Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

A Method Development Study of the Production of Albumin from Animal Blood by Ion-Exchange Chromatography

G. Leaver[ab]; J. R. Conder[a]; J. A. Howell[ac]
[a] Chemical Engineering Department, University College of Swansea, Swansea, Wales [b] Biotechnology Separations Division, Warren Spring Laboratory, Herts, United Kingdom [c] Chemical Engineering Department, University of Bath, Bath, United Kingdom

To cite this Article Leaver, G. , Conder, J. R. and Howell, J. A.(1987) 'A Method Development Study of the Production of Albumin from Animal Blood by Ion-Exchange Chromatography', Separation Science and Technology, 22: 8, 2037 — 2059
To link to this Article: DOI: 10.1080/01496398708057626
URL: http://dx.doi.org/10.1080/01496398708057626

SEPARATION SCIENCE AND TECHNOLOGY, 22(8-10), pp. 2037-2059, 1987

A Method Development Study of the Production of Albumin from Animal Blood by Ion-Exchange Chromatography

G. LEAVER,* J. R. CONDER,† and J. A. HOWELL‡

CHEMICAL ENGINEERING DEPARTMENT
UNIVERSITY COLLEGE OF SWANSEA
SINGLETON PARK, SWANSEA SA2 8PP, WALES

Abstract

The conditions of operation of ion-exchange chromatography are examined for the production of albumin from bovine serum and plasma. The ion-exchanger is a Vistec-DEAE cellulose. Of the various modes of chromatographic operation available, the most suitable for proteins is shown to be adsorption onto the whole column followed by stepwise elution. The method is developed by studying, experimentally, the effect of major parameters so as to point the way to optimization. Prior dialysis and dilution of the feed are beneficial. Other conclusions apply to the large-scale separation of proteins in general. In particular, column length, eluent velocity, feed band width, and packing rigidity are parameters where trade-offs will be needed to maximize performance. If suitable precautions are taken in column design, scale-up can be achieved by increasing the column diameter alone. Albumin separated chromatographically is superior in electrophoretic purity to that produced by the commercial Cohn precipitation method.

INTRODUCTION

Large-scale chromatographic separation is a major field of current interest in the downstream processing of biological macromolecules.

*Present address: Biotechnology Separations Division, Warren Spring Laboratory, Gunnels Wood Road, Stevenage, Herts. SG1 2BX, United Kingdom.

†To whom correspondence should be addressed.

‡Present address: Chemical Engineering Department, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom.

Chromatographic techniques are especially useful for materials such as proteins and polypeptides that are difficult to isolate in high purities by conventional methods.

At present, potentially important sources of protein are wasted in the milk processing industry (cheese whey) and meat process industry (animal blood). The resulting process effluents are major pollutants. It is an attractive proposition to recover the protein by using ion-exchange, size exclusion or affinity adsorbents for efficient separation of proteins from other waste constituents. This can be achieved either by the batch, stirred-tank method (*1–5*) or by using the adsorbent as a fixed bed in a chromatographic column. The stirred-tank approach has been adopted in full scale ion-exchange plants currently producing powdered whey protein in the British Isles and USA. To produce high added-value proteins, however, requires protein fractionation and this cannot be achieved efficiently in a stirred tank. Particularly when different proteins desorb under rather similar processing conditions, a chromatographic column is more easily controlled and automated so as to resolve the proteins according to their affinity for the adsorbent. Chromatographic separation also has other advantages. Liquid holdup and protein wastage are less. There is no shear imposed on the adsorbent medium or protein to cause attrition of particles of the medium or denaturation of the protein. Additionally, solid-liquid separation procedures are not needed and aseptic conditions are more easily maintained (*6*, *7*).

Several types of liquid chromatography are available for production-scale protein separations. Size exclusion (gel filtration) chromatography (*8*) is unable to separate proteins of different charge unless they also differ in size. Its main disadvantage for scale-up is poor throughput compared with ion-exchange chromatography, due to the relatively poor mechanical properties of the column packing (*6*). The most promising methods are affinity chromatography and ion-exchange chromatography. Affinity chromatography is a powerful separating tool because the ligand is normally specific to a single protein. Its disadvantages are that the ligand media can be expensive, have low adsorptive capacities, and often tend to be unstable, producing a contaminated product (*9–11*). For purification of high value products such as enzymes, these disadvantages may be outweighed by the many advantages conferred by the specificity of the ligand (*12*). For other protein separations, ion-exchange chromatography may offer lower costs and longer working life.

All three forms of chromatography have been used in large-scale processes devised for the separation of albumin from human blood plasma (*13–20*). Janson (*6*, *21–23*) has reviewed production chromatography of proteins but, until recently, most published work was con-

fined to qualitative descriptions of the process. Aspects of the design of affinity chromatographs, however, have been examined by Graves and Wu (*24*), Chase (*7*, *25*, *26*), Arnold et al. (*27*), and Hamman and Calton (*28*). Here we report a method development study of the separation of proteins by ion-exchange chromatography. The separation investigated was that of albumin from bovine serum or plasma. The aim was to select the most suitable form of chromatographic operation, examine the effect of the main variables on performance, and deduce how these variables are likely to interact in the overall design problem. The results are applicable to 1) recovering animal blood proteins for use in veterinary medicine, as substitutes for human plasma fractions and as protein boosters, stabilizers, and specific amino acid sources in foodstuffs (*29*, *30*); 2) fractionating human plasma, which presents a similar chromatographic separation problem to animal plasma; and 3) separating other proteins by production chromatography.

MODES OF CHROMATOGRAPHIC OPERATION

There are several ways of operating an ion-exchange or affinity column to separate proteins. In *elution* chromatography, a discrete sample or batch of mixed protein solution is introduced to the column inlet and carried through the column by the flowing mobile phase. Different affinities (in the general sense of the word) of different proteins for the packing cause them to migrate at different rates, allowing them to be collected successively. In *frontal* chromatography, a continuous stream of protein solution is admitted to the column. The frontal boundaries of an n-component mixture emerge (break through) in succession, forming a stepped chromatogram, but only the first component to break through can be collected in pure form. Once all the boundaries have emerged, the column is in equilibrium with the feed stream and no further separation occurs. *Overload elution* and *eluto-frontal* chromatography (*31*, *32*) are hybrids in which passage of a front (adsorption) boundary is more or less rapidly followed by passage of a rear (desorption) boundary. This permits separation of all n components from larger batches than elution chromatography. A different approach to separation is to adsorb on the whole column and then desorb under different conditions of mobile phase composition (or temperature or pressure), choosing these conditions so as to achieve selective desorption of one component at a time. This *adsorption-desorption* approach, sometimes confused with frontal chromatography, is not truly chromatographic since it does not involve

differential migration, but is often regarded as a form of chromatographic operation. *Displacement development* chromatography is a further technique, relying on competitive adsorption, which has yet to be exploited for large-scale protein separations (*33*, *34*).

