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Effect of cell exposure to top or bottom phase prior to cell partitioning in dextran-poly(ethylene glycol) aqueous phase systems: erythrocytes as a model

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Cells exposed to dextran (Dx)-rich bottom phase prior to cell partitioning in Dx-poly(ethylene glycol) (PEG) aqueous two-phase systems have lower partition ratios than cells exposed to PEG-rich top phase. Aspects of this previously observed phenomenon were explored. In the present work charge-sensitive phases made with Dx T500 and PEG 8000 were used exclusively. It was found that: (1) even on countercurrent distribution (CCD) red cells (RBC) loaded in bottom phase have a lower apparent partition ratio, G, than the same cells loaded in top phase; (2) when part of the same cell population is loaded into top phase and part into bottom phase of the same load cavities for CCD, with the cells loaded into top or bottom bearing an isotopic tracer (^{51}Cr), the cells loaded into top phase have a higher G value than the cells loaded into bottom phase; (3) the shift in the CCD curves of human or of rat RBC between cells loaded in top or bottom phase using systems having the same polymer concentration (though different salt compositions) shows no striking difference and is, for the number of experiments run, not statistically significant; (4) when the quantity of cells loaded for CCD is reduced from 10^9 to 10^8, the G value of cells loaded in top phase is reduced slightly while that of cells loaded in bottom phase is diminished more appreciably; (5) increasing polymer concentrations yield larger differences in G values between (rat) RBC loaded in top or bottom phase; (6) when cells exposed to top or bottom phase, respectively, are centrifuged and suspended in bottom or top phase, respectively, their CCD patterns are qualitatively similar to cells exposed to these latter respective phases initially; (7) rat RBC populations containing ^{59}Fe-labeled cells of different but distinct age are fractionated on CCD irrespective of whether loaded in top or bottom phase. An exception are populations containing very young mature labeled cells (e.g., 4-d old) which are resolved when loaded in top phase but not in bottom phase. Thus cell populations exist which can be resolved by CCD when loaded in one of the phases but not when loaded in the other. Glutaraldehyde-fixed rat RBC containing 4-d old labeled cells are fractionated by CCD irrespective of whether loaded in top or bottom phase.

Introduction

Partitioning in two-polymer aqueous phase systems is an established method for the separation and fractionation of biomaterials including cells, organelles and membranes [1,2]. Adsorption of macromolecules on surfaces, when it occurs, is generally a much more rapid process than is desorption. The observation that erythrocytes exposed to dextran (Dx) have a lower partition ratio, P, in Dx-poly(ethylene glycol) (PEG) phase systems than cells not so exposed has previously been made [3] and is, most likely, a consequence of the adsorption of Dx to the cell surface and the preference of 'Dx-coated' cells for the Dx phase. The adsorption of dextrans to (red) cell surfaces has been much studied [4–7] and is the subject of a recent critical review [8]. PEG is also adsorbed on cells and, apparently, even more so than Dx (Brooks, D.E., personal communication). In the work reported here we examine whether cell surface changes due to exposure of cells to the bottom, Dx-rich, phase prior to CCD (in comparison to the 'standard' manner of exposing cells to and loading cells in top, PEG-rich, phase [9]) (a) affect cell separability, (b) are cell surface-specific and (c) are reversible. Such information may provide an additional parameter

Abbreviations: CCD, countercurrent distribution; Dx, dextran; G, the apparent partition ratio, obtained from the location of the peak in a CCD curve and equal to $r_{max}/(n-r_{max})$ where r_{max} is the cavity number of the peak of the distribution and n is the total number of transfers carried out; P, partition ratio, is the quantity of cells in a bulk phase as a percentage of total cells added; PEG, poly(ethylene glycol); RBC, red blood cells.

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for manipulating the P values of cells in Dx-PEG phase systems and optimizing conditions [10,11] for separation and fractionation of cell populations.

Materials and Methods

Reagents

Dextran (Dx) T500 (lot No. 01 06905) was obtained from Pharmacia LKB (Piscataway, NJ) and poly(ethylene glycol) 8000 (PEG, 'Carbowax 8000') from Union Carbide (Long Beach, CA). Glutaraldehyde was purchased from Ladd Research industries (Burlington, VT). [^{59}Fe]Ferrous citrate was a product of NEN (Boston, MA) and sodium [^{51}Cr]chromate was from ICN (Irvine, CA). All salts used were of reagent grade.

Collection of rat and human blood

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 400 g were bled by heart puncture. 10 ml of blood were collected in 3 ml acid-citrate-dextrose (ACD) anticoagulant. Human blood from presumably normal individuals was obtained by venipuncture using the above-indicated ratio of blood to anticoagulant. Red blood cells were used in experiments within one week of collection.

In vivo radioisotopic labeling of rat erythrocytes of different ages with ^{59}Fe

Some of the rats were injected with 10–20 μCi of [^{59}Fe]ferrous citrate via the saphenous vein. These were then bled at different times following injection giving rise to cell populations in which cells corresponding in age to the time elapsed between injection and bleeding were isotopically labeled [12]. Aliquots of erythrocytes were washed three times with at least ten times the cell volume of isotonic phosphate-buffered aqueous salt solution, pH 7.0 (PBS) before being used in the countercurrent distribution (CCD) experiments described below.

In vitro radioisotopic labeling of human or rat erythrocytes with ^{51}Cr and preparation of mixtures of labeled and unlabeled cells

Labeling of erythrocytes with [^{51}Cr]chromate has previously been described in detail [13]. Approximately 10–20 μCi ^{51}Cr were used per ml of an aliquot of anticoagulated human or rat blood. Labeled human or rat blood as well as an aliquot of unlabeled human or rat blood were then washed five times with PBS.

To examine the extent of separation of human plus rat erythrocytes as a function of phase (top or bottom) in which loaded (see below), a mixture of ^{51}Cr-labeled rat erythrocytes and an excess of unlabeled human red blood cells was prepared [14]. Into a centrifuge tube containing 4 ml of PBS were pipetted 0.5 ml of washed, packed, labeled rat erythrocytes and 2 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 the CCD experiments outlined below.

Fixation of rat erythrocytes with glutaraldehyde

^{59}Fe-labeled rat erythrocytes were fixed in glutaraldehyde [15]. The washed, packed cells were pipetted into 10 times their volume of a 280 mosM sodium phosphate buffer (pH 7.4), containing 1.85% (w/v) glutaraldehyde while mixing. Aliquots of fixed cells were washed two times with at least ten times their volume of an aqueous isotonic salt solution (saline) and three times with PBS prior to use in the CCD experiments described below.

