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Cross-Axis Synchronous Flow-Through Coil Planet Centrifuge Free of Rotary Seals for Preparative Countercurrent Chromatography. Part II. Studies on Phase Distribution and Partition Efficiency in Coaxial Coils

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Abstract

Potential capability of the apparatus in performing countercurrent chromatography has been examined with three different types of coiled columns, all coaxially mounted around the holder. In single-layer coils, typical solvent systems display characteristic hydrodynamic distribution which ensures a stable retention of the stationary phase against heavy sample loading in preparative separations. Direct observation of the hydrodynamic motion in the rotating spiral column revealed vigorous mixing of the two solvent phases throughout the area which promises a high partition efficiency of the present method. Gram-quantity preparative separations were performed in the multilayer coil with two different sets of test samples: Isocratic elution of dinitrophenyl amino acids yielded high partition efficiency of 1600 theoretical plates while the versatility of the method was demonstrated on gradient elution of dipeptides including two pairs of sequential isomers.

INTRODUCTION

As described in Part I, the cross-axis synchronous flow-through coil planet centrifuge produces a novel pattern of the centrifugal force field which strongly suggests a high performance in countercurrent chromatography (CCC).

In the present paper the potential capability of the apparatus for CCC is evaluated by a series of experimental studies using coiled columns coaxially mounted on the holder. These studies are divided into the following three categories:

1) Retention of the stationary phase in single-layer coils with different helical diameters
2) Stroboscopic observation on hydrodynamic motion of colored solvent phases in a rotating flat spiral column
3) Partition efficiency studies on a multilayer coil

The results of the above studies have clearly demonstrated a great potential capability of the present CCC scheme in gram-quantity preparative separations.

EXPERIMENTAL

Apparatus and Coiled Columns

The apparatus used in the present studies is the first prototype of the cross-axis synchronous flow-through coil planet centrifuge described in Part I. It is a compact table top model with a 10-cm revolutional radius (see Fig. 2 in Part I). The column holder of the apparatus is interchangeable to accommodate various types of coiled columns to facilitate comparative studies.

Three types of coiled columns were prepared each from 2.6 mm i.d. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, New Jersey) as schematically illustrated in Fig. 1.

Single-layer coils (Fig. 1, left), used for phase distribution studies, were prepared by winding the same tubing, 250 cm long and 15 mL capacity, directly onto the holder-hub with three different diameters measuring 5, 10, and 15 cm, yielding β values ($\beta = r/R$, where r is the coil radius and R is the revolutional radius) of 0.25, 0.5, and 0.75, respectively. The multilayer coil (Fig. 1, center) for preparative separations was prepared by winding an about 75 m length of the tubing onto a 10-cm diameter holder-hub making multiple coiled layers between the two flanges spaced 5 cm apart. The β values of the coil range from 0.5 at the internal terminal to 0.8 at the external terminal. The total column capacity measures approximately 400 mL. The flat spiral column (Fig. 1, right) was designed for stroboscopic observation on hydrodynamic motion and distribution of colored solvent phases during the centrifuge run. The

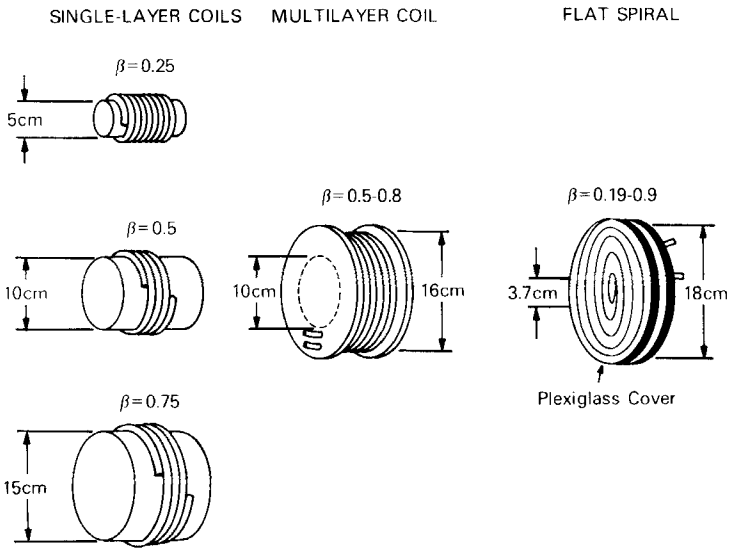

FIG. 1. Three types of coils used in the present studies.

holder consists of a pair of large flanges spaced 3 mm apart to accommodate a single-layer spiral column snugly between them. The column was attached at the tip of the holder shaft which extends through a pair of bearing blocks so that the whole area of the spiral column becomes visible from the outside under stroboscopic illumination. In order to facilitate observation, the front piece of the flanges was made from clear Plexiglas while the surface of the other flange (aluminum) was painted white to increase the color contrast. The β values of the spiral column range from 0.19 at the internal terminal to 0.9 at the external terminal while deviation (l) of the column position from the center of the holder axis is 6.25 cm.

All three types of coiled columns were connected to a pair of flow tubes, 0.85 mm i.d. and each about 1 m long, to facilitate continuous elution during the centrifuge run. As described in Part I, these flow tubes can rotate freely around the centrifuge axis without twisting, thus totally eliminating the need for the rotating seal device.

Reagents

Most of the organic solvents, such as *n*-hexane, ethyl acetate, chloroform, *n*-butanol, *sec*-butanol, and methanol, are glass-distilled chroma-

tographic grades (Burdick & Jackson, Muskegon, Michigan). Other reagents including glacial acetic acid (J. T. Baker Chemical Co., Phillipsburg, New Jersey), hydrochloric acid (Fisher Scientific Co., Fair Lawn, New Jersey), dichloroacetic acid (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin), ammonium formate (Matheson, Coleman & Bell, Norwood, Ohio), and various dinitrophenyl (DNP) amino acid and dipeptide samples (Sigma Chemical Co.) are reagent grades while two dyes, Sudan III and acid fuchsin (Allied Chemical and Dye Corp., New York), are practical grades.

