Modification of Membrane Lipid: Physical Properties in Relation to Fatty Acid Structure†

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ABSTRACT: Differential scanning calorimetry (DSC) and electron spin resonance (ESR) measurements were made to characterize how modifications in the fatty acid composition of *Escherichia coli* affected the thermotropic phase transition(s) of the membrane lipid. When the fatty acid composition contained between 20 and 60% saturated fatty acids, the DSC curves for isolated phospholipids and cytoplasmic membranes showed a broad (15–25 °C) gel to liquid-crystalline phase transition, the position of which depended on the particular fatty acid composition. Utilizing multiple lipid mutants, enrichment of the membrane phospholipids with a single long-chain *cis*-monoenoic fatty acid in excess of that possible in a fatty acid prototroph led to altered physical properties. A sharp thermotropic phase transition (1 °C) appeared at saturated fatty acid levels <20% and gradually replaced the broad peak as the *cis*-monoenoic fatty acid content increased. These results were obtained with phospholipids, cytoplasmic membranes, and whole cells. With these same phospholipids, plots of 2,2,6,6-tetramethylpiperidinyl-1-oxy partitioning and ESR order parameters vs. $1/T$ revealed discontinuities at temperatures 40–60 °C above the calorimetrically measured gel to liquid-crystalline phase transitions. Moreover, when the membrane phospholipids were enriched with certain combinations of *cis*-monoenoic fatty acids (e.g., *cis*-Δ^9-16:1 plus *cis*-Δ^{11}-18:1), the DSC curves showed a broad gel to liquid-crystalline phase change below 0 °C but the ESR studies revealed no discontinuities at temperatures above those of the gel to liquid-crystalline transition. These results demonstrated that enrichment of the membrane lipids with molecules in which both fatty acyl chains are identical *cis*-monoenoic residues led to a distinct type of liquid-crystalline phase. Furthermore, a general conclusion from this study is that *Escherichia coli* normally maintains a heterogeneous mixture of lipid molecules and, by so doing, prevents strong lipid–lipid associations that lead to the formation of lipid domains in the membrane.

The lipid composition of naturally occurring membranes is generally quite heterogeneous. At the present time, the relationship between specific lipid composition and membrane structure and catalytic activity is not well understood. Furthermore, little is known about the regulatory mechanisms governing membrane lipid composition. As one approach to understanding the normal complexity of membrane lipids, attempts have been made to simplify the lipid composition and then to determine the effect of such alterations on membrane structure and biological function. *Escherichia coli* is well suited for such studies. It has only surface membranes and these are characterized by relatively simple lipid compositions. Moreover, the biochemical mechanisms that conserve the properties of membrane lipids essential to physiological function can be genetically manipulated. The cytoplasmic membranes of *E. coli* contain two major glycerophosphatides, phosphatidylethanolamine and phosphatidylglycerol; the former comprises approximately 70–75% of the phospholipids. These glycerophosphatides have one major naturally-occurring saturated fatty acid, 16:0, and two naturally occurring unsaturated fatty acids, *cis*-Δ^9-16:1 and *cis*-Δ^{11}-18:1. If only phosphatidylethanolamine is considered, nine possible arrangements (molecular species)[1] are possible. Three of these, $16{:}1_{c_1}$-$16{:}0_{c_2}$, $16{:}1_{c_1}$-$18{:}1_{c_2}$, and $18{:}1_{c_1}$-$16{:}0_{c_2}$, are not found in large amounts, since the enzymes that catalyze the attachment of the acyl chains virtually exclude these arrangements (Van Golde and Van Deenen, 1967; Silbert et al., 1973). Therefore, the principal molecular species are six in number: I, $16{:}0_{c_1}$-$16{:}0_{c_2}$; II, $16{:}0_{c_1}$-$16{:}1_{c_2}$; III, $16{:}0_{c_1}$-$18{:}1_{c_2}$; IV, $16{:}1_{c_1}$-$16{:}1_{c_2}$; V, $18{:}1_{c_1}$-$16{:}1_{c_2}$; VI, $18{:}1_{c_1}$-$18{:}1_{c_2}$.

The first *E. coli* mutants available for modifying fatty acid composition were unsaturated fatty acid auxotrophs (see references in Silbert, 1975). With these strains (and derivatives containing a β oxidation defect in addition to the biosynthetic mutation) the fatty acid composition of the phospholipids in the cell could be simplified and many types of membrane lipid alteration could be induced with unsaturated fatty acid analogues as growth supplements. However, since these mutants still synthesized saturated fatty acids, they retained an important means for regulating the properties of the membrane lipid during the growth period. There are now available *E. coli* strains containing a number of mutations that restrict rather completely the cell's ability to modulate its lipid composition (Davis and Silbert, 1974; this paper). In these strains, the molecular species of phospholipid synthesized during growth, as well as the overall content of a particular acyl group, can be controlled by nutritional means. With a double mutant de-

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[1] Abbreviations used are: short hand designations for molecular species, e.g., $16{:}1_{c_1}$-$16{:}0_{c_2}$ refers to a phospholipid with 16:1 and 16:0 fatty acids on the 1 and 2 positions, respectively; 16:1 and 18:1 are *cis*-Δ^9-16:1 and *cis*-Δ^{11}-18:1, respectively; NADH, reduced nicotinamide adenine dinucleotide; ESR, electron spin resonance; DSC, differential scanning calorimetry; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EDTA, (ethylenedinitrilo)tetraacetic acid.

fective in total fatty acid synthesis and in fatty acid degradation, Davis and Silbert (1974) demonstrated that extensive enrichment of the membrane lipid with unsaturated fatty acids led to altered cell growth, loss of NADH oxidase activity associated with the cytoplasmic membranes, and increased passive permeability. Although an analysis of composition in terms of molecular species was not made, they showed that passive permeability of *o*-nitrophenyl thiogalactoside (ONPG) increased abruptly at compositions of unsaturated fatty acids, and in particular of *cis*-Δ^{11}-18:1, beyond those found in the β oxidation defective control cells. Loss of NADH oxidase activity was correlated with an increased content of *cis*-Δ^{11}-18:1 from 42% in the control strain to approximately 90% in the double mutant. These results suggested that certain functions in the *E. coli* cytoplasmic membrane were dependent for normal activity on a heterogeneous mixture of phospholipids and that excessive enrichment of this membrane with species such as $18{:}1_{c_1}$–$18{:}1_{c_2}$ had deleterious effects.