Electronic cell counting

In some of the experiments aliquots of washed cells, suspended in saline were electronically counted using a Celloscope (Particle Data, Elmhurst, IL) operating on the Coulter principle and fitted with a 76 μm orifice tube. Countercurrent distributions of glutaraldehyde-fixed cells were also analyzed by electronic cell count (see below).

Preparation of two-polymer aqueous chase systems

Aqueous phase systems, selected and prepared as previously described, were used [9]. Phase system 1 contained 5% (w/w) Dx, 3.9% (w/w) PEG and 0.11 M Na-phosphate buffer (pH 6.8). Phase system 2 contained 5% Dx, 3.9% (w/w) PEG, 0.09 M Na-phosphate buffer (pH 6.8), and 0.03 M NaCl. System 3 contained 5% (w/w) Dx, 4.3% (w/w) PEG and the same ionic composition and concentration as system 2. System 4 was similar to system 3 except that it contained 4.5% (w/w) PEG instead of 4.3% PEG. These phase systems have an electrostatic potential between the phases (top phase positive) and are charge-sensitive (see Refs. 9 and 16 for a detailed discussion of their physical properties).

Each phase system was permitted to equilibrate in a separatory funnel at 4–5°C. Top and bottom phases were then separated. A small aliquot of top and of bottom phase was brought to room temperature for the cell incubation experiments described in the next section.

Incubation of erythrocytes in top or bottom phase

In our early experiments aliquots of washed, packed cells were suspended usually in ten times their volume of top or bottom phase and incubated at room temperature for 30 min prior to initiation of the experiments described below. The 30 min incubation period was subsequently found to be unnecessary. Cells exposed to

top or bottom phase without extended incubation yielded identical results.

Cell concentrations in top or bottom phases were adjusted to those used for loading the CCD apparatus (see below) and these 'load mixes' were returned to the cold room (4–5°C). In some experiments cells were centrifuged, washed once with saline, and resuspended at the desired concentration in the phase opposite to the one to which they had been initially exposed. In other experiments cells were centrifuged and resuspended without washing in the phase opposite to the one to which they had initially been exposed.

Countercurrent distribution (CCD) of erythrocytes

Our CCD apparatus (Workshop, Chemical Center, University of Lund, Sweden) consists of two circular Plexiglas plates, one stator and one rotor, having 120 cavities with a bottom phase capacity of 0.7 ml [17]. The 120 cavities enable us to carry out 30-transfer CCDs on four different cell populations simultaneously and without overlap. Each cell population to be studied was loaded into three adjacent cavities (i.e., 0–2, 30–32, 60–62 or 90–92) as described below for the different experiments. All CCDs were carried out at 4–5°C, with the phase system compositions indicated in the figures and tables, using 0.5 ml bottom phase, 0.9 ml top phase, a 6 min settling and a 22 s shaking time.

For the experiments depicted in Figs. 1, 2A and B, 3, 4 and Table I, load mixes (of the cells or cell mixtures indicated in the respective figures or table) made in top phase contained 0.25 ml packed red cells + 2.75 ml of top phase. Load mixes made in bottom phase contained 0.25 ml packed red cells + 1.5 ml of bottom phase. The three adjacent cavities which were to be loaded with top phase load mixes (e.g., 0–2, 60–62, see above) first received 0.5 ml of bottom phase. Nine-tenths ml of top phase load mix was then added to each of these cavities. For experiments in which bottom phase load mixes were to be used, 0.5 ml of these was first pipetted into three adjacent cavities (e.g., 30–32, 90–92) followed by 0.9 ml of top phase. All other cavities received 0.5 ml bottom phase and 0.9 ml top phase.

For the experiment shown in Table II load mixes (of the indicated cell mixture) made in top phase contained $2.5 \cdot 10^9$ or $2.5 \cdot 10^8$ red cells in 3 ml of top phase. Load mixes made in bottom phase contained $2.5 \cdot 10^9$ or $2.5 \cdot 10^8$ cells in 1.75 ml of bottom phase. These four cell suspensions were loaded onto the CCD apparatus as described in the previous paragraph.

Experiments with cells incubated in top or bottom phase (as in Figs. 2A and B) but subsequently centrifuged, washed once with PBS and then suspended and loaded in top phase (using the same cell and phase volumes as described for Figs. 2A and B), are presented in Figs. 2C and D.

For the experiments depicted in Figs. 5A and B, load mixes of human ^{51}Cr-labeled or unlabeled erythrocytes made in top phase contained 0.125 ml packed red cells + 2.75 ml of top phase. Load mixes made in bottom phase contained 0.125 ml packed red cells + 1.5 ml of bottom phase. Loading of the CCD apparatus was as follows: Cavities 0–2 received 0.5 ml of the human unlabeled erythrocyte load mix made in bottom phase and also 0.9 ml of the human ^{51}Cr-labeled erythrocyte load mix made in top phase; cavities 30–32 received human ^{51}Cr-labeled erythrocyte load mix made in bottom phase and also 0.9 ml of human unlabeled erythrocyte load mix. The other cavities (except for 60–62 and 90–92, see below) all received 0.5 ml bottom and 0.9 ml top phase.

For the experiments depicted in Figs. 5C and D, load mixes of human ^{51}Cr-labeled or unlabeled erythrocytes were incubated in top or bottom phase. After incubation the cells were centrifuged, the supernatant solution discarded and, without washing, load mixes were made using the phase opposite to the one in which the cells had been incubated. Cell and phase volumes were as described in the previous paragraph. Loading of the CCD apparatus was as follows: cavities 60–62 received the human ^{51}Cr-labeled erythrocytes incubated in top phase and load mix made in bottom phase and also human unlabeled erythrocytes incubated in bottom phase and load mix made in top phase; cavities 90–92 received human unlabeled erythrocytes incubated in top phase and load mix made in bottom phase and also human ^{51}Cr-labeled erythrocytes incubated in bottom phase and load mix made in top phase.

Analysis of cells after CCD

After CCD the erythrocytes in each cavity were collected, by means of a fraction collector, directly into plastic centrifuge tubes. Saline (0.7 ml) was added to each tube thereby reducing the polymer concentrations and giving rise to a single homogeneous supending medium. Cells in each three adjacent cavities (sometimes more at the ends) were pooled. They were centrifuged at $1200 \times g$ for 10 min, the supernatant solution was discarded, and the cells were lysed in 3 ml of 20 mosM sodium phosphate buffer (pH 7.2–7.4). The tubes were then centrifuged at high speed to remove the stroma. Aliquots were analyzed for hemoglobin absorbance (540 nm) on a Gilford spectrophotometer and ^{59}Fe or ^{51}Cr radioactivity was determined on a Beckman scintillation well-counter.

