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A STUDY OF CHANGES IN SURFACE AREA AND MOLECULAR INTERACTIONS IN PHOSPHOLIPID VESICLES BY CONDENSED PHASE RADIOLUMINESCENCE

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Summary

(1) The radioactive decay of tritium was used to excite 12-(9-anthroyloxy)-stearic acid. The resulting radioluminescence was observed by single-photon counting. A signal can only be observed if the emitting tritium is close enough to the absorbing fluorophore. This is accomplished by condensing the emitter and absorber into a lipid membrane. Therefore, we call the technique Condensed phase radioluminescence (CPR).

(2) We present the theoretical background for the observed CPR signals.

(3) We observed a large CPR signal when tritiated oleic acid was added to 12-(9-anthroyloxy)stearic acid micelles.

(4) The phase transition of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in unilamellar vesicles can be monitored with CPR, and the relative intensity change observed is directly related to the relative surface change at the centre of the bilayer.

(5) Oleic acid and 12-(9-anthroyloxy)stearic acid exchange between dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles. The labels show no real preference for the fluid phase of the dimyristoyl phosphatidylcholine vesicles.

(6) CPR is a powerful method for studying distance and binding relationships in membranes.

Abbreviation: CPR, Condensed phase radioluminescence.

Introduction

Scintillation is widely used in biochemistry, mainly to count the amount of radioactive label in the system [1,2]. In scintillation, the energy of the decaying particle is transferred to a fluorescent dye by appropriate solvents. Without the use of such solvents, the dye can be excited directly by the highly energetic particles after the decay but the fluorescence emission is hardly measurable. The excitation process of radioluminescence is very complex [3] and some aspects of it will be discussed in this article.

Here we present a new way of using radioluminescence in lipid membranes where a fluorescent probe is excited by the β-decay of tritium. This excitation only occurs with reasonable probability if the dye and the tritium, hereafter called emitter, are close. In contrast to light, only selected areas are 'illuminated'. This situation is achieved if the dye and the emitter are 'condensed' into a phospholipid bilayer. The emitter could be any tritiated lipid or other tritiated molecule which is preferentially located in the bilayer. We call the method, using radioactive decay to excite a fluorophore where the emitter and absorber are not randomly distributed in the solvent but condensed in a supporting system (membrane, protein, etc.), Condensed phase radioluminescence (CPR). The CPR intensity is measured in cpm.

In this paper we first demonstrate that by using tritiated oleic acid and 12-(9-anthroyloxy)stearic acid the formation of dye micelles can be qualitatively followed. We then derive the quantitative relationships that are tested by trapping the emitter and absorber in phospholipid vesicles.

We show how CPR can be used to measure changes in the surface area of phospholipid vesicles (for example, during a gel-to-liquid crystalline phase transition), and to follow exchange of labels between vesicles.

Having first tried the qualitative method for checking micelle formation, we adopted the quantitative analyses for the data obtainable from dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles. In these vesicles we can verify that the basic relationships for CPR developed here are correct. The radius of a vesicle is changing at the phase transition and the thickness of the membrane is reduced by the passage from the gel to the liquid-crystalline state [4]. CPR can provide us with information about the surface changes because, as the distance between the emitter and absorber increases, the intensity will decrease.

The parameters that govern CPR

The CPR intensity is dependent on the specific activity of the sample (a) and the cross section ($\vec{A}$) of the dye for the emitted electron which is dependent on the velocity of the electron. The vector character of this quantity reflects the properties of the mean transition dipole moment of the dye [5]. The distance between the emitter and absorber is $\vec{r}$ and the steric angle under which the cross section is seen by the emitter is:

$$\frac{1}{4\pi} \cdot \frac{\vec{A}\vec{r}}{r^3}$$

Since the β-particles are stopped in the solvent, the probability that the elec-

trons reach a distance r depends on their initial energy. When they reach the dye they have a new energy E. To account for these two effects we have to multiply the term above by the relative amount of electrons $f(r,E)$ that reach a distance r and, having reached the target, have the energy E. As the β-particles are not monoenergetic, one must integrate over all energies of the β-spectrum. The number of excited states corresponds to the sum over all possible absorbers and emitters. The decay of these excited dyes is described in the same way as in fluorescence by the quantum yield ϕ. The intensity in CPR can then be described by Eqn. 1:

$$I = a\phi/(4\pi) \cdot \Sigma_{\text{emitter}} \Sigma_{\text{absorber}} \int_{E=0}^{E=\max} (\vec{A} \cdot \vec{r}/r^3) f(r, E)\, dE \qquad (1)$$

We shall see that this general equation reduces to more manageable relationships when applied to specific problems. The most suitable β-decay for CPR is from tritium which has the lowest endpoint energy. Therefore, the electrons only reach a limited distance. The second advantage of using tritium is its short lifetime. The specific activity is high and the amount of tritium used to get a signal above the noise level can be kept to reasonable amounts. Because of the low endpoint energy of tritium the stopping radiation ('bremsstrahlung') is very low and causes few problems. As this stopping radiation is a limiting factor for CPR resolution, we only used tritiated probes. CPR intensities are of the order of the stopping radiation and therefore we need single-photon counting for signal detection.

