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Studies on a new cross-axis coil planet centrifuge for performing counter-current chromatography

I. Design of the apparatus, retention of the stationary phase, and efficiency in the separation of proteins with polymer phase systems

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ABSTRACT

An improved model of the cross-axis synchronous flow-through coil planet centrifuge has been designed in light of previous studies. The apparatus has a versatile feature in that both analytical and preparative columns can be accommodated in both off-center and central positions. Each has merit in separations.

Retention of stationary phase was examined with various two-phase solvent systems used for the separation of biopolymers. Both analytical and preparative columns showed satisfactory retention of the stationary phase under optimum conditions. The apparatus was evaluated in separation of a set of protein samples using a polyethylene glycol–potassium phosphate biphasic system. In both types of columns all proteins were resolved with partition efficiencies of 260 to 670 theoretical plates. Further studies indicated that the relatively low partition efficiency of proteins is mainly attributed to their high molecular mass or molecular heterogeneity within each species rather than due to the high viscosity of the polymer phase system.

INTRODUCTION

Counter-current chromatography (CCC) has been increasingly used for the separation and purification of various natural and synthetic products and biological samples [1,2]. In the past, development of CCC technology has been focussed mainly on improvement of the retention of stationary phase, peak resolution and separation times using organic–aqueous two-phase solvent systems. Among various CCC models developed, the high-speed CCC centrifuge has proven most useful since it provides advantages in peak resolution and separation times in addition to durability and stability of the instrument [3]. On the other hand, the apparatus shows poor retention of the stationary phase when using the aqueous–aqueous polymer phase systems [4] so useful for the separation of macromolecules. This problem is apparently caused by violent mixing of the two phases that tends to

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produce emulsification; the result is loss of stationary phase from the column.

The cross-axis coil planet centrifuge (CPC) introduced in the mid-1980s has a unique mode of planetary motion such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge [5,6]. This motion allows much better retention of the stationary phase. The apparatus has been successfully used for preparative-scale separations of natural and synthetic products [7].

Recent studies have shown that retention of the stationary phase in the cross-axis CPC can be further improved by shifting the column holder laterally along the rotary shaft. In this position, the column is subjected to a strong lateral centrifugal force component that acts across the diameter of the tube to suppress emulsification. The proper combination of coil orientation and elution mode provides satisfactory retention of the stationary phase for viscous, low-interfacial-tension solvent systems [8].

Using aqueous–aqueous polymer phase systems, a standard mixture of stable proteins [9], lipoproteins [10] and recombinant enzymes from *Escherichia coli* lysate [11] have been separated using the type-XLL cross-axis CPC with a 10-cm revolution radius equipped with a pair of multilayer coils at an off-center position 20 cm from the mid point of the rotary shaft. In this work it was found that the ratio between the revolution radius (X) and the lateral deviation (L) of the column is an important parameter in retention of the stationary phase. In general, an increase in ratio L/X resulted in higher retention of stationary phase by reducing the phase mixing effect.

The present paper describes the design and performance of a new cross-axis CPC which can accommodate a pair of column holders in two different positions, the off-center position ($L/X = 1.5$) or the central position ($X = 0$). The apparatus was evaluated for retention of the stationary phase of four solvent systems each providing a specific merit on the separation of macromolecules as shown in Table I. Under the optimized conditions for polyethylene glycol–phosphate two-phase system (solvent 2), the partition efficiency was measured in the separation of a set of stable proteins using both analytical and preparative coiled columns.

EXPERIMENTAL

Apparatus

The cross-axis synchronous flow-through CPC used in the present studies was designed in our laboratory and constructed at the NIH machine shop. Both analytical and preparative columns

TABLE I

FOUR TYPICAL SOLVENT SYSTEMS FOR PARTITION OF MACROMOLECULES

HPC = Hexadecylpyridinium chloride; PEG = polyethylene glycol.

No.	Solvent systems	Target samples	Characteristic features
1	*n*-Butanol–0.13 *M* NaCl (1:1) containing 1.5% (w/v) HPC	Polysaccharides: chondroitin sulfate, heparin, hyaluronic acid	No other efficient solvent system available
2	12.5% (w/w) PEG 1000, 12.5% (w/w) K_2HPO_4	Proteins	High retention, good efficiency
3	4.4% (w/w) PEG 8000, 7.0% (w/w) dextran T500	Proteins	High solubility for proteins
4	4.0% (w/w) PEG 8000, 5.0% (w/w) dextran T500	Nucleic acids, cell particles	Physiological pH and osmotic pressure for cell separation

can be accommodated in two different positions, off-center ($X - 1.5L$) and central (L) locations. Each position has its own merit on the separation of macromolecules with aqueous–aqueous polymer phase systems.

Fig. 1 illustrates the design of the apparatus. The motor (not shown in the diagram) drives the central shaft and the rotary frame around the central axis of the centrifuge. The rotary frame consists of two pairs of side-plates: a pair of inner side-plates is bridged by a pair of horizontal plates at the upper and lower edges. These rigidly support the outer side-plates with a set of links. Column holders and counter-rotating tube holders are mounted between the inner and outer side-plates in two different positions on each side of the rotary frame, *i.e.*, an off-center position ($X - 1.5L$ position) as shown in Fig. 1 and a central position (L position). The planetary motion of the column holder is provided by a set of miter gears and countershafts as follows: a stationary miter gear (45°) is rigidly mounted coaxially around the central shaft on the bottom plate of the centrifuge. This stationary gear is engaged to an identical gear affixed at the proximal end of the countershaft radially mounted on each side of the rotary frame. The above engagement produces synchronous rotation of the countershaft on the rotating rotary frame. This motion is further conveyed to each column holder by coupling a pair of identical pulleys, one on the distal end of the countershaft and the other on the column holder shaft using a toothed belt.

In order to prevent the flow tubes from being twisted, a pair of counter-rotating tube holders is placed one on each side of the rotary frame. The plastic gear (10 cm in pitch diameter) mounted on each tube holder is engaged to an identical gear affixed on the neighboring column holder so that the tube holder synchronously rotates with the column holder in the opposite direction. The positions of the column holder and the tube holder are easily interchanged by loosening the screws on each bearing block from the rotary frame: When the column holder is mounted at the off-center position (as shown in Fig. 1), the tube holder is placed at the central position and *vice versa*. The layout of the flow tubes shown in Fig. 1B connects the pair of separation columns in series and permits continuous elution through the running columns without the use of a rotary seal device. Each flow tube is lubricated with grease and individually protected with a short sheath of Tygon tubing at each projecting hole to prevent direct contact with the metal parts.