In this paper, differential scanning calorimetric (DSC) and electron spin resonance (ESR) studies were undertaken to determine how the physical properties of the membrane phospholipids were effected by enrichment with cis unsaturated fatty acids. In these experiments we used phospholipids from a β oxidation defective control strain, largely containing molecular species of types II and III, and from fatty acid synthesis–β oxidation defective multiple mutants, specifically enriched with molecular species of types V or VI. Molecular species V and VI were chosen since type VI was shown to affect membrane activities (Davis and Silbert, 1974). DSC studies were performed first in order to characterize the gel to liquid-crystalline transitions (Steim, 1968; Ladbrooke and Chapman, 1969) that have been shown to affect specific membrane functions (Linden et al., 1973; Fox, 1975) and to obtain quantitative data on the strength of lipid–lipid interactions (Ladbrooke and Chapman, 1969). The ESR spin-labeling technique, which is sensitive to physical changes such as "cluster" formation (Lee et al., 1974) that occur at temperatures above the gel to liquid-crystalline transition, was then used to further characterize the structure of the membrane lipids.

Materials and Methods

Fatty acids were obtained from Nu Check Prep (Elysian, Minn.). Brij 58 and Unisil were purchased from Sigma and Clarkson Chemical Companies, respectively. Tempo was a gift from Dr. Chien Ho and 5-doxylstearic acid was purchased from Synvar. Dioleoyllecithin was a gift of Drs. F. Dombrose and C. M. Jackson.

Strains and Media. Bacterial strains L51, L8-2, and L8-37 are all *E. coli* K-12 derivatives. Strains L51 (formerly AB1623 fadE) and L8-2 (formerly LA2-22 fadE) have been described previously (Davis and Silbert, 1974). Strain L51 has a β-oxidation defect (fadE), while strain L8-2 contains a temperature-sensitive mutation affecting total fatty acid synthesis (fabE1), as well as fadE. Strain L8-37 is a derivative of strain L8-2 that contains not only fabE1 and fadE but also fabB, a mutation eliminating unsaturated fatty acid synthesis. Its construction will be described elsewhere.

Unless otherwise noted, cells were grown overnight at 30 °C and subcultured at 37 ± 0.3 °C in Medium 63 (Pardee et al., 1959) containing 0.4% glycerol, 5 mM glutamate, 1 μg/ml of B_1, and 0.5 μg/ml of yeast extract. Brij 58 was supplied at a final concentration of 1 mg/ml. Growth was monitored with a Klett-Summerson colorimeter at 660 nm.

Preparation of Cytoplasmic Membranes. Membranes were prepared by the lysozyme–EDTA treatment as described by Osborn and co-workers (Osborn et al., 1972).

Lipid Extraction and Analysis. Cells were harvested during logarithmic growth and washed two times at room temperature with Medium 63 containing 800 μg/ml of Brij 58. Total lipid extracts were obtained by the method of Bligh and Dyer (1960) and fractionated into neutral lipids and phospholipids on a silica-gel column (Unisil, 100–200 mesh), with chloroform and then chloroform:methanol (3:2). The phospholipid fraction was concentrated and an aliquot was taken for further purification by thin-layer chromatography (Silbert et al., 1973). The fatty acid composition of this latter material was determined utilizing methanolysis and gas-liquid chromatography as described earlier (Silbert et al., 1973).

Calorimetry: Preparation and Analysis. Phospholipids that had been isolated by column chromatography were dried with a stream of nitrogen and then under vacuum over P_2O_5. Aqueous dispersions of these phospholipids were prepared by adding a fivefold excess (w/w) of either distilled water, 0.05 M Tris-HCl, 10 mM NaCl, pH 7.0, or 0.1 M KPO_4, pH 6.9 (the results obtained were independent of the solvent used). The dispersions were heated under a nitrogen atmosphere to 60 °C and vortexed in the presence of glass beads. After vortexing, the mixtures were placed in aluminum pans and centrifuged, and the excess solvent was removed. The pans were then hermetically sealed and analyzed.

Cytoplasmic membranes or whole cells in either 50% ethylene glycol or 0.05 M Tris-HCl, 10 mM NaCl, pH 7.0, were prepared by centrifugation at 105 000*g* for 2 h onto an aluminum block that was placed at the bottom of a conical Teflon insert (as suggested by Dr. Leonard Banaszak). The excess solvent was removed and the wet pellet was placed in the aluminum pans, sealed, and analyzed.

Differential scanning calorimetry was performed with a DuPont 990 thermal analyzer. Reference pans were prepared by adding alumina so that the total weight of the reference pan was within 5–10% the weight of the sample pan. The samples were cooled to −40 °C and allowed to equilibrate for 20–40 min to reach an isothermal state. Then, several cooling and heating curves were run at rates of 2 or 5 °C/min. The temperature scale and heat determinations were standardized with stearic and palmitic acids and were accurate to within 5%. However, reproducibility from sample to sample was only precise to about 10%.

In analyzing the thermal transitions, the beginning and end of the phase transitions were taken from the onset temperatures of the heating and cooling curves, respectively. The widths of the phase transitions measured from only the heating or cooling curves were broader than the true values. Since the observed width was influenced by sample size and heating rate, the discrepancy noted here would appear to be caused by temperature gradients across the bottom and side of the samples. The reported values were averages of two or three runs on at least two separate samples.

The cooperative units are the ratios of the enthalpies derived from the van't Hoff equation to the calorimetric enthalpies. We calculated the van't Hoff enthalpies (ΔH_{vH}) from the expression (Tsong et al., 1970)

$$\frac{d\gamma}{dT} = \frac{\gamma(1-\gamma)\Delta H_{vH}}{RT^2} \quad (1)$$

where T is the absolute temperature, R is the gas constant, and γ is a measure of the fractional completion of the phase transition. The expression assumes that the process is a two-step transition and that the heat absorption, γ, is a linear measure

TABLE I: Fatty Acid Composition of Strains L51, L8-2, and L8-37 Grown with Various Fatty Acid Supplements[a]

Strain and Supplement	Fatty Acid Composition: Saturated 14	Saturated 16	Saturated T[c]	Monounsaturated[b] 16	Monounsaturated[b] 17	Monounsaturated[b] 18	Monounsaturated[b] T[c]	Extent of Growth[d] (Generations)
1. L51 None	2	27	29	37		34	71	2.8
2. L51 *cis*-Δ^{11}-18:1	3	20	23	24		53	77	3
3. L51 16:0	2	56	58	28		14	42	1
4. L51 16:0	2	63	65	32		3	35	3
5. L51 16:0	1	57	58	39		3	42	9
6. L8-2 16:0 + *cis*-Δ^{9}-16:1	3	35	38	59		2	61	3
7. L8-2 *cis*-Δ^{9}-16:1 + *cis*-Δ^{11}-18:1	1	6	7	42		51	93	2.6
8. L8-2 *cis*-Δ^{11}-18:1	2	26	28	36		36	72	<1
9. L8-2 *cis*-Δ^{11}-18:1	1	8	9	20		71	91	1.5
10. L8-2 *cis*-Δ^{11}-18:1	–	3	3	5		92	97	>3
11. L8-37 *cis*-Δ^{11}-18:1	1	5	6	5		89	94	>3
12. L8-37 *cis*-Δ^{9}-18:1	2	7	9	4		87	91	>3
13. L8-37 *cis*-Δ^{10}-17:1	2	4	6	4	90		94	>3

