Biochimica et Biophhysica Acta, 455 (1976) 8–23

BBA 77485

PARTITION IN TWO-POLYMER AQUEOUS PHASES REFLECTS DIFFERENCES BETWEEN MEMBRANE SURFACE PROPERTIES OF ERYTHROCYTES, GHOSTS AND MEMBRANE VESICLES

HARRY WALTER and EUGENE J. KROB

Laboratory of Chemical Biology, Veterans Administration Hospital, Long Beach, Calif. 90822 *and the Department of Physiology, University of California, College of Medicine, Irvine, Calif.* 92717 *(U.S.A.)*

(Received April 7th, 1976)

SUMMARY

Partition of cells (defined as the percentage of added cells in the top phase) in dextran-polyethylene glycol aqueous two-phase systems is an extremely sensitive indicator of cell surface properties. By appropriate choice of polymer concentrations and salt composition and concentration one can, to some extent, select the membrane properties (e.g. charge (Walter, H. (1975) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. 9, pp. 25–50, Academic Press, New York), lipid composition (Walter, H, Krob, E.J. and Brooks, D.E. (1976) Biochemistry, 15, 2959–2964)) that determine the partition behavior of cells (or membranes). In the present experiments we have obtained by partition information on separability, alteration and ensuing heterogeneity during preparation of human erythrocytes, ghosts and rightside-out and inside-out vesicles from human erythrocyte membranes. A phase system in which partition is greatly (although not exclusively) determined by membrane charge was used. It was found that:

1. The partition coefficients (and hence the membrane surfaces) of ghosts and of rightside-out vesicles differ from those of the erythrocytes from which they are derived.

2. Rightside-out and inside-out vesicles have different countercurrent distribution patterns with the rightside-out vesicles having the higher partition coefficient (probably reflecting higher membrane charge of the latter).

3. Rightside-out vesicles are highly heterogeneous membrane populations as evidenced by broader than theoretical countercurrent distribution curves which, in some experiments, even split into two populations outright. This heterogeneity is particularly interesting since the enzyme markers used to establish vesicle sidedness (Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 2, pp. 245–281, Plenum Press, New York) indicate "pure" rightside-out preparations.

4. Inside-out vesicles could, under no conditions tested, be rendered totally free of rightside-out vesicles according to the enzyme markers used to indicate vesicle sidedness (Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 2, pp. 245–281, Plenum Press, New York).

A discussion of factors involved in the partition of the vesicles and ghosts together with analogies to the partition behavior of stored or in vitro modified human red blood cells is presented.

INTRODUCTION

When aqueous solutions of dextran and of polyethylene glycol are mixed above certain concentrations immiscible, liquid two-phase systems are obtained [1], with a polyethylene glycol-rich top and a dextran-rich bottom phase. Such systems, can be rendered isotonic by incorporation of salts and are suitable for the partition and separation by countercurrent distribution, of cells and membranes [2–4]. The partition coefficient of cells or membranes (defined as the percentage of added cells or membranes in the top phase) depends on subtle differences in their surface properties. Depending on the polymer concentrations and salt compositions and concentrations chosen (see below) the membrane surface properties measured by partition can be as diverse as membrane charge [3] or the ratio of membrane polymonounsaturated fatty acids [5]. Furthermore, the incorporation of small quantities of a polymer-ligand (which favors one of the phases) into the aqueous two-phase system permits extraction of cells or membranes which interact specifically with the ligand by affinity [6–9]. Aqueous phase systems are thus, at present, the most versatile tool available for the separation of cells and membranes.

Even though both dextran and polyethylene glycol are non-ionic polymers, some salts (e.g. phosphate) distribute unevenly in dextran-polyethylene glycol aqueous phase systems [10] giving rise thereby to an electrostatic potential difference between the phases [11]. This phase charge interacts with membrane surface charge-associated properties of the cells or membranes added to the system [3]. Charge is thus a major determinant of the cell's partition coefficient. Incorporation of a salt which itself partitions equally between the phases (e.g. NaCl) results in the absence of such a potential difference between the phases and most cells, under these conditions, adsorb at the interface (i.e. their partition coefficient as defined above is zero). If the polymer concentrations are reduced in such a system (e.g. from 5 % dextran, 4 % polyethylene glycol to 5 % dextran, 3.5 % polyethylene glycol) so that the system is closer to the critical point (the component concentrations below which a homogeneous solution occurs) the partition coefficient of all cells increases and has been shown, at least for erythrocytes from different species, to have an excellent correlation to the cell's membrane poly/monounsaturated fatty acids [5].

When small quantities of a polyethylene glycol-ligand (e.g. the ester of palmitic acid and polyethylene glycol, polyethylene glycol-palmitate, which itself favors the top phase) are incorporated into a dextran-polyethylene glycol phase system containing NaCl (i.e. no electrostatic potential difference between the phases) and at some distance from the critical point (e.g. polymer ratio 5 %/4 %), only those cells or membranes that interact with the ligand will be "pulled" into the top phase [6–8]. An affinity partition method thus results.

Since human (and beef) erythrocytes, their ghosts and membrane vesicles are models in membrane structure studies we became interested in testing the homogeneity of such preparations by partition in two-polymer phases. We have previously

reported that, although all beef erythrocytes have the same electrophoretic mobility [12], they fall into three partition classses, those with low, intermediate or high partition coefficients in a phase system with an electrostatic potential difference between the phases [12]. Cells with high partition coefficient release far more sialic acid when treated with neuraminidase or trypsin than do the other two classes. More recently, the countercurrent distribution patterns of sealed rightside-out and inside-out vesicles from human erythrocyte membranes were examined in a dextran-polyethylene glycol phase system containing NaCl and small quantities of polyethylene glycol-palmitate [7]. Rightside-out vesicles were found to have a higher partition coefficient than inside-out vesicles reflecting difference in available hydrophobic residues on these two membrane surfaces.

In the present work we present results on the partition behaviour of human erythrocytes, ghosts, and rightside-out and inside-out vesicles in phases with an electrostatic potential difference. Information was obtained on separability, alteration during preparation and ensuing hetereogeneity of the preparations. The heterogeneity established for rightside-out vesicle populations was particularly interesting because they were "pure" according to the enzyme markers used to establish vesicle sidedness.

