# Phase Transitions in Cells, Membranes, and Lipids of *Escherichia coli*. Detection by Fluorescent Probes, Light Scattering, and Dilatometry†

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ABSTRACT: A fatty acid auxotroph of *Escherichia coli* K12 was supplemented with either *cis*-$\Delta^9$-octadecenoate, *trans*-$\Delta^9$-hexadecenoate, or *trans*-$\Delta^9$-octadecenoate. Arrhenius plots of *in vivo* *o*-nitrophenyl galactoside hydrolysis and other transport parameters show breaks at about 16, 31, and 37° for *cis*-$\Delta^9$-$C_{18:1}$-, *trans*-$\Delta^9$-$C_{16:1}$-, and *trans*-$\Delta^9$-$C_{18:1}$-supplemented cells, respectively. Analysis of the isolated phospholipids, of membranes and whole cells by a variety of physical techniques (dilatometry, 90° light scattering, monolayers at an air–water interface, and fluorescence probing with 8-anilinonaphthalene-1-sulfonate (ANS) or *N*-phenyl-1-naphthylamine (PhNap)) reveals that the transition temperatures of transport lie at or near the transition temperatures, $T_t$, of the crystalline–liquid crystalline phase transition of the lipids. $T_t$ values at 14–17, 25–30, and 35–42° are found in the *cis*-$\Delta^9$-$C_{18:1}$, *trans*-$\Delta^9$-$_{16:1}$, and *trans*-$\Delta^9$-$C_{18:1}$ system, respectively, depending on the preparation (total phospholipids, membranes, or cells), method, and ionic conditions used. The range of the transition, $\Delta T$, is narrow for lipids and membranes containing trans-unsaturated fatty acids (4–8°) and broader for *cis*-$\Delta^9$-$C_{18:1}$-containing lipids (11–13°). The use of the hydrophobic dye PhNap provides a simple method for detecting phase transitions. Whereas low concentrations ($<10^{-5}$ M) of this label do not influence the transition temperature, higher concentrations ($10^{-5}$–$5 \times 10^{-4}$ M) decrease $T_t$ by 3–7° in membranes and phospholipids suggesting that the dye penetrates into the hydrophobic part of the lipid phase and interferes with the orderly packing of the hydrocarbon chains. Fractionation of the *trans*-18:1-containing lipid into its major components (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin) and subsequent analysis of their phase behavior by 90° light scattering showed that the transition temperature of the mixture is mainly determined by the predominant component phosphatidylethanolamine. Moreover, the decrease of $T_t$ in membranes and phospholipids by an increase in ionic strength in the aqueous phase can be attributed to the negatively charged cardiolipin and phosphatidylglycerol.

The occurrence of thermotropic phase transitions is characteristic for phospholipid–water systems such as lipid dispersions, black lipid films (Krasne *et al.*, 1971; Stark *et al.*, 1972), or monolayers at an air–water (Chapman *et al.*, 1966) or water–organic solvent (Sackmann and Träuble, 1972) interface. In dispersions of synthetic lipids this crystalline–liquid crystalline phase transition has been observed by a variety of physical techniques, *e.g.*, X-ray diffraction (Luzzati, 1968), differential thermal analysis (Chapman *et al.*, 1967), dilatometry (Träuble and Haynes, 1971), electron spin (Hubbell and McConnell, 1971) or nuclear magnetic resonance (Veksli *et al.*, 1969; Barker *et al.*, 1972; Lee *et al.*, 1972), and laser Raman investigations (Lippert and Peticolas, 1971, 1972). Most recently, the phase transition has been revealed by several convenient optical methods using 90° light scattering, absorption, or fluorescent probing (Träuble, 1971; Sackmann and Träuble, 1972; Lussan and Faucon, 1971). As a result of these studies it became apparent that below the transition temperature, $T_t$, characteristic for each lipid, the hydrocarbon chains in the bilayer are in the all-trans conformation forming a hexagonal closely packed array. The transition is accompanied by a loss of order in the two-dimensional lattice, a sudden increase in chain mobility, a lateral expansion in the plane of the membrane, a decrease in the bilayer thickness, and a net increase in volume per lipid molecule. The fluid state ($T > T_t$) permits rapid lateral diffusion of the lipid molecules in the plane of the membrane (Träuble and Sackmann, 1972; Devaux and McConnell, 1972). The transition is highly cooperative and, therefore, a large number of lipid molecules in a continuous phase are a prerequisite for its occurrence.

