The Structure of a Model Membrane in Relation to the Viscoelastic Properties of the Red Cell Membrane

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ABSTRACT The molecular arrangement within a lamellar structure composed of human erythrocyte lipids is determined. The 45 A thick lipid layer, in water, is filled in the interior with a liquid-like configuration of the hydrocarbon chains of phospholipid molecules and is covered on both sides by their hydrophilic polar groups. Cholesterol is located so that part of its steroid nucleus is between the polar groups of the phospholipid molecules while the rest of the molecule extends into the inner hydrocarbon layer. This lipid leaflet would be expected to have the mechanical properties of a purely liquid surface, as other authors have shown for the "black" lipid membranes. Data are presented which demonstrate that the intact erythrocyte membrane is a tough viscoelastic substance with a Young's modulus of 10^6–10^8 dynes/cm^2 and a viscosity of 10^7–10^{10} poises. The parameters and the kinetics of membrane breakdown are incompatible with the model system of pure lipid. Caution must be exercised in applying various data on the model systems to intact membranes.

I would like to refer to two sets of experimental data that have been obtained from erythrocyte membranes. One set provides structural resolution of the molecular arrangement within a lipid lamellar layer in bulk solution, a structure which may well be the same as that of the black lipid membranes that Dr. Tien and Dr. Finklestein have just talked about. The second set of data, dealing with the mechanical properties of intact membranes, show that these are viscoelastic substances and not purely viscous, as the model membranes are. These two sets of data, taken together, suggest that extreme caution must be used in applying to intact cell membranes results that have been obtained with model systems. In fact, all three speakers before me have expressed the idea that the model system that they are working with may or may not have anything to do with the intact cell membrane. It is my impression that reluctance to apply various kinds of data on models to intact, fully hydrated membranes is fairly recent, and as a result it is dogma today,

at least to people outside the field, that biological cell membranes have as a basic structure a bimolecular layer of lipid. I am sure Dr. Korn will provide us with a critical review of the evidence on which this dogma rests.

I shall start first with experiments dealing with a model system. This is an investigation, using X-ray diffraction, of the structures formed by lipids, extracted from human erythrocytes, in water. I did this work with Dr. Vittorio Luzzati in France, to whom I owe my thanks for close collaboration and for offering facilities in his laboratory. Without going into the background of this work, it is now well established that the lamellar sheets formed by lipids, or by many amphiphilic molecules, i.e. molecules having both a hydrophilic and hydrophobic part to them, is only one of a number of structures that form in water (1–3). The results to be described here were obtained on a lamellar structure formed by erythrocyte lipids and have led to resolution of the molecular arrangement within the lipid layer and, more particularly, to determining the position of cholesterol within a bimolecular layer of phospholipids. Full details of the technique and analysis are described elsewhere (4), but they will be very briefly outlined here.

First, standard techniques for the extraction and analysis of erythrocyte lipids were used, with a chloroform-methanol solvent system. Then a number of samples at different concentrations of lipid in water (dry weight per cent) were prepared, and the equilibrium structures that these samples form at various temperatures were characterized by use of small-angle X-ray diffraction. The concentration and temperature ranges over which a single lamellar phase exists were delimited; the lamellar phase being a structure in which there is a regular periodic repeat of electron density in one dimension and no correlation of electron density in the other two dimensions. The spacings in the X-ray diagrams give the dimension of the repeat period, d. The volumetric concentration of lipids in a sample can be determined, and, on the assumption that the lipid molecules aggregate in sheets to the mutual exclusion of water, and that the repeat period is therefore made up of one layer of water and one layer of lipid, then, for each sample, the thickness d_1 of the lipid sheet in the repeat period can be calculated. Justification of the above assumption is made in the final analysis. Fig. 1 shows the dimensions of the repeat period d, obtained from the X-ray diagrams, and the calculated thickness of the lipid layer d_1, over the entire concentration range where the single lamellar phase exists. d_1 is constant at 45 A, and this provides strong evidence that the lipid layer in the repeat unit is the same over this concentration range and that the variation in d is a result of variation only in the thickness of the water layer in the repeat unit. This is a necessary condition in order to proceed further with the X-ray data and to analyze the structure of the lipid layer. In fact, under these conditions, the amplitudes of the intensities of the X-ray reflections for the lamellar phase are given by the Fourier

transform of the electron density distribution through the lipid layer. The procedure then is to determine an electron density model for the lipid layer which will account for the X-ray intensities. Such a model has been constructed on the basis of knowledge of the chemical make-up of the erythrocyte lipids and therefore of their electron content. Again reference should be made to a more detailed account of this analysis, but Fig. 2 shows the crystallographic verification for the molecular model illustrated in Fig. 3. It is