Preparation of Two-Phase Solvent Systems

Nine two-phase solvent systems with a wide spectrum in hydrophobicity were chosen for phase distribution studies in single-layer coils. Their compositions are, in the order of the hydrophobicity of the nonaqueous solvent, *n*-hexane/water, *n*-hexane/methanol, ethyl acetate/water, ethyl acetate/acetic acid/water (4:1:4), chloroform/water, chloroform/acetic acid/water (2:2:1), *n*-butanol/water, *n*-butanol/acetic acid/water (4:1:5), and *sec*-butanol/water.

For stroboscopic observation in the flat spiral column, a small amount of dye was added to the above solvent system to exclusively color one of the phases used as the mobile phase. In general, Sudan III was used to color the nonaqueous phase and acid fuchsin to color the aqueous phase.

For preparative separations with the multilayer coil, two different types of solvent systems were employed: chloroform/acetic acid/0.1 *N* hydrochloric acid (2:2:1) was used for separations of DNP amino acid samples and a pair of solvent systems, *n*-butanol/dichloroacetic acid/0.1 *M* ammonium formate (100:1:100) and *n*-butanol/0.1 *M* ammonium formate (1:1), was used for gradient elution of a dipeptide mixture.

All these two-phase solvent systems were thoroughly equilibrated at room temperature in a separatory funnel by repeating vigorous shaking and degassing many times and then separated shortly before use.

Measurement of Stationary Phase Retention in Single-Layer Coils

The experiments were performed with the single-layer coils of 5, 10, and 15 cm helical diameters illustrated in Fig. 1, left. Stationary phase retention of each coil was measured for the nine pairs of solvent systems (see Preparation of Two-Phase Solvent Systems) according to the procedure reported earlier (*1*).

For each measurement, the coil was first entirely filled with the stationary phase. Then the apparatus was run at a desired revolutional speed while the mobile phase was pumped through the coil at 120 mL/h with a Chromatronix Cheminert Pump. The effluent from the outlet of the coil was collected into a 25-mL-capacity graduated cylinder to measure the volume of the stationary phase eluted from the coil as well as the total volume of the mobile phase eluted. The run was continued until the effluent volume reached 20 mL or more. Then the apparatus was stopped and the coil was emptied by connecting the inlet of the coil to an N_2 line pressured at 80 psi. The coil was then flushed with several milliliters of methanol miscible with both phases. Finally, the coil was again flushed with several milliliters of the stationary phase to be used in the next experiment. During emptying and flushing the coil with N_2, the apparatus was rotated at a moderated speed of 100–200 rpm in a direction making the coil outlet the head to promote the drainage of the coil contents.

For each solvent system, the measurement was repeated by changing various operational conditions such as revoluational speed (200, 400, 600, and 800 rpm), mobile phase (upper and lower phases), and elution mode (head to tail and tail to head elution). In each experiment the volume of the stationary phase retained in the coil was calculated from the volume of the stationary phase eluted from the coil and expressed as a percentage of the total capacity of the coil according to the expression, $100(V_c + V_f - V_s)/V_c$, where V_c denotes the total capacity of the coil, V_f is the free space in the flow tubes, and V_s is the volume of the eluted stationary phase.

Using the retention data thus obtained, the hydrodynamic distribution of the two solvent phases in the coil was summarized in a phase distribution diagram which was constructed by plotting the percentage retention of the stationary phase as a function of revolutional speed for each mobile phase. A group of retention curves produced by different elution modes but otherwise identical experimental conditions can be illustrated in the same diagram.

Procedure for Stroboscopic Observation

The experiment was performed to observe hydrodynamic motion of two immiscible solvent phases directly in the rotating flat spiral column (Fig. 1, right) which was coaxially mounted on one end of the holder shaft at a distance 6.25 cm from the center of the holder (l = 6.25 cm, see Part I, Fig. 7).

In each experiment the column was first entirely filled with the stationary phase. This was followed by rotation of the column at 700 rpm. Then the darkly colored mobile phase was introduced into the column at a flow rate of 120 mL/h. After a steady-state hydrodynamic equilibrium was reached, hydrodynamic motion and distribution of the two solvent phases in the column was observed under stroboscopic illumination which fixed the image of the rotating column at any desired position. During stroboscopic observation the stationary image of the rotating spiral column was photographed by adjusting exposure time to 1/8 of a second (at 700 rpm) so that the photograph was taken from a single light pulse emitted from the stroboscope. This provided most reliable information on the hydrodynamic process taking place in the rotating column.

Various two-phase solvent systems were examined using both the head and the tail phases as the mobile phase. For each mobile phase the following four different elution modes were observed: the internal head to the external tail, the internal tail to the external head, the external head to the internal tail, and the external tail to the internal head.

In each run the effluent from the outlet of the column was collected into a 50-mL graduated cylinder to determine the percentage retention of the stationary phase relative to the total column capacity as in the phase distribution studies described earlier.

Procedures for Preparative Separations with Multilayer coil

Partition capability of the present CCC scheme has been evaluated with a multilayer coil (Fig. 1, center) on separations of two sets of test samples, i.e., (1) isocratic elution of DNP amino acids with chloroform/acetic acid/0.1 *N* hydrochloric acid (2:2:1) and (2) gradient elution of dipeptides with *n*-butanol/dichloroacetic acid/0.1 *M* ammonium formate (100:1:100 → 100:0:100).

