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TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY IN THE AFFINITY PARTITIONING OF NICOTINIC CHOLINERGIC RECEPTOR ENRICHED MEMBRANES

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ABSTRACT

A variation on the aqueous polymer phase partition method, affinity partitioning, has proved suitable for the preparative scale purification of binding site enriched membrane fragments. The full resolving potential of the affinity partitioning technique often requires the utilization of multiple extraction procedures such as countercurrent distribution. In this report, we evaluate the combination of a newly developed countercurrent purification technique, toroidal coil chromatography, with affinity partitioning. This approach provides an efficient method for purification and characterization of membrane bound nicotinic cholinergic receptors. The relative merits of the toroidal coil chromatography technology and the more conventional thin-layer countercurrent distribution techniques are compared.

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INTRODUCTION

Affinity chromatography, which relies upon the bio-specific binding properties of enzymes, receptors and other binding proteins to accomplish separation, has proven highly useful in the purification of soluble proteins or membrane bound proteins, once they are solubilized by the action of detergents. On the other hand, the application of affinity chromatography to the fractionation of intact cells and cell particles has met with several difficulties including problems in achieving specific retention onto affinity matrices and the converse difficulty of eluting bound cells or membrane fractions without the use of denaturing conditions. These problems appear to be due to the inherent limitations of the solid affinity matrices. Affinity partitioning (1-4) is a promising new affinity separation technique based upon the phase partition method for purification of biological particles in aqueous polymer phase systems (5), thus avoiding the problems associated with solid affinity matrices.

Aqueous polymer two-phase systems provide a gentle non-denaturing milieu for cells, subcellular particles and enzymes; near complete recoveries of biological activities are routinely achieved after distribution of heterogeneous biological material among the two aqueous phases and the interface between them. The phase systems are formed upon mixing solutions of water-soluble polymers, e.g. poly(ethylene oxide) [poly(EtO)] and dextran, a polymer pair most frequently used for such separations.

Several physical chemical parameters of proteins and cell particles are known to correlate with and, therefore, presumably influence their distribution among the phases and the interface. In the case of subcellular particles, these parameters include a particle's charge density (6), lipid composition (7), and tendency to interact with the constitutent polymers of the phase system (8). The latter parameter may be turned to advantage by synthesizing ligand-polymers that specifically interact with biological

particles containing biospecific binding sites. The two-phase systems can then be used to purify membranes enriched in specific binding sites in much the same way that affinity chromatography has been utilized to purify soluble proteins.

Typically, the specific ligand is covalently attached to one of the polymers that make up the phases, e.g to the ends of the linear poly(EtO) molecule. This variation on the phase partition method, designated affinity partitioning, has proved useful in the purification and characterization of Torpedo electroplax membranes enriched in nicotinic cholinergic receptor sites (3,4). In affinity partitioning, binding of a ligand-polymer to membrane fragments possessing specific binding sites influences their distribution to a degree that varies directly with the binding density per surface area. Thus, subcellular particles, which are often intrinsically heterogeneous, may be purified on the basis of their specific binding site content.

In purifying receptor enriched membranes, affinity partitioning has advantages over affinity chromatography where often, biological particles may be bound irreversibly to the affinity matrix. This is likely due to the multivalent character of an affinity matrix bead, which usually contains multiple covalently bound ligands arrayed such that simultaneous binding of matrix ligands to multiple cell surface sites occurs. In affinity partitioning, binding of ligand-poly(EtO) is essentially non-cooperative and ligand-polymers are generally reversibly bound and readily removed by centrifugation (4). The virtues of the affinity partitioning technique, when applied to the purification of subcellular particles, are similar to those of affinity chromatography when applied to the purification of soluble proteins: the binding of ligand-polymers provides specificity for the separation technique.

