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LIPID- AND TEMPERATURE-DEPENDENT STRUCTURAL CHANGES IN *ACHOLEPLASMA LAIDLAWII* CELL MEMBRANES

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SUMMARY

The lipids in cell membranes of *Acholeplasma laidlawii* were enriched with different fatty acids selected to produce membranes showing molecular motion discontinuities at temperatures between 10 and 35 °C. Molecular motion in these membranes was probed by ESR after labelling with 12-nitroxide stearate, and structure in these membranes was examined by electron microscopy after freeze–etching.

Freeze–etching and electron microscopy showed that under certain conditions the particles in the *A. laidlawii* membranes aggregated, resulting in particle-rich and particle-depleted regions in the cell membrane. Depending upon the lipid content of the membrane, this aggregation could begin at temperatures well above the ESR-determined discontinuity. Aggregation increased with decreasing temperature but was completed at or near the discontinuity. However, cell membranes grown and maintained well below their ESR-determined discontinuity did not show maximum particle aggregation until after they had been exposed to temperatures at or above the discontinuity.

The results show that temperatures at or near a phase transition temperature can induce aggregation of the membrane particles. This suggests that temperature-induced changes in the lipid phase of a biological membrane can induce phase separations which affect the topography of associated proteins.

INTRODUCTION

Structural changes associated with phase transitions in the lipid bilayer portion of biological membranes have been studied using X-ray diffraction, ESR and differential scanning calorimetry (DSC) measurements[1–9]. In single lipid systems the phase transition has been associated with disorder–order transitions in which the hydrocarbon chains of lipids go from a liquid-like state to a more regular, ordered conformation[10]. However, recent discussions[6,11] point out that the term phase transition is appropriate only when dealing with pure chemical compounds and that "phase separations" may be a better term to describe the transitions which occur at a characteristic temperature in mixed lipid systems. Since biological membranes contain a mixture of

Abbreviation: DSC, differential scanning calorimetry.

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lipids we should not be surprised if temperature-induced changes in the lipid phase are associated with phase separations which also affect the order, mobility and topography of associated proteins. Order and mobility in the hydrophobic region of the membrane have been studied by monitoring the effects of altered temperature and lipid composition with physical techniques[1–3,7–9,12]. By extending these observations to include ultrastructural studies, it should be possible to determine the effect of phase separations on the topography of associated proteins.

Membranes of altered fatty acid compositions have been studied by freeze–fracture electron microscopy[5,9,13,14]. This technique splits membranes and exposes extensive face views of the hydrophobic interior of the lipid bilayer continuum[15,16]. The bilayer appears to be interrupted by particles which have been interpreted as the morphological manifestation of proteins which are intercalated into and may traverse the membrane[17]. In *Acholeplasma laidlawii* enriched with a saturated fatty acid (stearic acid, 18:0) these particles frequently appear aggregated in the plane of the membrane while in membranes enriched with unsaturated fatty acids (oleic acid, 18:1 and linoleic acid, 18:2) the particles are dispersed[9,13]. *A. laidlawii* membranes from cells grown at 37 °C and enriched in stearate show an ESR- and DSC-determined transition at or near their growth temperature[7,9], while those enriched in oleate have an X-ray- and DSC-determined transition well below their growth temperature of 37 °C[2,7]. Taken together with the physical measurements, the freeze–etch observations suggested that the distribution of the particles which are intercalated in the *Acholeplasma* cell membrane may be responsive to a transition in the lipid bilayer.

We therefore set out to determine: (1) if growth at a temperature below a phase separation temperature is in fact responsible for the kind of particle aggregation observed in stearate-enriched *A. laidlawii* membranes, and (2) what properties of fatty acids are important in determining the morphology of the membranes into which they are incorporated. During the course of our investigation Verkleij *et al.*[18] and Speth and Wunderlich[19] have published observations suggesting that temperature-induced phase transitions control particle aggregation in *A. laidlawii* and *Tetrahymena*.

