Coupling of Glycosaminoglycans to Agarose Beads (Sephacrose 4B)

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1. Heparin, heparan sulphate, chondroitin sulphate and dermatan sulphate were covalently attached to beads of agarose activated by cyanogen bromide. The bond is probably mediated by the amino group of a serine or peptide residue at the reducing end of the polysaccharide chain. 2. The uptake of glycosaminoglycan during the coupling procedure is about 0.9 mg/ml of wet gel. However, direct analysis of washed and freeze-dried gels reveals that only about one-third of this amount is firmly attached to the gel. 3. The use of the gels for polysaccharidase analyses is exemplified by a hyaluronidase assay. Further applications, e.g. interaction studies and preparative purposes, are discussed.

Connective-tissue glycosaminoglycans are negatively charged polyelectrolytes with ion-exchange properties (Laurent, Wasteson & Öbrink, 1969). The presence of charges might account for certain biological effects, e.g. the anticoagulant property of heparin and the liberation of lipoprotein lipase into the blood after heparin injection (Brimacombe & Webber, 1964). Further, it has been stated that the charged glycosaminoglycans are involved in the fibrillogenesis of collagen (Jackson & Bentley, 1968) and the development of atherosclerosis (Muir, 1964). However, on the whole our knowledge in this field is scanty.

Several methods have been used to study the electrostatic interaction between proteins and polyelectrolytes. Interactions giving rise to insoluble precipitates can be detected by assays of the supernatants or by turbidity measurements (Cornwell & Kruger, 1961). However, such techniques do not reveal the formation of soluble complexes. Further, the ratio between the protein and the polysaccharide concentrations determines the composition of the complexes, which in turn affects their solubility (Cornwell & Kruger, 1961). Electrophoresis has been used to detect the formation of soluble complexes (Pigman, Gramling & Holley, 1961), but the interpretation of such experiments has been criticized (Davies, Nichol & Ogston, 1963). Analytical ultracentrifugation (Janado & Nishida, 1967) is an elaborate method that suffers from the same drawbacks as electrophoresis. The interaction between tropocollagen and glycosaminoglycans has been studied by light-scattering (B. Öbrink, unpublished work). Soluble complexes may be detected by this method, which

avoids the limitations of the dynamic methods of electrophoresis and ultracentrifugation. Finally, a column-chromatographic technique has been employed by Öbrink & Wasteson (1971), who studied the interaction between tropocollagen and chondroitin sulphate by using a granulated gel of tropocollagen cross-linked with glutaraldehyde.

The methods reviewed above have been used to investigate the interaction between free polysaccharide chains and other substances. Such experiments differ from conditions *in vivo*, where the glycosaminoglycans, except heparin and hyaluronic acid, are immobilized by attachment to a protein core (e.g. see Balazs, 1970).

A new approach to interaction studies was introduced by Bettelheim, Laurent & Pertoft (1966), who used granulated gels of chondroitin sulphate cross-linked with epichlorohydrin for ion-exchange chromatography. However, the introduction of cross-links interferes with the polysaccharide structure. The covalent coupling of various substances to insoluble polymers that have been activated by cyanogen bromide has proved a useful and mild method for proteins (Axén, Porath & Ernback, 1967). Glycosaminoglycans liberated from tissues by proteolysis have a serine or peptide residue at the reducing end of the polysaccharide chain and thus possess a free amino group (e.g. see Balazs, 1970). Therefore it has been possible to use the cyanogen bromide method for attaching glycosaminoglycans covalently to agarose beads (Sephacrose 4B). The present paper describes the preparation and characterization of some glycosaminoglycans and their conjugates with Sephacrose 4B.

EXPERIMENTAL

Materials

Crude heparin (prepared from pig intestinal mucosa; anticoagulant activity, 126 U.S. Pharmacopeia units/mg) was purchased from Wilson Laboratories, Chicago, Ill., U.S.A.

Human aortas were obtained from autopsy cases. No strict selection was made with regard to age or state of health, but severely atheromatous vessels were not used. The vessels were kept frozen until preparation. Bovine aortas were obtained from a slaughterhouse and cooled to 4°C within 1½ h after death.

