MAGNETIC RESONANCE STUDIES ON DRUG-MEMBRANE INTERACTIONS

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INTRODUCTION

ONE of the most important problems facing mankind today concerns with the understanding of the biological response of drug molecules at a molecular level. Linked to this is the problem of designing compounds having a stronger and more selective biological response. Most drug molecules bind to some highly specialised and selective sites in tissues. These sites which form part of the three-dimensional pattern of a specific macromolecule are called receptors. The drug-receptor interactions lead to a chain of events finally resulting in the response at a macroscopic level. Only a few molecules are sufficient to invoke response at the level of the whole body. Thus the engagement at molecular level is capable of massive amplification.

The drug-receptor interactions involve the consideration of the following points:

(a) the ability of the drug molecule to reach the receptor sites in the system
(b) the geometrical and electronic structure of the drug which has to be complementary to the binding sites in the receptor
(c) knowledge of the topography of the receptor
(d) nature of the forces responsible for the formations of drug-receptor complex
(e) changes in the structure subsequent to binding
(f) the resulting biological response, and
(g) excretion of the drug.

While the precise biological and pharmacological actions of drug molecules are largely undetermined, there is a widening belief that interactions with membranes play an important role. For example the first problem outlined above is ultimately linked to transport of the drug across biological membranes. After administration, the drug usually enters the blood stream rapidly and then gets circulated through the body. It can be removed from circulation by metabolism, excretion or accumulation. In these processes the drug molecule has to permeate through biomembranes. The permeability of the drug is determined by its interaction with membrane lipids—a major constituent of biomembranes. Very little is known about the structure and size of receptors despite serious efforts towards their isolation and characterisation. The most common receptors are constituents of biomembranes, proteins and nucleic acids. In the former case, a direct drug-receptor binding on the membrane surface can bring about the desired response. However, an alternative mode of action may involve changes in membrane-stereodynamics in the vicinity of the receptor which may signal a "switching on" of the desired receptor-response.

Certain drugs directly attack on the membranes. Examples are certain anti-infectious agents such as polymyxin, bacitracin and colistin. Whatever may be nature and site of the receptor, it must be remembered that when a drug molecule diffuses to a cell to provoke or prevent some cellular activity, it first encounters the cell membrane. It then interacts with a receptor on the cell membrane or pass through it in order to interact with a receptor inside the cell. It is for these reasons that it is important to know how drugs interact with biological membranes.

MEMBRANE ORGANISATION AND FLUIDITY

The major components of biomembranes are proteins and phospholipids. In mammalian cells, small amount of carbohydrates are also present in the form of glycoproteins or glycolipids[1–3]. Due to the amphiphatic character of phospho-

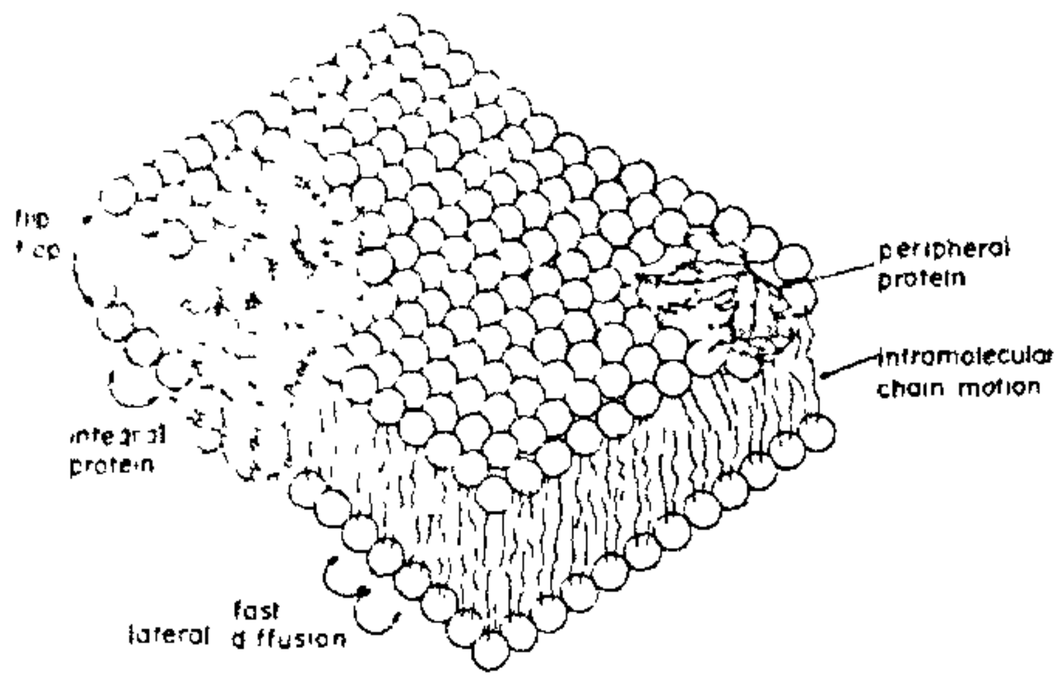

Figure 1. Model of biological membrane showing various types of molecular motions present in the liquid crystalline phase.

lipids, these molecules acquire a bilayer structure (figure 1) such that the hydrocarbon chains are aligned parallel to one another with the polar heads submerged in water on either side of the bilayer. The membrane proteins are either loosely bound to the surface of lipid bilayers (peripheral proteins) or deeply embedded into the lipid matrix (integral proteins). The molecular architecture of biomembranes is stabilised by lipid-lipid, lipid-protein and protein-protein interactions.