Materials and Methods

Dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine were from Koch-Light Ltd. and were used without further purification. 12-(9-Anthroyloxy)stearic acid was synthesized in this laboratory [6]. [9,10-^{3}H(n)]oleic acid (2.1 Ci/mmol) was bought from the Radiochemical Centre, Amersham. Samples of a specific activity of 50 Ci/mmol were purchased from the Commisariat a l'Energie Atomique, Siren, France.

Phospholipid vesicles were prepared by cosonicating the probes with the lipids into 10 mM Hepes buffer adjusted to pH 7.0 [7]. Labels added after the vesicle preparation were in an ethanolic solution.

An apparatus with a temperature-controlled stopped-flow cell (5 ml) in front of an RCA 8850 phototube was constructed specially for measuring self-emitting solutions [8]. The phototube had the sensitivity to count single photons. The pulses were amplified by an Ortec Timing Filter Amplifier (model 454). The low-intensity pulses were discriminated by an Ortec Timing SCA (model 420a) and the remaining pulses were counted in Multichannel Analyzer (Ortec Model 6220). The data thus obtained were transferred to a PDP 11/05 computer and stored on papertape. The dark counts of the phototube were further reduced by cooling the phototube with gas from boiling liquid nitrogen. A dark count level of 25 cps was obtained with the shutter closed. A χ^2 test was used to make sure that the signal was random. Having done this, we know that the signals will obey pure counting statistics. The ideal reference to com-

pare the data from instrument to instrument is the amount of stopping radiation. This is proportional to the activity in the cell and is, in an aqueous solution, not significantly dependent on the solute. In our instrument we measure a stopping radiation of 1.23 ± 5% cps/μCi per ml in a glass cell. This was determined with tritiated water and with the samples before any dye was added. To compare this value with that from another apparatus one must also know the spectral-response curve of the phototube. The CPR signal will differ from one machine to another according to the ratio of their stopping radiation. Cell constants and geometrical aspects will all be included in such a comparison.

Theoretical Section

The β-decay of tritium and the effect of inelastic scattering on CPR

Not all electrons released by the tritium after a β-decay have the same velocity, they have a range of kinetic energies. A common method for presenting spectra are Fermiplots [9]. $(N/f)^{1/2}$ is plotted against E (N = number of electrons with an energy, E; f = Fermifunction) [10]. The Fermiplot for tritium gives a straight line and has been measured to a high degree of accuracy at energies higher than 1 keV. The endpoint energy is 18.65 keV which corresponds to the fastest possible velocity of an electron released from tritium. From the linear Fermiplot we calculated N for $E > 1$ keV [11]:

$$N(E) \sim (18.65 - E)^2/[1 - \exp - (2.15/E)] \tag{2}$$

where E is the initial energy of the electron.

If the distance r between the emitter and absorber is increased, a certain number of electrons will no longer reach the absorber. Therefore, we want to know what percentage of electrons can reach the absorber. The range of penetration only depends on the density of the matter. The range R (mg/cm^2) can be approximated from measurements made in aluminium [12–14]. For energies below 20 keV, we linearly extrapolated the graph described by Ref. 13 and used a density of 1 g/cm^3 for our solvent.

$$\log (r(\text{Å})) = 2.139 + 2.092 \log (E(\text{keV})) \qquad 1 < E < 20 \text{ keV} \tag{3}$$

We call the number of electrons travelling further than 100 Å 100% and calculate from Eqns. 2 and 3 the relative amount which reaches a distance greater than r (Fig. 1b). A change of 10% in r for $r \leqslant 1000$ Å causes less than 1% effect on the number of electrons reaching the absorber, but causes 10% change in steric angle.

The problem becomes more serious if we focus our attention on the cross-section for inelastic scattering. On a quantum-mechanical basis, $\vec{A}$ could be calculated for a specific dye [5].

$$\frac{\vec{A} \cdot \vec{r}}{r_2} = 8(e/\bar{h}v)^2 \, dx^2 \log(\beta v \bar{h}/e^2) \tag{4}$$

where dx^2 is the projection of the mean transition dipole moment of the dye on to the direction of flight of the particle. β is a dimensionless constant which cannot be calculated in general, v is the velocity of the electron and e the charge of electron. Only the general shape of the cross-section is relevant to us.

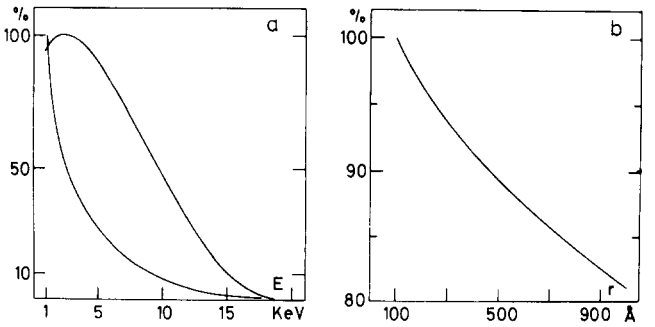

Fig. 1. (a) β-spectrum for $^3H(\beta^-)^3He$ decay calculated from the Fermiplot (upper curve). The β-spectrum was multiplied by the calculated inelastic cross-section (lower curve). (b) Relative amount of electrons reaching a distance larger than r compared to those reaching 100 Å.