Crude papain type II (EC 3.4.4.10), deoxyribonuclease I (EC 3.1.4.5) from ox pancreas, ribonuclease A type I-A (EC 2.7.7.16) also from ox pancreas, and bovine testicular hyaluronidase type I (EC 3.2.1.35) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Bovine testicular hyaluronidase in ampoules containing 100 V.R.U. (viscosity-reducing units) was also supplied by Dr B. Högborg, AB Leo, Hälsingborg, Sweden (lot 15573). Chondroitin sulphate lyase ABC (EC 4.2.99.6) was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Sepharose 4B (lots 4088, 5214 and 5742), Sepharose 6B and Sephadex G-200 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Cyanogen bromide was obtained from Schuchardt, Munich, Germany, and ethanolamine and acetic acid anhydride were from E. Merck, A.-G., Darmstadt, Germany.

Analytical methods

Hydrolysis before hexosamine analysis was performed in sealed glass tubes at 100°C for 14 h. After hydrolysis, the samples were evaporated, dissolved in water and applied to micro-columns (5 mm × 35 mm) of Dowex 50W (X8; 200–400 mesh; H⁺ form). The columns were rinsed with 3 ml of water before the hexosamines were eluted with 1.5 ml of 2 M-HCl. The samples were analysed for total hexosamine or for glucosamine/galactosamine ratios. The former analysis was performed by a modification of the Elson–Morgan method (Gardell, 1953) with no correction for destruction during hydrolysis. The latter analysis was performed by either of two g.l.c. methods (Radhakrishnamurthy, Dalferes & Berenson, 1966; Hase & Matsushima, 1969).

Uronic acid was determined by the carbazole reaction as modified by Bitter & Muir (1962), with glucuronic lactone as standard. By comparing the colour yields obtained in the presence and in the absence of borate respectively, the reaction was also used to identify different glycosaminoglycans (Bitter & Muir, 1962). Sulphate was determined by the method of Antonopoulos (1962). The contents of sulphaminohexose in heparin and heparan sulphate were measured by the method of Lagunoff & Warren (1962), with glucosamine as standard. Samples were dried *in vacuo* at 60°C over P₂O₅ to determine their dry weight.

Specific optical rotations of glycosaminoglycans in aqueous solution were measured in a Perkin–Elmer 141 polarimeter at 25°C and at 589 nm (kindly performed by Dr L. Ohlsson at the Institute of Organic Chemistry, University of Uppsala). Electrophoresis of polysaccha-

rides was carried out on strips of cellulose acetate in 0.1 M-barium acetate (Wessler, 1968) or in 0.1 M-HCl (Wessler, 1971). To improve the separation between chondroitin 4-sulphate and chondroitin 6-sulphate, the duration of electrophoresis was increased to 12 h. Reference polysaccharides were kindly supplied by Dr E. Wessler of this Institute.

Average molecular weights of the polysaccharides were determined by analytical gel chromatography on columns of Sephadex G-200 or Sepharose 6B, calibrated with well-characterized fractions of chondroitin sulphate (Wasteson, 1971).

Preparative procedures

Proteolysis of tissue. Human or bovine aortas were freed from adhering tissues, cut into pieces and defatted at 4°C with several changes of acetone and ether. Dry defatted material was digested with papain for 24 h at 65°C in 0.05 M-sodium acetate (pH 5.5)–0.3 M-NaCl–0.01 M-EDTA–0.01 M-cysteine hydrochloride. Papain (1 g) and 150–200 g of dry tissue were added to each litre of buffer. After 12 h the amounts of papain and cysteine hydrochloride were doubled. The final digests were filtered through glass-wool and then centrifuged to remove insoluble residues.

Other enzymic procedures. Nucleic acids were removed by digestion for 20 h at 37°C with ribonuclease and deoxyribonuclease (10 mg of each/l) in 0.05 M-tris-HCl buffer (pH 7.0)–0.02 M-MgCl₂–0.15 M-NaCl. Digestion with hyaluronidase (Sigma) was performed in 0.1 M-sodium acetate buffer (pH 5.0)–0.15 M-NaCl for 48 h at 37°C and an enzyme concentration of 0.4 g/l. Hyaluronidase-resistant material was digested for 38 h at 37°C in 0.05 M-tris buffer, pH 8.0, containing chondroitin sulphate lyase ABC (20 units/l) and bovine serum albumin (100 mg/l) (Yamagata, Saito, Habuchi & Suzuki, 1968).

