New Approach to Aqueous Gel Permeation Chromatography of Nonderivatized Cellulose

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Synopsis

A novel approach to the gel permeation chromatography (GPC) of nonderivatized cellulose is reported using Sepharose CL-6B as the column packing material, 0.5*N* NaOH as the eluent, and cadoxen as the cellulose solvent. The traditional approach to GPC of cellulose has been to convert the cellulose to its nitrate thereby making it soluble in the solvent tetrahydrofuran. The circumvention of the need to derivatize the cellulose in the new system results in considerable saving of time. The new system gives good fractionation for cellulose. It also provides excellent separation of polystyrene sulfonate and dextran standards thereby making the system amenable to calibration. The effect of the particle size distribution of the column packing material on the efficiency of separation is discussed. Potential applications for this new method include studies on both acidic and enzymatic hydrolysis as well as fine structure of cellulose, starch, and other polymers capable of forming stable alkaline solutions.

INTRODUCTION

Gel permeation chromatography (GPC) of cellulose has traditionally been carried out using crosslinked polystyrene as the chromatographic support and tetrahydrofuran (THF) as the eluent. In this approach the cellulose is derivatized in the nitro,[1–6] acetyl,[5,7,8] or carbanil[9] form to make it soluble in THF. GPC is then carried out by dissolving the derivatized sample in THF, injecting the sample onto the column, and eluting with THF. The carboxymethyl derivative of cellulose has been used in aqueous GPC.[10] Segal has reviewed the subject of GPC of cellulose and its derivatives.[11]

An alternate approach uses the cellulose solvent cadoxen as eluent and polyacrylamide,[12] agarose,[13,14] or another suitable gel[15] as the chromatographic support. Since cellulose is soluble in cadoxen (containing 5–7% CdO in 28% aqueous ethylenediamine),[16,17] the cellulose is not derivatized prior to analysis. This saves considerable time and cellulose sample and minimizes any changes in the cellulose due to sample preparation. The disadvantage of this method is that large quantities of a rather expensive and difficult to prepare solvent (i.e., cadoxen) are needed.

Research on cellulose solvents in the Laboratory of Renewable Resources Engineering (LORRE) has resulted in a new approach to dissolving cellulose in cadoxen and keeping it in solution even upon 20-fold or higher dilution with 0.5*N* aqueous sodium hydroxide (NaOH). This phenomenon has been developed into a novel method for the GPC of cellulose. Here, the cellulose is dissolved in a small amount of cadoxen and subsequently injected onto a column and eluted with 0.5*N* NaOH. Hence, the cellulose is not derivatized prior to analysis. An

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added advantage is that the eluent is more readily available and easier to handle than cadoxen. The development of this exciting new tool is timely for the study of ways in which cellulose, one of nature's most abundant renewable resources, is broken down to give fermentable sugars and subsequently liquid fuels and chemicals.

BACKGROUND

The solvent cadoxen was discovered by Jayme and Neuschaffer[18] in 1957. It is a clear, colorless, and stable solvent for cellulose of degree of polymerization (D.P.) up to 10,000. Cellulose can be dissolved in this solvent for long periods of time with little, if any, degradation.[16,17]

The use of this solvent for GPC of bleached sulfite pulp of D.P. 780, 1180, and 2000 was reported by Eriksson et al.[12] Polyacrylamide gel was used as the chromatographic support with a 1:1 dilution of aqueous cadoxen as the eluent. Other chromatographic supports applicable for GPC with cadoxen include agarose gel[13,14] and an unspecified inorganic gel.[15]

Further development of this tool was limited by several factors. The high viscosity of solutions of cellulose in cadoxen caused separation inefficiencies due to the phenomenon known as viscous fingering. In the GPC of samples of high viscosity, the rear boundary of the elution profile of a polymer is unstable as the solvent finds the easiest pathway through the packing and this causes extensive tailing in the chromatograms. A detailed description of this phenomenon has been given by Moore.[39] In addition, a large quantity of cadoxen is required for each analysis and since cadoxen is difficult and time-consuming to prepare in large quantities, its use as the GPC eluent is less than favorable.

