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Transport System Assembly and the Mobility of Membrane Lipids in *Escherichia coli*†

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ABSTRACT: Arrhenius plots describing the logarithm of the rate of β-galactoside transport *vs.* the reciprocal of the absolute temperature of transport assay are biphasic in slope with intersects (transport transition temperatures) at 30 and 13° for cells of an *Escherichia coli* unsaturated fatty acid auxotroph grown at 37° in medium supplemented with elaidic acid and oleic acid, respectively. When transport is induced at 37° for a short time period (less than one-eighth of a generation of growth) after shifting from growth with an oleic acid supplement to growth with an elaidic acid supplement or *vice versa*, a single transition temperature for β-galactoside transport is detected in each case, and the temperatures at which the transitions are observed indicate that transport is primarily influenced by the average fatty acid composition of the membrane lipids. If transport induction proceeds at 25° after the fatty acid shift, however, the Arrhenius plots are triphasic. The two observed transport transition temperatures reflect the properties of cells with membranes composed primarily of oleic acid or elaidic acid derived lipids. When the cells induced at 25° are subsequently incubated at 37° before commencing transport assay, only a single, new transport transition is detected and the transition temperature is similar to that observed with cells induced at 37° after the fatty acid shift. We interpret these results to indicate that under the conditions where two transport transition temperatures are observed, a portion of the newly formed transport protein is incorporated into membrane with, and influenced by, the lipids synthesized concomitantly. Subsequent incubation of these cells at elevated temperature leads to a mixing of the preformed and newly synthesized lipids within the membrane bilayer matrix. Thus the mobility of lipids in membranes may, at least in certain instances, be grossly restricted below a defined critical temperature.

Several independent studies point to an interrelationship between protein and lipid biosynthesis during the morphogenesis of certain microbial transport systems. Following a period of starvation for an essential fatty acid, induction of a fully functional lactose transport system in unsaturated fatty acid auxotrophs of *Escherichia coli* is blocked, even though the induction of β-galactosidase and thiogalactoside transacetylase proceeds normally (Fox, 1969; Overath *et al.*, 1971a). Addition of an essential fatty acid subsequent to the starvation period does not result in a recovery of transport activity (Fox, 1969). Induction of functional lactose transport system activity in *E. coli* and *Staphalococcus aureus* is blocked when glycerol auxotrophs of these organisms are starved for a source of glycerol or glycerol phosphate for complex lipid biosynthesis. The transport proteins, however, are incorporated into membrane and exhibit normal binding activity in *E. coli*, and phosphotransferase activity in *S. aureus* (Hsu and Fox, 1970; Mindich, 1971). Transport can be activated by subsequent growth in the presence of glycerol in *S. aureus*, but not in *E. coli*. Uptake and accumulation of amino acids are defective in pantothenic acid deficient *Lactobacillus plantarum* (Holden and Bunch, 1972). Transport activity is restored to normal when the pantothenate-starved cells are incubated with long-chain fatty acids.

Properties of transport systems induced in unsaturated fatty acid auxotrophs of *E. coli* have been exploited in the study of membrane structure–function relationships (Schairer and Overath *et al.*, 1970; Wilson and Fox, 1971; Esfahani *et al.*, 1971) and membrane assembly mechanisms (Fox, 1969; Wilson and Fox, 1971; Tsukagoshi *et al.*, 1971; Overath *et al.*, 1971a; Robbins and Rotman, 1972; Tsukagoshi and Fox, 1973). A report from this laboratory provided evidence supporting the view that assembly of the lactose-transport system proceeds by a coordinated incorporation of newly syn-

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thesized lipids and proteins into membrane (Wilson and Fox, 1971). Serious objections to this interpretation have been raised by Overath *et al.* (1971a), who favor a model in which phospholipids are first synthesized by membrane-associated enzymes and then randomized throughout the lipid phase by a lateral diffusion process, the transport protein being incorporated into an essentially homogeneous lipid phase. We report here evidence which indicates that the lactose transport system is likely to arise by coupled insertion of newly synthesized lipids and proteins, followed by a temperature-dependent randomization of newly formed and preformed membrane constituents.

Methods

Procedures for growth of the unsaturated fatty acid auxotroph of *E. coli* K12, strain 30E, were as described in the accompanying report (Tsukagoshi and Fox, 1973). Unless otherwise stated, growth was in liquid medium consisting of medium A (Davis and Mingioli, 1950), supplemented with 1% Difco casamino acids, 5 μg/ml of thiamine·HCl, 0.5% Triton X-100 (Rohm and Haas), and the essential fatty acid at 0.02%. Procedures for induction of proteins of the lactose operon, for the assay of transport, β-galactosidase, and protein, for lipid extraction and thin-layer chromatography, and for the determination of radioactivity were as described in the accompanying report (Tsukagoshi and Fox, 1973). The sources of all materials employed are likewise described therein. The units for transport activity are nmol hr^{-1} mg^{-1} of protein at the specified temperature. Turbidimetric estimation of cell growth was made with a Klett colorimeter utilizing a KS-42 (400–450 nm) filter.

