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ASSEMBLY OF LIPIDS AND PROTEINS IN ESCHERICHIA COLI MEMBRANES

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Presently there is much interest in the relationship between the structure and function of biological membranes. An approach to the understanding of this relationship has been the study of the effect of the modification of the membrane lipids on the function of membrane-associated activities. In our laboratories we have modified the apolar portion of the membrane lipids of unsaturated fatty-acid auxotrophs of Escherichia coli and investigated the effect of such modifications on enzymes of the electron-transport system. From these studies we were able to conclude that E. coli regulates the relative fatty-acid content of its phospholipids and maintains a certain membrane fluidity necessary for proper membrane function (1–3). We have also proposed that lipids are heterogeneously distributed within the membrane in domains of differing fluidity (4). The studies of McConnell, Chapman, and others (5–13) have corroborated these concepts and extended them to other biological and model membranes. In this paper we review some of our previous results and present evidence to show how NADH and D-lactate oxidases of E. coli membranes are influenced by the fluid states of membrane phospholipids. Preliminary evidence is also presented to show that biogenesis of membranes probably occurs by independent insertion into the membranes of lipids and proteins which upon subsequent interaction with each other form the functional lipoprotein units.

REGULATION OF FATTY-ACID INCORPORATION INTO THE PHOSPHOLIPIDS OF E. COLI MEMBRANES

The growth requirements of unsaturated fatty-acid auxotrophs can be met by a variety of unsaturated fatty acids. When each member of a structurally homologous series of cis-unsaturated fatty acids serves as a growth factor for the auxotroph, the percentage of incorporation of unsaturated acids into phospholipids increases with increasing chain length or decreasing number of double bonds in the apolar chain of the fatty-acid supplements (Table I). Increasing the chain length from cis-Δ^9-$C_{14:1}$ to cis-Δ^9-$C_{18:1}$ results in an

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TABLE I. Effect of Exogenous Unsaturated Fatty Acids on the Percentage of Saturated and Unsaturated Fatty Acids of Phospholipids of Ol_2^- Strain

Fatty acid added to growth medium	Fatty acids in phospholipids (%)		
	Saturated	Unsaturated	Unknown
cis-Δ^9-$C_{14:1}$	70	29	1.4
cis-Δ^9-$C_{16:1}$	52	46	1.5
cis-Δ^9-$C_{18:1}$	45	53	2.0
All cis-$\Delta^{9,12}$-$C_{18:2}$	60	37	2.5
All cis-$\Delta^{9,12,15}$-$C_{18:3}$	66	30	4.4
cis-Δ^{12}-$C_{18:1}$	45	53	2.3
trans-Δ^{11}-$C_{18:1}$	29	69	1.6
trans-Δ^9-$C_{18:1}$	27	65	7.4

increase in the relative amounts of incorporated unsaturated fatty acids from 29% to 53%. Likewise, the percentage of unsaturated fatty acids in the lipid decreases from 53% to 30% with a corresponding increase in the number of double bonds from one to three of the supplemental fatty acids. Furthermore, there is no difference in the ratio of saturated to unsaturated fatty acids in the phospholipids isolated from cells grown on fatty acids of the same chain length but differing only in the position of the unsaturated bond as found in cis-Δ^9-$C_{18:1}$ to cis-Δ^{11}-$C_{18:1}$.

The effect of chain length and degree of unsaturation of fatty acids on the physicochemical characteristics of phospholipids have been well documented (14, 15). In these studies, shortening the chain length or increasing the degree of unsaturation in the fatty-acyl residues results in increasing fluidity of the phospholipids and lowering of the temperature at which gel–to–liquid-crystalline phase changes are observed (16, 17). Thus, in the E. coli membranes the expanding effect of the unsaturated fatty acid is counterbalanced by the condensing effect of the increased level of saturated acids. Physical properties of phospholipids containing trans-unsaturated acids are intermediate between those containing saturated fatty acids and ones containing the corresponding cis-unsaturated acids (18). Thus, in the presence of trans-unsaturated fatty acids the organism incorporates significantly higher levels of this acid into the membrane phospholipids – for example, strain civ-2 fao-6 (a derivative of strain Ol_2^- unable to oxidize fatty acids) incorporates from the medium up to 87% of the fatty-acid content of the phospholipids as elaidate.

Cis-eicosenoic acid ($\Delta^{11}C_{20:1}$) can also meet the unsaturated fatty acid requirement of the auxotroph strain Ol_2^- (Table II). However, in doing so this acid is extensively degraded to the corresponding C_{16} and C_{18} unsaturated homologs prior to its incorporation into phospholipids (1, 2). In comparison strain civ-2 fao-6 when grown on eicosenoic acid incorporates only 30% of the acid into the membrane phospholipids (3, 19). However, in order to compensate for the condensing effect of eicosenoic acid, the cells increased the myristic acid content of the phospholipid to the unprecedented level of 25%. In this way the organism was able to regulate not only the amount of fatty acids taken up from the medium and incorporated into phospholipids but also the chain length of fatty acids synthesized by its synthetase. To achieve this type of regulation one would have to assume

TABLE II. Fatty-Acid Composition (Percentage) of Phospholipids of Ol_2^- and Civ-2 Fao-6 Strains Grown on cis-Δ^{11}-eicosenoic Acid

Fatty acids in phospholipids	Ol_2^- strain (%)	civ-2 fao-6 strain (%)
$C_{14:0}$	2	25
$C_{14:0}$	41	46
$C_{16:1}$	10	–
$C_{18:0}$	2	–
$C_{18:1}$	31	–
$C_{20:1}$	12	30
Unknown	2	–

that the enzymes responsible for lipid synthesis respond to the physical properties of the lipids produced and are juxtapositioned with the fatty-acid synthetase. They are thereby able to regulate the latter by incorporating into the lipid the synthesized fatty acid when it is at the C_{14} state and not at the usual C_{16} chain length. For the moment it is not obvious how this is achieved, but it is reasonable to assume that the fatty-acid synthetase is located very close to the phospholipid synthesizing enzymes and hence near or on the cytoplasmic membranes, where it can readily supply the required acid for lipid synthesis.

The activity of the fatty-acid synthetase in vivo appears to be in tune with the overall requirements of the cell for fatty acids (2). Resting cells of strain civ-2 fao-6 supplemented with elaidate require a relatively low amount of saturated fatty acids (Table I). The activity of the fatty-acid synthetase, measured by the incorporation of ^{14}C-acetate into fatty acids, is less than it is for cells grown on linolenate, which have been shown to require five times the relative amount of saturated fatty acids (Table III). Likewise, resting cells grown on oleate, which incorporate an intermediate amount of saturated fatty acids, display an intermediate level of fatty-acid synthetase activity. Therefore, the amount of fatty acid synthesized from ^{14}C-acetate by resting cells was highest in those cells which contain the lowest level of unsaturated fatty acid in the phospholipid. These results suggest again the existence in E. coli of a regulatory mechanism between fatty-acid synthetase and the enzymes of phospholipid synthesis.

TABLE III. Incorporation of ^{14}C-Acetate into Fatty Acids by Resting Cells of Civ-2 fao-6 Mutants

Fatty acid added to growth medium	^{14}C-fatty acid synthesized (cpm/mg cells; dry weight)	Unsaturated fatty acids in phospholipids (% of total)
Elaidic	10,500	87
Oleic	17,800	59
Linolenic	42,000	37

EFFECT OF VARIOUS UNSATURATED FATTY ACIDS ON ACTIVITIES OF MEMBRANE-ASSOCIATED ENZYMES

Further studies were conducted to determine the effect of lipid alterations on the activities of membrane-associated enzymes (20–22). In these studies membrane vesicles were derived from cells grown on various fatty acids, and the activities of some of their enzymes were measured in response to changes in temperature. The resulting Arrhenius plots of the activities exhibited discontinuities reflecting changes in the activation energies of the reactions. The important feature of this type of measurement is that the temperature of discontinuity is directly related to the fatty-acid supplement utilized for growth. As can be seen in Fig. 1, Arrhenius plots of NADH oxidase activity showed transition temperatures of 32°, 29°, 19–21°, and 18–20°C for membrane vesicles (strain Ol_2^-) grown on palmitelaidate, elaidate, oleate, and linolenate, respectively (20). Similar discontinuities appear with the D-lactate oxidase activity of the same membrane vesicles, giving transition temperatures of 32°, 32°, 19–21°, and 18–19°C, respectively (21). Reconstitution studies with the succinate-ubiquinone reductase have presented direct evidence that these discontinuities are indeed due to the respective lipid components of the membrane (22). Further investigations have shown this to also be the case for the D-lactate oxidase (21).

The lower activity of NADH and D-lactate oxidases in vesicles grown on elaidate and palmitelaidate, compared with the activity of those derived from cells grown on oleate and linolenate, should be noted (Fig. 1). The higher activity for enzymes associated with oleate- and linolenate-containing membranes is consistently observed and may reflect a difference in the effect of trans acids on protein conformation as monitored through these measurements. The lower activity may be due to the condensing effect of the trans acid present in the membrane phospholipids. The effect of trans acids on cellular activities appears to be of a more general nature. Mutants grown on elaidate, for example, are temperature-sensitive and undergo rapid loss of viability and eventual lysis when they are shifted from 37° to 27°C. At the lower temperatures, the biosynthesis of both DNA and RNA in these cells is also adversely affected (2).