With both conventional and affinity aqueous phase partitioning techniques, purification is based upon differential distributions of the constituents in a mixture among the three

compartments of the two phases, i.e., the poly(EtO)-rich phase, the dextran-rich phase and the interface. If the constituents differ greatly in their distribution, then substantial purification may be achieved by performing a single or a few extractions, which are easily performed manually; but more often, the full separatory potential of the phase partition method may only be approached by performing multiple extractions using the countercurrent distribution (CCD) technique (5). Usually, adequate separation requires thirty or more transfers, which are best performed with an automated CCD apparatus.

The typical CCD apparatus designed for the purification of antibiotics or peptides in organic-aqueous phase systems is less suitable for aqueous polymer phase systems. This is because the settling times for aqueous polymer phase systems are substantially longer than for aqueous-organic phase systems. Since the settling time varies directly with the thickness of the phase layers, a useful approach for decreasing the phase settling time is to utilize thin phase layers with a proportionally greater cross-sectional area. Albertsson introduced a thin-layer CCD apparatus (9) that incorporates the required features in the form of two circular Plexiglas plates. As discussed below, the thin-layer CCD technology may have substantial limitations when applied to the affinity partitioning of membrane fractions.

A radically different approach was first developed (10) to increase the effectiveness of fractionation based upon distribution between aqueous-organic phase systems. A helically wound tube is located circumferentially on a spinning disc. The radial acceleration field holds the heavier phase stationary in the outer part of the coil, while the lighter phase is pumped through the stationary phase such that mixing occurs. A sample injected with the mobile phase travels through the coils at a rate dependent upon its distribution between the phases. This process, Toroidal Coil Chromatography (TCC), is analogous to a continuous form of

liquid-liquid chromatography, but without the associated disadvantages of a solid support. Alternatively, it can be considered as a continuous form of CCD as opposed to a discrete one.

The application of TCC to separations using polymer phase systems has so far been limited to gradient techniques. The initial success of these studies suggested that the TCC technology might offer substantial advantages for the purification of membranes fragments or subcellular particles when compared with the thin-layer CCD technique, both in terms of speed and efficiency of the separation and the avoidance of nonspecific interactions of particles with the Plexiglas surface. This paper describes the application of the TCC technique to the separation of subcellular particles containing nicotinic cholinergic receptors prepared from the electroplax of Torpedo californica, and provides a direct comparison with the results obtained using conventional CCD technology.

METHODS

Affinity Partitioning of Torpedo Membranes

Membranes enriched in nicotinic cholinergic receptor from Torpedo californica electric organ were partially purified by sucrose density gradient centrifugation (11) with the initial homogenization media containing EDTA and phenylmethylsulfonyl fluoride as described previously (4). For all experiments, membranes were analyzed using the nicotinic cholinergic ligand-polymer, hexaethonium-poly(ethylene oxide), [Et_3N-Me_6-NEt_2-poly(EtO)]. Single phase system extractions, countercurrent distribution, and membrane prelabeling with ^{125}I-α-bungarotoxin were performed as described previously (4).

Toroidal Coil Centrifuge Configuration

The toroidal coil rotor was constructed on a principle which eliminates the use of rotating seals as described by Ito and

Bowman (10,12). The coil was prepared by winding 18-gauge PTFE tubing (i.d. 1.07 mm) around a 5 mm diameter cylindrical nylon core. The core was formed into a 36 cm diameter torus and connected to the sample loop and a fraction collector, as shown in Figure 1. The dead volume from the end of the sample loop until the beginning of the coil was 0.4 ml. The sample coil contained a total volume of 8.5 ml, and the output dead volume was 0.7 ml. The toroidal coil rotor was mounted in a PR-2 International centrifuge located in a cold room (3.5°C). The centrifuge refrigeration system was set to maintain the chamber air temperature to within 0.5°C of the cold room temperature. In initial experiments, the cycling of the centrifuge refrigeration system was monitored, using a thermistor placed within the centrifuge chamber. The period of the refrigeration system cycle was 11 min with a 0.3°C variation in chamber air temperature.

