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A Spin Label Study of the Organization and Fluidity of Hydrated Phospholipid Multibilayers—a Model Membrane System*

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Abstract

The essential theory of the spin label method is presented and applied to the case of a rigid spin label localized in an oriented phospholipid smectic mesophase (hydrated phospholipid multibilayers). The fatty acid chains of the phospholipids in a model membrane of egg lecithin are shown to make an average angle of 24° with respect to a perpendicular to the bilayer plane. Addition of cholesterol stiffens the bilayers and decreases this angle to 10° at 55% cholesterol. The various requirements of a sterol for this condensing effect are explored, and the thesis is developed that one of the roles of sterols in membranes is to increase organization and rigidity of fatty acid chains, thus decreasing passive permeability and regulating lipid-lipid and lipid-protein interactions. Ions, proteins, anesthetics, antibiotics, and decouplers of oxidative phosphorylation are shown to have profound effects on the structure of lipid bilayers. Some extensions of the technique to real biological membranes are described, and the future for this application is predicted to be excellent.

Introduction

In 1965 the first paper on the spin label method appeared from the group of Professor H. M. McConnell (1). Since then the method and its applications have grown enormously. Several review articles describe the developments up to the present (2–7). I shall outline here application of the technique to the study of oriented multibilayers of phospholipids, a model membrane system. This application was developed at the National Research Council by Drs. H. Schneider, K. Butler, H. Dugas, J. C. Hsia, and myself, and independently by the research group of Professor O. H. Griffith at the University of Oregon.

The Electron Spin Resonance Spectra of Free Radicals

In a strong magnetic field the two spin states of an unpaired electron diverge in energy, Figure 1. Qualitatively, these energy states correspond to having the magnetic moment of the electron parallel or antiparallel to the magnetic field. Transitions between these energy levels may be induced by application of electromagnetic radiation of the appropriate energy. The relationship between the magnetic field and the required frequency of radiation is $h\nu = g\beta H$, where h is Planck's constant; ν is the frequency; g is the g-value for the electron in that particular molecule; β is the Bohr magneton, a fundamental constant for the electron; and H is the magnitude of the applied magnetic field. In principle any values of ν and H can be chosen. The higher the frequency, the greater is the theoretical sensitivity of the instrument, since the intensity of the transition is determined by the population difference between the two states. The most common conditions are $\nu = 9 \times 10^9$ Hz, $H = 3300$ gauss; a few spectrometers operate at $\nu = 35 \times 10^9$ Hz, $H = 13.200$ gauss, but they are difficult to use for aqueous samples.

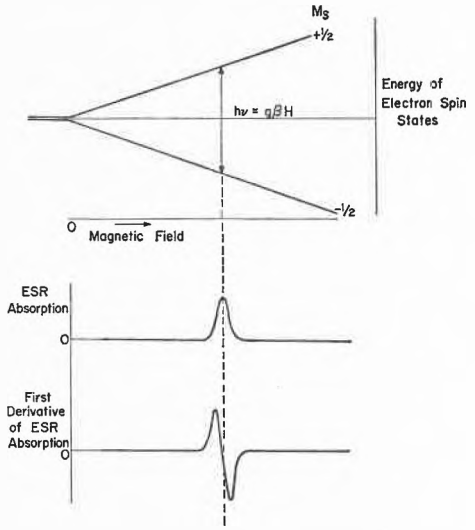

Fig. 1. The ESR experiment for a free electron. The quantum numbers ± ½ designate the value of the electron spin angular momentum in the direction of the applied magnetic field. The magnetic field is swept, while the microwave frequency is held constant. Most instruments present the first derivative of absorption

The ESR spectra become more complex, and consequently more useful, when the unpaired electron can interact with a magnetic nucleus. For example, ^{14}N has a nuclear spin of one unit. In qualitative terms, the magnetic moment of ^{14}N can be aligned parallel, antiparallel, or perpendicular to the magnetic moment of the unpaired electron. Thus, each of the two electron

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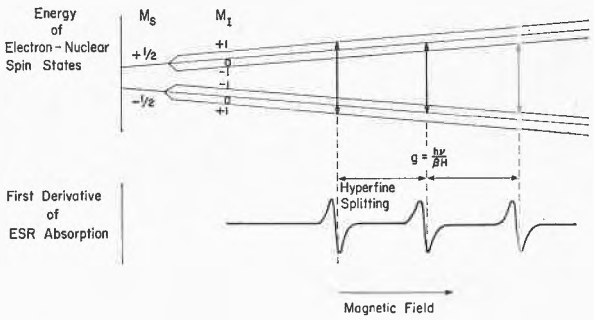
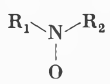


Fig. 2. The ESR experiment for a species containing an unpaired electron and a nucleus of spin 1 (^{14}N). The separations between the resonance lines yield the hyperfine splitting constant

energy levels is split into three, one of which has the same energy it would have in the absence of the interacting nuclear moment. Six ESR transitions are apparently possible. However, only those which result in a change in the polarization of the electron magnetic moment with no change in the polarization of the nuclear moment are readily observable (Figure 2). Thus, within a first order approximation, one would observe three ESR absorptions, whose separations are equal and known as the hyperfine splitting of the nitrogen nucleus. Due to coupling between the spin angular momentum and orbital angular momentum of an unpaired electron in a molecule containing nuclei and other electrons, the g-value can deviate significantly from the free electron value. The g-value is obtained from the ESR spectrum by measuring accurately the magnetic field at the center of the spectrum and the microwave frequency $\left(g = \frac{h\nu}{\beta H}\right)$.

The Spin Label Method

The object of the spin label method is to make a biological system of interest studiable by ESR spectroscopy. The high sensitivity of ESR spectroscopy to the concentration (presently concentrations of about 10^{-7} M spins in aqueous solution can be studied) and environment *(vide infra)* of the free radicals make it potentially very powerful in structural studies. The difficulty lies in making the biological system paramagnetic with a minimal disturbance of its structure. This can be done by synthesizing a stable free radical whose chemical structure is very similar to that of some component of the biological molecules. This free radical must then be inserted into the biological system. A vast number of nitroxide spin labels containing the paramagnetic moiety



now have been made and attached covalently or by intermolecular forces to the biological macromolecules (2–7).

The Electron Spin Resonance Spectra of Nitroxide Spin Labels

If a nitroxide is present in a single crystal (which is magnetically dilute so as to avoid magnetic interactions between nitroxides), its ESR spectrum will depend upon the angles between the magnetic field and the principal axes of the nitroxide moiety (Figure 3). The largest hyperfine splitting is observed when the magnetic field is parallel to the nitrogen π-orbital (the z-axis). Note that both the hyperfine splitting (A) and g-value are dependent on these angles. At angles between the three principal directions, the spectrum will be somewhere between the three principal spectra shown in Figure 3.