CHANGE IN D-LACTATE AND NADH OXIDASE ACTIVITIES AFTER A FATTY-ACID SHIFT

In a previous communication (4) evidence was presented for the presence of a non-uniform viscosity or a heterogeneity in the distribution of lipids along the bilayer of plasma membrane. Recent studies on model systems of mixtures of phospholipids and membrane preparations have provided further evidence for the cluster formation and phase segregation of lipid components within the membrane (6, 8, 10, 21–25). Thus, it was of interest to determine if cells grown on one fatty-acid supplement, washed free of the acid, and then shifted to a growth medium containing a second fatty acid would maintain two separate phases, yielding discontinuities in the Arrhenius plots indicative of the transition temperature obtained in membranes containing each of the individual fatty acids (20, 21). Indeed it was found that the NADH oxidase activity of vesicles isolated from cells grown first on elaidate and then transferred to media containing

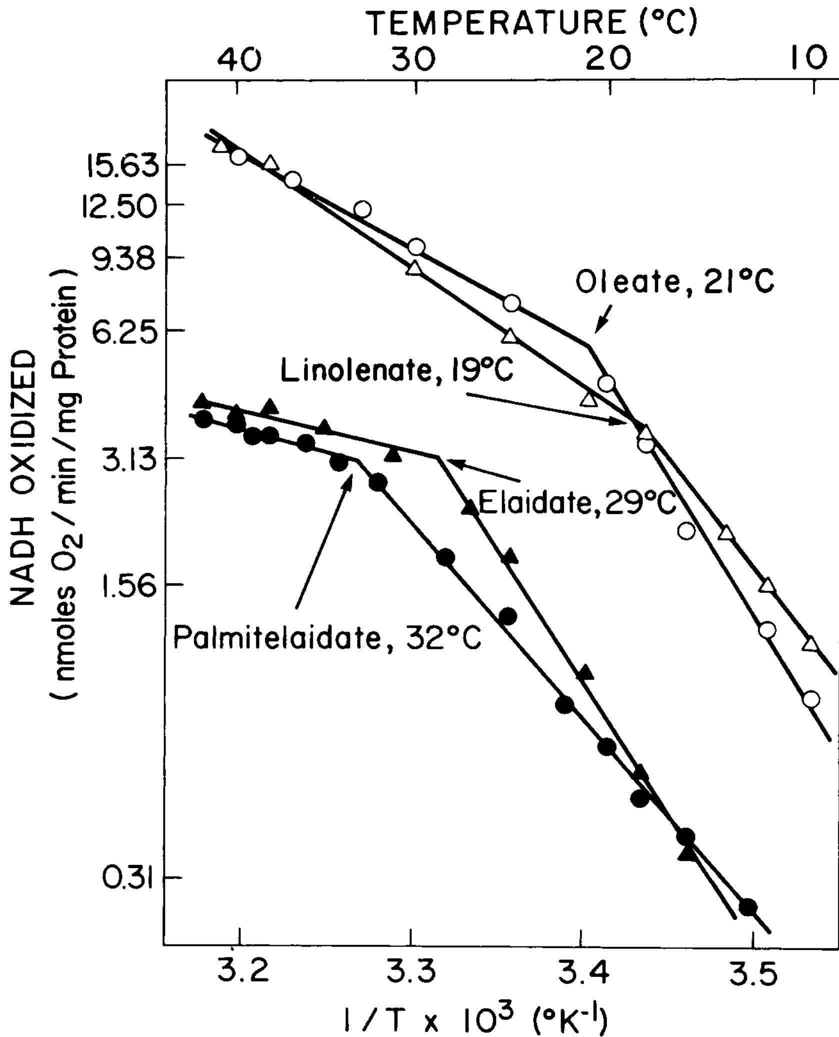

Fig. 1. Arrhenius plots of NADH oxidase activities of membrane vesicles prepared from cells supplemented with oleate (○), linolenate (△), elaidate (▲), or palmitelaidate (●). Membrane vesicles were prepared according to the procedure described in Ref. 3. Each reaction vessel contained 150 μmoles of potassium phosphate, pH 7.5 and 5.5 μmoles of NADH and 0.2–0.4 mg of membrane protein. The assay was initiated by the addition of substrate, and the temperature was controlled to within ±0.1C.

linolenate and allowed to grow for 0.5, 2, or 4 hr exhibited in the Arrhenius plots a second discontinuity at 19°C which corresponded to that obtained in vesicles isolated from cells grown on linolenate only. As shown in Fig. 2, growth on linolenate for periods of 0.5 and 2 hr yielded triphasic curves with discontinuities indicative of the transition temperatures obtained in pure linolenate membranes. However, after prolonged incubation (4 hr) in the linolenate growth medium the initial transition temperature obtained with elaidate-associated protein is completely replaced by a transition characteristic for the

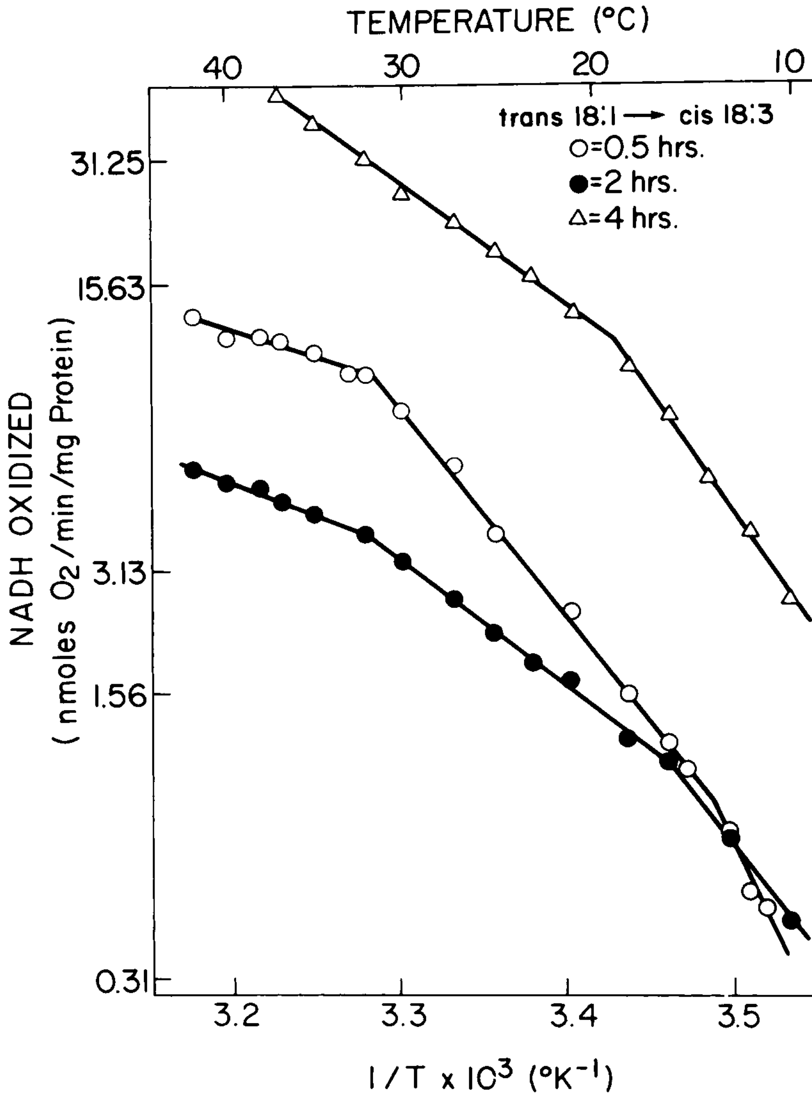

Fig. 2. Arrhenius plots of NADH oxidase activities of membrane vesicles prepared from cells grown first on elaidate and then transferred to media containing linolenate. The cell cultures were incubated at 37°C for 0.5 hr (○), 2 hr (●), and 4 hr (△) before the cells were harvested and membrane vesicles prepared. The NADH oxidase was assayed exactly as described in Fig. 1.

new species (Fig. 2). When this occurs the percentage of elaidate in the phospholipids of the membrane has dropped from the initial value of 70% to 10%, whereas that of linolenate has increased from zero to 28%. The advent of the linolenate-associated protein can also be noted by the increase in overall enzyme activity from the normally low elaidate-containing membrane to the corresponding higher linolenate-enzyme activity (Figs. 1 and 2). Similar results were also obtained for the D-lactate and NADH oxidase activities of

vesicles derived from cells which had undergone shift experiments like the one described above but using a variety of unsaturated fatty acids. Full reports on these experiments will be published elsewhere (20, 21).

These types of shift experiments have also been conducted by Fox and Overath in their studies of the lactose-transport system in unsaturated fatty-acid auxotrophs of E. coli (26–29). Fox and co-workers have found that when the β-galactoside transport system is induced at 37°C following a shift of the cells from growth on oleate to growth on elaidate, a single transition temperature is observed which is intermediate between that obtained for cells grown on either oleate or elaidate (26, 28). However, when the induction was carried out at 25°C after the shift to elaidate, triphasic Arrhenius plots were obtained with transition temperatures of 14.6°C and 26°C, corresponding to those temperatures obtained with cells induced during growth on oleate and elaidate, respectively. Heating these cells for 10 min at 37°C resulted in mixing of the lipid phases and the formation of one transition temperature intermediate between the two temperatures, similar to that obtained at 37°C after the shift to elaidate. In similar types of experiments Overath and colleagues (29) showed that continued growth of the auxotrophs in a second fatty acid at 37°C yielded cells with transition temperatures for the β-galactosidase activity intermediate between those grown on each of the individual acids separately. The actual temperature of the transition varied depending upon the mole ratio of the two unsaturated fatty acids present in the membrane lipids. These results could be explained by the assumption that the presynthesized fluid lipid and the newly formed solid lipid domains coexisted in the same membrane at the lower temperature. However, at the higher temperature of 37°C the lipids were converted to the fluidus state and were randomized into a single phase. Thus the insertion of the newly synthesized lipid at temperatures below its transition leads to its segregation from the preexisting phase. Thus, the membrane proteins are either distributed among the two lipid phases or are somehow influenced by them in such a way as to give two transition temperatures.

