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# SEPHACRYL GELS: PHYSICAL PROPERTIES AND EVALUATION OF PERFORMANCE IN GEL FILTRATION

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## ABSTRACT

Sephacryl is a gel filtration medium composed of allyl dextran and N,N'-methylene bisacrylamide. Sephacryl S-200 SF has a fractionation range similar to that of Sephadex G-150. Sephacryl S-300 SF has a fractionation range more similar to Sephadex G-200 or Sepharose 6B, with an exclusion limit for globular proteins of about $10^6$ daltons. In typical applications, excellent resolution of protein mixtures was obtained at flow rates up to 30 cm/hr. Good correlation was established between elution volumes of proteins and their molecular weights. The gels contain few ionic groups, as determined by titration. Studies using Sephacryl S-200 at different pH values indicate that, above pH 3, protein does not interact with Sephacryl S-200 at moderate ionic strengths. However, below pH 3, protein binds strongly to the gel through hydrophobic interactions and/or hydrogen bonding. Most separations using Sephadex G-150 or Sephadex G-200 could be duplicated using Sephacryl S-200 or S-300 at much higher flow rates.

## INTRODUCTION

The ability to separate proteins or other macromolecules by gel filtration depends on selection of the proper gel as well as the proper experimental conditions. The ideal gel filtration medium would be capable of high resolution, rigid, and would not otherwise interact with the sample. Many hydrophilic gels composed of agarose, dextran, and/or polyacrylamide perform excellently. However, many of the more porous gels, particularly those synthesized for fractionating molecules

over about 10,000 daltons, are relatively soft gels. When these mechanically weak gels are used in long gel beds, inconvenient slow flow rates are often required, since the gels are not rigid enough to withstand the pressure required to increase the flow rate.

To counteract the disadvantages of softer gels, much work has been justifiably expended on developing more rigid hydrophilic gels. This report serves to inform investigators of the unique advantages and properties of Sephacryl S-200 SF (Superfine) and Sephacryl S-300 SF. These are relatively rigid, hydrophilic gels suitable for separating macromolecules such as proteins in the 5,000 - 1,000,000 dalton size range.

## MATERIALS

Sephacryl is a gel permeation medium prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide. A dry bead of Sephacryl S-200 is shown in Figure 1. For a comparison with Sephadex, see (1). Sephacryl S-200 is obtained already swollen with a wet bead diameter range of 40 - 105 μ, averaging 70 μ (3). Sephacryl S-300 had similar specifications. Sephacryl and Sephadex gels are available from Pharmacia Fine Chemicals (Piscataway, N.J.). Polyacrylamide-agarose composite gels (Ultrogel) are products of LKB - Industrie Biologique Francaise.

Tris(hydroxymethyl)aminomethane, abbreviated as Tris, was obtained from Fisher Scientific Company. Purified proteins used in this study were obtained from Pharmacia gel filtration calibration kits or as previously described (2).

## METHODS

### Packing Procedures

Gels were packed in Pharmacia columns with diameters ranging from 1.0 to 5.0 cm and with lengths varying from 20 to 100 cm. All columns were equipped with flow adaptors. Columns were packed with gel following each manufacturer's instructions. The best resolution with Sephacryl S-200 was obtained if it was packed under a constant pressure source of 0.5 - 1.0 atmospheres for 1-2 hours. A packing pressure of 0.5 atm is recommended for columns under 2.0 cm or over 5.0 cm in

