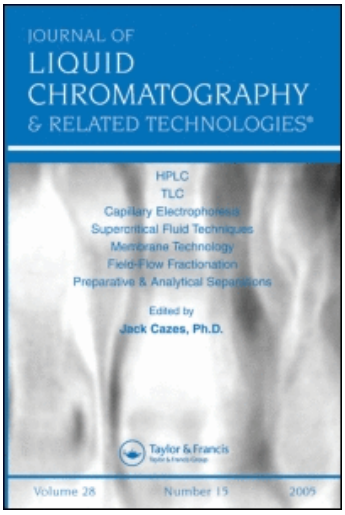

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Cell Separations on the Countercurrent Chromatograph

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CELL SEPARATIONS ON THE COUNTERCURRENT CHROMATOGRAPH

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ABSTRACT

Separation of cells differing only subtly has been achieved by partitioning between the two phases formed by solution of dextran and polyethylene glycol in water. Cell populations which have related, but not identical, surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, repeated partitions are carried out via countercurrent distribution or countercurrent chromatography to effect the separation. Potential advantages of countercurrent chromatography are its ease and rapidity of operation. In the present work we describe our approach to determining and possibly improving the efficiency of polymer phase partitioning by countercurrent chromatography.

INTRODUCTION

Modern biomedical research depends heavily upon effective techniques of separation and purification. An important prerequisite for studying the normal or pathological activity of any biological system, whether molecular or cellular, is the ability to isolate its components from one another so that they can be characterized, manipulated, and recombined under controlled

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conditions. Separation methods for molecular biology are well developed. However, the preparation of homogeneous, functionally specific populations of living cells remains one of the chief obstacles to progress in cell biology today. Among the most widely used techniques of cell purification are velocity sedimentation and isopycnic centrifugation, which operate entirely on the basis of such physical characteristics as cell size and density. Other separation methods employ the selective killing of unwanted cells within a culture, or the tendency of certain cells to attach themselves to solid substrates. While these are useful procedures in many cases, a number of biologically important cell types are not amenable to such separations. For the most part, the methods described lack the power to select a subpopulation of cells differing only subtly from contaminating cell types. Cell types may differ from one another only in the nature or amount of a single surface membrane constituent, for example, a characteristic that cannot be detected by the separation techniques in ordinary use.

A need exists for a cell purification technique that is able to sort a heterogeneous mixture of cells on the basis of specific, subtle features of the cell surface, and can do this for cells about which one may have very little specific biochemical information. A simple and sensitive method that shows promise of fulfilling these criteria is phase partitioning in aqueous polymer systems.

When aqueous solutions of two different polymers are mixed above certain concentrations they frequently form immiscible, liquid, two-phase solutions. Each of these phases usually consists of more than 90 percent water and can be buffered and made isotonic and hospitable to cells, organelles, and biomolecules by addition of low molecular weight species. If a cell or particle suspension is added to such a system, then shaken, the cells (upon re-equilibration) are frequently found to have partitioned unequally between one of the phases and the interface. This preferential partition behavior can be used as the basis of

a separation procedure for differing cell populations since partition in these systems is determined directly by cell membrane properties such as charge, lipid composition, and specific cell proteins (1,2).

The polymers typically used for phase partitioning are polyethylene glycol (PEG) and dextran. Solution of these polymers above certain concentrations (approximately 5% by weight for PEG-6000 and dextran-500,000) produces two, immiscible aqueous phases in which the top layer is PEG-rich and the bottom is dextran-rich. The concentration at which the two phases become immiscible is known as the critical point. By convention, the concentrations of dextran and PEG in a phase system are represented as a ratio; for example, a 5/4 system is one containing 5% dextran and 4% PEG. Some important physical characteristics of these phase systems are as follows: densities, upper 1.01 g/mL and lower 1.03 g/mL to 1.08 g/mL; interfacial tensions, 0.007 dyne/cm for a 6/4 system, 0.0031 dyne/cm for a 5/4, and 0.00046 dyne/cm for a 5/3.5 (1); and viscosities, upper 4 cP and lower from 44 cP for a 5.6/4 system to 27 cP for a 5/4 to 23 cP for a 5/3.5 (3). Density differences, interfacial tensions, and lower-phase viscosities decrease as polymer concentrations are lowered and approach the critical point; buffer variations also affect these same parameters.

