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COUNTERCURRENT CHROMATOGRAPHY

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INTRODUCTION

Historically, the separation of similar molecules by their differential solubility between two immiscible phases has provided the basis of several highly selective separatory techniques such as paper chromatography, countercurrent distribution, and liquid-liquid column chromatography. The great selectivity of these techniques arises from the fact that the partition coefficient is a fundamental reflection of intermolecular forces which can be strongly modified by conditions of temperature and phase composition. Thus, with judicious choice of conditions, highly specific interactions can often be exploited which will permit the resolution of selected components from even very complex mixtures.

Paper chromatography has long been used as a highly selective separatory method. However, sample sizes are limited and separations are of relatively low efficiency. Until the fairly recent advent of "high resolution" column chromatography, countercurrent distribution was probably the method of choice for high resolution separations based upon the liquid-liquid partition principle. The

advantages of large sample capacities, an almost unlimited choice of solvent pairs, and easy description of separations tended to outweigh the concomitant disadvantages of expensive, cumbersome apparatus and long separation times. Although the newly developed techniques of high pressure liquid chromatography, which afford highly efficient separation, have all but replaced countercurrent distribution techniques for separations involving small molecules, they do not, as yet, afford a panacea to the problems of separation. The choice of phases is rather limited by the requirements of retention (adsorption or chemical bondability) to the support which, itself, must be inert. In particular, these problems are more acute for highly polar molecules, for which the availability of suitable phases is rather limited, and for biological macromolecules for which suitable phases are virtually non-existant.

We have been experimenting with a liquid-liquid partition technique which combines the selectivity afforded by paper chromatographic and countercurrent distribution techniques with the efficiency and speed afforded by column chromatographic techniques. This technique, which has been called countercurrent chromatography, is a continuous flow technique which, although employing much of the instrumentation of column chromatography, does not utilize a solid support.[1-5] Thus, the choice in phase composition equals or exceeds that possible with paper chromatography or countercurrent distribution. Additionally separations on both an analytical and preparative scale are possible.

In order to eliminate the solid support, it was necessary to overcome four problems.

1. How to retain the stationary phase while the mobile phase is eluted.
2. How to divide the column space into numerous partition units and reduce laminar flow spreading of sample bands.
3. How to increase interfacial area.
4. How to mix each phase to reduce mass transfer resistance.

The following model system describes one method of achieving this which overcomes the above problems.

THE FLOW-THROUGH COIL PLANET CENTRIFUGE

Principles of operation

The model consists of a helically coiled tube mounted with its long axis perpendicular to the gravitational field. The coil is slowly rotated about its long axis. (The speed of rotation is slow enough so that centrifugal forces may be ignored). This model reproduces much of the essential operating phenomena, and is easier to visualize than the centrifuge device which is used for separations.

First, the coil is completely filled with a single solute. During rotation, the solvent simply follows the tubing around. The superimposed gravitational field has no effect because the solvent is of homogeneous density. Next, some air bubbles and plastic beads denser than the solvent are introduced. Now, the solvent still follows the tubing around, but the air bubbles and beads experience buoyant forces as well. The air bubbles and beads are observed to migrate toward the same end of the column, which will be called the head. The motion of the suspended objects results from interplay of buoyancy, which tends to keep them in the top or bottom of the coil, and hydrodynamic drag which tends to move them with the solvent. In fact, any suspended object, whether heavier or lighter than the solvent, migrates toward the head of the column. In the extreme case where the buoyant forces overpower the hydrodynamic drag, e.g. large, heavy beads, the bead remains at the bottom while the coil undergoes helical screw motion past the bead. Hence, for every revolution, the bead advances one turn of the coil toward the head. In the more general case, the suspended object tries to follow the solvent around, but is consecutively retarded and accelerated relative to the solvent by gravity. This time, a more gradual migration toward the head is observed with a number of revolutions being required to advance the object one turn of the helix.

If a drop of an immiscible liquid is suspended in the solvent it will also migrate toward the head of the column as did the bead.

Now the coil is initially filled with roughly equal amounts of two immiscible solvents such as water and butanol colored with a dye for contrast. The principle that suspended drops migrate toward the head still applies, but the question of which phase acts as the suspending medium depends on which phase is in local excess within various parts of the coil. After a few minutes of rotation, the two phases reach a dynamic equilibrium state where, starting from the head end, each turn of the coil is occupied by nearly equal volumes of the two phases. Any excess of either phase will be forced to the tail end of the coil, because any excess in a given coil unit will simply spill over into the next coil unit.

It is this effect which obviates the inert support; an excess of either phase can be introduced into the head end by means of a pump. If more upper phase is slowly pumped into the head end of the coil, a like amount of upper phase will emerge at the tail end leaving the lower phase within the coil as a stationary phase. Meanwhile, in each turn of the coil, the two phases are continually percolating through each other as the helix revolves. Consequently, a solute introduced into the coil at the head will be repeatedly partitioned between the two vigorously mixing phases, and will move toward the tail at a rate dependent upon its partition coefficient. Thus, one can carry out liquid-liquid chromatography without using a solid support.

In summary, the salient feature of this system is the segmentation of phases produced by the combination of the coil and the gravitational field. In theory, the model described above could be used as is; however for a practical system centrifugation must be used to enhance the gravitational field in order to permit reasonable flow rates with small bore coils.

Principles of construction

The first scheme utilizing the principles illustrated with the above model is the "flow-through coil planet centrifuge" which is illustrated diagrammatically in Figure 1. This device employs a vertical helical column in the centrifugal field. The column is

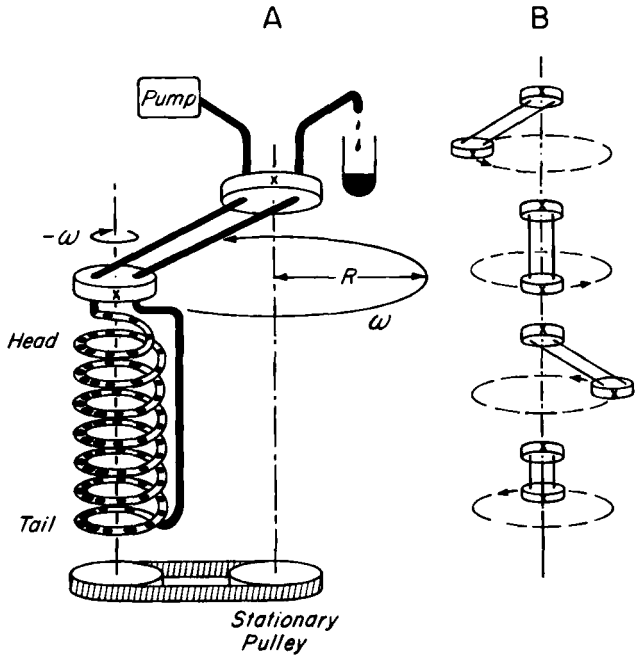

FIGURE 1
Principle of the flow-through coil planet centrifuge. (A) Helical separation column with flexible feed and return tubes supported by the moving and stationary disks. (B) Successive positions of the stationary and moving disks.

mounted in such a manner that it and the coil holder (the disk in Figure 1-A marked with the "x") make a single rotation about the coil axis for each revolution of the centrifuge. The requisite rotation can be provided by means of a belt on the helix holder which couples the lower moving disk to a stationary disk of equal diameter around the axis of rotation of the centrifuge. This coupling causes a counter-rotation of the helix to cancel out the rotation of the helix induced by the revolution of the centrifuge. As a consequence, the moving disk maintains the same face toward a stationary observer as shown in Figure 1-B. It is this feature which obviates rotating seals; the feed and return tubes do not twist because the moving disk does not rotate with respect to the stationary disks.

When the helical column is filled with two immiscible phases, such planetary motion can produce an equilibrium pattern where the multiple segments of the two phases are alternately arranged from the head end of the column and any excess of either phase remains at the tail. As with the previously discussed model, the mobile phase must be introduced from the head end as determined by both the handedness of the helix and the direction of planetary motion. Any solute introduced into the column will then be partitioned between the alternating segments of the two phases and eluted in the order of decreasing partition coefficient.

Apparatus

A "laboratory bread board" type of device has been constructed by modifying a conventional centrifuge as shown in Figure 2. The column holder and the counterweight are held by a pair of rotary arms with bearings which are equivalent to the previously mentioned "moving disk". A pair of pulleys of the same size, one at the bottom of the holder and the other at the stationary tube surrounding the axis of rotation of the centrifuge, are coupled with a timing belt to introduce the desired planetary motion to the coil holder.

