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Fluorescent probes in membrane studies

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A number of spectroscopic techniques are suitable for studying biological membranes. Of these, fluorescence has the sensitivity and time resolution for following membrane events associated with nerve excitation.

In this paper, the nature of the information derived from measurements of the fluorescence properties of externally introduced chromophores in membranes is examined. In particular, the locations of various probes are described on the basis of nuclear magnetic resonance (n.m.r.) experiments in model situations. Then the motional characteristics of the probe molecules (rotation and diffusion) are discussed. Finally, experiments designed to relate the detailed observations that can be made in lipid bilayers using n.m.r. and fluorescence measurements to those (more limited in nature) that can be made in membranes are described.

Introduction

The use of externally introduced fluorescent molecules to study 'structure–function' relationships in biological membranes is now widespread (Radda & Vanderkooi 1972). It is therefore not surprising that fluorescence techniques have not escaped the attention of neurophysiologists (Tasaki, Watanabe & Hallet 1972) who are now trying to define in molecular terms the role of excitable membranes in the many processes we heard discussed at this meeting. What of course makes fluorescence a particularly useful method of observation in this field is its sensitivity, the speed of detection and not least of all the way light emission can tell us something about the environment of the fluorescent chromophore.

It is this last feature of fluorescence measurements that I would like to discuss. It is relatively easy to demonstrate that some change has taken place as a result of a biological process but if the measurements are to provide any meaningful information at the molecular level this has to be extracted from a knowledge of the different fluorescence parameters.

The fluorescence process

The processes that take place after light is absorbed by a molecule are shown in a schematic way in figure 1. Light absorption is very rapid so that during this process no molecular motion takes place and only a change in electron (charge) distribution results. Before light emission occurs there is an interval of about 10^{-8} to 10^{-9} s characterized by the life-time (τ) of the excited state. The events that occur during this period modify the fluorescent properties of the molecule in a way that information about molecular motion and environment may be obtained. First, because of the altered charge distribution in the excited state the solvent (in our case the membrane) molecules rearrange to optimize the interactions. This solvent relaxation will then lower the energy of the excited state and therefore the emission spectrum is shifted to the red with respect to the absorption spectrum. The extent of this interaction depends on

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the dipole moment of the excited state (or strictly on the dipole moment difference between the ground and excited states), on the polarity of the solvent and on the rate at which solvent molecules can relax in comparison to the excited state life-time. Since the change in dipole moment and life-time can be measured independently we can define the polarity and motional characteristics of the environment.

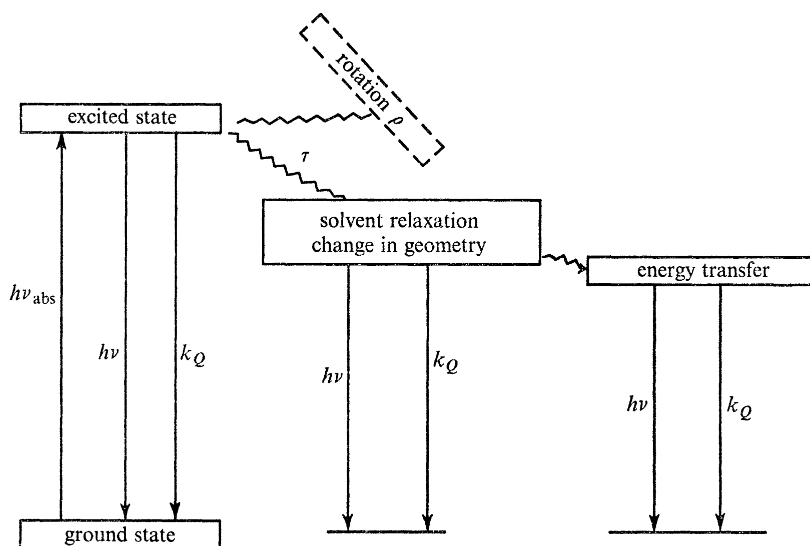

Figure 1. Schematic representation of processes that occur on electronic excitation (K_Q is the rate constant of radiationless deactivation, τ, the life-time of excited state).