To analyze the CCD of glutaraldehyde-fixed red cells, phases were similarly diluted with saline and adjacent tubes pooled. Cells were then counted electronically on the Celloscope and radioactivity was determined as described above.

Comparison of the magnitude of shift in apparent partition ratio, G, of erythrocytes as a function of whether cells are loaded in top or bottom chase

To assess and compare the magnitude of the shift of distribution curves of erythrocytes from different species (e.g., human, rat) as a function of whether cells were loaded in top or bottom phase (Table I, top), phase systems with identical polymer concentrations were used but with ionic conditions chosen [9] such that one of the distribution curves (i.e., cells loaded in top or bottom phase) for each species gave an apparent partition ratio, G, of approximately 1.0.

To compare the effect of polymer concentrations on the magnitude of the shift of a distribution curve of erythrocytes from a given species (rat), experiments, (Table I, bottom), were carried out in two phase systems in one of which the G value of the erythrocytes was 1.0 with cells loaded in bottom phase and the other with cells loaded in top phase.

Presentation of data

Distribution curves in Figs. 1–5 are given either in hemoglobin absorbance at 540 nm (obtained on lysis of erythrocytes) or in cell counts (of glutaraldehyde-fixed red blood cells) obtained in the different cavities along the extraction train. Isotope distributions are given in cpm. A relative specific activity is also shown and is defined [12] as:

$$\frac{\text{cpm/hemoglobin absorbance (or cell count) in a given cavity}}{\text{cpm/hemoglobin absorbance (or cell count) in the original unfractionated cell population}}$$

Table I shows the difference, in terms of number of cavities, in peak location on CCD for a number of experiments (each represented by an X) for the indicated cells loaded in top or bottom phase. Statistical significance of the observed differences was tested by use of the Mann-Whitney, non-parametric, rank-order test [18].

In Table II is recorded the mean number ± S.D. (for a total of seven separate experiments) of the peak cavity of human and of rat erythrocytes after CCD, as well as the numerical difference between these cavities, as a function of (a) total number of cells loaded (10^9 or 10^8) and (b) whether loaded in top or bottom phase.

Results and Discussion

Effect of phase in which loaded on the cell apparent partition ratio obtained on countercurrent distribution (CCD)

The CCD curves of rat erythrocytes presented in Figs. 1, 2A and B, 3 and 4 indicate that cells exposed to and loaded in top phase have higher apparent partition ratios (i.e., are further to the right) than the same cells exposed to and loaded in bottom phase (compare relative positions of curves given in hemoglobin absorbance or cell counts in the respective top and bottom halves of indicated figures). The most likely explanation for this phenomenon is the adsorption of the polymer to which the cells are first exposed on the cell surface and its retention during CCD. Cells 'coated' with top phase would tend to go more into the top phase while those 'coated' with bottom phase would have the opposite tendency.

Effect of phase in which loaded on the fractionation by CCD of rat red blood cells of different ages

Using a combination of isotopic labeling techniques and CCD of rat red blood cells it has previously been established that the G value of rat red blood cells changes as a function of cell age [9,12]. Reticulocytes have the lowest G value of any peripheral blood red cell, young mature erythrocytes the highest and erythrocytes of ever-increasing age have ever-diminishing G values. The partition ratios of the oldest erythrocytes are very close to those of reticulocytes. All of these experiments were conducted in the 'standard' manner with cells loaded in top phase [9] and it is thus of interest to examine whether the resolution of red cells of different ages is affected by the phase in which cells are first suspended.

We find that, at least qualitatively, the relative partitions of 18-h labeled red cells (reticulocytes), Figs. 1A and B, and 13-d old erythrocytes, Figs. 1C and D, are unaffected by the phase (top or bottom) into which cells are loaded. These results are representative of the resolution of red blood cells of different ages except for very young erythrocytes. Fig. 2 shows that the difference in G between young (e.g. 4-d old labeled) red cells and the bulk of erythrocytes readily observed when cells are loaded in top phase (Fig. 2A) is eliminated when an aliquot of the same population is loaded in bottom phase (Fig. 2B). Washing cells exposed to top or bottom phase with PBS and, subsequently, loading the washed cell populations in top phase (Figs. 2C and D) yields CCD patterns which are identical to each other and to the results depicted in Fig. 2A. Thus the shift in distribution curve and the elimination of the resolution (Fig. 2B) when loading cells in bottom phase are reversible.

Why of all rat erythrocytes only the young mature cells are not resolvable from the rest of the rat red blood cell population when loaded in bottom phase is not clear. However, these are the cells that have just matured from reticulocytes and have, during this process, undergone rapid changes in G value [12]. Perhaps they are more sensitive to the slight difference in one of the physicochemical properties [16] (e.g., pH, salt composition) between top and bottom phases than are the other red cells. Such a possibility is indicated by

the finding that glutaraldehyde fixation of rat erythrocyte populations containing 4-d old labeled cells permits their resolution when loaded either in top or bottom phase (Figs. 3A and B). Whatever the cause it is worthy of note that some cell populations that are resolvable when loaded in one phase are not necessarily resolved when loaded in the other.

Effect of phase in which loaded on the CCD pattern of a mixture of rat isotopically labeled red blood cells + human unlabeled erythrocytes

The CCD patterns of a mixture of ^{51}Cr-labeled rat red cells (1 part) + human unlabeled erythrocytes (4 parts) as a function of loading in top or bottom phase are shown in Fig. 4 (top and bottom, respectively). The cell separation, in this case, is more pronounced when the mixture is loaded in top phase which is most likely due to the relative G values of rat and human erythrocytes when loaded in top phase: one population has a higher (rat), the other (human) a lower G value than 1.0. The best separation of materials on CCD are obtained under these circumstances [10,11]. Thus the selection of the phase in which cells are loaded (i.e., top or bottom) provides another parameter (see Ref. 9 for others) for obtaining desirable partition ratios for their separation.

CCD patterns of human isotopically labeled erythrocytes loaded in one phase and human unlabeled cells loaded in other phase of the same cavities; reversibility of effect

When ^{51}Cr-labeled (human) erythrocytes are loaded in top phase and unlabeled erythrocytes from the same donor are loaded in bottom phase of the same cavities, the difference in the respective G values of cells loaded in top or bottom is maintained during the CCD (Fig. 5A) as is apparent from the displaced distributions of total and isotopically labeled cells. Labeling the cells that are loaded in bottom phase and loading unlabeled cells in top phase yields the anticipated reverse pattern (Fig. 5B).

