Biochimica et Biophysica Acta, 643 (1981) 435—448
Elsevier/North-Holland Biomedical Press

BBA 79217

THE EFFECT OF FATTY ACIDS ON THE SURFACE POTENTIAL OF PHOSPHOLIPID VESICLES MEASURED BY CONDENSED PHASE RADIOLUMINESCENCE

VINZENZ VON TSCHARNER and GEORGE K. RADDA

Department of Biochemistry, University of Oxford South Parks Road, Oxford (U.K.)

(Received December 17th, 1980)

Key words: Phospholipid membrane; Surface potential; Fatty acid; Carboxyl group; Condensed phase radioluminescence

Summary

(1) The carboxyl group of fatty acids has a very low pK value, which is shifted into the physiological pH range when they are incorporated in a phospholipid membrane. As a result of a pH increase the surface charge and surface potential of the membrane increase.

(2) The titration of the carboxyl group was observed with condensed phase radioluminescence. This technique uses the electron emitted by the tritiated membrane probe (oleic acid or cholesterol) to excite a fluorophore also incorporated in the bilayer.

(3) The phase transition of dimyristoyl phosphatidylcholine vesicles labelled with 12-(9-anthroyloxy)stearic acid was measured by condensed phase radioluminescence at different pH values.

(4) We related the condensed phase radioluminescence signal to the asymmetrical distribution of the fluorophore between the inner and outer layer of the lipid membrane which is induced by the repulsion of the negatively charged fatty acids.

(5) We showed that the condensed phase radioluminescence signal is proportional to the protonation of the carboxyl group. On this basis, the broadening of the titration curve can be explained as an effect of the self-induced membrane potential calculated using the Gouy-Chapman theory.

(6) Ca^{2+} drastically reduces the flip-flop rate of fatty acids across the membrane and also causes a decrease in the asymmetric distribution.

(7) We concluded that a fatty acid can act as a membrane surface buffer. The pK value of 12-(9-anthroyloxy)stearic acid in a dimyristoyl phosphatidylcholine membrane is 7.0 ± 0.3.

(8) We discuss the results with respect to aggregation, fusion and clustering.

Introduction

Biological membranes contain small amounts of free fatty acids and fatty acids can be incorporated into natural and synthetic lipid membranes. Fatty acids with a probe (e.g., spin label, fluorophore, deuterium) covalently attached to them are often used to monitor membrane properties. It is therefore important to know their lateral and transmembrane distributions as well as their dynamic properties. These properties may then be related to the biological functions that fatty acids may have in membranes. Free fatty acids affect the permeability of the mitochondrial membrane [1]. They induce Cl^- transport in corneal epithelium and alter the membrane fluidity [2]. Fatty acids also induce cell fusion [3,4]. The differences between saturated and unsaturated fatty acids with respect to membrane permeability were observed by Schaeffer and Curtis [5]. Complete deprotonation of the carboxyl group is only reached at pH 11. Fatty acids therefore add a variable amount of surface charge to the membrane according to their concentration and the degree of deprotonation [6]. The increase in the apparent pK for the carboxyl group was attributed to clustering of the fatty acids [7], though others believe that fatty acids usually disperse homogeneously [8]. The carboxyl group seems to play a major role in the organization of fatty acids in the membrane. It is therefore important to know more about the effect of the carboxyl groups at different degrees of deprotonation at the surface of a lipid membrane. The problem is best considered in two parts.

First, the lipid membrane acts as a solvent for the fatty acids and we observe, as a result of deprotonation, only those properties of the fatty acid such as the asymmetrical distribution between the two layers of the membrane, the clustering of the probes or the influence of the self-induced surface potential.

Second, the labelling alters the properties of the membrane such as fluidity, permeability and surface property resulting in structural changes and in differences in interaction with other membranes (e.g., aggregation, fusion or exchange of molecules).

In this paper we report studies on the first aspect but will also discuss the consequences of the second.

In spherical lipid vesicles labelled with different fatty acid-type probes, the carboxyl groups can laterally diffuse and swap from one layer to the other. Therefore, we have a conductance in the membrane. In an ideal spherical conductor all charges are located at the outer surface. A chemical potential results from the asymmetric distribution of the probe between the two layers. The electrical potential can be calculated from the Gouy-Chapman theory [9]. Its application to a biological membrane has been described [10,11] and the appropriate equations are summarized in the Appendix.

As a result of the deprotonation of the carboxyl group a surface potential is generated, which reduces the surface pH compared to the bulk pH. This effect depends on the surface charge density and the ionic strength.

This effect accounts quantitatively for the broadening of the titration curve of fatty acids in the lipid membrane.