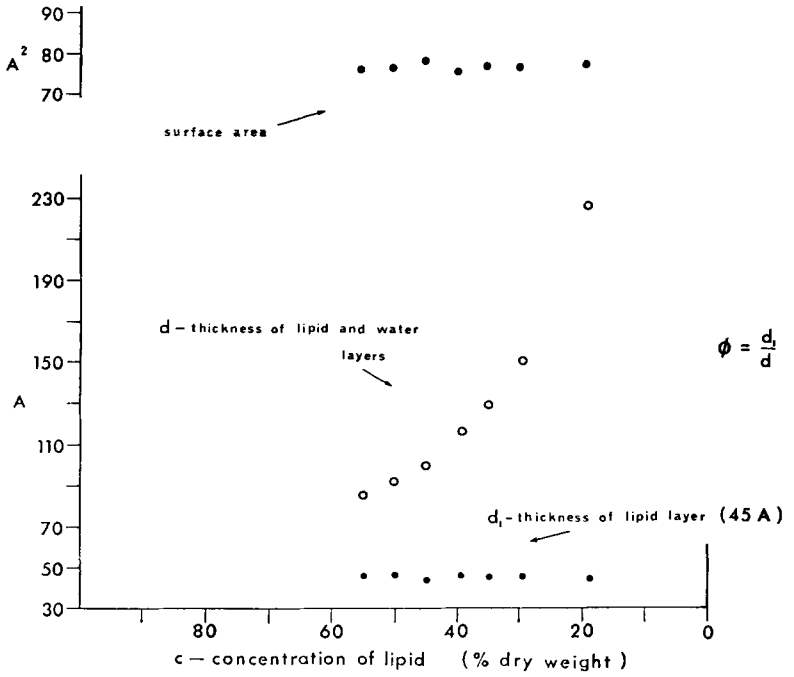


FIGURE 1. Dimensions of the lamellar phase at 0°C. The surface area represented is that occupied by 1 phospholipid molecule plus 0.7 cholesterol molecule on the surface of the leaflet. This is equivalent to 1 of each of these molecules occupying a total of 86 A^2. ϕ is the volumetric concentration of lipid in a sample. *Figure reprinted by permission from Biophysical Journal, 1968, 8:125.*

particularly difficult to find a model which gives the position of the three places where the X-ray intensities go to zero (dashed vertical lines in Fig. 2), and the model in Fig. 3 is the only one we have found which will do this.

Fig. 3 shows the electron density distribution through the lipid layer and the arrangement of molecules that gives this electron density distribution. Not surprisingly, the lipid layer is a bimolecular layer of phospholipids, the interior of which is filled with hydrocarbon chains. The interface between this hydrocarbon layer and the water layer is occupied by the hydrophilic groups of the phospholipid molecules. A characteristic feature of all the X-ray

diagrams of this structure is a diffuse band at 4.5 A, which is a result (1) of a high degree of disorder, or liquid-like or chaotic configuration of the hydrocarbon chains of the phospholipid molecules, as shown in the molecular arrangement in the model. What is interesting about this model is that it gives the position that cholesterol takes up in this lipid layer. In this particular preparation, there was 0.7 molecule of cholesterol for every molecule of phospholipid. The small hydrocarbon chain of cholesterol is in the interior