MATERIALS AND METHODS

Collection of blood. Blood was drawn from normal human donors by venous puncture using acid-citrate-dextrose solution as anticoagulant. The volume ratio of blood to anticoagulant solution used was between 4–5 and 1.

Preparation of hemoglobin-free ghosts from human erythrocytes. Hemoglobin-free ghosts were prepared from human erythrocytes by the method of Dodge et al. [13].

Preparation of rightside-out and inside-out vesicles from human erythrocyte membranes. Rightside-out and inside-out vesicles from human erythrocyte membranes were prepared by the method of Steck [14]. Sealed vesicles were obtained from the top of a dextran gradient, unsealed vesicles (when desired) were taken from the bottom of the gradient. Sidedness was established by measuring acetylcholinesterase (E C 3. 1. 1. 7) activity and glyceraldehyde-3-phosphate dehydrogenase (E C 1. 2. 1 12) activity. The former is a marker for the membrane's outer surface while the latter is located on the membrane's cytoplasmic side [14]. Activities were measured in the absence and presence of Triton X-100 in order to determine enzyme accessibility on the vesicles (activities without Triton give enzyme exposed on the vesicle's exposed surface while activities measured in the presence of Triton give total vesicle enzyme activities). The sealed rightside-out vesicle preparations were generally 100% rightside-out (except where indicated in the text); while inside-out vesicle preparations were between 30 and 85 % inside-out (as indicated in the specific experiments).

In vitro treatments of red blood cells. In some of the experiments human red blood cells were treated with neuraminidase to effect sialic acid removal as previously described [15]. Normal or neuraminidase-treated red cells were fixed with acetaldehyde [16] and aliquots of such acetaldehyde-fixed cells were extracted with ethanol to remove lipids [16]. In other experiments neuraminidase-treated erythrocytes were used in the preparation of rightside-out and inside-out vesicles (as above).

Preparation of two-polymer phase systems. Dextran-polyethylene glycol two-

phase systems containing buffers and salts (as indicated below) were used for erythrocyte, ghost, and vesicle partition and/or countercurrent distribution studies. Details of phase system preparation have been published [3] and actual phase system compositions are given in captions to figures and tables and in the text. Dextran T500, lot. No. 5996, was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., and polyethylene glycol was obtained under the trade-name "Carbowax" 6000 from Union Carbide, N.Y.

Partition of red cells, ghosts and fixed red cells in aqueous phase systems. The partition methods used for erythrocytes [3] and acetaldehyde-fixed erythrocytes [16] were the same as those previously described. Ghosts prepared by the method of Dodge et al. [13] were washed once in the top (polyethylene glycol-rich) phase of the system in which they were to be partitioned. The supernatant solution was discarded and the ghosts were resuspended in at least 10 times their volume of top phase. 2 ml of this supension was added to 2 ml of the corresponding bottom (dextran-rich) phase in a test tube. The phases were then mixed by inverting the tube 10 times and permitted to settle 20 min by the clock at which time 1 ml of top phase was removed. After appropriate dilution with saline, the absorbance of this ghost suspension was measured at 500 nm as was a suitably diluted 1 ml aliquot of the original suspension in top phase ("whole"). The quantity of ghosts in the top phase (percent of total ghosts added) was calculated.

Countercurrent distribution of erythrocytes, ghosts and rightside-out and inside-out membrane vesicles. An automatic thin-layer countercurrent distribution apparatus with 120 concentric cavities and a bottom phase capacity of 0.7 ml as described by Albertsson [17] and constructed by Incentive Research and Development AB. Bromma, Sweden, was used.

(a) Erythrocyte and ghost countercurrent distribution: Erythrocytes were washed three times in isotonic saline and once in the top phase of the system in which countercurrent distribution was to be carried out. Erythrocyte ghosts were washed three times in 20 mosM sodium phosphate buffer, pH 7.4 [13], twice more in saline and finally once in top phase. All operations were at 4–5 °C. Washings of ghosts consisted of suspension in the indicated solutions followed by centrifugation at 20 000 $\times g$ for 40 min. After the final wash 0.55 ml of "packed" erythrocytes or ghosts were suspended in 4.85 ml of top phase ("load mix"). Cavities 0–4 and 60–64 each received 0.5 ml of bottom phase and 0.8 ml of either the red cell or ghost "load mix" suspension. All other cavities received 0.6 ml bottom and 0.7 ml top phase. Loading in this manner results in 0.1 ml stationary top phase (see ref. 2 for discussion). 60 transfers were completed using a 6 min settling time, 20 s shaking, at 4–5 °C.

(b) Erythrocyte and membrane vesicle countercurrent distribution: Washed, packed erythrocytes were suspended in top phase as above. Vesicles obtained by Steck's method [14] from the dextran gradient (top or bottom depending on the experiment) were diluted with 0.005 M sodium phosphate, pH 8, and centrifuged 30 min at 30 000 $\times g$ at 4 °C. The vesicles were then washed once with a salt solution corresponding in composition to that of the phase system to be used in countercurrent distribution and once in top phase of that system. In early experiments 0.1–0.3ml of a vesicle preparation was suspended in 2–3 ml of top phase. In later experiments equal quantities of rightside-out and inside-out vesicles estimated by absorbance of aliquots of the vesicle suspension at 500 nm were used (approx. 9 absorbance units in 2 ml top

phase constituted the "load mix" (see above)). The quantity of vesicles loaded appeared to have no discernible influence on the distribution obtained. The distribution patterns of three preparations (e.g. erythrocytes, rightside-out vesicles, inside-out vesicles) could be compared at the same time by loading one of the suspensions into cavities 0 and 1, another into cavities 40 and 41 and the third into cavities 80 and 81. 40 transfers were then completed. In these experiments 0.5 ml bottom phase was put into the load cavities (i.e. Nos. 0, 1, 40, 41, 80, 81) as were 0.9 ml of one of the "load mixes". All other cavities received 0.6 ml bottom and 0.8 ml top phase. The settling time was 6 min, and the shaking time 25 s. As before the entire operation was at 4–5 °C.