We use a recently presented method (condensed phase radioluminescence) which is sensitive to distance changes. In this technique, a fluorophore,

attached in our case to a fatty acid, is excited by an electron released from a tritiated molecule (fatty acid or cholesterol) which is also located in the same membrane. This radioluminescence is a well known physical mechanism [12]. We can only observe a radioluminescence signal if the local concentration of electron emitter and absorber is large enough. This condition is fulfilled in a lipid vesicle, where the probes are condensed in the lipid phase. The radioluminescence signal is related to the geometry of the vesicle and the concentration of the probes. For an emitter and an absorber located on a sphere of radius R and homogeneously distributed, the following relationship for the condensed phase radioluminescence intensity I holds [13]:

$$I = Z\phi\bar{A}\nu \frac{C_D}{C_L} \frac{R_0^2}{f_0} \frac{1}{R^2} \tag{1}$$

where Z is a constant including properties of the measuring instrument. ν is the activity of the observed sample. $\bar{A}$ is the mean cross-sectional area for the electron absorbance by the dye. ϕ is the fluorescnece quantum yield of the absorber. C_D and C_L represent the dye and the lipid concentrations, respectively. R_0 is the vesicle radius in the fluid phase where f_0 is the surface area per lipid.

We can rewrite Eqn. 1 in terms of the surface density of the probe:

$$I = Z\phi\nu\bar{A} \frac{N}{2\pi R^2} \tag{2}$$

where N represents the number of dye molecules on the sphere with radius R. Eqn. 1 has the advantage over Eqn. 2 that it relates the intensities to molar concentrations, but Eqn. 2 is more suitable for discussing the experiments.

This formula applies for a sphere with an infinitely thin shell. In a vesicle, R is an averaged radius for the location of the dye and the emitter. For a homogeneous distribution of the probes between the inner and outer layer of the vesicle, R is the radius at the centre of the bilayer. If the probes are moved to the outer layer, this radius is increased and the signal decreased. If two vesicles fuse and the surface density remains constant, we expect no change in intensity. According to Eqn. 2, a change in the signal will only happen if the density is altered because of the redistribution of the probes within the bilayer.

This equation does not account for changes in condensed phase radioluminescence intensity resulting from the clustering of the probes, but from previous measurements on micelles [13], we expect large changes when clustering takes place. The distances between vesicles are large and no intervesicle excitation is observed. Aggregation of vesicles will of course result in such intervesicle excitation.

Materials and Methods

N-Stearoyltryptophan was a gift from K. Thulborn and 12-(9-anthroyloxy)-stearic acid and 1-palmitoyl-2-(12-(9-anthroyloxy)octadecanoyl)-*sn*-glycero-3-phosphorylcholine (ASPC) were synthesized in our laboratory by M. Buckland. [9,10(n)-^{3}H]Oleic acid was bought from the Commissariat à l'Energie Ato-

mique, Siren, France. Samples of a specific activity of 50 Ci/mmol were used. [1α,2α(n)-^{3}H]Cholesterol (46 Ci/mmol) was bought from the Radiochemical Centre, Amersham, U.K. Dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine were obtained from Koch Light, Ltd., and were used without further purification. The buffer was a mixture of 0.01 M piperazine, 0.01 M imidazole and 0.01 M barbital, adjusted to the desired pH with HCl.

Phospholipid vesicles were prepared by cosonicating the probes with the lipids into the buffer [14]. The pH was measured with a DATEX IM 555 pH-meter at the beginning and at the end of a measurement. Intermediate pH values were adjusted by adding calibrated amounts of HCl. Fluorescence measurements were made on a Perkin Elmer MPF 2A fluorescence spectrometer. Condensed phase radioluminescence measurements were made with a single-photon counting detector as described elsewhere [15].

Results

Fluorescence measurements. Dimyristoyl phosphatidylcholine vesicles (400 μM) were labelled with 12-(9-anthroyloxy)stearic acid (5 μM) and *N*-stearoyltryptophan. The latter molecules were excited by light of 292 nm wavelength and the emission was observed at the emission maximum for 12-(9-anthroyloxy)stearic acid (440 nm). The energy transfer from *N*-stearoyltryptophan to 12-(9-anthroyloxy)stearic acid obeys the Förster mechanism [16]. The molecules become more densely packed as the lipid phase changes from the fluid to the gel phase. Therefore, one observes a signal increase which is larger than in condensed phase radioluminescence because of the r^{-6} relationship of this mechanism. Above the phase transition, the intensity decreases linearly with temperature because of the change in quantum yield of 12-(9-anthroyloxy)-stearic acid [13]. The rate of decay was 1.2%/degree Celsius. Below the phase transition, the signal stays constant. A change of the pH from 9 to 5 results in a decrease in the signal of less than 5% at all temperatures. Addition of 1.1 mM $CaCl_2$ at pH 9 had no effect upon the signal. Dimyristoyl phosphatidylcholine vesicles were labelled with 12-(9-anthroyloxy)stearic acid as the fluorescent probe. The probe has a carboxyl group exposed to the solvent at the surface of the bilayer. A comparison of the fluorescence in the liquid crystalline phase of the vesicles (λ_{ex} = 364 nm; λ_{em} = 460 nm) showed no measurable difference in intensity at low and high pH values:

$$I\,(\mathrm{pH} \leqslant 4) = I\,(\mathrm{pH} \geqslant 10) \pm 3\%$$

The 3% uncertainty is mainly caused by the bleaching effect of the probes and is especially pronounced at low pH values [17]. From these two measurements we conclude that the dimyristoyl phosphatidylcholine vesicles do not drastically change their structure in the liquid crystalline phase by an alteration from pH 3 to 11, nor does the 12-(9-anthroyloxy)stearic acid probe change its fluorescence properties.

