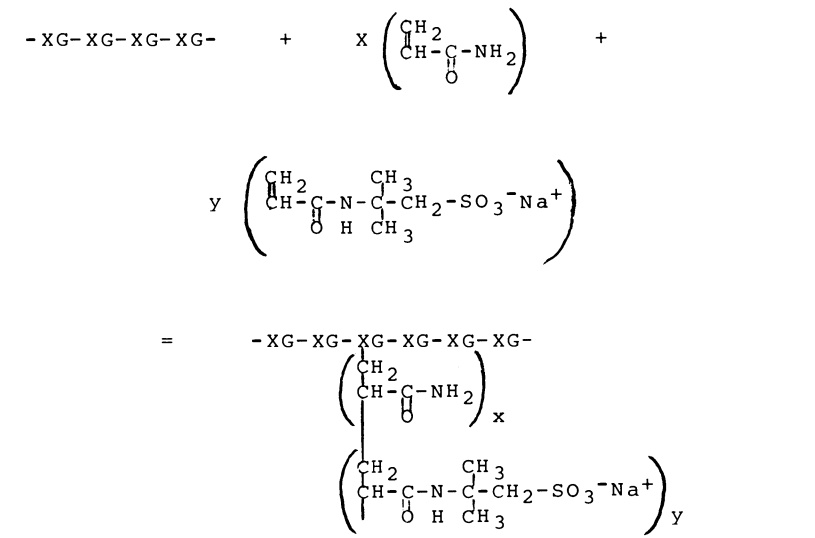
1) XANTHAN GUM-POLY(ACRYLAMIDE-CO-AMPSA)



2) SULFOMETHYLATED ZN:AM (1:1) GRAFT COPOLYMER

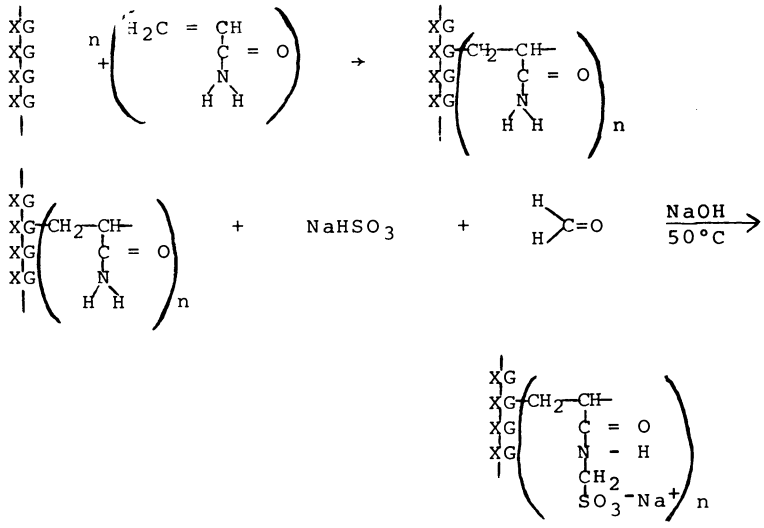


Figure 2. Polymerization reaction scheme

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TABLE I

Results of Graft Polymerization of AMPSA and Acrylamide onto Xanthan Gum

Example	Acrylic Monomer Weight (gm)	Weight (gm) 2-acrylamido-2-methyl-propane Sulfonic Acid	Weight (gm) copolymer	Content (%) Monomer	Content (%) 2-acrylamido-2-methylpropane sulfonic acid	Content (%) Xanthan gum	Viscosity of Copolymer (distilled water)	Viscosity of Copolymer (1% KCl solution)
1	4.6	1.7	19.1	25	9	66	520	480
2	4.2	2.1	18.9	22	11	66	520	480
3	2.1	4.2	19.2	11	22	66	500	480
4	10.6	1.9	26.4	42.5	7.5	50	350	340
5	9.3	3.1	25.7	37.5	12.5	50	350	340
6	8.4	4.2	26.2	33.3	16.7	50	250	200
7	6.2	6.2	27.1	25	25	50	250	250
8	4.2	8.4	28.1	16.7	33.3	50	250	230
9	3.1	9.3	26.5	12.5	37.5	50	300	300
10	0	12.5	23.4	0	50	50	300	290

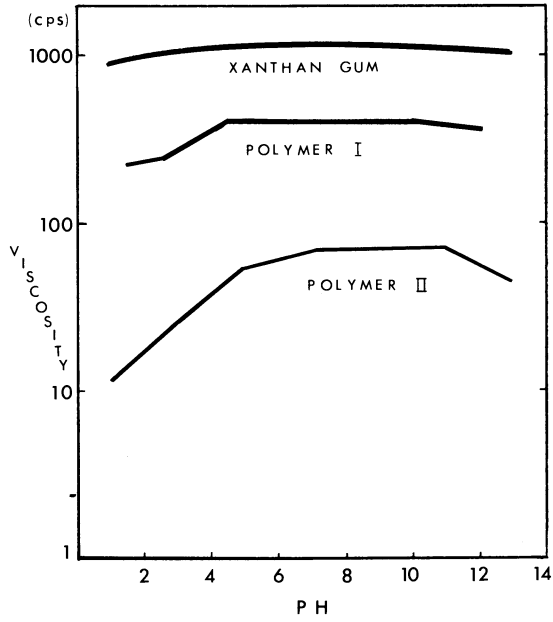

Figure 3. Viscosity vs. pH for xanthan gum and both polymers

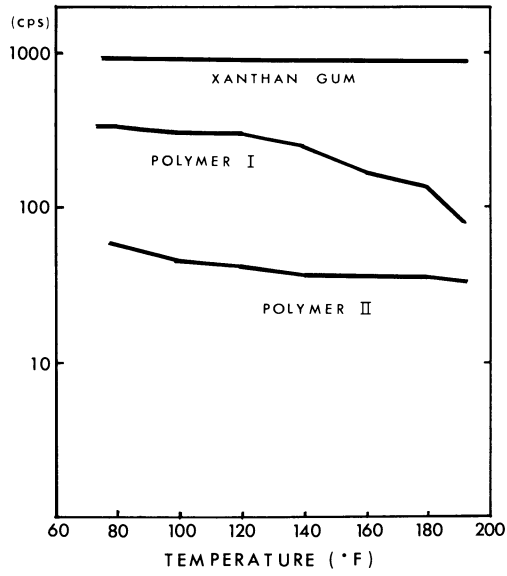

Figure 4. Viscosity vs. temperature for xanthan gum and both polymers

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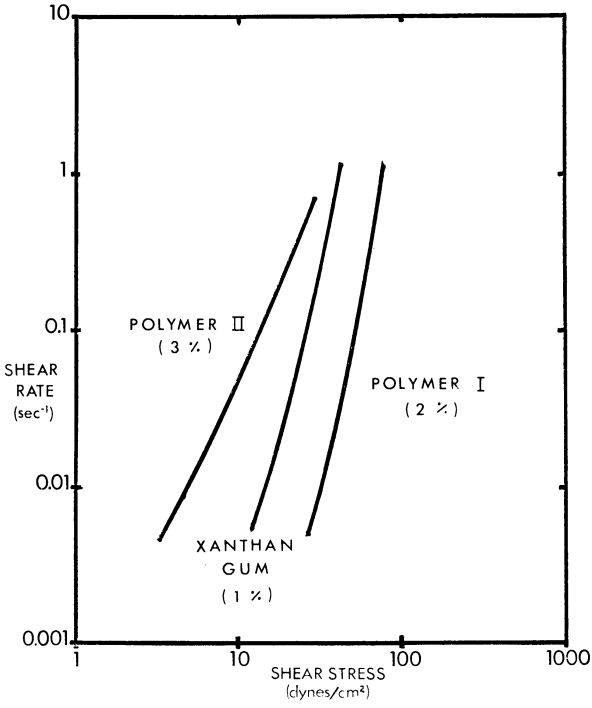

Figure 5. Low shear rate rheological properties of xanthan gum and both polymers. (These data were obtained using the Wells–Micro Brookfield Viscometer and the relaxation technique of Patton (12).)

Experimental

Xanthan Gum-Poly(acrylamide-co-AMPSA)

The samples were prepared in a 1000 ml reaction vessel equipped with a nitrogen inlet tube, a thermometer, and an addition funnel. For the first three samples (see Table 1), 500 ml of a fermentation beer and 6.3 g of total monomer (acrylic plus 2-acrylamido-2-methylpropane sulfonic acid) were combined. Examples 4-9 used 500 ml fermentation beer and 12.5 g total monomer. Example 10 combined 12.5 g of 2-acrylamido-2-methylpropane sulfonic acid and 500 ml of fermentation beer. The monomer was dissolved in 50 ml water. The fermentation beer contained 2.50 weight percent of Xanthomonas campestris colloid, as determined by isopropyl alcohol precipitation of colloid from a sample of the fermentation beer. The reaction mixture was then heated to 75°C, while the reaction vessel was purged with nitrogen gas for one hour. Thereafter, the reaction vessel was cooled to room temperature and 20 ml of a catalyst solution diluted with 50 ml of water was added over a period of 15 minutes while the contents were stirred. The catalyst solution was composed of 0.1 N ceric ammonium nitrate dissolved in 1 N nitric acid. This solution was made by dissolving 54.8 g of ceric ammonium nitrate in 1 N nitric acid and diluting to make 1 liter of solution. Following the addition of the catalyst, stirring of the reaction mixture was continued for two hours at a reaction temperature of 20 to 25°C, after which 2 g of p-methoxyphenol dissolved in 50 ml of isopropyl alcohol were added. The product was precipitated by the addition of excess isopropyl alcohol and was then collected and dried at 45-50°C for two hours.

The final weights of the products are shown in Table I. Viscosity of the copolymers was determined at 1% by weight solution in distilled water, using a Brookfield LVF viscometer at 60 rpm, No. 2 spindle. For purposes of comparison, Xanthomonas campestris colloid, which was obtained directly from the fermentation beer, had a viscosity at a concentration of 1% by weight in distilled water of 840 cps.

Sulfomethylated ZN:AM (1:1) Graft Copolymer - Polymer II

A graft copolymer is prepared by reacting equal parts by weight of xanthan gum and acrylamide according to the procedure given in Example 1 of U. S. patent

3,708,446. The resulting copolymer (1.44 g) is dissolved in 100 ml distilled water in a three neck, round bottom flask (250 ml) fitted with a thermometer, stirrer, and reflux condenser. To this solution is added 0.95 g $Na_2S_2O_5$ (0.005 mole), 0.80 g 50% NaOH (0.01 mole), and 0.81 g 37% CH_2O (0.01 mole). The solution is heated to 50°C and stirred at this temperature for 3 hours. At the end of this period, the mixture is cooled to room temperature (∿22°C), and the reaction product is recovered by addition of the solution to 2 volumes of isopropanol. The product is dried in vacuo at 40°C and milled through a 60 mesh (U.S. standard) screen. The results of this experiment are shown in Table II.

Table II. The Results of Sulfomethylation of Xanthan Gum-Acrylamide Graft Copolymer.

Yield (g)	2.13	
Theoretical yield (g)	2.58	
Yield (%)	82.60	
Amide Groups Reacted (%)	60.53	
Viscosity (cps), 1%	90	
KCl Viscosity (cps), 1%	87	Brookfield LVF Viscometer, 60 rpm

Abstract: Two novel graft copolymers of xanthan gum have been prepared by graft copolymerizing 2-acrylamido-2-methylpropane sulfonic acid and acrylamide onto xanthan gum and by graft polymerizing acrylamide onto xanthan gum followed by sulfomethylation. The preparation and properties of these two polymers are discussed.

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Sulfated Polysaccharides Metabolized by the Marine *Chlorophyceae*—A Review

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During the past twenty years my colleagues and I have investigated the polysaccharides of nine different genera of green seaweeds. In every case we have been able to separate at least one water-soluble polydisperse heteropolysaccharide substituted by half ester sulfate groups. That means not only that each seaweed metabolises molecules containing more than a single sugar but that each contains a mixture of molecules all built up on the same general plan but which differ in the fine details of structure. I should emphasise that the results are only the average of all the molecules present. Each weed presented its own particular problems, but certain groups of polysaccharide have emerged. From the Cladophorales and Codiales a sulfated xylogalactoarabinan, and from the Ulvales, Acrosiphoniales and Ulotrichales a glucuronoxylorhamnan, sometimes containing a small proportion of glucose, have been separated. The polysaccharides in each group appear to be built up on the same general plan, but differ in the proportions of the sugars present, and in the fine details of structure.

The polysaccharide from the unicellular green alga, Acetabularia, a member of the Dasycladales, resembles the latter group in containing rhamnose, xylose and glucuronic acid, but its major sugar is galactose and its 4-O-methyl derivative (1).

General Structural Studies

Details of polysaccharides from the first group of seaweeds are given in Table I. The proportions of sugars present in Cladophora and Chaetomorpha polysaccharides differ in the smaller proportion of galactose present in the latter, and both genera are devoid of 3,6-anhydrogalactose. Caulerpa polysaccharide appears to be the odd man out in that it contains a considerable proportion of mannose residues which defied fractionation, and this is true for two other species of Caulerpa we have examined. Nevertheless all four genera contain approximately the same proportion

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Table I. Water-soluble Polysaccharides from

Constituent Sugars	Cladophora rupestris	Chaetomorpha capillaris	Codium fragile	Caulerpa filiformis
L-Arabinose	xxxxx	xxxxx	xxxx	xx
D-Galactose	xxxxx	xxx	xxxxx	xxxxxx
D-Xylose	xx	xx	xx	xxx
D-Mannose	-	-	- -	xxx
3,6-Anhydrogalactose	-	-	+	+
Sulfate %	16	15	17	17.5
$[\alpha]_D$	ca.+53°	+70°	+46°	+13°

Table II. Water-Soluble Polysaccharides

Algal Species	Time Place	Approx. propor. Sugars	$AgNO_3$ on Papers	GLC Ratio
Cladophora rupestris	various	Gal	+++	1.0
		Ara	+++	1.0
		Xyl	+	0.4
Cladophora sericea*	Scotland	Gal	++	1.0
	Aug. 1969	Ara	++++	1.27
	April 1970	Xyl	++	0.80
Cladophora laetevirens	Scotland	Gal	++	1.0
	Sept.1969	Ara	+++++	1.8
	April 1970	Xyl	++ ++	0.9
Rhizoclonium riparium	Scotland	Gal	++++	
	1969	Ara	++	-
		Xyl	++	
Chaetomorpha melagonium	Scotland	Gal	++	1.0
	1969	Ara	+++	1.2
		Xyl	+	0.45
Chaetomorpha linum	Scotland	Gal	++	
	1969	Ara	+++	-
	1970	Xyl	+	

Key: Gal = galactose; Ara = arabinose; Xyl = xylose

*May have been contaminated with C. laetevirens.

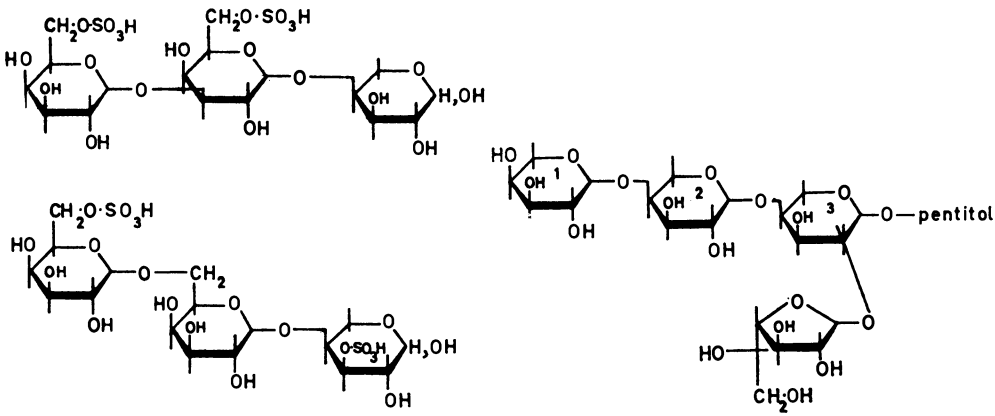

Figure 1. Structural features in Cladophora rupestris *(left); after reduction and desulfation (right)*

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Table IV. Water-Soluble Polysaccharides from

Constituent Sugars	Acrosiphonia centralis	Enteromorpha compressa	Ulva lactuca	Urospora penicilliformis	wormskioldii
L-Rhamnose	xxx	xxxxx	xxxxx	xxx	xxxx
D-Xylose	xxx	xxx	xx	xxxx	xxx
D-Glucose	xx*	x	x	-	-
D-Glucuronic Acid %	19	18	21	17	16
Sulphate %	7.8	16	17.5	17-22	11
$[\alpha]_D$	-31°*	-87°	-74°	-90°	-47°

*probably contaminated with starch-type polysaccharide $[\alpha]_D$ + 150°.

of half ester sulfate and all have a positive specific rotation.

Examination of the constituents of a number of different seaweeds belonging to the Cladophorales shows (Table II) a wide variation in the proportions of the sugars present (6).

Table III gives the linkages present in Cladophora rupestris polysaccharide and Figure 1 some of the structural features

Table III. Linkages present in Cladophora rupestris

1,3- and 1,6-linked galactose; 1,4-linked xylose;
1,4- and triply linked arabinose;

that have been determined (7) for this polysaccharide. Although tentative evidence of similar linkages in other genera of this group have been obtained these have not been firmly established.

In contrast to the arabinans the glucuronoxylorhamnans all have negative specific rotations (Table IV). Furthermore, the proportions of the sugars in the different species are very similar especially, if it is remembered that these figures are only approximate and are the average of all the molecules present. They are determined on hydrolysates of the polysaccharides, and the aldobiouronic acid, 4-O-β-D-glucuronosyl-L-rhamnose, is invariably present and not included in these proportions. While this prevents accurate determination of the proportions of the constituents its occurrence in the hydrolysates from all the species examined is evidence of the overall similarity of their polysaccharides. A striking difference is the lower proportion of sulfate in Acrosiphonia and Urospora wormskioldii. All the sugars in the polysaccharides in this group are linked in the same way (Table V).

Table V. Linkages Present in Glucuronoxylorhamnans

1,3- and 1,4-linked and end group rhamnose; 1,3- and 1,4-linked xylose, 1,3-linked glucose (when present); 1,4-linked glucuronic acid

However, the proportions of the different types of linkages appear to vary in the different species.

Structural features found in the polysaccharide from Ulva lactuca (13) are:

GA(1→4)R; GA(1→3)Xy; GA(1→4)Xy; G(1→3)Xy
2
S

GA(1→4)R(1→3)GA(1→3)Xy; R(1→4)Xy(1→3)G/GA.

GA = D-glucopyranuronic acid; R = L-rhamnopyranose; Xy = D-xylopyranose; G = D-glucopyranose; S = S-O- (O, O)

The Site of Sulphate Groups

These polysaccharides exist as mucilages in the plant and the presence of sulfate groups must play a vital role in their conformation and physical properties. It is important, therefore, to determine the site of these groups. This has been achieved in a variety of ways, depending upon the particular polysaccharide.

1. Cleavage by Alkali. Alkali will cleave sulfate groups from a polysaccharide if there is a free adjacent hydroxyl group trans to the sulfate, this occurs via an epoxide ring and Walden inversion of the hydroxyl carrying the sulfate group. Cleavage of the epoxide ring can occur in two ways and two sugars usually result. Cleavage also occurs with sulfate on C-6 of the hexose provided the hydroxyl on C-3 is free. In this event removal of the sulfate results in the formation of the 3,6-anhydride of the particular hexose.

In both the Ulvales and the Cladophorales this method of removal of sulfate has been particularly useful. The sulfate content in the rhamnan from U. lactuca was reduced from 14.1 to 12.5% by the action of alkali, and the hydrolysate of the recovered polysaccharide was found to contain arabinose (14). This could only have arisen from sulfated xylose (Figure 2). It could have come from xylose 2- or 3-sulfate. However, treatment of the polysaccharide with sodium methoxide gave 3-O-methylarabinose which could only have been derived from xylose 2-sulfate. Similarly the treatment of the xylogalactoarabinan from Cladophora rupestris with sodium methoxide gave mainly 2-O-methyl-L-xylose and little 3-O-methyl-L-arabinose (3) indicating that the sulfate was initially linked to C-3 of arabinose. Partial hydrolysis studies confirmed this result.

The removal of sulfate with alkali and the formation of 3,6-anhydrogalactose was demonstrated in the sulfated polysaccharide from Codium fragile (4). The sulfate decreased from approximately 13% to 9% and the 3,6-anhydrogalactose content increased from 1% to 3% on this treatment (Figure 3).

