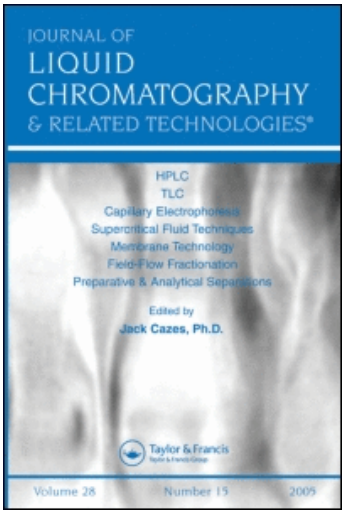

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CELL SEPARATION WITH COUNTER-CURRENT CHROMATOGRAPHY AND THIN-LAYER COUNTERCURRENT DISTRIBUTION IN AQUEOUS TWO-PHASE SYSTEM

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ABSTRACT

When mixed in aqueous solution at low concentrations the neutral polymers dextran and poly(ethylene glycol) (PEG) form a two-phase system consisting of a PEG-rich phase floating on a dextran-rich phase. These polymer phase systems have previously been shown to selectively partition cells and other particles between the phases and their interface on the basis of various surface properties of the particles. In combination with automated countercurrent techniques, partitioning in polymer phase systems gives rapid and sensitive fractionation of particle mixtures. This article contrasts separations obtained with mixtures of erythrocytes in polymer phase systems, using a nonsynchronous coil planet centrifuge (NSCPC - a version of Ito's

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countercurrent chromatograph) and in three different thin-layer countercurrent distribution (CCD) instruments. The results from CCD are shown to be in accord with simple theory and thus provide assistance in interpreting the theoretically complex results from the NSCPC. For polymer phase systems, the NSCPC was found to have a fractionation capacity similar to a CCD device providing twenty to thirty transfers.

INTRODUCTION

Advances in biomedical technology have increased the need for techniques capable of efficiently separating cells and other biological particles on the basis of their surface properties. For this reason partitioning with polymer phase systems is currently receiving widespread biotechnical interest (1-4). When mixed in aqueous solution at low concentrations the neutral polymers dextran and poly(ethylene glycol) (PEG) rapidly form two immiscible phases, with a PEG-rich upper phase and a dextran-rich lower phase. Aqueous two-phase systems can be buffered and made isotonic by adding salts or small molecules. When macromolecules or biological particles such as cells are added to these systems and the phases mixed and allowed to separate, the added substances often partition differently between the two phases, or in the case of particles, between one phase and the interface. Manipulation of phase-system components allows separations on the basis of properties such as surface charge, hydrophobicity, and affinity for a specific ligand (1-8). Some separations can be achieved in a single step, but difficult separations such as those of subpopulations of lymphocytes (9) or of cancer cell lines (10,11) usually require multi-step countercurrent techniques (4,9-11).

Instruments used for countercurrent distribution of aqueous two-phase systems can be divided into five basic groups. Classical CCD instruments (12) have been designed to partition soluble substances between two phases which differ greatly in density and have a high interfacial tension. These instruments are not suitable for polymer phase systems which separate slowly because of small differences in phase densities, high viscosity, and low interfacial tension (see Materials and Methods). Reasonable times for phase separation are obtainable by use of thin chambers or centrifugation.

A thin-layer CCD apparatus for polymer phase systems was first developed by Albertsson (4). The principle of operation is illustrated in Figure 1. Lower and upper halves of rectangular chambers, cut in half along their long axes, are machined in a circular pattern around the periphery of two round plexiglass plates. The lower plate is stationary and the upper plate can rotate. The countercurrent process proceeds stepwise; each step involves mixing the phases by shaking the plates, phase separation, and transferring upper phase onto fresh lower phase. The position of the interface between phases within the chamber is adjusted so that with each transfer the interface is either carried with the top phase, or in the most usual case, retained with the lower phase. In addition to partitioning particles on the basis of surface properties, the thin-layer CCD apparatus can also be used to separate particles on the basis of differential sedimentation (4,14).

