Membrane Surface Properties Other Than Charge Involved in Cell Separation by Partition in Polymer, Aqueous Two-Phase Systems†

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ABSTRACT: When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above certain concentrations, immiscible, liquid two-phase systems are obtained which are useful for separating cells by partition. Some salts partition unequally between the phases, giving rise to an electrostatic potential difference between them. Partition of cells has therefore been thought to depend predominantly on membrane charge. We now report two instances in which membrane charge either does not determine or is not the main determinant of cell partition. (A) Cell partition coefficients in phase systems approaching the critical point (the component concentrations below which a homogeneous solution occurs) increase, even in phase systems in which the phase potential difference is practically zero. Furthermore, in such systems, the partition coefficient of (human) erythrocytes is not reduced by complete removal of sialic acid. (B) Rat and mouse erythrocytes have sizable partition coefficients in a phase system away from the critical point with no potential difference between the phases. Cell surface interaction with the polymers is probably responsible for cell partition in these cases. Partition studies on erythrocytes from nine mammalian species in phases near the critical point with and without electrostatic potential differences reveal major species-specific differences in the membrane charge/noncharge components. A correlation has been found, in phases near the critical point that have essentially no electrostatic potential difference, between partition coefficient and the ratio of poly/monounsaturated fatty acids in the membranes of red cells from different species. Our present results thus provide parameters for the separation of cells by partition in addition to or instead of membrane charge depending on the polymer and salt composition and concentration selected.

When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above critical concentrations, immiscible, liquid two-phase systems are obtained with a poly(ethylene glycol)-rich top and a dextran-rich bottom (Albertsson, 1971). Such phase systems can be buffered and made isotonic and are highly useful in separating cells by partition and in gaining certain information relating to the surface properties of the cells examined (Walter, 1975).

Some salts (notably phosphate) partition unequally between the phases (Johansson, 1970), even though both dextran and poly(ethylene glycol) are nonionic polymers, giving rise to an electrostatic potential difference between the phases (Reitherman et al., 1973). Thus, the separation and subfractionation of various cell populations by partition are often determined by the interaction of membrane surface charge with the potentials of the phases used (Walter et al., 1967, 1970, 1972, 1973; Brooks et al., 1971).

In the present paper we report two instances, one of general applicability and one a more specific illustration, in which membrane charge either does not determine or is not the main determinant of cell partition.

Experimental Methods

Chemicals. Dextran T500 (lot No. 5996) was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Poly(ethylene glycol), trade name "Carbowax 6000", was the product of Union Carbide, New York, N.Y. Salts were of analytical reagent grade. Neuraminidase (from *Vibrio cholerae*) was purchased from Behringwerke, Marburg Lahn.

Erythrocytes. Fresh blood was collected in acid-citrate-dextrose (ACD)[1] and used in the partition or countercurrent distribution experiments to be described within 24 h. Beef, horse, lamb, and pig blood was obtained from local meat packing houses; rat and mouse blood was obtained by heart puncture; rabbit blood was obtained from the ear marginal vein; and human and dog blood by venipuncture.

In Vivo Labeling of Rat Erythrocytes of Different Ages. Some rats were injected with 8–10 μCi of ^{59}Fe ferrous citrate via the saphenous vein. These rats were bled by heart puncture *either* 18 h *or* 3 days following isotope injection, yielding red blood cell populations in which the youngest reticulocytes or youngest mature erythrocytes, respectively, were isotopically labeled (Walter et al., 1971).

Neuraminidase Treatment of Human Erythrocytes. In some experiments human red blood cells were treated with neuraminidase to effect removal of the main surface charge component of the red cells (sialic acid) as previously described (Walter and Coyle, 1968), except that the incubation time used was 1 h.

Phase Systems. Two-polymer aqueous phase systems having different properties were prepared as described earlier (Walter, 1975). Essentially isotonic phase systems with the following compositions (abbreviations in parentheses) were used: (a) 5% (w/w) dextran–4% (w/w) poly(ethylene glycol) containing 0.11 M sodium phosphate buffer, pH 6.8 (5:4 # 1); (b) same polymer concentrations but 0.09 M sodium phosphate buffer

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[1] Abbreviations used: ACD, acid-citrate-dextrose; Hb, hemoglobin; Hb abs, hemoglobin absorbance.

