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The Influence of Lipid State on the Planar Distribution of Membrane Proteins in *Acholeplasma laidlawii*

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The distribution of surface proteins within the plane of the cell membrane was investigated using a biotin–avidin–ferritin reagent. This reagent reacts specifically, in the case of *Acholeplasma laidlawii*, with proteins, and permits their visualization in the electron microscope. *A. laidlawii* membranes were prepared which had different lipid phase transition-temperature ranges as determined by X-ray diffraction. These membranes were labeled at temperatures above, below and in the middle of their transitions, where the states of the lipids were, respectively, smectic, paracrystalline or a mixture of the two phases. In membranes labeled in either the smectic or paracrystalline phase (above or below the transition), the labeled sites were relatively dispersed, whereas in membranes labeled at temperatures in the mixed phase (mid-transition), patches of high and low density of label were found. In all cases, the distribution of the proteins was dependent upon the temperature relative to the lipid transition rather than the absolute temperature. In addition, the patching phenomenon was found to be freely reversible in experiments in which the temperature was shifted after labeling. To explain these observations, a model is proposed which suggests a phase separation of lipid and the preferential association of protein with either the smectic or paracrystalline state. In order that such a change in protein distribution can occur, protein molecules must be mobile through both the paracrystalline and smectic lipid phases over the time-scale of the experiments. These results suggest that temperature-induced changes in the physical state of membrane lipids can cause changes in the relative location of protein components parallel to the membrane surface.

1. Introduction

The topological and structural paradigms for the molecular profiles of biomembranes have been explored by a number of methods, but relatively little information exists which contributes to an understanding of membrane planar organization. In this study we examine the influence of the physical state of the lipid components on the planar distribution of membrane proteins by labeling the proteins with a specific ferritin reagent and visualizing the distributions in the electron microscope.

X-ray diffraction (Engelman, 1970), differential scanning calorimetry (Steim *et al.*, 1969), and electron spin resonance (Metcalfe *et al.*, 1972) studies of *Acholeplasma laidlawii* membranes have shown that, as the temperature is decreased, membrane

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lipids undergo a thermal phase transition from a disordered smectic state to a paracrystalline state at temperatures dependent on the nature of the hydrocarbon chains of the lipids. Since lipids undergo such a temperature-induced change, the associated phase separation may affect the distribution of proteins in the membrane.

The topological organization of *Acholeplasma* membrane proteins perpendicular to the membrane surface has been examined with various proteolytic enzymes and chemical labels (Amar *et al.*, 1974) and their general localization using X-ray diffraction (Wise, 1974). In order to investigate the organization of proteins in the plane of the membrane, the biotin–avidin–ferritin procedure for electron microscopy (Heitzmann & Richards, 1974) was used in this work. The membrane-permeable reagent biotinyl-*N*-hydroxysuccinimide reacts specifically with primary amino groups located in the membrane. The membrane is subsequently treated with ferritin covalently coupled to the protein avidin which has a high affinity for biotin. Ferritin is a protein of about 120 Å diameter which contains an electron-dense iron core (diameter ~65 Å) that is visible in unstained preparations in the electron microscope as a discrete dark spot.

A. laidlawii was selected for this study because it possesses a single membrane, the lipids of which undergo a phase transition which may be monitored by a number of physical techniques. The organism has no pathways for the synthesis of cholesterol and can be grown in its absence, and the lipid fatty acid composition can be altered by supplementation of the growth medium to form a membrane of fairly homogeneous fatty acid composition (in most cases 70 to 90% of a single species depending on the particular fatty acid supplement used). As a result, the phase transition range may be relatively narrow and the temperature at which it occurs is controllable. Sodium dodecyl sulfate/gel electrophoresis has been used to show that both the molecular weight distribution of the proteins and the protein to total lipid ratio of these membranes remains constant with variation in lipid composition (Pisetsky & Terry, 1972). The membrane lipids contain no primary amino groups (Shaw *et al.*, 1968) and the membrane carbohydrates contain few, if any (Gilliam & Morowitz, 1972). Thus, the labeled sites are almost exclusively confined to membrane proteins, which is the basis of our interpretation.

The only previous evidence for the distribution of protein within the plane of the *Acholeplasma* membrane is from freeze-fracture electron microscopy (Verkleij *et al.*, 1972; James & Branton, 1973). In one paper, James & Branton proposed, primarily on the basis of studies with myristate-supplemented *Acholeplasma*, that there exists a relatively random distribution of intramembrane particles above the phase transition temperature, whereas these particles are aggregated or patched below the transition as a result of "squeezing out" of proteins by lipids as they undergo phase separation. Our results with specific labeling of surface proteins seem to differ from their interpretation, although the two sets of results are not necessarily mutually exclusive since our observations are of surface sites rather than inner membrane particles.

In this paper we report studies which reveal the distribution of surface protein sites at temperatures above, below and in the middle of the range of the lipid phase transition. The states of the membrane lipids are, respectively, smectic, paracrystalline, and a mixture of these two phases. The results show that the sites are dispersed when the lipids are in either the smectic or paracrystalline phase (above or below the phase transition) but are patched into regions of dense sites and sparse sites in the instance where two phases exist within a single membrane (mid-transition).