[a] Cells of the strains shown in the table were grown with the indicated fatty acid supplements and lipid analyses were performed as described under the Materials and Methods. All values are expressed as weight percentage of total fatty acids in the phospholipids. Any components that were ≤0.5%, such as 18:0, are not included in the Table. [b] Unsaturated fatty acids and their cyclopropane derivatives are included together. [c] T is total saturated or monounsaturated fatty acid percentages. [d] Refers to number of generations of growth before harvest.

of the extent of the phase transition. To calculate the ΔH_{vH} for a single phase transition, a series of γ_n was determined as follows: the sharp transitions were divided into 10 strips each of 0.2 °C, while the broad transitions were divided into 30 strips each of approximately 1 °C; the area of each strip was measured with a planimeter or by counting squares; and then γ_n was calculated from

$$\gamma_n = \frac{\sum_{i=1}^{n} a_i}{A_{\text{total}}} \qquad (2)$$

where γ_n is the fractional completion of the phase transition through the nth strip, a_i is the area of the ith strip and A_{total} is the total area under a thermotropic phase transition. The γ_n's were then plotted against the temperature T_n's, where T_n was the temperature corresponding to the midpoint of the nth strip. From the plots of γ vs. temperature, the slope $d\gamma/dT$ was determined at a particular value of γ and T. Using the values of $d\gamma/dT$, γ, and T, ΔH_{vH} was calculated from eq 1. As a test of the starting assumptions that the transition was a two-step process and that γ was a linear measure of the completion of the transition, ΔH_{vH}'s were calculated and compared for several values of T, γ, and $d\gamma/dT$ from a single thermogram. If the values of ΔH_{vH} were constant throughout the transition, the values of the cooperative units were taken to be a meaningful measure of the extent of lipid–lipid interactions.

Electron Spin Resonance Spectroscopy. For the ESR measurements, phospholipid dispersions in 0.05 M Tris-HCl, 10 mM NaCl, pH 7.0, or 0.1 M KPO_4, pH 6.9 (see under calorimetry above), were labeled with either Tempo or 5-doxylstearic acid. The labels were dissolved in ethanol and evaporated to dryness with a stream of dry N_2. The molar ratio of the phospholipid per label was >100:1. After adding the aqueous lipid dispersion, the samples were mixed by vortexing and drawn into a melting point capillary tube. Spectra were obtained on a Bruker x band (Model B-ER 4185) electron paramagnetic resonance spectrometer equipped with a variable temperature controller.

The Tempo partition coefficients between the lipid and aqueous phases were determined according to Shimshick and McConnell (1973). The amplitudes H and P (see Figure 1 in Shimshick and McConnell (1973)) are proportional to the amounts of Tempo in the lipid and aqueous phases, respectively. The parameter α was calculated from the ratio H/P.

Results

Fatty Acid Content of fadE Control Cells and fab fadE Mutant Strains Grown with Various Fatty Acid Supplements. Previous studies with cells of the control strain L51 showed that phospholipids from cells cultured with either 16:0 or *cis*-Δ^{11}-18:1 were enriched in these fatty acids (Davis and Silbert, 1974). The fatty acid compositions were maintained within the range of 20–60% saturated and 40–80% unsaturated fatty acids, and the cells showed the same rate and extent of growth despite these differences in composition (Davis and Silbert, 1974).

The growth pattern of the temperature-sensitive fatty acid biosynthetic mutant (strain L8-2), however, depended on the added fatty acid (Davis and Silbert, 1974). Strain L8-2 grown at 30 °C and subcultured at 37 °C with certain combinations of long-chained saturated and *cis*-monoenoic fatty acids (e.g., 16:0 + *cis*-Δ^{9}-16:1, 17:0 + *cis*-Δ^{10}-17:1) grew at a normal rate and maintained the relative amounts of saturated and unsaturated fatty acids within the range accommodated by strain L51. Cultivation of strain L8-2 with *cis*-Δ^{9}-16:1 led to growth for only one generation and to limited incorporation. Supplementation of strain L8-2 with 16:0 led to enrichment of this fatty acid in excess of that found in the L51, but growth was terminated after approximately one generation due to cell lysis. On the other hand, cultivation of strain L8-2 for several generations at 37 °C with *cis*-Δ^{11}-18:1 led to extensive growth, but at a rate slightly less than normal, and to incorporation of this fatty acid far in excess of that observed in the control strain grown under similar conditions.

Table I, lines 1–6 and 8–10, shows the fatty acid composition of phospholipids isolated from cells of strains L51 and L8-2 supplemented with fatty acids shown in column 2 and grown for the number of generations indicated in the final column.

These data are in agreement with the previous findings of Davis and Silbert (1974) and are presented to show the composition of the phospholipids used in the DSC and ESR studies presented below. Table I, line 7, gives the fatty acid composition of phospholipids from cells of strain L8-2 grown at 37 °C with *cis*-Δ^9-16:1 plus *cis*-Δ^{11}-18:1. Under these conditions, the unsaturated fatty acid content exceeded 90% but was almost equally divided between *cis*-Δ^9-16:1 and *cis*-Δ^{11}-18:1. Moreover, these cells showed a normal rate and extent of growth (data not shown). Extensive enrichment of membrane phospholipids with long-chain *cis*-monoenoic fatty acids other than *cis*-Δ^{11}-18:1 can be achieved by cultivating strain L8-37 with *cis*-Δ^9-18:1 or *cis*-Δ^{10}-17:1 (Table I, lines 11–13). This strain contains fabB as well as fabE1 and fadE mutations so that the synthesis of *cis*-Δ^9-16:1 is virtually eliminated. Growth of strain L8-37 with *cis*-Δ^9-18:1, *cis*-Δ^{10}-17:1, or *cis*-Δ^{11}-18:1 is identical to that of strain L8-2 with *cis*-Δ^{11}-18:1.

Differential Scanning Calorimetry of Membrane Lipids from Strain L51 Cells Grown with either cis-Δ^{11}-18:1 or 16:0. The observations on fatty acid composition presented above demonstrated that the various fatty acid supplements supported normal physiological functions as long as the relative fatty acid composition remained within certain limits. As a first approach to understanding the physical significance of these restrictions, DSC studies were carried out with lipids and cytoplasmic membranes from the fadE control cells grown under conditions that produced different fatty acid compositions within the range discussed above (Table I, lines 1–5). The thermotropic phase transitions of the membrane lipids from strain L51 grown at 37 °C unsupplemented or supplemented with either 16:0 or *cis*-Δ^{11}-18:1 were all between 15 and 25 °C in width. These broad transitions are similar to those observed previously with *E. coli, Acholeplasma laidlawii,* and rat liver mitochondria (Steim et al., 1969; Ashe and Steim, 1971; Blazyk and Steim, 1972; McElhaney, 1974) and are different from the sharp gel to liquid-crystalline transitions observed with pure synthetic phospholipids (Ladbrooke and Chapman, 1969; Sturtevant, 1974). Moreover, the broad melting curves indicate the presence of two domains (gel and liquid-crystalline) throughout the transition and imply that the lipid–lipid interactions of the lipids from strain L51 are less than those found in pure synthetic phospholipids.