EXPERIMENTAL

Dilution Properties of Cellulose in Cadoxen

Cadoxen is thought to dissolve cellulose by forming a complex with cellulose.[19] The presence of 0.5*N* NaOH in cadoxen is known to increase the solvent's cellulose-dissolving capacity.[20] This observation suggested that cellulose dissolved in cadoxen might withstand dilution by NaOH without causing the cellulose to reprecipitate.

The effect of adding aqueous NaOH to cellulose dissolved in cadoxen was tested using 0.2, 0.4, 0.5, 0.6, and 0.8*N* NaOH solutions. Avicel, a microcrystalline cellulose (PH 101, 210 D.P., FMC Corp., Philadelphia, PA) was dissolved in cadoxen to give a 2% solution. The cellulose solution was then diluted 20-fold with aqueous NaOH. The onset of turbidity (i.e., cellulose reprecipitation) was monitored by measuring the change in optical density at 500 nm as a function of time with a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY).

The results of these experiments are shown in Figure 1. While there was no turbidity increase (i.e., cellulose reprecipitation) for 0.5*N* NaOH, all other NaOH concentrations caused cellulose reprecipitation. This experiment was repeated for 1 and 3% Avicel solutions as well as for 1, 2, and 3% CF-11 cellulose solutions (Whatman Ltd., Kent, England), and the same results were obtained. On the

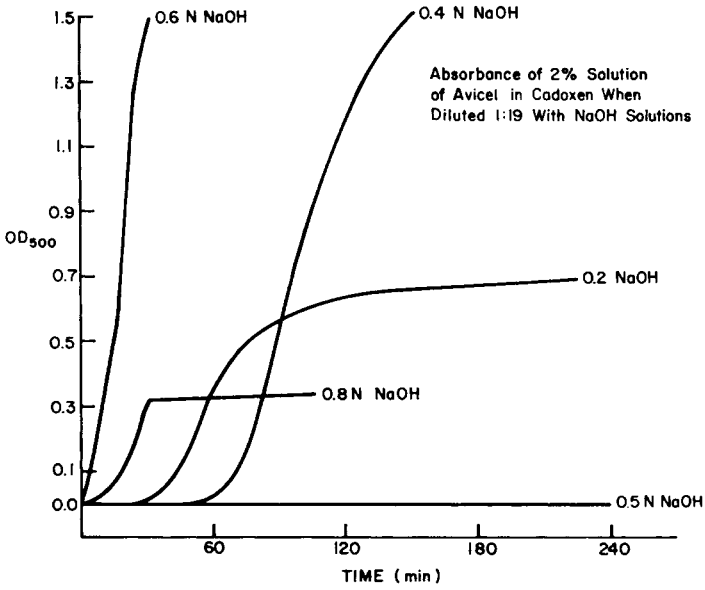


Fig. 1. Cellulose reprecipitation as a function of time.

basis of these results, 0.5*N* NaOH was chosen as a candidate for eluting cellulose dissolved in cadoxen.

Selection of Chromatographic Support

Silica- and alumina-based supports commonly used for GPC are not suitable when aqueous NaOH is the eluent. These supports are soluble in base and therefore mechanically and chemically unstable. Supports such as Sepharose CL-6B,[21] Agarose 5M,[22] and Sephadex G-150,[23] which have a carbohydrate backbone, are reported to be base stable. Hence, these materials were tested for stability with respect to 0.5*N* NaOH.

These materials were packed in small columns of dimensions (0.4 cm × 10 cm) and exposed to a continuous flow of 0.5*N* NaOH for nine days. The eluent was monitored continuously for dissolved carbohydrate using a standard colorimetric carbohydrate assay (24). The results, summarized in Table I, show that Sepharose CL-6B is quite stable showing no loss after one day. The other gels exhibited more dissolution than Sepharose CL-6B.