(1) DNP Amino Acid Separation. Two sets of sample mixture were prepared: Δ-*N*-DNP-L-ornithine (DNP-orn), *N*-2,4-DNP-aspartic acid (DNP-asp), *N*-2,4-DNP-DL-glutamic acid (DNP-glu), *N,N*-di-(2,4-DNP)-L-cystine (diDNP-$(cys)_2$), and *N*-2,4-DNP-L-alanine (DNP-ala) at 1:2:2:1:4 in weight for the elution with the upper aqueous phase mobile and *N*-2,4-DNP-L-leucine (DNP-leu), *N*-2,4-DNP-L-proline (DNP-pro), *N*-2,4-DNP-β-alanine (DNP-β-ala), *N,N*-di-(2,4-DNP)-L-cystine (diDNP-$(cys)_2$) and *N*-2,4-DNP-DL-glutamic acid (DNP-glu) at 1:2:2:1:4 in weight for the elution with the lower nonaqueous phase mobile. Each mixture was

dissolved in the stationary phase or a mixture of both phases to make the total concentration 5 g%.

In each separation the column was first completely filled with stationary phase. This was followed by injection of the sample solution above (2, 10, 20, or 40 mL) through the sample port. Then the apparatus was run at 700 rpm while the mobile phase was pumped into the column at 120 mL/h in the proper elution mode predetermined in the phase distribution studies. Effluent from the column outlet was continuously monitored with an LKB Uvicord S at 278 nm and fractionated with an LKB fraction collector. After all peaks were eluted from the column, the apparatus was stopped and, by connecting the column inlet to a pressured N_2 line (80 psi), column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column. After N_2 appeared through the outlet of the column, the apparatus was slowly rotated in such a direction that the outlet becomes the head. This process promotes recovery of the column contents up to 99%, thus providing accurate retention values of the stationary phase.

An aliquot of each fraction was mixed with a known amount of methanol and the absorbance was measured at 430 nm with a Zeiss spectrophotometer. From the obtained chromatogram the partition efficiency was computed according to the conventional gas chromatographic equation.

$$N = (4R/w)^2 \tag{1}$$

where N denotes the column efficiency expressed in theoretical plates (T.P.), R is the retention volume referred to the peak maximum, and w is the peak width expressed in the same unit.

(2) Dipeptide Separation. The sample mixture consisted of the following six dipeptides, all containing a tyrosine moiety: L-tyrosyl-L-alanine (tyr-ala), L-valyl-L-tyrosine (val-tyr), L-tyrosyl-L-valine (tyr-val), L-leucyl-L-tyrosine (leu-tyr), L-tyrosyl-L-leucine (tyr-leu), and L-tryptophyl-L-tyrosine (trp-tyr), at 4:4:4:3:3:2 in weight. The sample mixture was dissolved in the upper stationary phase of the starting phase system at a total concentration of 2.5 g%.

For the gradient elution a pair of two-phase solvent systems was prepared: The starting phase system was composed of *n*-butanol/dichloroacetic acid/0.1 *M* ammonium formate (1:0.01:1) and the ending phase system of *n*-butanol/0.1 *M* ammonium formate (1:1). A proportional gradient in decreasing concentration of dichloroacetic acid in the

lower aqueous phase was formed with a varigrad gradient device (Buchler Instruments, Fort Lee, New Jersey) by filling the first and second compartments each with 300 mL of the lower aqueous phase from the starting and the ending phase system, respectively.

For each separation the column was first filled with the upper nonaqueous stationary phase of the starting phase system followed by sample injection (4, 20, or 40 mL) through the sample port. Then the apparatus was spun at 800 rpm while the mobile phase was pumped into the column at a flow rate of 120 mL/h in the tail to head elution mode, the gradient being started immediately. After 600 mL of the gradient elution, the elution was continued with the lower phase of the ending phase system until all six peaks were eluted from the column. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and then fractionated with an LKB fraction collector. An aliquot of each fraction was mixed with a known volume of methanol and the absorbance was measured at 280 nm with a Zeiss spectrophotometer.

After the run, retention of the stationary phase was determined according to the method used in the DNP amino acid separation.

RESULTS AND DISCUSSION

Phase Distribution Studies on Single-Layer Coils

Figure 2 illustrates a set of phase distribution diagrams for nine volatile two-phase solvent systems in the single-layer coils with three different helical diameters. These diagrams are divided into four groups according to the major organic solvent and arranged in columns from left to right in the order of hydrophobicity. For each solvent group, the top three rows indicate the retention of the lower phase with the upper phase mobile, and the bottom three rows indicate the retention of the upper phase with the lower phase mobile, while each row was assigned for a particular helical diameter or β value as labeled on the left side. Two retention curves drawn in each diagram indicate the elution mode of the mobile phase: the solid line for the head to tail elution and the broken line for the tail to head elution.

Let us first observe the top three diagrams of hexane/water located at the left end. All these diagrams clearly show that high retention of the lower phase is attained by the tail to head elution (broken lines) of the upper mobile phase while no retention results from the head to tail elution (solid lines). The above retention profile of the lower phase