The column is prepared by winding Teflon tubing onto a plastic core to make a column unit. Multiple column units are interconnected in series and tightly mounted into the peripheral grooves of the holder. Both feed and return tubes are passed through the center hole at the top of the holder and then supported at a height of about 25 cm above the center of the apparatus. These tubes, protected with a piece of greased silicone rubber tubing at the holes, have not failed or stretched appreciably even after having been used many times. The radius of revolution is adjustable to 30.7, 20.2, 13.6, and 8.6 cm. The revolutional speed is continuously regulated up to 700 rpm at the 30.7 cm radius.

To operate the instrument, the column is filled with the stationary phase and a sample solution is introduced through the

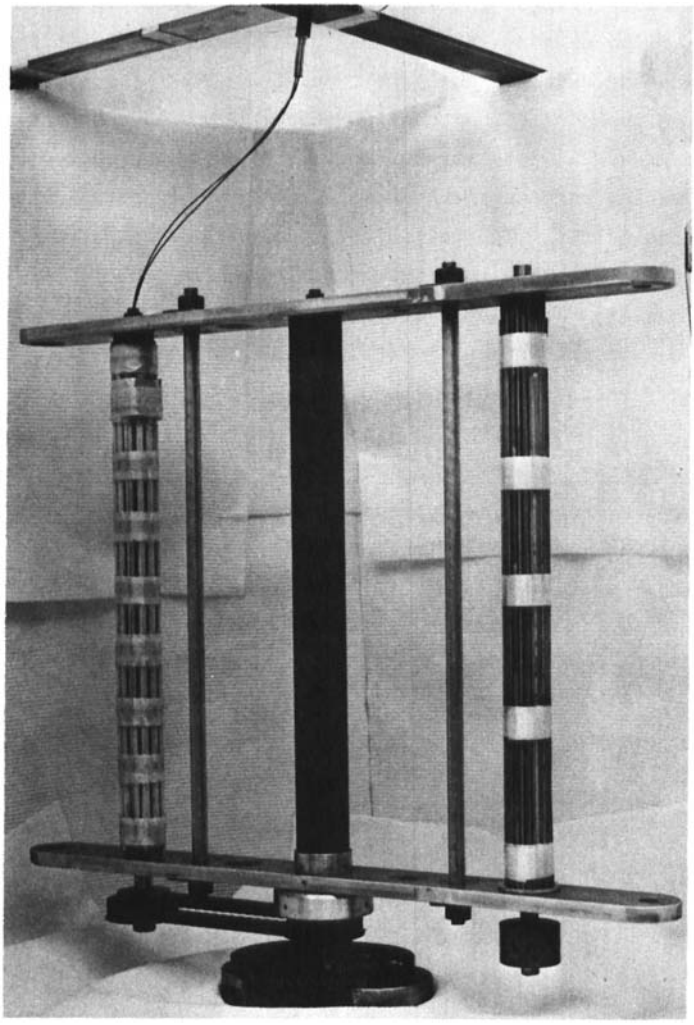

FIGURE 2
The flow-through coil planet centrifuge. The column unit is to the left. Note the coupling pulley at the bottom.

feed tube. After the desired revolutional speed is established, the mobile phase is pumped through the feed tube while the eluate is monitored with an LKB UV monitor at 280 nm. It should also be noted that it is not necessary to maintain a constant composition

for the mobile phase. In fact, a gradient in the composition of a third component can often be employed advantageously to improve resolution and decrease analysis time.

Three types of column configurations which have been used are shown in Figure 3. The straight helix column was prepared by winding tubing onto a rigid rod which was mounted parallel to the axis of the holder. The coiled helix column was prepared by winding a tube onto a flexible tube which was coiled around the holder at various angles. The flat coiled helix column was prepared by winding a tube onto a plastic strip which was coiled around the holder at various angles.

Factors affecting distribution of phases in coil

Efficient separation depends on satisfying two requirements for phase behavior in the equilibrated coil. The first requirement is to retain an adequate amount of the stationary phase in the column,

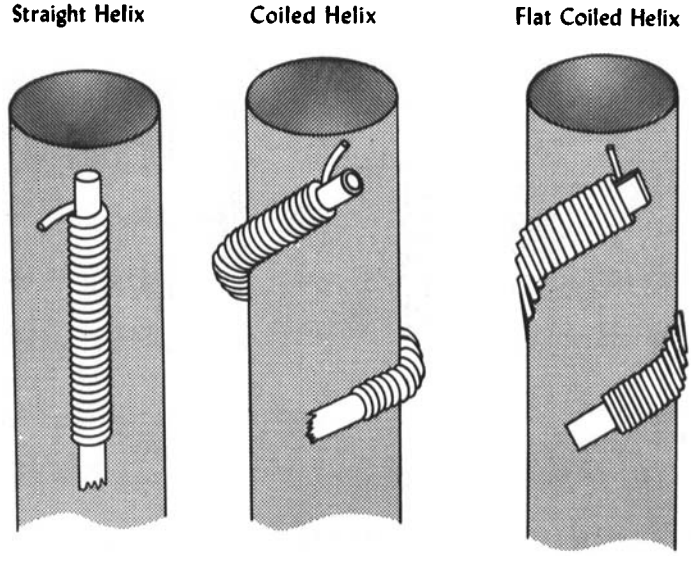

FIGURE 3
Three types of column configuration used with the flow-through coil planet centrifuge.

and the second requirement is to obtain segmentation of the phases adequate to produce a large number of partition units with high interfacial area. For a given solvent system, these requirements can usually be satisfied by choosing an appropriate combination of instrumental variables such as column configuration and inside diameter, revolutional speed, radius, flow rate, etc. The number of variables is considerable, and thus, in order to facilitate this choice it is usually necessary to resort to the phase distribution diagram. The phase distribution diagram describes the steady state relative volumes occupied by the two phases as a function of revolutional speed for a given set of other variables.

Figure 4 shows a phase distribution diagram of Hexane/water with a straight helix column.[4] The ordinate indicates the percentage of the column space occupied by the aqueous phase and the abscissa the applied revolutional speed in rpm at 30.7 cm radius. Two points are measured for each speed: light circles for the aqueous phase used as the mobile phase and black circles for the aqueous phase as the stationary phase. As the flow rate is decreased, these two points move closer together, and finally reduce to one point which represents the true equilibrium composition under no flow conditions. A pair of curves drawn through each point shows some common features among various phase systems examined. The upper curve starts to move down earlier and displays a sharp spike before it becomes nearly horizontal, whereas the lower curve starts to rise later and smoothly approaches the upper curve.

Thus, the phase diagram may be conveniently divided into three regions as shown in Figure 4: the "plug flow" region, the "spike" region, and the "equilibrium" region. The plug flow region is found at low speeds and is characterized by complete displacement of the stationary phase (plug flow). In the spike region, plug flow is induced only if the nonaqueous phase is used as the mobile phase. Although retention does occur in this region when the aqueous phase is the mobile phase separations are of low efficiency.

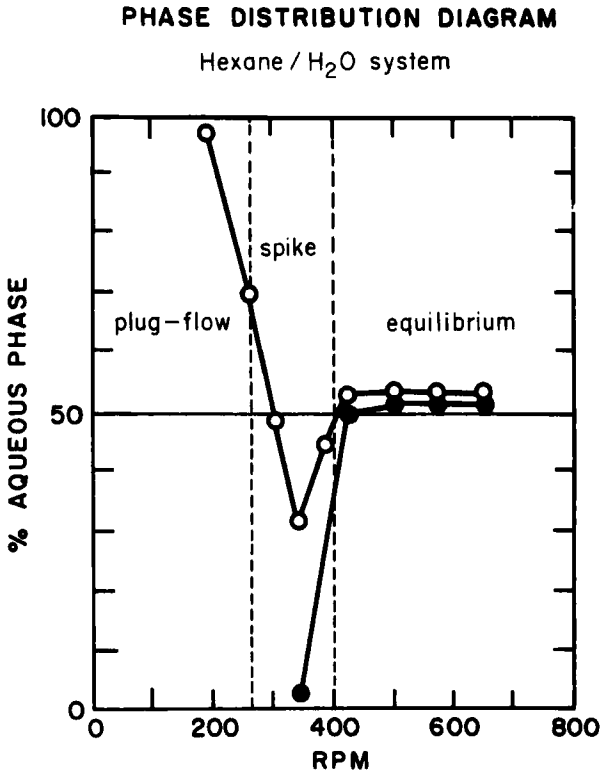

FIGURE 4
Phase distribution diagram of hexane/water phase system.

The relatively flat equilibrium region has been used most widely for separations. The phase distribution is relatively insensitive to speed variations, either phase can be used as mobile phase, and separations are of high efficiency.