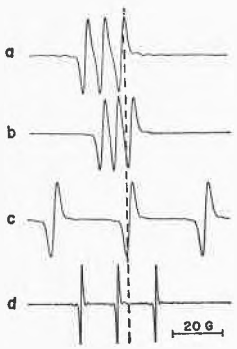


Fig. 3. ESR spectra of di-*t*-butyl nitroxide in a single crystal of tetramethyl-1,3-cyclobutanedione.
a) With the magnetic field along the x-axis (parallel to the N—O bond). b) Along the y-axis (perpendiculat to the N—O bond and the nitrogen π-orbital). c) Along the z-axis (parallel to the nitrogen π-orbital). d) Dissolved in di-*t*-butylketone, 10^{-5} M, room temperature. The dotted line represents a field corresponding to $g = 2.00036$ (2,2-diphenylpicrylhydrazyl). From reference (3)

If the crystal is dissolved in a solvent of low viscosity, the ESR spectrum will be similar to that of Figure 3 d. The observed hyperfine splitting and g-value are now the averages of the three principal values. This is because the nitroxide is undergoing rotational reorientation at a rate that is rapid relative to the frequency differences between the transition energies of the various possible ESR spectra ($\gg |A_{zz} - A_{xx}| \approx 73 \times 10^6$ sec^{-1}; $\gg |g_{xx} - g_{zz}| \beta H h^{-1} \approx 29 \times 10^6$ sec^{-1}). If the rate of rotational reorientation becomes comparable to these frequency differences, however, useful linewidth effects in the ESR spectra become apparent (Figure 4). From the relative heights of the ESR lines in spectra such as Fig. 4A–4C, it is possible to estimate the correlation time for rotational reorientation (1), but this is at best an estimate due to various assumptions involved in the formalism. If the label reorients very slowly, the ESR spectrometer is able to observe an entire ESR spectrum before the label changes in its orientation in space. But, since essentially all orientations are represented in an isotropic medium, the spectrometer will see a different spectrum from every nitroxide in the sample, and the overall spectrum of the solution will be the sum of all

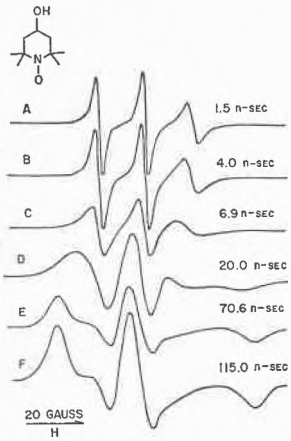


Fig. 4. ESR spectra of a nitroxide in solvents of increasing viscosity. The numbers to the right of the individual spectra are estimates of the times characterizing rotational reorientation (rotational correlation times) in nanoseconds (10^{-9} sec). From reference (8)

possible spectra. This spectrum will be similar to that of a magnetically-dilute powder of nitroxides, and so is called a powder spectrum. It will be similar to that in Figure 4F, but with even broader lines.

I have described here mostly limiting examples of ESR spectral behaviour. All intermediate cases are of course possible, and some of these will be encountered in the examples considered later. A particularly vexing case is that of partial orientation of a spin label undergoing anisotropic motion about the orientation axis.

The Membrane Problem

Biological membranes are composed of neutral lipids (such as cholesterol), phospholipids (such as phosphatidyl choline), and proteins (such as adenosine triphosphatase). The function and location of most of these components remains a mystery. It has recently been demonstrated by *X*-ray diffraction (9) that much of the lipid in some membranes exists in a bilayer structure, such as one of the bilayers shown in Figure 5.

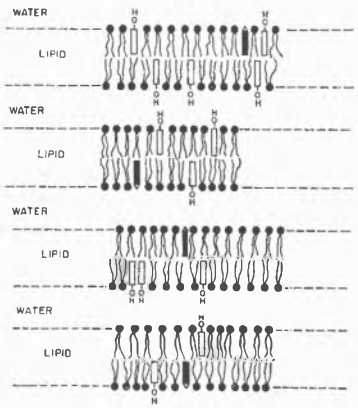


Fig. 5. Schematic of hydrated phospholipid multibilayers. The existence of such structures has been demonstrated recently by *X*-ray diffraction studies (10,11). The rectangle with attached —OH is a schematic representation of cholesterol. The black pencil represents a probe molecule (spin label). Figure courtesy of Dr. H. SCHNEIDER

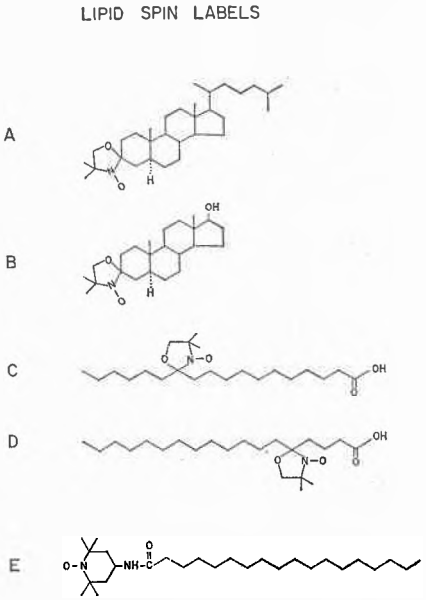


Fig. 6. Lipid spin labels used to study the organization and fluiditiy of phospholipid multibilayers (12). References are to papers describing syntheses of these labels. A. 3-spiro[2′(N-oxyl-4′,4′-dimethyloxazolidine)] cholestane (13.) B. 3-spiro[2′-(N-oxyl-4′,4′-dimethyloxazolidine)]-17β-hydroxy-androstane (14). C. 12-spiro[2′-(N-oxyl-4′,4′-dimethyloxazolidine)] stearic acid (15). D. 5-spiro[2′-(N-oxyl-4′,4′-dimethyloxazolidine)] stearic acid (16). E. 4-stearamide-1-oxyl-2,2,6,6-tetramethylpiperidine (17)

We have undertaken studies of hydrated phospholipid multibilayers, whose structure has recently been shown to be similar to that of Figure 5 (10, 11) in order to explore systematically the roles and interactions of various membrane components, and the influence of various membrane-active substances on the organization and mobility of the phospholipids.

The multibilayers are formed by evaporation of lipid mixtures into the flat sides of a standard ESR aqueous sample cell. Lipid spin labels at low concentration are incorporated into the lipid mixture before evaporation. Some of the lipid labels we have used are shown in Figure 6. It can be seen that some of them (A, B, and E) should be useful for exploring the polar head group region of bilayer structure, while the others (C, D) penetrate to various degrees into the lipid interior. After evaporation of the organic solvent, and pumping under hard vacuum to remove all traces of solvent, the films are hydrated by filling the cell with aqueous salt solutions. Under these conditions ESR spectra can only be taken with the external magnetic field parallel or perpendicular to the plane of the multibilayer film (using a conventional rectangular ESR cavity). If the aqueous solution is drained off after equilibration, the films can be examined at all angles.

Some types of spectral behaviour to be expected from phospholipid multibilayer films are summarized in Figure 7. Here we shall use the cholestane spin label (CSL); it has the particular advantage that the nitroxide moiety is firmly attached to the steroid nucleus, and

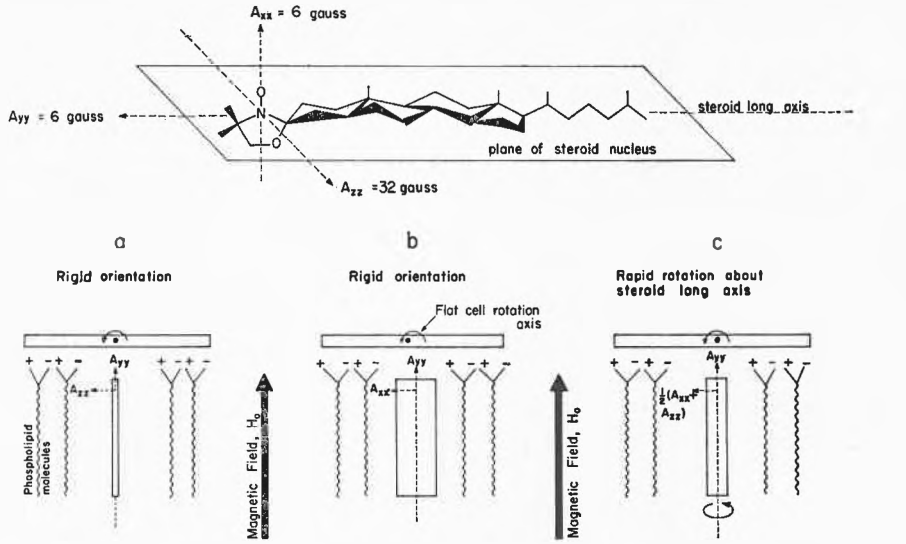


Fig. 7. Schematic representation of possible orientations and corresponding ESR hyperfine splittings of the cholestane spin label in multibilayer films. In (a), (b) and (c) the steroid backbone is represented as a plane. In (c) the narrower plane indicates that rapid rotation about the steroid axis averages the hyperfine splittings along the x- and z-axis

cannot undergo any motion relative to the steroid. Thus, the ESR spectra of CSL are indicative of the motion of the entire steroid nucleus. The hydrophilic nitroxide in all likelihood is located in the polar head group region of the films, and so explores organization to a depth of approximately 15 Å.