2. Materials and Methods

(a) *Organism and growth conditions*

A. laidlawii B was grown statically at 37°C in a medium consisting of 20 g Fisher tryptose, 4 g Tris, 5 g NaCl, 10 g glucose, 0·2 ml (50,000 units) of penicillin, and 4 g of sterile fatty-acid-poor bovine serum albumen (Goodman, 1957) in 1 l of deionized water at pH 8·4. Each liter of medium was supplemented with 120 μmol of palmitic, erucic, myristic or elaidic acid in ethanolic solution (McElhaney & Tourtellotte, 1969). Cells were harvested in mid-log phase, as evaluated by absorbance at 550 nm.

(b) *Isolation and characterization of membranes*

Membranes were prepared by osmotic lysis in 1:20 β buffer (Razin *et al.*, 1965), consisting of 7·8 mM-NaCl, 2·5 mM-Tris, 0·5 mM-β-mercaptoethanol in deionized water, pH 7·4. They were pelleted at 48,300 *g*, and subsequently washed 3 times in this buffer at 4°C. The low ionic strength of this buffer results in the loss of extrinsic membrane proteins as defined by Singer & Nicolson (1972). The final pellet was resuspended in 0·1 M-$NaHCO_3$ and washed twice with the bicarbonate.

Protein was assayed according to the Folin phenol method of Lowry *et al.* (1951) using bovine serum albumen as a standard.

The membrane lipid was extracted from the isolated membrane pellet with $CHCl_3$:MeOH (2:1) under N_2 to prevent oxidation. Methyl esters of the fatty acids were prepared according to the BF_3/MeOH method of Morrison & Smith (1964). The methyl esters were extracted from the reaction mixture with spectrograde hexane and analyzed by gas/liquid chromatography using a Perkin Elmer model 990 instrument.

(c) *X-ray determination of phase transition temperatures*

Membranes were pelleted in deionized water and sealed in 1-mm glass capillaries. X-ray analysis was preformed on an Elliot toroid point focusing camera (Elliot, 1965) fitted with a thermally-controlled sample holder regulated by external water circulation. Temperature control was to ± 1 deg. C. X-ray patterns were recorded on Ilford industrial G X-ray film. Exposures of about 4 h with CuK_α radiation were used. The range of the transition temperature was determined by the first appearance of the broad 4·6 Å diffraction ring of the disordered phase and the disappearance of the sharp 4·15 Å reflection associated with the paracrystalline phase (Engelman, 1970).

(d) *Differential scanning calorimetry*

Palmitic membranes at a concentration of 5·4 mg lipid/ml in deionized water were heated at a rate of 0·3 deg/s from 5°C to 55°C in an adiabatic differential scanning calorimeter. The sample was cooled and reheated to examine the behavior of the lipids minus protein denaturation. The instrumentation and procedures in obtaining excess specific heat (cal/deg g lipid) were identical to those described by Melchior *et al.* (1970).

(e) *Synthesis of reagents*

Biotinyl-*N*-hydroxysuccinimide was synthesized according to the method of Becker *et al.* (1971).

Avidin-ferritin was synthesized as described by Heitzmann and Richards with the following modifications: the recrystallized ferritin was freed from Cd^{2+} by exhaustive dialysis in the cold *versus* 0·9% NaCl, followed by 0·1 M-Na phosphate, pH 7·0. The Av-F† preparation was filtered through a Millipore filter (0·45-μm pore diameter) and concentrated to about 30 mg ferritin/ml.

(f) *Labeling of membranes*

One ml of isolated membranes (1 mg protein/ml in 0·1 M-$NaHCO_3$) was reacted with 0·1 ml biotinyl-*N*-hydroxysuccinimide (20 mg/ml in dimethyl formamide) for 1 h at 20°C (Heitzmann & Richards, 1974). Membranes were washed twice with 0·1 M-$NaHCO_3$.

† Abbreviation used: Av-F, avidin covalently coupled to ferritin.

(g) *Preparation of grids*

Membranes were labeled with Av-F on 200-mesh Formvar-coated copper grids using a temperature-controlled stainless steel holder in which 100% humidity was maintained during preparation (Fig. 1). Temperature control was maintained by immersing the holder in a water-bath which controlled temperature to ± 1 deg. C as measured with a thermistor probe embedded in the holder. To the grid was applied 3 μl of biotinyl-*N*-hydroxysuccinimide-labeled membranes (~5 mg protein/ml) which was left on the grid for 5 min. The grid was flushed in sequence with 0·1 M-$NaHCO_3$ for 2 min, wet N_2 gas, 3 μl of Av-F solution (30 mg/ml), for 5 min, and finally deionized water for 10 min. The membranes were fixed by drying in dry N_2 gas. It is assumed that fixation occurs upon drying as it was possible to observe shifts of ferritin patterns by shifting the temperature at any point in the process prior to this stage (see Results), but not at any point subsequent to drying.

On occasion a 0·5% solution of glucose in deionized water was added for 5 min prior to drying, as described by Unwin & Henderson (1975).

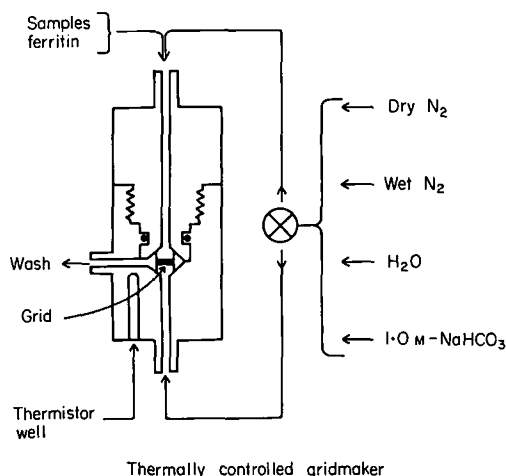


FIG. 1. Design of a temperature- and humidity-controlled gridmaker. The grid is held in a small central chamber in a large stainless steel block. The block is immersed in a water-bath to regulate the temperature at which the labeling sequence is carried out (see text).

