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Partitioning of cells in dextran–poly(ethylene glycol) aqueous phase systems

A study of settling time, vessel geometry and sedimentation effects on the efficiency of separation

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ABSTRACT

The effect of prolonged settling times (up to 2 h), in high- and low-phase columns, on the cell partition ratios measured and on the separability of cell populations was examined. With closely related cell populations, modelled by rat erythrocytes in which subpopulations of red blood cells of distinct age were labeled isotopically, it was found that partitioning proceeds over the entire time period examined as evidenced by the continuous change in relative specific activity of cells in the top phase as the partition ratio falls. In control cell sedimentation experiments in top phase there was almost no change in the quantity of cells present when vertical settling (*i.e.*, high-phase columns) was used and no separation of specific subpopulations was found. In the horizontal settling mode the initially higher cell partition ratio, as compared to vertical settling, decreased to a greater extent with longer time intervals; a given purity of cells only being obtained at a lower partition ratio than in the vertical settling mode. Cell sedimentation in top phase was appreciable with time in the horizontal settling mode but did not result in a separation of cell subpopulations.

The effect of relative cell partition ratios and sizes in high- and low-phase columns on the efficiency of separation was examined by use of rat or sheep ^{51}Cr-labeled red cells mixed with an excess of human unlabeled erythrocytes. Rat and sheep red cells are appreciably smaller than human erythrocytes. Rat red cells have higher, and sheep red cells lower partition ratios than human erythrocytes. With vertical settling, over a 2-h period, there is no appreciable contribution to the change in relative specific activities by cell sedimentation. However, the more rapid sedimentation of the larger human red cells has, with time, a measurable effect on the relative specific activities obtained during cell partitioning when run in the horizontal mode: enhancing the rat–human and diminishing the sheep–human cell separations.

Partitioning cells in high-phase columns is of advantage with respect to increasing separation efficiency and virtually eliminating the influence of other physical parameters (*e.g.*, cell size). Since the cell partitioning process continues for long periods of time, yielding ever-lower partition ratios with increasing proportions of cells with higher P values, a time may be selected which balances desired relative cell purity and yield.

INTRODUCTION

Partitioning in dextran–poly(ethylene glycol) aqueous-phase systems is an established, sensitive method for the separation and fractionation of cell populations

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based on differences in their surface properties (for some recent reviews see refs. 1–3). Unlike the partitioning of soluble materials which occurs between the bulk phases and depends on the materials' relative solubility, the partitioning of cells generally takes place between one of the bulk phases and the interface and is time-dependent [4,5]. Early events in the partitioning process which involve characteristic kinetics of cell- and phase-specific interactions and phase separation have previously been described [4,6,7]. A relation between phase-column height, a factor in the rapidity of phase settling, and the efficiency of cell separation has also been detailed [8]. In the current work we explore the effect of prolonged settling times, in high- and low-phase columns, on the cell partition ratios measured and on the separability of closely related cell populations as well as of cell mixtures. The contribution of cell sedimentation on the partitions obtained is also examined. Our results indicate that partitioning proceeds over a period of (at least two) hours; that with time one can obtain more purified cells at lower yield; that a given purity of cells is attained at higher yield when phase columns are high rather than low, and that cell sedimentation has no appreciable effect on the yield or separation obtained with high-phase columns but can affect both with low-phase columns.

The reported enhancement of cell separation by partitioning by extending settling times in low-phase columns [9,10] is thus: (a) a consequence of cell sedimentation superimposed on the partitioning process and (b) not to be taken as a general rule since a reduction of a partitioning separation by sedimentation can occur equally well.

EXPERIMENTAL

Reagents

Dextran T500 (lot No. 01 06905) was obtained from Pharmacia LKB (Piscataway, NJ, U.S.A.) and poly(ethylene glycol) 8000 (PEG, "Carbowax 8000") from Union Carbide (Long Beach, CA, U.S.A.). [^{59}Fe]Ferrous citrate and [^{51}Cr]-sodium chromate were products of ICN (Irvine, CA, U.S.A.). All salts used were of reagent grade.

Bleeding of rats, sheep and of human donors

Male Sprague–Dawley rats (Charles River, Wilmington, DE, U.S.A.), weighing between 250 and 400 g, were bled by heart puncture. Sheep blood (UCI Medical Center, Orange, CA, U.S.A.) was obtained by use of an indwelling catheter in the femoral vein or by venipuncture of the jugular. A 10-ml volume of blood was collected in 3 ml acid–citrate–dextrose (ACD) anticoagulant. Human blood from presumably normal individuals was obtained by venipuncture using the same ratio of blood to anticoagulant as indicated for rat and sheep blood. Red cells were used in experiments within one week of collection.

In vivo radioisotopic labeling of rat erythrocytes of different age

Some of the rats were injected with 10–20 μCi [^{59}Fe]ferrous citrate via the saphenous vein. These were then bled at 4, 12 or 13 and 42 or 43 days after injection. This gave rise to erythrocyte populations in which cells, corresponding in age to the time which elapsed between injection and bleeding, were radioactively labeled.

Aliquots of the erythrocyte populations were washed three times with at least ten times the cell volume of isotonic aqueous salt solution (saline) before being used in the partitioning experiments described below.

In vitro radioisotopic labeling of rat or sheep erythrocytes and the preparation of mixtures of rat or sheep labeled red cells plus human unlabeled erythrocytes to test cell separation efficiency by partitioning

Labeling of erythrocytes with [^{51}Cr]sodium chromate has previously been described [11]. Approximately 10–20 μCi ^{51}Cr was used per ml of an aliquot of anticoagulated rat or sheep blood. Labeled rat or sheep blood and an aliquot of unlabeled human blood were then washed five times with saline.

