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AMINO GROUPS AT THE SURFACES OF PHOSPHOLIPID VESICLES

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SUMMARY

Reactivity of the amino groups of three nitrogen-containing phospholipids dispersed in aqueous salt solution in the form of liquid crystalline vesicles was studied by microelectrophoresis. The reactivity against 2,4,6-trinitrobenzenesulfonic acid (TNBS) and formaldehyde after incubation was assessed on the basis of changes in their electrophoretic mobility.

It was found that pure phosphatidylethanolamine did not react with TNBS but rather with formaldehyde. If phosphatidylethanolamine was mixed with phosphatidylcholine, which was not reactive alone, with either reagent, the reactivity of phosphatidylethanolamine with both TNBS and formaldehyde was greatly enhanced.

It was also found that phosphatidylserine had limited reactivity with either reagent, whether in pure form or in mixtures with phosphatidylcholine.

It was concluded that the limited reactivity of the primary amino groups at the surface of phospholipid liquid crystalline vesicles dispersed in saline is due to inter- and intramolecular salt linkages with adjacent phosphate groups. The effect of ΔpH at the surface is also discussed as a possible explanation for the lack of reactivity.

Finally the implications of these findings are discussed in terms of some of the proposed models for biological cell membrane structure.

INTRODUCTION

In a previous paper[1] experiments were described in which four types of mammalian cell were treated with four reagents known to react with positively charged groups associated with proteins. The observation that this treatment was not associated with changes in cellular electrophoretic mobility led to the conclusion that positively charged groups associated with protein were not present in detectable amounts at the electrokinetic surfaces of the cells examined.

At that time, no comment could be made on the presence or absence of detectable amounts of phospholipid amino groups at the cellular electrokinetic surface, as little was known about interactions of these groups with different reagents used to treat the cells.

Abbreviation: TNBS, 2,4,6-trinitrobenzenesulfonic acid.

The presence of cationic groups at a cell surface could considerably influence its contact reactions with the environment. A number of recent models proposed for cell membranes, including those of BENSON[2], GREEN *et al.*[3] and LENARD AND SINGER[4] indicate the presence of the polar heads of phospholipids at the interface between cells and their environment. Therefore it seemed of interest to study the reactivity of purified phospholipids of known constitution and molecular orientation and to compare these reactions with previously reported similar reactions with cells, in order to clarify the molecular structure of some cell peripheries.

Recent studies of the properties of purified phospholipids in the form of liquid crystalline vesicles suspended in water[5–8] suggested that such a system would be ideally suited for an investigation of the reactivity of amino groups exposed to the aqueous phase. This present communication describes the effect of formaldehyde and 2,4,6-trinitrobenzenesulfonic acid (TNBS) on the electrophoretic mobility of three different nitrogen-containing phospholipids. The data obtained are discussed both in terms of inter- and intramolecular bonding of amino groups and in terms of the possible orientation of phospholipid head groups at cell surfaces.

MATERIALS AND METHODS

Phosphatidylcholine isolated from egg yolk and phosphatidylserine isolated from bovine brain were chromatographically pure materials prepared and characterized as described earlier[6]. Phosphatidylethanolamine was obtained from Applied Science (State College, Pa.) and derived from plant sources. It gave one spot on thin-layer chromatography. Phosphatidylserine (bovine) was obtained from Applied Science. All the lipids were stored at −10° in chloroform under N_2. For preparing the vesicles, aliquots were taken from these solutions and were evaporated to dryness under vacuum. For preparing mixed vesicles, both lipid species were mixed in chloroform solution and were evaporated and suspended together. Hanks' balanced salt solution[9,*] (pH 7.2–7.6) with or without 1.26 mM Ca^{2+} and 0.89 mM Mg^{2+} (see footnote) was added to the dry material to give a concentration of 1 μM phospholipid phosphate per ml. The mixture was suspended by rotating for 5 min with a Vortex mixer at room temperature. The phospholipid suspensions were mixed with an equal volume of phosphate-buffered saline containing either 20 μg/ml TNBS or 8% formaldehyde. The reaction mixtures were incubated at 37° for 1 h and subjected to electrophoresis at 30° in a cylindrical cell apparatus described by BANGHAM *et al.*[10].

TNBS was obtained from Eastman Organic Chemicals. Formaldehyde was freshly generated from paraformaldehyde by heating for 30 min at 60° in physiological saline (pH 7.2–7.6).

