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THE ACTIVATION ENERGY OF INCORPORATION OF EXTRINSIC PROBES IN MODEL VESICLES

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Time dependence of fluorescence enhancement of probes after addition to lipid vesicles has been used to investigate the position of chromophores in the lipid bilayer. Incorporation studies of a series of *n*-(9-anthroyloxy) fatty acids (*n* = 2, 2, 12 and 16) and 1,6-diphenylhexatriene in dipalmitoyl phosphatidylcholine vesicles are described. The activation energies for incorporation of these several lipid-mimic type fluorescent probes have been measured. Results show that the activation energy is a function of the distance of the anthracene moiety (chromophore) from the polar end of the probe and the length of the acyl portion of the probe. An average insertion energy of 0.6 kcal/carbon is seen for these fatty acid probes. The activation energy of 1,6-diphenylhexatriene, a factor of 2 greater than that of 16-(9-anthroyloxy)palmitic acid, is consistent with locating 1,6-diphenylhexatriene in the middle of the bilayer.

Introduction

The incorporation of extrinsic fluorescent probes by model or real membranes is an ill-defined process. It is known that the final location of the probe within the bilayer is often dictated by the hydrophobic or hydrophilic character of the probe. This has been established by careful analysis of fluorescence emission and time-resolved fluorescence techniques. Some work has also been carried out on the measurement of the rate of incorporation of the probe. For example, interaction of 1-anilino-8-naphthalene sulfonate with erythrocyte membranes has been extensively studied in the last several years. A biphasic interaction was attributed to the presence of 'fast' and 'slow' sets of binding sites in erythrocyte membranes. That corresponded to interactions at the outside and diffusions into the membrane. However, only the fast phase was observed with sonicated membranes and membrane proteins. The interaction was observed to be strongly pH-dependent and was sensitive to changes in ionic strength brought about by addition of NaCl [1].

Further investigations [2] were concerned with the fluorescent enhancement of 1-anilino-8-naphthalene sulfonate added to erythrocyte ghosts, at room temperature, in terms of 'fast', 'medium' and 'slow' phases. It was suggested that most 1-anilino-8-naphthalene sulfonate binding sites are similar on the molecular level. However, the sites on the outer side of the membrane always give a fast 1-anilino-8-naphthalene sulfonate response, whereas the response from sites within the permeability barrier may be fast, medium or slow depending on the state of the membrane.

Later work by Fortes and Hoffman [3] on 1-anilino-8-naphthalene sulfonate, showed that the slower process had a half-time of about 8 min, independent of 1-anilino-8-naphthalene sulfonate concentration, in contrast with the rate of interaction of 1-anilino-8-naphthalene sulfonate with erythrocyte ghosts under

Abbreviations: 2-AP, 2-(9-anthroyloxy)palmitic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; 2-AS, 2-(9-anthroyloxy)-stearic acid; 12-AS, 12-(9-anthroyloxy)stearic acid; DPH, 1,6-diphenylhexatriene.

similar conditions where equilibrium was reached within a few seconds [1,2]. Fortes et al. [3] found that 1-anilino-8-naphthalene sulfonate was a permeant anion and a potent inhibitor of anion permeability in red cells. The inhibitory effects, regardless of the actual mechanisms involved, indicated that 1-anilino-8-naphthalene sulfonate and related compounds are not inert in their interactions with membranes. Hence, care must be excercised when interpreting observations made in the presence of a probe where the probe itself alters the characteristics of the membrane.

In a study of the microsomal drug metabolism and the interaction of fluorescent probes with microsomes at different temperatures, Lang et al. [4] observed that the fluorescent enhancement lasted about 4 min at 38°C in the case of 12-(9-anthroyloxy)stearic acid in microsomal suspensions as opposed to at least 35 min at 10°C.

Another probe that has been used extensively in labelling membranes is 1,6-diphenylhexatriene. Esko et al. [5] reported a rapid increase in fluorescence intensity as the dye was incorporated into the membrane. The uptake of the dye into the cells appeared to saturate after about 20 min. They reported apparent activation energies for membranes from choline and ethanolamine-supplemented cells of 7.7 and 8.7 kcal/mol, respectively.