The apparatus measures 60 cm wide, 60 cm long and 34 cm in height. The speed of the apparatus is regulated up to 1000 rpm by a speed control unit (Bodine Electric, Chicago, IL, USA). In the earlier model of the cross-axis CPC a set of metal gears produced noise and vibration. This problem has been largely eliminated by replacing the miter gears with plastic gears and restricting the up-and-down movement of the centrifuge shaft with shims. Consequently, the noise level of the present apparatus became acceptable, provided that the applied speed is away from the resonance points ranging between 650 and 700 rpm.

Preparation of coiled columns

The measurement of stationary phase retention in the preparative columns was performed using short coils of 2.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA). Two columns were prepared by winding the tubing directly around the holders of 5- and 10-cm hub diameters forming a single-layer coil with a total capacity of 19.8 ml and 41.0 ml, respectively. Both right-handed and left-handed coils were used. Each coiled column was firmly affixed on the holder with several pieces of fiber-glass reinforced adhesive tape. Each end of the column was connected to a 0.85 mm I.D. PTFE flow tube by inserting a series of smaller-diameter PTFE tubing into one another.

The protein separations were performed by three types of coiled columns shown in Fig. 2. Column I is a preparative-scale multilayer coil which consists entirely of left-handed coils of 2.6 mm I.D. PTFE tubing measuring 5 to 10 cm in diameter. Each multilayer coil was prepared by winding a single piece of PTFE tubing directly onto the holder hub making a tightly packed coil between the pair of flanges spaced 7.6 cm apart. After completing each coiled layer, the whole

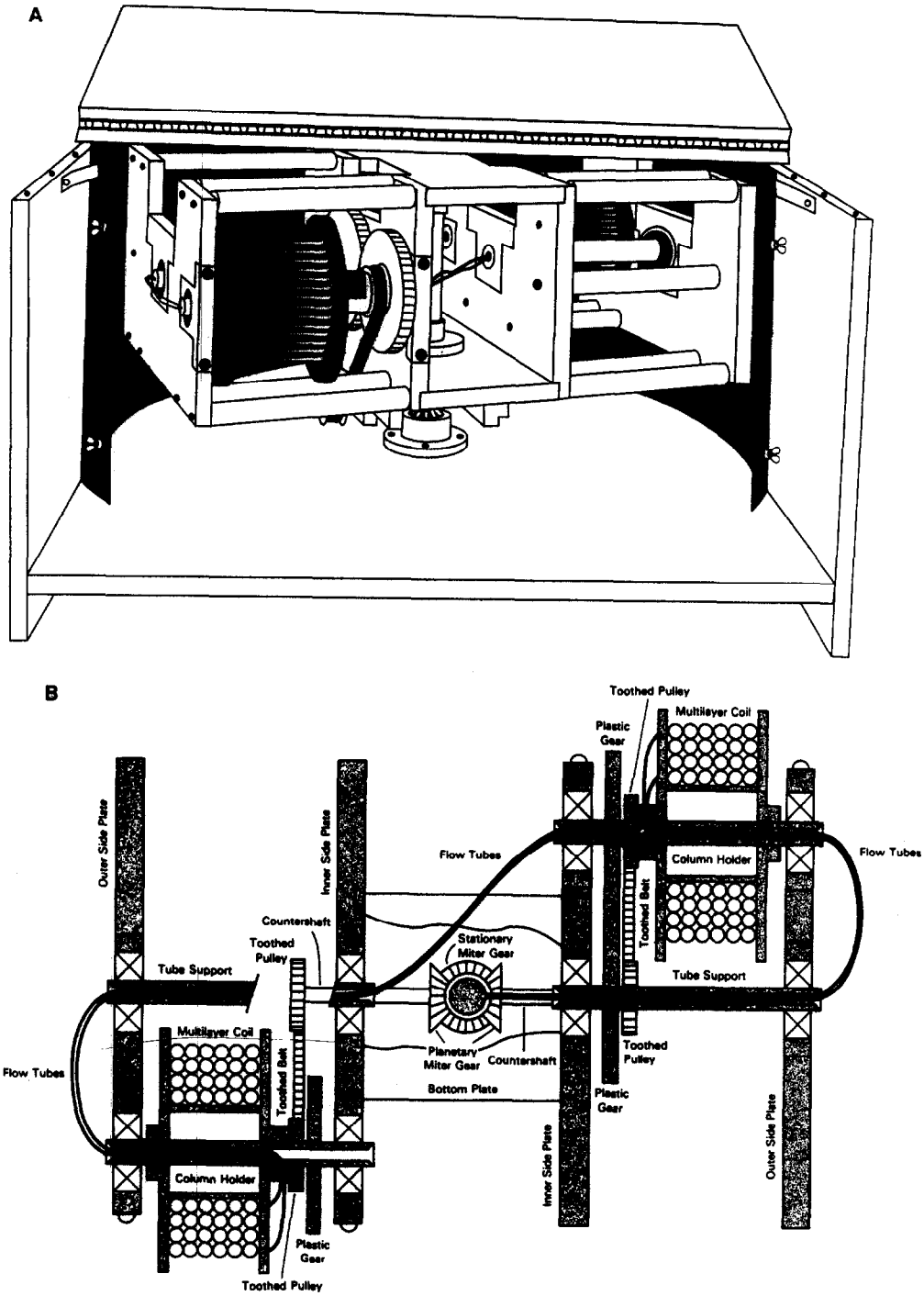


Fig. 1. Design of the apparatus. (A) Front view; (B) horizontal cross-sectional view through the axis of the column holder.