2. The Site of Sulfate from Periodate Oxidation Studies. Treatment of the rhamnan from U. lactuca with dry methanolic hydrogen chloride reduced the sulfate to 5% and periodate oxidation of the initial and the desulfated polysaccharide at 2° in buffered solution stopped after 70 hours. It was found that the desulfated polysaccharide had reduced twice as much periodate as the initial polysaccharide (14). Parallel oxidation experiments were carried out at room temperature. Again, the desulfated polysaccharide reduced approximately twice as much periodate as the initial polysaccharide. From this it can be deduced that removal of sulfate groups led to the formation of additional α-glycol groups. It is known that, in buffered solution at low temperature, vicinal cis hydroxyl groups in sugars are selectively oxidised by periodate. We were able, therefore, to conclude that desulfation furnished cis rather than trans glycol groupings. The only sugar present in

the polysaccharide in which cis-glycol groupings occur is L-rhamnose at position 2 and 3. It may be presumed therefore that the sulfate groups are linked either to C-2 or C-3 of rhamnose. Infrared analysis of the polysaccharide gave a strong peak at 850 cm^{-1}, characteristic of axial sulfate in galactose or glucose(15). If this is applicable to L-rhamnose, the majority of the sulfate in this polysaccharide is linked to C-2 of the L-rhamnose in its most stable 1C conformation.

In similar periodate oxidation experiments on Enteromorpha compressa polysaccharide, the relative proportions of the uncleaved sugars in the initial polysaccharide and in the desulfated polysaccharide after oxidation are given in Table VI.

Table VI. Enteromorpha Sulfated Polysaccharide

	Percentage Uncleaved:		
	Glucose	Xylose	Rhamnose
Initial Polysaccharide	26	16	58
Desulfated Polysaccharide	40	16	44

The amount of rhamnose and, to a lesser extent, of xylose was reduced in the desulfated polysaccharide. From these results it was calculated that about 22% of the rhamnose in the initial polysaccharide was cleaved by periodate and about 40% in the desulfated polysaccharide, that is an increase of 18% of free hydroxyl groups in the rhamnose residues proving that a high proportion of the sulfate groups are attached to this sugar residue. Infrared analysis of the initial polysaccharide gave a peak at 850 cm^{-1} which in galactose indicates axial sulfate. Again, if this is applicable to L-rhamnose which is in its stable 1C conformation then position 2 has an axial hydroxyl, and this would therefore, as in the Ulva polysaccharide, be the site of sulfate.

3. The Site of Sulfate from Methylation Results. Methylation before and after desulfation and comparison of the two sets of methylated sugars in the respective hydrolysates has not proved a very satisfactory method for determining the site of the ester sulfate in these polysaccharides. Their high sulfate content makes complete methylation impossible and so the effect of the removal of the sulfate groups cannot be properly assessed. However, with the glucuronoxylorhamnan from Urospora penicilliformis it was found (11) that the hydrolysate from the methylated desulfated polysaccharide contained considerably more 2,4-di-O-methylrhamnose than the hydrolysate from the initial methylated polysaccharide while the proportion of both the monomethyl and free rhamnose in the latter were reduced to trace amounts. From these results it was possible to deduce that the 1,3-linked rhamnose was monosulfated at C-4 and also disulfated at C-2 and C-4. The

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proportions of the different methylxyloses and glucuronic acids were the same in the two hydrolysates indicating that both of these residues are essentially devoid of sulfate.

From the Codiolum, the unicellular stage in the life history of Urospora wormskioldii, a sulfated mannan in addition to the glucuronoxylorhamnan, was separated (12). This on methylation and hydrolysis gave 2,3,4,6-tetra-, 2,4,6-tri, 3,6- and 2,3-di-O-methylmannoses in the relative proportions of 1:13:1.5:1. This mannan is, therefore, 1,4-linked, sulfated and/or branched at C-2 and C-6 and has an average chain length of 16. After desulfation with 0.08 M-methanolic hydrogen chloride, the sulfate content decreased from 6.5 to 0.6%. The desulfated material was methylated and hydrolysed and the above methylmannoses were obtained in the approximate relative proportions of 1:13:0.2:1. The fact that the 3,6-di-O-methylmannose had almost disappeared in the desulfated material and the quantity of 2,3-di-O-methylmannose remained virtually unchanged provided evidence that the mannan is sulfated on C-2 and branched at C-6.

4. The Site of Sulfate from Partial Hydrolysis. This has also been useful for the determination of the site of the sulfate groups in the Chlorophyceaean polysaccharides. For example, hydrolysis of the Codium polysaccharide with N-sulphuric acid for one hour at 100° led to the separation of galactose 4- and 6-monosulfates (4). These were characterised as follows: Each had a DP of 1. On hydrolysis they both gave only galactose. The molar ratios of ester sulfate to galactose were 1.15:1.0 for the 4-sulfate and 1.04:1.0 for the 6-sulfate. Methylation gave respectively 2,3,6- and 2,3,4-tri-O-methylgalactoses providing unequivocal proof of the site of ester sulfate groups as galactose 4- and galactose 6-sulfate.

Similar hydrolysis of the xylogalactoarabinan from Cladophora rupestris (3) led to the separation of arabinose 3-sulfate and galactose 6-sulfate. The latter was characterised in the same way as this derivative from Codium. The former after methylation gave 2,4- and 2,5-di-O-methylarabinoses, showing that C-3 involved in linkage to sulfate, thus confirming the results arrived at by alkali treatment of this polysaccharide.

Biological Implications

Unlike the galactans, none of these polysaccharides, after extraction, gives gels in aqueous solution, the most that can be said is that in some cases the solutions are somewhat viscous. The late Dr. Haug (16) reported that an aqueous solution of Ulva polysaccharide gave a strong gel if it was treated with borate and calcium ions at a concentration and a pH found in seawater. He experimented with a wide variety of metallic ions found in the sea, but this was the only combination with which Ulva polysaccharide gave a gel. He postulated that the C-2 and C-3 in the 1,4-linked rhamnose units complex with borate and that the Ca ions

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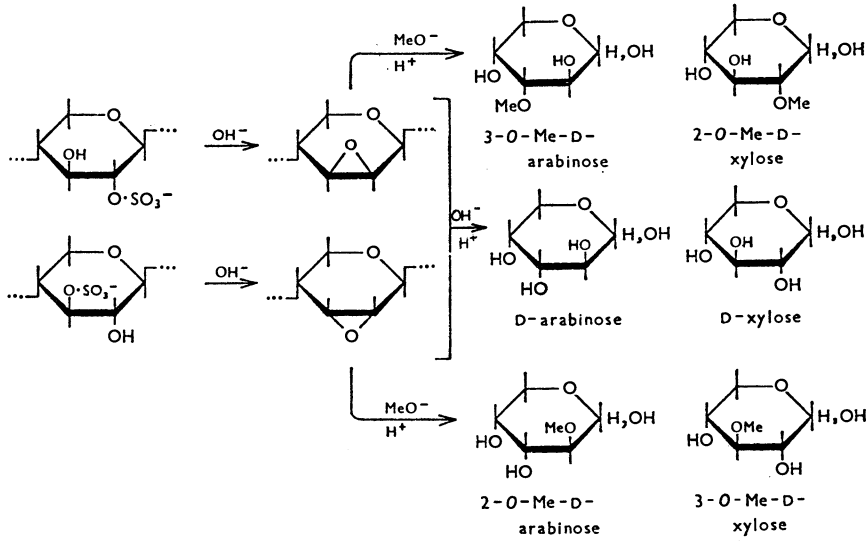

Figure 2.

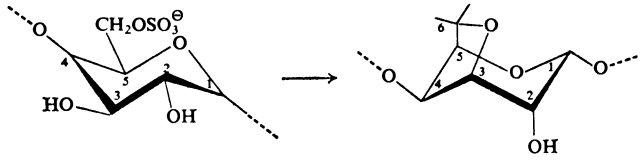

Figure 3. Galactose 6-sulfate (left) and 3,6-anhydrogalactose (right)

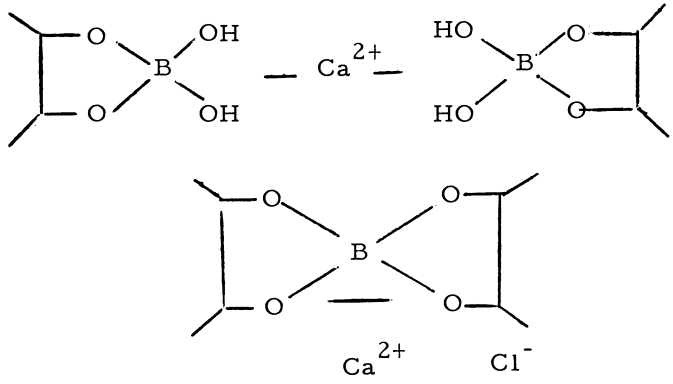

Figure 4.

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form bridges or act as stabilising influences (Figure 4). When C-2 of these rhamnose units are sulfated complex formation with borate is prevented, and it may be that this is the way in which Ulva lactuca regulates the extent of complexing with borate and hence, the stiffness of the polysaccharide gel.

Carrying out similar studies on the Cladophora rupestris polysaccharide, it was found that calcium ions alone produced a strong gel at the pH of seawater (17).

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Sulfated Polysaccharides of the *Rhodophyceae* – A Review

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General Structural Features

The major polysaccharides of the Rhodophyceae are galactans which carry varying proportions of half ester sulfate linked to one or more of the free hydroxyl groups of the galactose residues. They are readily extracted from the seaweeds by hot water, and research has shown that they all appear to consist of chains of alternating units of 1,3-linked β-galactose and 1,4-linked α-galactose, that is alternating A and B units (Figure 1). However, some of the units may be masked by modification or by substitution. Extracts from different seaweeds vary in the amount of D- and of L-galactose, in the extent to which these residues are modified to the 3,6-anhydrosugar, by the extent and position of half ester sulfate and methoxyl groups and by substitution with pyruvic acid (Figure 2). It is clear that there are a large number of ways in which the extracts from the different weeds differ from one another. It is these differences in fine structure which determine the conformation or shape of the molecules and, hence, the physical properties of the various extracts.

The members of the Rhodophyceae can be divided into those genera which synthesise agar-type molecules, that is alternating 1,3-linked D-galactose residues and 1,4-linked L-residues, others that metabolise carrageenan-type molecules in which all the units are D-galactose and galactans which show affinities to both agar and carrageenan (Table I).

Table I. Seaweeds Metabolising Polysaccharides of:

Agar-type	Carrageenan-type	Mixed-type
Gelidium	Chondrus	Porphyra
Gracilaria	Gigartina	Laurencia
Phyllophora	Eucheuma	Bangia
Pterocladia	Furcellaria	Gloiopeltis

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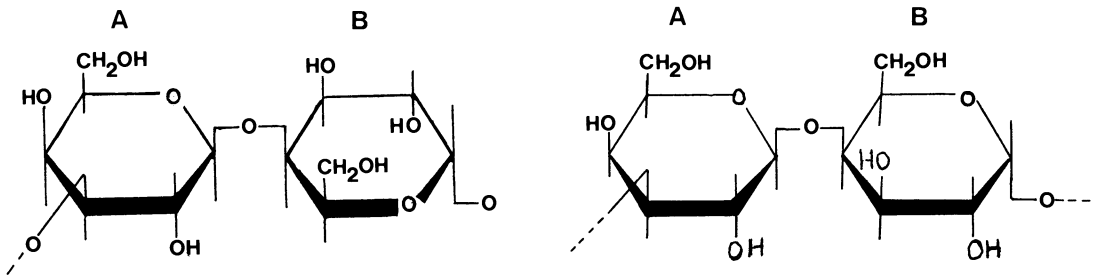

Figure 1. L-*Galactose (left) and* D-*galactose (right)*

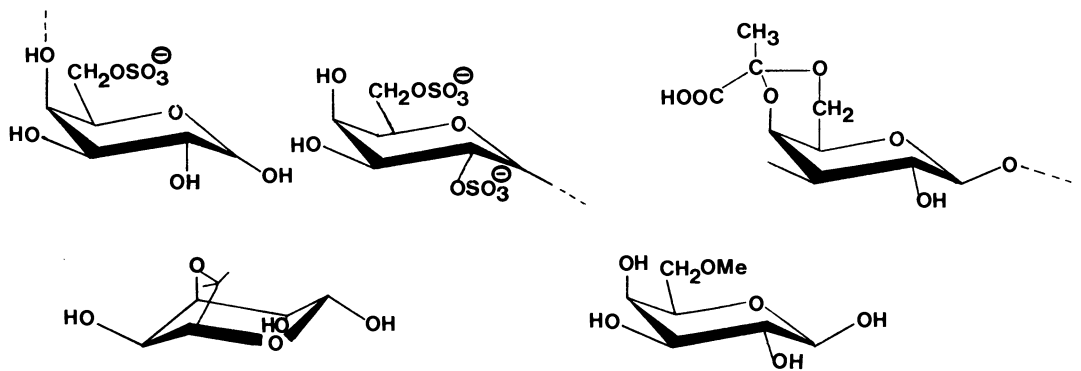

Figure 2. (left to right, top to bottom) D-*Galactose 6-sulfate,* D-*galactose 2,6-disulfate, galactose 4,6-0-1-carboxylethylidene,* α*-1,4-linked 3,6-anhydro-*L*-galactose, and 6-0-methyl-*D*-galactose*

In every case, the polysaccharides contain a family of molecules which differ in the fine details of structure detailed above. It has been possible to separate the extremes of the structures in some instances. The separation of agarose (Figure 3), a non-sulfated gelling fraction from agar, where the chains consist of alternating 1,3-linked D-galactose residues and 1,4-linked 3,6-anhydro-L-galactose, leaving agaropectin, a mixture of variously sulfated molecules, behind is one example. The precipitation by potassium ions of kappa-carrageenan (Figure 4) where the 1,3-linked units carry sulfate groups at C-4, also a separation of a gelling fraction from a whole extract of Chondrus crispus or Gigartina species is another example of this fractionation. Again, in carrageenan the material left in solution, the so-called "lambda"-carrageenan, a non-gelling fraction, is a mixture of variously substituted molecules with a low 3,6-anhydro content. In recent further fractionation work, Dr. Rees (1) suggests that the term lambda-carrageenan should be restricted to one fraction of this material (Table II).

Methods for the Determination of the Site of Sulfate Groups

It is important to know the position of the sulfate groups on the individual galactose residues and this has been determined by (1) removal of the sulfate by alkali, (2) methylation of the polysaccharide, (3) partial acid hydrolysis of the polysaccharide, and (4) infrared spectroscopy.

1. Action of alkali. - Early studies on model sulfated monosaccharides revealed that sulfate could be removed on treatment with alkali if there was an adjacent trans free hydroxyl group on the sugar moiety (2), then cleavage occurred with Walden inversion. If there is no free trans hydroxyl groups, then the sulfate is stable to alkali. At the same time, any sulfate linked to C-6 of the sugar residue will be cleaved by alkali if the hydroxyl group on C-3 is free. Walden inversion does not occur in these circumstances, but a 3,6-anhydro-ring is formed (Figure 5). This later state of affairs occurs frequently in these galactans. This was of commercial importance during the last war when the supply of agar from Japan was cut off, and carrageenan, which is isolated from Chondrus crispus and Gigartina species, was the source in Canada and Great Britain of material to replace agar. Carrageenan has a lower gelling strength than agar, but it was found that treatment with alkali increased the gel strength. Research later showed that the reaction just outlined had taken place, and it has since been shown that all polysaccharides with a high 3,6-anhydrogalactose content give strong gels. It is common practice in Japan at the present time to increase the gel strength of extracts of Gracilaria species, a somewhat low gel strength agar, in this way. In the plant an enzyme brings about this change so that the seaweed is able to regulate the proportion of galactose 6-sulfate and 3,6-anhydro-

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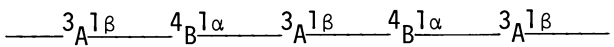
Table II. Repeating Units of Carrageenans



A UNITS	B UNITS	NAME
D-Galactose 4-sulphate	D-Galactose 6-sulphate	mu
	D-Galactose 2,6-disulphate	nu
	3,6-Anhydro-D-galactose	kappa
	3,6-Anhydro-D-galactose 2-sulphate	iota
D-Galactose 2-sulphate	D-Galactose 2-sulphate	xi
	D-Galactose 2,6-disulphate	lambda
	3,6-Anhydro-D-galactose 2-sulphate	theta

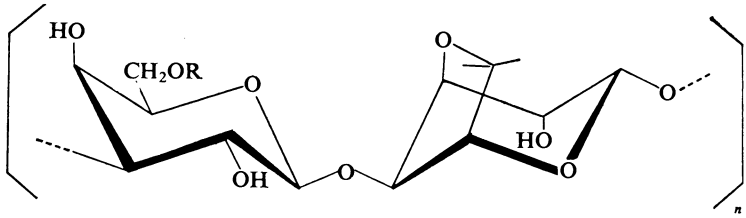


Figure 3. Agarose R=H or OMe

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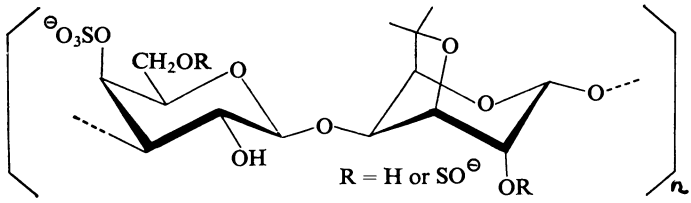

Figure 4. κ-Carrageenan

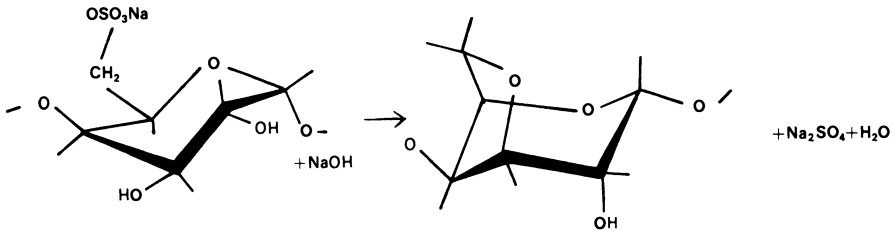

Figure 5.

galactose in any polysaccharide.

2. Methylation. - Methylation has also been used to determine the position of sulfate groups. As long ago as 1947, E. G. V. Percival and his colleague (3) found that 2,6-di-O-methylgalactose was the major methylated sugar in the hydrolysate after methylation of carrageenan from Gigartina stellata. This indicated that C-3 and C-4 were involved in linkage either to other residues or to sulfate. These authors had previously shown that the sulfate was stable to alkali and, therefore, it was not linked to C-3, and they were able to deduce that the galactose residues were mainly 1,3-linked with sulfate on C-4. Confirmation of this conclusion was obtained in 1966 (4) by the isolation and characterisation of O-α-3,6-anhydrogalactopyranosyl (1-3) galactose 4-sulfate from a partial enzymic hydrolysate.

Dolan and Rees (5) used methylation studies in a different way to provide evidence of the position of sulfate groups in "lambda"-carrageenans. These authors found that four treatments with 1.5%-methanolic hydrogen chloride at 35° for 48 hours reduced the sulfate content from 31 to 2%, and they recovered the desulfated polysaccharide in 60% yield. Methylation of the original polysaccharide and the desulfated material and comparison of the methyl sugars in the hydrolysates of the two products allowed assignment of the sulfate groups. From these results, it was followed that 45% of the 1,4-linked residues are sulfated at C-2 and C-6 and a high proportion of the 1,3-linked residues also carry sulfate at C-2 and, in contrast to kappa-carrageenan, only a small proportion of these residues are sulfated at C-4.

3. Partial Acid Hydrolysis. - Acid hydrolysis of these polysaccharides rarely gives any sulfated fragments, the glycosidic links and the sulfate groups having similar lability. Painter (6), however, found that autohydrolysis of the free acid form a variety of carrageenans in a dialysis sac surrounded by distilled water containing a suspension of barium carbonate gave a mixture of galactose and galactose monosulfates. From this mixture he was able to separate and characterise galactose 2-, 4-, and 6-monosulfates, again confirming the deductions from methylation.

4. Infrared Spectroscopy. - This has proved a useful diagnostic tool in the allocation of the site of sulfate groups. Study on model galactose sulfates (7) has shown that peaks at 820 cm^{-1} are characteristic of sulfate at a primary alcoholic group, that is at C-6; at 830 cm^{-1} of equatorial sulfate, that is at C-2 and at 850 of axial sulfate, that is at C-4.

Types of Carrageenans

By means of these and similar studies, two major groups of carrageenans have been recognised (8). In the first, the 1,3-linked units are sulfated in the 4-position, while in the second, the sulfate is in position 2 (Table II). This first group is subdivided according to the nature of the 1,4-linked units.

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These may be present as galactose 6-sulfate (mu-carrageenan) or galactose 2,6-disulfate (nu-carrageenan) or as the corresponding 3,6-anhydrides in kappa- and iota-carrageenan. In nature the 3,6-anhydrides of kappa- and iota-carrageenan are formed by enzymatic elimination of the 6-sulfate from the mu and nu forms, but the conversion is not always complete. In some seaweeds, these carrageenan types can be isolated in almost pure form, while in others they exist as copolymers.

In the second group, the 1,4-linked units are sulfated in the 2-position. In lambda-carrageenan, the 6-position is also sulfated while in theta-carrageenan, it is not.