The second process that modifies the emission spectrum in some cases is when the geometry of the molecule in the equilibrium excited state is different from that in the Franck–Condon state. If the environment restricts this geometrical change we can learn something about environmental constraint (Radda 1971).

The other dynamic events we can observe are rotation of the chromophore during the life-time of the excited state which results in depolarization of fluorescence (Weber 1953) and molecular diffusion (Radda & Vanderkooi 1972). This latter process can be used to measure diffusion rates of small molecules that quench the excited state (thus reducing its life-time and the intensity of emission) by a collisional mechanism that leads to an increase in the rate of reactions (k_Q) that compete with light emission.

Finally if we have several chromophores with particular spectral properties the energy of the excited state can be transferred to a neighbour. The efficiency of this transfer depends, among other things, on the separation between the two groups. We therefore have a method for measuring intermolecular distances up to about 8 nm in quite complex systems (Förster 1959).

The location of probes in membranes

With an understanding of the spectroscopic properties of particular fluorescent probe molecules we can proceed to the next stage of the investigations. If we are to extract meaningful information about membranes we have to know what region of the membrane is selected by the probe molecule. This is principally governed by the structure and solubility properties of

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the probe. I shall first show the conclusions and then take some examples of how we can determine the location of non-covalently linked fluorescent molecules.

Figure 2 shows the way we can look at a membrane. We can take chromophores such as *N*-phenyl-1-naphthylamine (which is essentially insoluble in water) and disperse them in the non-polar phase of the membrane (Radda 1971). When the chromophore is attached to a fatty acid chain in different positions the polar carboxyl group will anchor the probe at the interface so that we can sample different regions of the membrane. The positions of the emission maxima already tell us something about the 'polarity' of the environment around the chromophore

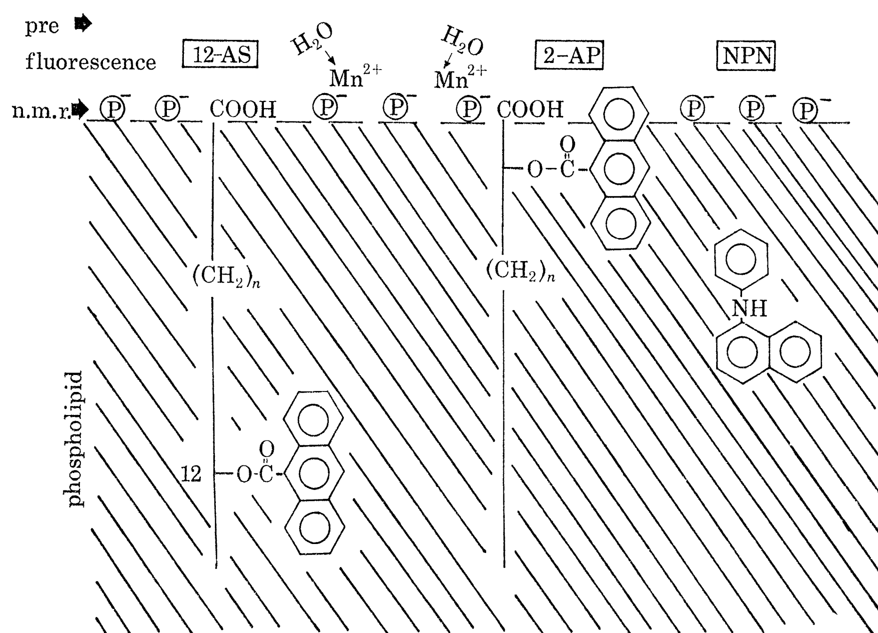

FIGURE 2. Some examples of probes used in membrane studies.

