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The Dynamic Properties of Biological Membranes

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The dynamic properties of biological membranes

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Biological membranes must be viewed as highly dynamic, undergoing continuous structural fluctuations and changes in response to external perturbations. The study of liposomes by ^{31}P n.m.r. and fluorescence can reveal some of the motional characteristics of the different regions in a bilayer. Asymmetric lipid distribution and how this depends on the environment is also observed by n.m.r.

The nature of the interaction of amine anaesthetics and of polypeptide antibiotics with membranes is discussed in relation to their perturbing effect.

The role of lipid mobility in modulating hormone–receptor interaction is discussed with reference to the binding of thyroid stimulating hormone.

Introduction

Membranes are responsible for a whole variety of biological phenomena. They provide a barrier towards diffusion of small and large molecules into the cell, are involved in concentrating metabolites and metal ions, are intimately associated with processes that are responsible for energy transduction, information transfer and recognition.

It is now generally accepted (though not proven) that membranes consist of phospholipid bilayers or regions of bilayers with proteins occupying both sides of the membrane, some penetrating into the bilayer and others possibly spanning the width of the membrane. Whatever the structural details, there is now considerable evidence that membranes must be viewed as highly dynamic, undergoing continuous structural fluctuations and also structural changes in response to the particular demand that the biological processes impose on the system.

The different forms of molecular motion we have to consider include: (i) rapid segmental motion within the individual membrane components (and particularly in the phospholipids). (ii) Lateral diffusion of phospholipids and proteins. (iii) Lateral diffusion of small molecules within the membrane phase. (iv) The translation of components and small molecules from one side of the membrane to the other. (v) Rotational motion of some proteins. (vi) The establishment of charge or ion gradients in or across the membrane, which often controls membrane linked activities.

In the time available all I can do is to try and give you an impression of how we can study some of these motional characteristics and how in some cases they could relate to particular biological functions and properties.

Methods

I will restrict myself to two methods (both spectroscopic) but we now have many other ways of looking at these problems. In the first method we rely on the use of fluorescent molecules which can be introduced into membranes and as a result of their particular structures occupy different positions. Thus we can sample specific regions of the membrane as has been discussed elsewhere (Radda & Vanderkooi 1972; Radda 1975).

The essence of using fluorescence to study molecular motion is that there is a short but measurable interval between the absorption and emission of light and during this period (characterized by the fluorescence life-time) the molecule can rotate, interact with its lattice or collide with other molecules. The first of these modifies the polarization of emission, the second modifies the spectrum and the third changes the intensity and life-time of fluorescence.

The second approach involves nuclear magnetic resonance (n.m.r.) of nuclei like ^{31}P and ^{1}H. Here we have a very similar situation. When nuclei with spins of say 1/2 are placed in a magnetic field they can take up one of two orientations. Initially both states are equally populated. this, however, represents an unstable situation. The equilibrium position is governed by the Boltzmann distribution law, and the rate at which it is established is characterized by a time

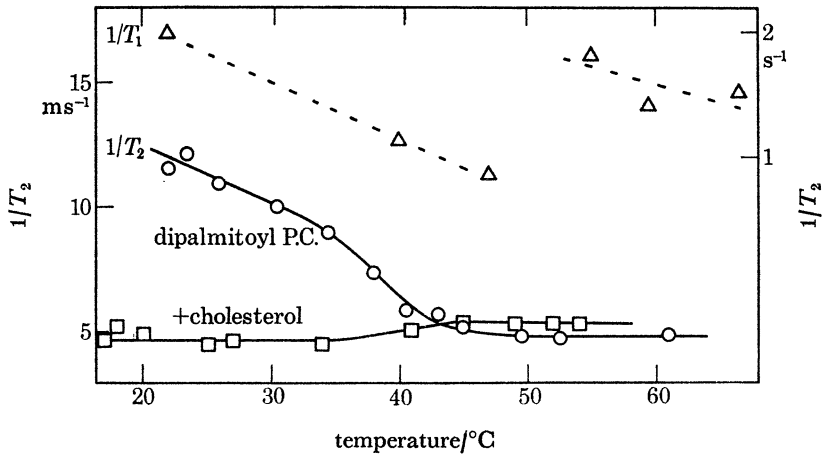

FIGURE 1. 84 MHz ^{31}P relaxation times of dipalmitoyl-DL-phosphatidylcholine dispersions with and without cholesterol.

constant (T_1) referred to as the spin-lattice relaxation time. Besides spin-lattice relaxation other processes have the effect of varying the relative energies of the spin levels, rather than their life-times. Such processes are characterized by a relaxation time T_2, often called the spin–spin relaxation time. Methods (based on pulsed n.m.r.) are available for measuring both relaxation times directly although T_2 can be derived from the observed widths of the magnetic resonance lines. A detailed comparison of the two relaxation times allows one to obtain, with the aid of known theories, quantitative information about parameters of molecular motion. This is because thermal motion is an important factor in modulating both the life-times and energy levels of the spin states.