Physical Properties and Conformational Effects

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Many of these extracts have the ability to form reversible gels which liquify when heated and set when cold. Some give stiff gels in dilute solution, that is give gels which retain a definite shape even though they consist of 99.9% water and others have no gelling properties at all. These properties depend largely on the presence or absence of sulfate groups in certain positions on the sugar residues, and the theory of change from random coil to double helix conformation in the sol gel transformation, developed by Dr. D. A. Rees and his colleagues (1), has gone a long way to explain this. X-ray diffraction studies of orientated fibers of kappa- and modified iota-carrageenan indicated two parallel 3-fold helical chains, each twisted around the other and having a rise of 26A° per turn. The two chains move past each other from relative positions that are perfectly general to form a special arrangement that is exactly staggered with identical groups moved to half the initial spacing (Figure 6). Only the presence of the 3,6-anhydro-ring allows the sugar ring to be so constrained as to have three equatorial C-H bonds, an arrangement that increases the flexibility of the chain and allows winding and unwinding of the double helix. The conformation is such that hydrogen bonding takes place between the O-2 and O-6 of galactose residues in different stands of the same double helix. Thus, every unsubstituted hydroxyl group in iota-carrageenan is engaged in hydrogen bonding, making the conformation very stable.

On the other hand, a sulfate group on the O-2 of the 1,3-linked galactose residue as in lambda-carrageenan will inhibit double helix formation. Even after alkali treatment to convert the 1,4-linked residues into 3,6-anhydrogalactose, this carrageenan will not gel. Rees by means of optical rotation studies (9), confirmed that when the molecules are in solution they exist in random coil formation and when the solution is cooled, the chains link by this double helix formation to give a three dimensional framework (Figure 7), the interstices of which are occupied by water. Iota-carrageenan is a copolymer of D-galactose 4-sulfate and 3,6-anhydro-D-galactose 2-sulfate with masking by replacement of 1/10th of the anhydride by D-galactose 2,6-disulfate

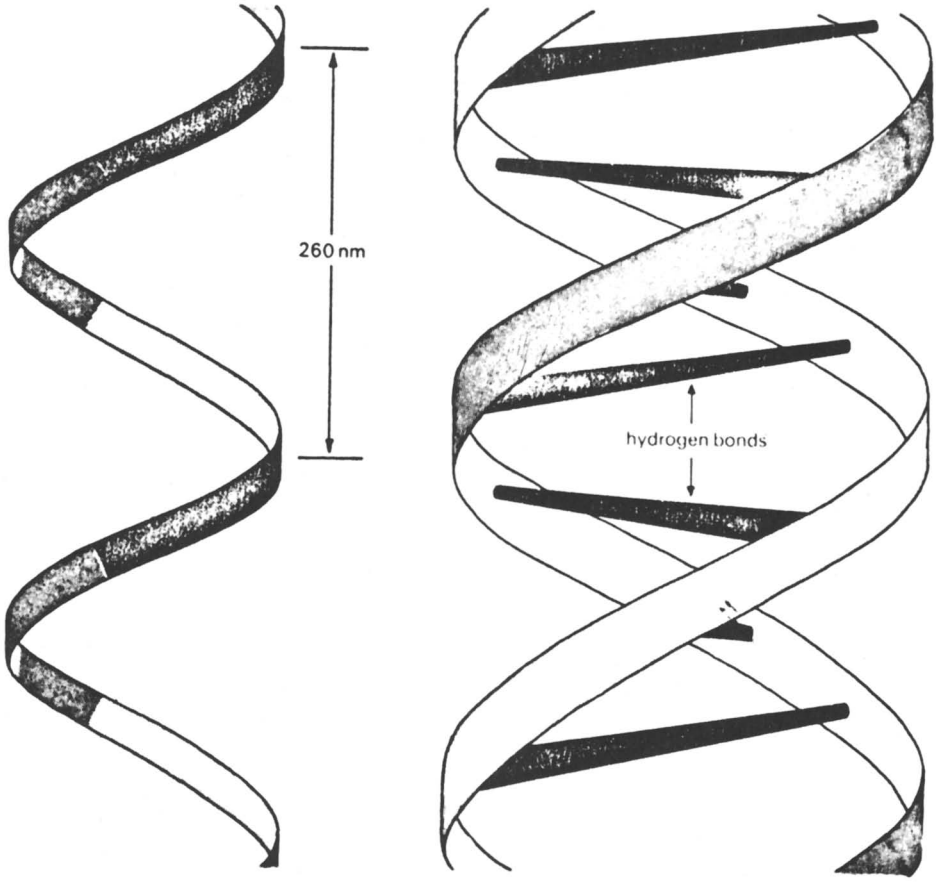

Figure 6. Helical structure

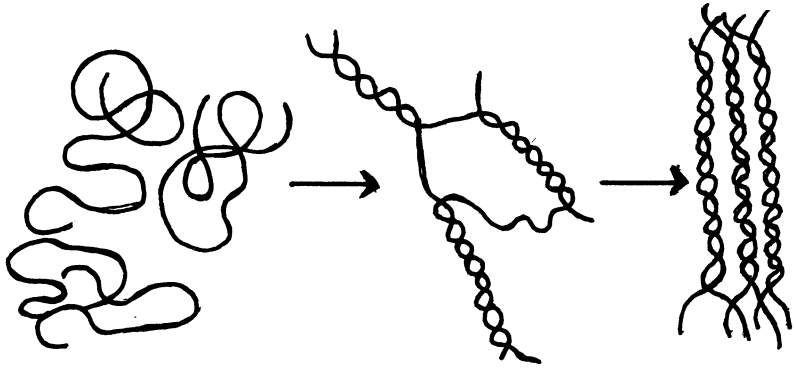

Figure 7.

and a much smaller proportion by D-galactose 6-sulfate (Figure 8). The masking residues cause kinking of the helical carrageenan chain. Thus, the physical and biological function of these residues is to serve as helix-breaking interruptions that cause each chain to enter into double helical association with more than one partner (Figure 7). This is necessary if a 3-dimensional network is to be formed rather than a collection of isolated chain pairs.

It is thought that in gel formation the aggregation of groups of double helices into parallel bundles takes place (Figure 7) as a secondary process and adds to the strength of the gel. 4-O-sulfate groups in the 1,3-linked residues and 2-O-sulfate groups in the 3,6-anhydro residues are situated on the outside of the helices and can, therefore, be expected to affect the degree of aggregation. Thus, the increasing sulfate in the series: agarose → furcellaran → kappa-carrageenan → iota-carrageenan coincides with increasing elasticity and decreasing brittleness of the gels.

Effect of Metallic Ions

It is understandable that the nature of the metal ion combined with the sulfate groups will influence the degree of aggregation of the helices. For example, the sodium salt of kappa-carrageenan gives little or only weak gelling products; whereas, potassium gives a strong gel. In contrast, the gel strength of iota-carrageenan with potassium is comparatively weak, whereas, with calcium it gives a strong elastic gel. Commercially, the manufacturer combines different extracts to give the appropriate properties required by the buyer.

Extracellular Polysaccharides from Microscopic Red Algae

Recent studies on the highly viscous extracellular polysaccharides isolated from cultured samples of the unicellular microscopic red algae, Rhodella maculata (11), Porphyridium cruentum (12-14) and Porphyridium aerugineum (14) reveal complex sulfated polysaccharides. Not only galactose, but considerable proportions of xylose, glucuronic acid and glucose are constituents of these mucilages. Both Rhodella and P. aerugineum contain appreciable amounts of 3-O-methylxylose (11,14) and 3- and 4-O-methylgalactoses are constituents of both Porphyridium species (14). In addition, P. aerugineum contains as much as 10% of 2,4-di-O-methylgalactose. 2-O-Methylglucuronic acid has also been reported for P. cruentum polysaccharide (13).

In both Porphyridium species the sulfate groups in the polysaccharides are labile to methylation and periodate oxidation conditions, although in cruentum mucilage they are somewhat more stable than in aerugineum. While infrared analysis of Rhodella polysaccharide (15) gave peaks at 1200 cm^{-1}, 874 cm^{-1} and 950 cm^{-1} which disappear on desulfation, the two Porphyridium polysaccha-

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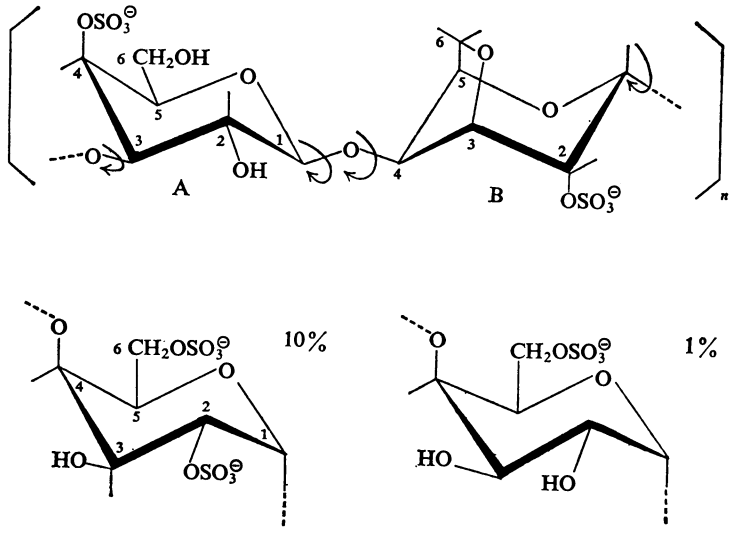

Figure 8.

rides give the characteristic peaks for galactose sulfates, that is broadish bands, at 820-830 cm^{-1} and a sharper band at 850 cm^{-1} (14) indicating that primary, equatorial and axial sulfate may be present. All the sulfate in P. cruentum polysaccharide is stable to alkali whereas in that from P. aerugineum the sulfate is decreased from 9% to 3.7%. Infrared analysis of a sulfated fragment isolated from an autohydrolysate of the free acid form of P. aerugineum (14) gives a peak at 830 cm^{-1}, indicative of equatorial sulfate. This has been characterised.

Conclusions

Although chemical studies have been made on only a minority of known species of red algae, it appears that the type of water extractable polysaccharides present follows the botanical classification into genera. There are distinct groups of polymers which, although having much in common, differ in detail, the proportion and position of sulfate groups being an important variable.

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Sulfated Fucose-Containing Polysaccharides from Brown Algae: Structural Features and Biochemical Implications

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The cell wall of plant cells is an intriguing biochemical system that continues to defy definitive characterization. Peter Albersheim and his co-workers recently have completed the most comprehensive characterization of the composition of the wall of tissue cultured cells (1,2,3). In addition, Albersheim has suggested a model for the structural arrangement of these polymers in the wall and in some cases, has suggested specific roles for particular polysaccharides (4). Nevertheless, the exact roles of the various fractions is not well understood. Moreover, information on the site and pathways of biosynthesis of wall polymers is very imcomplete (5,6).

The cell walls of algal cells have a unique composition and also present, in certain systems, a unique opportunity for the study of cell wall polysaccharide biosynthesis and assembly. The fertilized eggs of the brown alga Fucus appear to be ideal for the study of cell wall composition, biosynthesis, and control of cell wall formation (7,8,9). Wall-less eggs can be fertilized in a controlled manner and the development of the wall followed in the synchronously developing population (10).

The composition of the cell wall in the brown algae (Phaeophyceae) is made up of two fibrillar or structural polymers, cellulose and alginic acid, and matrix polymers. These matrix polymers are a complex array of fucose-containing sulfated polysaccharides (11). Mian and Percival (12) concluded that these polymers represented a spectrum from high uronic acid low sulfate-containing polymers to a relatively pure fucan sulfate.

The abundance of sulfated polysaccharides in all three classes of marine algae, Chlorophyceae (green), Rhodophyceae (red), Phaeophyceae (brown), while being essentially absent in land plants, has invited speculation as to the physiological function of these components. The two most commonly suggested roles are (1) involvement with selective cationic transfer in the saline medium; (2) prevention of desiccation when the plants are exposed to drying conditions such as low tides (13). A recent report of the presence of a sulfated polysaccharide as a major

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component in the cell wall of a halophilic bacteria (14) lends support to these suggestions. However, definitive data on the specific biochemical role of sulfate in these polymers has been lacking.

This report will examine the composition of the sulfated fucose-containing polysaccharides from brown algae in terms of their structural characteristics and possible roles in cell development and cell wall formation and function.

Polysaccharide Composition and Structure

The early work on the chemical composition of the fucose-containing polymers in brown algae centered on the material called "fucoidan", a hygroscopic sulfated polysaccharide fraction first isolated by Kylin in 1913. This material seemed to be ubiquitous in brown algae, but the most extensive studies were done on material isolated from Fucus vesiculosus and Ascophyllum nodosum by dilute acid or water extraction. Although small amounts of xylose and galactose were always found associated with "fucoidan", it was generally considered to be a unique substance. Its basic structure was shown by Conchie and Percival (15) to be composed primarily of L-fucopyranose units linked α-(1 → 2) with sulfate groups primarily located at position 4. Small amounts of single unit branches at C-3 and C-4 also were detected (15,16,17).

Larsen, Haug and Painter (18) showed that crude alginic acid preparations from Ascophyllum nodosum were contaminated by small amounts of a fucose-containing material which was not "fucoidan." This material was named ascophyllan and was composed of fucose (25%), xylose (26%), glucuronic acid (27%) and 13% sulfate. Traces of galactose and mannose also were found. Data from partial base hydrolysis of the molecule suggested a uronic acid "backbone" with branches containing xylose and fucose. Later work (19) led to the isolation of a significant quantity of 3-O-β-D-xylopyranosyl-L-fucose from a mild acid hydrolyzate of ascophyllan. This led to the conclusion that both sugars were part of the same branched chain.

Percival (20,21) also isolated and partially characterized a sulfated polymer fraction from A. nodosum which contained fucose (49%), xylose (10%) and glucuronic acid (11%). These data indicate that this was a different polymer fraction from ascophyllan. It was shown to be a highly branched structure with end-group and (1→4)-linked xylose, end group and (1→2)-linked fucose (some units were singularly branched at position 3 or 4 and some doubled branches were also indicated) and (1→4)-linked glucuronic acid. 3-O-(β-D-Glucopyranosyluronic acid)-L-fucose was a significant structural entity and evidence for (1→3)-linked fucose was also found. Sulfate groups were located on position 4 of fucose residues.

Larsen, et. al. (22) confirmed that ascophyllan was not the only "nonfucoidan" fucose polymer in brown algae. They showed

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that both F. vesiculosus and A. nodosum contained other polysaccharide "complexes" and suggested that perhaps "fucoidan" itself did not exist on these algae in the native state.

More recently, Percival's group studied the carbohydrates in several species of brown algae from different families of plants (12,23,24). They were unable to separate the fucose-containing fractions into unique entities but did accomplish separation into a series of fractions ranging from a nearly pure fucan sulfate to one with high uronic acid and low sulfate. These were considered to be a more-or-less continuous spectrum of structures. All species studied had essentially the same pattern and the structural data suggested the same highly branched characteristics as previously reported for "fucoidan" from F. vesiculosus (15) and the glucuronoxylofucan from A. nodosum (20,21). One of the species, Desmarestia aculeata, was shown to have a much higher proportion of galactose in its "fucans" (24), particularly in the material extracted with dilute alkali. This crude extract had galactose, fucose, xylose and glucuronic acid in the molar proportions of 2:1:0.13:1.7. The galactose was D-galactose and methylation studies indicated it was present in the polymer primarily as end-group and (1→3)-linked residues. Still other variations in composition have been found in partially purified extracts from Padina pavonia (25).

In light of the apparent complexity of the fucose-containing polymers in brown algae, there is a need for a more definitive characterization of these polymers in order to study their possible role in cell development. Medcalf and Larsen (26,27) have reexamined the fucose-containing sulfated polysaccharides from two well-studied brown algal species, Ascophyllum nodosum and Fucus vesiculosus. An attempt was made to assure minimum degradation during extraction in order to increase the reliability of the chemical and biological correlations. Extraction of dried weed with water at pH 2 or 0.1M EDTA at pH 7.5 gave identical results as shown by both free boundary electrophoresis at pH 2 and pH 7 and cellulose acetate electrophoresis at pH 7.5. Previous work had shown that the fucose-containing polymers were all relatively highly charged with uronic acid and/or sulfate groups. Thus, electrophoresis should be a reliable technique to indicate the complexity of the extracts and to follow the success of attempts to separate the mixtures into individual components.

A fractionation procedure developed by Larsen, et al. (18,22) was adapted for use in this study. It involved fractional precipitation with ethanol of polymer solutions containing varying concentrations of magnesium or calcium chloride. Cellulose acetate electrophoresis was used to follow small scale trial fractionations. Preparative scale fractionations were done using optimum conditions and the fractions characterized by both free boundary (pH 2) and cellulose acetate (pH 7.5) electrophoresis. Isolated fractions were refractionated until free boundary electrophoresis indicated at least 95% homogeneity.

General fractionation schemes for the crude extracts from A. nodosum and F. vesiculosus are shown in Figure 1. Figures 2, 3, 4, and 5 show the results of the purification efforts as indicated by electrophoresis. Four electrophoretically pure and distinct fractions were isolated from A. nodosum (Frac. I-4, Table I) and two from F. vesiculosus (Frac. 1-2, Table II). In each case an additional mixed fraction was obtained. However, the homogenious fractions accounted for almost 90% of the crude extracts from each species. Therefore, these fractions represent unique polymer molecules present in these algae which can be extracted with very dilute acid. They are probably present as matrix polymers in the cell wall. Quatrano (28) has shown that dilute acid extracts from the cell walls of 24 hour embryos of these species have an electrophoretic pattern very similar to those shown here.

Tables I and II give the analytical data for these fractions. Fraction 1 from Ascophyllum was very similar in composition to ascophyllan and was identical in electrophoretic mobility to an ascophyllan sample isolated previously (18). Hydrolysis of this fraction with dilute base followed by dialysis and analysis for fucose, uronic acid and unsaturated uronic acid gave the results shown in Table III. A significant portion of the remaining uronic acid and the unsaturated uronic acid produced via β-elimination were in the dialyzable fraction while the fucose remained essentially in the non-dialyzable portion. These data suggested that the majority of the fucose molecules were in relatively large fragments that did not contain uronic acid. This is consistent with a molecular structure having a majority of the uronic acid units in a main chain to which are attached fucose-containing side chains. While a complete structural analysis has not been done, these data plus the earlier data of Larsen (19) suggest that a significant portion of the ascophyllan structure can be represented as shown in Figure 6. The nature of the linkages in the main uronic acid chain and the linkage of fucose to xylose have not been determined for ascophyllan. The structure in Figure 6 uses information obtained by Percival (21) for these linkages. The ascophyllan-like polymer isolated by Medcalf and Larsen (27) contained mannuronic and guluronic acid in addition to glucuronic acid in contrast to both the data of Percival (20, 21) and earlier work by Larsen, et.al. (18,22) where only glucuronic acid was detected. This observation was confirmed by Larsen (29) on a new Ascophyllum sample. Further work is needed to ascertain whether these uronic acids are part of a single molecule, or whether there are several types of ascophyllan-like molecules, having the same basic structure, but differing in the relative amounts of each uronic acid.

Fraction 1 from Fucus was similar to ascophyllan in electrophoretic mobility. It differed somewhat in composition, primarily in an increased fucose content. However, its properties suggested a structure closely related to that given for ascophyllan.

Fractions 2 and 3 from Ascophyllum and Fraction 2 from Fucus

were complexes which could be easily hydrolyzed in very dilute acid at 80° to two new polysaccharides. These polymers could be distinguished electrophoretically as shown in Figure 7 for the Ascophyllum fractions. The two new polymers were separated by fractional precipitation with ethanol from a $MgCl_2$ solution. Compositional data for these fractions are shown in Table IV. The insoluble (slower) fraction was very similar to ascophyllan in both electrophoretic mobility and composition. However, only mannuronic acid could be detected in significant amounts (27). Base hydrolysis of the complex from Ascophyllum gave a pattern of dialyzable and non-dialyzable fucose, uronic acid and unsaturated uronic acid which was the same as found for ascophyllan (Table III). Fraction 2 from Fucus gave very similar electrophoretic results after dilute acid hydrolysis. The major difference between the "complexes" from the two species seemed to be that the Fucus fraction, on hydrolysis, gave more of the faster moving fucan polymer and less of the ascophyllan-like fraction. No fraction from either species corresponding to "fucoidan" could be detected in the original extract. It was concluded that "fucoidan" is not a native polymer in these species, but is a product of the hydrolysis of the fucose rich "complexes."

The two complex fractions from Ascophyllum were shown by a time-course study of the acid hydrolysis at 75° to give a gradual increase in the mobility of the original band and a simultaneous gradual increase in the concentration of the slower component (Figure 8). These data were interpreted to suggest that the fucan portion formed the backbone of the molecule, and the ascophyllan-like components were attached as branches by acid-labile linkages. The various complexes differ from each other only in the number of ascophyllan-like molecules attached to the fucan backbone. A proposed structure is shown in Figure 9. The fucan is shown with α - (1 → 2)-linkages based on data from Conchie and Percival (15). The nature of the linkage between the two polysaccharides in the complex is not known. Medcalf and Larsen (27) showed it did not involve a peptide bridge.