In our studies of the D-lactate and NADH oxidases of membrane vesicles isolated from cells grown on fluid fatty acids (i.e., oleate or linolenate) and then shifted to media containing more solid acids (elaidate or palmitelaidate), we obtained triphasic Arrhenius plots even though the growth temperature was 37°C before and after the shift (20, 21). Further heating of the vesicles at 37°C for 10 min prior to assay did not alter these profiles. However, each of the transition temperatures became less apparent and disappeared after prolonged growth on the second fatty acid, presumably because of the decrease in the mole ratio of the first acid due to dilution. These results suggested that a tight lipid–protein association exists for these enzymes that did not allow randomization to occur under these conditions. Randomization of membrane lipids may be a function not only of temperature but also of lipid–protein interactions within the membrane. Evidence for the presence of two types of physically distinct lipids in membranes, protein-bound and bulk lipids, has been presented by Jost et al. (11, 30) from electron paramagnetic resonance measurements of the interaction of beef-heart cytochrome oxidase and phospholipid. These investigators found that within the time span of their measurement there is a layer of immobilized lipid immediately surrounding the protein and separating it from the adjacent fluid-lipid bilayer. The size of this immobilized lipid is independent of the volume of the bulk-lipid bilayer. Similarly, the D-lactate and NADH oxidases of E. coli membranes may be surrounded by immobilized boundary lipids which

do not readily randomize with the bulk lipids. This behavior may be a characteristic of enzymes of the electron-transport system that distinguishes them from those of sugar-transport carriers. In the latter complex, translocation of substrate is an essential function of the system, requiring more mobile lipids which are readily randomized by temperature and other agents. In the electron-transport system, however, no such mobility is required; mixing of lipids would not be obligatory, and they are less apt to mix. However, randomization of the lipids of this system may be achieved when the membranes are energized by sonic irradiation, as will be shown later.

EFFECT OF INHIBITION OF PROTEIN SYNTHESIS ON CHANGES IN OXIDASE ACTIVITIES AFTER A FATTY-ACID SHIFT

It is evident from the data presented above that the discontinuities observed in the Arrhenius plots of NADH or D-lactate oxidase activities of membrane vesicles are characteristic of the unsaturated fatty acid utilized during growth of the cells and the relative amounts incorporated into the membrane lipids. It was of interest to determine whether the newly acquired transition temperature after a fatty-acid shift is also dependent upon protein synthesis (20). To answer this question, cells were first grown on elaidate and then shifted to linolenate-supplemented medium which did not contain the required amino acids threonine or leucine but did contain chloramphenicol at a concentration of 70 μg/ml. After 120 min incubation in this medium, the cells were harvested, washed, and utilized for membrane-vesicle preparation. The linolenate composition of the membrane phospholipids increased from 0 to 7%, whereas the elaidate content of the phospholipid decreased from 70% to 57% after the incubation. The NADH oxidase activity of the membrane after the incubation gave a triphasic Arrhenius plot with discontinuities at 32°C and 20°C, analogous to those temperatures obtained previously (Fig. 3). The extent of the incorporation of linolenate into the membrane phospholipids in the presence of chloramphenicol was significantly less than that in cells grown for the same length of time in media devoid of chloramphenicol and supplemented with the required amino acids. Protein synthesis as measured by the incorporation of ^{14}C-lysine into total cellular proteins was found to be only 3% of that obtained in cells grown in normal media, indicating that protein synthesis was almost completely inhibited under these experimental conditions. The fact that fatty-acid incorporation into the membrane lipids continued at a significant rate despite the inhibition of protein synthesis suggests that the newly synthesized lipids are free to intercalate into the membrane irrespective of protein synthesis. Once inserted into the membrane, these new lipids may penetrate the boundary lipids of various proteins and interact with the preexisting enzymes in a manner that alters their catalytic activities. It may also be possible that the functional lipoprotein complexes contain lipids of different fluidities, some of which constitute a microenvironment of solid and fluid lipids and affect enzyme activity by virtue of their close proximity to the active center. Changing the temperature of the membrane would alter the fluidity of these lipid domains, which in turn would influence the conformation of the protein and hence its catalytic activity. The protein, however, may also play a role in determining the volume of the boundary lipid and the size and nature of the fluid and solid domains within this layer of lipids. Hence, there is a two-way relationship between lipids and proteins, each influencing the physicochemical properties of the other.

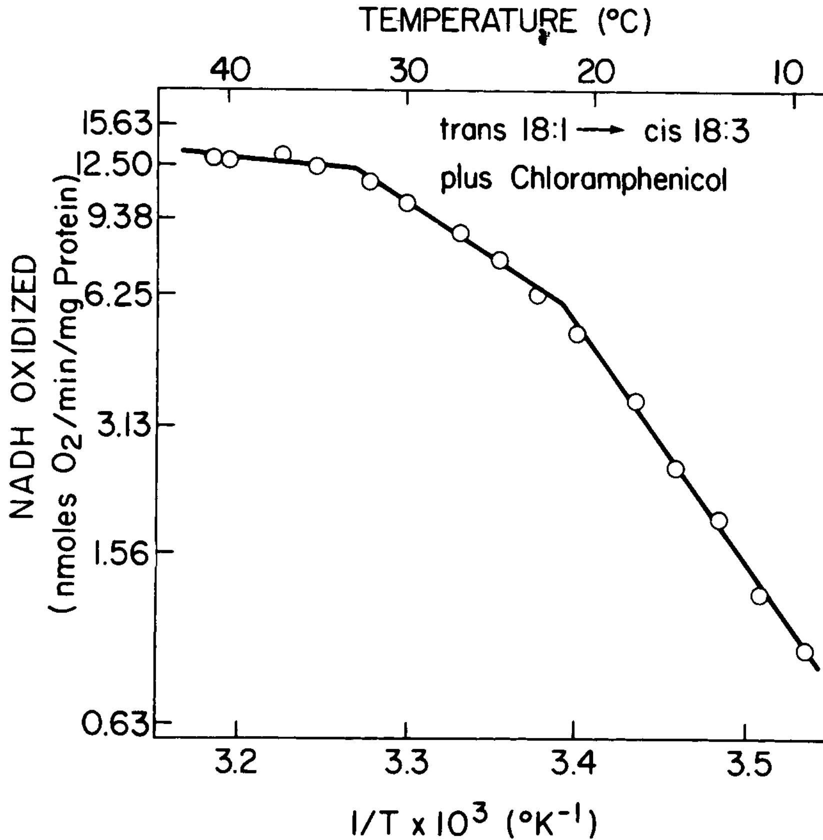

Fig. 3. Arrhenius plots of NADH oxidase activities of membrane vesicles prepared from cells grown on elaidate and then shifted to medium containing linolenate, chloramphenicol, and all other components except threonine and leucine (Ref. 3). The cells were incubated for 120 min and then harvested and used for the isolation of vesicles. The NADH oxidase was assayed as described in Fig. 1.

EFFECT OF SONIC IRRADIATION ON TRANSITION TEMPERATURES OF MEMBRANE VESICLES ISOLATED FROM CELLS AFTER A FATTY-ACID SHIFT

The double discontinuities observed in Arrhenius plots of the D-lactate and NADH oxidase activities of membrane vesicles derived from cells after a fatty-acid shift were explained by the segregation of the lipids associated with each of the oxidases. The lipid phases appeared to be stable enough to resist randomization when either the cells were grown at 37°C in the second fatty acid or the vesicles were incubated at 37°C for 10 min. Sonic irradiation of these vesicles, however, yielded particles having biphasic Arrhenius plots, as shown in Fig. 4. In these experiments cells were grown on oleate, washed, and shifted to medium containing palmitelaidate. The cultures were allowed to grow at 37°C until an increase in turbidity of 36% was attained. Cells were harvested and vesicles were prepared as usual (3, 4). Analysis of the fatty-acid composition of the phospholipids of the isolated vesicles showed that growth on the fatty-acid supplement resulted in an

