These data again point out that the coumarins, in addition to their effect on prothrombin synthesis, have profound effects on the metabolism of vitamin K. Not only was the interconversion of vitamin and oxide interferred with, but coumarin administration caused an increase in the amount of vitamin K metabolites which could not be identified as either vitamin K or vitamin K oxide. In normal rats, roughly 50% of the activity from injected vitamin K can be identified as these two compounds (Table II), and coumarin administration reduces this to about 25%. The other metabolites have not been identified, but are presumably more polar, as they are not extracted from the tissues by hexane.

Although these data do indicate that vitamin K oxide is not the antagonist of vitamin K when coumarins are administered, they do not furnish any indication of what the significance of this compound might be. The presence of an oxidase and reductase in liver, and the observations (Willingham and Matschiner, 1974) that the level of at least the epoxidase may change with vitamin K status of the animal would suggest that this interconversion between the two forms of the vitamin might in some way be related to the physiological actions of the vitamin.

References

Bell, R. G., and Matschiner, J. T. (1970), *Arch. Biochem. Biophys. 141,* 473–476.

Bell, R. G., and Matschiner, J. T. (1972), *Nature (London) 237,* 32–33.

Bell, R. G., Sadowski, J. A., and Matschiner, J. T. (1972), *Biochemistry 11,* 1959–1961.

Campbell, H. A., and Link, K. P. (1941), *J. Biol. Chem. 138,* 21–33.

Lowenthal, J., and Birnbaum, H. (1969), *Science 164,* 181–183.

Mameesh, M. S., and Johnson, B. C. (1959), *Proc. Soc. Exp. Biol. Med. 101,* 467–469.

Matschiner, J. T., Bell, R. G., Amelotti, J. M., and Knauer, T. E. (1970), *Biochim. Biophys. Acta 201,* 309–315.

Matschiner, J. T., Zimmermann, A., and Bell, R. G. (1974), *Thromb. Diath. Haemorrh., Suppl.* (in press).

Olson, R. R. (1970), *in* The Fat-Soluble Vitamins, DeLuca, H. F., and Suttie, J. W., Ed., Madison, Wis., University of Wisconsin Press, pp 463–489.

Shah, D. V., and Suttie, J. W. (1972), *Arch. Biochem. Biophys. 150,* 91–95.

Shapiro, S. S., and Waugh, D. F. (1966), *Thromb. Diath. Haemorrhag. 16,* 469.

Suttie, J. W. (1975), *Thromb. Diath. Haemorrh., Suppl.* (in press).

Willingham, A. K., and Matschiner, J. T. (1974), *Biochem. J. 140,* 435–441.

Clusters in Lipid Bilayers and the Interpretation of Thermal Effects in Biological Membranes†

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ABSTRACT: The partitioning of the spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo), has been studied in a number of aqueous phospholipid dispersions. In dioleoyllecithin bilayers the formation of quasicrystalline clusters has been detected at temperatures below *ca.* 30°, as shown by the exclusion of Tempo from the clusters. A pure complex of dioleoyllecithin with the [Ca^{2+}; Mg^{2+}]ATPase from sarcoplasmic reticulum has been prepared, and a break in the Arrhenius plot observed at 29°. Similar evidence is presented for cluster formation in native sarcoplasmic reticulum membranes at temperatures below *ca.* 25°. The possibility is discussed that breaks in Arrhenius plots at *ca.* 20° for a number of other enzyme systems also correspond to cluster formation rather than to a lipid liquid-crystalline to crystalline phase transition.

In the last few years much attention has been focused on the effects of temperature on biological membranes, in the hope of being able to correlate known changes in the physical properties of the lipid component with a corresponding change in some membrane property. The major change occurring in a lipid–water system as the temperature is varied is the crystalline to liquid crystalline transition, also called the order–disorder transition, corresponding to a "melting" of the lipid–hydrocarbon chains. This change has been detected by thermal calorimetry, X-ray diffraction, electron spin resonance, and nuclear magnetic resonance (see, for example, Chapman *et al.,* 1967; Levine, 1972). In the liquid crystalline state, a lipid has considerable freedom of motion, with extensive internal motion (Hubbell and McConnell, 1971; Levine *et al.,* 1972), rapid anisotropic rotation of the whole lipid molecule (Lee *et al.,* 1974b), and rapid diffusion in the plane of the bilayer (Träuble and Sackmann, 1972; Devaux and McConnell, 1972; Lee *et al.,* 1973), probably by a vacancy diffusion process (Lee *et al.,* 1974a). Although less is known about mobility in lipids below the crystalline to liquid-crystalline transition temperature, it is clear that many of these motions are severely restricted (Lee *et al.,* 1974a).

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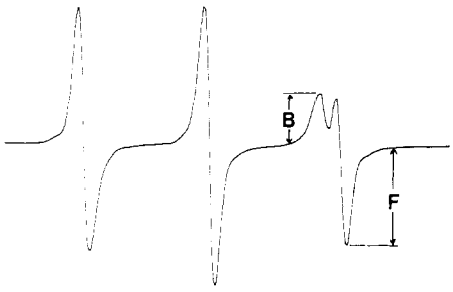
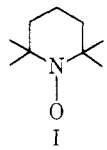


FIGURE 1: Paramagnetic resonance spectrum of Tempo dissolved in an aqueous dispersion of dimyristoyllecithin, showing the peaks attributed to Tempo in the bilayer (B) and free in solution (F).