Cell populations which have related, but not identical, surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, many repeated partitions are carried out via countercurrent distribution (CCD) or countercurrent chromatography (CCC) to effect the separation. The usual automated device for performing repetitive partitioning is the Albertsson CCD apparatus (1). In this machine 60 to 120 thin vessels are arranged around the periphery of a disc. Each vessel is loaded accurately with top and bottom phases, and the sample is introduced into a small number (usually three) of the adjacent vessels. The entire disc is then shaken to promote mixing of the phases and partitioning of the sample.

After settling has brought about phase separation (typically 15-20 minutes) the disc is rotated so that top halves of the vessels are shifted to join with adjacent bottom halves. The interface and a small portion of the bottom layer are carried with the top vessel half, or alternatively, the interface and a portion of the top phase remain with the bottom half. The result of repetition of this process is a countercurrent movement of top and bottom phases and a consequent countercurrent distribution of the partitioned material. The prime limitations of CCD for polymer-phase partitioning are the laboriousness of accurately and individually loading the 60-120 vessels and the long delay necessitated for phase separation after each mixing (e.g., 60 transfers at 15 minutes per transfer equals 15 hours per run).

Phase partitioning of cells can also be performed by CCC with the potential advantage of avoiding the above limitations of CCD. However, injection of particles the size of cells (> 1 μM) into the typical countercurrent chromatograph in which a centrifugal force is applied to the medium results in the particles being pressed against the column walls and immobilized. This immobilization can be eliminated by applying a second, counter rotation to the coils as in the nonsynchronous coil planet centrifuge (NSCPC) of Ito, Figure 1. Ito and Sutherland have demonstrated that cells can be separated on this device (4-6). The major barriers to broad application of the NSCPC to these purifications are the lack of demonstrated broad applicability of the device and the lack of precise information about the efficiency of these separations. To date only a single work has been published on cell purifications by polymer phase partitioning on the NSCPC; this pioneering work was done by Sutherland and Ito, and involved separations of fresh red blood cells (RBC's) of sheep, human, and dog (6).

Unlike the Albertsson CCD device, the NSCPC has the capability to perform separations based upon cell size and sedimentation velocity in addition to the two-phase partitioning technique described above. In this mode, the coils of the NSCPC column are

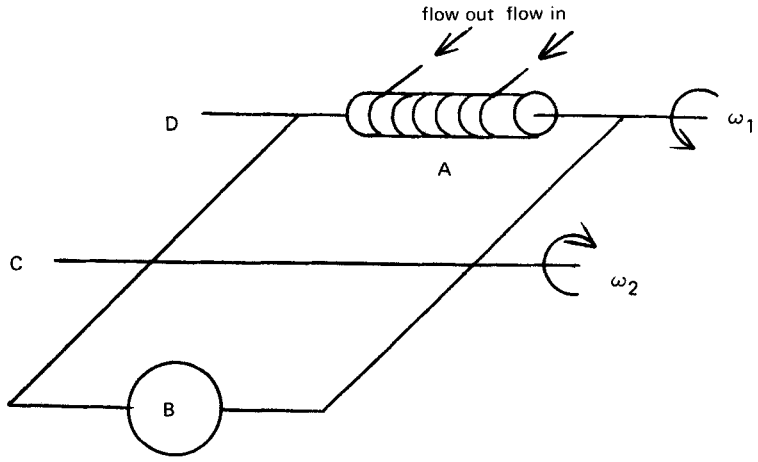

Figure 1. Schematic drawing of the Ito nonsynchronous coil planet centrifuge. A - helical coil (column), B - counter weight, C - central rotation axis, D - planetary rotation axis.

filled with a single solution of physiological saline or some similar isotonic eluent (5).

SEPARATION EFFICIENCY - INITIAL CONSIDERATIONS

Although Sutherland and Ito achieved good resolution in their separations, there is reason to suspect that polymer phase partitioning may be relatively inefficient on the NSCPC as compared to CCD. First, as described in the accompanying article by Sutherland and coworkers (7), polymer phase mixing in CCC is apparently inefficient. These workers have used an elegant apparatus to view PEG mobile phase streaming through dextran stationary phase with little mixing; what mixing does occur takes place at the interface where the PEG phase leaves the dextran phase, and is suggestive of the turbulence at the foot of a waterfall. Presumably this inefficient mixing is due to the high viscosity of the dextran phase.