Quenching studies of aromatic hydrocarbons have also been exploited to obtain information about the permeability of membrane-like systems [6–9]. Tsong [10] reported an apparent activation energy of 1-anilino-8-naphthalene sulfonate transport in dimyristoyl phosphatidylcholine of 240 kcal/mol in the first half of the transition and –96 kcal/mol at the second half. The time scale used was up to 60 s. The negative activation energy seems to be strange. However the author attributed that to the nonreproducibility of the results due to the slow fusion of the vesicles.

Pyrene binding to dimyristoyl phosphatidylcholine bilayers has been studied by Tsong [10]. Although the kinetics were complex, the half-time of the binding showed no abnormalities in the phase transition temperature of the phospholipid vesicles.

To gain more insight into the process of incorporation, the activation energies for incorporation of several lipid-mimic type fluorescent probes into dipalmitoyl phosphatidylcholine vesicles have been measured. The results of this study are presented here.

Experimental

Dipalmitoyl phosphatidylcholine was obtained from Sigma Chemical Co. The fluorescent probes, 12-(9-anthroyloxy)stearic acid, 2-(9-anthroyloxy)-stearic acid, 16-(9-anthroyloxy)palmitic acid and 2-(9-anthroyloxy)palmitic acid were obtained from Molecular Probes, Inc. and used as received. 1,6-diphenylhexatriene was obtained from Aldrich Chemical Co. Water for the buffer solutions was deionized and then distilled over $KMnO_4$ to remove any traces of fluorescent impurities and millipore filtered. The structures of the probes are shown for clarity in Fig. 1.

The vesicles (minimum size) were prepared by the injection method [11] and were characterized by transmission electron microscopy. The vesicle diameters were found to range from 200 to 700 Å. All vesicle preparations were made immediately prior to the fluorescent measurement. The final vesicle suspension consisted of $1.7 \cdot 10^{-4}$ M phospholipid in a phosphate buffer with an acid salt concentration (NaH_2PO_4) of 0.012 M and pH 7.4.

The vesicle suspension in the phosphate buffer was stirred at approx. 5 Hz with a teflon coated stir-

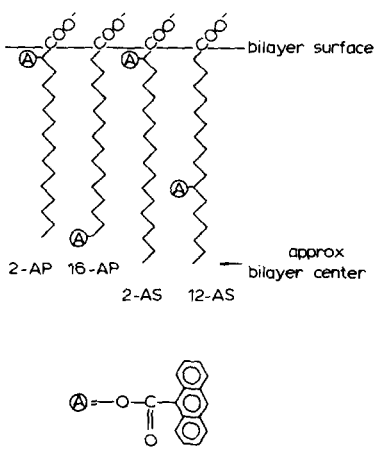

Fig. 1. The *n*-anthroyloxy fluorophore structures as located in a biological membrane. The format is similar to that in Ref. 17 because of the clarity provided by such a presentation.

rer within a thermostatically controlled housing in the sample compartment of the fluorometer over the course of each experiment. Stirring of the suspension was found to be essential to the measurement of reproducible incorporation curves. A Bailey Instrument Co. thermocouple thermometer was used for temperature measurement. The jacket was thermostatically controlled sufficiently well that the maximum temperature excursion of the sample over the course of an individual incorporation measurement did not exceed ±0.1°C.

Fluorescence measurements were made on a home-built fluorescence spectrometer with 0.45 meter excitation and emission monochromators (16 Å/mm reciprocal dispersion). The excitation source was a 500 watt xenon arc lamp and the detection system included a single photon counting apparatus. The entire fluorometer was automated using an Apple II Plus microcomputer.

The wavelength settings of the excitation and emission monochromators are given in Table I. These wavelengths were chosen such that the maximum intensity of the fluorescence was obtained with little or no interference from Raman bands associated with the H_2O solvent or the vesicles themselves. Raman emission proved to be of considerable relative strength at the low total emission intensities obtained. It was soon apparent, therefore, that emission filters would not be nearly selective enough. The band pass of the emission monochromator was typically 3.2 nm while that of the excitation monochromator was typically 2.4 nm.

