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Surface properties of crosslinked erythrocytes as studied by counter-current distribution in aqueous polymer two-phase systems

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Abstract

The bifunctional imidoester dimethyl suberimidate hydrochloride can stabilize rat red blood cells (RBCs) by membrane protein crosslinking, and in that way they can be used as carrier systems for exogenous substances. Counter-current distribution fractionation in charge-sensitive dextran–polyethyleneglycol two-phase systems has been used to detect slight changes in surface charge in stabilized cells. A decrease in the surface charge of crosslinked RBCs and an apparent masking of the age-related cell surface properties have been found to result from the protein crosslinking. Digitonin treatment used to permeabilize crosslinked RBCs produces a significant decrease of the cell surface charge while the age-related surface properties do not seem to be modified by the treatment.

1. Introduction

The possible use of erythrocytes from animals or humans as carrier systems for exogenous substances under certain clinical situations is being studied [1]. Normal or carrier red blood cells (RBCs) can be stabilized by protein crosslinking (C-RBCs). Dimethyl suberimidate hydrochloride (DMS) is a membrane permeable bifunctional imidoester that crosslinks the free primary amino groups in membrane proteins of cells [2,3]. As an extension of the crosslinking approach, crosslinked-permeabilized RBCs (CP-RBCs) can be prepared by delipidation of the DMS-crosslinked RBCs with digitonin [4–7]. Our present aim is to study the basic age-related surface properties of C-RBCs and CP-RBCs, in an attempt to understand their behaviour as potential organ-targeted systems. Age-related alterations in the physical and chemical properties of the RBC membrane may be responsible for the recognition of senescent erythrocytes by the mononuclear phagocytic system [8].

Multiple partitioning in aqueous dextran–polyethyleneglycol (D–PEG) two-phase systems using a thin-layer counter-current distribution (CCD) apparatus [9], is a method to fractionate RBCs on the basis of changes in their surface properties occurring during ageing [10]. In rats, RBC ^{59}Fe-labelling studies in vivo [10,11] and the decrease in the specific activity of age-dependent enzymes [12,13] have shown the fractiona-

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tion of maturing reticulocytes and ageing erythrocytes after CCD in charge-sensitive two-phase systems. These studies were successfully extended to rat erythrocytes during animal development (i.e. RBC switching) in order to distinguish between embryonic, fetal and adult cells [14]. Finally, this approach was used in a comparative study on adult rat and human (normal and pathological) erythrocytes using the decline in the specific activity of glycolytic enzymes [15,16]. CCD-fractionation in charge-sensitive D–PEG two-phase systems is employed here to detect alterations in the charge surface properties of C-RBCs and CP-RBCs compared to control cells. The specific activity of two age-dependent enzymes, acetylcholinesterase (AChE) and pyruvate kinase (PK) [8,15,16], is determined in the fractionated cells in order to study the cell surface properties related to RBC ageing.

2. Experimental

Blood from male Wistar rats (150–200 g body weight) was collected into heparinized (10 U/ml) tubes and filtered through α-cellulose–cellulose type 50 (mean size, 50 μm) filters (Sigma, St. Louis, MO, USA) to obtain erythrocytes [17]. Three washings (400 *g*, 10 min) were carried out with phosphate buffered saline (PBS) (136.8 m*M* NaCl, 3.5 *M* KCl, 0.5 m*M* $MgCl_2$, 1.47 m*M* KH_2PO_4, 8.09 m*M* Na_2HPO_4 and 1 U/ml heparin, pH 8).

Erythrocytes were stabilized by crosslinking with dimethyl suberimidate hydrochloride (DMS) (Pierce, Rockford, IL, USA) and permeabilized with digitonin (Sigma) as previously described [4–7]. RBCs in PBS (pH 8) (1 ml, 1:1, v/v) were incubated with a fresh DMS solution (24 mg in 7 ml 100 m*M* triethanolamine, pH 8.7, and 75 m*M* NaCl) for 30 min at 37°C. Cells were washed (1000 *g*, 10 min) with 10 volumes of PBS at 4°C. Crosslinked RBCs (0.5 ml) were suspended in a mixture of 9.5 ml of water plus 0.5 ml of 1% digitonin in ethanol for 10 min at 25°C. Cells were washed twice (1000 *g*, 10 min) with PBS (pH 8) at 4°C and resuspended until use.