Condensed phase radioluminescence measurements. Dimyristoyl phosphatidylcholine vesicles labelled with 12-(9-anthroyloxy)stearic acid and tritiated oleic acid were titrated from pH 10 to 3. The change in the condensed phase radioluminescence signal is caused by the protonation at the carboxyl group of

the probe. The condensed phase radioluminescence intensity increased with decreasing pH (up to pH 5.5), but below this value no further change was observed (Fig. 1). The midpoint of the titration was at pH 7.3 ± 0.3. The shape of the titration curve is asymmetric and broad and cannot be fitted with a single equilibrium equation. A practically identical titration curve was obtained when the tritiated oleic acid was replaced by tritiated cholesterol, which is not charged. Addition of CH_3NH_2 (16 mM), which abolishes the pH gradient across the vesicles, had no effect on the titration curves. The membrane potential will vanish with time, yet the observed signal is stable for hours. The change in signal with pH is not caused by a pH gradient or a membrane potential. Replacing 12-(9-anthroyloxy)stearic acid by the uncharged ASPC and using tritiated cholesterol as an emitter resulted in no change in intensity between pH 9 and 5. Under these conditions, the vesicles tended to aggregate, but shaking the samples after 20 min brought the signal down to the initial value. The initial values are represented in Fig. 1. The error bars represent the large scattering of the measurement caused by aggregation. A small change in intensity was observed when 60 mM NaCl was added at pH 9 before the titration (+5%).

The change in condensed phase radioluminescence signal with temperature reflects not only the structural changes of the vesicles but is also caused by the change in fluorescence quantum yield (Eqn. 1). The quantum yield of 12-(9-anthroyloxy)stearic acid is not temperature dependent when the probe is in the gel phase of a phospholipid vesicle, but decreases linearly when the probe is in the fluid phase. The phase transition of dimyristoyl phosphatidylcholine vesicles measured with condensed phase radioluminescence at pH 9 shows the characteristic change expected from the variation in quantum yield at the phase transition. A nearly constant intensity can be observed below and a linear decay is seen above the critical temperature. No intensity change related to the radius change was observed (see Eqn. 1 and Fig. 2). After cooling to 15°C, a steady increase in signal was observed over 1 h. The cooling curve remained nearly parallel to the heating curve; a difference in slope is observed between the heating and cooling cycle in the gel phase only. In contrast, the condensed phase radioluminescence intensity at 35°C remained constant to within 1% over

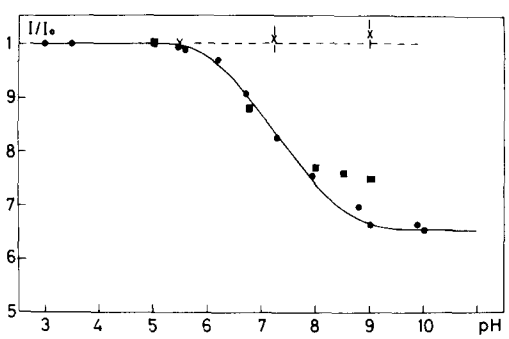

Fig. 1. Titration of dimyristoyl phosphatidylcholine (400 μM) vesicles labelled with: •, 5 μM 12-(9-anthroyloxy)stearic acid and 46 μCi/ml oleic acid; ▪, 5 μM 12-(9-anthroyloxy)stearic acid and 60 μCi/ml cholesterol; ×, 5 μM 1-palmitoyl-2-(12-(9-anthroyloxy)octadecanoyl-*sn*-glucero-3-phosphorylcholine, and 53 μCi/ml cholesterol observed by condensed phase radioluminescence. I_0 is the intensity when the solution was acidic. The solid line is calculated from the Gouy-Chapman theory with c = 15 mM, T = 308 K, C_D/C_L = 5/400, pK = 7.0.