MOTIONS IN PHOSPHOLIPID SYSTEMS

Let me illustrate the two methods on a well known example. Taking a phospholipid like dipalmitoyllecithin we can make multilamellar structures which undergo a phase change (melting) at round 40 °C. In this system if we look at the ^{31}P n.m.r. of the phosphate head group we can see that the phase transition imparts increased mobility to this group, although even below the transition temperature the head has considerable motional freedom as can be derived from measuring the different relaxation times in the unsonicated samples (Barker, Bell, Radda & Richards 1972).

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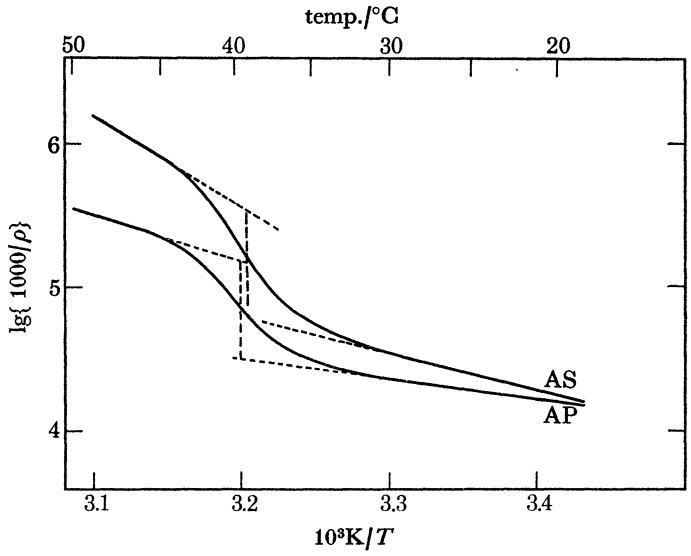

FIGURE 2. Apparent rate of rotation (ρ) of 12-(9-anthroyl)-stearic acid (AS) and 2-(9-anthroyl)-palmitic acid (AP) in dipalmitoyl-L-phosphatidylcholine bilayer vesicles.

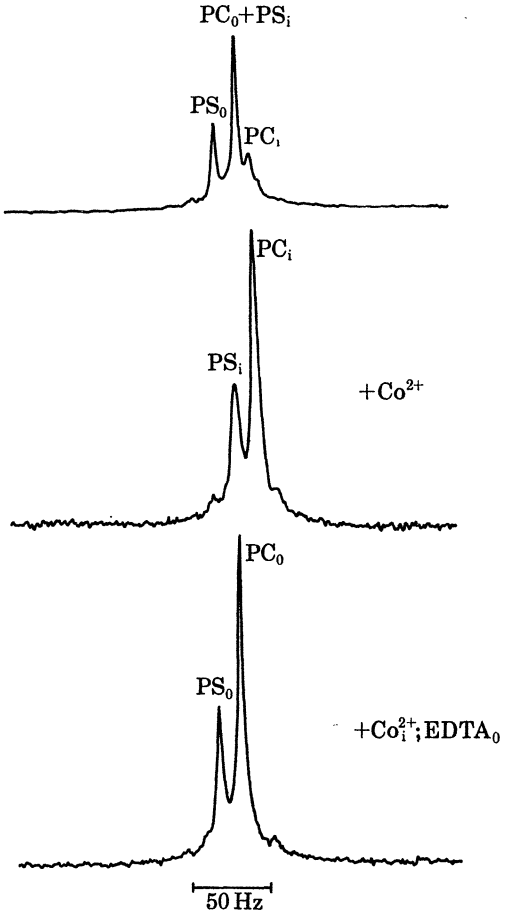

FIGURE 3. ^{31}P n.m.r. spectra (at 36.4 MHz) of phosphatidylcholine (PC)/phosphatidylserine (PS) vesicles (50 mg/ml lipid) in 20 mM sodium acetate–1 mM EDTA (pD 7.2) at 26 °C. The delay time between subsequent pulses was 15 s. Upper spectrum: no additions; middle spectrum: 3 mM $CoCl_2$ added; lower spectrum: after resonication in the presence of 3 mM $CoCl_2$, 4 mM EDTA was added to the outside medium.

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The 'rate of rotation' of some fluorescent molecules in bilayer vesicles (prepared by sonication) as a function of temperature also significantly increases around the transition temperature. The two fluorescent probes used in these experiments sample the bilayer in different regions. In 2-(9-anthroyl)-palmitate the anthracene group is located close to the phospholipid head-groups while in 12-(9-anthroyl)-stearate this chromophore is near the hydrocarbon ends in the middle of the bilayer (Radda & Vanderkooi 1972). It is clear that above the transition temperature the mobility of the probe molecule in the interior of the bilayer is considerably more than that of the chromophore close to the phosphate groups, in agreement with the idea of the 'flexibility gradient' in lipids previously demonstrated by other techniques (McConnell & McFarland 1972).