The following stock solutions were used to prepare aqueous two-phase systems: 20% (w/w) dextran T-500 (D) (Pharmacia, Uppsala, Sweden), standardized by polarimetry, 40% (w/w) polyethyleneglycol 6000 (PEG) (Serva, Heidelberg, Germany), 0.2 *M* sodium phosphate buffer (pH 6.8) and 1 *M* sodium chloride. Charge-sensitive two-phase systems formed by 5% (w/w) D–4% (w/w) PEG or 5.6% (w/w) D–4.6% (w/w) PEG, 0.03 *M* sodium chloride and 0.09 *M* sodium phosphate buffer were prepared by weight from the above stock solutions. The phase systems were allowed to equilibrate for 48 h at 4°C (separatory funnel) before the top PEG-rich and bottom D-rich phases were separated [9,10].

A thin-layer counter-current distribution apparatus [9] with two thin-layer units (60 concentric cavities) made at the University of Sheffield (Sheffield, UK) (Bioshef TLCCD, MK 3), each unit formed by two circular plates (bottom, or stator plate and top, or rotor plate), was used. The volume of the bottom units was: 0.85 ml (unit 1) and 0.81 ml (unit 2). Experiments were carried out in both units after appropriate corrections were made to take into account differences in the bottom volumes.

(a) Experiments with 5.6% D–4.6% PEG: Three adjacent cavities (0–2) each received a mixture of 0.46 ml (unit 1) or 0.42 ml (unit 2) of bottom D-rich phase plus 0.1 ml of the RBC suspension, whereas the remaining cavities (3–60) each received 0.56 ml (unit 1) or 0.52 ml (unit 2) of the D-rich bottom phase. The cavities on the bottom plates were then filled to about 64% of their total capacity. The space left for cell adsorption at the interface was 0.29 ml. A 0.77-ml (unit 1) or 0.72-ml (unit 2) volume of the PEG-rich top phase was added to the 60 cavities. The top-/bottom-phase volume ratio was $L = 0.77/0.56$ (unit 1) or $L = 0.72/0.52$ (unit 2), which both equal 1.4.

(b) Experiments with 5% D–4% PEG: Three adjacent cavities (0–2) each received a mixture of 0.66 ml (unit 1) or 0.62 ml (unit 2) of bottom D-rich phase plus 0.1 ml of the RBC suspension, whereas the remaining cavities (3–60) each received 0.76 ml (unit 1) or 0.72 ml (unit 2) of the above D-rich bottom phase. The cavities on the

bottom plates then contained about 89% of their total capacity and the space left for the cell adsorption at the interface was 0.09 ml. A 0.99-ml (unit 1) or 0.94-ml (unit 2) volume of the PEG-rich top phase was added to the 60 cavities. The top-/bottom-phase volume ratio was $L = 0.99/0.76$ (unit 1) or $L = 0.94/0.72$ (unit 2), which both equal 1.3.

A partitioning step consisted of a 20-s shaking period followed by a 6-min settling period and a transfer of the top (rotor) plate. With each transfer the cells in the top phase are carried forward where they are re-extracted with fresh bottom phase whereas the cells partitioned at the interface were left behind to be re-extracted with fresh top phase. Sixty transfers were performed at 4°C.

After a cycle of multiple partitions (know as a distribution run), 1 ml of PBS (pH 8) was added to all cavities in order to transform the two-phase system to a single phase, and the content of each cavity was collected separately. The distribution profile for each RBC population is given by the hemoglobin absorbance at 540 nm against the cavity number of the distribution unit. Cells with affinity for the top phase (high distribution coefficient, G) were distributed as fast-moving cells in the fractions with the highest number, i.e. towards the right-hand side of the distribution profile. Cells with affinity for the interface (lower G values) tended to remain in the fractions with the lowest number, as slow-moving cells, i.e. towards the left-hand side of the distribution profile.

Enzyme activity of total RBC populations or pooled adjacent CCD-fractions (as indicated in the figures) were measured in hemolysates prepared by hypo-osmotic shock with two volumes of stabilizing solution (2.7 mM EDTA-Na_2–0.7 mM mercaptoethanol, pH 7), followed by freezing and thawing. Pyruvate kinase (EC. 2.7.1.40; PK) and acetyl cholinesterase (EC 3.1.1.7; AChE) activities were measured as previously described [15,16]. To maintain the stability of the C-RBC and CP-RBC suspensions, 7% Ficoll was added to the cuvette [4,5]. The hemoglobin concentration and cell number were determined in a Serono-Baker Diagnostics Systems 9000 + coulter counter and the specific enzyme activity was expressed as U/g Hb or U 10^{-10}/cell. Reagents, substrates, cofactors and auxiliary enzymes were from Sigma and Boehringer (Mannheim, Germany).