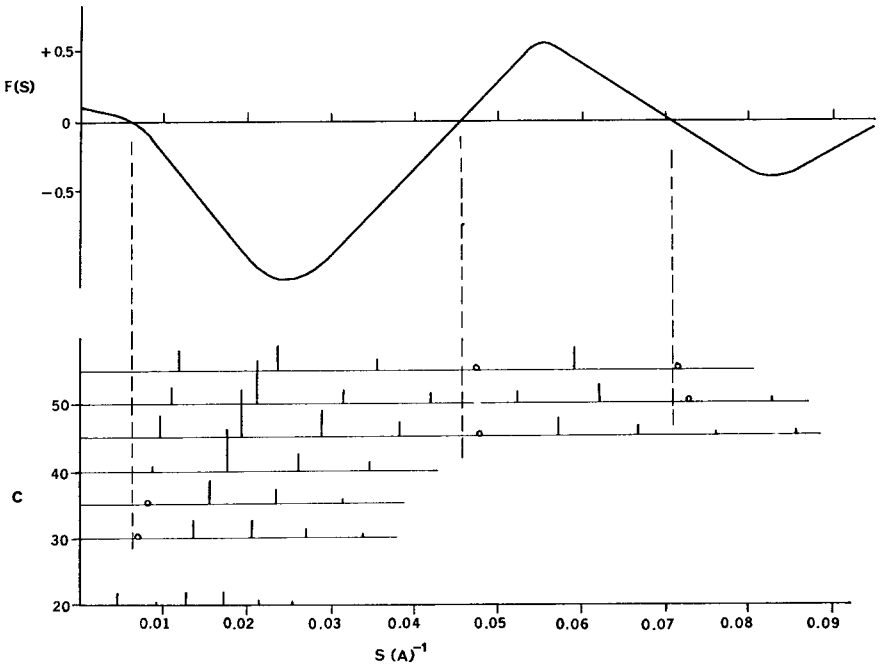


FIGURE 2. Crystallographic verification of the model shown in Fig. 3. Lower: the ordinate represents the concentration of the sample, and the abscissa represents the reciprocal space parameter, s. The vertical lines give the intensity (estimated visually) of the X-ray reflections. Therefore each horizontal line represents the X-ray diagram for that concentration. Upper: $F(s)$ is the Fourier transform of the electron density distribution of Fig. 3 (lower). *Figure reprinted by permission from Biophysical Journal, 1968, 8:125.*

of the lipid layer; the steroid nucleus is pointing toward and "intercalated" between the polar groups of the phospholipid molecules. The small hydrophilic hydroxyl group of the steroid nucleus is very weakly exposed to the water layer. This verifies a number of hypotheses about where cholesterol would situate itself within a phospholipid layer (5, 6). Our data do not provide any more specific or higher-resolution information on the position of cholesterol than is shown in the model, nor do they suggest any specific configurational interactions between the phospholipid molecules and choles-

terol. These in fact are probably not fixed, since cholesterol is in a liquid environment or "dissolved" in a highly mobile aggregate of phospholipid molecules.

Another interesting parameter that can be obtained from the X-ray data is shown in Fig. 1: 1 cholesterol molecule plus 1 phospholipid molecule occupy an area at the surface of the lipid layer of 86 A^2. If, as is suggested by

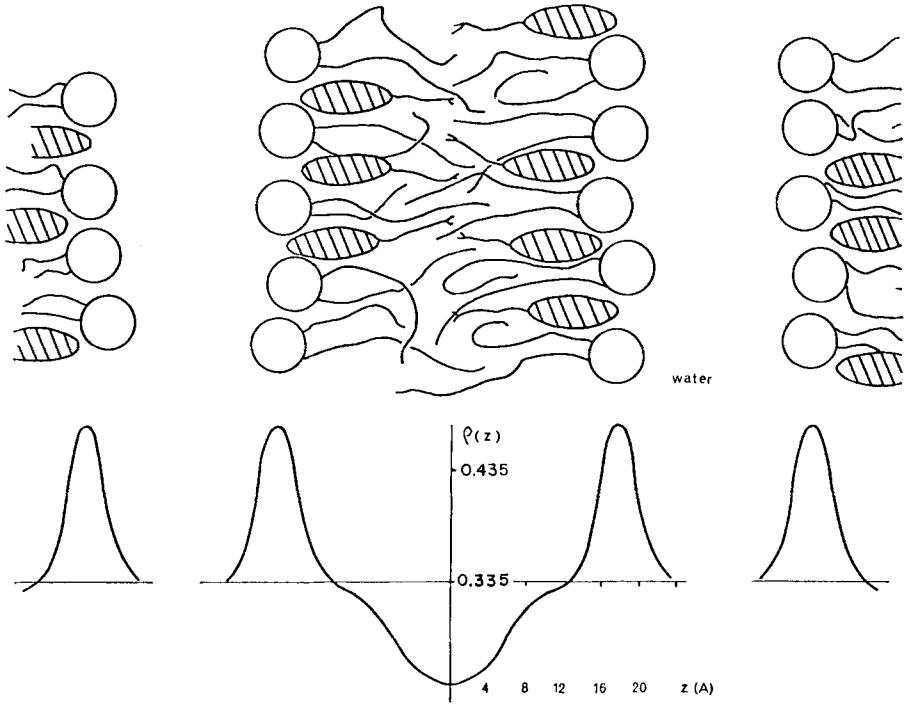


FIGURE 3. Upper: schematic representation of the structure of the lamellar phase. The circles represent the polar groups of the phospholipid molecules. The curved lines represent the hydrocarbon chains. The hatched group represents the steroid nucleus of the cholesterol molecule. Lower: the electron density distribution through the lipid leaflet, indicating the absolute levels of electron densities and the dimensions, z, from the center of the leaflet. *Figure reprinted by permission from Biophysical Journal, 1968, 8:125.*