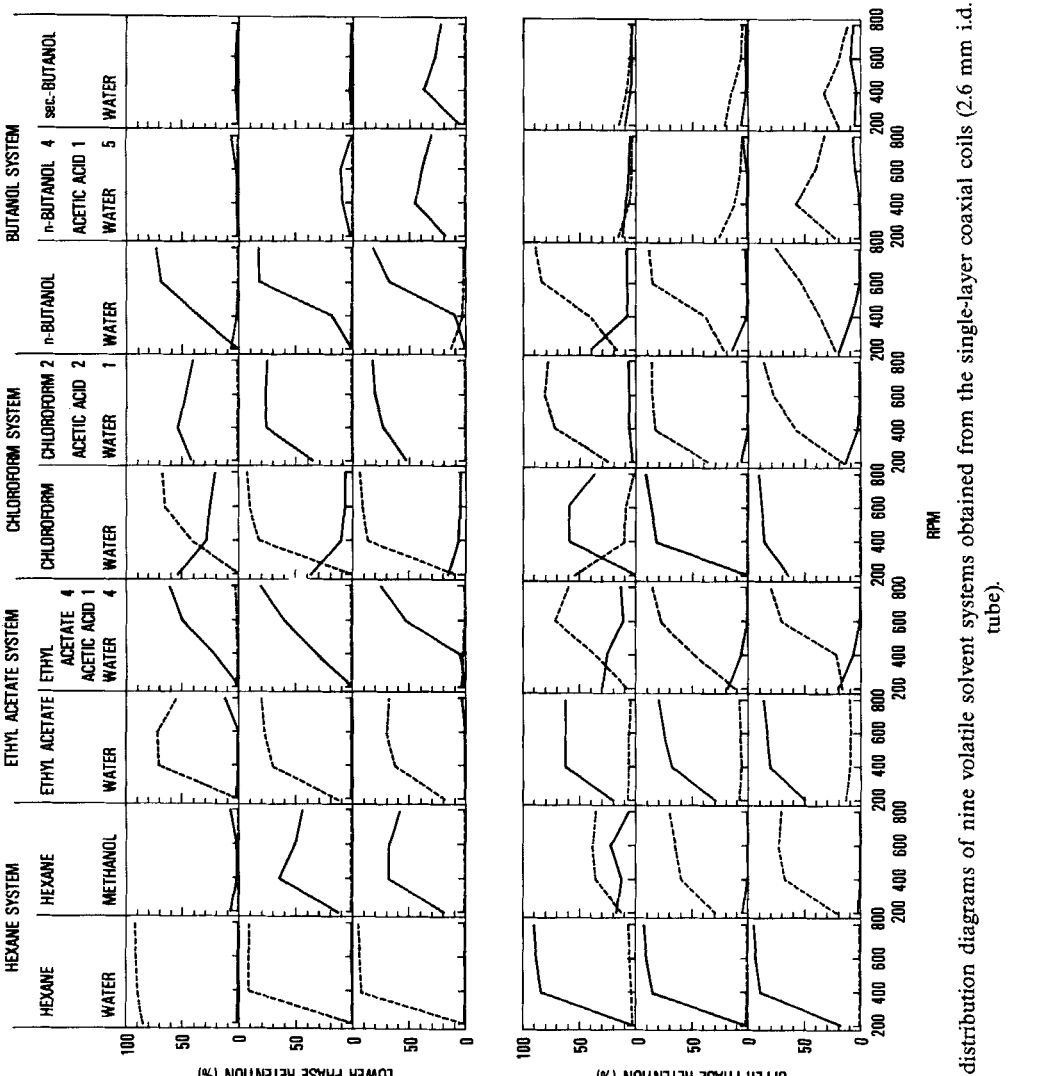

FIG. 2. Phase distribution diagrams of nine volatile solvent systems obtained from the single-layer coaxial coils (2.6 mm i.d. tube).

suggests that the two solvent phases display unilateral distribution in the coil where the upper phase distributes on the head side as the head phase and the lower phase on the tail side as the tail phase. Thus the upper phase introduced from the tail (broken lines) can move quickly through the lower phase toward the head, leaving a large amount of the lower phase stationary in the coil. On the other hand, the upper phase, if introduced from the head (solid lines), will tend to stay permanently on the head side and steadily push the entire segment of the lower phase out from the coil, resulting in no retention. The above reasoning is further supported by the retention profile of the upper phase shown in the bottom three phase distribution diagrams of hexane/water where the relationships between two retention curves are completely reversed. Under these circumstances, introduction of the lower tail phase from the head (solid lines) results in high retention where the reversed elution mode (broken lines) causes total loss of the stationary phase from the coil.

This retention profile of hexane/water as described above is considered to be ideal because either the upper or the lower phase can be used as a mobile phase with high retention of the stationary phase. Among the phase distribution diagrams in Fig. 2, similar retention curves are found in two other binary phase systems, ethyl acetate/water and chloroform/water. These three binary systems are characterized by high hydrophobicity of the nonaqueous phase and share common physical properties of high interfacial tension and low viscosity. In these hydrophobic solvent systems the upper phase becomes the head phase in a broad range of β values applied in the present experiments.

The rest of the two-phase solvent systems possess various degrees of reduced hydrophobicity and interfacial tension. All these solvent systems display a reversed hydrodynamic trend, i.e., retention is attained either by head to tail elution (solid lines) of the upper phase or by tail to head elution (broken lines) of the lower phase, indicating that the lower phase is the head phase in these solvent systems. Most of these solvent systems yield satisfactory retention (over 50%) of the stationary phase above $\beta = 0.5$ except for the most hydrophilic butanol solvent systems including *n*-butanol/acetic acid/water (4:1:5) and *sec*-butanol/water which show a significant degree of retention only at the highest β value of 0.75.

The phase distribution diagrams described above reveal one important advantage of the present scheme (Type X) over other high performance CCC schemes (Type J and Type J-L) in that Type X permits most useful solvent systems with moderate hydrophobicity to maintain stable retention in the coil against the heavy sample loading necessary for preparative separations.

In the phase distribution diagrams obtained from the Type J scheme, the two-phase solvent systems are classified into three categories according to hydrophobicity of the nonaqueous phase which determines hydrodynamic distribution of the solvent phases in the coil (*1*, *2*). Hydrophobic solvent systems such as hexane/water, ethyl acetate/water, and chloroform/water always distribute the upper phase toward the head regardless of the applied β values. In contrast to the above, hydrophilic solvent systems such as *n*-butanol/acetic acid/water (4:1:5) and *sec*-butanol/water always distribute the lower phase toward the head. In the rest of the solvent systems with moderate hydrophobicity (intermediate solvent systems), the head phase is determined by the β values, i.e., under large β values the upper phase becomes the head phase as in the hydrophobic solvent systems while under small β values the lower phase becomes the head phase as in the hydrophilic solvent systems. Further studies have shown that these hydrodynamic trends are also closely related to the physical properties of the solvent system, particularly viscosity and to a lesser extent interfacial tension and density difference between the upper and the lower phases. In other words, the hydrodynamic trend of a given solvent system may be greatly affected by addition of a substance which significantly alters the viscosity and other physical properties of the solvent system.

