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EFFECT OF pH ON THE SURFACE CHARGE DENSITY OF PLANT MEMBRANES

COMPARISON OF MICROSOMES AND LIPOSOMES

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The surface potential of microsomes of horse bean roots was compared to the one of liposomes prepared from the whole phospholipid extracts. The surface potential was determined from the affinity of the membranes for the anilinonaphthalene sulphonate dye. The effect of pH was studied at two KCl concentrations. It appeared from this comparison that the surface charge density was nearly the same on both materials in the neutral pH range. The isoelectric point was pH 1.7 for the liposomes and pH 4.0 for the microsomes. The implication of these observations is that the surface charge density of microsomes is nearly the same above the lipid and protein components of the membrane. This hypothesis was checked by measuring the activity of a microsomal enzyme with an anionic substrate, while modifying the net surface charge of the membrane. The biological significance of the results is discussed.

Introduction

In contrast with most animal cells, plant cells do not live in an homeostatically controlled environment. Their membranes have to deal with somewhat large fluctuations of the ionic medium. More specifically, their plasma membranes as well as their microsomal membranes which line the various vacuolar compartments must face pH changes. It has been shown that the roots of the calcicole leguminosae horse bean lose the control of their passive permeability at pH < 4. Under this same condition, the roots of the calcifuge leguminosae yellow lupine behave normally [1]. The unsaturation level of the fatty acids and the amount of acidic phospholipids (phosphatidic acid, diphosphatidylglycerol, phosphatidylserine), of neutral lipids and glycolipids [2,3] are markedly different between the two plants. It has been proposed that the two former charcteristics are important for determining the proton permeability of horse bean phospholipids liposomes via the fluidity of the bilayer [4]. Furthermore, the amount of the three acid phospholipids has been shown to determine the response of the microviscosity of horse bean liposomes to pH [3]. All these results show the importance of the surface interactions which may be considered as the first step in the mediation of the effect of the ionic medium on the membranes. They emphasize the role of the phospholipids. On the other hand, the analysis of membrane proteins show generally a large excess of acidic amino acids [5,6]. The estimation of the surface charge density of membrane proteins in situ is experimentally difficult and the information about it is scarce. We present here a comparative study of the surface charge of horse bean lipo-

Abbreviations: ANS, 8-anilino-1-naphthalene sulphonate; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid; SDS, sodium dodecyl sulphate.

somes and microsomes. This leads to a tentative estimation of the surface charge density of microsomal proteins. We have studied the effect of the pH on membranes from horse bean roots in order to determine their electrostatic behaviour under the conditions which are known to open the permeability barrier in vivo.

Materials and Methods

Membrane preparation

The roots were excised from 8-day-old seedlings of horse bean (*Vicia faba* L., var. *minor*) grown on dilute saline solution [7]. Homogeneization of the roots was performed with a triple-roll mill (Pascall Engineering Co., U.K.) at 4°C. The homogenization medium contained 0.4 M sucrose, 50 mM Tris (pH 7.4) and 10 mM mercaptoethanol. A heavy membrane fraction was first discarded at $3 \cdot 10^5$ $g \cdot$ min. Microsomes were then sedimented at $3 \cdot 10^6$ $g \cdot$ min and washed twice in the same medium as above plus 0.1 M KCl. The sucrose was omitted for the second wash. The final pellets were resuspended in 10 mM Hepes (pH 7.4)/10 mM mercaptoethanol. The liposomes were obtained as described elsewhere [3]. The amount of membranes was determined by assaying for proteins in the case of microsomes [8] and for phosphorus in the case of liposomes [9].

Fluorescence measurements

Fluorescence measurements were performed on a Jobin-Yvon JY3D spectrofluorimeter. The excitation and emission wavelengths were 390 and 480 nm, respectively. The experimental procedure for the fluorescence intensity measurement has been described elsewhere [10].

The pH was adjusted with small aliquots of HCl or KOH and was continuously monitored with a combination pH microprobe (Microelectrodes, Inc., U.S.A.). As sonication did not modify the fluorescence intensity at that time, we consider that we measured the mean fluorescence intensity of ANS adsorbed on both the outside and the inside surfaces of the membranes [11–13].