(Waggoner & Stryer 1970). The difficulty in this method is that environmental constraint also alters the spectrum and it is not always possible to separate the two effects. In an alternative method we rely on observations based on nuclear magnetic resonance methods that can tell us that the paramagnetic Mn^{2+} ion binds to the external phosphate groups of phospholipids in lipid vesicles and membranes and how molecules in the membrane are located in relation to these sites. If for example we take the fluorescent probe 2-(*N*-methylanilino)-naphthalene-6-sulphonate (MNS) the proton magnetic resonance spectrum of the probe can be completely assigned as shown (figure 3). In the presence of lecithin vesicles the spectrum is broadened because the observed spectrum now represents a time-average between free and bound probe molecules (figure 4). If we now bind Mn^{2+} ions to the lipid phosphates the spectrum is broadened further but this paramagnetic effect is selective in that the hydrogen close to the sulphonate moiety is much more affected than for example the protons on the phenyl group of the molecule. These data can be quantitated but the details of the method are inappropriate to discuss here. Suffice it to say that we have to measure different relaxation times, temperature dependence, we have to know the amount of probe and Mn^{2+} bound and we have to use the theory of spin–spin interactions. We can nevertheless construct a model (which is consistent with X-ray diffraction studies on lipid bilayers (Lesslauer, Cain & Blasie 1972)) which implies that MNS

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binds close to the phosphate head-group of the lipid with the phenyl group of the probe just penetrating the hydrocarbon core (figure 5). Incidentally this figure also shows that incorporation of cholesterol into the vesicles appears to push the probe molecules farther into the membrane.

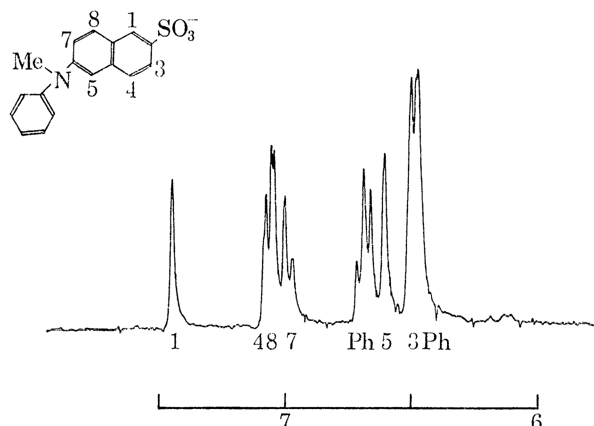

FIGURE 3. High resolution proton n.m.r. spectrum of MNS at 270 MHz.

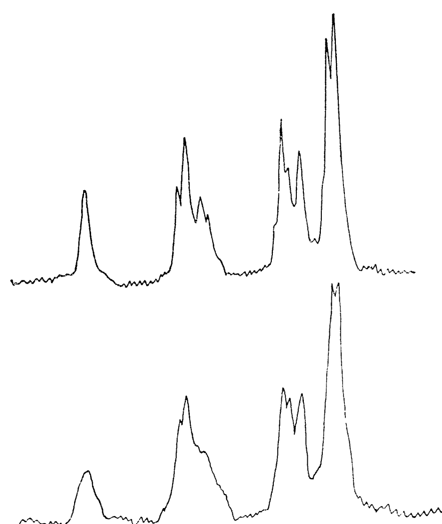

FIGURE 4. High resolution proton n.m.r. spectrum of MNS and 270 MHz in the presence of 0.4 mg/ml dipalmitoyllecithin (DPL) vesicles and after addition of 10 μM Mn^{2+} to this system.

