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PARTITIONING BEHAVIOUR IN AQUEOUS TWO-PHASE SYSTEMS AND FRACTIONATION BY COUNTER-CURRENT DISTRIBUTION OF CHICK-EMBRYO ERYTHROCYTES WITH NUMERICAL RESOLUTION OF DISTRIBUTION CURVES

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SUMMARY

The partition of chick-embryo and young-chick erythrocytes in dextran–poly(ethylene glycol) two-phase systems depends on the interfacial tension and electrical potential differences between the phases. Counter-current distribution with charged 5% dextran–poly(ethylene glycol) systems has proved to be an adequate method for the separation of primitive and definitive erythrocytes present in chick embryos when a phase settling time of 20 min is used. The computer-aided numerical resolution of experimental curves has shown the existence of subpopulations which could not have been detected by using conventional methods.

INTRODUCTION

Morphologically distinct red blood cells (RBCs) are present during the development of chick embryos and young chicks, reaching a single population in adult chickens[1–4]. The successive erythroid cells derive from the same ancestral stem cell or they are the progeny of independent stem cells arising successively at different steps of development[1–5]. As primitive RBCs are being replaced by definitive ones, differences in surface glycoproteins[3,6], globin genes transcribed and peptide chain produced (four haemoglobins in primitive and two in definitive RBCs)[3,7–10] and organic phosphates involved in the control of haemoglobin oxygenation (2,3-bisphospho-glycerate in embryos and inositol-pentaphosphate after hatching)[11–13], have been described. The separation of definitive from primitive RBCs is then a prior objective for studies of RBC switching.

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A suitable separation procedure is based on the partition ratio of cells into the upper phase and the interface of aqueous systems formed by a dextran (D)-rich lower phase and a poly(ethylene glycol) (PEG)-rich upper phase[14–18]. A study of basic partitioning parameters responsible for both the interfacial tension (cell partition due to surface hydrophobicity) and the electrical potential difference between the phases (cell partition on the basis of surface charge-related properties), should be made prior to cell separation by D–PEG partitioning. The first objective of this work was then to study, in single-tube experiments, the dependence of the partition of embryo and young chick erythrocytes on the interfacial tension and the electrical potential difference between the phases generated by different phosphate and/or polymer concentrations.

Albertsson's thin-layer counter-current distribution (CCD) procedure for multiple partitions can then be applied for the fractionation of cells with divergent partition values[14–20]. Using this procedure with charged 5% D–4% PEG systems, cell fractionation according to age of the single RBC populations present in adult chickens was shown[15], and the replacement of primitive by definitive RBCs during the developmennt of young chicks was demonstrated by a combination of CCD fractionation with radioactive labelling (^{59}F) of cells[16]. The separation of these two RBC populations in young chicks was achieved after increasing the settling time (t = 20 min) allowed for the phases to be separated after each mixing step during the CCD procedure[16–18]. The second objective of this paper is then to demonstrate the presence of both RBC populations in 16-day-old embryo chicks. To show cell separation with charged 5% D–4% PEG systems, decreasing upper/lower phase volume ratios in the cavities of the CCD rotor and increasing settling times were tested.

A computerized study of theoretical and experimental CCD curves[18,21] was made in order to establish the number of RBC subpopulations in chick embryos. Results from this work and others[14,16–19,22–24] show an improvement in the efficiency of CCD fractionation of heterogeneous cell populations after increasing the settling time with charged two-phase systems and suggest that this is a determinant mechanism of cell fractionation by CCD.

EXPERIMENTAL

Four-day-old chicks and 16 embryos of White Leghorn chickens were obtained from a local Poultry farm (Avicola Grau, Madrid, Spain). Blood was collected and erythrocytes were prepared as before[18].

Two-phase systems were prepared as described[18,24] from the following stock solutions (in water): 20% (w/w) Dextran T-500 (D) (Pharmacia, Sweden), 40% (w/w) poly(ethylene glycol) (PEG-6000) (Serva, F.R.G.), 1 M sodium chloride and 0.2 M sodium phosphate buffer (NaPB), pH 6.8. Systems were prepared in sufficient quantity (800 g) for several experiments. They were allowed to equilibrate (24 h, 4°C) and the upper and lower phases were then separated.

