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Phase Transitions and Heterogeneity in Lipid Bilayers

Abstract. *The optical reflectivity of several well-characterized lipid bilayer systems has been correlated with calorimetric studies of the membrane components. There is a large increase in mean membrane thickness when a bilayer is cooled below the transition temperature of the membrane lipid. Similar studies on membranes generated from a mixture of two lipids possessing different degrees of unsaturation suggest that between the characteristic transition temperatures of the two lipids, the bilayer contains clusters of gel and liquid crystalline lipid which coexist within the plane of the membrane.*

One explanation for the heterogeneity in the lipid composition of natural membranes may be that the different lipid species are required for the generation of a mosaic lipid membrane in which particular regions of the membrane are enriched with certain lipids. Indeed, recent physical studies suggest that some natural membranes contain heterogeneous lipid domains (*1*). According to this view of membrane structure, specific membrane functions would be gained by local interactions of the lipid mosaic with other membrane components. Thus it would be of considerable interest to examine the intrinsic physicochemical properties of lipid bilayer membranes in which heterogeneity within the plane of the membrane was established and subject to control. We now present evidence for the construction of a planar lipid bilayer membrane containing heterogeneous lipid (gel and liquid crystalline) domains. Evidence in support of this structure comes from measurements of the reflectivity of the membrane and from a correlation of the membrane composition with differential scanning calorimetric (DSC) studies of the membrane components.

Planar bilayer membranes were formed by standard techniques (*2*) in an all-quartz water-jacketed cell from *n*-hexadecane (*n*-C_{16}) solutions of (i) glyceryl monooleate (GMO), (ii) glyceryl monostearate (GMS), or (iii) a mixture of GMO and GMS (1 : 1) at concentrations of 10 mg of monoglyceride per milliliter of solvent. These systems were chosen for study because the bilayers that form spontaneously from them are chemically well defined, the number of molecules of monoglyceride and neutral hydrocarbon per unit area of membrane having recently been determined with the use of radioactively labeled components and a special sampling technique (*3*). The GMO and GMS were obtained from Sigma Chemical Co., and *n*-C_{16} was obtained from Koch Light Ltd. Membranes formed from solutions (ii) or (iii) were generated above 65° to 70°C and were subsequently cooled to the desired temperature.

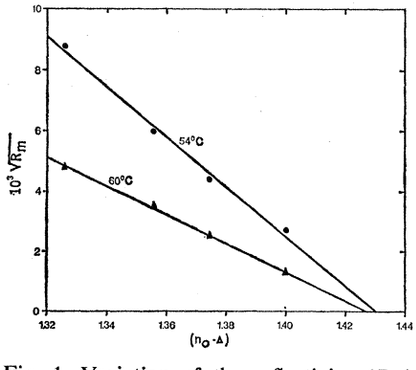


Fig. 1. Variation of the reflectivity (R_m) of bilayers formed from glyceryl monostearate (GMS) and *n*-hexadecane with refractive index ($n_o - \Delta$) at 60° and 54°C.

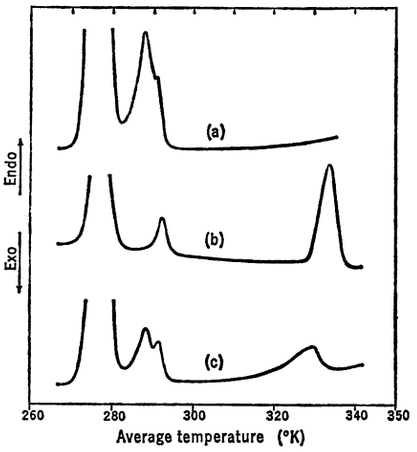
The thicknesses of the lipid membranes were determined from reflectivity measurements by a modification of the technique developed by Cherry and Chapman (*4*), in which the membranes were illuminated at near normal incidence by light from a helium-neon laser, and the membrane reflectance was obtained by comparing the reflected intensity with that of a quartz plate placed in the same aqueous solution. The reflectivity of the membrane, R_m, is related to its thickness by the previously derived equations (*4*)

$$\frac{\lambda R_m^{1/2}}{2\pi d} = \bar{n} - n_o + \Delta \quad (1)$$

$$\Delta = (\bar{n} - n_o)/(\bar{n} + n_o) \quad (2)$$

where λ is the wavelength of the incident light, d is the total membrane thickness, $\bar{n}$ is the mean refractive index in the plane of the membrane, and n_o is the refractive index of the aqueous phase, which is varied by forming the membranes in different solutions of sucrose or NaCl. By plotting $R_m^{1/2}$ against n_o, an approximate value of n is obtained. This value was used to calculate the correction term Δ; the final values of d and $\bar{n}$ were obtained by plotting $R_m^{1/2}$ against $(n_o - \Delta)$. For each of the systems examined, R_m was determined at four values of n_o, each value of R_m being the average of five measurements.