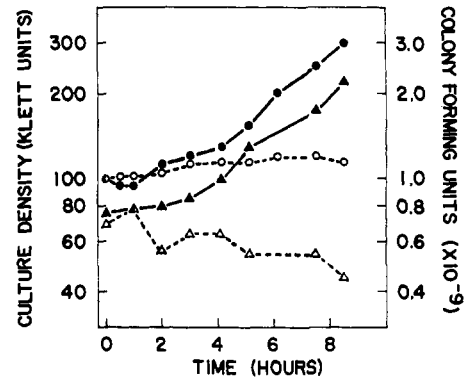

FIGURE 1: Growth of cells of an unsaturated fatty acid auxotroph at 25° in medium containing oleic or elaidic acid supplements, after initial growth at 37° in medium containing an elaidic acid supplement. A culture of strain 30E was grown at 37° to a culture density of approximately 5×10^8 cells/ml in medium supplemented with elaidic acid (Methods). The cells were collected by centrifugation at room temperature (22°), washed once with medium containing no unsaturated fatty acid supplement, and suspended in fresh medium supplemented with either elaidic acid or oleic acid. The growth of both cultures was then commenced at 25° and monitored by recording the change in turbidity (Klett units) or by determining the viable cell count (colony-forming units). For the determination of the viable cell count, suitable dilutions of the culture were plated on a solid medium consisting of medium A (Davis and Mingioli, 1950), 1.5% Difco agar, 0.02% oleic acid, 0.05% Triton X-100, and 5 μg/ml of thiamine·HCl. Samples of the cells which survived (or grew) after 8 hr were also plated on this same solid medium with 1% glucose substituted for oleic acid and Triton X-100. The number of revertants to prototrophy for unsaturated fatty acid synthesis detected by this procedure accounted for less than 0.01% of the viable cells in the populations: Klett units in oleic acid medium (●) and in elaidic acid medium (○); colony forming units in oleic acid medium (▲) and in elaidic acid medium (△).

Results

The critical temperature below which bacterial growth ceases is determined by the fatty acid composition of membrane lipids. For cells of an unsaturated fatty acid auxotroph of *E. coli*, growing in medium supplemented with elaidic acid, the critical temperature for growth correlates well with the transport transition temperature (Overath *et al.*, 1970). The experiments described in the accompanying report (Tsukagoshi and Fox, 1973) indicate that the failure in growth below the critical temperature may arise from defective membrane assembly. The experiment described in Figure 1 shows that the growth stasis encountered below 30° for cells grown in medium supplemented with elaidic acid can be averted by substituting oleic acid as the essential fatty acid source. Cells of the unsaturated fatty acid auxotroph 30E were first grown at 37° in medium supplemented with elaidic acid, and then shifted to 25° for further incubation in medium supplemented with either elaidic or oleic acid. The cells incubated at 25° in medium supplemented with elaidic acid exhibited no further growth by criteria such as culture turbidity and viable cell count. Other workers have observed extensive cell lysis under similar conditions (Esfahani *et al.*, 1971), but this is not a characteristic of the strain employed here. For cells shifted to incubation at 25° in medium supplemented with oleic acid, on the other hand, growth resumed after a lag period of approximately 2 hr. In a similar experiment in which cells were first grown at 37° in medium supplemented with oleic acid and then shifted to incubation at 25° in medium supplemented with either oleic or elaidic acid, the following observations were made. (1) Cells shifted to incubation at 25° in medium supplemented with oleic acid continued growing for ten doublings of cell mass, at which point the experiment was terminated. (2) Cells shifted to incubation at 25° in medium supplemented with elaidic acid ceased growing after approximately one doubling in cell mass. These experiments are summarized in Table I.

These data suggest that assembly of a fully functional membrane requires mobile lipids, the fatty acid composition of membrane lipids being a determinant of their mobility. Lipids derived from either elaidic or oleic acid endow the membrane with fluid, mobile characteristics at 37°. At 25°, lipids derived from oleic acid retain these characteristics,

TABLE I: Effects on Growth When Cells Grown Initially at 37° in Medium Supplemented with Elaidic or Oleic Acid Are Incubated at 25° with or without a Concomitant Fatty Acid Shift.

Fatty Acid Supplement		Growth at 25° [a]	
During Initial Growth at 37°	During Subsequent 25° Incubation	Short Term	Long Term
Elaidic	Elaidic	−	−
Oleic	Oleic	+	+
Elaidic	Oleic	+	+
Oleic	Elaidic	+	−

[a] Growth as indicated by increases in turbidity and colony forming units is denoted by (+), and lack of growth by (−). See text and Figure 1 for details.

whereas those derived from elaidic acid are rigid, imparting less lateral mobility to the membrane bilayer. In case 1 (Table I) where cells with elaidic acid derived lipids are shifted to incubation at 25° with no concomitant shift in the fatty acid supplement, membrane assembly steps required for cellular growth are aberrant. This is a general example of abortive membrane assembly which has been demonstrated in a specific case for the β-galactoside transport system (Tsukagoshi and Fox, 1973) and indicates that insertion of functional membrane proteins is defective when both pre- and newly synthesized phospholipids have restricted fluidity owing to the physical properties of their essential fatty acid precursors. Examination of cases 2–4 shows that insertion of newly synthesized membrane components can proceed in a fashion which allows cellular growth when either the pre- or newly synthesized lipids, or both, are in the fluid state at 25°. Case 4 deserves further comment. In this example, aberrant membrane assembly, as assessed from the onset of growth stasis, was encountered after approximately one generation of growth. The onset of growth stasis is probably a response to the altered physical properties of the membrane lipids.

