CALORIMETRIC EVIDENCE FOR THE LIQUID-CRYSTALLINE STATE OF LIPIDS IN A BIOMEMBRANE*

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Abstract.—Both membranes of Mycoplasma laidlawii and water dispersions of protein-free membrane lipids exhibit thermal phase transitions that can be detected by differential scanning calorimetry. The transition temperatures are lowered by increased unsaturation in the fatty acid residues, but in each case they are the same for membranes and lipids. The transitions resemble those observed for synthetic lipids in the lamellar phase in water, which arise from melting of the hydrocarbon chains within the phospholipid bilayers. Such melts are cooperative phenomena and would be greatly perturbed by apolar binding to protein. Thus the identity of membrane and lipid transition temperatures suggests that in the membranes, as in water, the lipids are in the bilayer conformation in which the hydrocarbon chains associate with each other rather than with proteins. Observations of morphological changes indicate that osmotic imbalance occurs when the membrane transition temperature exceeds the growth temperature, and that for transport processes to function properly the hydrocarbon chains must be in a liquid-like state.

After many years of research, knowledge of the molecular organization of biological membranes remains meager. Although the concept of a phospholipid bilayer bounded on each side by protein is accepted by some investigators, others question the basic assumptions of the bilayer model and suggest alternative models in which the association between lipid and protein is hydrophobic rather than polar.[1] If in fact lipids exist in membranes as bilayers, some unique property of a bilayer array might be detectable in membranes by a direct physical technique. Such a property is the reversible thermotropic gel-liquid crystal phase transition observed in phospholipid myelin forms in water. It has been studied by differential scanning calorimetry, differential thermal analysis, nuclear magnetic resonance spectroscopy, X-ray diffraction, and light microscopy; it arises from the melting of the hydrocarbon interiors of lipid bilayers.[2-4] Unlike transitions between liquid-crystalline phospholipid mesophases,[5] the melt does not result in a molecular rearrangement and the lipids exist in the lamellar conformation both above and below the transition temperature. As in the case of bulk hydrocarbons, the melting point varies with unsaturation and chain lengths of the fatty acids in the phospholipids. Because cholesterol interferes,[4] to detect a phase change above the ice point in a membrane an organism containing rather saturated fatty acids but little or no cholesterol must be chosen.

The membranes of Mycoplasma laidlawii satisfy these requirements. Previous studies of this organism have shown that the cell membrane contains no choles-

terol and that the fatty acid composition of the membrane polar lipids can be varied over a wide range of saturation by supplying the appropriate fatty acids in the growth medium.[6, 7] When the membranes contain large amounts of long-chain saturated fatty acids, morphological effects that suggest an *in vivo* transition are observed.[6–8] Cells grown in unsaturated, branched-chain, and cyclopropane fatty acids are filamentous, while those grown with long-chain saturated acids become coccoid and eventually swell and lyse. Swelling is osmotic, since it can be reversed in 0.3 *M* sucrose.

In this communication we report the direct calorimetric observation of a phase transition that occurs at the same temperature in *M. laidlawii* membranes, whole cells, and membrane lipids dispersed in water.

Materials and Methods.—*M. laidlawii* (strain B, PG9) were grown at 37° for 24 hr in tryptose medium, unsupplemented or containing added fatty acids, and were lysed in deionized water.[7] The membranes were sedimented and washed in deionized water or 0.25 *M* NaCl. Such preparations show the typical "unit membrane" appearance in the electron microscope.[9]

Samples of 90–100 mg of sedimented membranes or cells (10–15% dry weight) were sealed in sample pans and scanned in a Perkin-Elmer DSC-1B differential scanning calorimeter at 1 mcal/sec full-scale sensitivity and a heating rate of 5° per minute. Membranes prepared from cells grown in unsupplemented tryptose and in tryptose supplemented with stearate were examined in water. For both membranes and whole cells supplemented with oleate, the solvent was 50% ethylene glycol containing 0.15 *M* NaCl. The pellets of sedimented membranes contained free water, since representative portions of each sample showed a freezing endotherm. Membrane samples used for transition studies were not frozen prior to calorimetry.