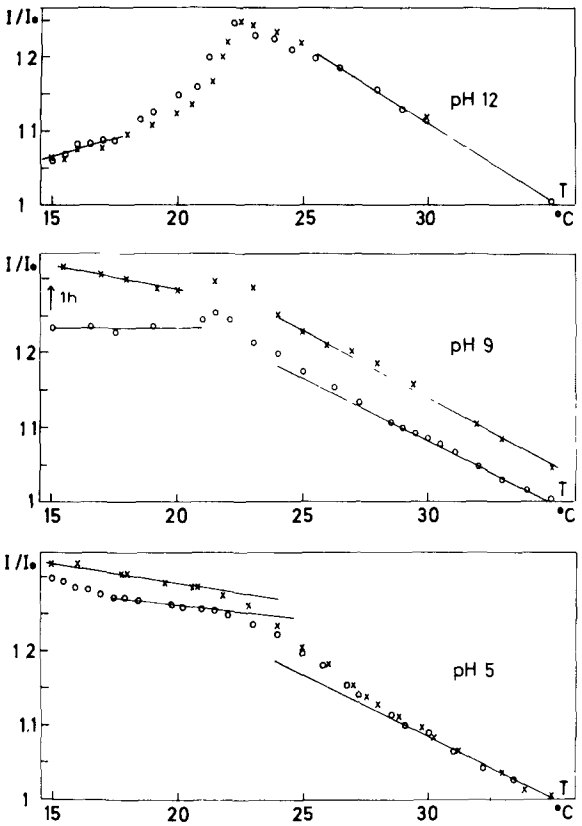

Fig. 2. The phase transition of dimyristoyl phosphatidylcholine (200 μM) vesicles labelled with 12-(9-anthroyloxy)stearic acid (5 μM) and tritiated oleic acid at three different pH values. I_0 is the intensity at 35°C. ○, cooling; ×, heating.

15 h. No aggregation of the vesicles was observed. At pH 10 and above, a signal increase at the phase transition is superimposed on the quantum yield change of 12-(9-anthroyloxy)stearic acid (illustrated in Fig. 2 for pH 12 only). A very different pattern is observed at pH 5. Below the phase transition the curve is nearly horizontal, but as the temperature is further decreased to 15°C, the signal slowly increases. In the heating cycle the intensity regains the initial values above the phase transition. This small effect could be attributed to some sort of clustering of the probe or to a structural change in the lipid phase [18].

At the critical temperature, the signal reflects the change caused by the variation of the surface area induced by the change in phase [13]. The relative change of the intensity measured over the transition is about 7%, which corresponds to the value measured at pH 7. At pH 5, 4 mM $CaCl_2$ at 35°C did not change the condensed phase radioluminescence intensity at all over the next hour. There is no binding site for Ca^{2+}, because all carboxyl groups are protonated.

This Ca^{2+} binding is different at high pH values. At pH 8.1 a change in the condensed phase radioluminescence signal after addition of Ca^{2+} can be ob-

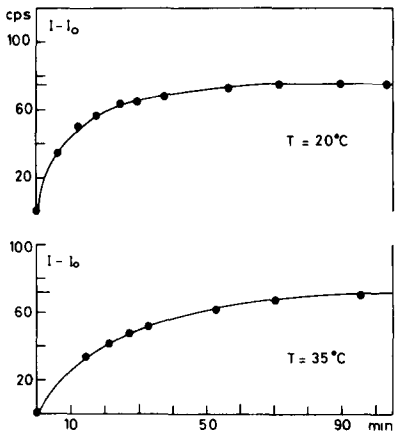

Fig. 3. The time course of the condensed phase radioluminescence signal after addition of 1 mM Ca^{2+} to dimyristoyl phosphatidylcholine (600 μM) vesicles labelled with 4.1 μM 12-(9-anthroyloxy)stearic acid and tritiated oleic acid (95 μCi/ml).

served (Fig. 3). An exponential increase with a τ value of 15 min was found in the gel phase (20°C) and with a τ value of 30 min in the liquid crystalline phase (35°C). After the equilibrium value was reached, a further addition of more than 1 mM $CaCl_2$ induced no change. An increase in acidity to pH 5 caused a further increase. The kinetics of this last effect were too fast to be observed with our instrument ($\tau < 60$ s).

The normalized plots of the condensed phase radioluminescence intensity vs. temperature measurements above the critical temperature were equal for solutions with and without Ca^{2+}.

Discussion

We have seen that a pH titration of dimyristoyl phosphatidylcholine vesicles labelled with 12-(9-anthroyloxy)stearic acid showed no change in fluorescence quantum yield nor in energy transfer measurements from *N*-stearoyltryptophan to 12-(9-anthroyloxy)stearic acid. We conclude from these measurements that the vesicles are stable over a pH range from 3 to 11 and that no structural change altering the packing density of lipids had occurred. The only technique which responded to the pH change was condensed phase radioluminescence measurement. Hauser and Guyer [6] reported from electrophoretic measurements that the carboxyl groups of fatty acids in vesicles are not fully deprotonated up to pH 11. There has been no attempt to calculate quantitatively the broadening of the titration curve resulting from the clustered fatty acids.