To test the efficiency of the separation of human plus rat or sheep erythrocytes by partitioning under various conditions (see below) a mixture of ^{51}Cr-labeled rat or sheep erythrocytes and an excess of unlabeled human erythrocytes was prepared. Into a centrifuge tube containing four ml of saline was pipetted 0.5 ml of washed, packed, labeled rat or sheep red cells and 2.0 ml of washed, packed, unlabeled human erythrocytes. The tube was capped, inverted a few times to mix the cells, and centrifuged. The supernatant solution was discarded and the packed cell mixture used in some of the partition experiments described below.

Preparation of two-polymer aqueous phase systems

Four dextran–PEG aqueous two-phase systems having different polymer and/or salt compositions were prepared as previously reported [5]. System I was composed of 5% (w/w) dextran, 3.6% (w/w) PEG, 0.15 *M* sodium chloride and 0.01 *M* sodium phosphate buffer, pH 6.8; system II contained 5% (w/w) dextran, 4.4% (w/w) PEG and 0.11 *M* sodium phosphate buffer, pH 6.8; system III, 5% (w/w) dextran, 3.4% (w/w) PEG with the same salt composition as system I; and system IV, 5% (w/w) dextran, 3.9% (w/w) PEG and the same salt composition as system II. Systems II and IV in which phosphate predominates have an electrostatic potential difference between the phases (top phase positive) and are deemed charge-sensitive [5]. Phases in which sodium chloride is the main salt have virtually no potential difference between the phases and are non-charge-sensitive [5].

Partitioning of erythrocytes in aqueous two-phase systems as a function of vessel geometry and time

The phase system which was to be used for partitioning, at 21–24°C, was mixed and about 12 ml were poured into each of 14 partition tubes (*i.e.*, calibrated tubes, 125 mm × 16 mm). The tubes were centrifuged to speed-up phase separation and the top- and bottom-phase volumes were adjusted to be equal at 5 ml. A 0.05-ml volume of washed, packed rat erythrocytes (containing ^{59}Fe-labeled red cells of distinct age) or a rat or sheep (^{51}Cr-labeled) plus human (unlabeled) erythrocyte mixture which was to be partitioned was added to each of the fourteen tubes containing the same phase system and mixed. As soon as a tube had been mixed the timing of its cells' partitioning commenced. One set of seven tubes was permitted to remain in a vertical position while the phases settled and the tubes were sampled at different times. A 4.5-ml volume of the top phase was withdrawn at 20, 30, 40, 50, 60, 90, or 120 min. The second set of seven tubes was capped (with parafilm) and permitted to settle in a horizontal position. One

tube was gently raised to a vertical position (without agitating the contents) after 7.5, 15, 30, 45, 60, 90 or 120 min and permitted to settle for one additional min. A 4.5-ml volume of top phase was then withdrawn. The quantity of cells in each top phase was determined by lysing the cells and measuring the hemoglobin absorbance at 540 nm as previously described [12]. The quantity of cells initially added to the partition tubes was similarly determined. Aliquots of the lysates were counted on a Beckman scintillation well-counter using the ^{59}Fe or ^{51}Cr setting (depending on the isotope used in the experiment).

Sedimentation of erythrocytes in top phase as a function of vessel geometry and time

Phase systems, at 21–24°C, in a separatory funnel, were mixed and permitted to settle overnight. Top and bottom phases were then separated. Top phase was centrifuged in a Sorvall RC-5 centrifuge (set to the same temperature range) at 12 000 *g* for 10 min to remove any remaining bottom-phase droplets. A 5-ml volume of bottom phase was pipetted into each of fourteen partition tubes as used above. A 0.75-ml volume of washed, packed erythrocytes (*i.e.*, aliquots of the same cells as used in the partitioning experiments described in the previous section) was pipetted into 70 ml of the centrifuged, absolutely clear top phase. The top phase was gently shaken so as to obtain a homogeneous cell suspension. A 5-ml volume of the latter was carefully layered over each of the bottom phases in the tubes without mixing or agitation. (This is best done by holding the tubes at an angle and permitting the cell suspension in top phase to run slowly down the inner wall of each tube.) Seven tubes were left standing in a vertical position and were sampled at various time intervals and were analyzed in a manner analogous to that described for vertical tube partitioning. Seven tubes were capped and carefully placed in a horizontal position (again without mixing or agitating the systems) and were then sampled at various time intervals and were analyzed in a manner analogous to that described for horizontal tube partitioning.

Presentation of data

The cell partition ratio, *P*, is defined as the quantity of cells in the top phase, at the time of sampling, as a percentage of the total quantity of cells added [5]. The relative specific activity (RSA), a measure of the relative extent of labeled and unlabeled cell separation in a cell population or mixture, is [6].

$$\frac{\text{counts/min per hemoglobin absorbance in the top phase}}{\text{counts/min per hemoglobin absorbance in the original, unfractionated cell population (or mixture)}}$$

In the figures we present plots of the partition ratio *vs.* the RSA value obtained at the different times of phase sampling. Sedimentation results are given in an analogous manner: percentage of added cells found in the top phase *vs.* RSA at the different sampling times. The figures show data typical of that which was obtained in at least three separate experiments using blood from different animals and individuals (with the exception of sheep blood with which only two experiments were conducted).