Phase systems for eluting the TCC were prepared with an LKB Ultrograd gradient maker using a control valve (Fig. 1). This arrangement allowed variation of phase system ligand-polymer composition using an additional valve not shown and the inclusion of controlled amounts of dextran-rich bottom phase emulsified in the poly(EtO)-rich top phase. The mixing device on the LKB Ultrograd produced a fine emulsion containing the ratio of bottom phase suspended in top phase indicated in the Figure Legends.

Sample Loading and Analysis of Results

Operation of the TCC was begun by filling the coils at 1 x g with bottom phase. The rotor was then brought up to 1,200 rpm, and pumping of the emulsion commenced at a flow rate of 14 ml/hr. Prior to connecting the gradient maker output to the TCC, the LKB Ultrograd system was initiated until a uniform flow composition was achieved.

Membranes (0.36 mg protein containing 460 pmol receptor and prelabeled with ^{125}I-αBgt 45nCi, 0.22 pmol) were added to a volume

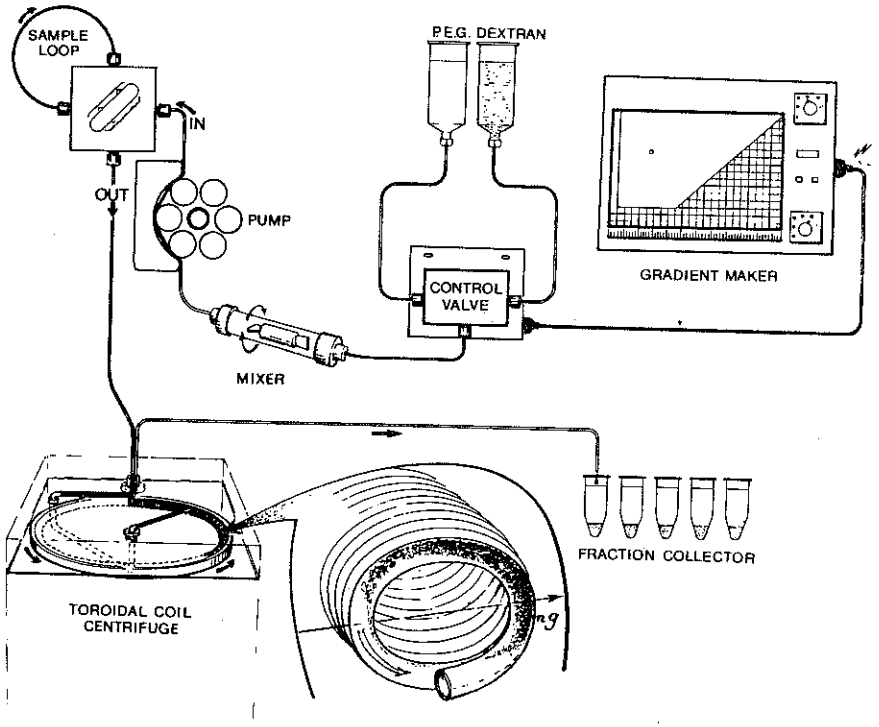

FIGURE 1

Toroidal Coil Chromatography Operating System

Schematic layout of operation system showing the method for generating an emulsion of the bottom (dextran enriched) phase in the upper (poly(EtO) enriched) phase. The enlargement of a single coil unit illustrates the retention of bottom phase in the outer segment of the coil, while the top phase percolates through it.

of the emulsion prepared by removing 20% of the water by evaporation under a stream of N_2 yielding the identical polymer composition but a sodium phosphate concentration at 2 mM higher than the phase system. The sample was suspended in the emulsion (0.5 ml) and loaded into the sample loop. The ratios of top phase to bottom phase volumes (V_r) were analyzed by measuring the height of the total phase volume and the interface in each of the frac-

tions (70 drops or 1.33 ml/fraction), which were collected in 1.5 ml plastic centrifuge tubes (Sarstedt No. 72.696). The relationship between the heights and the phase volumes was calibrated, and the conversions made to determine the phase volumes.