The demonstration of lipid phase transitions in natural membranes provides a convincing argument that part of the phospholipids form a continuous phase with direct hydrocarbon chain interaction, most likely a bilayer (Wilkins *et al.*, 1971). In *Mycoplasma laidlawii*, *Micrococcus lysodeicticus*, and mammalian membranes phase transitions have been observed by differential scanning calorimetry (Steim *et al.*, 1969; Reinert and Steim, 1970; Melchior *et al.*, 1970; Ashe and Steim, 1971; Blazyk and Steim, 1972). In *Mycoplasma* membranes Engelman (1970, 1971) and Abramson and Pisetsky (1972) have detected the thermal transition by X-ray diffraction and turbidimetry, respectively.

Since biological membranes generally contain lipids with cis-unsaturated hydrocarbon chains, phase transitions are expected to occur at relatively low temperatures. It was therefore of considerable importance that mutants auxotrophic for unsaturated fatty acids were isolated by Silbert and Vagelos (1967) which permitted a defined alteration of the hydrocarbon chain composition of the membrane phospholipids by supplementing fatty acids to the growth medium (Silbert *et al.*, 1968; Schairer and Overath, 1969; Esfahani *et al.*, 1969; Silbert, 1970). In particular, the replacement of the cis- by trans-unsaturated fatty acids was expected to cause an in-

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crease in the transition temperature of membrane phosphotransport lipids. An analysis of the temperature dependence of several processes and other physiological properties in *Escherichia coli* auxotrophs grown in the presence of various fatty acids revealed characteristic breaks at temperatures that were dependent on the fatty acid composition in a similar way as the $T_t$ of synthetic lipids (Schairer and Overath, 1969; Wilson *et al.*, 1970; Overath *et al.*, 1970, 1971a,b; Esfahani *et al.*, 1971; Rosen and Hackette, 1972). The suggestion that these breaks were the result of a lipid phase transition in the membrane which in turn impaired membrane protein-dependent functions is so far supported by two types of experiments. Overath *et al.* (1970) isolated the main phospholipid, phosphatidylethanolamine, from *E. coli* cells and showed that monolayers of these lipids were unable to form a condensed state for $T \geqslant T_t$, where $T_t$ denotes the breaks in transport observed *in vivo*. Phillips and Chapman (1968) had previously observed in the case of dipalmitoyllecithin that the temperature above which no condensed phase could be formed in monolayers appears to be identical with the critical temperature, $T_t$, of the crystalline–liquid crystalline phase transition of fully hydrated dispersions. More recently and more directly, several groups have demonstrated lipid phase transitions in *E. coli* membranes (Esfahani *et al.*, 1971; Shechter *et al.*, 1972; Dupont *et al.*, 1972; D. Engelman, personal communication) by X-ray diffraction. These results will be discussed in more detail below.

The purpose of this study was the development of convenient and fast optical methods for the detection of phase transitions in lipids, membranes, and whole cells of *E. coli*. The relation of the lipid phase transition to protein-dependent membrane functions will be discussed.

## Materials and Methods

*Organisms and Growth Conditions. E. coli* strain K1062 (Overath *et al.*, 1971a) was grown in Cohen-Rickenberg (CR) mineral salts medium (Anraku, 1967) supplemented with 0.5% glycerol, 0.3% Casamino Acids (Difco, vitamin free), 2% Brij 35 (polyethylene glycol–monolauryl ether), and 0.02% of either *trans*-18:1 (C. Roth, Karlsruhe, Germany), *cis*-18:1 or *trans*-16:1 (both from Hormel Institute, Austin, Minn.). *trans*-18:1-supplemented cells were grown at 39°. A growth temperature of 37° was used for the other fatty acid supplements; 750-ml batches of cells were grown in 2-l. Fernbach flasks with slow rotatory aeration to an optical density of 5–6 at 420 mμ. After harvesting the cells, they were thoroughly washed several times with CR buffer in order to remove fatty acids adsorbed to the cells. CR buffer (12 g of $K_2HPO_4$, 3 g of $KH_2PO_4$, 2 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per l. of water) has an ionic strength of 0.28 and a pH of 7.3.