Even for simple organic molecules the actual process of melting is generally very complex (Ubbelohde, 1965). Both premelting and prefreezing phenomena are common, the premelting phenomena making the solid more "liquid-like" and the prefreezing phenomena making the liquid more "solid-like." Prefreezing arises from a gradual extension of short-range order in the liquid as the temperature of the melt is lowered toward the freezing point. Studies of the heat capacities and viscosities of many organic liquids have led to the concept of small quasicrystalline clusters of molecules present in the liquid phase together with freely dispersed molecules (Ubbelohde, 1965; Davies and Matheson, 1967; Ertl and Dullien, 1973). With increasing temperature, these clusters gradually break up to give monomeric, freely dispersed molecules.

We now report on experiments which suggest that clusters of lipids occur in dioleoyllecithin bilayers up to *ca.* 30°. These experiments involve study of the distribution of the spin label 2,2,6,6-tetramethylpiperidine-1-oxyl or Tempo (I) between the



I

aqueous and fluid hydrophobic phases present when phospholipids are dispersed in water. It has previously been shown that Tempo will partition into the fatty acid chain region of phospholipid bilayers, the partition coefficient depending on the "fluidity" of the chains (Hubbell and McConnell, 1971; Metcalfe *et al.*, 1972; McConnell *et al.*, 1972; Linden *et al.*, 1973; Shimshick and McConnell, 1973a,b). In particular, it has been shown that Tempo will partition into the fatty acid chains of phospholipids when the phospholipids are in a liquid-crystalline state, but that in the crystalline state, the Tempo solubility is greatly reduced (Hubbell and McConnell, 1971; Metcalfe *et al.*, 1972; Shimshick and McConnell, 1973a,b). We show here that Tempo solubility can be similarly used as a probe for the presence of quasicrystalline clusters in lipid bilayers in the liquid-crystalline state. We also show that spin-labeled fatty esters incorporated into the lipid bilayer are sensitive to the presence of these quasicrystalline clusters.

The presence of clusters in dioleoyllecithin bilayers up to 30° could account for the change in enzymatic activity at this temperature of a defined complex of dioleoyllecithin with the [Ca^{2+}; Mg^{2+}]ATPase from sarcoplasmic reticulum membranes: the liquid-crystalline to crystalline phase transition in dioleoyllecithin occurs at −22°. Similar clusters are also probably present in the complex lipid mixture of the native sarcoplasmic reticulum membrane, and could account for the change in activity of the ATPase in the native membrane at 26°. In a number of other cases, membrane-bound enzymes also show changes in activity at about 25° although the liquid-crystalline to crystalline phase transitions of the associated lipids are expected to occur at much lower temperatures.

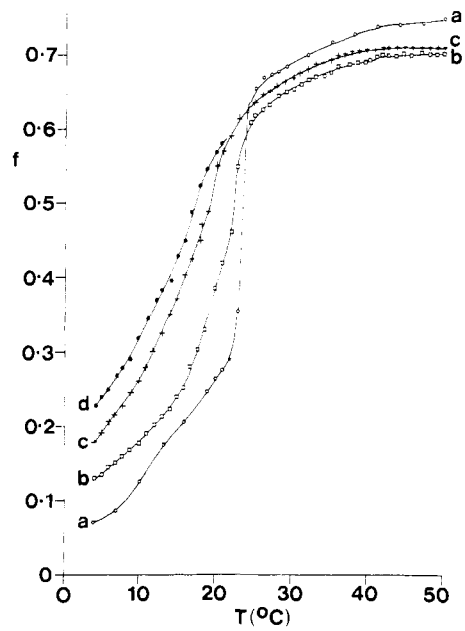


FIGURE 2: The spectral parameter *f vs.* temperature for aqueous dispersions of dimyristoyllecithin and for dimyristoyllecithin-dioleoyllecithin mixtures (all at a total lipid concentration of 100 mg/ml). The data for 80:20 dimyristoyllecithin-dioleoyllecithin is similar to that for 97:3 dimyristoyllecithin-dioleoyllecithin above 25°: (a) dimyristoyllecithin; (b) 97:3 dimyristoyllecithin-dioleoyllecithin; (c) 90:10 dimyristoyllecithin-dioleoyllecithin; (d) 80:20 dimyristoyllecithin-dioleoyllecithin.

The word cluster is used in this paper in the sense that it is used in physical-chemical studies, to mean a short-lived, more densely packed arrangement of molecules within an environment of freely dispersed molecules. The term cluster has, however, also been commonly used in membrane and lipid studies to mean a segregated pool of lipid, generally in cases where a complex lipid mixture has segregated into pools containing predominantly a single lipid class. A lipid cluster, in the sense of a lipid pool, may, or may not, be a cluster in the physical-chemical sense. Further, there is no *a priori* reason why a lipid cluster in the physical-chemical sense should contain only lipids of a single class.