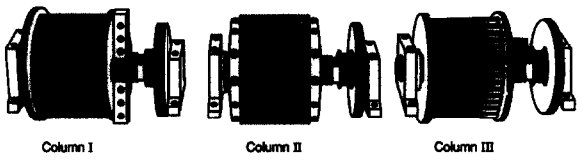


Fig. 2. Three coiled columns used for protein separations. I = Preparative multilayer coil; II = analytical eccentric coil assembly (2.0 cm core diameter); III = analytical eccentric coil assembly (5 mm core).

layer was wrapped with a piece of adhesive tape and the tubing was directly returned to the original side to start the next layer by winding the tube over the interconnection tube. In order to prevent excessive distortion of the coiled column, interconnection tubes were evenly distributed around the holder with minimum overlapping. Two identical multilayer coils were connected in series to make up a total capacity of 575 ml. Columns II and III are the eccentric coil assemblies for analytical separations. Column II consists of 8 column units each prepared by winding a 0.85 mm I.D. PTFE tube onto a 7.6 cm long, 2 cm diameter stainless-steel core forming left-handed coils. Column III consists of 32 units which are similarly prepared by winding a 0.85 mm I.D. PTFE tube onto a 7.6 cm long, 5 mm diameter nylon pipe making tight left-handed coils. In each analytical column assembly, the set of coil units is symmetrically arranged around the holder in parallel to and at the same distance (5 cm in column II and 4 cm for column III) from the holder axis. Each pair of the coil assemblies is connected in series. The total column capacities of these analytical columns are 35.4 ml for column II and 34.0 ml for column III.

Reagents

n-Butanol was chromatographic grade and purchased from Burdick & Jackson (Muskegon, MI, USA). Polyethylene glycol (PEG) 1000 (M_r 1000), PEG 8000 (M_r 8000), cytochrome *c* (horse heart), myoglobin (horse heart), ovalbumin (chicken egg), hemoglobin (bovine), serum albumin (human and bovine) and 1-hexadecylpyridinium chloride were purchased from Sigma (St. Louis, MO, USA); dibasic potassium phosphate and sodium chloride from J.T. Baker (Phillipsburg, NJ, USA); potassium dichromate from Fisher Scientific (Fair Lawn, NJ, USA); fumaric acid and maleic acid from Chem Service (West Chester, PA, USA) and dextran T500 (M_r 500 000) from Pharmacia (Sollentuna, Sweden). All reagents are of reagent grade.

Preparation of two-phase solvent systems and sample solutions

The composition of the four pairs of two-phase solvent systems used in the present studies are shown in Table I. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after the clear two phases has been formed.

Samples were prepared by dissolving a mixture of cytochrome *c* (horse heart), myoglobin (horse heart), ovalbumin (chicken egg) and hemoglobin (bovine) in about equal volumes of the upper and lower phases of the two-phase solvent system.

Measurement of retention of stationary phase

A series of experiments was performed to measure the volume of the stationary phase retained in the column using single-layer coils of 2.6 mm I.D. PTFE tube mounted on the holders with 5 cm and 10 cm diameter.

In each measurement, the entire column including the space in the flow tubes was filled with the stationary phase. Then, the apparatus was rotated at the desired speed while the mobile phase was pumped into the column at a given flow-rate using a metering pump (Milton Roy Minipump, UDC, FL, USA). The effluent from the outlet of the column was collected in a graduated cylinder to measure the volume of the stationary phase eluted from the column as well as the total elution volume. The experiment was continued until the total elution volume exceeded the capacity of the column. During the experiment, the temperature inside the centrifuge was controlled within 28 ± 2°C by placing a dry ice bag over the top plate of the centrifuge. After the run was terminated, the column contents were emptied into a graduated cylinder by connecting the inlet of the column to a nitrogen line (*ca.* 80 p.s.i.; 1 p.s.i. = 6894.76 Pa). Then the column was washed with *ca.* 100 ml of

distilled water and flushed with the stationary phase to be used for the next experiment.

The measurements of the stationary phase retention were performed with four different solvent systems at the maximum revolution speed of 800 rpm and at a flow-rate of 3.0 ml/min. The retention of each solvent system was measured under eight different experimental conditions, *i.e.*, all possible combinations of the direction of revolution (P_I = counterclockwise; P_{II} = clockwise), the head–tail elution mode (H = head to tail; T = tail to head), and inward–outward elution mode (I = inward; O = outward). The inward–outward refers to the direction of the elution along the holder axis: "inward" is from the peripheral toward the promixal against the action of the centrifugal force field and "outward" is in the opposite direction. These studies required the use of both right-handed and left-handed coils. The eight elution modes at the off-center position ($X - 1.5L$) are summarized in Table II. The elution modes applied to the central position (L) are similarly defined by modifying the orbit of the column rotation in P_I and P_{II} shown in Table II.

After choosing the experimental conditions producing the highest retention of stationary phase in each handedness of the coil, the retention of the stationary phase was further studied under both reduced speeds (600, 400 and 200 rpm at a fixed flow-rate of 2.0 ml/min) and flow-rates (2.0, 1.0 and 0.5 ml/min at a fixed 800 rpm of revolution). Retention data for each mobile phase from each solvent system are summarized in a diagram (Figs. 3 and 4) where the percentage retention of the stationary phase is plotted against either the applied revolution speeds or flow-rates.

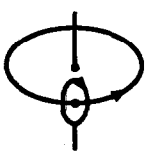
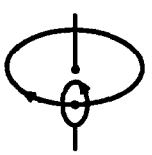
Similar experiments were performed to measure the retention of the stationary phase in the analytical columns (column II).

CCC separation of proteins

A set of stable proteins, including cytochrome *c*, myoglobin, ovalbumin and hemoglobin, was selected as test samples in the present study. Using a two-phase solvent system composed of 12.5% (w/w) PEG and 12.5% (w/w) dibasic potassium phosphate in distilled water, the partition efficiency of both analytical and preparative columns was evaluated at the two different column positions.

TABLE II

EIGHT DIFFERENT ELUTION MODES AT OFF-CENTER COIL POSITION ($X - 1.5L$)

Planetary motion	Head–tail elution mode	Inward–outward elution mode (handedness of coil)[a]	Combined elution mode[b]
P_I 	Head→tail	Inward (R)	P_I-H-I
	Head→tail	Outward (L)	P_I-H-O
	Tail→head	Inward (L)	P_I-T-I
	Tail→head	Outward (R)	P_I-T-O
P_{II} 	Head→tail	Inward (L)	P_{II}-H-I
	Head→tail	Outward (R)	P_{II}-H-O
	Tail→head	Inward (R)	P_{II}-T-I
	Tail→head	outward (L)	P_{II}-T-O

[a] R = Right-handed; L = left-handed.

[b] H = head→tail; T = tail→head; I = inward; O = outward.