In recent years there has been a fundamental change in our understanding of membrane organisation with the realisation that they have a highly mobile structure in the temperature range over which the living cells function. Basically three types of molecular motions have been detected. The hydrocarbon chains of phospholipids show intramolecular chain motions involving rotations around single bonds. The lipids and proteins on each side of the lipid-bilayer can diffuse laterally at a relatively faster rate. On the other hand, the rate of exchange across the two surfaces of the bilayer (flip-flop exchange) is relatively slow. Thus, the cell-membranes show a liquid-crystalline behaviour. The phospholipid bilayers exhibit a characteristic temperature (Tm) below which they undergo a phase transition from liquid crystalline to gel phase as a consequence of the 'freezing' of molecular motions discussed above.

There is a 1:1 correspondence between the fluidity and permeability across biomembranes. Thus, the transport properties are directly linked to the mobility of membranes. Further, it has been discovered recently that many of the membrane bound enzymes are switched on or off as the system goes from gel to liquid crystalline phase or *vice versa*.

Magnetic resonance techniques, electron spin resonance (ESR) and nuclear magnetic resonance (NMR) are ideally suited to investigate the molecular motions and phase transition in model and natural membrane system[4-6]. We wish to discuss here how these techniques can be used to investigate problems related to drug-membrane interactions.

SPIN LABEL AND DETECTION OF PHASE TRANSITIONS

In the spin label method, a free radical with a suitably designed functional group is attached to selected molecules (*e.g.* fatty acids, steriods, phospholipids, etc.)[6]. These molecules with free