The effects of a temperature shift, with or without a simultaneous shift of the essential fatty acid supplement, on the efficiency of membrane assembly were determined for the specific case of β-galactoside transport system assembly. As described in the accompanying report (Tsukagoshi and Fox, 1973), assembly of the lactose transport system is virtually blocked when cells of an unsaturated fatty acid auxotroph growing in medium supplemented with elaidic acid are shifted from 37 to 25°, but proceeds with only a small decrease in efficiency when cells growing in medium supplemented with oleic acid are subjected to the same temperature shift. The data summarized in Table I suggest that transport system induction would proceed efficiently for a significant period of time during the 25° incubations described in parts 3 and 4 of Table I. When the fatty acid supplement was shifted simultaneously with the 37–25° shift in culture incubation temperature, the ratio of β-galactosidase:transport activities formed after the shift remained constant for at least one hour of induction at 25° (data not shown). For cells shifted from medium supplemented with oleic acid to medium supplemented with elaidic acid, the β-galactosidase:transport ratio is approximately 10, identical with that of cells maintained entirely in medium supplemented with oleic acid (Tsukagoshi and Fox, 1973). By comparison, for cells subjected to an elaidic to oleic acid shift before inducation at 25°, the overall rate of lactose operon expression is reduced over threefold. The ratio of β-galactosidase:transport activities, however, is approximately 17, a value similar to that observed when the lactose operon is induced at 37° in cells grown entirely in medium supplemented with elaidic acid (Tsukagoshi and Fox, 1973). The effects of essential fatty acid source and temperature on efficiency of transport system induction are thus similar to the effects of these conditions on short-term growth (Table I). Induction with reasonable efficiency proceeds in all cases except where the cells are incubated both before and after the temperature shift in medium supplemented with elaidic acid.

The fact that transport system induction proceeds with essentially normal efficiency in the example where cells growing at 37° in medium supplemented with elaidic acid were induced at 25° in medium supplemented with oleic acid, but is virtually blocked when the cells are maintained throughout in medium supplemented with elaidic acid, gives further support to our concept of coupled insertion of newly formed lipids and proteins of the β-galactoside transport system into membrane (Fox, 1969; Hsu and Fox, 1970; Wilson and Fox, 1971). Stronger support for this hypothesis follows from a detailed examination of the properties of the transport system induced after essential fatty acid shifts (Figures 2 and 3).

Figure 2 describes the properties of β-galactoside transport induced at 37 or 25° after an oleic to elaidic acid shift. The durations of the incubation periods for induction at 25 and 37° after the essential fatty acid shift were systematically adjusted so that equal amounts of [^{3}H]elaidic acid were incorporated into the total phospholipid fractions. The amount of [^{3}H]elaidic acid incorporated into the total phospholipid fraction for both conditions of induction (Figure 2A,B) was 12 ± 1.5% of that incorporated into the total phospholipid fraction of a culture grown with a [^{3}H]elaidic acid supplement for four generations, the comparisons being based on the units: cpm of [^{3}H]elaidic acid incorporated into lipid per 1×10^9 colony forming units. The Arrhenius plot for transport induced at 37° is biphasic with an intersection (transition temperature) of 18.0° (Figure 2A). This indicates that the transport system is influenced by a mixed phase of lipids synthesized before and after the essential fatty acid shift since the transition temperature is intermediate between those obtained with cells grown entirely in medium supplemented with oleic or elaidic acids, where the transition temperatures are 13 and 30°, respectively (Wilson and Fox, 1971a). We are therefore in general agreement with the experiments of Overath *et al.* (1971a), who have performed similar experiments which employ an essential fatty acid shift where the incubation temperature is maintained at 37° throughout the course of the experiment. When induction proceeded at 25° after the essential fatty acid shift, on the other hand, the Arrhenius plot for transport was triphasic, and transition temperatures of 26.0 and 14.6° were observed (Figure 2B). Similar results were obtained in two identical independent experiments. In both of these, the Arrhenius plots were clearly triphasic, and the transition temperatures were within 0.5° of those shown. From the data in Figure 2B it appears that a major portion of the transport activity, that with a transition temperature of 26.0°, is influenced primarily by lipids newly synthesized from elaidic acid at 25° after the simultaneous essential fatty acid and temperature shifts. The remaining transport activity, that exhibiting a transition temperature of 14.6°, appears to be influenced primarily by lipids formed before the fatty acid shift, *i.e.*, those derived from oleic acid.