They attributed the p*K* shift of the carboxyl group to the p*K* change caused by clustering of the fatty acids. In the liquid phase the relative changes of intensities measured by all these techniques (fluorescence, condensed phase radioluminescence and energy transfer) are equal (1.4 ± 0.2%/degree Celsius) within the experimental limits. In all three cases the change results from the fluorescence quantum yield change of 12-(9-anthroyloxy)stearic acid. If the

clusters melt and the relative distances change between the probes, we would observe a different dependence on temperature by each technique. Passing from the fluid to the gel phase the membrane clusters would remain or even increase [7], especially in the low pH range where no more electrostatic repulsion is present. If the quantum yield change represents the property of the inside of the clusters, then we would expect that this property would at least partly continue in the gel phase. This is similar to immiscible lipids where each type melts at its typical temperature, in spite of the presence of the other. Because we see a clear break at the lipid phase transition, this gives us no evidence for clustering. In the neutral to acidic pH region, cooling curves are horizontal in the gel phase in fluorescence, condensed phase radioluminescence and energy transfer measurements. Therefore, the same argument as before is valid and their is no immediate formation of clusters. Within 1 h the intensity increases in the gel phase. At pH 5 this increase disappears again by reheating the sample. This is the type of property expected for the clustering of the probes. We therefore conclude from our measurement that in the liquid crystalline phase we have no clustering of 12-(9-anthroyloxy)stearic acid. This is in agreement with photobleaching data showing that clustering of 12-(9-anthroyloxy)stearic acid does not happen in the fluid phase [17] and calorimetric data which show that fatty acids do not cluster [8].

The binding of Ca^{2+} does not alter the change in quantum yield observed in the fluid lipid phase. We conclude that Ca^{2+} binding does not induce clustering of the probe. In summary, we have no firm evidence for any probe clustering of 12-(9-anthroyloxy)stearic acid and oleic acid or cholesterol in either phase of dimyristoyl phosphatidylcholine vesicles.

In a neutral to acidic environment, the change in condensed phase radioluminescence signal observed around the phase transition temperature was attributed to the change in size of the vesicles. The relative change in intensity is theoretically equal to the relative change of the surface area if the emitter and absorber are thought to be homogeneously distributed over a sphere.

We can therefore conclude that the condensed phase radioluminescence signal increase observed with decreasing pH is not a result of clustering, nor of a quantum yield change or of the binding of the emitter to the absorber. We have to find another explanation.

We believe that the change in signal is caused by a redistribution of the probes between the inner and the outer layer of the membrane after a change in protonation of the carboxyl groups. The negatively charged carboxyl groups in a vesicle behave like the charges on a conducting sphere; they are forced by their mutual repulsion to stay in the outer layer. By protonating these groups some of the fatty acids swap to the inner layer of the membrane, thereby reducing the asymmetric distribution. The mean radius of the sphere formed by the dyes which are attached to the fatty acids becomes smaller. Therefore, we expect, according to Eqn. 2, a large increase in condensed phase radioluminescence intensity with protonation of the carboxyl groups. Superimposed would be the effect of a change in quantum yield, but because the environment of the dye is very much the same in the inner and the outer layer, there is no change in quantum yield.

First, we derive how condensed phase radioluminescence signal changes with

deprotonation. N_0 is the number of uncharged dyes and their mean radius is R_0. $N_1 = N - N_0$ is the number of charged dyes and their mean radius is R_1. If we split Eqn. 2 into two components, one for the charged particles and one for the uncharged, we find:

$$I = Z\nu\phi\bar{A}(N_0/2\pi R_0^2 + N_1/2\pi R_1^2) \tag{3}$$

$$I = \frac{Z\nu\phi\bar{A}N}{2\pi}\left(\frac{1}{R_1^2} + \frac{N_0}{N}\left(\frac{1}{R_0^2} - \frac{1}{R_1^2}\right)\right)$$

where N_0/N is the degree of protonation, and Eqn. 3 indicates that the condensed phase radioluminescence intensity increases linearly with protonation. The titration curve is too broad and asymmetric to be fitted by a simple equilibrium of the protonated and deprotonated form. This distortion is caused by the charged carboxyl groups forming a surface charge density which changes the pH at the surface. According to the Gouy-Chapman theory, which was successfully applied to lipid membranes [10], a surface potential is generated. The surface potential gradually decreases the pH at the membrane surface compared to the bulk pH. This self-induced pH change can only occur if the charges remain in the membrane plane. The solid line in Fig. 1 is calculated from the Gouy-Chapman theory and the agreement with the measurement is obtained without further assumptions (see Appendix). We applied the Gouy-Chapman theory as it was derived for a flat surface. A spherical surface can be treated like a flat one if the Debye length [9] is small compared to the radius of curvature. With the concentration of salt we used, the Debye length is of the order of the membrane thickness. Therefore, we think that the principal feature of the titration curve as calculated for a flat surface would not be significantly different if calculated in spherical coordinates. Most of the errors will be based upon the fact that we did not include the gradual change in the asymmetric distribution in the calculation for the surface charge changes (Fig. 4). All these refinements can be done, but would lead to a cumbersome analysis without any further improvement in understanding the events.