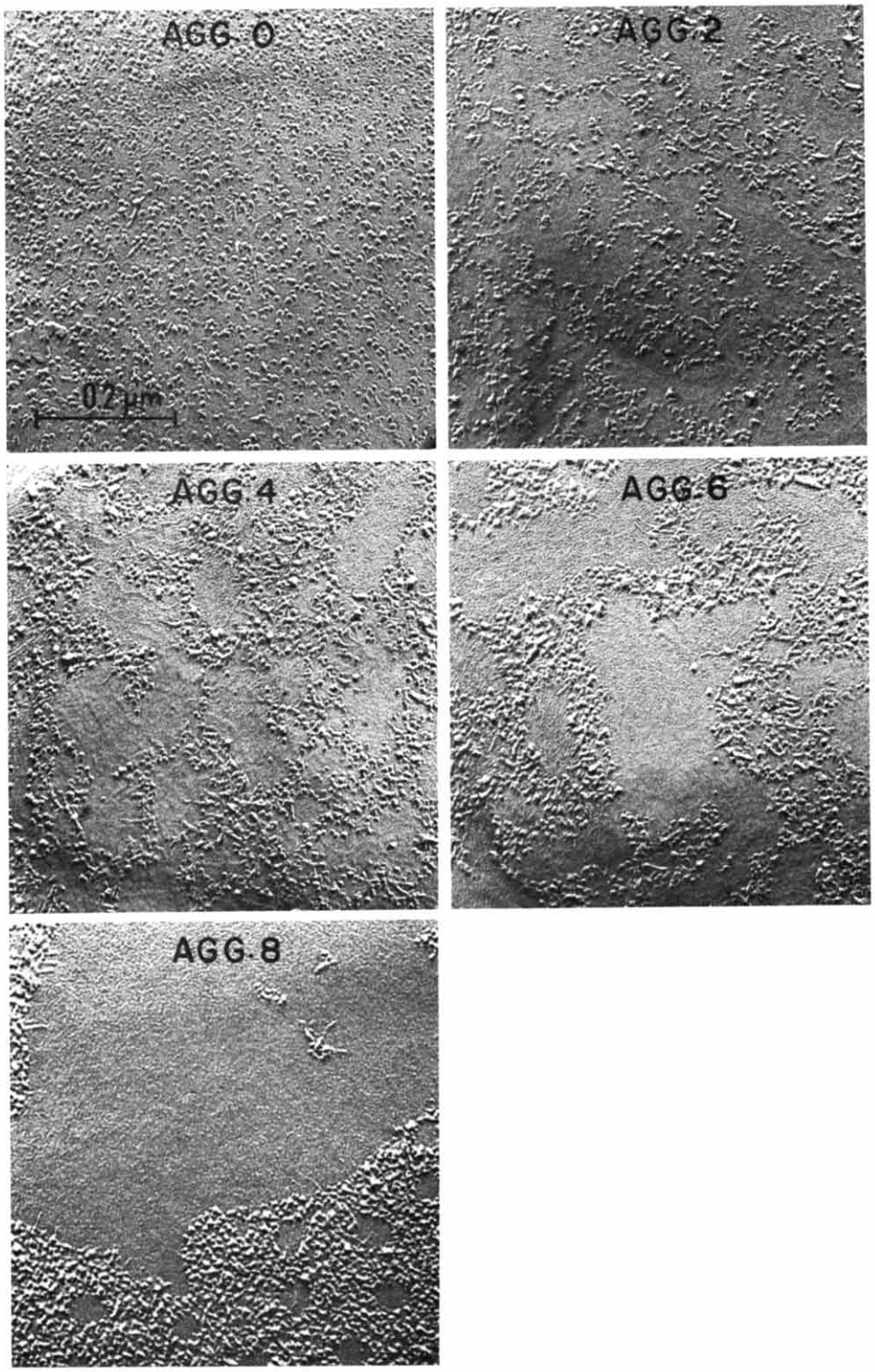
MATERIALS AND METHODS

Chemicals

Stearolic acid (18:1$^{\equiv}$) was purchased from Lachat Chemicals, Inc., Chicago Heights, Illinois. It was 99% pure, or better, as determined by gas–liquid chromatography of the methyl ester. Elaidic acid (18:1 *trans*), eicosenoic acid (20:1 *cis*) and myristic acid (14:0), were purchased from the Hormel Institute, Austin, Minnesota. The purity was described as 99% or better and was not further tested. The spin label 12-nitroxide stearate was synthesized and purified by Dr Alec Keith.

Culture conditions and cell harvest

A. laidlawii B., previously called *Mycoplasma laidlawii* B., was grown statically at various temperatures in 10 to 600 ml of medium in glass tubes or erlenmeyer flasks. The medium was modified only quantitatively from that of Razin *et al.*[20]; 1 l contained the following: 20 g Difco Tryptose, 10 g glucose, 5 g NaCl, 3.7 g Tris, 4 g bovine serum albumin very poor in fatty acid (Grade B, Cal-Biochem), and 100000 units potassium penicillin G. The unadjusted pH of this medium was 8.3.