The second consideration suggestive of inefficiency in the NSCPC derives from a theoretical consideration of cell partition-

ing and comes from the work of Brooks and coworkers (8). In this work an expression is derived relating the effects of factors such as interfacial free energies, interfacial potential, and surface charge density of particles on partitioning. Surprisingly, a bacterial partitioning experiment carried out to test this theoretical construct could only be explained if it was assumed that the temperature of the experiment had been equal to 10^5 Kelvin. Obviously, the expression fails to take account of some powerful randomizing factor. These workers suggest that this randomizing energy comes from the fluid shear stresses that occur during phase separation following phase mixing. If this conjecture is correct, then the high G field present in the NSCPC might be expected to enhance shear forces and cell randomization, leading to a reduction in the quality of separations achieved.

EXPERIMENTAL MEASUREMENT OF EFFICIENCY

Efficiency in the NSCPC can be determined by use of the Craig equations for CCD, eqs. 1-3, where p and q are the mole

$$(p + q)^n = 1 \quad (1)$$

$$K = p/q = p/(1 - p) \quad (2)$$

$$T_{n,r} = \frac{n(n - 1)(n - 2)\ldots(n - r + 2)}{(r - 1)!}(1/ K + 1)^n K^{r-1} \quad (3)$$

fractions of soluble sample in upper and lower phases, respectively, n is the number of transfers, r is the tube number, and $T_{n,r}$ is the amount of sample in the rth tube after n transfers. Insertion of the proper partition coefficient K and the proper n into eq. 3 permits reproduction of experimental sample distribution. Application of CCD equations to separations on the NSCPC is appropriate because of the manner in which the cell separations are performed. The column is initially filled with the more dense dextran stationary phase, rotation is begun and approximately one milliliter of mobile PEG phase is pumped onto the column. The sample, dissolved in mobile phase, is then injected and mobile phase is pumped for the remainder of the run. Rather than allowing the run to continue in this fashion until all cells have elu-

ted, the rotation is halted just before the sample reaches the end of the column, a technique found by Sutherland and Ito to optimize resolution (6). After spinning is stopped, the column contents (resembling a CCD distribution) are pumped from the machine and fractionally collected.

To evaluate the efficiency of phase mixing on the NSCPC in the absence of shear forces on particles, we have determined the distribution of albumin using the same protocol as used for cell separations (see Experimental), Figure 2. Note that the experimental curve can be reproduced effectively by eq. 3 assuming that $\underline{K} = 3.0$ and $\underline{n} = 20$. Thus under these conditions, the equivalent of 20 transfers (or mixing events) has occurred. However, since the NSCPC column contains 600 coils, almost all of which have been shown (see below) to retain some stationary phase throughout the run, this instrument should be able to deliver the equivalent of around 600 individual transfers ($\underline{n} = 600$). Apparently phase mixing under these conditions is indeed poor.

Another indication of poor mixing is provided by comparison of the theoretical $\underline{K}$ value of 3.0, Figure 2, with the experimental value of 0.38 for a single tube partition. Such a high theoretical $\underline{K}$ could result if the albumin, injected dissolved in the PEG mobile phase, moved through the column without interacting with the dextran stationary phase.

In applying the Craig equations it is necessary to include the volume ratios of stationary and mobile phases (V_s/V_m). For the sake of simplicity we have assumed this ratio is unity. In fact it varies throughout the column as revealed by pumping off the column contents (see V_s/V_m, Figure 2) but typically averages about 0.8.

Application of the Craig equations to cell separations is not entirely satisfactory, since the cell samples are heterogeneous in size and possibly in cell surface properties as well. It is important to recall that cell size affects retention time on the NSCPC. Consequently cells will be smeared across the column and provide a poor measure of the number of transfers involved. Also it is

