Subcellular fractionation of rat liver homogenates using two-polymer phase systems in a toroidal-coil centrifuge

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The principal organelles of rat liver homogenates were fractionated by two-phase partition chromatography using toroidal-coil centrifugation with a mixture of dextran T 500 and poly(ethylene glycol) 6000 in 0.26 M-sucrose containing 10 mM-sodium phosphate/phosphoric acid buffer, pH 7.4. The effects of varying the following parameters on organelle elution profiles, as reflected by their marker-enzyme activities, were studied: centrifuge speed; the composition and relative proportion of dextran-rich and poly(ethylene glycol)-rich phases in the eluent; flow rate; sample volume; homogenate concentration; helix diameter; tubing bore and the number of loops in the coil. Optimal resolution of the organelles was achieved with a toroidal coil of internal diameter 1.07 mm with a 4.55 mm helix diameter on a 0.42 m-diameter rotor running at 1000 rev./min. The eluent was prepared by combining, in a ratio of 93:7 (v/v), the poly(ethylene glycol)-rich upper phase and dextran-rich lower phase obtained from a phase mixture containing 3.3% (w/w) dextran and 5.4% (w/w) poly(ethylene glycol). The flow rate of the eluent was 14 ml/h. Optimal conditions for separation of the organelles were evaluated. Resolution of plasma membrane and lysosomes was achieved. Separation of endoplasmic reticulum, which showed marked heterogeneity, from plasma membrane was also demonstrated. DNA and marker enzymes for peroxisomes, mitochondria and cytosol showed distinct elution profiles.

Partition in aqueous two-phase polymer systems has been used for the separation of macromolecules, cell particles and intact cells (Albertsson, 1971). Depending on the choice of phase system, separation can occur on the basis of surface charge, hydrophobic properties or ligand affinity (Albertsson *et al.*, 1982; Fisher, 1981). This provides an alternative means of organelle isolation to that of other techniques, including centrifugation. In particular, two phase partition offers the advantage of not exposing the organelles to the high osmotic and hydrostatic pressures encountered in high-speed-centrifugation procedures (Bronfman & Beaufay, 1973; Wattiaux, 1974).

Rat liver homogenates have been fractionated by two-phase partition using a discrete counter-

current-distribution technique (Morris & Peters, 1982). The apparatus used (Pritchard *et al.*, 1975) is limited in the number of possible transfers and the amount of tissue that may be fractionated. It is also time-consuming to use, but the results were encouraging in that organelles that are difficult to separate by centrifugation techniques were readily resolved by two-phase partition. A recent development, the toroidal-coil centrifuge, has been designed to allow a continuous flow method of countercurrent partition (Sutherland *et al.*, 1983). This differs from discrete transfer-step countercurrent-distribution systems in having a high theoretical number of transfers and the potential for a fully automated partitioning system capable of processing large quantities of sample.

In the present study, rat liver homogenate has been fractionated with the toroidal-coil centrifuge and the effects of varying operating conditions on resolution examined. When fully developed, the

Abbreviations used: PTFE, polytetrafluoroethylene; PEG, poly(ethylene glycol).

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technique should prove useful for the isolation of various membranous components and studies on organelle alterations in experimental liver disease.

Experimental

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, U.K.). All other reagents were standard AR grade.

Preparation of phase mixtures

Phase systems (150 g) were prepared containing dextran T 500, 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. When a phase system had equilibrated to 4°C, it was shaken and allowed to separate overnight into two phases.

Previous work (Morris & Peters, 1982) has shown that optimum fractionation of rat liver homogenate is obtained with concentrations of 5.8% (w/w) dextran and 4% (w/w) PEG 6000 in the phase system. This phase mixture yields an almost equal volume of dextran-rich lower phase and PEG-rich upper phase. This is wasteful for use in the toroidal-coil system, where only a small volume of the dextran-rich phase is necessary. A tie-line§ of the phase system was constructed (Hartman *et al.*, 1974) in order to determine a composition that gives the required volume ratio (1:4). The concentration of dextran T 500 was varied from 2.67% (w/w) to 3.5% (w/w) while that of PEG was kept at 5.4% (w/w). This was necessary, since points on Morris & Peters (1982) original tie-line did not give an adequate organelle separation, owing to changes in dextran batch. The standard phase system chosen for studies reported in the present paper contained 3.3% (w/w) dextran T 500 and 5.4% (w/w) PEG 6000.

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 between use. An accurate determination of the dextran concentration was obtained by measuring optical rotation (model 141 polarimeter; Perkin-Elmer, Beaconsfield, Bucks., U.K.).