Fraction 4 from Ascophyllum had a neutral sugar composition quite different from any fraction previously reported for this species. It contained about equal amounts of fucose and galactose, some uronic acid, and 14% sulfate. Only two galactose rich polymers previously have been identified in brown algae (24,30) and only Percival and Young (24) have published structural data. Their fraction, isolated by alkaline extraction, was described earlier. Medcalf, Schneider and Barnett (31) reisolated fraction 4 in highly purified form by repeated fractionation with ethanol from $CaCl_2$ solutions. It gave a single band on electrophoresis and had the composition shown in Table V. The uronic acid was shown to be glucuronic and only traces of neutral sugars other than galactose and fucose were found in the purified material. The galactose was shown by D-galactose oxidase to be primarily L-galactose. Only two major methylated

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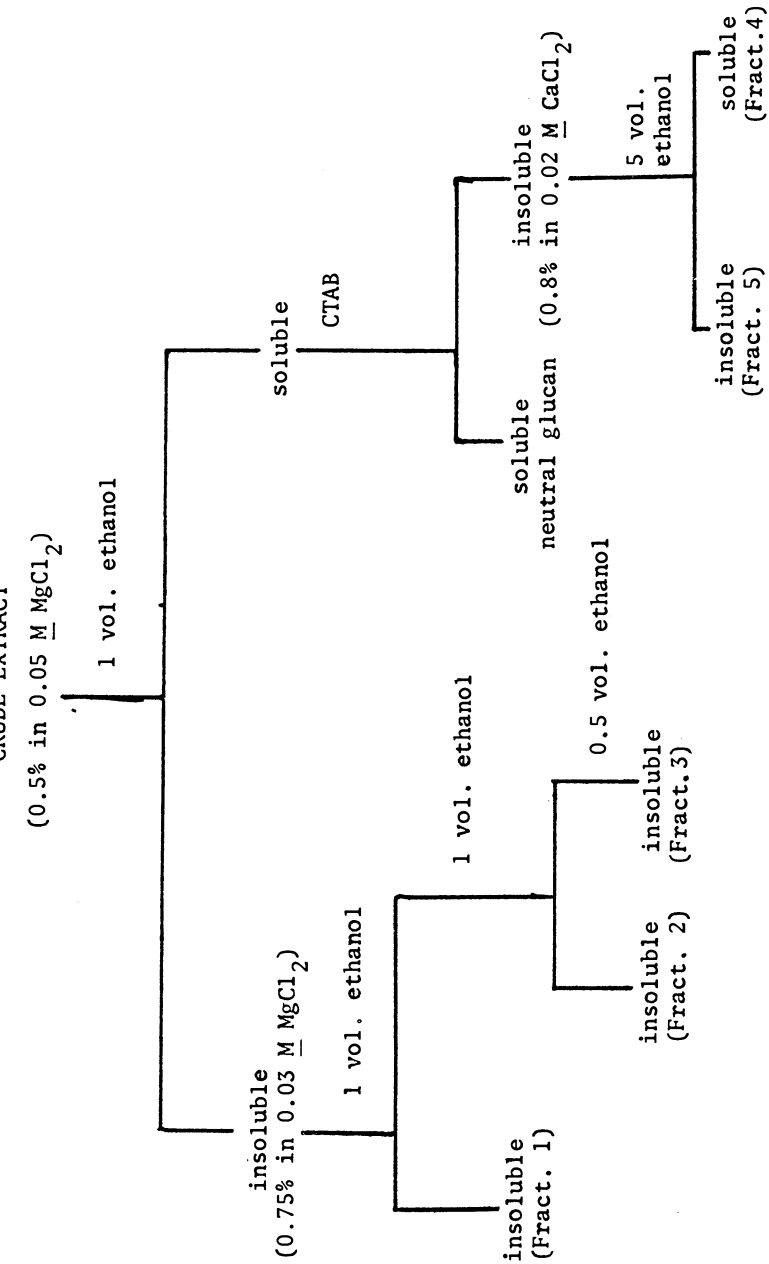

Figure 1. Fractionation scheme used for the isolation of electrophoretically pure fractions from A. nodosum *dilute acid extracts*

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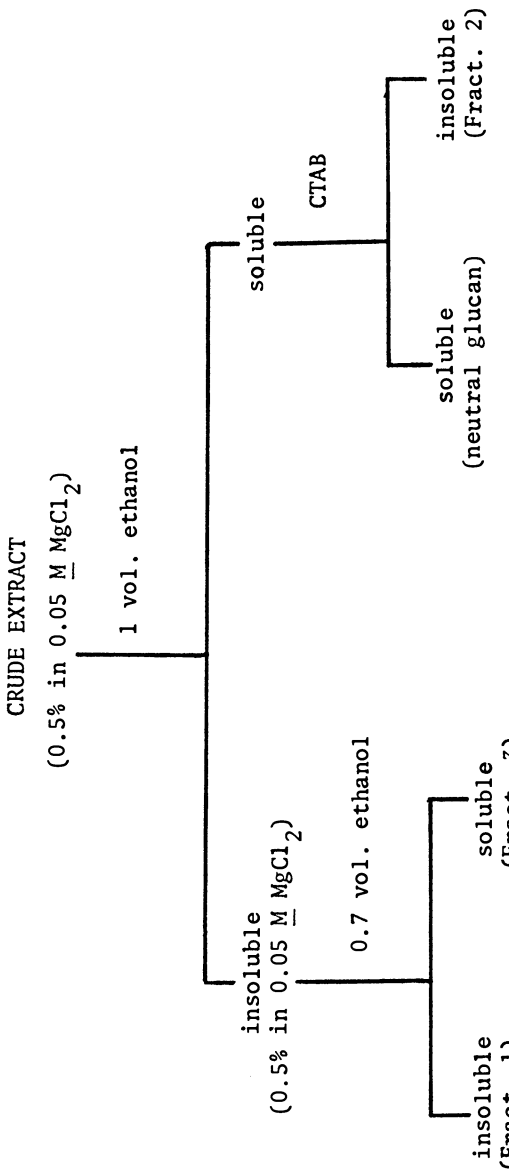

Figure 1. Fractionation scheme used for the isolation of electrophoretically pure fractions from F. vesiculosus *dilute acid extracts*

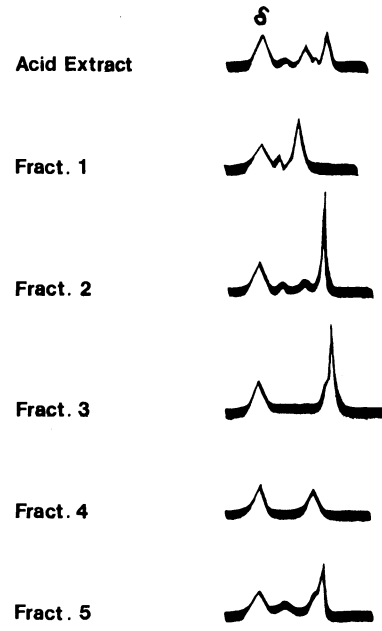

Figure 2. Ascending pattern at pH = 2 of the original extract and purified fractions from A. nodosum

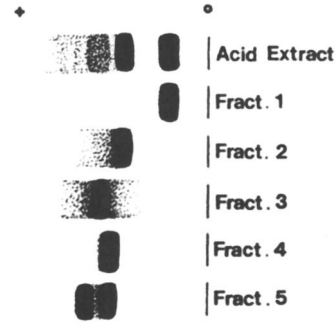

Figure 3. Cellulose acetate electrophoresis, toluidine Blue stain, at pH = 7.5 of the original extract and purified fractions from A. nodosum

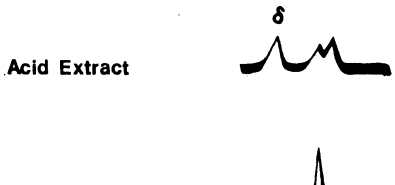

Figure 4. Ascending pattern at pH = 2 of the original extract and purified fractions from F. vesiculosus

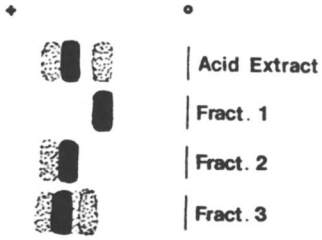

Figure 5. Cellulose acetate electrophoresis, toluidine Blue stain, at pH = 7.5 of the original extract and purified fractions from F. vesiculosus

TABLE I MAJOR FRACTIONS FROM A. nodosum

Fraction	Yield[a] (%)	Protein (%)	Uronic Acid (%)	Sulfate (%)	Fucose (%)	Neutral Sugar distribution[b] (%)				
						Fuc	Xyl	Mann	Gal	Glu
1	31	3.6	26.4	12.8	15	37	29	21	3	11
2	29	2.4	15.8	20.9	33	73	11	10	2	5
3	18	1.8	6.4	25.2	40	81	9	4	2	4
4	11	8.5	7.1	14.7	19	34	14	15	27	10
5	11	3.1	7.4	8.1	36	71	7	4	14	4

[a]Based on recovered material only.

[b]Calculated from gas chromatograms where total area under the five peaks equals 100%.

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TABLE II. MAJOR FRACTIONS FROM F. vesiculosus

Fraction	Yield[a] (%)	Protein (%)	Uronic Acid (%)	Sulfate (%)	Fucose (%)	Neutral Sugar distribution[b] (%)				
						Fuc	Xyl	Mann	Gal	Glu
1	21	6.3	21.9	4.1	18.1	50	15	17	4	14
2	66	5.4	5.6	25.4	48.1	70	7	4	8	11
3	13	3.0	13.9	12.7	40.4	70	7	9	5	9

[a]Based on recovered material only.

[b]Calculated from gas chromatograms where total area under the five peaks equals 100%.

TABLE III. COMPOSITION OF FRACTIONS 1 AND 2 AFTER BASE HYDROLYSIS

	Total Carbohydrate (mg/ml sample)	Fucose (mg/ml sample)	Uronic Acid (mg/ml sample)	Unsaturated Uronic Acid (mg/ml sample)
Fraction 1				
Original	0.70	0.12	0.242	0.0
Base-hydrolyzed	0.76	0.13	0.206	0.042
Base-hydrolyzed-dialyzed	0.62	0.11	0.153	0.028
Fraction 2				
Original	0.44	0.22	0.128	0.0
Base-hydrolyzed	0.46	0.23	0.098	0.028
Base-hydrolyzed-dialyzed	0.38	0.20	0.067	0.017

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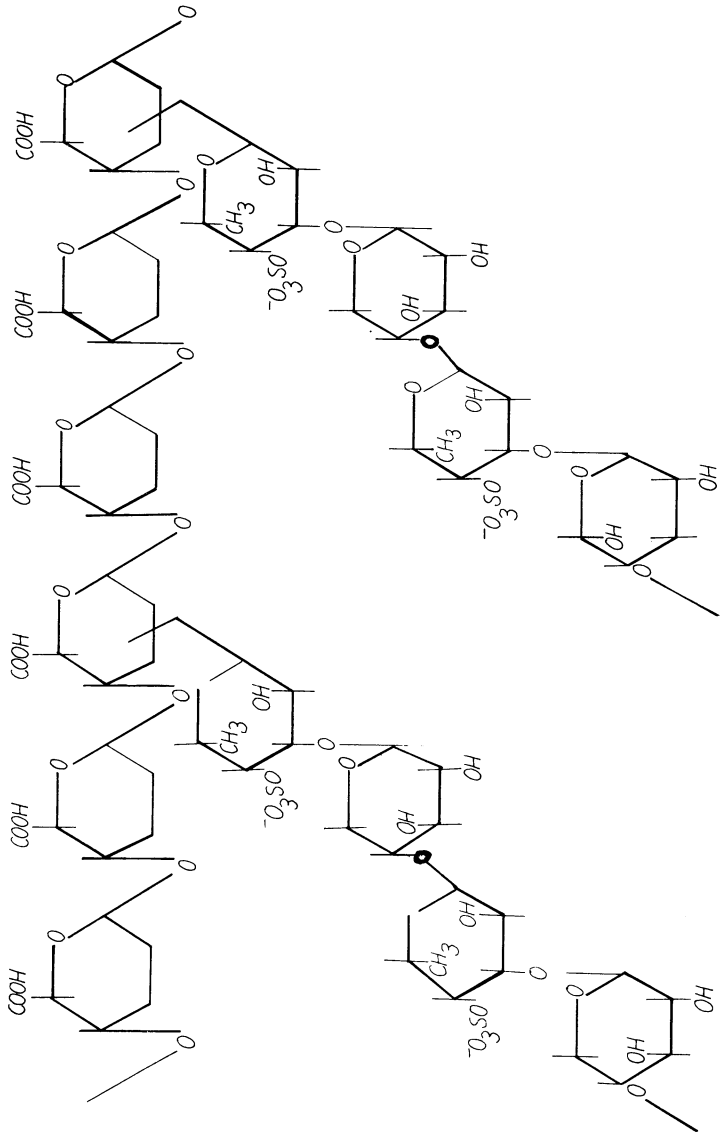

Figure 6. Structural features of ascophyllan

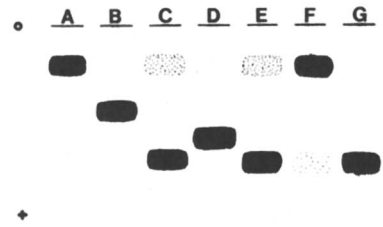

Figure 7. Cellulose acetate electrophoresis, toluidine Blue stain, at pH = 7.5; (A) ascophyllan; (B) Fraction 2; (C) Fraction 2, hydrolyzed; (D) Fraction 3; (E) Fraction 3, hydrolyzed; (F) hydrolyzed Fraction 2, ethanol-insoluble fraction; (G) hydrolyzed Fraction 2, ethanol-soluble fraction

TABLE IV. FRACTIONS FROM MILD ACID HYDROLYSIS OF A. nodosum COMPLEX

Fraction	Yield[a] (%)	Protein (%)	Uronic Acid (%)	Fucose (%)	Neutral sugar distribution[b] (%)				
					Fuc	Xyl	Mann	Gal	Glu
2(complex 1)	--	2.4	15.8	33	73	11	10	2	5
Insoluble	32	6.8	24.1	11	28	22	28	6	17
Soluble	68	1.6	4.1	42	77	8	8	1	6

[a]Based on recovered material only.

[b]Calculated from gas chromatograms where the total area under the five peaks equals 100%.

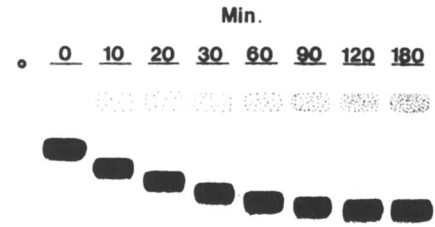

Figure 8. Cellulose acetate electrophoresis, toluidine Blue stain, at pH = 7.5. Mild acid hydrolysis (0.02M HCl, 75°C) of Fraction 2.

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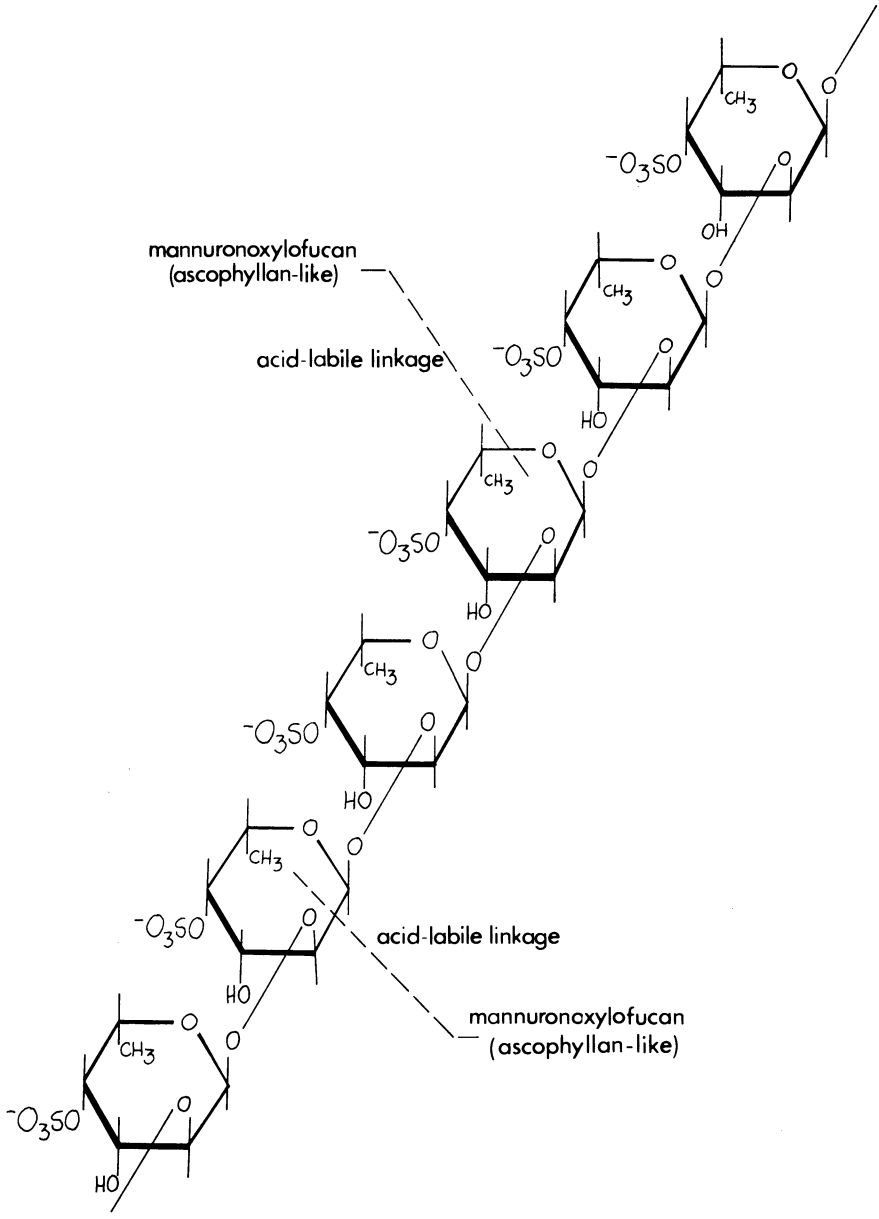

Figure 9. Structural features of a fucan "complex"

sugars were found after hydrolysis of the uronic acid reduced and methylated polymer; 2,3,4-tri-O-methylfucose and 3,6,-di-O-methylgalactose. Much smaller amounts of 2,3,4,6-tetra-O-methylglucose and mono-methylfucose were also detected. These data, along with data on periodate oxidation from both the original and desulfated polymers, suggested the partial structure shown in Figure 10.

Biochemical Role

The early stages of growth of fertilized Fucus zygotes are shown in Figure 11 (7). A localized protuberance or rhizoid appears 12-16 hours after fertilization. This is subsequently partitioned from the rest of the cell by the first division at 20-24 hours. The rhizoid cell becomes the site of attachment to the substratum (32) and considerable data has been accumulated to show that this cell is both morphologically and chemically distinct from the other cell of the two-celled embryo (7,32,33). One of the distinguishing characteristics of the rhizoid cell is its accumulation of a sulfated fucose-containing polysaccharide. The properties of this polymer fraction and its role in rhizoid formation and cell differentiation have been studied extensively by Quatrano and co-workers (32,34,35,36).

Both audioradiographic techniques with ^{35}S, and toluidine Blue O cytochemically, showed that the sulfated fucan initially is observed around the rhizoid-half of the nucleus radiating toward the site of rhizoid initiation, and eventually becomes localized in the region of the cell wall protuberance. After cell division this material is found in the cell wall of the rhizoid cell (32,37). Quatrano and Crayton (32) showed that sulfation of the preformed polymer began at about the time of rhizoid initiation. It was postulated that the synthesis of the highly charged fucan sulfate might be the cause of polarity in the zygote and lead to rhizoid formation. However, some very elegant further work by Crayton, Wilson and Quatrano (34) proved that the establishment of a polar axis and initiation of rhizoid formation could occur even though sulfation of the fucan polymer was prevented. Very recently, Hogsett and Quatrano (36) isolated a plant lectin from Ricinus seeds which would bind to both sulfated and desulfated fucans. This lectin was galactose specific, but showed affinity for the fucans found in Fucus. These authors attribute this binding to galactose residues associated with these fractions. By attaching a flourescent dye to the lectin, they were able to use it as a cytochemical marker for the fucan polymers. The data obtained clearly showed that the unsulfated fucan does not accumulate in the rhizoid region. The polymer was available but sulfation was required for its localization and incorporation into the cell wall of the rhizoid cell. This appears to be the first clear documentation of a specific biochemical role for sulfate groups in polymers of brown algae.