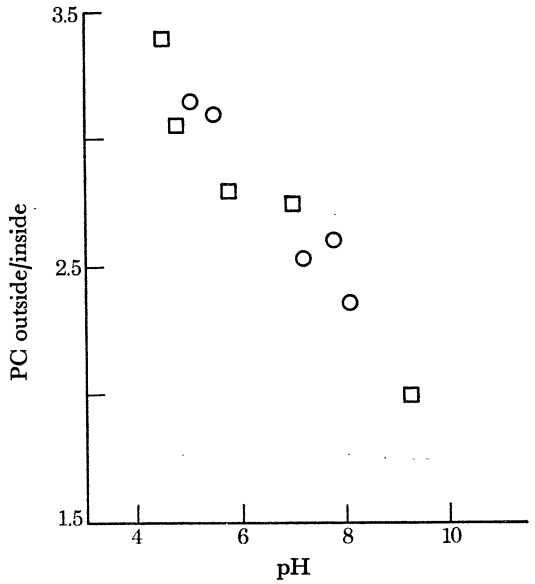


FIGURE 4. pH dependence of phosphatidylcholine outside/inside distribution in phosphatidylcholine/phosphatidylserine vesicles. Lipids (20 mg/ml) in 20 mM sodium acetate. The phosphatidylcholine distribution was measured from the ^{1}H n.m.r. spectra in the presence of 250 mM ferricyanide (see Berden *et al.* 1975). □, vesicles sonicated at various pH values; ○, the pH of the original samples was changed by addition of DCl or NaOD and after 2 h at 25 °C ferricyanide (250 mM) was added and the spectra measured.

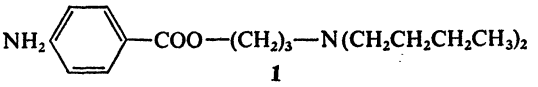
In small bilayer vesicles the ^{31}P n.m.r. relaxations are also dependent on vesicle tumbling (Berden, Barker & Radda 1975). This, however, can be separated out from the effect of local motion (Berden *et al.* 1974) and thus we can study how in vesicles containing mixed phospholipids the packing effects motion (Michaelson, Horwitz & Klein 1974; Berden *et al.* 1975). For example, phosphatidylserine (PS) forms vesicles in which the lipid head-groups are more tightly packed than in vesicles of phosphatidylcholine (PC). In mixtures of these two lipids the ^{31}P resonances can be resolved into three lines corresponding to the two different phosphate groups and the 'inside' and 'outside' resonances in the vesicle (with the inside PS overlapping with outside PC). Addition of Co^{2+} to a suspension of vesicles broadens out the ^{31}P signals from the outside of the bilayer (figure 3*b*) while if Co^{2+} is placed inside the vesicles (by preparing them in the presence of this ion and removing the Co^{2+} outside by addition of EDTA) only the 'outside' resonances are observed (figure 3*c*). These experiments enable us to calculate the distribution of phospholipids on the two sides of the vesicles. In the particular case shown in figure 3 phosphatidylserine shows a preference for the inside of the vesicle. This *asymmetric*

distribution depends on the size of the vesicles (Berden *et al.* 1975) presumably since in the small, highly curved internal surface the more efficient packing of PS is an advantage even at pH values where the phosphatidylserine is entirely neutralized. As one might expect the asymmetry also depends on the pH of the medium. In figure 4 the ratio of the outside to inside phosphatidylcholine in vesicles is plotted as a function of pH in a system containing equimolar mixtures of phosphatidylcholine and phosphatidylserine. Since here the ratio of total phospholipid outside to inside is about 1.8 it is clear that more PS is placed on the inside of the bilayer as the pH is decreased. This figure contains one further interesting observation. The □ symbols represent the situation where the vesicles were prepared (by sonication) and the indicated pH. The ○ symbols give the results of experiments where vesicles were first prepared at neutral pH and then the pH was adjusted to the required value. In both sets of experiments the same lipid distribution is obtained, but in the latter case the equilibrium value is reached only after 1 to 2 h. This indicates that in response to a 'constraint' imposed by the new pH the phospholipids can 'flip' across the bilayer (in the situation shown relatively slowly).

Membrane perturbations

We know very little how important 'membrane fluidity', a general term that is used to describe the different forms of molecular mobilities in membranes, is in the different biological functions. One approach that biochemists have used for a long time is that of perturbing the system in some way and to correlate the observed effect on function with changes in structure and dynamics of the structure. This approach has proved to be particularly useful in circumstances where the detailed structure is not known and where the available methods are not capable of defining the structure precisely but can give useful information about structural changes.

For example, the interaction of amine anaesthetics with membranes has been employed as such a tool. In many systems butacaine (**1**) causes well defined perturbations. It accelerates



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energy linked Ca^{2+} uptake in mitochondria (Chance *et al.* 1969), in nerves it interferes with Na^+ and K^+ transport (Dettbarn 1962) by inducing a structural reorganization of the membrane as a result of interaction with the membrane phospholipids. We can look at the interaction of butacaine with phospholipid vesicles as follows.