RESULTS AND DISCUSSION

Phospholipid systems

The phospholipid particles used in this study are spheroidal and consist of a number of concentrically arranged lamellae of bimolecular thickness[5,6]. Within each

* Hanks balanced salt solution contains the following (g/l): NaCl (8.0), KCl (0.40), $CaCl_2$ (0.14), $MgSO_4 \cdot 7\ H_2O$, $MgCl_2 \cdot 6\ H_2O$ (0.1), $Na_2HPO_4 \cdot 2\ H_2O$ (0.06), KH_2PO_4 (0.06), glucose (1.00), $NaHCO_3$ (0.35).

lamella, individual phospholipid molecules are oriented normal to the plane with their polar groups facing the water phase. Each phospholipid molecule occupies a minimum of approx. 60 $Å^2$ (assuming close packing), and thus the polar groups could be in a proximity favorable to intermolecular bondings.

When TNBS reacts with amino groups to form *N*-trinitrophenyl groups, a yellow color develops which is due to the delocalization of electrons over the whole aromatic system, including nitro groups which lower the energy of the excited state. This provides a simple independent check on the sensitivity of the electrophoretic technique in detecting the reaction of amino groups with TNBS. The findings with phosphatidylcholine are consistent in that no change was observed in either the color or the electrophoretic mobilities, as expected from the low reactivity of the quaternary ammonium group. In the phosphatidylethanolamine/phosphatidylcholine vesicles, a deep yellow color was observed with a mean increase of approx. 110 % in net surface negativity. With phosphatidylserine and phosphatidylserine/phosphatidylcholine, a faint yellow color was observed which was not associated with a significant change in electrophoretic mobility; phosphatidylethanolamine vesicles showed faint yellow coloration associated with a consistent increase in surface negativity which was statistically significant. The experiments with phosphatidylethanolamine and phosphatidylserine indicate that the technique of particle electrophoresis used by us is incapable of detecting statistically significant changes of less than 5 % in surface net negativity.

The presence of divalent metals (Ca^{2+} and Mg^{2+} in concentrations of 1.26 and 0.89 mM, respectively) does not have any appreciable effect on the reactivity of either phosphatidylserine or phosphatidylethanolamine with TNBS, although it binds to the surface as indicated by a decrease in electrophoretic mobilities (Table I).

Phosphatidylcholine vesicles which are normally isoelectric show no changes in mobility after incubation with either formalin or TNBS (Table I). This negative result serves as control for the possibility of nonspecific adsorption of the reagents on the phospholipid surface.

When phosphatidylcholine is mixed with stearylamine (10 % stearylamine, molar basis), the particles become strongly positively charged. The addition of TNBS results in a large decrease in surface positivity, producing an increase of 75 % in net negativity. This clearly indicates the effectiveness of this reagent in reacting with amino groups exposed to the aqueous environment, presumably at the electrokinetic slip plane.

Phosphatidylethanolamine vesicles gave rather conflicting results (Table I). Pure phosphatidylethanolamine shows only limited reactivity to TNBS (5–8 % increase in net surface negativity), while the same lipid particles are very reactive with formaldehyde which produces changes in the range of 150 %. This finding is not entirely unexpected, since there is some evidence that the amino group of phosphatidylethanolamine interacts with the diesterified phosphate possibly by sharing a proton[11]. Certain properties of phosphatidylethanolamine liquid crystals were interpreted[6] as due to phosphate–amino group interactions.

It should be noted that TNBS exhibits low reactivity toward amino groups of proteins before denaturation[12]. It has also been shown that hydrogen bonded amino groups of polyriboadenylic acid are not reactive with formaldehyde[13]. In this case, protonated amino groups of adenine were considered to interact with phosphate groups of adjacent chains in a salt linkage.

TABLE I

ELECTROPHORETIC MOBILITY MEASUREMENTS FOR VARIOUS PHOSPHOLIPID LIQUID CRYSTALLINE PARTICLES

Bulk phase was phosphate-buffered saline (Hanks solution) at pH 7.2–7.6 with or without Ca^{2+} (1.6 mM) and Mg^{2+} (0.9 mM) as indicated. Mobility measurements were taken at 30°. Asterisk after the abbreviated name of the phospholipid in the first column indicates that the preparation used was isolated and purified in our laboratory. Values between parentheses in the fourth column indicate number of observations.