With $\beta = 1$ and $\bar{h}c/e = 137$ we get:

$$\vec{A}\vec{r}/r \sim c^2v^2 \log(137v/c) \tag{5}$$

The β-spectrum multiplied with the cross-section is shown in Fig. 1a, lower curve. We realize that the slowest electrons are the most efficient in exciting our dye. This is again a good reason for using tritium and not another atom to excite the dye. As the electrons slow down with increasing distance from the emitter, their energy spectrum is continuously changing and the cross-section is increasing. The loss of electrons reaching the absorber and the increase in the cross-section caused by the slowing down of the electrons will to some extent compensate. What then happens to the slow electrons? Their mean path is not straight anymore. Most of the electrons have passed the absorber when they are stopped in the solvent. They cannot interact a second time with the absorber but are absorbed by the buffered solvent. Some of the electrons which remain close to the dye and its environment could form hydrated electrons [3]. Their lifetime is much longer than that of a bare electron and therefore can react with the dye or surrounding molecules. However, the percentage of this occurrence is so low that, unless some inconsistency is observed, we can neglect this effect.

Results and Discussion

Micelle formation observed by CPR

12-(9-Anthroyloxy)stearic acid molecules surrounded by water have a lower fluorescence quantum yield than in the hydrophobic surrounding which they create by forming micelles. When 12-(9-anthroyloxy)stearic acid is added to water (to a concentration of 20 μM) (λ_{ex} = 370 nm, λ_{em} = 450 nm), there is a fast initial jump in fluorescence in less than 30 s with no further change in the following hour. This does not necessarily mean that micelle formation is rapid. When a mixture of tritiated oleic acid and 12-(9-anthroyloxy)stearic acid is added to water we observe a very slow increase in CPR intensity with time (Fig. 2). The increase in intensity is biphasic and neither phase is exponential. Only after about 20 h is equilibrium nearly attained. The change in intensity

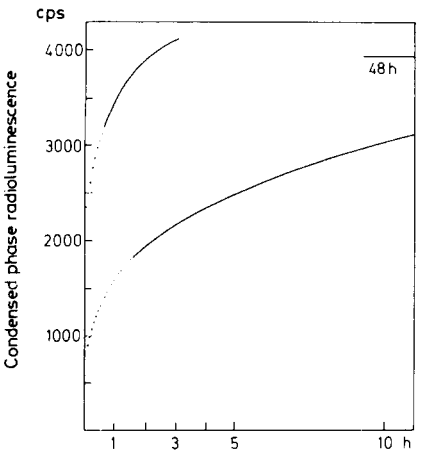

Fig. 2. Time course of the CPR intensity of 12-(9-anthroyloxy)stearic acid (40 μM) and radioactive oleic acid (4 μM) added to the buffer (lower curve). Radioactive oleic acid added to the equilibrated 12-(9-anthroyloxy)stearic acid micelles (upper curve). T = 20°C.

arises from the incorporation of tritiated oleic acid into the micelles, increasing the probability of excitation of the closely positioned dye molecules. As more dye molecules surround the emitter this probability increases further and, therefore, the intensity reflects the size of the micelle. One decay can even excite more than one dye. This can be seen by filtering out double-photon pulses with the discriminator. The dense clustering makes a quantitative treatment of the data difficult. The intensity increase is not linear with size.

To make sure that we have not just observed the uptake of tritiated oleic acid by some rapidly formed micelles, we first equilibrated the dye micelles for 20 h and then added the emitter. After 2 h the same intensity is reached as that obtained after 20 h by mixing tritiated oleic acid and 12-(9-anthroyloxy)stearic acid right from the beginning. Most of the signal occurred within the mixing time. Adding tritiated H_2O of the same activity instead of tritiated oleic acid results in a negligible signal, proving that tritiated oleic acid has been taken up by the micelles. We have shown that the signal increase in Fig. 2 (lower curve) is not caused by uptake of the emitter. The biphasic and non-exponential behaviour suggests a complex process including the possibility that an excited 12-(9-anthroyloxy)stearic acid molecule dimerises with an unexcited one [15]. Photodimerisation can be induced by irradiating the sample with light of 360 nm and the dimers can be dissociated by irradiation with light of 250 nm. Tritiated oleic acid added to 12-(9-anthroyloxy)stearic acid micelles which have been exposed to 250 nm light for 2 min, the period after which the fluorescence was at its maximum, showed a rapid intensity increase within the mixing time followed by a slow decrease within 5 min. The intensity was recovered by exposing the sample again to light of 250 nm. The β-particles stimulate several excited states of 12-(9-anthroyloxy)stearic acid and can, like ultraviolet light of 360 nm, induce dimer formation. The dimer is not fluorescent [15]. The dissociation of 12-(9-anthroyloxy)stearic acid dimers can also be induced by the β-particles. Depending upon the cross-section for the two processes, one or the other will preferentially occur. Thus, in 12-(9-anthroyloxy)stearic acid micelles,

the radioactive decay of tritium brings the 12-(9-anthroyloxy)stearic acid dimerisation reaction into an equilibrium state. Addition of phospholipid vesicles to the equilibrated 12-(9-anthroyloxy)stearic acid micelles labelled with tritiated oleic acid results in a drastic decrease in the CPR signal. Presumably, oleic acid and 12-(9-anthroyloxy)stearic acid are taken up by the vesicles and are spread over the whole surface of the bilayer, increasing the distance between emitter and absorber. The decrease in CPR intensity is biphasic. A very rapid decrease to half the expected starting signal within the first 20 s is followed by a slower one and the equilibrium is reached after about 2 h. The decay cannot be quantitatively related to the amount of 12-(9-anthroyloxy)-stearic acid taken up because of the unknown mechanism of excitation of the dye in the micelles, but it appears that about 2 h are needed to label vesicles with 12-(9-anthroyloxy)stearic acid which is comparable to the time course observed by direct fluorescence measurements.