Materials and Methods

Sarcoplasmic reticulum was prepared from rabbit muscle by the method of Robinson *et al.* (1972) and ATPase was purified by the method of Warren *et al.* (1974). The lipid:protein molar ratio in the purified ATPase is *ca.* 25:1. More than 99% of the endogenous lipids associated with the purified protein were replaced by dioleoyllecithin by incubating the ATPase in a mixture of cholate and dioleoyllecithin. After equilibration of the lipid pools, excess lipid and cholate were removed by sedimenting the substituted ATPase through a detergent-free sucrose gradient in the presence of a high centrifugal field (Warren *et al.*, 1974).

ATPase activity of the sarcoplasmic reticulum was estimated using a coupled enzyme assay in a medium containing 100 mM triethanolamine hydrochloride-KOH (pH 7.2), 5 mM $MgSO_4$, 2 mM ATP, 0.1 mM $CaCl_2$, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 3 ml at 37° (Warren *et al.*, 1974).

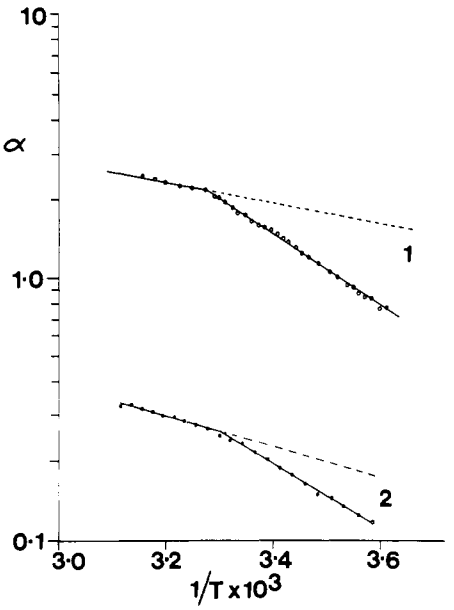


FIGURE 3: The spectral parameter α *vs.* $1/T$ for an aqueous dispersion of dioleoyllecithin: 1.100 and 2.9.6 mg/ml.

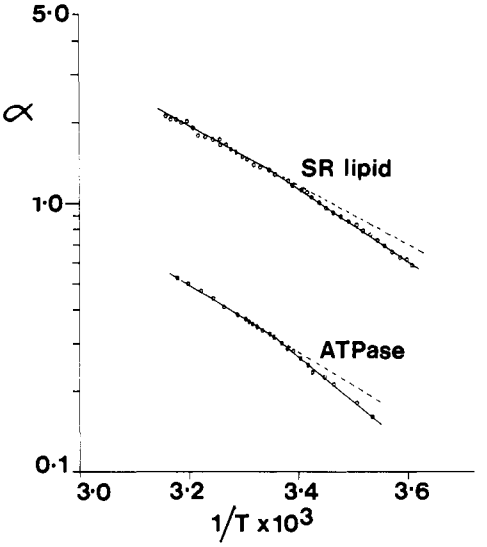


FIGURE 4: The spectral parameter α *vs.* $1/T$ for an aqueous dispersion of lipid (100 mg/ml) and for the purified ATPase-lipid complex (70 mg of protein/ml) from sarcoplasmic reticulum.

Dioleoyllecithin and dimyristoyllecithin were synthesized by the method of Robles and Van den Berg (1969). The lecithin samples gave a single spot on thin-layer chromatography (silica gel G, chloroform–methanol–water (65:35:4) and chloroform–methanol–7 N NH_4OH (69:27:4.5)). Gas–liquid chromatographic analysis of the fatty acid methyl esters derived from dioleoyllecithin showed that the lecithin contained 98.7% 18:1, 0.8% 16:0, and 0.5% 18:0. Lipids were extracted from the sarcoplasmic reticulum by homogenizing in 20 volumes of chloroform–methanol (2:1, v/v). After filtration, the extract was washed in one-fifth volume of 1 mM $MgCl_2$, and the lower phase was evaporated to dryness under N_2 in a rotary evaporator.

Electron spin resonance (esr) spectra were obtained using a Varian E3 spectrometer with a variable temperature controller, at Tempo concentrations of 1×10^{-4} M. Lipid concentrations of between 10 and 100 mg/ml were used, so that at all times the concentrations of Tempo partitioned into the lipid were low.

Results

A typical first derivative electron spin resonance spectrum of Tempo in an aqueous dispersion of dimyristoyllecithin is shown in Figure 1. The spectrum is a superposition of the spectrum of Tempo in an aqueous environment and in a lipid environment. Small changes in the isotropic hyperfine coupling constants and g factors for Tempo result in the partial resolution of the high-field hyperfine line, as shown. The amplitudes B and F of the high-field signals are measured (Figure 1), where B is approximately proportional to the amount of Tempo dissolved in the lipid and F is approximately proportional to the amount in the aqueous phase. The ratio $\alpha = B/F$ is then approximately equal to the ratio of the amounts of Tempo bound to the membrane and free in solution. A parameter f can also be defined as $f = B/(F + B)$ and is approximately equal to the fraction of spin label dissolved in the lipid (Shimshick and McConnell, 1973a). In Figure 2, the Tempo parameter f is plotted against temperature for aqueous dispersions of dimyristoyllecithin and for various mixtures of dioleoyllecithin and dimyristoyllecithin.

