Randomization of Membrane Lipids in Relation to Transport System Assembly in *Escherichia coli*†

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ABSTRACT: The distribution of newly synthesized lipid molecules in the pre-existing lipid phase of the membrane was studied in whole cells of the fatty acid requiring *Escherichia coli* strain K1062. The fluorescence probe *N*-phenyl-1-naphthylamine revealed reversible lipid phase transitions in cells supplemented with *cis*-Δ^9-octadecenoate (transition temperature T_t = 14°C; width of the transition ΔT = 13°C) or *trans*-Δ^9-hexadecenoate (T_t = 27°C; ΔT = 7°C). Cells were first grown in the presence of *cis*-Δ^9-octadecenoate at 37°C and subsequently for various periods in the presence of *trans*-Δ^9-hexadecenoate at 37 or 22°C, i.e. above or below the transition of the newly formed lipids. Reproducible phase transitions with single, well-defined T_t values between 14 and 27°C were observed under both conditions. β-Galactoside transport induced in a similar experiment before or during a change in the fatty acid composition showed a single change in activation energy at a temperature close to the lipid transition temperature, T_t. Starvation of *cis*-Δ^9-octadecenoate-supplemented cells for this fatty acid led to a gradual rise in the transition temperature, due to an increase in the percentage of saturated acyl chains in the membrane lipids. It is concluded that under all conditions investigated a mixed lipid phase composed of newly synthesized and pre-existing lipid molecules is formed in the membrane. Since conserved domains of newly synthesized lipids surrounding simultaneously formed transport proteins could not be demonstrated, the results do not support a membrane assembly mechanism proposed by N. Tsukagoshi and C. F. Fox [(1973), *Biochemistry 12*, 2822–2829]. It rather appears that newly formed lipid molecules are continuously released from their sites of synthesis into the lipid matrix by a rapid diffusion-controlled process.

The isolation of mutants of *Escherichia coli* requiring unsaturated fatty acids for growth (Silbert and Vagelos, 1967) provided a novel approach to investigate the structure–function relationship of membrane phospholipids. The composition of the fatty acid side chains of the phospholipid moieties in these mutants could be manipulated by merely supplementing the growth medium with a particular long chain length monocarboxylic acid (Silbert et al., 1968; Schairer and Overath, 1969; Esfahani et al., 1969; Fox et al., 1970; Silbert et al., 1974). Using cells and isolated cytoplasmic membranes with defined phospholipid hydrocarbon chain composition, several groups (Schairer and Overath, 1969; Overath et al., 1970, 1975; Wilson et al., 1970; Esfahani et al., 1971; Overath and Träuble, 1973; Sackmann et al., 1973; Linden et al., 1973a,b; Linden and Fox, 1973; Haest et al., 1974; Shechter et al., 1974) have attributed changes in the activation energy of enzymatic and transport activities to the occurrence of order↔disorder lipid phase transitions.

In particular, cells, membranes, and lipid extracts containing *trans*-Δ^9-18:1,[1] *trans*-Δ^9-16:1, or *cis*-Δ^9-18:1 as predominant acyl chain components revealed distinct and reversible lipid phase transitions when analyzed by several methods (dilatometry, fluorescent and spin-label probes, wide-angle x-ray diffraction). A good correlation has been established between the lipid transition temperature, T_t, characteristic for each fatty acid supplement and a corresponding temperature, T_c, where a change in slope of the Arrhenius plot of β-galactoside transport occurs (Overath and Träuble, 1973; Sackmann et al., 1973; Overath et al., 1975).

The mutants proved useful in studies on the mechanism of membrane assembly (Fox, 1969; Wilson and Fox, 1971; Overath et al., 1971a,b; Robbins and Rotman, 1972; Tsukagoshi and Fox, 1973a,b; Nunn and Cronan, 1974). The questions underlying these experiments were: (a) are newly synthesized lipid molecules arranged in patches or growth zones within the plane of the membrane and (b) are newly formed membrane proteins integrated into the membrane together with simultaneously synthesized lipid molecules (coupled insertion)?

A distinction between newly synthesized and previously formed, "old" lipid molecules can be achieved by growing the cells first in the presence of one fatty acid and then in the presence of a second, different fatty acid. Results obtained in two laboratories (Overath et al., 1971a; Tsukagoshi and Fox, 1973b) indicated that the β-galactoside transport protein, inserted into the membrane before or after such a fatty acid shift, was always present in a mixed phase of "old" and "new" lipid molecules when the temperature throughout the experiment assured a fluid state of all lipid components. This randomization of the lipid phase could be readily accounted for by the rapid lateral diffusion of lipid molecules in the plane of the membrane (Träuble and Sackmann, 1972; Devaux and McConnell, 1972; Sackmann et al., 1973; see also Edidin, 1974, for a review). It therefore appeared that lateral diffusion precluded the demonstration of domains of newly synthesized lipids and, consequently, of a coupled insertion mechanism.