For single-tube experiments, charged systems (5 g) containing increasing polymer concentrations (see section 1a for composition) or low-polymer systems of decreasing charge (see section 1b for composition) were individually prepared by weighing adequate quantities of the above stock solutions and water, into 10-ml graduated tubes. The phases were allowed to equilibrate (4°C) and then vigorously

shaken and allowed to settle (24 h, 4°C). Finally, if the volumes of the phases were remarkably different from each other, as is normal when the polymer concentration increases, enough of the upper and lower phases was carefully removed so as to make them nearly equal.

Partition was measured by adding 0.02 ml of RBC suspension to each system. After mixing (60 inversions), the systems were allowed to settle for 20 min at 4°C. Aliquots (0.2 ml in duplicate) were removed from the upper phase. Haemoglobin was measured by a standard method. The partition ratio, P, is given by the quantity of haemoglobin in the upper phase as a percentage of the total cells added.

For CCD experiments (section 2), charged 5% (w/w) D–4% (w/w) PEG systems were used. The CCD has been described and an automatic version of the thin-layer CCD apparatus with a 60-cavities rotor was used[14,18,19]. Each cavity received a constant volume of the lower phase (0.7 ml), except cavity 1 which received 0.1 ml of cell suspension plus 0.6 ml of lower phase. Different volumes of the upper phase (0.9, 0.7 or 0.3 ml) were then added. This means that the total volume of the phase system was 1.6, 1.4 or 1.0 ml and the ratio, L, between the volumes of the upper and lower phases was $0.9/0.7 = 1.3$, $0.7/0.7 = 1$ or $0.3/0.7 = 0.45$, respectively. Taking into account the volume of the lower plate cavities, *i.e.*, stationary phase, 0.8 ml, the volume of the mobile upper phase was 0.8, 0.6 or 0.2 ml and the ratio between the volumes of the mobile and stationary phases was $0.8/0.8 = 1$, $0.6/0.8 = 0.75$ or $0.2/0.8 = 0.25$, respectively. The settling time and collection of cells were as before[18]. CCD profiles are given in terms of the absorbance (540 nm) in each cavity of the rotor.

The computation was described previously[18]. The numerical resolution of the CCD curves was developed by using the theory described by Blomquist and Wold[21], who linearized the non-linear problems of estimating the parameters which minimized a weighted sum, U, of squares of deviation between theoretical and experimental concentrations; U is a function of the tube with the maximum concentration and expresses the goodness of fit between theoretical and experimental values. The flow-chart in Fig. 1 shows the optimization strategy followed by Blomquist. We replaced the plot option in the original program by the program "6D" of the statistical package "BMPD". All computations were performed on a computer Cyber-180. The U values were calculated from eqn. 4 in ref. 18.

RESULTS AND DISCUSSION

Cell partition experiments

The partition ratio of RBCs from 16-day-old embryo and 4-day-old chicks has been studied in (a) highly charged systems containing increasing polymer concentrations, and (b) low-polymer systems with decreasing concentrations of phosphate. A general observation from these experiments is the variation in P values observed (Figs. 2 and 3). This was also the case for the cell population of rat bone marrow[20] as a consequence of the influence of the settling time on the partitioning behaviour of heterogeneous cells.

Partition in highly charged systems as a function of increasing total interfacial tension. Charged systems, *i.e.*, with an electrical potential difference between the phases are formed by the asymmetric distribution of phosphate towards the lower phase but the symmetric distribution of chloride between both phases, such that the