Three versions of the thin-layer CCD apparatus are commercially available; two of these (one from

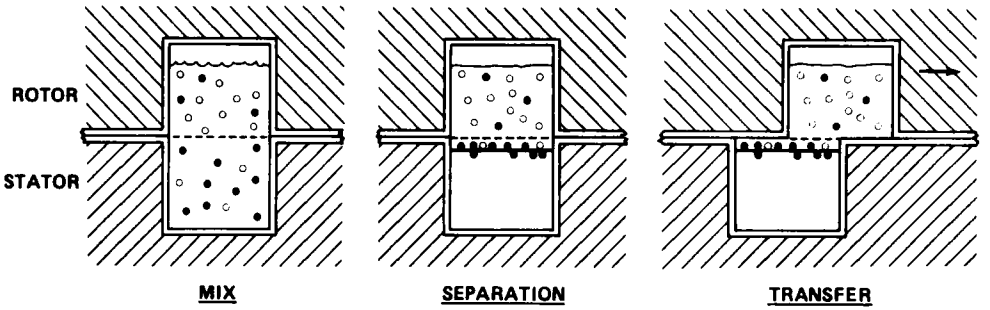

FIGURE 1. Thin-layer countercurrent distribution (CCD) in aqueous two-phase systems. The phases are mixed, allowed to separate and material distributed between both cavitites is separated by transfering the upper cavity onto a new lower cavity. The process is then repeated over and over.

Albertsson's laboratory) are automated, while the third requires manual shaking and plate advancement (see below).

Two types of thick-layer CCD instruments have been developed that use centrifugal force to provide rapid separation of the phases (7,15). Since centrifugation also increases the rate of particle sedimentation, these devices are best suited for separating soluble substances such as enzyme mixtures (7) or particles such as subcellular organelles (15).

Ito has developed two forms of the helical-coil countercurrent chromatograph (CCC) which have proven useful with polymer phase systems. These are the toroidal coil chromatograph (TCC) (8,15,17) and the nonsynchronous coil planet centrifuge (NSCPC) (17-19). Both instruments use a helical coil which is centrifuged to retain one phase while the second, mobile phase is pumped through the coil. Ideally each loop in the helical column can act as a partition

chamber providing one "theoretical plate." In the TCC the helical column is formed into a torus which is attached to the periphery of a metal disc mounted in a centrifuge (8,15,17). In the NSCPC the coil is wound around a cylinder fitted into a planetary holder. The holder is rotated at speeds up to approximately 1000 rpm around a central shaft, while a counter rotation of approximately 50 rpm is applied to the column in the holder. This second rotation counteracts the radial centrifugal force, which in the TCC tends to trap particles such as cells against the tubing wall, and permits separations of cells on the NSCPC (18,19).

Recently Flanagan et al. (8) and Sutherland et al. (15) carried out polymer two-phase separations of membrane fragments (8) and organelles (15) using the TCC and CCD. Both groups concluded that mixing on the TCC was only about 5% efficient, so that a 600 loop column provided only thirty theoretical plates. Nevertheless, both groups found the TCC to be a viable alternative to thin-layer CCD for these purifications. Both techniques yielded separations unobtainable by density gradient centrifugation (15).

In our own work with cell separations and with albumin partitioning on the NSCPC (19), we concluded that the 600 coils of our column provided twenty theoretical plates when polymer phase systems were used. For the cell separations, however, this conclusion was based on the tenuous assumption that the ratio of upper-phase volume to interface volume is unity. The present work was undertaken to provide a direct comparison, under similar conditions, of cell separations on thin-layer CCD devices and the NSCPC. We are particularly interested in using the

theoretically simpler CCD devices, in which phase mixing, separation, and transfer are efficient and in which the number of theoretical plates equals the number of transfers, to test our previous conclusions regarding the complex mixing events in the NSCPC. In addition, this work allowed us to compare three different commercial CCD instruments. These separations have been done with dog, sheep, human, and mice erythrocytes since they represent cells of similar size (30-90 μm^3) (21) and surface charge (mobility = 1.15 +/- 0.09 $\mu m\ sec^{-1}\ V^{-1}$ cm in standard saline) (22).

MATERIALS AND METHODS

Unless specified otherwise, all reagents are ACS grade or better quality from commercial sources.

Red Blood Cells

Blood samples were obtained by venipuncture from healthy normal individuals; 10 mM EDTA was used as an anticoagulant. Samples were stored at 4 C until just before use when one mL aliquots were washed four times by suspension in twenty volumes of isotonic buffer (Isoton II, Coulter), centrifuged for ten minutes at 1000 X g, and the supernatant and "buffy coat" removed. The samples were then washed once in the appropriate upper phase before being suspended in fresh upper phase prior to phase partitioning.

