Biochimica et Biophysica Acta 860 (1986) 650–661
Elsevier

BBA 73249

Effect of surface modification of rat erythrocytes of different ages on their partitioning behavior in charge-sensitive two-polymer aqueous phases

Harry Walter [a,b,*], Eugene J. Krob [a], Ali Pedram [a], Cherry H. Tamblyn [c] and Geoffrey V.F. Seaman [c]

[a] *Laboratory of Chemical Biology, Veterans Administration Medical Center, Long Beach, CA 90822,* [b] *Department of Physiology and Biophysics, University of California, Irvine, CA 92717 and* [c] *Department of Neurology, Oregon Health Sciences University, Portland, OR 97201 (U.S.A.)*

(Received February 10th, 1986)
(Revised manuscript received May 28th, 1986)

Key words: Cell surface; Aging; Phase partitioning; (Erythrocyte)

Partitioning differences between cells in two-polymer aqueous phase systems originate from subtle differences between the surface properties of cells. Because of the exponential relation between the parameters affecting the partition ratio (*P*) and the *P* itself, differences in membrane components suspected of effecting the differential partitioning of closely related cell populations cannot be directly established by conventional chemical assay techniques. In order to study the chemical nature of the components responsible for the age-related changes in surface properties of rat red cells we have devised an approach which uses a combination of isotopic labeling of erythrocyte subpopulations of distinct cell age with different enzyme and/or chemical treatments followed by countercurrent distribution in charge-sensitive two-polymer aqueous phase systems. These studies show that: (a) neuraminidase-susceptible sialic acid is not responsible for the cell age-related surface differences detected by partitioning; (b) the component(s) responsible for the cell age-related surface differences can be extracted (from aldehyde-fixed red cells) with ethanol or cleaved with dilute sulfuric acid. Our data are consistent with the hypothesis that ganglioside-linked sialic acid is the chemical moiety responsible for the cell charge-associated surface differences among rat red blood cells of different ages.

Introduction

By a combination of radioisotope labeling techniques and cell partitioning in two-polymer aqueous phase systems we have previously established that rat erythrocytes undergo systematic changes in their surface charge-associated properties as a function of cell age [1–3]. We have now endeavored, by examining the effect of selected modifications of the rat erythrocyte surface, to obtain information on the chemical component(s) responsible for the alteration in red cell age-related surface charge.

Dextran and poly(ethylene glycol) when dissolved in water above certain concentrations give rise to liquid, immiscible two-phase systems with a poly(ethylene glycol)-rich top and a dextran-rich bottom phase [3]. Such phases can be buffered and made isotonic and are not deleterious to cells partitioned in them [4–7]. By partitioning cells in such systems one can obtain information on subtle differences or alterations in their surface proper-

* To whom correspondence should be addressed at: Laboratory of Chemical Biology-151, VA Medical Center Long Beach, CA 90822, U.S.A.

ties [1–7]. Changes in polymer concentration, ionic composition and electrolyte concentration have marked effects on the physical properties of the phase systems [8] which, in turn, influence their interaction with biological cells. In the current experiments phase system compositions were used in which the cell partition ratio, P (i.e., the relative affinity of cells for the top or bottom phase or their adsorption at the interface) reflects surface charge-related properties.

Injection of rats with radioactive iron (^{59}Fe-ferrous citrate) followed by bleeding animals at different times thereafter gives rise to red blood cell populations in which erythrocytes of different but distinct cell age, corresponding to the time elapsed between injection and bleeding, are labeled [1–3]. By subjecting such labeled cell populations to countercurrent distribution (CCD, i.e., a multiple extraction procedure) in two-polymer aqueous phase systems, and determining the relative positions (G * values) of the labeled and unlabeled cells, we have established that rat reticulocytes have the lowest G value; that the G value increases rapidly, in a matter of hours, as the reticulocytes mature to become the youngest erythrocytes; and that the G value of erythrocytes then diminishes over the entire life-span of the rat red cell [1–3]. Near the end of the life-span of the cells, their G value is close to that of the reticulocytes.

In the present experiments we have treated rat red blood cell populations containing isotopically labeled cells of different but distinct ages with selected reagents or enzymes prior to countercurrent distribution. The effect of the treatment on the relative G values of labeled and unlabeled cells was then determined. These results suggest that ganglioside-bound sialic acid is the most likely component responsible for the charge-associated differences between rat red cells of different ages.

Materials and Methods

Animal injection and bleeding

Male Sprague-Dawley rats (Hilltop Laboratory Animals, Scottdale, PA), weighing between 300 and 500 g, were used. In isotope experiments they were injected with about 10 to 15 μCi of ^{59}Fe-ferrous citrate (Mallinckrodt, St. Louis, MO) via the saphenous vein. The animals were exsanguinated, at different times after injection, by heart puncture and the blood collected in the anticoagulant ACD (acid-citrate-dextrose). This gave rise to red cell populations in which the cells that were labeled corresponded in age to the time elapsed between isotope injection and animal bleeding.

In vitro treatments of rat red blood cells containing isotopically labeled subpopulations of different, distinct cell age

(a) Glutaraldehyde fixation. An aliquot of the red blood cells was washed three times with phosphate-buffered saline (pH 7.0). The washed, packed red cells were then slowly pipetted into a 280 mosM sodium phosphate buffer solution (pH 7.4) containing 1.85% (w/w) glutaraldehyde (Ladd, Burlington, VT) while swirling the flask. The ratio of cell volume to fixative was 1 : 10.

(b) Treatment of fresh or glutaraldehyde-fixed erythrocytes with neuraminidase (removal of neuraminidase-susceptible sialic acid). 2 ml of packed red blood cells were washed three times with 10 vol. of isotonic cacodylate buffer (pH 6.4). A 0.5-ml aliquot of such washed red cells + 3.5 ml cacodylate buffer was put into a 40 ml glass centrifuge tube and incubated together with 200 μl (1 I.U./ml) of neuraminidase (*Vibrio cholerae*, Calbiochem-Behring, San Diego, CA) at 37°C for 90 min. At the same time a similarly treated aliquot of the same cell population was incubated in the absence of neuraminidase. After incubation the cell suspension was centrifuged ($1200 \times g$ for 10 min) and the supernatant fluid removed for assay of sialic acid on duplicate aliquots. The treated cells were washed three times with cacodylate buffer. (In preliminary experiments it had been established by prolonged incubation with larger quantities of neuraminidase that all neuraminidase-susceptible sialic acid is released by neuraminidase under the conditions used.)