The phase distribution pattern varies both with phase composition parameters and instrumental variables. Figure 5 shows the phase distribution diagrams for 9 phase systems in relation to the internal diameter of the straight helix column.[4] In each diagram, the flat equilibrium region close to the 50% line represents the optimal situation. Note that as the coil diameter decreases, the equilibrium region is shifted to higher speeds and

that for some solvent systems, the equilibrium region does not appear on the phase diagram. This factor is related to the interfacial tension which is the major solvent variable affecting the phase distribution. Some appreciation of the effect of interfacial tension can be gained from Figure 5. The high interfacial pairs such as hexane/water or ethyl acetate/water exhibit an equilibrium region at high speeds, while for low interfacial tension solvents, the equilibrium region appears at lower speeds. In some of these however, there is no true equilibrium region because of the formation of an emulsion. It can also be seen that addition of substances soluble in both phases tends to reduce interfacial tension while the addition of substances, such as salts, which are soluble in one phase only tends to increase it.

These factors combine to produce two technical difficulties. When high interfacial tension phase systems are used in fine bore columns, plug flow persists to higher speeds. This can be overcome to some extent by increasing the revolutional speed. It should be noted that the separation efficiency is inversely related to the tube diameter; thus for high resolution separations as small a diameter as possible is desirable. On the other hand, when low-interfacial tension phase systems are used with large bore columns, the phase diagram passes from plug flow to emulsification without going through a stable equilibrium region.

This is especially serious because low interfacial tension phase systems such as 2-butanol/water and 1-butanol/acetic acid/water systems have been widely used to separate biological macromolecules. However, this problem was solved by using the coiled helix column configuration at 60° angle as shown in Figure 6.

The three column configurations described above were employed with several aqueous/organic solvent systems. The columns are made from 0.85 mm i.d. teflon tubing, and the mobile phase is pumped at the rate of 12 ml/hr using a Harvard syringe pump. The radius of the centrifuge arm was set at 30.7 cm. As can be seen both coiled helix columns show excellent flat equilibrium regions close to the 50% line for low interfacial tension phase systems. Careful studies

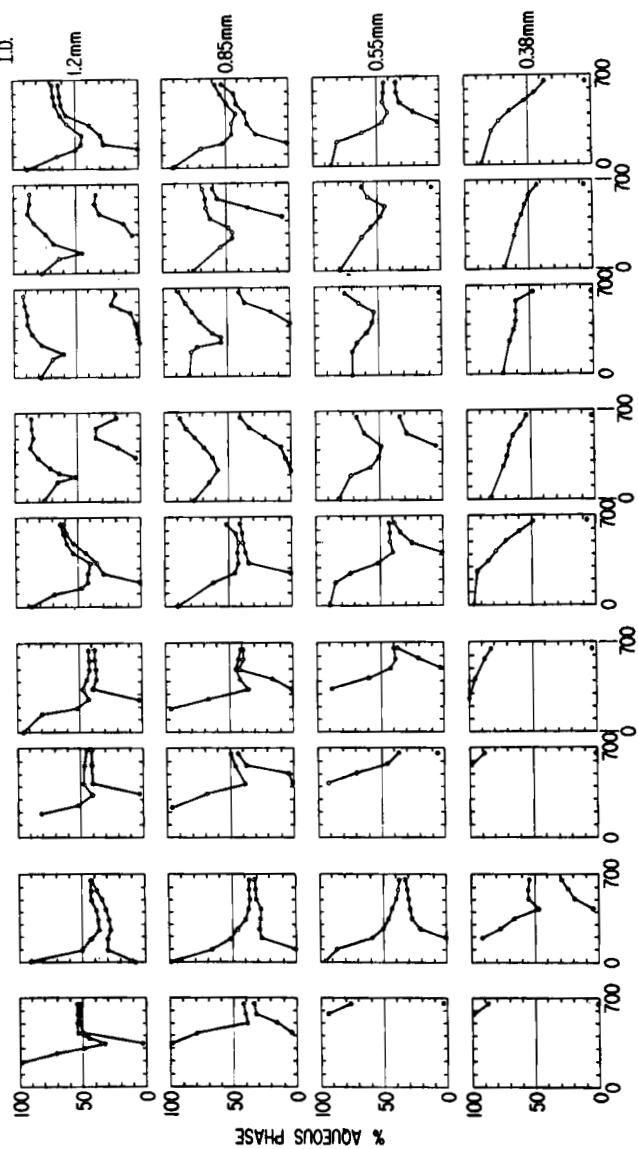

FIGURE 5. Phase distribution diagrams of nine phase systems as a function of the internal diameter of the column. The flat equilibrium region represents the optimal phase distribution for separations. The effect of the interfacial tension can be seen from the diagram. Hexane/water and ethyl acetate systems have a high interfacial tension and, hence, give plug flow in small bore tubing. The chloroform systems, which would be expected to behave similarly, show good phase retention due to the reduction of interfacial tension by the addition of acetic acid. Note the effect of the addition of acetic acid to the low interfacial tension butanol systems. Further reduction of the already low interfacial tension leads to emulsification. Note the improvement in phase retention in the 2-butanol systems effected by the addition of salt, which increases interfacial tension.

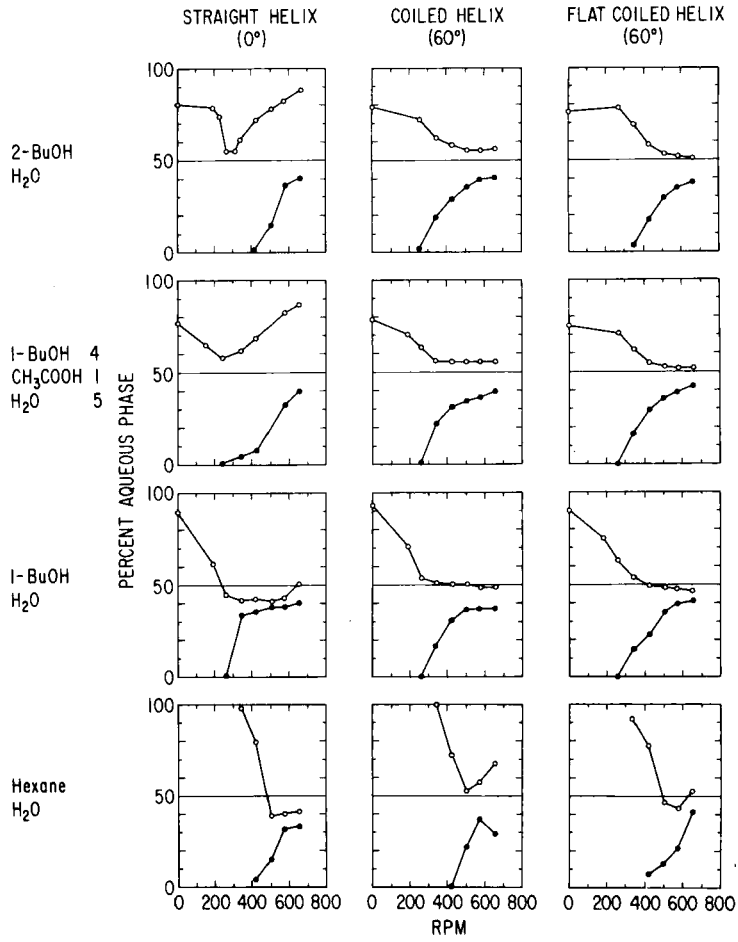

FIGURE 6
The effect of column configuration on phase retention. Note the improvement in phase retention shown for the top two phases in the coiled helix and flat coiled helix columns.

have shown the flat helix to be superior to the ordinary coiled helix for stabilization of the interface, however. Since these columns have very similar efficiency a flat coiled helix column was employed for low interfacial tension phase systems which tend to emulsify.

Factors affecting choice of phase systems

These factors have been considered at length elsewhere[4] and will be reviewed only briefly here. The major factors affecting the choice of phases have already been discussed above. An absolute requirement is retention of stationary phase. In general, retention of at least 30% is mandatory; thus, the solvent composition, speed, and tubing diameter must be chosen with this in mind. Although, the single most important physical property of the solvent pair is the interfacial tension, the viscosity also plays a role in that it is more difficult to obtain adequate retention for viscous phases. It also appears that the efficiency of separation decreases with viscosity. Resolution is the product of both efficiency and selectivity factors, and thus the latter must also be considered. Few rules can be given, except to observe that separations dependent primarily upon polarity differences are rarely as selective as those dependent upon some specific interaction, such as between dichloroacetic acid and the peptide bond.[6] It should also be noted that partition coefficients in the range 5.0 - 0.2 provide the best separations.