TABLE I
Stability of Gels in 0.5*N* NaOH

	Cumulative amount of packing dissolved, %				
	1 Day	2 Days	3 Days	5 Days	9 Days
Sepharose CL-6B	0.07	0.07	0.07	0.07	0.07
Agarose 5M	1.1	1.2	1.2	1.2	1.2
Sephadex G-150	5.8	5.9	5.9	5.9	5.9

Column Packing Procedure

Sepharose CL-6B, while stable to base, is mechanically friable due to its soft organic gel structure. Hence, special packing techniques are required to obtain stable columns of this material. Based on experience attained in fabricating low-pressure liquid chromatography (LPLC) columns using gel-type ion exchangers,[25,26] a technique for Sepharose CL-6B was developed.

The most important step in the packing procedure is the preparation of the Sepharose CL-6B. The gel is supplied by the manufacturer (Pharmacia Chemicals, Piscataway, NJ) dispersed in water. Equilibration of the gel with 0.5*N* NaOH was carried out in a series of sequential washing steps (0.025, 0.05, 0.15, 0.25, 0.35, 0.45, and finally 0.5*N* NaOH). This procedure was chosen to minimize rupture of gel particles by osmotic pressure difference due to sudden exposure to 0.5*N* NaOH of high ionic strength.

Once equilibrated, an elutriation technique was used to fractionate the gel to obtain a more uniform particle size distribution. The technique consisted of dispersing the gel in 0.5*N* NaOH in a 2-liter graduated measuring cylinder and allowing it to settle partially. The smaller gel particles which were still suspended in the upper section of the liquid in the cylinder were withdrawn by siphoning. Particle size distribution was established by observing the dispersed gel under a microscope at 400× magnification. This fractionation procedure was repeated a number of times until fractions in the desired particle size ranges were obtained.

Three particle size fractions resulted (Fig. 2): "coarse" (ca. 75–150 μm), "fine" (ca. 30–70 μm), and "very fine" (ca. 25–50 μm). Subsequent packing and testing of all three fractions showed the resolution obtained with the "fine" fraction to be much better than for the "coarse" fraction. The "very fine" fraction also showed an improvement over the "fine" fraction. Columns packed with either "fine" or "very fine" gels were used in most of the subsequent work.

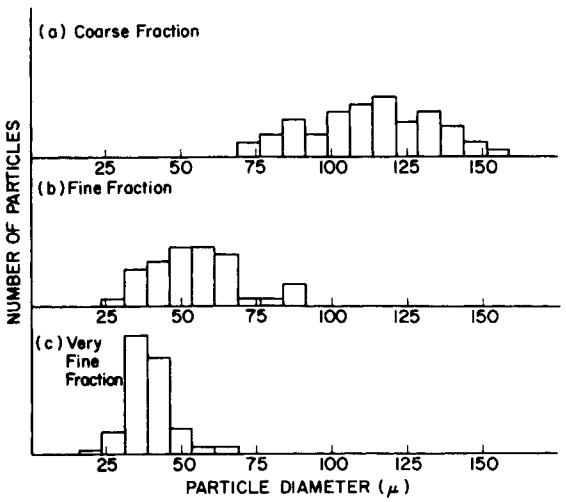


Fig. 2. Particle size distribution of column packing Sepharose CL-6B as determined by counting dispersed samples under microscope at 400× magnification.

The fractionated gel was packed in a 40 cm × 6 mm (I.D.) stainless steel column capped with 10-μm endfittings (Waters Associates, Milford, MA). Small (0.75 ml, settled volume) amounts of gel were suspended in 0.5*N* NaOH and allowed to flow into the column under gravity at room conditions. This procedure was repeated until the column was completely full of gel. Sodium hydroxide was then allowed to flow under gravity through the column for 12 hr. The column was then capped off with a 10-μm endfitting and connected to the liquid chromatography apparatus. Our experience indicates that a column packed in this manner has an operational stability of at least six months.

GPC Setup

The hardware for the GPC was assembled from individual components. These include (Fig. 3) (1) a 4-liter, constantly stirred flask for the solvent reservoir; (2) a Milton–Roy minipump (model 396, Laboratory Data Control, FL); (3) a pulse-dampener (Waters Associates, Milford, MA); (4) a model 7120 loop injector (Rheodyne Corp., Berkeley, CA); (5) the column; (6) a model 401 differential refractometer (Waters Associates, Milford, MA) thermostated to 32°C with a Haake model FE circulating waterbath (Haake Co., Bound Brook, NJ); and (7) a Heath/Schlumberger model SR-204 recorder (Heath/Schlumberger, Benton Harbor, MI). All connecting tubing between components (4) and (6) was 0.022 cm I.D. stainless tubing (Waters Associates). Small-bore connecting tubing is necessary for minimizing sample dispersion.