Surface potential (ψ_0) and surface charge density (σ)

It has been previously shown that the fluorescence properties of ANS adsorbed on membranes allow one to determine their affinity by titrating the fluorescence intensity (FI) of a fixed amount of sites with increasing ANS concentrations. The apparent dissociation constant, K, determined from a Scatchard plot (FI/[ANS] vs. FI), can be used to calculate ψ_0 [14,15]:

$$\psi_0 = \frac{kT}{Ze} \log \frac{K}{K_i} \tag{1}$$

where Z is the valency of the ion, e is the elementary charge, k and T have their usual meaning and K_i is the intrinsic affinity (the affinity when $\psi_0 = 0$). The σ value was calculated from the Gouy-Chapman relation for 1 : 1 electrolytes:

$$\psi_0 = \frac{2kT}{e} \sinh^{-1}\left(\frac{\sigma}{\sqrt{[C]\, 8\epsilon N k T}} \right) \tag{2}$$

where ϵ is the dielectric constant, N is the Avogadro number. K_i was determined by the following procedure: eliminating ψ_0 between Eqn. 1 and 2 gives a new relation between K_i, K, σ and [C]. This relation is applied to two experimental measurements of K with different KCl concentrations. K_i is eliminated and σ calculated from the two numerical relations. ψ_0 is then obtained from Eqn 2 and used to calculate K_i from Eqn. 1. K_i was 71 μM for liposomes and 83 μM for microsomes. The surface ionic concentrations in the vicinity of the surface $[C]_0$ were calculated from the Boltzman law

$$[C]_0 = [C] \exp\left(- \frac{Ze}{kT} \psi_0 \right) \tag{3}$$

where [C] is the bulk ionic concentration.

NADH-cytochrome c oxidoreductase (EC 1.6.99.3) activity

The reaction medium contained 0.05 mM cytochrome c, 10 mM Hepes, (pH 7.4), 1 mM KCN, 0.1 M KCl and approx. 0.05 g/l proteins.

The reaction was initiated by adding various NADH concentrations. The reduction of cytochrome c was monitored with a Varian Cary spectrophotometer at 550 nm. The initial rate of the reaction was estimated from the slope of the graph with an extinction coefficient. of 18.5 $mM^{-1} \cdot cm^{-1}$ (reducted minus oxidized form) for cytochrome c.

The NADH substrate was considered as being a univalent anion [16]. In this condition the value of the apparent Michaelis constant (K_m) was related to ψ_0. As its intrinsic value was unknown, we just compared two apparent K_m values:

$$(\psi_0)_1 - (\psi_0)_2 = \frac{kT}{Ze} \log \frac{(K_m)_1}{(K_m)_2} \quad (4)$$

where the subscripts 1 and 2 refer to two different experimental conditions giving the two values $(\psi_0)_1$ and $(\psi_0)_2$.

Results

Effect of pH and KCl concentration on the surface potential

The titrations of microsomes and liposomes by ANS were performed at various pH values over the range pH 1–11, for two KCl concentrations (0.1 and 1.5 M). The surface potentials determined in these conditions are shown in Fig. 1. The increase of the KCl concentration from 0.1 M to 1.5 M reduced the absolute value of ψ_0, although the depolarization was not complete.

The liposomes were systematically more polarized than the microsomes. The isoelectric point of the two materials was respectively at pH 1.7 and pH 4.0. For both materials the curves of ψ_0 vs. pH presented a quasi-plateau in the middle range values of pH.

Fig. 2 shows the values of the surface charge density calculated from the results of Fig. 1 and Eqn.2. It appears that σ is systematically higher in 1.5 M than in 0.1 M KCl. The values of the surface pH (pH_0) were obtained by introducing the bulk H^+ concentrations and the observed ψ_0 value into Eqn. 3.