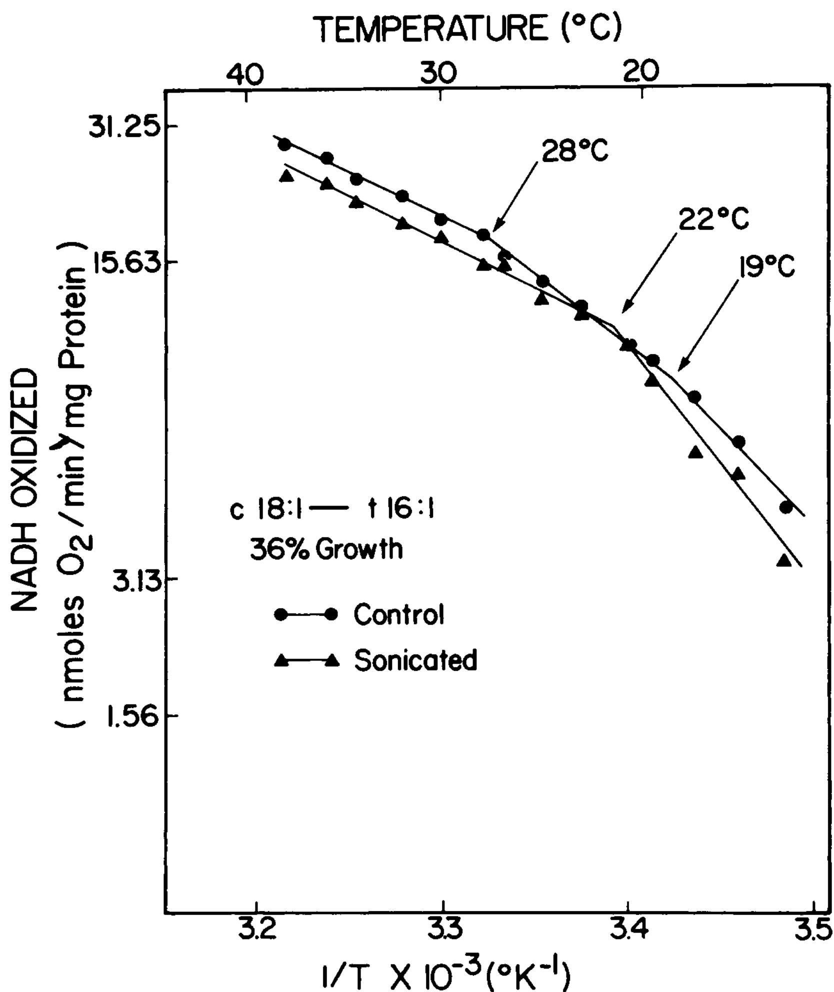

Fig. 4. Arrhenius plots of NADH oxidase activities of membrane vesicles prepared from cells grown on oleate and then shifted to palmitelaidate-containing medium. Cell cultures were incubated at 37°C until 36% increase in optical density was achieved. The cells were then harvested and vesicles were prepared (Ref. 3). NADH oxidase activity of the vesicles was measured before (●) and after (▲) sonication, as described in Fig. 1.

incorporation of 25% palmitelaidate into the membrane phospholipids and that the oleate content dropped from the initial level of 63% to 31% in the new membrane (Table IV). An Arrhenius plot of the NADH oxidase activity of these vesicles yielded the expected triphasic curve with discontinuities at 28° and 19°C (Fig. 4). When these vesicles were subjected to 30-sec pulses of sonic irradiation (a Branson Sonifier and a probe were employed at a power setting of 3) for a total of 4 min at 0°C, the NADH oxidase gave a biphasic Arrhenius plot with a transition temperature of 22°C (Fig. 4). Similar results were obtained with vesicles derived from cells grown first on linolenate and then shifted to elaidate. As can be seen in Fig. 5, the transition temperatures for the NADH oxidase

TABLE IV. Fatty-Acid Composition (Percentage) of Membrane Phospholipids

Fatty-acid Supplement									
Initial	Final	Unknown	12:0	14:0	16:0	16:1	18:0	18:1	18:3
Oleate	–	–	–	2	35	–	–	63	–
Oleate	Palmitelaidate (36% growth)	9	–	2	34	25	–	31	–
Palmitelaidate	–	6	–	–	14	80	–	–	–
Palmitelaidate	Oleate (55% growth)	2	8	–	21	37	2	27	–
Linolenate	–	–	–	2	81	–	–	–	17
Elaidate	–	–	–	1	6	–	–	93	–
Linolenate	Elaidate (55% growth)	–	–	1	36	–	–	54	9

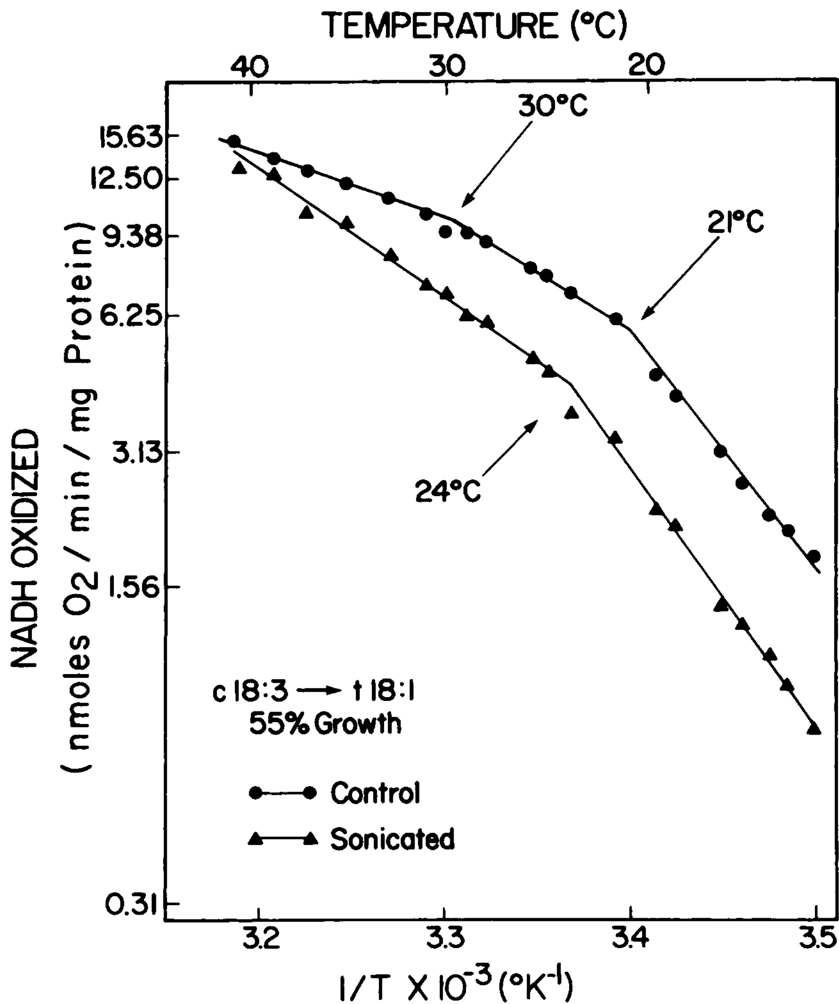

Fig. 5. Arrhenius plots of NADH oxidase activities of membrane vesicles prepared from cells grown on linolenate and then shifted to elaidate-containing medium. Cell cultures were incubated at 37°C to an increase of optical density of 55%. Cells were harvested and vesicles prepared as usual. The NADH oxidase activity of vesicles were measured before (●) and after (▲) sonication, as described in Fig. 1.

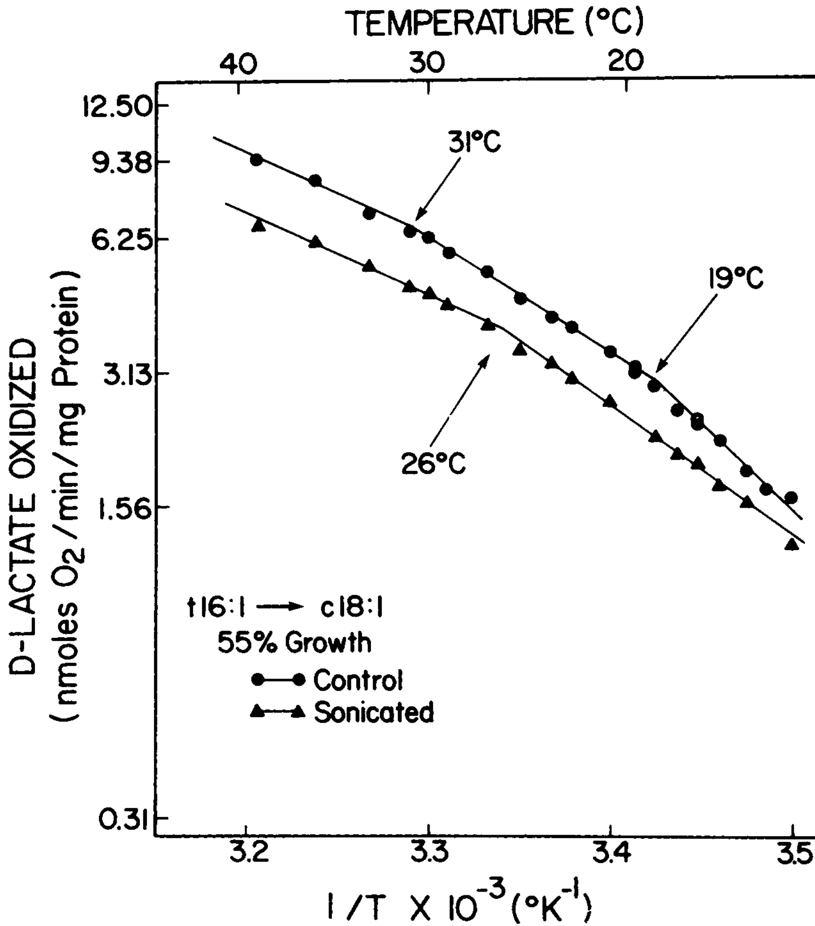

Fig. 6. Arrhenius plots of D-lactate oxidase activities of membrane vesicles prepared from cells grown on palmitelaidate and then shifted to oleate-containing medium. Cell cultures were incubated at 37°C to an increase in optical density of 55%. Cells were harvested and vesicles prepared as usual. Each reaction vessel contained 150 μmoles potassium phosphate, pH 7.5, 200 μmoles D-lactate, and 0.4–0.8 mg membrane protein. The assay was initiated by the addition of substrate, and the temperature was monitored to within 0.1°C. The D-lactate oxidase activity was measured before (●) and after (▲) sonication.

prior to sonication were 30° and 21°C, and after sonication a single transition at 24°C was obtained. It seems that sonic irradiation causes randomization and mixing of phospholipid phases, hence the advent of an intermediate transition temperature for enzyme activity.