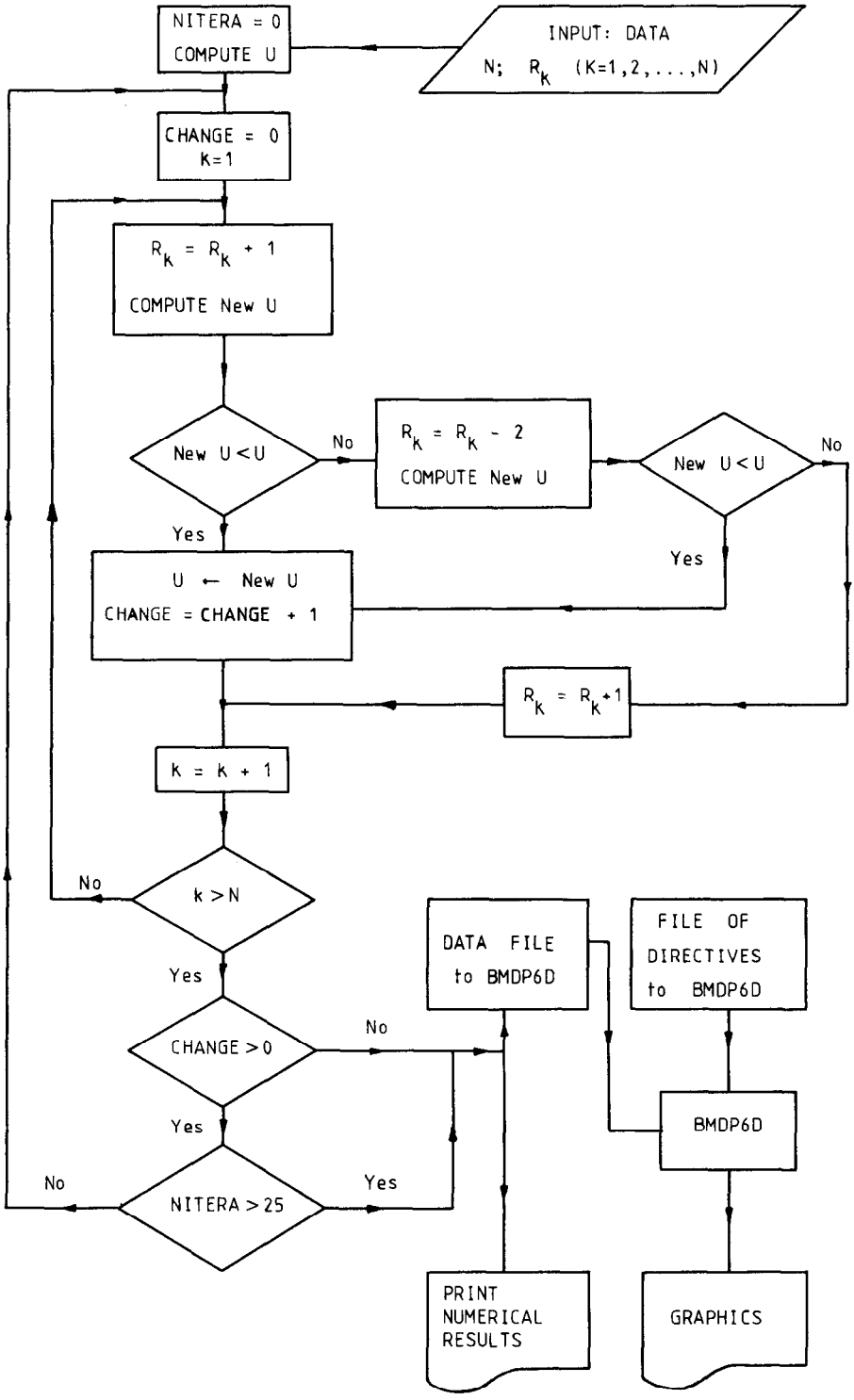

Fig. 1. Flow-chart of the computation[21] described in ref. 18. The goal is to estimate the location (R_k) of the tubes that present the maximum concentration for the N different components. U measures goodness of fit in the model used in order to estimate the parameters.