Effect of the surface charge on the activity of the NADH-cytochrome c oxidoreductase

We attempted to estimate the surface potential

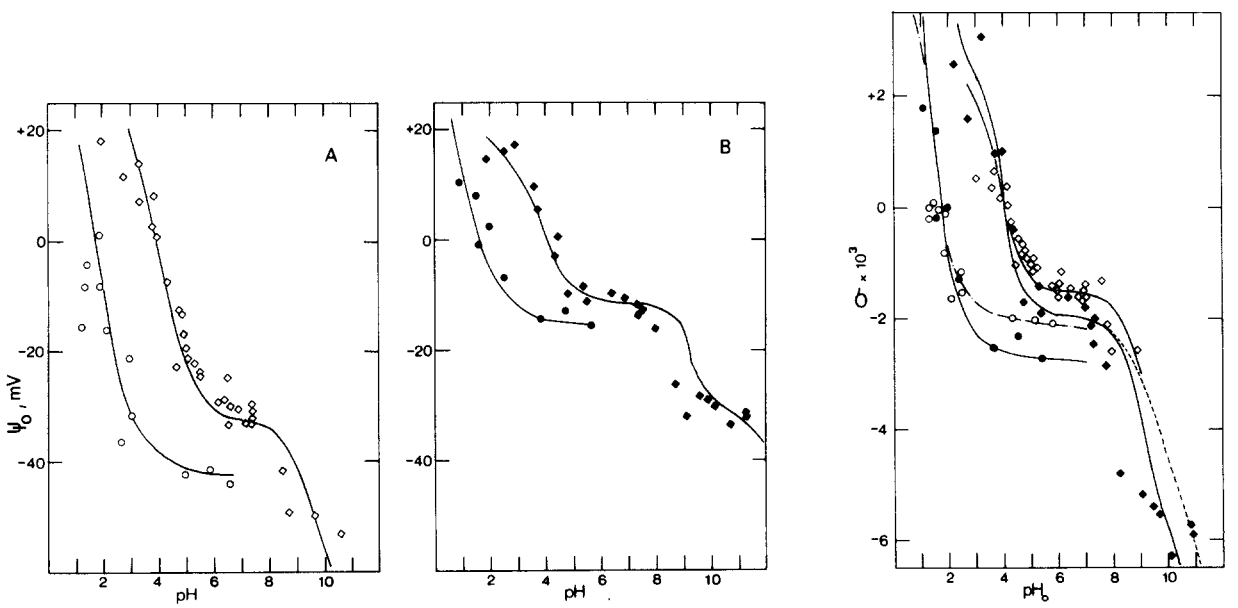

Fig. 1. (Two left-hand panels.) Surface potential ψ_0 of horse bean liposomes (○, ●) and microsomes (◇, ◆) as a function of the bulk pH estimated from the apparent affinity for ANS. A, in 0.1 M KCl; B, in 1.5 M KCl. The theoretical curves are calculated with the parameters listed in Table I.

Fig. 2. (Right-hand panel.) Surface charge density (elementary charge $\times \text{Å}^{-2}$) of liposomes (○, ●) and microsomes (◇, ◆) as a function of the surface pH (pH_0). σ was calculated from the ψ_0 values of Fig. 1 and the Gouy-Chapman relation. The mediums contain 0.1 M KCl (open symbols) or 1.5 M KCl (closed symbols). The theoretical curves are calculated with the parameters listed in Table I. The pK attributed to the positive protein charges is 9.5 (———) or 10.5 (- - - - -).