If all the carboxyl groups are in the outer membrane, the surface charge is

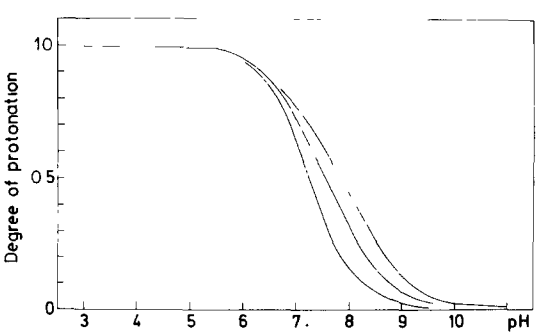

Fig. 4. Degree of protonation calculated from the Gouy-Chapman theory for the titration of the carboxyl group at the membrane surface. pK = 7.25, T = 308 K, n = 30 mM, C_D/C_L = 0, 5/400, 10/400, respectively.

twice as large as the one we would find for a homogeneous distribution. This is probably the reason why the measured values at large pH are slightly greater than those calculated.

The ratio of the intensities of the fully protonated form (I_1) to the fully deprotonated form (I_0) is according to Eqn. 3:

$$I_1/I_0 = R_0^2/R_1^2 \tag{4}$$

We measured $I_1/I_0 = 0.7 \pm 0.05$ and from Eqn. 4 we find $R_0/R_1 = 0.84$. This corresponds to a difference between R_1 and R_0 of about 19 Å in a vesicle of $R_0 = 100$ Å (see Table I). Because half the thickness of the bilayer is the maximum possible difference between R_0 and R_1, the results show that most (more than 80%) of the charged labels are located in the outer membrane.

The small change in intensity observed in the energy transfer measurements between *N*-stearoyltryptophan and 12-(9-anthroyloxy)stearic acid is a result of two compensating effects. First, all molecules are deprotonated and form a single layer. Then, as a result of protonation, this layer is split in two. This causes a decrease in intensity. Second, the newly formed layer has a smaller radius and, therefore, the intensity should increase. The calculation in Appendix II shows that under certain conditions the two effects compensate. We did not try to find conditions where a significant change in signal could be observed because very little new information could have been obtained. A major change in the intensity is expected, if the labels cluster.

There is a less asymmetric distribution of the dye in a large vesicle than in a small one because in the limit of an infinite radius the layers are parallel and cannot have any asymmetric distribution. The density of the dye can increase with growing size of the vesicles. This effect is observed at the phase transition when all carboxyl groups are deprotonated. The radii of the vesicles increase on going from the gel to the liquid crystalline phase. The condensed phase radioluminescence signal should decrease, according to Eqn. 2, and increase because there is a change in the asymmetric distribution.

Over the phase transition at pH 9 the two processes compensate each other. Above pH 9 the change in asymmetric distribution becomes dominant and at pH 5 we mainly observe the change related to the size of the vesicle (Fig. 2). We attributed the difference between the heating and cooling curves at pH 9 (Fig. 2) to the fusion of vesicles forming larger unilamellar vesicles and changing the asymmetric distribution. In these larger vesicles the asymmetric distribution is reduced and the intensity therefore increases. This conclusion is reached because of the following: (i) clustering, or structural changes, would disppear by reheating; (ii) possible aggregation of vesicles can be eliminated by shaking the sample, which has no effect on the observed curves; (iii) we have already eliminated the possibility of oleic acid binding to 12-(9-anthroyloxy)-stearic acid.

The charged carboxyl groups are not preventing vesicle fusion but do prevent aggregation. To explain why fusion occurs only with the presence of charged groups, one could imagine two vesicles colliding and at the point of collision the charges have to leave the area of inflection because of the electrostatic repulsion. Their diffusion is hindered in the gel phase and a rupture of the membrane is the consequence of the collision. The vesicles then immediately

fuse together. According to the presented model the addition of salt or calcium does not increase the condensed phase radioluminescence signal in the acidic pH region but does increase it in the basic pH range. The flip-flop rate induced by a pH jump was too fast to be resolved (40 s minimum accumulation time). The binding of calcium to the carboxyl groups changes the surface charges. These carboxyl groups would tend to swap, but because of the bulky positively charged calcium, the flip-flop rate is much slower than for the neutral protonated form. From the faster rate observed in the gel state we conclude that fluidity is obviously not the major parameter for the mechanism following calcium binding.

Conclusion

We have seen that the fatty acids we used behave like 'ordinary fatty acids' [7] in the sense that their pK is shifted to the physiological pH range and that their protonation takes place over a wide pH range. We have no indication for clustering in the liquid phase of the membrane. Only in that sense do they resemble the 'spin-labelled probes' described by Hauser et al. [7]. The fatty acids we used behave to a large extend according to their electrostatic interaction, and so do — we believe — the ordinary fatty acids in general.