The studies discussed so far have inferred the distribution of lipid molecules in the membrane indirectly from the temperature characteristic of transport. A sensitive fluorimetric technique for detecting lipid phase transitions in whole cells (Overath and Träuble, 1973) enabled us to study, in a more direct way, the distribution of newly formed lipid molecules within the pre-existing lipid matrix. Our results indicate that a mixed lipid phase originates under all applied condi-

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tions, notably those recently used by Tsukagoshi and Fox (1973b) for restating their hypothesis of a coupled insertion mechanism of membrane assembly.

Materials and Methods

Strain and Growth Conditions. The fatty acid requiring *E. coli* strain K1062 (Overath et al., 1971a) was grown in Cohen-Rickenberg (CR) mineral salts medium (Anraku, 1967) supplemented with 0.5% glycerol, 0.3% casamino acids (Difco, vitamin-free), 0.5% Brij 35, and 0.01% of either *cis*-Δ^9-18:1, *trans*-Δ^9-16:1, or *trans*-Δ^9-18:1. Cells were grown in 500-ml batches in 2-l. Fernbach flasks with slow rotary aeration. Cells were harvested at an optical density between 1 and 3 at 420 nm by centrifugation and washed at least 5 times in 15 ml of CR buffer without supplements. All harvesting procedures were carried out strictly at 18–20°C. The cells were resuspended in CR buffer to an optical density between 20 and 30 and kept at a temperature of 18–20°C.

For a fatty acid shift, cells were rapidly centrifuged, washed twice with 15 ml of CR buffer containing 0.5% Brij 35, at a temperature of 18–20°C, and resuspended in growth medium, preincubated at the desired temperature. The lactose operon was induced by adding 0.5 m*M* iPrS-Gal.[1]

For fluorescence measurements freshly harvested cells were diluted in CR buffer to an optical density of 0.5–1 at 18–20°C. After addition of a methanolic solution of PhNap[1] to a final concentration of 0.7–1 × 10^{-5} *M* the cell suspension was slowly cooled to 0–4°C and either stored at this temperature or used directly for the fluorescence measurements. The cold cell suspension was transferred to a cuvette and the change in fluorescence was monitored between 0 and 45°C as described before (Overath and Träuble, 1973). The transition temperature obtained in this first increasing temperature scan is designated T_t^1. T_t^2 refers to the transition temperature of the second scan. The transition temperature, T_t, and the width of the transition, ΔT, were obtained as illustrated in Figure 1, curve 5.

β-Galactoside transport was measured as described before (Overath et al., 1971a). The fatty acid composition was determined as described previously (Schairer and Overath, 1969). Chemicals were obtained from the same sources as described before (Overath and Träuble, 1973).

Results

The use of whole cells in the fluorescence measurements reported below deserves brief comment. The transition temperature, T_t, as well as the width of the transition, ΔT (see Figure 1 for definition), as measured with PhNap as a probe, agree remarkably well in whole cells, cytoplasmic membranes, and total lipid extracts (Overath and Träuble, 1973). The change in PhNap fluorescence in both cytoplasmic and outer membrane fractions has been correlated to the order↔disorder transition of the hydrocarbon chains of the phospholipids. The contribution of the outer membrane to the total fluorescence change observed with whole cells was estimated to be ≤20%, because this layer of the cell envelope has a relatively low lipid content and a smaller fraction of the hydrocarbon chains take part in the phase transition (Overath et al., 1975). Moreover, changes in the hydrocarbon chain composition of the phospholipids of both membranes are expected to occur in parallel (see below). The use of whole cells has the advantage that a freshly harvested cell suspension can be analyzed under exactly the same conditions for the temperature dependence of both PhNap fluorescence and β-galactoside transport.