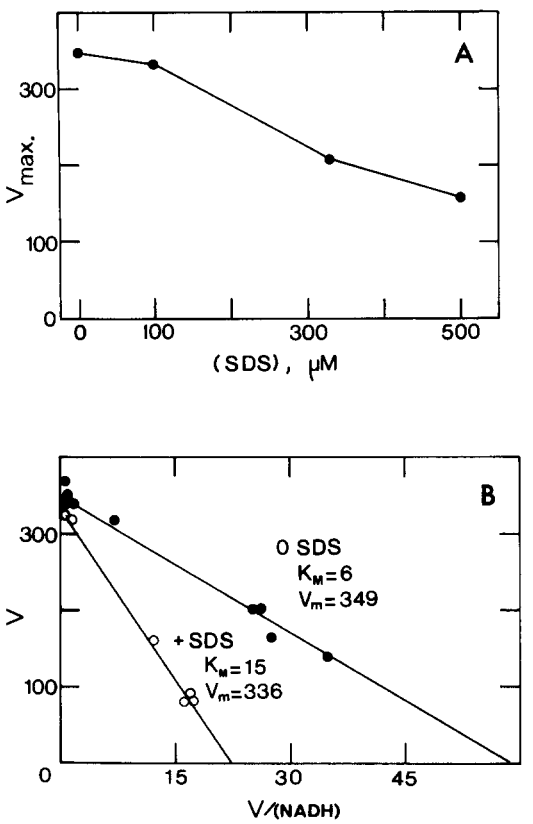

Fig. 3. Effect of low SDS concentration on the activity of the microsomal NADH-cytochrome *c* oxidoreductase. *V*: initial reaction rate, μmol/min per g protein. [NADH]: concentration of the substrate (μM)., The activity was measured by monitoring the kinetic of absorbance change at 550 nm in a medium containing 10 mM Hepes (pH 7.4), 1 mM KCN, 0.1 M KCl and approx. 0.05 $g \cdot l^{-1}$ microsomal proteins. A: maximum specific activity in function of the concentration of SDS. B: Eadie-Hofstee plot of the activity measured with SDS 100 μM (○) and without SDS (●).

in the vicinity of the membrane proteins by determining the effect of the KCl concentration on the activity of the microsomal NADH-cytochrome *c* oxidoreductase. The kinetic analysis of the results revealed that both parameters, V_m and K_m, were affected in a complex way at pH 7.4 by the KCl concentration (results not shown). The variation of V_m indicated that some conformational change may occur. Thus, there is no objective reason to admit that the variation in the apparent K_m was only due to that in the repulsive electrostatic interaction between the substrate and the protein (Eqn. 4).

Another attempt to modify the local potential was made by using very low SDS concentrations. It has been shown that this anionic surfactant increases the absolute value of the surface charge of various membranes without disorganizing them when applied in the range 0–100 μM [19]. The enzymatic kinetics were studied at pH 7.4 in 0.1 M KCl plus 1 mM KCN. The concentration of SDS was increased to 500 μM. Fig. 3 shows that V_m was not affected by 100 μM SDS but that K_m increased from 6 to 15 μM. Titrations of microsomes by ANS were performed in parallel runs. From the values of K_m introduced in Eqn. 4, the variation of ψ_0 due to SDS was −20 mV. From the values of apparent affinity for ANS it was −19 mV.

Theoretical analysis

Liposomes

The phospholipids of horse bean liposomes contained five major dissociable functions, whose

TABLE I

COMPOSITION OF THE PHOSPHOLIPID EXTRACTS IN DISSOCIABLE GROUPS (ROOTS OF HORSE BEAN)

The number, *n* of each dissociable group is expressed per 100 mol of phospholipids. The percentages of the polar heads were:phosphatidylinositol, 7.9; phosphatidylglycerol, 6.7; phosphatidylcholine, 44.5; phosphatidylethanolamine, 30.7; phosphatidylserine, 4; diphosphatidylglycerol, 2.5; phosphatidic acid, 3.7; (from Ref. 3). The p*K* values were obtained from Refs. 17 and 18.

Parameter	Dissociable group				
	$-PO_4^-$	$-COO^-$	$-PO_4^{2-}$	$-NH_3^+$	$-N^+(CH_3)_3$
n	102.5	4.0	3.7	34.7	44.5
p*K*	1.2	3.5	8.0	9.0	12.0

frequencies in the extract are given in Table I. The corresponding pK values allow one to calculate the surface charge of liposomes. The net surface charge density, σ, may then be calculated from estimation of the mean molecular area of phospholipids. We considered this latter as a semi-adjustable parameter. We retained the values which gave the best adjustment to results of Fig. 2. The mean molecular area was taken as 130 Å^2 for 0.1 M KCl and 100 Å^2 for 1.5 M KCl treatments. As it will be discussed later, there are indeed theoretical reasons for believing that an increase of the ionic strength may lead to a shrinkage of the membranes.

The curves of σ vs. pH_0 were computed from these areas and data of Table I. Fig. 2 shows that they correspond well with the theoretical curves for the σ values obtained from the experimental results of Fig. 1.