Similar results were obtained for the D-lactate oxidase activity of vesicles isolated from cells grown initially on palmitelaidate and then shifted to oleate-containing medium. In this instance the cells were incubated at 37°C and allowed to grow to an optical-density increase of 55%. Sonic irradiation of the vesicles resulted in membranes with a biphasic profile for D-lactate oxidase activity. The discontinuity occurred at a temperature of 26°C, which is intermediate between those of 19° and 31°C obtained with the same vesicles

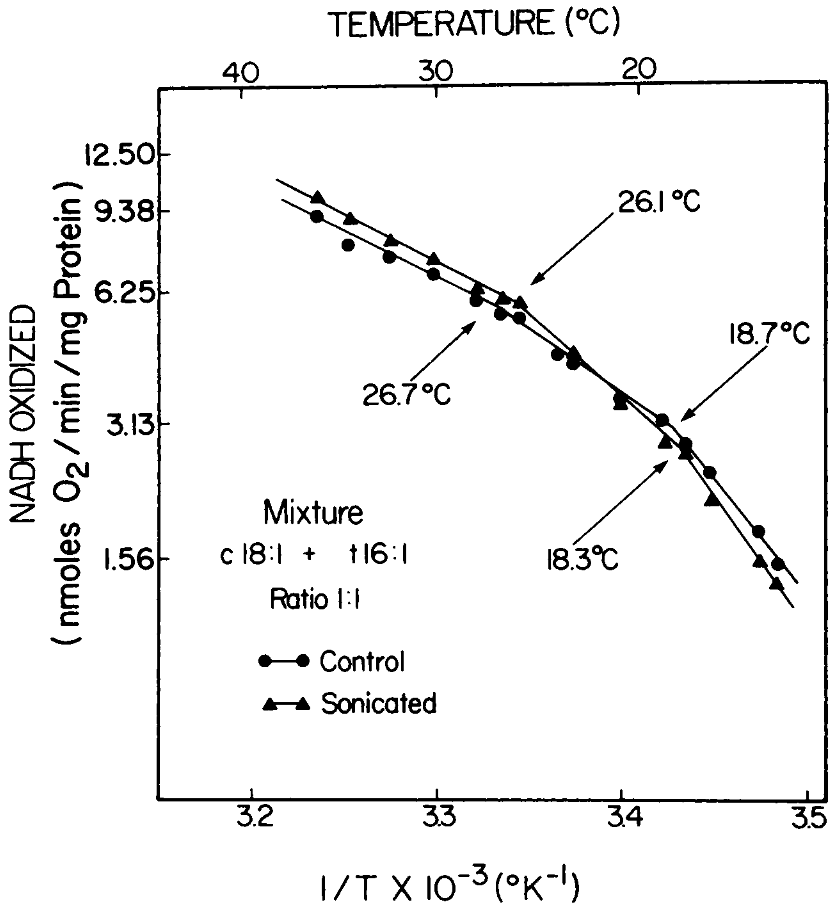

Fig. 7. Arrhenius plots of NADH oxidase activity of a mixture of membrane vesicles prepared from cells grown with oleate or palmitelaidate supplementation. Equal amounts of protein of each type of membrane vesicle were mixed immediately prior to assay as described in Fig. 1 (●). An aliquot of this mixture was subjected to short-term sonication as described in the text and then assayed (▲).

prior to sonic irradiation (Fig. 6). Analysis of the fatty-acid composition of the phospholipids of the vesicles before and after the shift to oleate showed that initially the membrane contained palmitelaidate as the only unsaturated acid present and that after the shift the phospholipids contained both palmitelaidate and oleate (Table IV). Sonic irradiation of the vesicles did not alter these ratios.

The triphasic Arrhenius plots for the NADH oxidase could be reproduced by mixing equal amounts of two vesicle preparations derived from cells grown separately on palmitelaidate or oleate, as shown in Fig. 7. Sonic irradiation of this membrane mixture did not alter the activity profile, and essentially the same transition temperatures were obtained (7). These results showed that under these conditions no mixing of the two lipid-associated NADH oxidases had occurred, presumably because of the existence of the oleate- and palmitelaidate-associated oxidases in two separate vesicles. Mixing or

randomization of particular lipids which were initially associated with the two types of oxidases would require their coexistence within the same membrane bilayers. The latter state was achieved during growth of the cells in the presence of the second fatty acid. Sonication of a mixture of two suspended vesicles each containing different oxidase-associated lipids did not randomize the lipids, presumably because the two membranes were not fused in a single bilayer under these conditions.

FUSION OF PHOSPHOLIPID INTO MEMBRANE VESICLES

The exhibition of two transition temperatures by NADH or D-lactate oxidases when measured in membranes derived from cells grown on one fatty acid and then shifted to another is thought to be due to the presence of oxidases associated with two types of lipids within the same membrane. Some oxidase molecules are associated with lipids containing the fluid fatty acids (oleate and linolenate), and others are associated with lipids containing the solid fatty acids (elaidate and palmitelaidate). The distribution of oxidase into the two lipid types appeared to be independent of protein synthesis but was proportional to the relative amount of each of the lipid types present within the membranes. Further evidence to substantiate this hypothesis was obtained from experiments involving fusion of phospholipid vesicles into membrane vesicles derived from cells grown on various unsaturated fatty acids. The plots illustrated in Fig. 8A showed that whereas vesicles derived from cells grown on oleate gave a biphasic plot with the expected transition temperature of 20°C, fusion of these membrane particles with vesicles prepared from total lipids extracted from cells grown on palmitelaidate gave a triphasic plot with transition temperatures of 18.5° and 30°C. Similar plots were obtained when fusion was made with vesicles prepared from palmitelaidate phospholipids instead of from the total extracted lipids (Fig. 8B). These results were similar to those obtained with membrane vesicles derived from cells grown on oleate and then shifted to a growth medium containing palmitelaidate and chloramphenicol but devoid of the required amino acids (Fig. 3). It would appear, therefore, that vesicles prepared by sonicating phospholipids are incorporated into the membrane vesicles, modifying their oxidase activity by associating with the enzyme protein or one of its carrier components.

The phospholipid vesicles were prepared by a modification of McConnell's procedure (31, 32). A suspension of 5–8 mg of phospholipid in 1 ml of 0.1 M potassium phosphate, pH 6.6, was sonic irradiated under N_2 for 30 min at 37°C. A Branson Sonifier was employed at a power setting of 3.5. The resulting solution was centrifuged, and the clear supernatant (sometimes slightly opaque) was removed and used for fusion. This treatment should produce single-compartment bilayer vesicles (31, 32). In determining optimum conditions for fusion, we have found that fused vesicles always have lower activity than the original membrane vesicles. The reason for this decrease in activity is not apparent. However, the decrease in activity was dependent upon the relative amounts of phospholipid vesicles used during fusion, as shown in Fig. 9. The higher the ratio of phospholipids to membrane protein, the more inhibition was obtained. In most experiments we arbitrarily used a mixture of 1:1 (w/w) of phospholipid to membrane protein. At this ratio we found that fusion occurred best at 37°C, a temperature which is above the transition temperature for either NADH or D-lactate oxidase activities regardless of the

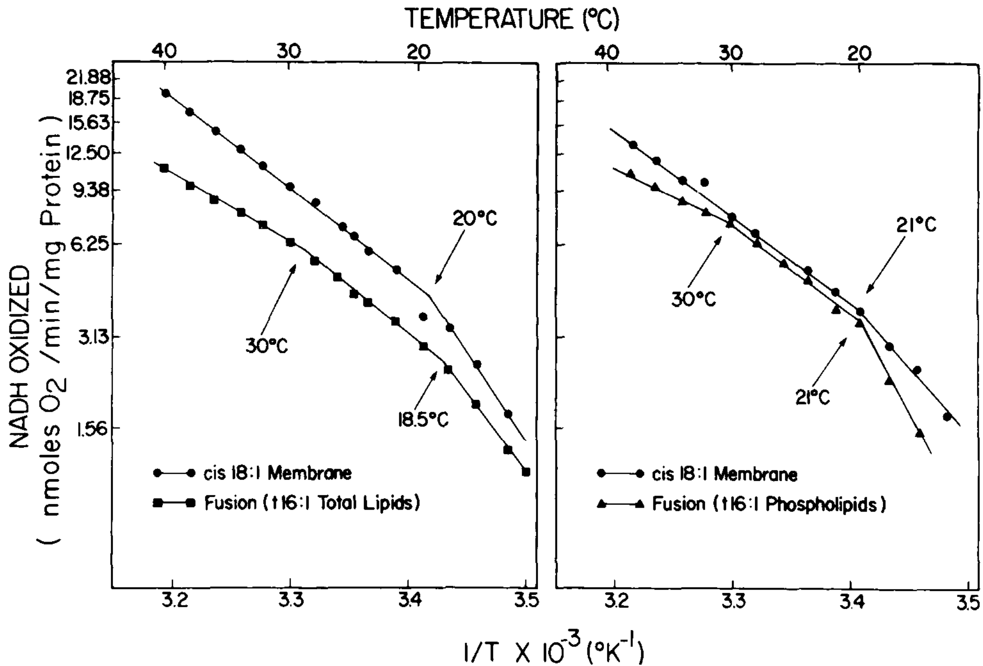

Fig. 8. (Left) Arrhenius plots of NADH oxidase activity of membrane vesicles prepared from oleate-supplemented cells before and after fusion with palmitelaidate total membrane lipids. The lipids were extracted from cells grown on the indicated fatty acid, and the phospholipids were further isolated by thin-layer chromatography (Ref. 3). Fusion was carried out as described in the text. Equal amounts of membrane proteins were incubated with lipids at 37°C for 60 min. NADH oxidase activity was determined with fused (■) and nonfused (●) vesicles as described in Fig. 1.