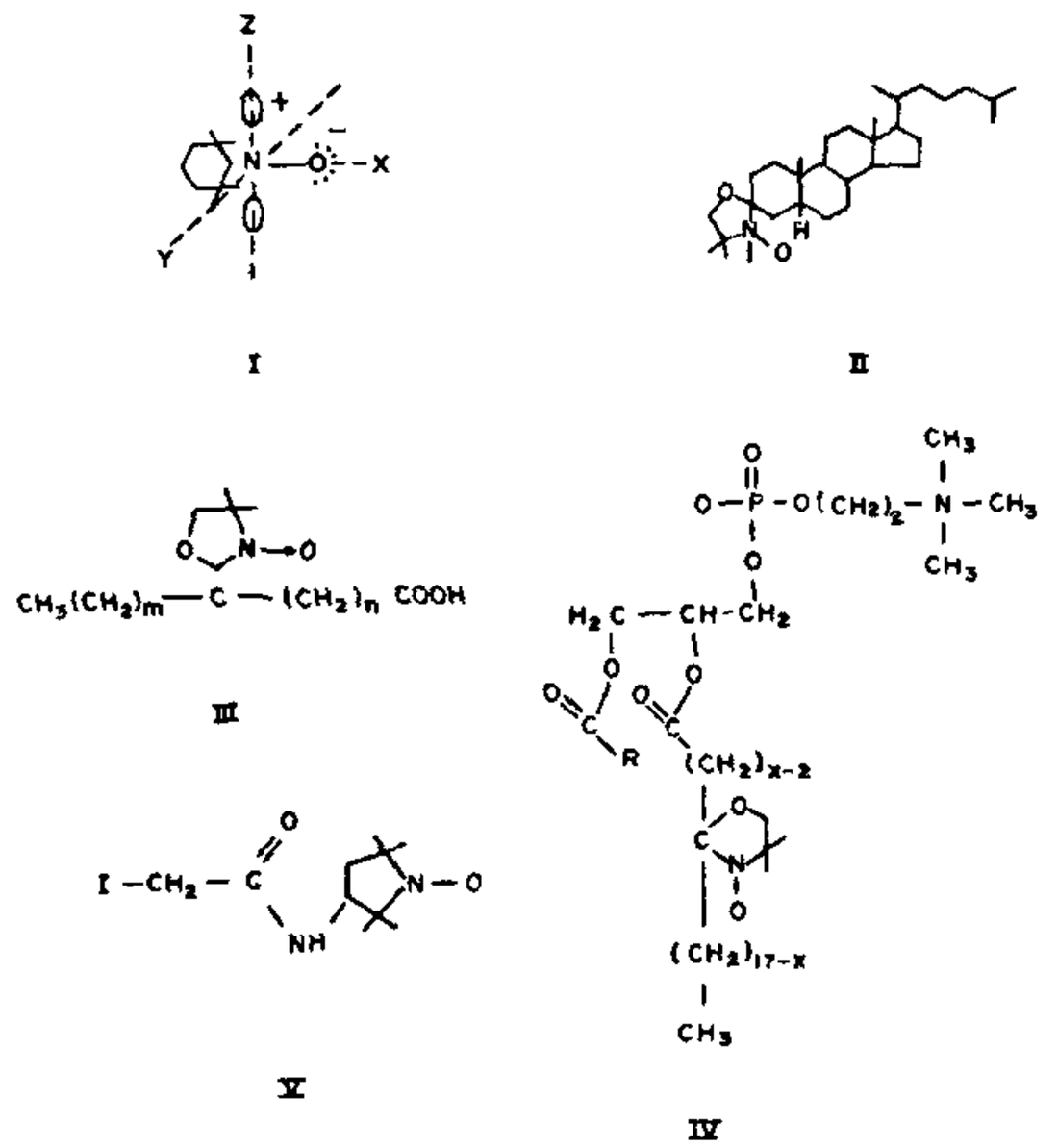

Figure 2. Various spin labels used in ESR studies of membrane fluidity. **I.** 2,2,6,6-tetramethylipiperidine (TEMPO). **II.** 3-doxylcholestane (CSL). **III.** stearic acid labelled at position (m, n). **IV.** phosphatidyl choline labelled at 5th carbon atom position from glycerol backbone along fatty acid chain. **V.** Iodoacetamide spin label.

radical moieties are intercalated into the system. The most commonly used free radical in membrane studies are nitroxide radicals. Figure 2 shows some typical spin probes; the utility and function of these probes are different. Spin label TEMPO(I) is spherical in shape and possesses the property of partitioning into lipid and water phase. The probe is particularly useful, as described in detail later, in studying phase transitions. The steroid spin probe (II) resides at the bilayer surface and reports the head group conformation of membrane constituents. The fatty acid probe (III) and phospholipid probe (IV) can map out the fluidity and permeability gradient across the lipid bilayer by moving the nitroxyl moiety along the fatty acid chain. Probe IV is preferred to probe III as phospholipids are natural constituents of biological membranes, but are not commonly used in practice because of difficulties in synthesis. Spin label V is used to probe the protein part of the membranes. Methods