and 0.03 M NaCl (5:4#2); (c) same polymer concentrations but 0.06 M sodium phosphate buffer and 0.075 M NaCl (5:4#3); (d) same polymer concentrations but 0.03 M sodium phosphate buffer and 0.12 M NaCl (5:4#4); (e) same polymer concentrations but 0.01 M sodium phosphate buffer and 0.15 M NaCl (5:4#5); (f) 5% (w/w) dextran–3.5% (w/w) poly(ethylene glycol), containing 0.11 M sodium phosphate buffer (5:3.5#1); (g) same polymer concentrations as in f, but 0.01 M sodium phosphate buffer and 0.15 M NaCl (5:3.5#5); and (h) 7% (w/w) dextran–4.4% (w/w) poly(ethylene glycol) containing 0.11 M sodium phosphate buffer (7:4.4#1).

Potential of Top Phase Relative to Bottom Phase. Bulk phase potential differences were measured essentially as described by Reitherman et al. (1973), except that a high impedance millivoltmeter (Vibran 33B Electrometer, R in $>10^{15}\Omega$) or Systron Donner Digital Multimeter (Model 7205, R in $>10^{10}\Omega$) was used. Each value reported is the mean of at least five determinations.

Partition of Erythrocytes. Five milliliters of bottom phase (dextran-rich solution) and 5 ml of top phase [poly(ethylene glycol)-rich solution] of a given phase system were pipetted into a series of test tubes. Each tube then received 0.1 ml of washed, packed red blood cells from a given species. The test tubes were inverted for mixing ten times and the phases were then permitted to settle. In the case of the 5% dextran–4% poly(ethylene glycol) phase systems, the settling time chosen was 20 min by the clock with the tubes in vertical position. For the 5% dextran–3.5% poly(ethylene glycol) phase systems, the settling time was 7 min by the clock with the tubes capped and in horizontal position (to speed settling by reducing the height of the phase column). At the end of the settling time (the horizontal tubes were gently brought to the vertical position without agitation), a 1-ml aliquot was carefully pipetted from the top phase of each tube and placed into a centrifuge tube. Nine milliliters of water was added to these tubes to lyse the cells and the stromal residue was subsequently removed by high-speed centrifugation. The absorbance of each lysate was determined on a Gilford spectrophotometer (at 410 or 540 nm). The Hb abs in the entire top phase was calculated by taking into account the fraction of top phase taken and the dilution. The total quantity of cells added to each phase system (in terms of Hb abs) was obtained independently by analysis of 0.1 ml of packed cells pipetted into 9.9 ml of Drabkin's solution. In each case the partition coefficient was calculated as described below. The entire procedure was carried out at 22–24 °C.

Countercurrent Distribution of ^{59}Fe-Labeled Rat Erythrocytes. A thin-layer countercurrent distribution apparatus with 120 cavities as described by Albertsson (1970) was used. In a typical experiment, 0.55 ml of washed, packed red blood cells were suspended in 4.85 ml of top phase of phase system 5:4#5 (see above). Cavities 0 to 4 received 0.5 ml of bottom phase; cavities 5 through 119 received 0.6 ml of bottom phase. Cavities 0 to 4 received 0.9 ml of the above indicated red cell suspension in top phase; cavities 5 to 119 received 0.8 ml of top phase. Since the bottom phase capacity of our countercurrent unit is 0.7 ml, loading in the indicated manner results in 0.1 ml of stationary top phase (Albertsson and Baird, 1962). Sixty transfers were then completed in the cold room (4–5 °C) using a shaking time of 25 s and a settling time of 6 min. For a detailed discussion of countercurrent distribution procedures with cells, see Walter (1975).

Analysis of Cells after Countercurrent Distribution. Cells were collected after countercurrent distribution directly into plastic centrifuge tubes. Isotonic aqueous salt solution (0.5 ml) was added to each tube to break the phase system. In some cases a few adjacent tubes were pooled. The tubes were centrifuged at low speed, the supernatant solution was discarded, and the cells were lysed by adding a known volume (3 ml) of Drabkin's solution. The tubes were then centrifuged at high speed to remove stromal material and the absorbance and ^{59}Fe radioactivity were determined as previously described (Walter et al., 1971).

Presentation of Data. Countercurrent distribution curves are presented in terms of hemoglobin absorbance (Hb abs) (at 540 nm) in the different cavities of the extraction train; the isotope distribution is in terms of counts per minute. A relative specific activity is also plotted. This is defined as:

$$\frac{\text{cpm/Hb abs in a given cavity}}{\text{cpm/Hb abs in the original cell population}}$$

Partition of cells in single tube experiments is given as the quantity of cells in the top phase (percent of total cells added). The electrostatic potential of top phase relative to bottom phase is given in millivolts.