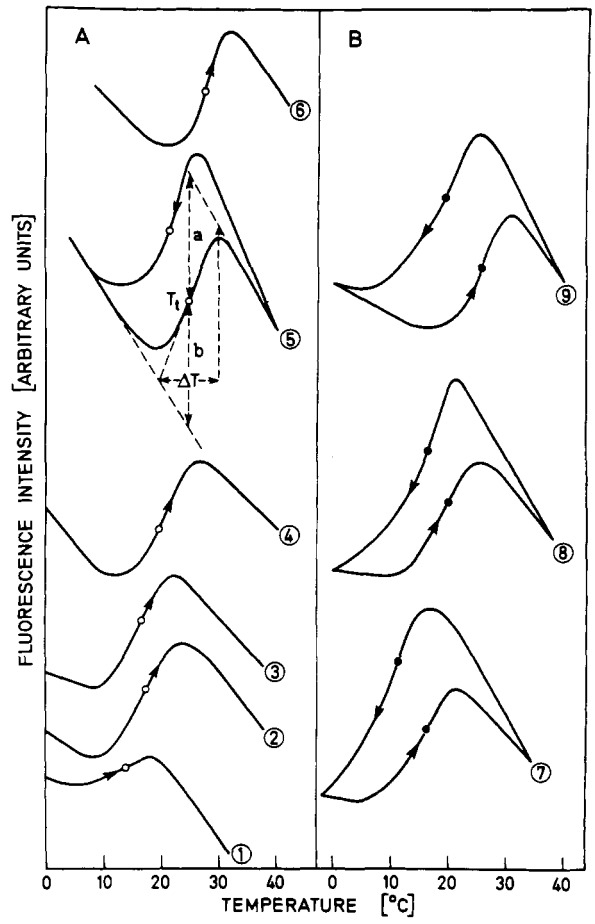

FIGURE 1: PhNap fluorescence as a function of temperature indicates the phase transition in whole cells. The measuring conditions were: excitation at 350 nm; emission at 450 nm; PhNap, 6.7 × 10^{-6} *M*, cell concentration of optical density 0.5 at 420 nm in CR buffer. The temperature was varied at 1–2°C per min. Arrows indicate measurement during an increase or decrease in temperature. The evaluation of the transition temperature, T_t, is obtained as illustrated in curve 5 where $a = b$. ΔT, as indicated in curve 5, is a measure for the width of the phase transition. Curves 1 and 6 refer to cells grown with *cis*-Δ^9-18:1 or *trans*-Δ^9-16:1, respectively. The other curves were obtained for cells which had been shifted from *cis*-Δ^9-18:1 supplement at 37°C to *trans*-Δ^9-16:1 at 37°C (part A, curves 2–5) or at 22°C (part B, curves 7–9). The time of harvest of the samples is indicated by arrows in the inset to Figure 2.

Curves 1 and 6 in Figure 1A show increasing temperature scans for cells grown in the presence of *cis*-Δ^9-18:1 (curve 1, T_t = 14°C, ΔT = 13°C) or *trans*-Δ^9-16:1 (curve 6, T_t = 27°C, ΔT = 7°C), respectively. T_t and ΔT values are always given for increasing temperature scans, since downward scans proved less reproducible.

Fatty Acid Shift from cis-Δ^9-18:1 to trans-Δ^9-16:1. These experiments are illustrated in Figures 1 and 2. A culture is first grown at 37°C in the presence of *cis*-Δ^9-18:1, as shown in the inset to Figure 2. The cells are then transferred to a *trans*-Δ^9-16:1-supplemented medium and fur-

[1] Abbreviations used are: 12:0, dodecanoic acid; 14:0, tetradecanoic acid; 16:0, hexadecanoic acid; 18:0, octadecanoic acid; *cis*-Δ^9-18:1, *cis*-Δ^9-octadecenoic acid; *trans*-Δ^9-18:1, *trans*-Δ^9-octadecenoic acid; *trans*-Δ^9-16:1, *trans*-Δ^9-hexadecenoic acid; PhNap, *N*-phenyl-1-naphthylamine; iPrSGal, isopropyl 1-thio-β-D-galactopyranoside; NphGal, *o*-nitrophenyl β-D-galactopyranoside.

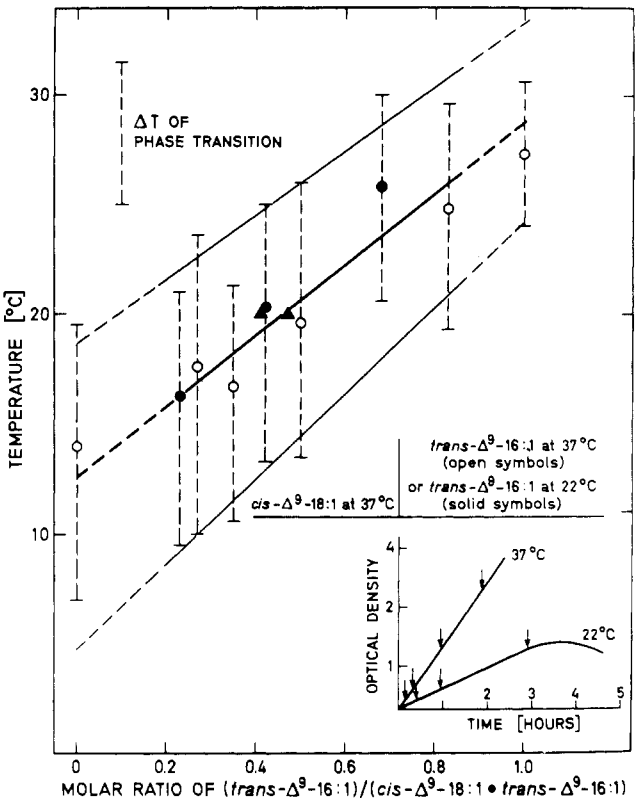

FIGURE 2: The phase transition temperatures from Figure 1 are plotted as a function of the acyl chain composition in the membrane lipids. Cells were grown with *cis*-Δ^9-18:1 fatty acid supplement. At time 0 as indicated in the inset, the cells were shifted to a medium containing *trans*-Δ^9-16:1 as supplement at either 37°C (open symbols) or 22°C (closed symbols). Arrows indicate when samples were taken for fluorescence measurements and fatty acid analysis. The transition temperature was measured as illustrated in Figure 1. The fatty acid composition is given in Table I. The solid triangles represent the experiments from Figure 4. A best fitting straight line is shown for all T_t values except those obtained for cells grown only in the presence of *cis*-Δ^9-18:1 or *trans*-Δ^9-16:1. The upper and lower straight lines are best fitted through the upper and lower ends of ΔT, respectively.

ther grown at 37°C (fraction A) or 22°C (fraction B). Both cultures immediately resume exponential growth. Three hours after transfer fraction A continues to grow exponentially while fraction B stops growing.

