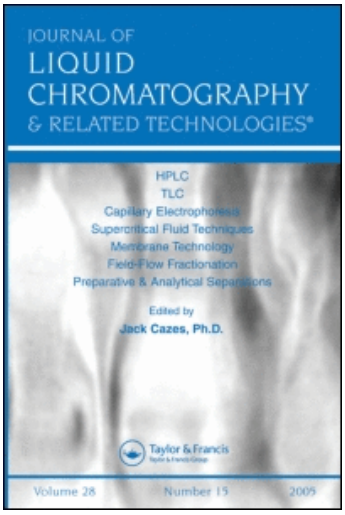

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COUNTERCURRENT CHROMATOGRAPHY USING A TOROIDAL COIL PLANET-CENTRIFUGE: A COMPARATIVE STUDY OF THE SEPARATION OF ORGANELLES USING AQUEOUS TWO–PHASE PARTITION

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ABSTRACT

A toroidal coil planet centrifuge is described and compared with other countercurrent chromatography (CCC) and countercurrent distribution (CCD) techniques. The basis of separation is partition in aqueous two-phase polymer systems, with each method assessed by fractionating rat liver organelles. The size and ease of operation of the toroidal coil planet centrifuge gave significant advantages over conventional CCD systems achieving equivalent resolution in a fraction of the time.

INTRODUCTION

The separation of viable biological material on the basis of partition between two immiscible aqueous polymer phases is a

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sensitive technique which is complimentary to both electrophoresis and centrifugation. Recent reviews (1,2) suggest that the approach is increasingly used to separate closely related material.

Aqueous polymer two-phase systems provide a gentle isotonic environment for cells, subcellular particles and enzymes; near complete recoveries of biological activities are routinely achieved. Separation can be based on a particle's charge density (3), lipid composition (4), and tendency to interact with the constituent polymers of the phase system (5). The latter may be turned to advantage by synthesizing ligand-polymers that selectively interact with biological particles containing biospecific binding sites (6,7).

With both conventional and affinity aqueous phase partitioning techniques, purification is based upon differential distributions of the constituents in a mixture between the three compartments of the two phases, the upper phase, the lower phase and the interface. If the constituents differ greatly in their distribution, then substantial purification may be achieved by performing single extractions. Usually the differences in the distributions are small, and multiple extractions are necessary.

Thin layer countercurrent distribution (TLCCD) was developed by Albertsson (8) and has now become an accepted method of separating material of similar partition coefficients. There are numerous applications cited in the literature and these are currently being reviewed by Walter, Brookes and Fisher (9). The Albertsson TLCCD device is still the preferred method, although a competitive device has recently been developed (10) which is claimed to have an improved rotor design. While the major advantage of CCD is its theoretical predictability, its high labour content and long separation times have led to attempts to develop new methods that are more suited to the modern laboratory and eventual automation in industry. These methods fall into two

categories: those based on countercurrent distribution using the discrete mixing settling-transfer-approach, and those based on continuous flow countercurrent chromatography.

Continuous countercurrent chromatography has been developed (11,12) to increase the speed and effectiveness of fractionation based upon distribution between double aqueous phase systems. A helically wound tube is located circumferentially on a rotating disc. The radial acceleration field holds the heavier phase stationary in the outer part of the coil, in such a way that when the lighter phase is pumped through the coils it mixes with the stationary phase without displacing it. Sample components injected with the mobile phase travel through the coils at a rate dependent upon their distribution between the phases. This process, Toroidal Coil Chromatography (TCC), is analogous to a continuous form of liquid-liquid chromatography.

A direct comparison of results obtained using TCC and CCD technology were reported (12) showing that TCC was not only faster but gave significantly greater resolution. Recent work by Johansson (13) using the enhanced gravity CCD apparatus developed by Akerlund (14) has established the importance of short separation times in avoiding degradation and changes in partition with time. Furthermore it has been observed (15) that synaptic membranes change their surface properties after prolonged contact with polymer phase systems.

While a rotating helically wound piece of tubing is simple to envisage, in reality fluids have to pass from a stationary to a rotating frame of reference. Ideally this is achieved without rotating seals in order to preserve sterility and maintain sample viability. This can result in an elaborate rotor design requiring a floor standing centrifuge. Recently Ito (16) has developed a new motion that avoids rotating seals and is simpler to construct. He first used toroidal coils on this device (17,

18,19) and later multilayer coils. He named the process multilayer coil planet centrifugation and is successfully applying the technique to countercurrent chromatography with aqueous/organic phase systems (20).

This paper describes a new method of Toroidal Coil Countercurrent Chromatography which combines the advantages of the Toroidal Coil Centrifuge described previously (12) with the simplicity of the Ito device. The method, whereby a toroidal coil is mounted on an epicyclic coil planet centrifuge, is evaluated by examining the fractionation of rat liver homogenate in comparison with similar separations performed on a Bioshef unit gravity TLCCD apparatus (10), a Pritchard 17-transfer enhanced gravity CCD device (21) and the Toroidal Coil Centrifuge (12).