Precipitation of polysaccharide with cetylpyridinium chloride. Precipitation of polysaccharide with cetylpyridinium chloride (Scott, 1960) was performed at the ionic strengths of the respective buffers (*I* 0.3 or less) with the precipitant present in a threefold excess. The polysaccharides were converted into their sodium salts by dissolving the cetylpyridinium–polysaccharide precipitates in 2 M-NaCl at 37°C, followed by precipitation with 3 vol. of 95% (v/v) ethanol. This procedure was repeated twice. Chloride-free polysaccharide precipitates were obtained by dropwise addition of 4 M-sodium acetate to a solution of polysaccharide in 80% (v/v) ethanol. The precipitates were washed with acetone and dried under a stream of air.

RESULTS

Isolation and characterization of glycosaminoglycans

Heparin. Crude heparin was repeatedly precipitated from 1.2 M-sodium chloride by adding cetylpyridinium chloride as described by Lindahl, Cifonelli, Lindahl & Rodén (1965). On electrophoresis in barium acetate most of the material migrated as heparin, but a faint spot was also seen in the position of dermatan sulphate. This impurity was also detected by g.l.c. of the hexosamines,

Table 1. *Analysis of polysaccharide fractions*

Percentage values are expressed on a dry-weight basis. The sulphate/disaccharide molar ratios were calculated by comparing measured sulphate contents with theoretical values for polymers containing repeating disaccharide units only. $\bar{M}_w$ is the weight-average molecular weight and $\bar{M}_n$ is the number-average molecular weight.

Fraction	Uronic acid (%)	Hexose-amine (%)	Sulphate (%)	Sulphamino-hexose (%)	Sulphate/disaccharide molar ratio	Carbazole (E_{530}) borate/no borate ratio	$[\alpha]_D^{25}$	$\bar{M}_w$	$\bar{M}_n$
Heparin (Hep-II)	34	20	38	28	2.3	1.65	+54	12 800	10 500
Heparan sulphate (HS-II)	40	34	10	13	0.46	1.55	+95	58 300	40 500
Chondroitin sulphate (CS-I)	34	27	20	—	1.1	2.04	-24	37 100	27 900
Dermatan sulphate (DS-III)	29	30	21	—	1.1	2.83	-62	41 500	32 600

which were shown to contain about 4% of galactosamine. The degree of sulphation, as determined by electrophoresis in 0.1M-hydrochloric acid, was the same as that of a reference sample of heparin. The results of the analyses are shown in Table 1.

Heparan sulphate. Dried human aorta (4.5 kg) was digested with papain. The polysaccharides were isolated from the digest by precipitation with cetylpyridinium chloride and then subjected to nuclease digestion. Cetylpyridinium chloride precipitation of the nuclease digest yielded 45 g of crude polysaccharides, which on electrophoresis in barium acetate showed components having mobilities similar to those of chondroitin sulphate, dermatan sulphate and heparan sulphate. The crude polysaccharide was further digested with hyaluronidase in 2.5 litres of sodium acetate buffer, precipitated with cetylpyridinium chloride and finally treated with chondroitin sulphate lyase ABC in 1 litre of tris buffer. The material (5.9 g) obtained after a final cetylpyridinium chloride precipitation behaved as pure heparan sulphate on electrophoresis in barium acetate. G.l.c. of the constituent amino sugars showed that glucosamine was the only detectable component, confirming the purity of the preparation. Electrophoresis in 0.1M-hydrochloric acid showed a homogeneous spot with a mobility about half that of monosulphated chondroitin sulphate. A sulphate-disaccharide molar ratio of 0.46 confirmed the result of the electrophoresis. Additional analytical results are given in Table 1.