If this interpretation is correct, the properties of transport induced at 25° should be altered when the cells are subsequently incubated at a temperature above 30° to allow mixing of lipid regions derived primarily from oleic or elaidic acid. A sample of the cells induced at 25° was therefore incubated for 10 min at 37° prior to assays of transport rate as a function of temperature (Figure 2C). This Arrhenius plot, like that obtained with cells induced at 37° after the essential fatty acid shift (Figure 2A), is biphasic; and the new transition temperature (18.1°) is identical within the limits of experimental error to that shown in Figure 2A. We interpret these data to indicate that transport proteins which were sequestered in regions of membrane consisting primarily of lipids formed either before or after the essential fatty acid shift exist in a mixed bulk lipid phase after the 37° incubation, and that the mixing process is exquisitely temperature dependent. In membranes containing a mixture of elaidate- and oleate-derived lipids, incubation at 25° might lead to a "freezing out" of regions con-

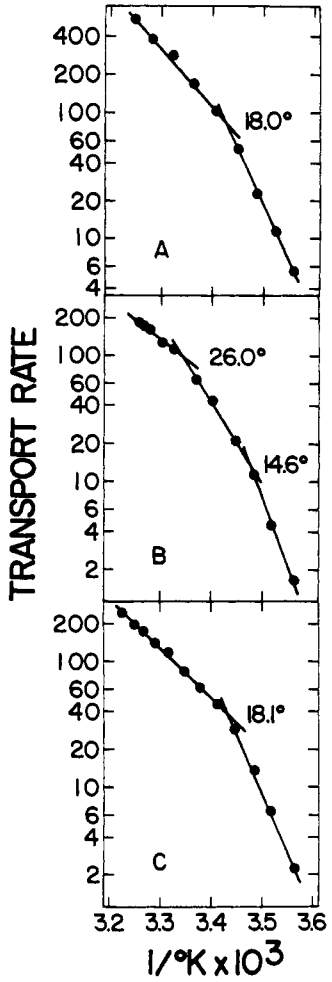

FIGURE 2: The influence of temperature on β-galactoside transport induced after shifting from growth in medium supplemented with oleic acid to growth in medium supplemented with elaidic acid. Cells of strain 30E were grown at 37° in medium supplemented with oleic acid to a culture density of 5×10^8 cells/ml. The cells were harvested by centrifugation and washed once at room temperature in medium supplemented with elaidic acid. The cells were then suspended in medium supplemented with [^{3}H]elaidic acid at the cell density described above and split into two portions. (A) A flask containing one portion of cells was incubated with rotary agitation in a water bath shaker maintained at 37°. After a 5-min incubation, induction of the lactose operon was initiated, and the cells were incubated for an additional 15 min. At this point, chloramphenicol was added at 50 μg/per ml and the culture was quickly chilled to ice bath temperature. The cells were collected by centrifugation, washed once with medium A containing 50 μg/ml of chloramphenicol, and suspended in medium A–chloramphenicol. Samples of cells were removed for lipid extraction (Tsukagoshi and Fox, 1973), and for transport assays (Methods) at the temperatures indicated in the figure. Aliquots of the chloroform phase of the lipid extract were applied to silica gel thin-layer plates which were developed as described in the accompanying report (Tsukagoshi and Fox, 1973). The areas of silica gel containing phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, and cardiolipin were removed by scraping and combined. The silica gel was then placed in a fritted glass filter, and the lipids were eluted with 2:1 (v/v) chloroform–methanol. The eluate was evaporated to dryness, and radioactivity estimated by scintillation counting (Tuskagoshi and Fox, 1973). (B) A flask containing the second portion of cells was incubated with rotary agitation in a water bath shaker maintained at 25°. After 5 min, induction of the lactose operon was initiated, and the cells were incubated for an additional 30 min. At this point, chloramphenicol was added, and the cells were treated at described in part A. (C) A portion of the washed cells suspended in medium A–chloramphenicol from part B was incubated at 37° for 10 min with no aeration before commencing transport assays.