Whereas the widths of the melting curves of phospholipids from strain L51 were not markedly affected by fatty acid composition, the shapes and positions of the transitions were dependent on the added fatty acid. In contrast to the symmetrical melting curves for phospholipids from strain L51 grown without supplement or with *cis*-Δ^{11}-18:1 (Figure 1a,b), the DSC curves of phospholipids isolated from cells enriched with 16:0 were nonsymmetrical (Figure 1c–f). The values of ΔH_{vH} calculated from these latter thermograms were not constant throughout the transition (see Materials and Methods). This latter fact indicates that the phase transition was not a simple two-step process in this particular mixture. These findings suggest that the transition had intermediate phases (gel or liquid-crystalline) or that the membrane lipids did not co-crystallize. If the latter explanation is true, the melting curve was a composite of separate overlapping gel to liquid-crystalline transitions from domains that differ in their lipid composition. Although the data do not distinguish between the two possibilities, studies with lipid mixtures of known composition (Phillips et al., 1970) favor the latter explanation. In addition to the nonsymmetrical shapes, the completions of the transitions were shifted to temperatures above 37 °C (growth temperature). The fadE control cells tolerate a percentage of their membrane lipids in the gel state without apparent effect on physiological function. These results are in agreement with observations made on membrane lipids from similar cultures of *Acholeplasma laidlawii* (McElhaney, 1974). An accurate estimate of the amount of gel present could not be determined from the calorimetric data because the observed enthalpy was not linearly related to the temperature, as shown by the variation of ΔH_{vH} throughout the heating curve (see Materials and Methods). The fadE control cells appear to set an upper limit on the saturated fatty acid composition and the amount of gel state in the membrane lipid (Table I, lines 3–5; Figure 1c–f). The cells appear to initially overshoot this limit and then return to it: compare fatty acid composition and DSC curves obtained for the lipids isolated from cells grown for three generations with 16:0 (Table I, line 4; Figure 1d) with that for the lipids from cells grown for one and nine generations with this supplement (Table I, lines 3 and 5; Figure 1c,e).

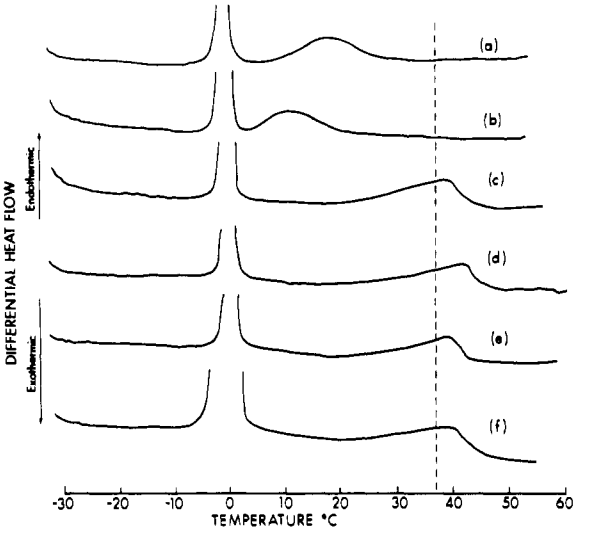

FIGURE 1: DSC curves for phospholipids and cytoplasmic membranes from the fadE control cells (strain L51). Experimental conditions are described under Methods. The data are plotted as excess specific heat vs. temperature. The large endothermic transition centered at 0 °C is due to the melting of the excess water. The DSC curves are for phospholipids from cells grown (a) without supplement for 3 generations, (b) with *cis*-Δ^{11}-18:1 for 3 generations, or (c), (d), (e) with 16:0 for 1, 3, and 9 generations, respectively; (f) is for cytoplasmic membranes from the control cells grown with 16:0 for 1 generation.

Differential Scanning Calorimetry of Membrane Lipids From Strain L8-2 Grown with Various Fatty Acid Supplements. In the course of the composition studies, we had observed that the cells of strain L8-2 had normal growth patterns only if certain combinations of saturated and/or unsaturated fatty acids (e.g., 16:0 and *cis*-Δ^9-16:1 or *cis*-Δ^9-16:1 and *cis*-Δ^{11}-18:1) were supplied. Phospholipids and cytoplasmic membranes isolated from cells of strain L8-2 grown at 30 °C and subcultured at 37 °C with the above combinations had broad thermotropic phase transitions with widths of 19.5 °C (Figure 2a,b) and 12.5 °C (Figure 2c), respectively (see also Table II, lines 5,6). The positions of the thermograms again reflected the overall fatty acid composition and were completed at temperatures below the growth temperature.

Whereas the widths of the thermotropic phase transitions for lipids isolated from cells that showed normal growth were uniformly broad, indicating heterogeneous mixtures of weakly interacting lipids, the widths of the melting curves of lipids from strain L8-2 supplemented with *cis*-Δ^{11}-18:1 were sharp or broad depending on the fatty acid composition (Figure

TABLE II: Thermodynamic Data for the Gel to Liquid-Crystalline Transitions of Phospholipid Dispersions from Strains L51, L8-2, and L8-37 Grown with Various Fatty Acid Supplements[a]

Strain and Supplement	Extent of Growth[b] (Generations)	Temperature of Phase Transition (°C)	ΔT (°C)	ΔH (kcal/mol)	Cooperative Unit (η)
1. L51 None	2.8	12 to 28.5	16.5 ± 1	4.1	20
2. L51 *cis*-Δ^{11}-18:1	3	6 to 20	20 ± 1	4.3	17
3. L51 16:0	1	23 to 43	20 ± 1	4.6	N.D.[c]
4. L51 16:0	3	23 to 48	25 ± 1	4.2	N.D.[c]
5. L51 16:0	9	21 to 42	21 ± 1	3.8	N.D.[c]
6. L8-2 16:0 + *cis*-Δ^{9}-16:1	3	10 to 29.5	19.5 ± 1	3.7	18
7. L8-2 *cis*-Δ^{9}-16:1 + *cis*-Δ^{11}-18:1	2.6	−23 to −10.5	12.5 ± 1	6.5	22
8. L8-2 *cis*-Δ^{11}-18:1	<1	15 to 30	15 ± 1	4.1	15
9. L8-2 *cis*-Δ^{11}-18:1	1.5	−14 to −13	1	8.4	125
		10 to 28	18 ± 1	ND[c]	ND[c]
10. L8-2 *cis*-Δ^{11}-18:1	>3	−14 to −13	1	8.4	140
11. L8-37 *cis*-Δ^{11}-18:1	>3	−14 to −13	1	8.3	129
12. L8-37 *cis*-Δ^{9}-18:1	>3	−15 to −13.8	1.2	9.0	105
13. L8-37 *cis*-Δ^{10}-17:1	>3	−18 to −16.9	1.1	9.2	118

[a] Cells of the strains in the Table were grown with the indicated fatty acid supplements and the thermodynamic parameters (ΔT, ΔH, and η) were determined as described under the Materials and Methods. [b] Refers to the number of generations of growth before harvest. [c] These values were not determined (ND) for reasons stated in the Materials and Methods.