monolayer work, cholesterol itself occupies a minimum area of 35 A^2, then in this structure 1 phospholipid molecule occupies a maximum area of 51 A^2, or 25 A^2 per hydrocarbon chain. Now in all the other phospholipid systems, which are cholesterol-free, the hydrocarcarbon chains occupy invariably an area of 30 A^2 (2, 3). Therefore cholesterol shows the same condensing effect for the fully hydrated bimolecular layer as it does on a monolayer of similar phospholipids (7). Now, if we assume that the structure of this lipid layer in bulk phase is the same as the layer that Dr. Finkelstein was dealing with,

then the observation of the condensing effect of cholesterol ties in very nicely with his observation that the permeability to water of a phospholipid layer in which there is cholesterol is less than in the cholesterol-free system. Dr. Luzzati first suggested to me that "pores" could be considered to exist in such a thin lipid layer whose hydrocarbon chains were in liquid configuration or highly mobile. The obvious interpretation of the difference in permeability mentioned above would be that the condensing effect of cholesterol restricts the motion of the hydrocarbon chains, thereby restricting the passage of substances through this layer.

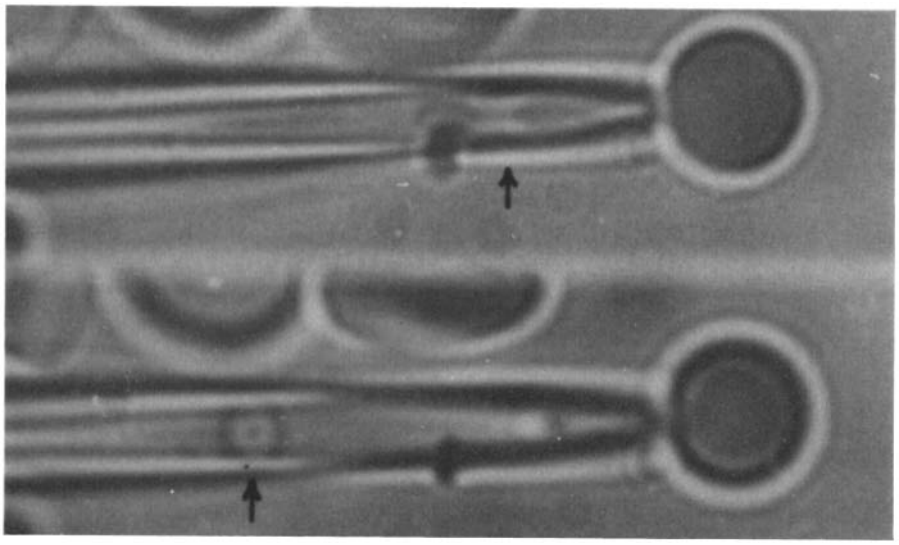
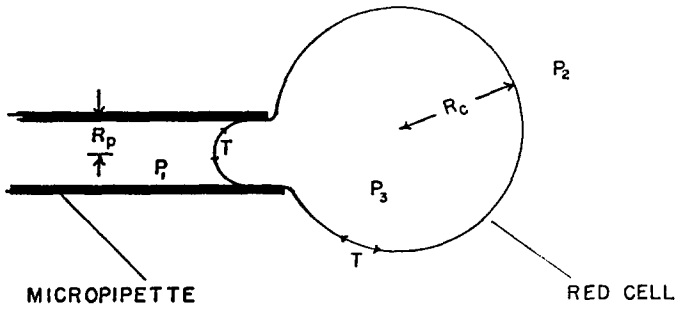


FIGURE 4. Illustrating the spontaneous collapse of the cell membrane on itself when pulled into a long cylinder. Arrows point to the beginning of collapse at a neck of the cylinder and to a refractile globule which finally forms, separating from the rest of the cell. The outer spherical portion of the cell is about 3 μ in diameter. *Figure reprinted by permission from Biophysical Journal, 1964, 4:303.*

I think it is not a difficult assumption to make that the structure in the bulk phase is the same as the structure of the black membrane referred to in the last two papers. In the bulk phase at a high degree of hydration, or in low lipid concentration, there is as much as a 200 A water layer between adjacent lipid layers, and this might be considered to be equivalent to one lipid layer separating two aqueous compartments, the interaction between adjacent lipid layers being very low. The one reservation about this is that, although the lipid layers are separated by a large distance, there must be some sort of interaction in order for them to pile up into large, very regular arrays, even with this degree of separation. Perhaps the interaction is not one that would change the structure within each lipid layer. On the other

hand, the mesomorphic transitions that have been demonstrated in bulk solution (1) may of course take place in a two-dimensional leaflet.