Results and Discussion

Short Summary of Some Properties of the Phase Systems. In order to present the results of the current experiments in a comprehensible manner, a few of the properties of the phases must first be recounted. When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above critical concentrations, two immiscible phases result. When buffered with phosphate, a measurable electrostatic potential difference between the phases results (Reitherman et al., 1973), because of the unequal partition of the phosphate between them (Johansson, 1970). The partition coefficient of the phosphate depends, among other things, on the concentration of the polymers constituting the phase system (Brooks et al., in preparation). Thus, near the critical point, the phosphate will distribute more equally between the phases than in phases further removed from the critical point. The measurable, electrostatic potential difference between the phases therefore increases with increasing polymer concentration (Table I).

The partition of cells [e.g., red blood cells (Walter et al., 1967; Brooks et al., 1971) or liver cells (Walter et al., 1973)] in a phase system containing phosphate depends to a great extent on their membrane surface charge. If sodium chloride is substituted for the phosphate in the phase system, there is no potential difference between the phases (Reitherman et al., 1973) since this salt partitions almost equally (Johansson, 1970), and cells from most sources collect at the interface in such a system. Cells that do partition in such a phase system must partition due to surface properties not related to their membrane charge and some examples of this phenomenon will be given below.

It has long been known (Albertsson and Baird, 1962) but not further explored that the partition coefficient of cells increases when (in the presence of constant salt composition and concentration) the polymer concentration is reduced. Since we now know that the electrostatic potential difference between the phases diminishes with reduction of polymer concentration (Table I), the species-specific increase in partition coefficients of cells must be due to membrane surface properties other than charge. Data indicating that this is so and that the properties measured are useful in separating cells and in gaining information on membrane surface will be presented.

Specific Examples of Cell Partition Not Due to Membrane Charge. If red blood cells from a number of species are partitioned in phase systems containing 5% dextran–4% poly(eth-

TABLE I: Electrostatic Potential Difference between Top and Bottom Phases (Top Phase Positive).

Phase System[a]	Potential Difference (mV)
5:3.5#1	+0.68 ± 0.09
5:4#1	+1.13 ± 0.26
7:4.4#1	+2.50 ± 0.14
5:3.5#5	+0.04 ± 0.01
5:4#5	+0.04 ± 0.06

[a] Phase compositions were as follows: (5:3.5#1) 5% dextran, 3.5% poly(ethylene glycol), and 0.11 M sodium phosphate buffer, pH 6.8; (5:4#1) 5% dextran, 4% poly(ethylene glycol), and the same salt concentration and composition as above; (7:4.4#1) 7% dextran, 4.4% poly(ethylene glycol), and the same salt concentration and composition as above; (5:3.5#5) and (5:4#5) contained 5% dextran, 3.5% *or* 4% poly(ethylene glycol), respectively, and 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 6.8.

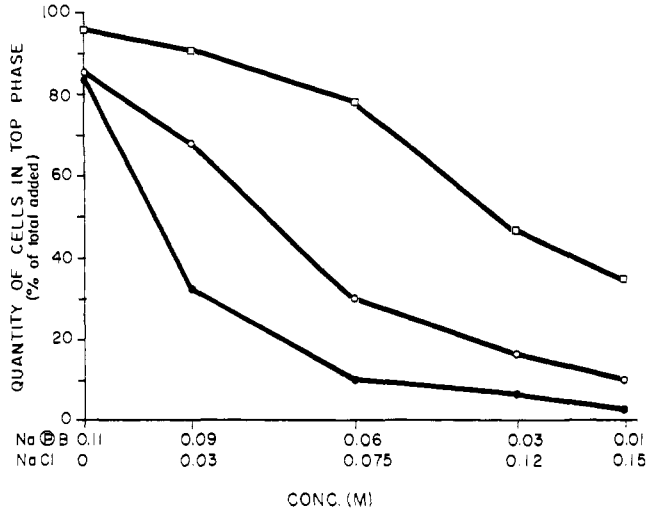

FIGURE 1: The quantity of mouse (□), rat (○), and dog (●) erythrocytes found in the top phase of a system containing 5% (w/w) dextran and 4% (w/w) poly(ethylene glycol) at different sodium chloride and sodium phosphate buffer concentrations. The phase system containing phosphate has the highest electrostatic potential difference, and the phase system containing sodium chloride has essentially no potential difference between the phases. For discussion, see text.