(Right) Arrhenius plots of NADH oxidase activity of membrane vesicles isolated from cells before (●) and after fusion with palmitelaidate phospholipids (▲). Fusion and assay conditions were the same as those described above except for the use of phospholipids instead of total lipids.

fatty-acid supplement. The effect of time on fusion, as measured by the decrease in the specific activity of NADH oxidase, is shown also in Fig. 9. As can be seen, incubation for 60 min is sufficient to lower the activity of the enzyme to the fused level. However, incubating the membrane at 37°C for the same length of time in the absence of phospholipid did not substantially alter enzyme activity (Fig. 9). Another factor important in these types of experiments is the nature of the fatty acid present in both the membrane vesicles and liposomes. We have always found that it is technically easier to fuse solid lipids into fluid-lipid–containing membranes, partly due to the lowering of enzyme activities by the fusion process itself and partly due to the generally lower activities of these enzymes associated with solid-containing phospholipids (trans-unsaturated fatty acids) in comparison to those containing fluid phospholipids (cis- and polyunsaturated acids) (Fig. 1). When linolenate-containing vesicles were fused with elaidate lipids a single transition temperature at 25°C was obtained (Fig. 10), presumably either because mixing had occurred or because the lower transition temperature due to linolenate is so low that

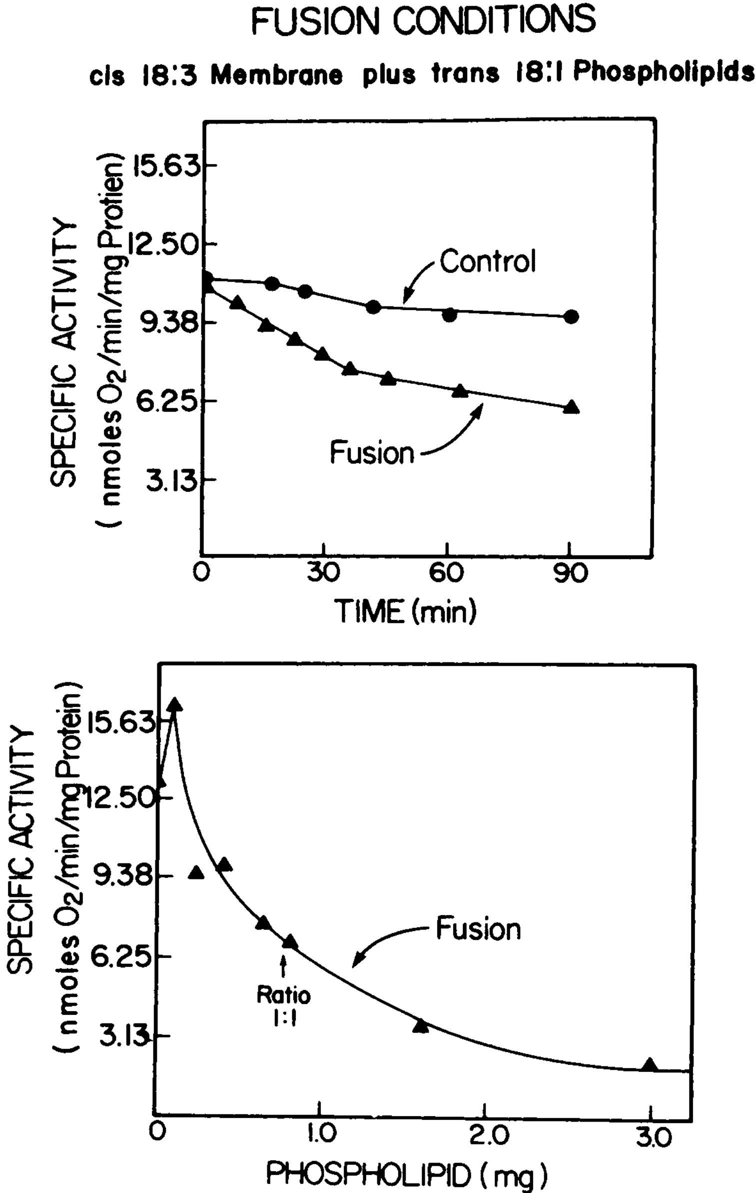

Fig. 9. (Top) The NADH oxidase activity of membrane vesicles prepared from cells grown on linolenate and fused with elaidate phospholipid vesicles. Fusion was carried out by incubating equal amounts of vesicle protein and phospholipid at 37°C. Samples were withdrawn for NADH oxidase assay at the indicated times. In the control experiment vesicles were incubated in the absence of phospholipids in the same manner.

(Bottom) NADH oxidase activity of membrane vesicles prepared from linolenate-supplemented cells and fused with vesicles prepared from elaidate phospholipids as described in the text. Membrane vesicles (0.78 mg) were used with indicated amounts of phospholipid vesicles and assayed for NADH oxidase as described in Fig. 1.

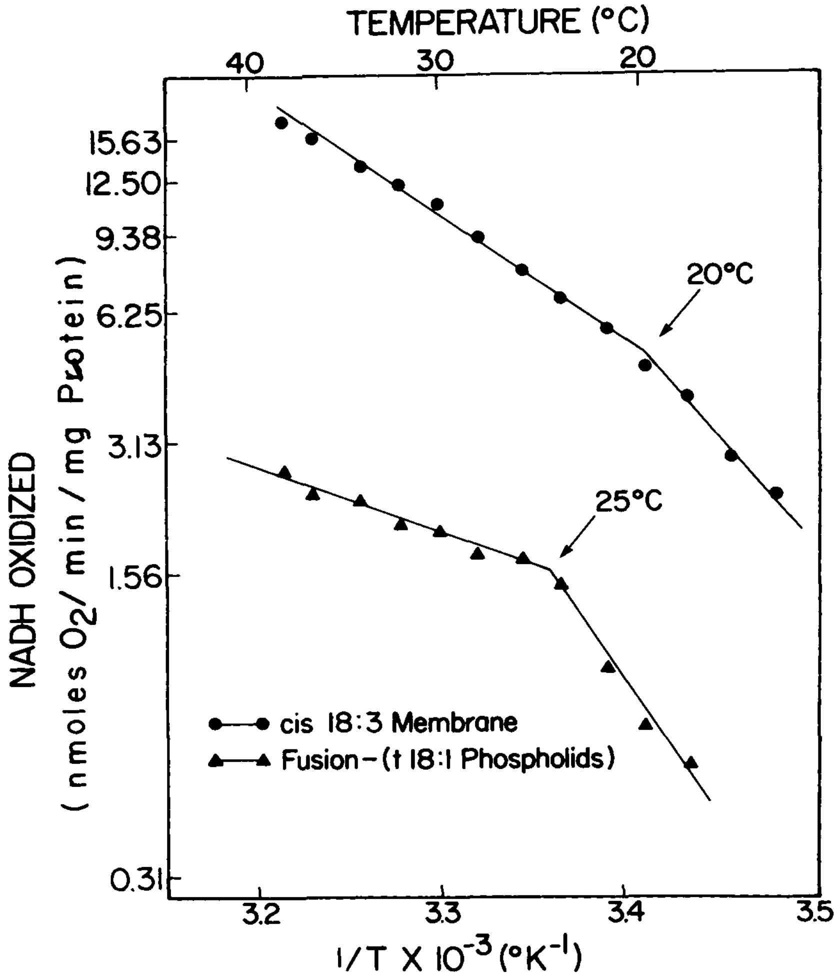

Fig. 10. Arrhenius plots of NADH oxidase activity of membrane vesicles prepared from linolenate-supplemented cells (●) or linolenate membrane vesicles fused with elaidate phospholipids (▲). Fusion and assay conditions were the same as those described in Fig. 8, except elaidate phospholipids and linolenate-containing membrane vesicles were used.

it could not be determined with any degree of accuracy. Another reason for our inability to detect the lower transition temperature due to linolenate lipids could be the dilution of the linolenate-containing lipids to such a low concentration that the oxidase is primarily associated with the fused elaidate lipids. Clearly, further experiments are needed in order to clarify this point.