Both the X-ray data and Dr. Tien's data show that the lipid layers, if they are the same, are essentially liquid in character; the black lipid membranes obey surface tension laws, and the X-ray data show that the hydrocarbon chains are disordered or liquid-like in configuration. This point brings me to my second set of data, which suggest that intact membranes, mechanically at least, are indeed not liquid or as liquid in character as the pure lipid systems, and for this reason I have reservations about the relevance of these



$$P_3 - P_1 = \frac{2T}{R_p} \qquad P_3 - P_2 = \frac{2T}{R_c}$$

$$\therefore \; P_2 - P_1 = 2T\left(\frac{1}{R_p} - \frac{1}{R_c}\right)$$

FIGURE 5. Schematic diagram of a swollen red cell that has been drawn into a micropipette until the outer part is the portion of a sphere. Application of the law of Laplace allows the tension in the membrane to be calculated. *Figure reprinted by permission from Biophysical Journal, 1964, 4:303.*

pure lipid-water model systems to the structure of intact cellular membranes. The mechanical properties of lipid membranes and intact membranes are quite different. Experiments on the mechanical properties of intact cell membranes were done in the laboratory of Dr. A. C. Burton, to whom I owe the idea of attempting to micromanipulate individual red blood cells. Micromanipulating red blood cells is somewhat like palpating a baby with a hockey stick, but I do think some useful information can be obtained in this kind of experiment. Again, these data have been published elsewhere, and reference should be made to the original publication for details (8).

First let me describe one situation in which the membrane can be shown to behave as a liquid. This is shown in Fig. 4. If a red blood cell is pulled into a very small micropipette, and if it has enough area compared to its volume so that a long tongue can be sucked into the micropipette, this long cylindrical

tongue will collapse and reseal itself with the formation of a very tiny "cellule" and a smaller red blood cell left on the outside. Collapsing of the long cylinder is typical of the instability of a liquid cylinder.

However, if the cells are swelled, not enough to hemolyze them but to increase their ratio of volume to area, then a long tongue cannot enter the pipette before the outer part becomes the portion of a sphere. Then, in order for the cell to undergo any further deformation, the membrane area must increase. At this point tension in the membrane goes to very high values, and, from the geometry and the application of the law of Laplace to the con-

$$\frac{1}{T} = \frac{1}{S_c d}\left[\frac{1}{Y_1} + \frac{1}{Y_2}\left(1 - \exp\left(-\frac{Y_1}{\eta_1} t\right)\right) + \frac{1}{\eta_2} t\right]$$

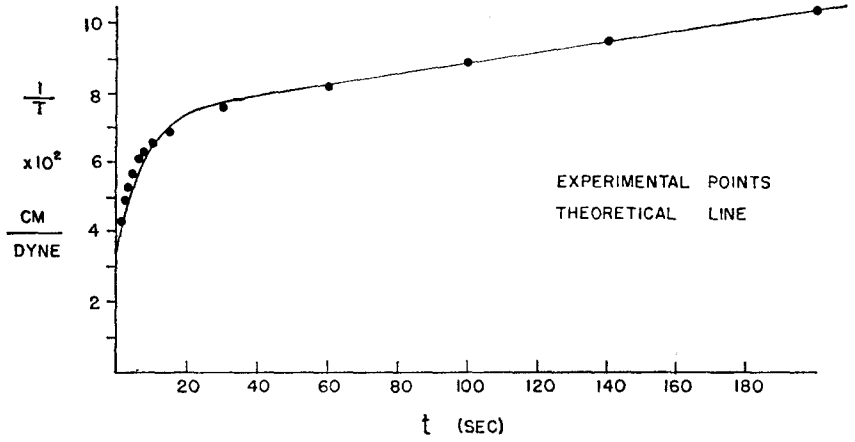


FIGURE 6. Plot of the reciprocal of the membrane tension vs. time for hemolysis. The experimental points are means from the results using five different micropipettes. The theoretical line was obtained from the postulated viscoelastic model of Fig. 7, whose kinetics are shown in the equation, using the elastic and viscous parameters given in the text. *Figure reprinted by permission from Biophysical Journal, 1964, 4:303.*