AGG 0
0·2 µm
AGG 2
AGG 4
AGG 6
AGG 8

Fatty acid supplements were added as ethanol solutions to give a final concentration of 120 μM fatty acid. The ethanol in the medium did not exceed 0.5%. Cells were harvested during late log phase by centrifugation at $13000 \times g$ and were washed twice in β-buffer (Razin *et al.*[20]). To obtain isolated membranes, cells were lysed by squirting the washed cells into β-buffer diluted 1:20 and incubated in this mixture for 30 min at 0 or 37 °C. After incubation, the suspension was centrifuged for 20 min at $40000 \times g$, and the resulting pellet was then washed twice in β-buffer, diluted 1:20. To remove unlysed cells, the membrane preparation was centrifuged at $8000 \times g$ for 5 min and the pellet was discarded. The supernatant contained isolated membranes. The cells or their isolated membranes were then used for: (1) lipid analysis, (2) ESR studies, or (3) freeze–fracture. For experiments where temperature was critical all harvest and isolation procedures were carried out at the growth temperature.

Freeze–fracture and electron microscopy

Samples from the various pellets were pipetted onto 3-mm cardboard planchets and frozen in liquid freon cooled by liquid nitrogen. In those cases where the cells were frozen directly from 37 °C, the cells, planchets, and all transfer equipment were maintained in a water-saturated 37 °C atmosphere until a fraction of a second before the cells entered the liquid freon. When the cells were frozen from 21 or 4 °C, they were first equilibrated at the selected temperature and all operations were carried out in a room at 21 or 4 °C. Samples were freeze–fractured at −110 °C with no etching[15,21]. Replicas were viewed in a Siemens Elmskop 1, using direct magnifications of 12000–30000. Replicas were scanned and areas to be photographed were selected solely on the basis of membrane concentration and replica quality.

Two methods were used to determine the membrane particle distribution. In many cases both methods were applied and gave similar results. The first method consisted of comparing electron micrographs to a predetermined set of standard pictures showing various degrees of particle aggregation. These standards are shown in Figs 1a–1e. Fig. 1a shows no aggregation and was given the numerical value 0 while each succeeding standard shows more aggregation and is assigned a numerical value increasing by twos. This scale of 0, 2, 4, 6, and 8 was used to evaluate photographs of at least 25 cells for each condition. Cells falling between two standards were assigned the odd number between standards. A numerical average, referred to as the aggregation value, was thus obtained for each experimental condition. A low value indicates little aggregation and a high value indicates great aggregation. The second method involved the analysis of particle distribution on prints (total magnification 72000) of the electron micrographs, which were overlaid with a grid of 1-cm squares. The particles in each of 100–150 1-cm squares overlaying 10–20 cells were counted by hand. The counts are presented by plotting the number of particles counted in areas of varying density.

Fig. 1. Freeze–fractured membranes of *A. laidlawii* cells showing various degrees of particle aggregation found under a variety of different conditions. The photographs have been arranged in a sequence to show gradually increasing aggregation and to provide a set of standards with arbitrarily assigned aggregation (AGG) values of 0, 2, 4, 6 and 8.

Electron spin resonance

Labelling with 12-nitroxide stearate was carried out *in vitro* by addition of 12-nitroxide stearate in ethanol directly to cell pellets. This method has been shown to label the membrane lipids and allow detection of changes in molecular motion (τ_0)[22–25]. Molecular motion is reported as τ_0, an empirical molecular motion parameter which was calculated from the expression

$$\tau_0 = KW_0\left[\left(\frac{h_0}{h_{-1}}\right)^{\frac{1}{2}} - 1\right]$$

where the numerical value of K is unimportant for relative values, W is line width, h is line height, and 1, 0, and -1 refer to low-, mid-, and high-field lines. The derivation of the equation employs the spectral parameters of Griffith *et al.*[26] and Lorentzian line shapes and is based on work by Kivelson[27]. The derivation is identical to that reported by Henry and Keith[12].

A Varian Model 4500 EPR spectrometer was used. This instrument was equipped with a Varian temperature accessory calibrated with an iron constantan thermocouple accurate to approximately ± 1.5 °C. A detectable destruction of the spin label immediately after labelling indicates that the methyl ester of 12-nitroxide stearate penetrates the cell quickly, allowing incorporation into the membrane lipids.