Of course this kind of measurement is not possible in many biological situations and certainly would be impossible with the present techniques if, for example, we wanted to decide whether the probe molecule moved into a new position in response to say an action potential in nerve membranes. Fortunately we have a method which is less precise in information content but can be used in rapid optical measurements. It is illustrated in figure 6. The three molecules shown all fluoresce approximately twice as well in D_2O as in H_2O. This is probably because water is a better collisional quencher of the excited state (possibly acting as a proton donor) than D_2O. When the different probes are bound to phospholipid vesicles the extent to which their fluorescence is enhanced by D_2O compared to H_2O is a measure of the accessibility of the chromophore to water (Radda 1971). It is clear from the diagram that ANS 'sees' quite

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a lot of water, MNS is only about 60 % exposed and *N*-phenyl-1-naphthylamine is unaffected by water, consistent with the expectation that the first two probes bind to the lipid–water interface and the last one in the hydrocarbon interior. We have been able to use this kind of measurement to study the exposure of probe molecules in different membranes and in relation to biological activities (Ballard *et al.* 1972). If the kinds of responses that are observed during action potentials represent some displacement of probes of this type further into the membrane the study of the solvent isotope effect on the fluorescence change may be interesting to measure.

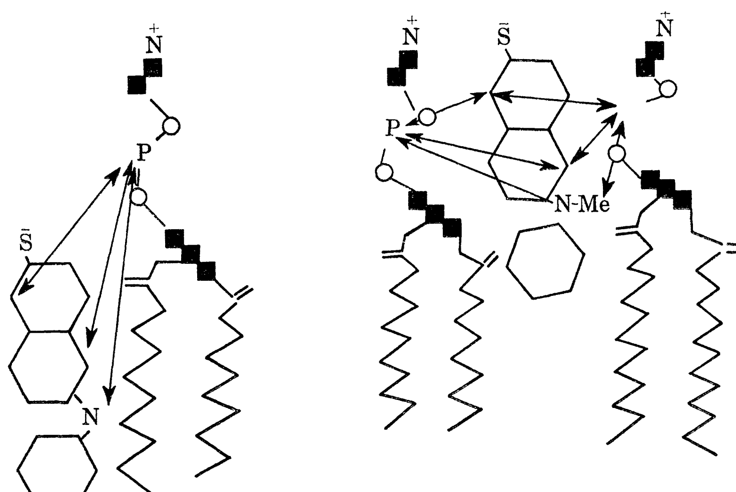

FIGURE 5. Model for location of MNS in lipid bilayers. Left hand model, in presence of cholesterol; right hand model, without cholesterol.

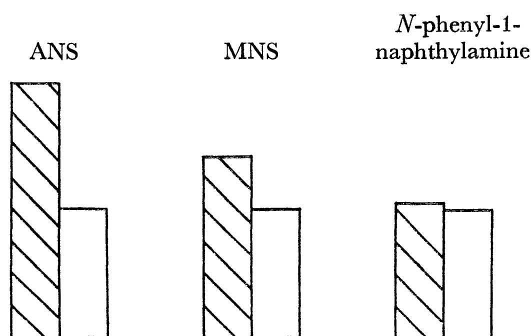

FIGURE 6. The effect of D_2O on the fluorescence intensity of probes (10 μM) in lecithin vesicles. ▧, fluorescence in D_2O; □, in H_2O.

So far I have not considered whether the probes that bind at the head-group region of phospholipid vesicles and membranes are distributed on both sides of the membrane. Certainly in relation to fluorescence responses to membrane potentials we have to know whether the probe can rapidly penetrate across a lipid bilayer. Obviously conductivity measurements across black lipid membranes in the presence of anionic probes can give this information. An alternative and more direct method is illustrated in figure 7. The head-group $-\overset{+}{N}Me_3$ proton resonances in phospholipid vesicles give a sharp, well defined line. On addition of ANS this line is split into two resonances. The most likely cause of this is proximity of the aromatic rings of ANS to the $-\overset{+}{N}Me_2$ group causing a ring current shift (for our present purposes knowledge of the exact mechanism of the shift is not essential). The proportion of the shifted to unshifted

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FIGURE 7. The effect of ANS (4–8 mM) on the *N*-methyl proton resonances of lipid vesicles. Top curve: egg lecithin-phosphatidic acid (1:1) (total lipid 10 mM), pH 7.0, 50 mM NaCl; middle curve: same with 4 mM ANS (5 min after addition); bottom curve: same as top curve with 8 mM ANS (1 h after addition).