Dermatan sulphate. The papain digest from 3.6 kg of dried bovine aorta was precipitated with cetylpyridinium chloride. The precipitate was dissolved in 900 ml of 2M-sodium chloride and centrifuged. The clarified solution was mixed with 2 vol. of 95% (v/v) ethanol. After centrifugation, the supernatant (A) was retained for isolation of chondroitin sulphate as described below. The

precipitate was subjected to ethanol fractionation in 1 litre of calcium acetate buffer by the procedure of Meyer, Davidson, Linker & Hoffman (1956). The fraction that was precipitated by raising the ethanol concentration from 0% to 25% (v/v) was converted into the sodium salt. This preparation contained nucleic acids, as indicated by the u.v. spectrum, and was therefore digested with nuclease. After the digestion, the polysaccharide was finally precipitated with cetylpyridinium chloride, giving 4.8 g of dried preparation. Electrophoresis in barium acetate showed a single spot with a mobility slightly greater than that of the dermatan sulphate standard. Galactosamine was the only hexosamine detected by g.l.c. On electrophoresis in 0.1M-hydrochloric acid, the polysaccharide behaved like monosulphated standards of chondroitin or dermatan sulphate. Additional analytical results are given in Table 1. The ratio of the extinctions obtained from the carbazole reaction with and without borate was 2.83. Since the ratio for dermatan sulphate from other sources has been found to be about 3.6 (Bitter & Muir, 1962; P.-H. Iverius, unpublished work) and that of pure chondroitin sulphate 2.0, the value of 2.83 is compatible with a hybrid structure containing both L-iduronic acid and D-glucuronic acid (Fransson & Havsmark, 1970).

Chondroitin sulphate. Supernatant A, obtained during the fractionation of glycosaminoglycans from bovine aorta (see above), was mixed with another 2 vol. of 95% (v/v) ethanol. The resulting precipitate was also subjected to ethanol fractionation in 1 litre of calcium acetate buffer by the method of Meyer *et al.* (1956). The fraction obtained by raising the ethanol concentration from 25 to 40% (v/v) was converted into the chloride-free sodium salt and dried. This fraction, which weighed 8.9 g, behaved as pure chondroitin sulphate on barium acetate electrophoresis. Galactosamine

was the only hexosamine detected by g.l.c. The electrophoretic mobility in 0.1M-hydrochloric acid was the same as that of reference monosulphated chondroitin sulphate. Electrophoresis for 12h in barium acetate, which separates chondroitin 4-sulphate from chondroitin 6-sulphate, established that chondroitin 4-sulphate was the only component. The chemical analyses are presented in Table 1.

Binding of glycosaminoglycans and ethanolamine to Sepharose 4B by covalent linkage

Sepharose 4B was activated by the cyanogen bromide method (Axén *et al.* 1967) as described by Kato & Anfinsen (1969). Activated gel (generally 75ml) was mixed with polysaccharide (150–160mg, dry wt.) and the volume was made up to 150ml with 0.1M-sodium hydrogen carbonate. The reaction mixture was stirred at 4°C. After 16h, 0.5ml of the supernatant was withdrawn for analysis and remaining active groups on the gel were eliminated by stirring for 4h with 7.5ml of ethanolamine. The gel was finally transferred to a sintered-glass filter and washed consecutively with 1 litre of water, 0.5 litre of 0.5M-sodium chloride and 3 litres of water. Parallel to the coupling of the glycosaminoglycan to activated gel, a reference experiment was performed in which non-activated gel was substituted for the activated gel. Also gels containing only ethanolamine were prepared by incubating activated gel with ethanolamine for 16h.

Glycosaminoglycan contents of different gel batches

The glycosaminoglycan contents of the gels were analysed in three different ways.

One value was based on the transfer of uronic acid from the liquid phase to the gel during the coupling procedure. The samples withdrawn from the supernatants, as described above, were diluted 20-fold before analysis. The ratio between the extinction in the experiment with activated gel and the control experiment with non-activated gel represents the proportion of the added polysaccharide that remained unattached. Since the amount of added polysaccharide was known in each experiment, the amount bound/vol. of wet gel could be calculated. Dry-weight contents were calculated from the wet gel contents by using the value 10.5mg of dry gel/ml of wet gel. This value was obtained from a dry-weight determination of the gel heparan sulphate II–Sepharose 4B-I fraction (HS-II–Seph. 4B-I).

Two other values were derived from the sulphate and hexosamine contents of the freeze-dried gel. The samples for sulphate assays, which varied between 3.32 and 10.2mg dry wt. depending on the sulphate contents, were hydrolysed in sealed glass tubes with 2ml of 25% (v/v) formic acid. The hexosamine determinations were performed on samples of about 10mg dry wt. that were hydrolysed with 2ml of 4M-hydrochloric acid.