When ^{51}Cr-labeled cells in top phase are centrifuged, resuspended and loaded in bottom phase and unlabeled cells in bottom phase are centrifuged, resuspended and loaded in top phase, the CCD patterns obtained are the same as if the labeled cells had only been in bottom and unlabeled cells only in top phase

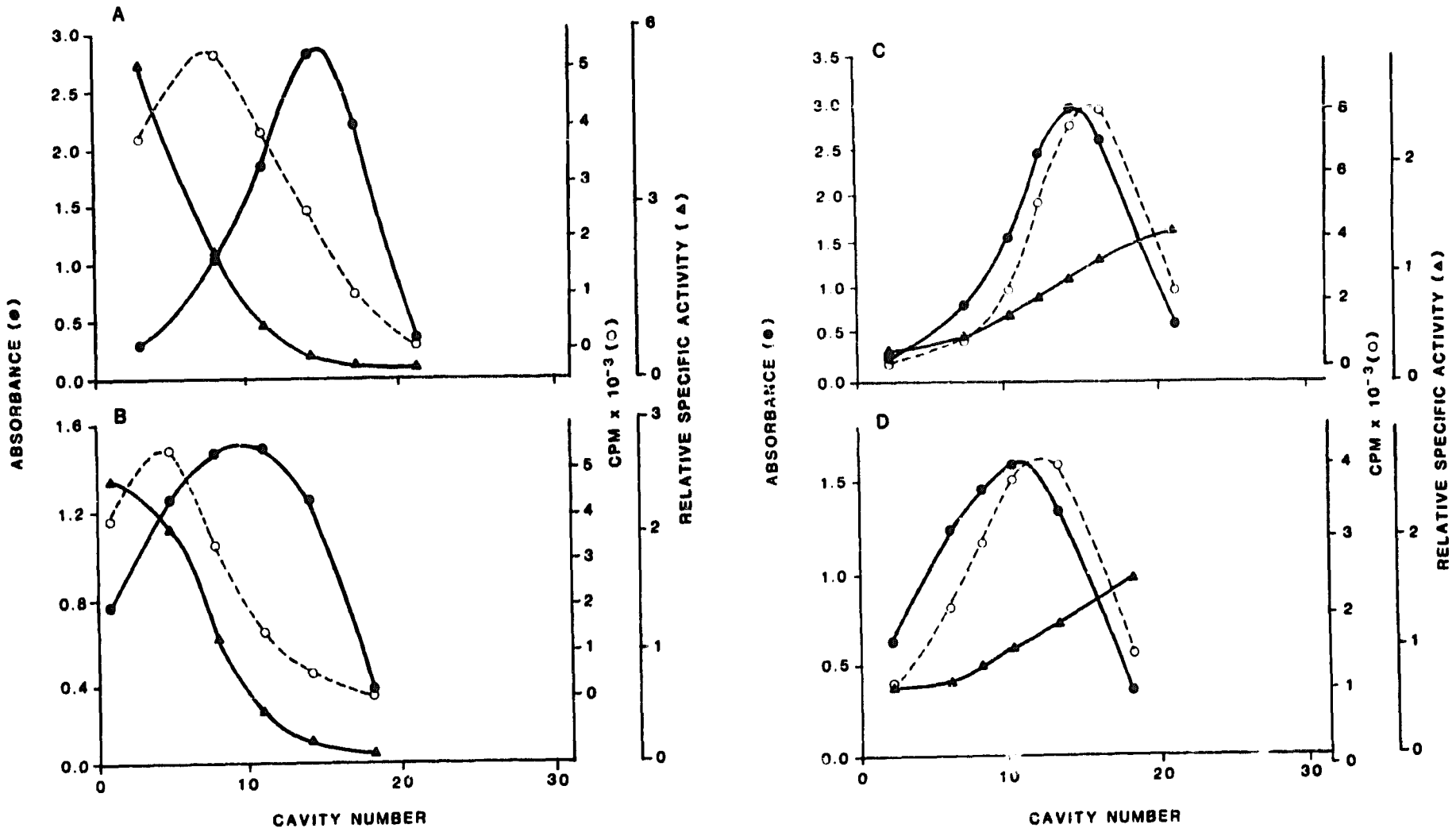

Fig. 1. Countercurrent distribution patterns of rat red blood cells obtained from animals which had been injected with [^{59}Fe]ferrous citrate either 18 h (rat 1990-7) or 13 d (rat 1990-2) prior to bleeding. The cell populations thus contain, respectively, reticulocytes or 13-d old erythrocytes that are labeled. (A) Rat 1990-7 cell population exposed to and loaded in top phase onto the CCD apparatus; (B) cell population as in (A) but exposed to and loaded in bottom phase; (C) rat 1990-2 cell population exposed to and loaded in top phase; (D) cell population as in (C) but exposed to and loaded in bottom phase. 30 transfers were carried out at 4–5°C using phase system 3 with a settling time of 6 min and a shaking time of 22 s. Distribution curves (●) of the red blood cell populations are given in terms of hemoglobin absorbance. The distributions of labeled cells of distinct cell age (i.e., 18-h or 13-d old) are depicted in cpm (○). A relative specific activity (▲) is also shown. Note that cells loaded in bottom phase have lower G values (i.e., are to the left) than the same cell population loaded in top phase while reticulocytes have lower and 13-d old erythrocytes higher partition ratios than the bulk of the red blood cells irrespective of phase in which loaded. See text for additional details and discussion.