For dimyristoyllecithin, as previously reported by Shimshick and McConnell (1973a), there is an abrupt decrease in f at 23°, corresponding to the temperature of the liquid-crystalline to crystalline transition. Below this main transition, there is a second, broader, transition, possibly corresponding to the "pre-transition" observed with differential scanning calorimetry (Hinz and Sturtevant, 1972). For dioleoyllecithin no abrupt transitons are observed in the temperature range above 4°, consistent with the reported liquid-crystalline to crystalline transition temperature of −22° (Phillips *et al.*, 1972). However, there is a marked decrease in Tempo binding as the temperature is lowered from 50 to 4°. Plots of log α against $1/T$ for dioleoyllecithin at concentrations of 100 and 9.6 mg/ml are shown in Figure 3. They are clearly nonlinear and both show a discontinuity at *ca.* 30°. A plot of the ATPase activity of the complex of dioleoyllecithin with the $[Ca^{2+};Mg^{2+}]$ATPase from sarcoplasmic reticulum also shows a discontinuity at 29° (Figure 5).

Plots of log α against $1/T$ for aqueous dispersions of the total lipid extract from sarcoplasmic reticulum and for vesicles of intact sarcoplasmic reticulum are shown in Figure 4. Although the nonlinearity in these plots is less pronounced than in

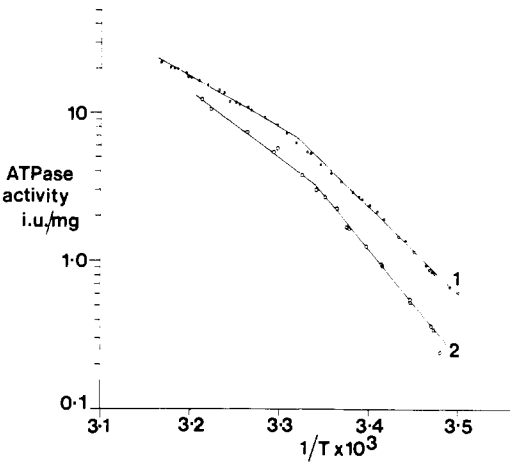


FIGURE 5: Specific activity of the $[Ca^{2+}; Mg^{2+}]$ATPase-lipid complex from sarcoplasmic reticulum *vs.* $1/T$: (1) for dioleoyllecithin-substituted ATPase; (2) for purified ATPase.

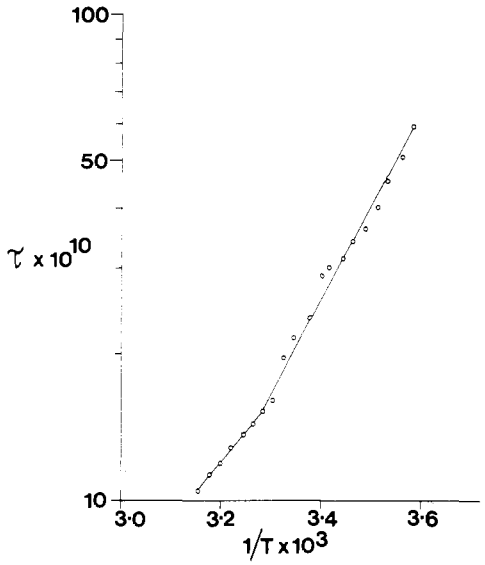


FIGURE 6: Estimated "correlation time" τ (seconds) for methyl 12-nitroxide stearate incorporated into bilayers of dioleoyllecithin *vs.* $1/T$.

the case of dioleoyllecithin, both show discontinuities at *ca.* 25°. A similar discontinuity occurs in plots of the activity of the purified ATPase as a function of temperature (Figure 5).

The data obtained for aqueous dispersions of dioleoyllecithin containing the *N*-oxyl-4,4′-dimethyloxazolidine derivative of methyl 12-ketostearate in a molar ratio of 100:1 are presented in Figure 6. The "correlation time" τ has been calculated from the simplified expression (Sinensky, 1974)

$$\tau = (6.45 \times 10^{-10})[\sqrt{h(0)/h(-1)} + \sqrt{h(0)/h(1)} - 2]\Delta H(0)$$

where $h(0)$, $h(1)$, and $h(-1)$ are the amplitudes of the center, low-field, and high-field lines, respectively, and $\Delta H(0)$ is the width of the center line in gauss. Although this expression is strictly only valid for $\tau < 10^{-9}$ sec, such expressions have previously been used as an empirical parameter to characterize esr

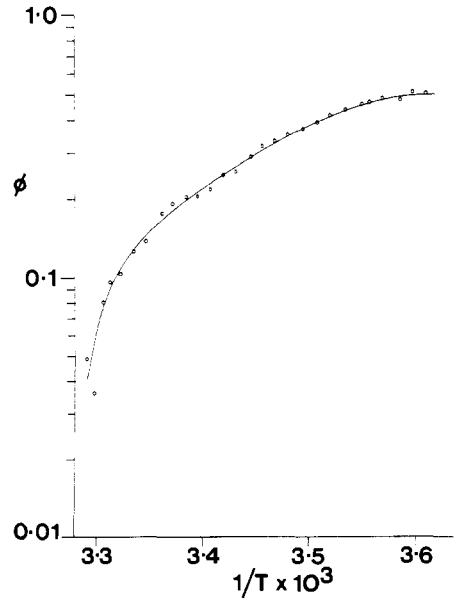


FIGURE 7: The estimated fraction of lipid present as clusters in dioleoyllecithin bilayers *vs.* $1/T$.

spectra (see Eletr *et al.*, 1974). Despite the relatively large errors associated with the measurement of the line-width $\Delta H(0)$, there is a clear discontinuity in the Arrhenius plot at *ca.* 30°, in the direction expected for increased viscosity.