If the spin label were able to tumble freely and rapidly in the films, the ESR spectra would be independent of the angle between the film and the magnetic field. The relative widths of the hyperfine lines would be indicative of the rate of tumbling e.g. Figure 4B. If it were reorienting very slowly, the spectra would be angular-independent, and similar to Figure 4F—a powder spectrum.

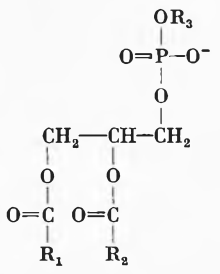
Suppose, however, that the long axis of CSL is arranged parallel to the long axis of the phospholipids in a well organized bilayer, and that no motion of the spin label is possible (Figure 7a,b). In this case the steroid plane can still have any angle with respect to rotation about the steroid long axis, since the lipids have essentially cylindrical symmetry. If we were to look perpendicular to the plane of the film, i.e. along the steroid long axis, we would observe only the yy component of the hyperfine and g-tensors. Thus, we would see an ESR spectrum of three narrow lines separated by 6 gauss. If we looked parallel to the film plane, i. e. perpendicular to the steroid long axis, we would observe some nitroxides with the magnetic field along the x-direction (6 gauss splitting) some with it along the z-direction (32 gauss splitting) and some with it at any intermediate angle (any splitting between 6 and 32 gauss). Thus we would expect an envelope of all spectra with splittings between 6 and 32 gauss, and g-values between 2.0089 and 2.0027. This would be somewhat similar to a powder spectrum, but less asymmetric (see the section on egg lecithin).

Another interesting case is if CSL is in a situation similar to that just described, but rotating rapidly about the steroid long axis. In the direction perpendicular to the plane of the film we should still see a spectrum of three narrow lines separated by 6 gauss. The rapid rotation will average out the difference between the x and z direction, and therefore when we apply the magnetic field parallel to the plane of the film, we should observe three narrow lines separated by the average of 6 and 32 gauss, 19 gauss. If the rate of rotation is comparable to the frequency equivalent of the difference between 6 and 32 gauss ($\approx 73 \times 10^6$ sec^{-1}), the lines will be separated by approximately 19 gauss, but will be of different widths. The relative widths will enable calculation of the effective rotational reorientation rate about the steroid long axis.

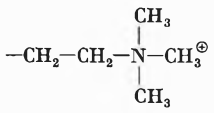
If the long axis of CSL is not perfectly oriented perpendicular to the plane of the film, then the ESR spectra with the magnetic field parallel and perpendicular to the plane of the film will be more complex than above. I shall discuss several examples of this with egg lecithin and the phospholipids from ox brain.

Phospholipids

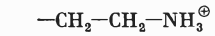
The molecular composition of phospholipids can be described generally by the formula



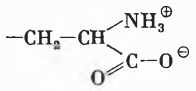
Usually R_1 and R_2 are long hydrocarbon chains, often containing some double bonds. R_3 may be one of



Phosphatidyl Choline



Phosphatidyl Ethanolamine



Phosphatidyl Serine

or one of a series of more complex groups. The phosphatidyl choline group of phospholipids are commonly referred to as lecithins, e.g. if R_1 and R_2 contained fifteen saturated carbon atoms, and R_3 were choline, we would have dipalmitoyl lecithin. I shall concentrate mainly on data obtained for various lecithins and for the more complex lipids from ox brain (which contain lecithins, neutral lipids such as cholesterol, acidic lipids such as phosphatidyl serine, cardiolipin, and sulfatides).

Egg Lecithin

The lecithin obtained from egg yolk contains a variety of R_1 and R_2, but in general R_1 is saturated and R_2 unsaturated. The chain lengths are from 13 to 17 for R_1, and 17 for R_2 with one double bond (between positions 9 and 10 of the oleic acid) or two double bonds (between 9 and 10, and 12 and 13).

When dry films are prepared from pure egg lecithin containing CSL we obtain the ESR spectra shown in Figure 8a (18). The hyperfine splitting and line shapes are independent of the direction of the applied magnetic field. The splitting of 15.7 gauss is indicative of a nitroxide undergoing isotropic rotational diffusion. The relative heights of the three hyperfine lines indicate that this motion is characterized by a correlation time of 1×10^{-8} sec. (1). But this is a solid unhydrated film! Thus we have a demonstration of the phenomenon of microscopic viscosity—although the film looks like a powder, it is liquid as far as the immediate environment of CSL is concerned.

Hydration of the above film with aqueous salt solution results in the spectrum shown in Figure 8b. A large difference in the hyperfine splittings in the parallel (magnetic field parallel to the plane of the film) and perpendicular directions is manifest (17 gauss and 10 gauss, respectively). From the discussion of the previous section and Figure 7, it is clear that the CSL is oriented with its long steroid axis approximately perpendicular to the plane of the film, and that CSL is undergoing rapid rotational reorientation about the long steroid axis at a rate of approximately 10^8 sec^{-1}. By implication the phospholipid molecules must be arranged with their fatty acid chains approximately parallel to one another and perpendicular to the plane of the film. Thus, hydration of the egg lecithin film has induced a liquid crystalline state of the type represented in Figure 5. The presence of the first broad line at low field in Figure 8b (dashed spectrum), indicates that the ordering is not perfect—for in the ideal case only three narrow lines separated by about 6 gauss would be observed.

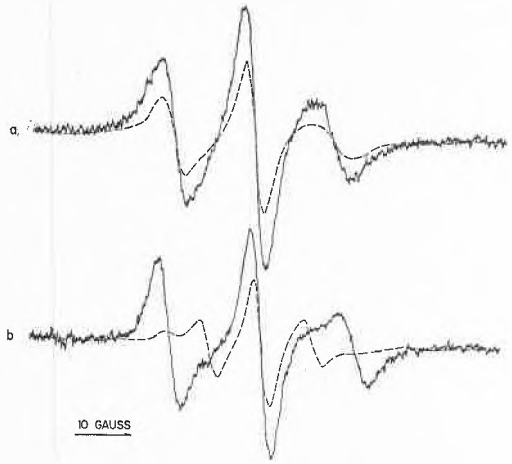


Fig. 8. ESR spectra of CSL in cholesterol-free films of egg lecithin. The solid and dashed spectra were taken with the magnetic field parallel and perpendicular to the plane of the film, respectively. a) dry film b) sample responsible for a) but hydrated with aqueous salt solution. Spectra from (18)

If cholesterol is present in the egg lecithin films the spectral behaviour is different. With a dry film, a considerable degree of spectral anisotropy is observed, Figure 9a. The separation between the hyperfine extrema in the solid line spectrum (magnetic field parallel to film) is approximately 64 gauss—the line shapes and hyperfine separation are those expected for random distribution of the hyperfine tensor components A_{xx} (6 gauss) and A_{zz} (32 gauss) around a perpendicular to the plane of the film (see Figure 7). In the perpendicular direction, dashed spectrum Figure 9a, we have three much narrower hyperfine lines separated by approximately 7 gauss. This spectral behaviour corresponds to that expected for approximate orientation of the CSL long axis perpendicular to the plane of the film, with no motion about the long axis of CSL—that is CSL has a random distribution of orientations about the long axis, but a definite ordering of the long axis.

Hydration of the cholesterol-containing egg lecithin film causes a dramatic change in the ESR spectra of the included CSL (Figure 9b). In the parallel direction (solid lines) the hyperfine lines are narrower and separated by only about 19 gauss—what we expect if rotational diffusion of CSL about its long axis averages the 6 and 32 gauss hyperfine splittings. The relative heights of the hyperfine lines indicate that this rotational diffusion takes place at a rate of about 10^8 sec^{-1}. In the perpendicular direction we observe three narrow hyperfine lines separated by about 6 gauss, corresponding to almost perfect orientation of the CSL long axis perpendicular to the plane of the egg lecithin multibilayer film.