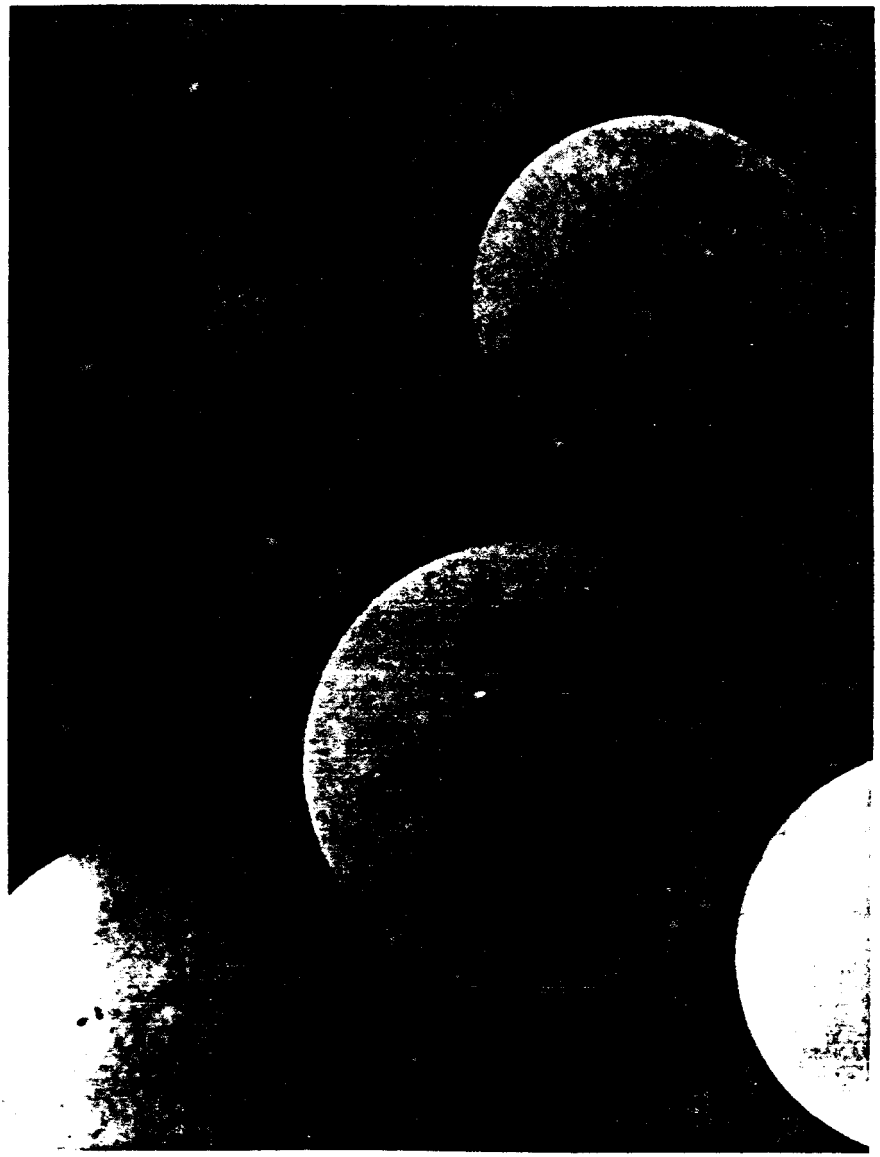

Figure 1. A dry bead of Sephacryl S-200 as seen in a scanning electron microscope. Magnification about 1000 times originally (about 800 times at presend). (Microscope Laboratories, Red Bank, N.J.) A comparison with Sephadex G-200 can be seen in Reference 1.

diameter or for columns over 50 cm long. Results equivalent to constant pressure packing were obtained by equilibrating gel beds using a peristaltic pump to produce a linear flow rate of about 40 cm/hr (40 ml/cm/hr) for one hour. Sephacryl S-300 was packed as described for S-200 but at about 2/3 the pressure or flow rate described for S-200. Good packing was also obtained by shrinking gel in 80% ethanol. After the gel was packed at a low flow rate in 80% ethanol, the ethanol was eluted slowly with buffer. The reswollen gel had properties identical to pressure-packed gel.

Pressure-Flow Determinations

Columns were filled with gel to a bed height of about 40 cm and connected to a constant pressure tank (Amicon Corp.). Flow rate was measured at about 60 min intervals following pressure increases. All experiments used water as eluant at 22°C. Flow rates were normalized to 40 cm long columns assuming that flow rate was inversely proportional to column height.

Calculations

Separation efficiency of a given gel under stated conditions was given as the "height equivalent to a theoretical plate (HETP)" calculated as:

$$HETP = \frac{H}{16\ (v_e/w)^2}$$

where H is the length of the gel bed in cm, $v_e$ is the elution volume in ml, and w is the width of the eluted peak at baseline in ml.

Separation of two adjacent peaks was given in terms of "specific resolution factor" calculated as:

$$R_{sp} = \frac{2(v_{e2} - v_{e1})}{(w_1 + w_2)} \cdot \frac{1}{\log_{10}\ (MW_1/MW_2)}$$

where $v_{e1}$ and $v_{e2}$ refer to the two elution volumes of the peaks, and $w_1$ and $w_2$ refer to their corresponding peak widths, and $MW_1$ and $MW_2$ refer to their corresponding molecular weights.

Chromatographic Method

Samples were generally chromatographed in 2.6 cm diameter columns packed with gel equilibrated with 0.1 M Tris/HCl (pH 8.4) - 0.1 M NaCl - 0.02% sodium azide, at 22°C. Samples were injected using a 1 ml sample loop containing a small air bubble to minimize mixing of

the sample with eluant. The eluant was monitored using Pharmacia ultraviolet (UV-2) monitors at 280 nm. Fractions were collected by fraction collector or manually, and yields were estimated on the basis of $A_{280}$.

Titration of Sephacryl S-200 SF

Sephacryl S-200 SF was washed on a fritted glass funnel with 0.1 M NaOH - 0.5 M KCl, then with 0.5 M KCl, and then titrated using HCl solutions containing 0.5 M KCl. A blank solution was also titrate which contained the same amount of KCl solution as in the gel suspensi The difference between amount of acid used for the sample and the blan was plotted vs. pH. A similar procedure for the basic side of the titration curve was followed, using gel washed in 0.1 M HCl - 0.5 M KC The titration was conducted at 22°C using a Sargent-Welch model NX pH meter.

Gel Filtration Using Sephacryl S-200 at Different pH Values

Aliquots of Sephacryl S-200 and Sephadex G-25 were equilibrated overnight with 1 M glycine/HCl (pH 3), 1 M sodium acetate (pH 5.5), or 1 M Tris/HCl (pH 8). The gels were then washed with the same buffers diluted to 50 mM and packed in 2 ml plastic columns.

Proteins were dissolved in the selected buffers at 1 mg/ml and applied to each column. Each column was rinsed with enough buffer to obtain the fraction just prior to the elution position of the protein. Protein was then eluted by the addition of 1 column volume of buffer and yield estimated by $A_{280}$. In other experiments, protein left bound to the column after this procedure was eluted by additional rinses with buffer or buffer containing detergents or other reagents. All solution were adjusted to the pH studied before use.

## RESULTS AND DISCUSSION

Pressure-Flow Characteristics of Sephacryl S-200

The flow rate obtained with any gel is generally a direct function of the operating pressure on the gel as supplied by a hydrostatic pressure head, a constant pressure source, or from a pump. A complicating factor is that walls of chromatographic columns can provide considerable support to soft gel beds. As a consequence, narrow columns

may allow higher flow rates with soft gels than columns of larger diameters. Using the softer Sephadex and Sepharose gels, we have found these wall effects most apparent in columns less than 2 cm in diameter.