The availability of synchronously developing populations of

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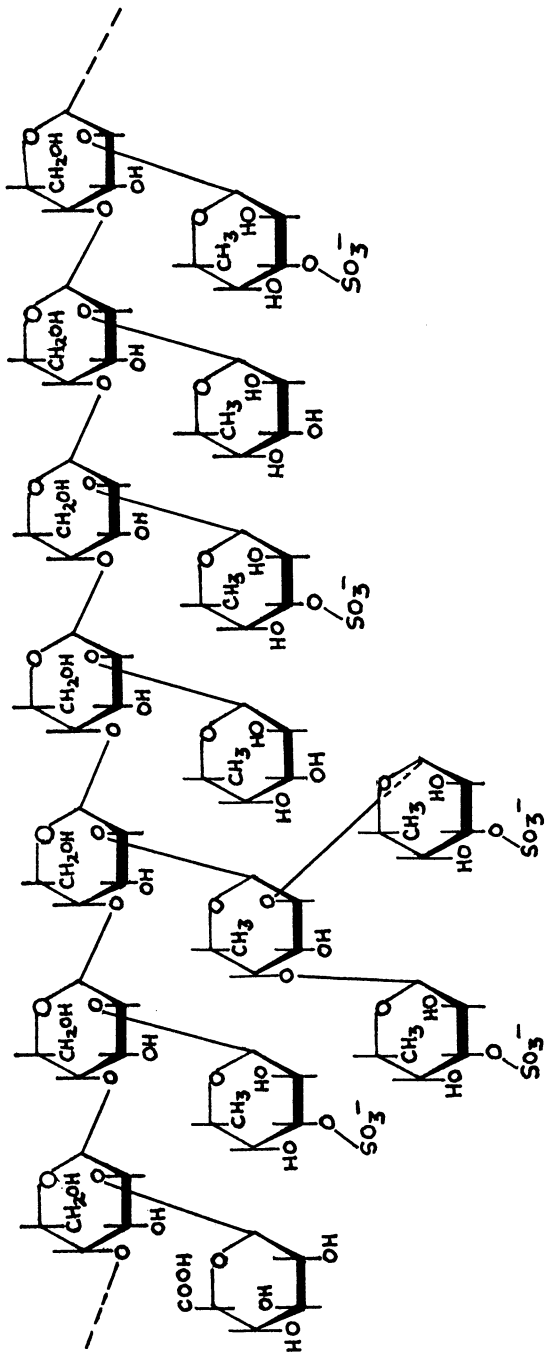

Figure 10. Structural features of the galactose-rich polymer from Ascophyllum

TABLE V. GENERAL CHARACTERIZATION OF THE GALACTOFUCAN POLYMER

		Molar ratio	
$[\alpha]_D^{25}$	-54^o	Galactose	1.0
Sulfate (%)	15	Fucose	1.1
Uronic Acid (%)	8	Xylose	0.1
Protein (%)	7	Mannose	0.1
		Glucose	trace

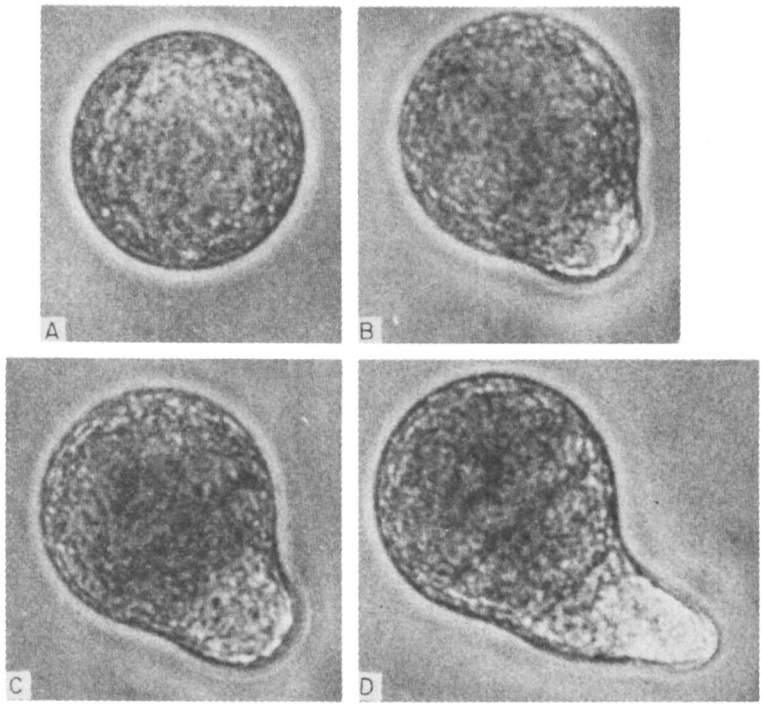

Figure 11. Stages in the development of Fucus *embryos. (A) 0–12 hr, (B) 12–16 hr, (C) 16–20 hr, (D) 30–36 hr. ×600 (7). (Photomicrographs courtesy of G. B. Bouck)*

Fucus zygotes from eggs which have no detectable cell wall provides a unique system for the study of wall formation (8,9). The appearance of various wall fractions can be followed and these data may suggest specific roles for individual wall polysaccharides. Quatrano and Stevens (9) used this system to follow wall development in *Fucus vesiculosus* zygotes. Cell wall formation could be observed as early as 10 to 15 minutes after fertilization. However, wall birefringence was not observed until 60 minutes after fertilization. Complete birefringence (all the population) did not occur for four hours. The wall was composed of alginate and cellulose in about equal amounts at 30 minutes (Figure 12). Fucans were not detected until four hours after fertilization when they represented about 25-30% of wall carbohydrate. While the total carbohydrate in the wall continued to increase, particularly during rhizoid elongation (16-24 hours), the proportion of the major polysaccharide groups remained relatively constant. Embryos at 24 hours consisted of 60% alginate, 20% cellulose and 20% fucose-containing polymers. The fucans were composed of two electrophoretically distinct fractions; F_1, a slow moving component, which very likely was the ascophyllan-like Fraction 1 from *Fucus* described by Medcalf and Larsen (26); and F_2, a faster moving fraction having a higher fucose content than F_1. This corresponded to the complex (Fractions 2 and 3) reported by Medcalf and Larsen (26,27).

The two fucan polymers did not appear in developing walls at the same time. Only F_1 was detected prior to 12 hours after fertilization. By 12 hours, the developing wall acquired F_2 which corresponded to the fraction which was sulfated and associated with rhizoid formation. The role these polymers play in the developing wall is only speculation at this time. However, Quatrano and Stevens (9) have suggested that the appearance of F_1 as the earliest matrix component in the developing wall, coincident with the acquisition of structural integrity and birefringence of the wall, argues for its basic structural role in wall assembly and function. F_2, which appeared at the time of rhizoid formation, and which had to be sulfated before it was incorporated (37) may be responsible for cell adhesion. Zygotes in which sulfation of F_2 was inhibited, formed rhizoids but did not adhere to the substratum (32).

The requirements of sulfate groups for adhesion is consistent with data suggesting that the presence of anionic groups at the surface plays an important role in barnacle adhesion (38).

The data on the polysaccharides found in the primary cell wall of suspension-cultured sycamore cells has been proposed as a model by which to compare information on cell walls from other systems (3). While algal systems have unique features, such as structural polymers other than cellulose and high degrees of sulfation, there is a similarity of structures which can be noted. Table VI is an attempt to show possible relationships between the detailed wall information from sycamore cells (4) and the data

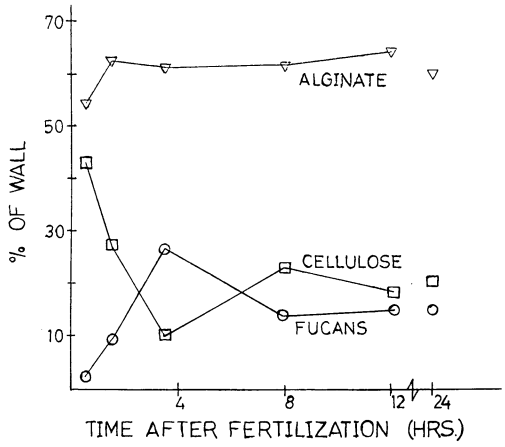

Figure 12. Changes in the major polysaccharide composition of isolated cell walls from F. vesiculosus *at different times after fertilization* (9)

TABLE VI. COMPARISON OF CELL WALL POLYSACCHARIDES

	% of Total Carbohydrate	
	Sycamore Cells[a]	Brown Algae[b]
Structural Polymers	Cellulose, 27%	Cellulose, 20% Alginic Acid, 60%
Pectic Polymers	Arabinogalactan, 22%	Glucuronoxylofucan (ascophyllan-like) 9%
	Rhamnogalactan, 18%	Glucuronogalactofucan, 1%
Matrix Polymers	Xyloglucan, 23%	Fucan complexes, 10%

[a]Taken from Albersheim (4)

[b]Composite of data from Medcalf and Larsen (26) and Quatrano and Stevens (9).

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obtained by Medcalf and Larsen (26) and Quatrano and Stevens (9) from brown algae. While the proposed relationships are oversimplified, they do suggest the possibility that plant cell walls, from whatever the source, including algae, have more similarities than may previously have been recognized. Alginic acid may serve both structural and pectic roles in the brown algae and thus could be listed in both fractions in the table.

Summary

The complicated nature of the sulfated fucose-containing polysaccharides from brown algae have been reviewed. For Ascophyllum nodosum and Fucus vesiculosis, a series of electrophoretically homogeneous fractions have been isolated and partially characterized. These include a xylofucoglucuronan (ascophyllan or ascophyllan-like), complexes which have ascophyllan-like molecules attached to a fucan backbone, and in Ascophyllum, a novel glucuronogalactofucan. Information on the biochemical role of major fucose-containing polymers in Fucus have been reviewed. Work by Quatrano and co-workers indicated that these polymers have an important role in cell wall development. The fucan complexes, when sulfated, are localized in the rhizoid cell and are required for adhesion of the embryo to the substratum.

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Co-Ion and Counter-Ion Interactions with Sulfonated Polysaccharides

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With the advent of the Debye-Hückel theory and its subsequent scrutinization to experimental testing, a physical picture emerged for the behavior of simple electrolyte solutions along with its limiting laws which are valid for very dilute solutions. This model, along with its further development by Onsager, and the Bjerrum ion-pair concept prove useful. It should be noted that more recent theories have extended our understanding of electrolyte solutions to higher concentrations. With the particularly high electrostatic potential on linear polyelectrolytes due to constrained charges along the chain, it is presently believed that a rod-like or cylindrical model for the polyion with its ionic atmosphere is the appropriate representation for the linear polyelectrolyte in solution. The most comprehensive modern theory of polyelectrolyte behavior in solution is by Manning (1). His line charge model for the polyelectrolyte results in limiting laws for thermodynamic (2), mass (2) and electrical transport (3,4,5) properties. Generally, the infinite line charge is thought to interact with simple ions via two phenomena, by ion-atmosphere interaction and by condensation (site-binding) of counterions onto the polyion. If the charge density of the polyelectrolyte, which is proportional to the dimensionless parameter ξ

$$\xi = \frac{e^2}{\varepsilon k T b} \qquad (1)$$

where e is the protonic charge, ε is the dielectric constant of the medium, k is the Boltzmann constant, T is the absolute temperature and b is the average distance between adjacent charge groups on the polyelectrolyte, is less than a critical charge density ξ_c, which is given $\xi_c = |z_1 z_p|^{-1}$, where z_1 is the charge of the

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counterion and z_p is the charge of a single charge-site on the polyion, no condensation of counterions occurs and both counterions and coions (if a simple salt is present) interact with charges on the polyion by Debye-Hückel forces, i.e., the potential at any point in the ionic atmosphere surrounding the polyion is given by the Debye-Hückel screening potential. If ξ_c is greater than ξ for a polyelectrolyte, an instability is shown to exist which is relieved by condensation of counterions onto the polyion so as to reduce ξ to its effective value ξ_c. All uncondensed counterions and coions in the ionic atmosphere interact with the polyion by Debye-Hückel forces. Since $|z_p| = 1$ for almost every polyelectrolyte, then for $\xi > |z_1|^{-1}$, and the fraction of condensed (site-bound) counterions is $(1-|z_1|^{-1}\xi^{-1})$.

The Manning model has been tested experimentally using different polyelectrolytes (6-23). Since the model demands a rod-like geometry for the polyion, ionic polysaccharides have been used because of their relatively stiff structure as compared to the more flexible synthetic polyelectrolytes. For sulfonated polysaccharides, activity coefficient measurements for the simple ions gave excellent agreement with Manning's theoretical results over a large concentration range for sodium chondroitin sulfate (12), the sodium salt of sulfated proteoglycan from bovine intervertebral disc (12), dextran sulfate (11,22,23) and iota, kappa and lambda carrageenan salts of sodium, potassium and calcium (19). Self-diffusion measurements of Na^+, Ca^{+2} and Sr^{+2} in the presence of chondroitin sulfate have established the validity of the condensation phenomena predicted by Manning's theory (10,24). Similar studies with sodium iota carrageenan in aqueous solutions of NaCl, Na_2SO_4 and $Na_4Fe(CN)_6$ indicate that while the Manning theory correctly predicts the self-diffusion coefficients for monovalent Cl^- ion, it clearly overemphasized the electrostatic interactions between the divalent SO_4^{2-}, the tetravalent $Fe(CN)_6^{4-}$ and the polyion, except for the good agreement at low ratios of polyelectrolyte to simple salt concentrations (6). With our recent emphasis on the interactions of coions with polyelectrolytes, it was of interest to examine the effect of the charge density of several coions on this interaction by potentiometric and self-diffusion techniques. Here we report results for single ion activity coefficients for Na^+, Cl^-, Br^- and I^- in aqueous solutions of sodium iota carrageenan (NaCarr) and sodium dextran (NaDS) at 25°C. The results will be discussed in light of modern Manning theory of polyelectrolyte solutions.

Experimental Section

Materials. Sodium iota-carrageenan, an extraction of the red algae *Eucheuma Spinosum* was kindly supplied by Marine Colloids, Inc. It consists of β-D-galactose-4-sulfate and 3,6-anhydro-α-D-galactose-2-sulfate residues with an average distance between charges on the chain of b = 4.4Å. Analysis of the carrageenan (sample Re 7275) by the manufacturer indicated that this sample has a weight average molecular weight of 291,000 and a number average molecular weight of 191,000. No contaminating cations or anions were present in the sample; the criteria for contamination being that the cation or anion is present in the sample in excess of 0.5%. The sample has an equivalent weight of 255.2 ± 0.1g. Chemical analysis of the purified iota-carrageenan gave a sulfate-hexose ratio of 0.99; therefore, one sulfate group is assumed per sugar unit. The sample was purified by the manufacturer by washing of the unpurified carrageenan with a 1 *N* NaCl solution in 50% isopropyl alcohol. This procedure was repeated four times. This was followed by four plain alcohol washes to remove residual salt. The product was then dried and ground. Solutions were prepared by adding weighed amounts of the carrageenan, which were dried in vacuo at 40°C for at least 24 hours, to a volumetric flask and an aliquot of the appropriate salt from a stock salt solution. The flask was diluted to the mark with deionized water. Dried reagent grade NaCl, NaBr, and NaI were used to prepare all solutions.

Dextran sulfate, a sulfated derivative of dextran, was kindly supplied by Pharmacia Fine Chemicals, Inc. The sample had a molecular weight of 500,000. Foreign ions were removed from the sample by passing the sample through appropriate ion exchange columns. The equivalent weight of the sample was found to be 180.5 ± 0.5 g/equiv., indicating 2.06 sulfate groups per sugar unit. This gives an average spacing between charges on the NaDS sample of b = 2.5Å (11). The concentration of the NaDS solutions were determined by converting the NaDS to the hydrogen form followed by titration.

Electromotive Force Measurements. The counterion activities of the ionic polysaccharide solutions with added simple salt were determined using the Orion Model #94-11 sodium selective solid-state electrode in conjunction with a calomel electrode. Coion activities of the ionic polysaccharide solutions with added simple salt were determined using the appropriate anion selective solid state electrode. Orion Model #97-17 chloride

ion electrodes, Orion Model #94-35 bromide ion electrodes and Orion Model #94-53 iodide electrodes were used to determine the appropriate coion activities. All the above anion electrodes were used in conjunction with a standard calomel electrode.

The electromotive force was measured utilizing a Corning Model 12 pH meter or an Orion Model 801A pH meter with an accuracy of ± 0.1 mv. All measurements were carried out in a constant temperature water bath thermostated at 25.00 ± 0.01°C. Air was bubbled through all solutions. A constant potential reading over a 20 minute period was regarded as a reliable emf. Stable potentials were obtained in 2 minutes for dilute polyelectrolyte solutions with added salt and 10 minutes for the more viscous solutions. Reproducibility of ± 0.1 mv was obtained which corresponds to an uncertainty of ± 0.002 activity coefficient units. The calibration of electrodes was carried out repeatedly during each run using solutions of the appropriate simple salt without polyelectrolyte present. In the concentration range studied, the slopes of the emf in mv vs. log a_+ were found to always give close to Nernstian behavior. The data is reported as γ_i/γ_i^0, the ratio of the experimental ion activity coefficients in the presence of polyelectrolyte to that in the absence of polyelectrolyte.

Results and Discussion

A principal aim of this study was to examine the effect of the charge density of the coion on its interaction with the polyion. Thus the single ion activity coefficients for Cl^-, Br^- and I^- ions were determined in aqueous solutions containing NaCarr and NaDS at 0.00100 *M*, 0.00500*M* and 0.0100*M* NaCl, NaBr and NaI at 25°. The results are given in Tables I and II for NaCarr and NaDS, respectively, where γ_2/γ_2^0 is the ratio of the coion activity coefficients in the presence of polyelectrolyte to that in the absence of polyelectrolyte and $X = n_p/n_s$, where n_p and n_s are the equivalent concentrations of polyelectrolyte and simple salt, respectively. The general trend for the NaCarr results shows that γ_2/γ_2^0 is fairly constant at unity for $X < 1$ and decrease gradually to fairly constant values for $X > 4$, with the exception for 0.0010*M* NaCl and NaBr where the γ_2/γ_2^0 ratios are constant at about 0.9 for $X < 0.5$ prior to their gradual decrease. The γ_2/γ_2^0 ratios for 0.00100*M* and 0.00500*M* NaCl and NaBr are almost identical at corresponding simple salt concentrations and X values, indicating that the Cl^- and Br^- ions interact with the

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Table I. The Coion Activity Coefficient Ratio γ_2/γ_2^0 Dependence on X for Sodium Iota Carrageenan.

NaCl				NaBr				NaI			
X	0.00100*M*	0.00500*M*	0.0100*M*	X	0.00100*M*	0.00500*M*	0.0100*M*	X	0.00100*M*	0.00500*M*	0.0100*M*
0.080	0.914	1.003	1.000	0.10	--	0.957	1.040	0.12	1.004	1.015	1.012
0.28	0.894	1.003	1.000	0.22	0.907	1.000	1.028	0.28	0.965	1.036	1.047
0.48	0.883	0.962	0.958	0.48	0.900	0.985	1.025	0.52	1.012	1.036	1.044
0.70	0.845	0.962	0.958	0.80	0.896	0.966	1.000	0.80	1.004	1.009	1.004
0.98	0.835	0.928	0.922	0.96	0.888	0.949	0.996	1.00	1.004	1.000	1.000
1.48	0.831	0.926	0.902	1.60	0.854	0.947	0.981	1.60	1.004	0.976	0.978
1.96	0.809	0.913	0.891	1.92	0.854	0.942	0.961	2.00	1.004	0.961	0.966
3.00	0.803	0.880	0.868	3.20	0.822	0.925	0.942	3.00	0.985	0.957	0.955
4.00	0.800	0.878	0.860	4.00	0.725	--	--	4.00	0.965	0.938	0.948
6.00	0.765	0.893	0.863	6.00	0.765	0.882	0.981	6.00	0.906	0.938	0.948
8.00	0.741	0.863	0.879	8.00	0.773	0.888	0.927	8.00	0.884	0.938	0.948

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Table II. The Coion Activity Coefficient Ratio γ_2/γ_2^0 Dependence on X for Sodium Dextran Sulfate.

X	0.00100M			0.00500M			0.0100M		
	Cl^-	Br^-	I^-	Cl^-	Br^-	I^-	Cl^-	Br^-	I^-
1.00	1.014	1.014	0.872	1.011	1.088	1.054	0.985	1.002	1.003
2.00	1.011	0.983	0.889	0.981	1.074	1.011	1.012	0.986	0.977
3.00	1.014	0.986	0.869	0.979	1.103	0.997	1.031	1.002	0.981
4.00	1.011	0.979	0.875	0.983	0.098	0.989	1.054	1.030	0.995
5.00	1.027	0.961	0.879	1.028	1.096	1.002	1.047	1.012	0.992
7.50	1.022	0.968	0.862	1.029	1.115	1.047	1.073	1.074	0.992
10.00	1.026	0.972	0.826	1.063	1.188	0.995	1.086	1.048	0.994

NaCarr polyion to the same extent. Also, for the 0.00100*M* NaCl and NaBr solutions, the γ_2/γ_2^0 ratios are consistently lower at each *X* value when compared to the 0.00500*M* and 0.0100*M* solutions, which is probably due to less screening of the charges on the polyion at the lowest simple salt concentration. The γ_2/γ_2^0 ratios for the three NaI concentrations closely approximate one another for each *X* value and the ratios for the 0.0100*M* NaI are close to the ratios for 0.0100*M* NaBr and NaCl for each *X* value. At the two lower simple salt concentrations the Cl^- and Br^- ions interact with NaCarr to the same extent, while at the highest simple salt concentration the Cl^-, Br^- and I^- ions interact to the same extent with this polyelectrolyte. Except for 0.00100*M* NaCl and NaBr, the γ_2/γ_2^0 ratios approximate unity at low *X* values, indicating that the coion interacts in solution as if the polyelectrolyte were *not* present. It should be noted that coion activity coefficients were also determined for NaCarr in 0.000500*M* simple salts. They were found to be close to the values found for 0.00100*M* simple salts and will be discussed below.