The high resolution proton resonance spectrum of butacaine together with the assignments is shown in figure 5. This figure also shows that in the presence of phospholipid vesicles the resonances are considerably broadened. A further increase in the line-widths is produced by introducing a spin-labelled stearic acid (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl) into the lipid vesicles. The qualitative observations of figure 5 already tell us that butacaine interacts with the vesicles, that probably it is rapidly exchanging between the bound and free forms, that on binding it is considerably immobilized and that in the membrane it is sufficiently close to the spin label to experience dipole–dipole interaction between protons on the molecule and the electron of the radical.

To analyse the results qualitatively we have measured the spin-lattice relaxation times (T_1) at two frequencies (90 and 270 MHz) of all the protons in butacaine in the different situations represented in figure 5.

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For free butacaine in solution the motional characteristics of the molecule can be derived by the method described earlier (Coates *et al.* 1973). In the absence of paramagnetic impurities the spin-lattice relaxation time of a proton in a molecule is usually dominated by intramolecular dipole–dipole interactions. The magnitude of T_1 depends on both the molecular motion and the mean inverse sixth power ΣR^{-6} of the distance between the various proton dipoles. Assuming that all the interproton vectors reorient isotropically the ratio of T_1 values at two frequencies may be used directly to find a correlation time characterizing this motion (Coates *et al.* 1973) and from the appropriate equations the distance term can then be derived. Comparison of this term with the theoretical intra-group ΣR^{-6} term calculated from bond length data serves as a test for the isotropic motion model. The correlation times for all protons of butacaine lie between 0.9 and 2.4×10^{-10} s and are shorter than the bulk rotational correlation time of the

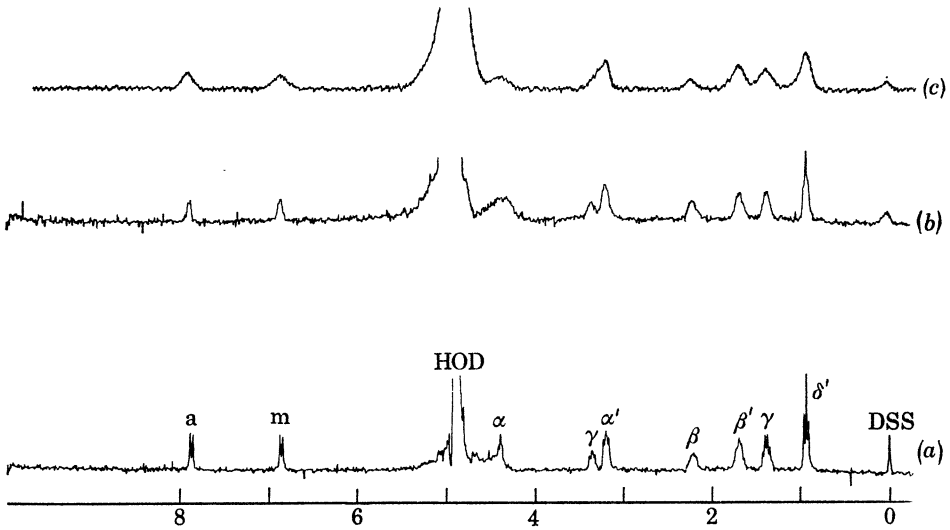

FIGURE 5. High resolution proton resonance spectrum of butacaine in the presence and absence of phospholipid vesicles. (*a*) 40 mM butacaine in D_2O (pH 7.0 experimental); (*b*) 20 mM butacaine with 25 mg/ml dipalmitoyl phosphatidylcholine; (*c*) as *b* but with 1:25 (mole:mole) spin label to lipid (spin label: 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl).

molecule that can be calculated from the Stokes–Einstein equation. Hence we must assume that the result obtained describes anisotropic segmental motion of the hydrocarbon chain. Comparison of the experimental and theoretical intra-group ΣR^{-6} terms suggests that anisotropic motion is important for the methylene and methyl protons of the hydrocarbon chain (Barker *et al.* 1974). In the latter case the method of Woessner (1962) elaborated by Coates *et al.* (1973) can be used to find values of the isotropic motion correlation time (τ_c) and the anisotropic correlation time (τ_R) for the methyl protons.

The same treatment is not strictly valid for the methylene protons, since it requires the assumption (i) of rotation around a single axis and (ii) that only protons attached to the same atom are important. We can, however, use the results of this treatment to compare qualitatively the motional freedom of methylenes in different parts of the molecule (table 1). We find that while the calculated isotropic correlation times (τ_c) are not very different for the various protons, the anisotropic correlation times characterizing segmental motion vary widely in the molecule. The most restricted motion is shown for the $C_\alpha H_2$ and segmental motion becomes more significant on moving towards the terminal methyl group.