have been developed to study *in vivo* drug absorption, distribution and excretion and drug metabolism by synthesising spin labelled drugs. Such methods however have limitations particularly because the drugs many a times show decreased pharmocological activity on account of the presence of bulky nitroxyl group[6]. Spin trapping technique promises a powerful probe into the problem of carcinogenesis[7]. Feldman *et al* have reported *in vivo* ESR studies in the liver of rats[8].

The first quantitative measurements of the fluidity of lipid bilayer vesicles were made using ESR spin label techniques. Figure 3 shows typical ESR spectra from a nitroxide spin label; it is characterised by the g value and the hyperfine splitting constant A due to interaction of the free radical with ^{14}N nucleus. Hyperfine splittings are different when the spin probe is oriented parallel (a) and perpendicular (b) to the applied magnetic field. Similarly the spectra possess different characteristics when the probes are rigidly immobilized and randomly oriented (c) or when undergoing isotropic rapid and random tumbling (d). Because of this anisotropic nature of the A tensor this method is useful in studying various aspects of molecular motion such as esti-

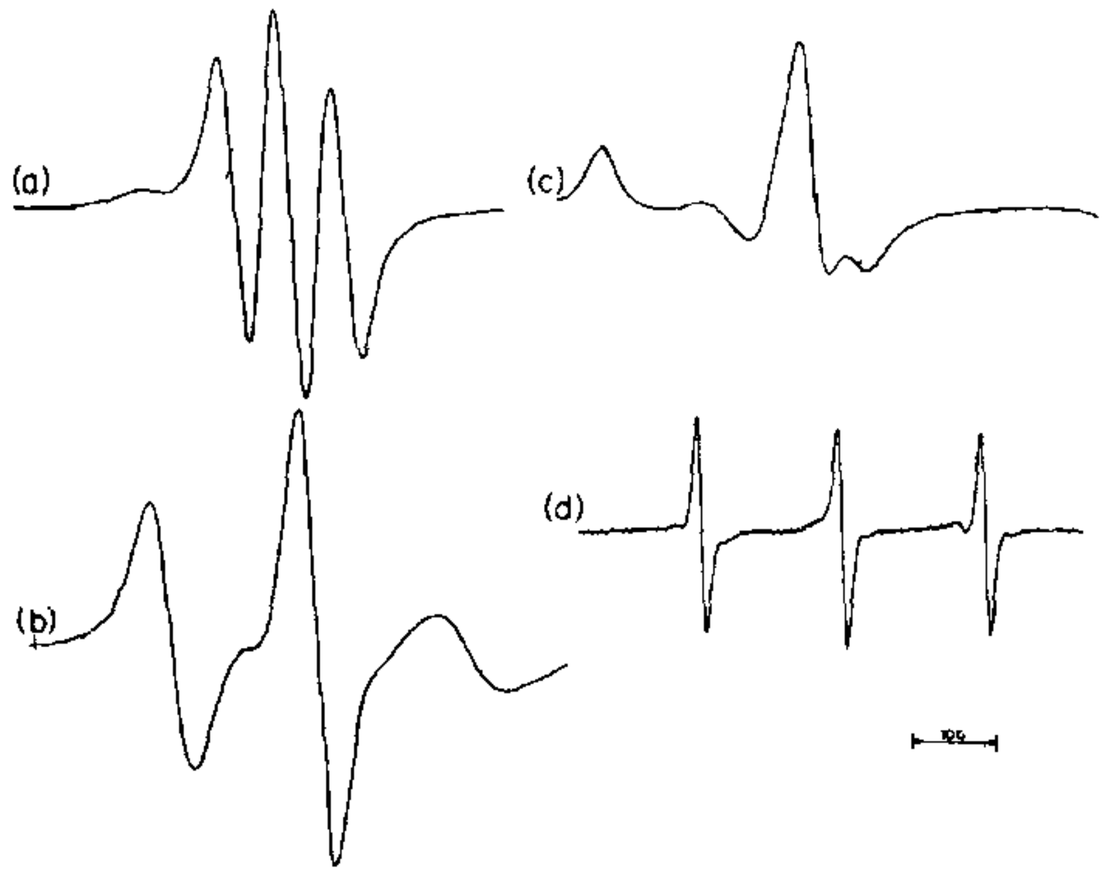

Figure 3. ESR spin label spectra of 5-doxyl stearic acid spin label (5-SASL).