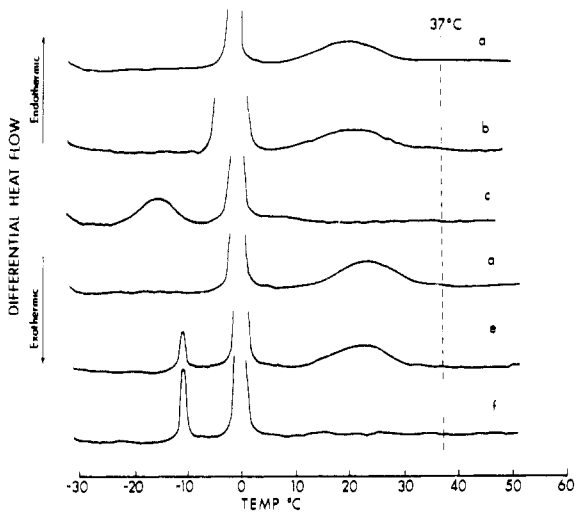

FIGURE 2: DSC curves for isolated phospholipids and cytoplasmic membranes from strain L8-2. Experimental conditions are given under Methods. The data are plotted as excess specific heat vs. temperature. The phase transition at 0 °C is due to the melting of the excess water. The DSC curves are (a) for phospholipids and (b) for cytoplasmic membranes from cells grown with 16:0 plus *cis*-Δ^{9}-16:1 for 3 generations; (c) for phospholipids from cells grown with *cis*-Δ^{9}-16:1 plus *cis*-Δ^{11}-18:1 for 2.6 generations; and (d), (e), (f) for phospholipids from cells grown with *cis*-Δ^{11}-18:1 alone for <1, 1.5, and 3 generations, respectively.

2d–f). During the first generation of growth with *cis*-Δ^{11}-18:1 as supplement, the fatty acid composition of the phospholipids from strain L8-2 was not unlike that of the membrane lipid from strain L51 grown without supplement (compare Table I, lines 8 and 1). The DSC curves for the phospholipids from strain L8-2 showed a broad thermotropic phase transition similar to the melting curve of phospholipids from the control cells (compare Figure 2a and 1a). In contrast to this, the DSC curve of the phospholipids isolated after one generation had two thermotropic transitions, a broad melting species between 10 and 28 °C and a sharp melting species between −14 and −13 °C (Figure 2e). The occurrence of two transitions indicated the presence of two phases that did not co-crystallize. After three generations, a single sharp transition was observed (Figure 2f) that had the same position and width as the −14 to −13 °C transition in Figure 2e. Since the fatty acid composition (Table I, line 10) of strain L8-2 grown for three generations at 37 °C with *cis*-Δ^{11}-18:1 was over 90% *cis*-Δ^{11}-18:1, the lipids were predominately of molecular species type VI ($18{:}1_{c_1}$–$18{:}1_{c_2}$). This homogeneity was reflected in the sharpness of the melting curve (Figure 2f) and is similar to the transitions observed for pure synthetic phospholipids (Sturtevant, 1974; Ladbrooke and Chapman, 1969). The identical positions and widths of this melting curve with the −14 to −13 °C transition of Figure 2e suggest that these phase changes were due to similar molecular species. This would, therefore, imply that the lipid component observed after one generation was also made up mostly of molecular species $18{:}1_{c_1}$–$18{:}1_{c_2}$.

Differential Scanning Calorimetry of Isolated Membranes and Whole Cells from Strain L8-2 Grown for Three to Four Generations at 37 °C with cis-Δ^{11}-18:1. Since the results above were obtained with isolated phospholipids, one could argue that they were an artifact of isolation and did not reflect the physical state of the lipids in the whole cell. Because of the large amount of water, the studies with whole cells were done in the presence of 50% ethylene glycol. Ethylene glycol appeared to have no observable effect on the phase transitions of the isolated membranes: the width, shape, and position of the melting curve of the membranes suspended in ethylene glycol (Figure 3b) were identical with those of cytoplasmic membranes in aqueous media (Figure 3a). The thermograms for the *whole cells* of strain L8-2 that were grown at 37 °C in *cis*-Δ^{11}-18:1 and then suspended in ethylene glycol demonstrated the presence of a reversible phospholipid transition (−14 to −13 °C) and an additional very large, broad, irreversible transition due to protein denaturation (Figure 3c,d). The width, shape, and position of the reversible endothermic curve obtained with the isolated cytoplasmic membranes (Figure 3a) were in agreement with those seen with the isolated lipids (Figure 2f). These findings support the interpretation that the physical properties of the isolated phospholipids or cytoplasmic membranes reflected those of the lipids in situ.

Differential Scanning Calorimetry of Isolated Phospholipids from Cells of Strain L8-37 Highly Enriched with Different Long-Chain Unsaturated Fatty Acids. To test the gen-

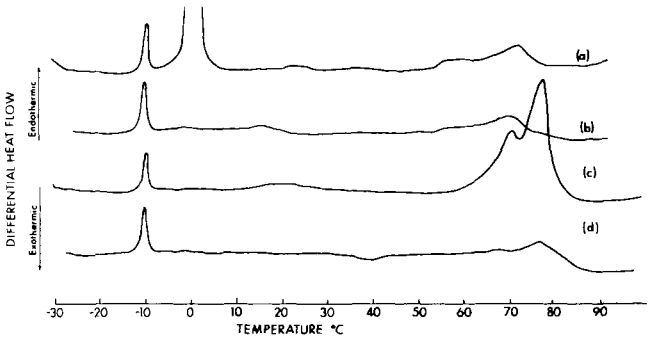

FIGURE 3: DSC curves for cytoplasmic membranes and cells from strain L8-2. Experimental conditions are described under Methods. The data are plotted as excess specific heat vs. temperature. (a) and (b) are for cytoplasmic *membranes* isolated from strain L8-2 grown with *cis*-Δ^{11}-18:1 for >3 generations: (a) was taken in water and the excess endothermic transition at 0 °C was from the excess water; (b) was taken in 50% ethylene glycol; (c) was for *cells* from strain L8-2 grown under the same conditions as in (a) and (b) and taken in 50% ethylene glycol; (d) was a second heating of (c) after protein denaturation.