We have observed a large increase in the membrane reflectance when lipid bilayers formed from GMS and *n*-C_{16} at 65°C are slowly cooled below 55°C. This increase in reflectivity occurs rapidly (< 1 second for a bilayer of area 0.03 cm^2) at ~54.5°C, and the resulting membrane is extremely fragile. In several instances it was possible to reheat such a membrane without causing membrane rupture, and the membrane reflectivity was observed to return to its lower initial value. This phenomenon has been studied quantitatively as outlined above, and the data are summarized in Fig. 1. For each membrane R_m was measured at 60° and 54°C. From Eqs. 1 and 2 and the data in Fig. 1, we calculated that, on cooling, the membrane thickness, d, increased from 45 ± 1 Å to 77 ± 4 Å, while the



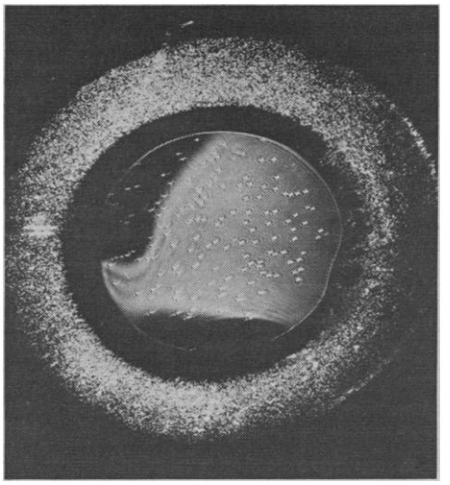


Fig. 2 (left). Differential scanning calorimetry thermograms of (a) GMO plus n-C_{16}; (b) GMS plus n-C_{16}; and (c) a mixture of GMS and GMO (1 : 1) plus n-C_{16}; all in excess water. Fig. 3 (right). Photograph of a planar bilayer membrane formed from a mixture of GMS and GMO (1 : 1) and n-hexadecane at 60°C.

mean refractive index (*5*) of the membrane is essentially unchanged (1.427 ± 0.004 at 60°C as compared to 1.430 ± 0.007 at 54°C). From the DSC heating curve of GMS and n-C_{16} in H_2O shown in Fig. 2b, three endothermic transitions are seen (*6*). These correspond to the melting of ice at 273°K, the melting of pure n-C_{16} at 292°K, and the gel-liquid crystal transition for GMS at 328°K (55°C). Thus we conclude that the large increase in membrane reflectance corresponds to the gel-liquid crystalline phase transition occurring in the planar lipid bilayer.

Since the maximum length of two fully extended molecules of GMS is only about 46 Å, the large increment in membrane thickness at the phase transition must be due to a rearrangement of the molecules of GMS and n-C_{16} within the bilayer. If it is assumed that the neutral hydrocarbon is interdigitated among the side chains of GMS when the bilayer is in the fluid, liquid crystalline state, then, on formation of the rigid gel phase, a two-dimensional crystallization of GMS would cause the hexadecane to be squeezed out either into a continuous hydrocarbon layer in the hydrophobic region of the membrane or into microlenses. In either case, both the mean membrane thickness and the number of molecules of GMS in the bilayer would increase below the transition temperature.

No change in the reflectivity of bilayer membranes formed from GMO and n-C_{16} could be detected between 20° and 70°C. This is consistent with DSC measurements on this mixture (Fig. 2a), showing that the phase transitions for both GMO and n-C_{16} are below 20°C (*7*).