mation of amplitude and rate of motion, translational diffusion, intermolecular separations and transport etc. We shall restrict ourselves to the elucidation of fluidity, permeability and phase transition characteristics of membranes on drug incorporation.

Phase transition properties are conveniently studied by using spin label TEMPO which partitions into lipid and water[9] as shown in figure 4(a). The relative partitioning depends upon the state of the lipids. The solubility of TEMPO in the lipid phase increases as one goes from gel to liquid crystalline phase. Consequently the TEMPO spectral parameter $f = H/(H + P)$ increases with temperature (figure 4(b)) and gel-liquid crystal phase transition is reflected by an abrupt change in the f value. The characteristics of phase transition curve change when a drug *e.g.* propranolol or a vitamine E(α-tocopherol) is incorporated in the membrane. In the case of propranolol a β-adrenergic blocking drug the phase transition temperature is lowered by about 10° C. This indicates that the drug is fluidizing the membrane[10]. In case of vitamin E the sigmoidal nature of phase transition is totally lost indicating that the membrane is heterogeneous. There are regions of membrane which are rich and regions which are poor in drug content. The transition curve that one obtains is an overall sum of curves from different regions and hence does not show characteristic sigmoidal nature.

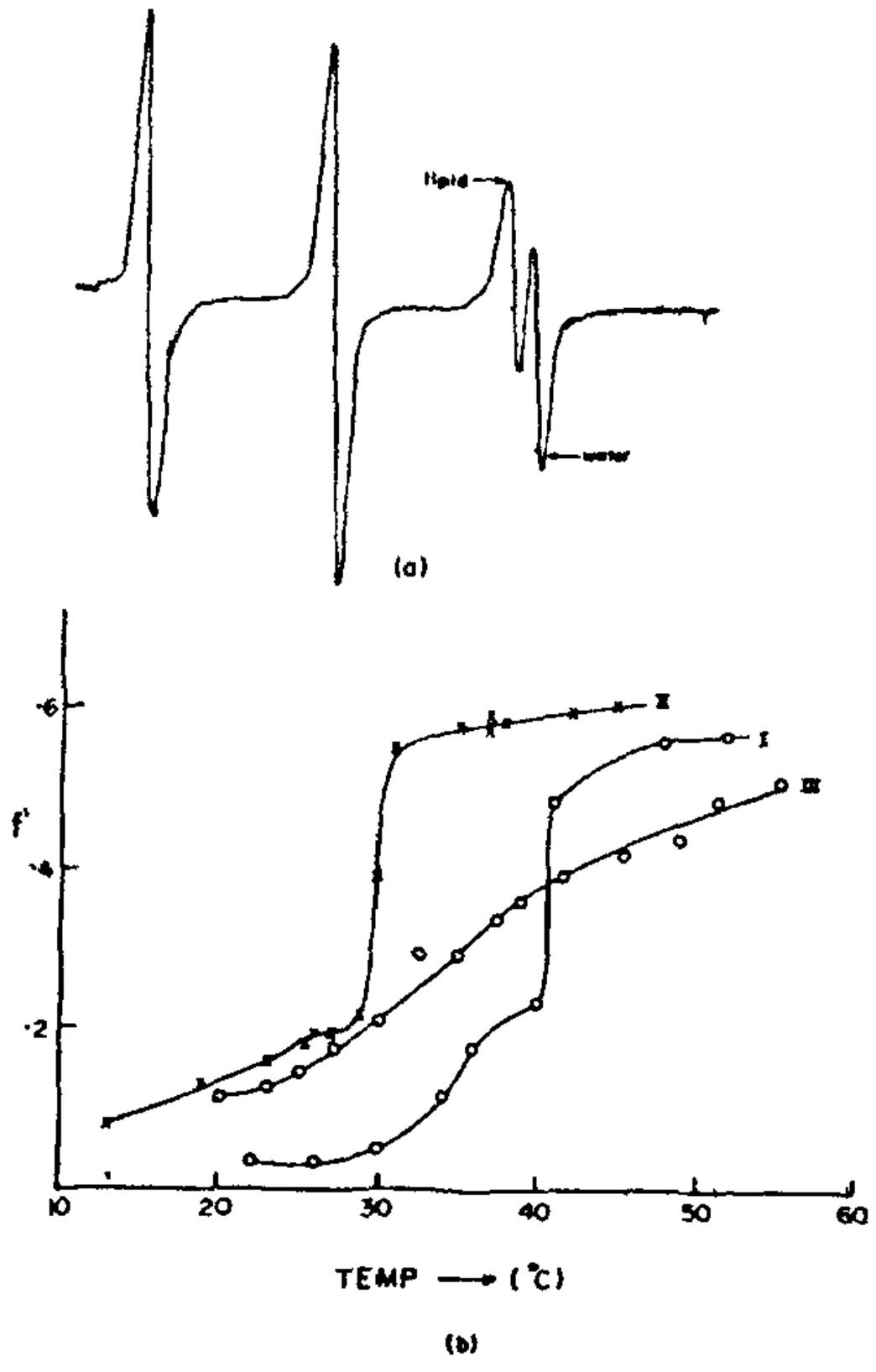

Figure 4. (a) ESR spectrum of TEMPO (5×10^{-5} M) dissolved in suspension of dipalmitoyl phosphatidylcholine (DPPC) vesicles in water. The high field component of the three line spectrum splits into a doublet corresponding to TEMPO in lipid and water phase. (b) spectral parameter $f = H/(H + P)$ where H = signal due to TEMPO dissolved in lipid environment; P = signal due to TEMPO dissolved in water as a function of temperature. **I.** DPPC (100 mM). **II.** DPPC (100 mM) + propranolol (50 mM). **III.** DPPC (100 mM) + α-tocopherol (20 mM).

ORDER PARAMETERS

Another type of ESR experiment whereby molecular ordering can be monitored is through measurements of order parameters[12]. It is a measure of the degree of lipid organization within the bilayer. Molecular orientations are expressed relative to some reference axis. The axis is usually chosen to be the normal to the membrane surface. For motions that are fast on ESR time scale, the hyperfine splittings are solely determined by geometric considerations. If the instantaneous orientations of nitroxide X, Y, Z axis (figure 2) relative to the bilayer normal are θ_1, θ_2, θ_3 the angular amplitudes of motion can be expressed as

$$S_{ii} = \tfrac{1}{2}(3 < \cos^2\theta_i > - 1), \quad i = 1, 2, 3.$$

This is related to the spectral parameter by

$$S_{33} = A_{11} - A_1 \Big/ A_{zz} - A_{xx}$$

where A_{11} and A_1 correspond to the hyperfine splittings when the applied magnetic field is respectively parallel and perpendicular to bilayer normal. The order parameter is often used as a tool to estimate the effect of the drug on membrane. Alcohols for example, have been found to cause ordering in reconstituted lipid membranes derived from human red cell ghosts at low concentrations of alcohol. However, above a certain concentration (C_i) the membranes show a continuous decrease in the order parameter. The value of C_i for alcohols of different chain lengths shows a direct correlation with the osmotic hemolysis of the red cell[13].

Another interesting technique for the study of order-parameter is through the use of ^{2}H NMR (deuterium NMR)[14]. Lipids are selectively deuterated at different sites in the hydrocarbon chains or in the polar group. The spectra contain information in the form of residual quadrupole splittings ($\Delta\nu$) in the ^{2}H NMR. The deuterium quadrupole splittings for the C–D vector depends on the orientation of the vector with respect to the direction of the normal to the bilayer and the anisotropy of the motions. For a fully ordered system, the quadrupole splitting attains a maximum and decreases as the randomness in the orientation of the vector increases. Thus

$$\Delta\nu = 3/4(e^2qQ/2)\,S_{CD},$$

where S_{CD} is the order parameter and (e^2qQ/h) is the quadrupole coupling constant. Its value is about 170 kHz for methylene groups in hydrocarbon chains. Measurements of $\Delta\nu$ in selec-

tively deuterated lipids thus permit the estimation of the order at different points in the molecule when embedded in membranes. Quadrupole splittings of deuterated lipids decrease upon the addition of anesthetics like procaine and tetracaine. This has been ascribed to the increased separation between lipid molecules due to intercalation of anesthetic[15]. The anesthetic molecule itself can be selectively deuterated at different positions and used as ^2H probe.

FLUIDITY MEASUREMENTS THROUGH RELAXATION TIMES

One of the difficulties with ESR techniques is that it is not known how much the bulky spin-label perturbs the motion of molecules in membranes. Measurement of the spin-lattice (T_1) and spin-spin (T_2) relaxation times of the natural (nuclear) spin-labels such as ^1H, ^{13}C or ^{31}P help to remove this difficulty. Through proper pulse sequences in FT NMR, the relaxation times can be measured for each resolved resonance line in ^{13}C, ^1H or ^{31}P spectra. The qualitative interpretation of the ^{13}C T_1 are particularly simple since the relaxation is dominated by the ^{13}C–^1H intramolecular dipole-dipole interaction and are sensitive to intramolecular motions. In the temperature ranges of our interest, a higher value of T_1 corresponds to a higher intramolecular chain motion and a lower value of correlation time (τ_c). As in the case of ESR and ^2H NMR results, T_1 results show an increasing chain motion as one goes towards the centre of lipid bilayers[14].