Once connected, the column was equilibrated for 72 hr at a flow rate of 6.0 ml/hr using 0.5*N* NaOH as eluent. The pump was set such that the flow rate through the column was equivalent to that which would have been obtained with a 1-m eluent head above the column. This was done to prevent compaction of packing material under pressure. The RI detector attenuation was set at 2×.

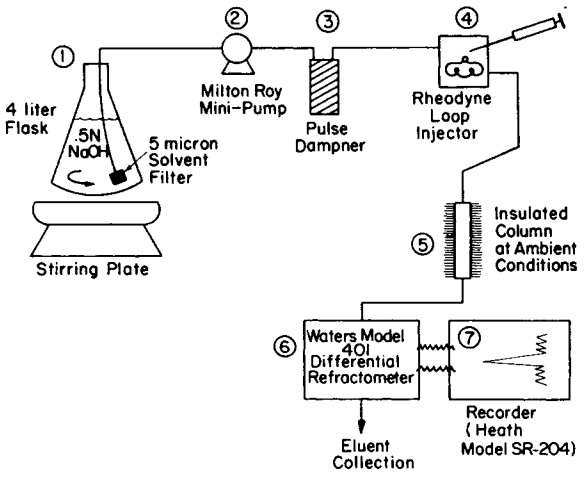


Fig. 3. Component assembly for low pressure GPC.

RESULTS

Fractionation of Polymer Standards of Known Molecular Weight Distribution

Fractionation efficiency of the system was checked using standard polymer fractions of known molecular weight distribution. Polystyrene sulfonate (PSS) standards (Pressure Chemical Co., Pittsburgh, PA) and dextrans standards (Pharmacia Fine Chemicals, Piscataway, NJ) were used for this purpose.

$\overline{MW}_w$, $\overline{MW}_n$, and polydispersity ratio $\overline{MW}_w/\overline{MW}_n$ of PSS standards PSS4, PSS16, PSS65, PSS195, and PSS690 as provided by the manufacturer are given in Table II. From the closeness of the polydispersity ratio of these standards to 1.00, it is evident that these standards have very narrow molecular weight distribution. Very sharp peaks were obtained when 50 μl of 0.25% solutions of each of these polymer standards in 0.5*N* NaOH were injected on to the column. The resultant chromatograms are shown in Figure 4. Chromatograms for the standards PSS16 and PSS690 as supplied by the manufacturer are also included in Figures 4(a) and 4(b) for the purpose of comparison. Next, a mixture of all five standards at a concentration of 0.1% each was applied to the column. The chromatogram is shown in Figure 5. A plot of log $\overline{MW}_w$ against elution time at the peak gives a straight line indicating that the column is capable of performing excellent fractionation of the polystyrene sulfonates. The salt peak seen in the chromatogram is due to the Na_2SO_4 that is present as an impurity in the standards.

Dextran standards have been used extensively in aqueous GPC.[27-29] Relevant properties of dextran standards are listed in Table III. These standards have relatively broad molecular weight distribution. The fractionation of dextrans T-10, T-40, and T-70 resulted in symmetrical curves as shown in Figure 6. Dextran T-500 was only partially resolved since a portion of it had a molecular weight higher than the exclusion limit of the column. That is the reason why T-500 exhibits a bimodal distribution. Plot of log $\overline{MW}_w$ versus elution time at peak is also a straight line as shown in Figure 6. Sample of 0.1% glucose in 0.5*N* NaOH was injected on the column for the determination of plate count. It was approximately 6000 plates/meter for the column packed with "very fine" gels.

Chromatography of Celluloses

In order to demonstrate the ability of the column to fractionate cellulose, the following cellulose samples were used: (a) dilute acid hydrolyzed cotton linter pulp to its leveling-off degree of polymerization (LODP) prepared by Dr. M.