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 peri-

lobular tissue was minced with a scalpel blade. The minced tissue was then disrupted in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) 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 liver tissue. The tissue received 10 strokes of a loose-fitting (type A) pestle followed by 10 strokes of a tight-fitting (type B) pestle. Fibrous material was removed by passing the homogenate through a 50 μm -mesh gauze filter. This homogenate was mixed with dextran-rich lower phase in the same proportion as in the eluting mixture. Routinely 1 ml of this preparation was used as the sample. The effects of increasing the weight of tissue in the homogenate from 0.25 g to 2.5 g, and decreasing the sample volume from 1 ml to 0.2 ml, were explored.

Operation of toroidal-coil centrifuge (Fig. 1)

The toroidal coil consists of PTFE tubing [internal diameter (i.d.). 1.07 mm] helically wound on to a former (outer diameter 4.55 mm). The coil, approx. 550 loops, is mounted around the circumference of a rotating plate (diameter 0.42 m). A greater number of loops can be obtained by increasing the number of circumferences covered by the coil.

The standard operating conditions were as follows. Initially, with the plate stationary, the coil was filled with dextran-rich lower phase. The plate was then rotated at 1000 rev./min in a refrigerated centrifuge bowl (J6; Beckman, High Wycombe, Bucks., U.K.) at 4°C. PEG-rich upper phase containing 7% (v/v)-dextran-rich lower phase was then pumped at 14 ml/h into the rotating coil with a multichannel peristaltic pump (Gilson Minipulse 2; Anachem, Luton, Beds., U.K.); the proportion of the two phases was determined by the relative cross-sectional areas of the peristaltic tubing. Mixing of the two phases occurred in a fine-bore (0.5 mm) counterflow mixing chamber; the resulting mixture will be referred to as the 'eluting mixture'. To ensure that the eluting mixture contained no air and was of stable composition, the pump was run for about 20 min before connection to the coil.

The eluting mixture displaced only some of the more-dense dextran-rich phase, a proportion being retained by centrifugal action. When the system reached equilibrium (i.e. the proportion of phases in the output mixture was similar to that in the input mixture), the sample was injected into the coil by means of a four-way valve (Altex; Anachem). As the eluting mixture flowed through the coil it mixed with, and separated from, the retained dextran-rich phase in each loop in a similar way to a single-step of a countercurrent-partition experi-

§ A line on the phase diagram along which the composition of the two phases remains the same but their volume ratio varies.

Fig. 1. Schematic lay-out of toroidal-coil centrifuge operating system
 ng is relative centrifugal acceleration, typically $n = 250$ and $g = 9.81 \text{ m} \cdot \text{s}^{-2}$.

ment. Thus organelles in the sample with a high partition coefficient flowed through with the eluting mixture and were eluted first, whereas the elution of those with a low partition coefficient was delayed. A fraction collector (Gilson TDC80; Anachem) was used to collect the eluent in 1 ml fractions. After collection of 30–40 ml, the centrifuge was stopped and the coil contents pumped out with water. The volume of each phase in each fraction was determined by measurement of interface position and total sample height. The fractions were stored at -20°C for subsequent analysis. All apparatus was maintained in a cold-room at 4°C .

The following operating conditions were varied in turn: proportion of dextran-rich phase in eluting mixture from 0 to 14.5% (v/v); flow rate from 6.5 to 28 ml/h; rotational speed of coil from 0 to 1500 rev./min; tubing i.d. from 0.71 to 1.38 mm; helix diameter from 2.94 to 6.46 mm; number of

loops in coil from 550 to 1400. When each parameter was varied the remaining ones were as described above, except in two cases. When the number of loops was increased, a tubing i.d. of 1.38 mm was used. Where the tubing i.d. was altered, a constant linear velocity through the coil was maintained by adjusting the flow rate. The equipment used was the same during the study of all variables, except for the flow-rate variations when two high-pressure liquid-delivery modules (Gilson 302; Anachem) were used. This was necessary, since flow-rate alterations on the peristaltic pump led to a variation in the proportion of dextran-rich phase in the eluting mixture due to pressure changes. The high-pressure liquid-delivery modules were also used when the number of loops in the coil was increased. Full details of the technical aspects of the equipment are reported elsewhere (Sutherland *et al.*, 1983).