RESULTS

Our studies were undertaken with closely related cell populations (rat erythrocytes containing ^{59}Fe-labeled cell subpopulations of different but distinct age) and with cell mixtures (*i.e.*, ^{51}Cr-labeled rat or sheep erythrocytes plus human red blood cells). From previous studies [13,14] it is known that, in both non-charge-sensitive and charge-sensitive phase systems, rat young and middle-aged (*e.g.*., 4 and 12–13 days old) red cells have higher, and old (*e.g.*, 42–43 days) cells have lower partition ratios than the mean partition ratio of the whole cell population. Rat red cells have higher partition ratios than human erythrocytes in both non charge-sensitive and charge-sensitive phases while sheep red cells have much lower partition ratios than those from humans in non charge-sensitive phases and slightly lower partition ratios in charge-sensitive phases [5]. Furthermore, rat and sheep erythrocytes have appreciably smaller volumes than human red blood cells [15,16].

Partition and sedimentation studies on rat red blood cell populations containing ^{59}Fe-labeled erythrocytes of distinct age. Experiments in vertically-placed tubes (high-phase columns)

In the figures we show the effect of sampling time on the percentage of cells in the top phase and on the cells' RSA value. Figs. 1 (top left panel) and 2 (top left and right panels) depict results obtained with rat red cell populations containing isotopically labeled 4, 13 and 42 days old cells, respectively, partitioned in non-charge-sensitive phase system I. The points in each graph represent, in sequence from right to left samples obtained after 20, 30, 40, 50, 60, 90 and 120 min of vertical-phase settling. As the time is extended, the percentage of cells in the top phase diminishes from about 65 to 20% while the RSA values continue to change (increasing from 1.04 to 1.62 with erythrocytes containing 4 days old labeled cells, 1.00 to 1.23 with 13 days old cells, and decreasing from 0.88 to 0.60 with 42 days old cells). The ever-changing RSA values (in the absence of appreciable cell sedimentation, see below) indicate that cell partitioning continues over the entire time course of the experiment.

In Fig. 1, upper right panel, the results of a sedimentation experiment with rat erythrocytes containing 4 days old labeled cells are depicted. Cells suspended in top phase (system I) were permitted to stand in a vertical position and sampled at times and analyzed in a manner analogous to that described above for the partitioning experiments. The percentage of cells in the top phase hardly diminishes (from about 97 to 90%) over the 2-h sedimentation period and the RSA values remain virtually constant at 1.00. Identical results on sedimentation are obtained with rat red blood cells irrespective of the age of the labeled cell subpopulation they contain. Thus there is no evidence of separation in the different-aged rat erythrocytes upon sedimentation in the top phase.

Figs. 3 (top left panel) and 4 (top left and right panels) present results analogous to those in Figs. 1 and 2, respectively, except that charge-sensitive phase system II was used. In these experiments the percentage of cells in the top phase diminishes, with time, from about 60 to 50% while the RSA values change (increasing from 1.51 to 1.67 with erythrocytes containing 4 days old labeled cells, 1.50 to 1.57 with 12 days old cells, and decreasing from 0.70 to 0.56 with 43 days old cells). Thus, the initial RSA values are much higher (lower in the case of 43 days old cells) than in the non charge-sensitive