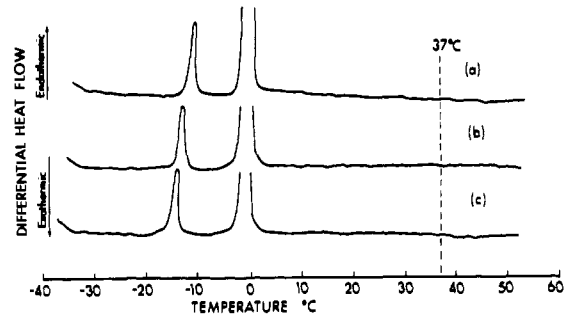

FIGURE 4: DSC curves for phospholipids from strain L8-37. Experimental conditions are described under Methods. The data are plotted as excess specific heat vs. temperature. The DSC curves are for phospholipids from strain L8-37 grown for >3 generations with (a) *cis*-Δ^{11}-18:1, (b) *cis*-Δ^{9}-18:1, and (c) *cis*-Δ^{10}-17:1.

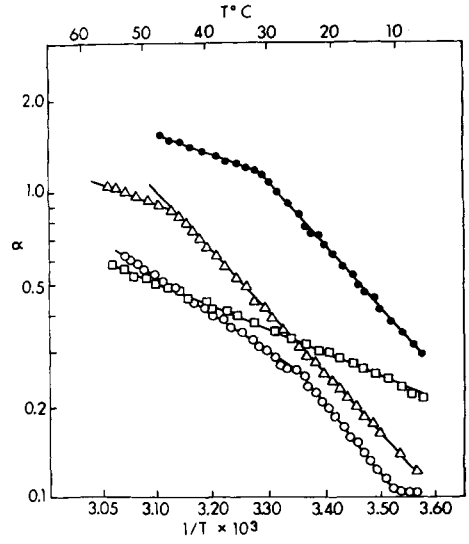

FIGURE 5: Tempo partitioning as a function of $1/T$ for aqueous dispersions of synthetic phospholipids and phospholipids isolated from strain L8-2. Experimental conditions are described under Methods. α is the Tempo solubility parameter (see Methods). The ESR data are for (●) synthetic dioleoyllecithin; and for phospholipids from the double mutant strain L8-2 grown (○) with 16:0 plus *cis*-Δ^{9}-16:1, (□) *cis*-Δ^{9}-16:1 plus *cis*-Δ^{11}-18:1, and (△) *cis*-Δ^{11}-18:1. The plot for the fadE control cell (strain L51) grown without supplement is virtually identical to (○).

erality of the above results, DSC curves of phospholipids isolated from cells of strain L8-37 grown at 37 °C in the presence of *cis*-Δ^{11}-18:1, *cis*-Δ^{9}-18:1, or *cis*-Δ^{10}-17:1 were measured (Figure 4a-c). The phase transitions of lipids from cells supplemented with *cis*-Δ^{11}-18:1, *cis*-Δ^{9}-18:1, or *cis*-Δ^{10}-17:1 were all sharp (approximately 1 °C wide) and occurred at −14 to −13, −15 to −13, and −18 to −17 °C, respectively.

Thermodynamic Parameters. Table II summarizes the thermodynamic parameters taken from the DSC data presented above. In the last two columns we have measured the enthalpies and calculated the cooperative units as described under the Materials and Methods. The cooperative units for the phospholipids from cells of strain L51 enriched with 16:0 were not tabulated, since ΔH_{vH} was found to vary throughout the heating curve. Inspection of the ΔT's, ΔH's, and η values indicated a good correlation between the presence of sharp transitions and increased enthalpies and the size of the calculated cooperative units. Phospholipids that had ΔT's of approximately 1 °C gave η values at least tenfold greater than phospholipids that had ΔT's of 15-25 °C. These findings showed that enrichment of the phospholipids with a single long-chain unsaturated fatty acid led to greater cooperativity in the melting behavior of the molecules.

Melting Behavior of Phospholipids of Cells from Strains L51, L8-2, and L8-37, as Determined by Tempo Partitioning. In order to obtain a picture of the physical state of the membrane lipids at temperatures above the gel to liquid-crystalline transition, spin-labeled Tempo partitioning studies were carried out. In Figure 5, the logarithm of $\alpha = H/P$ is plotted as a function of temperature for aqueous dispersions of dioleoyllecithin and of phospholipids isolated from strain L8-2 grown with various fatty acid supplements. As previously reported by Lee et al. (1974), there was a discontinuity[2] in the plot of Tempo partitioning vs. $1/T$ for dioleoyllecithin at approximately 30 °C. This transition was clearly above the reported gel to liquid-crystalline transition at −22 °C (Phillips et al., 1969) and has recently been associated with the breakup of quasicrystalline structures (clusters) that are believed to be present in the liquid phase of certain polymers (Lee et al., 1974). Plots of the log α vs. $1/T$ for the phospholipids from strain L51 grown without supplement (data not shown but see legend to Figure 5) and strain L8-2 grown with the combination 16:0 and *cis*-Δ^{9}-16:1 were nonlinear, showing abrupt changes at approximately 11 and 26 °C. These discontinuities corresponded to the onset and completion of the phase transitions observed with DSC (Figures 1a, 2a; Table II, lines 1 and 5) and are, therefore, associated with the gel to liquid-crystalline phase transitions. The phospholipid dispersion isolated from cells of strain L8-2 grown at 37 °C with the unsaturated fatty acids *cis*-Δ^{9}-16:1 and *cis*-Δ^{11}-18:1 did not have any apparent discontinuities in the plot of the partition coefficient vs. $1/T$ (Figure 5). Whereas the plots discussed above were continuous above the growth temperature, the phospholipid dispersion obtained from strain L8-2 enriched with *cis*-Δ^{11}-18:1 had a break at about 46 °C (Figure 5). This abrupt change in the slope of the Tempo partition coefficient was about 59 °C above the calorimetrically measured transition and reflects a physical change unrelated to the gel to liquid-crystalline transition (Figure 2f). The fatty acid composition of the lipids of these cells (Table I, line 10) and the sharpness of the thermotropic phase transitions (Table II, lines 10,11) suggest that the physical properties should be those of a pure

[2] In keeping with current usage, discontinuity has been used to describe temperatures at which the measured parameters show changes in slope or are undefined.