Microsomes

For the microsomes, the ψ_0 values obtained with ANS may result from the lipidic charges, or the proteic ones, or from both of them, according to the nature of the binding site of the probe. The site of ANS on natural membranes is known to be mainly lipidic [15,20–24]. On the other hand, the well demonstrated energy transfer between the tryptophan residues of the membrane proteins and ANS indicates that it may be in a close vicinity of the proteins [15,22,23]. This hypothesis is strengthened by the results of Fig. 2, which show that the apparent pK near pH 2 in liposomes was shifted to pH 4 in microsomes. This latter pK indicates the presence of carboxylic acid groups in the vicinity of the sites (Table I). Since the phospholipid spectra were the same in the liposomes and the microsomes [2], the simplest explanation is that the ANS experienced the excess of carboxylic acid charges brought by the protein components of microsomes.

Neither the number of the ionic groups nor the area of microsomal proteins is known. We retained as a first approximation the basic assumption of the Gouy-Chapman model, i.e., an uniformly smeared surface charge. This assumption implies that the local charge density of the protein and lipid components of the membrane, as experienced by ANS, remains undistinguishable for each pH value. This crucial assumption will be discussed later. Furthermore, we assumed that the ratio of the areas of both components was the same as the ratio of their weight (protein : phospholipids : neutral lipids and glycolipids = 43 : 47 : 10). In these conditions the surface charge density of microsomes should be described by the following relation:

$$\sigma_{micros.} = 0.43\,\sigma_{prot.} + 0.47\,\sigma_{pl} \qquad (5)$$

where prot. is protein and pl phospholipids.

Introducting the experimental values of $\sigma_{micros.}$ and σ_{pl} into Eqn. 5 gave the $\sigma_{prot.}$ values shown in Fig. 4. From the shape of the graph it appears that in the pH range 1–7 the protein charges are due to one kind of anionic group, the pK of which is 4, plus a fixed amount of dissociated basic groups. The Hasselbach-Henderson relation which de-

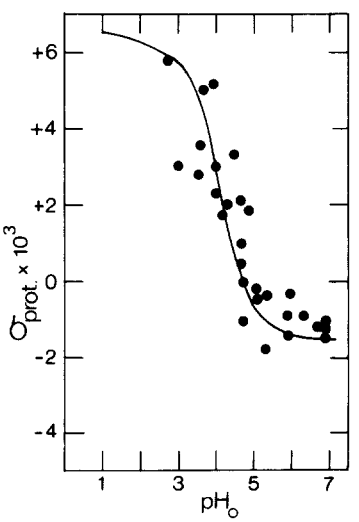

Fig. 4. Tentative estimation of the surface charge density of the protein component ($\sigma_{prot.}$) of the microsomes (elementary charge $\times$Å^{-2}) as a function of the surface pH (pH_0). $\sigma_{prot.}$ is calculated from the relation: $\sigma_{micros.} = f_{prot.}\sigma_{prot.} + f_{pl} \cdot \sigma_{pl}$ where $\sigma_{micros.}$ and σ_{pl} are the values determined for microsomes and phospholipid liposomes respectively, and where $f_{prot.}$ and f_{pl} are the fractional areas attributed to the protein and phospholipid components of microsomes on the basis of their respective amounts. The theoretical curve is calculated from the Hasselbach-Henderson relation with a pK of 4.0 for the anionic groups. The pH_0 values are calculated from the Boltzman law and the ψ_0 values of Fig. 1.

scribes this situation is

$$\sigma_{prot.} = \frac{\sigma^{+}_{max}}{1 + 10^{pH_o - pK^{+}}} - \frac{\sigma^{-}_{max} \cdot 10^{pH_o - 4}}{1 + 10^{pH_o - 4}} \quad (6)$$

where pK^{+} is higher than 8 and σ^{+}_{max} and σ^{-}_{max} refer to the maximum surface densities of positive and negative protein charges. The curve on Fig. 4 was computed from Eqn. 6 with the arbitrary values $\sigma^{+}_{max} = 6.5 \cdot 10^{-3}$ and $\sigma^{-}_{max} = 8 \cdot 10^{-3}$ (elementary charges per Å^2). Finally, the curves for $\sigma_{micros.}$ (Fig. 2) were obtained from relations 5 and 6. It appeared that pK^{+} was probably in the pH range 9.5–10.5. The corresponding curves for ψ_0 are shown in Fig. 1.