Cell Counting

Blood cells were identified, on the basis of size, and counted with an impedance cell counter (Coulter)

equipped with a 100-channel size analyzer interfaced to an Apple II+ computer. The mean volumes of the erythrocytes used were found to be close to the literature values (21): human 87 μm^3, dog 66 μm^3, mouse 49 μm^3, and sheep 31 μm^3.

Preparation of Two-Phase Systems

Polymer phase systems were prepared as described previously (3,5,6,13) by mixing appropriate weights of the following aqueous stock solutions (all weight %): 20% dextran T500 (M_w = 472,000, M_n = 174,000, Pharmacia, lot IE 32126); 30% PEG 8000, Union Carbide, lot B529-9104; 0.6M NaCl, and 0.22M Na_2HPO_4, 0.07M NaH_2PO_4, pH 7.2 buffer. PEG 6000 was recently redesignated PEG 8000 by Union Carbide. Dextran concentrations were determined polarimetrically (4,12), and PEG concentrations were determined gravimetrically. Once prepared the phase systems were filtered through a 0.45 μM filter and allowed to settle overnight in a separatory funnel at the experimental temperature. The phases were then separated and used immediately.

Phase systems were characterized at room temperature by measuring the potential difference between the phases, phase viscosities, and interfacial tensions as described previously (3,5,13). The potentials (top phase positive), viscosities and tensions given below represent the mean and standard deviations of ten independent measurements. Unless stated, values for these parameters are similar to those given for other systems.

Four polymer phase systems were used in this work:

(a) (5,4)I: A system containing 5% dextran, 4% PEG,

109 mM Na_2HPO_4, 35 mM NaH_2PO_4, pH 7.2. Potential 2.23 +/- 0.13 mV, tension 8.28 +/- 0.11 μN/m, viscosity 3.30 +/- 0.01 cP top phase and 30.14 +/- 0.14 cP bottom phase, density 1.016 g/mL top and 1.057 g/mL bottom.

(b) (5,4)II: 5% dextran, 4% PEG, 50 mM NaCl, 73 mM Na_2HPO_4, 23 mM NaH_2PO_4, pH 7.2. Potential 1.93 +/- 0.08 mV.

(c) (5,4)V: 5% dextran, 4% PEG, 150 mM NaCl, 7.3 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, pH 7.2; potential 0.28 +/- 0.14 mV, tension 4.99 +/- 0.06 μN/m.

(d) (5, 3.5)V: Similar to the (5,4)V system except 3.5% PEG; potential 0.23 +/- 0.06 mV, tension 0.71 +/- 0.28 μN/m.

Partitioning Experiments

Automated countercurrent distribution experiments were performed as described previously (3,5,10,11) using a 60-chamber Biosheff MK II machine (Biochemistry Department, University of Sheffield, Sheffield, UK) at room temperature, or a 60-chamber Albertsson device (IRD, Bromma, Sweden - currently produced by the Biochemistry Department, University of Lund, Lund, Sweden). Manual CCD experiments were performed with a twenty chamber handheld apparatus produced by Fractionation Technology, PO Box 7042, Huntsville, AL 35807.

The Biosheff lower cavity volume is 850 μL; the upper cavity is larger to facilitate mixing. Dye partitioning was performed with a (5,4)V system by adding 850 uL of lower phase to all chambers and 850 uL of top to all chambers except numbers 0/60 and 30, which received 850 μL of upper phase containing 0.2

mg/mL of trypan blue (Aldrich). Duplicate experiments of thirty transfers were undertaken at the same time using twelve seconds shaking and eight minutes settling. Cell separations were performed by adding 800 uL of lower phase to all chambers. For runs involving twenty-one transfers 900 µL of upper phase was added to all cavities except 0/60, 1 and 30 and 31, which received 900 µL of upper phase containing $1x10^8$ cells/mL. Again twelve seconds shaking and eight minutes settling were used. Thirty-transfer runs were conducted in the same manner except that cavities 59, 0/60, 1 and 29, 30, and 31 were loaded with cells. The sixty chambers could be loaded in about five minutes by use of a Gilson Repetman.