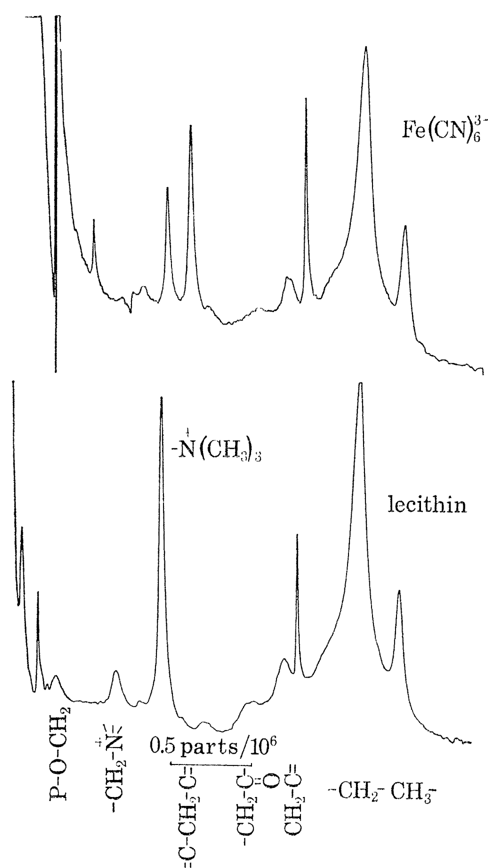

FIGURE 8. High resolution proton n.m.r. spectra of lecithin vesicles (at 270 MHz). Lower curve: small vesicle fraction from sepharose 4B (lecithin 130 mM) in 20 mM sodium acetate (pD 8.4); upper curve: the same with 160 mM $K_3Fe(CN)_6$ added.

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resonance is very similar to that of the 'outside/inside' ratio of the $-\overset{+}{N}Me_3$ groups measured by an independent method (see below). This implies that during the 5 min period that the measurements required ANS only occupied to outside surface of the phospholipid vesicle. All the groups were affected after 1 h suggesting that either ANS slowly penetrated across or that it broke up the lipid vesicles at the relatively high probe concentrations we had to use for the n.m.r. measurements. (It is relevant that the hydrophobic permeant anion tetraphenylboron shifted all the resonances immediately after addition to the vesicles.) Figure 8 shows how we can measure the outside/inside lipid ratios in phospholipid vesicles. At 270 MHz the n.m.r. spectrum of the lipids is well resolved. On addition of ferricyanide a proportion of the $-\overset{+}{N}Me_3$ resonances is shifted to lower field. The ratio of the areas then gives the ratio of the two types of head-groups. (We have been able to study the 'sidedness' of lipids in mixed phospholipid vesicles as a function of vesicle size and lipid composition by this and similar methods using ^{31}P n.m.r.)

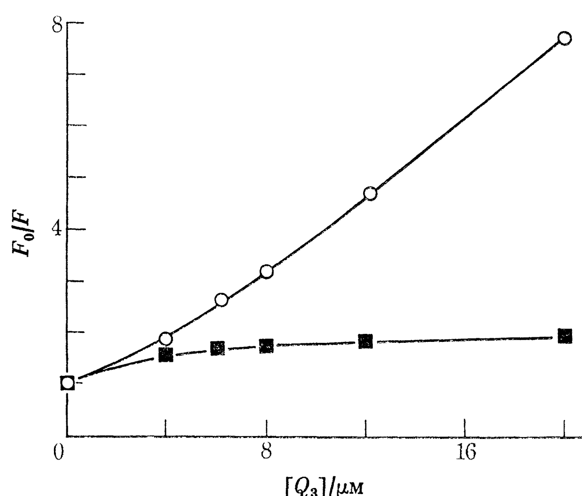

FIGURE 9. Stern–Volmer plots for ubiquinone-3 quenching of 12-(9-anthroyl)-stearic acid fluorescence in lipid micelles. Medium: 10 mM phosphate, temp. 24 °C, pH 7.0, 5 μM probe, 500 μM lecithin (○), or lecithin with cholesterol (170 %:30 % mole/mole) (■). F_0, fluorescence intensity in absence of quencher; F, fluorescence intensity in presence of quencher.