Lipids were extracted from membranes with chloroform-methanol 2:1 v/v and repeatedly dried in a rotary evaporator to denature proteolipid protein, which was removed by filtration. No protein was detectable by the Folin procedure.[10] Samples were dried over P_2O_5 in a vacuum, sealed in sample pans with the same solvents used for the membranes from which they were extracted, and allowed to equilibrate several hours at room temperature before being examined in the calorimeter. Lipid concentrations were 10–25% by weight.

The Perkin-Elmer differential scanning calorimeter records the differential power input necessary to heat both the sample and a thermally inert reference (i.e., showing no transition) at the same rate. If no transition occurs in the sample, a straight base line is obtained; if an endothermic transition occurs, more heat must be applied to the sample than to the reference, and a peak is recorded with area proportional to the heat of transition. Both the temperature scale and peak areas can be calibrated with a compound of known melting point and heat of transition, such as stearic acid or water. Since commercially available sample pans are too small (15 μl) for biological materials, we used specially constructed, sealed, stainless steel sample pans of 100-μl capacity. Stability and signal:noise ratio were improved by mounting the calorimeter cell in a brass cylinder immersed in a dry ice-ethanol bath. With this arrangement repetitive scans of reversible transitions are superimposable.

Results.—Figure 1 shows representative endothermic transitions of lipids, membranes, and whole cells of *M. laidlawii*. For the lipids, heats of transition are 2–3 calories per gram. Lipids from cells grown in tryptose supplemented with stearate are shown in Figure 1, curve *a*, while curve *b* is a scan of intact membranes from the same cells. Curves *c* and *d* are from lipids and membranes of cells grown in unsupplemented tryptose. Transitions of lipids and membranes rich in oleate are shown in *e* and *f*. The transition for whole cells, shown in *g*,
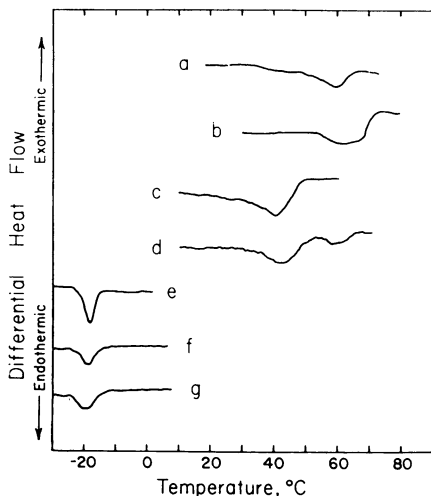
FIG. 1.—Calorimeter scans of *M. laidlawii* lipids, membranes, and whole cells. (*a*) Total membrane lipids from cells grown in tryptose with added stearate; (*b*) membranes from stearate-supplemented tryptose; (*c*) total membrane lipids from cells grown in unsupplemented tryptose; (*d*) membranes from unsupplemented tryptose; (*e*) total membrane lipids from cells grown in tryptose with added oleate; (*f*) membranes from oleate-supplemented tryptose; (*g*) whole cells from oleate-supplemented tryptose. The first four preparations were suspended in water; for the latter three scans, the solvent was 50% ethylene glycol containing 0.15 M NaCl.

indicates that the effects observed are not artifacts introduced during the preparation of membranes. For both membranes and lipids with transitions above the ice point, the transitions are the same in distilled water, 0.25 M NaCl, and 0.05 M MgSO$_4$. In 50 per cent ethylene glycol transition temperatures are depressed 3–5°, but the heats of transition are unaffected within the limits of experimental error.

Lipid transitions in membranes, as in protein-free membrane lipids, are reversible. Transition temperatures and peak shapes are independent of scan rate. In Figure 1, curve *d*, the smaller transition at higher temperature probably arises from protein denaturation[11] because it is irreversible, occurs at approximately the same temperature for all membranes regardless of lipid composition, and is greatly decreased by treatment of the membranes by pronase. In Figure 1, curve *b*, the protein transition is obscured by the lipid transition. In such cases, where protein and lipid transitions overlap, the peak area on the initial scan is always greater than on subsequent scans. This effect would be expected from irreversible protein denaturation.