Phospholipid	*Suspending fluid*	*Incubated with*	*Mobility* ($\mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$ ± *S.E.*)	*Change in net negativity* (%)	*t-test P*
PC (100%)	PBS (CMF)	Control	Isoelectric (50)		
	PBS (CMF)	TNBS	Isoelectric (52)	No change	
PC* (100%)	PBS (CMF)	Control	Isoelectric (60)		
	PBS (CMF)	TNBS	Isoelectric (58)	No change	
	PBS (CMF)	Formaldehyde	Isoelectric (52)	No change	
PE (100%)	PBS (CMF)	Control	−2.123 ± 0.025 (50)		
	PBS (CMF)	TNBS	−2.306 ± 0.027 (50)	+ 8.6	<0.001
	PBS (Ca/Mg)	Control	−1.877 ± 0.013 (64)		
	PBS (Ca/Mg)	TNBS	−2.01 ± 0.007 (68)	+ 7	<0.001
	PBS (CMF)	Control	−2.291 ± 0.026 (60)		
	PBS (CMF)	TNBS	−2.407 ± 0.031 (54)	+ 5.0	$0.01 > P > 0.001$
	PBS (CMF)	Control	−1.292 ± 0.028 (50)		
	PBS (CMF)	Formaldehyde	−3.317 ± 0.055 (50)	+156	<0.001
	PBS (CMF)	Control	−1.761 ± 0.048 (50)		
	PBS (CMF)	TNBS	−2.357 ± 0.032 (50)	+ 33.8	<0.001
	PBS (CMF)	Formaldehyde	−4.129 ± 0.099 (51)	+134.5	<0.001
PE/PC (10%)	PBS (Ca/Mg)	Control	−0.362 ± 0.009 (50)		
	PBS (Ca/Mg)	TNBS	−0.755 ± 0.016 (58)	+108	<0.001
	PBS (Ca/Mg)	Control	−0.470 ± 0.013 (50)		
	PBS (Ca/Mg)	TNBS	−1.010 ± 0.010 (50)	+114	<0.001
	PBS (CMF)	Control	−0.426 ± 0.008 (50)		
	PBS (CMF)	TNBS	−0.893 ± 0.014 (50)	+110	<0.001
	PBS (CMF)	Control	−0.521 ± 0.041 (50)		
	PBS (CMF)	TNBS	−1.261 ± 0.050 (53)	+142	<0.001
	PBS (CMF)	Formaldehyde	−1.896 ± 0.058 (58)	+264	<0.001
PS (100%)	PBS (Ca/Mg)	Control	−2.373 ± 0.031 (50)		
	PBS (Ca/Mg)	TNBS	−2.304 ± 0.023 (51)	− 2.9	$0.10 > P > 0.05$
	PBS (CMF)	Control	−2.943 ± 0.070 (60)		
	PBS (CMF)	TNBS	−3.312 ± 0.060 (51)	+ 12.6	<0.001
PS* (100%)	PBS (CMF)	Control	−3.926 ± 0.041 (50)		
	PBS (CMF)	TNBS	−3.691 ± 0.050 (50)	− 6	<0.001
	PBS (CMF)	Formaldehyde	−3.396 ± 0.026 (49)	− 13	<0.001
	PBS (CMF)	Control	−3.985 ± 0.034 (50)		
	PBS (CMF)	TNBS	−4.201 ± 0.041 (50)	+ 5.4	<0.001
	PBS (CMF)	Formaldehyde	−3.698 ± 0.037 (52)	− 7.2	<0.001
PS/PC (10%)	PBS (Ca/Mg)	Control	−0.677 ± 0.015 (50)		
	PBS (Ca/Mg)	TNBS	−0.626 ± 0.013 (50)	− 7.5	$0.02 > P > 0.01$

(*continued Table I*)

Phospholipid	*Suspending fluid*	*Incubated with*	*Mobility* ($\mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$ ± *S.E.*)	*Change in net negativity* (%)	*t-test* *P*
PS/PC (10%)	PBS (CMF)	Control	−0.933 ± 0.021 (50)		
	PBS (CMF)	TNBS	−0.883 ± 0.016 (51)	− 5.4	$0.10 > P > 0.05$
	PBS (CMF)	Control	−0.767 ± 0.022 (50)		
	PBS (CMF)	TNBS	−0.805 ± 0.028 (51)	+ 4.5	$0.3 > P > 0.2$
	PBS (CMF)	Formaldehyde	−0.825 ± 0.021 (50)	+ 7	$0.1 > P > 0.05$
St-am/PC (10%)	PBS (CMF)	Control	+1.205 ± 0.023 (52)		
	PBS (CMF)	TNBS	+0.298 ± 0.011 (50)	+ 75	< 0.001

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PBS (CMF) phosphate buffered saline, Ca^{2+} and Mg^{2+} free; PBS (Ca/Mg), phosphate-buffered saline with 1.26 mM Ca^{2+} and 0.89 mM Mg^{2+}; St-am, stearylamine.