The sulphate contents due to the presence of glycosaminoglycans were obtained by subtracting from the total sulphate contents the sulphate contents of a gel of the same batch, to which ethanolamine had been linked (5.2, 3.2 and 4.8 µg/mg for lots no. 4088, 5214 and 5742 respectively). Since the sulphate contents of the glycosaminoglycans were known, the polysaccharide contents of the gels could easily be calculated.

Table 2. *Analysis of gels containing glycosaminoglycans*

The amount of polysaccharides taken up by the gels during the coupling procedure was calculated (A) from uronic acid analyses of supernatants of reaction mixtures (see the text) and also from (B) hexosamine and (C) sulphate analyses of the lyophilized gels. The conversion factor required for each polysaccharide constituent was computed from Table 1. The nomenclature of the fractions is indicated in Table 1 and the text.

Fraction	Lot no. of gel	Glycosaminoglycan content			
		(mg/ml of wet gel)	(μg/mg of dry gel)		
			(A)	(B)	(C)
Hep-II-Seph. 4B-I	4088	1.21	115	39.1	34.6
Hep-II-Seph. 4B-II	4088	1.14	109	41.4	32.8
Hep-II-Seph. 4B-III	4088	0.91	86.7	27.8	21.6
Hep-II-Seph. 4B-IV	4088	0.71	67.6	14.9	12.8
Hep-II-Seph. 4B-V	5214	0.65	61.9	30.1	28.1
Hep-II-Seph. 4B-VI	5742	1.29	123	43.8	36.2
HS-II-Seph. 4B-I	5742	0.88	83.8	25.9	26.0
CS-I-Seph. 4B-I	4088	0.70	66.7	28.2	21.5
CS-I-Seph. 4B-II	4088	0.59	56.2	25.8	21.1
CS-I-Seph. 4B-III	5742	0.90	85.7	32.0	20.9
DS-III-Seph. 4B-I	4088	0.72	68.6	15.1	15.6
DS-III-Seph. 4B-II	5742	0.79	75.2	26.7	21.5

The hexosamine values were all corrected by subtraction of $0.53 \mu\text{g}/\text{mg}$. This value was obtained by analysis of a gel to which ethanolamine only had been linked. Cross-linked dextran, treated with cyanogen bromide, gives a positive Elson-Morgan reaction after hydrolysis (P.-H. Iverius, unpublished work). The calculations were then analogous to those based on the sulphate analyses. All analyses of the polysaccharide-substituted gels are presented in Table 2. The polysaccharide contents of the extensively washed, freeze-dried gels varied from about 10 to $40 \mu\text{g}/\text{mg}$ of dry gel (columns B and C). Generally, these values were only one-third of the amount of polysaccharide initially transferred from the liquid to the gel phase during the coupling procedure (columns A).

Inhibition of glycosaminoglycan binding to gels by blocking of reactive groups

Chondroitin sulphate was acetylated essentially by the procedure of Fraenkel-Conrat (1957). Chondroitin sulphate (25 mg) was dissolved in 2 ml of half-saturated sodium acetate and chilled in an ice bath. Four portions of acetic acid anhydride ($50 \mu\text{l}$) were added at 15 min intervals. Then, 15 min after the last addition, the sample was dialysed against 0.1 M-sodium hydrogen carbonate at 4°C . After dialysis, the volume of the sample was adjusted to 5 ml.

Activated Sepharose 4B (5 ml) was transferred to a 10 ml graduated flask. Another 10 ml flask was charged with 5 ml of non-activated gel, then 2.4 ml of the acetylated chondroitin sulphate solution was added to each flask, and the volumes were adjusted to 10 ml with 0.1 M-sodium hydrogen carbonate. The contents of the flasks were stirred for 16 h, after which the supernatants were diluted 20-fold and subjected to uronic acid analysis. In another experiment the activated gel was treated with 0.5 ml of ethanolamine in 1 ml of 0.1 M-sodium hydrogen carbonate before the non-acetylated chondroitin sulphate was added.