The rationale for these experiments can be explained with reference to curves 1 and 6 in Figure 1A. At a growth temperature of 37°C lipids derived from either supplement are in a fluid state. At a growth temperature of 22°C, the lipid phase of *cis*-Δ^9-18:1 supplemented cells is in a fluid state while the lipid phase of *trans*-Δ^9-16:1 supplemented cells is in an ordered state. Therefore, after the fatty acid shift, fraction A is supplemented with *trans*-Δ^9-16:1 at a temperature above, and fraction B below the phase transition of the newly synthesized lipids. Care was taken during the harvesting of the cells (see Materials and Methods) to ensure that the samples of fraction B were not heated above the growth temperature of 22°C before the fluorescence measurements.

Figure 1 shows that samples taken from both fractions reveal single, smooth, and reproducible phase transitions independent of growth temperature. No second phase transition at 27°C indicating separate domains containing only *trans*-Δ^9-16:1 derived lipids was observed. In Figure 2, the transition temperatures obtained from the increasing temperature scans are plotted as a function of the molar ratio

Table I: Fatty Acid Composition (Mole Percent) of Cells Supplemented with *cis*-Δ^9-18:1; Thereafter with *trans*-Δ^9-16:1, Cf. Figures 1 and 2.

No. of Sample[a]	12:0	14:0	*trans*-Δ^9-16:1	16:0	*cis*-Δ^9-18:1
1	4.7	14.7	<1	27.9	52.7
2	4.9	13.6	15.0	25.9	40.6
3	4.8	13.2	20.1	25.0	36.9
4	3.7	12.7	27.3	29.3	27.0
5	3.8	8.3	55.0	21.3	11.6
6	4.7	11.5	64.2	17.3	2.3
7	4.8	13.6	12.8	26.2	42.6
8	4.3	12.8	24.6	24.6	33.7
9	4.3	11.7	39.7	25.4	18.9

[a]Numbers refer to samples indicated in Figure 1.

(*trans*-Δ^9-16:1)/(*cis*-Δ^9-18:1 + *trans*-Δ^9-16:1) using the data reported in Table I. The best fitting straight line through values for both fractions A (open symbols) and B (solid symbols) extrapolates to the transition temperature of cells grown with *cis*-Δ^9-18:1 or *trans*-Δ^9-16:1 as supplement only. Figure 2 also gives best fitting straight lines through the upper and lower ends of the transition width, ΔT. The decrease in ΔT as the percentage of *trans*-Δ^9-16:1 increases indicates that lipids formed after the shift effectively randomize in the pre-existing lipid phase. Cytoplasmic and outer membranes obtained from cells after a fatty acid shift according to the method of Osborn et al. (1972) reveal that the transition temperature in both layers of the cell envelope changes in parallel. The transfer from *cis*-Δ^9-18:1 to *trans*-Δ^9-16:1 supplemented medium at both 37 and 22°C did not change the polar head group composition of the phospholipids (phosphatidylethanolamine, 85%; phosphatidylglycerol, 10%; cardiolipin, 5%). Comparison of the growth curve at 22°C (see inset to Figure 2) and the transition range of the corresponding samples suggests that normal growth takes place when part of the lipids form ordered domains. However, when T_t becomes equal to or higher than the growth temperature, i.e. when a high proportion of the lipids is in an ordered state, further growth is inhibited.

A shift experiment similar to that described in Figure 2 from a *cis*-Δ^9-18:1-supplemented culture grown at 39°C to a *trans*-Δ^9-18:1-supplemented medium at 25°C also indicates the formation of a mixed lipid phase. The latter temperature is 12°C below the transition temperature of cells grown in the presence of *trans*-Δ^9-18:1 only.

Tsukagoshi and Fox (1973b) have also performed the reverse experiment by shifting *trans*-Δ^9-18:1-supplemented cells grown at 37°C to a *cis*-Δ^9-18:1-supplemented medium at 25°C, i.e. a growth temperature $T < T_t$ of the *trans*-Δ^9-18:1-derived lipids. Under similar conditions strain K1062 showed a 6-hr lag period before growth resumed. Therefore, further experiments under these rather unphysiological conditions were abandoned.

Starvation for Unsaturated Fatty Acids. *cis*-Δ^9-18:1-supplemented mutant cultures derive 40–50% of their fatty acids from the growth medium; the remaining 60–50% are saturated fatty acids synthesized endogenously. About the same ratio of mono-cis unsaturated to saturated fatty acids is found in wild type *E. coli* (see, for instance, Silbert et al., 1968). There are three possible ways to decrease this ratio: (1) starvation of a previously *cis*-Δ^9-18:1-supplemented mutant culture (e.g., strain K1062 used in this study) for

Table II: Fatty Acid Composition (Mole Percent) of Cells, First Supplemented with *cis*-Δ^9-18:1, Then Starved for Unsaturated Fatty Acids; Cf. Figure 3.