(c) Lipid-extraction from glutaraldehyde-fixed red blood cells. Red blood cells fixed with glutaraldehyde as described above were washed twice with 10 vol. of saline and twice with distilled water.

* The apparent partition ratio, G, is defined as $r_{max}/(n - r_{max})$, where r_{max} is the number of the peak cavity of the distribution curve and n is the total number of transfers [3].

They were then suspended in 10 vol. of absolute ethanol, centrifuged, the supernatant solution removed and the cells were resuspended in a further 10 vol. of absolute ethanol. The cells were left standing in the ethanol for 1 h at room temperature and were then centrifuged and washed twice with 5 vol. of ethanol. The ethanol supernatant fluids were combined and evaporated to dryness for sialic acid analysis. The cells were washed twice with distilled water.

(d) Sulfuric acid-treatment of glutaraldehyde-fixed red blood cells (removal of total sialic acid). Red blood cells fixed with glutaraldehyde as described above were washed three times with distilled water. A 0.3-ml aliquot of packed cells was then suspended in 4 ml of 0.05 M H_2SO_4 and heated in a water bath for 1 h at 80°C. After neutralization with an equal volume of 0.05 M dibasic sodium phosphate [9] the cell suspension was centrifuged and duplicate aliquots of the supernatant fluids were used for the determination of sialic acid. The cells were washed twice with water and once with cacodylate buffer.

Determination of sialic acid released from glutaraldehyde-fixed rat erythrocytes

Prior to treatment with neuraminidase, 0.05 M H_2SO_4 or ethanol, fixed red blood cell suspensions were counted by electronic particle size analysis equipment using an Electrozone/Celloscope (Particle Data, Elmhurst, IL). Volume concentrations of the erythrocyte suspensions were calculated from microhematocrit readings obtained after 10 min centrifugation at $15\,000 \times g$, assuming a packing factor of 0.60 for fixed erythrocytes.

After treatment with neuraminidase, 0.05 M H_2SO_4 or ethanol, fixed red blood cell suspensions were centrifuged and duplicate aliquots of the supernatant fluids assayed for the quantity of sialic acid released. The thiobabituric acid method of Aminoff [10] was used for the sialic acid assay and calculations were made as outlined by Seaman et al. [11]. Sialic acid was released for assay from the ethanol extracted gangliosides by digestion in 0.05 M H_2SO_4 for 1 h at 80°C, followed by neutralization with dibasic sodium phosphate [9].

Electrophoretic mobility measurements on treated rat erythrocytes

Treated rat red blood cells were washed three times in phosphate-buffered saline (0.145 M NaCl/0.01 M sodium phosphate buffer (pH 7.4)) and suspended in this medium for examination by analytical particle electrophoresis. The electrophoretic mobilities of the erythrocytes suspended in phosphate buffered saline were measured at 25.0 ± 0.1°C in a cylindrical glass chamber equipped with Ag/AgCl reversible electrodes using the method described by Seaman [12].

Selection and preparation of dextran-poly(ethylene glycol) aqueous phase systems

Three different two-polymer aqueous phase systems, prepared as described by Walter [3], were used. Their compoisitions were as follows: system 1 contained 5% (w/w) dextran T500, Lot no. 11648 (Pharmacia Fine Chemicals, Piscataway, NJ), 4% (w/w) poly(ethylene glycol) 6000 ('Carbowax 6000', recently renamed '8000', Union Carbide, NY), 0.09 M sodium phosphate buffer, pH 6.8 (composed of equimolar quantities of mono- and dibasic phosphates) and 0.03 M NaCl; system 2 contained 7% (w/w) dextran, 4.4% (w/w) poly(ethylene glycol) and 0.11 M sodium phosphate buffer (pH 6.8); system 3 contained the same polymer concentrations as system 2 with 0.1 M sodium phosphate buffer (pH 6.8) and 0.015 M NaCl. All three systems have electrostatic potential differences between their top and bottom phases [8] and the partition behavior of cells in these systems reflects charge-associated surface properties. A major difference between system 1 and the other systems is that the latter have higher interfacial tensions. System 3 has a lower electrostatic potential difference between the phases than does system 2. The reasons for selecting these systems will be discussed below.

The phase systems were equilibrated overnight at 4 to 5°C in a separatory funnel and top and bottom phases were then separated.

Countercurrent distribution (CCD) of labeled untreated and variously treated rat red blood cells

Our thin-layer CCD plates [13] have 120 concentric cavities (Workshop, University of Lund, Sweden). The bottom plate cavities have a capac-

ity of 0.7 ml. Washed, packed red blood cells (whether fresh or treated) were suspended in ten times their volume of top phase ('load mix') of the system in which CCD was to be carried out. 0.5 ml of bottom phase was delivered into each of the 120 cavities. When four different preparations were to be examined by CCD concurrently, 0.7 ml of one 'load mix' was pipetted into cavities 0–2, a second 'load mix' into cavities 30–32, a third into cavities 60–62, and the fourth into cavities 90–92. All other cavities received 0.7 ml top phase. When only two populations were to be compared cavities 0–2 and 60–62 received the two load mixes. In this manner 30 transfers could be carried out on four preparations and 40 on two preparations simultaneously and without overlap. In some experiments only one preparation (ethanol-extracted glutaraldehyde-fixed erythrocytes) was subjected to CCD. In such cases cavities 0–4 received the load mix and 60 transfers were completed. A settling time of 6 min and a shaking time of 22 s was used with phase system 1 while a 4.5 min settling time and 30 s shaking time was employed with systems 2 and 3. (Systems 2 and 3 being further from the critical point settle more rapidly than system 1 but their higher viscosity requires a longer mixing time.) Runs were performed at 4–5 °C. At the end of a run cells were collected directly into plastic centrifuge tubes and pooled by threes after adding 0.7 ml saline to each tube. The cells were centrifuged, the supernatant solution discarded and the cells analyzed as indicated below.

Analysis of cells following CCD

(a) Cell concentration. Erythrocytes, both untreated and neuraminidase-treated, were lysed in a known volume of 20 mosM sodium phosphate buffer, pH 7.2. The stroma were removed by high-speed centrifugation (12000 × *g* for 10 min) and the hemoglobin absorbance measured at 540 nm [1–3]. Glutaraldehyde-fixed erythrocytes, as well as glutaraldehyde-fixed erythrocytes which had undergone additional treatments (see above), were suspended in a known volume of water and their concentration determined by turbidity measurement in a Klett-Summerson photoelectric colorimeter [14].