Analytical methods

Sucrose solution (0.25M, 0.15ml) was added to each fraction before analysis. The distribution of organelles was determined by assaying the following marker enzymes in alternate fractions: γ -glutamyltransferase, EC 2.3.2.2. (plasma membrane) (Smith *et al.*, 1979); β -N-acetyl-D-glucosaminidase, EC 3.2.1.30 (lysosomes); neutral α -D-glucosidase, EC 3.2.1.20 (endoplasmic reticulum); lactate dehydrogenase, EC 1.1.1.27 (cytosol); malate dehydrogenase, EC 1.1.1.37 (cytosol/mitochondria); and catalase, EC 1.11.1.6 (peroxisomes) (Peters & Shio, 1976). DNA was assayed by the method of Kapuscinski & Skoczylas (1977). All assays, with the exception of those for γ -glutamyltransferase and DNA, were performed with an Automatic Chemistry Unit (Aura System; Pye-Unicam, Cambridge, Cambs., U.K.).

Results

Figs. 2–8 show the effects of altering the value of various parameters on the distribution profiles of the marker enzymes for plasma membrane, lysosomes, endoplasmic reticulum and cytosol. These enzymes were chosen because they mark for organelles with high, low and intermediate partition ratios in the phase system used. The sample was injected corresponding to fraction number 0 in all cases, and P represents the point at which the coil was brought to rest and the entire coil contents pumped out and collected. The recovery of enzyme activities was 70–100% and tended to be lowest in the triple-coil experiments, suggesting that loss of activity may be due to denaturation or adsorption of protein to the coil surface.

Effect of polymer concentration on elution profiles (Fig. 2)

The effect of increasing the concentration of dextran in the phase mixture on the partition of the organelle marker enzymes is shown in Fig. 2. At higher dextran concentrations a second component of the endoplasmic-reticulum marker, with a lower partition ratio, was resolved. The partition ratio of this component progressively decreased as the dextran concentration was raised, to the extent that at 3.5% (w/w) dextran it had not eluted by the time the entire coil contents were pumped out. Optimal resolution was achieved with a 3.3% (w/w) dextran concentration, a value that was used in all subsequent experiments.

Effect of rotor speed on organelle resolution (Fig. 3)

Fig. 3 compares the distribution of organelle marker enzymes after toroidal-coil chromatography in which the rotor was run at various

Fig. 2. Effect of phase-mixture composition on organelle resolution

The percentage of recovered enzyme activity was plotted against fraction (1.0ml) number. P corresponds to pump-out, while the coil is stationary. Marker enzymes, with organelle shown in parentheses, are: ----, γ -glutamyltransferase (plasma membrane/biliary canaliculi); ····, β -N-acetyl-D-glucosaminidase (lysosomes); - · - ·, lactate dehydrogenase (cytosol); and □, neutral α -D-glucosidase (endoplasmic reticulum). Extra abbreviation used: D, dextran.

speeds. With the rotor stationary, the elution profiles of the four marker enzymes were identical, since partitioning cannot take place without lower-phase retention. Optimal resolution of the organelles, particularly of the plasma-membrane and lysosomal markers, occurred with rotor speeds of approx. 1000 rev./min. Increasing the rotor speed had little effect on the peak positions, although at the highest speed the plasma-membrane marker peak was delayed, causing a loss in resolution of

Fig. 3. Effect of rotor speed on marker-enzyme elution profiles
Details are as in Fig. 2.

Fig. 4. Effect of proportion of dextran-rich phase in eluting mixture on organelle distributions
Details are as in Fig. 2.

plasma-membrane and lysosomal markers. Also, greater amounts of membrane-bound activity were retained in the coil, probably accounting for the decreasing peak size of the early-eluted neutral α -D-glucosidase peak. In all subsequent experiments a rotor speed of 1000 rev./min was used.

Effect of proportion of dextran-rich phase in eluting mixture on organelle resolution (Fig. 4)

Fig. 4 shows the profiles when eluting mixtures containing between 0 and 14.5% dextran-rich phase were used. Material with low partition was retained in the coil when there was no dextran-rich phase in the eluent. Also, the enzyme marker peaks at the front of the extraction chain were not well separated. As the proportion of dextran-rich phase in the eluting mixture was increased, the low-partition-ratio endoplasmic-reticulum marker

peak was eluted progressively earlier. Optimal resolution of the organelles, particularly of endoplasmic reticulum, was obtained with a PEG/dextran-rich phase ratio of 93:7 (v/v).

Effect of flow rate on elution profiles (Fig. 5)

Fig. 5 shows the enzyme distribution in the coil fractions when various pump rates were employed. The increasing flow rate progressively decreased the span of sample elution. At low flow there was least resolution between lysosomal and plasma-membrane marker enzymes due to the earlier elution of the lysosomal marker. At high flow there was movement of the low-partition-ratio material (endoplasmic reticulum) to a much earlier elution and high-partition-ratio material (plasma membrane and lysosomes) to a slightly later elution, causing a crowding of peaks and loss of resolution.