Fatty acids analysis

After washing and pelleting, the cells for lipid extraction were weighed wet, and 10 vol. methanol (vol./cell wet wt) were added to the cells in glass centrifuge tubes. The cells were macerated with a glass rod and then incubated at 65 °C for 5 min. 20 vol. chloroform (vol./cell wet wt) were added, and the suspension was incubated at 65 °C for 20 min, with intermittent maceration[28]. The suspension was then filtered through lipid-extracted Whatman No. 1 filter paper, and the filtrate was washed two times with chloroform–methanol (2:1, v/v). Lipid extracts were then evaporated to less than 1 ml and applied to a thin-layer chromatography plate (Silica Gel G, Brinkman Instruments, Inc.). The phospholipids were then separated from neutral lipids and free fatty acids by thin-layer chromatography according to the methods of Keith *et al.*[29]. The phospholipids were saponified and methyl esters of the fatty acids produced according to Bottcher *et al.*[30]. Analysis of the methyl esters was performed on a Varian Model 600 gas chromatograph fitted with a polar column (DEGS), flame ionization detector, and disc integrator.

RESULTS

In order to study the roles of fatty acid composition and temperature on particle aggregation, we started by growing cells at 37 °C with a variety of different fatty acid supplements. The physical properties of these membranes at different temperatures were then examined by ESR (to detect discontinuities in a molecular motion parameter) and by freeze–fracture electron microscopy (to examine topographical features). The fatty acids were chosen to produce membranes showing molecular motion discontinuities at different temperatures between 10 and 35 °C.

TABLE I

EFFECT OF FATTY ACID SUPPLEMENT ON MEMBRANE PHOSPHOLIPID COMPOSITION

Fatty acid supplement	*Growth temperature* (°C)	*Fatty acid composition (moles per 100 moles)*		
		Supplemented fatty acid	*Other fatty acids*	
			Saturates	*Unsaturates*
a. 14:0	37	83.2	11.5	5.3
b. 18:1*trans*	37	78.0	20.2	1.8
c. 20:1*cis*	37	67.1	26.1	6.8
d. 18:1≡	37	45.6	48.7	3.0
e. 14:0	25	70.6	20.4	9.0

TABLE II

PARTICLE DISTRIBUTION AS A FUNCTION OF FATTY ACID SUPPLEMENT AND GROWTH TEMPERATURE

All cells grown at 37 °C; observations made on freeze–fracture whole cells. Arrows and temperatures indicate the ESR-determined Arrhenius-plot discontinuity for each enrichment as taken from Figs 2a–2d.

Fatty acid supplement	*Frozen from (°C)*	*Aggregation value*
a. 14:0	37	7.9
	34 °C ◀	
	25	7.5
b. 18:1*trans*	37	6.7
	22 °C ◀	
	21	7.0
	4	8.0
c. 20:1*cis*	37	1.7
	21	3.8
	13 °C ◀	
	4	7.0
d. 18:1≡	37	2.5
	23 °C ◀	
	21	7.5
	4	7.5

Previous work with stearate(18:0)-supplemented cells[9,13] has shown that cells enriched in saturated fatty acids and harvested at or just below their ESR-determined discontinuity showed marked particle aggregation. The ESR- and DSC-determined transition for 18:0-enriched cells occurs around 39 °C, making it impossible to grow these cells above the transition. For comparison we therefore initiated our study by examining myristate(14:0)-enriched cells (Table I, a) which at 37 °C were growing 5 °C above the Arrhenius-plot discontinuity of their membranes (Fig. 2a). The freeze–etch results (Fig. 3 and Table II, a) show aggregation similar to that reported for stearate-enriched cells growing just below their Arrhenius-

plot discontinuity and demonstrate that particle aggregation may occur above this discontinuity.

This conclusion is reinforced by our examination of other supplementation regimes using both *trans*- and *cis*-unsaturated fatty acids (Table I, b and c and Figs 2b and 2c). In both 18:1*trans*- and 20:1*cis*-enriched membranes aggregation was