(b) ^{59}Fe assay. Known aliquots of the above-indicated lysates or suspension of fixed erythrocytes were also counted in a Beckman scintillation well-counter to determine the ^{59}Fe counts per min.

Presentation of data

Distribution curves are given either in hemoglobin absorbance at 540 nm (obtained on lysis of fresh or neuraminidase-treated erythrocytes) or Klett units (of the turbididty of suspended aldehyde-fixed cells or differently treated aldehyde-fixed cells) obtained in the different cavities along the extraction train. Isotope distribution is given in cpm. A relative specific activity is also presented and is defined as [1]:

(cpm/unit hemoglobin absorbance (or Klett unit) in a given cavity) ÷ (cpm/unit hemoglobin absorbance (or Klett unit) in the original unfractionated cell population)

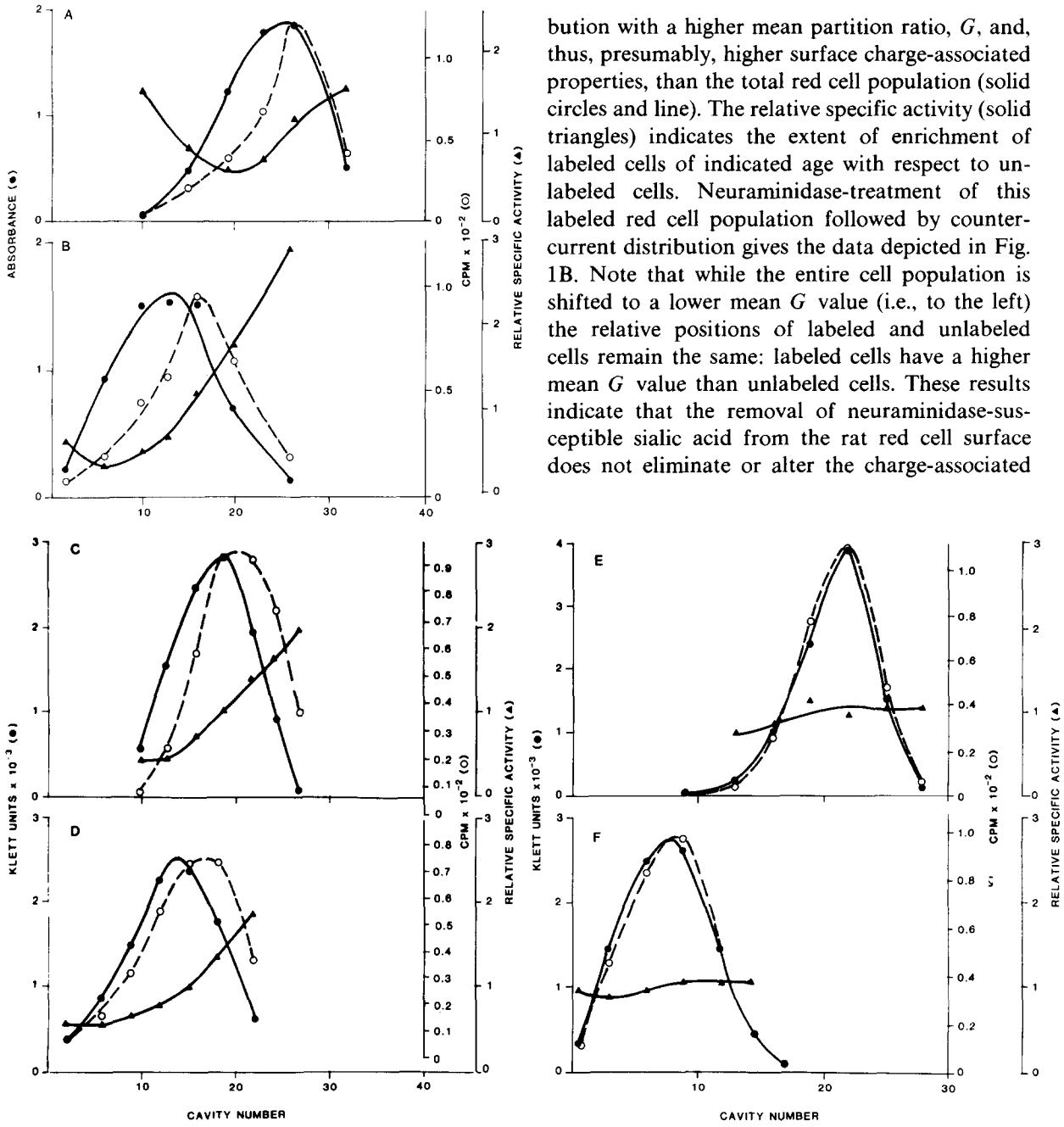
Sialic acid is given as femtogram (fg) per cell. Red blood cell electrophoretic mobilities are given as $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$.

Results

Effect of selected in vitro treatments on the relative surface charge-associated differences of rat erythrocytes of different ages

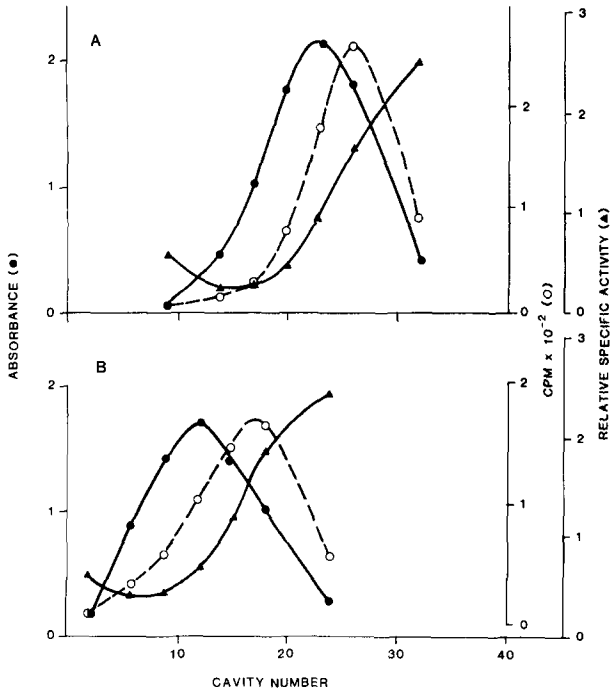
Rats were injected with ^{59}Fe-ferrous citrate and were bled at different times thereafter. Erythrocyte populations were thus obtained in which cells corresponding in age to the time elapsed between injection and bleeding were radioactively labeled [1–3]. Such cell populations were subjected to CCD in a dextran-poly(ethylene glycol) aqueous phase system which reflects surface charge associated surface properties [3]. From an extensive series of such experiments we present in Figs. 1–4 results obtained when rats are bled at 42 h, 13 days or 41 days after injection. These represent typical results with cell populations in which the mature youngest erythrocytes are labeled (Fig. 1, Fig. 4 top) young erythrocytes are labeled (Fig. 2, Fig. 4 middle) and old erythrocytes are labeled (Fig. 3, Fig. 4 bottom).

