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COUNTER-CURRENT CHROMATOGRAPHY

APPLICATIONS TO THE SEPARATION OF BIOPOLYMERS, ORGANELLES AND CELLS USING EITHER AQUEOUS–ORGANIC OR AQUEOUS–AQUEOUS PHASE SYSTEMS

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SUMMARY

Counter-current chromatography is a form of liquid–liquid chromatography which uses low-speed centrifugation to hold one phase of an immiscible liquid pair stationary while the other is eluted through it.

Two types of countercurrent chromatography are described: one suitable for preparative/analytical separation with aqueous–organic phase systems and the other for analytic fractionations using aqueous–aqueous phase systems.

Applications of both processes are described, ranging from the purification of antibiotics, pesticides, and peptides to the fractionation of whole cells.

INTRODUCTION

Counter-current chromatography can be considered either as liquid–liquid chromatography without a solid support or as a continuous form of liquid–liquid extraction. Since it was first introduced in the 1970s, the process has been through a number of development phases which have improved its design and efficiency. With few exceptions, all the schemes involve continuous coils of PTFE tubing, rotating in some form of planetary motion without rotating seals. The process is now being used for the analytical/preparative separation and purification of a wide range of natural products and soluble biopolymers with aqueous–organic phase systems and for analytical fractionations of membranes and organelles with double aqueous phase systems.

There are two essential requirements for successful countercurrent chromatography: the retention of one of the phases in the coil and adequate mass transfer of sample constituents, as the other mobile phase passes through and mixes with the retained phase. Successful retention is largely a function of the physical properties

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of the phase systems and interactions with the walls of the coils. Mass transfer depends on effective mixing, which is determined by hydrodynamic factors.

This paper reviews the latest counter–current chromatography techniques used for separations with both aqueous–organic and aqueous–aqueous phase systems.

COUNTER-CURRENT CHROMATOGRAPHY WITH AQUEOUS–ORGANIC PHASE SYSTEMS

All advanced forms of counter-current chromatography require some form of enhanced gravity to hold one phase of an immiscible liquid pair stationary while the other is eluted through it. Mixing takes place either by synchronous rotation of the

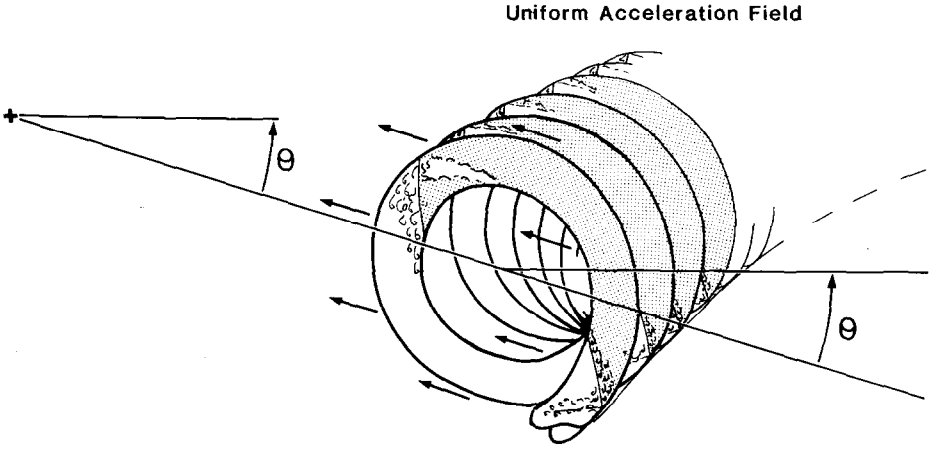


Fig. 1. Synchronous coil planet centrifuge—cascade mixing.