Within the categories of elution, overload elution, and eluto-frontal operation, there is the additional option of keeping the mobile phase composition constant (isocratic elution), or changing it steadily with time (gradient elution) or in discrete steps (stepwise elution).

In most chromatographic separations the choice between these alternative modes of operation is made on the assumptions that 1) the adsorption and desorption processes are sufficiently rapid for the adsorbate to be equilibrated between the moving and stationary phases, and that 2) there is no effective limit to the capacity of the packing for the materials being separated. These assumptions do not hold generally for proteins on ion-exchange and affinity adsorbents. It is characteristic of these systems, first, that the adsorbent has a relatively low capacity for protein and, second, that the distribution coefficient of the protein between the adsorbent and mobile phases changes from zero, through finite values, to infinity as the mobile phase pH or salt concentration changes. Only over a relatively narrow range of mobile phase conditions are the adsorption and desorption of a protein in equilibrium, permitting the normal chromatographic process of differential migration. Peterson (*35*) calls this condition finite adsorption equilibrium. A different condition, tight binding (*35*), exists when the eluent composition makes for a very low probability of simultaneously breaking all the bonds between a protein molecule and the packing. In this condition the protein molecule is immobilized on the packing and will not elute. Some authors describe the condition as irreversible adsorption since the protein, once adsorbed from the solution passed into the column, does not desorb when the solution is replaced by protein-free eluent of the same pH and salt concentration. Desorption can, however, be effected by changing the eluent conditions.

The binding of protein to the adsorbent is highly sensitive to variation of eluent conditions within a certain range characteristic of the protein and adsorbent. If the eluent composition is chosen to permit finite adsorption equilibrium of one protein, other proteins in the mixture are likely to be either tightly bound or not sorbed at all. The appropriate method of protein separation will, therefore, usually be the adsorption-desorption technique rather than one based on differential migration. A stream of mixed protein is first adsorbed onto the column under conditions of tight binding, and the eluent composition is then changed

in a series of steps to desorb each required protein in turn by bringing it into a condition of finite adsorption equilibrium or nonsorption. It is also possible, however, to view this, more broadly, as a form of nonisocratic chromatographic operation in which, first, the normal finite range of distribution coefficients encompassed by the mixture has been expanded to cover the range zero to infinity and, second, the width of the band of protein feed is sufficient to occupy the whole length of the column. In fact, this is perhaps a more fruitful way of viewing the process because it draws attention to two aspects of optimization strategy which are not so obvious outside the chromatographic context.

First, in a chromatographic separation, the width of the protein feed band is a variable to be optimized; the production rate is known to be very sensitive to this variable in gas or liquid chromatography of small molecules (*36*).

Second, nonisocratic operation can be conducted either by gradient elution or stepwise elution. Thus, tightly-bound proteins are eluted successively either by a progressive change in the mobile phase composition or by stepwise changes. Edwards and Wellington (*37*) have compared the two methods for the same system as used in the present study, the separation of bovine blood proteins on a Vistec cellulose ion exchanger. Stepwise elution was found to give better separation and resolution than gradient elution. This is as expected for a mixture of adsorbates whose distribution coefficient between phases changes drastically with eluent composition.

Occasionally, a protein separation may involve key components which are in finite adsorption equilibrium under similar eluent conditions. In such a case the adsorption-desorption approach to separation is very inefficient and only chromatographic separation under isocratic eluent conditions is appropriate. More commonly in protein separations, it is expected that eluent conditions can be chosen so that only one key component at a time is in finite adsorption equilibrium. This case is typified by the separation of albumin from bovine serum or plasma on a Vistec cellulose ion exchanger. We have, therefore, adopted the adsorption-desorption mode of chromatography with stepwise changes of eluent composition in the desorption stage. In developing the method, the two principal parameters studied were the feed band width and protein feed concentration, which can be controlled by dilution or ultrafiltration. In addition, we investigated the effects of dialysis, mobile phase velocity, eluent composition program, choice of serum or plasma as starting material, and scale-up on performance.

EXPERIMENTAL

Equipment

Two columns, of 9 and 75 mm diameter, were used. Their packed heights (and volumes) were, respectively, 0.30 m (19 mL) and 0.34 m (1500 mL). The general arrangement of the equipment is shown in Fig. 1. The eluent flow for the 9-mm column was small enough to be directed entirely through the UV detector.

The 9-mm column was supplied by Pharmacia Fine Chemicals, Sweden. The larger column was constructed from QVF glass tubing and Corning flange pieces; polypropylene end-plates carrying the inlet and outlet connectors were separated by a shallow (~2 mm) chamber from an 8-hole flow distributor, in turn separated from a coarse (~2 mm) mesh backing a fine (~80 μm) mesh to retain the packing. This arrangement produced a good distribution of liquid flow over the cross section.

Flow was controlled by peristaltic pumps. The protein concentration at the column outlet was monitored continuously with a Pye Unicam UV20 detector, with a 10 mm × 1 mm flow cell, at a wavelength of 280 nm. The detector gave a very nonlinear response, the calibration curve for blood serum albumin following the equation $y = 1.63x/(12.1 - x)$, where y is the concentration (kg/m^3) of albumin and x the absorbance height (cm) on the pen recorder. The chromatograms in subsequent figures are therefore increasingly compressed as the concentration rises.

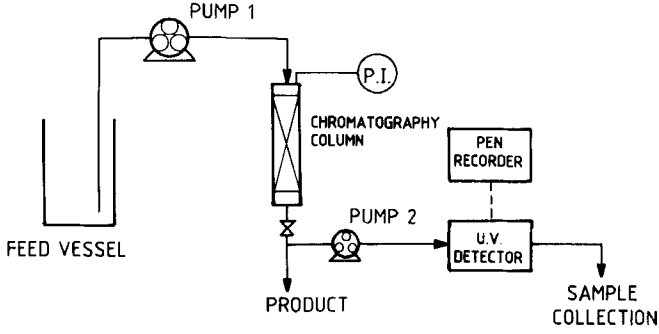

FIG. 1. Flow diagram of apparatus. P.I. = pressure indicator.

Materials

Widely-used ion-exchangers based on dextran, agarose, or cellulose offer a good capacity for protein but suffer from compressibility with increasing flow rate (*21*, *38*). We used a Vistec DEAE ion-exchanger (Koch-Light, Ltd.), a granular medium made from crosslinked, regenerated, natural cellulose pulp (*3*). This is less compressible than most exchangers based on polysaccharide matrices. Three grades of Vistec medium are available, of which D1 and D2 were selected as having the highest adsorption capacity (*39*).