^{125}I-αBgt labeled membranes were counted in a Biogamma II counter for 5 min. Protein analysis of the fractions was performed by a modification of the Bradford (13) protein assay method. To minimize the degree of precipitation of membranes and phase components, 600 µl aliquots of effluent from the TCC were preincubated with 200 µl 0.5 M H_3PO_4 at 50° for 3 hr prior to addition of 530 µl 1:2 diluted Bradford reagent (Bio-Rad). Blanks and protein standards contained the identical phase system polymers and salt composition as the individual TCC fractions. The blank absorbance was observed to drift 0.025 OD over the measurement of forty fractions, and a proportional correction was made prior to calculation of the protein values per fraction.

RESULTS

TCC Elution Procedures

In earlier applications of the TCC, utilizing aqueous-organic solvent systems in the fractionation of dinitrophenylated amino acids, elution of the top phase (aqueous) through the bottom phase (organic solvent), proved suitable for achieving excellent separation (10). The elution of retained bottom phase with pure top phase was also utilized in the separation of various strains of E. coli in aqueous polymer phase systems. The various strains were separated by eluting with a gradient prepared with pure top phases containing varying concentrations of NaCl, which yielded fractionation on the basis of cell surface charge composition (14). The latter application employed a dextran-poly(EtO) phase system further from the critical point of the phase diagram than has proved useful in affinity partitioning applications (4).

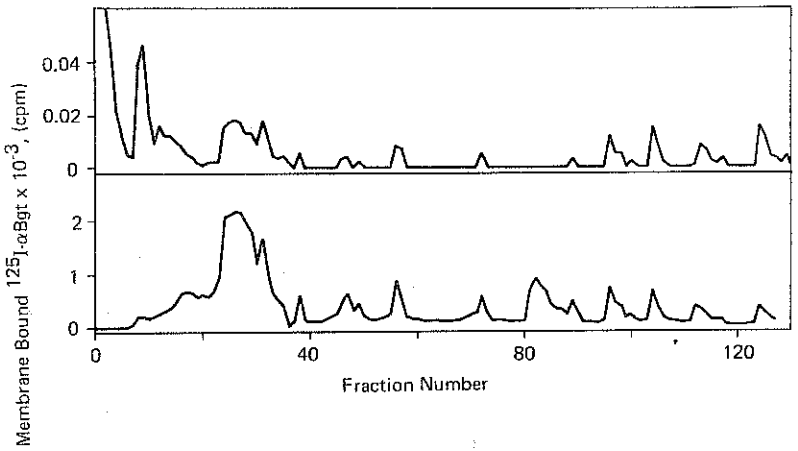

FIGURE 2

Chromatograms of Torpedo Membranes Obtained by Elution with Homogeneous Poly(EtO) Phase through Toroidal Coil

Torpedo membranes, enriched in nicotinic cholinergic receptor, were prelabeled with ^{125}I-αBgt at 0.05% of stoichiometric binding levels and loaded onto the TCC in pure poly(EtO)-rich phase. Elution was continued with pure top phase. An erratic profile of eluted bottom phase (plotted as $1/V_r$, bottom phase vol./top phase vol.) coincides with the elution of membrane bound cholinergic receptor. Phase system composition is the same as Figure 3.

In attempting to utilize a phase system close to the critical point, we typically observed results shown in Fig. 2 where the TCC apparatus was loaded with pure bottom phase and elution attempted by pumping pure top phase through the coil. Membrane fractions were eluted in a profile of narrow peaks that initially appeared to provide an exceptional degree of resolution of the membrane populations. Unfortunately, such fractionation profiles are not reproducible from one run to the next. An explanation for the erratic elution profiles was provided by measurements of the amount of bottom (dextran-rich) phase eluted in each fraction of the run (displayed as the volume ratio), which correlated remarkably well with the elution of membrane-bound ^{125}I-αBgt into

each fraction. This unpredictable "stripping" of the bottom phase was not due to temperature fluctuations, because the elution pattern did not correlate well with TCC refrigeration cycle of 11 min.