The distinction between selectivity and efficiency should be noted. Selectivity, which is measured by the relative separation of the peaks or band centers of two solutes, is governed by the difference in partition coefficient of the respective solutes. To a first approximation this reflects fundamental molecular properties and is insensitive to instrumental performance factors while efficiency, on the other hand, and is governed primarily by these instrumental performance factors. The two factors combined discribe the resolution which is measured by the overlap of two solute bands.

Applications

DNP Amino Acids:[1] A set of nine dinitrophenyl amino acids were separated using a two-phase solvent system composed of chloroform/acetic acid/0.1 N aqueous HCl (2:2:1V/V/V). The partition coefficients of the various DNP amino acids in this phase system varied from 100 to .18. Analytical and preparative

chromatograms are shown in Figure 7. In each case, the nine DNP amino acids are well resolved in order of decreasing partition coefficient. The efficiency of separation was estimated using a formula from gas chromatography, $N = (4R/w)^2$, where N is the number of theoretical plates (TP), R is the retention time referred to the peak maximum, and w is the peak width in the same units. By this measure, the analytical column achieved 10,000 to 3,000 TP for the various peaks, while the preparative column achieved 2,000 to 500 TP. The time required per transfer was 0.7 to 2.4 seconds for the analytical column and 3 to 12 seconds for the preparative column, compared to several minutes per transfer required for countercurrent distribution method. To assess the relative performance of countercurrent chromatography and countercurrent distribution, the following rough comparison can be made. If two solutes are barely resolved in a countercurrent chromatography separation of 100 theoretical plates, an equivalent separation by countercurrent distribution would require migration of the solute peaks through 100 tubes using the same solvent system.

Catecholamine Metabolites:[2] The analysis of catecholamine metabolites from urine is shown in Figure 8. The metabolites (3-methoxy-4-hydroxyphenyl)hydroxyacetic acid (VMA) and (3-methoxy-4-hydroxphenyl)-acetic acid (HVA) were well separated from each other. HVA was completely separated from other u.v. absorbing components in urine. VMA elutes close to several unknowns which, however, do not interfere with colorimetric assays for VMA. Characteristic profiles are obtained for various neurosecretory tumors, and the technique does not require preliminary concentration or purification of the urine sample.

Dipeptides:[4] A mixture of depeptides was separated using a 1-butanol/aqueous dichloroacetic acid solvent system with results as shown in Figure 9. Note the resolution of sequential isomers. As previously mentioned, it is possible to change the composition of the mobile phase during elution. A gradient in dichloroacetic acid concentration (0 -2% W/V) was employed to achieve the separation of the same dipeptides with results as shown in Figure 10.

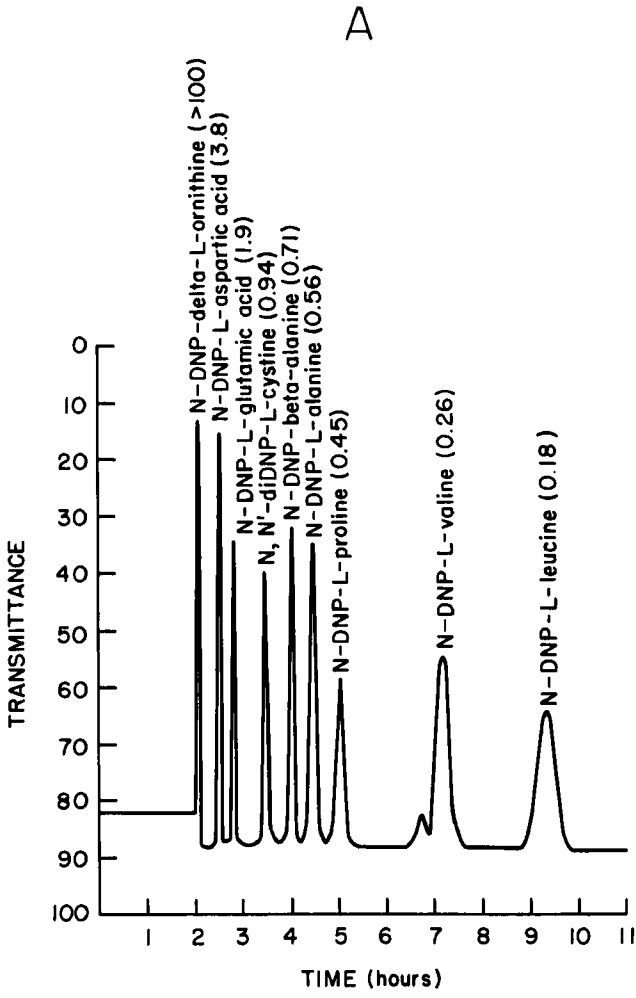

FIGURE 7

Separation of DNP amino acids. (A) Analytical column: straight helix wound from 100 meters of 0.30 mm i.d. Teflon tubing, helix diameter 5 mm, total capacity 8 ml. 10 µl sample contained about 1% (W/V) concentration of each component (where solubility permited), dissolved in upper phase of solvent system. Speed 550 RPM, radius 30.7 cm., elution with upper phase at 2.4 ml/hr. Phase system was chloroform/acetic acid/0.1N HCl (aqueous) (2:2:1 V/V/V). (B) Preparative column: straight helix wound from 100 meters of 1.4 mm i.d. Teflon tubing, helix diameter 1 cm, total capacity 140 ml. Sample: 100 ul of sample solution used in A, diluted with upper phase to 2 ml (sample was diluted to facilitate VV monitoring) speed 520 RPM, radius 8.6 cm, flow rate 60 ml/hr.

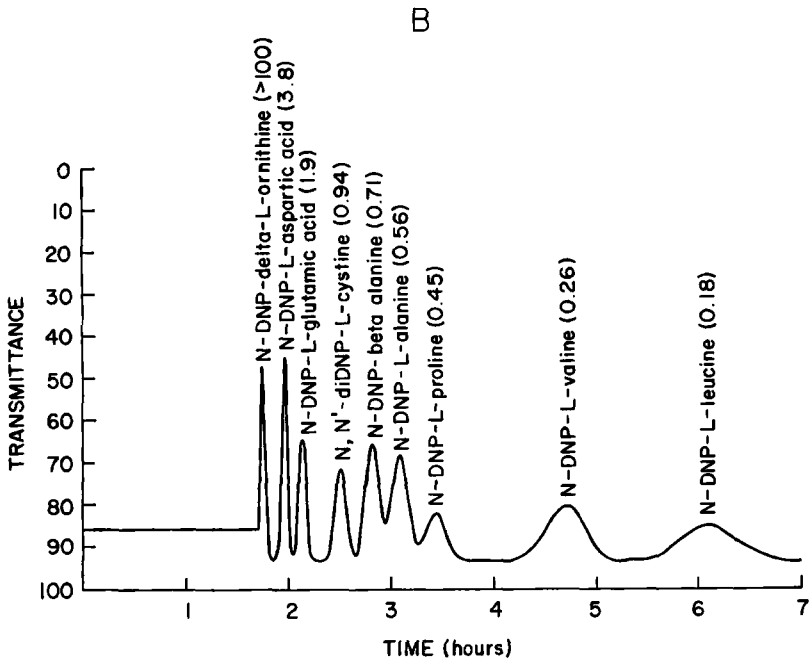

FIGURE 7. Continued.

Two features should be noted. Firstly, the analysis time has been reduced almost by a factor of three. Secondly, and equally importantly, the efficiency of the separation of the last peak has actually been increased by roughly the same factor. As a consequence, there is much less dilution of the later peaks. In fact, this phenomenon can be exploited to concentrate solutes if the solute has a high solubility in the initial stationary phase. This is illustrated in Figure 11 for the same dipeptides. Note that although the resolution of the early peaks is degraded, the resolution of the later peaks is unaffected by a 33-fold increase in sample size.