Analysis of the Tempo Data. The parameter α is given approximately by

$$\alpha = n_b/n_f \quad (1)$$

where n_b is the number of moles of Tempo dissolved in the lipid membrane and n_f is the number of moles of Tempo dissolved in the bulk aqueous phase. This equation is not exact because of the neglect of the problems associated with signal overlap, linewidth differences, and the underlying ^{13}C hyperfine satellites (Shimshick and McConnell, 1973a).

The partition coefficient K for Tempo into this system is defined by

$$K = (n_b/n_f)(V_{aq}/V_{lipid}) \quad (2)$$

where V_{aq} and V_{lipid} are, respectively, the volumes of the aqueous and lipid regions. We therefore have

$$\alpha = K(V_{lipid}/V_{aq}) \quad (3)$$

The partition coefficients for many small molecules between water and nonpolar solvents have been shown to follow a simple Arrhenius equation

$$K = K_0 e^{-\Delta G^\circ/RT} \quad (4)$$

where K_0 is a constant and ΔG° is the standard free-energy change between bulk water and bulk nonpolar solvent (see, for example, Johnson and Bangham, 1969; Galey *et al.*, 1973). For Tempo partition into a lipid bilayer therefore

$$\alpha = K_0(V_{lipid}/V_{aq})e^{-\Delta G^\circ/RT} \quad (5)$$

Equation 5 gives an accurate description of the temperature dependence of α for aqueous dispersions of dioleoyllecithin at temperatures above 32° (Figure 3). Below that temperature, however, the measured value of α is smaller than the extrapolated value, α_{extr}, which we attribute to the formation of lipid clusters.

At a given temperature T, we denote the fraction of lipid present as clusters as ϕ. The partition coefficient K_c for Tempo into the clusters is expected to be considerably smaller than K_m, the partition coefficient into the freely dispersed, monomeric lipid. We assume that the partition coefficient for a bilayer containing both freely dispersed lipid and lipid clusters can be written as a linear combination of the partition coefficients K_m and K_c

$$K = (1 - \phi)K_m + \phi K_c \quad (6)$$

This equation ignores any possible interactions between lipid clusters and freely dispersed lipid, and so is most likely to be accurate for relatively small ϕ. The measured value of α is then given by

$$\alpha_{meas} = \frac{[(1 - \phi)K_m + \phi K_c]V_{lipid}}{V_{aq}} \quad (7)$$

At any given temperature, the ratio $\alpha_{meas}/\alpha_{extr}$ at that temperature gives ϕ

$$\alpha_{meas}/\alpha_{extr} = 1 - [1 - (K_c/K_m)]\phi \quad (8)$$

The value of K_c/K_m is unknown, but it is probably small. Putting $K_c/K_m = 0$, we obtain minimum values for ϕ. Values of the cluster fraction ϕ obtained in this way for the dioleoyllec-

ithin bilayer at a concentration of 100 mg/ml are shown in Figure 7 and vary from 0 at 30° to *ca.* 0.5 at 2°. The results obtained for a dioleoyllecithin concentration of 9.6 mg/ml (Figure 3) are analogous to those obtained at the higher lipid concentration. The calculated values of ϕ are somewhat lower, but the data are likely to be less accurate because of the small size of the peak due to bound Tempo for the low lipid concentration.

Discussion

Lipid Clusters. Anomalies in a number of the physical properties of organic liquids near the freezing point have been attributed to cluster formation (Ubbelohde, 1965). These clusters are pictured as short-lived dynamic arrangements of adjacent molecules having coordinated movements. The mean molecular density within the cluster will be higher than for freely dispersed molecules, and internal rotational freedom is probably inhibited for molecules within the cluster. Although the lifetime for the formation and dispersal of the cluster may be short, if the relaxation time for the cluster is appreciably greater than that for some other characteristic movements of the molecule, then in respect of such movement, the cluster will behave as a colloidal particle. Thus, when a transient cluster is formed, the group of molecules involved will move as a whole during its lifetime, and, for example, the viscosity of the liquid will be greater than that of the hypothetical, cluster-free, liquid. Such increases in viscosity have been observed for a number of *n*-alkanes up to *ca.* 50° above the melting point (Ertl and Dullien, 1973). The fraction of clusters present in alkanes just above the melting point was estimated as *ca.* 10%, with the average cluster size being three–four molecules. The presence of clusters has also been invoked to explain anomalies in the heat capacity data for a number of *n*-alkanes (Davies and Matheson, 1967).

The crystalline to liquid–crystalline phase transitions in lipid bilayers have been shown by a variety of techniques to correspond to a "melting" of the lipid hydrocarbon chains. One convenient indicator of this transition is the marked decrease in the partition of the spin-label Tempo into the bilayer as the temperature is lowered through the transition temperature (Hubbell and McConnell, 1971; Shimshick and McConnell, 1973a). In this paper we report a discontinuity in the binding of Tempo to dioleoyllecithin bilayers at temperatures around 50° above the liquid–crystalline to crystalline phase transition. By comparison with the results obtained with simple organic molecules we attribute this to the formation of quasicrystalline clusters within the lipid bilayer. No comparable discontinuities have been observed for the partition of a number of small molecules between decane and water in the temperature range 20–55° (Johnson and Bangham, 1969). Within the cluster, the mean molecular density will be higher and the fluidity lower, so that Tempo binding will be reduced relative to freely dispersed lipid. The proportion of lipid present in clusters is estimated as zero at 30° and about 50% at 2°.