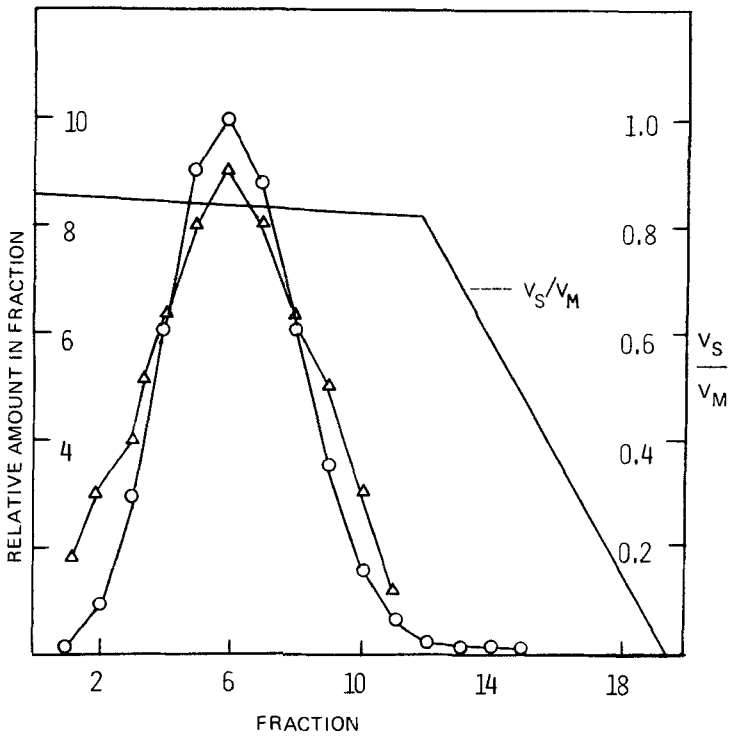

Figure 2. Theoretical (o) and experimental (Δ) curves for partitioning of albumin, and a plot of V_s/V_m as a function of fraction number (in phase system "D"). The calculated curve assumes $\underline{n}$ = 20 and $\underline{K}$ = 1.50; experimental $\underline{K}$ = 0.38.

important to note that cells are partitioned between the upper phase and the interface, and it is impossible at present to approxmate the ratio (needed for eq. 3) of interface volume to mobile-phase volume in the heterogeneous environment of the countercurrent chromatograph. With these limitations in mind, application of eq. 3 to two passes of fresh sheep cells through the NSCPC with differing phase systems is shown in Figure 3. Note that the theoretical $\underline{K}$ value is greater than the measured value and further that $\underline{n}$ is rather small. It is interesting that a significant

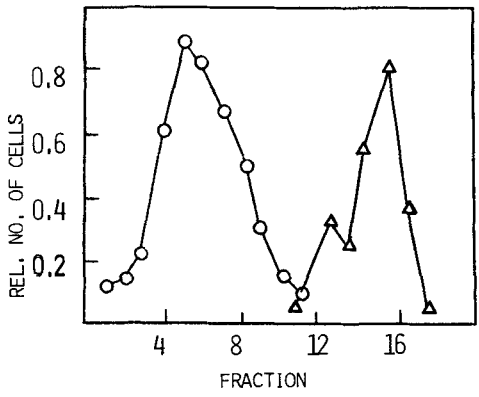

Figure 3. Plots for two runs of fresh sheep RBC's in phase system D (o) and phase system B (Δ). For D, $K_{exptl} = 0.31$, $K_{theor} = 0.80$ and $\underline{n} = 20$. For B, $K_{exptl} = 0.02$, $K_{theor} = 1.3$, and $\underline{n} = 30$. Theoretical curves using these parameters match the experimental curves well.

amount of band broadening occurs despite poor phase mixing, since under these circumstances one might expect the cells to remain in a tight band as they move through the machine. Possibly this broadening results from the increased shear forces characteristic of the NSCPC. On the other hand, as discussed above, it could result from the heterogeneity of the sample.

A more effective measurement of separation efficiency, and an evaluation of the factors involved in band broadening, may be obtained by direct comparison of cell experiments on the NSCPC and the CCD apparatus, since the number of transfers is known in CCD. Such experiments are currently in progress in our laboratory and that of D. E. Brooks (University of British Columbia).

IMPROVEMENT OF EFFICIENCY

The measurements of efficiency we have presented thus far are of course dependent on the specific machine and phase parameters utilized. It is important to explore the possibility of improving the efficiency of the NSCPC. Regarding machine para-

meters it is evident that increasing flow rate and reducing rotation rate results in rapid removal ("stripping") of the stationary dextran phase from the column. A flow of approximately 8 mL/hr and a rotation rate of approximately 750 rpm is ideal from this standpoint; changing the "small" rotation has no effect on stripping of stationary phase.

Since presumably the inefficient phase mixing on the NSCPC is largely influenced by the high differences in viscosity and density between the polymer phases and also possibly by variation in interfacial tension between polymer phases, one would suspect that varying the phase systems themselves would be likely to enhance phase mixing. We have preliminary evidence that this is the case. In figure 4 we present a comparison of RBC separations on the NSCPC under presumably identical conditions; experiment 4a was performed in our laboratory and experiment 4b was performed by Sutherland and Ito (6). It is important to note that the machine parameters are identical in the two experiments. In addition, the column on our machine is the same one which was used on the Sutherland-Ito machine. Our machine was constructed at NIH by Ito, who simply removed the column from his older instrument and transferred it to our newer instrument.