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Table 1. Correlation times for internuclear vectors in butacaine derived assuming anisotropic internal motion

group	$c/10^{-10}$ s	$R_0 t$/s
$C_\alpha H_2$	1.1	6.3×10^{-10}
$C_\beta H_2$	1.2	4.5×10^{-10}
CH_2	2.9	1.2×10^{-10}
$C'_\alpha H_2$	2.9	9.5×10^{-11}
$C'_\beta H_2$	1.9	8.5×10^{-11}
$C'H_2$	2.3	5.3×10^{-11}
$C'H_3$	1.7	1×10^{-12}

Errors estimated at ± 40%.

Before it is possible to find the relaxation times of butacaine bound to the lipid, it is necessary (i) to determine the fraction of the butacaine present that is bound and (ii) to show that the free and bound anaesthetic molecules are in fast exchange. The fraction of bound butacaine was measured by equilibrium dialysis and temperature dependence studies suggested (without proving), that fast exchange conditions held. The T_1 values for bound butacaine were then calculated (again at two frequencies) from the equation:

$$\frac{1}{T_1\,(\text{observed})} = \frac{\text{fraction bound}}{T_1\,(\text{bound})} + \frac{\text{fraction free}}{T_1\,(\text{free})}.$$

The results are compared with T_1 values for free butacaine in table 2. The analysis applied to free butacaine is not valid for the bound form partly because additional relaxation is caused by external protons (i.e. those of the lipid molecules) and partly because the nature of the molecular motion (modulating both the intra- and intermolecular interactions) could be quite complex. It can be seen, however, that all the relaxation times have shortened considerably while the ratios of the T_1 values at the two frequencies changed much less. In addition the largest change

Table 2. T_1 values of protons in free and bound butacaine at two frequencies

(Temp. 23 °C, 40 mM butacaine (pH 6.8), standard deviations ± 8 %.)

group	T_1 (270 MHz)/s	T_1 (90 MHz)/s	T_1 (270 MHz)/s	T_1 (90 MHz)/s
aromatic *m*-CH	2.18	1.66	0.14	0.10
aromatic *o*-CH	2.08	1.43	0.13	0.07
$C_\alpha H_2$	0.40	0.36	—	—
$C_\beta H_2$	0.36	0.34	0.27	0.16
CH_2	0.34	0.26	0.33	0.19
$C'_\alpha H_2$	0.37	0.27	0.32	0.16
$C'_\beta H_2$	0.45	0.39	0.30	0.14
$C'H_2$	0.80	0.58	0.29	0.09
$C'H_3$	1.35	1.11	0.17	0.12

in the relaxation times occurs for the aromatic protons where the effect on the T_1 ratios at two frequencies is smallest, while the most significant change in the T_1 ratios is for the methylene protons of the *n*-butyl group. Since qualitatively the T_1 ratios at two frequencies reflect molecular motion and the change in T_1 at a given frequency on binding depends on both motion and additional relaxations due to external protons we can conclude (i) that the butacaine molecule is most strongly restricted in the region of its positive charge, (ii) that all parts of the molecule are reorienting more slowly in the bound state, and (iii) that the aromatic part of the molecule is in close proximity to some of the phospholipid protons.

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To obtain a better idea of the positioning of the anaesthetic in the lipid bilayer the T_1 values of the butacaine protons in the presence of vesicles containing the stearic acid spin label (figure 5) were measured, again at two frequencies. As with proton–proton dipolar interactions, measurements of the contribution of proton–electron interaction to T_1 at two frequencies enable one to deduce both dynamic and positional information. The detailed theory used here is described elsewhere (McColl 1973) and it is sufficient to note that again the proportion of

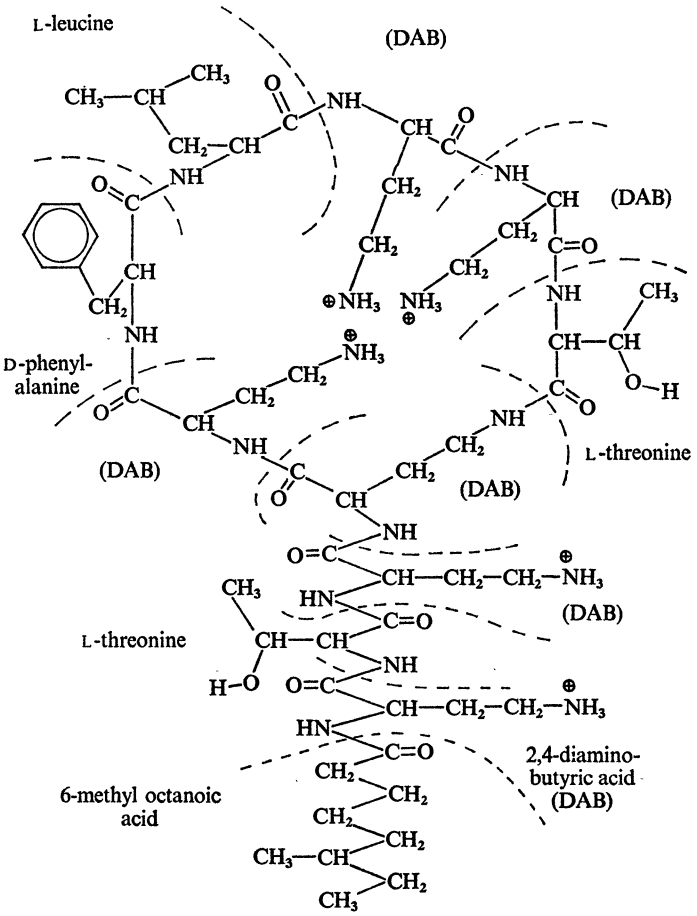

FIGURE 6. Primary structure of polymyxin B_1.