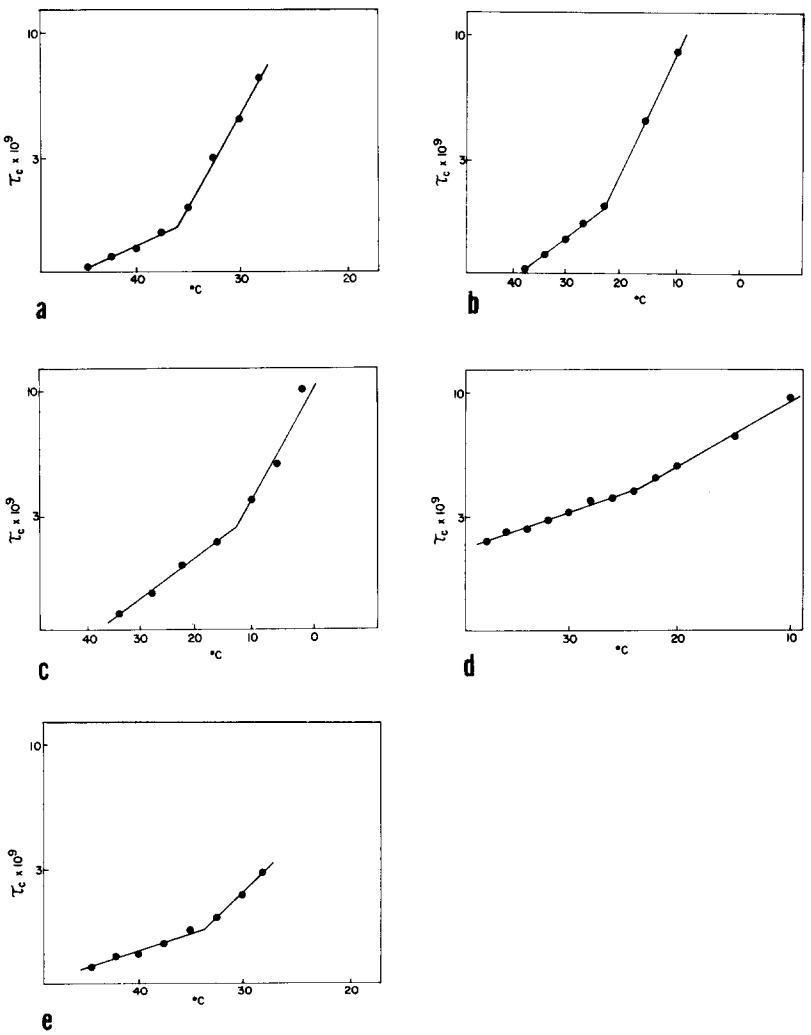
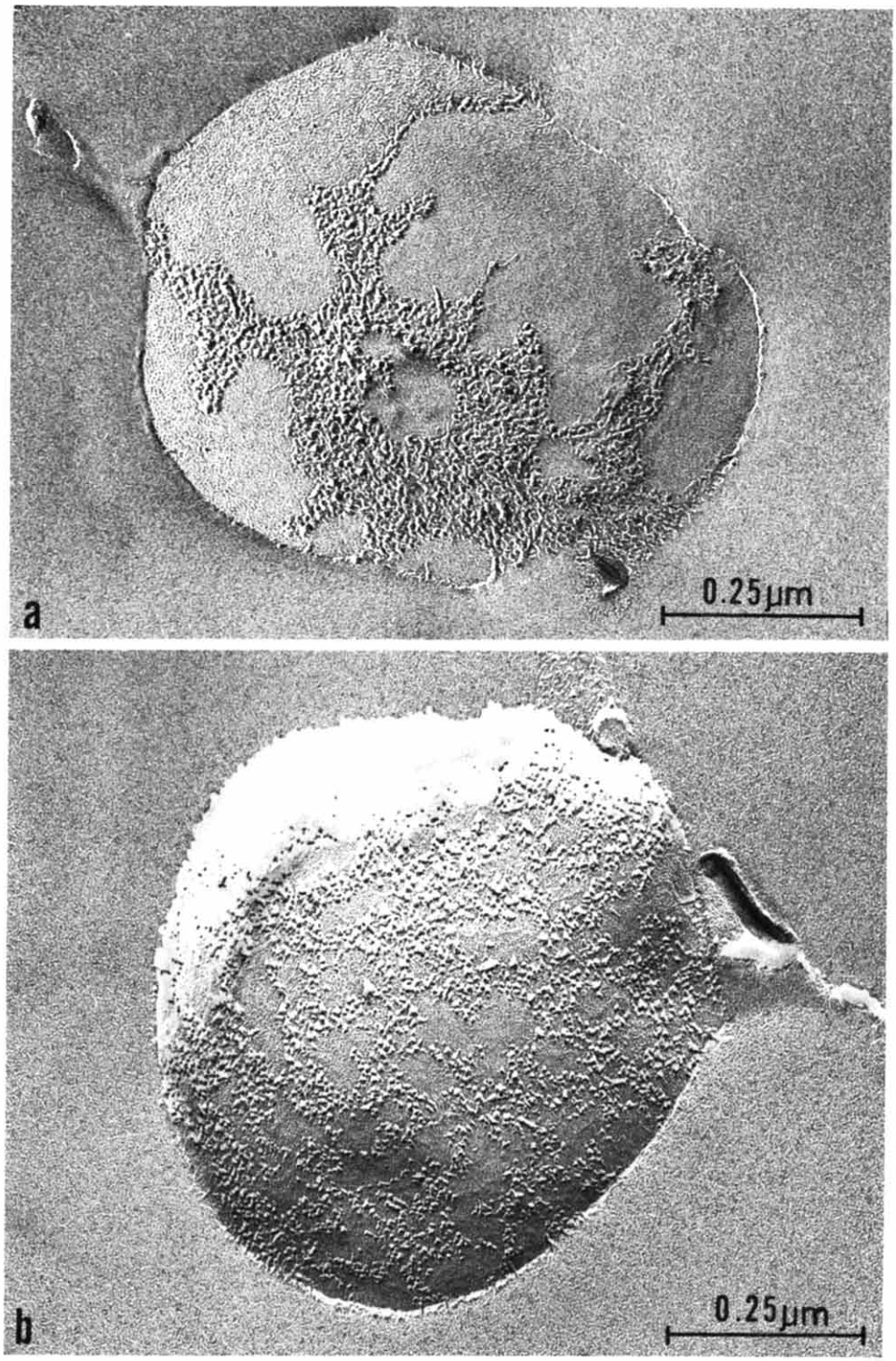