Fig. 1A shows that, as previously reported [1], the youngest mature erythrocytes (i.e., the labeled cells represented by the broken line) have a distri-



bution with a higher mean partition ratio, G, and, thus, presumably, higher surface charge-associated properties, than the total red cell population (solid circles and line). The relative specific activity (solid triangles) indicates the extent of enrichment of labeled cells of indicated age with respect to unlabeled cells. Neuraminidase-treatment of this labeled red cell population followed by countercurrent distribution gives the data depicted in Fig. 1B. Note that while the entire cell population is shifted to a lower mean G value (i.e., to the left) the relative positions of labeled and unlabeled cells remain the same: labeled cells have a higher mean G value than unlabeled cells. These results indicate that the removal of neuraminidase-susceptible sialic acid from the rat red cell surface does not eliminate or alter the charge-associated

Fig. 1. Countercurrent distribution patterns of rat red blood cells before and after different treatments and obtained from an animal which had been injected with ^{59}Fe-ferrous citrate 42 h prior to bleeding. The cell population thus contains very young mature erythrocytes that are labeled. (A) untreated cell population (except for incubation at 37°C for 90 min, see text); (B) neuraminidase-treated red blood cells; (C) glutaraldehyde-fixed red cells; (D) glutaraldehyde-fixed red cells subsequently treated with neuraminidase; (E) glutaraldehyde-fixed red cells subsequently extracted with ethanol; (F) glutaraldehyde-fixed red cells subsequently treated with dilute sulfuric acid. Forty transfers were carried out with cells in (A) and (B) using charge-sensitive phase system 1 and a settling time of 6 min and shaking time of 22 s. Forty transfers were carried out with cells in (C) and (D) and 30 transfers with those in (E) and (F). Charge-sensitive phase system 2 was used for all glutaraldehyde-fixed cells with a settling time of 4.5 min and a shaking time of 30 s. Distribution curves (●) of fresh or of neuraminidase-treated red cells (i.e., in A and B) are given in terms of hemoglobin absorbance; of all cells involving glutaraldehyde-fixation (C, D, E, and F) in terms of Klett (turbidity) units. The distribution of labeled cells of distinct cell age is depicted in cpm (○). A relative specific activity (▲) is also shown. See text for additional details and discussion.



surface differences between the mature youngest erythrocytes and the rest of the red blood cell population.

Looking at Figs. 2A, B and 3A, B, which present analogous experiments to those just described except with blood from animals bled at 13 and 41 days, respectively, we find similar results. 13-day labeled cells (young erythrocytes) also have higher mean G values than the whole red cell population [1] while 41-day labeled (old erythrocytes) have lower mean G values than the whole cell population [1]. In both cases neuraminidase-treatment results in cell distributions which, although the entire cell population is shifted to lower mean G values, maintain the relative position of cells of a given age (i.e., 13 days in Fig. 2B and 41 days in Fig. 3B) to the whole red cell population. We can thus conclude that, quite generally, removal of neuraminidase-susceptible sialic acid reduces the surface charge (and hence the mean G value) of rat red cells but does not affect the relative charge-associated differences among rat red blood cells of different ages.

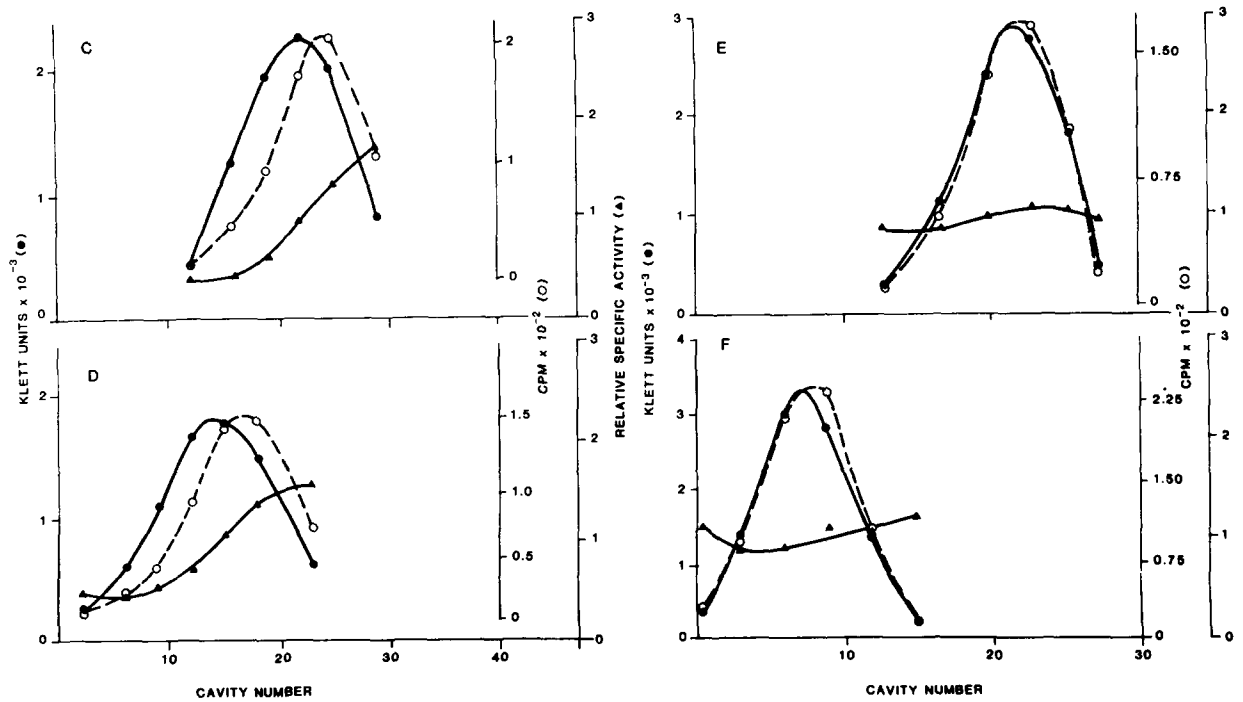
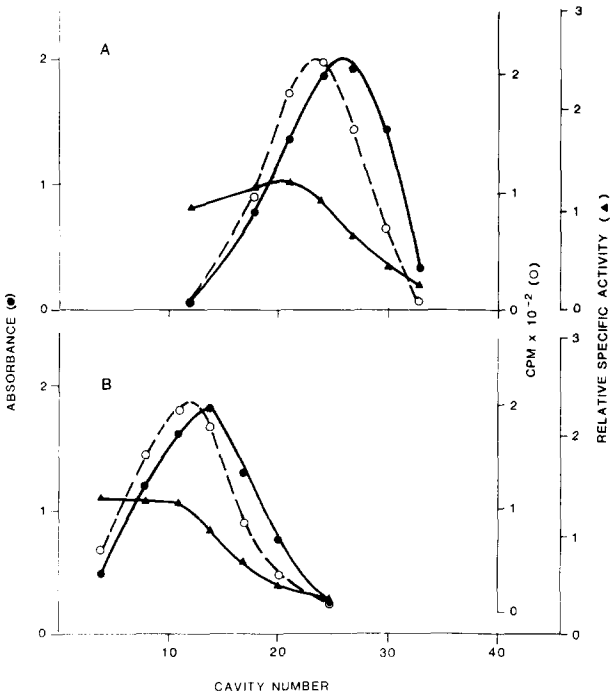