DESCRIPTION OF APPARATUS

Epicyclic Toroidal Coil Planet Centrifuge

The motion of the epicyclic coil planet centrifuge is best described by considering two identical gear wheels "A" and "B", with "A" stationary in a central position and "B" rotating around it with the gears meshed (figure 1). Rotation of gear "B" about the stationary gear "A" will result in two rotations of "B" to every rotation of the line joining the centres of the two gears "OC". Leads connecting "O" and "C" will not twist provided they come out of the paper at "O", rotate 180 degrees in a plane perpendicular to the paper and then return into the paper again at "C".

If a toroidal coil is mounted circumferentially around a drum attached to gear "B" then the locus of the point "P" on the periphery of the toroidal coil will describe a cardioid as illustrated in figure 2a. This produces an acceleration vector that varies in both magnitude and direction as shown in figure

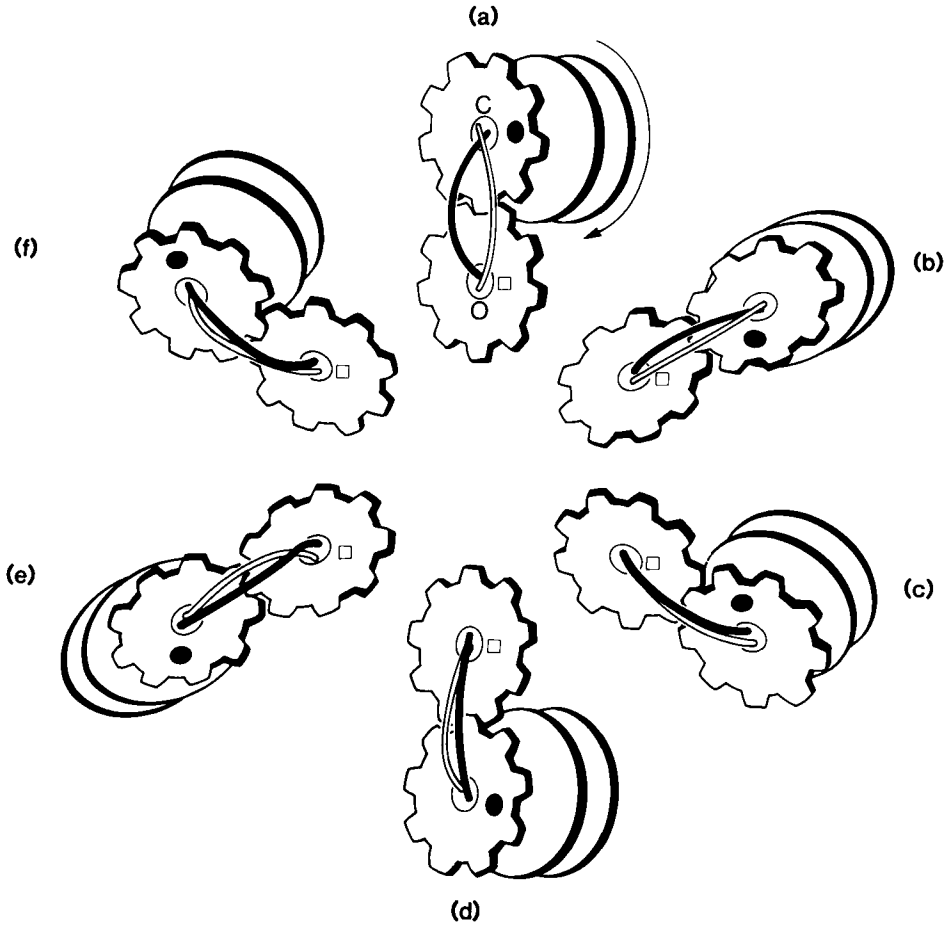

FIGURE 1

A sequence of diagrams showing the principle of seal-less connections from a fixed to a rotating frame of reference in the epicyclic toroidal coil planet centrifuge.