For positive staining, membranes were labeled in the usual manner with Av-F. After washing with deionized water, 3 μl of 1% uranyl acetate in water were added for 30 s prior to drying.

Membranes undergoing a temperature-shift after labeling were labeled with Av-F in the usual manner, washed with deionized water, and flushed with wet N_2. A 3-μl drop of deionized water was then added to the grid, the grid holder was placed in a bath at the new temperature and allowed to equilibrate, and flushed with nitrogen to dry.

Biotin-quenched membranes were prepared as above except that instead of deionized water, a 3-μl drop of a solution of biotin (10 mg/ml) in 0·1 M-$NaHCO_3$ was added to the grid before shifting the temperature.

(h) *Electron microscopy*

Grids were examined at 60 kV in a Phillips EM300 electron microscope fitted with an anticontamination cold trap, and were photographed on Kodak medium contrast plates with a 1-s exposure time. No movement or redistribution of ferritins was observed while viewing in the electron microscope. At least 2 grids prepared at each temperature were scanned visually and the patterns of ferritin dots observed and recorded: (1) approximately 400 membranes per grid that were not grossly folded or convoluted were viewed directly and evaluated visually in the electron microscope. In one typical experiment,

92% of the membranes examined under dispersing conditions displayed a dispersed distribution whereas 97% of the membranes examined under patching conditions displayed a patched distribution. (2) Of these approximately 4 membranes/grid that were representative of the population observed were photographed for a permanent record and the photographs assessed visually as to their labeling patterns.

(i) *Pronase digestion of membranes*

Myristate-supplemented membranes labeled with biotinyl-*N*-hydroxysuccinimide were incubated with 500 μg Pronase/ml at 48°C for 1·5 h, according to the method of Morowitz & Terry (1969). The washed digested membranes were then labeled with Av-F.

3. Results

Palmitate-enriched membranes, which had a phase transition range of 28 to 37°C, were labeled with Av-F at several temperatures above, below and mid-way through the transition (Fig. 2(a) to (c)). At both 19°C (below the transition) and 39°C (above) the distribution of ferritin-labeled protein sites was relatively dispersed, while in those membranes labeled at 32°C (middle) these sites formed patches of very low label density as well as patches of high label density (essentially close-packed ferritins). A patch is considered to be a region with a well-defined boundary which has a relatively uniform ferritin distribution within that boundary.

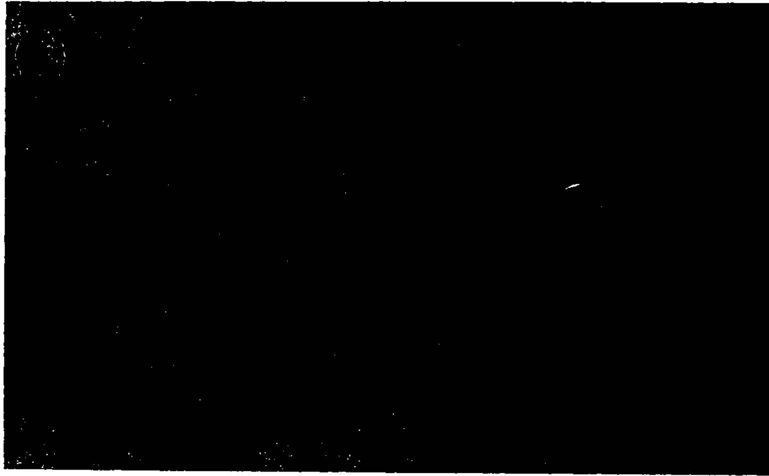
A substantially different phase transition range of 15 to 23°C was found for erucate-enriched membranes; these membranes were also labeled at temperatures corresponding to the three stages in the phase transition: 8°C (below), 19°C (middle), 26°C (above). A result similar to the palmitate case was obtained for the distribution patterns: (Fig. 3(a) to (c)) dispersed at temperatures above and below the transition, formed patches at temperatures intermediate in the transition.

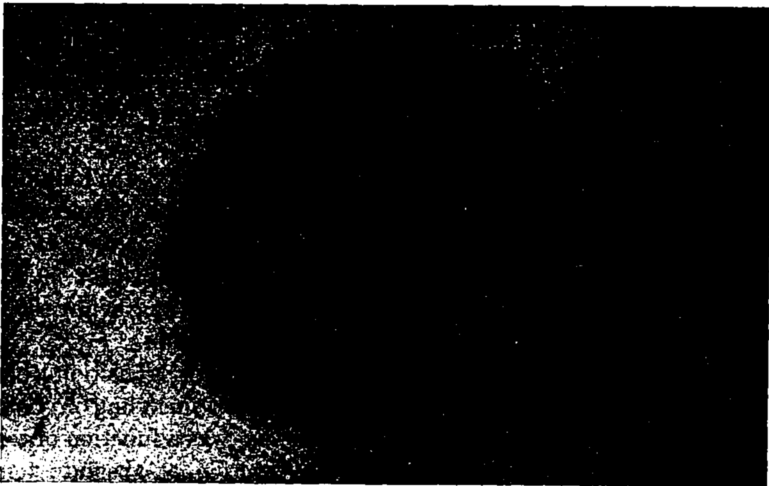
Results from other supplements with different phase transition temperatures (Table 1) were also consistent with these observations (including myristic, elaidic, palmitic plus elaidic, and palmitic plus oleic): patching was a function of temperature relative to the phase transition rather than a function of absolute temperature. Membranes were labeled at several additional temperatures at various points in the transition and also showed consistent results: above and below the phase transition, protein was dispersed in the membrane surface and in the transition the protein was patched (see for example, Fig. 4).