As shown in Figure 2, flow rates obtained using Sephacryl S-200 may exceed 50 cm/hr (50 ml/cm /hr) in 2.6 cm and 5 cm diameter columns, without collapse of the gel. Higher flow rates were obtained in 1.6 cm columns than were obtained in 2.6 cm columns under the same pressure. In comparison, Sephadex G-150 columns 40 cm in length reach a maximum flow rate of about 7 cm/hr under about 0.1 atmosphere of pressure. Higher pressures on Sephadex G-150 result in collapse of the gel and

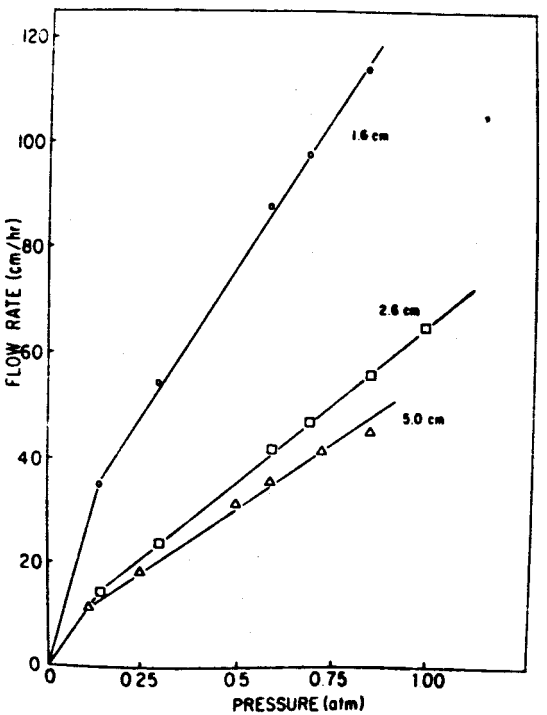


**Figure 2. Pressure-Flow Properties of Sephacryl S-200 Packed in Columns of Three Different Diameters. See Methods section.**

diminution of the flow rate. Results obtained with beds of Sephacryl S-200 twice as long, 80 cm, yielded flow rates nearly exactly half as obtained in 40 cm long beds tested at the same pressures.

A pressure-flow curve was also plotted for Sephacryl S-300 (Figure 3) using the same scale as for Sephacryl S-200 in Figure 2. Diminution of the flow rate, indicating impending collapse of the gel bed, was apparent at a pressure of about 0.7 atmospheres. The maximum flow rate for 40 cm long Sephacryl S-300 beds is about 30 cm/hr, although higher flow rates are obtainable in smaller diameter columns.

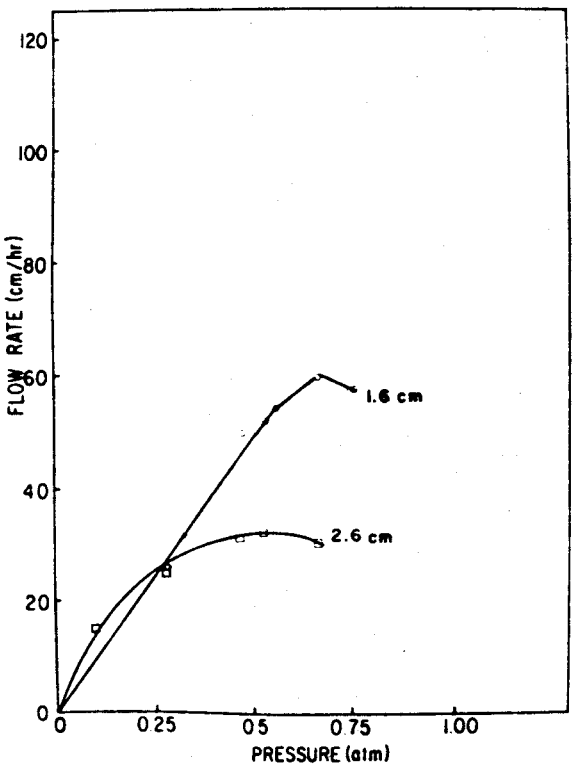


**Figure 3. Pressure-Flow Properties of Sephacryl S-300 in Columns of Two Different Diameters. See Methods section.**

At extremely high flow rates, Sephacryl can show symptoms of "overpacking". At flow rates over 60 cm/hr (40 cm long columns), peaks eluted asymmetrically with a large leading edge, a phenomenon we have labeled "heading" to distinguish it from the more readily observable "tailing" phenomenon (see Figure 4). We speculate that this "heading" results from the distortion of gel beads in the center of the gel bed, resulting in the core of the column having altered chromatographic properties. This occurs even though the gel bed does not compact and appears unchanged. This phenomenon is avoided by limiting the flow rates to about 40 cm/hr, or limiting the pressure differential to 0.5 atm. Similar, but 30% lower limits, apply to Sephacryl S-300.

Effect of Flow Rate on Peak Widths

Resolution in gel permeation chromatography can be defined by the difference in elution volumes between two peaks and the widths of the two elution peaks. Direct normalized comparison of peak widths obtained with several gels at a given flow rate can be obtained through

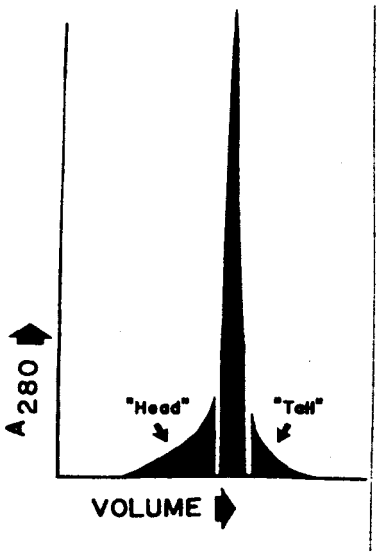


**Figure 4. Example of "Heading". Chromatograph trace taken from a 1.0 cm column packed with Sephacryl S-200 under 2 atmospheres pressure.**

calculation of the HETP (height equivalent to a theoretical plate) values. Low HETP values reflect narrow bands.