The 9-mm column was packed with grade D1 of nominal size range 120–200 μm, and the larger column with D2 grade of size 150–300 μm. The media were pretreated as directed in the Koch-Light literature by soaking in 15 volumes of 0.5 *M* HCl for 30 min, washing with distilled water to pH $\geqslant$ 4.0, soaking twice in 15 volumes of 0.5 *M* NaOH for 30 min each time, and finally washing with distilled water to pH $\leqslant$ 8.0. Each medium was slurry-packed, settling under gravity to a bulk density in the swollen state of 154 kg dry medium per m^3 bulk volume.

Cattle blood was collected freshly from an abattoir. Serum was produced by dicing the clotted blood, and filtering, centrifuging, and decanting the liquid released. To obtain plasma, sodium citrate granules (sufficient to give a concentration of 1% w/v) were added to the container prior to blood collection to prevent clotting, and the whole blood was centrifuged and decanted to remove the red cell concentrate.

For some experiments, serum or plasma was dialyzed by standing overnight in semipermeable Visking tubing in a sink of cold water.

Buffer solutions were made by titrating 0.01 *M* trishydroxymethylamine with hydrochloric acid to pH 7.5.

Method

To perform a run, buffer solution or distilled water was first passed through the column at the chosen flow rate. The feed pipe was directed from the buffer to the feed container for the desired time and then back to the buffer to elute nonsorbed proteins. Solutions of successively greater ionic strength were introduced to remove groups of proteins. Finally the salt solution was flushed with buffer ready for the next run. During a run, samples were taken from the column or detector outlet for determination of protein purity by electrophoresis and protein content by a Pye Unicam SP6 UV spectrophotometer.

RESULTS AND DISCUSSION

Figure 2(a) is a chromatogram of blood serum proteins separated on Vistec granular DEAE-cellulose ion exchangers under experimental conditions chosen to match those of Edwards and Wellington (*37*). The chromatogram is similar to theirs except that the time scale was shorter at 4 h instead of 12 h and Peak 5 was smaller. Superimposed on the diagram (broken line) is the concentration of sodium chloride in the eluent entering the column. The origin of the abscissa axis is the start of the serum feed to the column.

Peak 1 is the serum fraction not absorbed by the column and includes γ-globulin. The column voidage calculated from the time to the start of this peak is 0.4. The other proteins are initially tightly bound. Their peak maxima elute approximately 0.9 empty column volumes (2.3 packed column void volumes) after implementing the corresponding step change

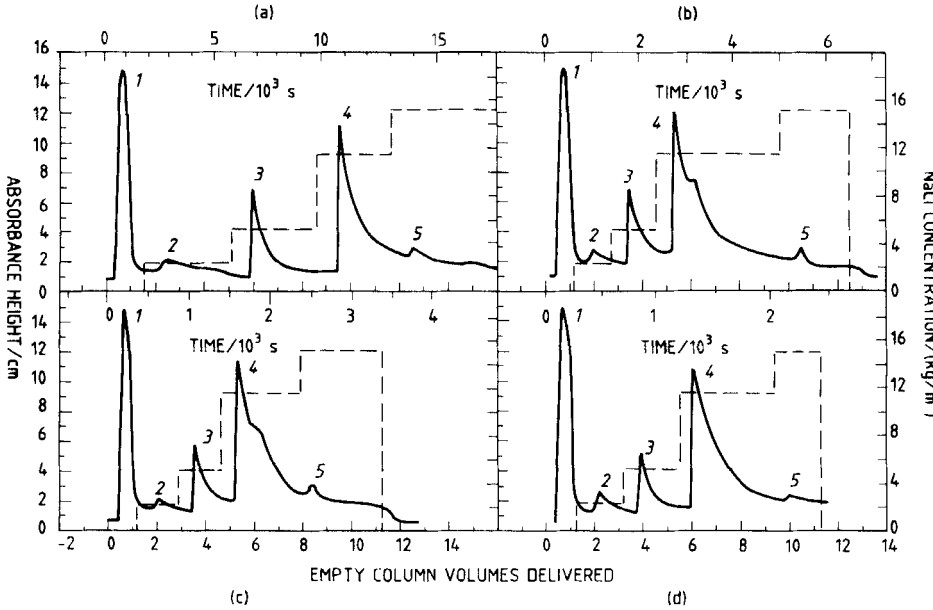

FIG. 2. Effect of superficial velocity: chromatograms of 0.21 empty column volumes of serum on 9 mm column with D1 medium. The solid line is the pen trace of the UV detector at 280 nm. The abscissa common to all four traces is the number of empty column volumes delivered. The abscissa is also shown in terms of time along the top edge of each plot. The stepped profile of salt concentration in the eluent is shown as a broken line. Superficial eluent velocities, u, are (a) 0.26, (b) 0.59, (c) 1.00, and (d) 1.47 mm/s.

in ionic concentration, implying a capacity factor (mass distribution coefficient, k') of about 1.3. This confirms that the ionic concentrations chosen for each step achieve a condition of finite adsorption equilibrium quite close to nonsorption for each eluted peak, so minimizing the cycle time.

This separation was therefore used as a basis for studying the effects of the principal operating parameters.

Effect of Superficial Velocity, *u*

Figures 2(a)–(d) show the effect of superficial eluent velocities of 0.26, 0.59, 1.00, and 1.47 mm/s, respectively. To facilitate comparison, the scale on the abscissa axis is normalized by showing empty column volumes (ecv) delivered instead of time (1 ecv is approximately equal to 2.5 packed column void volumes). The cycle time is inversely proportional to the superficial velocity. Figures 2(b)–(d) differ from Fig. 2(a) in that the process was further speeded up by implementing the step changes in ionic concentration at earlier stages in the elution cycle.

The shoulder of Peak 4 was only found in this series of experiments. It was probably a partially denatured version of the Peak 4 protein formed on keeping the serum for some time. The resolution of these two peaks is best in Fig. 2(b) and worst at the highest and lowest velocities. This indicates that the minimum in the plate height vs velocity curve occurs at about 0.26 mm/s for the purely isocratic separation of Peak 4 and its denatured satellite. The separation of the five major peaks, however, depends on nonisocratic, stepwise concentration changes rather than on the number of theoretical plates in the column. In this case it is evident that the superficial velocity can be increased without any apparent loss of resolution. Thus, the overall cycle time in Fig. 2(d) is 7 times smaller than in Fig. 2(a) owing to a combination of 5.7 times greater superficial velocity with earlier step changes.