To demonstrate the specificity of labeling, membranes treated with biotinyl-*N*-hydroxysuccinimide as well as untreated membranes were reacted with Av-F. No ferritin staining was seen with the untreated membranes, while the biotinyl-*N*-hydroxysuccinimide-treated membranes were heavily labeled in agreement with previous results of Heitzmann & Richards (1974). After Pronase treatment of the biotinyl-*N*-hydroxysuccinimide-labeled membranes under conditions which yield a 60% digestion of membrane protein but do not remove the polyhexosamine or lipid (Morowitz & Terry, 1969), no ferritin staining was observed (Fig. 5(a) and (b)).

Uranyl acetate staining of labeled membranes showed that the membranes were intact and that the patches containing little label were not regions where holes existed in the membrane (Fig. 5(c) and (d)).

Temperature-shift and biotin-quenching experiments demonstrated the reversibility of the labeling patterns and the absence of cross-linking by the (potentially) multivalent Av-F reagent. Myristate membranes were labeled with Av-F at 37°C under patching conditions, and then subjected to 4°C dispersing conditions before





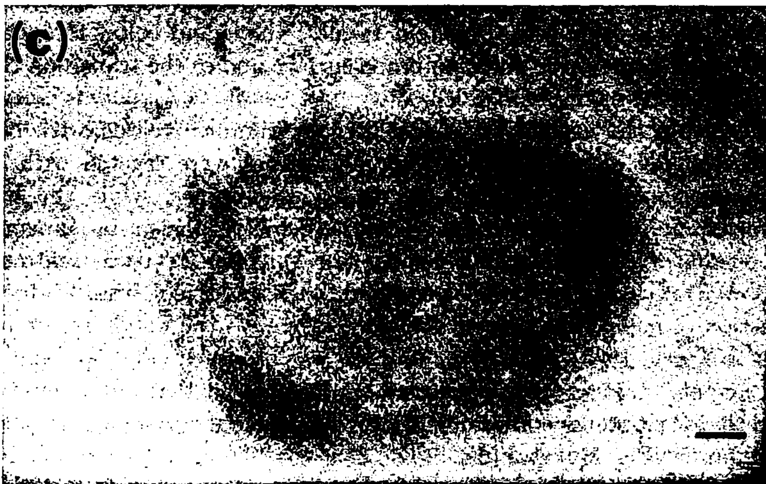
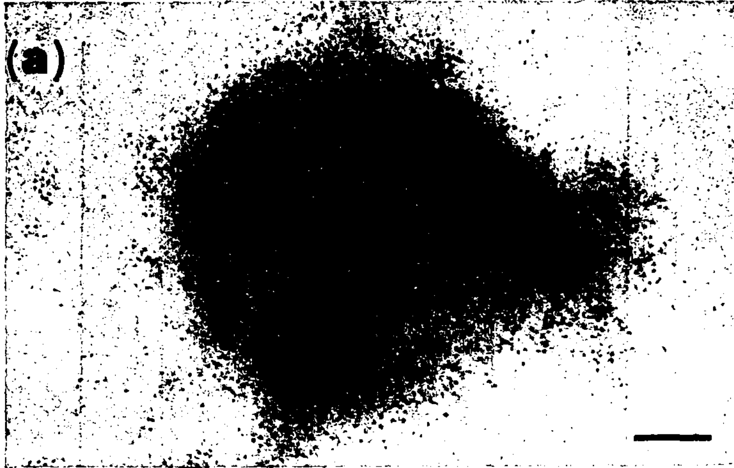


FIG. 2. Biotin-labeled palmitate-supplemented membranes treated with Av-F and dried at: (a) 19°C (below), (b) 32°C (mid) and (c) 39°C (above transition). The surface protein distribution is dispersed above and below the lipid transition and patched when the membrane lipids are partially through the transition. In all Figs, the bars represent 0·1 μm.



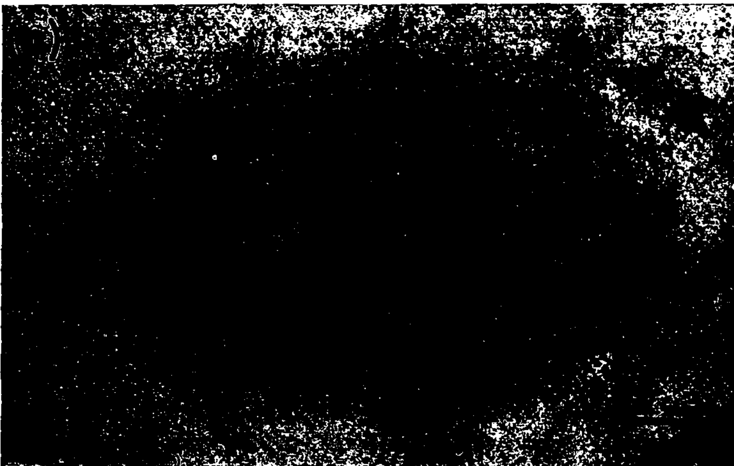


Fig. 3. Biotin-labeled erucate-supplemented membranes reacted with Av-F and dried at: (a) 10°C (below), (b) 19°C (mid), and (c) 26°C (above transition). When lipids exist in the homogeneous smectic or paracrystalline states, the distribution is dispersed; when lipids exist in a mixture of smectic and paracrystalline states, the distribution is patched.