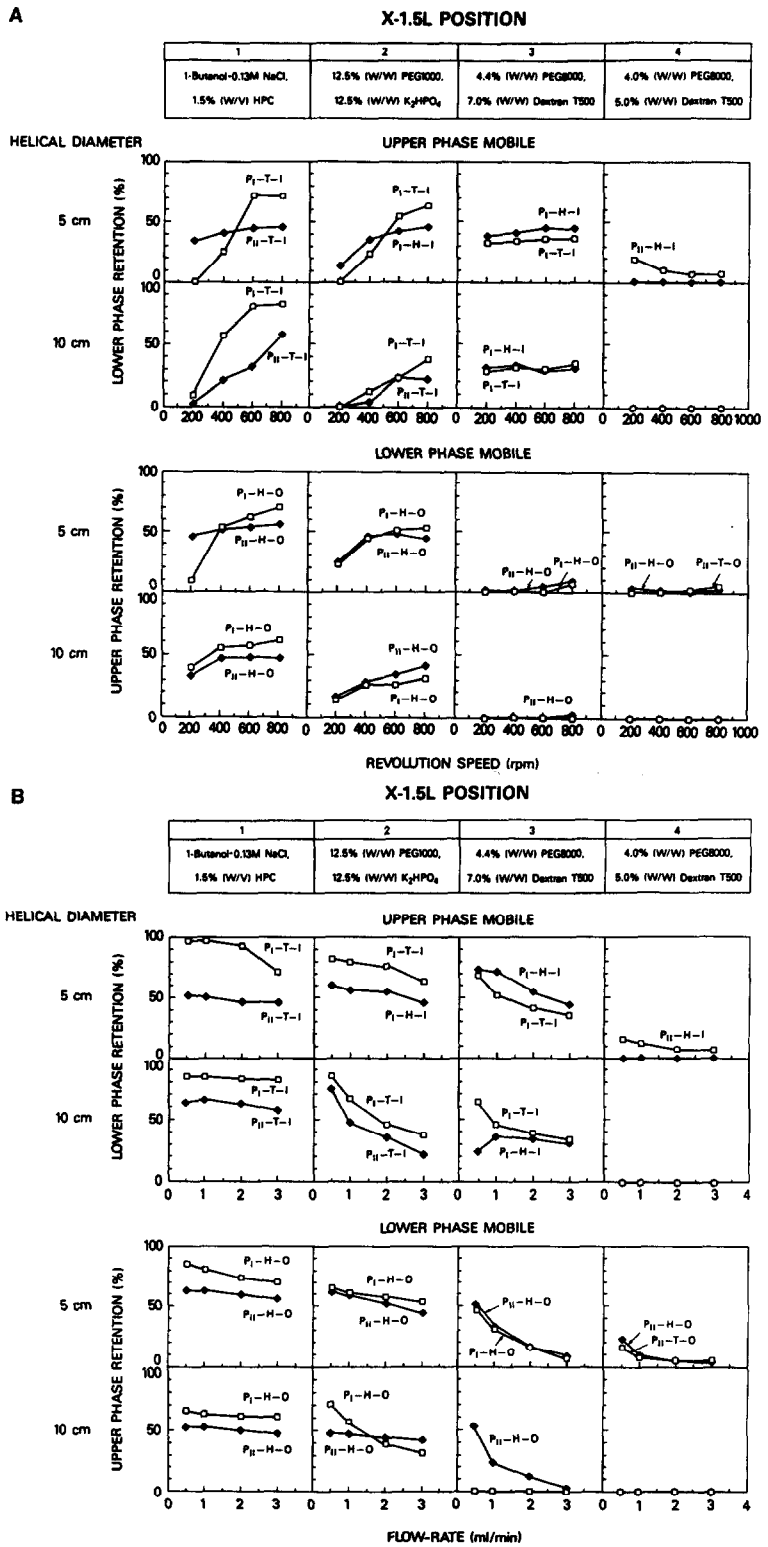


Fig. 3. Phase retention diagrams obtained from the coaxial single-layer coils (2.6 mm I.D.) at the off-center ($X - 1.5L$) position. (A) Effects of revolution on the retention of stationary phase; (B) effects of flow-rate on the retention of stationary phase.