TABLE III: Correlation of Transition Temperatures (°C)[a]

Physical Method	Synthetic Dioleoyllecithin	Phospholipids from Strain L8-2 or L8-37 Enriched with Fatty Acid(s) Shown: 16:0 + *cis*-Δ^9-16:1	*cis*-Δ^9-16:1 + *cis*-Δ^{11}-18:1	*cis*-Δ^{11}-18:1	*cis*-Δ^{10}-17:1
DSC	−22[b]	10 to 29.5	−23 to −10.5	−14 to −1[illegible]	−18 to −17
ESR-Tempo partitioning (5–55 °C)	30	11, 26	None	46	27, 40
ESR-5-nitroxylstearic acid order parameter (5–55 °C)	ND[c]	10, 25	ND[c]	45	ND[c]

[a] Summary of data presented in text. [b] Data taken from Ladbrooke et al., 1969. [c] ND, not determined.

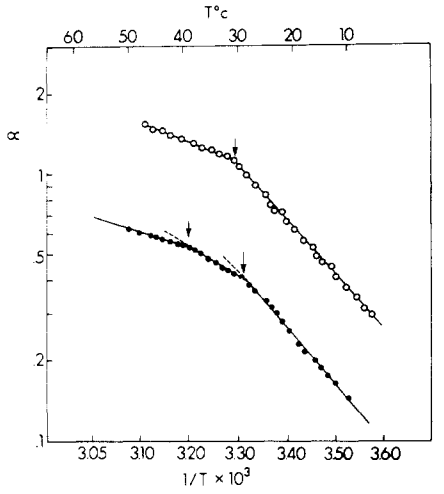

FIGURE 6: Tempo partitioning as a function of $1/T$ for aqueous dispersions of synthetic phospholipids and phospholipids from strain L8-37. Experimental conditions are described in the text. α is the Tempo solubility parameter (see Methods). (○) Data obtained with synthetic dioleoyllecithin; (●) measurements made on phospholipids from strain L8-37 grown with *cis*-Δ^{10}-17:1 for >3 generations.

synthetic phospholipid. Discontinuities at 27 and 40 °C were observed with strain L8-37 grown for three generations at 36 °C on *cis*-Δ^{10}-17:1 (Figure 6) and were, respectively, 44 and 57 °C, above the gel to liquid-crystalline transition. Table III summarizes the transition temperatures determined by DSC and ESR spectroscopy and described here and below for the different phospholipid preparations.

Physical Properties of the Phospholipids from Cells of Strain L8-2 as Determined by the Electron Spin Resonance Order Parameter. We have measured ESR spectra with 5-doxylstearic acid as an independent method of determining phase changes above the calorimetrically determined transitions. Figure 7 shows a representative ESR spectrum obtained with 5-doxylstearic acid incorporated into an aqueous dispersion of dioleoyllecithin. The spectrum is characteristic of rapid anisotropic motion about the molecular axis of the hydrocarbon chain from which the outer and inner hyperfine maxima ($2T_{\parallel}$ and $2T_{\perp}$) can be measured (Hubbell and McConnell, 1971). These can be related to the order parameter, S, that is a measure of local chain flexibility.

When $2T_{\parallel}$ was plotted as a function of temperature for aqueous dispersions of phospholipids isolated from strain L8-2 grown with 16:0 and *cis*-Δ^9-16:1, discontinuities were observed at 10 and 25 °C (Figure 8). These breaks were in agreement with the values obtained from DSC and Tempo partitioning studies (Figures 2a and 5; Table III). Furthermore, in the plot for the phospholipids from strain L8-2 grown with *cis*-Δ^{11}-18:1, a discontinuity occurred at 45 °C. This temperature was above that of the transition taken from the thermogram but again was in agreement with the Tempo partitioning data (Figures 2f and 5; Table III).

Discussion

Recently several groups (Lee et al., 1974; Wunderlich et al., 1975) have suggested the presence of short-lived quasicrystalline structures in the liquid-crystalline phase. On the basis of anomalies in the plots of the partitioning of Tempo between the aqueous and lipid phases and the ESR order parameter of 12-nitroxystearic acid vs. $1/T$, Lee et al. (1974) postulated the existence of clusters in aqueous dispersions of dioleoyllecithin and of membrane lipids from sarcoplasmic reticulum. Moreover, they pointed out the effect of such clusters on function by studying the (Ca^{2+}, Mg^{2+})ATPase of sarcoplasmic reticulum in which the natural membrane lipid was replaced by dioleoyllecithin. The Arrhenius plots of the ATPase activity showed a discontinuity at 29 °C which correlated with the observed change in the physical state of the lipid noted above. Wunderlich et al. (1975), on the basis of ESR, freeze-etch, and fluorescence data, suggested that clusters exist in the endoplasmic reticulum membranes of *Tetrahymena pyriformis* grown at 30 °C. They interpreted the discontinuities in the ESR and fluorescence data at 18 °C as due to phase separation of rigid clusters. In addition, ESR spectra of 12-nitroxylstearic acid indicate that more than one spin-label environment exists up to at least 27 °C. They suggested that above 18 °C the clusters are arranged randomly throughout the liquid-crystalline lipid bilayer. As the temperature is decreased, the cluster size increases and finally, at a temperature just above the observed discontinuity, the clusters reach a critical size and laterally segregate from the rest of the liquid-crystalline phase. This interpretation differs from Lee et al. (1974) who suggested that the discontinuities correspond to the onset of cluster formation and that at temperatures above these breaks no clusters are present. Hence, in the case of membrane lipids from sarcoplasmic reticulum, they imply that the clusters would exist at temperatures ranging from that of the endothermic phase transition to about 25 °C but not at the physiological temperature (37 °C). The results of the present study with *E. coli* membrane lipids demonstrate the presence of discontinuities in the plots of Tempo partitioning (Figures 5 and 6) and 5-nitroxylstearic acid order parameter (Figure 8) vs. $1/T$ at temperatures above the calorimetrically determined gel to liquid-crystalline phase change (Figures 1–4; Table III).

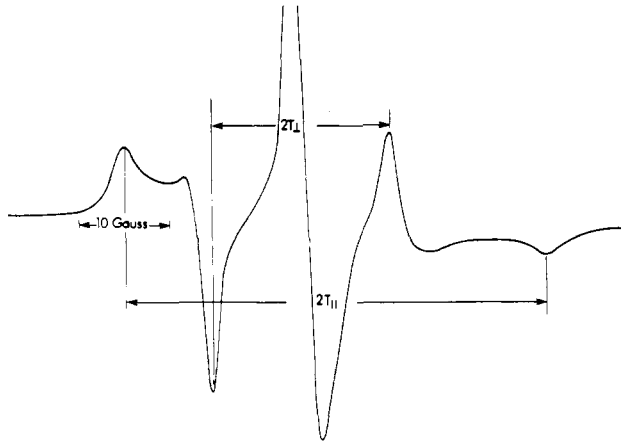

FIGURE 7: Electron spin resonance spectra at 25 °C of 5-nitroxylstearic acid incorporated into isolated phospholipids from strain L51. $2T_{\parallel}$ and $2T_{\perp}$ indicate the separation of the outer and inner hyperfine extrema, respectively (Hubbell and McConnell, 1971).