(c) Acetaldehyde-fixed red cell and membrane vesicle countercurrent distribution: Equal amounts (i.e. absorbances) of rightside-out and inside-out vesicles were loaded on the countercurrent plates as in b above. For comparison of vesicle distribution to that of red cells in hypotonic phase systems, fixed rather than fresh red cells had to be employed to avoid hemolysis. Fixed red cells, used not earlier than 2 weeks after fixation [16], were washed four times with water, once with top phase. Between 0.05 and 0.1 ml of packed, fixed red cells were suspended in 2 ml top phase and loaded into two cavities as described for the loading of vesicles in b above.

Collection and analysis of cells, ghosts and vesicles after countercurrent distribution. After the countercurrent run was completed, red cells, ghosts, vesicles, or fixed cells from each cavity were collected directly into plastic tubes. Every second or third tube containing ghosts or vesicles received a small, known aliquot of saline (usually 0.5 ml) to break the phase system (i.e. convert it to a one-phase suspending medium). Absorbance of ghost or vesicle suspensions was then measured at 500 nm on a Gilford spectrophotometer. In the case of red cells, saline was also added to break the phase system. The cells were then centrifuged, the supernatant solution discarded, the cells lysed in Drabkin's solution, centrifuged at high speed to remove stromal residue, and the hemoglobin absorbance measured at 540 nm. Fixed cell concentration was determined, after breaking the phase system by addition of saline, by measurement on a Klett-Summerson photoelectric colorimeter (filter No. 42).

In some experiments with rightside-out and inside-out vesicles those tubes that had not been used in absorbance measurements to obtain the distribution curve were pooled into a "left third", "middle third" and "right third" of the distribution curves after the phases were also broken by addition of a salt solution of the same composition as used in the phase system. The suspensions were then centrifuged (30 000 $\times g$ for 30 min at 5 °C). The supernatant solution was discarded and the vesicles washed twice more with the same salt solution. Each fraction was finally taken up in about 0.5 ml salt solution and acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase were assayed on aliquots of each fraction in the absence and presence of Triton X-100 as described by Steck [14] to establish sidedness of vesicles in different parts of the countercurrent distributions.

It should be stated that sealed vesicles always remain sealed during countercurrent distribution. This was checked by repooling all the vesicles from a countercurrent run and putting them once again on a dextran gradient [14]. All the vesicles were, once again, found near the top of the gradient.

Presentation of data. (a) Countercurrent distributions are given as red cell, ghost or vesicle absorbance (in the case of red cells hemoglobin absorbance) in

the different cavities of the extraction train. Fixed cell distributions are in Klett units.

(b) Partition coefficient of cells or ghosts is defined as the quantity of cells (or ghosts) in the top phase (percent of total cells (or ghosts) added). Partition coefficients in the Tables are given as the mean ± S.D. with the No. of experiments in parentheses.

(c) Enzyme (i.e. acetylcholinesterase or glyceraldehyde-3-phosphate dehydrogenase) determinations on rightside-out and inside-out vesicles are given as the percent of enzyme activity accessible in the absence of Triton X-100 to that assayed in the presence of Triton [14].

RESULTS AND DISCUSSION

Background

The properties of the membrane surface that determine the partition coefficient of membranes, cells or biological particles in aqueous two-polymer phase systems can, to some extent, be selected by appropriate manipulation of polymer phase composition parameters [3, 5, 7]. Thus, if phosphate is incorporated into the phase system membrane charge will be a major determinant of the suspended material's partition coefficient [3]. This is a consequence of the unequal partition of the phosphate itself in dextran-polyethylene glycol aqueous phases [10] a phenomenon which results in a measurable electrostatic potential difference between the phases [11]. The charge of the partitioned material interacts with the phase charge resulting in membrane charge-related partition coefficients. If NaCl, a salt which partitions equally between the phases, is selected as the salt instead of phosphate, there is no charge between the phases and most cells will accumulate at the two-phase interface. Reduction of polymer concentration (with accompanying reduction in interfacial tension) under these conditions results in species- and type-specific partition coefficients for (red blood) cells which depend on membrane lipid composition [5]. In addition to these at least partly defined contributors to partition (i.e. membrane charge, lipid composition) there are also other (as yet undetermined) interactions between the membrane surface and the polymers (e.g. the binding of phase polymer by stored erythrocytes [18]). Interactions of the last type have, so far, only been encountered with in vitro manipulated cell (or membrane) populations (e.g. cells prepared by disrupting tissue, stored cells). The basis for the partition of the red blood cells, erythrocyte ghosts and membrane vesicles to be described will be discussed and summarized in the concluding section below.

Partition and countercurrent distribution of human erythrocyte ghosts and the erythrocytes from which they were prepared

Ghosts from human erythrocytes were prepared by the method of Dodge et al. [13] and partitioned in the cold in two-polymer aqueous phase systems containing 5 % dextran, 4 % polyethylene glycol and either 0.11 M sodium phosphate buffer, pH 6.8, or 0.09 M sodium phosphate buffer and 0.03 M NaCl. Table I indicates a difference in partition behavior between ghosts and similarly partitioned red blood cells from which the ghosts were prepared. The ghosts always have the higher partition coefficient.

To determine whether additional alterations or heterogeneities could be detected in the ghost preparations, ghosts and erythrocytes were subjected simultaneously to countercurrent distribution on opposite halves of our circular counter-

TABLE I

PARTITION OF HUMAN ERYTHROCYTES AND HEMOGLOBIN-FREE GHOSTS FROM HUMAN ERYTHROCYTES IN TWO-POLYMER AQUEOUS PHASES

Partition is defined as the quantity of cells in the top phase (percent of total cells added).

Population	Partition in	
	5 : 4 (1)*	5 : 4 (2)*
Erythrocytes	38±8 (4)	11±2 (4)
Ghosts	83±3 (5)	58±4 (5)

* Phase composition is as follows: 5 : 4 (1) contained 5% (w/w) dextran, 4% (w/w) polyethylene glycol and 0.11 M sodium phosphate buffer, pH 6.8; 5 : 4 (2) contained the same polymer concentrations but 0.09 M sodium phosphate buffer, pH 6.8, and 0.03 M NaCl. Partition was carried out at 5 °C.