Fig. 2. Experiment as in Fig. 1 except that the rat was bled 13 days after isotope injection. Hence it is the young mature erythrocytes that are labeled.



Part C of Figs. 1–3 shows results obtained with glutaraldehyde-fixed erythrocyte populations in which, respectively, 42 h, 13-day or 41-day old red blood cells are labeled. Again it is clear that glutaraldehyde-fixation (while shifting the overall distribution curve, see Discussion) does not affect the relative positions of labeled and unlabeled cells (compare Figs. 1–3, parts A with their respective parts C). Glutaraldehyde-fixation thus does not affect the relative surface charge-associated differences among rat red blood cells of different ages.

Part D of Figs. 1–3 shows the results obtained when glutaraldehyde-fixed red cells are neuraminidase-treated prior to CCD. Such cells have lower G values than cells fixed with glutaraldehyde alone (compare parts C and D in Figs. 1–3). The relative positions of labeled and unlabeled cells are, however, not affected by the indicated treatments (compare respective parts A and D in Figs. 1–3). Our conclusions are therefore analo-

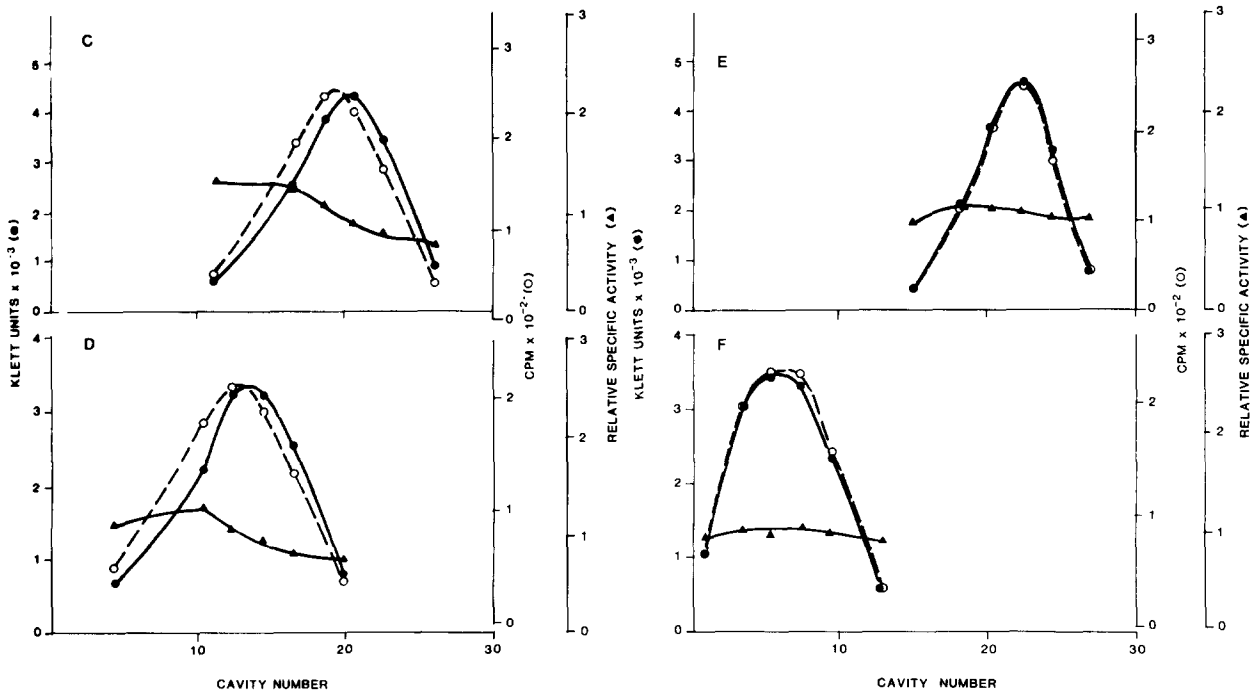


Fig. 3. Experiment as in Fig. 1 except that the rat was bled 41 days after isotope injection. Hence it is the old mature erythrocytes that are labeled. (Thirty transfers were carried out with cells in C and D.)

gous to those discussed above for parts C. Not shown but also examined were neuraminidase-treated red cells subsequently fixed with glutaraldehyde. The mean G value of these cells was lower than that of glutaraldehyde-fixed, neuraminidase-treated red cells but the relative positions of labeled and unlabeled cells were similar to those depicted in Figs. 1–3D.