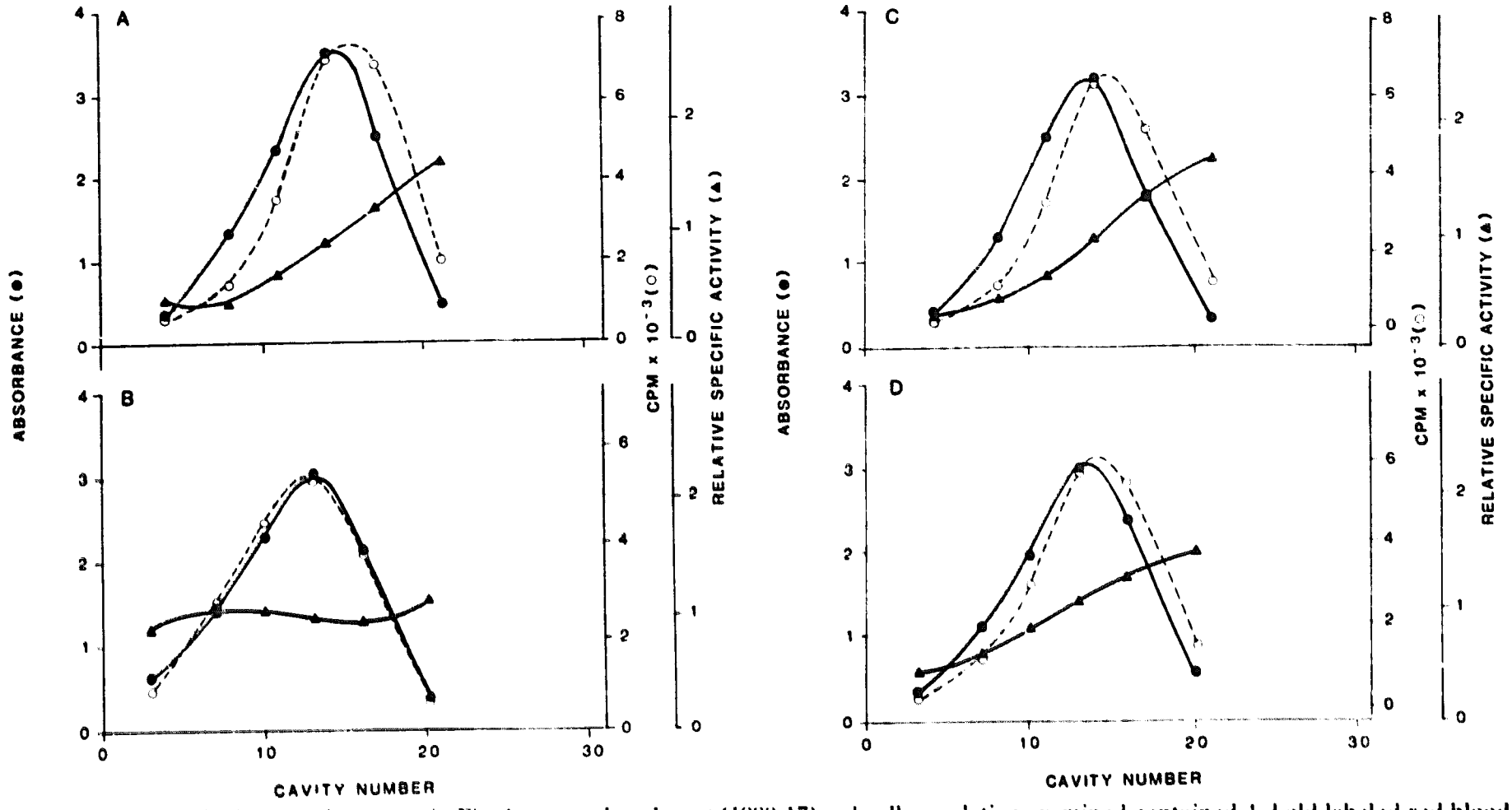

Fig. 2. (A) and (B) depict experiments as in Fig. 1 except that the rat (1990-17) red cell population examined contained 4-d old labeled red blood cells. Note that when these mature young erythrocytes are exposed to and loaded in bottom phase onto the CCD apparatus (B) there is no resolution of labeled erythrocytes and the bulk of the red blood cell population. When cells exposed to top or to bottom phase are centrifuged, washed once with PBS, resuspended and loaded in top phase the results depicted in (C) and (D) are obtained. The patterns obtained in (C) and (D) are similar to one another and to those in (A). This reflects (1) that the displacement to lower G value when loading cells in bottom phase is reversible when the cells are washed and resuspended and loaded in top phase and (2) that cells which are not resolved when loaded in bottom phase (B) are resolved after washing and loading in top phase (D). See text for additional details and discussion.

TABLE I

Number of cavities between the peaks of the distribution curves of erythrocytes loaded in top or bottom phases

In systems with the same polymer concentrations: Rat RBC, 5:3.9 No. 2, have G value of $0.98 + 0.12$ (13) when loaded in bottom phase. Human RBC, 5:3.9 No. 1, have G value of $0.98 + 0.08$ (10) when loaded in top phase.

Cavity difference	Species	
	rat	human
1		X
2		XXX
3	XXXXXXXXXXXX	XXXXX
4	XX	
	$P > 0.05$ (N.S.)	

In systems with different polymer concentrations: Rat RBC, 5:3.9 No. 2, have G value of 0.98 ± 0.12 (13), when loaded in bottom phase. Rat RBC, 5:4.3 No. 2, have G value of 0.95 ± 0.09 (32) when loaded in top phase.

Cavity difference	Phase system	
	5:3.9	5:4.3
2		XX
3	XXXXXXXXXXXX	XXXXX
4	XX	XXXXXXXXXXXXX
5		XXXXXXXXXXX
> 5		XX
	$P < 0.00003$	

(Fig. 5C). The inverse experiment is depicted in Fig. 5D. Thus the phenomenon described in Figs. 5A and B is reversible and it is only the last phase with which cells are in contact that determines their G value on CCD. The mechanism by which bound Dx is removed from the cell surface is not known [8]. Bound Dx does

TABLE II

*Relative shift in partition * of rat and of human erythrocytes (^{51}Cr-labeled rat + human red cell mixture) as a function of cell quantity and phase (top or bottom) in which loaded*

* Data are given in terms of the cavity in which the peaks of the human and rat erythrocyte distributions are found on countercurrent distribution, 30 transfers, in the same charge-sensitive phase system.

Cells loaded	Human	Rat	Difference
$(2-2.25)\cdot 10^9$ cells			
in Top phase	10.1 ± 3.2 (7)	18.1 ± 2.9 (7)	8.0 ± 2.1 (7)
in Bottom phase	7.6 ± 3.6 (7)	14.3 ± 2.9 (7)	6.7 ± 2.8 (7)
$(2-2.25)\cdot 10^8$ cells			
in Top phase	9.6 ± 3.6 (7)	17.0 ± 1.0 (7)	7.4 ± 2.4 (7)
in Bottom phase	5.4 ± 3.1 (7)	10.3 ± 3.9 (7)	4.9 ± 2.5 (7)
Ratios	$10^9/10^8$ cells		
	Human	Rat	Difference
Top/Top	1.05	1.06	1.08
Bottom/Bottom	1.41	1.31	1.39