DIFFUSION IN MEMBRANES

Turning to the question of diffusion within the lipid phase we were interested to look at the role of ubiquinone as a mobile carrier in mitochondrial electron transfer. These studies have been described elsewhere (Radda & Vanderkooi 1972; Barrett-Bee, Radda & Thomas 1972) and for the present discussion it is sufficient to refer to some of the related model studies. Using 12-(9-antroyl)-stearic acid as the fluorescent probe and ubiquinone-3 as a quencher the fluorescence of the probe bound to lecithin micelles is shown in figure 9 as a function of quencher concentration. The nonlinear Stern–Volmer plot is indicative of a complex quenching mechanism. However, a detailed comparison of changes in fluorescence life-times and intensities allows the separation by quenching of collisions and complex formation. The qualitative conclusion is that the rate of diffusion of ubiquinone in lecithin bilayers is relatively rapid while incorporation of cholesterol completely abolishes the collisional quenching but not that by the static mechanism (figure 9).

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Lipid–protein relations in membranes

While a whole range of studies on membrane lipids both in 'model' bilayers and biological membranes are beginning to define the structural and motional features of the lipid in relation to biological functions we know much less about the way membrane bound proteins and enzymes are arranged in the lipid matrix. So far fluorescence measurements have only made a limited contribution to the solution of this problem. Perhaps two approaches are worthwhile to consider. The first of these utilizes the transfer of excited state energy from tryptophan groups in membrane proteins to probe molecules located in the lipid region of the membrane. The second involves the introduction of a defined perturbation in the lipid and the observation of the effect of such perturbation on the properties of membrane proteins.

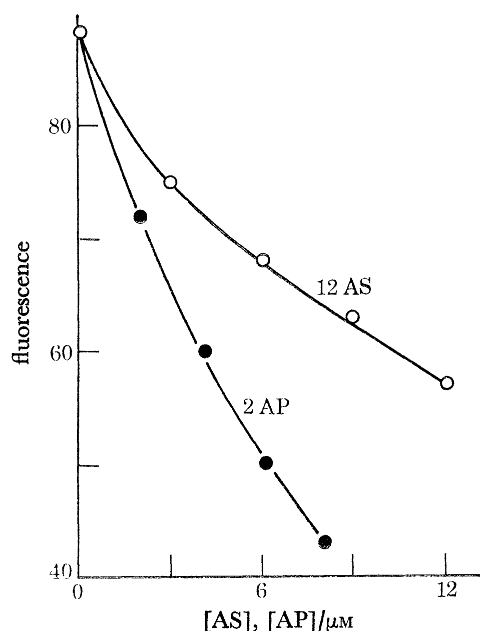

Figure 10. Quenching of the protein fluorescence of HDL_2 lipoprotein by 12-(9-anthroyl)-stearic acid and by 2-(9-anthroyl)-palmitic acid.

One can illustrate the first method by reference to a lipoprotein complex present in blood plasma, the so called heavy density lipoprotein. Introduction of both 12-(9-anthroyl)-stearic acid and 2-(9-anthroyl)-palmitic acid into this complex leads to a quenching or protein fluorescence (figure 10), the most likely quenching mechanism is energy transfer from protein to probe. The differential quenching effect of the two probes immediately tells us that the average distance between protein and the chromophore is larger for the former than for the latter probe. This observation is consistent with the proposed structure of the lipoprotein and the expected location of the fluorescent molecules (Scanu 1972). At the same time it must be emphasized that other structures are also consistent with these observations so that the method is more suitable for observing and describing changes rather than absolute structures.