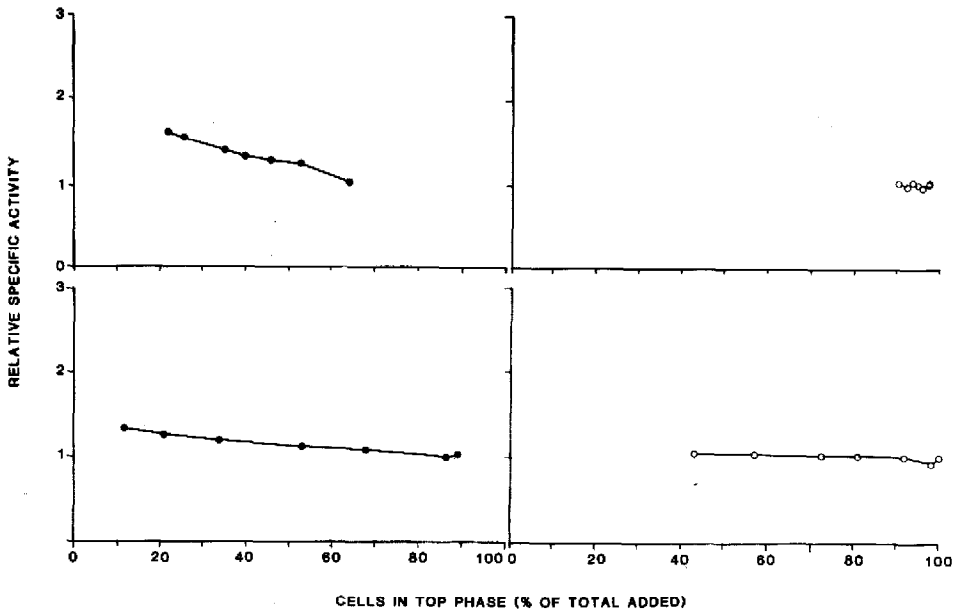

Fig. 1. Rats were injected with [^{59}Fe]ferrous citrate and bled at different times thereafter. This gave rise to red cell populations in which the bulk of labeled cells corresponds in age to the time elapsed between injection and bleeding. Rat erythrocytes of different ages have characteristic partition ratios (see text). Aliquots of such cell populations were partitioned in a dextran–poly(ethylene glycol) aqueous-phase system *or* permitted to sediment in the top phase of the same system. A series of tubes, seven in the vertical (high-phase column) and seven in the horizontal (low-phase column) mode, was used and the top phase withdrawn at different times (*i.e.*, at 20, 30, 40, 50, 60, 90 and 120 min for high- and at 7.5, 15, 30, 45, 60, 90 and 120 min for low-phase column experiments). The top phase was analyzed for the quantity of cells present as a percentage of the total cells added and for the cells' relative specific activity, RSA. In this figure data are shown in which rat blood obtained four days after isotope injection (rat No. 90-1) and non-charge-sensitive phase system I were used. The upper left panel gives results of partitioning cells in the vertical mode. The points indicate, from right to left, the time course of the decrease of cells in the top phase accompanied by an increase in RSA value (*i.e.*, enrichment of 4 days old rat red cells). The upper right panel shows the corresponding sedimentation experiment. Note that here the decrease of cells in the top phase is negligible and that the RSA value remains constant at 1.00. We conclude that cell partitioning continues over the entire 2-h period of the experiment. The lower left panel presents partitioning data in the horizontal mode. Again the time course of the decrease of cells in the top phase is accompanied by an increase in the RSA value. In the lower right panel, low-phase column sedimentation, the decrease of cells in the top phase is appreciable. The RSA value remains, however, constant at 1.00. Note that at any given percentage of cells in the top phase, the RSA value is higher when partitioned in high- rather than low-phase columns indicating a greater separation efficiency in the former mode (compare left top and bottom panels). See text for discussion.

system [Figs. 1 (top left panel) and 2 (top left and top right panels)] while the subsequent changes in RSA values are smaller.

Fig. 3, upper right panel, depicts the results of a sedimentation experiment with rat erythrocytes containing 4 days old labeled cells in phase system II and conducted as described for Fig. 1. The percentage of cells in the top phase again diminishes only slightly (from 100 to 97%) over the 2-h sedimentation period and the RSA values remain constant at 1.00. As with phase system I, identical results for sedimentation are obtained with rat red blood cells irrespective of the age of the labeled cell

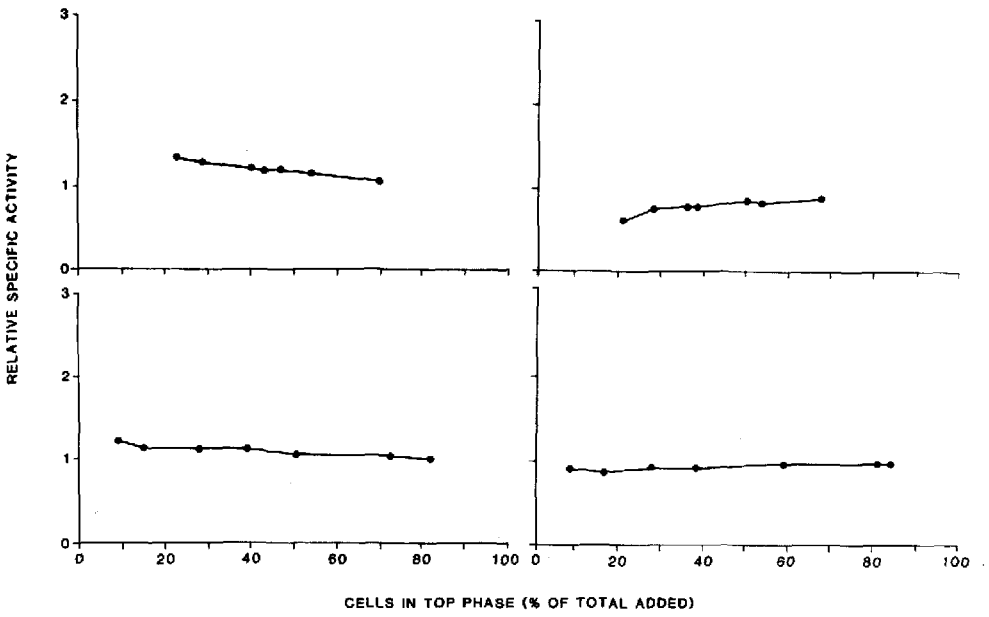

Fig. 2. Partitioning experiments (as in Fig. 1) with rat erythrocytes obtained 13 days (left panels, rat No. 90-5) or 42 days (right panels, rat No. 89-12) after ^{59}Fe injection. Top panels depict partitioning in high-phase columns; bottom panels partitioning in low-phase columns. See Fig. 1 and text for details.

subpopulation they contain. As in the case of the non-charge-sensitive phase system I, no fractionation can be detected for the different-aged rat erythrocytes upon sedimentation.

Partition and sedimentation studies on rat red blood cell populations containing ^{59}Fe-labeled erythrocytes of distinct age. Experiments in horizontally placed tubes (low-phase columns)

In the lower left panel of Fig. 1 and the lower panels of Fig. 2 the points represent, in sequence from right to left, partitioning samples in system I obtained after 7.5, 15, 30, 45, 60, 90 and 120 min of horizontal- plus 1 min of vertical-phase settling. As the sampling time is extented, the percentage of cells in the top phase diminishes markedly from about 85 to 10% while the RSA values change (increasing from 1.03 to 1.38 with erythrocytes containing 4 days old labeled cells, 1.00 to 1.23 with 13 days old cells, and decreasing from 0.98 to 0.89 with 42 days old cells).

In the lower right panel of Fig. 1 the results of a sedimentation experiment are depicted in which cells, suspended in top phase (system I), were permitted to be in a horizontal position and then in a vertical position for time intervals as described in the previous partitioning experiments and sampled and analyzed in a manner analogous to them. Unlike the small decrease in the percentage of cells in the top phase as a function of sedimentation when tubes are in the vertical position (Fig. 1, upper right), the decrease of cells in the top phase with tubes in a horizontal position is sizable over the 2 h period, 100 to 43%, and close in magnitude to the change in P values