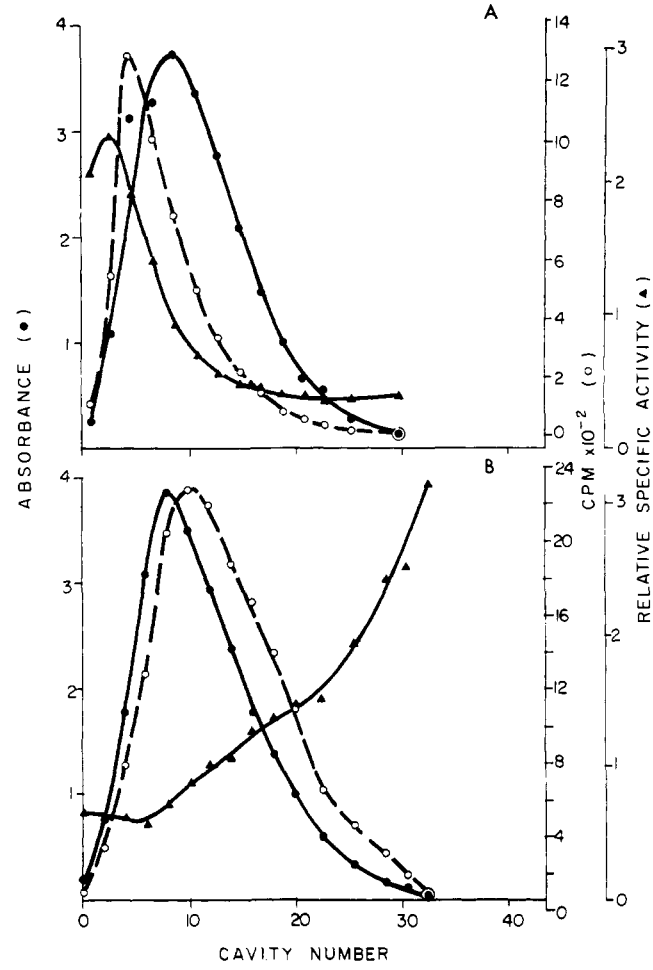

FIGURE 2: Countercurrent distribution of erythrocytes from rats injected with ^{59}Fe either 18 h (A) or 3 days (B) before bleeding. The cells labeled in A are the youngest reticulocytes, while those labeled in B are the youngest mature erythrocytes. Distribution of cells is given in terms of hemoglobin absorbance (●); distribution of radioactivity in terms of cpm $\times 10^{-2}$ (○); and a relative specific activity (▲) is also shown in which the activity of the original unfractionated cell population is 1.0 by definition. Phase system contained 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 0.15 M NaCl, and 0.01 M sodium phosphate buffer, pH 6.8. Sixty transfers were completed; run was at 4–5 °C. For other details, see text.

ylene glycol) made isotonic with phosphate buffer or with salt compositions of diminishing concentrations of phosphate and increasing concentrations of sodium chloride, one finds that the highest partition coefficient for cells is in the phase system with phosphate alone and the lowest is in the one with sodium chloride. This follows, as indicated earlier, from the fact that phosphate partitions unequally between the phases while sodium chloride does not. Thus, the phosphate system has the highest electrostatic potential difference between top and bottom phase of any in this series [i.e., of the order of 2 mV (Reitherman et al., 1973)], while the sodium chloride system has the lowest potential difference. Albertsson and Baird (1962) who measured the partition coefficients of human, dog, sheep, and rabbit red blood cells reported that the partition coefficient (i.e., the quantity of cells in the top phase, percent of total cells added) for all of these cells was zero in the sodium chloride system. We have now found, however, that mouse and rat red blood cells have a significant partition coefficient in a 5:4#5 (NaCl) phase system (Figure 1). Furthermore, the decrease in the partition coefficient (Figure 1) as one progressively replaces phosphate with sodium chloride is far less for mouse (and also rat) red blood cells than it is for dog red blood cells (which are representative of the red cells of the other mammalian species studied so far). It appears therefore that (a) as phosphate is replaced with sodium chloride in the phases something other than the membrane charge of the mouse and rat erythrocyte must affect their partition behavior and (b) that the partition coefficient in the NaCl system must depend on properties other than membrane surface charge since this phase system provides no electrostatic potential difference with which membrane charge could interact.