As membranes formed from a 1:1 mixture of GMO and GMS in n-C_{16} were slowly cooled (~0.5°C per minute) below 60°C, small highly reflecting regions about 30 μm in diameter were observed to appear (Fig. 3), the total number of "spots" being as many as 10^3 per square centimeter. These spots could be made to disappear by reheating the aqueous phase, and reappear by cooling the system again. No coalescence of the spots with each other or with the Gibbs border of the black film was ever observed, nor were such highly reflecting regions ever detected in membranes formed from n-hexadecane solutions of GMS or GMO alone. As the membranes were further cooled, no change in the appearance of the black film was detected until about 40°C, when the spots appeared to coalesce to form larger highly reflecting patches in the plane of the bilayer. This process usually started at the periphery of the black film and spread toward its center. At this stage the membrane was extremely fragile, but occasionally could be reheated to 65°C, where the surface appeared perfectly homogeneous under laser illumination.

DSC data for this mixture (Fig. 2c) shows that the transition temperature for the unsaturated component, GMO, is unchanged when mixed with GMS, whereas the transition for the fully saturated GMS is lowered and broadened. At temperatures intermediate to the transition temperatures of GMO and GMS, the presence of clusters of gel and liquid crystalline lipid is indicated, and we conclude that planar lipid bilayers of similar composition must similarly be heterogeneous at these temperatures. As the system is warmed above 40°C, a gradual mixing process occurs until the saturated compound (GMS) melts, giving rise to complete miscibility when both components are liquid crystalline.

In principle, it should be possible to rigorously identify the gel and liquid crystalline regions of the mosaic membrane in Fig. 3 by determining the ratio of intensities of the highly reflecting regions to the rest of the membrane, and comparing this data to the relative intensities of gel and liquid crystalline GMS bilayers given in Fig. 1. We have attempted to do this by photographing membranes such as those shown in Fig. 3, at different values of the refractive index of the aqueous phase, n_0, and taking densitometer tracings of the photographs. This analysis is complicated by the fact that the relative intensities of the various regions of the membrane may not be preserved during the stages of photographic development. While there is a good deal of scatter in the intensity ratios obtained by this method, the results were all about two to five times greater than the measured ratio obtained from the data in Fig. 1. Thus while the data for the GMO-GMS mixed system require the membranes to contain heterogeneous gel and liquid crystalline domains, we cannot at present exclude the possibility that the highly reflecting regions observed at low magnification (Fig. 3) are simply lenses of hydrocarbon solvent which are produced as a result of microscopic clustering of the monoglyceride species.

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index of GMO is substantially higher (1.463). However, when n-C_{10} was used as solvent the bilayer refractive index was only slightly less (1.429), although the refractive index of n-C_{10} itself is substantially lower (1.410). Hence it is likely that the refractive index of the membrane is largely determined by the lipid and is reduced from the bulk value by (i) anisotropic effects and (ii) hydration of the lipid polar groups. Similar effects were previously observed with egg lecithin bilayers (*4*).

6. Differential scanning calorimetric (DSC) measurements were made with a Perkin-Elmer model DSC-1B calorimeter for mixtures of monoglycerides and hexadecane in which the molar ratio of the components was approximately the same as those found experimentally (*3*) in bilayers generated from solutions (i), (ii), or (iii) (see text). Solutions were prepared for DSC by prior mixing of the components in the desired proportions in chloroform, evaporating off the chloroform at reduced pressure, adding an equal weight of distilled water, and thoroughly mixing the resulting paste.
7. We have compared the thickness obtained by the reflectance method with that calculated from the membrane capacitance for bilayers formed from GMO in n-decane, n-tetradecane, and n-hexadecane. The values are respectively 62, 62, and 49 Å from the optical technique, and 57, 49, and 41 Å from the capacitance data (*8*). A systematic error in the optical method was ruled out by verifying the previously published value of the membrane thickness for bilayers formed from egg lecithin in n-decane. Furthermore, the capacitance values of membranes formed in our laboratory were in essential agreement with those of Fettiplace *et al.* (*8*). Therefore, we conclude that the discrepancies in these two methods of determining membrane thickness must be due to the presence of microlenses of hydrocarbon solvent that are too thick to be measured in the membrane capacitance technique, but nevertheless contribute to the average bilayer thickness determined optically. The existence of such lenses has been inferred previously from electron micrographs (*9*) and composition studies (*3*) of the bilayer.
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Hyperinnervation of Skeletal Muscle Fibers: Dependence on Muscle Activity