No. of Sample[a]	12:0	14:0	*cis*-16:1	16:0	*cis*-Δ^9-18:1	18:0
1	7.7	15.7	3.6	28.8	37.1	7.1
2	7.3	18.4	6.0	31.8	32.6	3.9
3	7.1	19.8	3.3	33.1	34.6	2.1
4	10.0	25.9	4.8	31.8	24.7	2.8
5	8.2	28.9	3.5	33.2	23.7	2.5
6	10.4	32.8	3.5	31.3	18.9	3.1
7	13.4	33.2	6.0	31.4	12.2	3.8
8	12.0	38.5	2.8	30.0	14.5	2.2

[a] Numbers of samples are in the same order as indicated by arrows in the inset to Figure 3. The small amount of *cis*-16:1 found in the phospholipids presumably arises by partial degradation of *cis*-Δ^9-18:1. The amount of cyclopropane fatty acids formed was <1%.

this fatty acid (Silbert, 1970); (2) shift of a mutant (strain UC1098; see Cronan and Gelmann, 1973) with a thermosensitive lesion in unsaturated fatty acid synthesis to the nonpermissive temperature; (3) addition of 3-decynoyl-*N*-acetylcysteamine, a specific inhibitor of unsaturated fatty acid synthesis, to a culture of wild type *E. coli* (Kass, 1968).

All three procedures are equivalent in the sense that growth in the absence of unsaturated fatty acids leads to an increase in the percentage of saturated fatty acids in the membrane lipids. A decrease in the amount of unsaturated fatty acids below 15 to 20% of the total fatty acids causes growth inhibition and cell lysis (Cronan and Gelmann, 1973). A large number of studies dealt with the effect of starvation for unsaturated fatty acids on macromolecular synthesis (Henning et al., 1969; Fralick and Lark, 1973) and transport system formation (Fox, 1969; Overath et al., 1971a; Robbins and Rotman, 1972; Nunn and Cronan, 1974). We have analyzed the physical state of the lipid phase under such conditions (cf. Figure 3).

Cells of strain K1062 grown at 38°C in the presence of *cis*-Δ^9-18:1 were transferred to fatty acid free medium. After various growth increments at 38°C (see arrows on inset to Figure 3), samples were processed for fluorescence measurements and fatty acid analysis. All samples revealed a single, well-defined fluorescence change. Figure 3 shows the best fit straight line through the transition temperatures, T_t, as a function of the respective molar ratio of saturated to total fatty acids (cf. Table II). There is a continuous rise in the transition temperature from T_t = 14°C for the unstarved culture to T_t = 38°C, a value close to the growth temperature. T_t extrapolates to T = 52°C, a reasonable estimate for the transition temperature of lipids containing only saturated acyl chains.

This result is completely analogous to that of the experiment described in the previous section where cells were shifted to *trans*-Δ^9-16:1 at 22°C. Lipids containing saturated acyl chains, although synthesized throughout the starvation period, at a temperature below their transition temperature, mix with the existing *cis*-18:1-containing lipid phase. When T_t of the resulting lipid mixture rises above the growth temperature, further growth is inhibited and the cells begin to lyse. Table II indicates that the cells partially compensate for the decrease in fluidity of the membrane lipids by the formation of a higher proportion of 12:0 and 14:0 (Silbert, 1970).

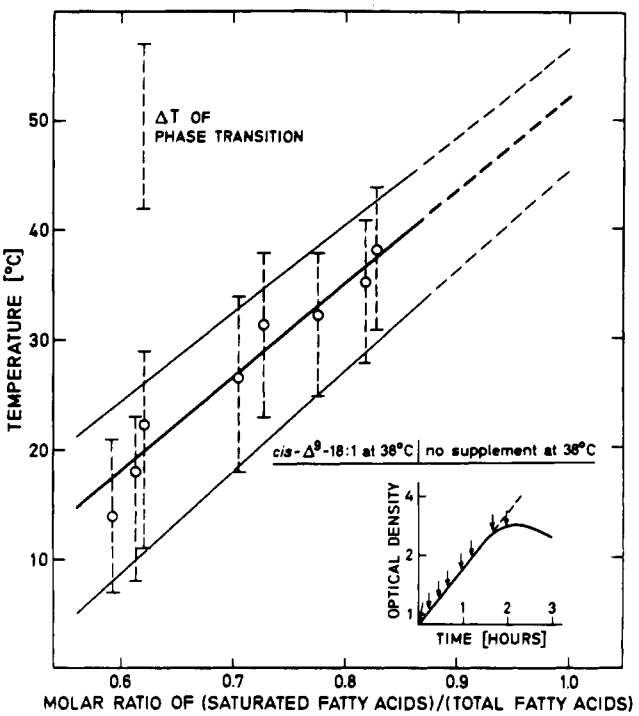

FIGURE 3: Transition temperature, T_t, as a function of increasing amounts of saturated acyl chains in the membrane lipids. Strain K1062 was supplemented with *cis*-Δ^9-18:1 at 38°C. At time 0 as indicated in the inset, the cells were transferred to a medium without a fatty acid supplement. After various growth increments at 38°C, samples were taken (arrows) for fluorescence measurements and analysis of fatty acid composition. Fluorescence was measured with whole cells as described in Figure 1. The detailed fatty acid composition of the samples is given in Table II. The best fitted straight line through the T_t values extrapolates to 52°C. Best fitting straight lines are also shown for the upper and lower ends of ΔT.