The results of ^{13}C T_1 measurements can be fruitfully used for building models of drug membrane complexes. Figure 5 shows the NT_1 values of propranolol in water solutions and when the drug is incorporated in lipid bilayers. The NT_1 values in aqueous solutions are in the range of 0.5 to 0.7 sec. The values are considerably smaller when the drug is bound to lipid bilayers. In particular, the aromatic moiety is highly immobilised with NT_1 values of the order of a few milliseconds. The terminal part of the oxypropanolamine group however retains considerable flexibility. The three-dimensional structure of

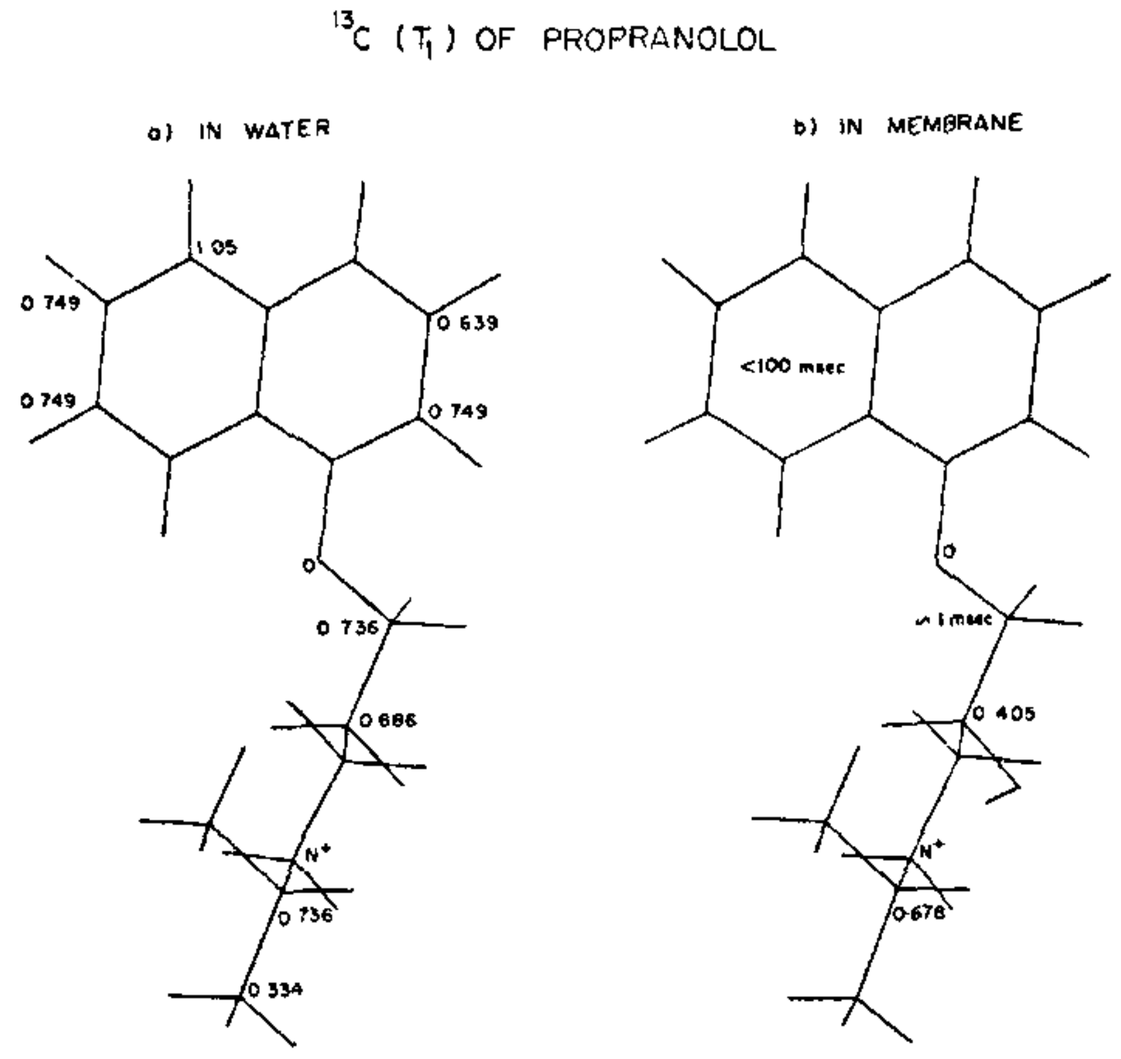

Figure 5. Values of ^{13}C spin lattice relaxation times (NT_1, where N is number of protons attached to carbon) **(a)** propranolol in water solution. **(b)** propranolol embedded into lipid bilayers.

propranolol is known to be extended[16] such that the hydrophobic aromatic moiety is well separated from the polar $-CH(OH)-CH_2-NH^+_2$ group. From these considerations one concludes that the drug binds to the lipid bilayers as shown in figure 6.