The mutual repulsion of the carboxyl groups results in an asymmetric distribution between the inner and the outer layer of the vesicle. This asymmetric distribution could be used to follow the protonation of the carboxyl groups. As a result of the deprotonation a surface potential is generated which induces a pH shift at the membrane surface. The intrinsic pK for 12-(9-anthroyloxy)-stearic acid is 7.0 ± 0.3. It is essential to realize that the fatty acids incorporated in a liquid layer have a pK which is about two pH units larger than the usual ones observed for carboxyl groups in water. It is not the clustering which causes the initial pK shift but the bipolar headgroup environment.

The wide physiological pK range obtained by the self-induced pH shift can be important in regulating the surface potential of a membrane. Fatty acids can work like a membrane surface buffer in the physiological pH range. Such a membrane buffer can be vital for a biological membrane. An example is the inhibition of the proton pump when the surface charge is shifted [19].

Calcium specifically binds to the carboxyl group of fatty acids. The resulting change in asymmetry implies that Ca^{2+} is carried through the membrane. The process is probably too slow to induce a biological effect.

Biological membranes which act as a barrier for calcium should contain very low amounts of free fatty acids to prevent a continuous calcium influx. This is in agreement with measurements made on chromaffin granules [20].

Appendix

The membrane potential ψ^0 can be calculated from the equation:

$$\psi^0 = \frac{2kT}{e} \operatorname{arc\,sinh}\left(\frac{\sigma}{c}\right) \tag{A1}$$

$$c = \frac{\epsilon}{2\pi} \frac{kT}{e} \hat{\chi} \tag{A2}$$

$$\hat{\chi}^2 = \frac{8\pi e^2}{\epsilon kT} n \quad \text{(for a monovalent salt)} \tag{A3}$$

where n is the concentration of the salt; e, ϵ, k, T have their usual meaning; σ represents the surface charge density and is related to the dye-to-lipid ratio and the degree of deprotonation α.

$$\sigma = \frac{C_D}{C_L} \frac{e}{f_0} \cdot \alpha \tag{A4}$$

$$\alpha = K_0/(K_0 + [H^+]_b \exp(e\psi^0/kT)) \tag{A5}$$

where K_0 is the equilibrium constant for the deprotonation. The calculation of α for a certain pH is found by an iterative procedure. We start with $\psi^0 = 0$ in Eqn. A5 and calculate ψ^0 from Eqn. A1. The new value of ψ^0 is then used in Eqn. A5. About 30 iterations are necessary. The results for three different values of C_D/C_L are shown in Fig. 4.

Appendix II

The estimation of the difference in energy transfer measurements by the change in the asymmetric distribution of the sensitizers (S) and the absorbers (A) in a lipid membrane can be derived as follows. The intensity (I) of a single layer of sensitizers and absorbers depends upon the mean distance between sensitizer and absorber. In a static model the mean distance between any label sensitizer or absorber is:

$$r = (N_S + N_A)/F)^{-1/2}$$

with $N = N_A + N_S$, $r = (N/F)^{-1/2}$, N_S and N_A represent the respective amount of sensitizer and absorber label on the surface F. $F = 2\pi R^2$, where R is the mean radius of the labels in the vesicle. The probability that the label in distance r from the sensitizer is an absorber is N_A/N. Assuming a Förster mechanism for the energy transfer, we find that for N_S sensitizer molecules the intensity obeys the proportionality:

$$I \sim \frac{N_S N_A}{N} \left(\frac{N}{F}\right)^3$$

For equal amounts of sensitizer and absorber molecules this becomes:

$$I \sim N^4/F^3 \tag{B1}$$

We now imagine this layer of labels to be formed by two layers, each containing half the labels. Then the intensity is divided in three terms, the two of each separate layer and one of the interaction between the two layers. The final intensity must be the same.

$$I_1 = I_1^{(1)} + I_1^{(2)} + I_1^{(12)} \tag{B2}$$

From Eqn. B1 we find:

$$I_1^{(1)}/I_1 = I_1^{(2)}/I_1 = \frac{(N/2)^4/F_1^3}{N^4/F_1^3} = 0.062$$

$$I_1^{(12)}/I_1 = (I_1 - I_1^{(1)} - I_1^{(2)})/I_1 = 0.87$$

The upper index represents the number of the layer. The lower index is unity if both layers are together and in the outer part of the bilayer. It is zero if the two layers are separated by the distance $d = 2(R_1 - R_0)$, where R_1 and R_0 are the same as in Eqn. 3. Now we calculate I_0 where the two layers are separated by the distance d. Half the layer remains in place and its intensity $I_0^{(1)}$ is not altered. The intensity of the second half increases according to Eqn. B1