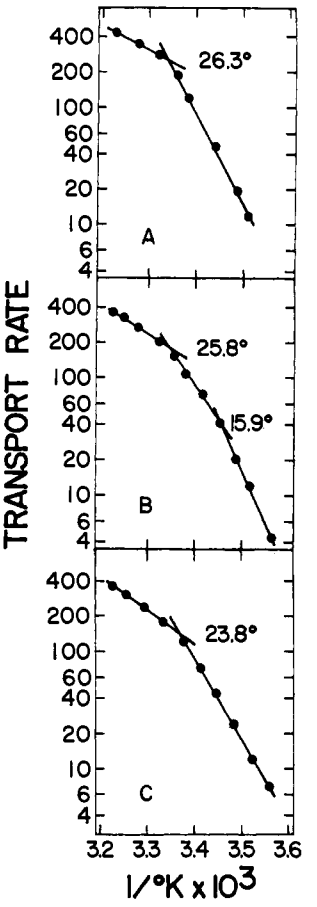

FIGURE 3: The influence of temperature on β-galactoside transport induced after shifting from growth in medium supplemented with elaidic acid to growth in medium supplemented with oleic acid. Cells of strain 30E were grown at 37° in medium supplemented with elaidic acid to a culture density of 5×10^8 cells/ml. The cells were harvested by centrifugation and washed once at room temperature in medium supplemented with oleic acid. The cells were then suspended in medium supplemented with oleic acid at the cell density described above and split into two portions. (A) A flask containing one portion of cells was incubated with rotary agitation in a water bath shaker maintained at 37°. After 5 min, induction of the lactose operon was initiated, and the cells were incubated for an additional 15 min. From this point, the procedure was as described for Figure 2A, except that no sample was removed for lipid analysis. (B) A flask containing the second portion of cells was incubated with rotary agitation in a water bath shaker maintained at 25°. After 35 min, induction of the lactose operon was initiated, and the cells were incubated for an additional 30 min. From this point, the procedure was as described for Figure 3A. (C) A portion of the washed cells suspended in medium A–chloramphenicol from Figure 3B was incubated at 37° for 10 min with no aeration before commencing transport assays.

sisting primarily of elaidate derived lipids. This, were it to occur, could give rise to the observations shown in Figure 2B. To test this possibility, a portion of the cells induced for transport at 37° (Figure 2A) was incubated at 25° for 60 min prior to determination of transport rate as a function of assay temperature. This 25° incubation had no effect, since the Arrhenius plot and transition temperature obtained after the cells had been incubated at 25° were identical within the limits of error to those in Figure 2A (data not shown). Thus the triphasic character of the curve shown in Figure 2B must arise as a consequence of events occurring during membrane assembly.

The complement to the experiments in Figure 2 is presented in Figure 3. Here, the order of growth with the two fatty acid

TABLE II: Estimation of Fraction of Transport Activity Induced at 25° with Characteristics Endowed by Lipids Synthesized before or after the Fatty Acid Shift.

Fatty Acid Supplement During Initial Growth at 37°	Fatty Acid Supplement During Subsequent Induction at 25°	Activn Energy from Curve above Higher Transition Temp (μ_1) (cal)	Activn Energy from Curve below Lower Transition Temp (μ_2) (cal)	Activn Energy from Curve between Higher and Lower Transition Temp (μ_3) (cal)	Fraction of Transport Act. between the Higher and Lower Transition Temp Displaying Characteristics of Membrane Regions Containing	
					Oleate-Derived Lipids (X)	Elaidate-Derived Lipids (Y)
Oleate	Elaidate[a] (35 min)	15,100	50,300	29,400	0.59	0.41[c]
Elaidate	Oleate (35 min)	11,600	37,500	27,400	0.39[c]	0.61
Elaidate	Oleate[b] (60 min)	12,900	42,400	28,300	0.48[c]	0.52

[a] From Figure 2B. [b] From Figure 3B. [c] Fraction of activity with characteristics endowed by lipids synthesized after the fatty acid shift. See text for details.

supplements was reversed, growth in medium supplemented with elaidic acid preceding growth in medium supplemented with oleic acid. The only other alteration in procedure was that the durations of the induction periods were not adjusted so that equal amounts of oleate-derived lipids were formed during incubation at 25 and 37°. The results described in Figure 3 are qualitatively the same as those shown in Figure 2. Induction at 37° after the essential fatty acid shift yields a biphasic Arrhenius plot, and induction at 25° yields a triphasic plot which becomes biphasic when the cells induced at 25° are incubated for 10 min at 37° prior to assay of transport rate as a function of temperature. The conversion of the Arrhenius plots shown in Figures 2B and 3B to those shown in Figures 2C and 3C was not detected when the cells induced at 25° were incubated at 37° for a period of only 1 min. Lipids in artificial bilayers have been reported to diffuse laterally at a rate sufficiently rapid for a single phospholipid molecule to be translated from one side of an *E. coli* cell to the other in a matter of seconds (Kornberg and McConnell, 1971; Devaux and McConnell, 1972; Sackmann and Träuble, 1972). Our observations indicate that not all the lipids in biological membranes may diffuse that rapidly. Thus conclusions drawn from lateral diffusion data obtained with artificial phospholipid bilayer systems should not be indiscriminately extrapolated to biological membranes.

We have treated the data in Figures 2B and 3B and an additional experiment to estimate the fraction of transport activity with characteristics endowed primarily by lipids formed prior to or after the fatty acid shift (Table II). In making these estimates, we equate the activation energy calculated from the curve between the two transition temperatures (μ_3) as being equal to the sum of the fraction of transport activity affected by elaidate-derived lipids (Y) times the activation energy calculated from the slope below the lower transition temperature (μ_2) where all transport activity can be considered as being influenced by lipids which have undergone a change in state from mobile to immobile, plus the fraction of transport activity affected by oleate-derived lipids (X) times the activation energy calculated from the slope above the higher transition temperature (μ_1) where all transport activity can be considered as being influenced by lipids which are entirely in the mobile state.

$$X\mu_1 + Y\mu_2 = \mu_3 \quad (1)$$

Since

$$X + Y = 1 \quad (2)$$

it is possible to solve for the fraction of transport activity which is influenced primarily by elaidate-derived (or oleate-derived) lipids.