figuration, shown in Fig. 5, the tension in the membrane can be calculated. Now the experiment to be performed is one of destructive testing. If a step function change in sucking pressure is applied to the cell in a micropipette, the cell does not change shape but after some time it suddenly disappears. It has been drawn into the micropipette, and the pressure can be reversed; the cell can be "spat" out and observed to be a very flexible, biconcave, hemolyzed red blood cell. The membrane has been stressed to the point of rupture, hemoglobin has leaked out of the cell, and the cell volume has gone down, enabling the cell to enter the pipette. An experiment consists of relating the stress or tension applied to the membrane and the time taken for the membrane to break down. These kinetics are shown in Fig. 6, the points on the curve of which give data averaged from experiments using five different

micropipettes. The relationship between time and tension in the membrane is typical of a viscoelastic solid rather than of either a liquid or a purely elastic substance. It shows that the membrane can withstand, for example, a tension of approximately 20 dynes/cm for a time of approximately 5–10 sec. If the membrane were purely liquid, the curve shown in Fig. 6 would be a straight line of constant slope passing through the origin. A mechanical analogue of the membrane is shown in Fig. 7. On the basis of the assumption that the membrane breaks down after a critical strain or a critical degree of increase in area of the membrane, we have attempted to estimate the viscosity and the Young's modulus of the membrane substance, i.e. to estimate η_1, η_2, Y_1, and Y_2 in Fig 7. These values are, for Young's modulus, between 10^6 and 10^8 dynes/cm^2 and, for the viscosity, between 10^7 and 10^{10} poises, and, when applied to the model of Fig. 7, give the theoretical fit to points of

$$S = F\left[\frac{1}{Y_2} + \frac{1}{Y_1}\left(1 - \exp\left(-\frac{Y_1}{\eta_1}t\right)\right) + \frac{1}{\eta_2}t\right]$$

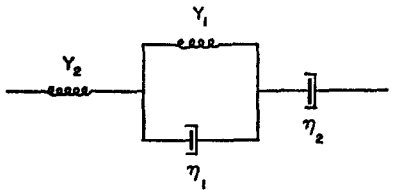


FIGURE 7. Mechanical model of the cell membrane used to describe the kinetics of the membrane breakdown and to obtain the theoretical line of Fig. 6. *Figure reprinted by permission from Biophysical Journal, 1964, 4:303.*

Fig. 6 and as well agree with the values obtained by Katchalsky et al. (9) for red cell membranes.

It is concluded that the intact erythrocyte membrane, far from being only a mechanically fragile, liquid, bimolecular lipid layer, is a tough viscoelastic substance. There must be some kind of strong bonding in the plane of the membrane other than weak van der Waals' interactions between hydrocarbon chains of phospholipid molecules. These bonds may be in the protein layers covering a bimolecular layer of lipids, as proposed in the Davson-Danielli model, but still the possibility is not ruled out that strong bonding may exist anywhere throughout the thickness of the membrane. The viscous and elastic parameters and the kinetics of membrane breakdown derived here are similar to those of a study by Biswas and Haydon (10) on the shear-stressing of a monolayer of bovine serum albumin on a petroleum ether surface, and unlike those of a monolayer of saturated phospholipid. Through a thorough study of the effects of pH, ionic strength, and temperature, Biswas and Haydon concluded that the serum albumin layer is primarily a hydrogen-bonded structure. This is just the kind of evidence that is lacking for intact cell membranes. Refinement of technique and further study of the mechani-

cal properties of red cells may lead to a more explicit idea of the kind of bonding in this membrane.

In summary, extreme caution must be taken in applying to intact cell membranes data pertaining to model systems of lipid and water: data on both structure and permeability. A high degree of structural resolution has been obtained for some lipid water systems, and shows that these are structurally very labile. Molecular arrangements other than the one described here are possible. However, the purely liquid character of the model membranes is incompatible with the mechanical properties of intact erythrocytes.

The author gratefully acknowledges the support of the Medical Research Council of Canada.

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Discussion

Dr. Mauro: Dr. Berton Pressman, who has done so much to open up the subject of valinomycin in connection with the mitochondrial system, would like to show three slides. This is relevant to the question that was asked of Dr. Finkelstein about the mode of action of valinomycin in promoting cationic selectivity in the lamellar film type of experiment.

Dr. Berton Pressman: I wanted to address myself briefly to the question of the origin of ionic specificity of the transport-mediating antibiotics with respect to the molecular dimensions of the hole in the interior. In Fig. 1, Discussion, we see the structure of

valinomycin which would accommodate in a reasonably close fit 1 hydrated potassium; however, we have evidence that the complex between this and potassium is with the unhydrated form of potassium.