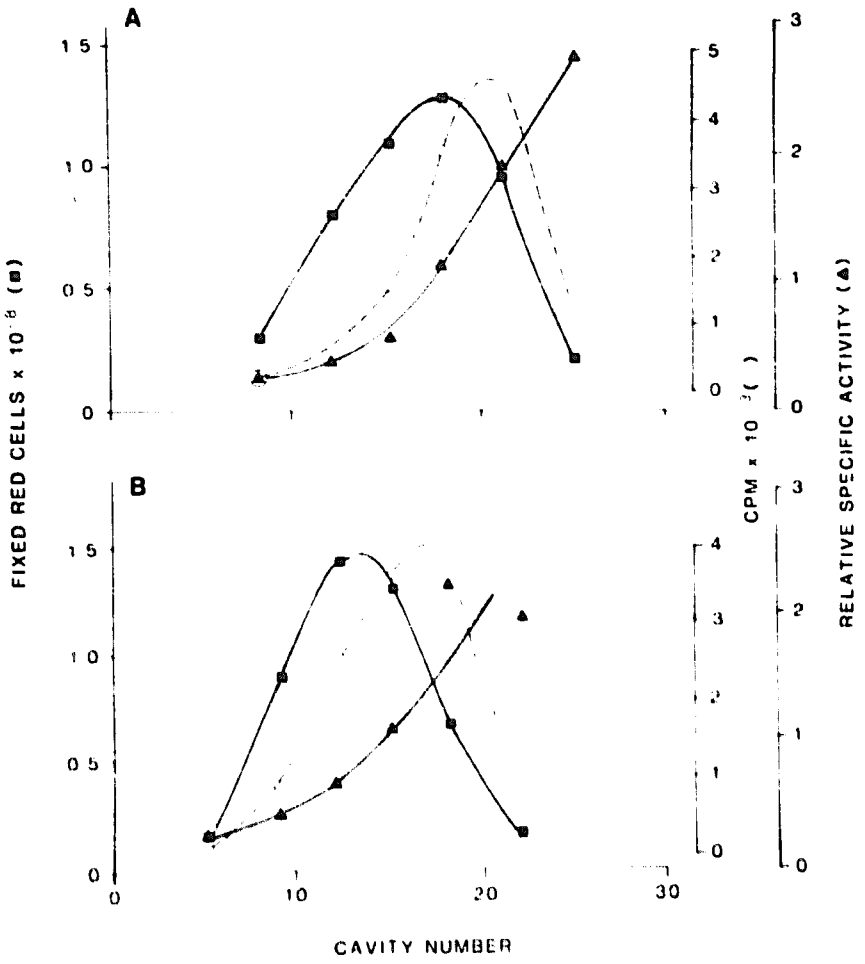

Fig. 3. Erythrocytes from rat 1990-17 (containing 4-d old labeled cells, see Fig. 2) were fixed with glutaraldehyde. Countercurrent distribution patterns of these fixed cells exposed to and loaded in top phase onto the CCD apparatus are depicted in (A); (B) shows the fixed cells as in (A) but exposed to and loaded in bottom phase. CCD procedures and symbols are as in Fig. 1 except that phase system 4 was used and that the distribution curves (■) of the red blood cell populations are given in terms of electronic cell counts. Note that the 4-d old cells in the fixed erythrocyte population loaded in bottom phase (B) have, unlike 4-d old fresh erythrocytes (Fig. 2B), a markedly different G value from the bulk of the red blood cells. See text for additional details and discussion.

not desorb significantly by exchange with free Dx as do bound proteins in the presence of free protein [4]. We are, however, not aware of polymer desorption experiments carried out in the presence of another polymer. Perhaps a polymer phase other than the one adsorbed to the cell surface can remove (or substitute for) the intially adsorbed phase from the cells. In the presence of both phases, as during the CCD extractions, the effect of the phase in which cells were initially loaded is maintained.

Relative shift in partition ratios (G) of erythrocytes from different species as a function of loading cells in the top or bottom phases of systems having (a) the same and (b) different polymer concentrations

In order to appropriately assess the relative shift in partition ratios of red blood cells from different species (human, rat), as a function of the phase (top or bottom) in which cells are loaded, the comparison must be carried out in a manner such that the phase system compositions differ only slightly from one another (i.e., that the physicochemical properties of the phases are reasonably close to one another) and that one of the distribution curves of cells from each species (i.e., cells loaded in top or bottom phase) has the same G value. For the study of human and rat erythrocytes phase systems with identical polymer concentrations were chosen (i.e., 5% Dx, 3.9% PEG) and salt compositions selected so that the G value of the cells would be close to 1.0 when the rat red cells were loaded in bottom phase of phase system 2 and when the human erythrocytes were loaded in the top phase of phase system 1 (Table I). Fourteen CCD experiments were carried out with rat and nine with human red cells. The number of cavities (after a 30 transfer CCD) between the peaks of the distribution curves obtained in each experiment with rat or human cells is indicated by an 'X' in the upper box of Table I. There is no striking difference in the shift observed on loading cells in bottom rather than top phase for these two species' red cells which differ appreciably in surface properties [9]. If a difference does exist it is very subtle and did not reach statistical significance for the number of experiments done ($P > 0.05$). A few experiments carried out with

