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Introduction of Specific Groups into Polysaccharide Supports for Liquid Chromatography

JAMES ELLINGBOE,* BJÖRN ALMÉ and JAN SJÖVALL

Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

A simple method is described whereby chlorohydroxypropyl derivatives of hydroxypropyl Sephadex (Sephadex LH–20) or cross-linked hydroxypropyl cellulose can be prepared with a controlled degree of substitution. The introduction of a halogen into the insoluble polymer makes it possible to perform further substitution reactions exemplified with the preparation of aminohydroxypropyl, aminoethoxyhydroxypropyl, *N*-hydroxyethylaminohydroxypropyl, lithocholamidohydroxypropyl and mercaptohydroxypropyl derivatives. The use of specifically substituted Sephadex for liquid-gel chromatography in organic solvents is discussed.

Insoluble polymers substituted with biologically active compounds are currently providing model systems for the study of enzymes, antigens, and antibodies.[1] Supports containing specific functional groups are also finding wider application in chromatographic and ion exchange processes.[2,3] In connection with studies of supports for use in liquid chromatography some procedures have been developed whereby cellulose, Sephadex, or other polysaccharide substances may be reacted to controlled degrees of substitution with a variety of functional groups.

The basic procedure has been applied to the synthesis of lipophilic-hydrophobic Sephadex derivatives.[4,5] Briefly, a hydroxyalkoxy derivative of the polysaccharide is prepared by well-known methods wherein propylene oxide or ethylene oxide is added under aqueous alkaline conditions, with or without the presence of cross-linking agents such as epichlorohydrin. The polysaccharide is thereby made somewhat lipophilic so that it can be reacted with epoxides in organic solvents under acidic conditions to yield products having a wide range of lipophilic or other special character.

When the epoxide used in the acid-catalyzed reaction is an epihalohydrin, the polysaccharide product should contain ether-linked halohydroxypropyl

* Present address: Department of Medicine, University of California, San Diego, La Jolla, California.

groups. Numerous possibilities for chemical substitution then arise. Under basic conditions vicinal halogen and hydroxyl groupings should undergo reactions typical of epoxides, whereas under other conditions, only the hydroxyl group or the halogen function may react.

The degree of substitution of the polysaccharide with halohydroxypropyl groups, or derivatives thereof, can be controlled by the amount of epihalohydrin used in the initial reaction. Likewise, the degree of lipophilicity may be controlled by the amounts of aromatic or aliphatic epoxides used in reactions before, during, or after the halohydroxypropylation. In order to establish the stoichiometric relationships for controlled substitution, the reactions of epichlorohydrin with hydroxypropyl derivatives of Sephadex were investigated as a function of relative amounts of boron trifluoride ethyletherate, epoxide and dextran derivative. Fig. 1 shows the results of these

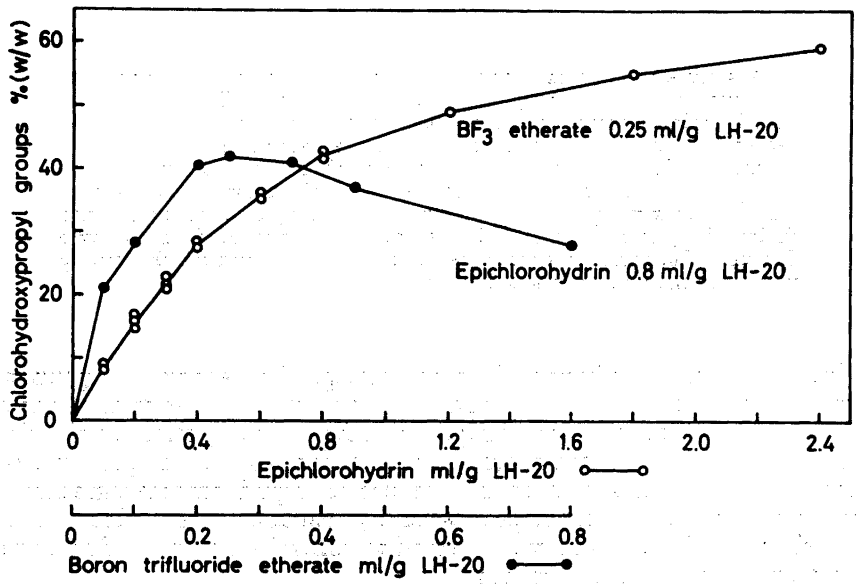

Fig. 1. Relationship between degree of chlorohydroxypropyl substitution and amount of epichlorohydrin and boron trifluoride ethyletherate (48 % BF_3) used in reactions with Sephadex LH—20. When the amount of epichlorohydrin was varied the amount of boron trifluoride ethyletherate was held constant at 0.25 ml/g Sephadex. A constant amount of 0.8 ml epichlorohydrin/g Sephadex was used when the amount of BF_3 was varied. Complete substitution of all hydroxyl groups in Sephadex LH—20 corresponds to about 45 % chlorohydroxypropyl group content.

reactions which were conducted essentially as described in the experimental section. The optimal amount of BF_3 was found to be 0.25 ml boron trifluoride ethyletherate (48 % BF_3) per g hydroxypropyl Sephadex. The epoxide was most conveniently added as a 10 % solution in dry dichloromethane. Reactions

with epibromohydrin gave essentially the same degree of substitution as when epichlorohydrin was used.