As the eluent velocity is increased, the bed contact time available for adsorption of protein decreases. Since the kinetics of adsorption are known to be slow (*39*), some loss of effective adsorption capacity might be anticipated with increasing velocity. This loss is barely detectable in a slight fall in the ratio of the heights of Peaks 4 and 1 from Fig. 2(b) to Fig. 2(d). The net effect of the change in cycle time and near-constancy of resolution and adsorption capacity is that the throughput increases almost linearly with velocity.

The superficial velocity is ultimately limited by the compressibility of the packing. With a nonrigid packing, as the pressure drop across the

column increases, the hydrostatic pressure at the column inlet reaches a point where the bed starts to compress rapidly and the flow rate falls off (*21*). This point was not reached with the Vistec D1 medium, even at the highest pressure gradient used, 500 kPa/m. For comparison, widely used but less rigid dextrose gel media, such as Sephadex G25 and G200, are limited to much lower maximum pressure drops of 20–60 kPa/m (*21*, *38*).

The permeability coefficient, B, of Vistec D1 medium was measured as 2.0×10^{-12} m^2.

Effect of Feed Volume, V_f

Figures 3(a) and (b) show chromatograms obtained with serum feed batches of 0.11 and 0.03 ecv, respectively. The previous experiments used 0.21 volumes. The major difference from Fig. 2 is the much reduced size of Peak 1 relative to the later-eluted peaks. This is attributable to protein, which would normally be adsorbed on the column under the initial eluent conditions, being eluted along with the nonadsorbable fraction when the feed volume is high. Confirmation was obtained by electrophoresis of collected peaks; the two smaller feed volumes gave one or two bands for Peak 1 whereas higher feed volumes gave several bands, increasingly resembling the pattern of a serum sample.

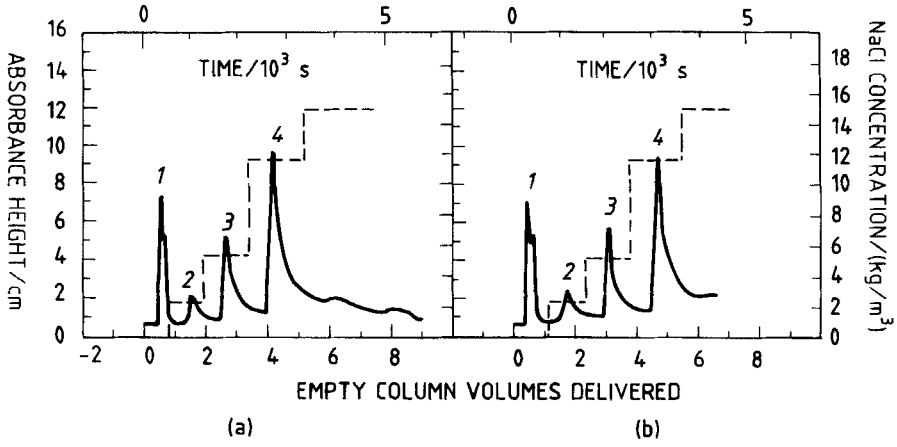

FIG. 3. Effect of feed band width, V_f, on chromatogram of serum on 9 mm column of D1 medium at u = 0.46 mm/s. (a) V_f = 0.11 ecv (empty column volumes). (b) V_f = 0.03 ecv.

The observed behavior can be explained by noting that the adsorption kinetics are relatively slow. When a differential bed is exposed to a continuous stream of albumin at constant concentration, it is found (*39*) that the adsorption rate falls off rapidly with contact time; the concentration of adsorbed protein reaches only $\frac{1}{2} - \frac{2}{3}$ of its equilibrium value even after 20 ecv of eluent have been passed, equivalent to about 1 ecv of the present column. In the present study the protein and salt concentrations are higher, preventing a direct quantitative comparison. Nevertheless, it is clear that the use of discrete feed bands limits the contact time available for adsorption as the band passes any point in the column. The effective adsorption capacity in each element of column length is less than the equilibrium capacity and varies with contact time and hence with band width and position of the element along the column. The amount of protein available in an elemental volume of serum exceeds the effective capacity available in the packing in contact with that volume. Only part of the protein in the feed band is taken up by the packing in the portion of column first occupied by the band; the band then passes on, delivering protein to successive portions of the column. If the amount of adsorbable protein in the feed band exceeds the effective capacity of the column overall, some protein will elute, unadsorbed, from the column at the same time as the nonadsorbable proteins.

The behavior of Peak 1 is explicable on the basis that narrow feed bands (0.03 and 0.11 ecv) contain insufficient protein to satisfy the effective overall adsorption capacity of the column. As the feed band is made wider, the effective column capacity (which increases less than proportionately because the adsorption rate falls off with contact time) proves insufficient to take up all the adsorbable protein in the feed. The excess adsorbable protein elutes as an addition to Peak 1.

An implication of this model is that protein can be fed either 1) as a wide band, sufficient just to occupy the whole free volume of the column, or 2) as a narrower band of similar protein mass but increased concentration. In the second case the protein will still be distributed over the whole column, as in the first case.

Purity of the Chromatographic Peaks

The efficiency of separation of 0.05 ecv of serum at a velocity of 0.88 mm/s was assessed by polyacrylamide gel electrophoresis. The results are shown in Fig. 4 for each of the major peaks numbered in the chromatograms. The photograph is arranged to show the migration of protein bands from top to bottom.

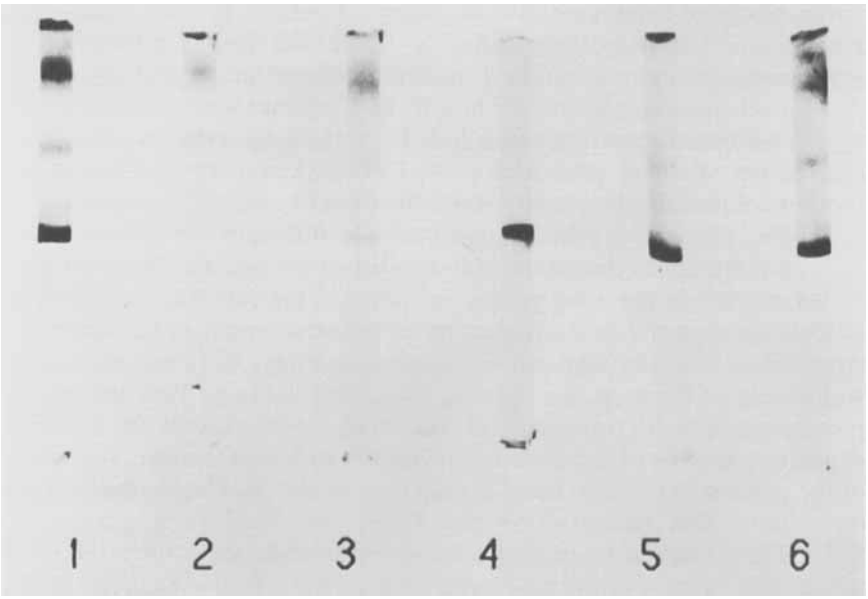

FIG. 4. Electrophoresis gels: (1) serum, (2) Peak 1, (3) Peak 3, (4) Peak 4, (5) albumin manufactured by Cohn precipation process, (6) plasma.