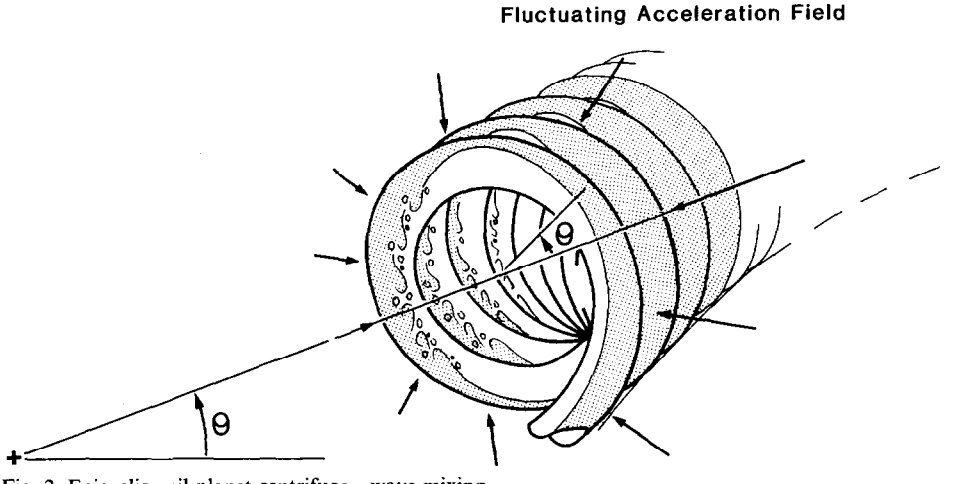


Fig. 2. Epicyclic coil planet centrifuge—wave mixing.

coils, which mixes the phases in a cascade fashion (Fig. 1) or by the relative stability of two layers of flow, as determined by a fluctuating force field (Fig. 2).

Up to the end of the 1970s the mixing methods were mainly of the synchronous variety[1–9]. Geometrical constraints limited retention to less than 50%, and the high mixing rates of these coil planet centrifuges, while enhancing resolution, were rather sensitive to carry-over and loss of the stationary phase. The process was therefore limited to analytical-scale, high resolution separations, biased towards the high-interfacial-tension range of phase systems.

By the late 1970s Ito had developed a new form of coil rotation (the epicyclic coil plant centrifuge), which fundamentally changed the mixing concept within each coil (Fig. 2). Instead of a succession of cascades, mixing was achieved between two thin layers of liquids in such a way that zones of mixing and settling travelled along the tubing coincident with areas of low and high force fields, set up by the epicyclic motion of the coils[10–12].

The time between successive mixing and settling cycles is typically between 50 and 100 ms, and the phase systems have to be able to respond to these fast changes without emulsification. Consequently, this particular scheme is limited to use with aqueous–organic phase systems where these physical properties at enhanced gravitational fields of between 100 and 200 g are such that these response times can be achieved.

The prime benefits of this new motion are:

(1) increased retention of stationary phase (up to 90%);

(2) faster flow-rates with minimal loss of retention;

(3) greater stability, allowing use of a wider range of lower interfacial tension phase systems;

(4) greater capacity, the sequence of phase mixing and settling is independent of tubing size and can therefore be scaled up;

(5) simplicity and ease of manufacture.

Description of motion

The motion of the epicyclic coil planet centrifuge is shown schematically in Fig. 3. Its major advantage is that the planetary mechanism is simple and can be mounted in a bench centrifuge.

The coil is mounted circumferentially around a drum, attached to gear "B" (Fig. 3a) such that the locus of the point "P" on the periphery of the coil prescribes a cardioid. This produces an acceleration vector that varies in both magnitude and direction, as shown in Fig. 3b[12]. The position of coils on the drum is defined by the β value (where $\beta = r/R$).

Liquids can be passed to and from the rotating coils without the use of rotating seals, provided the input/output tubes are passed through the center line of the centrifuge at "O" (Fig. 3a), and then turn through 180 degrees, reentering the planetary system along its center line at point "C".

The coils are initially filled with the intended stationary phase. Mobile phase is then pumped in, while the coils are rotating. Operation proceeds in a similar way to a standard chromatography process, the sample being injected with the mobile phase and eluted into a fraction collector.