As an illustration of usefulness of phase systems which separate cells on the basis of membrane properties other than charge, we have subjected red blood cells from rats injected with [^{59}Fe]ferrous citrate either 18 h or 3 days before bleeding to countercurrent distribution. It was found that the youngest reticulocytes were to the left, while the young mature erythrocytes were to the right of the distribution curve of the whole red cell population (Figure 2). Separation of cells of different

TABLE II: Effect of Removal of Membrane Surface Charge Components (Sialic Acid) from Human Erythrocytes on the Cells' Partition[a] in Different Aqueous Phase Systems.

Phase System[b]	Partition	
	Before Neuraminidase Treatment	After Neuraminidase Treatment[c]
5:4#1	54 ± 7 (6)	8 ± 2 (6)
5:3.5#5	59 ± 6 (6)	92 ± 4 (6)

[a] Partition is given as the quantity of cells in the top phase (percent of total cells added). [b] Phase compositions were as follows: (5:4#1) 5% dextran, 4% poly(ethylene glycol), and 0.11 M sodium phosphate buffer, pH 6.8; (5:3.5#5) 5% dextran, 3.5% poly(ethylene glycol), 0.15 M NaCl, and 0.01 M sodium phosphate buffer, pH 6.8. Physical properties of the phases are discussed in the text. [c] Approximately 80% of erythrocyte sialic acid is removed during neuraminidase treatment (Walter and Coyle, 1968).

TABLE III: Partition of Erythrocytes from Different Species in Two-Polymer Aqueous Phase Systems Measuring Different Membrane Surface Properties.

Species	Partition[a] in Phase System		
	5:4#1[b]	5:3.5#1[b]	5:3.5#5[b]
Beef[c]			
Class 1	32 ± 9	100 ± 5	4 ± 1
Class 2	56 ± 4	97 ± 12	3 ± 0
Class 3	81 ± 2	101 ± 14	3 ± 1
Dog	86 ± 9	95 ● 8	83 ± 10
Horse	51 ± 5	92 ± 7	15 ± 2
Human	54 ± 7	90 ± 2	59 ± 6
Lamb	40 ± 8	98 ± 4	9 ± 4
Mouse	97 ± 5	95 ± 6	95 ± 1
Pig	11 ± 2	90 ± 5	4 ± 1
Rabbit	5 ± 2	63 ± 3	67 ± 11
Rat	93 ± 6	89 ± 4	92 ± 7

[a] Partition is defined as the quantity of cells in the top phase (percent of total cells added). [b] Phase compositions were as follows: (5:4#1) 5% dextran, 4% poly(ethylene glycol), and 0.11 M sodium phosphate buffer, pH 6.8; (5:3.5#1) 5% dextran, 3.5% poly(ethylene glycol), and 0.11 M sodium phosphate buffer, pH 6.8; (5:3.5#5) 5% dextran, 3.5% poly(ethylene glycol), 0.15 M NaCl, and 0.01 M sodium phosphate buffer, pH 6.8. Properties of the phases and what they measure are discussed in the text. [c] Beef erythrocytes fall into three classes (Walter et al., 1972), those with low, intermediate, and high partition coefficients in phase system 5:4#1. The partition coefficient has been related to the presence of different quantities of sialic acid (and other membrane charge) components on these cells.

ages by countercurrent distribution in phase systems which have an electrostatic potential difference and in which the separation does depend on membrane charge has previously been reported (Walter et al., 1971).

General Examples of Cell Partition Not Due to Membrane Charge. Albertsson and Baird (1962) have previously shown that the quantity of cells in the top phase (percent of total cells added) increases in phases of constant salt composition and concentration as the critical point is approached. A phase system containing 5% dextran and 3.5% poly(ethylene glycol) is, at room temperature, close to the critical point (Albertsson, 1971). Since the potential difference between the phases diminishes as one approaches the critical point (Table I), it follows that the species-specific increase in partition in phases close to the critical point must be related to something other than membrane surface charge. It is most likely that the reduction in interfacial tension that accompanies reduction in polymer concentration (Ryden and Albertsson, 1971) results in less cell adsorption at the interface, and that the extent of the interaction of the cell surface with polymer [probably poly(ethylene glycol)] determines the cell partition coefficient.