In gel permeation chromatography, HETP values generally increase with increasing flow rate. A major cause of the increase in band width at high flow rates is the failure to appraoch an equilibrium state across the gel surface. The effect of increased flow rate upon HETP would be expected to be less dramatic with substances entirely excluded from the gel, since the gel surface would appear impermeable to the substance being chromatographed. A similar situation should exist for substances much smaller than the effective pore size of the gel, since the gel would be essentially "invisible" to the substance chromatographed. As shown in Figure 5, blue dextran (entirely excluded by Sephacryl S-200) and ribonuclease A (nearly entirely included by the gel) were not as effected by increases in flow rate as molecules intermediate in size. At flow rates below about 2 cm/hr, an increase in HETP might be expected due to the effects of longi-

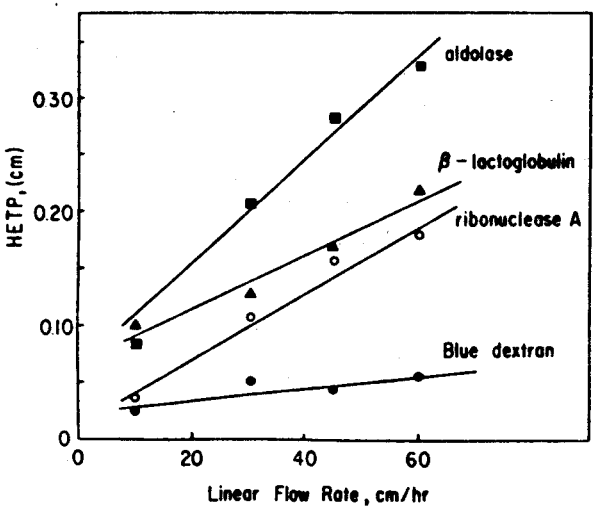


**Figure 5. Variation of HETP vs. Flow Rate of a Sephacryl S-200 Column. Data was obtained using a 2.5 cm x 24.1 cm column of Sephacryl S-200 in 50 mM Tris/HCl buffer (pH 8.0).**

tudinal thermal diffusion. However, in liquid chromatography, the use of such low flow rates is uncommon.

Comparison with Alternative Gels - HETP

Smaller particle sizes of gel generally produce better resolution at slow, as well as rapid, flow rates. Equilibrium between internal and external phases is reached more rapidly using small gel particles, and tighter packing of fine particles is usually obtained. With these factors in mind, we compared Sephacryl S-200 with other gels which cover the same fractionation range but which vary in their hydrated bead diameters and in the range of these diameters. Chromatographs (Figure 6) and data (Table 1) were obtained using a synthetic mixture of five purified proteins.

An interesting comparison can be made between Sephacryl S-200 and Sephadex G-150, both operating at a linear flow rate of 7 cm/hr (Table 1). HETP values using Sephacryl S-200 were about 7-fold lower than Sephadex G-150. This difference reflects that the average particle size of the Sephacryl S-200 was about 2.5 times smaller than the Sephadex G-150. The HETP values obtained using Sephacryl S-200 were slightly lower than those obtained with Sephadex G-200 Superfine, although the average particle sizes were about equal. The advantage obtained by Sephacryl S-200 probably reflects the relatively narrow particle size range of the gel.

Sephacryl S-200 was also compared with Ultrogel ACA-34, a polyacrylamide-agarose composite gel with a similar particle size distribution. Typical HETP's produced by Ultrogel average twice that of Sephacryl S-200. However, at flow rates of over 30 cm/hr, the HETP values produced by Ultrogel ACA-34 increased dramatically. This suggests that the gel was beginning to deform as its maximum operating pressure had been met.

Comparison with Alternative Gels -- $R_{sp}$

An excellent experimental value for evaluating a gel is the "specific resolution factor" ($R_{sp}$) between two suitable standards. $R_{sp}$ evaluates both peak widths and the difference in elution volumes, and is normalized for the difference in molecular weights of the samples. $R_{sp}$ values

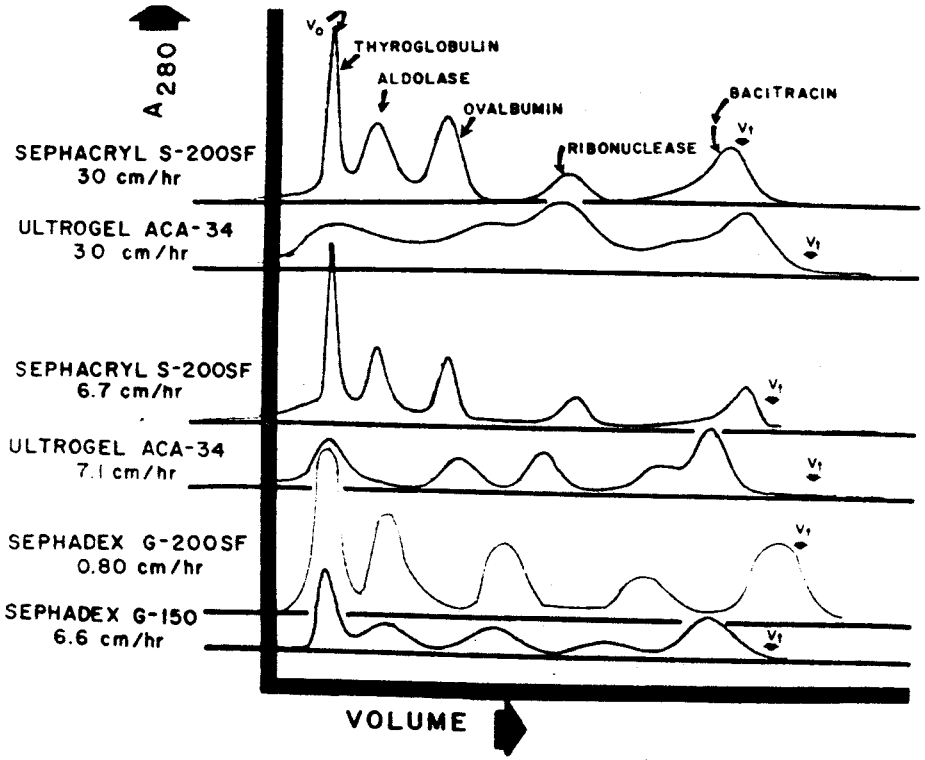


Figure 6. Comparison of Sephacryl S-200 with other Gels with Similar Fractionation Ranges: Separation of Proteins.