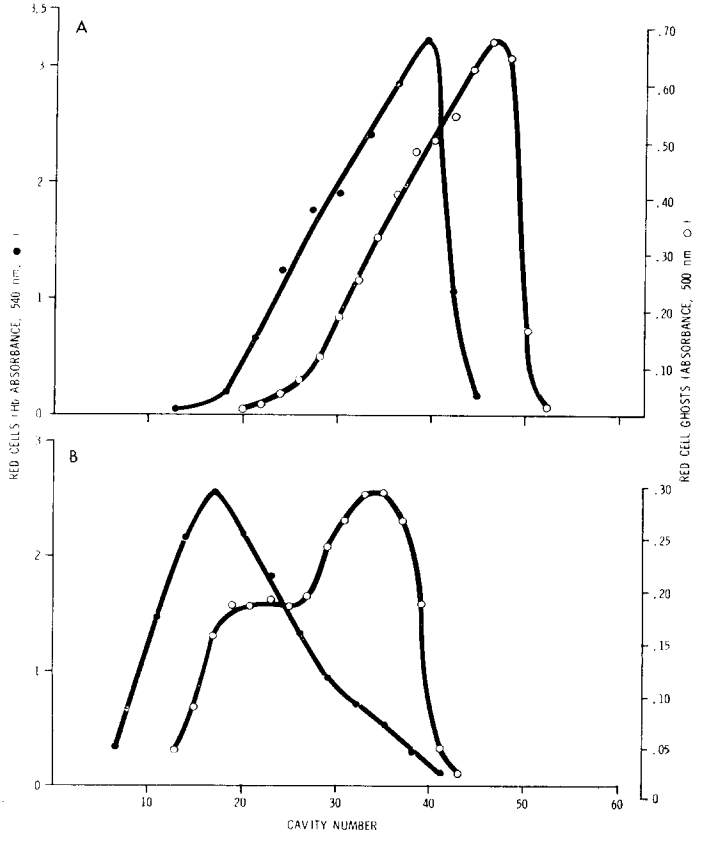

Fig. 1. Superimposed countercurrent distributions of human erythrocytes and erythrocyte ghosts in two different aqueous phase systems. ●, red cell distribution (in terms of hemoglobin absorbance); ○, ghost distribution (in terms of absorbance of ghost suspension). (A) Phase system contained 5% (w/w) dextran, 4% (w/w) polyethylene glycol and 0.11 M sodium phosphate buffer, pH 6.8. (B) Same polymer concentrations as A but 0.09 M sodium phosphate buffer and 0.03 M NaCl. 60 transfers were completed at 5 °C. For details see text.

current plates (see Materials and Methods) in one of the two phase systems described above. The superimposed distribution curves obtained after 60 transfers are depicted in Fig. 1. It appears that in both phase systems the ghost population is displaced to the right of the erythrocytes from which it is derived. In addition, the ghosts in the phase system containing both phosphate and NaCl show two distinct components, one more similar in partition behavior to the original red cells and one further displaced. The sequence of partition coefficients shown in Table I thus agree with the relative positions on countercurrent distribution of ghosts and red cells. The numerical values from single tube partition cannot, however, be used directly in estimating the apparent partition coefficient in a countercurrent distribution experiment for reasons previously discussed [3, 19].

The basis for the difference in partition deportment of red cells and their ghosts is not known but a number of possibilities to account for such a difference can be mentioned. Erythrocytes which have been stored over a period of 3 or more weeks have an increased partition coefficient as compared to their fresh counterparts [20]. This increase in partition coefficient has been traced to the strong adsorption of phase polymer to the stored cells' membrane although the membrane alteration causing this adsorption remains obscure [18]. Ghosts may analogously be metabolically depleted and have membrane surface properties similar to those of the stored red cell [21]. Another possibility lies in the analogy to acetaldehyde-fixed, lipid-extracted red cells. Acetaldehyde-fixed erythrocytes have partition coefficients identical with those of the red cells from which they are derived [16]. Lipid extraction, however, greatly increases the partition coefficient of the fixed cells [16]. Lipid loss, or possibly just "rearrangement", during erythrocyte ghost preparation may give rise to the observed increase in partition coefficient.

Since the original publication describing the preparation of hemoglobin-free red cell ghosts a number of papers, notably by Hanahan et al. [22, 23]. have appeared which show and discuss changes in and variability of erythrocyte membranes as a function of their preparation as measured by different biochemical parameters. The current experiments serve to emphasize that the membrane surface of the ghosts [13] and that of the red cells from which they are derived are different and that a physical separatory method can be applied to point up the difference, and more important, probe membrane sub-heterogeneities not possible by applying biochemical tests to the entire ghost population. Aspects of this will become more apparent in the presentation of the sub-fractionation of rightside-out and inside-out membrane vesicles below.

Countercurrent distribution of rightside-out and inside-out vesicles from human erythrocyte membranes

As an extension of the experiments in the previous section with human erythrocyte ghosts, we prepared and examined by countercurrent distribution rightside-out and inside-out vesicles from human erythrocyte membranes [14]. Not only were major differences found between rightside-out and inside-out vesicles but heterogeneities within each of the vesicle preparations were uncovered as well.