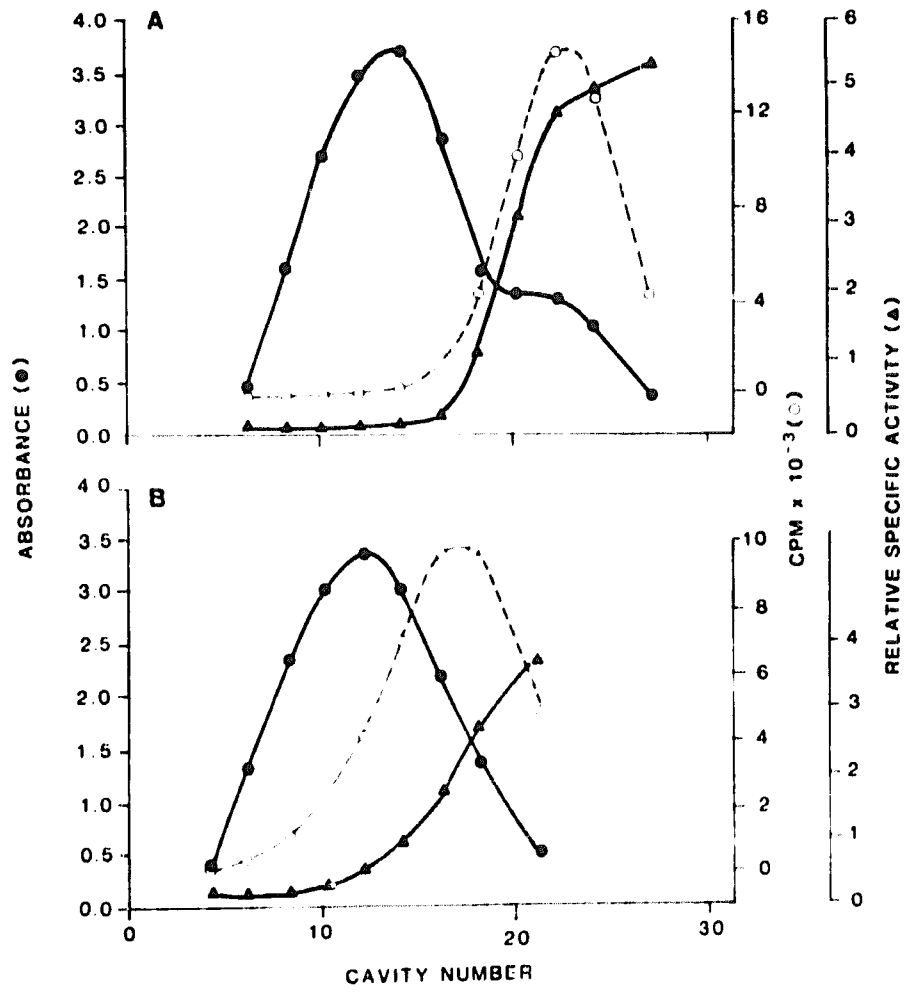

Fig. 4. Countercurrent distribution patterns of a mixture of rat ^{51}Cr-labeled erythrocytes + human unlabeled red blood cells (1:4) when loaded in (A) top or in (B) bottom phase onto the CCD apparatus. CCD procedure and symbols are as in Fig. 1 except that phase system 1 was used. Note that, in the example provided with the initial G values indicated, the shift in CCD curves obtained when loading cells in bottom vs. top phase results in different relative G values for the human (left peak) and rat (right peak) erythrocytes. A different degree of resolution of the two cell populations is obtained. Loading cells in top vs. bottom phase is thus another parameter with which cell partition ratios can be adjusted in Dx-PEG aqueous phase systems.

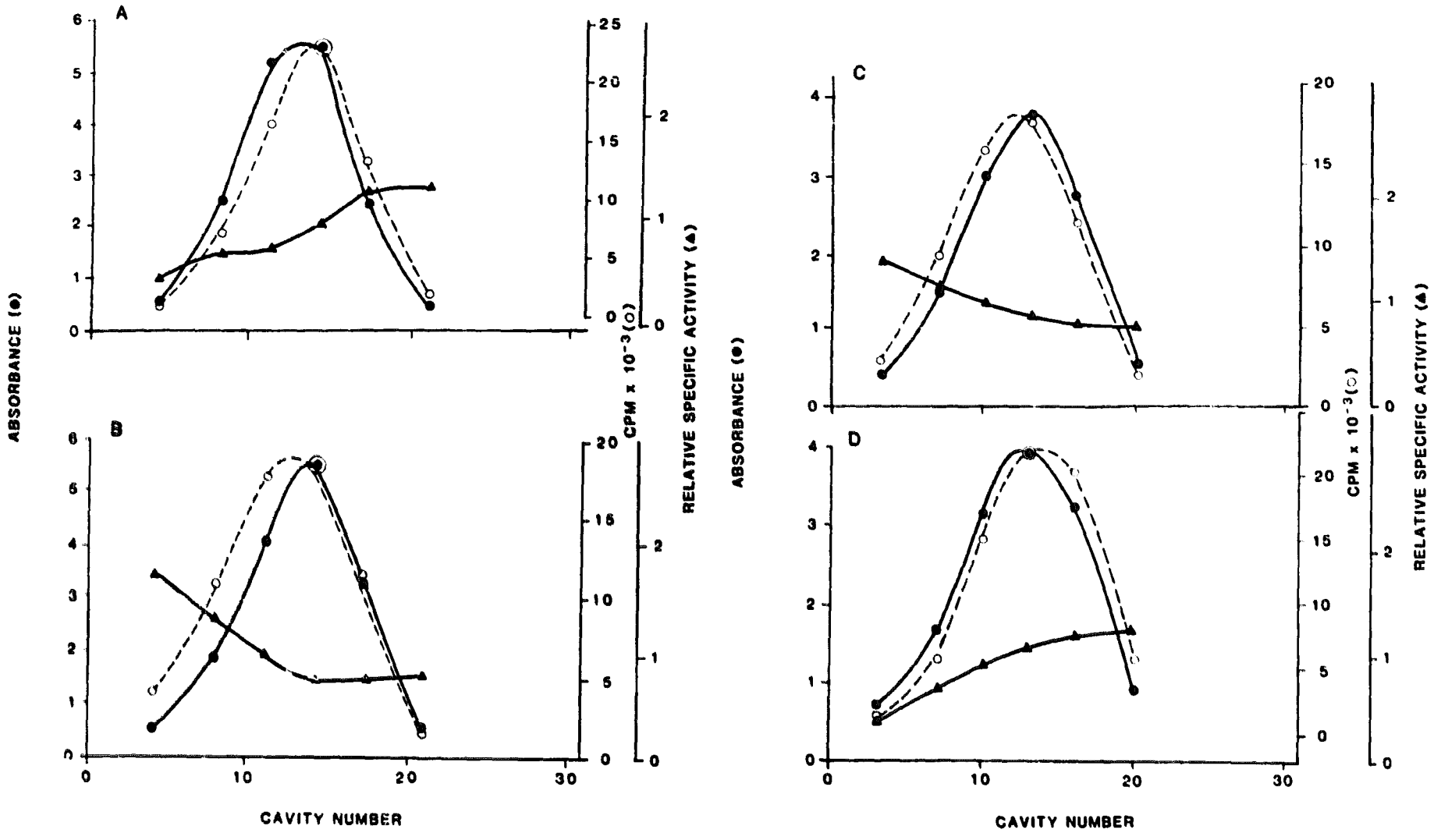

Fig. 5. Aliquots of a human erythrocyte sample were labeled with [^{51}Cr]chromate. These labeled cells were suspended in (A) top phase and loaded onto the CCD apparatus. Unlabeled cells from the same individual were suspended in bottom phase and loaded into the same cavities as the labeled sample. Equal quantities of labeled and unlabeled cells were loaded. CCD was carried out as in Fig. 1 except that phase system 1 was used. (B) Shows an experiment analogous to that in (A) except that the labeled cells were suspended and loaded in bottom phase and the unlabeled cells were suspended and loaded in top phase. Note that in each case cells loaded in bottom maintain a lower G value than the same cells loaded in top phase during the CCD run and in the presence of the cells loaded in top phase. (C) Shows results of an experiment in which labeled cells were suspended in top phase, then centrifuged and, without washing, suspended and loaded in bottom phase onto the CCD apparatus. Unlabeled cells were suspended in bottom phase, then centrifuged and, without washing, suspended and loaded in top phase into the same cavities as the labeled cells. Equal quantities of labeled and unlabeled cells were loaded. CCD and symbols are as in (A) and (B). (D) Shows an experiment as in (C) but with reversal of labeled and unlabeled cells. Note in (C) and (D) that the distribution patterns of cells due to cell exposure to a given phase (top or bottom), as illustrated in (A) and (B), is reversible when cells are removed from the initial suspending phase and suspended and loaded in the opposite phase. See text for additional details and discussion.

erythrocytes from sheep and from beef yielded analogous results.