Discussion

As shown above, the experimental results (Figs. 1 and 2) are correctly described by the theoretical curves obtained from the Gouy-Chapman model (Eqn. 2) and the law of mass action (Eqn. 6). The two main assumptions are (i) the mean molecular area of the phospholipids decreases at high ionic strength and (ii) the lipid and protein surface charges are homogeneously and uniformly dispersed.

It appears from Fig. 2 that the calculated surface charge density increases with the concentration of KCl for a given surface pH (pH_0). The theoretical treatment of the relation between electrostatic surface phenomena and the membrane structure predicts that an increase in the ionic strength will have two effects [25]. The first one is the dissociation of an additional amount of anionic groups, due to a shift of pH_0; this effect is not to be taken into consideration here because the variation of σ upon an increase of [KCl] was observed for given pH_0 values (Fig. 2). The second predicted effect is the attenuation of the repulsive lateral interaction of the phospholipids, which results in a decrease of their mean molecular area, thus in an increase of the absolute σ value. This is the theoretical basis of our assumption (i).

The assumption (ii) of a perfect mixing of the charges is inherent to the Gouy-Chapman model [26]. It is implicitly supported by the large amount of work which shows that this model describes correctly the surface electrostatic behaviour of the biological membranes [18,25,27,28]. Various methods have been used to show that the binding site of ANS on biological membranes is lipidic. In one of these studies [23] the pK of the main membrane group involved in the surface polarization (as seen by the probe) was shown to be 4.0. Furthermore, the chemical masking of the carboxylic function or the proteinase hydrolysis demonstrated that the probe actually monitored the protein charges.

The observed value of 4.0 for the pK of $\sigma_{proteins}$ estimated from the comparison of horse bean liposomes and microsomes (Fig. 4) is typical of amino acids. The fact that ANS detects the contribution of protein charges in spite of its lipidic localization may be understood by the close vicinity of the site to the proteins. More surprising is the finding that this calculated protein charge is close, at neutral pH_0 values, to the one of phospholipids. This was confirmed by the NADH-cytochrome *c* oxidoreductase experiments. The approach of its catalytic site by the anionic substrate appeared to be impeded by exactly the same electrostatic potential as the one predicted from ANS. The very good agreement between the estimation of ψ_0 from ANS and various enzymic activities has been already observed [19,29]. This suggests either that the catalytic sites perceive predominantly the surface charge of lipid components or that the value of protein and lipid charges are close at neutral pH values. From our comparison of the effect of pH on σ of liposomes and microsomes, it seems that the latter hypothesis is the one to be kept. Thus we are led to think that the surface charge density is uniform above both the membrane components.

The isoelectric points of various animal cells are in the pH range 4.4–6.9 [30]. Few data are available for plant cells; the value deduced from the stacking of thylakoids of spinach [31], horse bean and yellow lupine [32] is 4.7. The low value (4.0) we report here for the microsomes of horse bean roots is matched only by some viruses [30].

We have shown [10] that the quantum yield of the ANS bound on the membranes from horse bean roots is not modified when the pH is lowered from pH 8 to pH 2, and that the intrinsic affinity for the probe does not vary (unpublished data). These results indicate that there is no major structural change on the lipid bilayer, at least in that part which forms the ANS site. They suggest that

the electrostatic effects are predominant.

The total depolarization of the membranes at pH 4, which contrasts to the nearly constant surface charge density in the physiological pH range may be expected to have functional effects for two reasons. First, the membrane systems respond to local ionic concentrations which depend on the surface potential. The second reason is that the transmembrane diffusion potential itself is controlled by the surface potentials [33–36]. In highly charged membranes (as are our microsomes), this effect may be more important than that of the relative ionic mobilities [37]. In these conditions, it may be supposed that depolarizing the surface of the membrane has important effects on its functions. As was indicated above, this study was prompted by the finding of a drastic increase in the membrane permeability of the horse bean roots below pH 4 [1]. We do not known whether the coincidence of the two events is merely circumstancial or significant of a causal relation. This point deserves further experiment.

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