To demonstrate that something other than membrane charge determines cell partition in phases with zero potential difference close to the critical point, we have examined the behavior of human red blood cells in such phases before and after the cells were treated with neuraminidase. Sialic acid is the main surface charge component of human erythrocytes (Eylar et al., 1962; Seaman and Uhlenbruck, 1963) as well as the principal determinant of their partition coefficient in a charged phase system at some distance from the critical point (Walter and Coyle, 1968) (e.g., 5:4#1, see Table II). Partition of neuraminidase-treated human erythrocytes in a phase system having zero potential difference between the phases (5:3.5#5, Table II) not only does not result in a reduced quantity of cells in the top phase as compared with untreated red cells, but actually gives rise to a higher partition coefficient. Thus, surface charge appears not involved in this case and whatever membrane factor(s) does determine the partition behavior increases in potency as a consequence of sialic acid removal.

Table III shows results of partition experiments with red blood cells from nine different species in three phase systems: 5:4#1, 5:3.5#1, 5:3.5#5. The differences between these phases, as indicated earlier, are: 5:4#1 has the highest electrostatic potential difference and is farthest from the critical point; 5:3.5#1 has a lower potential difference and is closer to the critical point; 5:3.5#5 has virtually zero potential difference and is as close to the critical point as 5:3.5#1.

That the major determinant of cell partition in phase system 5:4#1 is due to membrane surface charge or charge-associated properties of the cells has been demonstrated and discussed earlier (Walter, 1975). The partition of cells in phase system 5:3.5#1 depends on a combination of charge (Table I) and other species-specific (noncharge) membrane properties (Table III) together with the decreased tendency of cells to be adsorbed at the interface. Human red cells have an increased partition coefficient in 5:3.5#1 as compared with 5:4#1 but a decreased partition coefficient in 5:3.5#5 as compared with 5:3.5#1. Both charge and other properties thus interact to give the higher partition coefficient in 5:3.5#1 and removal of the electrostatic potential difference in phase 5:3.5#5 results in a lower partition coefficient. Dog red blood cells, which have a relatively high membrane surface charge (Seaman and Uhlenbruck, 1963), have a high partition coefficient in phase systems 5:4#1 and 5:3.5#1. The high partition coefficient of dog erythrocytes in 5:3.5#5 must be due to a noncharge, major interaction with the phase polymer(s).

The difference in partition behavior of erythrocytes from different species in phase systems 5:3.5#1 and 5:3.5#5 (Table III) can yield qualitative information on whether membrane charge contributes to the partition of cells in 5:3.5#1. Charge is a major contributor to the partition of beef, horse, lamb, and pig erythrocytes in 5:3.5#1, less to that of human red cells, and little to that of rabbit red cells. In the cases of dog, mouse, and rat erythrocytes in which the quantity of cells in top phase is about 100% in both 5:3.5#1 and 5:3.5#5, no statement as to the relative contribution of charge to the partition can be made. Attempts to determine specifically the membrane properties (e.g., lipid composition) with which correlations to partition behavior can be found have proved rewarding. A