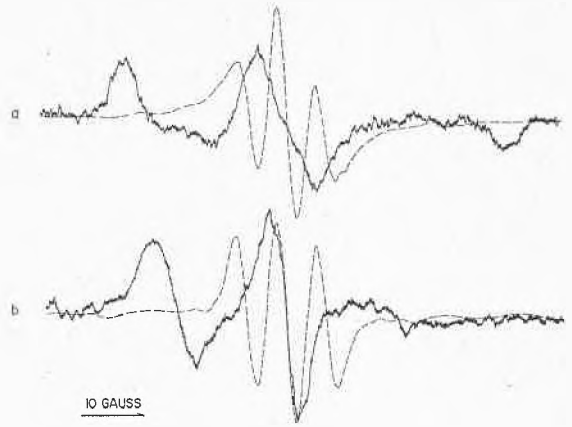


Fig. 9. ESR spectra of CSL in cholesterol-containing films of egg lecithin, 66 mole percent cholesterol. The solid and dashed spectra were taken with the magnetic field parallel and perpendicular to the plane of the film, respectively. a) dry film b) sample responsible for a) but hydrated with aqueous salt solution. Spectra from (18)

From the above experiments it is obvious that cholesterol has a very great effect on the microscopic viscosity of an egg lecithin film—large amounts of cholesterol causing a stiffening of the hydrocarbon chains. This is consistent with the results of surface pressure (19) and NMR data (20). Cholesterol also serves to induce a high degree of order into the lecithin film, with the long axes of the hydrocarbon chains approximately parallel. Hydration induces a high degree of order in dry cholesterol-free egg lecithin films, with a spacing between the hydrocarbon chains sufficiently large to allow rotation of CSL about its long axis. Hydration of the highly-ordered cholesterol-containing lecithin films also causes a rearrangement of intermolecular spacing such that rotation about the CSL long axis can take place.

In hydrated egg Lecithin multibilayers of varying Cholesterol content

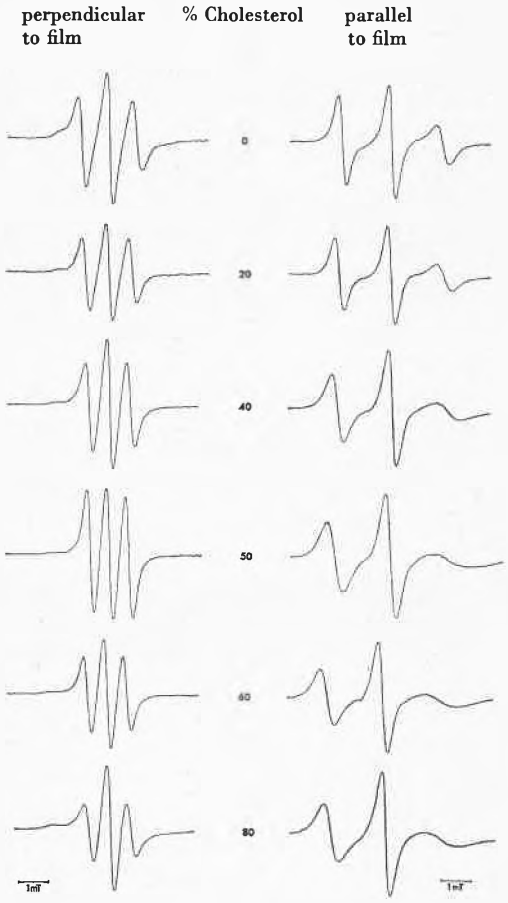


Fig. 10. ESR spectra of CSL in hydrated films of egg lecithin containing increasing amounts of cholesterol. Spectra from (21)

The Role of Cholesterol

The effects of steadily increasing amounts of cholesterol on hydrated egg lecithin films are shown in Figure 10. Up to 55 mole percent cholesterol the difference between the parallel and perpendicular splittings increases, and the asymmetry of the hyperfine lines in the parallel direction increases. This indicates an increasing degree of orientation of the CSL long axis perpendicular to the plane of the film, and a decreasing rate of rotation about the CSL long axis—the films are increasingly ordered and stiff. Above 55 mole percent cholesterol the degree of order begins to drop, and is very low for a film of pure cholesterol.

The ordering effect of cholesterol on egg lecithin films can be quantitated in terms of the behaviour shown in Figure 11. The long axes of the phospholipids are considered to be roughly parallel, and making an angle θ', with a perpendicular to the plane of the film. Due to the symmetry of the system this is equivalent to distributing the phospholipid (and CSL) long axes around the edge of a cone containing the angle $2\theta'$. With increasing cholesterol the effective cone angle decreases. An approximate treatment of this model is given in (22), and a more rigorous description in (21). The simulated spectra for this model system are shown in Figure 12. The most striking aspect of the system is that up to a tilt angle of 40° the ESR spectra are made up to three relatively narrow lines from which a hyperfine splitting can be measured. In the experimental data (Figure 10) the spectra for the magnetic field perpendicular to the cone axis are more complex than those shown for the simple model (Figure 12). This is due to an intermediate rotational diffusion rate of CSL about its long axis, which can be easily included in the simulation programme if desired. The derived angle of deviation from perpendicularity, θ', as a function of added cholesterol is shown in Figure 13. The degree of order increases with increasing cholesterol up to 55 mole percent cholesterol; the deviation angle varying from 24° in the absence of cholesterol to only 10° at 55%. Above this concentration the degree of order drops, becoming very low in a pure cholesterol film. This may be due to appearance of

a second phase, presumably cholesterol, in which the spin label is poorly oriented.

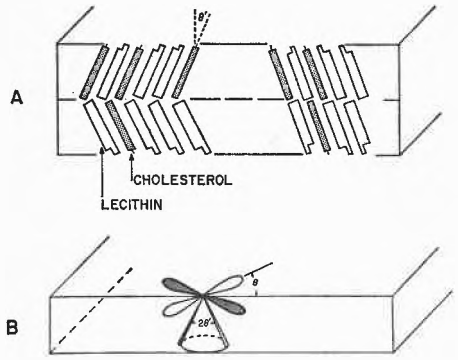


Fig. 11. a) Schematic representation of cholesterol and lecithin in the model multibilayer system. The long axes of the phospholipids, cholesterol, and CSL make an angle θ' with a perpendicular to the plane of the film. Increasing order results in a decreasing θ'. b) The cone defined by the long axes of the phospholipids, cholesterol, and CSL. The dumbell-shaped moiety is the p-orbital of the nitroxide nitrogen in two orientations around the cone (solid and open dumbells). Figure from (22)

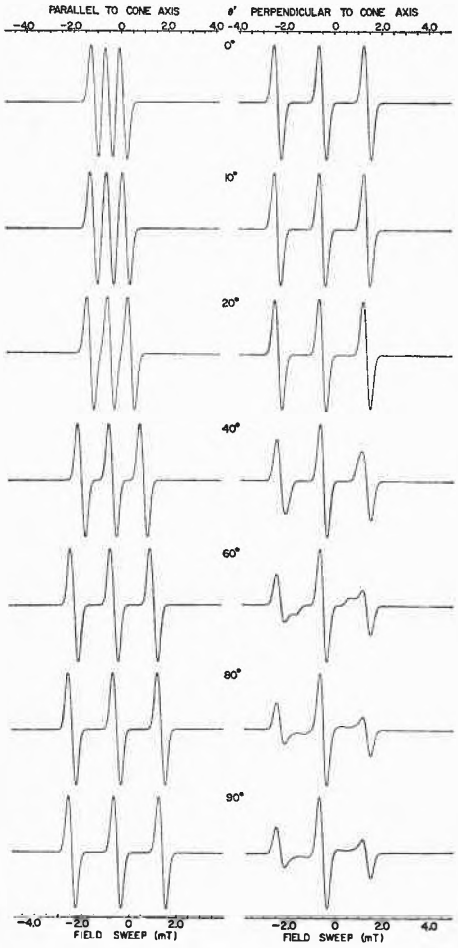


Fig. 12. Computer simulated ESR spectra for the cone model of Figure 11. The spectra on the left are for the magnetic field parallel to the cone axis, those on the right for the field perpendicular to the cone axis. The magnetic field is expressed in the new international units of milli Teslas—one Tesla being equal to 10000 gauss. Figure from (21)