CCD experiments were repeated at least four times with different RBCs. Enzyme activities were measured in duplicate for each CCD run. Results are expressed as mean ± S.E. (standard error of the mean). Mean values were compared by unpaired Student's t-test. Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results and discussion

Dimethyl suberimidate (DMS) crosslinked 60% of the RBC free amino groups with a cellular recovery of 80%, under the concentrations used here [7]. C-RBCs and CP-RBCs show a high hemolytic and mechanical resistance; they do not hemolyze in water, after freezing-thawing or after sonication [7].

The partitioning behaviour in D–PEG two-phase systems of C-RBCs and CP-RBCs has been previously studied in our group [18] by the appropriate manipulation of the polymer and/or salt concentrations, which allows charge and non-charge (hydrophobic) cell surface properties to be differentiated [10,18,19]. Since phosphate ions show a high affinity for the bottom phase, there is a Donnan potential between the two phases (top phase positive); however, NaCl has an essentially equal affinity for the two phases and there is thus virtually no potential difference between them [9,10]. The partitioning of erythrocytes (with negative cell surface charge) was carried out in a series of D–PEG two-phase systems ranging from low (5% D–4% PEG) to high (6% D–5% PEG) polymer concentration (i.e. increasing interfacial tension) in which the ratio of phosphate/NaCl is systematically reduced (while keeping constant the isotonicity, pH and temperature of the systems) [18]. In all charge- (phosphate rich) and non-charge- (phosphate poor) sensitive systems, control RBCs and C-RBCs showed a higher affinity towards the top

phase than the CP-RBCs, which are mainly distributed at the interface except in the 5% D–4% PEG charge-sensitive system in which about 50% of the CP-RBCs were in the top phase [18].

Control RBCs and C-RBCs show a relatively high affinity (60% of the cells) for the PEG-rich top phase, whereas CP-RBCs are mainly distributed at the interface of a 5.6% D–4.6% PEG charge-sensitive system. None of these three cell populations is partitioned in the equivalent non-charge-sensitive system (less than 10% of the cells in the top phase). Thus, the increase of the amount of cells in the top phase going from non-charge- to charge-sensitive systems can only be due to the cell surface charge [10,18,19]. On the other hand, having 60% of the C-RBCs in the top phase would make it possible to obtain centered CCD profiles which is desirable for good resolution of the cell fractionation [10]. Therefore, this polymer concentration was chosen to study CCD fractionation of C-RBCs and CP-RBCs as a function of cell surface charge properties.

Representative CCD profiles in the 5.6% D–4.6% PEG charge-sensitive two-phase system of the control RBCs, C-RBCs and CP-RBCs, are shown at the top of Figs. 1, 2 and 3, respectively. Slight differences between control RBC (Fig. 1, top) and C-RBC (Fig. 2, top) profiles are observed. Both profiles are homogeneous and the highest cellularity is found around cavity 38 and 33 respectively, i.e. the C-RBCs are fractionated towards the left-hand side of the control cells. This displacement means a slightly lower surface charge in crosslinked than in control cells which agrees with the slightly lower amount of sialic acid (the main compound responsible for the negative cell surface charge) released by neuraminidase in C-RBCs than in control RBCs [18]. The partially impeded access for neuraminidase to the sialic acid on the erythrocyte surface seems to be the cause for these differences [18]. Thus, control RBCs and C-RBCs would have a similar charge, but the charges on the cross-linked cells seem to be partially masked.

Permeabilization of C-RBCs with digitonin to obtain CP-RBCs significantly shifted the CCD