TABLE 1

Phase transition temperatures and fatty acid composition

Supplementation	Phase transition temperature range† (°C)	Fatty acid composition (%) 8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:0	22:0	22:1	Others
Palmitate (16:0)	28–37	—	—	1·09	2·45	—	78·60	—	1·96	11·01	0·86	—	—	—	4·03
Erucate (22:1)	15–23	—	—	4·11	9·35	0·72	21·06	2·11	2·57	5·24	1·32	1·50	1·20	42·87	7·94
Myristate (14:0)	34–42	—	—	2·3	73·3	5·7	4·0	—	10·0	1·2	—	—	—	—	3·5
Elaidate (18:1t)	23–30	—	—	1·97	2·00	—	5·57	—	1·76	87·10	—	—	—	—	1·60
Palmitate + oleate (16:0+18:1c)	23–45	4·96	1·48	5·84	9·16	—	54·41	—	1·77	21·23	1·15	—	—	—	—
Palmitate + elaidate (16:0+18:1t)	34–45	—	—	—	0·42	—	54·39	—	1·06	39·24	—	—	—	—	4·89

† Measured by X-ray diffraction.

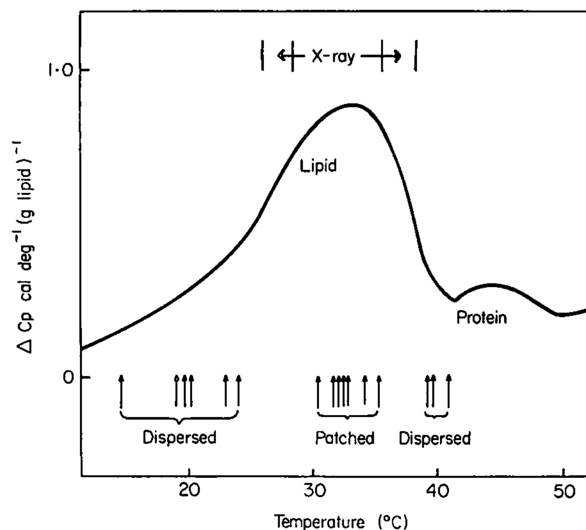


FIG. 4. Behavior of membrane proteins at different points within the phase transition. Differential scanning calorimeter plot of ΔCp *versus* temperature for palmitate-supplemented membranes. The lipid transition as detected by X-ray diffraction occurs between 28°C and 37°C, which corresponds to the differential scanning calorimetry-detected maximum. Protein denaturation occurs (above 42°C), so no labeling was done above this temperature. Membranes labeled below and above the transition have dispersed protein sites, while membranes labeled in the middle of the transition have patched protein sites. Each arrow indicates a separate experimental preparation.

drying at 4°C. A dispersed protein distribution was seen (Fig. 6(a) and (b)). Similar observations were made for palmitate membranes labeled under patching conditions and shifted to dispersing conditions. Furthermore, myristate membranes labeled at 4°C under dispersing conditions, quenched with excess biotin to saturate any unreacted avidin sites (to prevent cross-linking) and then held at 37°C before drying at 37°C, appeared patched as did the unquenched samples.

The presence of 0·5% glucose decreases the surface tension during the drying process and preserves structure in protein crystals (Unwin & Henderson, 1975). When membranes were dried in this manner the same patterns were obtained at various temperatures as for those membranes dried from deionized water.

4. Discussion

There is virtually no non-specific association of the ferritin–avidin complex with membranes unlabeled with biotin. As the lipid and polysaccharide components of the membrane contain no primary amino groups, one expects biotinyl-*N*-hydroxysuccinimide to react exclusively with protein sites. Pronase digestion indicates that the sites being labeled are protein, since under conditions of proteolysis which do not remove lipid or polyhexosamine, but do remove 60% of the protein from biotin-labeled membranes, few ferritin-binding sites remain. Thus, for the membranes of *A. laidlawii* the ferritin particles are attached to the protein components of the membrane, and the observed ferritin distributions reflect the distribution of these proteins. In this latter context it should be noted that the dissociation rate of the biotin–avidin complex is so slow (months or years) that the labeling is effectively irreversible and no exchange processes occur on any time scale of concern to this study.