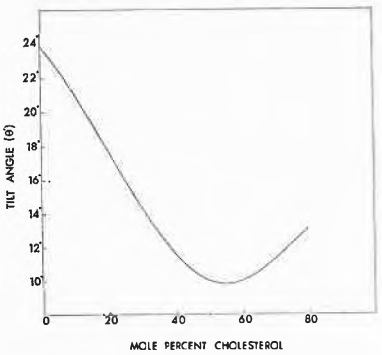


Fig. 13. Variation of the derived angle θ', describing deviation from ideal orientation, as a function of cholesterol concentration in egg lecithin films, from (21)

Similar behaviour has been observed on addition of cholesterol to films of dipalmitoyl lecithin (22). In this case the best degree of order is achieved at approximately 50% cholesterol, and the angle of deviation, 0°, is even less than that obtained with egg lecithin. This demonstrates that the double bonds in the hydrocarbon side chain of egg lecithin place an upper limit on the degree of order that can be achieved; in dipalmitoyl lecithin the saturated hydrocarbon chains have less difficulty in ordering themselves.

Using spin-labelled fatty acids in which the nitroxide is located at various positions along the fatty acid chain, the depth of the cholesterol condensing effect can be estimated. Spin labels *C* and *D* of Figure 6 were used, and their ESR spectra compared with those of CSL (23). The analysis of the spectra is more complex than that described previously, since the methylene groups of the fatty acids have a high degree of mobility. Thus, what one measures is the amplitude of the anisotropic motion of the nitroxide group. This may be expressed as an angle, but it is more related to the available space for anisotropic motion in a direction perpendicular to the fatty acid chains. With such an approach the cholesterol condensing effect is found to propagate as far as the 12 position of the fatty acid chains. In fact, the relative change in orientation caused by addition of cholesterol is greatest for the spin label with the nitroxide at the 12 position. This is because in the absence of cholesterol the degree of mobility of the hydrocarbon methylene groups increases from the head group to the terminal methyl group.

This remarkable influence of cholesterol on the degree of order in phospholipid multibilayer films led us to study the structural requirements of the sterol (24). In this study the lipids from the white matter of ox brain were used to form the multibilayers. This is because these lipids will not form ordered films in the absence of cholesterol, but form perfectly ordered films with 33 mole percent cholesterol, Figure 14. The molecular formulae of cholesterol and some derivatives are shown in Figure 15. Cholestanol, which differs from cholesterol

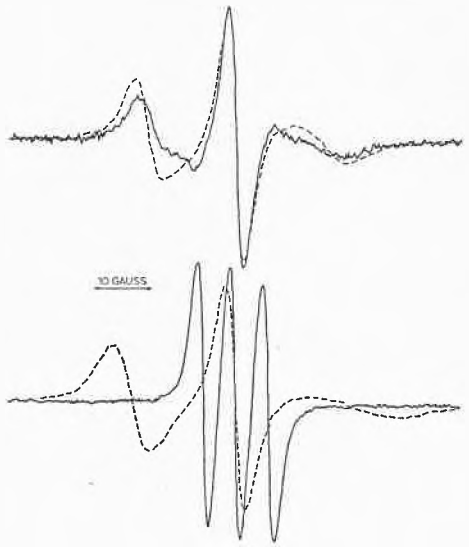


Fig. 14. ESR spectra of hydrated films of ox brain lipids in the absence (upper figure) and presence (lower figure, 33%) of cholesterol. The dashed and solid spectra are with the magnetic field parallel and perpendicular to the plane of the film. The ESR spectra of the upper figure are indicative of rapid almost isotropic, rotational reorientation of CSL. Spectra from (24)

only in being saturated at the 5–6 bond, induces order in films as well as cholesterol—thus, the 5–6 double bond is not essential. Coprostanol, which has the opposite configuration from cholestanol at the 5-position, is incapable of inducing order. Thus, a planar steroid nucleus is essential. Varying the methyl groups or double bonds in the steroid skeleton has little effect on ordering ability. Removal of the hydrocarbon chain at position 17 reduces the order-inducing ability somewhat. Cholestane induces no order, cholestan-3-one induces a slight degree of order. Inversion of the attachment of the 3-OH group, from β to α, removes ordering ability. Thus the hydroxyl group at position 3 is absolutely essential for ordering ability. β-Sitosterol, the 24-ethyl derivative of cholesterol commonly found in plants, was as effective as cholesterol. In fact cholesterol ordered plant lipids as well as did the naturally-occurring β-sitosterol.

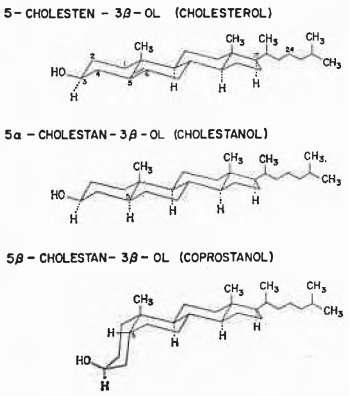


Fig. 15. Structural formulae of cholesterol, cholestanol, and coprostanol. Figure courtesy of Dr. H. SCHNEIDER

The effects of the various sterols in ordering the lipids from human erythrocyte ghosts were essentially the same as for the ox brain lipids. An excellent correlation exists between steroids which produce order in the lipid system and those which support mycoplasma growth (25), or alter some permeability properties of human erythrocytes (26). Thus, it appears that the ordering and stiffening effect of cholesterol on phospholipids is a general phenomenon. It seems probable therefore that at least one of the roles of cholesterol and related sterols in membranes is to regulate the spatial organization of the lipid components and their fluidities. The condensing effect tends to increase bilayer thickness and hence could regulate protein-lipid interactions. The increased bilayer dimension and decreased fluidity should result in a general decrease in permeability. Thus the passive permeability properties of a particular membrane could be determined by the steroid and lipid content, whereas some of the more delicate selective permeabilities are no doubt regulated by specific proteins or enzymes. These specific systems could be regulated secondarily by protein-lipid interactions, via the condensing effect of membrane steroid.

Ion Effects on Phospholipid Organization

The lipids from ox brain have a net negative charge. This suggests that the organization of the head group region should be sensitive to the cations in the solution bathing the phospholipid bilayers. In Figure 16 we have the ESR spectra of CSL in films of ox brain lipids hydrated with 5 and 50 mM NaCl. With no salts present, the spectra look similar to those of Figure 14 (upper figure). Addition of 5 mM NaCl produces some spectral anisotropy, but 50 mM is much more effective.