Gel 1 shows the multiple bands obtained from a serum sample. In Gel 2 (= Peak 1), the faster migrating bands are absent, a result found only when the chromatographic feed band is sufficiently narrow. Wide chromatographic feed bands cause Peak 1 to show all the serum electrophoretic bands, as already explained. Peaks 2 and 3 contained proteins (mainly transferrin) in the range between the chromatographically nonsorbed proteins at the top of the gel (Peak 1) and the albumin band in Gel 4. Peak 4 (Gel 4) contained only albumin. It may be compared with Gel 5, a commercial albumin sample produced by the Cohn precipitation process (*40*, *41*). The chromatographically separated albumin is evidently purer.

When Peak 5 (also albumin) had been eluted, the salt concentration was increased greatly to 58 kg/m^3 (1 *M*) to strip all remaining protein from the column. The resulting final peak gave a single band of albumin on electrophoresis. Clearly, albumin is the last protein to remain during the elution procedure and is not completely recovered by using the ionic concentration of Peak 4. (A similar effect is observed in the enhanced Peak 5 of Figs. 5a and 6a.) The two ionic concentration steps which give rise to Peaks 4 and 5 were adopted to conform with Edwards and

Wellington's (*37*) scheme, but could with advantage be replaced by a single step equal to that for Peak 5 or higher. The earlier scheme (*37*) appears to have been founded on the assumption that the tailing of chromatogram peaks was due to the presence of several proteins in each peak. The electrophoretic results make this unlikely for Peak 4 onwards. Our own studies of the equilibria and kinetics of adsorption of albumin on Vistec ion exchanger (*39*) indicate that the peaks would be expected to tail anyway because of the Langmuir-type curvature of the adsorption isotherm and slow adsorption (and hence, presumably, desorption) kinetics.

Electrophoresis thus reveals that the chromatographic separation conditions give a relatively crude group separation in which particular protein bands often appear in more than one peak. The encouraging fact, however, is the relative purity of the albumin fraction in Peak 4. The optimum recovery of this valuable protein from blood was the major object of the remaining experiments.

Modified Salt Concentration Steps

The cycle time can be reduced if all the nonalbumin proteins are removed in only one or two peaks before the albumin is recovered. Fig. 5(a) shows the separation obtained by changing the salt concentration to 5.3 kg/m^3, the previous Peak 3 value, immediately after the serum feed was added. It is evidently feasible to remove the nonalbumin fraction in a single peak. This is an extreme case giving minimum cycle time. An intermediate approach is to allow the first, nonadsorbed, peak (Peak 1) to develop fully before recovering the adsorbed nonalbumin fractions with 5.3 kg/m^3 salt solution; the nonalbumin material is recovered in two peaks rather than one or three. If the one-peak method is used, the contact time available for adsorption of albumin before the salt concentration is changed to a less favorable 5.3 kg/m^3 is restricted to the feed band width. The effective adsorption capacity for albumin becomes very sensitive to narrow feed bands. A higher adsorption capacity and recovery of albumin is obtained by removing the nonalbumin material in two peaks rather than one. This procedure was adopted in most subsequent runs.

Dialyzed Serum

In the experiments described so far, undiluted serum was applied directly to the top of the column. Even when the eluent is free of added

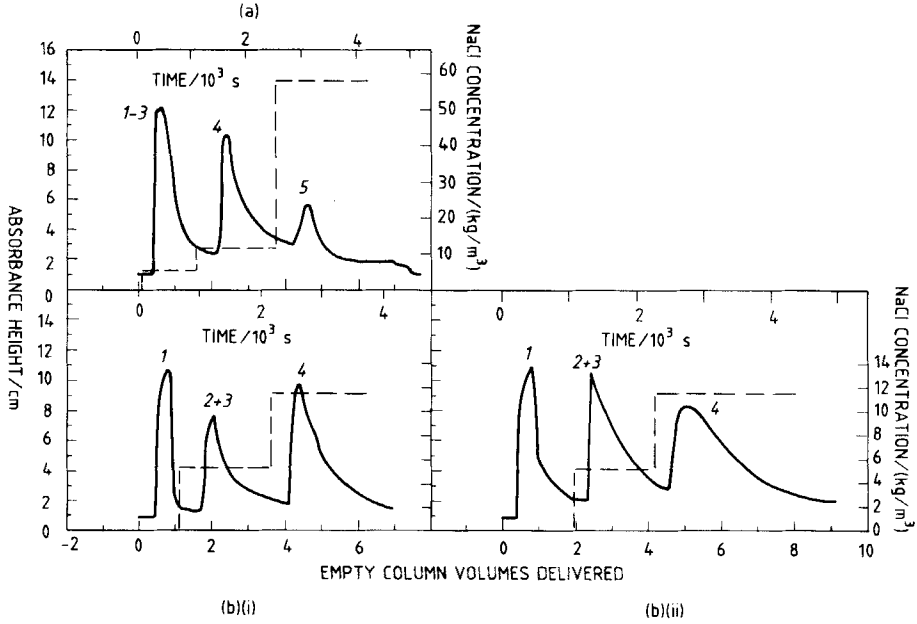

FIG. 5. Effects of (a) salt concentration step sequence and (b) dialysis on chromatogram of serum on 9 mm column with D1 medium. (a) $u = 0.45$ mm/s, $V_f = 0.05$ ecv. (b) (i) Nondialyzed serum, $u = 0.50$ mm/s, $V_f = 0.11$ ecv; (ii) dialyzed serum, $u = 0.55$ mm/s, $V_f = 0.21$ ecv.

salt, the serum itself contains salts which compete with protein for adsorption onto the ion exchanger. Experiments to improve the adsorption capacity were carried out using prior dialysis of the serum to reduce the salts content.