In Fig. 2, Discussion, we have an antibiotic, enniatin B, related to valinomycin, which has only one-half the ring size and yet functions very nicely as a transport-mediating antibiotic. Enniatin shows less preference for potassium over sodium than valinomycin.

Fig. 3, Discussion, shows the structure of the macrotetralide actins (R = H or CH_3), a group of non-nitrogen–containing antibiotics which have twice the hole size of the enniatins, and yet exhibit approximately the same ionic selectivity, and are effective at roughly the same concentrations. I think this clearly points out that the

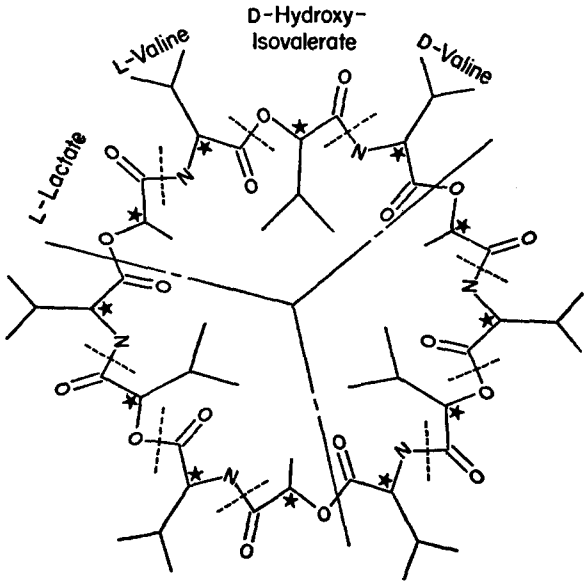

FIGURE 1. Valinomycin.

attempt to explain the activity and ion selectivity of the transport-mediating antibiotics on the basis of molecular fit is a great oversimplification. Other approaches that we are using will, we think, eventually explain the ionic selectivity.

Dr. Mauro: Is there a very brief comment or question with regard to this question?

Dr. Charles W. Wenner: The other point on which I'd like to question Dr. Finkelstein concerning the depsipeptides is: Have you looked at the effect of gramicidins in the artificial system? Gramicidins also have marked specificity in biological systems, and at least gramicidin A is a linear peptide. The explanation of a ring compound acting as a pore seems inadequate unless one infers that the gramicidin forms a cyclic configuration so that the specificity could be met. The question is: Have you looked at the effect of gramicidins?

Dr. Finkelstein: Yes, it does. There are several different kinds of gramicidin: A, B, C, and S. I don't recall which, but some gramicidins do cause cation selectivity, but

not very good selectivity between sodium and potassium. And there is speculation that even gramicidins can form ring structures.

Dr. Mauro: Is that an oversimplification of your question? Posing it that way?

Dr. Finkelstein: Probably Dr. Pressman could comment more on that.

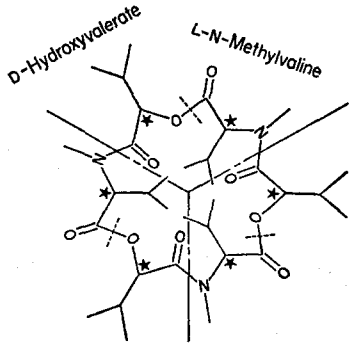

FIGURE 2. Enniatin.

Dr. Mauro: I might add that since these two sessions are related we might have a cross-discussion later on. Let's keep these comments very brief because we want Dr. Blank to get on with his fine program.

Dr. Pressman: Gramicidin S is cyclic and is chemically distinct from the

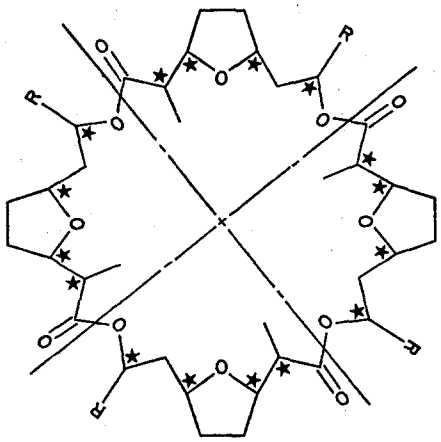

FIGURE 3. Macrolide actins.

other gramicidins; it is devoid of transport-mediating activity. Gramacidins A, B, and C, which are linear, are all active in the Rudin-Mueller model membrane system. As Dr. Finkelstein pointed out, they don't have the K/Na selectivity of the other transport-mediating antibiotics, and it is questionable whether they act either by forming a ring or merely by folding back on themselves. The requisite structure appears to be a central focus of electronegative groups. The ring per se is not the required structure in the transport-mediating antibiotics.