I shall illustrate the second approach with reference to the adrenaline storage vesicles (chromaffin granules) and membranes derived from them. The simplest perturbation we can introduce is that caused by changing the temperature. Figure 11 shows the temperature variation

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of the fluorescence polarization (plotted as $1/p$, this being related to molecular motion) of different probes with temperature. Two features of the membrane are discernible from these plots. The first is that molecular motion (probably involving a limited oscillation) increases as the probe penetrates further into the hydrocarbon core of the membrane showing the flexibility gradient previously demonstrated in lipid bilayers (McConnell & McFarland 1972). The

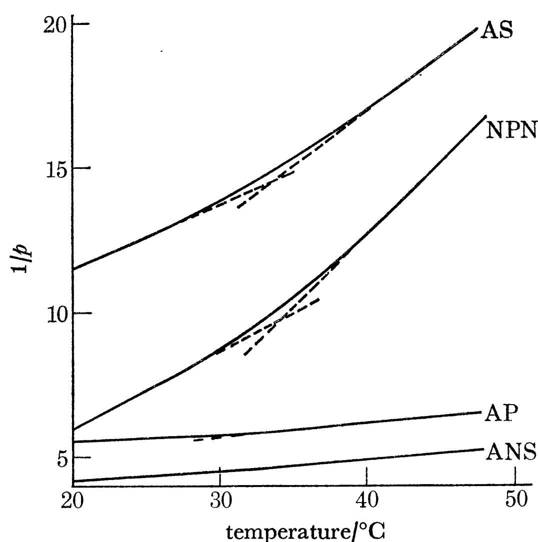

FIGURE 11. Fluorescence polarization (p) as a function of temperature of probes in chromaffin granule membranes. AS, 12-(9-anthroyl)-stearic acid (3 μM); NPN, N-phenyl-1-naphthylamine (3 μM); AP, 2-(9-anthroyl)-palmitic acid (3 μM); ANS, 1-anilino-naphthalene-8-sulphonic acid (3 μM) in 0.3 M sucrose, 10 mM tris-HCl buffer (pH 7.4).

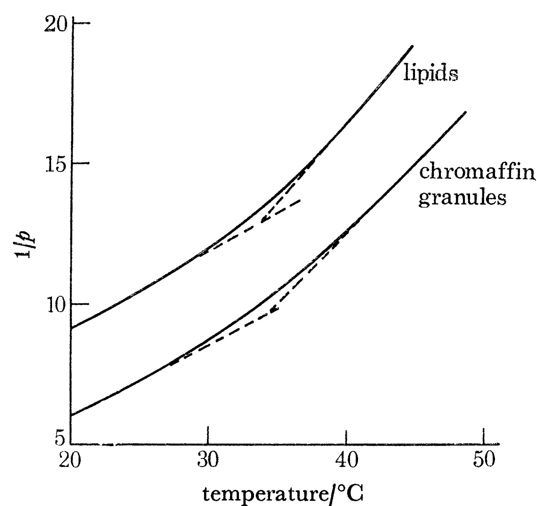

FIGURE 12. Fluorescence of N-phenyl-1-naphthylamine as a function of temperature in chromaffin granule membrane and in sonicated total lipid extract. (Condition as in figure 11.)

second point of note is the marked curvature of the plots for the two probes that sense the behaviour of the membrane interior (as opposed to the polar–nonpolar interface). This suggests that some kind of phase transition (or probably more correctly a phase separation) takes place which is centred around 35 °C. That this is indeed a property of the membrane lipid is demonstrated in figure 12 where a similar phase change is observed in the total lipid extract of