The Albertsson plates have a bottom cavity volume of 550 µL, hence these plates were loaded with 500 µL of bottom phase and 600 µL of top phase. Cells were placed in the same chambers as in the Biosheff, and a thirty second shake time (speed 3) and an eight minute settling time were used.

Manual twenty-transfer runs were performed with a Fractionation Technology instrument having a one mL lower cavity volume, so 1.1 mL of upper phase and 0.9 mL of lower phase were used. Cells were added to a single cavity, and a ten second shake time and a five minute settling time were used. Care was taken to keep the plates level during operation.

Single transfer partitions were used to determine partition coefficients. These experiments were performed as above except that cell concentrations were reduced to $1x10^7$/mL.

Countercurrent chromatography using the NSCPC was performed as described previously (19) using an

instrument provided by Y. Ito (18) containing 600 coils of 18 guage PTFE tubing. It should be noted that in CCC the particles with high partition coefficients are collected in the first- rather than in the last-numbered fractions as in the CCD experiments.

Trypan blue concentrations were determined by measuring the 590 nm absorbance of phase system fractions in which the phases had been "broken" by adding one mL of distilled water. Similarly, cell distributions were determined by breaking the phases in each fraction with isotonic buffer (Isoton II, Coulter Electronics) prior to cell counting. In both cases the distributions were expressed as a percentage of the total amount of material recovered.

CCD Data Analysis

Mathematical treatment of CCD experiments in terms of partition coefficient (K), phase volume ratio (b), and number of transfers (n) is straightforward and effective (4,12,16). The binomial nature of CCD partitioning leads to the prediction that the distribution peak of solute is given by:

$$r = bnK/(bK + 1) \qquad (1)$$

$$K = (r + 1)/b(n - r) \qquad (2)$$

The amount of substance (Y_x) in a chamber, $\underline{x}$ fractions from the distribution peak is related to the amount of material in the distribution-peak chamber (Y_o) by the following equation:

$$\ln Y_o/Y_x = X^2/F \quad (3)$$

where

$$F = 2nK/(K + 1)^2 \quad (4)$$

and

$$Y_o = F^{0.5} \quad (5)$$

These equations can be used to predict distributions if the parameters are known, or they can be used to calculate K values from CCD experiments to be compared with results from single-transfer experiments.

RESULTS AND DISCUSSION

Figure 2 presents the thin-layer countercurrent distribution of trypan blue in a (5,4)V phase system after thirty transfers in the Biosheff apparatus. The observed curve coincides in peak position and shape with that predicted from single-transfer partitions (K = 2.45, b = 1) This result is typical of those found with the other CCD instruments and confirms the discrete binomial nature of thin layer CCD (4,16) and the ability of thin-layer CCD devices to provide essentially complete phase transfer.

In spite of the complex nature of partitioning between one phase and the interface (3,4,23), the CCD equations also give excellent results for cell partitioning, if essentially complete transfer of top phase occurs (b = 1) (4). Figure 3 presents the results of twenty-one transfer countercurrent distributions of four different species' erythrocytes partitioned separately in the (5,4)I system. Despite