The aforementioned experiments indicated that not only could exogenous lipid vesicles be incorporated into the membrane vesicle but also that the membrane vesicle's integration can be measured functionally as a change in enzyme activity and the appearance of a transition temperature indicative of the new species of phospholipid-associated protein. At this time, the site of the phospholipid insertion is not known. Exogenous lipid may either

incorporate initially into the bulk phase and then diffuse laterally into the protein-associated lipid or insert itself directly into the boundary-lipid phase. It is also possible that both phenomena can occur simultaneously but at different rates. Of these two possible mechanisms, the first seems to be the most probable one, especially since Grant and McConnell (31) have observed with EPR measurements that in the fusion of dipalmitoylphosphatidylcholine into Acholeplasma laidlawii membrane, the new lipid incorporates in patches and then diffuses throughout the cell membrane. However, they also noted that exogenous dipalmitoylphosphatidylcholine readily diffusing laterally throughout the membrane does not necessarily imply complete lipid homogeneity. Our experiments support these conclusions and suggest that lipid heterogeneity may occur not only at the bulk-lipid areas but also at boundary-lipid layers.

We have also obtained evidence to show that membrane vesicles isolated after fusion exhibit a lower density due to the uptake of extra phospholipids. Membrane vesicles prepared from cells grown on linolenate-containing media were fused with ^{14}C-labeled elaidate-phospholipid vesicles as described above. The resulting particles were isolated and washed several times with phosphate buffer and layered on a 30–60% linear sucrose gradient (capacity, 30 ml). The sucrose gradients were then centrifuged at 0°C in a SW 27 rotor operating at 25,000 rpm. After 19 hr of centrifugation, equilibrium was attained, and the tubes were removed and 1 ml fractions were collected from the top of each tube and analyzed for protein and ^{14}C content. The membrane vesicles prior to fusion gave a peak of protein at a density of 1.26 g/cc, whereas fused vesicles gave a peak of protein at a significantly lower density (1.23 g/cc), as shown in Fig. 11. Moreover, all of the radioactivity was associated with the fused protein. The NADH oxidase was also associated with the protein of this peak. This was shown by pooling the fraction containing the peak protein and reisolating the particles by centrifugation and washing to remove the sucrose. The resulting particles contained most of the NADH oxidase activity of the preparation. The fused protein fractions also yielded an active particle, but its activity was one fourth of that of the control fractions. The lower value obtained is presumed to be due to the reduction of enzyme activity as often noted in fused membranes. The slight difference ($< 0.25\%$) between the sucrose gradients of the control and fused membrane preparations used in Fig. 11 is not sufficient to cause the observed shift in vesicle density from 1.26 to 1.23 g/cc. Therefore, it was concluded that the higher buoyancy of the vesicle was a consequence of the uptake of lipids during the incubation of membrane particles with the phospholipid vesicles.

Further evidence for such an uptake of lipids was obtained when ^{14}C-elaidate-containing phospholipid vesicles were fused with membrane particles isolated from cells grown on elaidate-supplemented medium. The NADH oxidase activity of the fused membrane was about 20% less than that of the original membranes. Again, the fused particles show a lower density (1.21 g/cc) than the original vesicles (1.23 g/cc) when analyzed by centrifugation in a sucrose density gradient of 40–60% (Fig. 12). The peak of ^{14}C-labeled elaidate phospholipids also coincides with the fused protein peak, as shown in Fig. 11. Analysis of the fatty-acid content of the lipids of the vesicles before and after fusion showed no difference in the relative content of palmitate and elaidate. These results are expected, since both lipids are essentially the same and the elaidate content is very high ($> 90\%$). Since the fused phospholipids contain ^{14}C-elaidate, it was estimated that 25% of the lipids of the fused vesicles are derived from the phospholipid vesicle.

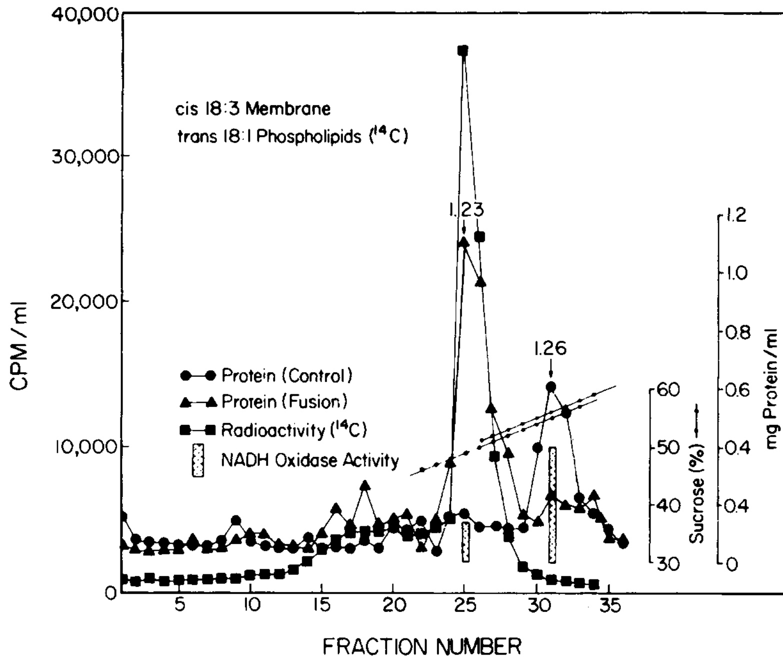

Fig. 11. Profile of sucrose gradient fractions obtained for linolenate-supplemented membrane vesicles (control) and linolenate membrane vesicles fused with liposomes prepared from ^{14}C-elaidate phospholipids (see text). Fractions were analyzed for protein content and radioactivity. Fractions 30 to 38 from the control tubes and 24 to 27 from the fused tubes were pooled separately. The particles were isolated by centrifugation, washed free of sucrose, and assayed for NADH oxidase activity. The cross bars represent the specific enzyme activities of 7.81 and 4.06 Nmoles · O_2/min/mg protein, respectively. The densities of the control and experimental sucrose gradients were compared for representative tubes (•— •), and the buoyant densities of peak tubes are indicated on the graph.

The NADH oxidase activity of oleate membranes was also measured before and after fusing with ^{14}C-labeled oleate phospholipid vesicles. In this experiment the Arrhenius plot of the fused vesicles showed the same profile as the nonfused particles, with a transition temperature of 22°C.

EFFECT OF CHOLESTEROL ON NADH OXIDASE ACTIVITY OF E. COLI VESICLES

Since E. coli. membranes do not contain any steroids, it is possible to study the incorporation of cholesterol into the membrane and its subsequent effect on membrane-associated enzyme activities. Lipid vesicles are prepared from sonic irradiation of a mixture of cholesterol and ^{14}C-elaidate phospholipids (1:1 w/w) as described above. Fusion was carried out by incubating equal amounts of the cholesterol-phospholipid vesicles and of the linolenate-containing membrane vesicles under the same conditions used previously. The fused particles were isolated, washed to remove excess lipids, and assayed for NADH oxidase activity at various temperatures. The results depicted in Fig. 13 showed that where-

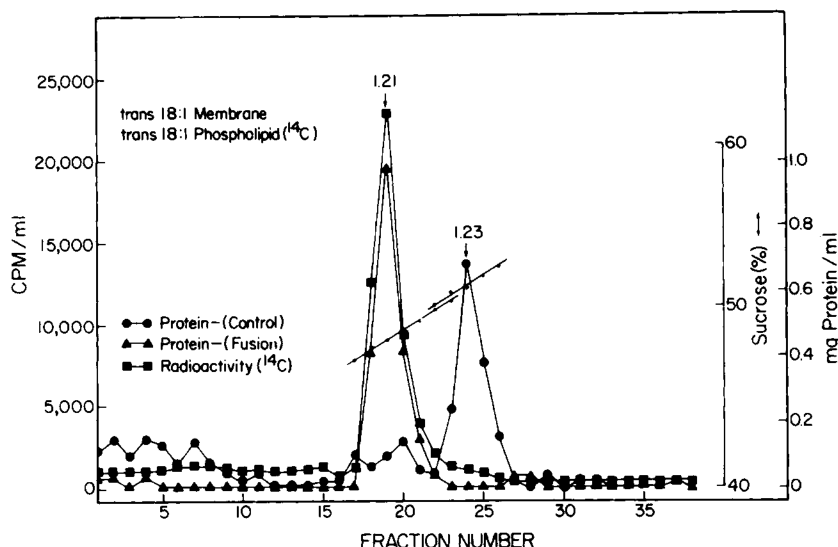

Fig. 12. Profile of sucrose gradient fractions obtained for elaidate-supplemented membrane vesicles (control) and elaidate membrane vesicles fused with ^{14}C-elaidate phospholipid vesicles. Protein, radioactivity, and the density of the sucrose gradients were measured as described in Fig. 11.

as control nonfused membrane vesicles gave the usual biphasic Arrhenius plots, with a transition temperature at 21°C, the elaidate-cholesterol fused vesicles had no apparent transition temperature. The transition temperature at 25°C noted earlier in membrane vesicles fused with elaidate was also absent when cholesterol was included with the elaidate lipids (Fig. 10). The disappearance of any transition for NADH oxidase between 15° and 38°C would suggest that cholesterol alters the lipid–enzyme association in some manner, either directly or indirectly yielding a phase whose fluidity varies proportionately throughout the temperature range of 15–38°C. It is also possible that cholesterol changed the transition temperature of the oxidase activity of the fused particles to levels outside the temperature range normally used in these studies. Further experiments are necessary in order to decide which of these two possibilities is the case. It is of interest that changes in the transition temperatures of membranes upon incorporation of cholesterol into their lipids have been found by other workers (8, 9, 34–39). Van Deenen and coworkers (37) found that the incorporation of cholesterol to the extent of 9% into the membrane lipids of Acholeplasma laidlawii during growth caused a decrease in the transition temperature of the membrane ATPase activity. Also Shimshick and McConnell (35) found that the phase transition as determined by electron paramagnetic resonance measurements due to the tempo dissolved in dipalmitoylphosphatidylcholine or dimyristoylphosphatidylcholine increased at relatively low cholesterol concentrations and disappeared when cholesterol was above a molar concentration of 20%.