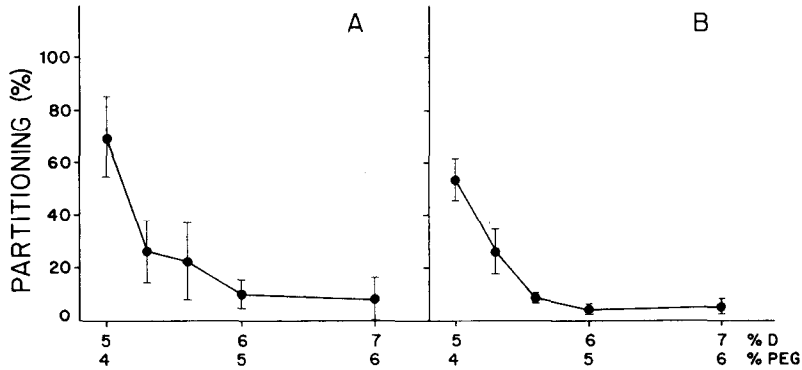

Fig. 2. Single partition experiments in highly charged systems as a function of increasing total interfacial tension. Partition ratio, P, of RBC populations from 16-day-old chick embryos (A) and 4-day-old chicks (B). Points represent the mean (S.E.) of four separate partition experiments, each sampled and measured in duplicate.

upper phase becomes relatively more positive than the lower one[14,19,20]. Systems with 0.09 M NaPB, *i.e.*, highly "charged" systems plus enough sodium chloride (0.03 M) to maintain the systems isotonic and increasing polymer concentration (5% D–4% PEG to 7% D–5% PEG) to increase the interfacial tension were used. Phosphate does not only increase the charge of the systems but also increases the interfacial tension[14,19,20]. These experiments then show the effect on RBC partition of both the high charge of the systems (due to phosphate) and the increase in total interfacial tension (because of phosphate plus the increase in D–PEG concentration). Results are given in Fig. 2A and B for chick-embryo and young-chick erythrocytes, respectively.

The effect of the high charge generated by phosphate is to increase the partition ratio, P, of cells while the progressive increase in total interfacial tension causes a decrease in cell partition. A decrease in P values as the D/PEG concentration increases is observed for both populations (Fig. 2A and B), which means that the effect on P of the charge of the system is diminished as the total interfacial tension increases. This behaviour is in agreement with theory and with results for other cell populations[14,19,20].

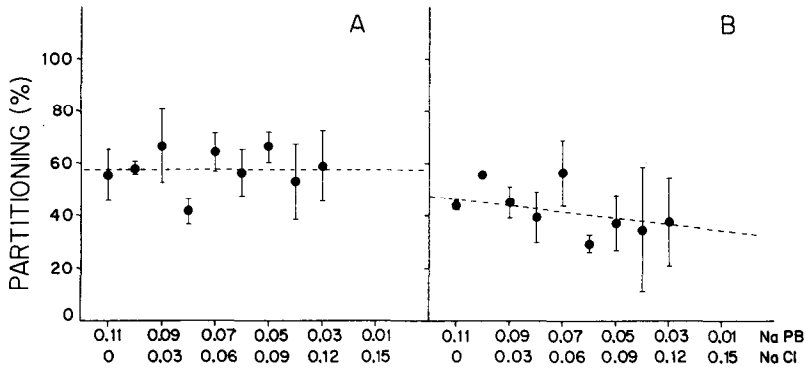

Fig. 3. Single partition experiments in low-polymer systems as a function of decreasing charge. Details and symbols as in Fig. 2.

The P values are higher in RBCs from chick embryos (Fig. 2A) than from young chicks (Fig. 2B). This suggests that the cell surface charge of chick-embryo RBCs is higher than that of young chick RBCs. Since the blood of chick embryos contains an higher proportion of primitive erythrocytes, the P values must be higher for primitive cells than for definitive ones. The difference in P values for the charged 5% D–4% PEG system (70%, chick embryos; 55%, young chicks, Fig. 2) can then explain the different location of primitive and definitive cells after CCD fractionation of cell populations from young chicks[13,16–18], or embryo chicks (Fig. 6, this work). Mainly because of their higher P values, primitive RBCs will be located towards the right-hand-side cavities of the CCD rotor, *i.e.*, distribution to the upper phase, while definitive RBCs will be distributed towards the left-hand-side cavities, *i.e.*, tendency towards the interface, lower P.