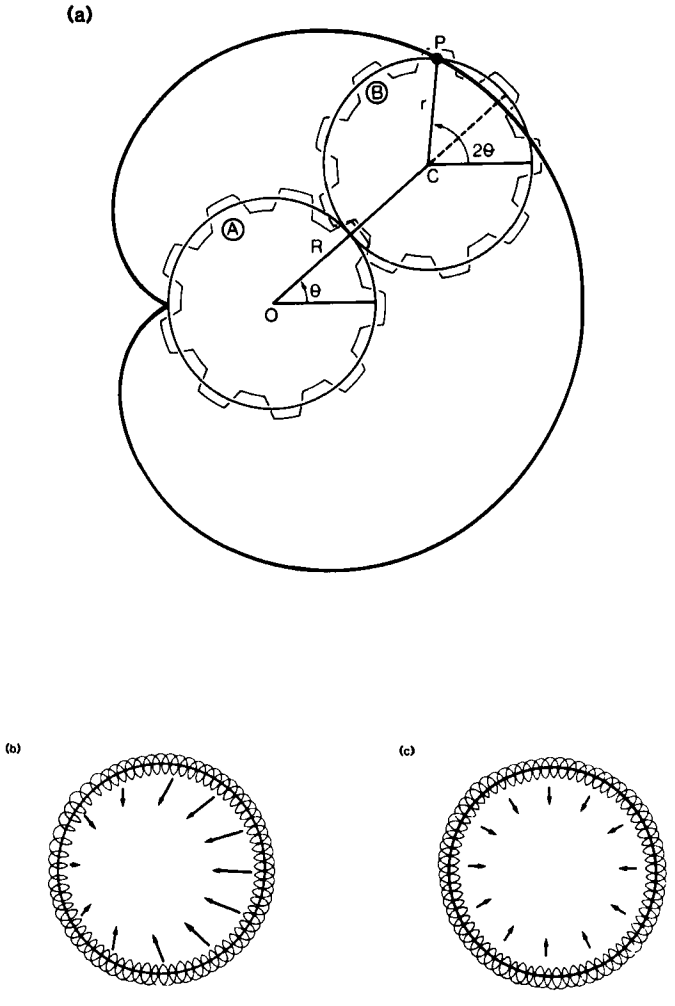

FIGURE 2

Schematic representation of the toroidal coil wound on the epicyclic coil planet centrifuge showing (a) the geometry of motion and the locus of the point "P" and (b) the associated acceleration field compared to (c) the uniform acceleration field on the conventional toroidal coil centrifuge.

2b (16). The acceleration vectors for the conventional toroidal coil centrifuge are shown for comparison in figure 2c. Here the vector is fixed relative to the coil and does not change in magnitude or direction. The heavier phase will be held in the outer segment of each coil unit by centrifugal action. Flow of the lighter phase will result in a series of cascades of one phase through the other. The same fluid behaviour mechanism will apply to both processes except that the epicyclic coil planet centrifuge will produce enhanced mixing of the phases that can be controlled by choice of the β value (the ratio of the coil radius "CP" to the planet radius "OC" in figure 2a). As the proportional variation of the direction and magnitude of the acceleration vector is greater at low β values (16) then a high β value will reduce mixing, while a low β value will increase mixing. In the limit as β becomes very large the epicyclic coil centrifuge will tend towards the toroidal coil centrifuge with its uniform acceleration field.

The toroidal coil is formed by winding PTFE tubing (1.65 mm external diameter and 1.07 mm internal diameter) into a 4.85 mm diameter flexible nylon former. The complete coil has 600 coil units and a total volume of 11 ml (excluding inlet and outlet leads). This is wound onto the 10 cm diameter drum of the epicyclic coil planet (figure 3) which has a rotational radius of 10 cm. The mean β value is 0.56 when allowance is made for the thickness of the toroidal coil. The rotor is mounted in a Beckman TJ6 refrigerated bench centrifuge (figure 4a). The rotor can operate at speeds varying from 400 to 1000 rev/min at 4°C.

Toroidal Coil Planet Centrifuge

A coil identical to that described above is mounted circumferentially on the rotating disc of a toroidal coil centrifuge (Sutherland (12) and Flanagan (22)), mounted in a floor standing Beckman J6 centrifuge (figure 4b). The effective radius is 21 cm, to give a uniform acceleration field (figure 2c) perpendicular to the coil axis.