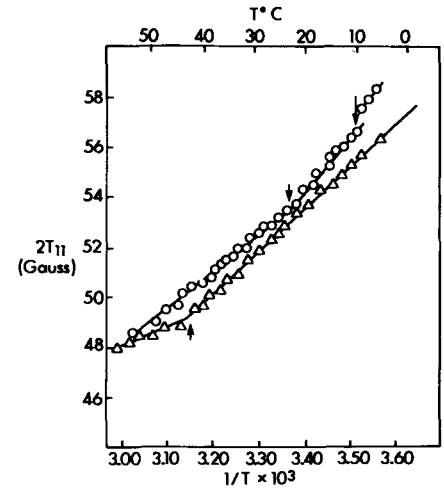

FIGURE 8: The separation of the outer extrema ($2T_{\parallel}$) of 5-nitroxylstearic acid as a function of $1/T$ for aqueous dispersions of phospholipids from strain L8-2. Experimental conditions are described in the text. (○) Data for phospholipids from strain L8-2 grown with 16:0 plus *cis*-Δ^9-16:1 for >3 generations; (△) data for phospholipids from strain L8-2 grown with *cis*-Δ^{11}-18:1 for >3 generations.

Since DSC measured the gel to liquid-crystalline transitions, the breaks in the plots of the Tempo partitioning and of the order parameter vs. $1/T$ for the phospholipids enriched with *cis*-Δ^{11}-18:1 or with *cis*-Δ^{10}-17:1 were due to some other temperature-dependent alteration of lipid structure. The suggestion of Lee et al. (1974) that quasicrystalline structures are present in the liquid-crystalline state of certain phospholipids is a possible explanation for these discontinuities. In the plots of Tempo partitioning vs. $1/T$ for phospholipids enriched in *cis*-Δ^{10}-17:1 there were discontinuities at 27 *and* 40 °C. The data do not presently support any definitive interpretation of such multiple breaks, although they might be indicative of the onset and completion of a lateral phase separation of clusters in keeping with the explanation of Wunderlich et al. (1975).

Regardless of the exact nature of the physical change of the lipids in the liquid-crystalline phase, the present results suggest that the physical change is related to the presence of lipid molecules with two identical cis unsaturated fatty acids. Phospholipids from the fadE control strain that were not enriched with respect to any one fatty acid beyond 60% (Table I, lines 1–5) did not show anomalies in the Tempo partitioning (Figure 5) above the DSC determined phase change. Furthermore, membrane lipids from the cells of strain L8-2 grown with *cis*-Δ^9-16:1 plus *cis*-Δ^{11}-18:1 did not undergo any detectable thermotropic change in the liquid-crystalline state. The latter is particularly informative, since these lipids contained over 90% cis unsaturated fatty acids (Table I, line 7) but retained the intramolecular heterogeneity of the acyl groups characteristic of phospholipids isolated from normal cells. The physical consequence of the loss of heterogeneity with respect to acyl groups on the same phospholipid molecule (Figures 2–4) can be seen by referring to the DSC curves and especially to the data presented in Table II. The widths of the transitions and the size of the cooperative units calculated for isolated phospholipids differing in their fatty acyl group structure (Table II) demonstrated that lipid–lipid interactions increased as intramolecular heterogeneity of acyl groups was reduced. Although the mole fraction of $18{:}1_{c_1}$–$18{:}1_{c_2}$ that precipitates cluster formation has not been determined, our thermodynamic observations suggest that clusters can exist at moderate levels of such molecules in the total membrane lipid (Table II).

From the calorimetrically determined enthalpies and from the enthalpies calculated from the Van't Hoff equation, we estimated that the clusters should be composed of at least 100–150 molecules. Assuming a diameter of 11 Å for a phospholipid molecule containing two *cis*-Δ^{11}-18:1 residues (diameter based on calculation from monolayer and x-ray diffraction data on cross-sectional area) and hexagonal packing, 100–150 molecules would form an area with a diameter of 13–16 nm. Since the van't Hoff calculation yields an estimate of the minimum number of interacting molecules, our data are in good agreement with the finding of Wunderlich et al. (1975) and suggest that cluster formation in *E. coli* membrane lipid mutants grown at 37 °C can be studied by freeze-etch electron microscopy.

Several laboratories (Overath et al., 1970; Esfahani et al., 1971; Linden et al., 1973; Beacham and Silbert, 1973; Morrisett et al., 1975) have found with *E. coli* unsaturated fatty acid auxotrophs that changes in membrane structure and associated activities can be induced at temperatures below the growth temperature by incorporating into the membrane lipid fatty acid analogues not found in the normal cell. Arrhenius plots of some of these activities have one or more discontinuities at temperatures corresponding to the onset, completion, or some intermediate point within the gel to liquid crystalline phase change. Furthermore, in some instances these plots show that the activation energy at temperatures above this transition is influenced by the particular fatty acid composition of the membrane phospholipid (e.g., Beacham and Silbert, 1973). The discontinuities have been also observed in the Arrhenius plots of glucose-6-phosphatase activity associated with membranes of *Tetrahymena pyriformis* (Wunderlich et al., 1975) but in this case they correlate with a physical change in the liquid-crystalline phase presumably related to the presence of lipid clusters. From observations on the correlation of the temperature at which the change in physical structure and the discontinuities in the Arrhenius plots occur, several laboratories (Morrisett et al., 1975) have suggested the existence of lipid domains in normal cells under physiological conditions. However, the data presented here suggest that the normal cell (fadE control), when grown without supplement, regulates its fatty acid composition so that it contains neither gel-like regions nor liquid-crystalline clusters at the growth temperatures (Figures 1a and 5). From previous studies (Davis and Silbert, 1974) and unpublished data, extreme enrichments with cis unsaturated fatty acids have serious effects on membrane

function and structure. Moreover, in contrast to the findings of other investigators, these changes are observed at the growth temperature. Enrichment of the fadE control cells with 16:0 created gel-like regions in the membrane during growth (Figure 1c–f). In strain L8-2, this modification in membrane lipid eventually led to cell lysis (Davis and Silbert, 1974). It is possible that β oxidation serves a regulatory function with respect to controlling the amount of exogenously supplied fatty acid that is incorporated into the phospholipids. In this context, $fadE^+$ fatty acid prototrophs might resist more completely than a fadE strain those changes in the physical state of the membrane lipid induced by growth of the cells with exogenous fatty acids. In any case, the present findings support the conclusion that *E. coli* normally maintains a heterogenous mixture of lipid molecules and, by so doing, prevents strong lipid–lipid associations that lead to the formation of either liquid-crystalline clusters or extensive gel-like domains.

Acknowledgments

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