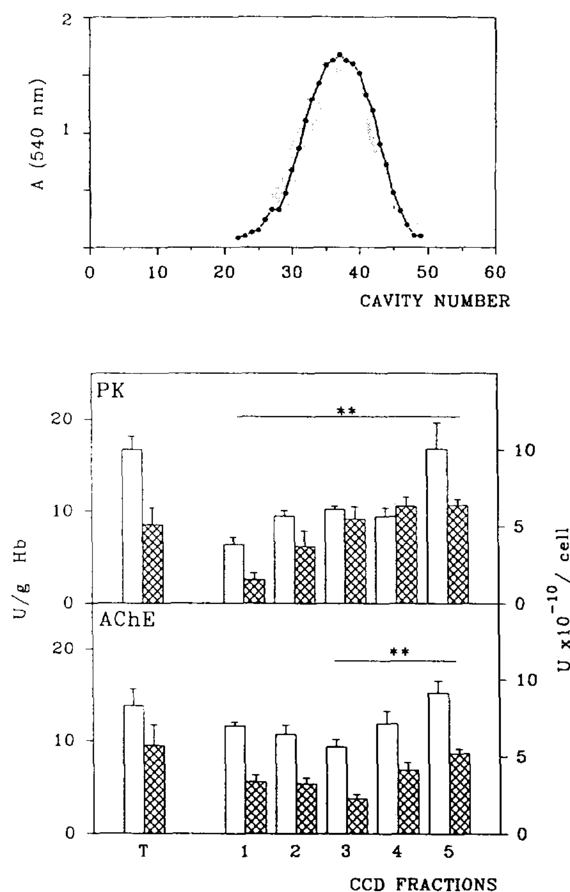
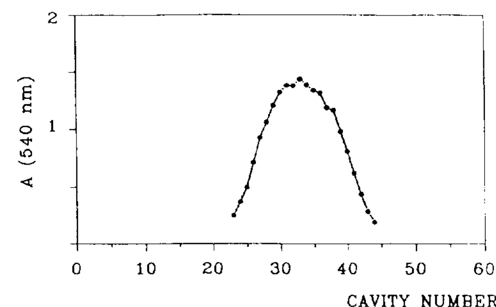


Fig. 1. Representative CCD profile (hemoglobin absorbance at 540 nm against cavity number) of control RBCs in a 5.6% D–4.6% PEG charge-sensitive two-phase system (top). PK and AChE specific activity (U/g Hb, white bars, and U · 10^{-10}/cell, striped bars) in the total cell population (T) and in the 5 pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E.** ($p < 0.01$).

profiles towards the first cavities: 1 to 10 (Fig. 3, top); this was expected because of the low partitioning ratio (around 10% of cells in the top phase) for the CP-RBCs in the 5.6% D–4.6% PEG charge-sensitive system used here.

Since the influence of cell sedimentation has been discarded [18], this CP-RBC fractionation behaviour can only reflect a large decrease in the cell surface charge. This would be explained by a significantly lower sialic acid release by neuraminidase, resulting from the loss of glycolipids–which contain sialic acid–during the permeabili-



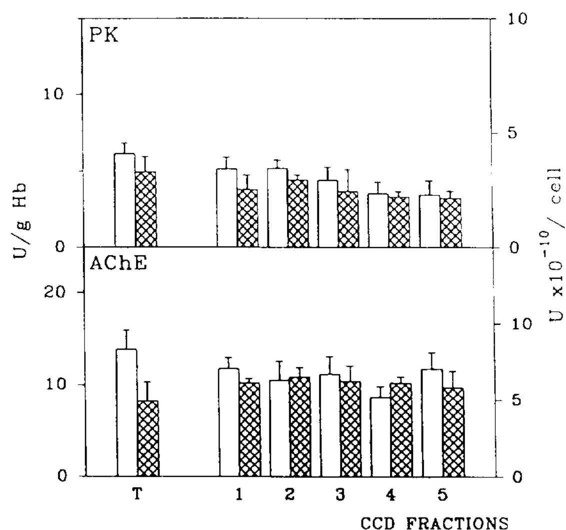
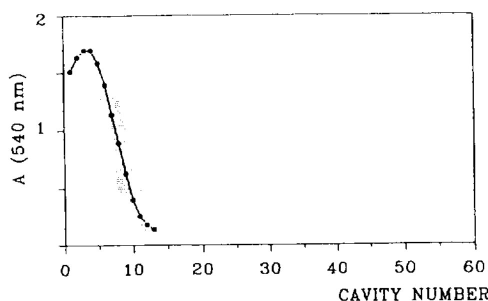


Fig. 2. Representative CCD profile (hemoglobin absorbance at 540 nm against cavity number) of crosslinked RBCs in a 5.6% D–4.6% PEG charge-sensitive two-phase system (top). PK and AChE enzymatic specific activity (U/g Hb, white bars, and $U \cdot 10^{-10}$/cell, striped bars) in the total cell population (T) and in the 5 pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E.