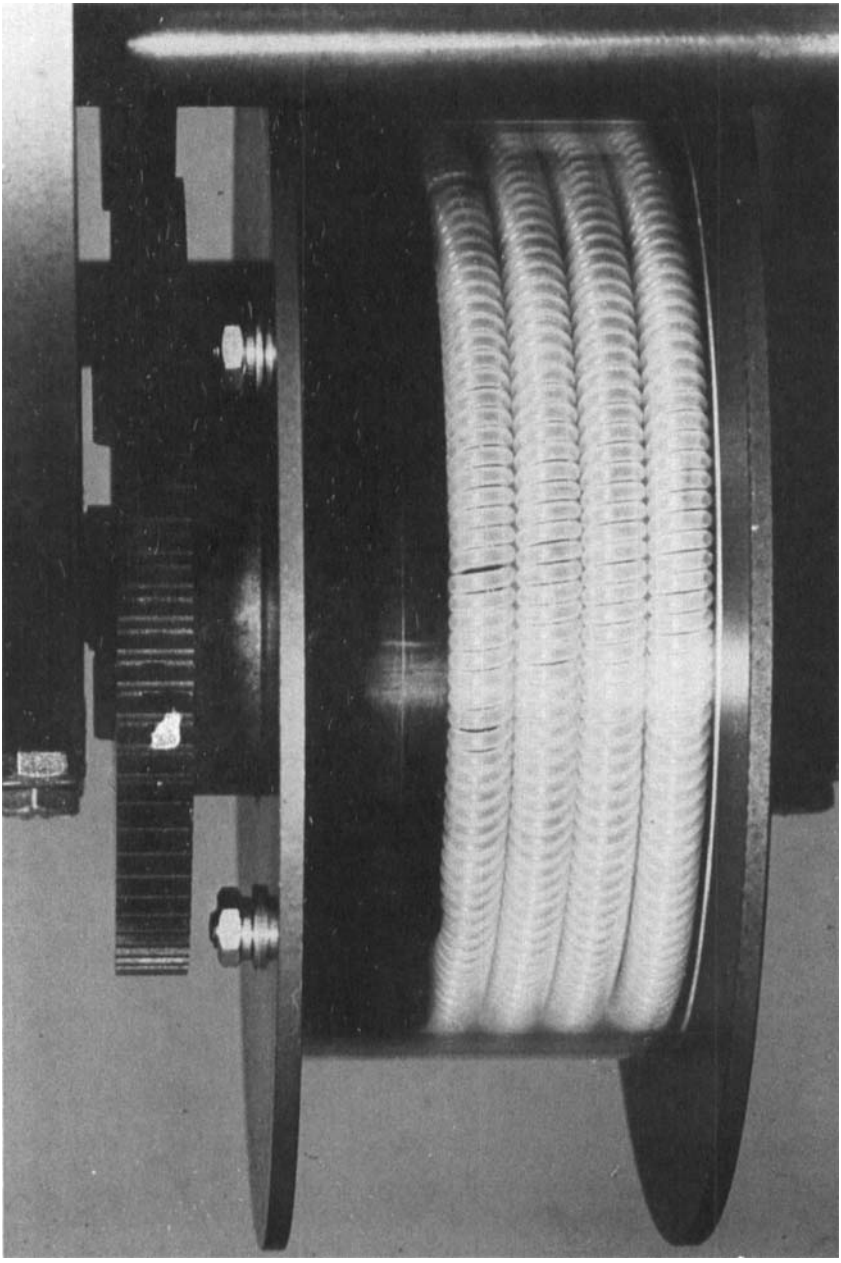

FIGURE 3 Detail of the mounting of the toroidal coil on the drum of the epicyclic coil planet centrifuge.

The Pritchard 17-transfer CCD Device

The Pritchard 17-transfer CCD device (21) is a small "gun-barrel" shaped unit with 18 coaxial chambers positioned circumferentially round a central axis (figure 4c). Mixing and transfer are accomplished manually while phase separation is enhanced by centrifugation. The device has been used by Morris (23) for membrane purification.

Bioshef Thin Layer Countercurrent Distribution Apparatus

The Bioshef Thin Layer Countercurrent Distribution Apparatus (TLCCD) is a unit gravity, 59 transfer unit providing phase separation times of up to 15 mins and mixing times of up to 60 secs (figure 4d). The design is based on the apparatus described by Albertsson (8) with the following additions. It can be operated with one or two sets of 60-chamber rotors for 59 automatically controlled partition steps.

A powerful hydraulic transfer mechanism allows the rotor halves to be clamped together firmly. This, combined with an improved rotor design which allows the contact surfaces to be lapped and polished, helps to prevent leakage between chambers. The whole apparatus is housed in a refrigerated cabinet.

MATERIALS AND METHODS

Materials

Dextran T 500 (batch 16027) was obtained from Pharmacia (Uppsala, Sweden). Breox poly(ethylene glycol) 6000 (PEG 6000) was obtained from Hythe Chemicals (Southampton, UK). All other reagents were standard AR grade.

Preparation of Phase Mixtures

Stock solutions of the polymers, 20% (w/w) dextran T 500 and 40% (w/w) PEG, were used to prepare the phase systems and stored at -20°C before use. An accurate determination of the dextran