TABLE II
Properties of Polystyrene Sulfonate Standards (PSS)[a]

Polymer	$\overline{MW}_w$	$\overline{MW}_n$	$\overline{MW}_w/\overline{MW}_n$
PSS690	663,762	592,644	1.12
PSS195	200,045	200,045	1.00
PSS65	61,335	68,296	0.90
PSS16	17,400	17,340	1.00
PSS4	approx. 4,000		

[a] Pressure Chemical Co., Pittsburgh, PA.

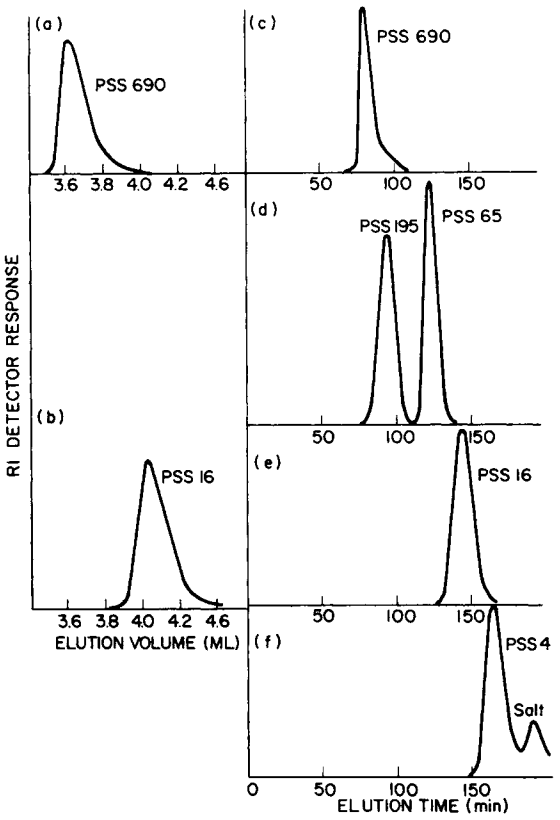


Fig. 4. (a) and (b) chromatograms of polystyrene sulfonate (PSS) standards obtained from manufacturer. (c)–(f) chromatograms of PSS obtained by column packed with "very fine" Sepharose CL-6B gels.

TABLE III
Properties of Dextran Standards[a]

Polymer	$\overline{MW}_w$	$\overline{MW}_n$	$\overline{MW}_w/\overline{MW}_n$
T 500	466,000	230,000	2.026
T 70	64,400	41,100	1.464
T 40	41,000	28,000	1.464
T 10	9,400	5,500	1.709

[a] Pharmacia Fine Chemicals, Piscataway, NJ.

Chang (LORRE, Purdue University) according to the procedure of Battista et al.[30]; (b) microcrystalline cellulose Avicel PH 101; (c) Whatman CF-11 cellulose powder.

Each cellulose sample was dissolved in cadoxen (prepared by method of Segal and Timpa[31]) at the concentration of 0.5%. The cellulose solution in cadoxen was then injected onto the column and eluted with 0.5*N* NaOH. The cadoxen showed up as a large peak following the polymer peak. Since the elution time for the cadoxen peak is constant for a particular column, the peak is only shown once in Figure 7(a).