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chromafin granule membranes. This phase change has a marked effect on the properties of at least two of the membrane bound enzymes. In the Arrhenius plots for the activities of the ATPase (figure 13) and the NADH oxidase (figure 14) the break-points also occur around 35 °C although when the latter enzyme is extracted from the membrane by detergent treatment the plot is linear over the whole temperature range. The observations clearly show that the lipid has a strong modulating influence on enzyme activities. It is also tempting to speculate about the biological significance of phase transitions that occur so close to the operating temperature of the system. If adrenaline release requires fusion between the vesicle and the plasma membrane as has been suggested (Douglas 1968), it is likely that fusion will take place more readily if the membrane lipid is in a fluid state. Perhaps the 'triggering' involves a shift

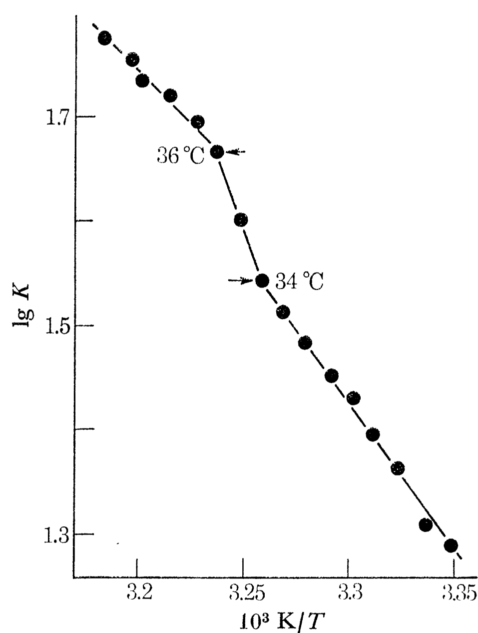

FIGURE 13. Arrhenius plot for chromaffin granule ATP-ase. Granule membrane: ATP, Mg^{2+}. Left hand scale: log activity.

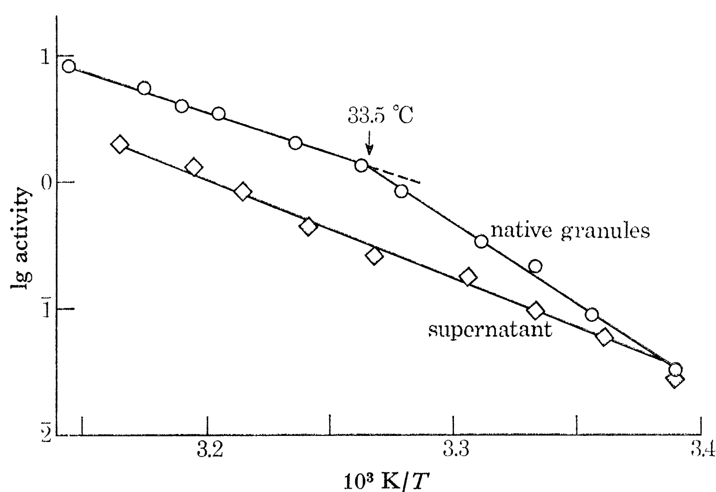

FIGURE 14. Arrhenius plots for membrane bound and Triton-X 100 extracted NADH oxidase from chromaffin granules. Sucrose 0.3 M, (tris-HCl 10 mM), pH 7.4. NADH: 25 μM.

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in the phase separation profile to bring the membrane into the more fluid state. We should note that the term phase change is used in a loose sense here. It refers to the 'anomalous' behaviour of the lipid at a given temperature range.

Conclusions

Fluorescence measurements provide us with some interesting dynamic and limited structural information. Naturally as with any probe method we have to be concerned about the extent of perturbation the probes introduce into the system of study. We can try and overcome this difficulty in two ways. First we should study model systems (e.g. lipid bilayers) where other non-perturbing methods (n.m.r. and X-ray) can be used with confidence and compare the results with those obtained from probe methods. We are then ready to study the biological situation (where the other methods are often difficult to use) where we have the additional test of observing the effect of probe on the biological functioning of the system. Nevertheless all the time we have to keep in mind that whenever possible 'direct' methods must remain superior to the use of probes.

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