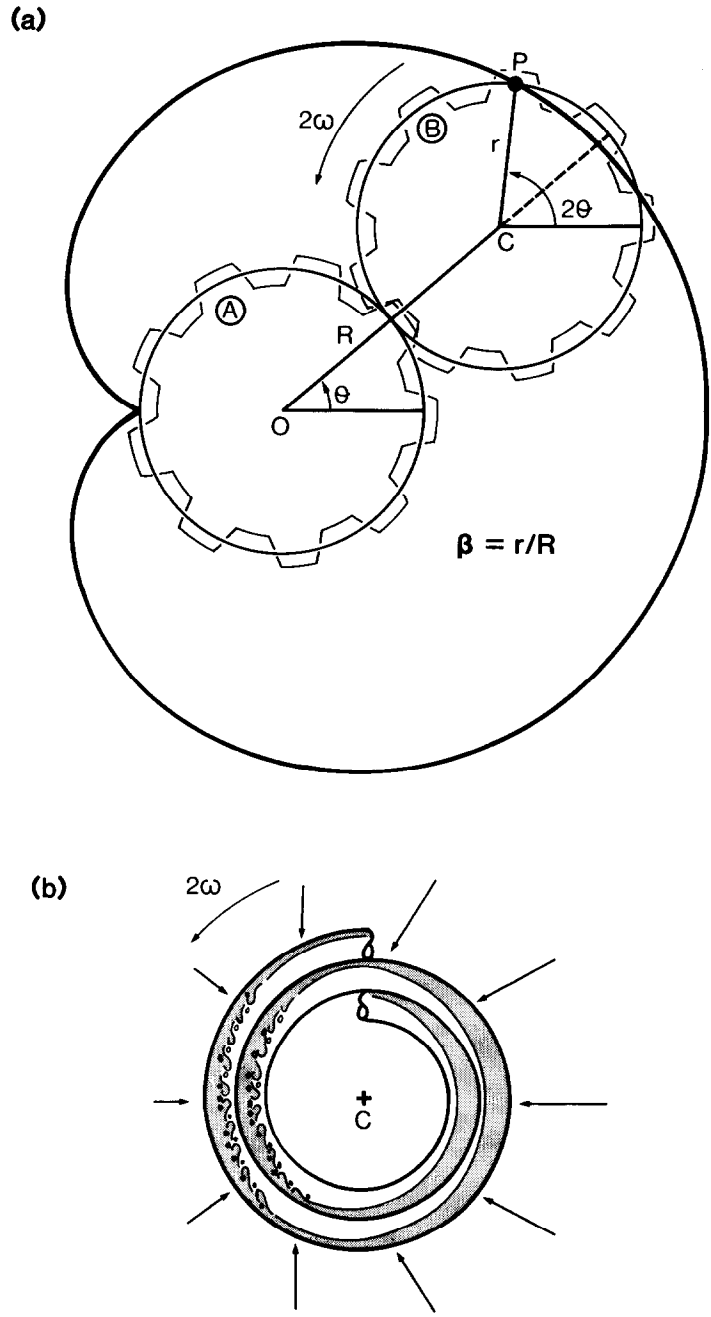
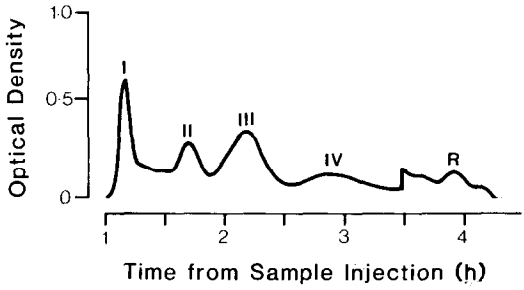


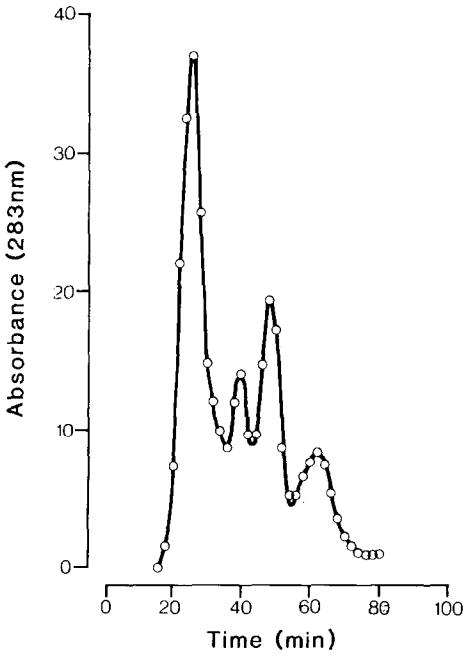
Fig. 3. The principle of motion showing (a) the cardioid locus of a point "P" and (b) the effect on the phase system of a fluctuating force field.