Condensed phase radioluminescence in vesicles

To relate CPR to the concentrations of lipids (C_L), dye (C_D) and the activity (a) as well as to the geometrical properties of the vesicles, we have to calculate the intensity, I, from Eqn. 1. The dye on the surface of the vesicle is assumed to be homogeneously distributed. Because of the spherical shape of the vesicles all the emitters will experience the same dye distribution. The number of absorbers in one vesicle is:

$$\frac{C_D}{C_L} \cdot \frac{4\pi R_o^2}{f_o}$$

where R_o is the radius of the vesicle and f_o the surface area per lipid in the gel phase. The dye density on the surface is then:

$$\frac{C_D}{C_L} \cdot \frac{R_o^2}{f_o R^2}$$

where R is the radius of the vesicle. $R = R_o$ in the gel phase. Instead of summing over all dyes in Eqn. 1 we can integrate over the surface area of the vesicle. Because all emitters have an equal position with respect to the dye the total intensity is found by multiplication with the number of emitters. The activity is equal to the specific activity times the number of emitters. We have seen in the previous section that for small distances we can use a mean cross-section, $\vec{A} = \int_E \vec{A}(E) f(r,E)\, dE$ (derived from the integral in Eqn. 1), without introducing too much error. When looking at large distances or distance changes, corrections are necessary.

The geometry of the measuring cell, the arrangement of the phototube and its response reduce the number of pulses counted compared to the number generated. Therefore, a machine constant, Z, has to be incorporated into our final equation. Eqn. 1 is now reduced to the following form where F represents the surface area and ν the total activity:

$$I = Z\phi\nu \frac{C_D}{C_L} \frac{R_o^2}{f_o} \frac{1}{R^2} \left[\frac{1}{4\pi} \oint_F \frac{\vec{A}\vec{r}}{r^3}\, dF \right] \qquad (6)$$

The remaining integration results in a constant factor (Appendix). If the direc-

tion of $\vec{A}$ is parallel to the surface vector this integral is equal to $\bar{A}$ and the final formula for the CPR intensity is:

$$I = Z \frac{\bar{A}}{2} \phi \nu \frac{C_D}{C_L} \frac{R_0^2}{f_o} \cdot \frac{1}{R^2} \tag{7}$$

Any other orientation of A will introduce a constant multiplication factor. Therefore, Eqn. 7 describes the properties of the intensity for all positions of A.

I is proportional to ϕ and fluorescence quenching effects are also reflected in CPR. If we want to follow the other parameters we need to use a fluorescent probe with a quantum yield which is relatively insensitive to environmental effects. 12-(9-Anthroyloxy)stearic acid shows only small changes in intensity at the phase transition of a phospholipid vesicle [7]. Up to the phase transition, ϕ is nearly constant and above the phase transition a linear decrease in ϕ is observed.

An interesting property of Eqn. 7 is that the intensity is not dependent upon the size of the vesicles as long as one compares vesicles in the same phase. The intensity only reflects a radius change if it is caused by an alteration in the membrane structure. Therefore, fusion of identical vesicles would not be observed in CPR. This simply means that if we have one emitter in two identical vesicles we get the same intensity as if we have two emitters in one large vesicle. The cross-section, A, depends on the energy levels of the different excited states of the dye. If the environment of the dye does not affect these energy levels, A is unchanged. Solvent-dependent shifts in fluorescence spectra do occur, but they are usually small compared to the quenching effects by the surrounding molecules. With the substitution:

$$i = \frac{\bar{A}}{2} \phi \frac{AR_0^2}{f_o}$$

Eqn. 7 becomes:

$$I = Zi\nu \frac{C_D}{C_L} \frac{1}{R^2} \tag{8}$$

This equation contains the parameters we are going to change.

Results of CPR and fluorescence measurements in vesicles

We first checked the linearity of Eqn. 8 with respect to the concentration of dye. A sample of 200 μM dipalmitoyl phosphatidylcholine was labelled with 30 μCi/ml tritiated oleic acid. Then successive amounts of 12-(9-anthroyloxy)-stearic acid were added, increasing the dye molarity by 2.5 μM each time. Because it takes a long time to reach equilibrium the vesicles were sonicated after each addition of dye. We observed a perfect linearity up to $C_D/C_L = 0.05$. The same results were obtained using tritiated cholesterol and without sonication. The amount of 12-(9-anthroyloxy)stearic acid remaining in the solvent is negligible. In contrast, when diphenylhexatriene is used as the fluorophore, a marked deviation from linearity as C_D/C_L increases is observed. There is a partitioning of this dye between the membrane and the solvent.