Insulin. Figure 12 compares the partition of bovine insulin in the 2-butanol/aqueous dichloroacetic acid system by countercurrent distribution and by countercurrent chromatography. The countercurrent distribution data reported by Harfenist and Craig[6] revealed a second component, identified as a deaminated insulin,

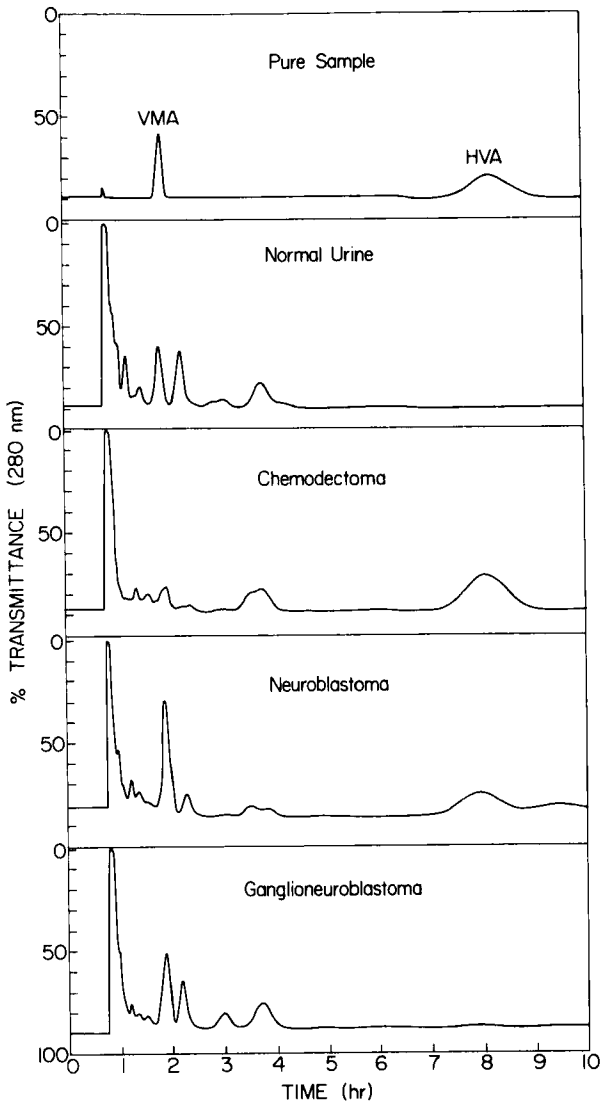

FIGURE 8

Analysis of catecholamine metabolites from urine. Phase system: ethyl acetate (5% W/V NaCl, 10% V/V Acetic acid, in H_2O) (1:1). Lower phase used as mobile phase. Straight helix column wound from 60 meters of 0.85 mm i.d. Teflon tubing. Sample: 0.9 ml of urine with 50 mg. of NaCl and 0.1 ml of glacial acetic acid added. Speed 720 RPM, radius 20.2 cm, flow rate 24 ml/hr.

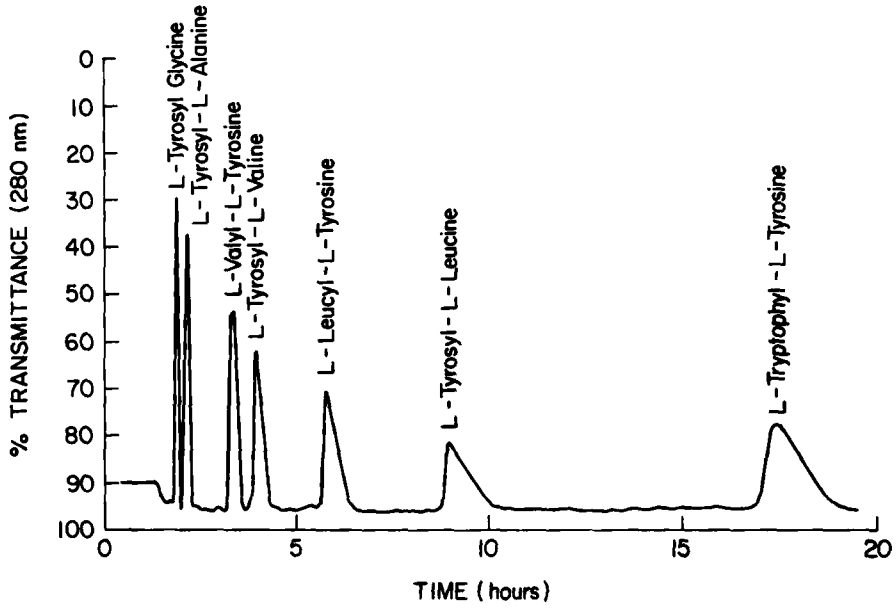

FIGURE 9
Separation of dipeptides by plain elution. Solvent system: 1-butanol/1% (W/V) aqueous dichloroacetic acid (1:1). Straight helix column wound from 140 meters of 0.55 mm bore tubing. Speed 720 RPM, 20.2 cm. radius, 0.3 ml. sample, flow rate 12 ml/hr.

which was not separable by either electrophoresis or ultracentrifugation at that time. Countercurrent chromatography shows a similar result.

Because of the low interfacial tension of this phase system, it was necessary to use a flat coiled helix column rather than the usual straight helix. The column was a flat coiled helix column wound from 90 meters of .55 mm i.d. Teflon tubing, using the phase system: 2-butanol/dichloroacetic acid/water (100:3:100 V/W/V). A sample of 0.5 ml of 1% solution of bovine insulin in lower phase was separated at a speed of 700 rpm at 30.7 cm radius and a flow rate of 6 ml/hr.

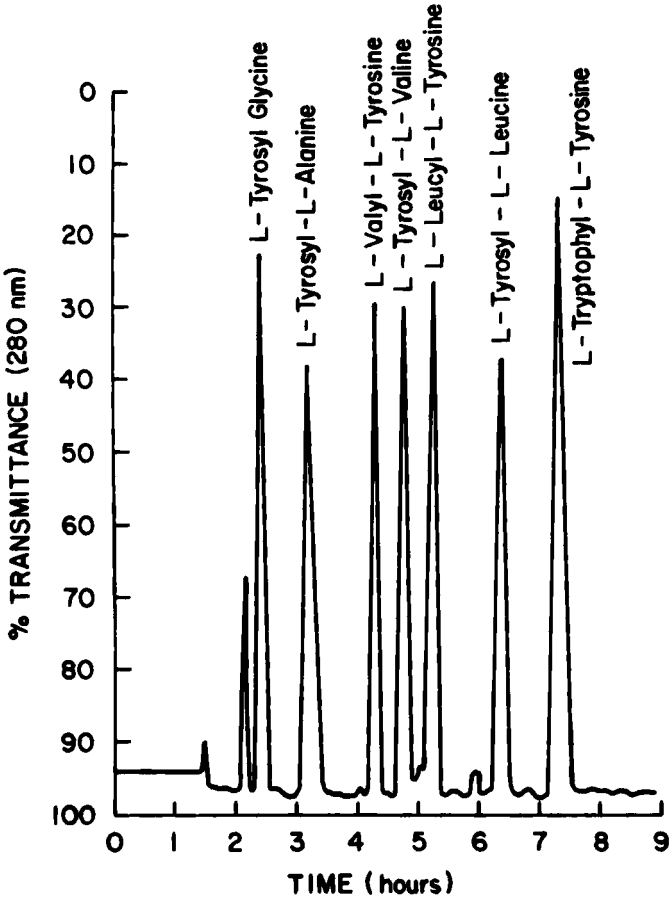

FIGURE 10
Separation of dipeptides by gradient elution. Gradient elution: solvent system: 1-butanol/0.1 M aqueous ammonium formate, gradient in dichloroacetic acid concentration from 1% (W/V) to 0%, with lower phase used as mobile phase. Straight helix column wound from 70 meters of 0.55 mm. i.d. tubing. Speed 720 RPM, 20.2 cm. radius, 0.3 ml sample, flow rate 6 ml/hr.

THE ELUTION CENTRIFUGE

Principles of operation

In addition to the "flow-through coil planet centrifuge," a second apparatus employing similar principles, the "elution cen-

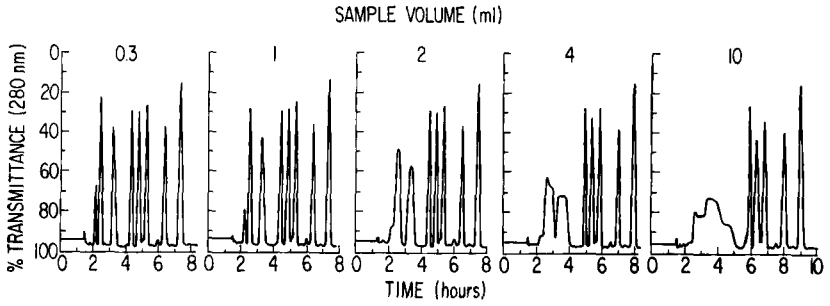

FIGURE 11
Effect of sample volume on gradient elution separation of dipeptides. To demonstrate the solute-concentrating capabilities of the gradient elution technique, the sample was diluted with mobile phase before loading. The earlier peaks become affected at 2 ml. However, efficiency of separation of later peaks is not degraded even at 10 ml, where the solute concentration at the peak maximum is estimated to be 10 times as high as that in the applied sample solution.

trifuge," has also been constructed.[3] A model similar to that used to describe the coil planet centrifuge can be used to describe its operation.