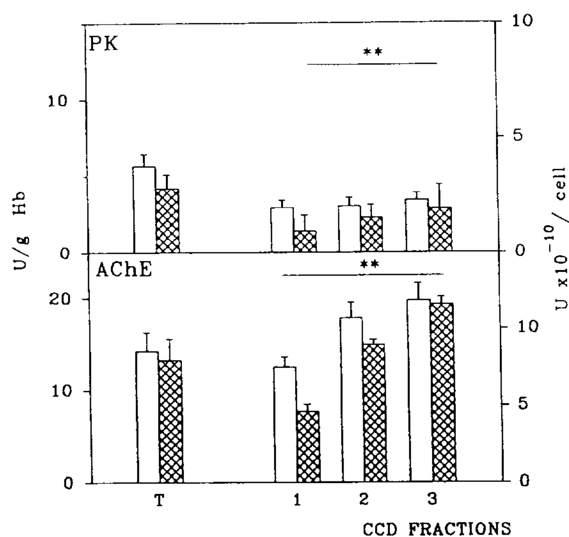


Fig. 3. Representative CCD profile (hemoglobin absorbance at 540 nm against cavity number) of crosslinked-permeabilized RBCs in a 5.6% D–4.6% PEG charge-sensitive two-phase system (top). PK and AChE enzymatic specific activity (U/g Hb, white bars, and $U \cdot 10^{-10}$/cell, striped bars) in the total cell population (T) and in the 3 pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E. ** ($p < 0.01$).

zation process [18]. The decrease in the partitioning ratio of control RBCs in charge-sensitive systems after neuraminidase treatment has been reported previously [10].

However, the shift in the CP-RBC CCD profiles towards the first cavities is too large to be entirely due to the influence of the sialic acid content. For example, the decrease in total lipids and cholesterol content observed in CP-RBCs [18] could cause membrane rearrangement during permeabilization accompanied by a change in the cell surface properties. This would agree with the eroded external appearance of stomatocytic CP-RBCs observed by scanning electron microscopy, which contrasts with the smooth surface of control and stomatocytic crosslinked cells [7]. Such a modification in the cell membrane structure seems to be reflected in the CCD by a decrease in cell partitioning. Thus, a correlation between the partitioning behaviour and the shape of human erythrocytes has been found [20]. When these cells are echinocytes partitioning is enhanced by the increase in membrane fluidity caused by lipid rearrangement [20]. De-

creased RBC deformability has been described in human erythrocytes crosslinked with DMS or glutaraldehyde. This was explained by crosslinking of membrane and cytoskeletal proteins, which implied decreased membrane fluidity [4,21–25].

To characterize the CCD fractionation of the stabilized cell populations, the specific activity of two age-dependent enzymes, pyruvate kinase (PK) and acetylcholinesterase (AChE), were measured in pooled CCD fractions (shadowed zones in the CCD profiles of Figs. 1–3, top). Because of the high hemolytic resistance of C-RBCs and CP-RBCs, it was difficult to determine the hemoglobin concentration, and thus, although the specific activity is expressed in both U/g Hb and U/cell, results are always discussed according to U/cell values.

PK is an intracellular allosteric enzyme that controls the glycolytic rate. Changes in enzyme activity would reflect the crosslinking effect in the intracellular proteins [4,5,7]. The specific activity of PK in the whole C-RBC (Fig. 2) and CP-RBC (Fig. 3) populations is lower than in the controls (Fig. 1) as a consequence of the effect of DMS (membrane permeable) on the enzyme structure [4,7]; its allosteric kinetic behaviour is lost (unpublished data) but its age-dependent character seems to persist (Figs. 3 and 4). AChE is a membrane enzyme the catalytic activity of which, directed to the extracellular space, does not seem not to be affected by DMS or digitonin treatment (Figs. 1, 2 and 3).

The specific activity of PK in the control cells as determined in five pooled CCD fractions (Fig. 1), shows a highly significant decrease ($p < 0.01$) from pool 5 to pool 1, suggesting age-related CCD fractionation. The highest specific activity, which is related to younger erythrocytes [8], is observed in cells located towards the right-hand side of the CCD profile, whereas the lowest specific activity, which is related to older erythrocytes [8], is observed in cells progressively located towards the left-hand side of the CCD profile. Such a fractionation is supported by the AChE specific activity, which shows a highly significant decrease ($p < 0.01$) from pool 5 to pool 3 (Fig. 1)