Extrapolation of the linear relationship to zero dye concentration yields the amount of dark counts of the machine together with the stopping radiation

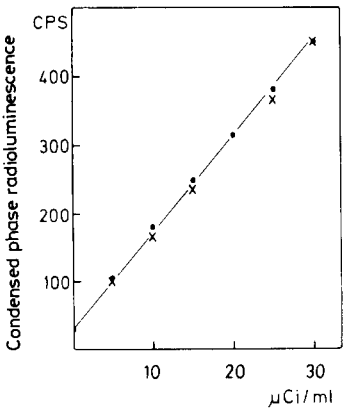

Fig. 3. CPR of dimyristoyl phosphatidylcholine vesicles labelled with 12-(9-anthroyloxy)stearic acid. •, increasing the activity; X, dilution of the final sample by adding buffer. $T = 30°C$.

caused by the energy loss of the electrons in the solvent. The linearity of Eqn. 8 as a function of the activity was checked by adding various amounts of tritiated oleic acid from an ethanolic stock solution to the dipalmitoyl phosphatidylcholine vesicles previously cosonicated with 12-(9-anthroyloxy)stearic acid. The uptake is completed within the mixing time (1 min) of the sample preparation.

Fig. 3 shows the expected linear increase in the CPR signal with activity. Dilution of the sample does not change C_D/C_L but the total amounts of dye and lipids are reduced. The reduction in intensity upon dilution should only be caused by the change in activity. The CPR intensity in the diluted samples (Fig. 3) follows a dependence on the activity identical to that of the undiluted sample. This experiment shows that the intensity is only dependent upon the ratio of dye to lipids. Another way of looking at this result is by saying: one vesicle with two tritiated labels emits the same amount of light as two of these vesicles each containing only one tritiated label.

The relative amount of oleic acid in the membrane can be decreased by increasing the lipid concentration at constant C_D/C_L. Addition of untritiated oleic acid to labelled vesicles does not affect CPR intensity. We can therefore conclude that no specific interaction between oleic acid and 12-(9-anthroyloxy)stearic acid takes place within the membrane, since otherwise untriated oleic acid would have displaced the tritiated one.

In different experiments, dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles with a total lipid concentration between 200 and 400 μM were prepared and cosonicated with 1—2.5 mol% of 12-(9-anthroyloxy)stearic acid. The amount of tritiated oleic acid used was between 30 and 90 μCi/ml. Typical phase transition curves for dipalmitoyl phosphatidylcholine and for dimyristoyl phosphatidylcholine vesicles are shown in Fig. 4. The phase transition in dimyristoyl phosphatidylcholine vesicles is broader than in dipalmitoyl phosphatidylcholine vesicles. No hysteresis was observed in a cooling-heating cycle in dipalmitoyl phosphatidylcholine vesicles but a difference was observed in dimyristoyl phosphatidylcholine vesicles especially in the gel phase. Below 18°C the signal sometimes significantly increased. Dimyristoyl phos-

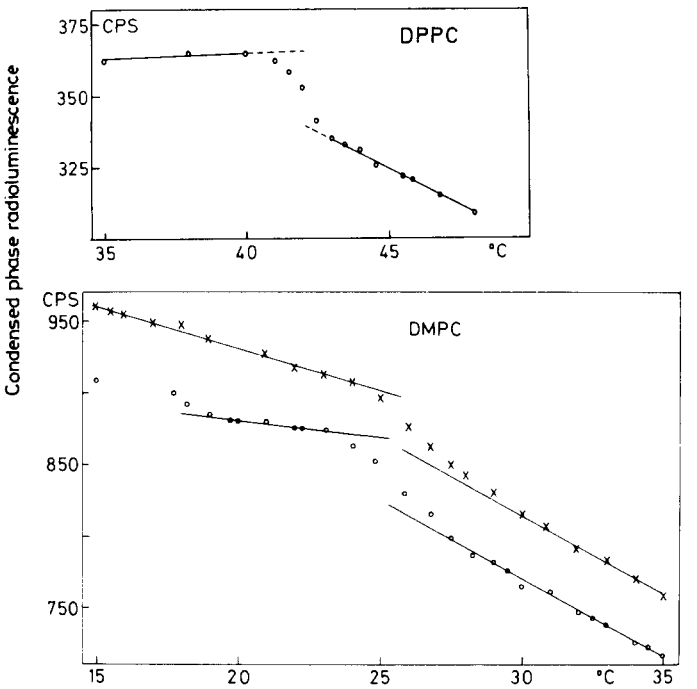

Fig. 4. (a) A typical phase transition as measured in CPR for dipalmitoyl phosphatidylcholine (DPPC) vesicles (200 μM) labelled with 12-(9-anthroyloxy)stearic acid (5 μM) at 30 μCi/ml activity. (b) The phase transition measured in CPR for dimyristoyl phosphatidylcholine (DMPC) vesicles (200 μM) labelled with 12-(9-anthroyloxy)stearic acid (5 μM) at 50 μCi/ml activity. ○, cooling cycle; X, heating cycle after 15 h rest at 35°C.

phatidylcholine vesicles in the liquid-crystalline phase showed a slightly increased signal after the heating-cooling cycle. A further increase was observed over a period of 15 h. The transition measured after 15 h showed the same broad transition characteristic for unilamellar vesicles and the relative change in intensity remained as before.