Applications

Using aqueous–organic phase systems, an example of the recent improvement in both resolution and throughput in countercurrent chromatography can be given for the work on purifying polyene macrolide antibiotics[13,14], where a separation of candicidin on an early synchronous coil planet centrifuge is compared with the same separation on the epicyclic coil planet centrifuge (Fig. 4a and b). Similar resolution



(a) Synchronous Coil Planet Centrifuge



(b) Epicyclic Coil Planet Centrifuge

Fig. 4. The fractionation of candicidin, a polyene antibiotic on (a) the synchronous coil planet centrifuge and (b) the epicyclic coil planet centrifuge. Conditions: (a) Solvent, chloroform–methanol–water (4:4:3); sample size, 0.3 ml; concentration, 1.0 mg/ml; speed, 500 rpm; flow, 60 ml/h; retention, 40%; column capacity, 110 ml. (b) Solvent, chloroform–methanol–water (4:4:3); sample size, 100 mg; concentration, 10 mg/ml; speed, 800 rpm; flow, 240 ml/h; retention, 57%; column capacity, 285 ml.

is achieved in a fraction of the time and with greater throughput by means of the new form of rotation.

Recently, the capability of the present method has been further demonstrated in separation and purification for broad spectrum of samples which include many other antibiotics[15,16], steroids[17], pesticides[18,19], and herbicides[20], plant hormones[21], pigment[22], tannins[23], DNP-amino acids[24,25], a variety of biologically active peptides[25–28], etc.

While preliminary studies[29,30] have shown that, in principle, scale-up is possible, the widespread application of the technique and full industrial scale-up will only be achieved if the basic factors affecting both resolution and throughput are more clearly understood. The studies of Ito and Conway on a wide range of phase systems in the epicyclic coil planet centrifuge[31] have identified viscosity as being an important factor determining which phase is retained in the coil. Nevertheless, studies on the hydrodynamics of the phase systems have been limited[32–34], and the fundamental reasons governing the retention of one phase in preference to the other are still not understood[30].

COUNTER-CURRENT CHROMATOGRAPHY WITH DOUBLE AQUEOUS-PHASE SYSTEMS

Principle

When certain polymers are mixed with water, two immiscible aqueous phases can be formed that can, with suitable additives, provide a hospitable medium for cells or organelles[35,36]. Unfortunately, these aqueous-phase systems have an increased viscosity, lower density difference, and considerably reduced interfacial tension, when compared to aqueous/organic phase systems. This makes them unsuitable for use in the conventional, high-resolution coils just described, due to their long mixing/settling cycle times. However, this does not preclude their use with countercurrent chromatography on an analytical scale. Special torodial coils can be mounted perpendicular to the force field. A mixing scheme similar to the one in Fig. 1 is used, except that the coils are fixed relative to the acceleration vector, and cascade mixing is produced simply by one phase flowing relative to the other[37].

Coils can be wound around the drum of an epicyclic coil planet centrifuge[38] or, alternatively, mounted circumferentially on a rotating disc (Fig. 5). The coil is initially filled with one of the phases. The plate is rotated at 1000 rpm while the other phase is pumped in. As the phases mix, centrifugal force ensures that the lighter and heavier phases are retained in the inner and outer halves of each coil unit, respectively. The pumped phase progressively displaces the other phase from the coils until an equilibrium is established. Continuous pumping of the mobile phase sets up a series of cascades (much like waterfalls) through the retained segments of the other phase in each coil unit.