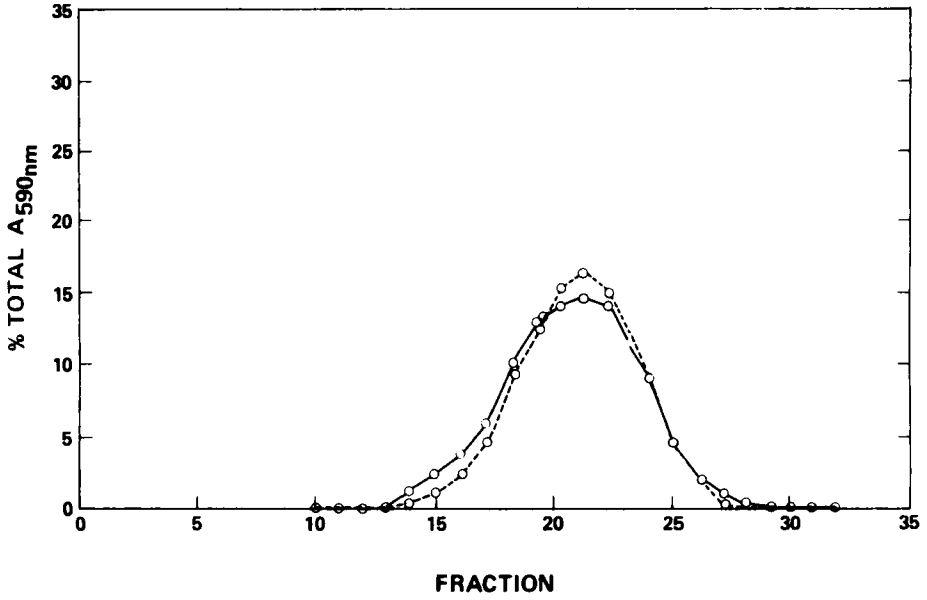

FIGURE 2. Thirty transfer thin-layer CCD of trypan blue dye in (5,4)V two-phase system (K = 2.45); observed result (——) and result predicted from single transfer experiments (- -).

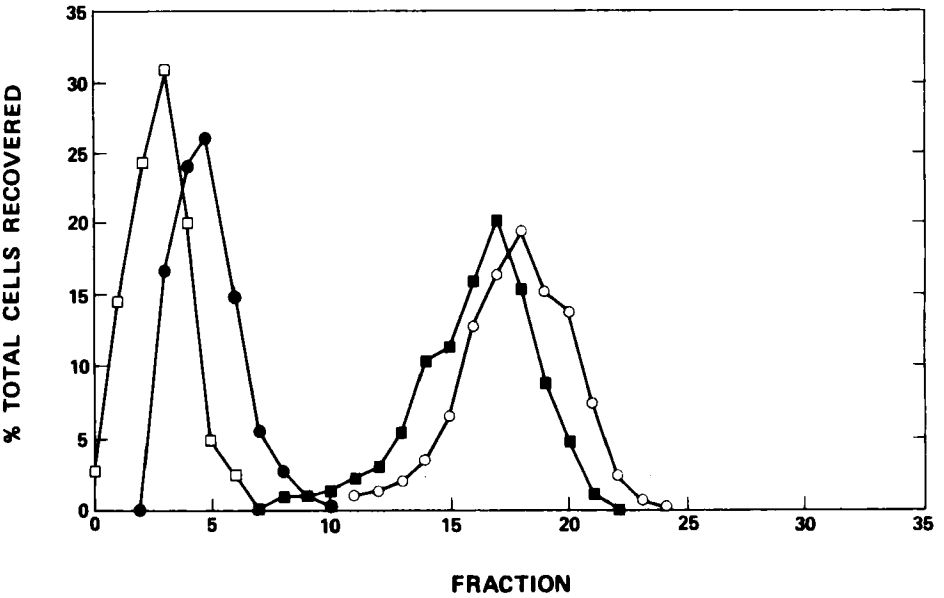

FIGURE 3. Separate twenty-one transfer CCD's of; 2 day-old sheep (K = 0.22, □), 1 day-old human (K = 0.38, ●), and 3 day-old dog (K = 4.50, ■) and mouse (K = 6.33, o) erythrocytes in (5,4)I system.

age heterogeneity among the cells in each sample (24), the distributions are very similar in shape to that in Figure 2. The partition coefficients given were calculated on the basis of peak position (eq. 2).

Particles partition independently of one another (4), so mixtures of erythrocytes should give results very similar to those displayed in Figure 3. Figure 4 presents the thirty-transfer results of an equal mixture of 2.8×10^8 dog and sheep erythrocytes in (5,4)I system. K values, calculated on the basis of peak position, were used to calculate theoretical curve shapes (equations 2-5) (dashed lines) which correspond well with the observed distributions. Figure 5 presents similar results for a mixture of 5.4×10^8 human and mouse erythrocytes. As can be seen, doubling the cell concentration results in peak broadening, probably because of interface overloading (3,4). Contaminating cells were not found in peak fractions in either case.

In accord with previous results, we have observed that K values, CCD peak positions, and base width increase with the time of storage of the cell samples (25). For instance the K value calculated from four CCD's of fresh human cells in a (5,4)I system was 0.45 +/- 0.15 while after sample storage for one week at 4 C in 10 mM EDTA the K value increased to 1.29 +/- 0.31. A similar result is seen for sheep cells in Figures 2 and 3. Single-transfer K values for fresh human cells averaged 0.50 +/- 0.09.