Similar results were obtained with the thermosensitive mutant, strain UC1098, mentioned above. This strain, kindly donated to us by Dr. J. E. Cronan, Jr., was grown at 30°C without any unsaturated fatty acid supplement and then shifted to a growth temperature of 34°C. After a growth increment of 350% at this temperature, the cells began to lyse. A crude membrane fraction of these cells obtained by sonication at 0°C displayed a smooth and reproducible thermal transition at T_t = 35°C, ΔT = 14°C. There was no evidence for a second transition at higher temperatures up to 70°C.

Induction of the Lactose Transport System before and after a Fatty Acid Shift from cis-Δ^9-18:1 at 38°C to trans-Δ^9-16:1 at 22°C. The protocol of this experiment (see inset at the top of Figure 4) is identical with that described above (cf. fraction B, Figures 1 and 2). In this instance, the inducer of β-galactoside transport, iPrSGal, was added to the cultures either after (Figure 4A) or before (Figure 4B) the shift to the *trans*-Δ^9-16:1-supplemented medium. After a 30% increase in optical density at 20.5 or 22°C, respectively, cells were harvested at 18–20°C. One-half of each batch was heated for 10 min at 37°C.

The upper parts of Figure 4 show Arrhenius plots of β-galactoside transport without (open circles) and with prior heating (crosses) of the cells to 37°C. Although leakiness of the heated samples causes larger scatter of the measurements at low temperatures, the values of both unheated and heated samples are clearly superimposable yielding extrapolated transition temperatures of T_c = 20 and 20.5°C for cells induced after or before the medium shift, respectively.

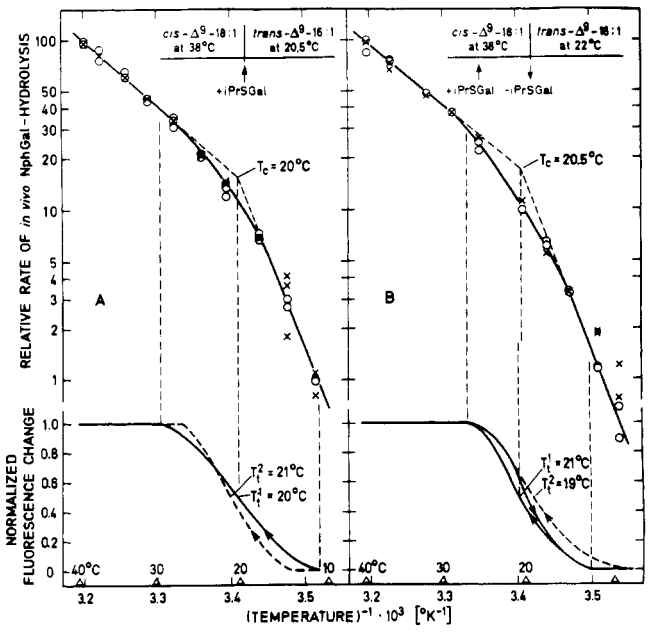

FIGURE 4: The temperature dependence of in vivo NphGal hydrolysis is compared with the phase transition as seen by PhNap fluorescence of whole cells. A culture was supplemented with *cis*-Δ^9-18:1 at 38°C and then transferred to a medium containing *trans*-Δ^9-16:1 as supplement at 20.5 or 22°C. The lactose transport system was induced by the addition of 0.5 m*M* iPrSGal, during a growth increment of 30% either in the presence of *trans*-Δ^9-16:1 (A) or in the presence of *cis*-Δ^9-18:1 (B) as indicated at the top of the graphs. Before measurements of both fluorescence and transport the cells were kept at strictly 18–20°C (open circles on transport curve). A fraction of the cells, however, was incubated at 38°C for 10 min, then again stored at 18–20°C until transport measurements (crosses). Fluorescence was measured as described in the legend to Figure 1. The transition curves were normalized by expressing the vertical distance from the lower extrapolate to the fluorescence curve as a fraction of the vertical distance between the lower and upper extrapolates (see curve 5, Figure 1). The temperature where this fraction is 0.5 is defined as the transition temperature, T_t. The first temperature scan gives T_t^1; the second scan gives T_t^2. The arrows on the fluorescence curves indicate the direction of the temperature scan. T_c gives the extrapolated transition temperature obtained from the transport measurements.