Fig. 2. Arrhenius plots of the molecular motion parameter (τ_0) of 12-nitroxide stearate-labelled cells enriched with (a) 14:0 and grown at 37 °C; (b) 18:1*trans* and grown at 37 °C; (c) 20:1*cis* and grown at 37 °C; (d) 18:1≡ and grown at 37 °C; and (e) 14:0 and grown at 25 °C.

Fig. 3. (a) Myristate(14:0)-enriched *A. laidlawii* cell grown at 37 °C and maintained at 37 °C until frozen. (b) Myristate(14:0)-enriched *A. laidlawii* cell grown at 25 °C and maintained at 25 °C until frozen. Note the evident particle aggregation in the membrane of cells grown at 37 °C in comparison to the more dispersed particle distribution in cells grown at 25 °C. Both × 110000.


a
0.25µm
b
0.25µm

observed (Table II, b and c) in cells frozen from temperatures above the ESR discontinuity. However in both cases aggregation increases as pre-freezing temperature decreases.

Stearolic acid (18:1≡) is an unsaturated fatty acid with a triple bond at the C-9 position. Cells enriched with this fatty acid (Table I, d) show minimal aggregation when frozen from 37 °C (Table II, d) which is above the Arrhenius-plot discontinuity (Fig. 2d). However, aggregation increases with decreasing temperature before freezing and is maximal near the Arrhenius-plot discontinuity (Table II, d).

In all of the above, where cells were grown at or above the temperature of their ESR-determined discontinuity, aggregation tended to increase with subsequent, post-growth decreases in temperature. However, when cells were grown with similar enrichments (Table I, e) below their ESR-determined discontinuity (Fig. 2e) and always maintained at these low temperatures during post-growth harvest, the freeze–fracture results showed far less particle aggregation than in the membranes of cells grown at or above their transition temperature (Fig. 3).

Cells grown at 25 °C and then incubated in growth medium at 37 °C for 2 h also look like those grown at 37 °C. Cells grown at 37 °C and then incubated in growth medium at 25 °C for 2 h have fracture faces which are the same as those for cels grown at 37 °C. Similar experiments with isolated membranes show similar results. These results suggest that the particle aggregation which occurs at 37 °C is not reversed of increased by incubation at 25 °C.

Experiments were carried out to determine the time course of the aggregation which occurs when membranes are transferred from 25 to 37 °C. Isolated membranes from myristate-enriched cells grown at 25 °C were incubated in a 1:20 dilution of