(A) *Countercurrent distribution in a high salt phase system.* Rightside-out and inside-out vesicles from human erythrocyte membranes were prepared by the method of Steck [14]. These vesicles as well as some of the erythrocytes from which they were prepared were subjected to countercurrent distribution in a phase system con-

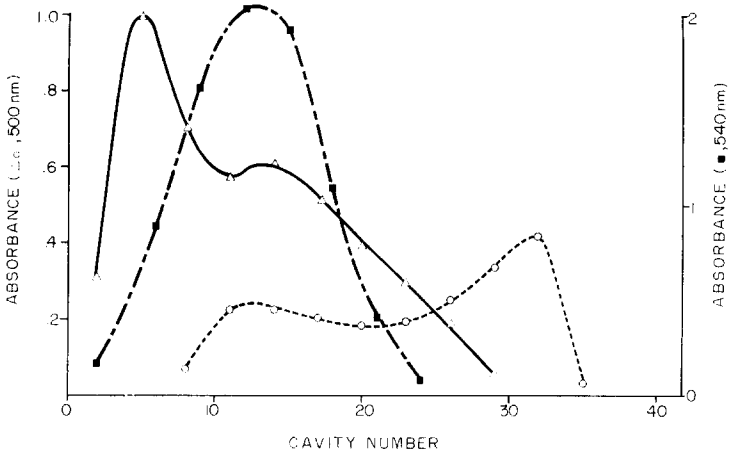

Fig. 2. Superimposed countercurrent distributions of human erythrocytes, rightside-out membrane vesicles and inside-out membrane vesicles. ■, red cell distribution (in terms of hemoglobin absorbance); ○, rightside-out and △, inside-out vesicle distributions (in terms of absorbance of vesicle suspensions). Phase system contained 5 % (w/w) dextran, 4 % (w/w) polyethylene glycol, 0.09 M sodium phosphate buffer, pH 6.8, and 0.02 M NaCl. 40 transfers were completed at 5 °C. For details see text.

taining 5 % dextran, 4 % polyethylene glycol, 0.09 M sodium phosphate buffer, pH 6.8, and 0.02 M NaCl, a phase system that is essentially isotonic for red cells and has previously been used in the study and sub-fractionation of mammalian blood cells [3, 19]. This phase system, furthermore, has an electrostatic potential difference between the top and bottom phases [11] and membrane surface charge-associated properties are a major determinant of the partition coefficient of cells or membranes added to the system [3, 18].

Fig. 2 shows the results of a countercurrent distribution experiment with vesicles and red cells. The inside-out vesicles are to the left, the rightside-out vesicles to the right and the red cells somewhere inbetween. Both rightside-out and inside-out vesicles are clearly heterogeneous as evidenced by the double peaks. After countercurrent distribution the tubes representing the left, middle and right thirds of either rightside-out or inside-out distributions were pooled and assayed for acetylcholinesterase activity in the absence and in the presence of Triton X-100. This enzyme is a marker for the membrane's outer surface [14]; activities without Triton give enzyme exposed on the vesicle surface while activities measured in the presence of Triton give total vesicle enzyme activities. All fractions obtained from the rightside-out distribution showed 100 % accessibility for this enzyme (i.e. activities were the same with or without Triton). Inside-out vesicles showed an increase of accessibility for this enzyme from left to right (34, 61 and 87 %) indicating that there were no "pure" inside-out vesicles even on the far left and that the quantity of contaminating rightside-out vesicles increased from left to right through the distribution curve. Glyceraldehyde-3-phosphate dehydrogenase activity, a marker for the membrane's cytoplasmic side [14], was not assayed in this experiment since this enzyme is washed away at the higher salt concentrations [14] used in this phase system.

The displacement of the rightside-out vesicles to the right of the inside-out vesicles is in line with the higher electronegativity of the membrane's outer surface (i.e. sialic acid residues are present only on the membrane's outer surface [14]) which fact has also led to an electrophoretic separation [24] of such vesicles. The additional displacement of the rightside-out vesicles to the right of the red cells from which they are derived (Fig. 2) is analogous to the displacement shown in Fig. 1 for erythrocyte ghosts.

(B) *Countercurrent distribution in a low salt phase system.* In order to subject the rightside-out and inside-out vesicles to as little change in ionic strength as feasible after preparation by Steck's method [14] as well as to keep the salt concentration during countercurrent distribution low so that glyceraldehyde-3-phosphate dehydrogenase could be assayed at the conclusion of a vesicle separation, we carried out experiments similar to those already discussed in a phase system of low salt concentration. This phase system contained 5 % dextran, 3.8 % polyethylene glycol, 0.001 M sodium phosphate, pH 8.0, 0.0015 M NaCl and 0.0001 M $MgSO_4$, and had an electrostatic potential difference between the phases. Since erythrocytes would lyse in such a low salt system we used acetaldehyde-fixed human erythrocytes as an indicator for the relative partitions of rightside-out, inside-out vesicles and red cells. Acetaldehyde-fixed erythrocytes have previously been shown to have partitions identical to fresh human red cells in isotonic phase [16].

Fig. 3 depicts a countercurrent distribution of sealed rightside-out, inside-out vesicles and acetaldehyde-fixed red cells. The rightside-out and inside-out vesicles are once again shown to be highly heterogeneous, with the rightside-out being to the right and the inside-out to the left. The acetaldehyde-fixed cells are inbetween.

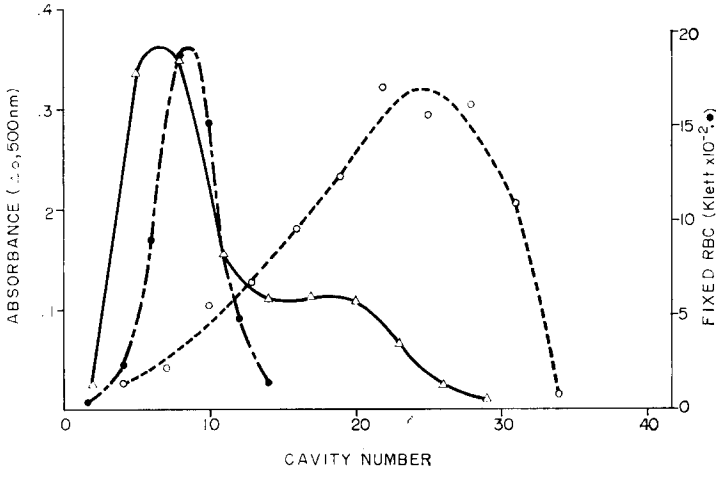

Fig. 3. Superimposed countercurrent distributions of acetaldehyde-fixed human erythrocytes, rightside-out membrane vesicles and inside-out membrane vesicles. ●, fixed red cells (in terms of Klett units); ○, rightside-out and △, inside-out vesicle distributions (in terms of absorbance of vesicle suspensions). Phase system contained 5 % (w/w) dextran, 3.8 % (w/w) polyethylene glycol, 0.001 M sodium phosphate, pH 8. 0.0015 M NaCl and 0.0001 M $MgSO_4$. 40 transfers were completed at 5 °C. For details see text.