An intuitive and successful solution to this problem was the elution of the TCC with an emulsion containing dextran-rich phase dispersed in poly(EtO)-rich phase. In this configuration, elution of bottom phase was observed to be continuous due to the flow of some dextran phase through the system and erratic changes in the elution of dextran phase were obviated. In the absence of nicotinic cholinergic ligand-polymer (Fig. 3A), ^{125}I-αBgt bound to cholinergic receptor distributes into the bottom phase and the interface (4). Because the bottom phase is the stationary phase, the membrane fractions are retarded during their elution through the toroidal coil. In the presence of ligand-polymer (Fig. 3B), the distribution of ^{125}I-αBgt labeled membranes is shifted to the mobile [poly(EtO)-rich] phase, thus eluting in the breakthrough volume.

Comparison of CCD and TCC Separations

For comparison with the TCC results, parallel thin-layer CCD runs were performed. The same two phase system conditions displayed in Fig. 3 were used to provide a direct comparison of the two countercurrent extraction technologies (Fig. 4). Fractions from the TCC are eluted in reverse order to the conventional fraction numbering system commonly used to display CCD data. Thus, the higher transfer number fractions contain the membrane fragments that partition almost exclusively into the poly(EtO)-rich phase. A very apparent difference between the TCC and CCD profiles is the sharpness of breakthrough peak from the TCC, when compared with the analogous CCD peak. The symmetry of the CCD profiles is due to fact that such profiles can be modeled as binomial distributions; while the TCC is analogous to other forms of liquid chromatography and is best described by a Poisson distribu-

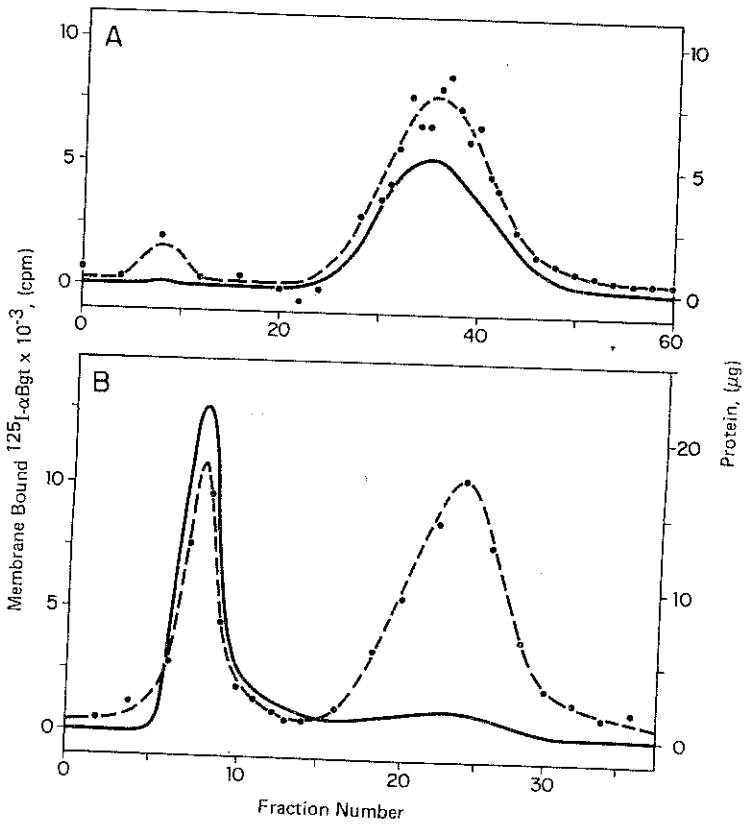

FIGURE 3

Chromatograms of Torpedo Membranes Obtained by Emulsion Elution through Toroidal Coil

Phase systems containing 4.6% (wt/wt) Dextran T-500 and 3.8% poly(EtO) 8000, 5 mM sodium phosphate pH 7.4, and 15 mM sodium chloride were prepared, the phases allowed to settle and the TCC system was loaded with bottom phase. Twenty-seven ml of emulsion containing dextran-enriched phase suspended in poly(EtO) rich phase [$1/V_r$ = 0.036, for A; 0.054, B] was then pumped through the rotating TCC apparatus. Torpedo membranes, suspended in the emulsion, were introduced into the emulsion flow at Fraction 1. Membrane bound ^{125}I-αBgt (——) was counted and protein assessments in the fractions performed (----). Profile A was observed with a phase system containing no ligand-poly(EtO), while B was obtained with a phase system in which 0.05% of the poly(EtO) was replaced with Et_3N-Me_6-NEt_2-poly(EtO). The flow rate was equivalent to 10 fractions/hr with sample loading as described in text.