The basic model consists of a horizontal coil as illustrated in Figure 13. This model differs from the previous one in two major details: the coil is not rotated with respect to the gravitational field, and the external flow is responsible for producing segmentation. In (a), the coil is filled with one of the two phases and the second is introduced as shown in (b). Now, if the flow is sufficiently low, the first phase will not simply be displaced. Because the flow displaces the heavier phase from its equilibrium position at the bottom of the coil unit, a net force is generated which prevents its displacement over the potential energy barrier represented by the top of the coil unit. Thus the net result of continued pumping is to produce a net flow of the second phase through the coil and past retained segments of the first phase. Solutes introduced into either phase will undergo separation, according to their partition coefficients, by partition between the stationary phase segments and the mobile phase which flows past it.

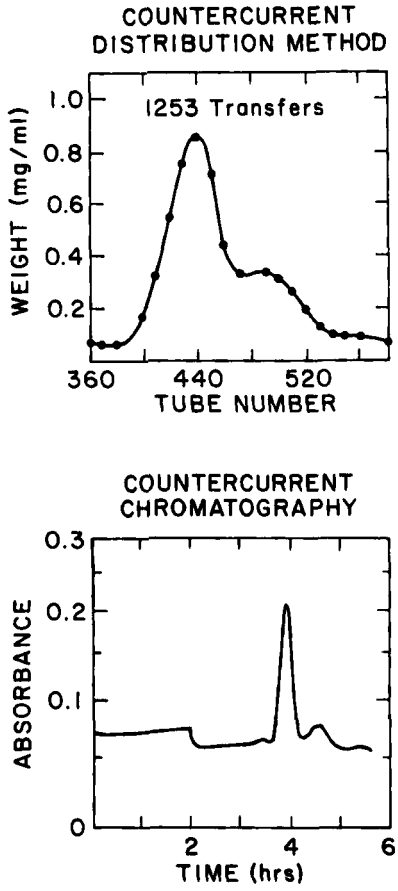

FIGURE 12

Partition of Bovine insulin-comparison with countercurrent distribution.[6] Countercurrent chromatography using flat coiled helix column wound from 90 meters of .55 mm i.d. Teflon tubing. Phase system: 2-butanol/dichloroacteic acid/water (1:0.03:1 V/W/V). Sample: 0.5 ml of 1% (W/V) solution of bovine insulin in lower phase, speed 700 RPM, 30.7 cm radius, flow rate 6 ml/hr.

Principles of construction

The above system is not practical for separation unless the number of coil units is large and the tube diameter small. In order to stabilize such a system, however, the acceleration field must be increased, and the "elution centrifuge" was developed for this purpose.

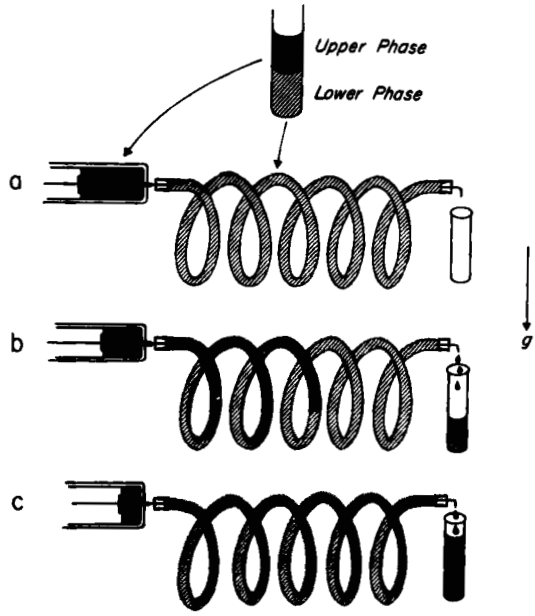

FIGURE 13
Stationary coil model for the elution centrifuge. (A) The coil is filled with stationary phase and mobile phase is introduced. (B) The mobile phase flows around the stationary phase is reached where only mobile phase is eluted. Solutes are partitioned between mobile phase and stationary phase segments.

The principle of the elution centrifuge is illustrated in Figure 14. A cylindrical holder containing the separation column is held in a horizontal position. Both feed and return tubes are led through the center of the holder and then are supported tightly at the center of the apparatus by a guide tube fixed to the top frame of the centrifuge. The holder revolves around the central axis of the apparatus in the horizontal plane and simultaneously rotates about its own axis at the same angular velocity in the indicated direction. This synchronous rotation unwinds the twist of the lead tubes caused by revolution, thus eliminating the need for rotating seals.

The synchronous rotation also adds a peculiar effect to the centrifugal force field at the column holder. Simple mathematical

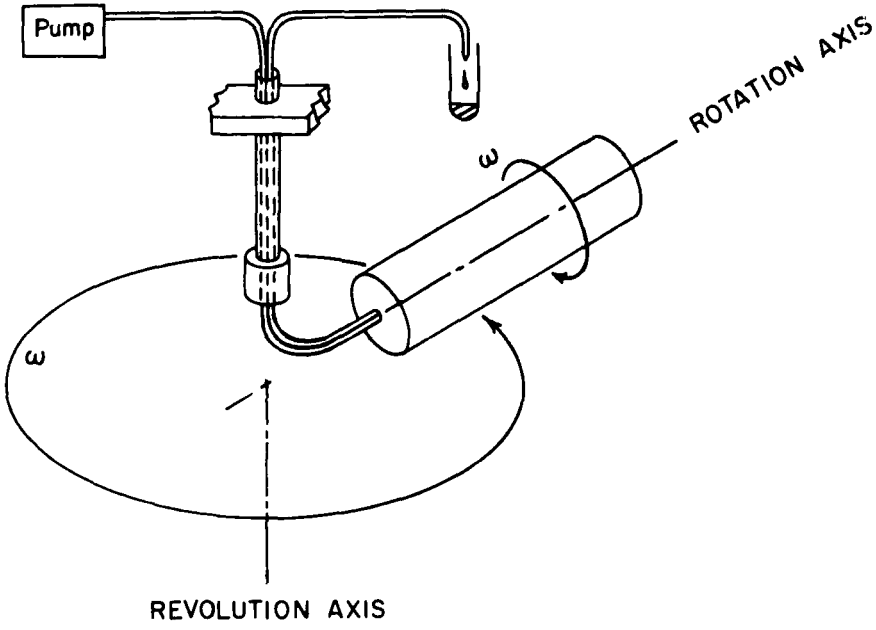

FIGURE 14
Principle of the elution centrifuge. A cylindrical holder containing the separation column is held in a horizontal position. Feed and return tubes pass through a guide tube from the center of the apparatus into the center of the holder. The holder revolves in the horizontal plane and about its own axis at the same angular velocity. This synchronous rotation unwinds the twist of the lead tubes caused by rotation of the centrifuge arm.

analysis[3] shows that any arbitrary points in the column holder except for those located on the axis of rotation are subjected to a periodic fluctuation of the centrifugal force. Under some conditions, the gravitational vector can even change sign and point toward the axis of the centrifuge. This rather startling result is produced whenever the radius of the coil holder is greater than or equal to one-half the radius of the centrifuge arm. For smaller ratios, the fluctuation of the centrifugal force vector does not reverse direction and diminishes in magnitude gradually. This has generally served to limit the radius of the coil holder to values less than one-tenth the centrifuge arm.

Apparatus

An apparatus to test these principles was constructed as shown in Figure 15 by modifying a conventional centrifuge. The centrifuge head was redesigned as a rectangular aluminum box with a center hole through the bottom plate to fit the motor shaft as shown in A. The two septa are placed to support the column holder and the counterweight horizontally. The rotation of the holder is accomplished as illustrated in B by applying a pair of pulleys of the same size, one fixed to the holder and the other to the motor housing at the center of the apparatus. These pulleys are coupled by a timing belt which passes over a pair of idle pulleys mounted on the bottom plate of the centrifuge head.

Again, the geometry of the column is important. Optimal column configuration were determined for both conventional and aqueous two-phase polymer systems developed by Albertsson using the three column configurations illustrated in Figure 16. The straight helix column was prepared by winding a tube onto a rigid rod and mounting it parallel to the axis of the holder. The twisted pair column was prepared by folding a tube in two and twisting along its length to make a rope-like configuration. The twisted tube is then wound around the column holder. The coiled helix column was prepared by winding a tube onto a flexible tube which is coiled tightly around the holder. Preliminary studies have indicated that the twisted pair gives the highest efficiency for the conventional phase systems while the coiled helix column is best for the polymer phase systems.