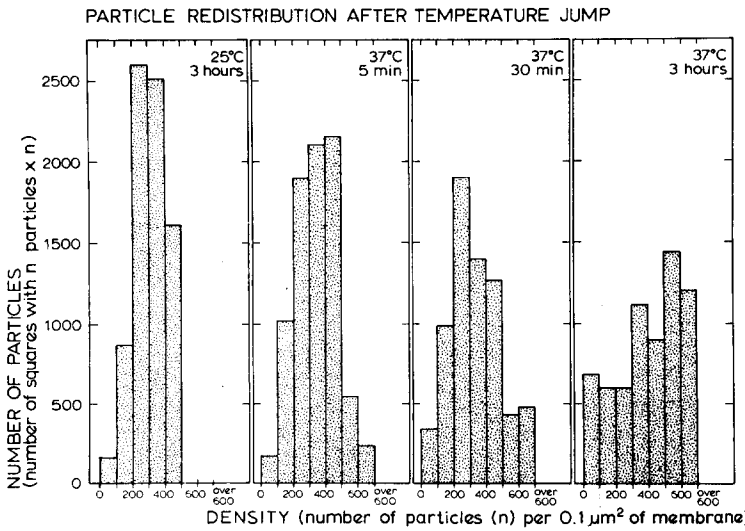


Fig. 4. Particle distribution on the freeze–fracture faces of membranes isolated from myristate-enriched cells grown at 25 °C. After isolation, during which all procedures were carried out at less than 25 °C, the membranes were incubated at 25 °C for 3 h; 37 °C for 5 min; 37 °C for 30 min; or 37 °C for 3 h before being frozen for freeze–fracture from the incubation temperature. The histogram shows the total number of particles found in regions of different density.

β-buffer at 37 °C for varying lengths of time, while a control was kept at 25 °C. Fig. 4 shows the distribution of particles in areas of various particle densities. The histograms show that membranes from cells grown at 25 °C and maintained at that temperature have most of their particles in areas where the particle density is 200–400 particles/μm^2. As early as 5 min after exposure to 37 °C, the particles have begun to aggregate. This is shown by a decrease in the median peak and the appearance of some areas where the particle density is greater than 5000/μm^2. This trend continues, and after 3 h there is a clear bimodal density distribution. Samples taken at other times (not shown) indicate that the change in particle distribution is near completion after exposure to 37 °C for 1 h. Using the standard picture scale (Fig. 1) to analyze the results of these experiments gave similar results (Table III).

Pinto da Silva[31] has reported that particles on the freeze-fracture face of red blood cells are aggregated by lowering the pH. Since the pH readings, after growth, of the media used for myristate(aggregated)-, stearate(aggregated)-[9,13], oleate(dis-

TABLE III

PARTICLE REDISTRIBUTION AS A FUNCTION OF POST-GROWTH TEMPERATURE

All cells grown in a myristate-supplement medium at 25 °C; all isolated cell membranes prepared at temperatures below 25 °C. The temperature is that at which either the whole cells or their isolated membranes were incubated for the incubation times indicated. Cells or membranes were frozen from the incubation temperature.

Sample	*Temperature (°C)*	*Incubation time*	*Aggregation value*
Whole cells	25	3 h	4.3
Isolated membranes	25	3 h	4.5
	37	5 min	4.8
	37	30 min	6.0
	37	1 h	6.4
	37	3 h	7.0

TABLE IV

PARTICLE DISTRIBUTION AS A FUNCTION OF POST-GROWTH pH

All cells grown in a myristate-supplemented medium at 25 °C. Aggregation values were determined on freeze-etched whole cells after a 3-h incubation at the pH values and temperatures indicated.

Incubation pH	*Aggregation value after incubation at 0 °C*	*Aggregation value after incubation at 37 °C*
5.2	5.4	6.5
6.2	4.4	7.1
7.2	5.0	7.1
8.2	4.8	7.5

persed)-[9,13], and linoleate(dispersed)-[13]enriched *A. laidlawii* cells were all 7.5 ± 0.2, pH cannot be responsible for the aggregation observed in these experiments. Furthermore, experiments were carried out to determine if alteration of pH could affect the aggregation phenomenon observed with myristate-enriched cells. Cells grown at 25 °C were exposed to different pH values at 37 °C for 1.5 h, while controls were exposed to the same pH values but maintained at 0 °C (Table IV). Cells kept at 0 °C, while incubated at pH 6.2, 7.2, and 8.2, all have membranes with aggregation values similar to that for cells grown and maintained at 25 °C while cells incubated at 37 °C at the same pH values all have membranes showing aggregation values similar to that of membranes exposed to 37 °C for 3 h. These results indicate that pH from 5.2 to 8.2 has no effect on the aggregation of particles which occurs at 37 °C.