Dr. Joseph F. Hoffman: I have two questions, one to Dr. Finkelstein and the other to Dr. Rand. I would like Dr. Finkelstein to comment on the mechanism of penetration of water through thin films. Does the mechanism of the water permeability change when one adds the system iodine plus iodide or nystatin or valinomycin? In other words, are the changes correlated with the changes in resistance? Also, can bulk flow be demonstrated under this circumstance?

Dr. Rand, for a number of years we've been playing what might be called the Gorter and Grendel game concerning whether or not there is enough lipid to cover the red cell in the form of a bimolecular leaflet. Gorter and Grendel's conclusions have in recent years been questioned on the basis of their surface area measurements and their lipid extraction procedure. Even so, the errors tend to cancel one another and the ratio remains qualitatively in support of the bimolecular leaflet interpretations. However, I wonder, from measurements such as Dr. Rand presented and from other estimates of the minimum areas occupied by phospholipid and cholesterol molecules that have been made in mixed films, whether or not the results are consistent with the bimolecular leaflet concept. When I make this type of calculation I invariably come out with only 60–80 % of the required amount of lipid to cover, in this case, the human red cell twice. I would like to hear Dr. Rand comment on this.

Dr. Rand: I agree completely with Dr. Hoffman's calculations on the amount of lipid available to fill the area of the whole erythrocyte membrane. If the condensing effect of cholesterol operates in the cell membrane, then there is only 60 % of the required amount of lipid. If the condensing effect does not operate, somehow cholesterol and phospholipids are separate, then there still is only 80 % of the required amount of lipid. My inclination now is to think that the errors involved in these types of measurements and calculations are sufficiently low to say that phospholipids and cholesterol themselves are not sufficient to cover the surface with a bimolecular layer.

Dr. Finkelstein: With regard to the water permeability, we've added EIM to these membranes and brought the resistance down by a factor of 1000, without any observable effects on the water permeability. Now, this may mean simply that one can have very few defects in the membrane and have very dramatic effects on the electrical properties. An analogy I like to think of is that if one has just a slab of wax and punches a little pinhole in it, then the resistance comes down from infinity to some reasonably finite value. And yet, for anything that was permeating by dissolving in and diffusing through the wax, this little pinhole would make a trivial contribution to its transport.

Dr. Mysels: In connection with Dr. Tien's discussion of optical thickness measurements, it may be worth mentioning that an extremely simple treatment is also available. It takes into account the sandwich structure of the film, and can be used for any number of layers in the sandwich. It is accurate as long as the layers are all very thin compared with the wavelength of light, which is indeed the case for both lipid and soap films.

A rigorous derivation is available (FRANKEL, S. P., and K. J. MYSELS. 1966. *J. Appl. Phys.* **37**:3725), but the results given below can be obtained by remembering only that the optical effectiveness of an elementary volume is proportional to its polarizability and, therefore, to $n^2 - 1$, where n is the refractive index. Hence, the

optical effect of two layers of thickness d_0 and d_1 will be the same when

$$d_0(n_0^2 - 1) = d_1(n_1^2 - 1) \quad (1)$$

provided they are thin enough to neglect secondary optical interactions. This means that a layer of surface molecules (d_1) can be replaced by an optically equivalent thickness of the core material (d_0) provided that its thickness is chosen to be

$$d_0 = d_1(n^2 - 1)/(n_0^2 - 1) \quad (2)$$

Similarly, layers having different refractive indices, n_i, and thicknesses, d_i, can be replaced optically one by one to give a total layer of the core material having the thickness

$$d_0 = [\Sigma d_i(n_i^2 - 1)]/(n_0^2 - 1) \quad (3)$$

If the film is observed not in vacuum but in a medium of refractive index l (e.g. water for lipid films), only the optical effectiveness of each layer above this medium has to be considered; it becomes $(n^2 - 1) - (l^2 - 1) = n^2 - l^2$, and the replacing thickness of the core layer becomes

$$d_0 = (n_1 - l^2)/(n_0^2 - l^2) \quad (4)$$

for a single layer instead of that given by equation 2. For multiple surface layers surrounded by such a medium, we obtain a formula analogous to equation 3, but with all the i's replaced by l's.