The sample is injected with the mobile phase, using a conventional liquid chromatography sample loop, and undergoes a series of mixing and settling steps before it is eventually eluted into the fraction collector. Sample components partitioned toward the mobile phase will be eluted early, while components favoring the stationary phase or interface will be retained. As there is no solid support, either phase can be used as the mobile phase or even a mixture of the two. Adding a small proportion of the stationary phase in the mixture in the above example would accelerate the

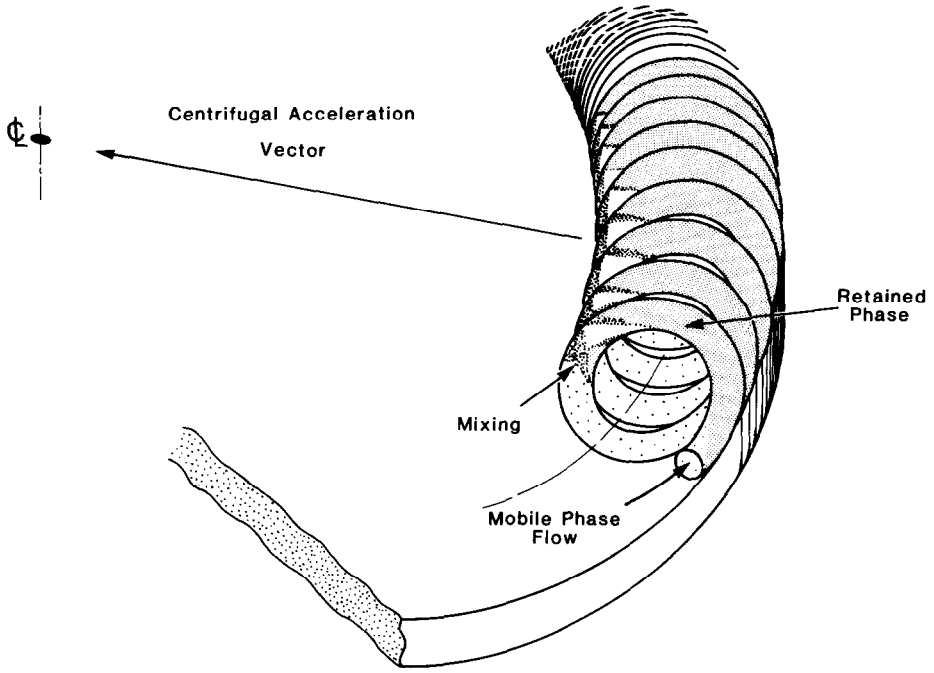


Fig. 5. Schematic diagram of the torodial coil rotor.

elution of all the retained components and clear the coil system for another sample loading.

Applications using double aqueous-phase systems

Rat liver homogenates. Both the toroidal coil and epicyclic coil planet centrifuges have been used for subcellular particle fractionations. For example, rat liver homogenate has been successfully fractionated on both machines, using a phase system containing 3.3% (w/w) dextran T 500, 5.4% PEG 6000, 10 m*M* sodium phosphate–phosphoric acid buffer (pH 7.4), 0.26 *M* sucrose, 0.05 m*M* Na_2EDTA, and 1 m*M* ethanol[38].

Sample preparation and enzyme assay procedures are outlined in detail by Heywood-Waddington *et al.*[39] and Sutherland *et al.*[37]. Fractionations in either machine are qualitatively similar, the plasma membrane being eluted early, lysosomes shortly afterwards, and endoplasmic reticulum spread over possibly three fractions. Heywood-Waddington *et al.*[39] used rat liver homogenate in a standard fractionation for studying a number of operating parameters, such as rotational speed, flow-rate, coil geometry, and sample loading. The process was shown not to be critical, and small changes in these parameters did not significantly affect the order of elution or resolution of the process, provided certain boundary conditions were met.

Torpedo membranes. Torpedo electroplax membranes, enriched in nicotinic cholinergic receptor sites have been successfully purified by Flanagan *et al.*[40] by affinity partitioning techniques with phase systems operating near the critical point.

Flanagan *et al.*[40] have elegantly demonstrated the power of linking affinity partition with counter-current chromatography and supports his case by performing a complementary study with thin-layer counter-current distribution techniques. He has also made major contributions by defining the procedures for operating the toroidal coil near the critical point, and examining the effects of sample loading. He found that it was essential to pump an emulsion of upper and lower phases (approximately in the ratio 10:1) to effect any elution of material at all, unlike in the phase system used for the rat liver fractionation, where it was optional. He also concluded that sample loading was limited not by the process but by the ability to obtain a sufficiently concentrated sample.