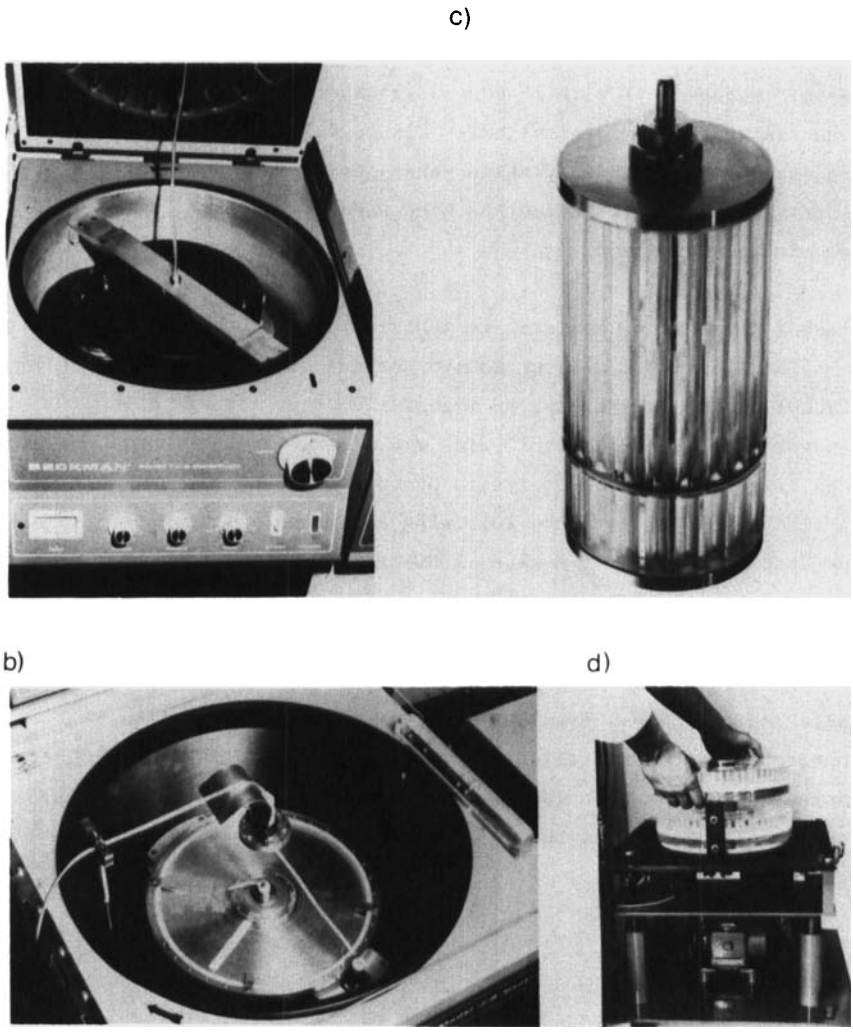

FIGURE 4

(a) The epicyclic toroidal coil planet centrifuge mounted in a Beckman TJ6 bench centrifuge; (b) The toroidal coil centrifuge mounted in a Beckman J6 centrifuge; (c) The Pritchard 17-transfer CCD device which is mounted in a Coolspin swinging bucket rotor (not shown) and (d) The Bioshef TLCCD rotors which are mounted in a refrigerated cabinet (not shown).

concentration was obtained by measuring optical rotation (model 141 polarimeter; Perkin-Elmer, Beaconsfield, Buckinghamshire, UK).

Phase systems (150 g) contained 3.3% (w/w) dextran T 500, 5.4% (w/w) PEG 6000, 10 mM-sodium phosphate/phosphoric acid buffer, pH 7.4, 0.26 M-sucrose, 0.05 mM-Na_2EDTA and 1 mM-ethanol. This phase system composition was established during previous studies on rat liver homogenate (23). The phase mixture was shaken and allowed to separate overnight at $4^{\circ}C$ into two phases. This phase system composition was used throughout except where otherwise stated.

Sample Preparation

Fed male Sprague-Dawley rats (150-200 g) were stunned and killed by cervical dislocation. The liver was immediately removed and 0.5 g of perilobular tissue was minced with a scalpel blade. The minced tissue was then disrupted in a medium sized (15 ml) Dounce homogenizer (Kontes Glass Co., Vineland, NJ, USA) in 10 ml of ice-cold PEG-rich upper phase. Water (0.4 g) had previously been removed by evaporation with a stream of N_2, to allow for the water content of the tissue. The tissue was disrupted with 10 strokes each of a loose-fitting (type A) pestle followed by a tight-fitting (type B) pestle. Fibrous material was removed with a 50 μm-mesh gauze filter.

Operation of the Epicyclic Toroidal Coil Planet Centrifuge

The coil was initially filled with dextran-rich lower phase and then rotated at 1000 rev/min. The mobile phase, comprising 94% PEG-rich upper phase, well mixed with 6% dextran-rich lower phase, was pumped into the coil at 14 ml/hr with collection of 1 ml fractions of the eluent. When the system reached equilibrium (i.e. when the eluent also contained 6% dextran-rich lower phase) the sample was injected via 4-way slider valve

(Altex). After collecting 40 fractions, the centrifuge was stopped and the contents pumped out with water. Fractions were stored at -20°C for subsequent analysis. All equipment was operated in a 4°C cold room.

The percentage of dextran T 500 in the phase mixture was changed from 3.3% (w/w) to 4% (w/w) to achieve a similar elution profile to that obtained with the toroidal coil (below). The β value (the ratio of the coil radius to planet radius) was 0.56.

Operation of the Toroidal Coil Centrifuge

The basic running procedure for the Toroidal Coil Centrifuge is the same as for the Epicyclic Toroidal Coil Planet Centrifuge. A complete description has been reported elsewhere (12).

Operation of the Bioshef 59-transfer TLCCD Apparatus

Each of the 60 chambers of the CCD rotor filled with 0.654 ml of equilibrated lower phase and 0.915 ml of the upper phase. Chambers 1 and 2 had an equivalent volume of homogenised sample added, instead of upper phase. A mixing time of 30 secs and a settling time of 7 mins were used. After 59 mixing-settling-transfer stages were completed, 0.25 M sucrose (0.654 ml) was added to each chamber to break the phase system. The rotor was shaken and the contents transferred to sample tubes. These were stored at -20°C for subsequent analysis. The CCD device was operated at a temperature of 4°C.