As for the *n*-alkanes, the presence of clusters in bilayers of dioleoyllecithin will cause an increase in viscosity within the bilayer. This increased viscosity is detected by spin-labeled fatty esters incorporated into the bilayer (Figure 6). Unfortunately it is not possible to estimate the cluster fraction for other lipids such as dimyristoyllecithin in this way, because of problems of spin-label reduction at high temperatures. However, it should be noted that the cluster fraction for dioleyllecithin might be unusually high because of the presence of the cis double bonds. Ubbelohde (1965) has stressed the effects of molecular entanglement on cluster formation.

There is also some information already in the literature consistent with the formation of clusters in pure lipid bilayers. Thus the mobility of a fatty ester spin label incorporated into bilayers of 1-palmitoyl-2-oleoyllecithin shows a discontinuous decrease as the temperature is lowered below 16° (Eletr and Keith, 1972). This temperature is considerably above that of the liquid–crystalline to crystalline transition temperature (3° in 1-stearoyl-2-oleoyllecithin; Phillips *et al.*, 1972). Similarly, in the liquid–crystalline system sodium decanoate–1-decanol–water, there is a change in order parameter for incorporated spin-labeled fatty acids at *ca.* 20° (Seelig, 1971).

The interpretation of Tempo binding data for lipid mixtures is more difficult. Figure 2 shows the data obtained for mixtures of dioleoyllecithin and dimyristoyllecithin. For pure dimyristoyllecithin the main crystalline to liquid–crystalline transition appears very clearly at 23°. Little can be said about the mixtures, however, except that even at very low concentrations, dioleoyllecithin has a very considerable "fluidizing" effect on the mixture, with the Tempo solubility being much higher than that expected from the equivalent concentrations of the two single lipids. Shimshick and McConnell (1973a) have shown how it is possible to construct a lipid phase diagram from Tempo partitioning into mixtures of two lipids, when the crystalline to liquid–crystalline phase transitions of the two component lipids can be separately observed. For each mixture of lipids, two breaks in the Tempo solubility curves are located, one corresponding to a point on the fluidus curve and one to a point on the solidus curve of the phase diagram. In the case of mixtures with dioleoyllecithin, the point corresponding to crystallization of dioleoyllecithin (−22°) is inaccessible. It is not then clear whether the breaks that are observed in plots of the Tempo spectral parameter against temperature correspond to a liquid–crystalline to crystalline transition, as is observed in pure dimyristoyllecithin, or to the onset of cluster formation, as is observed in pure dioleoyllecithin. Misleading results might then be obtained if such data were used to construct a phase diagram, using the method of Shimshick and McConnell (1973a). In this context, we note the general similarity between our data for mixtures of dimyristoyllecithin and dioleoyllecithin, and the data of Shimshick and McConnell (1973b) for mixtures of dimyristoyllecithin and cholesterol. Further, the agreement between the experimental data and the calculated phase diagrams for the latter system is poorer than for the other systems studied (Shimshick and McConnell, 1973a,b), so that the possible role of cluster formation in this system should be considered.

Interpretation of the Tempo binding characteristics of the complex lipid mixtures of most membranes is likely to be even more difficult (see below).

Effects of Lipid Clusters in Sarcoplasmic Reticulum. Evidence for the importance of lipid cluster formation on the activity of membrane bound enzymes comes from experiments on the $[Ca^{2+};Mg^{2+}]$ATPase of sarcoplasmic reticulum in which the exogenous sarcoplasmic reticulum lipid is completely replaced by dioleoyllecithin. The ATPase activity of the system shows a discontinuity at 29°, which is the same temperature at which lipid clusters appear in pure dioleoyllecithin bilayers, but is much above the liquid–crystalline to crystalline phase transition at −22° (Figure 5).

Data for intact sarcoplasmic reticulum are more difficult to interpret because of the complexity of the lipids present in the membrane. However, the Tempo binding data for sarcoplasmic reticulum lipid and for purified ATPase both show a discontinuity at *ca.* 25°. Since the lipids of sarcoplasmic reticulum are highly unsaturated with *ca.* 65% of unstaurated fatty acids

(Warren *et al.*, 1974), this discontinuity is unlikely to correspond to a liquid-crystalline to crystalline transition, but, by analogy with the data for dioleoyllecithin, could correspond to the onset of cluster formation. Corresponding to this change in the lipid component, there is a decrease in $[Ca^{2+};Mg^{2+}]$-ATPase activity at *ca.* 26°.