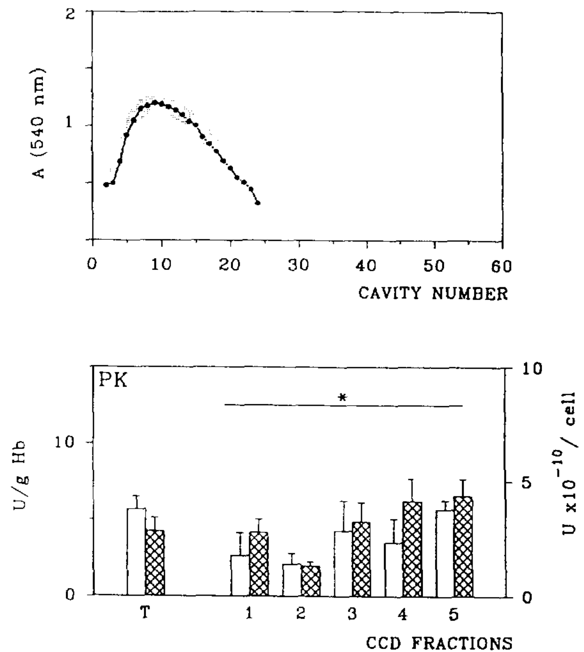


Fig. 4. Representative CCD profile (hemoglobin absorbance at 540 nm against cavity number) of crosslinked-permeabilized RBCs in a 5% D–4% PEG charge-sensitive two-phase system (top). PK enzymatic specific activity (U/g Hb, white bars, and $U \cdot 10^{-10}$/cell, striped bars) in the total cell population (T) and in the 5 pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E. * ($p < 0.05$).

and remains constant from pool 3 to pool 1. A similar fractionation by age, from the right- to the left-hand side of the CCD profile, has been described for control rat erythrocytes fractionated under different experimental CCD conditions [10,15].

The specific activities of PK and AChE are unchanged along the CCD profile of the C-RBCs (Fig. 2). These results support the non-efficient fractionation according to age of the C-RBCs. After the digitonin treatment to obtain CP-RBCs, the activities of PK and AChE significantly decrease ($p < 0.01$) from the right- to the left-hand side of the CCD profile (from pool 3 to pool 1, Fig. 3), suggesting fractionation according to age like that observed in control cells. Thus, DMS plus digitonin treatment does not seem to modify the age-related cell surface properties.

Because the partitioning ratio in the 5.6% D–4.6% PEG charge-sensitive systems for CP-RBCs is too low, a more appropriate polymer concentration was selected to fractionate this cell population by CCD. CP-RBCs are mainly distributed in the interface except in the 5% D–4% PEG charge-sensitive system in which about 50% of the CP-RBCs are in the top phase [18]. This polymer concentration was then chosen to displace the CCD profile of the CP-RBCs towards the more centrally located cavities. For this purpose, the bottom-phase volume was increased but the phase-volume ratio was kept at $L = 1.3$. These experimental conditions were used previously by our group to fractionate control rat RBCs [15].

The CCD profiles in a 5% D–4% PEG charge-sensitive system for CP-RBCs (Fig. 4, top), were displaced towards the right-hand side (cavities 1 to 25) and were broader than the one obtained above (Fig. 3, top). This means a higher resolution in the CCD fractionation. In agreement with our previous results [15], control rat RBCs fractionate into the last cavities, 40–60, when these CCD experimental conditions are used. Therefore, larger differences are observed in this case than before between control RBC and CP-RBC profiles. In contrast, C-RBCs fractionate into the same cavities (40–60) as control cells (results not shown) indicating that the previously observed differences in cell surface properties between either kind of cells are not detected under the experimental conditions using a 5% D–4% PEG system.

Characterization of CP-RBC CCD fractionation on the basis of the specific activity of PK was performed (Fig. 4). As can be observed, the PK activity significantly decreased ($p < 0.05$) from right- to left-hand side (from pool 5 to pool 1) of the CCD profile in a way like that previously observed for control rat RBCs under these experimental conditions [15]. Thus, CP-RBCs seem to be fractionated according to age. In agreement with the above results (Fig. 2), the PK activity was unchanged along the CCD profile of C-RBCs (results not shown), supporting the non fractionation by age of the C-RBCs population. The differences observed in cell surface properties of C-RBCs and CP-RBCs suggest their possible "in vivo" recognition by the mononuclear phagocytic system. Such an apparent inconvenience may serve to direct the carrier erythrocytes towards different organs and thus perform a selective therapeutic action.

In summary, CCD fractionation in charge-sensitive two-phase systems reveals a slightly lower surface charge in C-RBCs than in control cells, and suggests an apparent masking of the age-related cell surface properties as a consequence of DMS protein crosslinking. However, in spite of the important decrease in the cell surface charge detected by partitioning the CP-RBCs, these cells conserve their age-related surface properties.

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