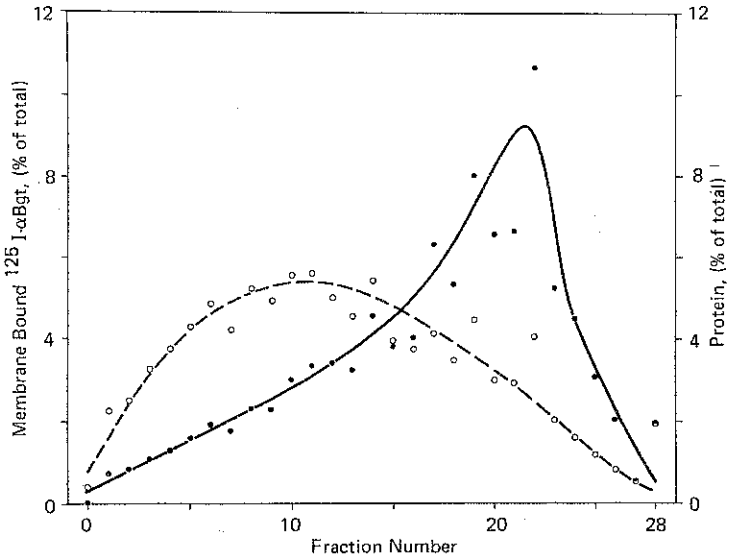

FIGURE 4

Thin-layer Countercurrent Distribution of Torpedo Membranes

Phase systems, containing 0.58% ligand-polymer, as described in Fig. 3, were used for CCD fractionation of membranes (1.8 mg protein). Profiles were determined by counting ^{125}I-αBgt (——) for receptor activity and Bradford assay (----) for protein. Note that membranes partitioning into the poly(EtO) enriched phase distribute into the higher CCD fractions, while during the TCC process, the order of fractionation is reversed.

tion. Both types of distributions approximate a Gaussian or normal distribution as the efficiency increases. A theoretical comparison (15) of the elution volumes of particles with differing partition coefficients shows that high partition components are concentrated in the early region of the TCC chromatogram, while for the CCD process the distributions are symmetrical.

Capacity of the TCC for Preparative Separation

The above results strongly suggest that affinity partitioning combined with TCC countercurrent chromatography provides a means

for the analysis of membrane heterogeneity, but its preparative scale utilization may be severely limited in the present configuration. However, it should be recognized that the inherent flexibility of the TCC would allow for modifications to increase its capacity. In order to provide a basis for further improvements in the technology, we were interested to determine the capacity of the present TCC coil configuration. The experiments described earlier were performed by loading 0.36 mg of protein (Fig. 3); upon application of 1.5 mg protein into the 0.5 ml sample loop of the TCC, essentially equivalent purification and TCC profiles were obtained. It is apparent that increasing the level of loading to as high as 3 mg/ml protein does not adversely influence the TCC process. Attempts to utilize higher protein concentrations in the sample loop were hindered, not by the capacity of the TCC, but rather by the difficulties in concentrating membranes samples.