In parts E of Figs. 1–3 we show the results obtained with glutaraldehyde-fixed cells from which lipids have subsequently been extracted with ethanol *. Such cells have mean G values that are greater than those of glutaraldehyde-fixed rat red cells (compare distribution curves in parts C and E). In this case, as shown by the constant value of 1.0 for the relative specific activities through the CCD curve, differences between labeled and unlabeled cells appear to be eliminated. Because at high G values cell populations may not be resolved, and the G value for ethanol-extracted glutaraldehyde-fixed rat erythrocytes are high, we examined these cells also in a phase system in which they have a lower G value (system 3). Results depicted in Fig. 4 show the overlap of labeled and unlabeled cell populations with only small deviations of the relative specific activities from 1.0 (again compare parts C in Figs. 1–3 with their respective parts E′ in Fig. 4). Ethanol-extractable lipids appear, therefore, to have a bearing on the surface charge-associated differences among rat red cells of different ages.

The final treatment of glutaraldehyde-fixed rat erythrocytes examined in the current study was the effect of dilute sulfuric acid which is known to split off the total sialic acid from the surface of the cells [9]. The data shown in Figs. 1–3 parts F indicate that complete removal of sialic acid results in very low mean G values for the cells (compare position of distribution curves in parts F

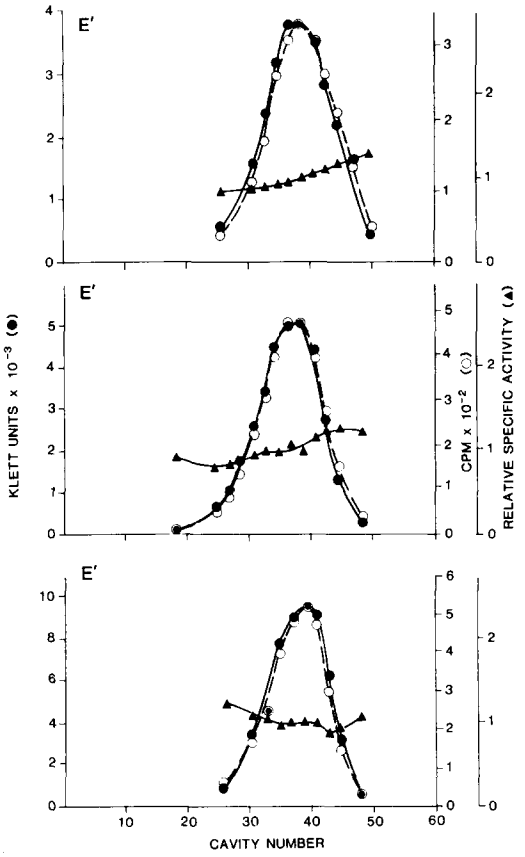


Fig. 4. Experiment as in parts (E) of Figs. 1–3 except that phase system 3 (in which the cells have a lower G value) was used, 5 cavities were loaded with 'load mix' and 60 transfers were carried out. Top, 42 h labeled cells; middle, 13-day labeled cells; bottom, 41-day labeled cells. Other conditions and symbols as in Fig. 1.

with those in parts C, D and E). Furthermore, complete removal of sialic acid results in elimination of differences among red blood cells of different ages (as again indicated by the constant relative specific activities of 1.0 through the CCD curves).

A summary of effects of the different treatments of rat erythrocytes on (a) the apparent partition ratio (G value) of the cells in charge-sensitive phase system 2 and (b) the relative charge-associated differences among cells of different ages is presented in Table I.

Effect of different treatments of glutaraldehyde-fixed rat erythrocytes on the quantity of sialic acid released and on the electrophoretic mobility of the cells

Glutaraldehyde-fixation of rat red blood cells,

* Ethanol-extraction of glutaraldehyde-fixed rat red blood cells which have been labeled in vivo with ^{59}Fe (see Materials and Methods) results not only in the extraction of lipid but also in the extraction of approx. 20% of the isotope. Systematic study with erythrocyte populations in which red blood cells of different and distinct age were isotopically labeled indicates that loss of isotope is independent of cell age (i.e., the same percentage of isotope is lost irrespective of the age of the cell subpopulation labeled). Hence the loss of isotope during ethanol extraction does not affect the results presented in this section.

TABLE I

SUMMARY OF EFFECT OF DIFFERENT TREATMENTS OF RAT ERYTHROCYTES ON THE APPARENT PARTITION RATIO (G) OF THE CELLS IN A CHARGE-SENSITIVE PHASE SYSTEM AND THE RELATIVE CHARGE-ASSOCIATED DIFFERENCES AMONG CELLS OF DIFFERENT AGES

G, the apparent partition ratio is derived from the location of the peak in a countercurrent distribution curve $= r_{max}/(n - r_{max})$, where r_{max} is the cavity number of the peak of the distribution and n is the total number of transfers carried out. G values are shown ± S.D. with the number of experiments in parentheses. The phase system is composed of 7% dextran, 4.4% poly(ethylene glycol), and 0.11 M sodium phosphate buffer (pH 6.8). Details of procedural aspects of the treatments employed are given in the text. N, neuraminidase treatment; GA, glutaraldehyde fixation; E ext., ethanol extraction of lipids; H_2SO_4, cleavage of total sialic acid by dilute sulfuric acid hydrolysis.

Treatment(s)	G value	Effect on realtive age-related charge-associated surface properties
N + GA	0.55 ± 0.12 (8)	None
GA	1.66 ± 0.38 (16)	None
GA + N	0.67 ± 0.10 (14)	None
GA + E ext.	2.65 ± 0.19 (8)	Dif. elim. [a]
GA + H_2SO_4	0.33 ± 0.06 (8)	Dif. elim.

[a] At high G values cell populations may not be resolved. Because the G value for ethanol-extracted glutaraldehyde-fixed rat erythrocytes is high in this phase system, the experiment was also run in phase system 3 in which the cells have a lower G value (approximately 1.75). The results obtained (Fig. 4) confirm the virtual elimination of relative charge-associated surface properties of glutaraldehyde-fixed rat erythrocytes of different ages by ethanol extraction.

under the conditions used, usually results in an unaltered or only minor increase in cell electrophoretic mobility. In Table II we show the effect of different treatments on the quantity of sialic acid released from glutaraldehyde-fixed rat red cells and on their electrophoretic mobility.