The differences between experiments 4a and 4b are obvious; the peak shapes and separation of 4b are far superior; in addition 4b reveals a different V_S/V_m pattern and different $\underline{K}$ values. Although the phase systems are supposedly the same, the difference between these two experiments lies in fact in the phase systems. Workers in the area of polymer phase partitioning are painfully aware of the difficulty in reproducing a particular phase system. Specific problems include variations in molecular weight distributions from batch to batch of the polymer, impurities, and determination of the water content of the hygroscopic polymer stock powders. Since the work of Sutherland and Ito (6), significant advances have been made in controlling these variables (see Experimental section for methods). (One problem which remains to be dealt with, however, is that of impurities). Discussions with

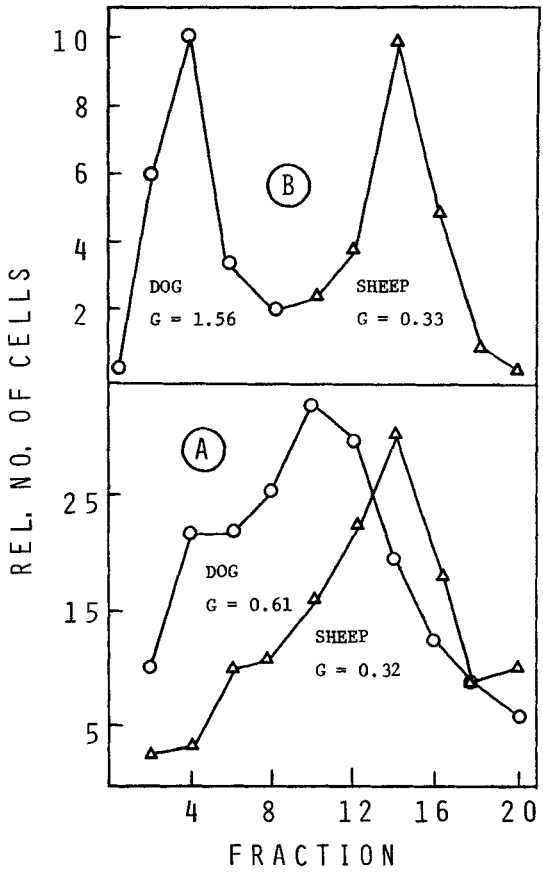

Figure 4. Separations of fresh sheep (Δ) and dog (o) as done by Sutherland and Ito (6) (plot B) and in our laboratory (plot A) using phase system "D".

Sutherland and Ito have made it evident that the water content of their polymers was not accounted for in their preparations of the 5/4 system used. If this is the case, we calculate that their phase system was in fact close to a 4.7/3.8 system. This concentration is nearer the critical point than the 5/4 system we used. Since interfacial tensions, phase-density differences, and phase-viscosity differences decrease as the critical point is approached,

phase mixing should be enhanced in a phase system close to the critical point. This effect could explain the superior separation results of experiment 4b. We are actively investigating this hypothesis with careful determination of polymer phase compositions.

EXPERIMENTAL

Polymer phase systems were prepared and separations on the NSCPC were performed as described previously (6). In a typical NSCPC run a large rotation rate of 750 rpm was used along with a small rotation rate of 10 rpm and a flow rate of 8 mL/min. PEG concentrations in stock solutions were determined by refractometry using an empirical relationship between refractive index and concentration derived with samples dried at 100°C for 24 hours; the PEG was also purified by precipitating from acetone with ethyl ether. Dextran concentrations in stock solutions were determined by polarimetry as described by Albertsson (1). PEG-6000 was purchased from Union Carbide as Sentry grade material. Dextran-500,000 was purchased from Pharmacia and used without purification. Phase system "B" contains 0.01M phosphate and 0.15M chloride, and system "D" contains 0.07M phosphate and 0.06M chloride (6). Partition coefficients were determined after phase settling had proceeded for 10 minutes rather than the three minutes used by Sutherland and Ito (6). Albumin concentrations were determined by absorbance at 280 ηM after dilution with water to assure a single phase system. A Coulter counter was used to determine cell numbers.

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