$$I_0^{(2)} = I_1^{(2)}(F_1^{(2)}/F_0^{(2)})^3 \tag{B3}$$

For parallel layers of sensitizer and absorber molecules the following equation holds [21]:

$$I_0^{(12)} = I_1^{(12)}\left(1 + \left(\frac{d}{d_0}\right)^4\right)^{-1} \tag{B4}$$

d is the distance between the teo layers and d_0 is the distance at which one out of two sensitizer molecules loses its energy by energy transfer to the acceptor. In our case each layer contains the sensitizer molecules to excite the absorber molecules in the other one. Using Eqns. B2—B4 we can calculate the ratio:

$$\frac{I_0}{I_1} = \frac{I_0^{(1)}}{I_1} + \frac{I_1^{(2)}}{I_1}(F_1^{(2)}/F_0^{(2)})^3 + \frac{I_1^{(12)}}{I_1}\left(1 + \left(\frac{d}{d_0}\right)^4\right)^{-1} \tag{B5}$$

In Table I we calculated the values I_1/I_0 for different radii. We can compare the maximum intensity ratios expected from condensed phase radioluminescence measurements with those expected from the energy transfer measurements. The changes in intensity expected from the energy transfer measurements are small compared to those expected from condensed phase radio-

TABLE I

The values for I_1/I_0 ET (ET, energy transfer) are listed for different radii. The I_1/I_0 ET values are smaller than I_1/I_0 condensed phase radioluminescence (CPR) for d_0 = 35 Å, 110 Å $\leq R_1 \leq$ 140 Å and $R_1 - R_0$ = 20 Å.

R_1	R_0	I_1/I_0 ET	I_1/I_0 CPR
110	90	0.75	0.67
115	95	0.84	0.68
120	100	0.92	0.69
125	105	0.99	0.70
130	110	1.05	0.72
135	115	1.12	0.73
140	120	1.17	0.73

luminescence measurements if we assume a reasonable d_0 value [21].

Eqn. B1 is only valid for calculating the intensity ratios of the two extreme cases. It is not meant to be used for intermediate label distributions. The only target of this calculation is to show that under certain reasonable conditions it is not surprising that our energy transfer measurements showed only small changes with pH.

References

1 Wojtzak, L. (1976) J. Bioenerg. Biomembranes 8, 293—311
2 Schaeffer, B.E. and Zadunaisky, J.A. (1979) Biochim. Biophys. Acta 556, 131—143
3 Ahkong, Q.F., Cramp, F.C., Fisher, D., Howell, J.I., Tampion, W., Verrinder, M. and Lucy, J.A. (1973) Nat. New Biol. 242, 215—217
4 Kantor, H.L. and Prestegard, J.H. (1975) Biochemistry 14, 1790—1794
5 Schaeffer, B.E. and Curtis, A.S.G. (1977) J. Cell Sci. 26, 47—55
6 Hauser, H. and Guyer, W. (1979) Biochim. Biophys. Acta 553, 359—363
7 Hauser, H., Guyer, W. and Howell, K. (1979) Biochemistry 18, 3285—3291
8 Kantor, H.L. and Prestegard, J.H. (1978) Biochemistry 17, 3592—3597
9 Kortüm, G. (1972) Lehrbuch der Elektrochemie, Verlag Chemie, Weinheim
10 Träuble, H. (1977) in Structure of Biological Membranes (Abrahamsson, S. and Pascher, I., eds.), Vol. 34, pp. 509—550, Nobel Foundation Symposium
11 Fromherz, P. and Masters, B. (1974) Biochim. Biophys. Acta 356, 270—275
12 Haissinsky, M. (1969) Actions Chimiques et Biologiques des Radiations, Masson, Paris
13 Von Tscharner, V. and Radda, G.K. (1980) Biochim. Biophys. Acta 601, 63—77
14 Bashford, C.L., Morgan, C.G. and Radda, G.K. (1976) Biochim. Biophys. Acta 426, 157—172
15 Harris, D., von Tscharner, V. and Radda, G.K. (1979) Biochim. Biophys. Acta 548, 72—84
16 Latt, S.A., Cheung, H.T. and Blout, E.R. (1965) J. Am. Chem. Soc. 87, 995—1003
17 McGrath, A.E., Morgan, C.G. and Radda, G.K. (1976) Biochim. Biophys. Acta 426, 173—185
18 Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 466, 381—392
19 Schaefer, G. and Rowohl-Quishoudt, G. (1976) J. Bioenerg. 8, 73—81
20 Buckland, R.M., Radda, G.K. and Shennan, C.D. (1978) Biochim. Biophys. Acta 513, 321—337
21 Weissberger, A. and Rossiter, B.W. (1972) Physical Methods of Chemistry, Part IIIB, Optical, Spectroscopic and Radioactivity Methods (Rossiter, B.W., ed.), pp. 600—605, Wiley-Interscience, New York