The γ_2/γ_2^0 results for NaDS are listed in Table II for 0.00100*M*, 0.00500*M* and 0.0100*M* NaCl, NaBr and NaI. With the exception of 0.00100*M* NaI, *all* the γ_2/γ_2^0 ratios closely approximate unity for the whole *X* range from one to ten. Similar results were obtained with sodium polyphosphate for all three simple salt concentrations (25). From these coion activity coefficient measurements, it appears that the monovalent coions interact with the polyion *only* at very low simple salt concentrations, where the Debye-Hückel atmosphere is largest.

The Manning theory for polyelectrolyte solutions gives for monovalent coion activity coefficients (2)

$$\gamma_2 = \exp\left[-\frac{1}{2}\,\xi^{-1}X/(\xi^{-1}X + 2)\right] \qquad (2)$$

where only Debye-Hückel interactions with the polyelectrolyte are considered. For a given polyelectrolyte ξ is fixed, so eqn (2) predicts that γ_2 decreases as *X* increases until high values of *X* cause γ_2 to level off. Since only 0.00100*M* NaCl and NaBr solutions of NaCarr indicate coion-polyion interaction over an extended range, these results will be used to test eqn (2), along with values for 0.000500*M* NaCl and NaBr. For 0.000500*M* NaI containing NaCarr it was found that γ_2/γ_2^0 approximated unity over the whole *X* range. The results for 0.000500*M* and 0.00100*M* NaCl and NaBr solutions containing NaCarr are presented in Figures 1 and 2, respectively, where $b = 4.4\text{Å}$ and $\xi = 1.62$. It should be noted that the small ion-small ion correction of Wells (26)

need not be applied because the ratio γ_2/γ_2^0 was used. Also, the somewhat difficult extrapolation of the data to zero ionic strength at a given X value suggests that only the lowest simple salt concentrations be correlated to the theory when an ionic strength dependence is present. The agreement between Manning's predicted values and experimental values is quite good over most of the range of X. Using cluster expansion theory for polyelectrolyte solutions, Iwasa and Kwak (27) recently predicted an appreciate difference between the activity coefficients of counterions and coions. From the contribution of higher order cluster terms to the activity coefficient of small ions in polyelectrolyte solutions, it was shown that for $\xi > 1$ considerable asymmetry between γ_1 and γ_2 values, with the values of γ_2 approximating unity. While this theory might better explain our results for γ_2, further evaluation awaits near future publications.

Chloride ion and sodium ion self-diffusion measurements were performed on aqueous solutions of NaCarr (6) and NaDS (28) in 0.00050*M*, 0.0010*M*, 0.0050*M* and 0.010*M* NaCl. The same ionic polysaccharide samples were used as those employed in the present study. When compared to the coion diffusion coefficients in the absence of polyelectrolyte D_2^0, the coion diffusion coefficients D_2 for Cl^- ion showed the same trend for NaCarr and NaDS. The D_2/D_2^0 ratios decreased from unity for $X < 1$ and leveled off at approximately $D_2/D_2^0 = 0.9$ for $X < 1$. The predicted values from Manning's theory were closely obeyed over the whole X range. It should be noted that for NaDS, the D_2/D_2^0 values were below unity for all NaCl concentrations, while the γ_2/γ_2^0 values were unity for almost every concentration. This probably reflects the greater sensitivity of the diffusion measurements in monitoring Debye-Hückel atmosphere interactions as compared to activity coefficient measurements. It is difficult to detect the effect of the interactions between coions and polyelectrolytes with activity coefficient measurements because of the high background level due to small ion-small ion interactions.

Sodium ion activity coefficients γ_{Na^+} were determined in aqueous NaCarr solutions containing 0.000500*M*, 0.00100*M*, 0.00500*M* and 0.0100*M* NaCl, NaBr and NaI at 25°C. The results are presented in Figures 3, 4 and 5, where it can be seen that for each simple salt concentration $\gamma_{Na^+}/\gamma_{Na^+}^0$ decreases as X increases and levels off approximately $X > 5$. While the experimental points are close in value for $X > 2$, the simple salt concentration dependence is evident for higher X values, where at a given X value γ_{Na^+} decreases as the normality of

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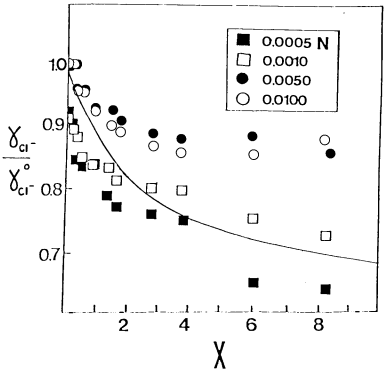

Figure 1. The dependence of the Cl^- ion activity coefficient ratio on X in NaCl solutions containing NaCarr. The solid line is predicted from Manning's theory.

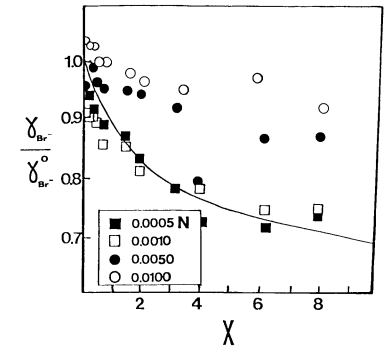

Figure 2. The dependence of the Br^- ion activity coefficient ratio on X in NaBr solutions containing NaCarr. The solid line is predicted from Manning's theory.

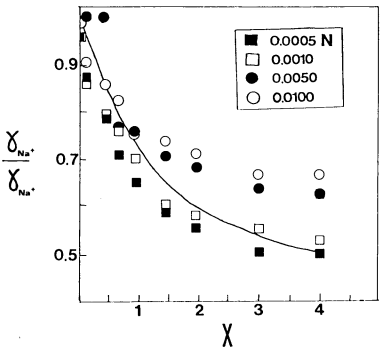

Figure 3. The dependence of the Na^+ ion activity coefficient ratio on X in NaCl solutions containing NaCarr. The solid line is predicted from Manning's theory.

simple salt decreases. Also note that γ_{Na+} is fairly independent of the nature of the coion at all concentrations. It is gratifying that the results for γ_{Na+} reported by Pass, Phillips and Wedlock (19) for 0.0050*M* and 0.010*M* NaCl are in excellent agreement with ours.

Sodium ion activity coefficients were determined for 0.00100*M*, 0.00500*M* and 0.0100*M* NaCl, NaBr and NaI solutions containing NaDS. The results are presented in Figures 6, 7 and 8, where it can be noted the experimental values of $\gamma_{Na+}/\gamma^0_{Na+}$ at the corresponding X values are practically superimposable for *all* three simple salts used. Thus, γ_{Na+} appears to be independent of the nature of the monovalent coion. Also, little ionic strength dependence is noted at each X value. As compared to $\gamma_{Na+}/\gamma^0_{Na+}$ values obtained for NaCarr, the values appear to be lower for NaDS at corresponding X values. This is probably due to the higher charge density of the NaDS chain with b = 2.5Å and ξ = 2.85.

To correlate the experimental data to the predicted values from the line-charge model of Manning, the results should be extrapolated to zero ionic strength. We avoided this because the two lowest simple salt concentrations used for NaCarr and for NaDS solutions were very close in $\gamma_{Na+}/\gamma^0_{Na+}$ values for each value of X. Also, extrapolating the data at these very low concentrations used, i.e., 0.000500*M* and 0.00100*M* salt, would give only a small correction.

The theoretical equation for the counterion activity coefficient γ_1 for $\xi > 1$ is (2)

$$\gamma_1 = (\xi^{-1}X + 1)(X + 1)^{-1}\exp\left[-\frac{1}{2}\,\xi^{-1}X/(\xi^{-1}X + 2)\right] \quad (3)$$

where $(\xi^{-1}X + 1)(X + 1)^{-1}$ represents the fraction of total uncondensed counterions. When compared to the experimental points for NaCarr, the solid theoretical lines in Figures 3, 4 and 5 indicate that excellent agreement is obtained for all simple salt concentrations below X = 1 and over the whole range of X for the two lowest simple salt concentrations employed. Similar findings are evident from Figures 6, 7 and 8 for NaDS at low X values, with small negative deviations from the theoretical values at the higher X values. Wells (11) reported that the thermodynamic measurements obtained for aqueous NaCl solutions containing NaDS were in excellent agreement with the theoretical values from the Manning theory. The NaDS sample he used was almost identical to ours with respect to charge density and molecular weight. Upon comparing the reported (11) NaCl mean activity coefficients to our experimental values for $(\gamma_{Na+}\gamma_{Cl-})^{\frac{1}{2}}$, we find very good agreement over the

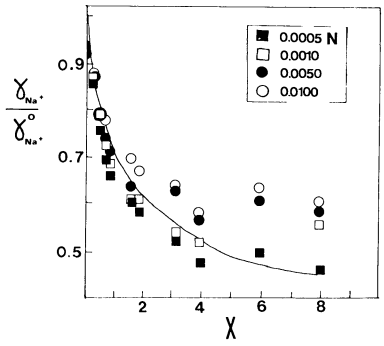

Figure 4. The dependence of the Na^+ ion activity coefficient ratio on X in NaBr solutions containing NaCarr. The solid line is predicted from Manning's theory.

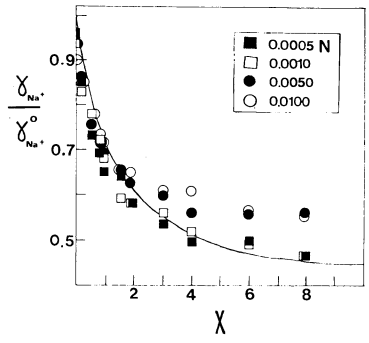

Figure 5. The dependence of the Na^+ ion activity coefficient ratio on X in NaI solutions containing NaCarr. The solid line is predicted from Manning's theory.

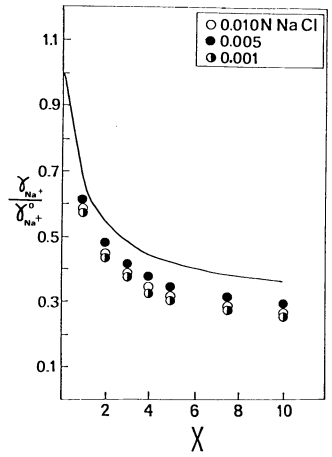

Figure 6. The dependence of the Na^+ ion activity coefficient ratio on X in NaCl solutions containing NaDS. The solid line is predicted from Manning's theory.

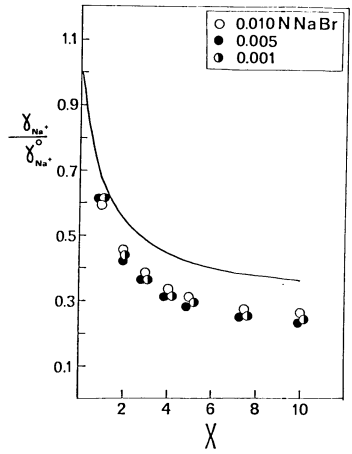

Figure 7. The dependence of the Na^+ ion activity coefficient ratio on X in NaBr solutions containing NaDS. The solid line is predicted from Manning's theory.

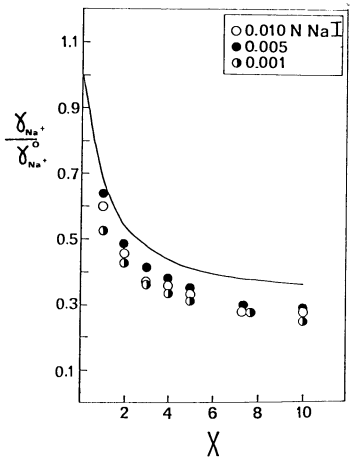

Figure 8. The dependence of the Na^+ ion activity coefficient ratio on X in NaI solutions containing NaDS. The solid line is predicted from Manning's theory.

same X range, giving substantiation to our single ion activity results.

The Na^+ ion self-diffusion coefficients for NaDS in NaCl solutions (28) follow the same trend as does $\gamma_{Na^+}/\gamma^0_{Na^+}$ vs. X in Figure 6. The sharp initial decline followed by a leveling off of $D_{Na^+}/D^0_{Na^+}$ is in very good agreement with the values predicted from Manning's theory. This is further evidence of the validity of the counterion condensation concept and the ionic atmosphere-polyion interaction concept which are paramount in the theory.

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Abstract. Chloride, bromide, iodide and sodium ion activity coefficients have been determined at 25°C for aqueous solutions of sodium iota carrageenan and sodium dextran sulfate over a large range of ionic polysaccharide concentrations for 0.000500*M*, 0.00100*M*, 0.00500*M* NaCl, NaBr and NaI. The equivalent concentration ratios of ionic polysaccharide to simple salt was varied from 0.10 to 8.0 for sodium iota carrageenan and 1.0 to 10.0 for sodium dextran sulfate. The experimental results are discussed in light of the monovalent coion and monovalent counterion interactions with the polyelectrolyte according to the modern theory of polyelectrolyte solutions by Manning.

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Interaction of Sulfated Polysaccharides with Counter-Ions

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The interaction between inorganic counterions and polyanions has been extensively studied with particular reference to the relative order of cation binding strengths. The interaction may be treated theoretically in terms of condensation of the cations on to the polyanion under the influence of the charge density of the polyanion and electrostatic interaction of uncondensed counterions with the polyanion[1,2]. The theoretical treatment has been applied to the interaction of counterions with a variety of polyanions[3-11], including sulphated polysaccharides, and is here applied to the carrageenans. The interaction of metal ions with carrageenan is of critical importance in controlling its behaviour as stabiliser in milk products. For this reason we have here investigated the interaction of sodium, potassium and calcium counterions with κ-, ι- and λ- carrageenan. An e.m.f. method, using ion selective electrodes, was utilised to yield activity coefficient data, both in the presence and absence of added simple electrolyte. We have extended the range of counterions for which a relative order of binding strength can be determined using a simple technique[12], in which a cationic dye, Methylene Blue, which undergoes a strong metachromatic spectral shift, is displaced by a competing metal counterion in the form of added simple electrolyte. The concentration of added simple electrolyte required for complete suppression of dye binding, and consequent reversal of metachromasia, gives a qualitative order of cation binding affinity[13]. Highly charged sulphated polyanions such as the carrageenans, with their associated counterions, cause electrostriction of large amounts of solvent water molecules[14]. The condensed counterions investigated here, namely calcium, potassium and sodium, can exist in three states of hydration[15,16]. Changes in the state of hydration cause volume changes. This fulfills the conditions required for a pressure change to cause perturbation of the equilibrium which can be monitored indirectly by ultrasound attenuation measurements[17]. Ultrasonic waves effect the perturbation on a time scale compatible with the kinetics of the forward and reverse reactions. In the method, excess absorption

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of a polyion salt compared with the absorption of the equivalent concentration of the tetramethylammonium salt form is measured. This bulky reference ion has a low affinity for the polyion and allows, to a reasonable approximation, a measure of the excess absorption, resulting from relaxational processes arising from the counterion binding phenomenon. Absorptions due to other processes such as polymer motions are effectively cancelled out. Excess absorptions so obtained can be related to the square of the volume change involed.

In addition to electrostriction of water by the intense electric field of the strongly electrolytic sulphate ester groups of carrageenan, hydration of carbohydrates[14] can arise also from interaction of solvent water with the polar -OH groups of the sugar units. This behaviour causes considerable amounts of water to be bound to the carrageenan[18], and is one of the major factors influencing the rheology of carrageenan solutions. This is another important factor in establishing the effectiveness of carrageenans as stabilisers in frozen milk products, since the resulting reduced diffusion[19] rate of water prevents ice crystal growth and changes in product texture. For this reason we have devised a differential scanning calorimetric method for measuring the non-freezing (i.e. bound) water in a series of solutions of sodium salts of κ- and λ- carrageenan.

Although protein-polysaccharide interactions have generally been widely studied, milk protein-stabiliser interactions have been neglected[20-23]. Such interactions also exert a major influence on the quality of frozen dairy products. Protein instability, during processing and storage of such products, is undesirable. Whereas carboxymethlcellulose, guar gum and locust bean gum tend to cause protein destabilisation, carrageenan can prevent it. κ- and λ- Carrageenans react in milk systems only with casein proteins and no complexes are detectable in the whey. This reaction is dependent upon the presence of calcium ions. The effect of several hydrocolloids on the stabilisation of α_s^1 casein and casein micelles [20,24,25] have been tested, for example, locust bean gum and guar gym of the neutral hydrocolloids; gum arabic, C.M.C., pectin, hyaluronic acid and alginates of the carboxylate polyanions and agarose, heparin, chondroitin sulphates, cellulose sulphate, fucoidan and carrageenan of the sulphated polyanions. Only carrageenan induced significant stabilisation against precipitation by calcium ions at pH 6·8, indicating its unique value within stabiliser mixtures. κ- Carrageenan was twice as effective as λ- carrageenan and complete stabilisation was effected at a ratio of 5:1 casein: κ- carrageenan and 2·5:1 casein : λ- carrageenan. The complex formed between α_s^{1-} casein and κ- carrageenan was the more stable and resisted the desruptive influence of calcium ions and heat to a greater extent than the corresponding α_s^{1-} casein / κ- casein complex. A variety of techniques have been used to study such milk protein - polyanion interactions, for example light scattering[26], polyacrylamide gel

electrophoresis[27], and rheological techniques[22]. Here we describe a new method using pulse radiolysis to study the interaction between α_s^1 casein and carrageenan in the presence of calcium ions. The method was previously used in our laboratory to study other polyanion - cation interactions[13].

Results and Discussion.

Measurements with Ion Selective Electrodes. Values of a_+, where a_+ is the cationic activity of a polyelectrolyte or polyelectrolyte/simple salt solution were obtained using calibrating solutions of the appropriate chloride salt and single cationic activity values for the chloride salts, calculated from the extended Debye-Hückel equation for aqueous solutions at 25°C, with the ion size parameters of Kielland[28]. Together with values of m_+, the molal concentration in equivalents of counterion /kg, the single ion activity coefficients are given by the usual relationship, eq (1).

$$\gamma_+ = \frac{a_+}{m_+} \quad (1).$$

For all carrageenan types, we find (Figs. 1, 2) that single ion activity coefficients of the monovalent ion salts in pure polyelectrolyte solution are greater for Na^+ than for K^+, which is in accord with previous measurements on sulphated polysaccharides[6,7,29].

The activity coefficients increase slightly with dilution for κ- carrageenans, but are relatively constant with dilution for ι- and λ- carrageenans in monovalent ion forms. The activity coefficient values at the most dilute concentration can be related to the linear charge parameter ξ, (Fig.3), calculated from eq(2), for monovalent-monovalent ion interactions.

$$\xi = \frac{e_o^2}{\varepsilon k T b} \quad (2).$$

with e_o = electronic charge, ε= dielectric constant of pure water, k = Boltzmann's constant, T = absolute temperature, b = average distance between charges. A value for b was obtained from measurements of molecular models in their most extended form, giving a mean value of 0·445nm for the length of a sugar unit in a carrageenan molecule, and taking into account the degree of substitution of the sample. The theoretical limiting value of the activity coefficient is given by the solid line when applying Manning's condensation treatment[2] as given by eq. (3) for univalent cations,

$$\gamma_+ = \frac{\exp(-\frac{1}{2})}{|z|\,\xi} \quad (3).$$

where $|z|$ is the magnitude of the charge on the counterion. Our results provide better agreement for potassium salts of the carrageenans with the theoretical limiting activity coefficient, than with the sodium salt forms. This would be anticipated since the nature of the selective interaction of the potassium and

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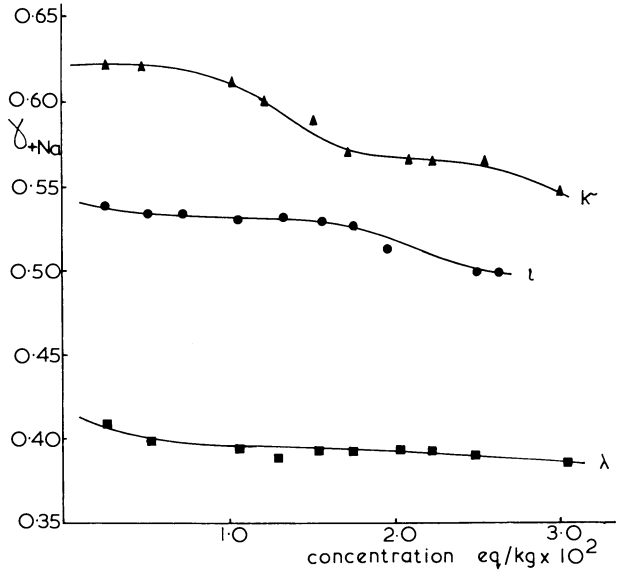

Figure 1. Activity coefficient of sodium counterions vs. concentration for κ, ι, and λ-carrageenan

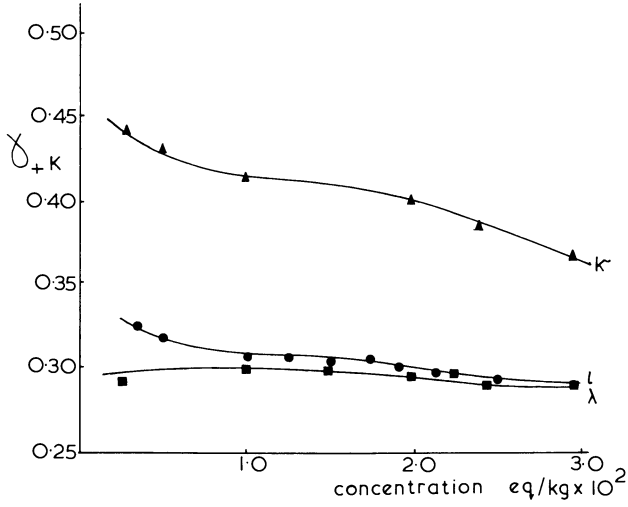

Figure 2. Activity coefficient of potassium counterion vs. concentration for κ-, ι-, and λ-carrageenan

sodium counterions is electrostatic ion binding in a mainly hydrated ion form. The smaller hydrated cation[28] K^+, should be more strongly bound than Na^+, and should coincide more nearly with Manning's limiting value of γ_+, as it approaches the point charge approximation of this theory. The relative constancy of activity coefficients as a function of concentration indicates that these polyanions approximate to a rigid line charge, not coiled, within the concentration range investigated.