All gels were swelled in 50 mM Tris/HCl - 0.1 M NaCl - 0.2% sodium azide at pH 7.5 and packed into Pharmacia K26 columns following the recommendation of each manufacturer. Each column was packed to a bed height of 50 cm + 5 cm. The mixture chromatographed contained exactly 9.87 $A_{280}$ units each of thyroglobulin, aldolase, ovalbumin, ribonuclease A, and bacitracin. Chart speeds were adjusted such that each chromatograph had approximately the same scale. Chromatograph of Sephadex G-200 SF was redrawn to the proper scale by hand.

TABLE 1

COMPARISON OF SELECTIVITY OF SEPHACRYL S-200 TO ALTERNATIVE GELS

| Gel | Flow Rate (cm/hr) | Hydrated Bead Diameter (microns) | Thyroglobulin | | | Aldolase | | | Ovalbumin | | | Ribonuclease | | | Bacitracin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $K_{av}$ | HETP (cm) | $R_{sp}$ | $K_{av}$ | HETP (cm) | $R_{sp}$ | $K_{av}$ | HETP (cm) | $R_{sp}$ | $K_{av}$ | HETP (cm) | $R_{sp}$ | $K_{av}$ | HETP (cm) |
| I. Flow Rate 30 cm/hr ± 10% | | | | | | | | | | | | | | | | |
| a. Sephacryl S-200 SF | 30.0 | 40-105 | 0.00 | 0.027 | 1.71 | 0.11 | 0.156 | 2.32 | 0.28 | 0.082 | 3.94 | 0.57 | 0.063 | 2.20 | 0.97 | 0.062 |
| b. Ultrogel ACA34 | 29.9 | 60-100 | 0.00 | 0.87 | 1.82 | 0.32 | 0.84 | 0.95 | 0.49 | 0.40 | 1.31 | 0.72 | 0.35 | 0.52 | 0.87 | 0.06 |
| II. Slow Flow | | | | | | | | | | | | | | | | |
| c. Sephacryl S-200 SF | 6.71 | 40-105 | 0.00 | 0.024 | 2.52 | 0.11 | 0.078 | 3.46 | 0.27 | 0.037 | 5.96 | 0.56 | 0.033 | 3.24 | 0.96 | 0.024 |
| d. Ultrogel ACA34 | 7.06 | 60-100 | 0.00 | 0.39 | 3.06 | 0.26 | 0.16 | 2.45 | 0.44 | 0.070 | 3.39 | 0.67 | 0.057 | 0.84 | 0.79 | 0.040 |
| e. Sephadex G-200 SF | 0.80 | 32-129 | 0.00 | 0.10 | 2.05 | 0.126 | 0.10 | 3.61 | 0.37 | 0.073 | 3.67 | 0.65 | 0.062 | 1.55 | 0.92 | 0.049 |
| f. Sephadex G-150 SF | 6.8 | 116-349 | 0.00 | 0.10 | 1.55 | 0.13 | 0.43 | 2.08 | 0.37 | 0.25 | 2.17 | 0.63 | 0.16 | 1.01 | 0.85 | 0.098 |

were calculated for 4 pairs of proteins covering the fractionation range of Sephacryl S-200 and alternative gels (Table 1). Generally, the evaluation of all gels conformed to findings determined by HETP alone; that is, the gels giving superior performance did so on the basis of narrow peak widths as opposed to increased elution volume between peaks. Note that the results obtained with Sephacryl S-200 were roughly comparable to the results obtained using a Sephadex G-200 SF gel at about 1/35 the flow rate (Table 1 and Figure 6). Sephacryl S-200 performed best using the proteins below 150,000 daltons.

Relationship between Molecular Weight and Elution Volume

The ideal gel permeation medium separates macromolecules only on the basis of molecular size; this property allows the gel to determine molecular size of unknowns. It is also well established that the correlation between molecular weight and elution volumes of proteins chromatographed on Sephadex gels is excellent (4). Molecular weight and molecular size of globular proteins generally correlate closely, and proteins which contain such high proportions of hydrophobic amino acids to cause adsorption effects are relatively rare (5). Using Sephacryl S-200, the correlation between elution volume, normalized to $K_{av}$ values, and the molecular weight of the tested proteins plotted on a logarithmic scale was also excellent (Figure 7). Furthermore, the relationship was approximately linear between about 2,000 and 100,000 daltons. As a reference, the selectivity curve obtained with Sephadex G-200 is given; the curve obtained with Sephadex G-150 proved virtually superimposable with Sephacryl S-200.

Sephacryl S-300 was more porous than Sephadex G-200 to proteins over 50,000 in molecular weight. The fit of proteins to a straight line was excellent over the range from 2,000 to about 700,000 daltons (Figure 8).

Provided that a proper pH and ionic strength are used in the elution buffer (see below), it would appear that Sephacryl gels, as well as dextran gels, can be recommended for estimating molecular weights of proteins. Since Sephacryl gels allow higher resolving power and higher flow rates than either dextran or agarose gels, such determinations should be completed much more rapidly using Sephacryl gels.