In practice, the above phenomenon may create a serious problem in large-scale preparative separations with the Type J or J-L CCC schemes. Intermediate solvent systems such as chloroform/methanol/water, *n*-butanol/water, etc. are most commonly used for CCC, and in a standard multilayer coil ($0.5 < \beta < 0.8$) they display a hydrodynamic trend similar to that in the hydrophobic solvent systems, i.e., the upper phase becomes the head phase. Thus the separations are performed by either pumping the upper phase from the tail or the lower phase from the head. However, introduction of a large amount of concentrated sample solution into the coil shifts the above hydrodynamic trend toward the transitional or further reversed state at the beginning of the coil due to alteration in the physical properties of the solvent phases in the sample compartment. This local change of the hydrodynamic trend will cause detrimental loss of the stationary phase, no matter which elution mode is applied to the coil. Under these circumstances, manipulation of various operational parameters such as centrifugal speed, flow rate of the mobile phase, etc. usually fails to improve the retention of the stationary phase. Although raising the column temperature may improve the situation by reducing the viscosity of the solvents, it might adversely affect the interfacial tension and density difference between the phases (*3*).

All these problems, which are inherent to the Type J planetary motion,

are largely alleviated in the present Type X scheme in which the intermediate solvent systems display the reversed hydrodynamic trend, i.e., the lower phase becomes the head phase as in the hydrophilic solvent systems in a wide range of β values ($\beta \leq 0.75$). Consequently, introduction of a large amount of sample does not alter the hydrodynamic trend in the sample compartment. Even though the high viscosity and lowered interfacial tension may substantially lower the retention of the stationary phase, these adverse effects can be effectively corrected by manipulating the operational parameters such as revolutional speed, flow rate of the mobile phase, etc. Thus a large-scale preparative separation is possible with the present CCC scheme with a minimum risk of carryover of the stationary phase.

On the other hand, the present scheme has a disadvantage over the Type J CCC scheme in that it necessitates application of lower flow rates of the mobile phase to produce a satisfactory level of stationary phase retention, thus requiring longer separation times. This difference is apparently caused by the altered centrifugal force field in the present scheme where the strong radial force component in the Type J scheme is reduced to form a laterally oscillating force field as described in Part I. As mentioned later, this oscillating force field produces efficient mixing of solvent phases to promote partition process, hence partially compensating for increased separation times. In addition, the retention capacity of the present scheme may be further improved by increasing the revolution speed and radius upon refinement of the present prototype.

Stroboscopic Observation of Hydrodynamic Motion of Two Solvent Phases in a Flat Spiral Column

Hydrodynamic motion and distribution of the two solvent phases in a rotating column was directly observed and photographed under stroboscopic illumination. The typical results obtained from the chloroform/water phase system are schematically illustrated in Fig. 3, left, in which the chloroform phase was eluted from the internal head terminal toward the external tail terminal through the aqueous stationary phase at 120 mL/h under 700 rpm. As shown in the diagram, the spiral column was divided into two areas: About one-third of the area near the central axis of the centrifuge shows vigorous mixing of the two phases forming coarse droplets throughout the area. This area extends toward the left slightly more in the lower half of the spiral segments than in the upper half as indicated in the diagram. In the rest of the area the two solvent phases form two layers, the lower nonaqueous phase along the outer portion and

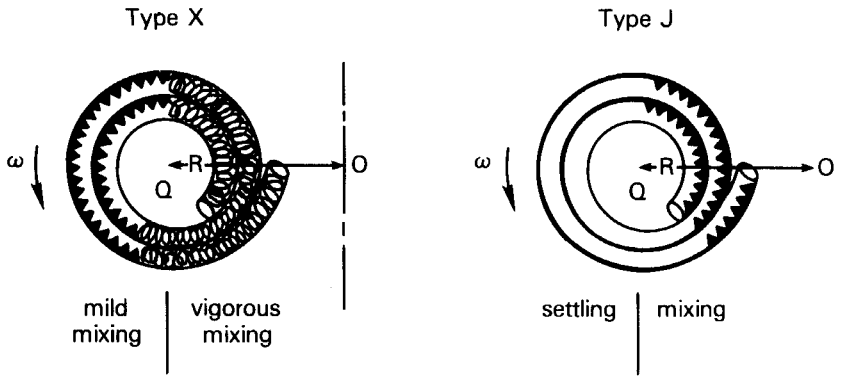

FIG. 3. Hydrodynamic motion of two solvent phases in the rotating flat spiral coil.

the upper aqueous phase along the inner portion of each spiral segment forming an undulating interface between the two layers. Similar findings were observed in many other solvent systems under proper elution modes.

The above findings are quite different from those observed from the Type J planetary motion reported earlier (*4*). As illustrated in Fig. 3, right, the Type J planetary motion forms two distinct zones in the spiral column: Mixing zone extends about one-quarter of the area near the center of the centrifuge where two solvent layers exhibit an undulating interface, indicating vigorous mixing, while the rest of the area, called the settling zone, shows two distinct layers with a smooth interface.

Comparison between hydrodynamic effects produced by these two schemes clearly shows the characteristic feature of the present Type X scheme. Probably due to the action of a laterally oscillating force field, the present scheme produces a more vigorous mixing effect which extends over the entire spiral segments. This indicates that solutes introduced into the column are subjected to a continuous partition process by alternating violent and less violent mixing between the two solvent phases and are efficiently separated according to their partition coefficients as the mobile phase steadily elutes through the column. Thus it is reasonable to expect that the present scheme can yield a higher partition efficiency than the Type J scheme which relies on an intermittent mixing process, provided that longitudinal spreading of the sample bands is insignificant during elution.