If the lipid content of the membranes is known, the heats of transition of lipids in membranes and in water dispersions can be compared by measuring peak areas for both systems. We have carried out such comparisons on oleate-rich membranes and lipids, using both lipid phosphorus determinations[12] and quantitative extraction and recovery of lipids. The enthalpy of transition of the lipids in the membranes was approximately 75 per cent of that observed for the isolated lipids. Thus the membrane transitions are not minor effects.

Discussion.—Transitions of *M. laidlawii* lipids in water show the same dependence upon fatty acid composition exhibited by synthetic lipids in the lamellar phase.[4] When negatively stained with phosphotungstic acid,[13] preparations of *M. laidlawii* lipids with transitions both above room temperature (stearate-enriched) and below room temperature (oleate-enriched) appear lamellar in the electron microscope. Similar smectic bilayer arrays are well established for other phospholipids in water at the concentrations used in these ex-

periments.[14]   When first placed in water at room temperature in a hot-stage microscope, lipids from stearate-enriched membranes appear as amorphous aggregates.   When the mixture is heated above the transition temperature, typical myelin forms are observed.   When the temperature is then lowered below the transition, the gross morphology of the myelin forms does not change, but the forms become rigid.   However, polarized light microscopy reveals an increase in birefringence at low temperature.   Lipids enriched in oleate produce myelin forms without heating above room temperature.   All these microscopic properties of the thermotropic gel-liquid crystal-phase transition are well established for synthetic phospholipids in water.   Thus the transitions observed with *M. laidlawii* lipids, like those of synthetic phospholipids, appear to arise from melting of the fatty acid chains within the interior of the bilayers.

The membrane transitions occur at the same temperatures as the lipid transitions.   They are reversible and remain after thermal protein denaturation or treatment with pronase.   There is little doubt that these reversible membrane transitions arise from a change in state of the lipids in the membranes, which is very similar to that observed with the protein-free membrane lipids dispersed in water.   Such melting of hydrocarbon chains is a cooperative phenomenon and should be greatly affected by any factors affecting the hydrocarbon chains.   For example, oleate-rich lipids from *M. laidlawii* melt 80° below the stearate-rich system, and synthetic dioleoyl lecithin melts 50° below dipalmitoyl lecithin.   Substitution of isopalmitic acid, with two terminal methyl groups, for palmitic acid lowers the transition temperature of *M. laidlawii* lipids by 20°.[8]   The addition of cholesterol to synthetic phospholipids decreases both the temperature and enthalpy of transition, until at $1/3$ mole per cent cholesterol the transition vanishes.   In membranes apolar binding of lipids to proteins via fatty acid hydrocarbon chains should produce similar effects: the transition would vanish or be profoundly perturbed.   It is reasonable to conclude that the majority of the lipids in *M. laidlawii* membranes are in a liquid-crystalline state in which the hydrocarbon chains associate with each other rather than with proteins.   It is possible that in the membranes the lipids could exist in one of several liquid-crystalline phases, but identical lipid and membrane transition temperatures suggest a bilayer as the most probable configuration.   Phases other than the lamellar have been demonstrated in phospholipids at very low water content (5%) and temperatures well above the physiological range.[5]   We suggest, therefore, that the transitions observed in membranes result from a melt of hydrocarbon chains within bilayers (Fig. 2).

If one assumes that in the membranes those lipids undergoing the phase change have the same enthalpy of transition as the free lipids in water, then comparison of areas of peaks for lipids and membranes indicates that approximately 25 per cent of the lipids in the membranes do not participate in the transition. These lipids could be hydrophobically bound to protein.