Fig. 5. Effect of flow rate on marker-enzyme elution profiles
Details are as in Fig. 2.

Subsequent experiments were performed with flow rates of approx. 13 ml/h.

Effect of sample volume and sample concentration on elution profiles (Fig. 6)

Fig. 6 shows the results of four experiments in which either the volume or concentration of

sample injected into the toroidal coil was varied. Increasing sample volume at the higher homogenate concentration led to some spreading of the distribution profiles, but at lower concentrations of liver homogenate good resolution was achieved even with the larger sample volume. Thus it is likely that resolution is more dependent on sample concentration than on sample volume. Distribution profiles (i) and (iv) show the same amount of tissue presented to the coil but in different concentrations. The larger volume of lower concentration gave better resolution.

Effect of coil geometry on elution profiles (Fig. 7)

Fig. 7 shows the results of a series of experiments in which either the internal diameter of the tubing or the helix diameter was altered. The results for variation of tubing diameter are not strictly comparable, owing to differences in the ratio of sample to total coil volume and the percentage of dextran-rich phase in the eluting mixture. However, it can be seen that the use of either the smallest i.d. tubing or the smallest i.d. helix adversely effects resolution, possibly by limiting the mixing of the two phases in each loop. Increasing the tubing or helix i.d. does not significantly affect the elution profiles of the organelles, with the exception of neutral α -glucosidase (endoplasmic reticulum), which is advanced.

Effect of coil length on elution profiles (Fig. 8)

Fig. 8 shows the effect of trebling the number of loops in which partition can occur. The separation of the peaks was clearly improved, particularly of the cytosolic marker enzyme from the plasma-membrane and lysosomal marker enzymes. The plasma-membrane marker profile has a shoulder, which may indicate potential for further resolu-

Fig. 6. Effect of sample volume and sample concentration on elution profiles
Details are as in Fig. 2.

Fig. 7. Effect of coil geometry on elution profiles
Details are as in Fig. 2. Abbreviation used: i.d., internal diameter.

Fig. 8. Effect of number of loops in coil on elution profiles
Details are as in Figure 2. The 1400-loop elution profile is an average result for three experiments. Additional enzyme markers (average result for two experiments) are: ▨, malate dehydrogenase (mitochondria/cytosol); ▤, catalase (peroxisomes); and ▩, DNA.

tion. An expected loss in peak sharpness can also be seen. Also distinct distribution profiles were determined for mitochondrial and peroxisomal marker enzymes and DNA in the longer coil.

Seven experiments were performed under identical conditions and showed high reproducibility (Sutherland *et al.*, 1983).

Discussion

The experiments described in the present paper have systematically investigated the relative importance of various parameters on the separation of rat liver organelles by two-phase partition by using toroidal-coil chromatography. The various organelles, as reflected by their marker enzymes, show a consistent pattern of elution, but their relative resolution is affected by alteration of the operating parameters. Plasma membrane shows frontal elution with therefore little tendency to partition into the dextran-rich phase; lysosomes are eluted next, although the degree of separation is variable. This separation represents a significant improvement on that achieved by discrete counter-current distribution, where the elution profiles of these two organelles were generally identical (Morris & Peters, 1982). The cytosolic fraction is usually eluted as a broad peak after the lysosomal fraction. The distribution of endoplasmic reticu-

lum shows marked heterogeneity with broad, frequently bimodal, distribution profiles. The reason for the heterogeneity of the endoplasmic reticulum is not clear. Although there is a partial localization of the neutral α -glucosidase to the cytosolic fraction, the bulk of the activity is recovered with both rough and smooth endoplasmic reticulum (Smith & Peters, 1980). It is therefore possible that the heterogeneity reflects the distribution of these organelle subcomponents. The finding that similar separations, with similar partition coefficients, are obtained with the toroidal-coil and discrete-step-partition apparatus, indicates that phase partition is the basis for these separations.