Table 1 summarizes retention of the stationary phase obtained from various solvent systems under different elution modes for each mobile

TABLE 1
Retention of the Stationary Phase in the Flat Spiral Column

	Solvent system and mobile phase									
	Hexane Water		Chloroform Water		Chloroform Acetic acid Water	2 2 1	*n*-Butanol Water		*n*-Butanol Acetic acid Water	4 1 5
Elution mode	Upper phase	Lower phase	Upper phase	Lower phase	Upper phase	Lower phase	Upper phase	Lower phase	Upper phase	Lower phase
Head → Tail Internal → External	1.2	79.8	17.1	75.0	52.9	12.6	50.5	27.1	34.0	15.5
Head → Tail External → Internal	0.5	88.1	9.5	82.1	16.7	35.7	10.2	73.8	49.5	0.7
Tail → Head Internal → External	92.9	0	92.9	0	46.0	14.3	60.7	10.7	0	57.1
Tail → Head External → Internal	91.7	0	21.0	42.0	0	53.6	11.9	53.6	0.9	22.4

phase. These retention values are the average of those obtained from various locations on the spiral column where outer spiral segments with higher capacity contribute greater proportions in the figure. In many cases, observation of the column after stopping centrifugation revealed that the distribution of the two solvent phases varied according to the β values. For example, in chloroform/acetic acid/water (2:2:1) with the lower phase mobile from the external head toward the internal tail with an average retention of 35.7% as listed, the retention was locally about 50% at $0.2 < \beta < 0.25$, less than 20% at $0.3 < \beta < 0.7$, and 80% at $\beta > 0.75$, while showing quite narrow boundaries of a few segments between these three regions. Nevertheless, these retention data provide valuable information on the hydrodynamic effects produced by the flat spiral column configuration.

The geometry of the spiral column creates a radial gradient in the centrifugal force field which favors the lower phase moving outward or the upper phase moving inward through the spiral column. This hydrodynamic trend has been demonstrated in a similar spiral column under the Type J planetary motion (*2*, *4*). However, the retention data obtained with the present Type X scheme show quite opposite results. With the exception of the hydrophilic *n*-butanol/acetic acid/water (4:1:5) phase system, all other solvent systems examined produced higher retention with outward elution (internal → external) of the upper phase or inward elution (external → internal) of the lower phase. In addition, comparison between head to tail elution (top two rows) and tail to head elution (bottom two rows) reveals an altered hydrodynamic trend of the *n*-butanol/water phase system with the upper phase being the head phase.

Although the cause of these modified hydrodynamic phenomena in the spiral column is presently unknown, they are most likely due to the presence of the lateral force field inherent in the present scheme. As described in Part I, Type X planetary motion generates an additional gradient in the lateral force field which is affected by deviation of the point along the holder axis. As the point moves away from the central plane of the holder, the internal area of the spiral gains relative strengths in the lateral force field over the external area, resulting in a hydrodynamic effect somewhat opposite to that produced by the radial force gradient. Consequently, phase distribution in the spiral column is governed by interplay between the above two gradient effects as well as by the head-tail hydrodynamic relationship.

Preparative Separations with a Multilayer Coil

(1) DNP Amino Acid Separation by Isocratic Elution

The results of DNP amino acid separations obtained with the multilayer coil are summarized in Fig. 4A where each chromatogram is arranged according to the sample size and the mobile phase as indicated in the diagram. The chromatograms obtained from the 100-mg sample size yielded the highest partition efficiency of 1600 T.P. (theoretical plates). The peak resolution shows no significant change until the sample size exceeds 500 mg (20 mL). Further increase of the sample size results in a gradual fall in partition efficiency, but even at the maximum sample size of 2 g (40 mL), the integrity of the individual peaks is well preserved in both mobile phase elutions.

The gradual loss of peak resolution with increased sample size is accompanied by reduced retention levels of the stationary phase as illustrated in Fig. 4B. In both mobile phase elutions the retention levels remain unchanged up to 500 mg sample size, followed by a gradual decline with further increase in the sample size. However, even at the maximum sample size (2 g), the retention levels stay within a satisfactory range of over 50% in both mobile phase elutions, indicating that a further increase of sample size may be feasible without serious loss of the retained stationary phase.

As described above, the highest partition efficiency obtained from DNP amino acid separations is 1600 T.P., which is expressed as an average of 8 T.P. per helical turn or 4.8 cm/T.P. (one theoretical plate per 4.8 cm length of tubing). The above partition efficiency appears to exceed those in similar separations obtained from the Type J multilayer coil planet centrifuge, although direct comparison is difficult due to the differences in the applied experimental conditions such as sample size, tube diameter, flow rate of the mobile phase, etc. (*5*, *6*). The high partition efficiency of the present scheme is apparently derived from characteristic hydrodynamic motion of the two solvent phases observed in the flat spiral column under stroboscopic illumination.

(2) Dipeptide Separations by Gradient Elution

In order to demonstrate versatility of the present scheme, gradient elution was performed for separation of six dipeptides, all with a tyrosine moiety and including two pairs of sequential isomers. The results obtained with three different sample sizes are illustrated in Fig. 5A.