Once again vesicles in tubes under the rightside-out and inside-out vesicle distribution curves were pooled into left, middle and right thirds. The rightside-out vesicles obtained from all parts of the distribution gave 100 % accessibility to acetylcholinesterase and 0 % to glyceraldehyde-3-phosphate dehydrogenase (i.e. they were "pure" rightside-out vesicles according to these marker enzymes). The inside-out vesicle preparation gave an increasing accessibility to acetylcholinesterase from left to right through the distribution (18, 46 and 84 %) and a decreasing accessibility to glyceraldehyde-3-phosphate dehydrogenase (58, 20 and 0 %). Thus the heterogeneity pointed up for the rightside-out preparation (i.e. it is much broader than a theoretical distribution curve) by countercurrent distribution is not detectable by marker enzyme assay. The heterogeneity of the inside-out preparation (i.e. the double peak) is due in part to the contamination of the inside-out preparation with rightside-out vesicles. There is, however, an additional sub-heterogeneity not so clearly evident by inspection of the distribution curve: even at the far left of the relatively symmetrical left-hand distribution curve of the inside-out vesicles (Fig. 3) only about 60 % of the vesicles are inside-out. One should add that even when inside-out vesicle preparations are subjected to 120 rather than just to 40 transfers during a countercurrent distribution experiment, no "pure" inside-out vesicles as judged by marker enzymes are obtained. One may conjecture, therefore, that some of the surface properties measured by partition of rightside-out and inside-out vesicles are similar and overlap on countercurrent distribution.

(C) *Countercurrent distribution of "leaky" rightside-out and inside-out vesicles.* One of our preparations of vesicles (presumably rightside-out and inside-out) which did not reseal (vesicles were at the bottom of the dextran gradient (ref. 14, see also Materials and Methods)) was subjected to countercurrent distribution. It was found (Fig. 4) that resealing is not essential for the separation of rightside-out and inside-out vesicles by partition. As before with sealed preparations, the rightside-out vesicles had a higher partition coefficient than the inside-out vesicles. Enzyme markers, in the case

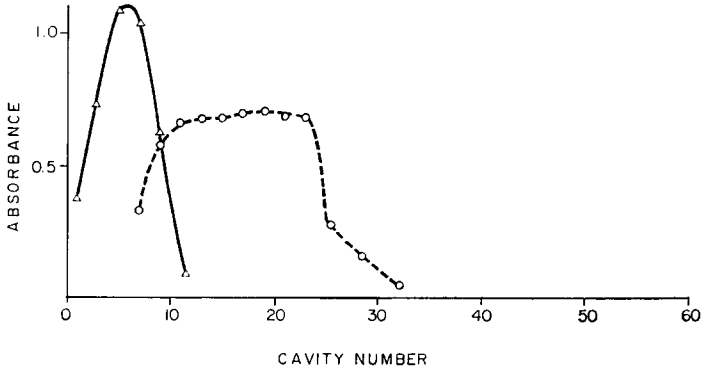

Fig. 4. Superimposed countercurrent distributions of "leaky" vesicles. ○, vesicles subjected to procedure making rightside-out vesicles and taken from bottom of dextran gradient [14]; △, vesicles subjected to procedure making inside-out vesicles and taken from bottom of dextran gradient (i.e. leaky). Phase system contained 5 % (w/w) dextran, 4 % (w/w) polyethylene glycol, 0.001 M sodium phosphate, pH 8, 0.003 M NaCl and 0.0001 M $MgSO_4$. 60 transfers were completed at 5 °C.

of "leaky" vesicles, cannot establish sidedness since marker enzymes located on both membranes surfaces are fully accessible.

Notes on variability of vesicle preparations and on the relative partition coefficients of rightside-out, inside-out vesicles and red cells

The mechanism underlying the preparation of sealed rightside-out and inside-out vesicles is far from well-defined[14]. In following Steck's procedures we were able to obtain vesicles of desired orientation most of the time (as evidenced by their partition behavior, see previous section), but obtained sealed vesicles in only about one-third of the runs. Although time consuming that is not otherwise serious since by dextran gradient and marker enzyme analyses one can readily determine whether suitable preparations have been obtained and, if so, proceed with subsequent countercurrent distribution experiments.

Countercurrent distribution of rightside-out and inside-out vesicles reveals, as indicated earlier, that rightside-out vesicles always have a higher partition coefficient than do inside-out vesicles. However, while the position of inside-out vesicles after countercurrent distribution is fairly constant that of rightside-out vesicles varies. This is readily apparent by comparing the distribution curves of (normal) rightside-out vesicles in Figs. 3 and 5. Extent of displacement of the rightside-out vesicles to the right of the red cells also varies therefore from major to minor. Rightside-out vesicles are sometimes found to give very broad distribution curves (and are hence clearly heterogeneous) and at other times to split into two peaks outright. The basis for these differences is not known but is probably related to slight, unintended variations in their preparation.

While the difference in partition behavior between rightside-out and inside-out vesicles is clear and obtained in every experiment, the comparative aspects between rightside-out and inside-out vesicles versus red cells require a few works. It is feasible to load equal quantities (i.e. estimated by absorbance) of rightside-out and inside-out vesicles for countercurrent distribution. The quantity of red cells loaded will, however, not be the same. In addition the size of vesicles and cells is different and could play a role in the relative partition coefficients observed. Relative size of cells and vesicles is probably not overly significant, however, in the currently reported experiments since the cells are found between the rightside-out and inside-out vesicle preparations. Previous experiments have indicated that membrane surface characteristics, at least with cells, outweigh size as a major determinant of partition coefficients [3].