the bound butacaine must be known and that fast lateral diffusion of the spin label during the life-time of butacaine bound to lipid is assumed in the calculations. The correlation times for proton–electron interaction and distances from the spin label to individual groups of protons in butacaine have been derived (Barker *et al.* 1974). The results were shown to be consistent with a model in which the anaesthetic lies horizontally in the lipid between the head-groups. This agrees with the observation that one butacaine molecule binds to two lipid molecules (Feinstein 1964) and with the data on the expansion of lipid monolayers on addition of procaine (Gershfeld 1962). Model building showed that if the spin-labelled stearic acid is incorporated in the bilayer so that the hydrophobic chain is aligned with those of the lecithin molecules, and the carboxyl group is level with the phosphate head-groups, then the spin-label is between 0.5 and 0.6 nm from the phosphate group where the anaesthetic can be postulated to bind.

It is possible to go further with this kind of approach to study problems which relate to the recognition of polypeptide antibiotics by cell membranes.

Polymyxin B has the primary structure given in figure 6 (Suzuki *et al.* 1964). It has virtually no effect on gram-positive organisms and gram-negative cocci, but most of the gram-negative bacteria are inhibited by it in concentrations of 0.02–5 μg/ml (Sebek 1967). It has been suggested that polymyxins combine with the cell membranes of gram-negative bacteria through association and orientation of the hydrophobic and hydrophilic regions of both the phospholipids and polymyxins (Newton 1954). The osmotic equilibrium of the cell is then disturbed and the cellular content is released (Newton 1953).

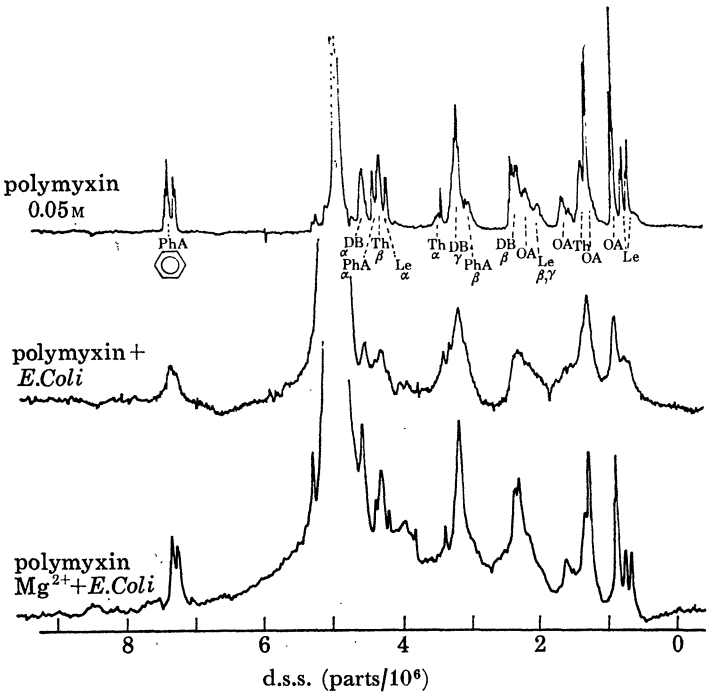

FIGURE 7. High resolution proton n.m.r. of polymyxin B in the absence and presence of *E. coli*. Le, L-leucine; PhA, D-phenylalanine; Th, L-theonine; DB, 2,4-diaminobutyric acid; OA, (+)-6-methyloctanoic acid.

The high resolution proton magnetic resonance spectrum of polymyxin B in D_2O, obtained by Fourier-transform spectroscopy is shown in figure 7. The spectrum can be assigned on the basis of (i) comparison with the spectra of the constituent amino acid; (ii) by taking spectra at varying field strengths (60, 90 and 270 MHz); (iii) by double irradiation and internuclear double resonance experiments and (iv) by evaluation of the integrated areas under the peaks. The assignments (some of which are still tentative) are given on the top line in figure 7.

The linewidths of all lines increase with increasing *E. coli* concentration, suggesting some sort of exchange mechanism for the *E. coli*–polymyxin interaction. More interestingly differential broadenings are observed. The lines being most affected are those of the hydrophobic groups: the methyl and methylene protons of 6-methyl octanoic acid, the phenyl protons of phenylalanine and the methyl protons of leucine.