Operation of the Pritchard 17-transfer CCD Device

The operating procedure devised by Morris (20) was used. Homogenized sample (0.65 ml) was added with dextran-rich lower phase (0.65 ml) to chamber number 1, while both PEG-rich upper phase (0.65 ml) and dextran-rich lower phase (0.65 ml) were added to the remaining 17 chambers. The transfer plane was arranged to leave a stationary interface with approximately 0.6ml

of upper phase transferred. The contents of the device were mixed by 20 inversions, each chamber (6.35 mm internal diameter) containing a 3 mm diameter nickel ball bearing to enhance mixing.

Phase separation was achieved by centrifugation at 250 g for 5 minutes in a Coolspin centrifuge (Measuring and Scientific Equipment, Crawley, Sussex, UK), fitted with a specially constructed swinging bucket rotor (r_{av} = 20.5 cm). After each centrifugation step, the upper section of the device was rotated until the index line was aligned with the next adjacent chamber.

When 17 transfers were completed, sucrose (0.25 M, 0.40 ml) was added to each chamber and the contents thoroughly mixed and stored at -20°C.

Analytical Methods

The resolution of the organelles in the different apparati was determined by assaying the following marker enzymes: γ-glutamyltransferase, EC 2.3.2.2 (plasma membrane) (21); N-acetyl-β-glucosaminidase, EC 3.2.1.30 (lysosomes); neutral α- glucosidase, EC 3.2.1.20 (endoplasmic reticulum); and lactate dehydrogenase, EC 1.1.1.27 (cytosol) (22). All assays, with the exception of γ-glutamyltransferase, were performed on an Automatic Chemistry Unit (Aura System; Pye-Unicam, Cambridge, Cambs., UK) (23). These marker enzymes were chosen to represent constituents covering a wide range of partition ratios.

RESULTS

Figure 5 compares the fractionation of rat liver homogenate obtained (a) with the Bioshef thin layer CCD apparatus and (b) with the Pritchard CCD device. The results are plotted in reverse to the usual order with upper phase transfer to the left. This is to facilitate comparison with countercurrent chromatography methods