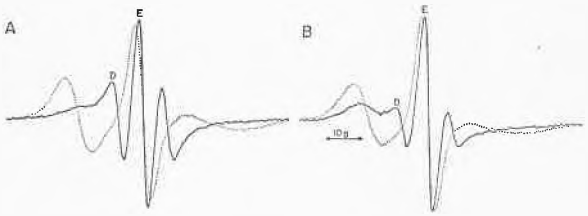


Fig. 16. ESR spectra of CSL in films of ox brain lipid (containing cholesterol). The solid and dotted spectra are with the film perpendicular and parallel to the magnetic field, respectively. A. Hydrated with 50 mM NaCl, B. Hydrated with 5 mM NaCl. Spectra from (27)

The ESR spectra of ox brain lipid films cannot be analyzed in terms of a simple cone model—they are better approximated by a distribution of cone angles, with a variable distribution width. A convenient spectral parameter however, is the ratio of peaks D and E in the spectra taken with the magnetic field perpendicular to the lipid film (Figure 16). For perfect order this ratio approaches one; for complete disorder it approaches

zero. Thus, in Figure 17, we plot this ratio versus concentration of various salts used to hydrate the films (27). It is obvious that the salt-induced order is dependent on the charge and concentration of the cation. Anions were found to have no differential effect. The effectiveness of cations in producing order increases as $Na^+ = K^+ = Li^+ < Mg^{2+} = Ca^{2+} < La^{3+} < Th^{4+}$. The phenomenon is not simply related to osmolarity, since equiosmolar solutions of LiCl and $CaCl_2$ have greatly different ESR spectral anisotropies.

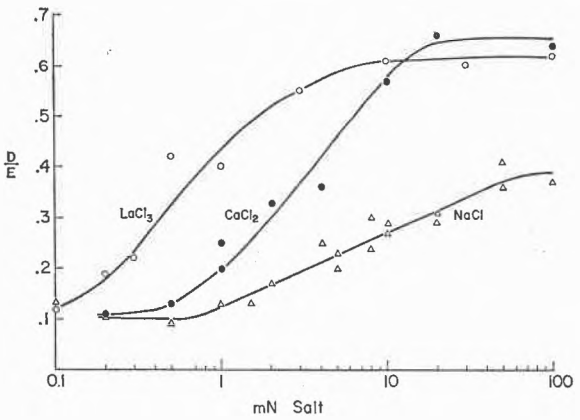


Fig. 17. Dependence of the order parameter D/E on the concentration of salts bathing the phospholipid film. A ratio of 1.0 would indicate perfect alignment in the film. Figure from (27)

Films of egg lecithin have no net charge at the *p*H of these experiments; they showed no sensitivity to the nature of the cations in the solution. Addition of dicetyl phosphate or phosphatidic acid to the films confers on them a net negative charge. Films of lecithin and phosphatidic acid (10% of total phospholipid) containing the sterol spin label *B* (Figure 6), with and without cholesterol, have been shown to respond to Ca^{++} ions in the same way as brain lipid films (23). The ordering effect of Ca^{++} could also be observed in lecithin-phosphatidic acid and brain lipid films when spin label *D* (Figure 6) was used, but only a slight response by label *C* was noted (23). Thus, in contrast to the condensing effect of cholesterol, the ion effect has its greatest magnitude near the head groups of the phospholipids.

The importance of charge balance on the bilayer surface for lipid organization has also been demonstrated by observing the ESR spectra of the androstanol spin label (B in Figure 6) in films of egg lecithin-phosphatidic acid and phosphatidyl ethanolamine as a function of *p*H of the hydrating solution (23). The data from the spectroscopic titration of phosphatidyl ethanolamine are shown in Figure 18. The degree of order is a maximum between *p*H 3 and 7, and falls off rapidly with extreme *p*H.

The influence of cations on the degree of organization of brain lipid films can be interpreted as a charge neutralization. In the absence of added salt, the negatively charged head groups of the brain lipids will repel one another, causing expansion of the film. Increasing cation

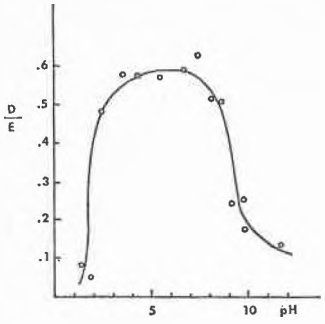


Fig. 18. Dependence of the order parameter D/E for the androstanol spin label (*B* of Figure 6) on the *p*H of solutions hydrating films of phosphatidylethanolamine (23)

concentration and charge should provide increasingly effective neutralization of the repulsive forces, and hence allow the head groups to come closer together and form a more organized structure. This contraction of the lipid-lipid distance will result in an increase in the width of the bilayer, and hence could be very significant in a biological membrane.

Lipid-protein Interactions

Biological membranes contain large quantities of protein. Some of the proteins have been found to have specific enzymic activities, such as adenosine triphosphatase; for many others no particular role has been found. There seems to be little doubt however that proteins play both structural and functional roles in membranes. It has been demonstrated by the spin label technique that some membrane protein conformations are independent of the presence of lipid, while others are not (28). Recently, recombination of spin-labelled lipids and proteins from human erythrocyte ghosts has demonstrated large conformational changes on complex formation (29). The nature of the assembled lipid-protein complexe in the membrane remains a mystery, however. The membrane structure theories of ROBERTSON (30) and GREEN (31) take entirely opposite points of view. The spin-labelled multibilayer technique seems promising in this respect, because it probes the *immediate* environments of the phospholipids or proteins in the complex.

For the protein study we have used the ox brain lipid system. These lipids, among which is cholesterol, form ordered multibilayers in solutions of ionic strength 0.01. In distilled water, however, no organization is manifest, and the films are very unstable. Figure 19 shows the effects of hydrating lipid films with protein or polypeptide solutions in distilled water (32). With lysine-phenylalanine copolymer at *p*H 5.6 no order is manifest in the ESR spectra. They remain the spectra due to CSL rotating slowly ($\approx 10^8$ sec^{-1}) in an almost isotropic fashion. Addition of RNase A at *p*H 2.6 results in spectra due to an almost perfectly organized film. Oxidized RNase A, which has the four disulfide bonds

broken and oxidized, is equally effective in producing order. At *p*H 7.6 RNase A induces some order, but not to the degree found at *p*H 2.6. The two latter results suggest that the protein is most effective at ordering the films when it is in a disrupted state (the *p*H range for optimum activity of RNase A is 5 to 7; it is inactive at *p*H 2.6).

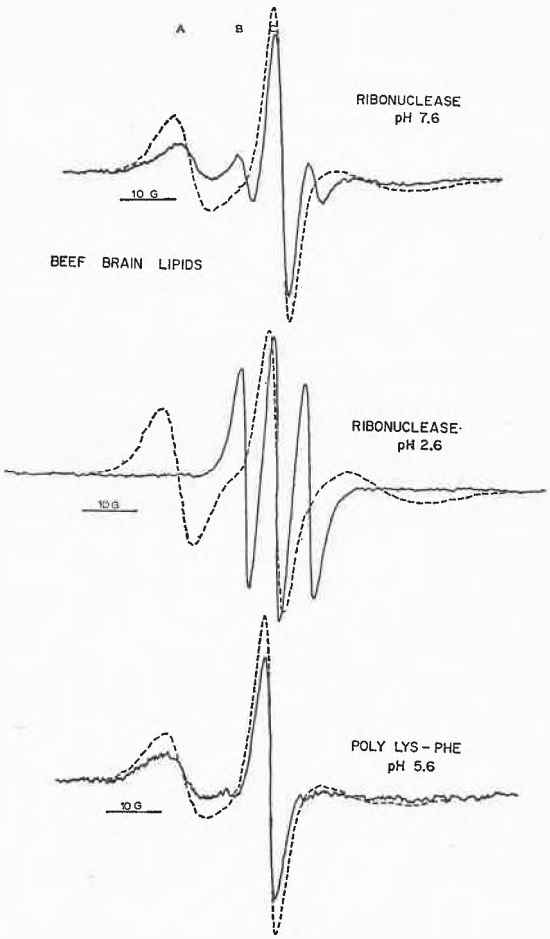


Fig. 19. ESR spectra of CSL in ox brain lipid films in the presence of proteins (10 mg/ml in solution). The solid and dotted spectra are with the magnetic field perpendicular and parallel to the plane of the film, respectively. Spectra from (32)

Another interesting result is that a film of ox brain lipids equilibrated with salt solution to give almost perfect spectral anisotropy loses all order when exposed to a similar solution containing 1 mg/ml of lysine-phenylalanine copolymer. Thus, proteins can also cause a large decrease in the order of the films. In this case it is very likely the presence of large $(Phe)_n$ regions in the copolymer that induce disorder, since polylysine normally has an ordering effect. Low angle *X*-ray diffraction studies on such disordered films, and on those with a high degree of order, indicate that under both conditions the lamellar structure is intact (45). Thus, the ordering and disordering effects of proteins are definitely due to changes in alignment and mobilities of the phospholipids within the bilayers.