$$Y = \frac{\mu_3 - \mu_1}{\mu_2 - \mu_1} \quad (3)$$

This treatment makes the tacit assumption that the μ_1 values are identical for cells containing only oleate-derived or elaidate-derived lipids, and that the μ_2 values are likewise identical when the same comparison is made. Though this is not strictly true in practice, we have never observed differences in either the μ_1 values or the μ_2 values which exceed a factor of 1.4. For example, a comparison of cases 1 and 2 (Table II), which represent the extremes of the data reported here, yields differences of a factor of 1.30 for μ_1, and 1.34 for μ_2. By way of contrast, the fraction of transport activity estimated to be endowed with characteristics reflecting the properties of elaidate derived lipids is 0.41 in case 1, where the fraction of membrane lipids derived from elaidic acid was 0.12. Since these values differ by a factor of greater than 3.4, the fraction of transport activity influenced by elaidate-derived lipids is certainly greater than that expected from the contribution of elaidate-derived lipids to the membrane lipid composition as a whole. One additional point of interest is that the fraction of transport activity influenced primarily by the lipids synthesized during induction at 25° remains relatively constant (Table II). In the three experiments described, this fraction varied from only 0.39 to 0.48. The value of 0.48 is the upper extreme of all our observations, and the true variation is probably somewhat less, for in four independent experiments identical to that of case 3, the values of Y ranged from 0.40 to 0.44. The possible significance of the apparent constancy of this fraction is treated in the discussion.

The rationale advanced for the experiments described up to this point stipulates that elaidate-derived lipids undergo a change of state at 30° from a mobile to an essentially immobile state, whereas oleate-derived lipids undergo this same transition at temperatures well below 25°. If this is indeed the case, then 30° might also represent the point above which

TABLE III: Transition Temperatures for β-Galactoside Transport Induced at Various Temperatures after Shifting from Elaidate to Oleate Medium.

Induction Temp (°C)	Transition Temp (°C)	
37.0[a]	26.3	
31.1[b]	26.8	
28.8[b]	25.1	16.4
25.0[a]	25.8	15.9

[a] From Figure 3. [b] Except for the induction temperatures employed, the procedure was that described for Figure 3B.

Arrhenius plots for transport are biphasic, and below which they are triphasic when induction commences after the essential fatty acid shift from oleate to elaidate or *vice versa*. Table III describes the results of experiments designed to test this hypothesis. These data support the hypothesis since biphasic Arrhenius plots are encountered below 28.8°, but not above 31.1°. We have calculated the activation energies for transport using the data from which the transition temperatures described in Table III were determined. From the calculated activation energies, we in turn estimated the fraction of transport activity influenced primarily by the newly synthesized lipids as described for Table II. This fraction appears to be slightly less for induction at 28.8° than in the case where induction was at 25.0° (Table IV). This difference seems to have less overall significance, however, when compared with the precipitous drop between 28.8 and 31.1°.

Discussion

The first direct correlation between physiological transitions in membrane functions and alterations in the physical properties of membrane lipids involved studies with phospholipid monolayers (Overath *et al.*, 1970). Alterations in the physical state of membrane lipids have also been detected by X-ray diffraction (Engelman, 1970) and differential scanning calorimetry (Steim *et al.*, 1969). The alterations which are detected by X-ray diffraction apparently do not in all cases occur at the same temperature or range of temperatures as do the transitions in transport kinetics (Esfahani *et al.*, 1971; D. Engelman and C. F. Fox, unpublished results). This discrepancy prompted one group to speculate that transport transitions do not arise from a physical alteration occurring in the bulk lipid phase (Esfahani *et al.*, 1971), although to draw this conclusion, it was necessary to ignore the data of Overath *et al.* (1970). Recent studies with other physical probes provide irrefutable evidence that the transport transitions arise from a physical alteration in the bulk lipid phase. Using lipid samples provided by Overath, Träuble has detected an alteration in the physical state of lipid–water dispersions by fluorescence measurements. The temperature at which the physical state of the lipids is altered correlates with the transition temperature for β-galactoside transport in the cells from which the lipids were extracted (Overath *et al.*, 1971b). Using *E. coli* inner membrane preparations (Tsukagoshi and Fox, 1971) correlations have been observed between the temperatures at which transitions in transport kinetics and alterations in the physical state of lipids are detected. Linden *et al.* (1973a), using a 5-nitroxide-substituted decane spin label, have detected what appears to be the onset of a shift in the distribution of the nitroxide moiety between the nonpolar and polar

TABLE IV: Fraction of Transport Activity Influenced Primarily by Newly Synthesized Lipid after Induction at Various Temperatures Following a Shift from Elaidate to Oleate Medium.[a]

Induction Temp (°C)	Fraction of Transport Influenced by	
	Newly Synthesized Lipid	Presynthesized Lipid
25.0	0.39	0.61
28.8	0.33	0.67
31.1	0	1.00
37.0	0	1.00

[a] From Figure 3 and Tables II and III; see text for details.

regions of the lipids in these membrane preparations. The onset temperature of this shift corresponds to the transport transition temperature characteristic of the cells from which the membranes were derived. Linden *et al.* (1973b) have studied the distribution of a different spin label (TEMPO)[1] between the aqueous environment of a membrane suspension and the hydrocarbon side chain milieu of the membrane. The studies with TEMPO have been discussed briefly in the accompanying paper (Tsukagoshi and Fox, 1973) and indicate that lateral phase separations begin at a critical temperature (t_h) and end at a second critical temperature (t_l). Above t_h all the fatty acid side chains of membrane lipids are apparently fluid and flexible. Between t_h and t_l less fluid and flexible patches of lipid separate laterally, and below t_l all the lipids are apparently in the less fluid and flexible state.