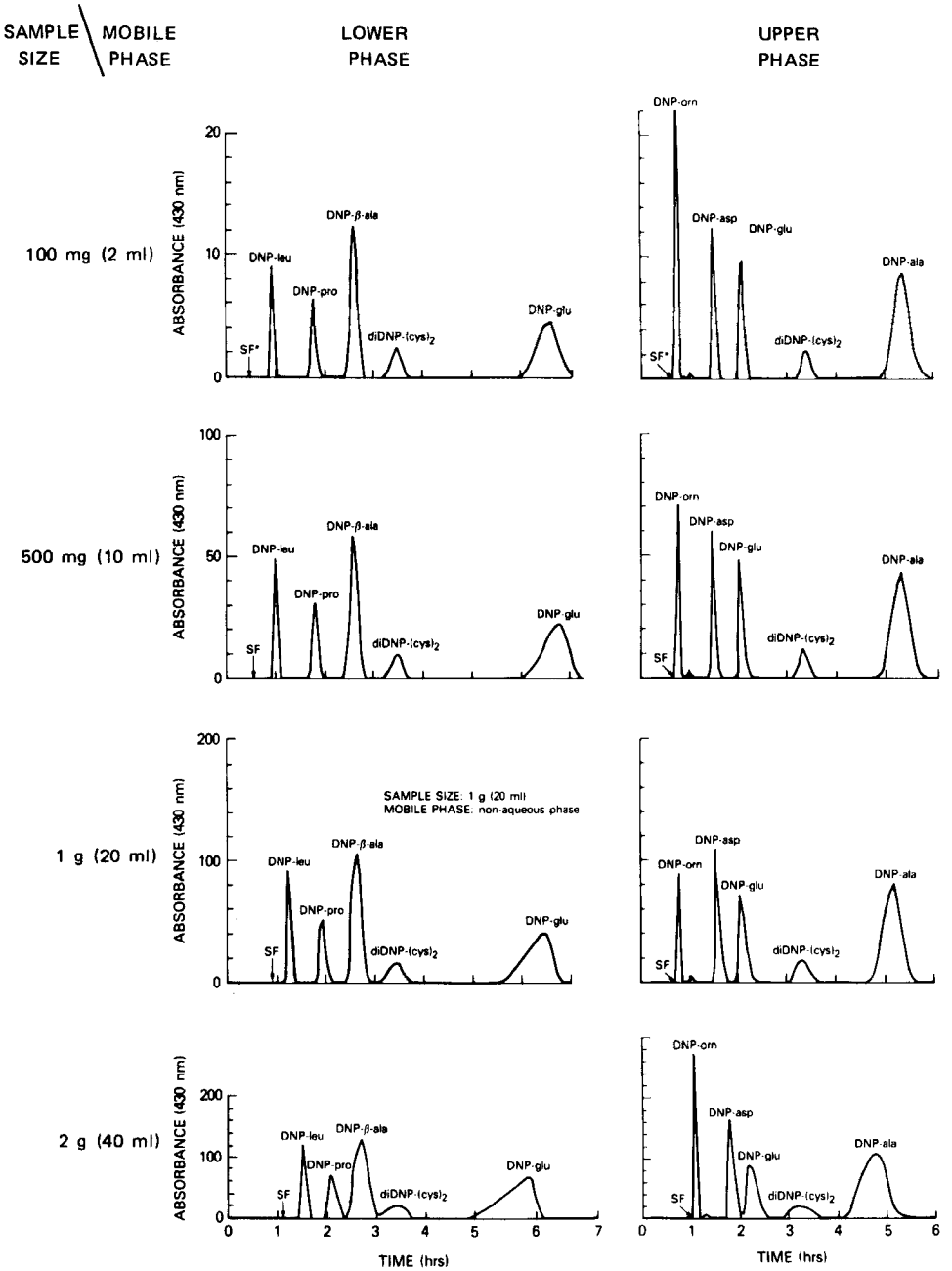

FIG. 4A. DNP amino acid separations by isocratic elution. Effects of sample size on peak resolution. *SF = solvent front.

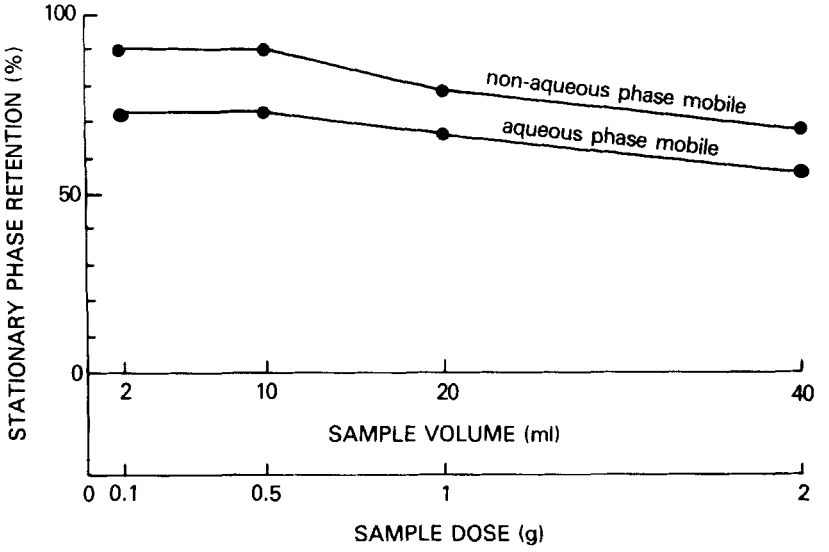

FIG. 4B. DNP amino acid separations by isocratic elution. Effects of sample size on stationary phase retention.

In the top chromatogram for the smallest sample size of 100 mg (4 mL), all peaks were well resolved into rather symmetrical shapes. As the sample size is increased, these peaks tend to display various degrees of skewing or deformation but most peaks maintain excellent resolution up to the maximum sample size of 1 g (40 mL).