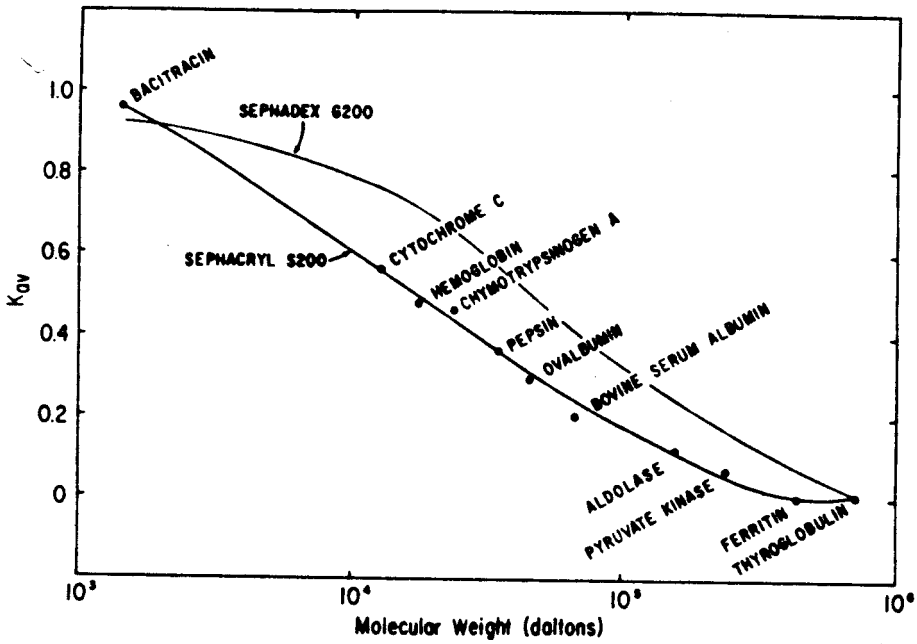


Figure 7. Semilogarithmic Plot of the Molecular Weight vs. $K_{av}$ of Proteins Chromatographed Using Sephacryl S-200. For details, see Methods section.

Example of Separation of a Natural Protein Mixture

An example of the resolution obtainable using Sephacryl S-200 is presented in Figure 9. A crude extract of crystallin proteins from bovine lenses was prepared and enriched for the smaller molecular weight crystallins by isoelectric precipitation of alpha crystallin from the mixture at pH 5 in ethanol. This extract was fractionated on a 67.5 cm long column of Sephacryl S-200. Eluted peaks of $B_H$ crystallin, $B_L$ crystallin, and gamma crystallin with molecular weights of 230,000, 80,000, 28,400, and 20,500 were all well separated. The $B_H$ peak appeared slightly asymmetric due to a small amount of alpha crystallin (670,000 daltons) present in the leading edge. All the crystallins can be purified in excellent yield free of contaminating crystallins (determined

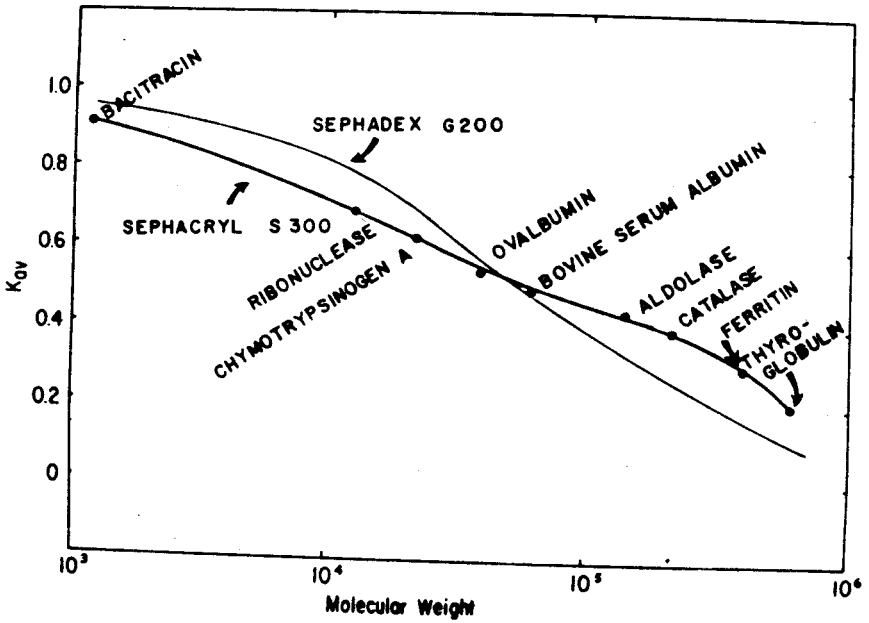


**Figure 8.** Semilogarithmic Plot of the Molecular Weight vs. $K_{av}$ of Proteins Chromatographed Using Sephacryl S-300. For details, see Methods section.

by analytical gel filtration experiments) using this single chromatographic step.

Absence of Ionic Groups in Sephacryl S-200

The ideal gel permeation medium contains no ionizable groups which can contribute to ion exchange, ion exclusion, or Donnan effects. Sephadex gels, for example, typically contain 10 - 20 μequivalents of carboxyl or sulfate groups per gram of dry gel. Generally, this low level of ionizable groups has no detectable effect except at very low ionic strengths with certain molecules. Titration of Sephacryl S-200 revealed less than 0.1 μequivalents per ml of gel of ionizable groups between pH 1.5 and 10 and about 0.1 μequivalents per ml of gel of a charged group with $pK_a$ of about 11.5 (Figure 10). This extremely low