The chromatographic separation studied (Fig. 5b) used two stages to remove the nonalbumin fraction by allowing the nonsorbed fraction to elute at zero added salt concentration before a concentration step was introduced to elute the combined Peaks 2 and 3. Material balances are shown for 0.11 empty column volumes of undialyzed and dialyzed feed serum in Table 1, Runs (a) and (b). There is an excess of total input over total output mass in both runs. This is not an accurate measure of protein unstripped at the final step concentration shown in Fig. 5(b)(i). The serum concentration values were obtained by UV measurement at 280 nm using bovine serum albumin as the calibrating protein. Since proteins differ in their adsorption characteristics, the data are useful as a

TABLE 1
Material Balances for Serum Proteins on 9 mm Column

Feed status and size[a]	Peak	Input (mg)	Output		Solid concentration (g protein/g dry medium)
			mg	% of total o/p	
(a) Undialyzed, 0.11	Serum	200			
	1		85	49	—
	2 + 3		24	14	0.008
	4		65	37	0.022
	Total	200	174	100	0.030
(b) Dialyzed, 0.11	Serum	162			
	1		24	18	—
	2 + 3		46	34	0.016
	4		65	48	0.022
	Total	162	135	100	0.038
(c) Dialyzed, 0.21	Serum	315			
	1		170	52	—
	2 + 3		64	19	0.022
	4		96	29	0.033
	Total	315	330	100	0.055

[a]Number of empty column volumes.

guide, rather than an absolute measure, to assess the effect of processing conditions.

Electrophoretic examination of Peak 1 in each run showed three bands for the undialyzed feed but only one band for the dialyzed feed. This indicates that the adsorbable protein content of Peak 1 was eliminated by dialyzing the feed, i.e., loss of this protein was prevented.

The reduced mass of protein in the dialyzed feed serum in Table 1(b) has two causes. First, some osmosis always accompanies dialysis, diluting the protein. Second, dialysis precipitated some of the protein because an adequate concentration of dissolved salts is needed to avoid denaturation. The amount of albumin adsorbed was, fortuitously, the same in both experiments. However, whereas in Table 1(b) albumin is present only in Peak 4, albumin is present in both Peaks 4 and 1 of Table 1(a). The increase in the total solid concentration in Table 1(b) is due to the contribution of the adsorbable proteins other than albumin.

To overcome the reduced amount of feed protein caused by osmosis and at the same time take up the increased column capacity created by

dialysis, the run was repeated with a larger serum feed of 0.21 ecv (Fig. 5bii and Table 1c). Electrophoresis revealed that Peak 1 included albumin and other adsorbable protein, the column's capacity again being exceeded with Peak 1 constituting about the same proportion of the output as in Table 1(a). Comparison of Runs (a) and (c) in Table 1, however, shows that the total capacity is increased nearly twofold by dialysis to 0.055 kg/kg, though the increase for the adsorbed albumin fraction is less, at about 50%.

Column Scale-Up for Plasma Fractionation

In the experiments so far the column diameter was 9 mm and the feed was serum. Isolation of albumin on a commercial scale will require columns of much greater diameter (>300 mm) and probably use plasma as starting material. Plasma has the advantage that the need to process clotted blood is avoided. Although the present study is concerned with method development rather than scale-up, the feasibility of using larger, 75 mm diameter columns was assessed with plasma as feed to see if any additional factors presented difficulties.

The electrophoresis bands of plasma (Fig. 4, Gel 6) are similar to serum (Gel 1) apart from the additional presence of fibrinogen, which accounts for 4% of the protein in plasma. The chromatogram of plasma on a 9-mm diameter column (Fig. 6a) is similar to that for serum, and electrophoretic analysis of Peak 4 showed the albumin fraction to have a purity comparable with serum-derived albumin.

As the diameter of a column is increased, separation efficiency is lost unless steps are taken to maintain a uniform distribution of flow over the cross section. Specially designed flow distributors were therefore used, as already described. A more difficult problem was expansion and contraction of the bed with changing salt concentration, leading to loss of separation efficiency and excessive pressure drop. This was overcome by two modifications to the experimental method. First, the column was run with an air gap between the liquid inlet and the top of the column. The gap could be controlled by adjusting the flow from the column exit with a valve since the liquid flow by gravity was reasonably good. Second, the salt concentration in the mobile phase was restricted to 11.7 kg/m^2 (0.7 *M*), which was sufficient to remove most of the final (albumin) peak leaving only a small proportion on the column. With these precautions, scaling up the column diameter at constant packed height caused no loss of separation efficiency.

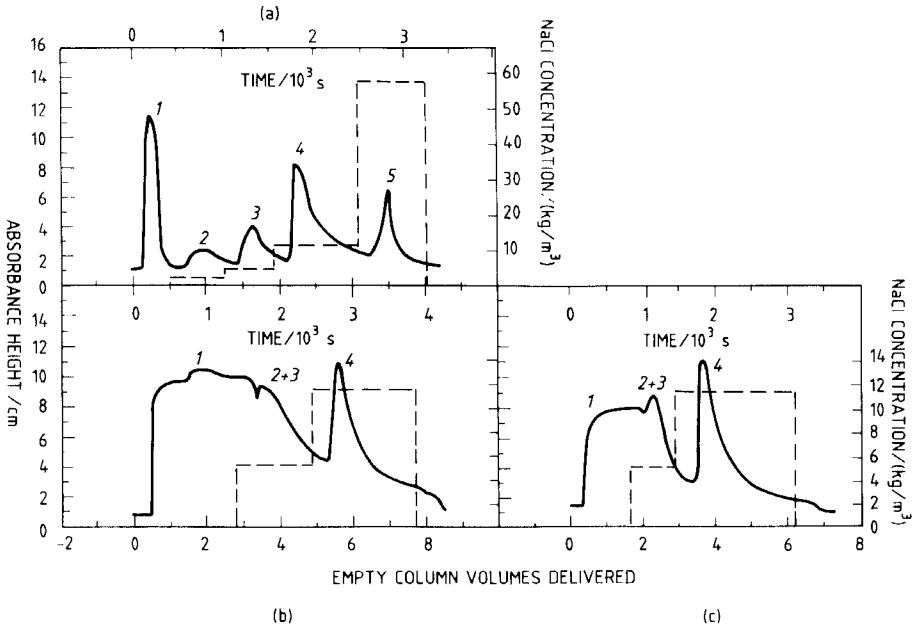

FIG. 6. Chromatograms for plasma separation. (a) 9 mm column of D1 medium, $u = 0.74$ mm/s, $V_f = 0.11$ ecv plasma. (b) 75 mm column of D2 medium, $u = 0.66$ mm/s, $V_f = 2.8$ ecv plasma at 1:10 dilution. (c) 75 mm column of D2 medium, $u = 0.69$ mm/s, $V_f = 1.7$ ecv dialyzed plasma at 1:10 dilution.

All the experiments described subsequently were carried out with plasma on a 75-mm column packed with Vistec D2 medium.