These T_c values agree with the transition temperatures T_t, obtained from the normalized fluorescence changes as shown in the lower part of Figure 4. Moreover, the T_t values obtained for the first (T_t^1 = 20°C, Figure 4A; T_t^1 = 21°C, Figure 4B) and second temperature scans (T_t^2 = 21°C, Figure 4A; T_t^2 = 19°C, Figure 4B) are the same within experimental error. Qualitatively similar results were obtained when the cells were induced during a shorter growth increment of only 15%. The change in activation energy from 17 to 50 kcal mol^{-1} is in agreement with that observed previously for cells grown in the presence of a single supplement (Overath and Träuble, 1973; Overath et al., 1971a). It occurs over a broader temperature range than reported previously (Overath and Träuble, 1973). However, this discrepancy may simply be due to the more extensive measurements presented in this paper.

These experiments do not support the contention of Tsukagoshi and Fox (1973b) that newly synthesized transport protein associates with phospholipids synthesized at the same time. These authors reported that the transport system induced under similar conditions as described in Figure 4A gave rise to a triphasic Arrhenius plot yielding two T_c values for molecules incorporated into separate lipid domains composed of molecules synthesized before or after the change in the supplement. Heating the cells then resulted in a biphasic Arrhenius plot with only one T_c value characteristic of a mixed lipid phase. Such a change in the temperature dependence is clearly not observed in Figures 4A or B suggesting that no further randomization of the lipid phase takes place during the 10-min long incubation at 37°C. In accordance with this result, the width of the fluorescence transition does not change significantly between the first and second increasing temperature scans. Sharpening of the transition would be expected if the first heating above the transition of *trans*-Δ^9-16:1-derived lipids (cf. Figure 1, curve 6) causes a randomization of the lipid phase.

Discussion

Membrane Assembly. One aspect of the complex biosynthetic process of membrane assembly (see Mindich, 1973, for review) is the insertion of newly made lipids and proteins into the membrane. Regarding the insertion of lipids, the present investigation attempts to differentiate between two mechanisms. (1) Since the enzymes responsible for phospholipid synthesis are themselves membrane bound (Kanfer and Kennedy, 1964; Bell et al., 1971; White et al., 1971), the most likely mechanism will be the continuous release of phospholipid molecules into the bulk lipid matrix by diffusion. (2) An alternative mechanism of lipid insertion has been proposed by Tsukagoshi and Fox (1973b; see also Fox, 1969; Wilson and Fox, 1971). These authors envision that newly synthesized lipid molecules first assemble in the form of (vesicular?) bilayers and are then integrated in a second step into the membrane in toto by an unknown process (membrane fusion?). Thus, newly inserted lipid molecules will form separate domains within the pre-existing lipid domains. Only in a subsequent, third step will lateral diffusion cause an intermixing of these domains provided both types of domains are in a fluid state. However, this randomization is thought to be prevented when the molecules in either the newly integrated or the pre-existing domains are in an ordered state below their respective transition temperature. This latter argument provides the rationale for the temperature decrease at the time of the medium shift from one to the other fatty acid supplement (cf. Figures 1, 2, and 4).

The lipid distribution is inferred from fluorescence measurements of lipid phase transitions. Under all conditions investigated (Figures 1–4) one observes smooth transition curves defining a single transition temperature, T_t, which depends on the molar ratio of the fatty acids in the membrane lipids. Therefore, these experiments give no indication for the occurrence of conserved domains of newly synthesized lipids whether the growth temperature is above or below the transition range of the newly formed lipids.

Another method to obtain information about the distribution of pre-existing and newly formed lipid molecules is by observing the behavior of a membrane protein as it reacts to temperature-induced changes in the surrounding lipid matrix. In the case of the first lipid insertion mechanism, a transport protein is simply inserted into a randomized lipid phase. According to the second lipid insertion mechanism as proposed by Tsukagoshi and Fox (1973b), the transport protein first associates with the newly formed lipid molecules and is then inserted into the membrane as a lipo-protein complex. It, therefore, appeared possible to deduce from the temperature dependence of transport if newly inserted lipid domains are conserved or not. Contrary to the findings of Tsukagoshi and Fox (1973b) we find that the

temperature characteristic of transport reveals a single extrapolated transition temperature, T_c, which is close to the transition temperature, T_t, of the order↔disorder transition. The temperature dependence is similar whether the transport protein is incorporated before or after the change in the lipid fatty acid composition and its shape remains unaltered by heating the cells to 37°C (cf. Figure 4). The experiments reported in this paper, therefore, do not support an assembly mechanism in which the transport protein is inserted into extended domains of lipid molecules synthesized at the same time.

The methods applied here are evidently inadequate to rule out the existence of an initial insertion complex if such a complex has a small lifetime and/or if the number of lipid molecules involved is too small to maintain separate domains in the ordered state. Furthermore, all arguments, pro and con, about a coupled insertion mechanism rest on the assumption that discontinuities observed in Arrhenius plots of NphGal hydrolysis reflect an effect of the lipid environment on the carrier protein itself.

The data reported in Figure 3 can be related to several experiments (Overath et al., 1971a; Robbins and Rotman, 1972; Nunn and Cronan, 1974) where the synthesis of the transport protein was induced during starvation for unsaturated fatty acids. Formation of a functional transport protein occurs as long as the lipid phase is fluid, i.e. the growth temperature is higher or equal to the midpoint of the phase transition, T_t. If T_t rises to or exceeds the growth temperature, assembly of a functional transport system becomes abortive (Tsukagoshi and Fox, 1973a). Evidently, these effects on transport system formation may be only one aspect of a more general breakdown of membrane assembly and function under these unphysiological conditions.