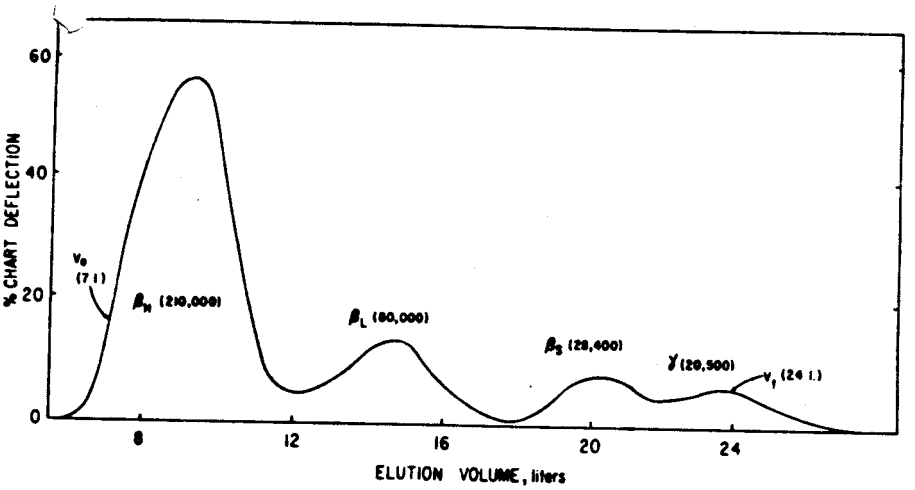


Figure 9. Separation of Crystallins Using Sephacryl S-200. A 1250 ml sample enriched for crystallins by the method of Papaconstantinous et al. (6) was applied to and eluted from a 21.5 cm x 67.5 cm column of Sephacryl S-200 equilibrated with 0.1 M Tris/HCl (pH 8.4) - 0.1 M NaCl - 0.02% sodium azide. Sample was eluted at 30 ml/min running upwards flow and monitored at 254 nm using a Pharmacia UV-1 monitor, with a full-scale sensitivity of 20 $A_{254}$.

level of ionizable groups suggests that Sephacryl S-200 is unlikely to act as an ion exchanger and that other ion effects are also unlikely to be detected. While the titration curve does not eliminate the possibility of charged groups with $pK_a$ values below 1, or over 12, the chromatographic behavior of the gel suggests few charged groups.

Gel Filtration Using Sephacryl S-200 at Different pH Values

A number of proteins with varying isoelectric point values (pI) were tested for adsorption onto Sephacryl S-200. These experiments provided guidelines for experimental conditions under which Sephacryl would serve as a gel permeation medium with a minimum probability for non-specific interactions.

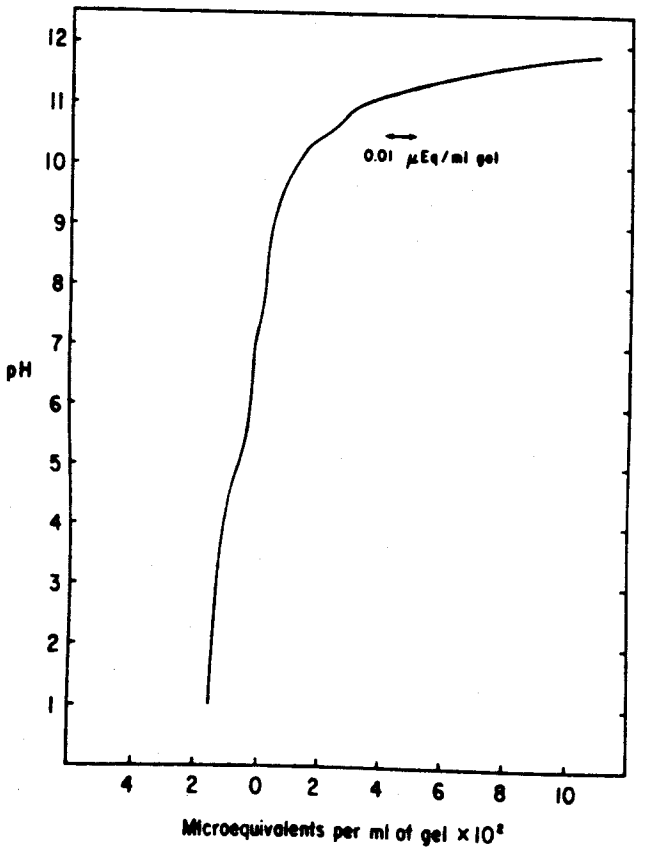


Figure 10. Titration of Sephacryl S-200. See Methods for experimental procedure.

As described by Belew, Porath, and Fohlman (7), Sephacryl S-200 showed virtually no adsorption effects between pH 5.6 and 7.5, even at low ionic strength. This was confirmed using three proteins with low, neutral, and high pI values in 50 mM sodium acetate (pH 5.6) with Sephacryl S-200 equilibrated with the same buffer (Table 2). At pH 8, the situation was somewhat more complicated. Using 1 mg aliquots of proteins chromatographed on 1 ml Sephacryl S-200 columns, adsorption occurred as evidenced by yields of proteins of only about 80%. In some cases, the yields obtained using Sephadex G-25 columns of the same size,

TABLE 2

YIELD OF PROTEINS ELUTING FROM SEPHACRYL S-200 SF COLUMNS AT pH 3.

| Protein | pI | Sephacryl[1] S-200 SF % Yield | Sephadex G-25 % Yield | % Yield from Sephacryl S-200 SF with Additional Eluants (2 × $V_t$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | pH 3 Buffer | 0.5 M NaCl | 3 M Urea | 3 M Guanidine/HCl | 1% Tween | 1% SDS |
| pepsin | 1-2 | 72 | 77 | - | - | - | - | - | - |
| ribonuclease $T_1$ | 2 | 34 | - | - | - | - | - | - | - |
| puruvate kinase | 6.6 | 7 | 97 | 0 | 0 | 64 | 91 | 100 | 9 |
| myoglobin | 6.7 | 0 | 89 | 5 | 14 | 5 | 59 | 24 | 65 |
| alcohol dehydrogenase | 6.8 | 14 | 96 | 2 | 2 | 4 | 16 | 100 | 26 |
| carbonic anhydrase | 7.3 | 3 | 97 | 4 | 1 | 3 | 78 | 100 | 70 |
| alpha-chymotrypsinogen | 9.1 | 0 | 80 | 14 | 69 | 7 | 2 | 22 | 59 |
| aldolase | 9.2 | 10 | 89 | 7 | 2 | 40 | 51 | 100 | 99 |
| cytochrome c | 9.5 | 5 | 88 | 23 | 84 | 84 | 7 | 10 | 54 |

Note: [1] Average of six experiments.