Other experiments on sarcoplasmic reticulum also indicate changes in membrane structure at similar temperatures. Inesi *et al.* (1973) have recently reported breaks in ATPase activity and in initial rate of Ca^{2+} uptake at *ca.* 20°; they attribute these breaks, however, to a lipid crystalline to liquid-crystalline transition. The partition of a spin label related to Tempo but containing a heptacosane ring into sarcoplasmic reticulum vesicles showed a discontinuous increase at 22°, and a fatty acid spin label showed a discontinuous decrease in motional freedom at 22° (Eletr and Inesi, 1972; Inesi *et al.*, 1973). The release of accumulated calcium from sarcoplasmic reticulum induced by the antibiotic X-537A also shows an abrupt decrease at 25°, although this was again interpreted in terms of a liquid-crystalline to crystalline phase transition in the membrane lipids (Scarpa *et al.*, 1972).

Since it is probable that the $[Ca^{2+};Mg^{2+}]$ATPase spans the membrane, it is not surprising that it is sensitive to the physical state of its lipid environment. Further evidence consistent with this is that a large decrease in ATPase activity has been observed on "freezing" of the lipid chains after substitution of endogenous lipid with dimyristoyllecithin or dipalmitoyllecithin (unpublished observations). The sensitivity to lipid environment shown by the decrease in activity on cluster formation in the dioleoyllecithin-substituted ATPase and probably in native sarcoplasmic reticulum is potentially important in attempts to explain the thermal effects observed in other membrane systems.

Thermal Effects in Other Biological Membranes. There have been a very large number of reports of thermal transitions in membranes, indicated by changes in enzyme activity or by changes in the esr spectra of spin labels incorporated into the membrane. Many of these changes occur at around 20°, and could be due to cluster formation in the membrane rather than to a liquid-crystalline to crystalline phase transition. Any definitive assignment is probably not possible in such complex systems, where, for example, segregation of the lipids into pools of differing lipid content is possible. However, there are a number of cases where an interpretation in terms of lipid clusters seems at least as likely as the more usual explanation in terms of an order-disorder transition.

A fairly broad lipid liquid-crystalline to crystalline transition has been observed in rat liver mitochondria by differential scanning calorimetry, centered at 0° and occurring between *ca.* −10 and 10° (Balzky and Steim, 1972). Spin-label studies, however, detect a change at *ca.* 25°, at which temperature there is also a discontinuity in an Arrhenius plot of the damped volume oscillations of rat mitochondria (Williams *et al.*, 1972; Tinberg *et al.*, 1972). Differential scanning calorimetric studies of rat liver microsomes also show a liquid-crystalline to crystalline transition occurring between −15 and 5°, with a peak at 0° (Balzky and Steim, 1972). Spin-label studies in guinea pig liver microsomes detect a transition at *ca.* 19°, which correlates with changes in the activities of microsomal glucose-6-phosphatase and UDP-glucuronyltransferase also at *ca.* 19° (Eletr *et al.*, 1973). Spin-label studies of lamb kidney microsomes and Arrhenius plots of $[Na^+;K^+]$ATPase activity both show discontinuities at *ca.* 20° (Grisham and Barnett, 1973). Both rabbit kidney $[Na^+;K^+]$ATPase (Charnock *et al.*, 1971, 1973) and rat brain $[Na^+;K^+]$ATPase (Gruener and Avi-Dor, 1966) also show discontinuities at 20°.

Microorganisms such as *Escherichia coli, Acholeplasma,* and yeast have attracted much attention because of the possibilities for manipulating the fatty acid composition of the membrane lipids. Membranes and lipids from *Acholeplasma laidlawii* grown on oleic acid have an order-disorder phase transition at *ca.* −20° as detected by calorimetry (Steim *et al.*, 1969). The high-temperature end of this transition for the membrane is detected at −15° by X-ray diffraction (Engelman, 1971). Fatty acid spin labels incorporated into this membrane, however, show rather complex changes at *ca.* 20° (Rottem *et al.*, 1970). Spin-label studies of yeast show a transition at 16° when grown on oleic acid (78% incorporation) and at 10° when grown on linolenic acid (73% incorporation) (James *et al.*, 1972; Eletr and Keith, 1972). Again, the possibility that the observed transitions are due to cluster formation should be considered.

Studies on essential fatty acid auxotrophs of *E. coli* illustrate some of the further complications that can appear. Thus Overath and Traüble (1973) have reported studies in which fatty acid auxotrophs of *E. coli* were grown on elaidic acid. Both the membranes and the extracted phospholipids showed fairly sharp transitions in dilatometric, 90° light scattering and fluorescence experiments, centered at between 37 and 42°, depending on the experimental conditions, the width of the transition being *ca.* 5°. Linden *et al.* (1973), however, interpret their Tempo binding studies for the same system not in terms of a single transition, but in terms of two sharp transitions at 37.7 and 30.7°. The correct interpretation is not clear. Although the lipid phase transition is very sharp in unsonicated aqueous lipid dispersions, in small sonicated lipid vesicles, where the cooperativity of the transition is less, the phase transition is broader and spreads over a range of some 10° (Lee *et al.*, 1974a). If the cooperativity of the lipid phase transition were small in complex lipid mixtures or biological membranes, then a broad transition would result. It would not then be clear if discontinuities observed in some physical parameter as a function of temperature were due to the two "ends" of a single transition or to two separate transitions.