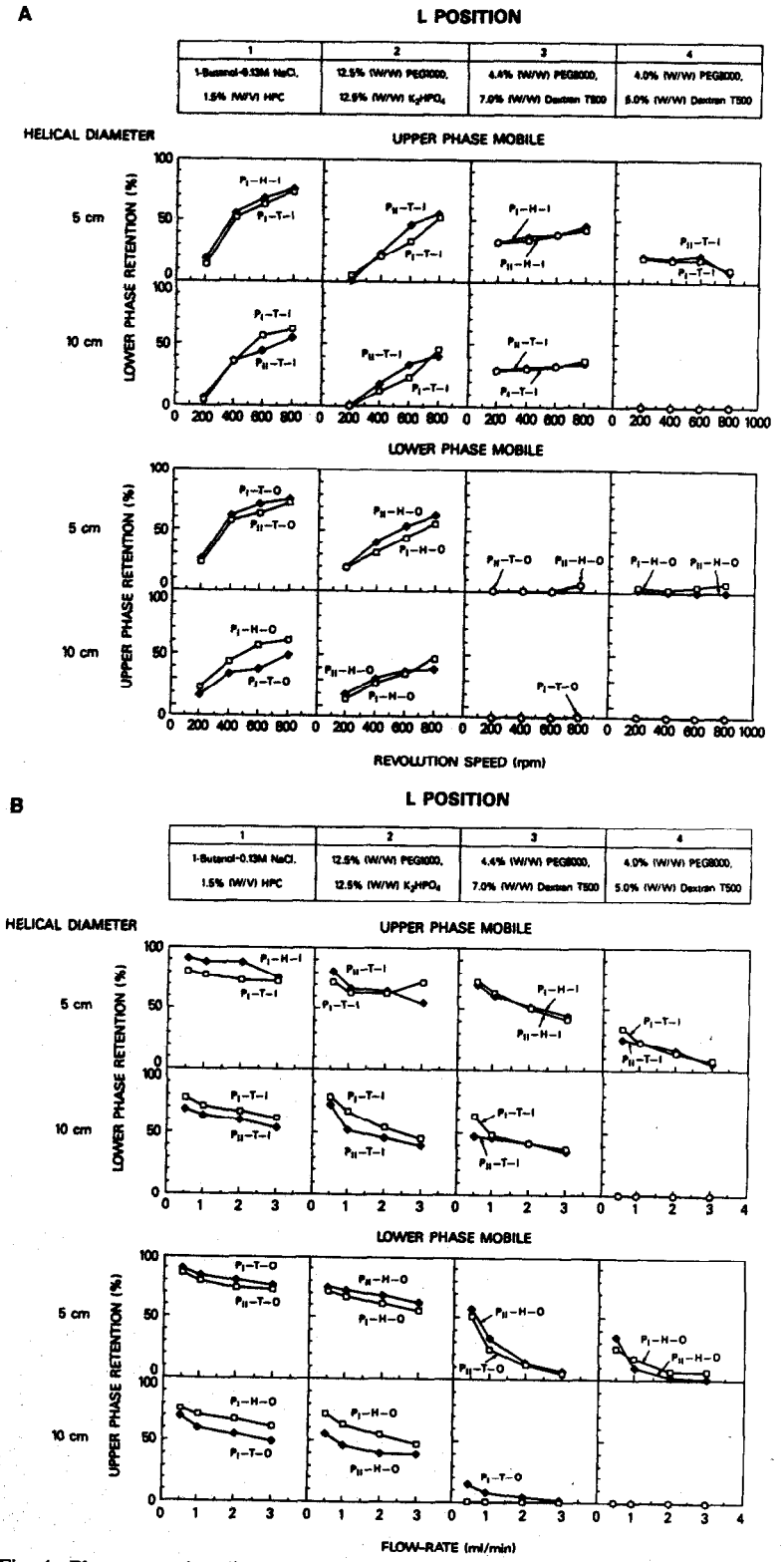


Fig. 4. Phase retention diagrams obtained from the coaxial single-layer coils (2.6 mm I.D.) at the central (L) position. (A) Effects of revolution on the retention of stationary phase; (B) effects of flow-rate on the retention of stationary phase.

For each separation, the coil was first completely filled with the PEG-rich upper stationary phase and an appropriate amount of the sample solution was charged into the column through the sample port. Then, the phosphate-rich lower mobile phase was pumped into the column at the optimum flow-rate (2.0 ml/min for column I and 0.2 ml/min for columns II and III), while the apparatus was rotated at a revolution speed of 800 rpm. The effluent from the outlet of the column was continuously monitored with an LKB (Stockholm/Bromma, Sweden) Uvicord S at 280 nm and collected into test tubes (4 ml per tube for column I and 0.4 ml/tube for columns II and III) using an LKB Ultrorac fraction collector.

Since the direct tracing of the elution profile by the uv monitor was often disturbed by carryover of the stationary phase, each collected fraction was manually analyzed with a Zeiss (Hanover, PA, USA) PM6 spectrophotometer at 280 nm.

Evaluation of partition efficiency

The partition efficiencies of separations can be computed from the chromatogram and expressed in terms of both the theoretical plate number (N) and peak resolution (R_s). Both values are based on the assumption that each peak represents the distribution of a single component. Among the four proteins in the sample mixture, ovalbumin produced an extremely broad peak which gave low partition efficiencies in both theoretical plate number and peak resolution. We later found that the ovalbumin used in the present studies produced two distinct bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Because of the heterogeneity of the ovalbumin (third peak), the partition efficiencies in the present studies are exclusively expressed as the theoretical plate number of the second peak (myoglobin), since the first peak (cytochrome *c*) is eluted close to the solvent front while the fourth peak (hemoglobin) is excessively retained in the column.

The theoretical plate number of the second peak was calculated from the conventional equation:

$$N = (4R/W)^2 \quad (1)$$

where R denotes the retention volume or time of the peak maximum and W the peak width expressed by the same unit as R.

RESULTS AND DISCUSSION

Studies on stationary phase retention

Fig. 3A illustrates a set of phase retention diagrams for four different solvent systems at the off-center position ($X - 1.5L$ position) obtained by varying the revolution speed at a given flow-rate of 3 ml/min. In this diagram, the upper panel shows the retention of the lower stationary phase obtained by eluting with the upper mobile phase and the lower panel, the retention of the upper stationary phase by eluting with the lower phase. In each panel, the retention diagrams in the first row were obtained from the coil mounted on the 5-cm diameter holder and those in the second row, from the coil mounted on the 10-cm diameter holder. Each diagram contains two best retention curves, one obtained from the right-handed coil and the other from the left-handed coil, each being selected among four possible combinations of the elution modes. In general, retention of over 50% produces an excellent result but 30% retention is considered satisfactory if carryover of the stationary phase is minimum.

The overall results of the experiments indicate that the stationary phase retention is improved by increasing the revolution speed up to the maximum of 800 rpm. The butanol solvent system (solvent 1) shows the highest retention where the head–tail elution mode plays a significant role in stationary phase retention. In the aqueous–aqueous polymer phase systems (solvents 2–4), the inward–outward elution mode plays the most important role in the retention of the stationary phase. The best retention is always achieved by eluting the upper phase inward or the lower phase outward through the coil. Different from the previous results with the X-LL cross-axis CPC [8], the left-handed coil (white square) yields only slightly higher retention of the stationary phase than the right-handed coil (black square) in the 5-cm helical diameter coil.

Among three polymer two-phase systems the PEG–phosphate system (solvent 2) produces the highest retention especially in the 5-cm helical diameter coil. This may be attributed to the physical properties of the PEG–phosphate system characterized by its relatively lower viscosity and greater differences in density and surface tension between the upper and lower phases. In the PEG–dextran systems (solvents 3 and 4) the retention of the lower phase (upper phase mobile) is always greater than that of the upper phase (lower phase mobile). This may be due to an extremely high viscosity of the lower dextran phase. In general the use of the less viscous phase as the mobile phase produces better retention of the stationary phase. This tendency is more pronounced in a small-bore analytical coil as described later.

Fig. 3B illustrates the retention diagrams for the same set of solvent systems at the off-center position ($X - 1.5L$ position) obtained by varying the flow-rate from 0.5 to 3.0 ml/min at 800 rpm. Among the two retention curves drawn in each diagram, one was obtained from the right-handed coil (black square) and the other from the left-handed coil (white square). The results indicate that the retention of the stationary phase is maximum at the lowest flow-rate of 0.5 ml/min and decreases rather sharply with the increased flow rate of the mobile phase. At a low flow-rate of 0.5 ml/min, the retention of the viscous PEG–dextran phase (solvent 3) is improved over 50% of the total column capacity.