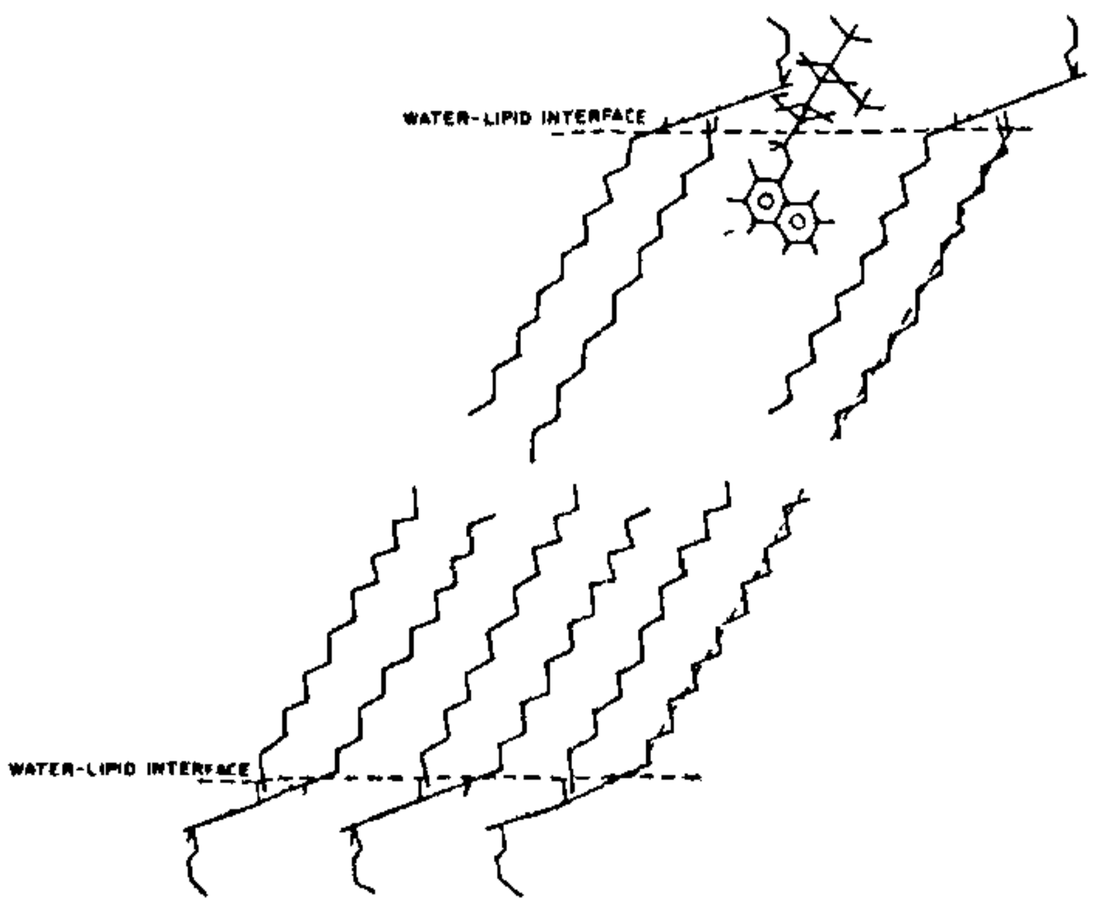

Figure 6. Model of interaction of propranolol with lipid bilayers. One notices a large void made by the drug in the region of its interaction which results in higher fluidity of the membrane.

While the ^{13}CNMR is sensitive to intramolecular motion, lateral diffusion is reflected in spin-spin interactions between protons on neighbouring lipid molecules. The lateral diffusion can thus be measured from T_2 values of protons. The diffusion constant in lecithin vesicles was thus estimated[17] to be 0.9×10^{-8} cm^2 sec^{-1}. Further, it has been found, in agreement with other techniques, that cholesterol (a neutral lipid) decreases lateral diffusion rates.

PERMEABILITY STUDIES

For a drug which acts on a receptor site inside the cell, an important consideration is the ease of transport of the drug across the membrane. Drugs can themselves bring about significant changes in transport properties of membrane by changing the membrane mobility. We have used a method based on ESR spin label method, for studying changes in permeability of reducing agents (reducing drugs, abscorbate ions etc.). The highly lipophilic spin label (5-SASL, labelled lipids etc.) is incorporated in phospholipid vesicles. The reducing agent is then put in the aqueous suspension of vesicles. As it permeates through the lipid bilayers, it reduces the spin-label thereby reducing the signal height in the ESR spectrum. The signal height decreases exponentially with passage of time, indicating that the permeation through phospholipid vesicles follows first order kinetics. One can see from figure 7 that the incorporation of propranolol in DPPC vesicles in liquid crystalline phase greatly assists the permeation of reducing agent—sodium ascorbate whereas α-tocopheral does not alter the permeation characteristics of DPPC vesicles significantly[11].