The data on *E. coli* grown on oleic acid are even less clear. Overath and Traüble (1973) interpret their data on both membranes and extracted lipid in terms of a single broad transition centered at between 11 and 17°, depending on the conditions used, with a width of *ca.* 12°. Linden *et al.* (1973) interpret their Tempo partition data in terms of two transitions, at 31.1 and 15.8° for the membrane, and at 27.3 and 8.9° for the extracted lipid. Overath and Traüble (1973) report a single break in sugar transport at 15–16°, whereas Linden *et al.* (1973) report three, at 26.0, 21.8, and 14.4°. Many of these temperatures are relatively high for a membrane whose fatty acids are *ca.* 50% oleic acid, and again the possibility that some are due to lipid cluster formation should be noted. We also note that Steim (1972) found that breaks in Arrhenius plots of the rate of galactoside transport in *E. coli* did not correlate with any unique portion of the lipid liquid-crystalline to crystalline transition for the membrane, as determined calorimetrically.

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References

Balzky, J. F., and Steim, J. M. (1972), *Biochim. Biophys. Acta 266,* 737.

Chapman, D., Williams, R. M., and Ladbrooke, B. D. (1967), *Chem. Phys. Lipid 1,* 445.

Charnock, J. S., Cook, D. A., Almeida, A. F., and To, R. (1973), *Arch. Biochem. Biophys. 159,* 393.
Charnock, J. S., Cook, D. A., and Casey, R. (1971), *Arch. Biochem. Biophys. 147,* 323.
Davies, D. B., and Matheson, A. J. (1967), *Discuss. Faraday Soc. 43,* 216.
Devaux, P., and McConnell, H. M. (1972), *J. Amer. Chem. Soc. 94,* 4475.
Eletr, S., and Inesi, G. (1972), *Biochim. Biophys. Acta 290,* 178.
Eletr, S., and Keith, A. D. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1353.
Eletr, S., Williams, M. A., Watkins, T., and Keith, A. D. (1974), *Biochim. Biophys. Acta 339,* 190.
Eletr, S., Zakim, D., and Vessey, D. A. (1973), *J. Mol. Biol. 78,* 351.
Ertl, H., and Dullien, F. A. L. (1973), *Proc. Roy. Soc., Ser. A 335,* 235.
Engelman, D. M. (1971), *J. Mol. Biol. 58,* 153.
Galey, W. R., Owen, J. D., and Solomon, A. K. (1973), *J. Gen. Physiol 61,* 727.
Grisham, C. M., and Barnett, R. E. (1973), *Biochemistry 12,* 2635.
Gruener, N., and Avi-Dor, Y. (1966), *Biochem. J. 100,* 762.
Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.
Hubbell, W. L., and McConnell, H. M. (1971), *J. Amer. Chem. Soc. 93,* 314.
Inesi, G., Millman, M., and Eletr, S. (1973), *J. Mol. Biol. 81,* 483.
James, R., Branton, D., Wisnieski, B., and Keith, A. (1972), *J. Supramol. Struct. 1,* 38.
Johnson, S. M., and Bangham, A. D. (1969), *Biochim. Biophys. Acta 193,* 92.
Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1973), *Biochemistry 12,* 1650.
Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1974a), *Methods Membrane Biol. 2* (in press).
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Roberts, G. C. K., and Warren, G. B. (1974b), manuscript in preparation.
Levine, Y. K. (1972), *Progr. Biophys. Mol. Biol. 24,* 1.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), *Biochemistry 11,* 1416.
Linden, C. D., Wright, K. L., McConnell, H. M., and Fox, C. F. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2271.
McConnell, H. M., Wright, K. L., and McFarland, B. G. (1972), *Biochem. Biophys. Res. Commun. 47,* 273.
Metcalfe, J. C., Birdsall, N. J. M., and Lee, A. G. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21,* 335.
Overath, P., and Traüble, H. (1973), *Biochemistry 12,* 2625.
Phillips, M. C., Hauser, H., and Paltauf, F. (1972), *Chem. Phys. Lipid. 8,* 127.
Robinson, J. D., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), *Biochemistry 11,* 2903.
Robles, E. C., and Van der Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.
Rottem, S., Hubbell, N. J., Hayflick, L., and McConnell, H. M. (1970), *Biochim. Biophys. Acta 219,* 104.
Scarpa, A., Baldassare, J., and Inesi, G. (1972), *J. Gen. Physiol. 60,* 735.
Seelig, J. (1971), *J. Amer. Chem. Soc. 93,* 5017.
Shimshick, E. J., and McConnell, H. M. (1973a), *Biochemistry 12,* 2351.
Shimshick, E. J., and McConnell, H. M. (1973b), *Biochem. Biophys. Res. Commun. 53,* 446.
Sinensky, M. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 522.
Steim, J. M. (1972), *Proc. FEBS (Fed. Eur. Biochem. Soc.) Meeting, 8th 28,* 185.
Steim, J. M., Tourtellotte, M. E., Reinert, J. C., McElhaney, R. N., and Rader, R. L. (1969), *Proc. Nat. Acad. Sci. U. S. 63,* 104.
Tinberg, H. M., Packer, L., and Keith, A. D. (1972), *Biochim. Biophys. Acta 283,* 193.
Traüble, H., and Sackmann, E. (1972), *J. Amer. Chem. Soc. 94,* 4499.
Ubbelohde, A. R. (1965), Melting and Crystal Structure, Oxford, Clarendon Press.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 622.
Williams, M. A., Stancliff, R. C., Packer, L., and Keith, A. D. (1972), *Biochim. Biophys. Acta 267,* 444.