A detailed study of many proteins has shown that the ordering effect depends on absolute and relative numbers of positive and negative charges on the protein, the number of hydrophobic amino acids, and the overall size of the protein. In general the extended (reversibly denatured) conformation of the protein is more effective at inducing order. The proteins are inseparable from the lipids in the complex by normal techniques, and the films are extremely difficult to remove from the quartz cell. This demonstrates the very favorable energies of interaction for the lipid-protein complexes.

The Influence of Anesthetics and Antibiotics

Having found such an organized structure in phospholipid multibilayers, one immediately wonders if membrane-active agents exert their effects by structural perturbations. We have studied a large number of such agents, and I shall describe the results for a few representative cases.

General anesthetics, such as chloroform (33) and butane (32) decrease the organization of lecithin or brain lipid films at very low concentrations. Local anesthetics, such as procaine and tetracaine, improve order at low concentration and decrease order at high concentrations (32). Figure 20 illustrates the ordering effect, and its reversibility. The spectra are taken with the magnetic field perpendicular to the plane of the film; for perfect order we expect three narrow lines separated by 6 gauss. In the absence of procaine the degree of order is very low, and we observe essentially a powder spectrum. Addition of 0.1 M procaine (which is used clinically in 1 to 20% solutions) results in the appearance of a 6 gauss splitting, but the induced order is not perfect since we still have a rather large spectral contribution 20 gauss to low field of the central hyperfine line. Pumping in buffer containing no anesthetic washes away the procaine and results in a reversal of the ordering effect.

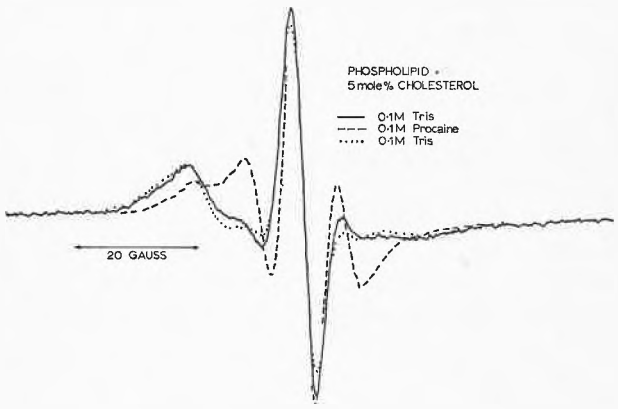


Fig. 20. ESR spectra of CSL in a hydrated film of ox brain lipids containing 5% cholesterol. The magnetic field is perpendicular to the film. The solid, dashed, and dotted spectra show the film before equilibration with procaine, in the presence of procaine, and after washing off the procaine with buffer (32)

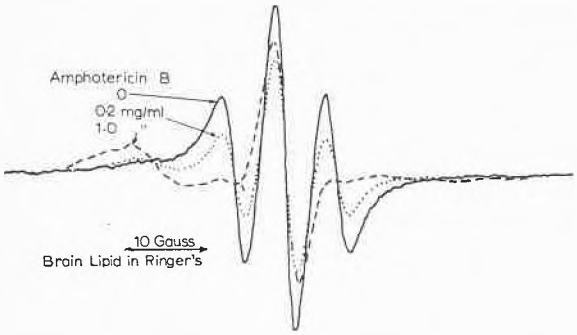


Fig. 21. Influence of the antibiotic amphotericin B on the ESR spectra of CSL in a hydrated film of ox brain lipids. The spectra are taken with the magnetic field perpendicular to the plane of the lipid film (32)

Antibiotics such as amphotericin B, nystatin, filipin, and myprozine all produced detectable changes in the ESR spectra of CSL in phospholipid multibilayers (33). Figure 21 shows the influence of amphotericin B on brain lipid containing cholesterol (32). In this case the ESR spectrum in the absence of antibiotic has the three lines separated by approximately 6 gauss that we attribute to a high degree of order. Addition of a solution containing 0.2 mg/ml of amphotericin B causes a decrease in the amplitude of the line 6 gauss to low field of the spectral center, and an increase in the component at 20 gauss to low field. Increasing amphotericin concentration magnifies this effect, 1.0 mg/ml (0.001 M) producing a powder spectrum. Thus, at a concentration which is similar to those used in practice ($L.D._{50}$ for mice is 280 mg/kg) amphotericin B can have a catastrophic effect on the organization of phospholipids in bilayers.

We have demonstrated that in some cases anesthetics and antibiotics can affect the degree of order of lipids in bilayers. The effects depend on the nature of the lipid and on the amount of cholesterol present. In other cases, however, we have found no effects whatsoever under pharmacological conditions. In theories of the mechanisms of these agents, the possible disruption of lipid organization must be taken into account. On the other hand, it is likely that many highly specific agents operate on specific proteins, or specific protein-lipid interactions.

The Effects of Decouplers of Oxidative Phosphorylation

Compounds which cause uncoupling of the complex chain of reactions involved in the process of oxidative phosphorylation have been shown to have very great effects on the electrical conductance of artificial bilayer membranes (34–37). It has been suggested that the uncoupler, which is usually a weak organic acid, carries current across the membrane in its anionic form or in a negatively-charged complex (36,38). We have investigated the influence of some of these compounds on the organization of phospholipid multibilayers (39).

In Figure 22 A we see the ESR spectrum of CSL in a film of egg lecithin containing 33 mole percent cholesterol, hydrated at *p*H 4.0. Equilibration for 30 minutes with the same buffer containing 2×10^{-5} M dinitrophenol (DNP) led to Figure 22 B. In this case the separations between the three sharp central lines are only slightly greater than in 22 A, but there is now a large component 20 gauss to low field of the spectral center. The essential constancy of the principal splitting indicates that most of the spin labels (and lipids) are unaffected by the DNP—the large component at low field indicates that a small fraction of the spin labels (and lipids) have been greatly affected. Thus, DNP at *p*H 4.0 causes a large local structural change in the lecithin multibilayers—a change from a highly-ordered to a highly-disordered state. The disruptive effect was a maximum at *p*H 4.0, the *p*K of DNP. Similar disruptive effects were observed with DNP and ox brain lipids, and with the decouplers dicoumarol and pentachlorophenol.

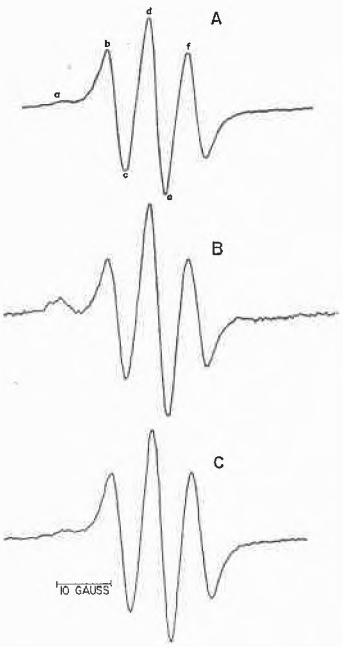


Fig. 22. ESR spectra of CSL in hydrated egg lecithin—cholesterol (33 mole percent) films A. *p*H 4.0 in absence of DNP, B. *p*H 4.0 in presence of 2×10^{-5} M DNP, C. *p*H 4.0 after removal of DNP. Spectra from (39)

The spin label reporter group has demonstrated that under the influence of compounds which cause large increases in electrical conductivity, large local changes in the state of organization of phospholipids in bilayers occur. These perturbed regions could have altered permeability for a variety of compounds, and hence could lead to a severe imbalance across a biological membrane. Such phenomena must be considered when constructing models for the mode of action of the decouplers.