Each 1 ml column was equilibrated with 50 mM glycine/HCl as described in Methods section of report. 1 mg of each protein was applied to each column and eluted with 1 × $V_t$ of buffer. Yields were determined by $A_{280}$. Control columns of Sephadex G-25 were treated in the same manner. Other eluants contained 50 mM glycine and were adjusted to pH 3.

yields were also substantially less than 100%. Proteins with high pI values tended to elute in higher yields, but not enough proteins were tested to establish this trend as a rule.

These results could indicate that Sephacryl S-200 weakly absorbed the proteins. Alternately, the gel might tightly adsorb protein but have a limited capacity. These alternatives were tested by using the same amount of protein and ten times as much gel. As shown in Table 2, this increase in the amount of gel generally did not increase the amount of protein bound. It appeared most likely that the protein was retarded rather than strongly bound, and this was confirmed. Washing columns with more buffer generally recovered all of the protein applied to the column.

Previous experiments have shown, on the basis of yield and absorption profiles, that inclusion of 0.1 M NaCl abolishes the absorption phenomenon seen at pH 8 at low ionic strength. This is also confirmed by the study of Belew, Porath, and Fohlman (7). The nature of the absorption was tested by attempting to elute protein bound at pH 3 with various agents, all adjusted to pH 3. Washing the columns with more pH 3 buffer generally eluted slightly more protein, indicating that some proteins were only weakly retarded by the gel. If the interaction were ionic, one would expect 0.5 M NaCl to elute most proteins. However, only chymotrypsinogen and cytochrome c were eluted with NaCl. 3 M urea or 3 M guanidine/HCl should effectively disrupt hydrogen bonds between gel and protein. As shown in Table 3, urea and guanidine were occasionally very effective in eluting protein, but urea was sometimes much more effective than guanidine, and vice versa. The effectiveness of a nonionic detergent, Tween-80, which should reduce hydrophobic interactions, was also quite erratic, eluting between 10% and 100% of applied protein. The ionic detergent sodium dodecyl sulfate was also tested. While it was overall the most effective eluting agent, it still failed to elute some proteins.

It must be concluded that the interaction of proteins with Sephacryl S-200 at pH 3 is relatively non-specific and may result by the combination of weak hydrogen bonding or hydrophobic interactions. The

TABLE 3

YIELD OF PROTEINS ELUTING FROM SEPHACRYL S-200 SF AT THREE DIFFERENT pH VALUES

| Protein | pI | Gel | 50 mM glycine pH 3 | sodium acetate pH 5.6 | Tris/HCl pH 8 | 10 ml Column pH 8 |
|---|---|---|---|---|---|---|
| pepsin | 1-2 | Sephacryl S-200 SF | 72 | 95 | 78 | 89 |
| | | (Sephadex G-25) | (77) | - | (76) | (97) |
| ribonuclease $T_1$ | 2 | Sephacryl S-200 SF | 34 | - | 71 | - |
| | | (Sephadex G-25) | - | - | - | - |
| pyruvate kinase | 6.6 | Sephacryl S-200 SF | 7 | - | 80 | - |
| | | (Sephadex G-25) | 97 | - | (99) | - |
| myoglobin | 6.7 | Sephacryl S-200 SF | 0 | 95 | 88 | 93 |
| | | (Sephadex G-25) | (89) | - | (98) | (96) |
| alpha-chymotrypsinogen | 9.1 | Sephacryl S-200 SF | 0 | 94 | 80 | 56 |
| | | (Sephadex G-25) | (80) | - | (97) | (76) |
| cytochrome c | 9.5 | Sephacryl S-200 SF | 5 | - | 89 | 87 |
| | | (Sephadex G-25) | (88) | - | (92) | (94) |

Each 1 ml or 10.6 ml column was equilibrated with 50 mM buffer as described in Methods section of report. 1 mg of protein was applied to each column and eluted with 1 x $V_t$ of buffer. Yields were determined by $A_{280}$. Control columns of Sephadex G-25 were prepacked PD-10 columns with a volume of 9.1 ml.

observation that a negative charge on the tested proteins may prevent absorption suggests that the gel contains a slight negative charge at pH 3, resulting in a repulsion overcoming the other interactions. Since no agent tested completely eliminates the absorption at pH 3, Sephacryl cannot generally be recommended for gel permeation chromatography at this pH.

It should also be noted that Sephacryl absorbs blue dextran (commonly used to estimate $V_o$) below pH 7 at low ionic strengths. Under these conditions, unsubstituted dextrans are more suitable to estimate $V_o$, but require refractive index monitors for detection. In addition, weak retardation of nucleosides and aromatic amino acids occurs on Sephacryl S-200 as it occurs on Sephadex gels, but nucleic acid polymers, in our experience, do not interact with Sephacryl S-200.

## CONCLUSIONS

Sephacryl gels, prepared by cross-linking allyl dextran with methylene bisacrylamide, were demonstrated to be significantly more rigid than other hydrophilic gels. Sephacryl gels permitted flow rates around 50 times faster than Sephadex G-200 SF, with no loss of resolution. Separations obtained using a gel such as Sephadex G-150 could be duplicated using Sephacryl S-200 at about 10 times the flow rate (c.f. Table 1 and Figure 6).

The usefulness of Sephacryl would be restricted if the gel interacted significantly with commonly separated biological molecules. Fortunately, the matrix does not interact significantly with proteins over a wide range of pH and ionic strength. The interactions observed at low pH suggest a slight hydrophobic character to the gel. This hydrophobic character could be the result of unreacted allyl groups or of groups which have undergone hydration to yield hydroxypropyl groups (8).

It should be noted that Sephacryl remains swollen in many organic solvents (3) and its use for fractionating various water-insoluble proteins has been demonstrated (2).

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