The total emission intensity of the chromophore emission relative to the Raman scattering intensity was low primarily because the probes were used at a low total concentration. This concentration was chosen so that the overall solution optical density at the λ_{max} of the probe was less than 0.04 per cm to eliminate problems associated with the inner filter effect. For example, the molar ratio of the probe to lipid concentration in case of 2-(9-anthroyloxy)palmitic acid was 0.035.

To monitor the rate of incorporation of the probe by the vesicles, a small amount (1.5 μl) of a concentrated solution of the probe in tetrahydrofuran was injected into 2.5 ml of vesicle suspension. The increase in the fluorescence intensity was then recorded by the photon-counting system as a function of time with a strip chart recorder. Some typical incorporation curves are shown in Fig. 2. The intensity is seen to increase exponentially and level off at long times or approach a slightly increasing but linear intensity with time. The linear long-time plot was extrapolated back to earlier times. From this straight line, a fluorescence intensity difference (extrapolated minus observed) was plotted against time to obtain the logarithmic incorporation curves shown in Fig. 3. Since these are so clearly logarithmic in functional form, it is clear that the incorporation process on this scale (i.e. $t \geqslant 1$ min) is first order. Fig. 3 does not

TABLE I

WAVELENGTH SETTINGS OF FLUOROMETER FOR DIFFERENT PROBES

Probe	Excitation (nm)	Emission (nm)
2-AP	363.5	442.5
16-AP	366	439
2-AS	362	446
12-AS	367	458
DPH	355	450

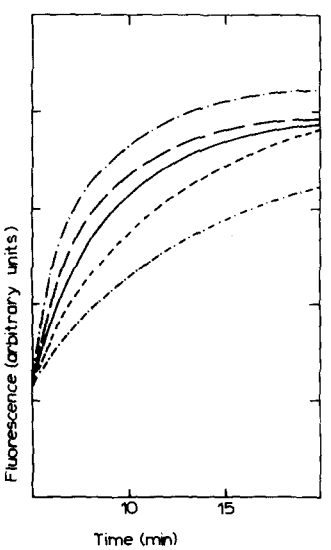

Fig. 2. The relative fluorescence intensity of 12-(9-anthroyloxy)stearic acid, measured at different temperatures of incorporation into dipalmitoyl phosphatidylcholine vesicles, as a function of incorporation time. ·····, temp. 20°C; - - - - -, temp. 25°C; ———, temp 35°C; – – –, temp. 38.5°C; – · –, temp. 40.5°C.

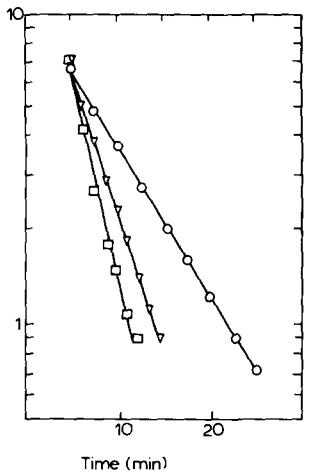

Fig. 3. Semi-logarithmic plots of the fluorescence intensity difference in the case of 12-(9-anthroyloxy)stearic acid incorporated into dipalmitoyl phosphatidylcholine vesicles at different temperatures, as a function of incorporation time. o———o, temp. 20°C; ▽———▽, temp. 35°C; □———□, temp. 40.5°C.

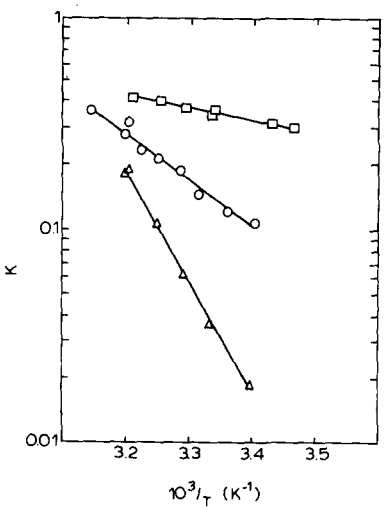

Fig. 4. Arrhenius plots of the rate constants for incorporation of 2-(9-anthroyloxy)palmitic acid (□———□); 12-(9-anthroyloxy)stearic acid (o———o); 1,6-diphenylhexatriene (△———△) into dipalmitoyl phosphatidylcholine vesicles.

show the data for times less than 5 min. The data was unreliable over the first 1–2 min because of turbulence in the mixing of the injected 1.5 μl of probe in tetrahydrofuran into the bulk solution. It should be noted that control studies in which the experiments were repeated but without vesicles present resulted in Arrhenius plots with slopes of approx. 0 K (no activation energies).