Sulfuric acid treatment of glutaraldehyde-fixed rat erythrocytes releases total sialic acid which is 8.1 fg/cell and reduces the cell electrophoretic mobility from -1.24 to -0.90 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ (i.e., about 27%). Neuraminidase-treatment releases about 5.8 fg sialic acid per cell (i.e., about 70% of total sialic acid [15]) and reduces the electrophoretic mobility to -0.88 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$. Sulfuric acid hydrolysis of neuraminidase-treated glutaraldehyde-fixed rat erythrocytes releases an additional 1.9 fg sialic acid per cell but has no additional effect on the electrophoretic mobility of the cells. Ethanol-extraction of glutaraldehyde-fixed erythrocytes yields 1.5 fg sialic acid per cell in the extract without affecting the electrophoretic mobility of the cells. Sulfuric acid digestion of ethanol-extracted red cells releases an additional 5.8 fg sialic acid per cell and reduces the cell electrophoretic mobility by the maximum amount of about 27% observed in these experiments.

TABLE II

EFFECT OF DIFFERENT TREATMENTS OF GLUTARALDEHYDE-FIXED RAT ERYTHROCYTES ON THE QUANTITY OF SIALIC ACID RELEASED AND ON THE ELECTROPHORETIC MOBILITY OF THE CELLS

Details of procedural aspects of the treatments employed are given in the text. Sialic acid release is given in femtograms (fg) per cell ± S.D. with the number of experiments in parentheses. The cell electrophoretic mobility is given in $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ ± S.D. with the number of experiments in parentheses.

Treatment(s)	Sialic acid released	Electrophoretic mobility
No additional	–	−1.24 ± 0.02 (8)
H_2SO_4 digestion	8.1 ± 0.3 (8)	−0.90 ± 0.05 (8)
Neuraminidase	5.8 ± 0.4 (8)	−0.88 ± 0.02 (8)
H_2SO_4 digestion after neuraminidase	1.9 ± 0.2 (8)	−0.94 ± 0.04 (8)
Ethanol extraction	1.5 ± 0.2 (8)	−1.21 ± 0.03 (8)
H_2SO_4 digestion after ethanol ext.	5.8 ± 0.2 (8)	−0.87 ± 0.04 (8)

Discussion

Background

Little is known about the chemical nature of the surface component(s) responsible for the age-related differences in charge-associated properties of rat red cells. Variables that affect the partition ratio are exponentially related to it. Chemical assays of different surface components of young and old red blood cells, undertaken to establish correlations with partitioning differences of erythrocytes of different ages, is thus not a sensitive enough method [16]. In our novel approach chemical and enzymatic treatments of red cell

populations containing ^{59}Fe-labeled subpopulations of distinct age (see above) are followed by CCD and analysis to determine whether the differences originally present between labeled erythrocytes (i.e., those of a particular age) and the total erythrocyte population is maintained or eliminated. Thus the involvement of surface components in establishing or contributing to age-related surface differences is tested. Previously [15] we found that neuraminidase removes approx. 70% of total rat red cell sialic acid and significantly reduces the *G* value of the cells, without affecting the relative partition ratios of erythrocytes of different ages. The results of our expanded studies are consistent with the hypothesis that ganglioside-bound sialic acid on the rat red cell surface is the component responsible for the age-related partitioning differences in charge-sensitive phase systems.

Effect of selected treatments on the relative surface charge-associated differences among erythrocytes of different ages

Young labeled cells (42 h, 13 days) are displaced to the right (i.e., higher partition ratio) and old (41 days) cells to the left of the distribution of total cells (Part A of Figs. 1, 2 and 3) [1,3]. Treatment with neuraminidase (part B) shifts the distribution curves to lower partition ratios, but does not change the relative positions of labeled and unlabeled cells (part B, Figs. 1–3). Hence neuraminidase-susceptible sialic acid is not responsible for the charge-related difference of rat red cells of different ages [15].

Glutaraldehyde-fixation (parts C) increases the partition ratio of rat red cells compared to that of the original unfixed cells. This is apparent when one considers that phase system 2 has a higher polymer concentration than phase system 1 and hence a greater interfacial tension [8]. The same cells partitioned in phase systems 1 and 2 will, generally, have lower partition ratios in system 2 than in system 1. Since the glutaraldehyde-fixed red cells (parts C) have about the same partition ratio in system 2 as unfixed cells (part A) do in system 1 (i.e., their peak is in about the same place) it appears that glutaraldehyde fixation alters the *G* value of the cells. (Phase system 2 (and also system 3, see below) were, in fact, selected for the study of the variously treated fixed cells because the *G* values of some of these would be too far to the right in phase system 1). While glutaraldehyde fixation changes the partition ratio of the rat red cell population as a whole, the relative positions of labeled and unlabeled cells (see parts C of Figs. 1–3 and compare to respective parts A) remains unaltered. Glutaraldehyde-fixation, therefore, does not affect the age-related surface differences of erythrocytes of different ages.

Neuraminidase-treatment of glutaraldehyde-fixed erythrocytes or glutaraldehyde-treatment of neuraminidase modified cells diminishes the *G* value of the cells because of sialic acid removal (compare parts D to respective parts C of Figs. 1–3).

From Figs. 1–3, it is apparent that ethanol extraction of glutaraldehyde-fixed rat red cells increases the partition ratio (compare parts E with C) while sulfuric acid treatment diminishes the partition ratio (compare parts F with C). The increases in partition ratio produced by ethanol extraction is likely related to changes in the surface resulting in exposure of additional charges to the charge-sensitive phase system. The reduction in *G* with sulfuric acid treatment most probably arises from the removal of total sialic acid. Both ethanol extraction of lipid and removal of total sialic acid (parts E and F of Figs. 1–3 and parts E′ of Fig. 4) eliminate virtually all differences between erythrocytes of different ages. This is indicated by the overlap of labeled and unlabeled cells in each case and a relative specific activity of close to 1.0 through each of these curves.

Effect of different treatments of glutaraldehyde-fixed rat erythrocytes on the quantity of sialic acid released and on the electrophoretic mobility of the cells

Rat erythrocytes, unlike human red cells, have an appreciable quantity (6.3%) of ganglioside-bound sialic acid which is not released by neuraminidase [17], but is removed by dilute sulfuric acid [9]. Sulfuric acid hydrolysis of glutaraldehyde-fixed rat erythrocytes yields about 8.1 fg/cell of sialic acid while only 5.8 fg sialic acid per cell is susceptible to neuraminidase cleavage (Table II). (These quantities of sialic acid released are identical to those obtained by analogous treatments

from fresh rat red cells [15].) Neuraminidase-resistant sialic acid on the fixed cell surface can be recovered by sulfuric acid hydrolysis which also eliminates age-related, charge-associated surface differences among rat red cells of different ages.