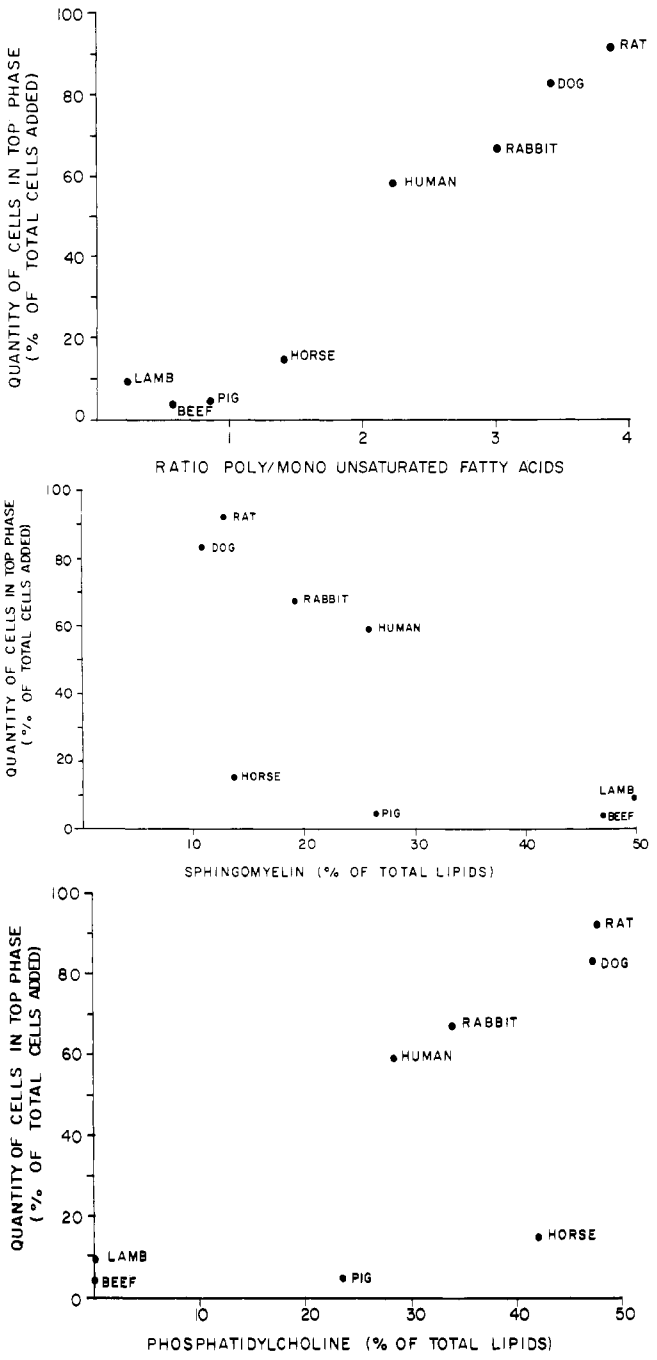

FIGURE 3: The quantity of erythrocytes from different species (percent of total cells added) found in the top phase of a system containing 5% (w/w) dextran, 3.5% (w/w) poly(ethylene glycol), 0.15 M NaCl, and 0.01 M sodium phosphate buffer, pH 6.8, plotted against various membrane lipid compositions as obtained from the literature. Partitions were at room temperature (22–24 °C). See text for discussion.

correlation exists between the partition coefficient of red blood cells from different species in 5:3.5 # 5 and the ratio of their membrane poly/monounsaturated fatty acids (Figure 3), the latter data being taken from the literature (Van Deenen and DeGier, 1974). Since with an increase in unsaturation the lipid chain packing becomes less efficient (Van Deenen and DeGier, 1974), it may be that the membrane under these circumstances can be intercalated by the polymer predominant in the top phase [poly(ethylene glycol)] leading to higher cell partition coefficients (Figure 3). There is also some correlation between the partition coefficients of erythrocytes from different species and their membrane's phosphatidylcholine and sphingomyelin content, increasing with the former and decreasing with the latter (Figure 3).

It has previously been shown that beef red blood cells from different animals fall into three partition classes in phase system 5:4 # 1 (Walter et al., 1972) of high, intermediate, or low partition coefficients (see Table III). The different behavior has been ascribed to the presence of different quantities of sialic acid or other membrane charge components on these cells. No difference in partition behavior between these three classes is discernible in phase systems 5:3.5 # 1 and 5:3.5 # 5. Since the partition coefficient in 5.3.5 # 5 is uniformly low for all classes of beef erythrocytes, one would expect that the partition coefficient of cells in 5:3.5 # 1 is due primarily to membrane charge. That no difference is found between the three classes in this phase is due to 100% of the cells being in the top phase for all three classes.

Conclusion

The bulk potential difference between aqueous polymer phases of constant salt composition (e.g., phosphate) decreases as the critical point is approached. At the same time the partition coefficient of cells increases in a species-specific manner. Thus factors other than membrane surface charge must be involved in cell partition under these circumstances. This is even more apparent when a phase system near the critical point is chosen having virtually no potential difference between the phases (e.g., a system containing sodium chloride). Partition coefficients in such a system cannot be due to membrane charge. Furthermore, in such a system, removal of sialic acid from human erythrocytes does not decrease their partition coefficient. Species-specific differences in the partition behavior of mammalian erythrocytes which depend primarily on membrane charge, partially depend on membrane charge, or do not depend on membrane charge point up not only the difference in membrane charge between such cells (Walter et al., 1967; Eylar et al., 1962; Seaman and Uhlenbruck, 1963) but also differences in other surface properties, some of which are associated with their membrane lipids.

Separation of cells and studies of their membrane properties by partition can, therefore, by appropriate selection of phase system, be based predominantly on membrane charge parameters, some membrane properties other than charge, or on both.