Recently reported evidence indicates that biological membranes and model phospholipid bilayers are best viewed as two-dimensional fluids (Frye and Edidin, 1970; Edidin, 1972; Singer and Nicolson, 1972; Kornberg and McConnell, 1971; Devaux and McConnell, 1972; McConnell *et al.*, 1972; Scandella *et al.*, 1972; Träuble and Sackmann, 1972). The membrane phospholipids and proteins are capable of rapid lateral diffusion within the plane of each monolayer face of the membrane. The experiments in Figures 2 and 3 are consistent with this view, but indicate that rapid lateral diffusion is severely restrained below t_l, the critical temperature which defines the low-temperature boundary of the lateral phase separations in membrane phospholipids. Our results are treated in the hypothesis shown in Figure 4. In a treatment of the data in Figure 2 the presynthesized phospholipids derived from oleate are depicted by open circles, and the newly synthesized lipids derived from elaidate, by filled circles. The β-galactoside transport system is depicted by the cross-hatched ellipsoidal figure. When induction proceeds at 37° (Figure 2A), both the oleate- and elaidate-derived lipids are above t_l and are thus laterally mobile. Induction at 37° after the shift from growth with one essential fatty acid to growth with the other therefore leads to the formation of a transport system with a kinetic response to assay temperature that reflects the properties of a mixed bulk lipid phase. If morphogenesis does proceed as indicated in Figure 4, where the transport system is inserted into membrane with lipids synthesized during transport system induction, rapid lateral diffusion of lipids could obscure detection of the initial insertion product. When transport is induced at 25° after the shift from growth in medium supplemented with oleic acid to growth in medium supple-

[1] Abbreviation used is: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

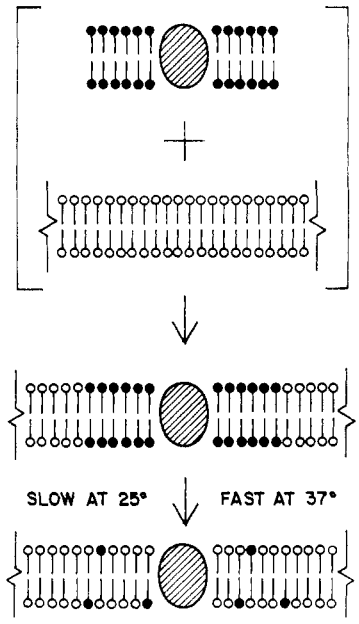

FIGURE 4: A hypothesis for assembly of the β-galactoside transport system (details in text).

mented with elaidic acid (Figure 2B), the initial product of the insertion process is stable, and exists as a solid patch of protein and elaidate derived lipid ($t_1 > 25°$) residing in a fluid milieu of oleate derived lipid ($t_1 < 25°$). The end product of induction at 37° may then be obtained by simply incubating cells (induced at 25°) at 37° to permit melting of the solid patches and subsequent lateral phospholipid diffusion to form a mixed bulk lipid phase (Figure 2A,C). In the reciprocal experiment (Figure 3A–C) where the shift is from growth in a medium supplemented with elaidate to growth in medium supplemented with oleate, the model depicted in Figure 4 still applies, but must be visualized in terms of the formation of liquid patches in a solid milieu.

In formulating this model, we have rejected the possibility that newly synthesized lipids are first formed randomly in the membrane and then subsequently coalesce at 25° by lateral phase separations to form patches into which the newly synthesized transport proteins are inserted. Were this the case, it should have been possible to generate the data in Figure 2B by incubating the cells used in the experiment described in Figure 2A at 25° prior to the assay of transport. Prior assembly of a lipoprotein complex containing the transport system and the subsequent insertion of this lipoprotein complex into membrane fit our data best.