Bacterial cells. Bacterial cells are approximately 1 μm in diameter, and their separation presents a borderline choice between the toroidal coil centrifuge or the non-synchronous coil planet centrifuge[41]. While these cells have been successfully separated on the toroidal coil planet centrifuge[42], the non-synchronous centrifuge was used in the following examples, on the purification of different strains of *Escherichia coli* and the separation of *Salmonella typhimurium* cells.

The partition behavior for two different strains of *E. coli* in a PEG–dextran

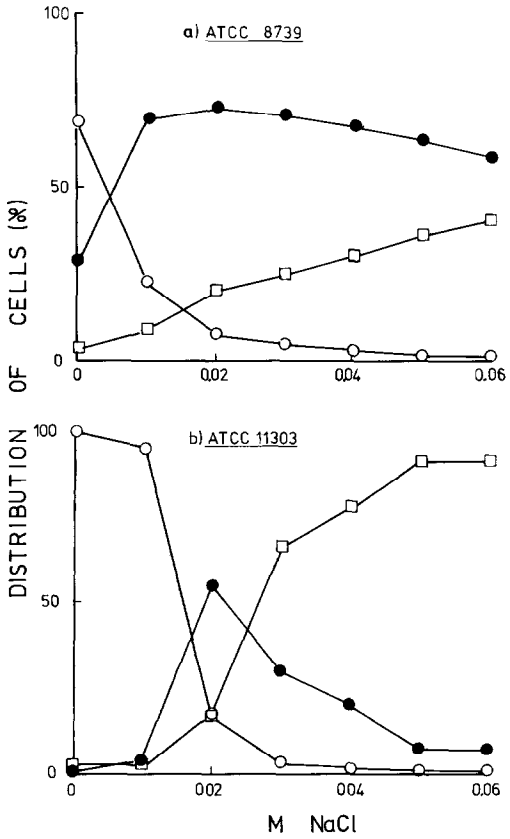


Fig. 6. The distribution of two different strains of *E. coli* in a two-phase polymer system. (○) In upper phase; (●) at interface; (□) in lower phase.

phase system is illustrated in Fig. 6 for various sodium chloride concentrations. The basic phase system used consisted of 5% (w/w) dextran 500, 4% (w/w) PEG 6000, and 0.01 *M* potassium phosphate (pH 6.9). Both strains were partitioned towards the upper phase at zero sodium chloride concentration. As the sodium chloride concentration increased, *E. coli* I started to partition predominantly toward the interface at a concentration greater than 0.005 *M*, while *E. coli* II exhibited the same behavior above a sodium chloride concentration of 0.015 *M*.

A 200-coil column of 1-mm I.D. PTFE tubing was used. It was initially filled with the heavier phase, and the centrifuge was set to rotate at 750 rpm with a coil rotation of 5.25 rpm. The flow-rate was 14 ml/h.

The *E. coli* cells of Fig. 6a, injected with the mobile phase, were eluted at the solvent front when no sodium chloride was present, but were retained in the column when a sodium chloride concentration of 0.06 *M* was used. This signified that a gradient separation was feasible. The column was then filled with the heavier phase at 0.02 *M* sodium chloride concentration. A mixture of the two *E. coli* strains (Fig. 6) was injected with the mobile phase. In order to produce a sodium chloride gradient (0.02–0.00 M), the mobile upper phase was made up with zero sodium chloride concentration and fed via a 5-ml continuously stirred mixing chamber (initially filled