Ethanol extraction yields 1.5 fg sialic acid per cell (Table II) and virtually eliminates the age-related, charge-associated surface differences between rat erythrocytes of different ages. Sulfuric acid digestion of ethanol-extracted glutaraldehyde-fixed cells releases the remaining 5.8 fg sialic acid per cell.

These data suggest that the 20–25% of the sialic acid removed by sulfuric acid (which is not susceptible to neuraminidase) or extracted by ethanol contains the charged groups responsible for the charge-associated differences among rat red cells of different ages detected by cell partitioning (Figs. 1–3). Ganglioside-bound sialic acid, known to be present on the rat erythrocyte, is the most likely component responsible for the age-related differences. It is not susceptible to neuraminidase cleavage [17], can be extracted with ethanol [17,18], and removed by sulfuric acid. Furthermore, it is interesting to note that human red blood cells, which have very little ganglioside-linked sialic acid [17], display no age-related charge-associated surface differences detectable by partitioning under conditions analogous to those used with rat erythrocytes [19,20].

The electrophoretic mobilities of glutaraldehyde-fixed rat erythrocytes and of these fixed cells after different treatments yield additional information on the surface properties of the cells (Table II). The mobility of fixed cells is, within experimental error, not appreciably different from that of fresh cells [19]. Removal of total sialic acid (sulfuric acid digestion) reduces the mobility from -1.24 to -0.90 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ (or about 27%). Partial removal of the sialic acid (about 72%) by neuraminidase reduces the electrophoretic mobility by the same amount as total cleavage of sialic acid by sulfuric acid indicating that the sialic acid which is not susceptible to neuraminidase does not contribute to the electrokinetic charge. A similar argument applies for the sialic acid extracted by ethanol. Since sulfuric acid treatment of fixed rat red cells reduces their G value more than does neuraminidase (Table I), the surface charge reflected by partitioning and that measured by electrophoresis is not the same. Electrophoresis measures charge at the electrophoretic surface while partitioning also measures charge deeper in the membrane [21].

Cell electrophoresis cannot detect differences between young and old rat erythrocytes [19]. However, measurement of electrophoretic mobilities of rat red cells from different cavities of the extraction train, known to contain cells of different ages [1–3], reveals increasing cell mobilities and G values to be concomitant [22,19,15]. This correlation is obtained with both fresh [22,19] and neuraminidase-treated cells [15] *. Furthermore, the mean electrophoretic mobilities of young and old red blood cells after countercurrent distribution (CCD) differ, with the young population having the higher mobility as well as G value [19].

Acknowledgments

This work was supported by the Medical Research Service of the Veterans Administration and by USPHS Grant HL24374 of the National Heart, Lung and blood Institute.

References

1 Walter, H. and Selby, F.W. (1966) Biochim. Biophys. Acta 112, 146–153
2 Walter, H., Miller, A., Krob, E.J. and Ascher, G.S. (1971) Exp. Cell Res. 69, 416–424
3 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
4 Brunette, D.M., McCulloch, E.A. and Till, J.E. (1968) Cell Tissue Kinet. 1, 319–327
5 Walter, H., Webber, T.J., Michalski, J.P., McCombs, C.C., Moncla, B.J., Krob, E.J. and Graham, L.L. (1979) J. Immunol. 123, 1687–1695
6 Walter, H., Graham, L.L., Krob, E.J. and Hill, M. (1980) Biochim. Biophys. Acta 602, 309–322
7 Miner, K.M., Walter, H. and Nicolson, G.L. (1981) Biochemistry 20, 6244–6250
8 Brooks, D.E., Sharp, K.A. and Fisher, D. (1985) in Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Applications to Biotechnology (Walter, H.,

* The basis for the difference in electrophoretic mobility of rat erythrocytes of different ages after CCD is not clear. It may be a consequence of differential interaction of these cells with phase polymer(s) [23].

Brooks, D.E. and Fisher, D., eds.), pp. 11–84, Academic Press, Orlando
9 Eylar, E.H., Madoff, M.A., Brody, O.V. and Oncley, J.L. (1962) J. Biol. Chem. 237, 1992–2000
10 Aminoff, D. (1961) Biochem. J. 81, 384–392
11 Seaman, G.V.F., Knox, R.J., Nordt, F.J. and Regan, D.H. (1977) Blood 50, 1001–1011
12 Seaman, G.V.F. (1975) in The Red Blood Cell (Surgenor, D. MacN., ed.), Vol. 2, pp. 1135–1229, Academic Press, New York
13 Albertsson, P.-A. (1970) Sci. Tools 17, 53–57
14 Walter, H., Krob, E.J., Brooks, D.E. and Seaman, G.V.F. (1973) Exp. Cell Res. 80, 415–424
15 Walter, H., Tamblyn, C.H., Krob, E.J. and Seaman, G.V.F. (1983) Biochim. Biophys. Acta 734, 368–372
16 Walter, H. and Krob, E.J. (1984) Biochem. Biophys. Res. Commun. 120, 250–255
17 Tenforde, T. (1970) Adv. Biol. Med. Sci. 13, 43–105
18 Korn, E.D. and Weisman, R.A. (1966) Biochim. Biophys. Acta 116, 309–316
19 Walter, H., Krob, E.J., Tamblyn, C.H. and Seaman, G.V.F. (1980) Biochem. Biophys. Res. Commun. 97, 107–113
20 Walter, H., Krob, E.J. and Ascher. G.S. (1981) Biochim. Biophys. Acta 641, 202–215
21 Walter, H., Tung, R., Jackson, L.J. and Seaman, G.V.F. (1972) Biochem. Biophys. Res. Commun. 48, 565–571
22 Brooks, D.E., Seaman, G.V.F. and Walter, H. (1971) Nature (London) New Biol. 234, 61–62
23 Brooks, D.E. (1971) Theoretical and Experimental Studies on the Interaction of Neutral Polymers with Cell Surfaces, Ph.D. Thesis, University of Oregon School of Medicine, Portland, OR