The triphasic nature of the curves in Figures 2B and 3B indicates that after the shift in essential fatty acid, a substantial portion of the transport system formed at 25° has kinetic properties endowed by the lipids synthesized before the shift. This indicates that: (1) the newly formed transport system can be inserted into membrane with either newly synthesized or presynthesized lipid, or (2) the transport system is always inserted into the membrane with newly synthesized lipids, but not all of these inserts are stable. Of these explanations, we prefer the second. The data in Table IV indicate that a larger fraction of the newly synthesized transport system reflects the properties of newly synthesized lipid when induction proceeds after the fatty acid shift at 25° than when it proceeds at 28.8°. This occurs in spite of the fact that less lipid was newly synthesized after the shift at 25° than at 28.8° (data not shown). The fact that transport kinetics reflect the properties of both newly synthesized and presynthesized lipids when induction proceeds at 25° after the shift in the essential fatty acid (Figures 2B and 3B) may be a result of insertion into membrane of newly formed lipoprotein complexes that are heterogeneous in size. Above some critical size, the newly formed structures maintain their stability (whether they exist as fluid patches in a solid milieu, or solid patches in a fluid milieu). Below this critical size, the curvature of the solid–liquid interface is so great that physical integrity of the two phases can not be maintained and rapid lateral mixing of the solid and liquid phases occurs. This explanation can account for the fact that, of the transport activity induced at 25° after the essential fatty acid shift, the fraction of activity with kinetic properties endowed by lipids formed after the shift remains relatively constant regardless of the order of growth with the essential fatty acids, or the extent of the induction period (Table II). This explanation can also account for the decrease in the fraction of transport activity influenced by newly synthesized lipid as the induction temperature is increased (Table IV). The Kelvin equation for curved surfaces provides a sound physical basis for this decrease mentioned above (see Tsukagoshi and Fox, 1973, and also, Kezdy, 1972).

The scheme for transport system morphogenesis depicted in Figure 4 might apply not only to the β-galactoside transport system, but also to other membrane systems composed (at least in part) of "integral" proteins (Singer and Nicolson, 1972). The studies on membrane assembly published to date largely indicate that the assembly process does not lead to segregation of newly synthesized and presynthesized domains (for a review, see Machtiger and Fox, 1973). In none of these studies, however, could the procedures employed have arrested the lateral mixing of presynthesized and newly synthesized domains. Thus even if such domains were formed, their detection would have been prevented by lateral diffusion leading to mixing of old and new membrane components. Our studies reported here indicate that, at least to a limited extent, presynthesized and newly formed domains do exist in membranes when morphogenesis proceeds under rigorously defined conditions. The model depicted in Figure 4 also establishes a rationale for a direct test by density labeling (Fox *et al.*, 1970; Tsukagoshi and Fox, 1971) to determine if coinsertion of newly synthesized lipids and proteins is a major mechanism of membrane morphogenesis.

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Phosphonoprotein. Characterization of Aminophosphonic Acid Rich Glycoproteins from Sea Anemones†

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ABSTRACT: Protein from the sea anemones *Metridium senile* and *Anthopleura xanthogrammica* was systematically examined for phosphonic acid compounds. Phosphonic acid compounds were isolated from the acid hydrolysates of fat-free whole body acetone powders of these animals by ion exchange chromatography and identified chromatographically. 2-Aminoethylphosphonate was found in the whole body protein of *M. senile* at a concentration 0.18 μmol/mg. No other phosphonic acid compound was detected in *M. senile* in a concentration greater than 0.3% of the 2-aminoethylphosphonate. 2-Aminoethylphosphate and *N*-methyl-2-aminoethylphosphate were found in the whole body protein of *A. xanthogrammica* at concentrations of 0.02 and 0.15 μmol/mg, respectively. No other phosphonic acid compound was detected in *A. xanthrogrammica* in a concentration greater than 0.3% of the combined 2-aminoethylphosphonic acid and *N*-methyl-2-aminoethylphosphate. Phosphonoprotein, protein rich in aminophosphonic acid, was isolated from the sea anemones *M. senile* and *A. xanthrogammica*. Phosphonoprotein retained its aminophosphonates through dialysis, gel filtration, electrophoresis, trichloroacetic acid precipitation, and solvent extraction. Phosphonoprotein isolated from *M. senile* contained 0.8–0.85 μmol/mg of 2-aminoethylphosphonate, 0.62 μmol/mg of neutral carbohydrate (glucose equivalents), and 0.36 μmol/mg of hexosamine, but no fatty acid or sialic acid. Electrophoresis indicated at least two components in this preparation. Its apparent mol wt by gel filtration was 2.4–3.0 × 10^5. Phosphonoprotein isolated from *A. xanthrogrammica* contained 0.03 μmol/mg of 2-aminoethylphosphonate, 0.37 μmol/mg of *N*-methyl-2-aminoethylphosphonate, 0.44 μmol/mg of carbohydrate (glucose equivalents), and 0.11 μmol/mg of hexosamine, but no fatty acid or sialic acid. The apparent mol wt by gel filtration was 2.5–3.0 × 10^5. Aminophosphonic acids were absent from some soluble proteins and from collagen of *M. senile*. The nonrandom distribution of aminophosphonic acids in protein fractions suggests that they may serve specific functions in a limited number of proteins which comprise 20–40% of the total body protein in these organisms.

Several aminophosphonic acids have been found in nature, *viz.*, 2-amino-3-phosphonopropionic acid, 2-aminoethylphosphonic acid, *N*-methyl-2-aminoethylphosphonic acid, *N*,*N*-dimethyl-2-aminoethylphosphonic acid and *N*,*N*,*N*-trimethyl-2-aminoethylphosphonic acid (see reviews by Quin, 1967; Kittredge and Roberts, 1969). Most of the work with these compounds has centered on their presence in lipid material. However, significant concentrations of 2-aminoethylphosphonic acid have been reported in proteinaceous fractions as well as lipid fractions from some protozoans (Horiguchi and Kandatsu, 1960), lower metazoans including sea anemones

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