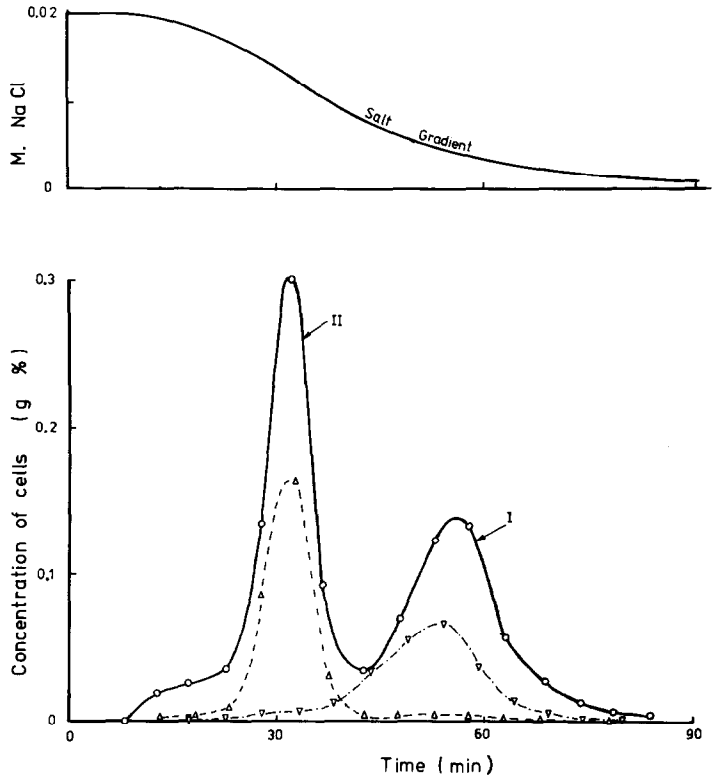


Fig. 7. A gradient separation of a mixture of two strains of *E. coli* cells by use of the non-synchronous coil planet centrifuge. Phase system, 5% PEG, 4% dextran. I = ATCC 8739; II = ATCC 11303. (○) Mixture of I and II; (▽) re-run of I; (△) rerun of II.

with upper phase containing 0.02 *M* sodium chloride concentration) before being pumped into the column.

The cells were eluted in two populations (Fig. 7). The most concentrated fractions from each peak were collected and rerun separately with up to 93% reproducibility in both cases. Earlier runs with the individual pure *E. coli* strains produced single peaks at the expected positions. Separations were repeated and found to be reproducible.

Counter-current chromatography on the non-synchronous coil planet centrifuge without rotating seals[43] was used to separate cells of *Salmonella typhimurium*, which have identical surfaces except for the proportion of lipopolysaccharide (LPS) molecules with long *versus* short polysaccharide chains[44].

Human erythrocytes. Sutherland and Ito have demonstrated that the non-synchronous coil planet centrifuge could be used for larger cells by separating different species of red blood cells[41]. They demonstrated that cells of varying size could be separated on the basis of their partition behaviour and were not affected by sedimentation. These cell separations were achieved under operating conditions similar to the ones previously described, except that the process was stopped before elution, and the coil contents were pumped out. When the process was extended beyond elution, peaks would spread out, possibly due to sedimentation effects in the outlet tubing.

Other research workers[45] have reported difficulties in reproducing these results, partly due to discrepancies in phase-system composition, and partly due to the fact that this is a non-elution process. Clearly, if these barriers were overcome, a promising new cell separation process could emerge.

CONCLUSIONS

Counter-current chromatography is easy to use, has a wide range of applications and is suited to automation. But its major asset is low cost and high sample recovery rates. The absence of a solid support minimizes adsorption problems and allows one set of coils to be used with a variety of different phase systems.

Counter-current chromatography with aqueous–organic phase systems has the potential of becoming a preparative-scale alternative to high-performance liquid chromatography for the separation of peptides and antibiotics. The principle of phase mixing and settling in the epicyclic coil planet centrifuge has the potential for further scale-up, once fundamental aspects concerning the hydrodynamics are understood.

The counter-current chromatographic techniques used for aqueous–organic phase systems cannot be applied directly for use with polymer phase systems, due to their high viscosity and low density difference. Consequently, its application has so far been restricted to analytical methods with toroidally wound coils that enhance retention of these viscous phases.

Counter-current chromatography is an emerging technique which is far from optimized. Phase system requirements are only beginning to be understood. One way for the technology to move forward is for new biocompatible phase systems to be identified with physical properties more suited for use with counter-current chromatography.

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