HYDROPHOBIC AFFINITY PARTITION IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING POLY(ETHYLENE GLYCOL)-PALMITATE OF RIGHTSIDE-OUT AND INSIDE-OUT VESICLES FROM HUMAN ERYTHROCYTE MEMBRANES

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1. Introduction

When aqueous solutions of D* and of PEG are mixed above certain concentrations immiscible, liquid two-phase systems are obtained [1], with a PEG-rich top and a D-rich bottom phase. These can be buffered and are suitable for the partition, and separation by CCD, of cells, membrane and macromolecules [1–4]. Some salts (e.g., phosphate) distribute unevenly [5] giving rise thereby to an electrical and a zeta potential between the phases [6,7]. This phase charge interacts with membrane surface-charge associated properties of cells or membranes added to the phases [8,9]. Thus the partition of suspended materials is greatly (although not exclusively) dependent on charge. D-PEG aqueous phase systems can be modified and adapted to specific separation requirements by incorporation of D- or PEG-bound ligand. Derivatives that have been used include DEAE-dextran [10,11] and trimethylamino-PEG or PEG-sulfonate [12] to enhance separations based on charge; triton X-100 (a PEG derivative) to effect purification (by hydrophobic interaction) of a hydrophobic protein [13]; diamidino-α,ω-diphenylcarbamyl-PEG which, as a strong competitive inhibitor of trypsin, permits the specific extraction of trypsin [14]; PEG-palmitate used in the extraction of serum albumin from plasma [15]; α,ω-bis-4-trimethylammonium (phenylamino)-PEG to purify acetylcholine receptor-enriched membranes [16]; and DNP-PEG used in the affinity partition of S-23 meyloma protein [17].

In the work presented here we have examined the CCD patterns of sealed RO and IO human erythrocyte membrane vesicles in a D-PEG phase system containing NaCl and small quantities of P-PEG (which itself favors the PEG top phase). The partition of suspended biological materials, in this system, depends on the hydrophobic interaction of the exposed membrane surface with the palmitoyl residue.

RO vesicles were found to have a higher partition coefficient than IO vesicles reflecting difference in available hydrophobic residues on these two membrane surfaces. The data clearly indicate (a) the hydrophobic asymmetry of the human erythrocyte membrane and (b) the usefulness of hydrophobic affinity partition in the separation of membranes (and cells).

2. Materials and methods

2.1. *Materials*

Dextran T500, lot no. 5996, was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. PEG (trade name 'Darbowax')6000 was obtained from Union Carbide, New York. The synthesis of P-PEG has been described [15]; it had an average of one palmitoyl residue per PEG molecule.

* *Abbreviations:* D, dextran T 500; PEG, poly(ethylene glycol) 6000; P-PEG, poly(ethylene glycol)-palmitate; RO, rightside-out membrane vesicles; IO, inside-out membrane vesicles; CCD, countercurrent distribution.

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2.2. *Preparation of sealed IO and RO vesicles from human erythrocyte membranes*

RO and IO vesicles from human erythrocyte membranes were prepared by the method of Steck [18]. Sealed vesicles were obtained from the top of a dextran gradient and sidedness was established by measuring the acetylcholinesterase (EC 3.1.1.7) activity and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) activity. The former is a marker for the membrane outer surface while the latter is located on the membrane's cytoplasmic side [18]. Activities were measured in the absence and presence of Triton in order to determine enzyme accessibility on the vesicle preparations (activities without Triton give enzyme exposed on the vesicle surface while activities measured in the presence of triton give total vesicle enzyme activities). Enzyme activities were measured prior to the vesicles' exposure to solutions of higher salt concentration (i.e., 0.05 M NaCl, see below) since glyceraldehyde 3-phosphate dehydrogenase is desorbed from the membrane at high ionic strength [18]. The sealed RO preparations were generally 100% RO; while the IO preparations were between 75–85% IO.

2.3. *Preparation of phase system*

The phase system was prepared as previously described [2]. It contained 5% (w/w) D, 4% (w/w) PEG, 0.05 M NaCl, 0.001 M Na-phosphate, pH 8, and 0.0007% (w/w) P-PEG. The phase system was brought to 5°C, mixed and permitted to settle in a separatory funnel. Top (PEG-rich) and bottom (D-rich) phases were then separated.

2.4. *CCD of RO and IO membrane vesicles*

The vesicles obtained after gradient centrifugation (see above) were washed twice (in the cold) with 0.5 mM Na-phosphate (pH 8), after which aliquots for