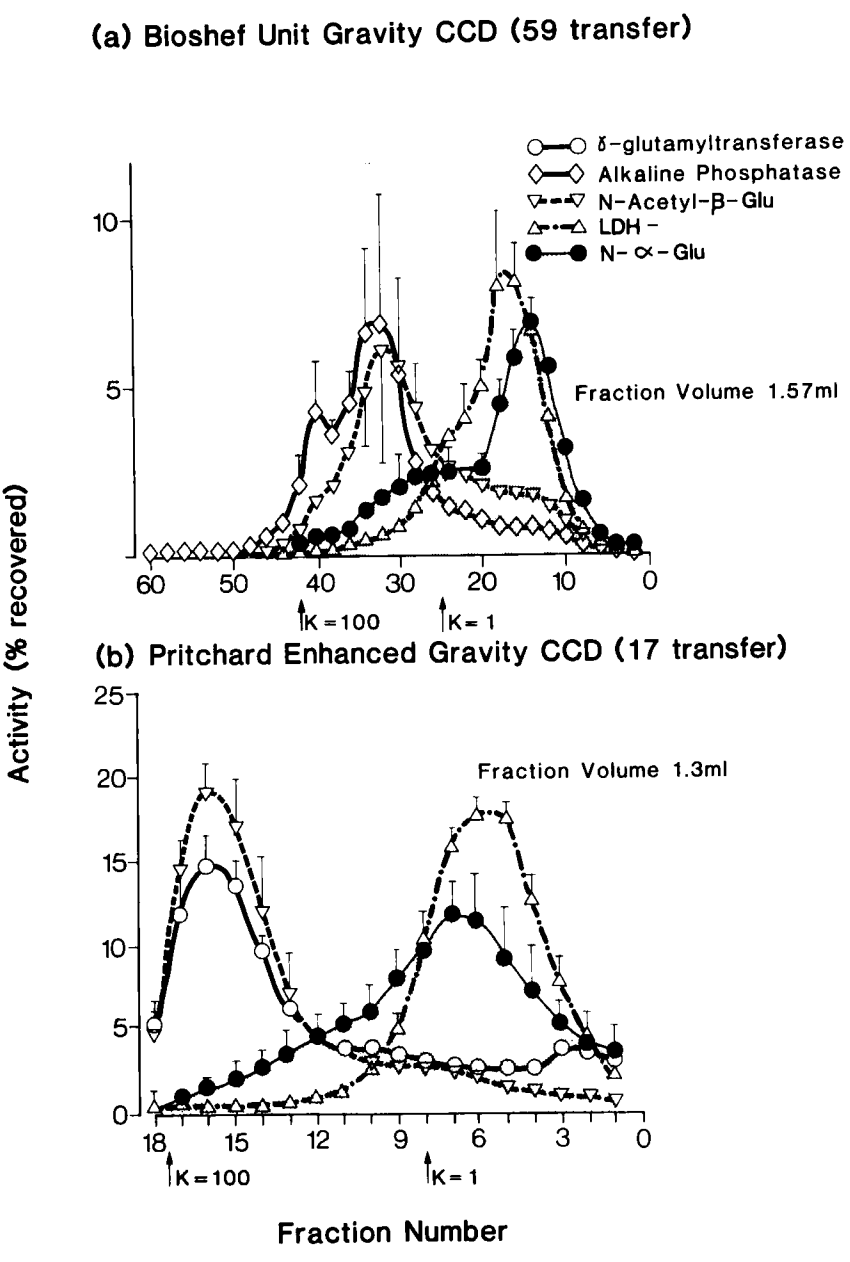

FIGURE 5

Fractionation of rat liver homogenate obtained using (a) the Bioshef thin layer CCD apparatus and (b) the Pritchard CCD device. Results show mean $\pm$ SD for (n) experiments with the Bioshef (n=3) and with the Pritchard (n=7). Recovered enzyme activities range from 70-90%.

which elute high partition coefficient material first. It is important to note the position of markers for k = 1 and k = 100. As the Bioshef is a thin film device, the proportion of upper phase retained with the lower phase following transfer is higher and so the progress of high k material along the transfer chain is reduced. The distribution of the marker enzymes are qualitatively similar but with the Bioshef profiles clearly showing greater resolution. The key points to note are the similarity between the peak positions of the plasma membrane and lysosome markers and the clear bimodality of the endoplasmic reticulum marker showing a component around k = 1 separate from its major low k component.

Figure 6 compares the same fractionation performed on (a) the toroidal coil centrifuge and (b) the epicyclic toroidal coil planet centrifuge. Note that the k = 1 and k = 100 markers are positioned differently from those for the CCD profiles. For CCD the k = 1 marker is approximately symmetrically placed between the k = 0 and k = ∞. As chromatographical methods are elution processes, the k = 1 point is biased more toward the point of elution (12). Allowing for this, qualitatively similar results are obtained with both CCC methods when compared to the Bioshef one. The high k component of the plasma membrane marker enzyme is lost in both CCC results due to the compression of the k scale, but the resolution between the plasma membrane and lysosome peaks is improved. The endoplasmic reticulum distribution is clearly divided into two or possibly three components.

It should be emphasised that when the identical phase system was used in the epicyclic toroidal coil planet centrifuge, all three particulate peaks eluted between fractions 5-10 with the soluble cytosol peak remaining in approximately the same position. The dextran content of the phase system was increased from 3.3% (w/w) to 4.0% (w/w) to obtain the result in figure 6(b). When the