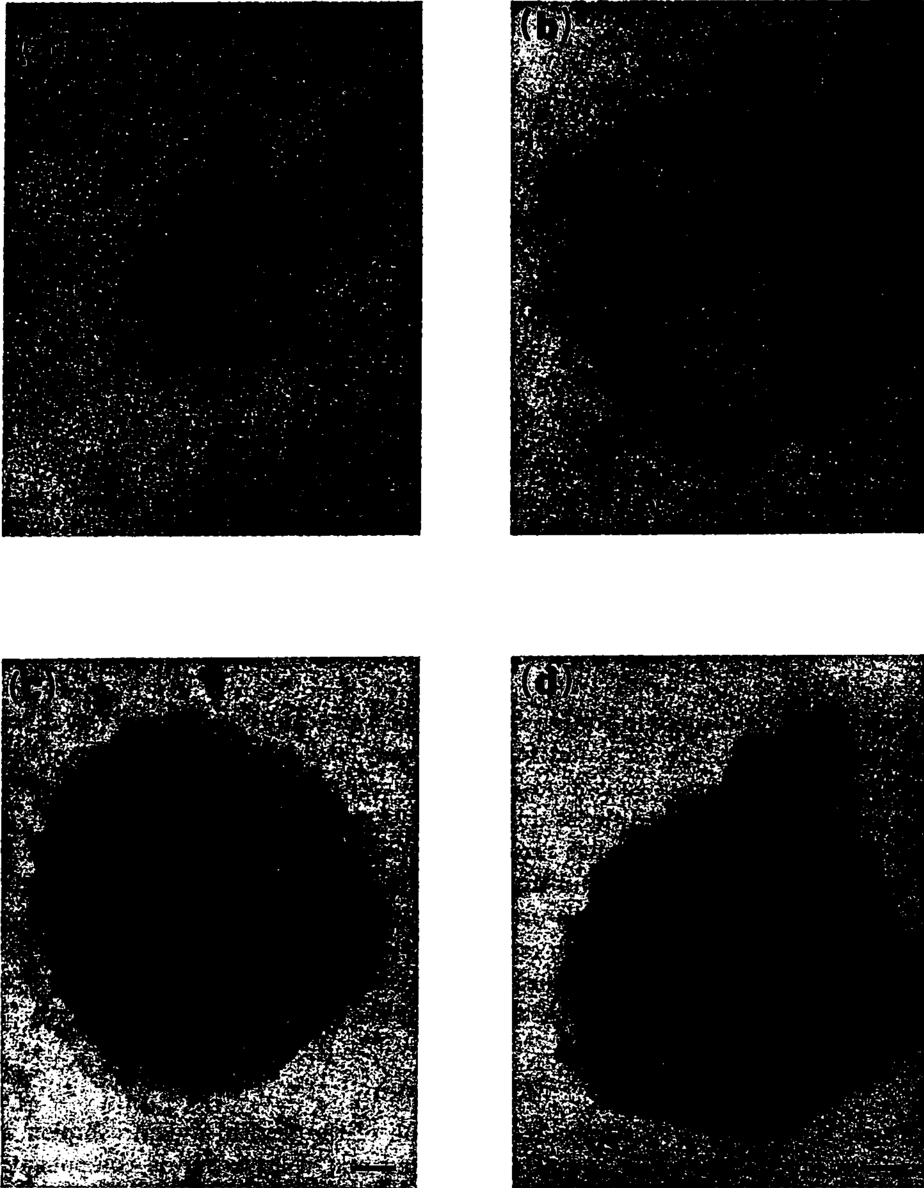
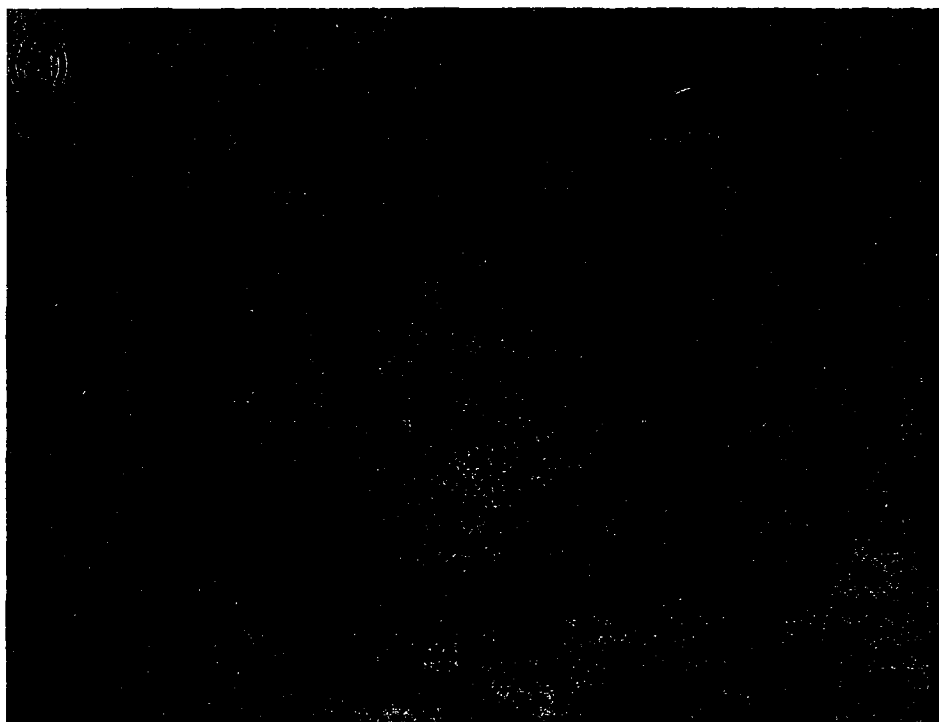


FIG. 5. (a) and (b) Pronase digestion.
Biotin-labeled myristate-supplemented membranes. (a) Membrane digested with Pronase followed by treatment with Av-F at 4°C. (b) Control membrane (no Pronase) treated with Av-F at 4°C. Virtually no labeling of the Pronase-treated membrane is seen, supporting the contention that labeled sites are proteins.

(c) and (d) Uranyl acetate stain.
Biotin-labeled myristate-supplemented membranes treated with Av-F and subsequently positive stained with 2% aqueous uranyl acetate at (a) 4°C (below) and (b) 37°C (mid-transition). Membrane is present in the patches containing few ferritin sites. The patches are not physical holes.