By normalizing the fluorescence and the CPR intensities vs. temperature plots at a temperature above the critical one, one can show that the percent change for the fluorescence intensity is −1.6%/°C while the change in CPR intensity is −1.4%/°C. Because the two changes are identical within the accuracy of the measurements we conclude that the observed change in the CPR intensity with temperature in the liquid-crystalline phase is a result of the change in the fluorescence quantum yield of the dye.

Since there is no quantum yield change in the gel phase, we expect a constant CPR intensity at low temperatures, but we observe a slight linear decrease with increasing temperature. The slope of this change is more pronounced at lower dye concentrations. To determine the relative change in intensity at the phase transition we extrapolated the straight-line portions of the plots to the midpoint of the transition. The vertical distance between the two lines at T_m is ΔI. The total intensity at T_m is the measured intensity minus the dark counts of the machine and the stopping radiation (1.23 cps/μCi per ml). The intensity changes were: $\Delta I/I = 7.5\% \pm 1.5$ in dimyristoyl phosphatidylcholine vesicles

and $\Delta I/I = 8.8\% \pm 1.5$ in dipalmitoyl phosphatidylcholine vesicles. Assuming that the probes are distributed homogeneously between the inner and outer layers of the membrane, R in Eqn. 8 represents approximately the radius of the vesicle at the center of the bilayer. The relative change in intensity is equal to the relative change in surface area as can easily be predicted from Eqn. 8:

$$\Delta\frac{I}{I} = \frac{\Delta F}{F} = 2\frac{\Delta R}{R} \tag{9}$$

To compare this change in surface area with other measurements we note the following relationship which also holds for a spherical shell:

$$\frac{\Delta F}{F} = \frac{\Delta V}{V} - \frac{\Delta D}{D} \tag{10}$$

The relative change in volume, V, of the membrane is the sum of the relative change in thickness of the bilayer (D) and the relative surface change. The volume change is known to be very small, 1.4% [16], 3.2% [17]. The published values for the thickness change vary between 5 and 8 Å, [4,18]. From neutron diffraction studies we calculated that between the P_β phase (38°C) and I_α phase (50°C) in dipalmitoyl phosphatidylcholine bilayers, a change in relative thickness over the whole membrane of 13.8% occurs [4]. By inserting these values in Eqn. 10 we find a maximum value for $\Delta F/F = 10.6\%$. This value corresponds to values found at the air/water interface but is slightly larger than our values [19].

The exchange of oleic acid and fluorescent dyes between vesicles

Next we will see if 12-(9-anthroyloxy)stearic acid and oleic acid are exchanged between vesicles. Dipalmitoyl phosphatidylcholine vesicles labelled with 1,6-diphenylhexa-1,3,5-triene were mixed with vesicles labelled with tritiated oleic acid. Only a rapid signal increase within the first 40 s is observed (Fig. 5, lower curve). Dipalmitoyl phosphatidylcholine vesicles labelled with 1,6-diphenylhexa-1,3,5-triene in tritiated water of the same activity as tritiated oleic acid show only the stopping radiation. Therefore, an exchange of oleic acid or 1,6-diphenylhexa-1,3,5-triene must have taken place. Since the uptake

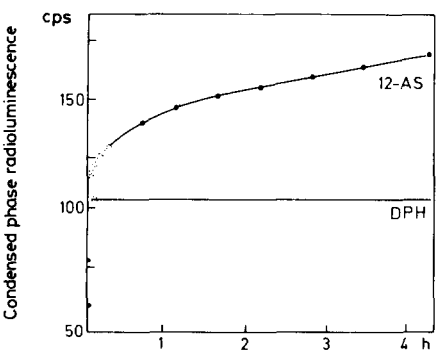

Fig. 5. The time course of CPR for dipalmitoyl phosphatidylcholine vesicles (200 M) labelled with 12-(9-anthroyloxy)stearic acid (12-AS) or 1,6-diphenylhexa-1,3,5-triene (DPH) observed after mixing with vesicle labelled with oleic acid. The final activity was 21 μCi/ml.

of 1,6-diphenylhexa-1,3,5-triene by dipalmitoyl phosphatidylcholine vesicles is relatively slow [20], oleic acid must have exchanged rapidly. Once the oleic acid has equilibrated no further change is expected in the CPR signal even if the dye is not yet in equilibrium.

A similar experiment was carried out using 12-(9-anthroyloxy)stearic acid (Fig. 5, upper curve). As already explained, once oleic acid has rapidly exchanged no further increase in CPR intensity should take place even if 12-(9-anthroyloxy)stearic acid exchange followed. The observed slow increase (which follows second-order kinetics) cannot therefore result from the exchange of 12-(9-anthroyloxy)stearic acid between vesicles.