Partition in low-polymer systems as a function of decreasing charge. Single partitions were carried out in 5% D–4% PEG systems, *i.e.*, the lowest polymer interfacial tension system, with decreasing phosphate concentrations from 0.11 to 0.02 M NaPB plus sodium chloride (0 to 0.135 M) to maintain the system isotonic. The results are shown in Fig. 3.

The main point here is again the higher P values observed for chick-embryo (Fig. 3A) than for young-chick RBCs (Fig. 3B). This is in agreement with the results shown for the 5% D–4% PEG system in both Fig. 2A (P near 70%) and Fig. 2B (P near 55%), and with CCD experiments shown in Figs. 6 and 7. It is then again suggested that the surface charge of primitive RBCs is higher than that of definitive RBCs.

Because of the strong influence of phosphate, increasing the interfacial tension at the low polymer concentration used here, the systems of decreasing charge in Fig. 3 are at the same time systems of decreasing interfacial tension (decreasing phosphate plus low polymer concentration). However, the drop in both interfacial tension and charge of the system does not seem to affect significantly the partition ratio of chick-embryo (Fig. 3A) or young-chick RBCs (Fig. 3B). The 5% D–4% PEG systems are so close to the critical point that the dependence of the decreasing charge of the system on cell partition[14,19] is not observed. As confirmed for both homogeneous (erythrocytes) or heterogeneous (bone marrow) cell populations[20], a D/PEG ratio higher than 5%/4% is required for this effect. This is also a consequence of the long settling time in charged 5% D–4% PEG systems of nucleated chicken RBCs[26].

CCD experiments

A similar study to that for heterogeneous cell populations from young chicks[16,18] and rat bone marrow cells[24] has been made to demonstrate the presence of definitive and primitive RBC populations in the blood of 16-day-old embryos. Two different parameters have been varied during the CCD procedure: the upper/lower phase volume ratio in the cavities of the CCD rotor, $L = 1.3$, 1 and 0.45, and the settling time allowed for the phases to be separated after each mixing step, $t = 5$, 10, 20 and 30 min. The results are shown in Fig. 4 only for the two more significant settling times, 5 and 20 min.

Calculations on the basis of CCD theory[14,19] show that a decrease in L value or the ratio between the volumes of the mobile and stationary phases displaces the position of the CCD profiles towards the left. This displacement was repeatedly observed in our laboratory for both soluble substances (vitamin B_{12}) and homoge-

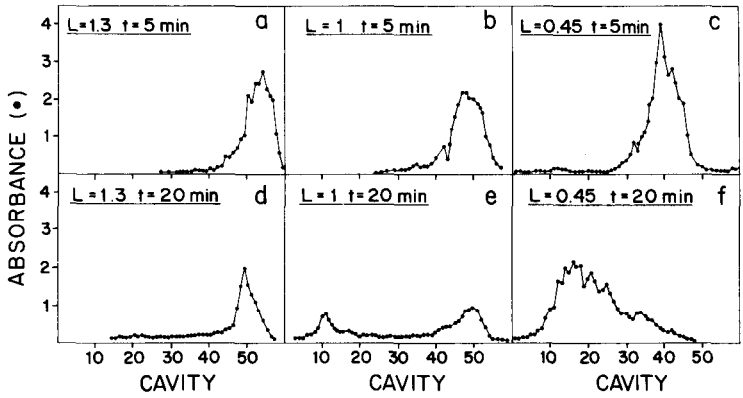

Fig. 4. Experimental CCD curves for different RBC populations from 16-day-old embryos. Experiments were carried out by varying the upper/lower phase volume ratio in each cavity of the CCD rotor, L, as well as the settling time, t, allowed for the phases to be separated after each mixing step during CCD.

neous cell particles (erythrocyte populations from adult rats and chickens)[26], as well as for heterogeneous populations of bone marrow cells[17,24] and young chick erythrocytes[17,18]. A displacement of the CCD profiles towards the left was observed as L was decreased from 1.3 (Fig. 4a and b) to 1 (Fig. 4b and e) and 0.45 (Fig. 4c and f). The fractionation in two peaks was observed for L-1 (but not for L-1.3 or 0.45) only when the settling time was increased to 20 min (Fig. 4e). Other settling times (10 and 30 min) gave rise to single-peak CCD profiles for all three L values tested (results not shown). As expected, single-peak CCD profiles were always observed for homogeneous RBC populations from adult chickens (results not shown).