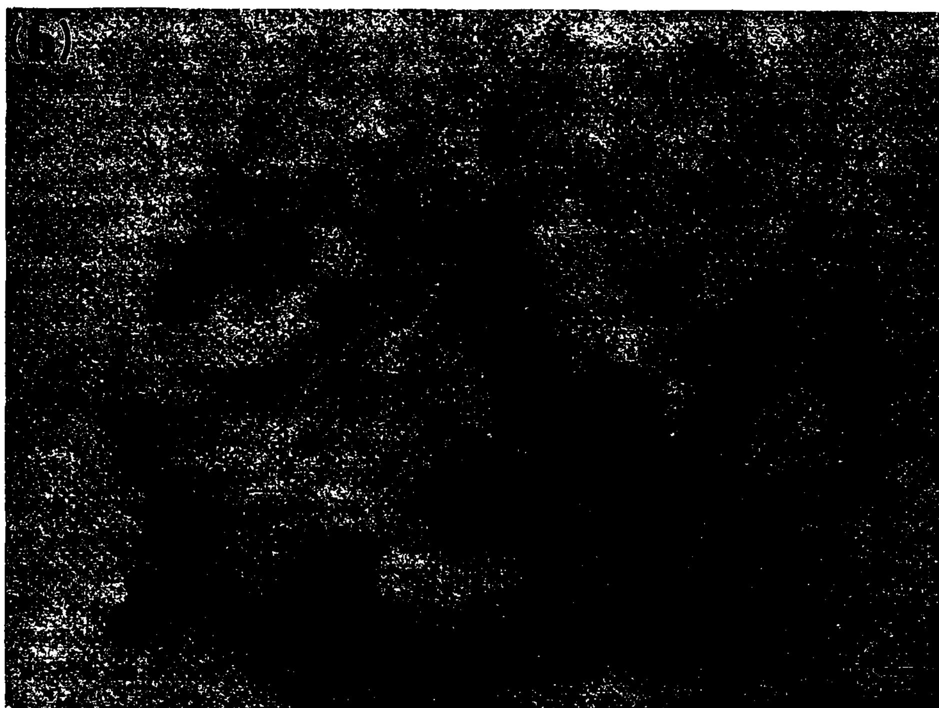


FIG. 6. Temperature-shift control.

Biotin-labeled myristic-supplemented membranes treated with Av-F at 37°C (mid-transition). Subsequently, membranes were incubated and dried from (a) 4°C (below) or (b) 37°C (mid-transition). The labeling is done under patching conditions; the patching may be reversed by cooling to 4°C to give a dispersed distribution.

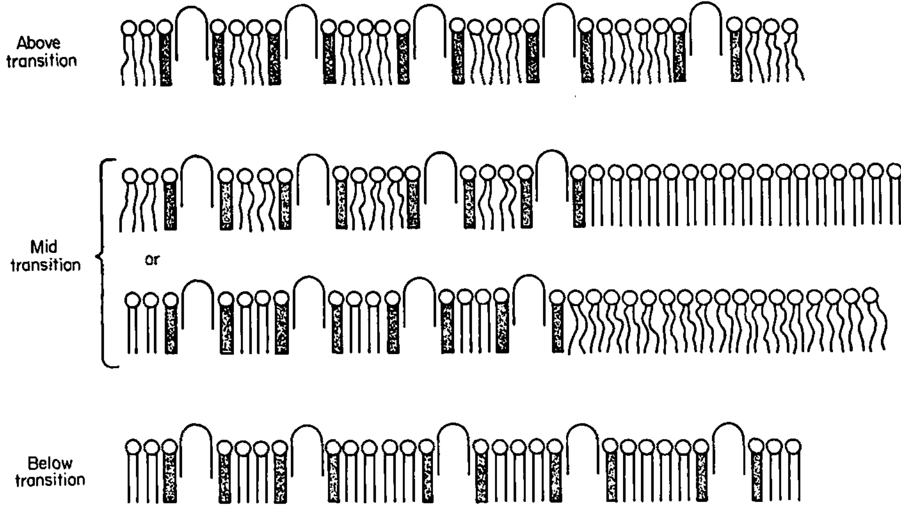


FIG. 7. Model for the effect of the reversible lipid transition on the distribution of protein within the plane of the lipid bilayer. The stippled fatty acid chains adjacent to the protein molecules indicate that their physical state may differ from that of the bulk phase lipids of the membrane. Above and below the phase transition, where the bulk of lipids exist as single phase, proteins are dispersed in the bilayer. In mid-transition where the lipids exist as a mixture of 2 phases, proteins exhibit a preferential association with one phase and form patches. The present experiments do not permit a distinction between the 2 alternatives for the mid-transition patches.

The possibility that drying artifacts produced the observed patterns is considered unlikely. We have no evidence that any redistribution of the ferritin molecules was caused by the drying procedures used. Treatment with a glucose solution prior to drying to decrease the surface tension, as suggested by Unwin & Henderson (1975), was also found to give identical results. Further the membranes are intact; the ferritin-free areas are not holes as shown by the uranyl acetate staining controls. The fact that two distinct patterns of organization are observed and that these can be repeatably and uniformly produced by manipulation of temperature and membrane composition requires that any artifact of drying has the property of a uniform, bimodal response to the experimental variables. We think that this is most improbable.

The membranes exhibit differences which depend on the phase transition of the lipids and not simply on temperatures thermodynamically favorable to protein association. At constant temperature the protein–protein interaction, if any, would be a constant factor. Palmitate-supplemented membranes, which are in the paracrystalline state at 19°C and erucic-supplemented membranes, which are in a heterogeneous mixed state at the same temperature (mid-transition) were both labeled with Av-F at 19°C. Ferritin sites on the palmitate membranes were dispersed; the sites on the erucate membranes were patched. Thus they exhibit patterns consistent with the state of the lipids as determined by X-ray diffraction rather than with the absolute temperature. This correlation was observed in all of the experiments performed in this study.