The activity coefficients of the calcium salts in κ- and λ-carrageenan are shown as a function of concentration in Fig.4. Again the λ form shows a greater constancy with concentration than the κ- form supporting the view that this fraction with its higher linear charge density, is more rod-like. Comparing the activity coefficients of the Na^+ and Ca^{2+} salts of κ- and λ- carrageenan (Fig.5) over a range of concentrations, indicates that they are in the ratio of ca. 2:1, which is in good agreement with the requirements of eq (3). Calcium and sodium were the ions used for the comparison since they have almost the same hydrated ion size[28]. It is observed that both κ- and λ- carrageenans exert a considerable calcium ion sequestering effect in solution.

The lower activity coefficient for the K^+ salt of each carrageenan compared to the Na^+ salt indicates electrostatic binding of the hydrated ion, when the smallest hydrated ion would be most strongly bound. This is in accord with the view that the interaction of the sulphate ester group with the cation is less than the interaction of water of hydration with the cation[30]. Consequently it is energetically unfavourable for the sulphate ester group to displace water from around a sodium or potassium cation. The results of our ultrasonic relaxation studies also support this conclusion.

Thus the smallest hydrated cation will bind most strongly, for cations of the same charge. The reverse situation would apply with carboxylic and phosphated polyanions, which would account for the now well established reversal of affinity sequence when comparing sulphate with phosphate and carboxylate polyanions[12]. Satake et al[5] and Noguchi et al[17], observed the same trend with sodium and potassium dextran sulphates.

We now consider polyelectrolytes with added simple electrolytes. Manning[2] derived an equation for single counterion activity coefficients of univalent ions in a polyelectrolyte plus added salt system for $\xi > 1$, eq.(4).

$$\gamma_+ = (\xi^{-1} X + 1)\ (X + 1)^{-1} \exp \frac{(-\frac{1}{2}\ \xi^{-1} X)}{(\xi^{-1} X + 2)} \quad (4)$$

Where $X = n_e/n_s$ is the ratio of the number of equivalents of polyelectrolyte counterion to the number of equivalents of simple electrolyte counterion in unit volume of solution. Fig.6 shows for ι- carrageenan that solutions most dilute in simple salt (5×10^{-3} m), where m is the molal concentration in eq /kg, give the best correspondence between theory and practice. Similar

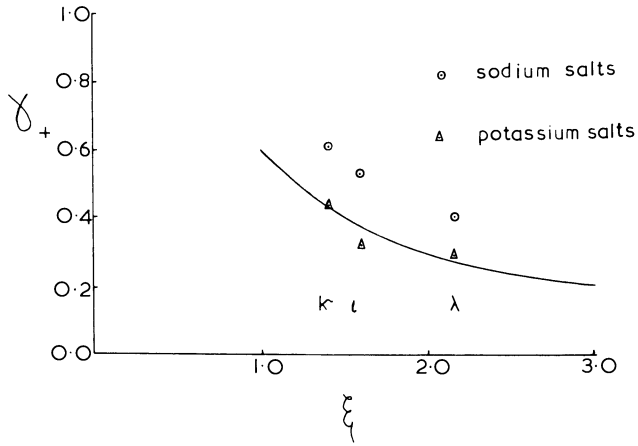

Figure 3. Activity coefficients of counterions in most dilute solution vs. linear charge parameter

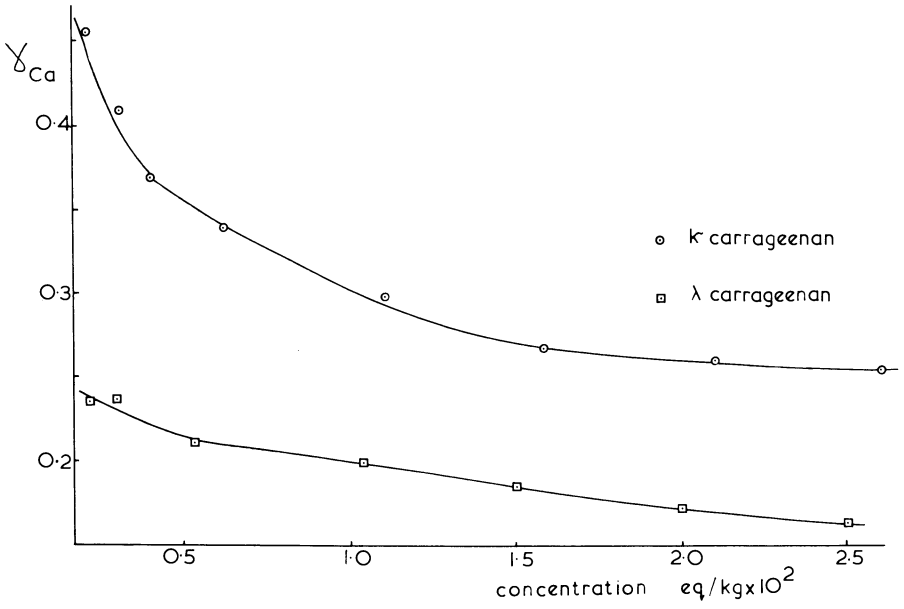

Figure 4. Activity coefficient of calcium counterion vs. concentration for calcium salts of κ- and λ-carrageenan

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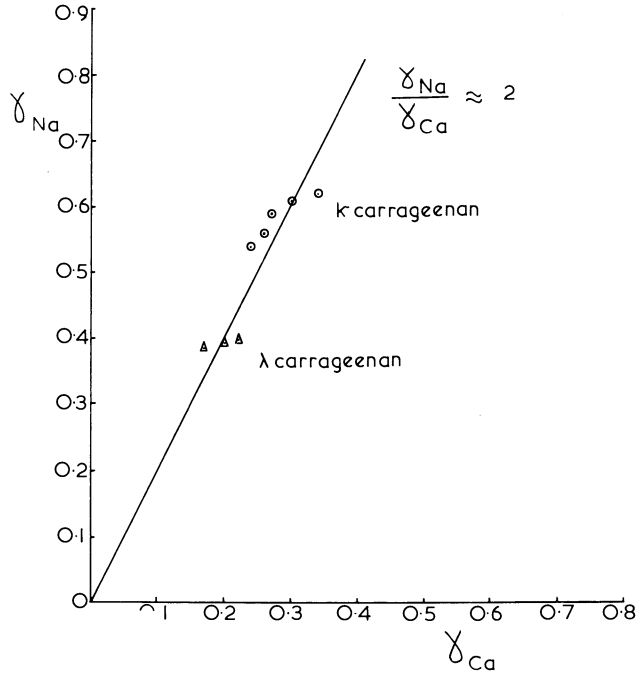

Figure 5. Activity coefficients of sodium counterions vs. activity coefficients of calcium counterions at identical concentrations for κ- and λ-carrageenans

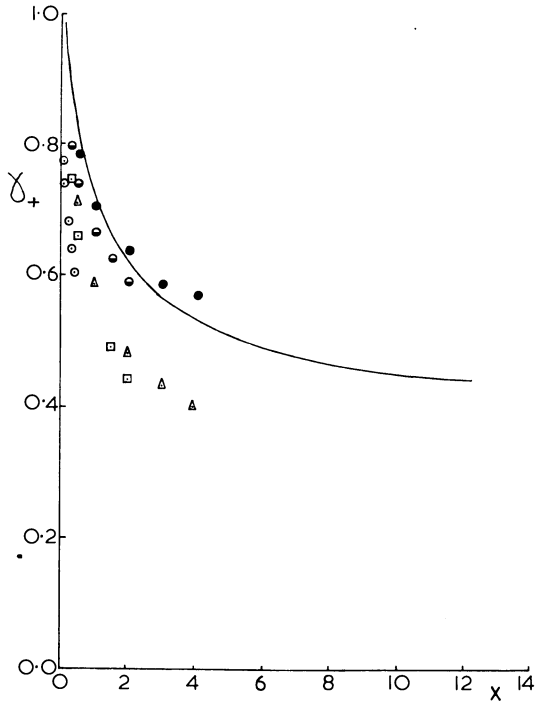

Figure 6. Activity coefficient of common cation in ι-carrageenan/simple electrolyte mixture, uncorrected for small ion–small ion interactions. Comparison with theoretical curve according to Equation 3. NaCl: (●) 5 × 10^{-3}m; (◒) 1 × 10^{-2}m. KCl; (△) 5 × 10^{-3}m; (⊡) 1 × 10^{-2}m; (⊙) 5 × 10^{-2}m.

results were obtained for κ- and λ- carrageenan[29]. The correspondence becomes less satisfactory as the concentration of simple salt increases. This could be due to the neglect of mutual interactions between ions of the simple electrolyte which is inherent in Manning's assumptions[2]. An empirical correction of mean activity coefficients has been proposed in which the mean activity coefficient of the ions in the pure simple salt solution is taken as a measure of their nutual interaction, the the presence of polyelectrolyte[31]. If a similar correction is applied to the single ion activity coefficient this may be written in the form, eq (5).

$$\ln \gamma_+ = \ln \gamma_+^{p} + \ln \gamma_+^{c} \quad (5)$$

where γ_+ is the single ion activity coefficient of the cation determined experimentally, γ_+^{p} is the single ion activity coefficient calculated according to Manning, γ_+^{c} is the single ion activity coefficient of the cation in the absence of polyelectrolyte and its counterions. At low concentrations of simple electrolyte, $\gamma_+^{c} \rightarrow 1$ and $\gamma_+ = \gamma_+^{p}$ in agreement with (Fig.6). The corrected activity coefficients are shown in (Fig.7), and provide much better agreement with the values calculated from Manning's theory. A selective ion binding process is still observed, even when there is considerable excess of simple electrolyte. This is further borne out by the results obtained using the Methylene Blue (MB^+) dye displacement technique[12]. It was found that the limiting salt concentration (LSC) required to remove completely the MB^+ from 2/1 site / dye ratio polyanion-dye complexes decreases in the order Li>Na>K>Cs and Mg>Ca>Sr for λ- and κ- carrageenans, where appropriate, (Table I). For κ- carrageenan, neither e.m.f. nor dye displacement measurements were carried out in the presence of potassium or caesium chloride because of the tendency of κ- carrageenan to gel in the presence of these electrolytes. Our results, therefore, again support the view that the strength of ion binding is greatest for the smallest hydrated ion. The strength of binding is, for both carrageenan fractions, in the order Sr>Ca>Mg and Cs>K>Na>Li, and selective ion binding interactions are still manifest in systems of high ionic strength. This observation could have biological significance, pointing to ionic interactions of glycosaminoglycans occurring in the connective tissue matrix.

Measurements were carried out using a calcium electrode on calcium salts of λ- and κ- carrageenan in the presence of calcium chloride. Here a useful parameter is the empirical rule of additivity of activities[32]. In summary this states that a counterion activity of a pure polyelectrolyte solution, a_+^{p}, can be added to a counterion activity in a simple salt solution, a_+^{s} to give a calculated activity for the mixture a_+^{calc}, not significantly different in many instances from the observed value a_+^{obs} for the mixture. Deviations from this rule can be expressed

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TABLE 1

Effect of inorganic ions on carrageenan-Methylene Blue interactions. Site/Dye ratio 2/1. Dye 1 x 10^{-5}M.

Lambda carrageenan

	Li	Na	K	Cs
LSC x 10^{-2}M	18.20	12.20	8.32	7.59
	Mg	Ca	Sr	
LCS x 10^{-2}M	11.50	8.75	6.03	

Kappa carrageenan

	Li	Na		
LSC x 10^{-2}M	24.40	14.45		
	Mg	Ca	Sr	
LSC x 10^{-2}M	19.95	13.18	8.13	

$$\Delta a_+ = \frac{a_+^{calc} - a_+^{obs}}{a_+^{obs}} \times 100$$

Although the rule is only empirical, many instances of very good agreement have been shown including monovalent ion carrageenan salts plus monovalent ion chlorides[33]. For sodium dextran sulphate and added sodium chloride, all deviation were within 1.5%[7]. However for our measurements of calcium salts of λ- and ϰ- carrageenan plus calcium chloride, Δa_+ values are significantly greater (Table II). For ϰ- carrageenan, in dilute simple salt solution, values of Δa_+ are 26%, greater than observed values of Δa_+ for λ- carrageenan. This does not conform to the general rule that additivity is more closely obeyed by polyions with lower degrees of substitution i.e. ϰ- carrageenan should give better agreement. It is therefore indicated that extra binding of calcium ions occurs with ϰ- carrageenan and probably a stoichiometry of one calcium ion per ester sulphate unit is being approached.

Lyons and Kotin[32] observed similar behaviour with the magnesium salts of DNA with added magnesium chlorides, where Δa_+ values up to ca. 30% were observed when the simple salt was present in low concentration. It is possible that calcium ions might be binding thus:

$$\left.\begin{matrix} OSO_3 & Ca^+ & Cl^- \\ OSA_3 & Ca^+ & Cl^- \\ OSA_3 & Ca^+ & Cl^- \end{matrix}\right\}$$

Here calcium ions are represented as site bound, with the result that the charge on the carrageenan is reversed. Viewed from the solvent the segment appears to be a polycation with negative univalent counterions. Such charge reversal phenonenon are comparatively common and have been involved to account for the reversal in the sign of electrophoretic mobility of polyvinylpyrdinium bromides in excess KBr[34].

Our results indicate that calcium ϰ- carrageenan has a greater tendancy to undergo site binding than calcium λ- carrageenan. These results are in agreement with the work of Hansen[20, 24, 25] who found that the stabilisation of α_S- casein by carrageenan against precipitation of calcium ions was greater with ϰ- carrageenan than with λ- carrageenan. The interaction between carrageenan and the casein proteins was shown to be dependent in some measure on the presence of calcium ions, in as much as the complex formed is a calcium / carrageenan / α_S- casein complex. If in the presence of excess calcium ions the ϰ- fraction shows an enhanced ability to complex calcium ions from solution, then it would aid formation of the protein-carrageenan complex. Moreover, our ultrasonic relaxation studies show an anomalous behaviour for the relative volume changes in calcium ϰ- and λ-

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TABLE II

Additivity of calcium ion activities for polyanion + salt

(a) Counterion activities in Ca λ- carrageenan - $CaCl_2$ systems

$m_+^p \times 10^2$	$a_+^p \times 10^2$	$m_+^s \times 10^2$	$a_+^s \times 10^2$	$a_{obsd} \times 10^2$	Δa_+ %
0.106	0.025	0.500	0.375	0.386	3.3
0.252	0.055	0.500	0.374	0.406	5.8
0.519	0.104	0.500	0.374	0.427	12.0
0.782	0.143	0.500	0.374	0.455	13.6
1.023	0.176	0.500	0.374	0.482	14.1

(b) Counterion activities in Ca κ- carrageenan - $CaCl_2$ systems

$m_+^p \times 10^2$	$a_+^p \times 10^2$	$m_+^s \times 10^2$	$a_+^s \times 10^2$	$a_{obsd} \times 10^2$	Δa_+ %
0.098	0.040	0.500	0.374	0.384	7.9
0.259	0.087	0.500	0.374	0.399	15.6
0.512	0.152	0.500	0.374	0.440	19.5
0.765	0.205	0.500	0.374	0.469	23.4
0.985	0.258	0.500	0.374	0.504	25.5

carrageenan ion binding processes, but can be explained by site binding in calcium κ - carrageenan. A unique calcium / κ - carrageenan / α_s casein interaction was also detected by pulse radiolysis supporting the view advanced here.

Ultrasonic Relaxation Measurements. Zana and Tondre[15, 16] have given the theory and assumptions underlying this technique. We have measured the quantity $\Delta\alpha/f^2$ as a function of f where, f= ultrasonic frequency and $\Delta\alpha$= ultrasound absorption coefficient of polyanion salt minus the ultrasound absorption coefficient of an equivalent concentration of the tetramethylammonium (TMA) salt form.

We ascribe any excess absorption of a polyion salt over its TMA salt form to a relaxation or relaxations arising from the counterion binding phenonenon (Figs. 8, 9). From a comparison of plots of proportional values of $\Delta\alpha/f^2$vf for monovalent and divalent ion salts of polyphosphate and carboxylic polyanions and carrageenans, it is evident that the carrageenans show the weakest relaxations. This a feature of sulphated polyanions, for example, sodium poly(ethylenesulphonate)[15]. The low frequency absorptions, below 4MHz are proportional to concentration (Figs. 10, 11). This is indication of the relaxation arising by monomolecular processes [15]. The low frequency absorption can be ascribed to a relaxation of the outer sphere/inner sphere equilibrium. Any relaxation can be characterised by a relaxation amplitude, A, and a relaxation frequency, f. As a result of practical difficulties in measuring values of $\Delta\alpha/f^2$ below 1MHz, actual values of the relaxation amplitude cannot be calculated. However, generally, values of the relaxational frequency for inner sphere complex formation do not show marked dependence on the nature of the counterion for counterions of the same valency and also show smaller differences than anticipated between monovalent and divalent counterions. The values of $\Delta\alpha/f^2$ at any particular frequency therefore, should be in proportion to the relaxation amplitude, given by eq.(6).

$$A = \Delta Vo^2 \quad \tau \quad f(k_1, k_{-1}, c_i). \quad (6)$$

Where ΔVo for the counterion binding process is essentially due to the volume change from release of electrostricted water by the polyion and water of hydration by the counterion. The relaxation time (τ) characterises the process and $f(k_1, k_{-1}, c_i)$, is a function of the forward and reverse rate constants for the equilibrium, and the concentration c_i of the participating species. From Figs. 8, 9 it can be seen that the total volume change on counterion binding to both λ - and κ - carrageenan, is in the order Ca>k>Na.