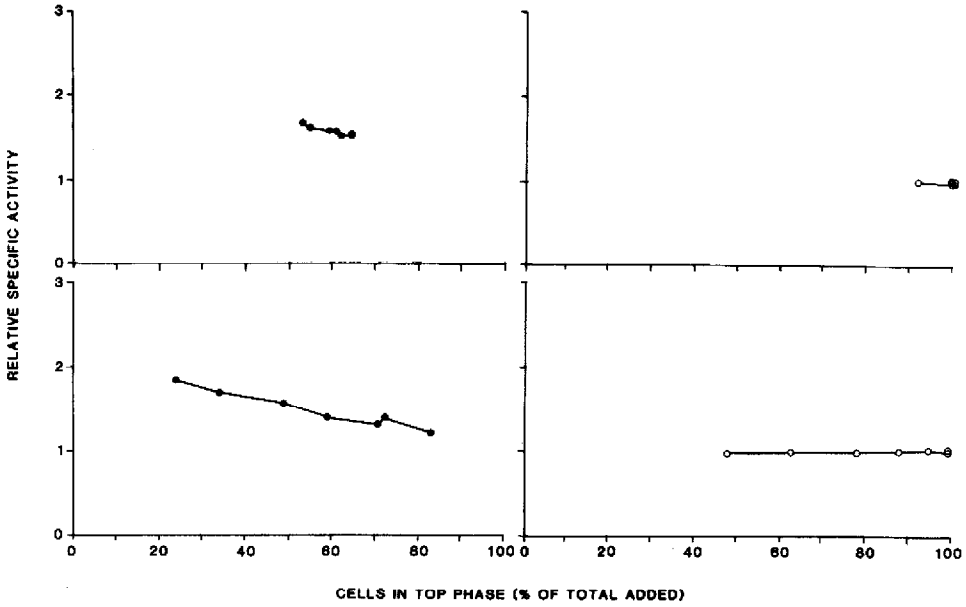

Fig. 3. Experiments as in Fig. 1 except that charge-sensitive phase system II was used. See discussion.

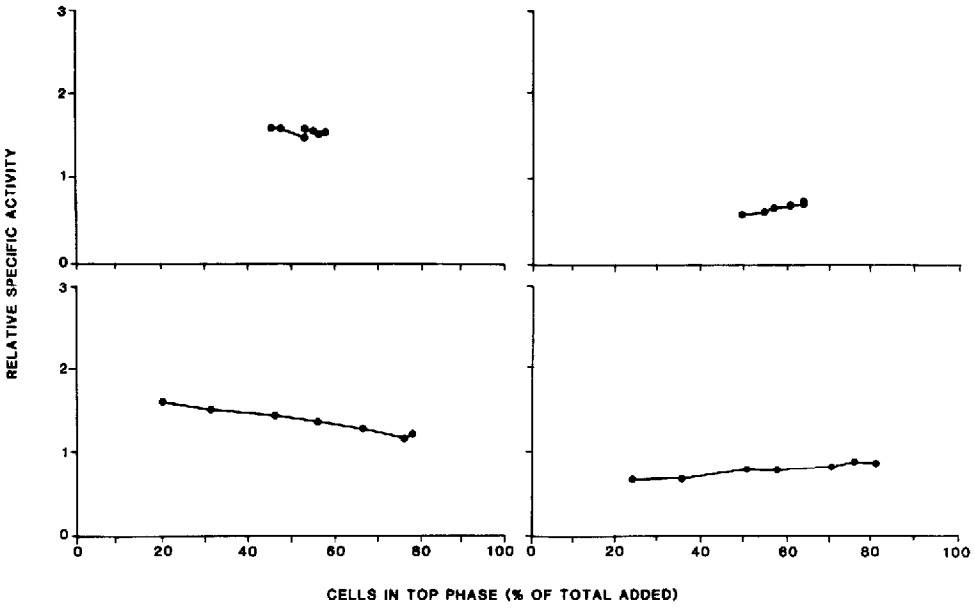

Fig. 4. Partitioning experiments similar to those in Fig. 2 except that charge-sensitive phase system II was used. Left panels: erythrocytes obtained 12 days after ^{59}Fe injection (rat No. 89-18) and, right panels, 43 days after isotope injection (rat No. 90-3). See Discussion.

observed with horizontal partitioning (compare Fig. 1, lower left and right panels). The RSA values remain constant at 1.00 again indicating that no fractionation of cells occurs during sedimentation.

In Figs. 3 and 4, lower panels, experiments analogous to those depicted in Figs. 1 and 2, respectively, were undertaken with the exception that charge-sensitive phase system II was used. The percentage of cells in the top phase again diminishes markedly with time, from about 80 to 20%, while the RSA values change (increasing from 1.39 to 1.85 with erythrocytes containing 4 days old labeled cells, 1.15 to 1.59 with 12 days old cells, and decreasing from 0.88 to 0.67 with 43 days old cells).

The accompanying sedimentation experiment (Fig. 3, lower right) again shows a decrease in the percentage of cells in the top phase over the time-course of the experiment, 100 to 48%, which is comparable in magnitude to that found on cell partitioning (Fig. 3, lower left), with the RSA values remaining constant at 1.00.

Partition and sedimentation studies on a mixture of rat ^{51}Cr-labeled red blood cells and an excess of human unlabeled erythrocytes. Experiments in high- and low-phase columns

Fig. 5 depicts superimposed partitioning and sedimentation results obtained with a mixture of ^{51}Cr-labeled rat red cells plus an excess of human unlabeled erythrocytes. In the top left panel the points represent, in sequence from right to left, samples obtained after 20, 30, 40, 50, 60, 90 and 120 min of vertical-phase settling in non-charge-sensitive phase system III. As in Figs. 1–4, the percentage of partitioned cells (solid symbols) in the top phase diminishes with time (from about 70 to 35%) while the RSA value increases dramatically (from 1.38 to 2.86). In the sedimentation experiment (open symbols), in which cells suspended in the top phase were permitted to stand in a vertical position and were sampled at time intervals and analyzed in a manner analogous to the partitioning experiment, the percentage of cells in the top phase diminishes very slightly during the 2-h experiment and the RSA values remain close to 1.00.