Dilution of Plasma

The dialysis procedure for albumin production adds another stage to the process. An alternative strategy is to dilute the feed to reduce the concentration of dissolved salts. This also dilutes the protein in the same proportion. However, there is little risk of losing throughput through reduced concentration of adsorbed protein because the undiluted feed has a protein concentration several times higher than required to satisfy the effective capacity of the ion exchanges, as already mentioned. It is necessary only to use a wider feed band in proportion to the dilution factor. The procedure used was to elute the nonalbumin fraction immediately after addition of plasma. Since the diluted plasma feed band

occupied at least 0.9 ecv (2.2 column void volumes), this procedure was equivalent to the previously described two peak method, though the two peaks were not separated in the chromatogram (e.g., Fig. 6c). Albumin was recovered after the development of this compound peak.

The material balance for three separations is shown in Table 2. The solid phase concentration of albumin achieved in Run (a) with undiluted plasma was only 0.009 kg/kg, a low figure compared with 0.022 for serum using the D1 medium and a similar feed band width. This is probably due to the addition of 1% sodium citrate at the blood collection stage. The beneficial effects of a 1:10 dilution of a given mass of feed protein are demonstrated in Run (b) by both the greater solid phase concentration (and hence amount of albumin recovered per batch) and the increased fraction (36%) eluted as albumin.

Dilution of a given mass of feed protein has three consequences. First, the lower protein concentration reduces the rate of uptake kinetics. Second, the greater volume gives a greater contact time between feed and each elemental length of column that it passes, so the time available for the adsorption process to proceed toward equilibrium is increased. Third, dissolved salts are also diluted, increasing the rate of uptake. Studies of the adsorption kinetics (*39*) show that the first factor outweighs the other two at sufficiently small contact times, with a net reduction in effective adsorption capacity; the reverse is true at larger contact times, giving a net increase in effective capacity. The results of the present dilution study show that the contact time with 0.09 ecv of undiluted feed is in the range where the effective capacity increases with dilution.

The effect of increasing the band width of diluted feed from 0.9 to 2.8 ecv (without further dilution) is shown by comparison of Runs (b) and (c) in Table 2. The solid phase concentration of albumin is doubled since the greater feed band width increases the effective adsorption capacity of the column by allowing a longer contact time. Consequently, the amount of albumin recovered is doubled. However, the proportion of feed albumin lost in the first peak increases substantially because the 3:1 times increase in feed protein exceeds the twofold increase in effective capacity. In addition, there is a time penalty as the chromatogram in Fig. 6(b) shows.

Dialysis and Dilution

Since both dialysis and dilution offer advantages with albumin adsorption, a combination of the two was tested. In particular, the presence of added salts in plasma should make dialysis worthwhile.

TABLE 2
Material Balances for Plasma Proteins on 75 mm Column

	Feed status	Feed size[a]	Component[b]	Input (g)	Output g	Output % of total o/p	Solid concentration (g/g)
(a)	Untreated	0.09	Plasma	14			
			C		11.9	86	—
			A		2.0	14	0.009
			Total	14	13.9	100	0.009
(b)	Diluted 1:10	0.9	Plasma	14			
			C		8.0	64	—
			A		4.5	36	0.019
			Total	14	12.5	100	0.019
(c)	Diluted 1:10	2.8	Plasma	44			
			C		33.2	79	—
			A		8.9	21	0.038
			Total	44	42.1	100	0.038
(d)	Dialyzed and diluted 1:10	1.7	Plasma	20			
			C		9.0	50	—
			A		9.1	50	0.040
			Total	20	18.1	100	0.040

[a]Number of empty column volumes.
[b]C = combined prealbumin fraction. A = albumin fraction.

The results are shown in Fig. 6(c) and Table 2(d) for a dilution factor of 1:10 in the dilution stage. They show that, compared with the run in Fig. 6(b), a similar albumin adsorption can be achieved at a reduced feed band width (1.7 instead of 2.8 ecv), eliminating the waste of feed and improving the cycle time by about 2 ecv. That all the albumin is adsorbed is shown by the fact that 50% of the output is adsorbed albumin, in good agreement with 49% usually quoted for bovine blood.

If one compares the figures for % albumin output and adsorbed albumin concentration in Tables 1 and 2, it is evident that combined dialysis and dilution offers better chromatographic performance than either dialysis or dilution alone. The benefit can be taken either as reduced albumin wastage for a given amount recovered, or (by increasing the feed band width) as increased amount recovered where loss of protein is tolerable.

CONCLUSIONS

There are a number of design and operating parameters which affect the throughput and purity achieved in separating proteins by ion-exchange chromatography. The parametric requirements for maximizing performance are often in mutual conflict. A more detailed study would be needed to predict the values of the parameters which give the optimum compromises for a particular protein separation. The present study, however, shows the effect of a number of the major parameters on performance and allows one to anticipate where compromises will arise.

For most protein separations, the adsorption-desorption mode of operation, which may also be regarded as an extreme form of nonisocratic eluto-frontal chromatography, is best (though displacement development may also be worth considering for possible scale-up (*33*, *34*)). In the desorption stage the ionic strength of the eluent should be changed stepwise in preference to gradient elution. When only a single protein fraction is to be isolated and this is the most strongly adsorbed, the most efficient program of concentration steps is a two-step sequence timed to elute the unwanted adsorbed fraction immediately after the nonsorbed fraction.

For blood proteins separated on a Vistec cellulose ion-exchanger, prior dialysis and dilution of the feed have a beneficial effect on effective adsorption capacity. The degree of benefit increases in the order: dilution, dialysis, dialysis and dilution combined. The disadvantage of dialysis, whether alone or in combination, is that it adds an extra, time-consuming step to the overall process.

The major area in which trade-offs are involved in optimizing performance is the choice of feed band width, column height, and eluent velocity. Considered as an independent variable, increased column height is beneficial because it permits greater feed band width, and so increased time for adsorption, at constant proportional protein wastage. Increased velocity is also beneficial, because protein throughput increases almost linearly with velocity, at least within the velocity range studied here. With a compressible medium, however, increased height can be attained only at the expense of decreased maximum velocity and vice versa. Since increased height and ultimately, one expects, velocity too are subject to diminishing returns, there will be an optimum combination of height and velocity for maximum throughput. The feed band width then depends on whether wastage of protein is tolerable. For example, with most medical products, no wastage could be tolerated, since yield is an important influence on economics. For animal blood the supply is plentiful, and wastage in the loading stage may be tolerated. Where wastage must be avoided, the optimum feed band volume for a given dilution is that which contains an amount of protein equal to the effective adsorption capacity of the whole column. Where wastage is tolerable, the optimum feed band width will be greater.