DISCUSSION

Our results on aggregation of membrane particles provide direct evidence for a phase separation of membrane components during temperature induced disorder–order transitions in the lipid components. Results on cells grown at 37 °C (Table II and Fig. 2) show that the separation of membrane components can begin at temperatures above the Arrhenius-plot discontinuity (14:0, 18:1*trans*, and 20:1*cis*) and particle aggregation and thus increases with decreasing temperature (18:1*trans*, 20:1*cis*, and 18:1≡). In addition these results suggest that the particle aggregation occurs mainly as temperature is decreased in the range above and near the Arrhenius-plot discontinuity and that separation is complete and maximal at or near this discontinuity. Previous results with 18:0-enriched cells growing at or near their Arrhenius-plot discontinuity also showed highly aggregated particles[9]. As shown in Table II (b and d), no significant increases in aggregation value occur once temperatures are well below the transition temperatures.

In fact when 14:0-enriched cells are grown at 25 °C, which is well below the ESR discontinuity, aggregation (Table III) is far less than in 14:0 cells grown at 37 °C (Table II). This finding suggests that at temperatures below the Arrhenius-plot discontinuity the mobility of the lipids is decreased to a point where it retards phase separation. It would therefore appear that as new membrane is synthesized proteins are not intercalated into the bilayer in an aggregated array, and that they assume this aggregated distribution only under certain conditions.

Recently, Verkleij *et al.*[18] have attributed particle aggregation in *Acholeplasma* to freezing from below the X-ray- and DSC-determined phase transition temperatures. Although direct comparison of ESR and X-ray data is not possible, several indirect comparisons suggest these techniques are measuring similar structural changes[1,2,7,14,32]. For 14:0- and 18:1*trans*-supplemented cells grown at 37 °C and frozen from 5 °C the aggregation of particles shown by Verkleij *et al.*[18] was similar to that shown here for similar supplements and freezing temperatures. However, the results of Verkleij of *et al.*[18] with cells frozen from 37 °C are somewhat different and not strictly comparable to those reported here. The fracture face they show for cells frozen from 37 °C is described as "net like" which in our interpretation would be considered aggregated and given a value of approximately 5 according to the scale presented in Fig. 1. But, since Verkleij *et al.*[18] have not presented a statistical survey of the fracture faces they observed, do not provide data on the degree of incorporation of

the supplemented fatty acids, and assume phase transitions based on literature citations for other batches of similar cells, elaborate comparisons between our results and theirs are not warranted. Verkleij *et al.*[18] also describe ridges which appear on the bilayer portion of the membrane when frozen from below the phase transition. Pinto da Silva[33] has reported that the appearance of ridges in lipid–water systems is enhanced by a slow rate of cooling prior to freezing for freeze–etching. Since Verkleij *et al.*[18] appear to have used a much slower, more carefully controlled cooling rate than we used, this probably accounts for the appearance of ridges on the fracture faces of their cells and the absence of these ridges in our cells.

Our temperature shifting experiments elucidate some of the factors which control the distribution of particles in the membrane. Isolated membranes derived from cells grown and maintained at 25 °C have an aggregation value of 5.0 (Table III and Fig. 4) while those shifted to 37 °C show a relatively slow but definite increase in the aggregation of the particle fraction of the membrane (Table III and Fig. 4). This indicates that a certain minimal temperature is required for a separation of membrane components to occur. 14:0-enriched cells which are grown at 37 °C and then shifted to 25 °C before freezing show no change in aggregation (Table II). The irreversibility of aggregation supports our conclusion that most of the phase separation occurs at temperatures near or above our ESR-determined discontinuity.

Differences in the proteins of the cell membranes do not appear to account for our observations of particle aggregation since Pisetsky and Terry[34] have observed that *A. laidlawii* cell membranes enriched with a variety of fatty acids all had approximately the same qualitative and quantitative distribution of protein components. Nor can metabolic phenomena account for our observations since particle aggregation was similar in intact cells and their isolated membranes. Hence, particle aggregation and the temperature dependency of this aggregation must be linked to the physical properties of the lipids, specifically their fluidity, mobility and phase separations.

It is interesting to note that 18:1*trans* cells frozen from 37 °C (15 °C above the discontinuity) show much more aggregation than do 20:1*cis* cells frozen from 21 °C (8 °C above the discontinuity). Also oleic acid(18:1*cis*)-enriched cells grown at 37 °C and frozen from 4 °C (19 °C above their reported discontinuity[9]) show little if any aggregation (James, R., unpublished). Why does aggregation occur so much further above the Arrhenius discontinuity in 18:1*trans* enriched cells than in the *cis* unsaturated 20:1 enrichments? Structural features of *cis* and *trans* unsaturates may account for these differences, but further experiments are needed to determine how the shape of the hydrocarbon chain is important in determining the temperature at which these phase separations begin to occur.

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