Inspection of morphological changes of the cells is informative.   Cells such as those grown in oleate (Fig. 1, curves *e*, *f*, and *g*), with melting points below growth temperature, are filamentous.   Cells grown within the region of transition, such as those from normal tryptose medium (Fig. 1, curves *c* and *d*), become
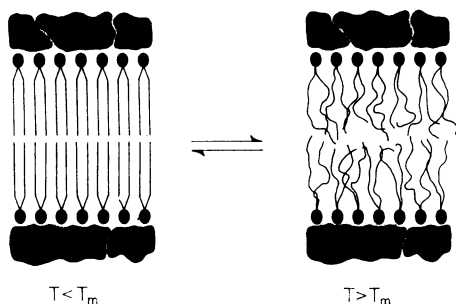
FIG. 2.—Schematic representation of the reversible thermal transition postulated for *M. laidlawii* membranes, showing the crystalline state of hydrocarbon chains below the transition temperature T_m, and the liquid-like state above the transition. Protein is shown covering the outside surfaces of the membrane. The polar ends of the lipids are shown as black dots.

$T < T_m$ $T > T_m$

coccoid, and cells with transitions above the growth temperature (Fig. 1, curves *a* and *b*) are swollen and eventually lyse. Swelling is reversed by 0.3 *M* sucrose. Cell division ceases at the coccoid stage. These results suggest that osmotic imbalance occurs when the membrane transition temperature exceeds the growth temperature, and that for the transport processes to function properly the hydrocarbon chains must be in a liquid-like state.

Finally, it should be noted that the gross physical properties of *M. laidlawii* membranes are similar to those reported for membranes from other organisms. The electron microscope reveals a unit membrane,[9] the optical rotatory dispersion spectrum indicates α-helicity and is bathochromically shifted,[15] no beta structure can be detected by infrared spectroscopy,[16] and reversible dissociation by detergents has been demonstrated.[9, 17]

Summary.—Scanning calorimetry of *M. laidlawii* membranes and dispersions of protein-free membrane lipids in water reveal thermal phase transitions that occur at the same temperature in both systems. Protein denaturation occurs independently of the lipid transitions. The effects are the same as those reported for synthetic phospholipids in the lamellar phase in water, which are known to arise from melting of the hydrocarbon chains within the phospholipid bilayers. Such transitions would be greatly perturbed by apolar binding to protein. Although the heats of transition of membranes suggest that some hydrophobic lipid-protein association may exist, the majority of the lipids appear to be in a liquid-crystalline state in which the hydrocarbon chains associate with each other rather than with proteins. In the membranes lipids could exist in one of several liquid-crystalline phases, but identical lipid and membrane transition temperatures suggest that in the membranes, as in water, the lipids are in the bilayer conformation.

[1] Korn, E. D., *Science*, **153**, 1491 (1966).
[2] Chapman, D., R. M. Williams, and B. D. Ladbrooke, *Chem. Phys. Lipids*, **1**, 445 (1967).
[3] Chapman, D., P. Byrne, and G. G. Shipley, *Proc. Roy. Soc. (London), Ser. A*, **290**, 115 (1966).
[4] Ladbrooke, B. D., R. M. Williams, and D. Chapman, *Biochim. Biophys. Acta*, **150**, 333 (1968).
[5] Reiss-Husson, F., and V. Luzzati, *Advan. Biol. Med. Phys.*, **11**, 87 (1967).
[6] Razin, S., B. J. Cosenza, and M. E. Tourtellotte, *J. Gen. Microbiol.*, **42**, 139 (1966).

[7] Razin, S., M. E. Tourtellotte, R. N. McElhaney, and J. D. Pollack, *J. Bacteriol.*, **91**, 609 (1966).

[8] Tourtellotte, M. E., R. N. McElhaney, and R. L. Rader, manuscript in preparation.

[9] Engelman, D. M., T. M. Terry, and H. J. Morowitz, *Biochim. Biophys. Acta*, **135**, 381 (1967).

[10] Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[11] Steim, J. M., *Arch. Biochem. Biophys.*, **112**, 599 (1965).

[12] Chen, P. S., T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

[13] Bangham, A. D., and R. W. Horne, *J. Mol. Biol.*, **8**, 660 (1964).

[14] Finean, J. B., *Biological Ultrastructure* (New York: Academic Press, 1967), 2nd ed., p. 267.

[15] Lenard, J., and S. J. Singer, these PROCEEDINGS, **56**, 1828 (1966).

[16] Steim, J. M., unpublished data.

[17] Terry, T. M., D. M. Engelman, and H. J. Morowitz, *Biochim. Biophys. Acta*, **135**, 391 (1967).