In a similar experiment, we mixed dimyristoyl phosphatidylcholine vesicles labelled with tritiated oleic acid with dipalmitoyl phosphatidylcholine vesicles labelled with 12-(9-anthroyloxy)stearic acid. The same pattern as in Fig. 5 was observed, but the slow rate was about twice that measured in the former system. After the equilibration we raised the temperature and observed two phase transitions corresponding to those of separate dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles (Fig. 6). The presence of oleic acid and 12-(9-anthroyloxy)stearic acid does not induce fusion as can be seen by the two distinctly separated phase transitions. Dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine do not fuse [21]. We can therefore conclude that 12-(9-anthroyloxy)stearic acid is exchanged between the two types of vesicle. We used the same amount of dimyristoyl phosphatidylcholine (DMPC) as dipalmitoyl phosphatidylcholine (DPPC) for this experiment and we have seen that the absolute intensity is independent of the radius of the vesicle (Eqn. 7). The CPR intensities in the two types of vesicle add together as follows:

$$I = \frac{i_b}{C_L}\left(\frac{C_D(\mathrm{DMPC})}{R^2(\mathrm{DMPC})}\cdot \nu(\mathrm{DMPC}) + \frac{C_D(\mathrm{DPPC})}{R^2(\mathrm{DPPC})}\cdot \nu(\mathrm{DPPC})\right) \qquad (11)$$

Between the two phase transitions a linear decrease in intensity of relative change 0.61%/°C is observed. This decrease is only caused by the change in the intensity from dimyristoyl phosphatidylcholine vesicles. The dipalmitoyl phosphatidylcholine vesicles are still in the gel phase and show no change in inten-

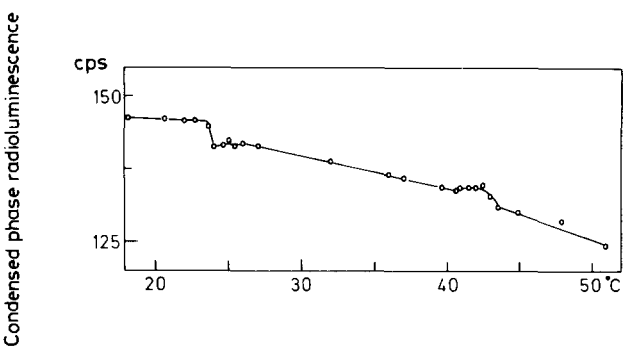

Fig. 6. CPR of the phase transitions of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles after the exchange of 12-(9-anthroyloxy)stearic acid and oleic acid.

sity. From the measurement of the dimyristoyl phosphatidylcholine phase transition we know that the relative change with respect to I (dimyristoyl phosphatidylcholine) should be 1.6%/°C. From these two values we can determine the ratio I (DMPC)/I (DPPC).

$$0.0061 = \frac{dI/dT}{I(\text{DPPC}) + I(\text{DMPC})} \quad 0.016 = \frac{dI/dT}{I(\text{DPPC})} \quad I(\text{DPMC})/I(\text{DPPC}) = 1.6 \qquad (12)$$

If we assume the radius (R) of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine to be equal we find using Eqn. 11:

$$\frac{I(\text{DMPC})}{I(\text{DPPC})} = \frac{C_D(\text{DMPC})}{C_D(\text{DPPC})} \cdot \frac{\nu(\text{DMPC})}{\nu(\text{DPPC})}$$

If we further assume:

$$\frac{C_D(\text{DMPC})}{C_D(\text{DPPC})} = \frac{\nu(\text{DMPC})}{\nu(\text{DPPC})}$$

then we find for the ratio of dyes:

$$\frac{C_D(\text{DMPC})}{C_D(\text{DPPC})} = 1.3$$

The probes are not excluded from the solid phase of the dipalmitoyl phosphatidylcholine vesicles, only an insignificant preference for the fluid phase of dimyristoyl phosphatidylcholine vesicles is detected.

It has been demonstrated that for mixed lipid systems dipalmitoyl phosphatidylcholine and dilauroyl phosphatidylcholine, 12-(9-anthroyloxy)stearic acid preferentially partitions into the liquid-crystalline phase [7]. Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine do not separate into two phases when mixed. It could therefore be that 12-(9-anthroyloxy)stearic acid is not excluded from the gel phase of dimyristoyl phosphatidylcholine because the two lipids are very similar to each other.

Conclusions

Condensed phase radioluminescence (CPR) yields detectable amounts of single photons if the local densities of the tritiated emitter and the fluorescent absorber molecules are large enough. This is achieved by 'condensing' a fluorescent probe and a tritiated fatty acid in a micelle or in a lipid vesicle. Since the CPR signal is related to the square root of the distance between emitter and absorber, changes in distances may be measured. Because of this distance relationship, unlike in fluorescence energy transfer measurements, a larger range can be covered by CPR. Electrons released by the β-decay of tritium can reach targets which are far away but the probability of hitting them is so low that a signal cannot be observed. The limitation of CPR is given by the stopping radiation which is part of the energy loss of the electron in the solvent. We suggest that CPR is useful only for distances less than 500 Å. When radioactive oleic acid and a fluorescent probe (e.g., 12-(9-anthroyloxy)stearic acid) are incorporated into phospholipid vesicles, the changes in CPR intensity can be used to follow phase transitions. This method, unlike the fluorescent probes [22—24],

measures the change in surface area during the transition and not the local environment of the probe. Clustering of oleic acid was suggested by Hauser and Guyer [25]. We have no evidence for clustering of the probes. This is in accordance with calorimetric studies which exclude a pure fatty acid phase [26].