To complement the linewidth measurements, we have also determined the proton high resolution T_1 (spin-lattice) relaxation times of polymyxin under different conditions. As for butacaine T_1 measurements can provide us with information about motion in specific parts of

the molecule. If we observe the resonances of the methyl protons of leucine and methyl octanoic acid at several frequencies, the correlation times are 5.0×10^{-10} s and 2.2×10^{-10} s respectively. This illustrates the larger mobility of methyl groups in residues on the side chain compared with those in the ring of polymyxin. Measurements of T_1 for all lines of the polymyxin spectrum on addition of *E. coli* and of the magnesium–*E. coli* complex show (Barrett-Bee, Radda & Thomas 1972) that the peaks most affected are again those of the hydrophobic region. We conclude that the hydrophobic groups of the antibiotic are more immobilized as a result of binding to the membrane than the polar regions. A likely implication of this is that the non-polar region penetrates into the membrane while the polar groups remain at the membrane–water interface.

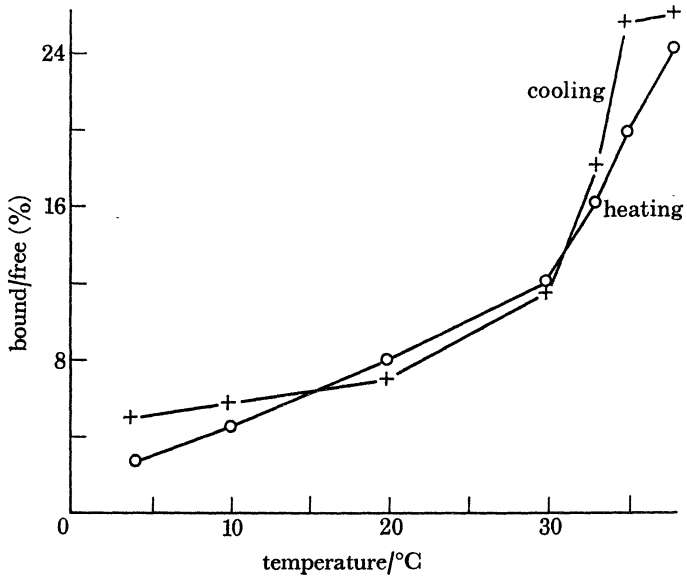

FIGURE 8. The effect of temperature on the binding of thyroid stimulating hormone to thyroid membranes.

HORMONE–MEMBRANE INTERACTIONS

Unfortunately the sensitivity of n.m.r. is not yet sufficient to look at situations where there are a small number of receptor sites, as is the case for hormone binding. We can, however, attempt to define the role of lipid mobility in the recognition process.

The binding of thyroid stimulating hormone (TSH) to plasma membranes of thyroid cells can be followed by radioactive techniques (Bashford, Harrison, Radda & Mehdi 1975). On increasing the temperature more hormone is bound and a relatively sharp increase in binding takes place at around 30 °C (figure 8). This kind of change is often attributed to a 'phase change' in the membrane.

In detailed binding studies we have demonstrated (Bashford *et al.* 1975) that additional hormone binding sites are uncovered above the transition temperature. The motional characteristics of the membrane lipids, as defined by fluorescence polarization studies on added probes, play an important role in modulating hormone binding. This can be seen not only by observing the relation between binding and lipid mobility as a function of temperature but also by the correlation found between binding and fluorescence polarization as the lipid mobility is altered on the addition of cations like Ca^{2+} (Bashford *et al.* 1975).

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Hormone release and membrane fusion

In discussing the dynamic properties of biological membranes we must consider membrane fusion. While we know very little about the mechanism of the process there is little doubt that it is important in several biological phenomena including virus action (Harris 1970) and the release of transmitter substances from intracellular storage vesicles (Douglas 1974). For example