In the lower left panel, the points represent, in sequence from right to left, samples obtained after 7.5, 15, 30, 45, 60, 90 and 120 min of horizontal- plus 1 min of vertical-phase settling. With longer settling times the percentage of partitioned cells in the top phase again diminishes (from about 90 to 25%) and the RSA values increase (1.11 to 2.48). In the corresponding sedimentation experiment the decrease of cells in the top phase, as they sediment in a low-phase column, is appreciable over the 2-h period (from about 100 to 30%) and is comparable in magnitude to the change in the P value observed with horizontal partitioning. Unlike the results obtained with horizontal sedimentation of closely related cell populations (Figs. 1 and 3) the RSA value of a mixture composed of cells of appreciably different size also changes (in the present case from 0.85 to 1.85). This reflects the more rapid sedimentation of the larger human red cells in the mixture yielding an increasing ratio of radioactively labeled rat to human erythrocytes. The more rapid sedimentation of the human red cells thus serves to enhance the separation of human and rat erythrocytes by partitioning.

The upper and lower right panels of Fig. 5 show experiments conducted as in the respective left panels of the figure except that charge-sensitive phase system IV was used. In the top right panel (high-phase columns) the percentage of partitioned cells (solid symbols) in the top phase diminishes with time (from about 68 to 48%) while the RSA values increase (from 1.45 to 2.00). In the corresponding sedimentation

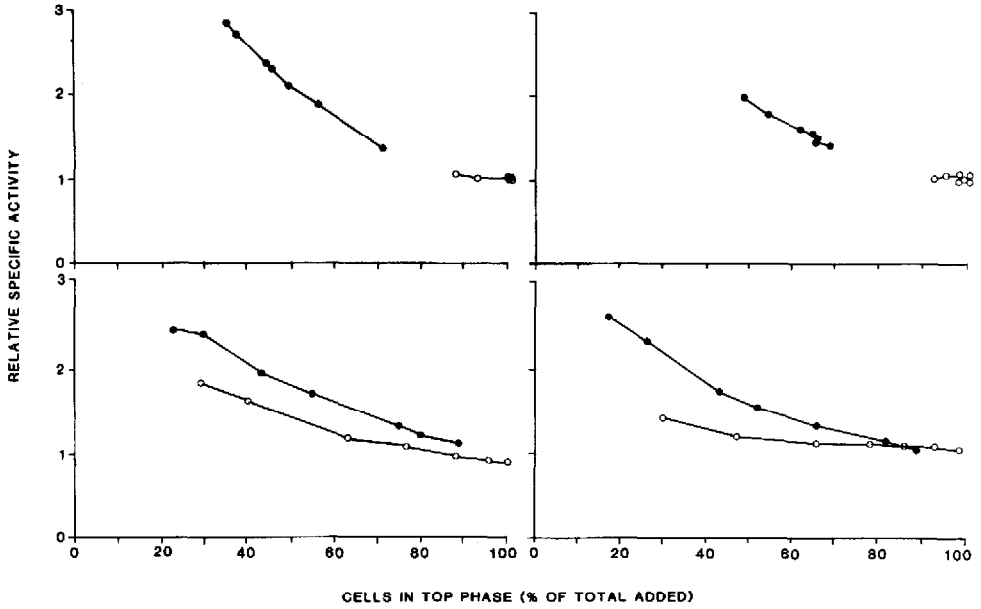

Fig. 5. Mixtures of ^{51}Cr-labeled rat red blood cells plus unlabeled human erythrocytes (1:4) were partitioned or permitted to sediment in a manner as described for Fig. 1. Partitioning (solid symbols) and sedimentation (open symbols) data are presented superimposed in the graphs. Left panels show experiments in non-charge-sensitive system III and right panels show experiments in charge-sensitive system IV. Top panels represent high-phase column partitioning or sedimentation; bottom panels, low-phase columns. Rat erythrocytes have higher partition ratios than human red cells in both systems III and IV. They are also smaller. Note that unlike the results obtained with the closely related rat red cells of different age (Fig. 1), sedimentation in low-phase columns also causes marked changes in the RSA values reflecting a more rapid sedimentation of human as opposed to rat red cells during the 2-h course of the experiment. For additional discussion see text.

experiment (open symbols), the percentage of cells in the top phase hardly diminishes over the 2-h experiment and the RSA values remain a constant 1.00.

In the bottom right panel (low-phase columns) the percentage of cells partitioned in the top phase again diminishes with time (from about 90 to 15%) and the RSA values increase (1.07 to 2.64). In the sedimentation experiment the decrease of cells in the top phase, as they form sediment in a low-phase column, is appreciable over the 2 h period (about 100 to 30%) and is comparable in magnitude to the change in the *P* value observed with low-phase partitioning. Again, as in Fig. 5, lower left panel, the RSA value of a mixture of cells of appreciably different sizes also changes on sedimentation (in the present case from 0.97 to 1.42) again reflecting a fractionation on the basis of size.