Applications

DNP Amino Acids. Using a twisted pair column consisting of 20,000 turns of Teflon tubing, .2mm x 60m, nine dinitrophenyl amino acids were separated with a phase system composed of chloroform/acetic acid/0.1 N HCL (2:2:1 V/V/V). As shown in Figure 17, all components were eluted out within 26 hours at efficiencies ranging from 6,000 to 2,500 theoretical plates. These results should be compared to those illustrated in Figure 7.

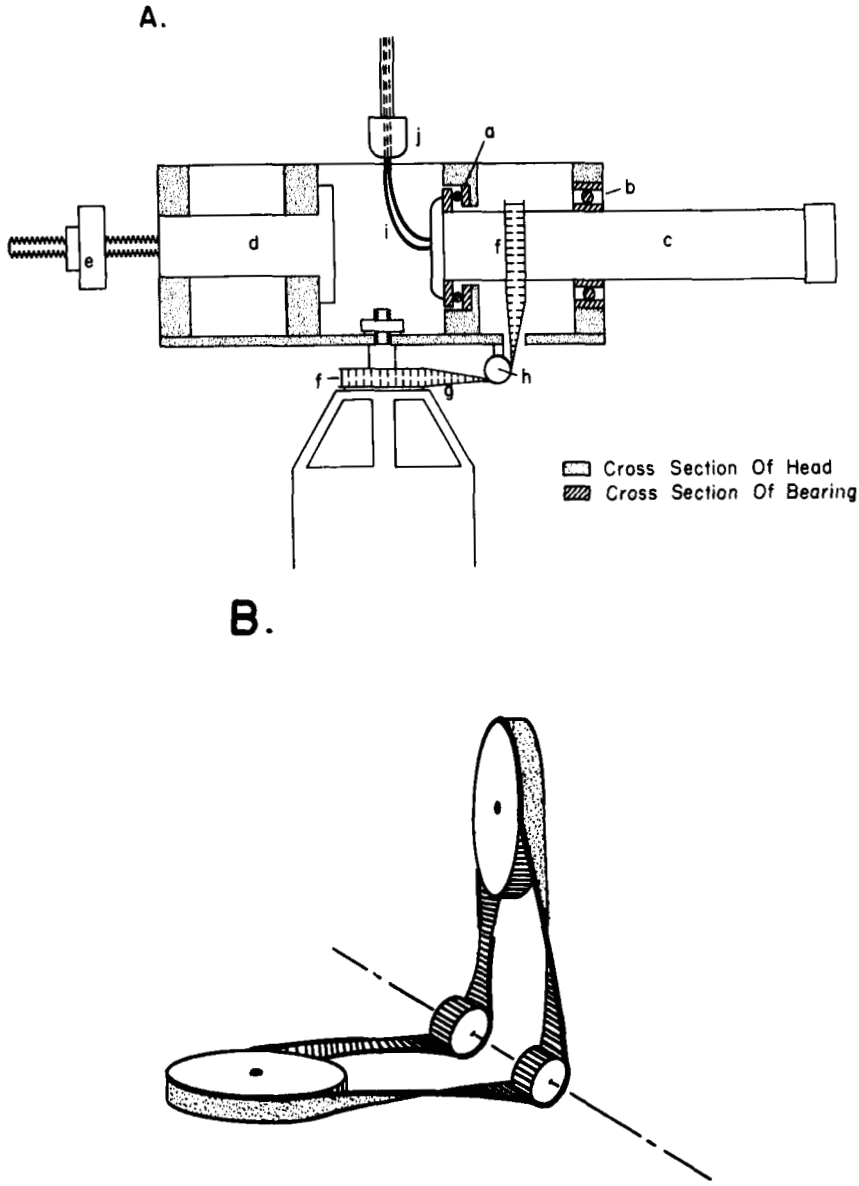

FIGURE 15

Design of elution centrifuge: (A) Column holder (c) horizontally supported by thrust bearing (a) and ordinary ball bearing (b) is counterbalanced by aluminum block (d) equipped with adjusting screw (e). A pair of toothed pulleys (f), one fixed to column holder and the other to motor housing at center, are coupled with toothed belt (g) through a pair of idle pulleys (h). Lead tubes (i) are supported by stationary guide pipe (j) projected down from top frame of centrifuge. (B) Belt scheme.

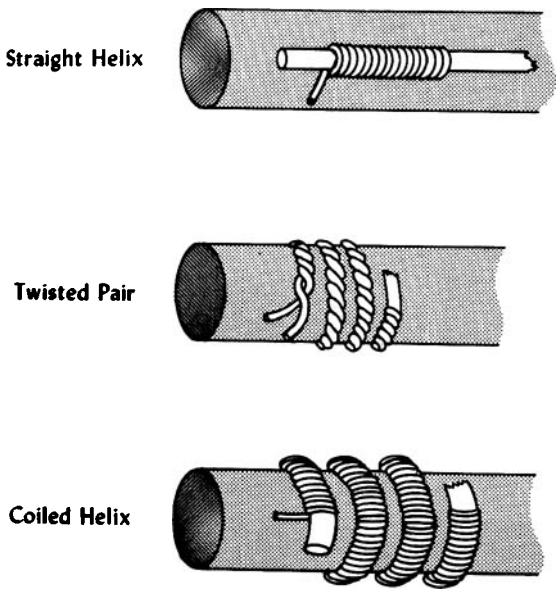

FIGURE 16
Three types of column configuration used with the elution centrifuge.

Polynucleotides. The elution centrifuge has been used with aqueous/aqueous polymer phase systems to partition polynucleotides. The phase system, developed by Albertsson,[7] consists of 5% Dextran T500 (Pharmacia), 4% polyethylene glycol 6000 (Union Carbide), and 10 mM Sodium phosphate of varing pH.

The three types of columns were tested for stationary phase retention and partition efficiency. All columns retained a sufficient amount of lower phase at speeds above 1000 rpm as shown in Figure 18A. The efficiency of these column configurations was investigated with the step-wise elution of polyuracil. Thus was accomplished by a discontinuous change in mobile phase to a higher pH. As shown in Figure 18B, the coiled helix column gave a much sharper peak than the other two columns. Note that phase retention is less dependent upon external conditions than for the coil-planet centrifuge.

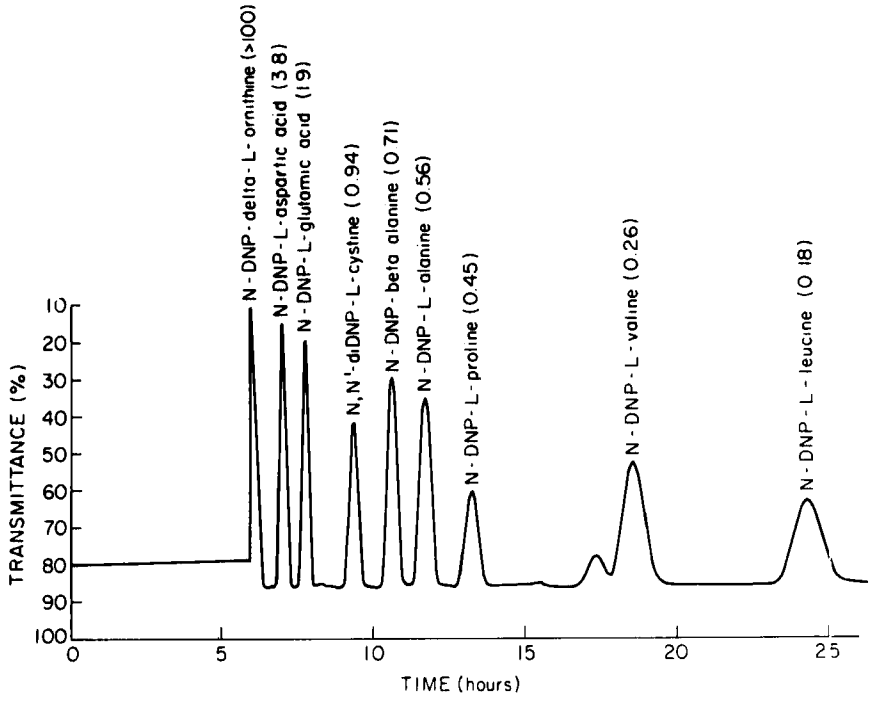

FIGURE 17
Separation of DNP amino acids using the elution centrifuge. Column: twisted pair of 20,000 turns of 0.2 mm i.d. 60 m long. Phase system: same as for Figure 7.

Using the coiled helix column, both stepwise and gradient elutions of Poly U, Poly C, Poly A, and Poly I were carried out with results as shown in Figure 19. The peaks from the different polynucleotides can be clearly resolved using either stepwise or gradient elution techniques.