DISCUSSION

Advantages of TCC and CCD Techniques: A Complementary Study

The thin-layer automated CCD technology provides substantially increased resolution over what may be achieved using manual countercurrent transfers. This apparatus has the virtue that mixing, transfer and separation of the phases is automated; however, loading and unloading of the phase systems is usually performed manually, and it is important to wash the plates extensively between runs. The choice of Plexiglas for construction of the thin-layer CCD apparatus was largely dictated by its water wettability properties, so that drop formation is avoided. It is also a suitable material for fabrication of the thin-layer CCD geometry, and, since it is transparent, the CCD process may be followed visually, especially in the separation of chloroplasts or erythrocytes. Another not so favorable property of Plexiglas thin-layer plates is that their brittleness makes them prone to impact damage and aging phenomena (small deformations) due to cycles of

contact with water or changes in temperature, making for a less effective seal between the chambers. The configuration of the thin-layer CCD plates limits the number of chambers to approximately two hundred, and results in extensive contact of biological particles with the Plexiglas surfaces.

Our experience in separation of electroplax membranes has indicated that nonspecific adsorption of membranes to the Plexiglas surface is a phenomenon that must not be ignored (4), especially when less than mg amounts of protein are loaded into the thin layer CCD apparatus. Such nonspecific adsorption phenomena could be due to deterioration of the surface properties of the Plexiglas during its normal use, but fortunately, this phenomenon was largely abolished by the addition of 2 mg/ml bovine serum albumin (BSA) to the phase systems (4). This, however, interferes with protein assays of the resulting fractions. In order to provide a direct comparison with the TCC technology, BSA was not added to phase systems used in the thin-layer CCD experiment illustrated in Fig. 4.

It is crucial for achieving a substantial affinity partitioning effect to utilize polymer systems near the critical point of the phase diagram (3,4), where the phases differ only slightly in composition and density. With these systems, the slight difference in densities and low interfacial tension between the phases lead to long settling times when compared to conventional aqueous polymer phase systems. Thus, high resolution CCD can take hours or even a day to achieve. Fortunately, the size of many subcellular structures is such that enhanced gravitation techniques can be considered to speed up the settling time of the phase system with minimal sedimentation effects on the sample itself. This, of course, would not be true for most cells. The simplest means to hasten phase separation is to centrifuge the phase system; this is especially useful when performing preparative scale separations involving a few extractions (4,16).

A second enhanced gravity approach is to place the thin-layer CCD apparatus in a centrifuge; increased leakage between the chambers is encountered, although these problems are now being approached with success (17).

A radically different approach was first developed (10) to increase the effectiveness of fractionation based upon distribution between phases (organic/aqueous or aqueous/aqueous polymer systems). In the latter application, the process is complicated by the viscous nature of the aqueous polymers used. The three most important factors for successful CCD separations are good mixing, settling and transfer. When these considerations are applied to the TCC process, settling and transfer are excellent with good retention of the stationary phase, but mixing can be poor as it is purely a function of flow phenomena (18). High flow will lead to good mixing, but at the expense of stationary phase retention. The retention volume is important in the fractionation of soluble constituents, which are not significantly retained in the interface; on the other hand, subcellular particles are significantly absorbed into the interface, and fractionation may depend primarily upon efficient contact of biological particles with the interface. In fact, Albertsson has shown for particles partitioning between the upper phase and the interface that the distribution is independent of the lower phase volume (5).

A theoretical advantage of the CCD technology is that the results of single tube partitioning experiments may be used to predict the countercurrent distribution behavior of presumably homogeneous particles. In turn, the results of CCD separations may be used to calculate the single tube distributions that should be obtained. Since a detailed understanding of partitioning of membranes into the interface can as yet not be translated into a membrane's TCC elution pattern. It is not clear just how single tube experimental results concerning the fraction of membranes that partition into the interface may be translated into a prediction of their behavior in the TCC; this is due to lack of

detailed knowledge of the geometrical considerations in separation of phases in the TCC coils. The empirically derived results displayed herein indicate that the membranes that partition only slightly into the phase system in single tube experiments are eluted early in the TCC run.

Future Improvements in TCC Technology

The TCC technique is a continuous flow separation technique and this facilitates the automation of the technique in a variety of ways. For example, it should be possible to adapt continuous flow protein and radioactivity analysis procedures for analyzing the membrane fractions. Also, the non-rotating seal platform used in the TCC design may accommodate multiple toroidal coils, facilitating simultaneous analysis of multiple samples or a single sample under several conditions.

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