Partition and sedimentation studies on a mixture of sheep ^{51}Cr-labeled red blood cells and an excess of human unlabeled erythrocytes. Experiments in high- and low-phase columns

Fig. 6 depicts superimposed partitioning and sedimentation data obtained in a manner similar to that described for Fig. 5 but with a mixture of ^{51}Cr-labeled sheep

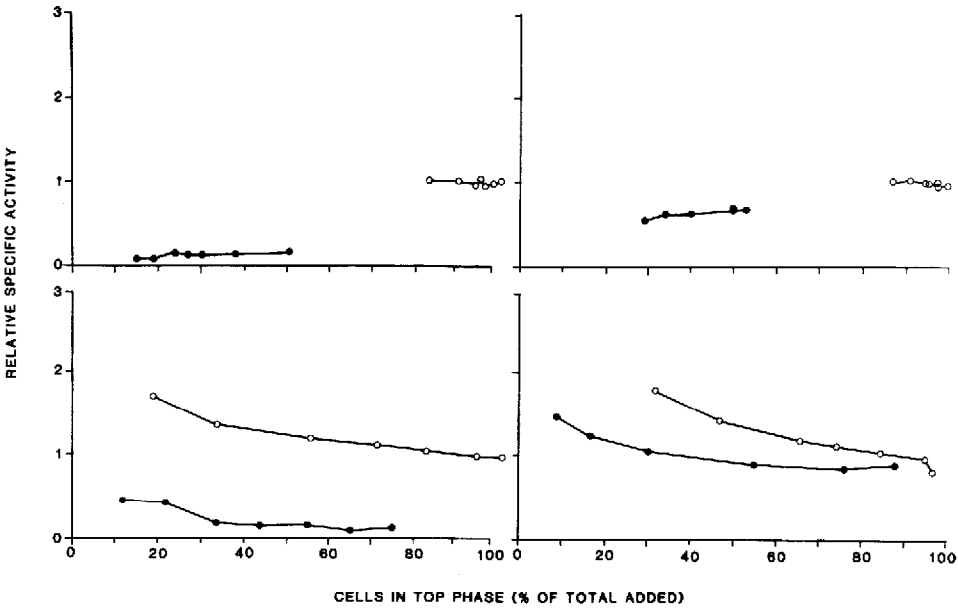

Fig. 6. Mixtures of ^{51}Cr-labeled sheep red blood cells plus unlabeled human erythrocytes (1:4) were partitioned or permitted to sediment in a manner as described for Fig. 1. Partitioning (solid symbols) and sedimentation (open symbols) data are presented superimposed in the graphs. Left panels show experiments in non-charge-sensitive system III and right panels show experiments in charge-sensitive system IV. Top panels represent high-phase column partitioning or sedimentation; bottom panels, low-phase columns. Sheep erythrocytes have much lower partition ratios than human red cells in system III and slightly lower partition ratios in system IV. They are also much smaller. Since the partition ratio of human red cells is higher than those from sheep but human red cells sediment more rapidly (shown by the increase in the RSA values on sedimentation in low-phase columns, lower panels), the initial low RSA value in the partitioning experiments increase with longer settling times (partitioning data in lower panels). For discussion see text.

red cells plus an excess of human unlabeled erythrocytes. In the top left panel vertical-phase settling results in non-charge-sensitive phase system III are shown. The percentage of partitioned cells (solid symbols) in the top phase diminishes with time (from about 50 to 15%) while the RSA value, reflecting the very low P value of the labeled sheep red cells, is and remains low (ranging from 0.17 to 0.08). In the sedimentation experiment (open symbols), the percentage of cells in the top phase diminishes only slightly over the 2 h experiment and the RSA values remain close to 1.00.

In the lower left panel, low-phase column partitioning and sedimentation data in system III are presented. With longer settling times the percentage of partitioned cells in the top phase again diminishes (from about 75 to 10%) and the RSA values at first remain low (0.12 to 0.20) but with extended sedimentation (*i.e.*, beyond 1 h) increase to 0.47. In the corresponding sedimentation experiment the decrease of cells in the top phase, as they sediment in a low-phase column, is appreciable over the 2-h period (about 100 to 20%). As with the results in Fig. 5, but unlike those in Figs. 1 and 3 which have closely related cell populations, the RSA value changes (in the present case from 0.99 to 1.69). This again reflects the more rapid sedimentation of the larger

human red cells in the mixture yielding an increasing ratio of radioactively labeled sheep to human erythrocytes. The increase in RSA values with prolonged partitioning times (*i.e.*, beyond 1 h) is due to the differential sedimentation of human and sheep red blood cells. Because both the sedimentation rate and the partition ratio of human red cells is greater than those of sheep erythrocytes, sedimentation tends to reduce the separation of human and sheep erythrocytes by partitioning. Sedimentation effects on the separation by partitioning of human and sheep red cells is shown even more dramatically in Fig. 6, right (see below).

The upper and lower right panels of Fig. 6 show experiments conducted as in the respective left panels of the figure except that charge-sensitive phase system IV was used. In the top right panel (high-phase columns) the percentage of partitioned cells (solid symbols) in the top phase diminishes with time (from about 55 to 30%) while the RSA values decrease minimally (from 0.68 to 0.54). In the corresponding sedimentation experiment (open symbols), the percentage of cells in the top phase hardly diminishes over the 2-h experiment and the RSA values remain a constant 1.00.

In the bottom right panel (low-phase columns) the percentage of cells partitioned into the top phase again diminishes with time (from about 90 to 10%) but, unlike the data in the upper right panel, the RSA values increase from less than to greater than 1.00 (0.90 to 1.46). (RSA values less than 1.00 reflect a smaller P value for sheep red cells than for human red cells while RSA values greater than 1.00 indicate the opposite.) The explanation for the change in RSA values is to be found in the corresponding sedimentation experiment in which the decrease of cells in the top phase, over the 2 h period in a low-phase column, is accompanied by an increase in RSA values all the way to 1.76. The increase in RSA values on prolonged sedimentation thus affects the apparent red cell P values. The larger human erythrocytes sediment more rapidly than the sheep red cells making it appear that the sheep red cell, which actually has a lower P value than the human red blood cell in phase system IV, has a higher P value.