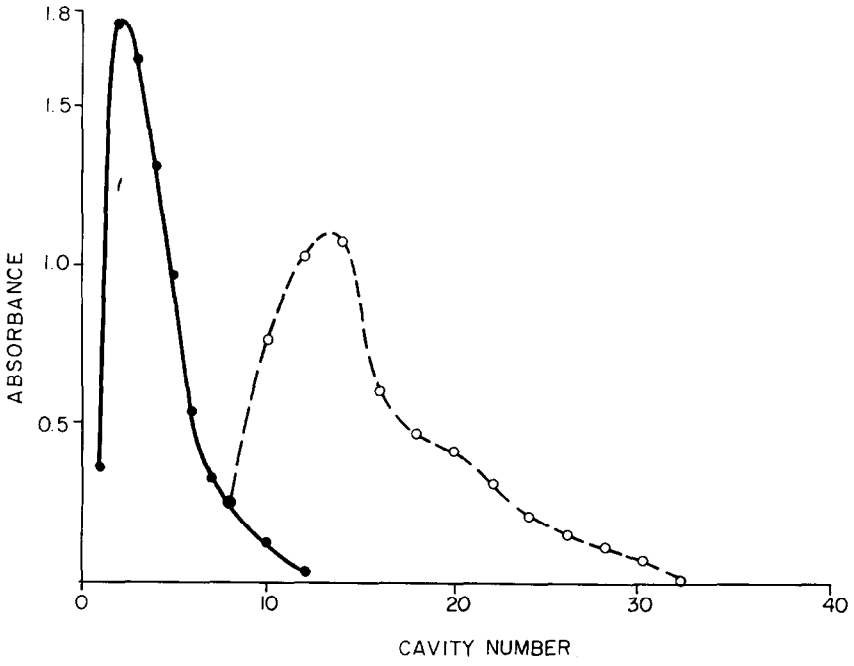

Fig.1. Superimposed countercurrent distribution patterns obtained with sealed rightside-out (○-○) and inside-out (●-●) vesicles from human erythrocyte membranes. Vesicles were prepared according to Steck [18] and tested for sidedness by measuring accessibility of marker enzymes. 40 transfers were completed at 5°C in a phase system containing 5% (w/w) PEG, 0.05 M NaCl, 0.001 M Na-phosphate buffer, pH 8, and 0.0007% (w/w) P-PEG. The partition of vesicles in this phase system depends on the hydrophobic interaction of the exposed membrane surface with the palmitoyl residue of P-PEG. For details see text.

enzyme determinations were withdrawn. The bulk of the vesicles were then washed further with a solution containing 0.05 M NaCl and 0.001 M Na-phosphate, pH 8, and finally with top phase of the two-phase system described above. 0.1 ml (or slightly less) of RO or IO membrane vesicles were suspended in 2 ml of top phase (load mix). A thin-layer CCD apparatus as described by Albertsson [19] and manufactured by Incentive Research and Development AB, Bromma, Sweden, was used in this work. The circular plexiglass plates have 120 concentric cavities, each with a bottom phase capacity of 0.7 ml. Cavities 0, 1 and 60, 61 each received 0.5 ml of bottom phase and 0.9 ml of either the RO or IO load mix. All other cavities received 0.6 ml bottom phase and 0.8 ml top phase. The unit was loaded in this manner so that a stationary interface would result (see [1] for full discussion). 40 transfers were completed using a settling time of 6 min, shaking time of 25 sec. The entire procedure was carried out at 5°C.

2.5. *Collection and analysis of membrane vesicles*

After completion of the countercurrent run, the samples were collected directly into plastic tubes. By adding saline to each tube, the two-phase system was converted to a single phase and the concentration of the membrane vesicles in selected tubes along the extraction train was obtained by measuring the absorbance at 500 nm.

3. Results and discussion

The D-PEG aqueous phase system selected for the hydrophobic affinity partition of RO and IO vesicles from human erythrocyte membranes contains NaCl. Sodium chloride partitions almost equally between the D and PEG phases [5] and this system therefore has virtually no electrical potential between the phases (see Introduction). If human red blood cells or membrane vesicles are added to such a system they collect at the interface. Incorporation of very small quantities of P-PEG (e.g., at a final concentration of 0.001% (w/w) or even less) is adequate to effect usable partition coefficients for most mammalian red cells. The PEG backbone of P-PEG assures that the P-PEG will be in the top (or PEG-rich) phase and thus 'pull' membranes (or cells) 'up' in accordance with the degree of hydrophobic interaction between the exposed membrane surface and the palmytoyl residue. That membrane charge is not involved in determining the partition of cells in this phase system (containing P-PEG and NaCl) is also shown by the fact that complete removal of human red cell sialic acid by neuraminidase treatment does not diminish the partition coefficient of erythrocytes [20].

It is evident from fig.1 that sealed RO and IO vesicles have different distributions with the RO vesicles having the higher partition coefficient (i.e., being further to the right in fig.1). One can therefore conclude (a) that separability of membranes (and cells) is feasible on the basis of difference in their hydrophobic surface and (b) that RO and IO vesicles have different exposed hydrophobic surfaces, indicative of membrane asymmetry (see review by Singer [21]). At present we do not know whether the separation in the present case is due to a difference in hydrophobic interaction between the palmitoyl residue of P-PEG and the RO or IO 'general' exposed surface, whether a more specific kind of palmitate receptor [22] on the RO vesicle surface is involved, or whether both of these interactions pertain.

CCD of erythrocyte membranes in phase systems containing D-PEG and phosphate have also permitted a separation of RO and IO vesicles [23]. In this latter case (as in the case of membrane electrophoresis [24]) the separation seems due to the membrane charge (i.e., sialic acid residues) present only on the membrane outer surface.

It appears from the data of others (see Introduction) as well as the current results that polymer-ligand in aqueous two-phase systems is a highly useful addition to the arsenal of affinity separatory procedures, especially as applied to cells, biological particles and membranes.

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