The similar sets of the phase retention diagrams for the central column position (L position) are illustrated in Figs. 4A and 4B. The results indicate that the coil mounted in the central position yields slightly higher retention of the stationary phase than the coil mounted in the off-center position. In the central coil position, the retention of the stationary phase is almost entirely governed by the inward–outward elution mode in all solvent systems including the butanol–aqueous solvent system (solvent 1) while other parameters such as direction of the revolution and head–tail elution mode only play a minor role in retention.

The retention of the stationary phase in the analytical coil (column II consisting of three coil units) was also studied with the PEG–phosphate systems (solvent 2) and PEG–dextran system (solvent 3). The results are summarized in Fig. 5A and B where four retention curves obtained from different elution modes are shown in each diagram. Different from the results obtained with the preparative coil, all elution modes produce similar retention patterns that are very sensitive to the flow-rate at a low range between 0 to 0.2 ml/min. The PEG–phosphate system (solvent 2) produces substantially higher retention than the PEG–dextran system (solvent 3) reaching 50% level at a low flow-rate of 0.1 ml/min. In both solvent systems, the use of the

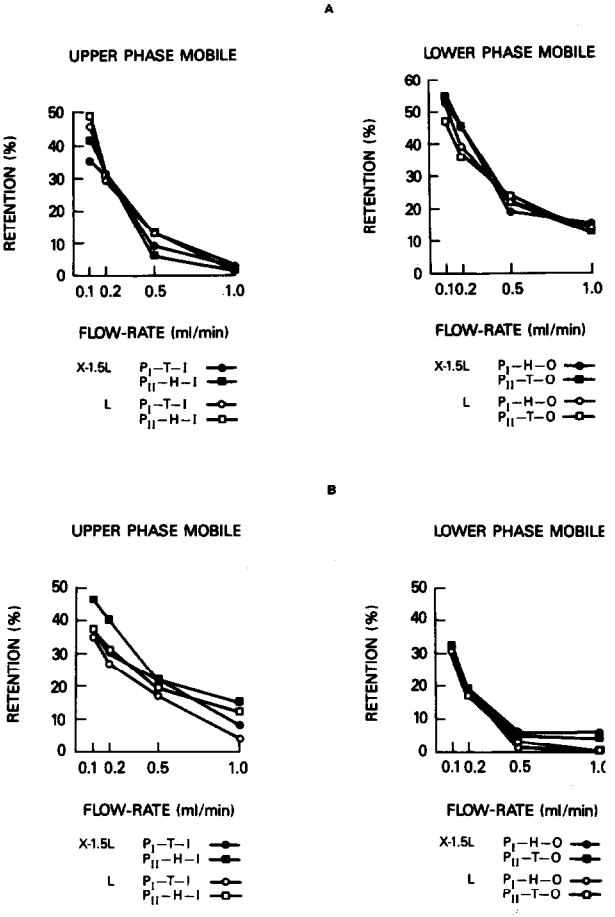


Fig. 5. Phase retention diagrams obtained from the analytical eccentric coil assembly (0.85 mm I.D. and 2 cm core diameter). (A) Effects of flow-rate on retention of 12.5% (w/w) PEG 1000, 12.5% (w/w) dibasic potassium phosphate; (B) effects of flow-rate on retention of 4.4% (w/w) PEG 8000, 7.0% (w/w) dextran T500.

less viscous phase favours the retention of the stationary phase, and this effect is clearly seen in the PEG–dextran solvent system (solvent 3) where the lower dextran phase has extremely high viscosity close to 100 cP.

Separation of proteins with polymer phase systems

The performance of the present apparatus was evaluated in the separation of a set of four stable proteins using both preparative multilayer coils (column I) and analytical composite coil assemblies (columns II and III) each at the off-center and central positions. A series of experimental runs was performed with an aqueous two-phase solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate using a phosphate-rich lower phase as the mobile phase.

Fig. 6 shows preparative separations of the standard protein mixture by the present apparatus equipped with a pair of multilayer coils (column I) with a total capacity of 575 ml. The separations were performed at 800 rpm and a flow-rate of 1.0 ml/min with the column held at the off-center position (A) and the central position (B). Cytochrome *c* and myoglobin were well separated at both column positions while myoglobin and ovalbumin were only partially resolved. The partition efficiencies computed from the second peak (myoglobin) are 202 theoretical plates (TP) for the column mounted at the off-center position (Fig. 6A) and 169 TP for the same column mounted at the central position (Fig. 6B). The difference in TP between the two coil positions indicates that the off-center position yields slightly higher partition efficiencies than the central position for the polymer phase system used in the present study. This may be mainly due to an efficient phase mixing produced by the strong rotating centrifugal force component acting on the off-center position (see Part II [12]). Although the central coil position produces less efficient separation compared with the off-center coil position, it provides the advantage of retaining a greater amount of the stationary phase in the column that allows application of higher flow-rates of mobile phase to shortening elution times without appreciable loss

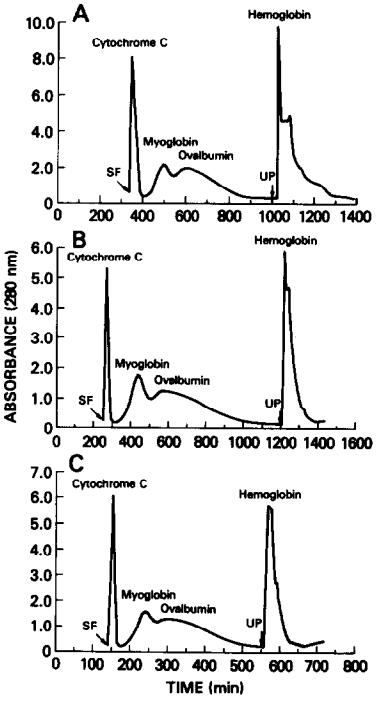


Fig. 6. Protein separations obtained from the preparative multilayer coils (column I) (A) at the off-center ($X - 1.5L$) position at 1 ml/min; (B) at the central (L) position at 1 ml/min; and (C) at the central (L) position at 2 ml/min. Other experimental conditions were as follows: solvent system, 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase, lower phase; revolution: 800 rpm. SF = Solvent front; UP = the column was eluted with the upper phase in the opposite direction.

in peak resolution. This effect was demonstrated by increasing and flow-rate to 2 ml/min in the central coil position but under the otherwise identical experimental conditions (Fig. 6C). In the chromatogram obtained at a higher flow-rate, the theoretical plates calculated from the myoglobin peak dropped to 104 TP while the peak resolution between the cytochrome *c* and myoglobin peaks was unaltered.