The different location of peaks in Fig. 4e, *i.e.*, increase in efficiency of fractionation, is in agreement with the results from cell partition experiments discussed above for young-chick and embryo RBCs. Because of their higher P values (see section on *Partition in highly charged systems*), primitive RBCs must be those located in Fig. 4e towards the right-hand-side cavities of the CCD rotor, *i.e.*, distribution towards the upper phase), while definitive RBCs are those distributed towards the left-hand-side cavities, *i.e.*, tendency towards the interface, lower P values.

The influence of the settling time results from the association of cells during phase separation with microscopic globules of the lower phase that persist in the PEG-rich upper phase for some considerable time after the interface has been formed (non-equilibrium conditions) and seems to be an important determinant mechanism for cell partition, as shown in single-tube partition experiments[14,22,23]. The results shown here are a confirmation of previous ones from our group[16–18,24]. An improvement in the efficiency of fractionation is observed for heterogeneous cell populations when the settling time is increased during the CCD procedure with charged two-phase systems. As a consequence, a modification of the above mechanism in terms of differences in sedimentation properties can be suggested.

Numerical resolution of distribution curves

The numerical resolution of experimental CCD curves has been carried out for

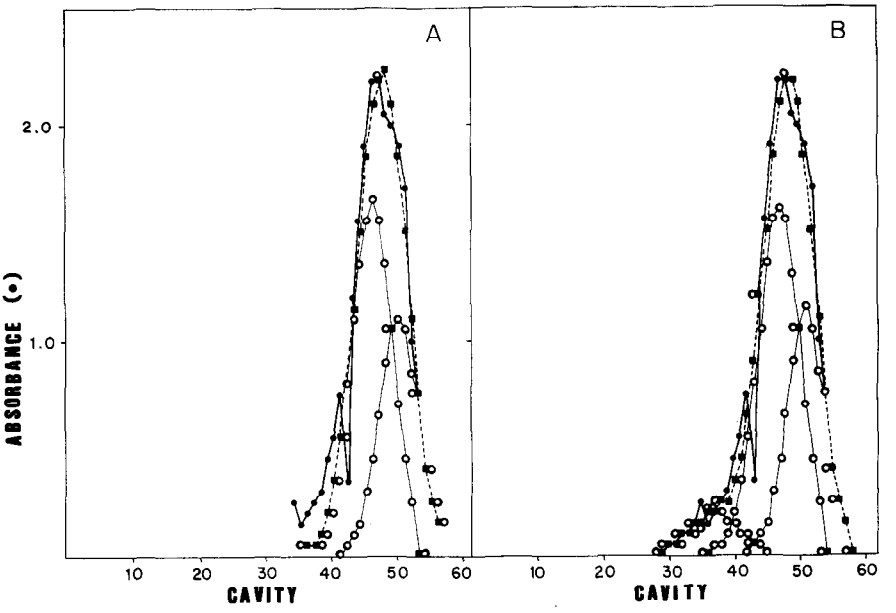

Fig. 5. Experimental and theoretical CCD curves obtained after fractionation at $L = 1$ and $t = 5$ min of a RBC population from 16-day-old embryos. A representative experimental CCD curve (●) is shown in comparison with its corresponding theoretical one (■) and with the CCD curves (○) calculated for the two cell components (A) or three cell components (B) assumed.