DISCUSSION

As mentioned earlier, countercurrent chromatography, in part, combines the selectivity and versatility in choice of phases characteristic of column chromatography. Although separations of small molecules can be performed very well with this technique, it is still slower in many respects than high-speed liquid chromatography. For solutes which can be separated by the latter tech-

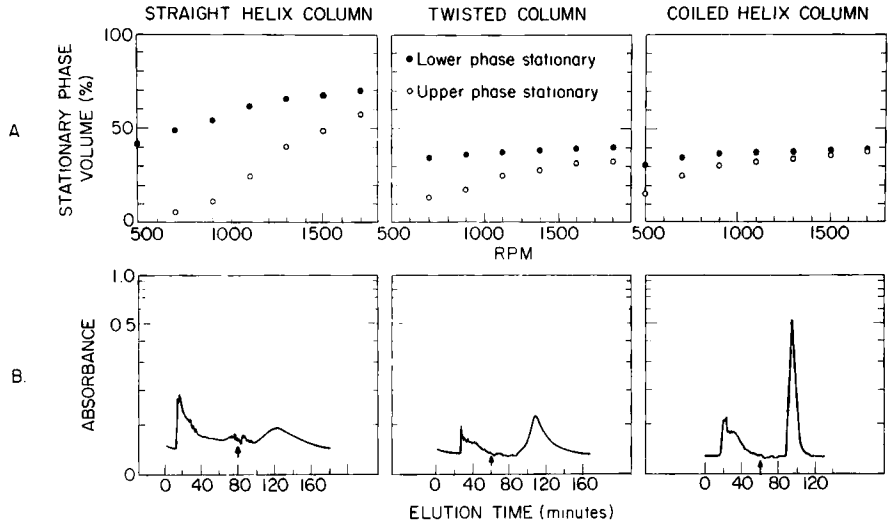

FIGURE 18

Performance of three types of separation column. (A) Retention of the stationary phase. All three columns show sufficient retention of over 30% above 1000 RPM, if the lower phase is stationary. (B) Column efficiency examined on stepwise elution. The first elution eliminates impurities while the second elution, indicated by arrows, elutes the poly U sample. Sharpness of the peak obtained by the coiled helix column indicates the highest efficiency.

nique, countercurrent chromatography will probably not be the method of choice. However, it should be noted that the analysis times of the DNP-amino acids reported in Figures 7 and 10 compare favorably with those afforded by amino acid analyzers. Thus countercurrent chromatography is comparable, insofar as analysis time is concerned, with ion-exchange techniques. For highly polar molecules, then, countercurrent chromatography at least offers an alternative.

With the exception of sample size, countercurrent chromatography compares most favorably with countercurrent distribution, and can provide comparable separations at 10 - 100 times the speed. In fact, almost any separation possible with countercurrent distribution should be feasible with countercurrent chromatography

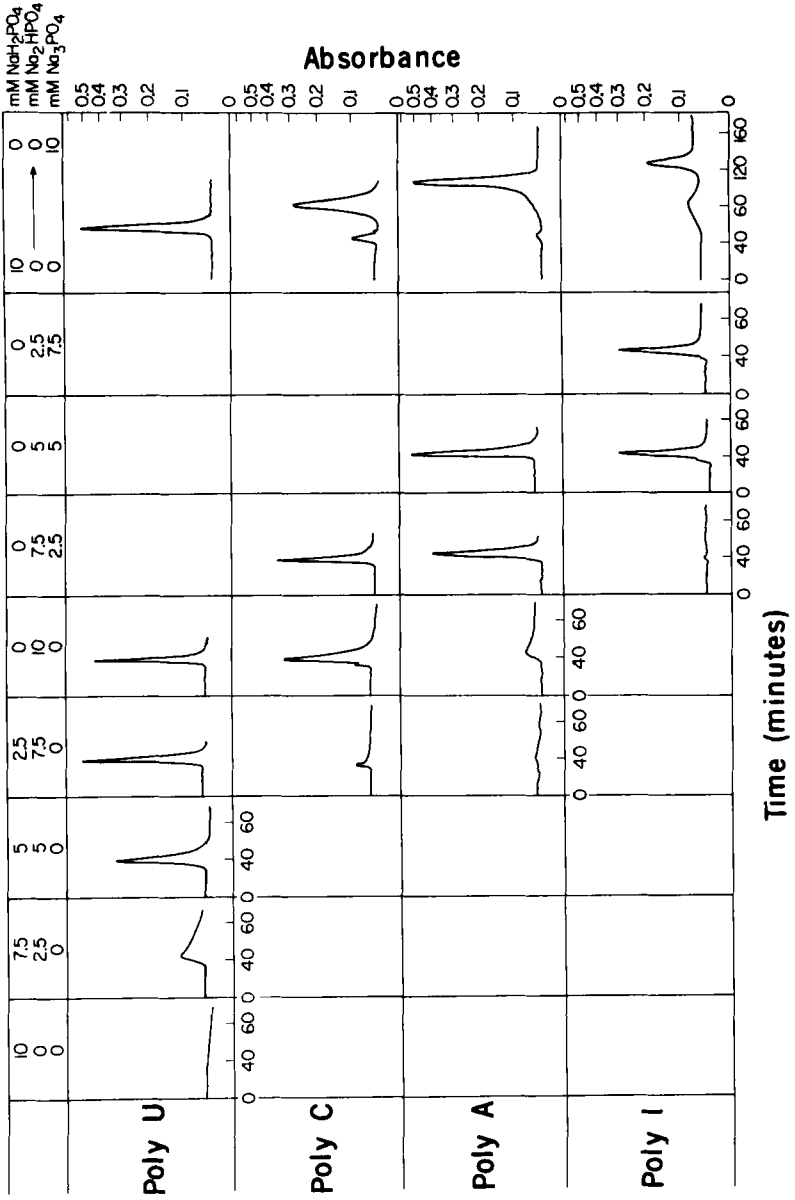

FIGURE 19. Countercurrent chromatograms fo polynucleotides with stepwise (left) and gradient (right) elution, using the phase systems composed of 5% (w/w) dextran T 500, 4% (w/w) polyethylene glycol 6000, and 10 mm sodium phosphate of the indicated compositions.

usually with the same solvent system. Additionally, utilization of a gradient elution scheme can be employed to reduce analysis times, increase resolution, expand greatly the range of partition coefficients over which high efficiency separations may be obtained, and even to concentrate certain solutes during the separation procedure.

It is with separations involving biological macromolecules, subcellular organelles, and intact cells that the method seems to offer the greatest promise. The above-mentioned separation of insulin demonstrates the potential for proteins. The dipeptide separation also suggests a further application in "fingerprinting" protein digests. Preliminary results also indicate that glycosaminoglycans can be separated with high resolution with a phase system consisting of 1-butanol/1% W/V aqueous hexadecylpyridinium chloride and a sodium chloride gradient. By making liquid-liquid methods for the high resolution separation of macromolecules possible, countercurrent chromatography offers at the very least a useful alternative to such techniques as electrophoresis and ion-exchange chromatography.

Although the separations using the Albertsson[7] two-phase polymer systems have been amply demonstrated, the lack of a technique for suitably exploiting small differences in partition coefficient has limited such studies to separations involving large differences in partition coefficient. The demonstration of the feasibility of using these phases in the elution centrifuge should permit high resolution separations of cells and subcellular organelles. In the case of cells, it has been shown that the partition coefficient reflects the membrane associated charge deep within the membrane.[8] The difference in aggultinability of normal and malignant cells has been attributed to membrane charge differences,[9] thus indicating a potential area of application. These phase systems can also be applied to the separation of subcellular organelles such as lysosomes. Appropriate high resolution separations of such particles could aid greatly in studies of the lysosomal storage disorders[10] (the mucopoly-

saccharidoses and glycolipidoses) in which abnoraml amounts of one or more metabolites are stored in the lysosome and for which existing separatory techniques are inadequate.

Future studies such as these should be faciliated by current instrumental developments. Each of the two schemes of counter-current chromatography has its own specific advantages as well as disadvantages. The flow-through coil planet centrifuge technique yeilds a high efficiency with a short separation time, but it requires a knowledge of the phase distribution pattern to determine the proper column configuration and operating conditions, which can limit the choice of phases. On the other hand, the elution centrifuge technique requires a longer separation time at somewhat lower efficiencies but the system provides a more stable acceleration field so that the choice of the phase system is unlimited. To combine the virtues of both instruments, a modified flow through coil planet centrifuge has been constructed in which the column can be tilted at 10^{o}, 20^{o}, or 30^{o} with respect to the vertical. Preliminary results indicate that stationary phase retention is adequate even for the polymer phase systems. Tests of partition efficiency are in progress.

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