After treatment of the acetylated polysaccharide with activated gel, the concentration of polysaccharide in the supernatant was 98% of that obtained with a non-activated gel. The corresponding value recorded after incubation of non-acetylated polysaccharide with activated and ethanolamine-treated gel was 99%. In contrast, only 60–70% of the polysaccharide remained in the liquid phase after the reaction of non-modified chondroitin sulphate with activated gel. It is therefore concluded that the covalent binding of glycosaminoglycans to activated agarose gels could be inhibited either by blocking the free amino groups of the glycosaminoglycan by acetylation or by blocking the active groups in the gel with ethanolamine.

Fig. 1. Release of uronic acid by hyaluronidase treatment of a gel containing chondroitin sulphate. (a) Various amounts of gel and 15 V.R.U. of enzyme were incubated in a final volume of 1.25 ml for 45 min at 37°C . (b) Enzyme was added in various amounts to a constant amount of gel (0.5 g) and the mixture incubated for 30 min at 37°C in a final volume of 1.25 ml as above.

Method for the assay of polysaccharidase

A gel containing chondroitin sulphate (fraction CS-I-Seph. 4B-I) was washed with 0.1 M-sodium acetate-sodium chloride buffer, pH 5.0. In one experiment various amounts (0–0.5 g) of packed gel were weighed into small test tubes. The tubes were placed in an ice bath and 15 V.R.U. of hyaluronidase (Leo) was added to each. Then the total volume of the contents was adjusted to 1.25 ml. Immediately after the contents had been mixed, the tubes were transferred to a water bath at 37°C . During the incubation the tubes were agitated at 5 min intervals. After 45 min the samples were chilled in an ice bath and centrifuged at 4°C , then the supernatants were analysed for uronic acid. In another experiment constant amounts of gel (0.5 g) were weighed into a series of tubes. Various amounts of

hyaluronidase (0–25 V.R.U.) were added and the volumes adjusted to 1.25 ml. The experimental procedure was otherwise identical with that described above, except that the incubation time at 37°C was 30 min.

The first experiment (Fig. 1a) shows that when the enzyme concentration in the assay is kept constant the activity observed depends on the amount of gel added. The second experiment (Fig. 1b) demonstrates how the enzyme activity observed varies with the enzyme concentration for a constant amount of gel. The graph seems to be approximately linear at the lower enzyme concentrations and should therefore be useful as a calibration curve in an enzyme assay. At higher enzyme activities, however, the graph is non-linear, presumably because the matrix-bound glycosaminoglycan substrate had been used up, since approx. 80% of the polysaccharide was liberated from the gel after incubation with 25 V.R.U. of enzyme for 30 min.

DISCUSSION

Cyanogen bromide activation provides a method for the coupling of compounds bearing free amino groups to a gel matrix (Axén *et al.* 1967). In the glycosaminoglycans the free amino groups of serine or peptide residues are available (see e.g. Balazs, 1970). The hexosamine residues also contain amino groups, but in the native state they are either acetylated or, in heparin and heparan sulphate, sulphated. The sulphamino groups are acid-labile and might be a potential source of free amino groups (Brimacombe & Webber, 1964). It thus seems reasonable to assume that covalent bonds are formed between the amino groups of the glycosaminoglycans and the cyanogen bromide-activated agarose. As acetylation primarily affects free amino groups (Fraenkel-Conrat, 1957), this assumption is further supported by the finding that acetylated chondroitin sulphate did not react with activated gel.

The glycosaminoglycan contents of the gels (Table 2) calculated from hexosamine (column B) and sulphate (column C) analyses are in fair agreement, although the values based on hexosamine determinations are generally somewhat higher. It is difficult to decide which analytical technique is better, since a relatively large blank correction had to be made in both methods. The values calculated from the transfer of glycosaminoglycan to the gel during the coupling procedure (columns A) are, however, about three times as great. The most probable explanation for this discrepancy is that many of the polysaccharide molecules are bound to 'brittle' parts of the gel beads and are thus

easily lost during the extensive washing that follows the coupling procedure.

The matrix-bound glycosaminoglycans were originally produced to provide a simple tool for studying the interactions between glycosaminoglycans and other substances such as, for example, human serum lipoproteins (P.-H. Iverius, unpublished work). The gels may also be used for preparative work. Lipoprotein lipase from bovine milk has been purified considerably by affinity chromatography on a heparin-containing gel (Olivecrona, Egelrud, Iverius & Lindahl, 1971). A third application, the assay of polysaccharidase activity, is described above.

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