Among the products which may conceivably be obtained from halohydroxypropyl-substituted polysaccharides are the primary, secondary, tertiary, and quaternary amines, which may be prepared by reaction with ammonia or substituted amines. By such procedures, which are analogous to those used in the preparation of anion exchangers from chloromethylated polystyrene,[6] it should be possible to prepare a series of anion exchangers ranging from the weakly basic primary amines to the strongly basic quaternary amines. The derivatives containing primary amino groups also offer potential use as matrices to which enzymes, antigens and antibodies may be bound.[1,7-9] By use of cyclohexylcarbodiimide it is possible to attach carboxyl group-containing compounds to the amino-substituted polysaccharides *via* amide linkage.

It should also be possible to attach compounds in ester or ether linkage, by reaction of the halohydroxypropylated polysaccharides with salts of carboxylic acids or with alcohols under anhydrous alkaline conditions. The halogen atoms of halohydroxypropyl polysaccharides may also be replaced by sulfhydryl groups when treated with a solution of alkali hydrosulfide or when reacted with thiourea and subsequently hydrolyzed by base. These derivatives show a strong affinity for mercury and they may be used for removal of mercurials from dilute aqueous solutions or for enrichment of mercurials for analytical purposes. The bifunctional mercurial, 3,6-bis(acetatemercurimethyl)-dioxane, used by Eldjarn and Jellum for the preparation of an organomercurial Sephadex [10] is also strongly bound.

Liquid-gel chromatography on hydrophobic Sephadex using miscible solvent systems has been found to be a simple and efficient method for separation of various lipids and steroids.[4,5] Depending on the composition of the solvent mixture, the compounds are eluted in order of increasing or decreasing polarity.[11] The solvent properties of the stationary phase are partly determined by the simultaneous presence of hydroxyl groups and long alkyl chains bound to the gel matrix. By using chlorohydroxypropylated Sephadex as a starting material it should be possible to synthesize stationary gel phases with covalently bound substituents showing greater selectivity towards solutes than the long alkyl chains. This would extend the scope of liquid-gel chromatography in organic solvents. Chlorohydroxypropyl derivatives of Sephadex or cellulose may also be useful intermediates in the synthesis of specific stationary phases for the separation of macromolecules.

SYNTHETIC PROCEDURES

Chlorohydroxypropoxypropyl Sephadex G—50. Hydroxypropylated Sephadex G—50® (10.0 g, superfine, bead form, prepared by addition of propylene oxide under aqueous alkaline conditions)[4] was soaked in 150 ml of dry dichloromethane. Boron trifluoride ethyletherate (1 ml, 48 % BF_3) was added and the mixture was stirred thoroughly for 10 min. While stirring at room temperature, epichlorohydrin (3.6 ml) was added slowly as a solution in 40 ml dry dichloromethane. After addition of the epoxide, the mixture was stirred for 20 min at room temperature. The product was filtered free of solvent, washed with chloroform followed by ethanol, then dried at 40° to a constant weight. The chlorohydroxypropyl content, as determined by weight and checked by Cl determination was 7.1 %. This product swelled in water, ethanol, and chloroform.

Chlorohydroxypropyl Sephadex LH—20. Sephadex LH—20 (170—240 mesh), 98.12 g, was soaked in 360 ml of dry dichloromethane. Boron trifluoride ethyletherate (25 ml, 48 % BF_3) was added and the mixture was stirred for 15 min. Epichlorohydrin (30.0 ml) was then added slowly as a solution in 50 ml dry dichloromethane. The mixture was stirred for 40 min, and the product was worked up as above. The chlorohydroxypropyl content was 22.9 % and the derivative swelled in ethanol, chloroform and benzene but not in water and heptane.