To test the effect of polymer concentration on the magnitude of the shift when cells are loaded in bottom vs. top phase we used rat erythrocytes and phase system compositions as indicated in the lower half of Table I. Rat RBC have a G value of about 1.0 when loaded in bottom phase of system 2 (as previously indicated) while a similar G value is obtained when these cells are loaded in top phase of phase system 3. The data in the lower box of Table I show a statistically significant increase in the number of cavities separating the peaks of the CCD of cells loaded in top or bottom phase with increasing polymer concentration.

Relative shift in partition of rat and human erythrocytes (mixture of rat ^{51}Cr-labeled + human unlabeled red cells) as a function of total cell quantity and phase (top or bottom) in which loaded

Table II indicates the peak cavities (mean ± S.D.) of the countercurrent distribution curves obtained for human and for rat erythrocytes when two different quantities of identical mixtures of these cells (with rat cells labeled with ^{51}Cr) are loaded in top or bottom phase. It is known that the cell G value decreases on CCD with diminishing quantities of cells when loaded in the 'standard' manner, i.e., in top phase [17]. However, from the ratios given in Table I it is apparent that the partition ratio of cells (both human and rat erythrocytes) diminishes much less with decreasing numbers of cells (10^9 to 10^8) when loaded in top rather than bottom phase (i.e., about 1.05 vs. 1.35). Thus the effect of Dx on shifting smaller quantities of cells to lower G values is more pronounced than that of PEG.

Conclusions

This paper examines aspects of the tendency of polymers to adsorb to cell surfaces and the effect of this phenomenon on the partitioning of cells in Dx-PEG phase systems. In phase systems containing the Dx (mol.wt. 500 000) and PEG (mol.wt. 8000) used in the

currently reported experiments, erythrocytes partition between the top phase and the interface [9]. Since cells loaded in bottom phase have lower G values than the same cells loaded in top phase it appears likely that, according to the previously described mechanism of cell partitioning (see Ref. 19 for discussion), cells 'coated' with bottom phase adsorb to the Dx droplets in top phase (following mixing of phases and initiation of the partitioning process) to a greater extent than do those 'coated' with top phase.

The fractionation of cells is usually (not always) similar irrespective of phase in which loaded (when G value in phase is taken into account).

CCD curves of cells with different surface properties (i.e., RBC from different species) appear to be quantitatively shifted to the same extent when loaded in bottom vs. top phase of systems having the same polymer concentrations and so selected, for appropriate comparison, that one of the CCD curves of each species has the same G value (see Results and Discussion). Erythrocytes with identical surface properties (i.e., cells from a given species) display increasing differences in G values of cells loaded in bottom vs. top phase in systems with increasing polymer concentrations and so selected, for appropriate comparison, that one of the CCD curves at each polymer concentration has the same G value (see Results and Discussion).

It is known that when smaller quantities of cells are loaded on a CCD their G value is lower. The current experiments reveal that as the quantity of cells loaded is reduced (i.e., from 10^9 to 10^8), a greater diminution in G value results with cells loaded in bottom than in top phase.

Polymer adsorption to cells is, as reflected by their partitioning behavior, at least qualitatively, reversible.

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References

1 Walter, H. and Johansson, G. (1986) Anal. Biochem. 155, 215–242.
2 Walter, H., Johansson, G. and Brooks, D.E. (1991) Anal. Biochem. 197, 1–18.
3 Hill, R.S., Norris-Jones, R., Still, B. and Brooks, D.E. (1986) Am. J. Hematol. 21, 249–257.
4 Rothman, S., Adelson, E., Schwebel, A. and Langdell, R.D. (1957) Vox Sang. 2, 104–109.
5 Brooks, D.E. (1973) J. Colloid interface Sci. 43, 701–713.
6 Chien, S., Simshon, S., Abbott, R.E. and Jan, K.-M. (1977) J. Colloid Interface Sci. 62, 461–470.
7 Buxbaum, K., Evans, E. and Brooks, D.E. (1982) Biochemistry 21, 3235–3239.
8 Janzen, J. and Brooks, D.E. (1991) in Interfacial Phenomena in Biological Systems (Bender, M., ed.), pp. 193–250, Marcel Dekker, New York, New York.
9 Walter, H. (1985) in Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Applications to Biotechnology (Walter, H., Brooks, D.E. and Fisher, D., eds.), pp. 327–376, Academic Press, Orlando, FL.
10 Walter, H., Brooks, D.E. and Fisher, D., eds. (1985) Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Applications to Biotechnology, Academic Press, Orlando, FL.
11 Albertsson, P.-Å. (1986) Partition of Cell Particles and Macromolecules, Wiley-Interscience, New York, NY.
12 Walter, H., Miller, A., Krob, E.J. and Ascher, G.S. (1971) Exp. Cell Res. 69, 416–424.
13 Walter, H. and Krob, E.J. (1983) Cell Biophys. 5, 205–219.
14 Walter, H. and Krob, E.J. (1984) Biochem. Biophys. Res. Commun. 120, 250–255.
15 Walter, H., Krob, E.J., Pedram, A., Tamblyn, C.H. and Seaman, G.V.F. (1986) Biochim. Biophys. Acta 860, 650–661.
16 Bamberger, S.B., Brooks, D.E., Sharp, K.A., Van Alstine, J.M. and Webber, T.J. (1985) in Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Applications to Biotechnology (Walter, H., Brooks, D.E. and Fisher, D., eds.), pp. 85–130, Academic Press, Orlando, FL.
17 Walter, H. and Krob, E.J. (1984) Cell Biophys. 6, 253–262.
18 Siegel, S. (1956) Nonparametric Statistics for the Behavioral Sciences, McGraw-Hill, New York, NY.
19 Fisher, D., Raymond, F.D. and Walter, H. (1991) in Cell Separation Science and Technology, ACS Symposium Series, Vol. 464 (Kompala, D.S. and Todd, P., eds.), pp. 175–188, American Chemical Society, Washington, DC.