Protein separations obtained by the analytical columns (column II in Fig. 2) are shown in Fig. 7. All separations were performed at 800 rpm and at a flow-rate of 0.2 ml/min. Among three chromatograms, chromatogram A was obtained from 27 mg of the protein mixture and chromatogram B from an increased sample size of 44 mg both using the column mounted at the off-center position. The partition efficiencies com-

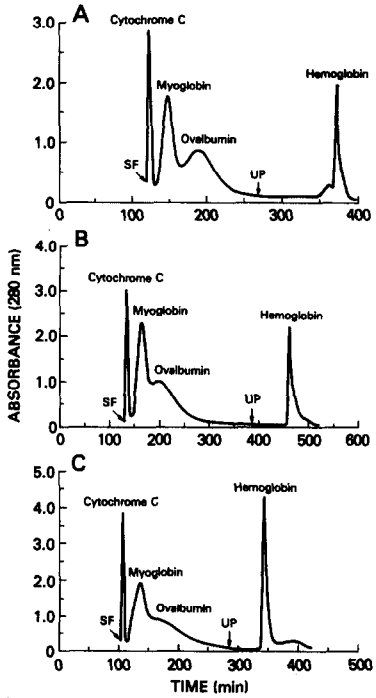


Fig. 7. Protein separations obtained from the analytical coil assembly (column II). (A) A 27-mg amount of sample mixture at the off-center ($X - 1.5L$) position; (B) 44 mg sample mixture at the off-center position; (C) 44 mg sample mixture at the central (L) position. Other experimental conditions were as follows: solvent system, 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase, lower phase; flow-rate, 0.2 ml/min; revolution: 800 rpm. SF = Solvent front; UP = the column was eluted with the upper phase in the opposite direction.

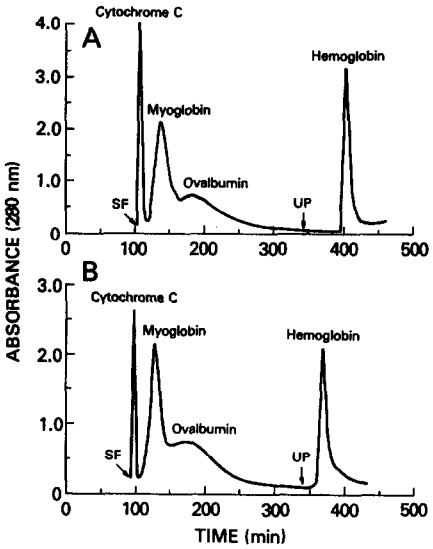


Fig. 8. Protein separations obtained from the analytical coil assemblies: (A) column II and (B) column III (see Fig. 2). Other experimental conditions were as follows: solvent system, 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase, lower phase; flow-rate, 0.2 ml/min; sample size, 44 mg; revolution, 800 rpm. SF = Solvent front; UP = the column was eluted with the upper phase in the opposite direction.

puted from the myoglobin peak are 670 TP for chromatogram A and 505 TP for chromatogram B. Chromatogram C was obtained from 44 mg of the protein mixture using the same column mounted at the central position (L position). In this case, the partition efficiencies measured from the second myoglobin peak is reduced to 228 TP.

The performance of the two analytical columns (columns II and III in Fig. 2) was compared by separations of 44 mg protein mixture at the off-center column position. Separations were performed under the optimum experimental conditions for each column. The result indicates that the chromatogram (Fig. 8B) obtained from the 5-mm-core-diameter analytical column (column III) gives substantially higher partition efficiencies of 402 TP for the myoglobin peak compared to 260 TP for the same peak in the chromatogram (Fig. 8A) obtained from the 2-cm-core-diameter analytical column (column II). This result indicates that the smaller diameter core produces a higher efficiency for a given length of tubing.

Comparative studies on partition efficiency

Partition efficiencies of protein separations in the polymer phase systems ranged from 100 to 200 TP which are considerably lower than those obtained from separations of small molecules in organic–aqueous biphasic systems. For example, a preparative column with a 570-ml capacity yielded 160 TP that correspond to 50 cm/TP (length of the column required to produce one TP) in protein separation whereas a similar column produced a much higher efficiency of 5 cm/TP in dinitrophenyl amino acid separations with chloroform–acetic acid–0.1 *M* HCl (2:2:1) [13]. The low partition efficiency in the protein separation may be attributed to high viscosity of

the polymer phase system and/or some particular nature inherent to the protein molecule. Further studies have been conducted to investigate the cause of low partition efficiencies in protein separation.

Using an analytical column (column III), a series of experiments was performed to measure partition efficiencies of various samples, including both low- and high-molecular-mass compounds, with the same polymer phase system under the identical experimental conditions. Since the TP values calculated from the same chromatogram tend to decrease with increased retention time of the solute, fair comparison of the partition efficiencies should be made from the samples with similar retention times. Therefore, we have selected a set of samples with similar partition coefficient values listed in Table III. It should be noted here that the majority of low molecular weight compounds distribute unilaterally in the PEG-rich upper phase and, therefore, the choice of the sample is extremely limited.

Fig. 9 illustrates analytical chromatograms of potassium dichromate (A), maleic acid (B) and fumaric acid (C) obtained with the polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate in distilled water. Separations were performed at a flow-rate of 0.2 ml/min at 800 rpm.

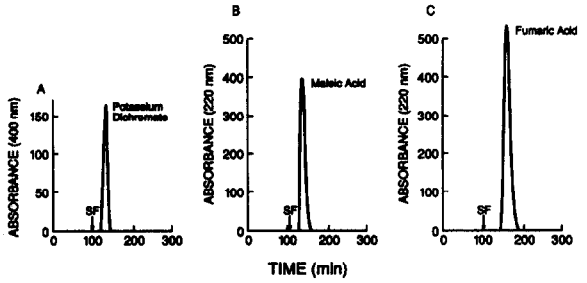


Fig. 9. Chromatograms of potassium dichromate (A), maleic acid (B) and fumaric acid (C) obtained from the analytical coil. Experimental conditions were as follows: solvent system, 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase, lower phase; flow-rate, 0.2 ml/min; sample size, 20 mg; revolution, 800 rpm. SF = solvent front.