Chlorohydroxypropoxypropyl cellulose. A sheet of filter paper weighing 49.0 g was cut into small pieces and soaked for 2 h in 500 ml of 10 % aq. sodium hydroxide. The excess aqueous phase was removed by suction and the wet alkaline paper pulp was hydroxypropylated and cross-linked by refluxing and stirring at 80°C for 6 h with a mixture of 400 ml ethylene chloride, 200 ml epichlorohydrin and 1 liter technical grade propylene oxide. The cellulose derivative was filtered free of solvents, washed with ethanol, water, and again with ethanol, then dried at 60°C. The product contained 28 % hydroxypropyl groups by weight. A portion of this product (28.1 g) was soaked in 200 ml dichloromethane. A solution of 40 ml boron trifluoride ethyletherate in 150 ml dichloromethane was then added with stirring. After 30 min a mixture of 50 ml epichlorohydrin and 100 ml dichloromethane was slowly added. After this addition was completed, the mixture was stirred for 25 min, filtered free of solvents, washed with ethanol, water and again with ethanol, then dried at 40°C. The product contained 14.3 % chlorohydroxypropyl groups by weight.

Aminohydroxypropoxypropyl Sephadex was prepared by first swelling 4.0 g of 10.9 % chlorohydroxypropylated Sephadex LH—20 for 5 min in 40 ml dioxane, then adding 60 ml concentrated aqueous ammonia and shaking for 60 h at room temperature. The product was collected on a filter, washed with water and ethanol and then dried at 40°. A test with ninhydrin in 95 % ethanol gave a positive reaction and nitrogen analysis showed 8.5 μg N/mg product.

Aminoethoxyhydroxypropoxypropyl Sephadex was prepared by first swelling 3.81 g of 9.0 % chlorohydroxypropylated hydroxypropyl Sephadex G—50 in 30 ml dioxane, then shaking for 22 h at room temperature with a mixture of 0.7 g potassium hydroxide in 30 ml ethanolamine. The product was collected on a filter and washed with water and ethanol. It gave a positive ninhydrin reaction and contained 12.1 μg N/mg. This corresponds to a conversion of about 90 % of the chlorohydroxypropyl groups into the amino derivative.

N-*Hydroxyethylaminohydroxypropoxypropyl Sephadex* was prepared by first swelling 12.6 g of 4.8 % chlorohydroxypropylated hydroxypropyl Sephadex G—50 in 250 ml of dioxane, then shaking for 15 h with 250 ml ethanolamine. The product was collected on a filter and washed with water and ethanol and was then dried *in vacuo*. The ninhydrin test was negative, indicating absence of free amino groups; nitrogen content was 1.9 μg N/mg product. This corresponds to a conversion of about 30 % of the chlorohydroxypropyl groups into the hydroxyethylamino derivative.

Lithocholamidohydroxypropoxypropyl Sephadex. By the same procedures described in the preceding examples, Sephadex LH—20 was chlorohydroxypropylated to 8.1 % on a weight basis and then treated with concentrated aqueous ammonia in dioxane to produce an amino derivative containing 5.5 μg N/mg. Lithocholic (3α-hydroxy-5β-cholanoic) acid was attached in amide linkage by adding a solution of 3.76 g lithocholic acid in 50 ml dry dichloromethane to 4.16 g of the dry amino-Sephadex derivative and then shaking for 70 h at room temperature with 3.0 g dicyclohexylcarbodiimide. The reaction product was collected on a filter and washed with dichloromethane, ethanol, 10 % ethanolic acetic acid, water, ethanol, acetone and was then dried to constant weight *in vacuo*. The increase of weight was 0.79 g, indicating full substitution of amino groups with lithocholic acid. The ninhydrin test was negative.

Mercaptohydroxypropoxypropyl derivatives of Sephadex and cellulose. 30 g of 10.5 % chlorohydroxypropylated Sephadex LH—20 was treated with a solution of 10 g sodium hydrosulfide, 100 ml absolute ethanol and 200 ml ethylene glycol for 2 h at 100°. The product was washed with water and ethanol and was then dried at 40°. The sulfur content was 14.3 μg/mg. Tests with phenyl mercuric hydroxide and mercuric acetate showed that the material had a strong affinity for mercury. The bound mercurial was stained with diphenyl carbazone.

By another procedure, similar to that described for substitution of chloromethylated polystyrene, 20 g of 10.5 % chlorohydroxypropylated Sephadex LH—20 was allowed to swell in 100 ml dioxane and was then heated on a steam bath for 8 h with 200 ml 10 % aqueous thiourea. The product of this reaction was washed with water and ethanol and was then hydrolyzed at room temperature by shaking with 200 ml of 10 % aqueous sodium hydroxide. The product was washed with water, ethanol and low boiling petroleum ether, then dried at 40°. The sulfur content was 8.1 μg/mg.

The sodium hydrosulfide procedure was used for the substitution of 14.3 % chlorohydroxypropylated hydroxypropyl cellulose with mercapto groups. The product showed a strong affinity for mercury indicating the presence of sulfhydryl groups. The sulfur content was 9.3 μg/mg.

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