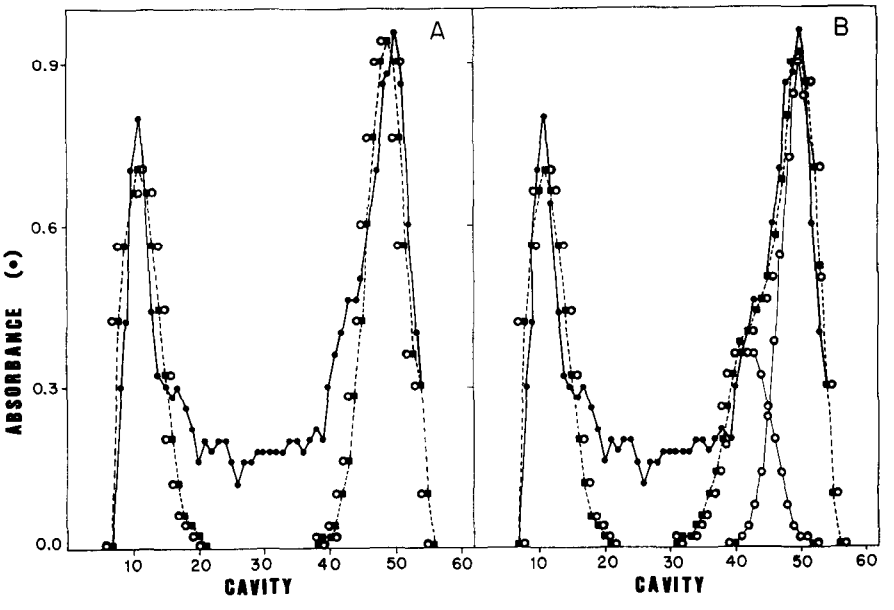

Fig. 6. Experimental and theoretical CCD curves obtained after fractionation at $L = 1$ and $t = 20$ min of a RBC population from 16-day-old embryos. Details and symbols as in Fig. 5.

TABLE I

U VALUES CALCULATED FOR DIFFERENT EXPERIMENTAL CONDITIONS, ASSUMING THE PRESENCE OF TWO OR THREE CELL COMPONENTS

$L = 1$.

Settling time (min)	*U*	
	Two components	*Three components*
5	0.1936	0.2024
20	0.1846	0.1156

$L = 1$ and $t = 5$ min, and $L = 1$ and $t = 20$ min. Experimental and theoretical CCD curves are compared in Figs. 5 and 6, and U values are summarized in Table I.

$L = 1$; settling time, 5 min. Two (Fig. 5A) or three cell components (Fig. 5B) are assumed for the single experimental CCD curve obtained for $L = 1$ and $t = 5$ min. By comparison of absorbance values for this CCD curve in Fig. 5A and B (a single peak with a maximum around cavities 46–48) with those for the theoretical ones in Fig. 5A (two cell components assumed), two gaps can be observed near cavities 35–42 and 49. The value of U is 0.1936 (Table I). When three ceell components are assumed (Fig. 5B), the gap observed around cavity 49 remains while the gap around the cavities 35–42 diminishes. This means that the CCD curve for this experimental condition is more in agreement with the theoretical one when three cell populations are considered. The similarity in U values for three (0.2024, Table I) and two (0.1936, Table I) cell components indicates that the differences between experimental and theoretical CCD curves are maintained when two populations as well as three are assumed. The fact that both U values are not elevated suggests a good agreement between experimental and theoretical CCD curves, although no resolution is achieved. It would mean the existence of very similar RBC subpopulations that cannot be separated under these conditions.

$L = 1$; settling time, 20 min. Results are shown in Fig. 6A and B for two and three cell components, respectively. By comparison of the experimental CCD profile in Fig. 6A and B (two peaks with maxima in cavities 11 and 49) with the theoretical CCD curve in Fig. 6A (two cell components assumed), a gap is clearly observed between cavities 16 and 45. The value of U is 1.846 (Table I). When three cell components are assumed (Fig. 6B), the gap between cavities 16 and 38 remains while the gap between cavities 38–45 disappears. The U value is lower (0.1156, Table I) than that for two cell components. This means, as for $L = 1$ and $t = 5$ min, that the CCD curves for longer settling times are more in agreement with theoretical ones when considering the presence of three cell populations. As discussed above, the first of these populations (towards the left of the CCD diagram) would be formed by definitive erythrocytes while the other two populations (towards the right) would be formed by closely related primitive erythrocytes.

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