Results and Discussion

Arrhenius plots of the rate constants for incorporation of three different probes (for example purposes) are shown in Fig. 4. The activation energies for the incorporation process for the five probes studied is summarized in Table II. The data reveal much about the energetics of the incorporation process at temperatures below the gel-to-liquid crystal phase transition. At temperatures above the phase transition, the incorporation was essentially instantaneous for the time scale of these experiments.

Examination of the data shown in Table II reveal the following. The insertion energy is lowest when the anthracene moiety (the chromophore) is nearest the polar end of the probe. Thus, the insertion energies for 2-(9-anthroyloxy)stearic acid and 2-(9-anthroyloxy)palmitic acid (5 and 2.6 kcal, respectively) are much lower than the insertion energies for probes in which the chromophore is well removed from the polar head group, as in 16-(9-anthroyloxy)palmitic acid and 12-(9-anthroyloxy)stearic acid (insertion energies of 12 and 10 kcal, respectively). This gives implicit credibility to the accepted idea that these probes align themselves with the lipids in the bilayer (as opposed to resting on the surface, for example). As further confirmation of the above, it is noteworthy that the insertion energy for 12-(9-anthroyloxy)stearic acid is lower than that for 16-(9-anthroyloxy)palmitic acid, although by only 2 kcal.

From this data it can be concluded that moving the chromophore 14 carbons down the lipid chain (16 vs. 2)-(9-anthroyloxy)palmitic acids increases the

TABLE II

CALCULATED ACTIVATION ENERGIES

Probe	E_{act} (kcal)
2-AP	2.6 ± 0.3
16-AP	12 ± 1
2-AS	5 ± 1
12-AS	10 ± 1
DPH	23 ± 2

insertion energy by about 0.7 kcal per carbon. A similar trend is seen when the insertion energies for 12 and 2-(9-anthroyloxy)stearic acids are compared. In that case, the energy increases by about 0.5 kcal per carbon. These two figures are well within experimental error of each other.

There remains, however, one discrepancy to be explained. That is the difference in insertion energies between 2-(9-anthroyloxy)palmitic acid (2.6 kcal) and 2-(9-anthroyloxy)stearic acid (5 kcal). 2-(9-anthroyloxy)stearic acid has a lipid chain two carbons longer than 2-(9-anthroyloxy)palmitic acid. This fact must account for at least some of the difference. Such a comparison of chain lengths cannot be made between the 12-(9-anthroyloxy)stearic acid and the 16-(9-anthroyloxy)palmitic acid because clearly the position of the chromophore is the dominant cause of the high or low insertion energy.

Finally, the above conditions shed some light on the result obtained for 1,6-diphenylhexatriene. The insertion energy for 1,6-diphenylhexatriene is a factor of 2 greater than that for 16-(9-anthroyloxy)palmitic acid. This is consistent with locating 1,6-diphenylhexatriene in the middle of the bilayer. It is tempting to conclude that 1,6-diphenylhexatriene must end up parallel to the plane of the bilayer since it requires only about 12 kcal to move an anthracene moiety to the vicinity of the middle of the bilayer (i.e. the case of 16-(9-anthroyloxy)palmitic acid) and 1,6-diphenylhexatriene is not so much larger than anthracene.

These results are tedious to obtain with any degree of reproducibility but provide data not available by any other methods. To wit, these results are now being used to obtain data on the occlusion of membrane surfaces by glycoproteins (Carraway, K., Rockley, M.G. and Najjar, D.S., unpublished results). This is information that cannot be obtained by fluorescence depolarization studies, for instance. Furthermore, the results reported here verify the binding constant efficiency data obtained in separate studies of the uptake and fluorescence quenching of *n*-(9-anthroyloxy) fatty acid probes [12,13].

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