DETECTION OF LIPID LATERAL PHASE SEPARATION

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The complex phase behavior of lipids in artificial lipid bilayer membranes and in some biological membranes has been determined through the use of several physical techniques. Since changes in several important membrane functions have been correlated with certain aspects of the phase behavior of the lipids in several biological membranes, considerable interest has recently arisen in this area of membrane biology (for a review, see "Phase Transitions in Model Systems and Membranes," by C. F. Fox in the MTP International Review of Science Biochemistry Series, Biochemistry of Cell Walls and Membranes, *C. F. Fox, editor, Butterworths, London, 1975). As a result, I thought that the Bioenergetics Subgroup of the Biophysical Society might also have some interest in this area and might benefit from a discussion of some of the physical techniques used for the detection of the phase behavior of lipids in biological membranes. The following papers by Doctors Engelman, Papahadjopoulos and Poste, and Grant provide a brief description of the utility of some of these physical techniques for this purpose with adequate references for the interested reader.*

THE USE OF X-RAY SCATTERING IN THE STUDY OF LIPID BILAYER PLANAR ORGANIZATION

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INTRODUCTION

Lamellar states of the lipids which occur in biomembranes consist of bimolecular planar aggregates. The tendency to form such arrays is a property which particularly accrues to the glycerolipids which have two hydrocarbon chains and a polar characteristic group. The tendency of such lipids to form membranous layers gave rise to many early speculations that lipid bilayers occur as the principal structural motif of mem-

branes, and recent evidence shows this view to be substantially correct. The issue has now become directed more toward the planar organization of membranes and, as an aspect of this general problem, the planar organization of lipid bilayers composed of a mixture of molecular species. In this brief discussion, I will outline the strengths and weaknesses of X-ray scattering as a method for the study of the planar separation of compositionally distinct regions of a mixed lipid bilayer.

POSSIBLE STATES OF A MIXTURE OF TWO PHOSPHOLIPIDS

A binary lipid mixture in a lamellar phase may exist in at least three distinct states with respect to the organization of the hydrocarbon chains. If both lipids are at a temperature above their transition temperatures, it is probable that they will be in a liquid state corresponding to a smectic mesophase of type A. In this phase the lipids may mix randomly or not, depending on the hierarchical influence of forces arising from charges, dipoles, counterions, hydrophylic and hydrophobic interactions, steric factors and dispersion forces. The relative influence of these is not presently understood for any single case let alone for the spectrum of lipids found in biomembranes, so that one cannot forcefully predict the miscibility of lipids in the smectic phase; nonetheless, one can expect that there will be instances of immiscible binary mixtures.

Similarly, the case where both lipids are below the transitions they exhibit in the pure state is one in which the full range of forces may be expected to govern the segregation of regions of different composition or the failure of such a segregation to occur. Again, it is to be expected that mixed and segregated cases will be found in different mixtures of lipids, and that other possibilities (such as the formation of specific heterologous aggregates) may also exist.

The third state is one which is sometimes found where the experimental temperature is between the transition temperatures of the two pure lipids of the mixture. In this region the lipid mixture may assume average properties or a separation of phases may occur (e.g., DeKruyff et al., 1974; Wu and McConnell, 1975). In a two-component mixture of lecithins, both averaging and separation effects can be seen as consequences of differences in the length and saturation of the hydrocarbon chains.

In the last case the separations can be identified by any method which is differentially sensitive to the presence of the paracrystalline and liquid packing of the fatty acids. One such method is X-ray diffraction, and the remainder of this note is concerned with the basis of such an approach. The first two cases, where the chains are all liquid or all paracrystalline, are more difficult to approach, and traditional methods are not applicable to their study.