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Inhibition of Estrogen-Receptor–DNA Interaction by Intercalating Drugs†

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ABSTRACT: Ethidium bromide, an intercalating drug, was shown to inhibit the in vitro DNA binding of the uterine estradiol-receptor complex. The inhibition was reversible, dose dependent, complete for total saturation of DNA intercalating sites by the dye, and proportional to the extent of intercalated drug. The binding of the receptor to phosphocellulose and poly(adenylic acid)-cellulose was not decreased by this drug. Similar inhibition was also obtained with 9-hydroxyellipticine. Denatured DNA was more efficient at binding the estrogen receptor than phosphocellulose or poly(adenylic acid)-cellulose but less efficient than native DNA. We conclude that the DNA binding of the estrogen receptor cannot be simply interpreted in terms of electrostatic interactions but requires a particular double-helical structure of DNA.

In the target cell estradiol translocates its cytosol receptor protein in the nucleus where the resulting complex interacts with nuclear acceptor(s) (Jensen and De Sombre, 1973). It has been postulated that DNA may represent at least a part of such an acceptor, since the binding of estradiol–receptor complexes to DNA has been demonstrated in vitro (Toft, 1973). However, the biological significance of such an interaction remains unclear, since no sequence DNA specificity has been proved. Ethidium bromide and 9-hydroxyellipticine (Figure 1) are cationic dyes that at low concentrations modify the three-dimensional structure of DNA by intercalating between base pairs; at higher concentrations, they bind weakly to phosphate groups of DNA (Waring, 1965; Le Pecq and Paoletti, 1967; Le Pecq et al., 1974). In the present study, we investigated the binding of the estrogen receptor to DNA after modifying its helical structure with intercalating drugs.

Experimental Procedure

Materials. [^{3}H]Estradiol, 60 Ci/mmol ($^3HE_2$), was obtained from CEA (Saclay, France), ethidium bromide from Boots Pure Drug Co. (Nottingham, England). The purity of E_2 and EB[1] was checked by thin-layer chromatography. 9-Hydroxyellipticine chlorohydrate was kindly supplied by Le Pecq and Paoletti (Villejuif, France; Le Pecq et al., 1974); 2 mg/ml solutions of the dyes were made in TE buffer and stored at 4 °C in the dark. *Escherichia coli* DNA labeled in vivo by ^{32}P was purified according to Marmur (1963), then centrifuged in a CsCl gradient, and eventually sonicated.

Calf thymus DNA (type 1) was purchased from Sigma, poly(adenylic acid) (poly(A)) from Calbiochem, and phosphocellulose (P 11) from Whatman. Phosphocellulose was treated before use as described (Burgess, 1969). DNA or poly(A) was adsorbed on Munktell's cellulose powder (Alberts and Herrick, 1971) and stocked dried at −20 °C.

Initial Steps. Frozen calf uteri or fresh uteri of 21 day old rats were cut in small pieces in five volumes of 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA buffer, and homogenized at 2 °C using a Virtis homogenizer or a glass/glass Potter-Elvehjem homogenizer, respectively. Cytosol was separated from particulate fractions by centrifugation for 70 min at 250 000*g*, diluted with TE buffer down to 2 mg/ml of protein, and then incubated at 2 °C with 2 or 3 nM $^3HE_2$ for ≥ 90 min. TE buffer or 10 mg of cellulose powder, containing or not containing DNA, was incubated batchwise with labeled cytosol under linear stirring (60 agitations/min) at 25 or 2 °C.

Measure of the E_2R–Polyanion Complexes. The binding of E_2R to *soluble* DNA was assayed according to the comigration of [^{3}H]estradiol with DNA by using either molecular sieving on Sepharose 4B (Musliner and Chader, 1972) or ultracentrifugation in a sucrose gradient (André and Rochefort, 1973). As shown previously, $^3HE_2$ did not comigrate with DNA when the receptor was denatured by heating or occupied by nonradioactive estradiol. By using [^{32}P]DNA we confirmed that the cosedimentation of $^3HE_2$ with DNA was not due to irreversible aggregates of the receptor, since the E_2R complex was completely liberated as an 8S peak after DNA hydrolysis by DNase (André and Rochefort, 1973a). The receptor in-

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[1] Abbreviations used are: EB, ethidium bromide; E_2, estradiol; R, estrogen receptor; E_2R, estrogen receptor complex; poly(A), poly(adenylic acid); EDTA, ethylenediaminetetraacetic acid disodium salt; TE buffer, 10 mM Tris, HCl, pH 7.4, 1.5 mM EDTA; EB/DNA, molar ratio of the concentration of total EB to the concentration of DNA phosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; OD, optical density; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole.