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Mechanistic Interpretation of the Influence of Lipid Phase Transitions on Transport Functions†

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ABSTRACT: In an attempt to understand the mechanism by which a structural change of membrane lipids affects transport functions, the temperature dependence of transport rate has been measured to below the low temperature end of the fluid↔ordered phase transition of the membrane lipids. The unsaturated fatty acid requiring *Escherichia coli* strain T105 was supplemented with either *trans*-Δ^9-octadecenoate or *trans*-Δ^9-hexadecenoate or supplemented with and subsequently starved for *cis*-Δ^9-octadecenoate. Fluid↔ordered phase transitions measured in whole cells using the fluorescence probe *N*-phenyl-1-naphthylamine were compared with the temperature dependence of β-glucoside and β-galactoside transport. In addition to the previously observed downward "break" in the Arrhenius plot of transport rate which occurred near the middle of the phase transition temperature range, a second upward "break" was observed which could be correlated with the low-temperature end of the phase transition. These experiments are interpreted in terms of a partitioning of transport proteins between ordered and fluid domains which is described by a lateral distribution coefficient, κ. This distribution coefficient varies with the membrane lipid composition as well as with the transport system. Values for κ suggest a 2-20-fold preference for the partitioning of transport proteins into the fluid parts of the membrane.

Temperature-induced fluid↔ordered phase transitions of phospholipids in biological membranes are useful to study the structure-function relationships between membrane proteins and their lipid environment. The influence of a phase change on the catalytic action of a membrane embedded protein manifests itself as a change in reaction rate and/or a change in slope of the corresponding Arrhenius plot. Using unsaturated fatty acid requiring mutants of *Escherichia coli* (Silbert et al., 1974; Silbert, 1975), such changes in slope or "breaks" have been attributed to lipid phase transitions for a number of functions associated with the bacterial membrane (Schairer and Overath, 1969; Wilson et al., 1970; Overath et al., 1970; Esfahani et al., 1971; Overath and Träuble, 1973; Sackmann et al., 1973; Linden et al., 1973; Haest et al., 1974; Shechter et al., 1974).

Any correlation between changes in the reaction rate of a membrane function and a fluid↔ordered transition of the lipids depends on an unambiguous determination of the phase transition. Although a large number of methods are now available for the measurement of thermal transitions in membranes (cf. Melchior and Steim, 1976, for review), the most direct method remains the use of wide angle x-ray diffraction (Engelman, 1971; Shechter et al., 1974). The x-ray measurements show that within a certain temperature range domains of fluid and ordered lipids coexist and by comparison with suitable lipid standards they define the proportion of lipids taking part in the membrane transition. The high- and low-temperature boundaries of the transition range, T_h and T_l, are given by the temperatures where all the lipid molecules taking part in the transition have entered the fluid and ordered states, respectively. The width of the transition range, ΔT,[1] is given by $\Delta T = T_h - T_l$; the mid-transition temperature, T_t, can be defined as the temperature where one-half of the lipid is in either phase. Whereas the x-ray method defines the phase transition on a molecular basis, the convenient fluorescence technique using *N*-phenyl-1-naphthylamine (PhNap)[2] as a probe gives similar results regarding ΔT and T_t (Overath and Träuble, 1973; Overath et al., 1975). This method enables us to measure the lipid phase transition in whole cells under exactly the same conditions as the temperature dependence of membrane functions (Thilo and Overath, 1976).

Previous studies on the influence of a lipid phase transition on transport rate across the bacterial membrane revealed a biphasic shape for the Arrhenius plots (biphasic: cf. Figure 6a; Wilson et al., 1970; Overath et al., 1970; Esfahani et al., 1971; Overath and Träuble, 1973; Shechter et al., 1974). The rather limited number of experimental points led to extrapolations which suggested that, at a critical temperature, T_c, there is an abrupt change of activation energy, i.e., a "break" in the sense of a discontinuity. In the case of β-galactoside transport, the

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[1] This definition of ΔT differs from that used previously (cf. Overath and Träuble, 1973).

[2] Abbreviations used are: *cis*-Δ^9-18:1, *cis*-Δ^9-octadecenoic acid; *trans*-Δ^9-18:1, *trans*-Δ^9-octadecenoic acid; *trans*-Δ^9-16:1, *trans*-Δ^9-hexadecenoic acid; *cis,cis,cis*-$\Delta^{9,12,15}$-18:3, *cis,cis,cis*-$\Delta^{9,12,15}$-octadecatrienoic acid; PhNap, *N*-phenyl-1-naphthylamine; iPrSGal, isopropyl 1-thio-β-D-galactopyranoside; NphGal, *o*-nitrophenyl β-D-galactopyranoside; NphGlu, *p*-nitrophenyl β-D-glucopyranoside; CR buffer, Cohen-Rickenberg mineral salts medium.

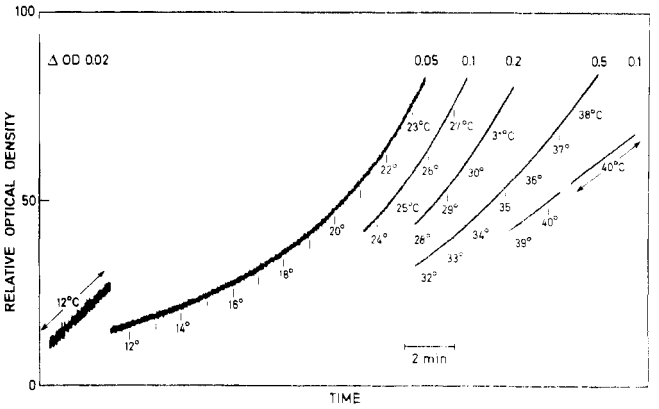

FIGURE 1: Temperature dependence of in vivo NphGlu hydrolysis. On the ordinate, 100 corresponds to a difference in optical density, ΔOD, as indicated. The rate of reaction is given by the slope of the curve. Evaluation at the different temperatures results in the Arrhenius plot of Figure 6b. Whole cells were used at an optical density of 16 at 420 nm. Substrate was added at a concentration of 4 mM.

extrapolated temperature, T_c, was found to correlate reasonably well with the transition temperature, T_t, of the lipids in the membrane (Overath and Träuble, 1973). Such a discontinuous change in the activation energy without a simultaneous drop in transport rate was difficult to understand. Firstly, it requires an isothermal phase change at T_c which is difficult to reconcile with the broadness of the transition. Secondly, in order to leave the rate constant at T_c unchanged, the change in activation enthalpy must be exactly compensated by a change in activation entropy (cf. Kumamoto et al., 1971).

In a more recent investigation (Thilo and Overath, 1976; see also Linden et al., 1973), the larger number of experimental points in the Arrhenius plots of sugar transport rate showed that there is a rather gradual, continuous transition from one slope to the other. A continuous change in slope in a biphasic Arrhenius plot of rate constants can be explained in terms of a cold inactivation of an enzyme (Han, 1972: Scheme III, Type II, Figure 2) or in a special case by a changeover between two parallel reactions of different efficiencies (Han, 1972: Scheme II, Figure 8). For the transport rates considered here, cold inactivation would have to be related to the lipid phase transition.

However, even the continuous transition of slope in a biphasic Arrhenius plot of transport rate occurred over a rather narrow temperature range which remained difficult to correlate with the significantly larger width, ΔT, of the lipid phase transition. It appeared possible that this change in slope indicated only the high temperature end of the thermal transition, while a second change in slope at the low temperature end had remained undetected. In almost all cases reported so far, the temperature dependence of transport rate has not been measured with sufficient accuracy below a simultaneously well-established lower end, T_l, of the lipid phase transition, to allow a decision as to whether the Arrhenius plot of such a function is actually triphasic or not. Therefore, we attempted a more accurate determination of the temperature dependence of transport rate down to and below the lower end of the lipid phase transition. Part of this work has been published in a recent communication (Overath et al., 1976).

Materials and Methods

Strain and Growth Conditions. Strain T105 is a derivative of the fatty acid requiring *E. coli* strain K1062 (Overath et al., 1971) selected for growth on salicin (Schaeffler, 1967). Strain T105 is therefore inducible for the β-glucoside transport system. The growth medium was Cohen-Rickenberg (CR) mineral salts medium (Anraku, 1967) supplemented with 0.5% glycerol, 0.3% casamino acids (Difco, vitamin-free), 0.5% Brij 35, and 0.01% of either *trans*-Δ^9-18:1, *trans*-Δ^9-16:1, or *cis*-Δ^9-18:1. The lactose transport system was induced by the addition of 0.5 mM iPrSGal for between one and two cell mass doubling times. For the induction of β-glucoside transport glycerol was omitted from the growth medium, while 0.5% salicin (Serva, Heidelberg) was added for a period of more than six generation times. Cell cultures (200–500 mL) were grown in 2-L Fernbach flasks at 39 °C with slow rotary aeration. Cells were harvested at an optical density at 420 nm between 1 and 2 and washed five times in 15 mL of CR buffer without supplements. The cells were then resuspended in CR buffer to an optical density between 20 and 30 and kept at room temperature. In case of cells induced for β-glucoside transport, 0.3% casamino acids were added after resuspension.

Measurement of Transport Rate. β-Galactoside transport was measured as described before using an Aminco DW-2 UV/Vis spectrophotometer (Overath et al., 1971). Since some samples had a high passive permeability for the substrate, NphGal, especially at low temperatures, care was taken that only the protein catalyzed reaction was measured. NphGal (2 mM) was first added to both cuvettes. After ensuring that the difference in change of optical density was negligible, 2 mM β-galactosyl 1-β-thiogalactoside was added to the reference cuvette in order to determine the rate of β-galactoside transport.

For the measurement of β-glucoside transport cells were diluted in CR buffer containing 0.3% casamino acids to an optical density of about 15. This cell suspension was then filled into two compartments of a multicompartment cuvette attached to a Gilford thermoprogrammer type 2527. After cooling the cuvette to the lower end of the temperature range to be covered by the measurement, the substrate NphGlu was added to one cuvette compartment at a concentration of 4 mM. The other compartment served as reference. The increase in optical density at 420 nm was measured in the Aminco spectrophotometer either at fixed temperatures or continuously at a heating rate of 1 °C/min. An example for the latter type of experiment is shown in Figure 1. The temperature dependence of the reaction rate was obtained from the slope of the curve at various temperatures. Completely congruent Arrhenius plots were obtained for a stepwise or continuous rise in temperature.

The lipid phase transition was measured for whole cells using the fluorescence probe PhNap as described before (Thilo and Overath, 1976). The temperature was increased at a rate of 1 °C per min; i.e., the conditions were the same as for the measurement of NphGlu transport. The *fatty acid composition* of the cells was similar to that described in previous publications.

Experiments. For the previously studied *lactose transport system* (Overath and Träuble, 1973), we now made extensive measurements on the temperature dependence of transport rate, especially at temperatures below the lower end of the lipid phase transition. Figure 2 shows an Arrhenius plot of transport rate (upper part) together with the fluorescence phase transition curve (lower part) for *trans*-Δ^9-16:1 supplemented cells. Both parts show the average of two independent experiments. A similar plot for *trans*-Δ^9-18:1 supplemented cells is shown in Figure 3. In this case the individual measurements are shown in order to give an indication of the reproducibility of the assays. This is important because the carrier mediated uptake

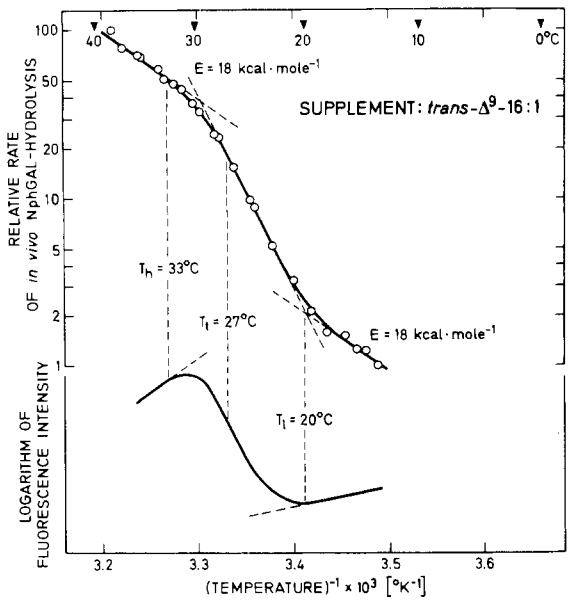

FIGURE 2: Temperature dependence of in vivo NphGal hydrolysis for *trans*-Δ^9-16:1 supplemented cells (upper part) in comparison with the lipid phase transition as measured by PhNap fluorescence in whole cells (lower part). Each point represents the average of at least four measurements, two from each of two independent experiments. Cells were used at an optical density of about 10 at 420 nm. Substrate concentration was 2 mM (see Materials and Methods).

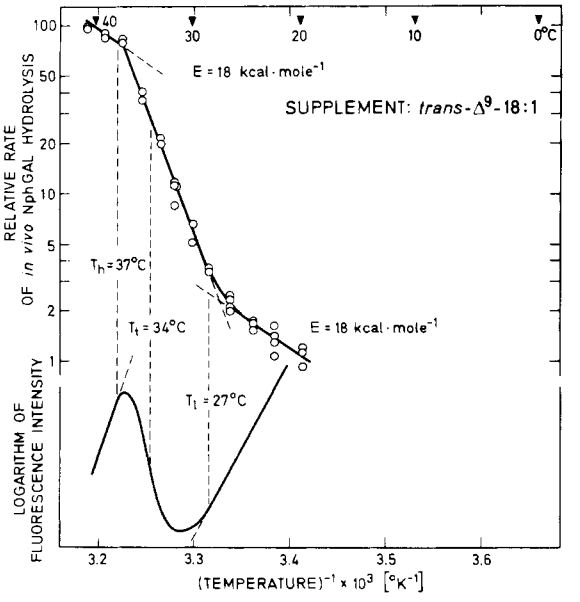

FIGURE 3: Temperature dependence of in vivo NphGal hydrolysis for *trans*-Δ^9-18:1 supplemented cells (upper part) in comparison with the lipid phase transition as measured by PhNap fluorescence in whole cells (lower part). Multiple rate measurements all belong to the same experiment.

of substrate is measured against a background rate of carrier-independent passive leakage, which, at low temperatures, may be two to five times faster than carrier-mediated uptake.

In both Figures 2 and 3 the Arrhenius plots of transport rate are clearly triphasic. The first change in slope occurs in the upper half of the phase transition as reported previously (Overath and Träuble, 1973). The second, upward change in slope correlates well with the low temperature end of the transition. As is indicated in Figures 2 and 3, activation energies are only

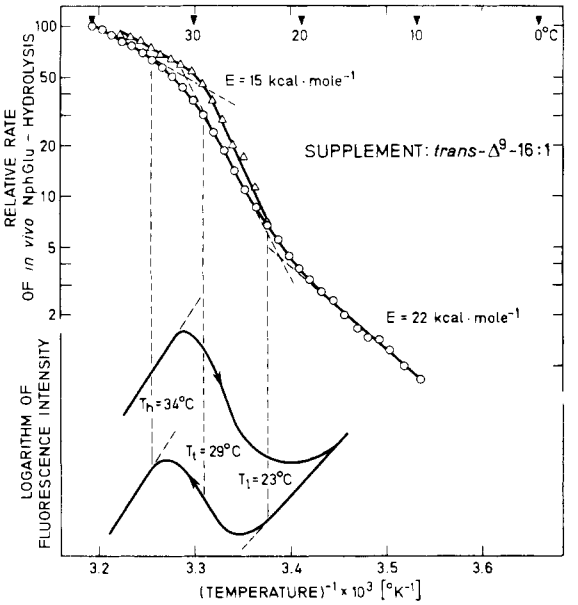

FIGURE 4: Temperature dependence of in vivo NphGlu hydrolysis for *trans*-Δ^9-16:1 supplemented cells (upper part) in comparison with the lipid phase transition as measured by PhNap fluorescence in whole cells (lower part). Transport rate measurements were obtained during a continuous increase (— O —) or decrease (— Δ —) in temperature (see Figure 1 and Materials and Methods). The points represent the average of three individual temperature scans. In the lower part the direction of temperature scans is indicated by the arrows.

assigned to those parts of the curves which are above and below the temperature range of the phase transition. A uniform activation energy of 18 kcal mol^{-1} for both the upper and lower ends of the curves fits all four cases within the experimental accuracy.

The apparent affinity of the transport protein for the substrate was determined at various temperatures throughout the phase transition. At all temperatures, plots of transport rate vs. substrate concentration are described by the Michaelis–Menten equation. Within the experimental accuracy, the K_M remains essentially unchanged, at about K_M = 1.2 mM which implies that the temperature-dependent changes in the rate constants entering the Michaelis–Menten equation compensate each other. According to this criterion, the binding of the substrate to the carrier appears to be unaffected by the phase transition.

The temperature dependence of *β-glucoside transport* has also been studied previously (Wilson et al., 1970; Linden et al., 1973). This system allows a continuous measurement of transport rate as a function temperature (cf. Figure 1). Figure 4 shows the temperature dependence of β-glucoside transport for *trans*-Δ^9-16:1 supplemented cells. The lower part of the figure indicates the fluorescence change during the lipid phase transition for both the upward and downward temperature scan. The observed hysteresis of about 3 °C has previously been reported (Overath and Träuble, 1973; Thilo and Overath, 1976). Hysteresis can also be detected for the high-temperature part of the Arrhenius plot as shown by the triangles in Figure 4.

The temperature dependence of β-glucoside transport was determined for cells of different hydrocarbon chain composition of the membrane lipids. Figure 5 shows the correlation between transport rate and lipid transition for *trans*-Δ^9-18:1 supplemented cells. In Figure 6a, cells were supplemented with *cis*-Δ^9-18:1. The lower end of the phase transition is below 8 °C and is difficult to locate by PhNap fluorescence in whole

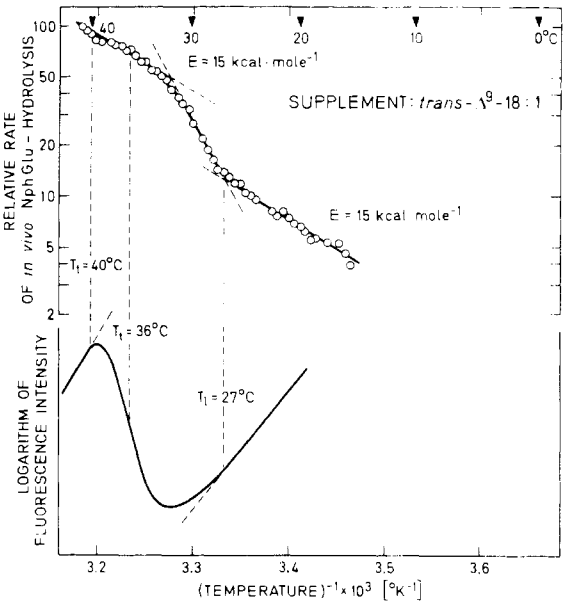

FIGURE 5: Temperature dependence of in vivo NphGlu hydrolysis for *trans*-Δ^9-18:1 supplemented cells (upper part) in comparison with the lipid phase transition as measured by PhNap fluorescence in whole cells (lower part). The points represent the average of three independent temperature scans.

cells. Correspondingly, we do not find the low temperature change in slope in the Arrhenius plot of sugar transport. The *cis*-Δ^9-18:1 supplemented cells were starved for unsaturated fatty acids. This leads to a gradual increase in the proportion of saturated acyl chains in the membrane lipids with a simultaneous rise in transition temperature (Thilo and Overath, 1976). Figures 6b and 6c show in their lower parts the phase transition curves after growth increments of 70 and 130%, respectively, and, in the upper part, the corresponding temperature dependences of transport.

Taken together, it is evident that, for all the cases presented in Figures 4 to 6, the Arrhenius plots of β-glucoside transport are triphasic. There is a good correlation between the low temperature end of the thermal transition and the temperature where an upward swing in slope occurs in the corresponding Arrhenius plots of transport. The same activation energy of 15 kcal mol^{-1} can be assigned to the upper and lower ends of the Arrhenius plots. The higher activation energy of 22 kcal mol^{-1} observed in Figure 4 is an exception (average of four experiments $E = 15 \pm 3$ kcal mol^{-1}; see also Figure 7, lower part).

The dependence of β-glucoside transport rate on substrate concentration follows the Michaelis–Menten equation only at temperatures below the phase transition of the membrane lipids. Above the phase transition, the transport rate increases linearly with increasing substrate concentration until a plateau is reached at a maximal rate, v_{max}. As a criterion for the substrate affinity of the carrier, we took the substrate concentration, c, where $v = 0.5v_{max}$. The following values were found for *trans*-Δ^9-16:1 supplemented cells: $c = 0.15 \pm 0.03$ mM for $T > T_h$, $c = 0.16 \pm 0.04$ mM for $T_l < T < T_h$ and $c = K_M = 0.04 \pm 0.01$ mM for $T < T_l$. The significance of this peculiar behavior is unclear. The change in saturation kinetics is related to the lipid transition: between 10 and 40 °C cells supplemented with *cis,cis,cis*-$\Delta^{9,12,15}$-18:3 reveal a linear dependence of transport rate on substrate concentration before reaching a plateau. Also, these cells show no lipid transition in this temperature region using PhNap as a probe. It should

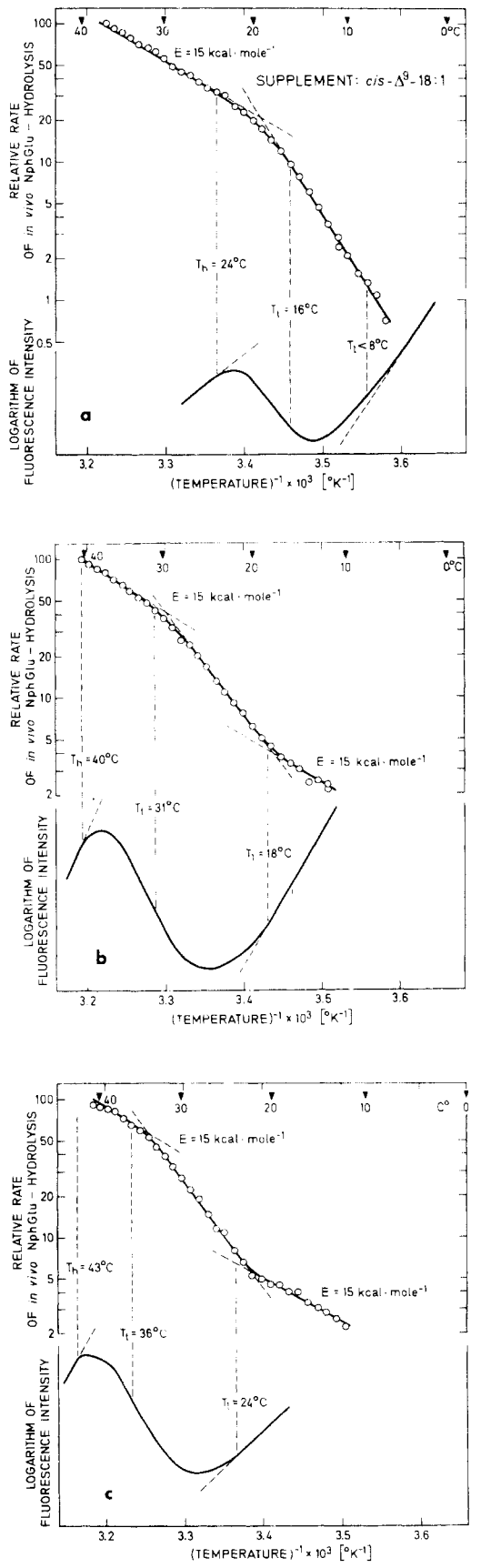

FIGURE 6: Temperature dependence of in vivo NphGlu hydrolysis for *cis*-Δ^9-18:1 supplemented cells (upper part, a). The cells were then starved for unsaturated fatty acids during a growth increment of 70% (upper part, b) and 130% (upper part, c). The proportions of *cis*-Δ^9-18:1 derived acyl chains were 45% (a), 29% (b), and 22% (c). The corresponding lipid phase transitions as measured by PhNap fluorescence in whole cells are shown in the lower parts of the figure.

be pointed out, however, that the data of Figures 4–6 were obtained at saturating substrate concentrations.

Theory. (i) Lateral Distribution Coefficient. As is suggested by x-ray measurements (Shechter et al., 1974; see also discussion in Overath et al., 1975, 1976), we consider the membrane within the transition range, ΔT, as being composed of ordered and fluid lipid domains. Let us first quantify the course of the lipid phase transition by a parameter, ρ, as

$$\rho = \frac{F_{fl}}{F_{fl} + F_{ord}} \tag{1}$$

with F_{fl} and F_{ord} denoting the surface area of coexisting fluid and ordered lipid domains, respectively. ρ changes from $\rho = 1$ at $T \geq T_h$ to $\rho = 0$ at $T \leq T_l$ according to the normalized change in fluorescence intensity at the phase transition (see, for example, Figure 7, upper part). The coexistence of fluid and ordered domains does not imply a stationary situation but can be regarded as fluid–ordered phase boundaries traversing the lipid matrix. At any given time, a single carrier protein will be in either a fluid or an ordered domain. Therefore, on the whole the carrier proteins will be distributed between fluid and ordered domains. This partitioning can be quantified by a lateral distribution coefficient, κ, as

$$\frac{P_{fl}}{P_{ord}} = \kappa \frac{F_{fl}}{F_{ord}} \tag{2}$$

where P_{fl} and P_{ord} denote the number of carrier proteins in the fluid and ordered domains, respectively.

The meaning of the coefficient, κ, is explained schematically in Figure 7. A value of $\kappa > 1$ implies preferential partitioning of the carrier molecules in the fluid, $\kappa < 1$ preferential partitioning in the ordered domains. For an individual carrier protein, $\kappa > 1$ implies that on the average the protein spends more time in a fluid lipid environment. In terms of the lipids, $\kappa > 1$ means that ordered domains tend to be formed in areas free of carrier protein. Since, in addition, the membrane proteins are undergoing lateral motion in the fluid parts of the membrane, then for $\kappa > 1$, this will result in large areas of ordered lipids which are free of proteins. Such lipid–protein separations are revealed by freeze-etch electron microscopic observations of membranes below the phase transition (for review, see Verkleij and Ververgaert, 1975).

(ii) Coupling between Lipid Phase Transition and Protein Function. The Arrhenius equation for the temperature dependence of transport rate, v, can be written in the usual way as

$$v = P\nu e^{-E/RT} \tag{3}$$

where P is the concentration of protein involved in the rate limiting step of transport. The preexponential factor, ν, has the dimension of a frequency and when multiplied with the exponential function gives the rate constant, k, of the rate limiting step. E is the activation energy, R the molar gas constant, and T the absolute temperature. The triphasic Arrhenius plots reported in this paper allow a mechanistic interpretation of the coupling between lipid phase transitions and transport functions. We assign activation energies, E_{fl} and E_{ord} to the linear parts of the plots above and below the lipid phase transition, respectively. The steep intermediate part of the plots will be interpreted as a change in the rate constant, k, and/or the effective carrier concentration, P, rather than as a higher activation energy.

(a) Change in Rate Constant, k. The carrier protein can function with one of two rate constants, k_{fl} or k_{ord}, depending on whether it functions in a fluid or ordered lipid domain, respectively. Therefore, the rate of transport can be expressed as the sum of two contributions

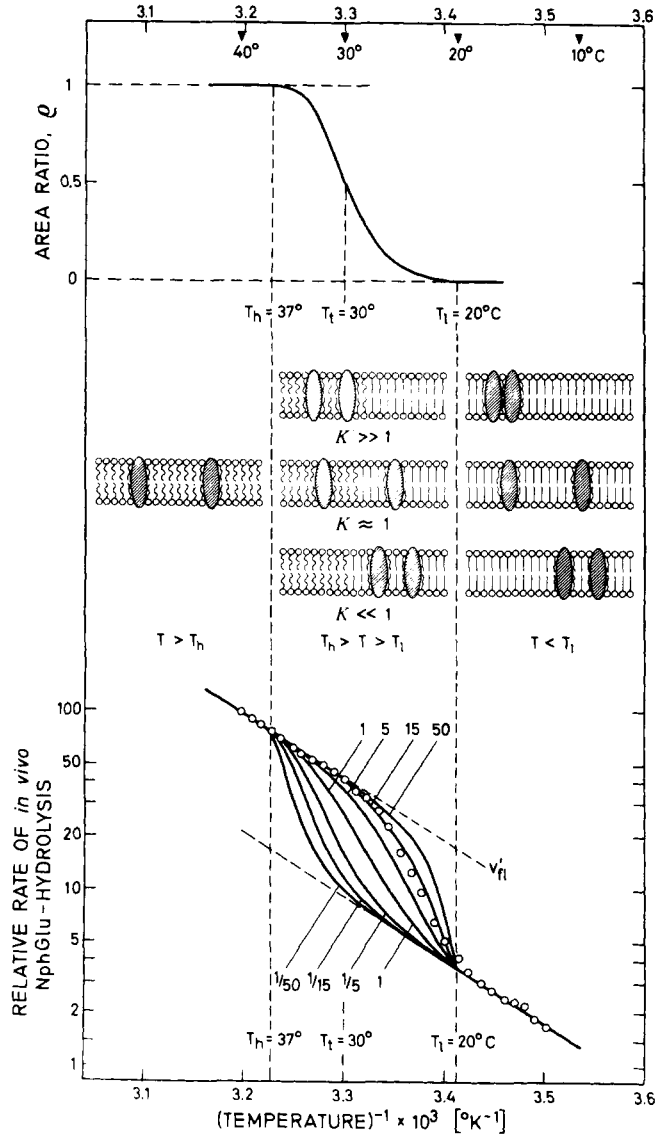

FIGURE 7: The distribution of carrier proteins between fluid and ordered lipid domains for different values of the lateral distribution coefficient κ (middle part, schematically). In the lower part, the temperature dependence of transport rate was calculated according to eq 5 with different values of κ as indicated. $E_{fl} = E_{ord} = 15$ kcal mol^{-1} and $\nu_{ord}/\nu_{fl} = 0.22$ were taken from the fit to the experimental values at $T > T_h$ and $T < T_l$. ρ was taken from the normalized change in fluorescence intensity as shown in the upper part. The temperature dependence of in vivo NphGlu hydrolysis is shown for whole cells supplemented with *trans*-Δ^9-16:1. Best fit between theory and experiment is obtained for $\kappa = 15$.

$$v = P_{fl}\nu_{fl}e^{-E_{fl}/RT} + P_{ord}\nu_{ord}e^{-E_{ord}/RT} \tag{4}$$

Equations 1–4 lead to an expression for the rate of transport, v, as a function of the fluid–ordered area ratio, ρ, viz.

$$v = \frac{v_{fl}'}{1 + \rho(\kappa - 1)}\left[\kappa\rho + (1 - \rho)\frac{\nu_{ord}}{\nu_{fl}}e^{(E_{fl}-E_{ord})/RT}\right] \tag{5}$$

where $v_{fl}' = P_{tot}k_{fl}$, is the rate when all carrier molecules, $P_{tot} = P_{fl} + P_{ord}$, function in fluid lipid domains (hypothetical for $T < T_h$).

(b) A change in the effective carrier concentration, P, will occur if the carrier proteins no longer function when they are in ordered lipid domains. In this case $k_{ord} = 0$, i.e., $\nu_{ord} = 0$. The remaining transport activity at $T < T_l$ can be accounted for

TABLE I: Lateral Distribution Coefficient.

Transport System	Fatty Acid Supplement	ΔT (°C)	κ
Glucoside	*cis*-Δ^9-18:1	>16	1.5[a]
	No supplement[b]	22	4
	No supplement[b]	19	10
	trans-Δ^9-16:1	11-17	4-15
	trans-Δ^9-18:1	13	20
Galactoside	*trans*-Δ^9-16:1	13	1.5
	trans-Δ^9-18:1	10	2.5

[a] Transport measurement was extrapolated below 7 °C with $E_{ord} = E_{fl}$ (Figure 6a). [b] Cells previously supplemented with *cis*-Δ^9-18:1 and then starved for unsaturated fatty acids for increasing lengths of time (cf. Figure 6b,c).

by the assumption that a residual number of carrier proteins, P_r, continues to function below the phase transition. If this fraction functions with the same rate constant, k_{fl}, as above the phase transition, a combination of eq 1-4 leads to

$$v = \frac{v_{fl}'}{1 + \rho(\kappa - 1)} \left[\kappa\rho + (1 - \rho) \frac{P_r}{P_{tot}} \right] \quad (6)$$

where now $P_{tot} = P_{fl} + P_{ord} + P_r$.

In the case when both parameters, k and P, undergo a change during the phase transition, this leads to an equation for the transport rate, v, which cannot be solved for κ with the use of the present experimental data.

The shape of Arrhenius plots which is predicted by eq 5 depends on the position of the two straight parts relative to each other, i.e., on k_{ord}/k_{fl} in the temperature range of the phase transition (cf. Han, 1972, Figures 5-7). If $|E_{fl} - E_{ord}|$ is significantly larger than RT_t, this again requires a certain degree of entropy-enthalpy compensation in order to prevent k_{ord}/k_{fl} from becoming incompatible with experimentally observed values (see introductory section).

(iii) Comparison between Experiment and Theory. In order to apply the theory to our experimental observations, we take E_{fl}, E_{ord}, and ν_{ord}/ν_{fl} or P_r/P_{tot} from the upper and lower linear parts of the Arrhenius plot and ρ from the normalized fluorescence curve. The distribution coefficient, κ, is then varied to obtain optimal fit with the experimental values of transport rate in the transition region of the Arrhenius plot. An example is given in Figure 7. A set of curves calculated for values of κ between 0.02 and 50 is compared with the measured temperature dependence of glucoside transport rate for cells supplemented with *trans*-Δ^9-16:1. The change in fluid-ordered area ratio, ρ, as measured for these cells was used for the calculations. The curve for $\kappa = 1$ is a direct transform of the change in ρ with temperature. For increasing values of κ, the calculated transition curves are shifted toward lower temperatures. In the given example, a value of $\kappa = 15$ results in a shift adequate to fit the experimental values. Values of κ obtained in this way for the various experiments are summarized in Table I together with ΔT, the width of the lipid phase transition.[3]

Our results show that, within experimental accuracy, $E_{fl} = E_{ord}$. In this case eq 5 and 6 differ only by the replacement of ν_{ord}/ν_{fl} with P_r/P_{tot}. Therefore, the evaluation according to eq 5 or 6 results in the same value for κ. On the basis of our present experiments, we can not distinguish between a change in rate constant or in effective carrier concentration.

[3] κ does not necessarily remain constant throughout the phase transition. Our experiments then yield average values of κ.

Discussion

Transport of β-galactosides is mediated by the y-gene product of the *lac*-operon and involves the concurrent translocation of a proton (see Simoni and Postma, 1975, for review). Transport of NphGlu, on the other hand, is mediated by the phosphotransferase system which couples phosphorylation of the sugar to translocation (Roseman, 1969; Rose and Fox, 1971). Since both transport systems cannot yet be studied in a purified form in defined lipid surroundings, it remains a reasonable, yet unproven assumption that the lipid phase transition directly affects a rate-limiting step during substrate translocation.

It should be recalled that the salient point for the interpretation of previously published Arrhenius plots (e.g., Overath and Träuble, 1973) was not their biphasic shape as such, but rather that the downward changes in slope could be correlated to the phase transition. The same argumentation is now applied to the upward changes in slope of the temperature characteristic observed at low temperatures in Figures 2-7; i.e., the correlation to the lower end of the phase transition, T_l, identifies these changes as a lipid effect on protein function. The transport proteins appear to behave like membrane embedded probe molecules sensing both the beginning and the end of the fluid↔ordered transition.

The values of κ are generally larger than one, implying that transport proteins partition preferentially into the fluid lipid domains. This result is consistent with freeze-etch electron microscopic data which reveal domains of densely packed particles and particle-free domains as the membrane is cooled below the thermal transition (see Verkleij and Ververgaert, 1975, for review; Shechter et al., 1974; Haest et al., 1974; Kleemann and McConnell, 1974; van Heerikhuizen et al., 1975). Especially in the case of the glucoside transport system, there is a tendency for κ to become larger when the width of the phase transition, ΔT, becomes smaller. This can be interpreted as a stronger exclusion of proteins from ordered lipid domains due to a more cooperative lipid-lipid interaction in the case of a sharper phase transition. For the glucoside transport system, the values of κ are markedly larger than in the case of the galactoside transport system. The ratio ν_{ord}:ν_{fl} or P_r:P_{tot} is about 1:10 in the case of β-galactoside transport and is similar for both fatty acid substitutions. The β-glucoside system shows a distinctly different behavior. Comparison of Figures 4 and 5 or Figures 6a-c reveals that ν_{ord}:ν_{fl} or P_r:P_{tot} becomes smaller upon an increase in the width of the transition, ΔT. At present we cannot explain the different behavior of the two transport systems.

Large values of κ may be simulated if the rate-determining step of a transport process depends on a diffusion-controlled interaction of two membrane proteins. (Compare, for example, the interaction of cytochrome b_5 reductase with cytochrome b_5 in dimyristoyllecithin liposomes; Strittmatter and Rogers, 1975.) For $\kappa > 1$ there will be an accumulation of membrane proteins in fluid domains which decreases the average distance between the interacting partners. This will increase the interaction rate and thereby counteract the decrease in transport rate due to the decreasing temperature. The result is a shift of the steep intermediate part of the Arrhenius plots toward lower temperatures which simulates an apparent value of κ which is larger than the true value. In addition, small values of ν_{ord}/ν_{fl} may be expected for large values of ΔT if more reaction partners become separated when trapped in the smaller and more numerous separated fluid domains which are generated during

a broader, less cooperative phase transition. These considerations may be relevant for the glucoside transport system.

In spite of a voluminous literature on temperature-dependent effects on a variety of membrane associated functions, there are very few studies which either support or contradict the experiments reported in this communication. Firstly, "breaks" in Arrhenius plots of membrane function observed in higher cells often appear to be related to "lipid cluster formation" rather than the ordered↔fluid transition considered here (Lee et al., 1974; Lee, 1975; Cannon et al., 1975; Davis et al., 1976). Secondly, a number of previous studies including some of our own work suffer one or several of the following shortcomings: (1) the order↔disorder transition was not established by or correlated with an unambiguous physical method such as wide-angle x-ray diffraction, calorimetry, or volumetry. (2) The low-temperature end, T_l, of the transition was below 0 °C. (3) The physiological parameter was not or could not be measured with sufficient accuracy above and below the limiting temperatures of the transition, T_h and T_l.

A suitable example for discussing these points is provided by the many reported experiments using *trans*-Δ^9-18:1-supplemented *E. coli* fatty acid auxotrophs or derived membrane preparations. By taking the average of several reported values obtained by independent methods (Esfahani et al., 1971; Linden et al., 1973; Kleemann and McConnell, 1974; Shechter et al., 1974; Haest et al., 1974; Overath et al., 1975; present communication), one arrives at a transition temperature, $T_t = 35 \pm 2$ °C, an upper end $T_h = 40 \pm 3$ °C, and lower end of the transition, $T_l = 27 \pm 4$ °C. Comparison of these values with the temperature range investigated in functional studies reveals that in some investigations a low temperature change in slope of Arrhenius plots should have been detected (e.g., β-galactoside efflux, Overath et al., 1970), whereas in other cases the number of measurements and/or the temperature range was insufficient (e.g., NphGal transport, Overath and Träuble, 1973; Linden et al., 1973; proline transport, Shechter et al., 1974). On the other hand, a triphasic Arrhenius plot was previously observed for the temperature dependence of respiration (Overath et al., 1970). In this context, it may be noted that we were unable to demonstrate any abrupt jumps in transport rate like those reported by Linden et al. (1973) and Linden and Fox (1973). In fact, strain 30 Eβox⁻, kindly provided by Dr. C. F. Fox, displayed a behavior for β-glucoside transport similar to that of strain T105 shown in Figures 4 and 5.

Recently, several reconstituted systems have become available in which the catalytic function of proteins is influenced by their lipid "annulus" (Warren et al., 1974; Houslay et al., 1975). If the protein is influenced solely by its lipid annulus and not by the bulk lipid phase an interpretation in terms of a lateral distribution coefficient becomes meaningless. On the other hand, the bulk lipid phase can be expected to exert an influence on the physical state of the lipids in the annulus and thereby on the protein (Marčelja, 1976). Other reconstituted systems (Wilschut et al., 1976; Strittmatter and Rogers, 1975) show, in fact, a triphasic temperature characteristic. These model systems can be useful for unravelling the molecular details of the coupling between lipid structure and protein function.

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Recognition of Different Pools of Phosphatidylglycerol in Intact Cells and Isolated Membranes of *Acholeplasma laidlawii* by Phospholipase A_2†

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ABSTRACT: Phospholipase A_2 (EC 3.1.1.4) from pig pancreas hydrolyzes phosphatidylglycerol in intact cells and isolated membranes of *Acholeplasma laidlawii.* Complete degradation of phosphatidylglycerol in intact cells at 37 °C does not result in lysis as shown by the retention of intracellular K^+ ions and the cytoplasmic glucose-6-phosphatase, as well as the inability to detect activity of membrane-bound intracellular NADH-oxidase. *A. laidlawii* was grown on linoleic acid. Phospholipase A_2 treatment of these cells at 5 °C, at which temperature the lipids are still in the liquid-crystalline state, results in a rapid breakdown of 50% of the phosphatidylglycerol. The residual phosphatidylglycerol can be hydrolyzed only at elevated temperatures and at much smaller rates, depending strongly on the incubation temperature. When membranes isolated from these cells are incubated at 5 °C, 70% of the phosphatidylglycerol is hydrolyzed immediately. The hydrolysis of the residual 30% is again strongly temperature dependent. Cells were grown on palmitate, elaidate, or oleate to investigate possible effects of the lipid phase transition on the accessibility of phosphatidylglycerol for phospholipase A_2. Under conditions in which all the lipid is in the solid state, no hydrolysis occurs. When solid and liquid-crystalline lipid phases coexist, a limited hydrolysis of phosphatidylglycerol can be observed. The results demonstrate the disposition of phosphatidylglycerol in three different pools in the membrane of *A. laidlawii.* Phospholipase A_2 has been used to discriminate between these pools and to estimate the amount of phosphatidylglycerol which is present in the liquid-crystalline phase. The present data, however, do not allow a definite localization of the phosphatidylglycerol pools.

Phospholipases have been used to determine the distribution of phospholipids between both layers of three types of natural membranes. Zwaal and co-workers (1973) demonstrated an asymmetric distribution of human erythrocyte phospholipids using a number of purified phospholipases of different origin. Using a similar approach, an asymmetric phospholipid distribution was found in rat liver microsome preparations by Depierre and Dallner (1975), in influenza virus membrane (Tsai and Lenard, 1975) and in other species of erythrocytes, although caution should be exercised in the application of phospholipases to elucidate membrane structure, as was shown by Martin et al. (1975).

Not only the localization, but also some physicochemical parameters of phospholipids in membranes, can be revealed by the action of phospholipases. Studying the surface pressure dependency of a number of enzymes for phosphatidylcholine spread on the air-water interface and extrapolating their results to the erythrocyte membrane, Demel et al. (1975) proposed a packing of phospholipids in this membrane which corresponds to a surface pressure between 31 and 34.8 dynes/cm. In model membranes, such as phosphatidylcholine liposomes, phospholipase A_2 can reveal the phase transition (Op den Kamp et al., 1975) and, finally, the inability of phospholipases to hydrolyze membranous phospholipids can point to a protective disposition of membranous proteins (Rottem et al., 1973; Gazitt et al., 1976).

Bearing these possibilities in mind, we studied the hydrolysis of phosphatidylglycerol in *Acholeplasma laidlawii.* This unicellular organism offers several advantages. It has no cell wall nor intracellular membrane systems (Razin, 1969); lysis is easily accomplished by washing in distilled water and the membranes do not vesiculate (Razin, 1969); only one of the lipids, phosphatidylglycerol, is susceptible to phospholipases (see Results); the lytic hydrolysis product of phospholipase A_2 action (lysophosphatidylglycerol) does not accumulate but is instantaneously hydrolyzed by a membrane bound lysophospholipase (van Golde et al., 1971); finally, the fatty acid composition of the lipids can be manipulated by the addition of fatty acids to the growth medium (McElhaney, 1974).

Materials and Methods

Cultivation of the Organism and Isolation of Membranes. Acholeplasma laidlawii strain B was grown on a lipid-depleted tryptose medium supplemented with palmitic acid, elaidic acid, oleic acid, or linoleic acid at a concentration of 0.12 mM (de Kruyff et al., 1972). Radioactive labeling of the lipids was obtained by the addition of 100 μCi of [^{32}P]orthophosphate per 100 mL of medium or by adding the fatty acids as 1-

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