Cholesterol has been shown to be an important component of many biological membranes (34). One postulated role for cholesterol is to control the fluidity of the hydrocarbon regions of the membrane, thereby stabilizing the bilayer with respect to temperature. In this manner Chapman (9) has described the effect of cholesterol as pro-

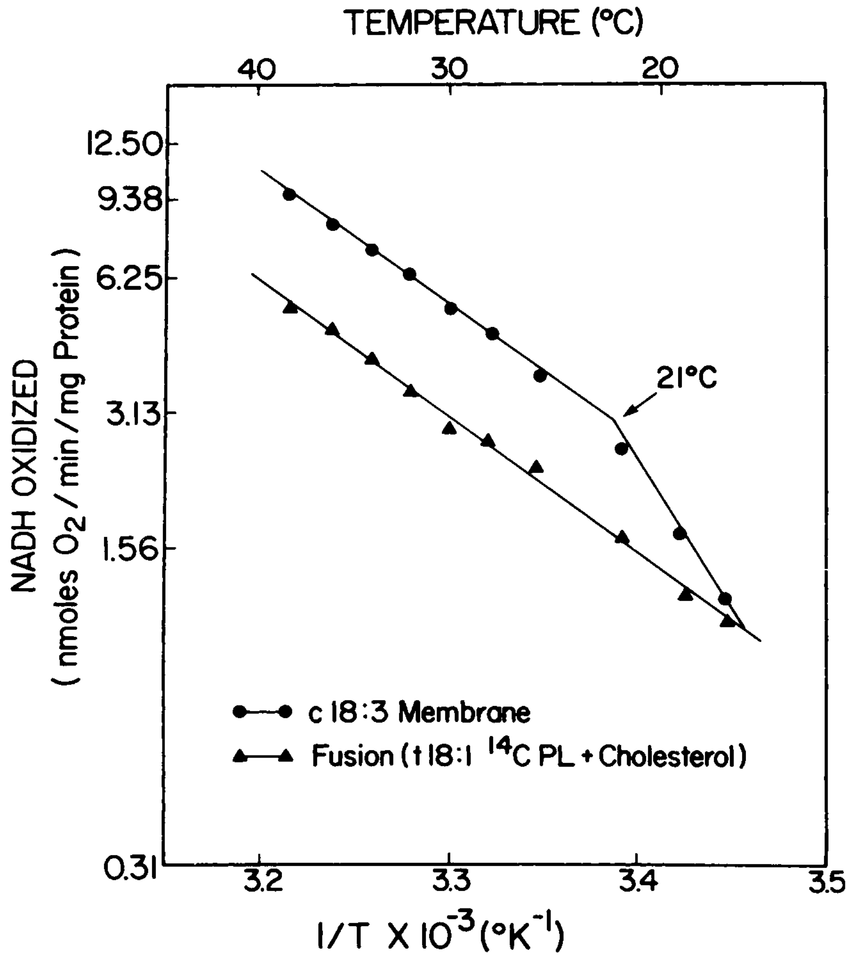

Fig. 13. Arrhenius plots of NADH oxidase activity of linolenate-supplemented membrane vesicles (●) and linolenate membrane fused with liposomes prepared from ^{14}C-elaidate phospholipids plus cholesterol at a ratio of 1 mg phospholipid to 1 mg cholesterol (▲) (see text). The NADH oxidase activity was measured as described in Fig. 1.

ducing an "intermediate fluid condition" in the membrane. In more rigid areas of the membrane where the acyl chains are below their gel–liquid-crystalline transition temperature, cholesterol interrupts lipid–lipid interactions, expands the membrane, and imparts a more fluid nature to that specific region. Conversely, in more fluid areas of the membrane where there is a localization of unsaturated fatty acids, cholesterol serves to decrease the motion of the fatty acyl chains, thus producing a condensing effect. Therefore, cholesterol probably stabilizes the membrane by placing regions of hydrocarbon with acyl chains which differ in molecular properties into the same intermediate fluid condition (36). In order for a membrane as a whole to experience a phase transition the individual hydrocarbon chains must act cooperatively. As noted by Rothman and Engelman (36), the fact that cholesterol produces an intermediate state at some sites along the bilayer

uncouples these lipids from interactions with their neighbors that are sterically not influenced by cholesterol. From this it follows that the nearest neighbors to cholesterol cannot experience a thermal change. Thus, depending on the molar concentration of cholesterol, it has been noted by several investigators using a variety of techniques in membranes and synthetic systems that cholesterol in association with the phospholipids disrupts their phase transitions (9, 34–38).

COMMENTS

In our previous studies with an unsaturated fatty-acid auxotroph of E. coli (4, 22) we proposed that the distribution of lipids in the membrane can be nonrandom. Furthermore, we proposed that one mechanism responsible for this heterogeneity is a temperature-dependent process based primarily on lipid–lipid interactions. The basis for this heterogeneity has been thought to be lateral diffusion and phase segregation of lipids (4, 9, 22, 39). Evidence presented in this paper and by others suggested that heterogeneity in the molecular organization of lipids may also be induced by relative immobilization of some lipid components by some membrane proteins (10). In our studies we used the transition temperatures observed in the Arrhenius plots as markers for studying lipid–protein interactions. This approach was justified by the observation that transition temperatures noted for succinate coenzyme Q reductase activity of E. coli vesicles were dependent on the nature of the fatty acid present (4, 26, 29). When lipids were removed by aqueous acetone extraction the discontinuity in the Arrhenius plots disappeared and was restored only when lipids were added back to the acetone-extracted particles (22). D-lactate oxidase and NADH oxidase activities of E. coli membranes gave Arrhenius plots with discontinuities at different temperatures, depending upon the fluid nature of the fatty acid present in the membrane lipids. When fluid unsaturated fatty acids were present in the membrane phospholipids (oleate or linolenate), the transition temperature was lower than when solid (elaidate or palmitelaidate) unsaturated fatty acids were present. When cells were grown on one fatty acid, washed, and then allowed to grow in media containing a second fatty acid, the D-lactate and NADH oxidases yielded activity profiles with two transition temperatures indicative of those obtained with membranes containing a single unsaturated fatty acid. These results were different from those reported for the β-galactoside transport system (26–29). In the latter studies, cells were first grown in oleate and then shifted to palmitelaidate-containing media. When the growth temperature in both media was 37°C, the resulting Arrhenius plots of the β-galactoside transport were biphasic, with the point of discontinuity falling between, rather than outside, the two extremes for oleate- or palmitelaidate-grown cells. If the growth temperature in the second growth medium of palmitelaidate was maintained at 25°C, the Arrhenius plots were triphasic, with transition temperatures similar to those obtained with cells grown on each of the individual acids. Incubating the cells at 37°C for 10 min shifted the transition temperatures to a single transition intermediate between those obtained for the individual acids. This change may be due to the effect of temperature in transforming the bulk of the oleate- and palmitelaidate-containing lipids to a liquid-crystalline state, thus establishing a relatively uniform viscosity along the lipid bilayer. If one assumes that the transport proteins are less tightly bound to their bounday lipids, allowing them to mix with the bulk

lipids, then the transport system would respond to the newly available homogenous lipids. This may be the case for the β-galactoside transport system and may not be applicable to other membrane enzymes. The D-lactate and NADH oxidases may be tightly bound to their boundary lipids via polar and/or apolar interactions and require higher energizing forces for randomization and mixing of their lipids. This view is supported by the observation that sonic irradiation of vesicles containing both fatty acids results in elimination of the two transition temperatures and the appearance of an intermediate one. Whether this behavior is confined only to enzymes of the electron-transport system or also to those involved in "vectorial metabolism" remains to be determined.

The segregation of the D-lactate and NADH oxidase activities among lipids of different fluidity proved to be useful in our studies of membrane biogenesis. Our concern has been to study the mode of incorporation of lipids and proteins into the membrane. Are lipoprotein complexes formed prior to their insertion into the membrane, or are proteins and lipids incorporated into the membrane independently of each other, followed by the formation of functional lipoproteins by specific and/or nonspecific interactions of these components? The results presented in this paper support the latter thesis – namely, that membrane biosynthesis occurs by the insertion of lipids and proteins independently of each other and that a functional unit is formed thereafter. This conclusion was based on the observation that insertion of lipids into the membranes either in vivo during the absence of protein synthesis or by fusion into isolated vesicles confers onto preexisting oxidases properties of the new lipids in a manner similar to that observed for cells grown on one fatty acid and then shifted to another. This finding also suggests that the functional interaction between certain membrane enzymes and phospholipids occurs, since active protein synthesis is not required in order to form the new association. Once the protein is in the membrane it may seek out and interact with its boundary lipids so as to form the functional complex. This interaction may be reversible and controlled by the relative amounts of lipid phases present within the membrane and by the number of phases in which the membrane exists.

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