Abstract. *After the motor nerve to the rat soleus muscle was blocked reversibly by local anesthesia, individual muscle fibers became innervated by a transplanted motor nerve without losing their original innervation. Such cross-innervation of the denervated soleus muscle by the same foreign nerve was largely reduced by direct electrical stimulation of the muscle. The results demonstrate the importance of muscle activity for synapse formation by a foreign motor nerve.*

Normally innervated and denervated skeletal muscle fibers of adult mammals differ in their ability to accept additional nerves and in their sensitivity to acetylcholine (ACh). Normal muscle fibers are usually innervated by a single motor nerve fiber. They will not normally accept additional motor innervation from a transplanted foreign nerve (*1*), and their sensitivity to the transmitter ACh is sharply localized in the end plate region. In contrast, after denervation the sensitive region expands to cover the entire surface of the muscle fiber (*2*), and the muscle can become innervated by the original nerve or by a foreign nerve. In certain experimental situations skeletal muscle fibers can accept foreign motor nerves in addition to their original innervation. This has been demonstrated after botulinus poisoning (*3*) and after local injury of muscle fibers (*4*). In both these cases the formation of new synapses is associated with supersensitivity to ACh.

From observations on frog sartorius, Miledi (*5*) argued that the spread of ACh sensitivity was due to the absence of a neural "trophic factor" in denervated muscles. Recently, however, Lømo and Rosenthal (*6*) showed that the ACh-sensitive area in rat soleus muscle fibers could spread in the presence of innervation after a prolonged, reversible block of impulse conduction in the soleus nerve. Furthermore, they showed that denervation supersensitivity could be prevented by direct electrical stimulation of the muscle. These observations suggest that muscle activity per se is an important factor controlling the extent of ACh sensitivity over the surface of the muscle fiber. The series of experiments reported here were undertaken to see whether the ability of muscle fibers to accept additional motor innervation might be similarly controlled by muscle activity.

The experiments were performed on young white rats, weighing about 200 g. The superficial fibular nerve and its branches to the peroneal muscles were dissected free, cut distally, and transplanted onto the proximal dorsal surface of the soleus muscle. The fascia over the soleus muscle was left intact to avoid any injury to the muscle fibers. The transplanted nerve was fixed in its new location by fibrin (*7*). After 2 weeks, impulse conduction was blocked reversibly in the sciatic nerve by application of a cuff containing a local anesthetic (Marcaine, Bofors) (*6*). The effectiveness of the nerve block was evidenced by clear signs of paralysis in the ankle muscles. The block was checked in the final experiment 1 week after the application of the cuff, after the nerve and muscle had been isolated from the leg. In successful experiments muscle contractions were elicited by nerve stimulation distal to but not proximal to the block.

In all six rats with successful nerve blocks we found that stimulation of the transplanted fibular nerve elicited visible contractions in the soleus muscle. As suspected, some of this was due to fibular innervation of muscle fibers which had lost their original innervation due to mechanical pressure from the anesthetic cuff with subsequent nerve degeneration. But in each of five rats we found many muscle fibers that could be activated both by stimulation of the original soleus nerve

Table 1. Effect of direct muscle stimulation on innervation by transplanted fibular nerve: F, fibular twitch, peak twitch tension to supramaximal single shock stimulation of fibular nerve; D, direct twitch, peak twitch tension to supramaximal direct stimulation of muscle; F/D, percentage ratio of fibular to direct twitch; ACh, peak tension of the muscle when suddenly exposed to acetylcholine (10^{-5} g/ml) in the bathing solution. When ACh was used, the contractions rose to a peak within 10 seconds and subsided slowly over the subsequent 60 to 90 seconds. Three of the five control muscles gave observable contractions even with an ACh concentration of 10^{-6} g/ml.

Rat No.	Stimulated muscle				Control muscle			
	F (g)	D (g)	F/D (%)	ACh (g)	F (g)	D (g)	F/D (%)	ACh (g)
1	0.04	5.8	0.7	0	6.0	11.4	52.4	5.1
2	0	6.1	0	0	0.9	15.1	5.4	1.8
3	0.02	15.8	0.1	0	1.2	6.0	20.0	2.4
4	0.2	17.0	1.2	1.4	2.0	14.3	14.0	2.9
5	0	24	0	1.1	0.7	9.0	7.8	2.7