HYDROCARBON STATES

In normal paraffins, three states of side-to-side association are distinguishable by X-ray scattering: crystalline, paracrystalline, and liquid. The liquid state is characterized experimentally by the presence of a broad band of scattered intensity over the region from about $(10\ \text{Å})^{-1}$ to $(3\ \text{Å})^{-1}$ with its peak at about $(4.6\ \text{Å})^{-1}$. In the early work of

Stewart (1928) it was shown that the shape of the peak and the position of the maximum are essentially the same over the range from 6 to 15 carbons in the normal paraffin chain. This shows that the observed diffraction arises from the side-to-side correlations of the hydrocarbon chains, since it is independent of length. In 1933, Warren developed an approximate theory to predict the scattering curve from a model structure consisting of a group of seven chains in contact at a separation of 5 Å, center-to-center. The agreement is reasonable, and shows that the scattering can arise from locally parallel packing of nearest neighbor chains. Luzzati et al. (1960) have compared the scattering curves of lamellar soaps with those of liquid paraffins and found them to be similar, and the scattering from the liquid-like chains of phospholipids in the smectic mesophase again has very nearly the same angular distribution. It follows that the scattering from smectic bilayers of membrane lipid has a component which resembles that of the liquid paraffins, and that the structural organization of the hydrocarbons may be similar to liquid paraffin in the side-to-side relationships of the chains. This statement is not the same as a statement that the hydrocarbon region is an isotropic liquid; it applies only to the nearest neighbor relations in the lipid and not to the overall net orientation of chains (the limits of this much-misunderstood statement are clear from the work of Stewart).

If paraffins of sufficiently long chain length are studied, a change of state occurs in a homogeneous bulk phase as the liquid is cooled. The diffraction pattern changes from a broad band to a series of sharp Bragg intensities arising from an hexagonal lattice (Müller, 1932). The intensities occur at roughly $(4.2\ \text{Å})^{-1}$, $(2.4\ \text{Å})^{-1}$, $(2.1\ \text{Å})^{-1}$, etc. and imply the existence of a parallel array of chains with an axial separation of about 4.8 Å. (The exact spacings are temperature dependent and may shift by 0.1 Å over a 25° range.) The strongest reflection by far is that at 4.2 Å. The chains are not systematically related to each other at the atomic level as in a true crystal, but appear to be rotationally disordered while remaining at well-defined lattice positions. Despite the rotational disorder, the sharpness of the reflections shows that the phase has a coherent structure over large distances. Perhaps the most apt description of this state is that it is a paracrystal, although it has been referred to as the gel, β, hexagonal, solidus, or crystalline state by various authors. This state of partial order has been observed as a property of the hydrocarbon chains of lipid bilayers (e.g., Luzzati, 1968) and in natural membranes (Engelman, 1970).

As the paraffin paracrystalline phase is further cooled, a second transition occurs to a true crystal. This normally is an orthorhombic lattice in which the symmetry excludes the 100 and 010 equatorial reflections, and the innermost equatorial reflections appear at 4.1 Å (110) and 3.9 Å (020). This crystalline state is seen in free fatty acids and alcohols, but with very few exceptions is not observed in two chain lipids which occur in membranes.

Thus there are three distinct organizational states of hydrocarbon chains which are separately identifiable by X-ray scattering. Of these the liquid state with its broad diffraction band centered at $(4.6\ \text{Å})^{-1}$ and the paracrystalline state with its series of

sharp lines are found in bilayers of membrane lipids. That they are so different is useful in assessing the relative amounts of material in each state (see below).

Another situation which deserves comment is the change in scattering of the liquid phase when cholesterol is added to a lipid bilayer (Levine, 1970). At a mole ratio of 1:1, egg lecithin:cholesterol, the wide angle scattering is a broad band with a slightly narrowed (about 20%) width at half-height and a shift of the maximum from about $(4.6\ \text{Å})^{-1}$ to $(4.75\ \text{Å})^{-1}$. This is a subtle change which would be difficult to exploit in a diffraction experiment on a mixed system of cholesterol-containing and cholesterol-deficient lipid regions.

INFORMATION OBTAINABLE

Where a mixture of lipids is being examined in the absence of other molecular species, both the diffuse band corresponding to the liquid state and the sharp line of the paracrystalline array can be well measured experimentally. Thus the presence of each state may be assessed. Unfortunately, it is not rigorously correct to compare the intensities of the two contributions to the wide angle scatter to obtain the relative population of the states; nonetheless, to a first approximation one may do this to obtain a general idea. More properly, one should explore a thermal range which drives the system entirely into each of the extreme states and compare each integrated intensity seen in an intermediate state to that of the appropriate homogeneous system. In this way a thermal profile can be obtained in which the relative proportion of each state of the lipids can be assessed at any temperature, and thus the degree and nature of phase transitions and separations can be described.