In order to investigate further the possibility of inter- and/or intramolecular amino–phosphate interactions, phosphatidylethanolamine was mixed with phosphatidylcholine in quantities of only 10% of total (percentage based on moles of phosphate). Electrophoresis of the phosphatidylethanolamine/phosphatidylcholine particles after incubation with either TNBS or formaldehyde indicates a high reactivity, reflecting increases of net surface negativity corresponding to mobility changes of 100% for the former and 200% for the latter reagent (Table I). The low reactivity of pure phosphatidylethanolamine compared with the phosphatidylethanolamine/phosphatidylcholine mixture can be accounted for in terms of intermolecular amino–phosphate interactions. When phosphatidylethanolamine is mixed in 10% amounts with phosphatidylcholine, the phosphatidylethanolamine molecules are expected to be statistically interdispersed with phosphatidylcholine molecules, an arrangement not allowing interaction of free amino groups of phosphatidylethanolamine with the phosphates of other phosphatidylethanolamine molecules. An alternative explanation could be that the presence of the phosphatidylcholine headgroups changes the orientation of the amino groups of phosphatidylethanolamine toward the aqueous phase and away from the phosphate. This latter possibility is not confirmed by studies on phosphatidylserine and phosphatidylserine/phosphatidylcholine.

The electrophoretic mobility of the purified phosphatidylserine is strongly negative as expected, because each molecule carries one net negative charge. As indicated in Table I, the phosphatidylserine obtained from Applied Science is less electronegative than the phosphatidylserine prepared by us. This is probably due to the presence of divalent metals (mainly Mg^{2+}), as shown by metal content analysis. Both TNBS and formaldehyde have only a small effect on the mobility of phosphatidylserine. The amino group does not appear to be available for reaction with either reagents, strongly suggesting inter- or intramolecular bonding particularly with phosphatidylethanolamine. It should be noted, however, that the mobilities of pure phosphatidylserine are very high, and it has been argued earlier[14,15] that under these circumstances, small changes in surface charge density might not be reflected in mobility measurements. This is unlikely because even when phosphatidylserine is

mixed with phosphatidylcholine (10% phosphatidylserine in phosphatidylcholine, Table I), with concomitant reduction in surface charge density, neither reagent significantly increases mobility. This result also argues against the relative TNBS susceptibility of phosphatidylethanolamine/phosphatidylcholine, compared to phosphatidylethanolamine, being due to orientation effects. It is therefore reasonable to suggest that amino groups in both phosphatidylethanolamine and phosphatidylserine form bonds probably by salt linkage to phosphates. In the case of phosphatidylethanolamine, these bonds would be intermolecular and in the case of phosphatidylserine, intramolecular.

Effect of surface pH on reactivity

Another explanation for the lack of reactivity of phosphatidylethanolamine and phosphatidylserine with formaldehyde or TNBS could be the effect of surface pH. The reaction of amino groups with both TNBS and formaldehyde is strongly influenced by pH, the reactive species being the uncharged amine[12,16]. Furthermore, the pH near a negatively charged surface is less than that in the bulk environmental phase[17], due to the asymmetric distribution of protons. Therefore it seemed possible that the failure of TNBS to react with phosphatidylethanolamine, phosphatidylserine and phosphatidylserine/phosphatidylcholine vesicles could be due to the expected lower pH near the vesicles surfaces.

The difference between "bulk-phase" pH (pH_B) and "surface" pH (pH_s) is known as Δ pH and may be calculated from ζ-potential according to the equation developed by HARTLEY AND ROE[18] and discussed by MCLAREN AND BABCOCK[19] and WEISS[20]. At 30° the Hartley–Roe equation may be written

$$pH_s = pH_B + \zeta/57$$

(note that ζ has a negative value).