Viable Biological Systems

I could not close and leave the impression that studies on such model systems will answer all the questions about phospholipid organization in biological mem-

branes. On the contrary, the many interactions we have seen in the multibilayer system have merely laid the groundwork for studies on more complex systems. One must really understand the isolated components before he has much hope of understanding the natural "Gemisch". In our laboratory, and in several others, people are inserting spin labels into biological membranes and attempting to explain the resulting ESR spectra. One simple approach is to intercalate lipid spin labels into the lipid region of the membranes—such as labels *B–E* of Figure 6 into red blood cell membranes (40, 41). We have been using this technique to study the organization of lipids in defective red blood cells such as spherocytes and sickle cells (41). Another approach is to introduce the spin label biosynthetically. This has been achieved in *Neurospora crassa* (42), *Mycoplasma laidlawii* (43), and the microsomal fraction from guinea pig liver (44). In each of these label *C* of Figure 6 was used and was shown to have been incorporated covalently into phospholipid.

Thus, the spin label technique, which has provided so much fundamental information about the organization and fluidity of phospholipid multibilayers in model systems, should now provide the same sort of information on the more complex biological membranes. The path will be difficult, and contains various potential pitfalls, but I feel that within the next several years the spin label technique will make a substantial contribution to our understanding of membrane structure and function.

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References

(1) T. J. STONE, T. BUCKMAN, P. L. NORDIO, and H. M. MCCONNELL, *Proc. Nat. Acad. Sci. U.S. 54* (1965) 1010.
(2) C. L. HAMILTON and H. M. MCCONNELL, in *Structural Chemistry and Molecular Biology*, edited by A. RICH and N. DAVIDSON W. H. Freeman, San Francisco 1968, p. 115.
(3) O. H. GRIFFITH and A. S. WAGGONER, *Acc. Chem. Res. 2* (1969) 17.
(4) H. M. MCCONNELL and B. G. MCFARLAND, *Quart. Rev. Biophys. 3* (1970) 91.
(5) I. C. P. SMITH, in *Biological Applications of Electron Spin Resonance Spectroscopy*, edited by J. R. BOLTON, D. BORG, and H. SWARTZ, John Wiley Interscience, 1971 (in press).
(6) P. JOST and O. H. GRIFFITH, in *Methods in Pharmacology*, Vol. 2, edited by C. CHIGNELL, Appleton-Century Crofts, New York 1971 (in press).
(7) I. C. P. SMITH, in *Free Radicals in Molecular Biology and Pathology*, edited by W. A. PRYOR and H. DEMOPOULOS, Academic Press (in press).
(8) L. H. PIETTE and J. C. HSIA, *Arch. Biochim. Biophys. 129* (1969) 296.
(9) M. H. F. WILKINS, A. E. BLAUROCK, and D. M. ENGELMAN, *Nature 230* (1971) 72.
(10) Y. K. LEVINE, A. I. BAILEY, and M. H. F. WILKINS, *Nature 220* (1968) 577.
(11) Y. K. LEVINE and M. H. F. WILKINS, *Nature 230* (1971) 69.
(12) Most of these spin labels are now available commercially from Syva Corporation, Palo Alto, California.
(13) J. F. W. KEANA, S. B. KEANA, and D. BEETHAM, *J. Amer. Chem. Soc. 89* (1967) 3055.
(14) W. L. HUBBELL and H. M. MCCONNELL, *Proc. Nat. Acad. Sci. U.S. 63* (1969) 16.
(15) A. S. WAGGONER, T. J. KINGZETT, S. ROTTSCHAEFFER, and O. H. GRIFFITH, *Chem. Physics Lipids 3* (1969) 245.
(16) W. L. HUBBELL and H. M. MCCONNELL, *Proc. Nat. Acad. Sci. U.S. 64* (1969) 20.
(17) J. C. HSIA, L. H. PIETTE, and R. W. NOYES, *J. Reprod. Fert. 20* (1969) 147.
(18) J. C. HSIA, H. SCHNEIDER, and I. C. P. SMITH, *Biochim. Biophys. Acta 202* (1970) 399.
(19) R. A. DEMEL, L. L. M. VAN DEENEN, and B. A. PETHICA, *Biochim. Biophys. Acta 135* (1967) 11.
(20) D. CHAPMAN and S. A. PENKETT, *Nature 211* (1966) 1304.
(21) R. D. LAPPER, S. J. PATERSON, and I. C. P. SMITH, *Can. J. Biochem. 1971* (in press).
(22) J. C. HSIA, H. SCHNEIDER, and I. C. P. SMITH, *Can. J. Biochem. 49* (1971) 614.
(23) S. SCHREIER-MUCCILLO, H. DUGAS, H. SCHNEIDER, and I. C. P. SMITH (manuscript in preparation).
(24) K. W. BUTLER, I. C. P. SMITH, and H. SCHNEIDER, *Biochim. Biophys. Acta 219* (1970) 514.
(25) P. F. SMITH, *Abhandl. Dtsch. Akad. Wiss. Berlin Kl. Med. 2* (1968) 37.
(26) K. R. BRUCKDORFER, R. A. DEMEL, J. DE GIER, and L. L. M. VAN DEENEN, *Biochim. Biophys. Acta 183* (1969) 334.
(27) K. W. BUTLER, H. DUGAS, I. C. P. SMITH, and H. SCHNEIDER, *Biochem. Biophys. Res. Comm. 40* (1970) 770.
(28) H. SCHNEIDER and I. C. P. SMITH, *Biochim. Biophys. Acta 219* (1970) 73.
(29) K. U. BERGER, M. D. BARRATT, and V. B. KAMAT, *Biochem. Biophys. Res. Comm. 40* (1970) 1273.
(30) J. D. ROBERTSON, *Biochemical Symposium 16* (1959) 3.
(31) G. VANDERKOOI and D. E. GREEN, *Proc. Nat. Acad. Sci. U.S. 66* (1970) 615.
(32) K. BUTLER, I. C. P. SMITH, and H. SCHNEIDER, unpublished results.
(33) K. BUTLER, J. C. HSIA, H. SCHNEIDER, and I. C. P. SMITH (unpublished).
(34) U. HOPFER, A. L. LEHNINGER, and T. E. THOMPSON, *Proc. Nat. Acad. Sci. U.S. 59* (1968) 484.
(35) E. A. LIBERMAN and V. P. TOPALY, *Biochim. Biophys. Acta 163* (1968) 125.
(36) E. J. A. LEA and P. C. CROGHAN, *J. Membrane Biol. 1* (1969) 225.
(37) U. HOPFER, A. L. LEHNINGER, and W. J. LENNARZ, *J. Membrane Biol. 3* (1970) 142.
(38) V. S. MARKIN, L. I. KRISTALIK, E. A. LIBERMAN, and V. P. TOPALY, *Biofizika 14* (1969) 256.
(39) S. P. VERMA, H. SCHNEIDER, and I. C. P. SMITH (unpublished results).
(40) W. L. HUBBELL, J. C. METCALFE, S. M. METCALFE, and H. M. MCCONNELL, *Biochim. Biophys. Acta 219* (1970) 415.
(41) P. HWANG, S. J. PATERSON, D. PHILLIPS, and I. C. P. SMITH (unpublished).
(42) A. D. KEITH, A. S. WAGGONER, and O. H. GRIFFITH, *Proc. Nat. Acad. Sci. U.S. 61* (1968) 819.
(43) M. E. TOURTELLOTTE, D. BRANTON, and A. D. KEITH, *Proc. Nat. Acad. Sci. U.S. 66* (1970) 909.
(44) N. Z. STANACEV, L. STUHNE-SEKALEC, S. SCHREIER-MUCCILLO, and I. C. P. SMITH, *Biochem. Biophys. Res. Comm. 1971* (in press).
(45) A. HANSON, K. BUTLER, H. SCHNEIDER, and I. C. P. SMITH (unpublished).