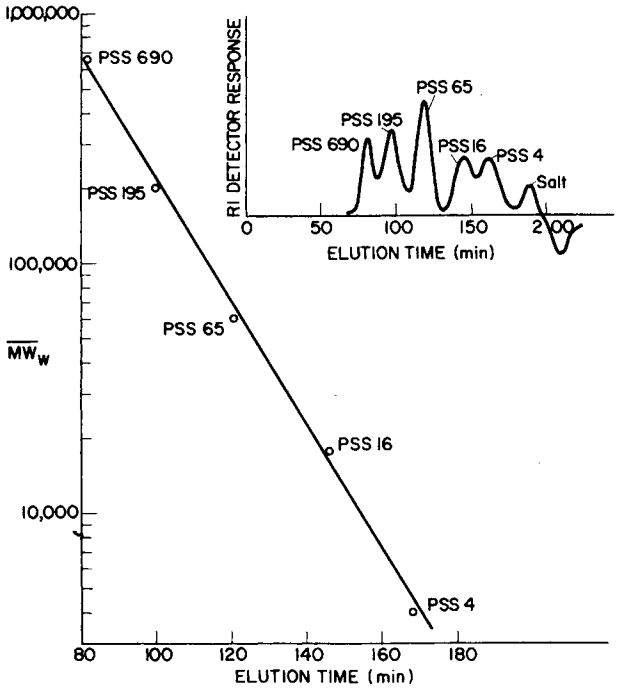


Fig. 5. Chromatogram of mixture of polystyrene sulfonate standards obtained by column packed with "very fine" Sepharose CL-6B gels, along with a plot of log $\overline{MW}_w$ versus peak elution time.

For comparison, each cellulose sample was trinitrated by the method of Alexander and Mitchell,[32] dissolved at a concentration of 0.25% in tetrahydrofuran (THF), and chromatographed on a series of μm-Styragel columns (Waters Associates, Milford, MA).

Chromatograms of each of the cellulose samples in 0.5*N* NaOH is shown in Figure 7(a), and that for the corresponding trinitrates is shown in Figure 7(b). The shape of the curves obtained by the former method is similar to that obtained by the latter. This establishes the feasibility of using 0.5*N* NaOH in the GPC of underivatized cellulose dissolved in cadoxen.

A carbohydrate balance was performed on the cellulose samples to verify that all the carbohydrate that was injected into the column was accounted for in the column effluent. This test was carried out by collecting the effluent from the column and measuring its volume and its carbohydrate concentration by the phenol sulfuric acid method.[24] The product of the volume and the concentration gave the total carbohydrate in the effluent. This was compared with the initial amount of carbohydrate injected onto the column. It was found that approximately 100% of the injected carbohydrate was recovered.

It is preferable to keep the cellulose concentration of the injected sample below 1%, preferably around 0.5%. If the cellulose concentration is higher than 1%, an anomalous peak is observed near the exclusion limit of the column. This is possibly due to aggregation of the cellulose molecules at high concentration in solution. A similar effect was observed in the case of detergents.[33]

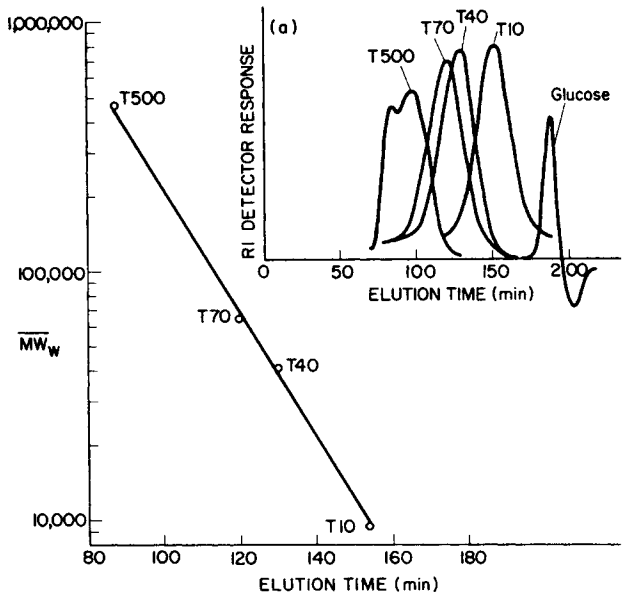


Fig. 6. Chromatograms of Dextrans obtained by column packed with "very fine" Sepharose CL-6B gels and the resultant calibration curve.

DISCUSSION

Column Characteristics

The fractionation efficiency of the columns packed with various particle size ranges was compared by calculating the plate count per meter for each of the columns. The results are shown in Table IV. Plate count was calculated by the following relationship:

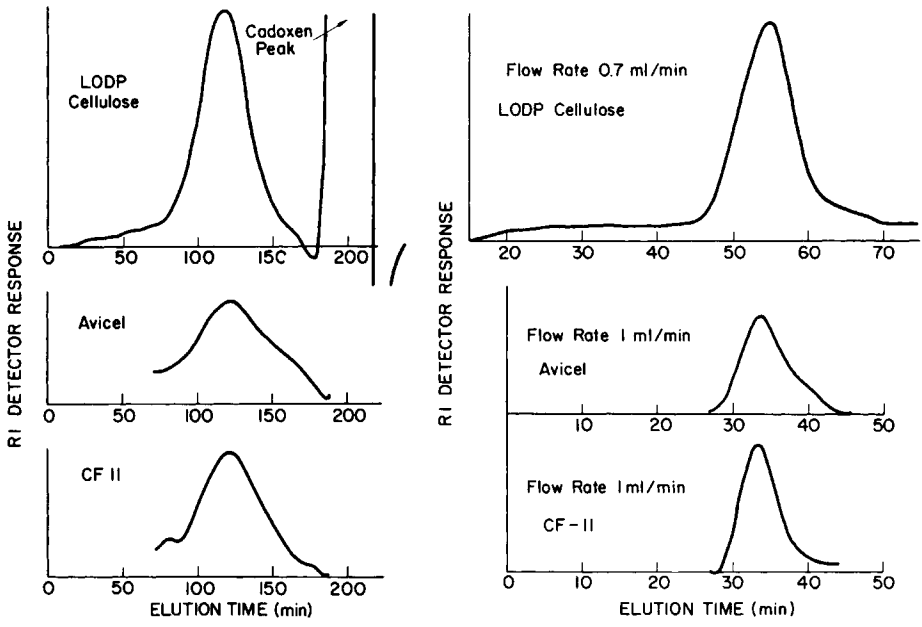
$$N = \frac{1600}{L}\left(\frac{V_R}{W}\right)^2$$

where L = column length in cm, W = the peak base width as determined from the intersections of the tangents drawn through the inflection points with the baseline, and V_R = the elution volume at the peak maximum. The increase in plate count with smaller particle size is consistent with theory.[35,36] The effect of various factors on zone spreading has been reviewed by Tung and Moore.[36]

The operational flow rate of various columns is also listed in Table IV. Because the fine and very fine columns gave low flow rates under gravity, they had to be operated at low flow rates to prevent compression of the packing. Each GPC run then took about 3 hr. This fractionation time is still an order of magnitude improvement over previous attempts at fractionating underivatized cellulose dissolved in cadoxen.[12,13]

Application

The GPC procedure using 0.5N NaOH for cellulose samples as described can be conveniently applied to studies on the hydrolysis of cellulose and starch by acids or enzymes.



(a) (b)

Fig. 7. (a) Chromatograms of cellulose samples obtained by column packed with "fine" Sepharose CL-6B gels. (b) Chromatograms of trinitrated cellulose samples obtained by μm-Styragel columns with tetrahydrofuran as eluent.

The results of the dilute acid hydrolysis[30] of cotton linter pulp obtained with the column packed with "coarse" gels are shown in Figure 8. The insoluble fraction of the pulp was withdrawn after various times of hydrolysis, washed free of acid, dried, dissolved in cadoxen, and injected onto the column. The chromatogram for the product of 10 min of hydrolysis shows a sharp peak at 21.5 min, which represents the fraction with high D.P. which was excluded from the column. As the reaction proceeded, the sharp peak quickly reduced in intensity, and after 30 min of hydrolysis a broad smooth curve was obtained. There was very little change in the distribution profile for samples hydrolyzed for a longer period of time. This result is consistent with previous observation.[30] The constant average D.P. value attained after a short period of hydrolysis is called the leveling-off degree of polymerization. This observation has also been used to interpret the molecular structure of cellulose.[37,38]

The position of the cadoxen peak on the chromatograms coincides with that of glucose and cellobiose. Consequently, the soluble fraction of the product of cellulose hydrolysis will have to be examined by LPLC.[25,26]

The change in molecular weight distribution of corn starch undergoing hy-

TABLE IV
Effect of Particle Size of Column Packing on Fractionation Efficiency of Column

	Particle size, μm	Plate count per meter	Flow rate, ml/hr
Coarse	75–150	3000	22
Fine	30–70	4500	6
Very fine	25–50	6200	5.4