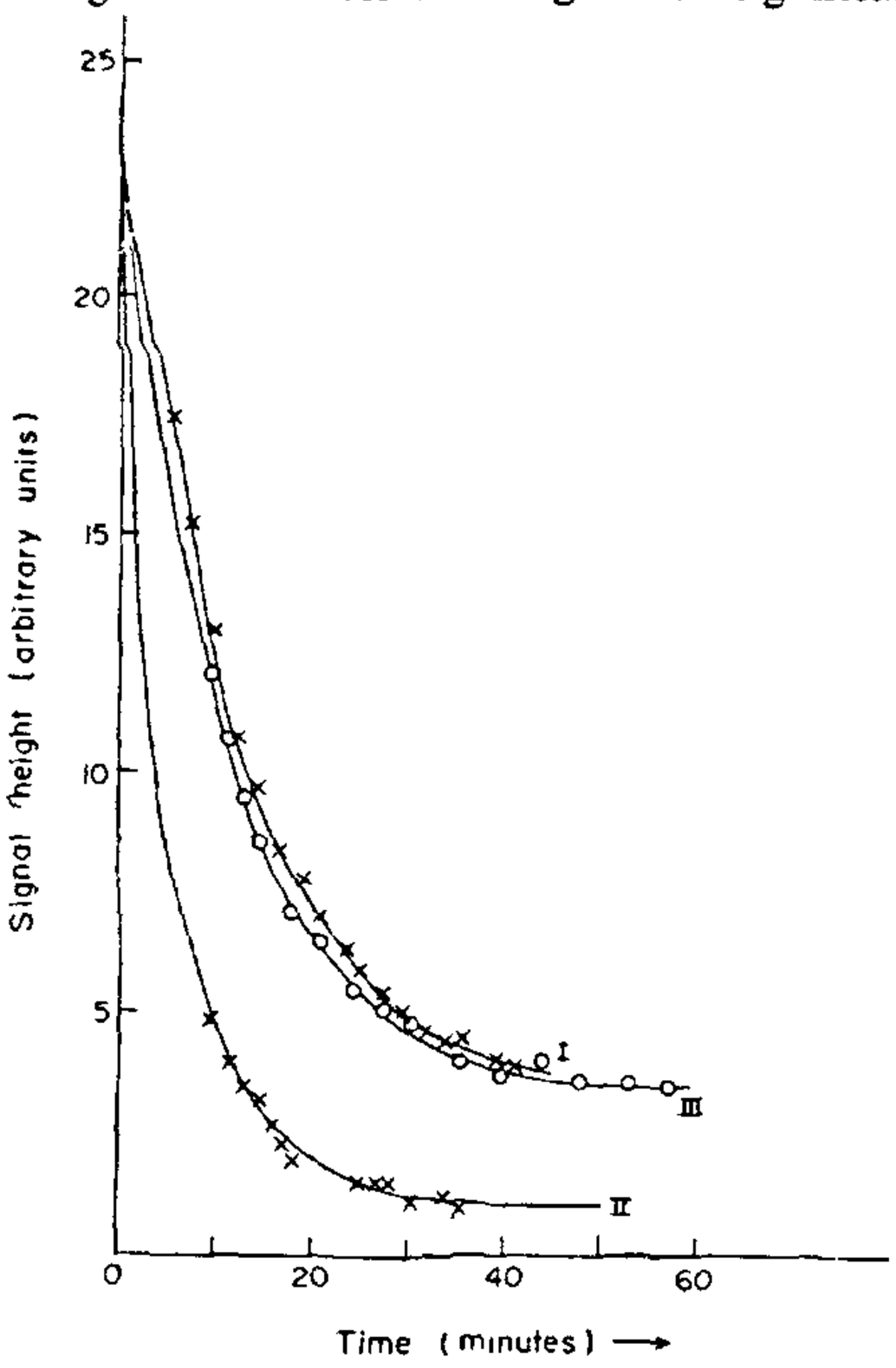

Figure 7. ESR signal intensity due to 5-SASL incorporated into lipid bilayers as a function of time when ascorbate ions are allowed to penetrate through the vesicles suspended in water. DPPC (100 mM), Temperature 46 ± 2° C. **I.** DPPC (Pure). **II.** DPPC + Propranolol (20 mM). **III.** DPPC + α-tocopherol (20 mM).

In the above experiments, we can monitore the passage of reducing agent through the lipid bilayers. A somewhat related experiment[18] is the one where the spin label is trapped in the internal aqueous compartment of the membrane and the reducing agent is added to the bulk aqueous phase. For example, it has been observed that monosodium urate does not affect the permeability casting doubt on a proposed mechanism where it was thought that the agent disrupts the membrane.

CONCLUSION

It is clear that magnetic resonance techniques provide valuable information on changes in organisation, fluidity, permeability and phase transition characteristics of model membranes following drug incorporation. So far, studies on drug interactions on intact cells, natural membranes or reconstituted membranes incorporating receptors have been lacking. Now that the potentialities of magnetic resonance techniques have been recognised, such studies are anticipated to make major contributions to our knowledge on how the drugs interact and alter properties of biological membranes.

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ANNOUNCEMENTS

FIRST SOLAR AIRPLANE

The first solar airplane powered by solar energy rose to a height of about 4,000 metres during a six and half hour flight at Shafter in California (USA) recently. The airplane which was designed by the American engineer Paul Mec Ready, is equipped with over 16,000 photo-electric cells which power a small motor directly without the aid of a battery.

COMPUTATIONAL PHYSICS ON THE DISTRIBUTED ARRAY PROCESSOR

The Computational Physics Group of the Institute of Physics is organising a conference, on Computational Physics on the Distributed Array Processor, to be held at the University of Glasgow on 5 and 6 August 1982. The Theme of the Conference will be user experience of converting, implementing and running computational physics codes on the DAP'.

Further information may be had from The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX.

INTERNATIONAL UNION OF BIOLOGICAL SCIENCES (IUBS)

The General Assembly Meeting of the IUBS will be held during 22–27 August, 1982 at Carleton University, Ottawa, Canada. A highly selected group of top scientists, teachers, and research administrators representing 36 countries from the Americas, Europe, Asia, Africa and Australia will participate. The range of interest of the participants at this meeting will be as diversified as the term "biology" implies.

Further information may be had from Prof. George Setterfield, Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6 Canada.