It appears that protein sites in the membranes of *A. laidlawii* are mobile, even in the paracrystalline lipid phase, and their relative positions within the membrane are affected by the physical state of the membrane lipids. We find that both above the phase transition, where membrane lipids are in a disordered smectic state, and below

the phase transition, where membrane lipids are in a paracrystalline state, the proteins are relatively dispersed within the plane of the membrane. At temperatures within the range of the phase transition, where the membrane consists of a mixture of some lipids in the paracrystalline state and some in the smectic state, regions of the membrane virtually devoid of sites are found, as well as regions in which the sites are patched into areas of high density (nearly close-packed ferritins). It would seem that the paracrystalline state is not so solid and resistant to diffusion as is widely supposed, for the protein must be capable of motion through this lipid phase in attaining a uniform distribution on lowering the temperature from the patched condition.

This latter observation, dispersal of patched areas, also indicates that the patching was not caused by cross-linking through the multiple biotin sites on a single Av-F particle. If this were the explanation, dispersal of the patches would have been impossible. In the reverse of this situation, the excess binding sites in a dispersed, labeled preparation can be quenched by adding saturating amounts of free biotin. Cross-linking is no longer possible. On adjusting the temperature to the mid-transition point patching is still observed. Both approaches clearly indicate that cross-linking between different Av-F particles is not the explanation of the observed patching phenomenon.

The patching phenomenon could be explained by a physical model (Fig. 7) with the following properties: (1) in both of the distinct lipid states, smectic or paracrystalline, the effective viscosity is low enough for the protein molecules to be mobile on a time-scale of tens of minutes; (2) the protein–lipid interaction energy is different for the two states of the lipid. The prediction for such a model would be that the proteins would be uniformly distributed whenever the lipid existed in one state. However, when both lipid states were present at the same time, the proteins would be found in that lipid phase for which the lipid–protein association constant was the highest. Only a very slight difference in interaction energy would be required to produce the type of patching observed. Although specific protein–protein interaction may also be an important part of membrane structure and may also be temperature-dependent, it would probably not be observed in this rather low resolution ferritin-labeling procedure unless the ordered protein aggregates were very extensive. The latter possibility appears unlikely in these *A. laidlawii* membranes. There is no data in the present study which would permit a decision as to which lipid phase has the higher affinity for the proteins.

Using the technique of freeze-fracture electron microscopy, James & Branton (1973) studied the distribution of intramembrane particles in *Acholeplasma* membranes. They found that the state of the lipid influenced the distribution of particles, but the details differ from those seen in our study. The freeze-fracture investigation showed an aggregation which began at a temperature well above the transition (measured by electron spin resonance) and increased with decreasing temperature. The aggregation was complete at the electron spin resonance discontinuity, and no dispersal of particles was seen as the temperature was lowered further. The main qualitative difference between this result and the one obtained from Av-F labeling is the state of aggregation at low temperatures. Although not necessarily explaining discrepancies between observations from the two techniques, variations in several experimental details should be noted. First of all, the two studies may have focused on two classes of membrane components which behave differently as a result of the

lipid phase transition; we observed the membrane proteins exposed, in part, on the membrane surface, while freeze-fracture studies viewed "intramembrane particles", reportedly corresponding to those proteins penetrating through the hydrocarbon interior of the bilayer. These classes should not be mutually exclusive, but the components may be affected in different ways by the lipid hydrocarbon chains and polar head groups as they undergo the transition. Secondly, the samples used may not have been identical. Although reportedly the same organism was used, a variation in strains between our two laboratories is possible. The phase transition temperature reported by James & Branton (1973) was 34°C as detected by electron spin resonance probes for myristic-supplemented whole cells. However, by X-ray diffraction and differential scanning calorimetry we found the upper end of the transition for membranes from cells grown in this supplement to be 42°C, and this difference would be significant in terms of the temperatures used in the two experiments. Although quite large for the difference between two preparations using the same supplement, the difference may indeed be real, or it may be due to a falsely low value from the electron spin resonance measurements caused by perturbation of the membrane by the probe. In addition, our study was performed on membranes harvested from cells grown to mid-log phase, whereas the membranes used in the freeze-fracture work were prepared from cells harvested in late-log phase. We observed increased fragmentation and holes in membranes from late-log phase cells, which prompted us to choose an earlier time of harvest. Finally, James & Branton did not clearly attribute the patching of the membranes they saw as specifically a phase-transition phenomenon, but reported that it was also affected by growth temperature.

We believe that the specificity of the Av-F label clearly defines a class of membrane proteins which behave in the same manner in many different types of supplemented membranes and that the behavior is due to the effect of the lipid phase transition on the membrane proteins.

Thus, the wide range of lipids found within each membrane in an otherwise fairly simple organism, may be a means of regulating the physical state of the total membrane lipids so that they may, in turn, affect the distribution of membrane proteins. This suggests a possible mechanism by which the cell may produce regions vastly different in composition, especially in the protein to lipid ratio, and in this way regulate the structure and perhaps the enzyme activity within a single membrane. While this is clearly not *required* for viability since the cells are capable of growth above, below or mid-way through their phase transition, it may offer a selective advantage. The mobility of the integral surface proteins suggests that a "fluid" model for the membrane would be appropriate for not only the smectic, but also the paracrystalline state, although the fluidity of the paracrystalline state may be much lower.

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