DISCUSSION

It is known that cell partitioning is a kinetic process which depends on cell- and phase-specific interactions wherein cells bind to droplets of one phase (in the present case, the dextran-rich phase) suspended in the other (PEG-rich phase) after mixing followed by the delivery of droplet-bound cells to the bulk interface [4]. Thus the partition ratio, P, of cells depends on the time chosen for analysis. Furthermore, since the height of the phase column influences the speed of phase settling, with smaller heights resulting in faster settling, cell separation by partitioning is also affected by the geometry of the vessel used. More efficient fractionations are obtained in high rather than small phase columns [8]. The reason suggested for this finding is that with low-phase column heights, phase separation proceeds too rapidly for cells to attach to the phase droplets. (Hence the use of a Craig counter-current distribution apparatus [17] with high-phase columns is of more advantage when fractionating cells than the more commonly used Albertsson thin-layer unit [18].)

In the present work we have further explored the efficiency of cell fractionation by partitioning in two-polymer aqueous-phase systems in both high- and low-phase columns *vs.* time. The contribution of cell sedimentation in the phases (in the absence

of partitioning) was also examined. We studied both closely related cells (*i.e.*, rat erythrocytes containing ^{59}Fe-labeled cells of different ages) and mixtures of red blood cells of different sizes (*i.e.*, human plus rat, human plus sheep) in which the smaller cells (sheep or rat erythrocytes) were isotopically labeled with ^{51}Cr and mixed with an excess of the larger cells. Efficiency of fractionation was determined by measuring the changes in relative specific radioactivity (RSA) with time.

A surprising result of our experiments is the fact that cel partitioning proceeds for (at least two) hours. This is indicated by the change in RSA value (*e.g.*, Fig. 1, top left) with time in the absence of cell sedimentation (*i.e.*, without an appreciable change in the percentage of cells present in the top phase, see *e.g.*, Fig. 1, top right) or even in the presence of cell sedimentation where the latter does not result in changes in RSA values (*e.g.*, Fig. 1, bottom right). A practical result of the finding that the partitioning process continues for a long period of time, yielding ever-lower P values with higher proportions of separated cells, is that a time interval may be chosen which balances relative cell purity in a given phase and yield.

With high-phase columns and closely related cells (*i.e.*, cells of similar size) cell sedimentation is negligible (over the 2-h period of the experiment) and no fractionation of the cells due to sedimentation is observed. With low-phase columns and closely related cells sedimentation is appreciable, although without cell fractionation since the RSA value remains constant at 1.0, (*e.g.*, Figs. 1 and 3, bottom right). The decrease in the percentage of added cells due to sedimentation does not differ much from the percentage of cells in the top phase found on partitioning (see Figs. 1 and 3, bottom). The partitioning and sedimentation processes are thus not "additive" and it is tempting to speculate that cell partitioning actually slows the sedimentation of some cells.

As previously reported [8] the RSA value obtained with the same cell population in the same phase system at a given partition ratio, P value, is always higher in vertical- (high) than in horizontal- (low) phase columns (see *e.g.*, Figs. 1, left; 2; 3 left). The current data show that one can, in some phases (see the left side of Fig. 3 as well as Fig. 4), attain the same RSA values with horizontal-phase settling as with vertical-phase settling but then only at a lower P value (*i.e*, lower cell yield).

With high-phase columns and mixtures of cells of different sizes, cell sedimentation is not significant (over the 2 h period of the experiment) and no fractionation of the cells due to sedimentation occurs (Figs. 5, top and 6, top). With low-phase columns and such cell mixtures, sedimentation is appreciable and affects cell separation. In the case of rat red blood cells which are smaller than human ones but have a higher partition ratio in these phases [5], the more rapid sedimentation of the larger human erythrocytes leaves more rat red cells in the top phase. Sedimentation, in this case, would thus serve to enhance the separation (Fig. 5, bottom). Sheep red blood cells are also smaller than human erythrocytes but have a slightly lower P value in charge-sensitive phases and a much smaller P value in non charge-sensitive phases [5]. The RSA value of sheep red blood cells should therefore be less than 1.0 and diminish with time. The removal of human red blood cells by sedimentation actually increases the observed RSA values (see Fig. 6, bottom).

The reports that increasing the settling time in low-phase columns (as on a thin-layer countercurrent distribution apparatus) improves the fractionation of cells by partitioning [9,10] was unexpected in light of the cell partitioning mechanism [8].

Our current studies show that the enhanced fractionation of bone marrow cells by partitioning [9,10] must be a function not of enhanced partitioning but of differential cell sedimentation. Such an improvement in separation cannot, however, be taken as a general rule [9,10], since a partitioning separation can equally well be diminished by cell sedimentation (see *e.g.*, Fig. 6).

CONCLUSIONS

Cell partitioning in two-polymer aqueous-phase systems proceeds for (at least two) hours. With high-phase columns one obtains, with time, ever-lower cell partition ratios containing greater proportions of cells with a higher P value. With low-phase columns the partitioning of cells of different sizes is affected by cell sedimentation with extended times. Depending on the relative partition ratios of the larger and smaller cells being separated, sedimentation can serve to enhance or diminish the separation by partitioning.

Extending the settling time of phases in cell separation procedures using low-phase columns (*e.g*, on a thin-layer counter-current distribution apparatus), as has been reported [9,10], must result, when working with populations of different cell size, in fractionations which depend on both cell surface properties (partitioning) and size (sedimentation). Such separations *cannot* thus be interpreted on the basis of cell surface properties [9,10].

Partitioning cells in high-phase columns is not only of advantage with respect to increasing separation efficiency [8] but also with respect to virtually eliminating the influence of other physical parameters (*i.e.*, cell size) on the fractionation.

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