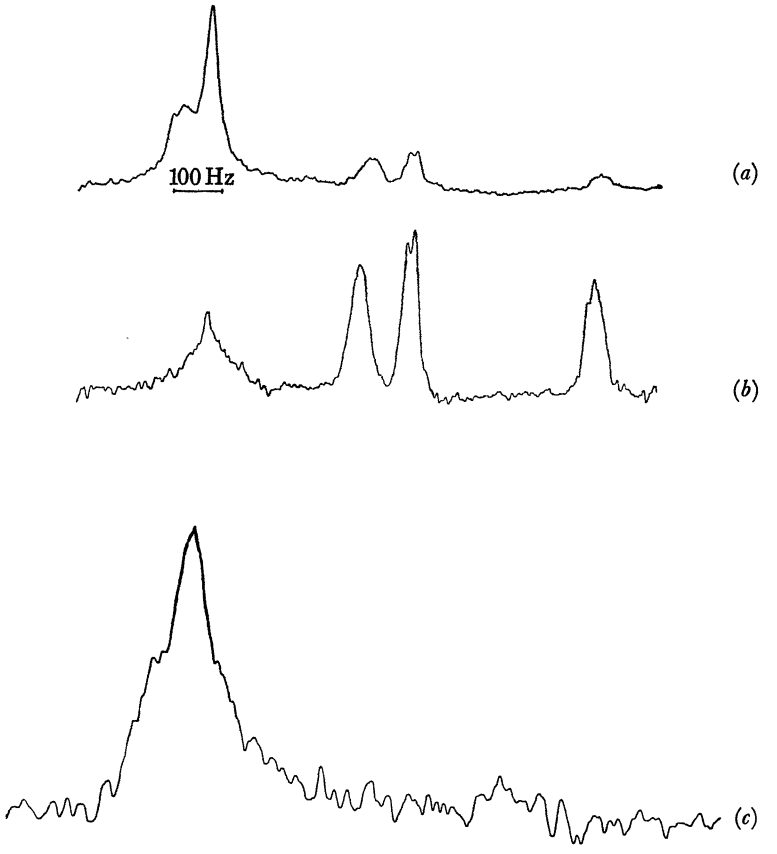

Figure 9. ^{31}P n.m.r. (at 36.4 MHz) spectra of chromaffin granules (*a*) of adrenal glands (*b*) and of adrenal glands after perfusion with Ca^{2+} and X537A (*c*).

catecholamines are stored in chromaffin granules inside the chromaffin cells of the adrenal medulla at concentrations of 0.4 M together with ATP (0.1 M) and some acidic proteins (Kirshner & Kirshner 1971). It is now generally believed that catecholamine release is accompanied by the extrusion of ATP and acidic proteins, indicating exocytosis (Douglas 1968). Some unusual properties of the chromaffin granule membrane that may be relevant to the release mechanism have been discussed previously (Radda 1975). We have also demonstrated that the chromaffin granule ATP-ase is sensitive to H^+ ionophores (uncouplers) (Bashford, Radda & Ritchie 1975) thus possibly being involved in the generation of a hydrogen ion gradient in or across the granule membrane. Such a movement of protons would lead to the development of both electrical and chemical potential within or across the membrane. This potential may well be the driving force for the uptake of catecholamines (Bashford, Casey, Radda & Ritchie, unpublished observations) but the details of this are not yet known. For our present discussion

we note that the experiments with uncouplers suggested (indirectly) an unusually low intravesicular pH in the isolated chromaffin granules. Since ion and charge gradients in or across membranes may be important in controlling different membrane functions, including transport and exocytosis, we need to be able to examine ion distribution and movements *in vivo*. Recently we have described how ^{31}P n.m.r. can be used to observe metabolites in intact biological tissue (Hoult *et al.* 1974) and how the positions of some of the resonances reflect the pH of the environment and complexation with metal ions like Mg^{2+}. The ^{31}P n.m.r. spectra of isolated chromaffin granules (figure 9*a*) show the resonances of ATP while when a whole adrenal gland is placed in the n.m.r. sample tube additional resonances (that of inorganic phosphate and possibly sugar phosphates) are observed (figure 9*b*). These additional peaks can be shown to arise from the adrenal cortex. The positions of the ATP resonances in the two situations (shown in figures 9*a* and *b*) are identical, implying a close similarity in the environments of the intravesicular ATP. In addition the resonance frequency for the γ-phosphate group of ATP corresponds to an intravesicular pH of below 6 in agreement with the results cited above (Bashford, Radda & Ritchie 1975). In view of the large concentration of catecholamines and acidic proteins inside the vesicles, however, the exact interpretation of this observation is not yet clear. Nevertheless ^{31}P n.m.r. gives us a new handle for observing intracellular events. Thus when the whole gland is perfused with Ca^{2+} in the presence of the ionophore X537A (Cochrane & Douglas 1974) all the ATP is released as can be seen in the spectrum shown in figure 9*c* which was recorded after the perfusion medium was washed out.

We can thus conclude on the optimistic note that many of the spectroscopic methods have now been developed sufficiently to study the complex dynamic properties of biological membranes not only in their isolated forms but hopefully also *in vivo*.

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Discussion

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One of Dr Radda's slides was a graph of the strength of binding of TSH to a membrane preparation from the thyroid gland, plotted against temperature (figure 8). The slope of the curve increased at about 30 °C, and he interpreted this as indicating that a 'phase change' occurred at that temperature. This seems to me most improbable, since a phase change would cause a discontinuity in the *actual value* of the ordinate, not merely in the *slope*.

G. K. Radda

Huxley's statement is only valid in restricted circumstances. First, I would like to make the distinction between first- and second-order phase changes. In the former the function ($\partial G/\partial$ variable) has a discontinuity while in the latter it does not. Order–disorder transitions in alloys or the appearance of molecular rotation in crystals of NH_4Cl are examples of second-order phase changes. In a membrane 'lateral phase separation' is in effect an order–disorder transition and would be expected to lead to the kinds of observations I have described.

There is one further point. In a *real system* (like a biological membrane) the distinction between the two types of phase changes is not always clear-cut because (i) the system is not infinitely large, (ii) it is likely to contain defects and (iii) is not uniform. In these circumstances even phase changes of the first kind will only produce curvature and not a discontinuity in the relevant functions. For these reasons I believe we are correct in using the terms phase and phase changes in membranes.

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