Finally, it should be emphasized that the possibility of a lipid–lipid separation occurring during the membrane assembly process must be clearly distinguished from a separation of different lipid molecules into ordered and fluid domains formed throughout the temperature region of a lipid phase transition as discussed for lipid mixtures and *E. coli* membranes by Shimshick and McConnell (1973), Linden et al. (1973b), and Kleemann and McConnell (1974). This proposal refers to the mechanism of the phase transition (see Overath et al. (1975) for an alternative interpretation) which is not directly relevant for the conclusions drawn in this paper.

Lipid Phase Transitions in Relation to Growth of E. coli. Figures 2 and 3 indicate that growth of *E. coli* is possible when part of the lipid phase is in the ordered state. However, when the ratio of fluid to ordered molecules falls below 1, i.e. when the growth temperature is equal to or smaller than T_t, further growth is inhibited. A similar response is observed in *Acholeplasma laidlawii* (Steim et al., 1969; McElhaney, 1974). Since integral proteins are excluded from regions of ordered lipid molecules (Verkleij et al., 1972; James and Branton, 1973; Kleemann and McConnell, 1974; Haest et al., 1974; Shechter et al., 1974), part of the membrane appears to be nonfunctional under such growth conditions.

On the other hand, it may be asked if domains of ordered lipid molecules in the membrane are *required* for normal function. The following reasoning argues against such a hypothesis. *E. coli* mutant cells grow with about the same generation time in the presence of *cis*-Δ^9-18:1, *trans*-Δ^9-16:1, or *trans*-Δ^9-18:1 at a temperature of 38–40°C. This temperature is 20°C above the high-temperature end of the transition in *cis*-Δ^9-18:1-supplemented cells (cf. Figure 1A), about 7°C above the transition in *trans*-Δ^9-16:1-supplemented cells (cf. Figure 1A), and within the upper part of the transition in *trans*-Δ^9-18:1-supplemented cells (Overath and Träuble, 1973). Therefore, at 38–40°C the percentage of ordered hydrocarbon chains will be very different in these three cell types. It appears unlikely that the cell could cope with such a variation if ordered domains play an important role in membrane assembly and function.

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Low and High pH Form of Cadmium Carbonic Anhydrase Determined by Nuclear Quadrupole Interaction†

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ABSTRACT: The pH dependence of the nuclear quadrupole interaction between the excited 247-keV state in ^{111}Cd bound to the active site in human carbonic anhydrase B and the nearest protein surroundings has been studied by means of the nuclear spectroscopic technique of perturbed angular correlation of γ rays. The enzyme has been studied in the pH region 5.6-11.0 at 22 and −196°C. The results show that the Cd enzyme changes from one form at low pH to another form at high pH both at 22 and −196°C. The pK of the transition is 8.9 ± 0.2 at −196°C and close to 9 at 22°C. Parallel to this transformation, the esterase activity of the Cd enzyme for the hydration of *p*-nitrophenyl acetate exhibits a pH dependency with a pK of 9.1 ± 0.2. The sulfonamide inhibitor acetazolamide completely inhibits this activity of the Cd enzyme. The quadrupole interaction parameters for the Cd enzyme are not significantly different at −196°C from those obtained at 22°C. A measurement at 0°C pH 5.7 shows, however, a form different from those at 22°C pH 5.6 and −196°C pH 5.7. The change in the quadrupole interaction with pH is, in a simple model, consistent with an ionization of a metal-bound water molecule.

Carbonic anhydrase is a zinc metalloenzyme which contains one tightly bound zinc ion essential for activity. The enzyme catalyses the reversible hydration of carbon dioxide, as well as the hydrolysis of esters and the hydration of aldehydes (for an extensive review see Lindskog et al., 1971). X-ray diffraction studies of human carbonic anhydrase B and C have established that the enzyme is almost spherical in shape with a radius of about 20 Å and that the Zn^{2+} ion is located at the bottom of the 12-15 Å deep active site cavity with three histidine residues and possibly a solvent molecule as its ligands (Kannan et al., 1975; Liljas et al., 1972). The essential Zn^{2+} can be replaced with other divalent metals, e.g., Co^{2+}, Mn^{2+}, Cd^{2+}, and Cu^{2+}. The Co and Mn enzymes are the only derivatives which so far have been shown to exhibit substantial esterase and CO_2 hydration activity (Lindskog et al., 1971; Lanir et al., 1975). However, as demonstrated in this paper, the Cd enzyme also exhibits activities but the pH-activity profile is shifted to higher pH value as compared to the Zn and Co derivatives.

Although numerous techniques have been employed to investigate the structure-function relationships of this enzyme (Lindskog et al., 1971), the mechanism of the enzymatic reaction has not been established with certainty (Lindskog and Coleman, 1973). It has been shown that a basic form of a group closely linked to the metal ion and having a pK near 7 is involved in catalysis. Spectroscopic changes with pH for various metal derivatives of carbonic

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