ζ-potential may be estimated from measurements of electrophoretic mobility, using the Helmholtz–Smoluchowski equation,

$$\zeta = \frac{4\pi\eta \cdot V}{\varepsilon}$$

where η = dynamic viscosity at the hydrodynamic slip plane of the vesicle; ε = dielectric constant in this region; and V = electrophoretic mobility.

The results of the calculations are shown in Fig. 1 and indicate that in the case of phosphatidylserine vesicles having a mobility of 4.0 $\mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$, Δ pH is calculated at 0.9 pH unit; in the case of phosphatidylserine/phosphatidylcholine vesicles having a mobility of 2.3 $\mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$, Δ pH is calculated to be 0.5 pH unit.

These calculations at best give the average decrease in pH near a surface carrying a net negative charge; they include neither the effects of positively charged groups at the interface which will attract OH^- nor discrete charge effects resulting in a non-Boltzmann distribution of counter ions. Therefore our calculated average values for Δ pH, where surface parameters are relevant, are over-estimates.

In order to investigate the reactivity of TNBS with amino groups at different values of bulk phase pH without the complications of surface pH, the following experiment was performed. Ethanolamine chloride and serine were mixed with TNBS

in phosphate-buffered saline which was titrated with HCl or NaOH to different pH values. The mixtures were allowed to interact at 37° for 1 h. The extent of the reaction was determined by recording the absorbance of the mixtures at 410 mμ. The results are given in Fig. 2. It appears from considerations of Δ pH (Fig. 1) that the surface

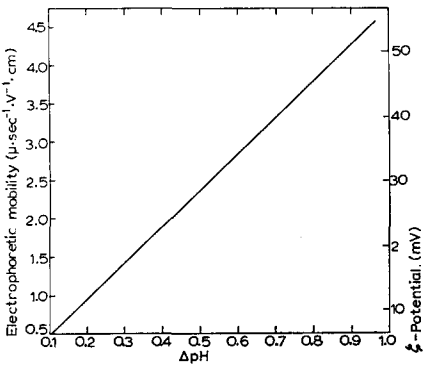

Fig. 1. Plot of the relationship between △ pH and ξ-potential (and electrophoretic mobility) based on the equation of HARTLEY AND ROE[18].

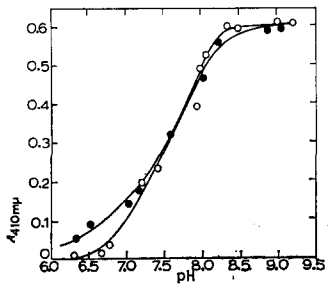

Fig. 2. Reactivity of ethanolamine and serine with TNBS at different pH values. 0.1 μmole of ethanolamine or serine was mixed with 20 μg of TNBS in 3 ml of phosphate-buffered saline titrated to different pH values. The mixtures were incubated for 1 h at 37°, and the absorbance was measured at 410 mμ. Titration of ethanolamine in the absence of TNBS shows a pK of 9.5. ○, reaction of ethanolamine with TNBS; ●, reaction of serine with TNBS.

pH of phosphatidylserine vesicles could be as low as 6.56 when the bulk-phase pH value is 7.4. As indicated in Fig. 2 at this pH and under the conditions employed, very little reaction with TNBS would occur (5 % of the value of pH 7.5). Nevertheless, this is the only case where Δ pH could justify the lack of reactivity observed with phospholipid vesicles with TNBS. Phosphatidylethanolamine with a surface pH of 7.0 and phosphatidylserine/phosphatidylcholine with a surface pH of 7.28 would be expected to yield 32 % and 63 % of the reaction yield (considering the yield at pH 7.5 as 100 %), respectively. As described in Table I, neither lipid exhibited appreciable reactivity with TNBS.

In order to strengthen the above arguments, additional experiments were made in which phospholipid vesicles were reacted with TNBS at bulk-phase pH values of 8.9. Measurements of electrophoretic mobility indicated an increase of 7.7 % ($P<0.001$) in phosphatidylethanolamine vesicles, an increase of 13 % ($P<0.001$) in phosphatidylserine/phosphatidylcholine (10 %) vesicles and an increase of 5.6 % ($0.02>P>0.01$) in phosphatidylserine vesicles. Except in phosphatidylserine/phosphatidylcholine vesicles, the electrokinetic changes resulting at pH 8.9, were within the range observed at pH 7.4 (Table I). Inspection of the curve showing the reaction of ethanolamine and serine with TNBS at different bulk-phase pH values (Fig. 2) shows that at pH 8.9, even allowing for the calculated effect of Δ pH (Fig. 1), the reaction of the lipids with the TNBS should be very close to maximal. Thus, the failure of TNBS to react appreciably with the vesicles at pH 7.4 is not readily explained in terms of the lower pH near their surfaces.