Cell partitioning is known to be very sensitive to potential difference and interfacial tension between the phases (3,4,5,12). Such an effect is illustrated in Figure 6, which presents a thirty-transfer countercurrent distribution of human erythrocytes in

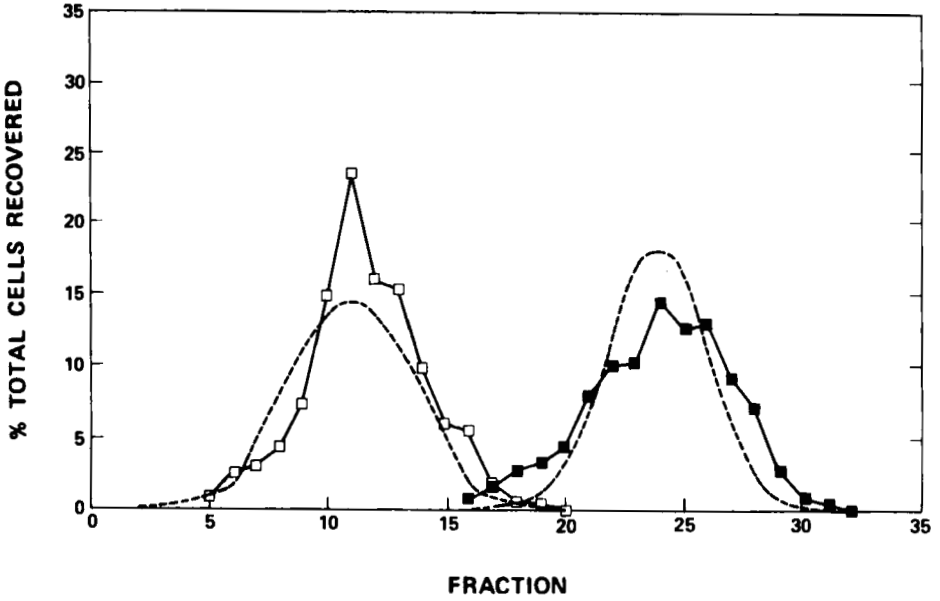

FIGURE 4. Thirty transfer CCD of an equal mixture of 7 day-old sheep (K = 0.63, □) and 4 day-old dog (K = 4.17, ■) erythrocytes in a (5,4)I two-phase system; observed (—) and predicted (- -).

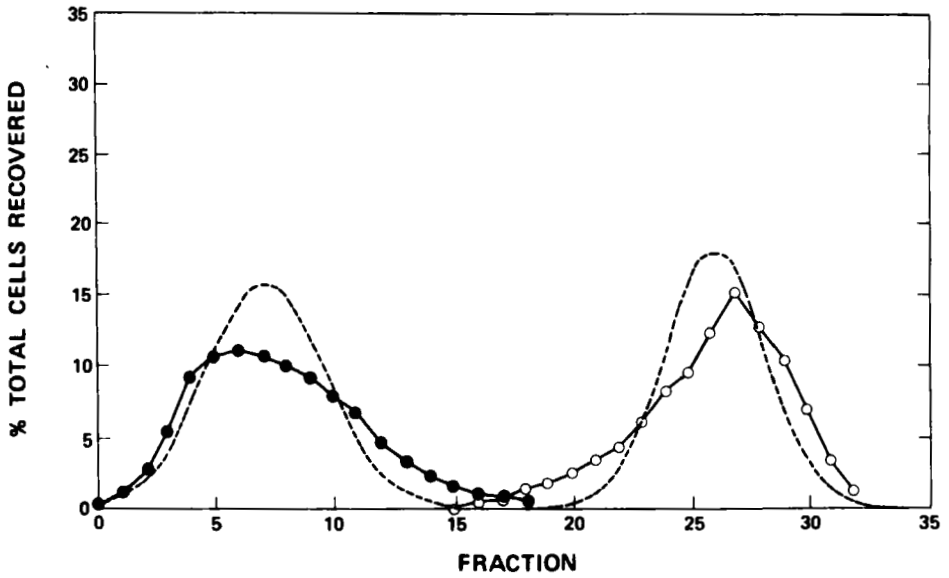

FIGURE 5. Thirty transfer CCD of an equal mixture of 4-hour-old human (K = 0.35, ●) and 7 day-old mouse (K = 6.75, ○) erythrocytes in a (5,4)I two-phase system; observed (—) and predicted (- -).

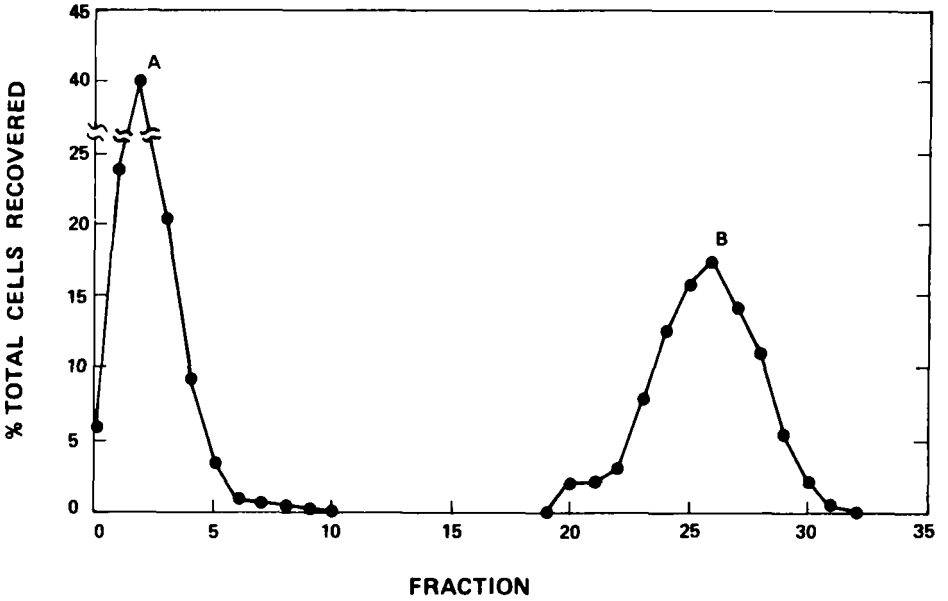

FIGURE 6. Thirty transfer CCD of fresh human erythrocytes in (5,4)V (K = 0.11, curve A) and (5,3.5)V (K = 6.75, curve B) systems.

the (5,4)V and (5,3.5)V systems using the Albertsson CCD device. Increasing polymer concentration increases interfacial tension and consequently lowers the K value and the fraction number of the peak. The effect of phase potential is illustrated by comparing Figures 5 and 6. The top phase is more positive for the system used in Figure 5, and as expected, higher K values and fraction number for the peak are observed (5).

Comparison of Figures 4 and 5 also indicates the ability of the CCD instruments to give experimental curves in accord with theory for cell distributions as well as for soluble substances.

The automated Albertsson (the most commonly used) and Biosheff instruments differ principally in the

maximum number of chambers in the plexiglass plates, the way in which the plates are held together, and the mechanism for advancing the plates. The Biosheff uses 60-chamber plates, is mounted in a variable temperature cabinet, and uses electrically-driven mixing and pneumatically-driven transfer mechanisms. The plates are held together by a bolt through the central axis. The Albertsson apparatus uses 120- or 60-chamber plates held together by four spring clamps. The machine operates at room temperature, and uses electrically driven mixing and transfer. The manual unit is made of solvent-resistant, autoclavable Delrin (Dupont) plastic plates, is approximately 25 cm in diameter by 5 cm thick, and contains twenty chambers.

The above results demonstrate the predictable nature of CCD experiments. If the K values, lower-cavity volumes, phase-volume ratios, and number of transfers are known, a theoretical curve can be derived which will closely correspond to the experimental curve (4). Consequently, we feel we are justified in using these experiments to aid in evaluating the less predictable CCC experiments (8,15). In our previous work with the NSCPC (19) we were forced to make a questionable assumption in order to approximate the number of theoretical plates achieved in our cell separations. For soluble molecules on the NSCPC, it is possible to approximate the phase-volume ratios (needed for calculation) simply by examining the column contents (by pumping off) at the completion of a run (18,19). However, cells partition between the interface and the top phase, and it is impossible (to our knowledge) to determine physically the actual interface/top-phase ratio for the NSCPC. Of course,

this same problem applies to CCD experiments, but the CCD equations are quite effective if one assumes the b value equals the amount of top phase transferred divided by the total volume of top phase (4). As a result we can use the well-behaved CCD experiments to test our previous conclusion (19) that mixing of polymer phase systems is inefficient in the NSCPC.

Figure 7 presents the NSCPC separation of dog and sheep erythrocytes obtained by Sutherland and Ito (18) in a system supposedly similar in composition to our (5,4)II system but believed to have somewhat lower polymer concentrations (19). We have used a (5,4)I system in our CCD runs to give K values similar to those of Ito and Sutherland. This system yields thin-layer CCD separations after twenty to thirty transfers (Figures 3 and 4) that are similar to those obtained in CCC experiments (Figure 7). We conclude, therefore, that the NSCPC also produced roughly twenty to thirty theoretical plates.

In view of our experience with both the CCD devices and the NSCPC it seems appropriate to comment on the relative advantages of the two types of instruments (8,15). A problem with CCD which one avoids with CCC is minor leakage of phase system between the plates. Separations are somewhat more rapid on the CCC. A twenty transfer CCD run takes about three hours, while the corresponding CCC run takes about two hours. CCC can be used with organic solvents which would dissolve the plexiglass CCD plates. We had initially expected accurate loading of the CCD chambers to be time consuming. However, use of repetitive pipetting equipment reduced the loading process to ten minutes. An advantage of the CCD is its relative mechanical

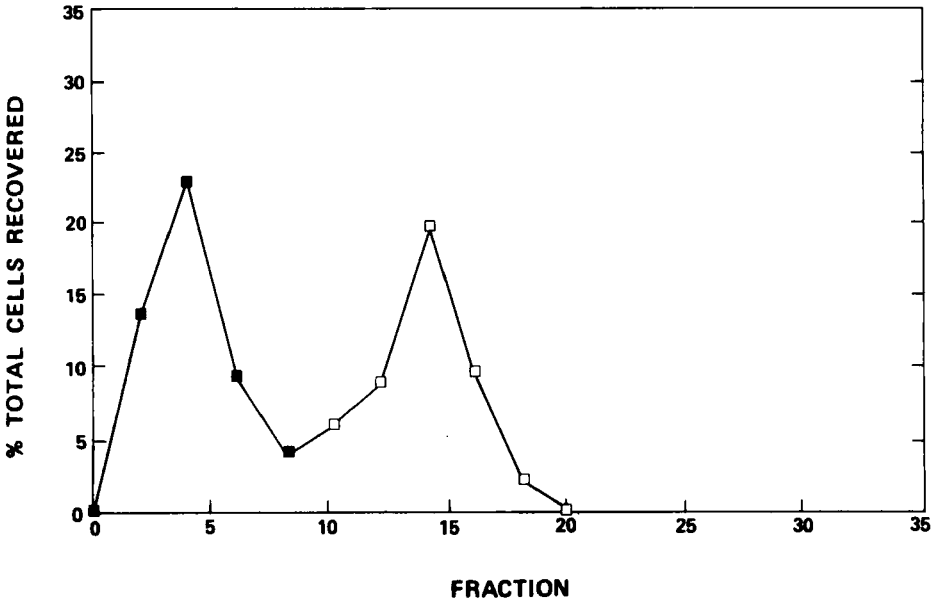

FIGURE 7. Nonsynchronous flow-through coil planet centrifuge (NSCPC) countercurrent chromatography (CCC) of a mixture of dog (■) and sheep (□) erythrocytes, as reported by Sutherland and Ito (18).

simplicity. Since the CCC must rotate at up to 1000 revolutions per minute it might be expected to be somewhat more prone to breakdowns; however, we had little trouble with our NSCPC over a two-year period. Probably the prime practical advantages we have noted with thin-layer CCD are its theoretical simplicity which permits single-tube partitioning to be used in a straightforward fashion in choosing a phase system for a particular application, and the ability to process two sample mixtures at the same time under identical conditions. With the more complex CCC it is somewhat difficult to predict the end result of a separation procedure and choose the proper conditions (rotation

speeds and pump rates). On the other hand, these variables provide additional power once their characteristics are understood.

ACKNOWLEDGEMENTS

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