One factor determining the elution profile, for a given phase system, is the proportion of lower phase retained in the individual loops (β). As the proportion is increased, any sample partitioning into the lower phase will take an increasing length of time to flow through the system. The simultaneous decrease in upper-phase volume causes organelles with a high partition ratio to move through the coil at a higher rate (see the Appendix). As the flow rate of the eluting mixture was increased, a decrease in proportion of lower phase retained in each loop was found. This provides an explanation for elution at the front of the distribution profile being delayed and that at the end being advanced as the flow rate was increased. A flow rate of 28 ml/h gave $\beta = 0.27$, which led to a predicted elution span of 26 ml, whereas a flow rate of 6.5 ml/h gave $\beta = 0.38$, leading to a span of 41 ml (see the Appendix). This is in agreement with experimental results, where the elution span decreased from over 41 ml to 30 ml as the flow rate was increased. Thus adjusting the flow rate appears to alter the resolution by changing the proportion of lower phase retained in the loops rather than by affecting the degree of turbulence and mixing in the loops.

The equation in the Appendix can also be used to predict the effect of varying the proportion of dextran-rich phase in the eluting mixture (α). Assuming a constant β of 0.32, the increase in α from 0.04 to 0.145 would delay the elution of high-partition-ratio organelles by 0.75 ml, whereas those with a low partition ratio would elute 50 ml earlier. This explains the large change in position of the second endoplasmic-reticulum marker enzyme peak while the peak positions of organelles with higher partition ratios remained unchanged as α was increased. When no dextran-rich phase was included in the eluting mixture the predicted elution volume for low-partition-ratio material was infinite and this was substantiated experimentally. Operating under these conditions produces reasonable resolution and has the advantage that only one pump is required.

The necessity for retention of lower phase in the loops for partitioning is confirmed by the total lack of fractionation seen when the coil was stationary. All the material eluted after a volume equivalent to that of the coil. Once running, the speed of the rotor has little effect on the resolution. However, the higher centrifugal force does tend to retain more unfractionated material, possibly due to sedimentation against the wall of the loops. No evidence was found for enhanced mixing at higher speeds.

The studies on sample volume and concentration indicate that the interface between the two phases can be saturated, leading to a loss of resolution. When handling higher quantities of tissue it appears better to spread the load over a number of loops rather than use a highly concentrated short dose.

The column length is an important design parameter in liquid chromatography. Resolution is only proportional to the square root of the column length, owing to the fact that zone spreading also increases (Hadden *et al.*, 1971). Therefore an improvement in resolution by a factor of 1.6 can be predicted on increasing the coil length from 550 to 1400 loops. This compares well with the experimental value of 1.8 ± 0.8 . Indications for potential further resolution are seen for the plasma-membrane marker and for the low-partition-ratio endoplasmic-reticulum marker-enzyme peak.

It is surprising that the wide variation in separation conditions are not associated with markedly differing elution profiles. This is reflected by the excellent reproducibility in separate experiments and would suggest that reasonably complete equilibration is achieved in the coil. However, it is possible that the coil geometry used limits the efficiency of mass transfer due to regions of poor mixing. This is improved to some extent by including a proportion of lower phase in the eluting mixture, but the high viscosity of the dextran-rich phase could still represent a barrier to efficient mass transfer. Adaptations of the coil geometry should improve the efficiency of the system and hence resolution.

The present approach has been to adopt an analytical fractionation procedure to explore the effect of technical variables on the organelle separations. Good separations both of plasma-membrane and endoplasmic-reticulum and of lysosome and mitochondrial markers have been achieved. These components have very similar equilibrium densities in sucrose gradients (Smith & Peters, 1980) and are therefore difficult to resolve by density-gradient centrifugation. Resolution of organelles separated by rate-zonal techniques is more limited, owing to the broad size distributions of organelles (Evans, 1982). Toroidal-coil chro-

matography also has the advantages of using an iso-osmotic bio-compatible medium, low-speed centrifugation and short processing times, which result in little damage to the organelles. Adaptation of the procedure to preparative fractionation should be relatively easy. Use of larger sample volumes and wide-bore tubing would greatly increase the amount of tissue which could be processed. It is unlikely, however, that this technique would prove of most value when used in conjunction with existing centrifugation procedures, e.g. for the subfractionation of the microsomal or mitochondrial fractions.

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APPENDIX

The effect of various coil operating conditions on the elution span can be predicted by using the following equations (Sutherland *et al.*, 1983):

$$V_U = V_{IO} + \frac{(1-\beta)V^*}{(1-\alpha)}$$

$$V_L = V_{IO} + \frac{\beta}{\alpha} V^*$$

where α = proportion of lower phase in eluting mixture; β = proportion of lower phase retained in loops; V_{IO} = volume of inlet and outlet leads; V^* = volume of coil; V_U = volume eluted until material partitioning totally into top phase is eluted; V_L = volume eluted until material partitioning totally into lower phase is eluted; $V_L - V_U$ = elution span.