The main practical limitations of the method are that a relatively high concentration of lipid is required and that other scatter from the sample may interfere. The concentrations required for the simultaneous measurement of both contributions to the scattering are greater than 10%. Lower concentrations do not allow the accurate characterization of the broad $(4.6\ \text{Å})^{-1}$ band against the broad, strong water band centered at about $(3.3\ \text{Å})^{-1}$. At the higher concentration the intensity of the broad band can be measured to $\pm 5\%$, at lower concentration it is much less accurate. Dilute systems (0.25–2%) may be examined in terms of the sharp $(4.2\ \text{Å})^{-1}$ reflection intensity alone, provided that it is confidently known that the low extreme temperature has placed all chains in the lipid in the paracrystalline state. Since the intensity is concentrated in a relatively narrow angular range, the peak is more easily measured against the diffuse water background. This is also the case where more complex systems such as biomembranes are studied. The additional complication in such a case is that other components of the membrane, especially proteins, contribute to the wide angle scattering. Here the use of the broad $(4.6\ \text{Å})^{-1}$ band is severely restricted and is reasonable only as a qualitative (i.e. $\pm 20\%$) measure of the state of the system, whereas the sharp $(4.2\ \text{Å})^{-1}$ line is still a distinct feature which can be measured accurately.

In all cases where the sharp line is seen, a rough idea of the extent of the ordered re-

gions can be obtained using the method of Stokes and Wilson (1942). The point is that as more chains are added to an array in the paracrystalline state the reflection becomes progressively narrower in angular half-width, eventually reaching the instrumental limit. If the ordered regions are in the range of roughly 30–200 Å in extent, their average size can be determined. Most often, however, the patches are found to be much larger than this and no practical statement can be made beyond the specification of the lower bound of size.

To summarize, one can hope to characterize the proportioning of lipids between the paracrystalline and liquid states using X-ray diffraction. The method has the advantages of being relatively nonperturbing and of being a direct measurement of the states at equilibrium. In a simple mixture of two lipids in an aqueous environment, a full characterization of the phase separation can be observed with considerable accuracy.

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REFERENCES

DEKRUYFF, B., P. W. M. VAN DIJCK, R. A. DEMEL, A. SCHUIJFF, F. BRANTS, and L. L. M. VAN DEENEN. 1974. Non-random distribution of cholesterol in phosphatidyl-choline bilayers. *Biochim. Biophys. Acta.* **356**:1.

ENGELMAN, D. M. 1970. X-ray diffraction studies of phase transitions in the membrane of *Mycoplasma laidlawii*. *J. Mol. Biol.* **47**:115.

LEVINE, Y. K. 1970. Ph.D. Thesis, University of London, King's College.

LUZZATI, V., H. MUSTACCHI, A. SKOULIOS, and F. HUSSON. 1960. La structure des colloides d'association. I. Les phases liquides-crystallines des systemes amphiphile-eau. *Acta Crystallogr.* **13**:660.

LUZZATI, V. 1968. *In* Biological Membranes. D. Chapman, editor. Academic Press, Inc., New York.

MÜLLER, A. 1932. An X-ray investigation of normal paraffins near their melting points. *Proc. R. Soc. (Lond.). A Math. Phys.* **138**:514.

STEWART, G. W. 1928. X-ray diffraction in liquid normal paraffins. *Phys. Rev.* **31**:174.

STOKES, A. R., and A. J. C. WILSON. 1942. A method of calculating the integral breadths of Debye-Scherrer lines. *Proc. Camb. Phil. Soc.* **38**:313.

WARREN, B. E. 1933. X-ray diffraction in long chain liquids. *Phys. Rev.* **44**:969.

WU, S. H., and H. M. MCCONNELL. 1975. Phase separations in phospholipid membranes. *Biochemistry.* **14**:847.