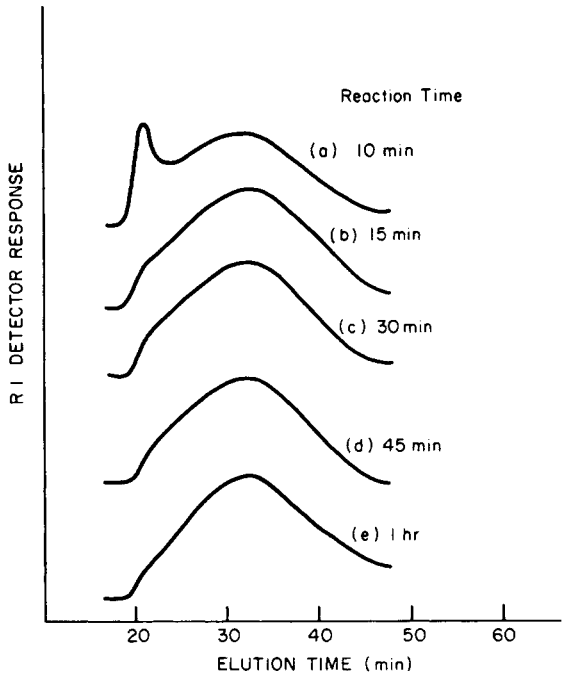


Fig. 8. GPC for dilute acid hydrolysis of cotton linter pulp obtained by column packed with "coarse" Sepharose CL-6B gels.

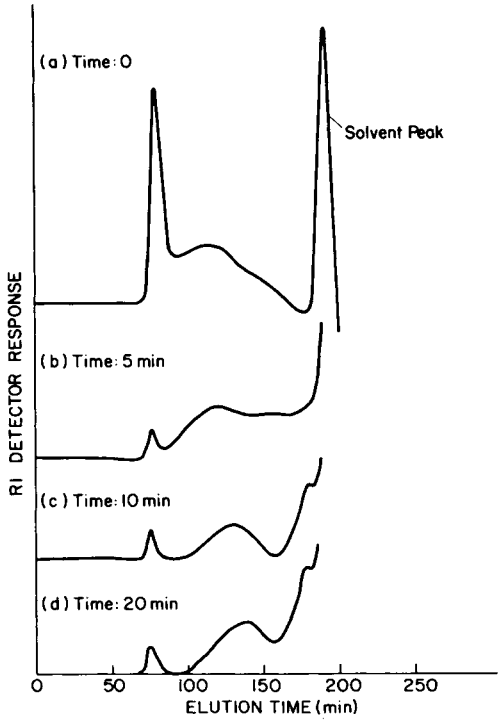


Fig. 9. GPC for enzyme hydrolysis of corn starch obtained by column packed with "very fine" Sepharose CL-6B gels.

drolysis by amylases was followed using this GPC setup. The starch samples are soluble in 0.5*N* NaOH. Hence, the samples were dissolved in 0.5*N* NaOH instead of cadoxen and loaded onto the column. The pattern of change of molecular weight distribution are shown in Figure 9.

The possibility of using this GPC technique for studies of any other polymer that forms a stable alkaline solution is evident.

CONCLUSIONS

The results reported in this paper establish the feasibility of carrying out GPC of nonderivatized cellulose using 0.5*N* NaOH as the eluent. In comparison to nitrated cellulose, the saving in time is considerable. The nitration of cellulose, the drying of nitrated samples, and their subsequent dissolution in tetrahydrofuran takes at least 30 hr. In contrast, the GPC of nonderivatized cellulose in 0.5*N* NaOH is completed in 4 hr. Since only a small amount of cadoxen is needed to dissolve the cellulose samples initially, the preparation of large quantities of cellulose solvent is no longer necessary. Aqueous NaOH solution has been allowed to flow through the cell of the RI detector for eight months without any damage to the cell or loss in sensitivity of the instrument. Excellent resolution of the polystyrene sulfonate standards was obtained on this system. Thus, these standards can be used to calibrate the column. This new technique has potential application in the studies of kinetics of acid and enzymatic hydrolysis as well as molecular structure of cellulose, starch, and other alkali-soluble polymers. The preliminary results presented in this paper on the hydrolysis of starch and cellulose demonstrated the capability of the technique.

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