Values of $\Delta\alpha/f^2$ for κ - carrageenan in the calcium and potassium salt forms are greater than those for λ - carrageenan at the same concentrations of counterion. It was found for poly(styrenesulphonate) (PSS) that values of $\Delta\alpha/f^2$ were less than

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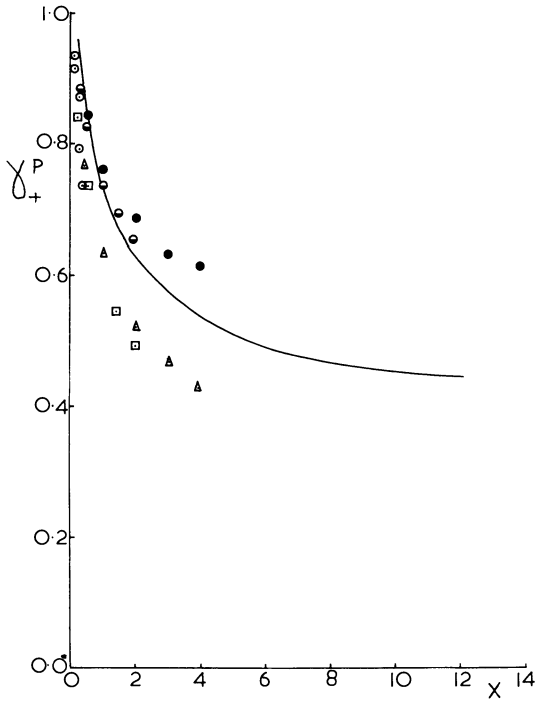

Figure 7. Activity coefficient of common cation in ι-carrageenan/simple electrolyte mixture, corrected for small ion–small ion interactions (Same symbols as for Figure 6)

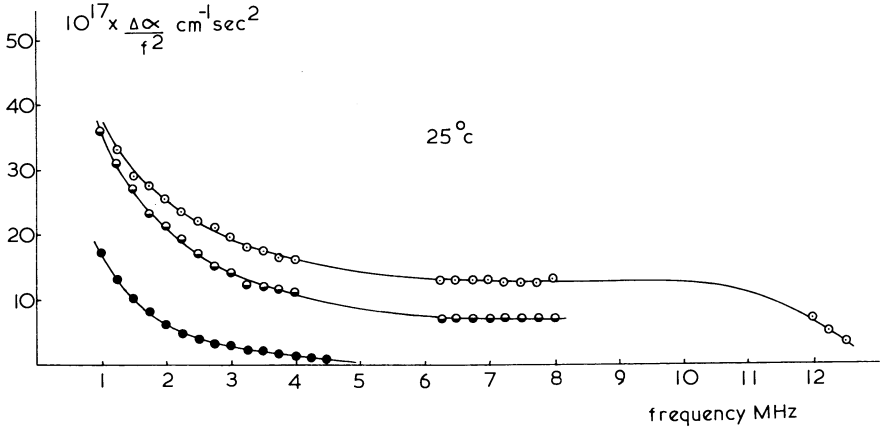

Figure 8. Plots of ultrasonic absorption $\Delta\alpha/f^2$ for 0.04N aqueous solutions of calcium (⊙), potassium (◒), and sodium (●) salts of κ-carrageenan

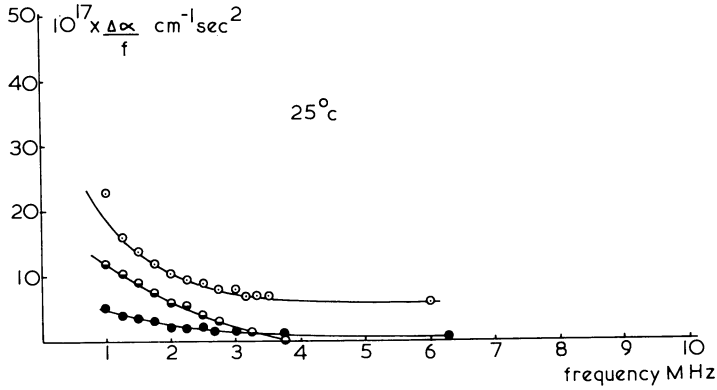

Figure 9. Plots of ultrasonic absorption $\Delta\alpha/f^2$ for 0.04N aqueous solutions of calcium (⊙), potassium (◒), and sodium (●) salts of λ-carrageenan

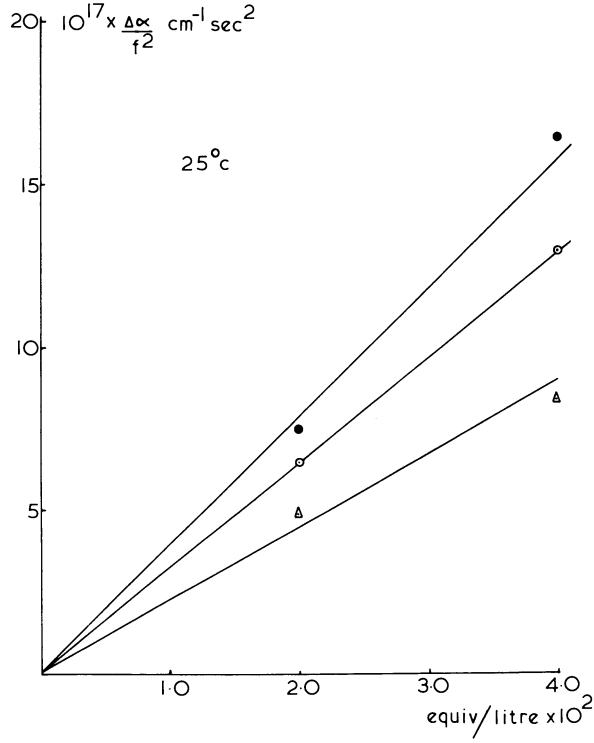

Figure 10. Variation of ultrasonic absorption $\Delta\alpha/f^2$ at particular frequencies for sodium salts of κ-carrageenan, as a function of polyanion concentration

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for poly(ethylenesulphonate) (PES)[16]. While the linear charge parameter of these two polyions is virtually the same, the minimum distance between two charges (d) on the rod-like model of these molecules is different: $d_{PES} > d_{PSS}$.

For samples of sodium carboxymethylcellulose of different degrees of substitution[35], $\Delta\alpha/f^2$ increased with increase in degree of substitution, and so relates to the decrease in minimum distance between charged sites. Since the expected trend $\Delta\alpha/f^2$ κ- carrageenan $< \Delta\alpha/f^2$ λ- carrageenan, anticipated by their relative degrees of substitution is not found, it would appear that the minimum distance between charges on λ- and κ- carrageenan are not solely related to the degree of substitution. Optical rotation studies of κ- carrageenan solutions have been interpreted on the basis of double helix formation and more recent work has also indicated that ι- carrageenan can form double helices in solution[36]. X-ray studies on oriented fibres of calcium salts of ι- carrageenan[37], show that the sulphate groups were in pairs unexpectedly close together. Moreover, ι- and κ- carrageenan have very similar conformational characteristics. At the concentrations used for this study, the onset of gel formation and hence double helix formation in solution is probable for κ- carrageenan[36]. Sulphate group proximities were established using crystalline samples of calcium ι- carrageenan in the double helical state, and these results were extrapolated to suggest that if double helix formation of ι- carrageenan occurred in solution cation fixation was necessary, with removal of water molecules from ionic hydration shells[37]. In this entropically unfavourable situation electrostatic attractions cannot completely offset such influences, and would not favour double helix stability. We, therefore, infer that in solutions of ι- and κ- carrageenans there is an equilibrium between random coil and double helical forms, with the fraction of polyanion in the double helix form being responsible for a very large volume change on counterion binding. This could be a satisfactory account for the relative magnitudes of $\Delta\alpha/f^2$ values for the κ- and λ- fractions and points to some site binding occurring in κ- carrageenan with those counterions most likely to cause gelling i.e. calcium and potassium.

Pulse radiolysis measurements.

For whole acid casein and carrageenan at pH6.6 a non-additive relationship is observed for k_1 values, (first order rate constants for the disappearance of the hydrated electron) in carrageenan and casein solutions, compared with mixtures with identical concentrations (k_1^{obs}). Interaction is, therefore, indicated[38].

A typical experiment is shown in Fig.12. After repeated experiments using a range of systems e.g. purified κ- carrageenan, purified λ- carrageenan and Gelcarin HMR (Algin Corporation of America), it was found that non reproducibility was a constant feature. This variability in k_1 values could be overcome if the

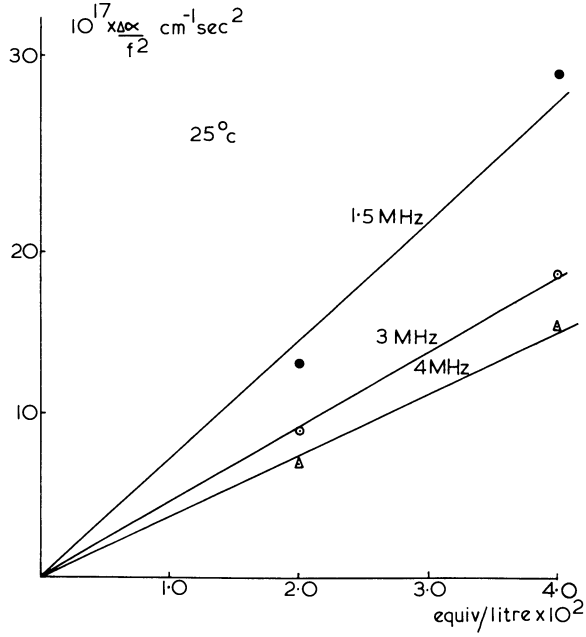

Figure 11. Variation of ultrasonic absorption $\Delta\alpha/f^2$ at particular frequencies for calcium salts of κ-carrageenan, as a function of concentration

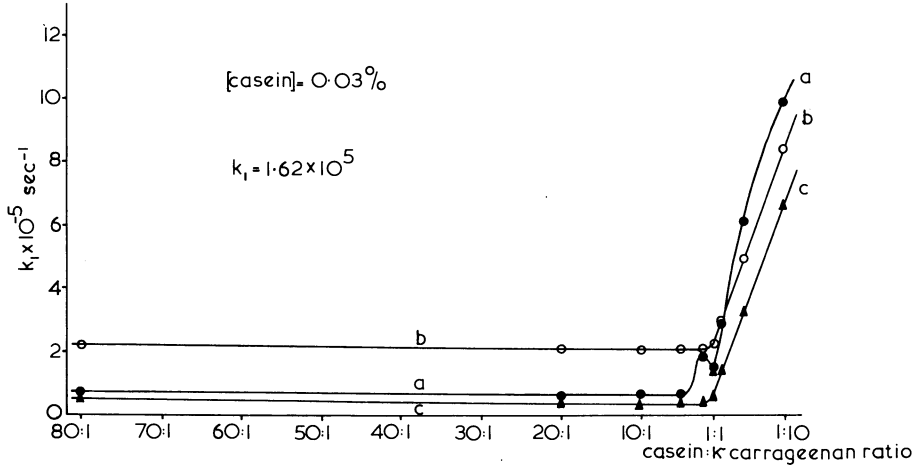

Figure 12. Plot of first-order rate constant for reaction of the hydrated electron with the casein: κ-carrageenan complexes

deoxygenation of the solution was carefully standardised. This mechanical perturbation influenced the polymeric systems. Fig.13, shows similar behaviour obtained for whole acid casein in the presence of CMC. Curve (a) $k_1$obs for mixture, curve (b) = k_1 protein +k_1 polysaccharide, and curve (c) =k_1 polysaccharide, applied throughout.

A linear increase in reactivity of casein towards e^-_{aq} with increasing sodium chloride concentration was found, but sodium chloride did not influence the reactivity of the polyanions. The interactions of carrageenan - casein and CMC - casein were unaffected by 10^{-1}M NaC1. Two types of interaction can be identified following the argon bubbling deaeration. 'Type A' occurs at protein: polyanion ratios of 80 : 1 to 1 : 1, leading to a decrease in the reactivity of the mixture towards e^-_{aq} (i.e. $k_1^{obs} < k_1^{protein} + k_1^{polyanion}$). 'Type B' interaction occurs at ratios of protein: polyanion from 1 : 1 to 1 : 10 and leads to an increase in e^-_{aq} reactivity of the mixture (i.e. $k_{obs} > k_{1protein} + k_{1polyanion}$).

Initially, based on previous experience[39, 40], 'Type A' interaction was concluded to be electrostatic in nature, due to interaction between negative sites on the polyanion and positive centres on the protein. 'Type B' was attributed to a structural change in the protein, making available more positive sites for e^-_{aq} attack as a result of interaction with the polyanion.

Later our experience indicated that at pH 6.6, where both polyanion and protein bear an overall negative charge (although positive sites are still available on the protein) electrostatic interactions of whole casein and α_s^1 casein with carrageenan and CMC do not occur. It is also doubtful whether structural changes in the protein are responsible for the 'Type B' interaction. It is more probable that the decrease in the reactivity of whole casein and α_s^1 casein towards e^-_{aq} with progressive argon bubbling is due to a surface phenonmenon.

When casein-carrageenan and casein-CMC interactions were studied using the standardised 'syringe technique' for deoxygenation[41], 'Type B' interactions were not found. Type 'A' interactions occurred at all ratios of protein : polyanion between 40 : 1 and 1 : 10. The interaction was unaffected by 10^{-1}M Na C1 indicating that 'Type A' interactions are not electrostatic in nature, which is additionally supported by our observation that 'Type A' interactions are present in succinyl casein and CMC mixtures. We are not able, therefore, to demonstrate by this technique that interation between positive sites on the protein and negative sites on the carrageenan occurs. 'Type A' interactions are observed at all concentration ratios investigated even in casein guar gum mixtures (Fig. 14) when components are not significantly charged.

We nevertheless find that the α_s^1 casein - carrageenan interaction is unique (Fig. 15) in that k_1 obs $\simeq k_1$ α_s^1 casein + k_1

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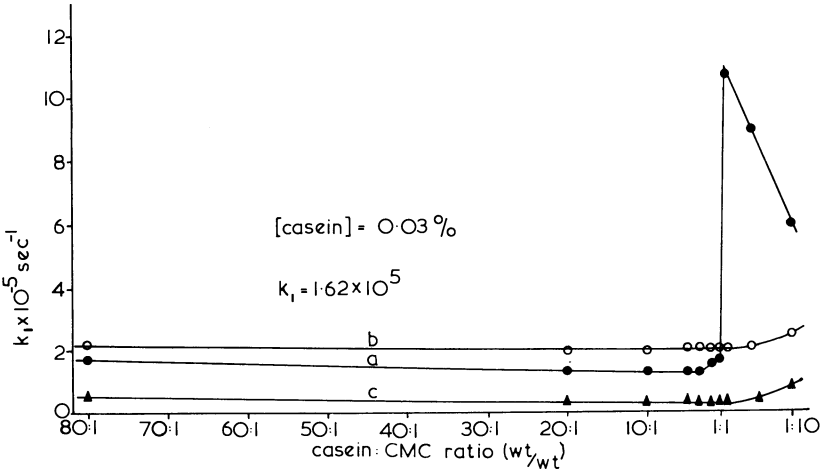

Figure 13. Plot of first-order rate constant for reaction of the hydrated electron with the casein: CMC complexes

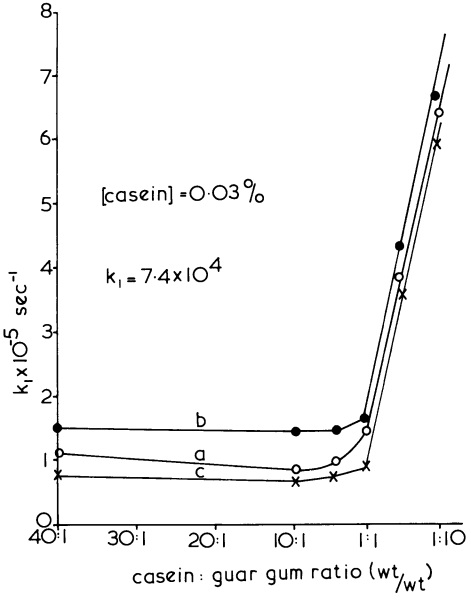

Figure 14. Plot of first-order rate constant for reaction of the hydrated electron with casein: guar gum complexes

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carrageenan at the protein : polyanion ratio of 1 : 5. In addition the admixture of 10^{-1}M $CaCl_2$ induces an increase in reactivity similar to the 'Type B' interactions observed in our initial experiments, although k_1 obs $\neq k_1\alpha_s^1$ casein + k_1 carrageenan. Lin and Hansen[25] have suggested that in the presence of Ca^{2+} ions, α_s^1 casein and carrageenan form micelles in a similar manner to α_s^1 casein and κ-casein. In the whole casein - CMC interaction at pH 6 · 6 it was shown that calcium ion bridges are not found since a 'Type A' interaction is observed both in the presence and absence of 10^{-1}M $CaCl_2$.

Therefore we conclude that polysaccharides generally affect the reactivity of milk proteins towards e^-_{aq}, but no electrostatic interaction can be demonstrated using pulse radiolysis. Since hydrophobic bonding is unlikely a satisfactory explanation for these effects must be awaited.

The thermodynamic incompatibility of high molecular weight species such as polyanions and proteins is a well established phenomenon[42]. A partial cause of this is 'volyme exclusion'[43] which renders the system less stable so that it can be totally destabilised, resulting in phase separation, by small changes in environment. The polysaccharide in solution is in a highly hydrated state. Fig.16 shows the results obtained with sodium salts of λ and κ- carrageenan for 'non freezing' water, sometimes termed 'bound water', using differential scanning calorimetry to determine the enthalpy of fusion of the carrageenan solutions[18]. Thus the polysaccharide occupies a certain, quite considerable, volume in the solution from which the protein is excluded. The effect is independant of pH, ionic strength and isoelectric point of the protein, but increases with the concentration of polysaccharide and the size of the protein. As the volume excluded increases, the activity of the protein solution increases and the protein approaches its solubility limit. Any effect which lowers the solubility further, e.g. heating or the presence of certain ions will then push the protein over its solubility limit resulting in phase separation.

It is not possible to explain our results completely in terms of volume exclusion, although it does undoubtedly contribute to these complex interactions which exert such a vital controlling influence on the quality of milk products during production and storage.

Experimental

E.m.f. procedures to give cationic binding data have been reported previously[29]. An E.I.L. model 1048-4 sodium ion selective electrode, a model 1057-2 potassium ion selective electrode and on Orion 92-20 calcium ion selective electrode were used with an E.I.L. model 7050 mV/pH meter. The dye displacement technique[12, 13] and the differential scanning calorimetric method for measuring water binding[14] have also been described.

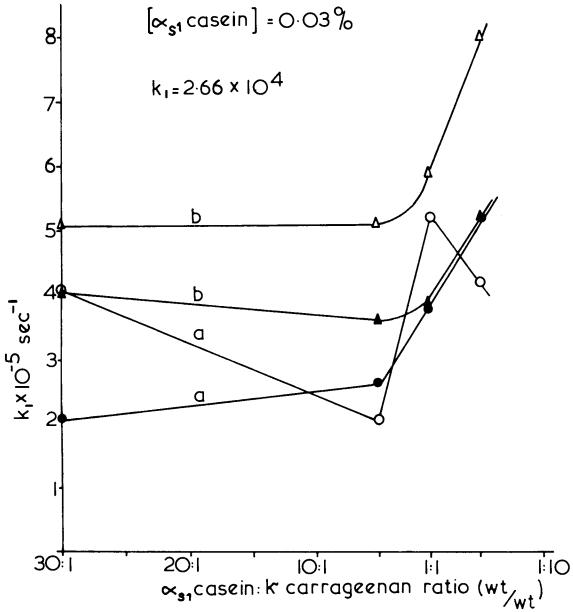

Figure 15. Plot of first-order rate constant for reaction of the hydrated electron with $\alpha_s 1$ casein: κ-carrageenan complexes

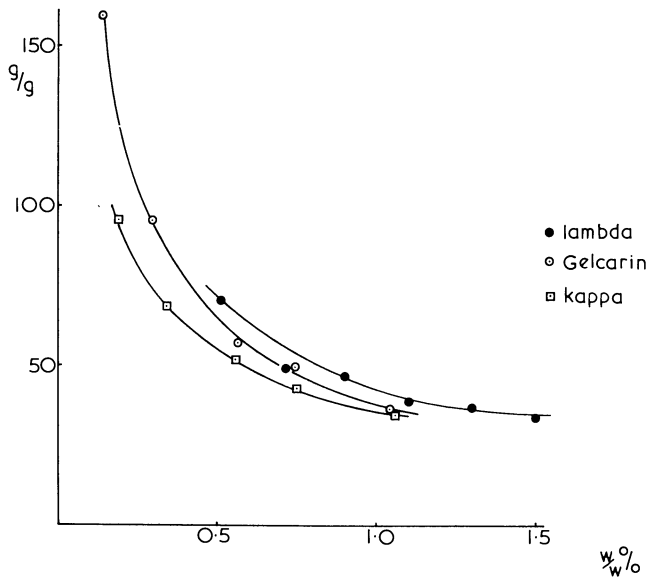

Figure 16. Plot of grams of unfrozen water/gram of carrageenan as a function of weight percentage of carrageenan in solution

Ultrasonic absorption was measured using the resonance interferometric technique of Eggers[44]. The electronic system was a modified version of the standard sweep level measuring set of Wandel and Goltermann[45].

Pulse radiolysis was carried out at the Christie Hospital and Holt Radium Institute, Manchester 20, U.K. A pulsed linear accelerator was used to delivery a 50n sec pulse of approximately 10MeV electrons to the radiation cell. A special deaeration procedure was used to prevent surface denaturation of protein which can cause error in the results[41].

Samples of λ, ι and κ - carrageenan were supplied by Copenhagen Pectinfabrik, κ - carrageenan used in the pulse radiolysis experiments, was a commercial sample (Gelcarin HMR) supplied by Dari Tech Corp, Atlanta, Ga.

We acknowledge the assistance of Dr. E. Wyn-Jones for providing facilities and interpreting the ultrasonic relaxation data.

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ABSTRACT

Our results show that the interaction of simple ions with the carrageenans can be explained in terms of electrostatic binding for λ - carrageenan, but some site binding is evident for κ - carrageenan, particularly the calcium salt of κ - carrageenan. Whereas there are indications from pulse radiolysis measurements that there is an interaction between the casein proteins and carrageenans, with a unique interaction between α_{s}^{1} casein and carrageenan, there is no evidence to suggest that these interactions are electrostatic in nature. Volume exclusion provides a partial explanation.

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