Because these peptide samples display a nonlinear isotherm in the *n*-butanol solvent systems, they tend to form characteristic skewed peaks in isocratic elution in which the elution with the lower aqueous phase produces skewing toward the left, leaving a tail on the right side, thus simulating the adsorption effect onto the solid surface. However, this skewing is corrected to some extent by gradient elution which tends to produce a similar deformation of the peaks in the opposite direction. Since the peak skewing is intensified with sample concentration, the interplay between these two mutually antagonistic effects creates various peak shapes in the chromatograms of 500 mg to 1 g sample size. The early-eluting tyr-ala peak is mainly affected by the skewing due to the nonlinear isotherm while later eluting peaks are affected by the gradient effects. The val-tyr peak eluting at the transitional position simultaneously exhibits these two effects, forming double peaks at the 500-mg sample size: The first peak is caused by the nonlinear isotherm and the

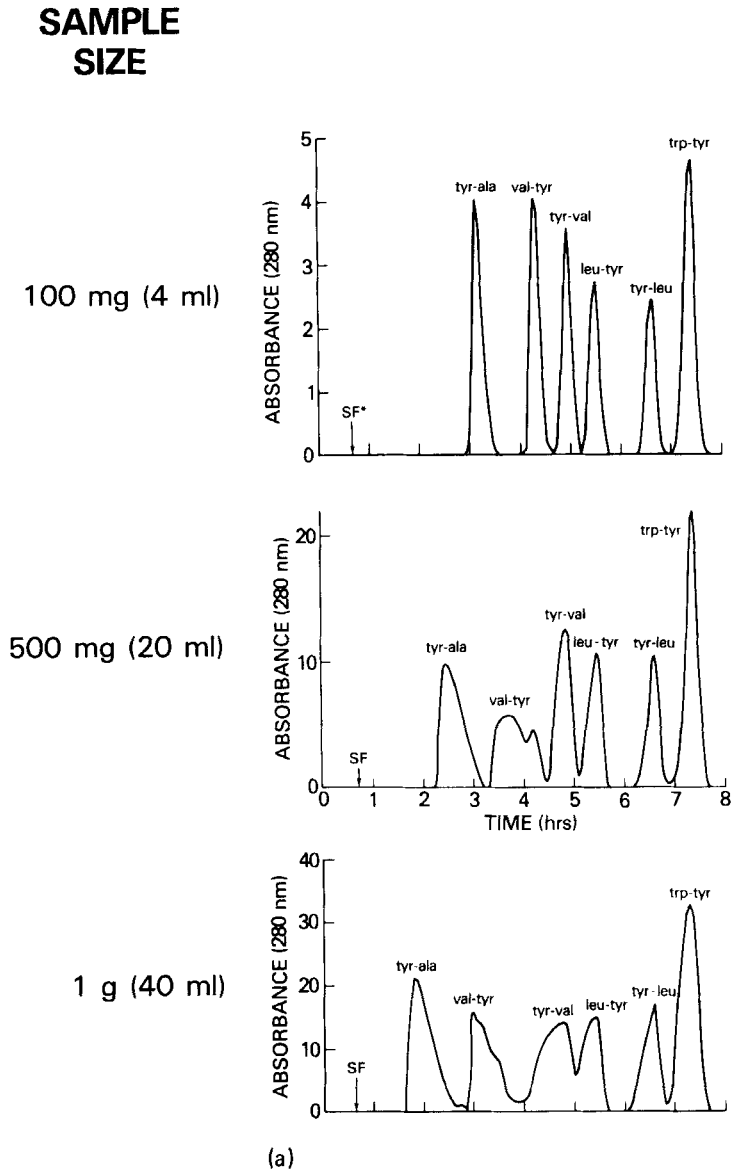

FIG. 5A. Dipeptide separations by gradient elution. Effects of sample size on peak resolution. *SF = solvent front.

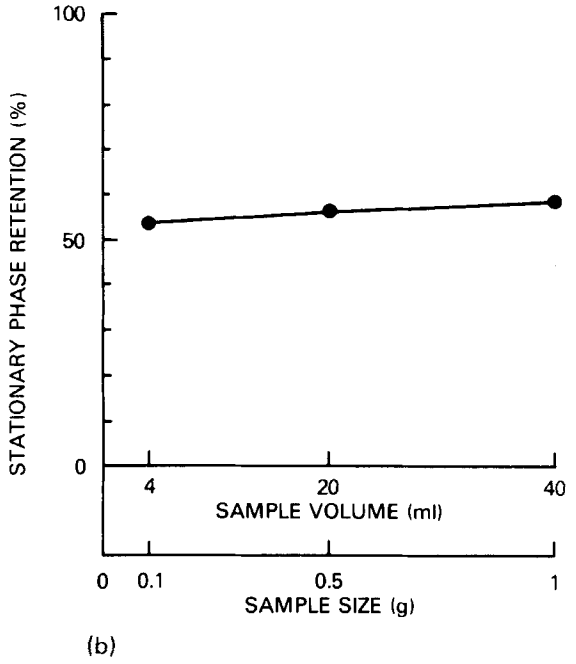

FIG. 5B. Dipeptide separations by gradient elution. Effects of sample size on stationary phase retention.

second peak is due to the gradient effect. These anomalies may be corrected by raising the dichloroacetic acid concentration in the starting solution, which results in augumentation of the gradient effect over the nonlinear isotherm.

In all these separations the solvent front emerged within 1 h. This was followed by substantial amounts of carryover of the stationary phase, which gradually tapered off as the elution proceeded. Thereafter, sporadic carryover spells were observed throughout the run. Nevertheless, the collection of the column contents revealed a satisfactory amount of over 50% stationary phase relative to the column capacity. Rather intensive carryover of the stationary phase observed after the solvent front may be largely eliminated either by reducing the flow rate or by increasing the revolutional speed and radius. The effects of sample size on retention of the stationary phase in these dipeptide separations are illustrated in Fig. 5B. It clearly indicates that the retention is not adversely affected by the sample size but is slightly improved by an increase of sample size. The maintenance of satisfactory retention under heavy

sample loading may well be the manifestation of the unique capability of the present scheme which is particularly suitable for large preparative-scale separations.

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