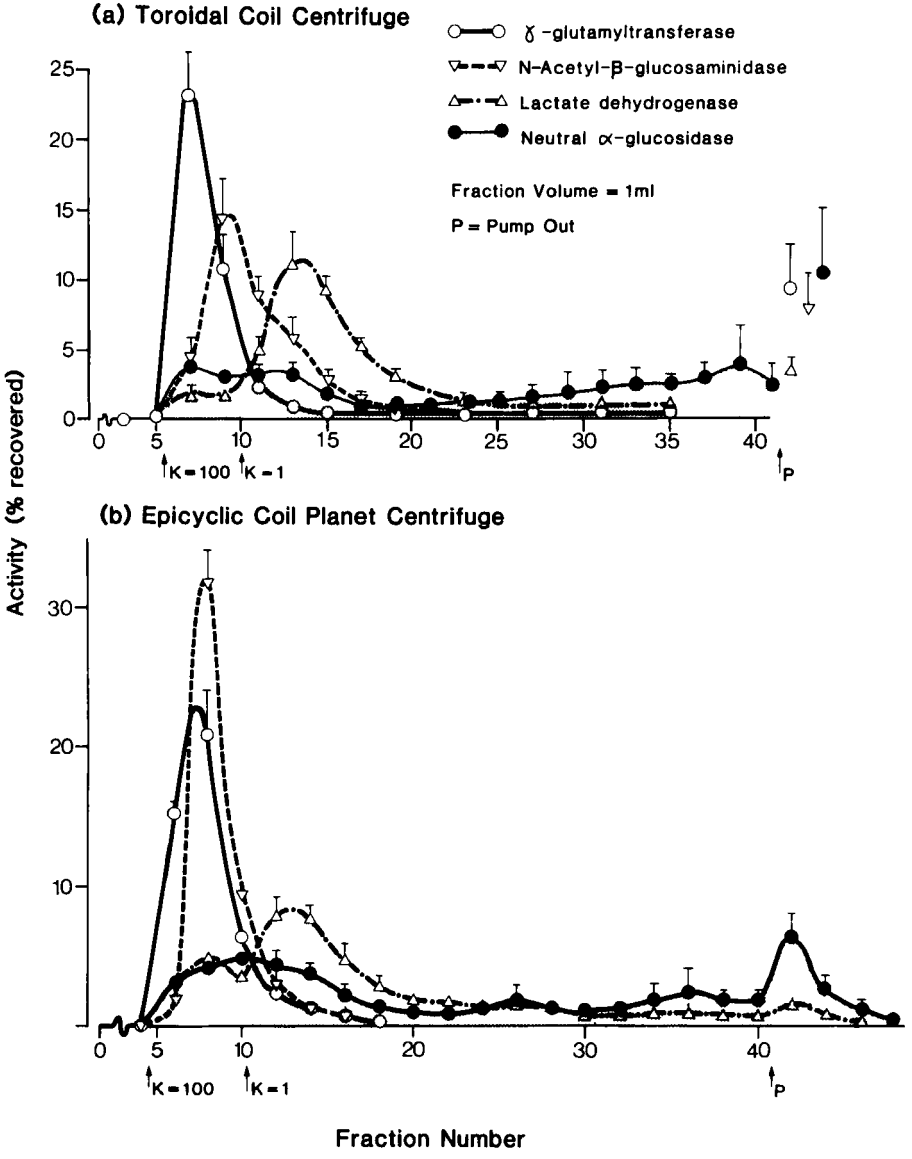

FIGURE 6

Fractionation of rat liver homogenate obtained using (a) the toroidal coil centrifuge and (b) the epicyclic toroidal coil planet centrifuge. Results shown mean $\pm$ SD for (n) experiments with the Toroidal Coil Centrifuge (n=3) and with the Epicyclic Toroidal Coil Planet Centrifuge (n=2). Recovered enzyme activities range from 70-90%.

epicyclic result is compared to the TCC one, the plasma membrane and lysosome peaks are sharper and less resolved while the endoplasmic reticulum profile appears resolved into even smaller subcomponents. Increasing the β value of the epicyclic CPC to 0.78 (result not shown) only affected the elution profile of the endoplasmic reticulum peak, with more activity eluting at higher fraction numbers.

DISCUSSION

Comparison of different two-phase separation processes is complicated by the recent observations by Raymond and Fisher (27) and Heywood-Waddington (28) that phase partition of particles is a dynamic process. For example, if the settling time for a given CCD experiment were lengthened particles in suspension in the upper phase attached to microdroplets would have more time to sediment to the interface resulting in a reduction in the apparent partition coefficient. The two CCD profiles shown in Figure 5 are in good qualitative agreement, but an analysis of the peak k values would reveal a number of quantitative differences that could be attributed to this dynamic effect.

This is an important consideration when assessing a continuous process like CCC where there is no settling time. The formation of droplets of one phase in the other and their re-formation is entirely a function of the mixing energies created by the fluid dynamic mechanism used. The fact that all particulate material eluted with the solvent front when the TCC phase system was used in the epicyclic coil planet centrifuge, suggests that the mixing energies are higher. This produces smaller sized microdroplets of dextran-rich phase which can be carried along by the mobile phase flow with loosely bound sample constituents. The fact that more endoplasmic reticulum is retained when the β value was increased to 0.78 (i.e. when the mixing energy is less due to a reduction in the magnitude and direction of the acceleration

vector) supports this theory. Increasing the dextran composition of the phase system increases the association of the sample with the interface resulting in a lower partition coefficient (29) and less loosely bound material on the microdroplets carried along with the mobile phase flow. The fact that retention of the endoplasmic reticulum can be obtained when the phase system is modified in this way suggests the epicyclic toroidal coil planet centrifuge is indeed separating organelles on the basis of the dynamic model of phase partition. Furthermore, the increased mixing of the phase systems appears to avoid the need for pumping a small proportion of the dextran-rich phase with the mobile phase resulting in a much simpler operating system. Whether this would apply to phase systems working very near to the critical point as discussed by Flanagan (21) remains to be tested.

Comparison of the toroidal coil results with the 59-transfer CCD suggests that an equivalent resolution of between 60-100 transfers is obtained with a 600 turn toroidal coil in either CCC system giving an overall efficiency of 10-16%. This low efficiency has previously been thought to be due to poor mixing in the toroidal coil (12), but the increased mixing in the epicyclic CPC does not seem to significantly improve resolution suggesting that some other mechanism may be causing this loss of resolution. Recently Heywood-Waddington (28) in her experiments on the dynamics of phase partition has found evidence of sample aggregation in the presence of the phase systems used suggesting another possible reason why resolution is reduced.

In conclusion, the epicyclic CPC offers a simpler method for continuous countercurrent chromatography, both in cost and in operation, which compares extremely favourably with CCD and TCC. This preliminary work indicates a need to concentrate future resources on developing the epicyclic toroidal coil planet centrifuge by thoroughly examining the separation variables and by developing phase systems that minimise aggregation problems.

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