Basis for separation and sub-fractionation of rightside-out, inside-out vesicles and erythrocytes (fresh or fixed) by countercurrent distribution

The dextran-polyethylene glycol two-phase systems containing phosphate used in the present experiments all have electrostatic potential differences between the phases. This phase charge interacts with the membrane charge of cells, particles or membranes added to the phase system and the separation of the latter is therefore greatly determined by surface charge [3, 18]. The charge measured by partition is not necessarily the same as that which determines electrophoretic mobility since the latter depends on the charge at the plane of shear while the former appears to gauge additional charge deeper into the membrane [12]. One may conjecture that charge is a principal determinant in the separation of inside-out and rightside-out vesicles.

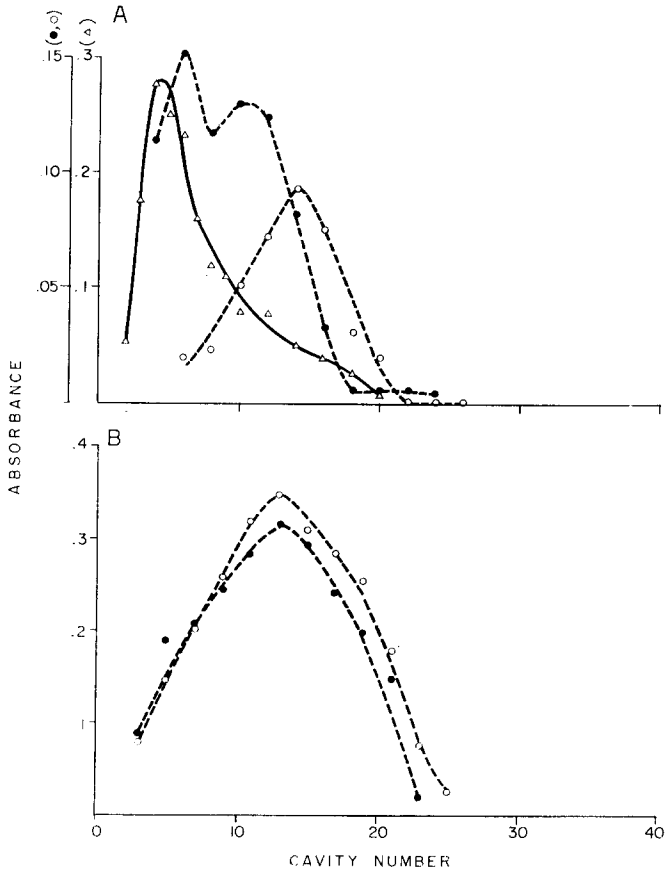

Fig. 5. Superimposed countercurrent distributions of membrane vesicles prepared from normal and from neuraminidase-treated erythrocytes. (A) Sealed vesicles: △, inside-out and ○, rightside-out vesicles from normal erythrocyte; ●, rightside-out vesicles from neuraminidase-treated red cells. (The rightside-out vesicle preparation from neuraminidase-treated red cells contained only 75 % rightside-out vesicles). (B) Leaky vesicles: ○, vesicles prepared by rightside-out vesicle procedure of Steck [14] from normal erythrocytes; ● similarly prepared vesicles from neuraminidase-treated erythrocytes. Phase system contained 5 % (w/w) dextran, 3.8 % (w/w) polyethylene glycol, 0.001 M sodium phosphate, pH 8, 0.0015 M NaCl and 0.0001 M $MgSO_4$. 40 transfers were completed as 5 °C. For details see text.

Rightside-out vesicles have sialic acid residues (the main determinant of red cell membrane charge) on their outer surface while inside-out vesicles do not [14]. This difference appears to be the basis for the electrophoretic separability of inside-out and rightside-out vesicles [24]. The facts that rightside-out vesicles (also inside-out vesicles) are heterogeneous as shown by countercurrent distribution and that the rightside-out vesicles (as well as erythrocyte ghosts) are displaced to the right of the red cells from which they are derived must reflect alterations in these membranes during their preparation.

The question as to whether such alterations are themselves related to membrane charge-associated and/or other surface properties is difficult to assess. We have recently found that, under certain conditions, the lipid composition of the erythrocyte

membrane influences the red cell's partition coefficient [5]. This involvement of membrane lipid-phase polymer interaction is most apparent in phases near the critical point without electrostatic potential difference between the phases (i.e. in uncharged phase systems such as those containing predominantly NaCl). Under some circumstances (e.g. with very high ratios of membrane poly/mono-unsaturated fatty acids) lipid may also play a role in partition in phases away from the critical point [5].

The displacement of the rightside-out vesicles (and also of the ghosts) to the right of the red cells on countercurrent distribution (Figs. 1–3) has an analogy, as indicated earlier, in the displacement of metabolically depleted, stored erythrocytes as compared to fresh red cells [18, 20]. In both cases membrane alterations may be involved which are related to metabolically regulated phosphorylation of components of the spectrin complex perhaps mediating some actomyosin-like interaction [21]. In any case the changed membrane of the stored red cell appears to bind phase polymer to its surface [18] which results not only in an increased partition coefficient but also in an elevated electrophoretic mobility [18, 25].

The heterogeneity and displacement of the rightside-out vesicles (and ghosts) may also be a consequence of the loss of membrane lipid during their preparation. Extraction of lipid from acetaldehyde-fixed red cells results in an appreciably higher partition coefficient for the cells [16]. This increase is probably primarily due to the exposure of additional charge to the phase sytem since no partition coefficient is in evidence in uncharged phase systems (i.e. no cells are in the top phase).

The partition behavior of neuraminidase-treated red cells and of the rightside-out vesicles prepared from such cells was examined. Sialic acid removal from red cells appreciably reduces both their electrophoretic mobility [26] and partition coefficient [15]. Fig. 5A shows a slight leftward shift of the rightside-out vesicles (75 % rightside-out preparation by enzyme marker analysis) from neuraminidase-treated red cells when compared to "normal" rightside-out vesicles. The bulk of the vesicles is distinct-

TABLE II

EFFECT OF DIFFERENT IN VITRO TREATMENTS ON THE PARTITION BEHAVIOR OF HUMAN ERYTHROCYTES

Partition is defined as the quantity of cells in the top phase (percent of total cells added). 5 : 4 (1) phase composition is 5 % (w/w) dextran, 4 % (w/w) polyethylene glycol, and 0.11 M sodium phosphate buffer, pH 6.8. Partition was at 21–24 °C.