A further trade-off arises in the selection of ion-exchange medium. An ideal medium would have both a high adsorption capacity and a high resistance to bulk density changes when pressure, eluent concentration, or pH are changed. Both these properties make for high throughput, the first directly and the second indirectly. With packings in general, however, as one property is improved, the other is degraded. The Vistec media appear to offer a good compromise between these properties in comparison with widely used less rigid cellulose media. The development of rigid packings with good adsorptive capacity will be an important factor in the development of production chromatography.

Acknowledgment

We thank the Science and Engineering Research Council for support of this work and for a research studentship awarded to G.L.

REFERENCES

1. R. A. Grant, *Proc. Biochem.*, 9(2), 11 (1974).
2. D. T. Jones, *Ibid.*, 9(10), 17 (1974).
3. D. T. Jones, in *Food from Waste* (G. G. Birch, K. J. Parker, and J. F. Worgan, eds.), Applied Science Publishers, Essex, 1976, Chap. 17.

4. D. E. Palmer, *Proc. Biochem., 12*(2), 24 (1977).
5. A. D. A. Kanekanian and M. J. Lewis, Progress in Food Engineering Symposium, Milan, Italy, June 3–5, 1981.
6. J-C. Janson and P. Hedman, *Adv. Biochem. Eng., 25,* 43 (1982).
7. H. A. Chase, *Chem. Eng. Sci., 39,* 1099 (1984).
8. R. A. M. Delaney, in *Applied Protein Chemistry* (R. A. Grant, ed.), Applied Science Publishers, Essex, 1980, Chap. 10.
9. P. Dunnill, in *Enzyme Engineering,* Vol. 13 (E. K. Pye and H. H. Weethall, eds.), Plenum, New York, 1978, pp. 207–215.
10. R. Eketorp, in *Affinity Chromatography and Related Techniques* (T. C. J. Gribnau, J. Visser and R. J. F. Nivard, eds.), Elsevier, Amsterdam, 1982, p. 263.
11. C. J. Bruton, *Philos. Trans. R. Soc. London, B300,* 249 (1983).
12. A. Atkinson, *Proc. Biochem., 8*(8), 9, 28 (1973).
13. J. M. Curling, L. O. Lindquist, and S. Erikson, *Proc. Biochem., 12,* 22 (1977).
14. J. M. Curling, J. Berglof, L. O. Lindquist, and S. Erikson, *Vox Sang., 33,* 97 (1977).
15. J. M. Curling, in *Chromatography of Synthetic and Biological Polymers* (R. Epton, ed.), Ellis Horwood, 1978, Chap. 6.
16. J. M. Curling, in *Methods of Plasma Protein Chromatography* (J. M. Curling, ed.), Academic, London, 1980, pp. 77–91.
17. J. Saint-Blanchard, J. M. Kirzin, P. Riberon, F. Petit, J. Fourcart, P. Girot and C. Boscetti, in *Affinity Chromatography and Related Techniques* (T. C. J. Gribnau, J. Visser, and R. J. F. Nivard, eds.), Elsevier, Amsterdam, 1982, p. 305.
18. J. Travis, J. Bowen, D. Tewkesbury, D. Johnson, and R. Pannell, *J. Biochem., 157,* 301 (1976).
19. R. Hanford, W. d'A. Maycock, and L. Vallet in *Chromatography of Synthetic and Biological Polymers* (R. Epton, ed.), Ellis Horwood, 1978, Chap. 23.
20. J. L. Tayot, M. Tardy, P. Gattel, R. Plan, and M. Roumiantzeff, in *Chromatography of Synthetic and Biological Polymers* (R. Epton, ed.), Ellis Horwood, 1978, Chap. 8.
21. J-C. Janson and P. Dunnill, in *Industrial Aspects of Biochemistry* (B. Spencer, ed.), (FEBS Meeting, 1973), North-Holland, 1974, p. 81.
22. J-C. Janson, in *Affinity Chromatography and Related Techniques* (T. C. J. Gribnau, J. Visser, and R. J. F. Nivard, eds.), Elsevier, 1982, p. 503.
23. J-C. Janson, *Trends Biotechnol., 2*(2), 31 (1984).
24. D. J. Graves and Y. T. Wu, in *Advances in Biochemical Engineering,* Vol. 12 (T. K. Ghose, A. Fiechter, and N. Blakebrough, eds.), Springer Verlag, 1979, p. 219.
25. H. A. Chase, *J. Chromatogr., 297,* 179 (1984).
26. H. A. Chase, *J. Biotechnol., 1,* 67 (1984).
27. F. H. Arnold, J. J. Chalmers, M. S. Saunders, M. S. Croughan, H. W. Blanch, and C. R. Wilke, *ACS Symp. Ser., 271,* 113 (1985).
28. J. P. Hamman and G. J. Calton, *Ibid., 271,* 105 (1985).
29. M. D. Rankin in *Applied Protein Chemistry* (R. A. Grant, ed.), Applied Science Publishers, Essex, 1980, Chap. 6.
30. D. Halliday, *Proc. Biochem., 8*(12), 15 (1973); *10*(4), 11 (1975).
31. J. R. Conder and J. H. Purnell, *Chem. Eng. Symp. Ser., 65*(91), 1 (1969).
32. J. R. Conder and J. H. Purnell, *Chem. Eng. Sci., 25,* 353 (1970).
33. C. Horvath, in *The Science of Chromatography (A. J. P. Martin Symposium)* (F. Bruner, ed.), Elsevier, Amsterdam, 1985, p. 179.
34. E. A. Peterson and A. R. Torres, *Methods Enzymol., 104,* 113 (1984).
35. E. A. Peterson, in *Cellulosic Ion Exchangers,* North Holland/American Elsevier, Chap. 3, pp. 255–270.

36. J. R. Conder and M. K. Shingari, *J. Chromatogr. Sci., 11,* 525 (1973).
37. W. G. Edwards and C. A. Wellington, *J. Chromatogr., 135,* 463 (1977).
38. J. Porath, in *Enzyme Engineering* (Biotech. and Bioeng. Symp. No. 3) (L. B. Wingard, ed.), Wiley-Interscience, New York, 1972, p. 145.
39. G. Leaver, J. R. Conder, and J. A. Howell, In Preparation.
40. E. J. Cohn, L. E. Strong, W. L. Hughes, D. J. Mulford, J. N. Ashworth, M. Merlin, and H. L. Taylor, *J. Am. Chem. Soc., 68,* 459 (1946).
41. E. J. Cohn, W. L. Hughes, and J. H. Weare, *Ibid., 69,* 1753 (1947).