The exchange of oleic acid and of the fluorescent probes between vesicles can also be studied by CPR. In mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles, oleic acid exchanges in less than 5 s. 12-(9-Anthroyloxy)stearic acid does not show preferential location in the gel phase of dipalmitoyl phosphatidylcholine or in the fluid phase of dimyristoyl phosphatidylcholine vesicles. The dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles do not fuse.

The CPR technique can clearly be extended for the study of a variety of biological systems. Radioactive labels can be incorporated through specific molecules that are present in or interact with membranes, and we have seen that the intrinsic fluorescence of tryptophan can also be excited by electrons. Thus, the interaction of molecules like hormones, drugs, etc., with membranes can be studied by CPR, including observation of the kinetics of the processes. Currently, the time resolution of the method is about 5 s.

Appendix

To calculate:

$$\frac{1}{4\pi} \oint_F \frac{\vec{A} \cdot \vec{r}}{r^3} \mathrm{d}F$$

we must make an assumption about the direction of $\vec{A}$. For simplicity we assume $\vec{A}$ to be parallel to the surface vector. This assumption is very useful because as we approach $r = 0$, the integral is not divergent. With any other angle we must assume a minimal approach distance for the emitter and absorber.

We define ϕ as the angle between $\vec{r}$ and the tangential plane at the location of the emitter. $\vec{r}$ can be expressed by the radius R of the vesicle and the angle ϕ.

$$\vec{r} = 2R \sin \phi e^{i\phi}$$

The surface unity vector can be expressed as:

$$\vec{F} = e^{i(2\phi - \pi/2)}$$

The product of those two values is:

$$\vec{F} \cdot \vec{r} = 2R \sin^2 \phi$$

The surface element is a ring seen under the angle ϕ.

$$\mathrm{d}F = 8\pi R^2 \sin\phi\cos\phi \mathrm{d}\phi$$

Our starting equation can be transformed into an integration over the angle ϕ.

$$\frac{\bar{A}}{4\pi} \int_{\phi=0}^{\phi=\pi/2} \frac{2R \sin^2\phi}{8R^3 \sin^3\phi} \cdot 8\pi R^2 \sin\phi\cos\phi \mathrm{d}\phi = \frac{1}{2} \int_{\phi=0}^{\phi=\pi/2} \cos\phi = \bar{A}/2$$

This integration adds a constant factor of $\bar{A}/2$ to Eqn. 8.

References

1 Birks, I.B. (1964) The Theory and Practice of Scintillation Counting, Pergamon Press, Oxford
2 Bransome, E.D. (1970) Liquid Scintillation Counting, Grune and Stratton, New York
3 Haissinsky, H. (1969) Actions Chimiques et Biologiques des Radiations, Masson, Paris
4 Buldt, G., Gally, H.H., Seelig, A., Seelig, I. and Zaccai, G. (1978) Nature 271, 182—184
5 Landau, L.D. and Lifshitz, E.M. (1975) Quantum Mechanics, p. 578, Pergamon Press, Oxford
6 Barratt, M.D., Badley, A., Leslie, R.B., Morgan, G.C. and Radda, G.K. (1974) Eur. J. Biochem. 48, 595—601
7 Bashford, C.L., Morgan, G.C. and Radda, G.K. (1976) Biochim. Biophys. Acta 426, 173—185
8 Harris, D., von Tscharner, V. and Radda, G.K. (1979) Biochim. Biophys. Acta 548, 72—84
9 Piel, F. (1975) Nucl. Phys. A 203, 369—381
10 Curran, S.C., Angus, J. and Cockroft, A.L. (1949) Philos. Mag. 40, 53—60
11 Fano, U. (1952) Appl. Math. Ser. 13, 1—19
12 Siegbahn, K. (1966) Alpha-, Beta and Gamma-Ray Spectroscopy, Vol. 1, North-Holland, Amsterdam
13 Katz, L. and Penfold, A.S. (1952) Rev. Mod. Phys. 24, 28—44
14 Marion, J.B. and Young, F.C. (1968) Nuclear Reaction Analysis, p. 10—11, North-Holland, Amsterdam
15 McGrath, A.E., Morgan, G.C. and Radda, G.K. (1976) Biochim. Biophys. Acta 426, 173—185
16 Träuble, H. and Haynes, D.H. (1971) Chem. Phys. Lipids 7, 324—335
17 Sirnivasan, K.R., Kay, R.L. and Nagle, J.F. (1974) Biochemistry 13, 3494—3496
18 Chapman, D., Williams, R.M. and Ladbrooke, D. (1967) Chem. Phys. Lipids 1, 445—475
19 Jahnig, F., Harlos, K., Vogel, H. and Eibel, H. (1979) Biochemistry 18, 1459—1467
20 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528
21 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4529—4537
22 Radda, G.K. (1972) Biochim. Biophys. Acta 266, 509—549
23 Sackmann, E. and Träuble, H. (1972) J. Am. Chem. Soc. 94, 4482—4491
24 Sackmann, E. and Träuble, H. (1972) J. Am. Chem. Soc. 94, 4492—4498
25 Hauser, H. and Guyer, W. (1979) Biochim. Biophys. Acta 553, 359—363
26 Kantor, H.L. and Prestegard, J.H. (1979) Biochemistry 17, 3592—3597