Implications for cell membrane models

In a previous paper[1] it was reported that treatment of human and mouse erythrocytes, sarcoma 37 cells, and cultured human tumor cells with either formaldehyde or TNBS produced no change in their electrophoretic mobilities. Positively charged amino (and other) groups would have been "neutralized" following these treatments, resulting in a gain of net negativity at the cell surface which should have been detectable as an increase in cellular electrophoretic mobility. It was therefore argued that within the limits of this experimental technique, none of the positively charged groups expected to be associated with proteins at the cell peripheries were detectable at their surfaces. The positively charged species were not concealed by either trypsin- or neuraminidase-susceptible groups.

In discussing these experimental observations, the possibility that phospholipid amino groups were located at the electrokinetic surfaces of the cells could not be commented upon, since with the exception of lecithin which was not expected to react with the reagents, little was known of the reactivity of the cationic groups at the polar heads of other phospholipids. In this connection it should be noted that BONTING AND BANGHAM[21] observed changes in the electrophoretic mobility of phosphatidylethanolamine particles after reaction with retinal. The lack of reactivity of phosphatidylserine with both TNBS and formaldehyde indicates that the presence of this phospholipid at cell surfaces could not be detected with these reagents. Phosphatidylethanolamine exhibits varying reactivity, depending on whether it is used in purified form or in mixtures with phosphatidylcholine. The reactivity of phosphatidylethanolamine in mixtures is higher; thus it would be more easily detected if it existed as such at cell surfaces. Unfortunately we have no knowledge of the distribution of the different phospholipid species at cell surfaces. We will assume in our further discussion that the reactivity of phosphatidylethanolamine at cell surfaces would be that of the pure phospholipid. Use of the minimal value for reactivity would result only in an underestimation of its reactivity in the cell periphery.

In the absence of analytical data on the composition of surface membranes of other cell types used in previous experiments, the next part of our discussion must be restricted to the human erythrocyte where reliable membrane analyses are available[22,23]. Calculations based on the published data show that phosphatidylethanolamine accounts for 6 % of the total dry weight of hemoglobin-free human erythrocyte ghosts. In this situation phosphatidylethanolamine represents 15.6 % of the total membrane lipids.

Of the various models suggested for membrane structure, we can consider the parallelopiped repeating unit of GREEN *et al.*[3] in which the membrane surface is visualized as consisting of the polar heads of phospholipids. In this model, the polar heads of phosphatidylethanolamine would account for approx. 15 % of the membrane surface area, assuming close packing and approximately similar area/weight ratios for all lipid components. This latter assumption appears justified considering monolayer studies[24,25] and X-ray diffraction data[26] on the packing of phospholipids and cholesterol, which make up approx. 85 % of the human erythrocyte lipids. At the other extreme, the model of DANIELLI AND DAVSON[27] assumed that no phospholipid head groups were present in the aqueous phase. "Intermediate" models, such as those proposed by BENSON[2] and LENARD AND SINGER[4], depict a cell surface composed of the head groups of phospholipids, as well as polypeptide chains and carbohydrates.

In these models it is impossible to exactly estimate the proportion of the cell surface occupied by phospholipid head groups, since an unknown amount of protein is considered to lie in the interior of the membrane. The thicker the membrane, the less the proportion of membrane protein at its surface and the higher the percentage of surface area occupied by phospholipid head groups.

When phosphatidylethanolamine vesicles are treated with formaldehyde, there is a 135 % increase in their electrophoretic mobilities and negative surface charge density. In the model of GREEN *et al.*[3], 15 % of the erythrocyte surface would be occupied by the polar heads of phosphatidylethanolamine, and an 135 % increase in their surface charge density would impart a 20 % increase in mobility to the whole cell. This was not observed in our previous experiments with erythrocytes. If the intermediate models are correct, it follows from calculations based on the 135 % increase that not more than 8 % of the cell surface can be occupied by the polar heads of phosphatidylethanolamine. It may be noted that of the intermediate models the one proposed by LENARD AND SINGER[4,28] is consistent with recent spectroscopic evidence on the conformation of membrane proteins.

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