Both potassium dichromate (M_r 294.22) and maleic acid (M_r 116.07) yield high TP values of 1340 and 1156, respectively. Fumaric acid, an isomer of maleic acid, gives a considerably lower TP value of 484, apparently due to the longer retention time resulting from its high K value (see Table III).

Fig. 10 shows a set of chromatograms of protein samples obtained from the same polymer phase system under the identical experimental conditions. Among those, ovalbumin (A), bovine albumin (C), and human albumin (D) have K values similar to that of fumaric acid whereas myoglobin (B) has K value close to those of

TABLE III

SUMMARY OF PARTITION STUDIES

All data were obtained from the analytical column (column III) and the solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate in distilled water.

Compounds	M_r	Mol. vol. (10^3 Å^3)	K (C_U/C_L)[b]	Retention time (min)	TP
Potassium dichromate	294.22	0.342	0.62	128	1340
Maleic acid	116.07	0.512	0.78	136	1156
Fumaric acid	116.07	0.512	1.37	154	484
Myoglobin	18 800	48.4 [6]	0.76	128	253
Ovalbumin	45 000	102 [7]	1.38	164	41[a]
Human albumin	68 000	180 [8]	1.66	158	119
Bovine albumin	68 000	180 [8]	1.75	160	92

[a] This low TP value is due to the presence of an impurity which was detected by gel electrophoresis (see text).
[b] C_U = Solute concentration in upper phase; C_L = solute concentration in lower phase.

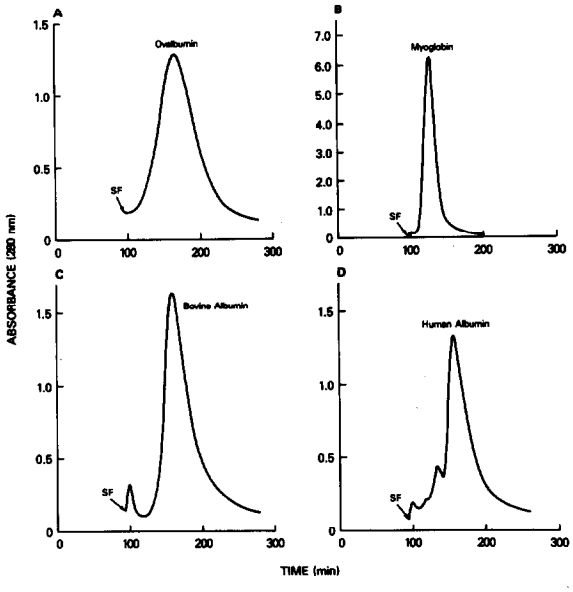


Fig. 10. Chromatograms of ovalbumin (A), myoglobin (B), bovine serum albumin (C) and human serum albumin (D) obtained from the analytical coil. Experimental conditions: solvent system, 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase, lower phase; flow-rate, 0.2 ml/min; sample size, 30 mg; revolution, 800 rpm. SF = Solvent front.

potassium dichromate and maleic acid (see Table III).

All protein samples yielded substantially lower TP values compared with those obtained from the low-molecular-mass compounds. Among these four proteins, myoglobin with a low K value (similar to that of maleic acid) produced the best TP value of 253, while other proteins with high K values (similar to that of fumaric acid) showed much lower TP values of 41 for ovalbumin (A), 92 for bovine albumin (C), and 119 for human albumin (D). The extremely low TP value of ovalbumin suggested a heterogeneous nature of the sample. This possibility was supported by the analysis of the original sample with sodium dodecyl sulfate gel electrophoresis that revealed two distinct bands at a calibration point corresponding to M_r 44 000. Further studies are being carried out to determine these two products.

The overall results of the present studies indicate that the low partition efficiency in protein separation is not primarily caused by the high viscosity of the polymer phase system but more likely due to the high molecular mass and/or some heterogeneity of the proteins.

CONCLUSIONS

The results of the present studies indicate that the new cross-axis CPC can be used for separation of proteins at both analytical and preparative scales. As briefly mentioned earlier, the four different two-phase solvent systems examined each provide a specific merit for separation of the biopolymers (Table I). Thus, solvent 1 (*n*-butanol–aqueous solution containing NaCl and hexadecylpyridinium chloride) has been effectively applied to the separation of mucopolysaccharides including heparin and chondroitin sulfate [14]. Because the hexadecylpyridinium chloride in the solvent system acts as a surfactant, the high-speed CCC centrifuge based on the type J planetary motion fails to retain a satisfactory amount of the stationary phase in the column, while the present apparatus produces excellent retention of the stationary phase.

Solvent 2 (PEG–potassium phosphate) systems have been most effectively used for separation of proteins [9–11]. In this polymer phase system, the low-molecular-mass compounds are generally partitioned unilaterally in the upper phase regardless of the pH and the polymer composition whereas the partition coefficients of the proteins are broadly adjusted by pH and the polymer composition.

Solvent 3 (7% PEG 8000 and 4.4% dextran T500) systems form two phases by themselves without an addition of salts. Although the systems have high viscosity and require an application of lower flow-rate of the mobile phase, it can be effectively used for separation of proteins and other macromolecules that tend to be salted-out by a high salt concentration of the PEG–phosphate systems (solvent 2).

Solvent 4 (4% PEG 8000 and 5% dextran T500) systems have a low interfacial tension and small difference in density between the upper and lower phases. This particular physical properties of the solvent system cause emulsification resulting in low retention of the stationary phases in the present apparatus. However, this

polymer phase system can be applied to separation of cell particles by adjusting the osmotic pressure and pH to meet the physiological requirements of the live cells. Since cell particles are mainly partitioned between the upper phase and the interface between the two phases, a large amount of the stationary phase is not required for the partition of cells. In this case, phase emulsification may give a beneficial effect for cell partitioning by providing a large interfacial surface. Therefore, the present apparatus also has a potential in cell partitioning with the polymer phase systems.

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