Sample	Partition in 5 : 4 (1)
Acetaldehyde-fixed human red cells	54±17 (3)[a]
Neuraminidase-treated red cells, then acetaldehyde fixed	14± 0 (3)
Acetaldehyde-fixed human red cells, then ethanol extracted[b]	88± 4 (3)
Neuraminidase-treated red cells, acetaldehyde fixed then ethanol extracted[b]	71±10 (3)

[a] Same partition as obtained with fresh human red cells [16].

[b] Partition of acetaldehyde-fixed red cells at least 2 weeks after fixation [16]. Similarly, ethanol extraction was done on cells that had beeh fixed with acetaldehyde at least 2 weeks prior to extraction.

ly to the right of the inside-out vesicles (included in the figure for comparison). In other experiments (e.g. Fig. 5B) there is no discernible difference between the two preparations. From these experiments it might appear that the rightside-out membrane charge does not appreciably influence the vesicle partition coefficient. Again it is useful to discuss this result by analogy. Table II shows that when neuraminidase-treated red cells are fixed with acetaldehyde their partition coefficient (like that of fresh erythrocytes) is lower than that of the untreated fixed red cells. If such neuraminidase-treated, fixed cells are extracted with ethanol, their partition coefficient goes up greatly (see Table II). It goes up so much, in fact, that it becomes greater than the partition coefficient of the original fixed (or fresh) red cells and comparable to that of acetaldehyde-fixed, lipid-extracted red cells. It is evident that the partition behavior, and hence the surface properties, of neuraminidase-treated red cells and of the right-side-out vesicles prepared from them are quite different.

CONCLUSION

Countercurrent distribution patterns of rightside-out and inside-out vesicles reveal major differences between these vesicles and, in addition, heterogeneities of each of the examined vesicle preparations. Rightside-out vesicles always have a higher partition coefficient (i.e. are further to the right in the figures) than inside-out vesicles. However, the difference is not always equally great and there is some variability in the observed partition behavior of vesicle preparations obtained at different times. Rightside-out vesicle preparations are generally "pure" when judged by assay of enzyme markers (i.e. acetylcholinesterase for the outer surface and glyceraldehyde-3-phosphate dehydrogenase for the cytoplasmic side), but are highly heterogeneous as evidenced by the very broad distribution curves obtained by countercurrent distribution. Inside-out vesicle preparations obtained by us are never "pure" (again as judged by analysis of the enzyme markers). Even at the far left end of the distribution curve (i.e. where the vesicles of lowest partition coefficient are), both inside-out and right-side-out vesicles are found. Hence, some of the surface properties measured by partition on rightside-out and inside-out vesicles overlap. Analysis of the two above-indicated enzyme markers on vesicles obtained from different parts of the countercurrent extraction train following distribution of "inside-out" preparations shows an increase in the contaminating rightside-out vesicles from left to right through the distribution.

Rightside-out vesicles generally have distribution curves which only partly overlap with those of the red blood cells (either fresh or fixed) and the bulk of which is displaced to the right of the latter. These results are similar to those observed with erythrocyte ghost preparations. These experiments (as well as others with rightside-out vesicles obtained from neuraminidase-treated red cells, see sections above) show that the rightside-out vesicles, though "pure" by the criterion of selected enzyme markers, have surfaces measurably altered and rendered highly heterogeneous when compared to those of the erythrocytes from which obtained.

Since a phase system with an electrostatic potential between the phases is required, at the polymer concentrations used to effect a separation of rightside-out and inside-out vesicles, we conclude that in the current experiments charge is a predominant factor in the separations obtained. Vesicle surface interaction with phase

Biochimica et Biophhysica Acta, 455 (1976) 8–23

BBA 77485

PARTITION IN TWO-POLYMER AQUEOUS PHASES REFLECTS DIFFERENCES BETWEEN MEMBRANE SURFACE PROPERTIES OF ERYTHROCYTES, GHOSTS AND MEMBRANE VESICLES

HARRY WALTER and EUGENE J. KROB

Laboratory of Chemical Biology, Veterans Administration Hospital, Long Beach, Calif. 90822 *and the Department of Physiology, University of California, College of Medicine, Irvine, Calif.* 92717 *(U.S.A.)*

(Received April 7th, 1976)

SUMMARY

Partition of cells (defined as the percentage of added cells in the top phase) in dextran-polyethylene glycol aqueous two-phase systems is an extremely sensitive indicator of cell surface properties. By appropriate choice of polymer concentrations and salt composition and concentration one can, to some extent, select the membrane properties (e.g. charge (Walter, H. (1975) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. 9, pp. 25–50, Academic Press, New York), lipid composition (Walter, H, Krob, E.J. and Brooks, D.E. (1976) Biochemistry, 15, 2959–2964)) that determine the partition behavior of cells (or membranes). In the present experiments we have obtained by partition information on separability, alteration and ensuing heterogeneity during preparation of human erythrocytes, ghosts and rightside-out and inside-out vesicles from human erythrocyte membranes. A phase system in which partition is greatly (although not exclusively) determined by membrane charge was used. It was found that:

1. The partition coefficients (and hence the membrane surfaces) of ghosts and of rightside-out vesicles differ from those of the erythrocytes from which they are derived.

2. Rightside-out and inside-out vesicles have different countercurrent distribution patterns with the rightside-out vesicles having the higher partition coefficient (probably reflecting higher membrane charge of the latter).

3. Rightside-out vesicles are highly heterogeneous membrane populations as evidenced by broader than theoretical countercurrent distribution curves which, in some experiments, even split into two populations outright. This heterogeneity is particularly interesting since the enzyme markers used to establish vesicle sidedness (Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 2, pp. 245–281, Plenum Press, New York) indicate "pure" rightside-out preparations.

4. Inside-out vesicles could, under no conditions tested, be rendered totally free of rightside-out vesicles according to the enzyme markers used to indicate vesicle sidedness (Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 2, pp. 245–281, Plenum Press, New York).