*Isolation of Phospholipids.* The total lipids extracted from the cells according to Ames (1968) were separated into nonpolar lipids and phospholipids by silicic acid chromatography (Sweeley, 1969). Cardiolipin was eluted with $CHCl_3$–$CH_3OH$ (9:1), phosphatidylethanolamine and phosphatidylglycerol appeared together with $CHCl_3$–$CH_3OH$ (4:1 and 3:2). All phospholipid-containing fractions were pooled and taken to dryness in a rotatory evaporator. Preliminary experiments with phospholipid labeled with [$^{14}$C]acetate *in vivo* revealed that the recovery of radioactivity from the column was essentially quantitative (>90%) and that an insignificant amount of labeled material (<5%) was eluted with $CHCl_3$ (quinones, etc.). The phospholipid fraction obtained in this way was used for the determination of their physical properties.

Separation of the various phospholipid classes was accomplished in the following way (Rouser *et al.*, 1969). Phospholipid mixture (490 mg), prepared as described above, was applied to a DEAE-cellulose column (2.6 × 20 cm, acetate form). Stepwise elution was carried out with $CHCl_3$ (1000 ml), $CHCl_3$–$CH_3OH$ (320 ml, 9:1), $CHCl_3$–$CH_3OH$ (1100 ml, 7:3), $CH_3OH$ (500 ml), and a mixture of 800 ml of $CHCl_3$, 200 ml of $CH_3OH$, 20 ml of ammonia, and 39 g of ammonium acetate. Phosphatidylethanolamine and small amounts of cardiolipin were eluted with $CHCl_3$–$CH_3OH$ (9:1 and 7:3). Phosphatidylglycerol and the bulk of cardiolipin were eluted with the ammonium acetate containing solvent mixture. The respective fractions were pooled and the solvent was removed in a rotatory evaporator. Ammonium acetate was removed by lyophilization. Subsequent silicic acid column chromatography (elution scheme as described above) separated phosphatidylethanolamine from cardiolipin and phosphatidylglycerol from cardiolipin.

The purification of the phospholipids was followed by thin-layer chromatography. Cardiolipin was free of both phosphatidylethanolamine and phosphatidylglycerol. Phosphatidylglycerol gave only one ninhydrin-negative spot. Phosphatidylethanolamine was free from cardiolipin, but minor contamination by phosphatidylglycerol could not be excluded.

By a combination of both thin-layer and column chromatography of radioactive lipid obtained by growth of the cells in the presence of [2-$^{14}$C]acetate and *trans*-16:1 as a supplement, the proportion of the various phospholipid classes in the crude lipid extract could be estimated. The values obtained (82 mol % phosphatidylethanolamine, 11 mol % cardiolipin, 7 mol % phosphatidylglycerol) are within the range estimated by other investigators (see Cronan and Vagelos, 1972, for review).

*Isolation of Membranes.* Membranes of strain K1062 were prepared according to the method of Kaback (1971). After washing with 0.1 M potassium phosphate–10 mM EDTA (pH 6.6), the membranes were taken up in CR buffer. Membrane protein was determined according to Lowry *et al.* (1951). The lipid content of the membranes was estimated by two independent procedures. (a) Pentadecanoate, a fatty acid not present in the lipids, was added as a standard to an aliquot of the membrane suspension. After saponification, extraction, and esterification, the absolute amount of the various fatty acids present in the sample could be calculated from the gas chromatogram (Schairer and Overath, 1969) by comparison with the peak of the standard. (b) An aliquot of the membrane preparation was extracted with $CHCl_3$–$CH_3OH$. After three washings with 2 M KCl, the amount of phosphate was determined in the organic phase by the method of Bartlett (1959). After correction for losses during the extraction, the lipid content was somewhat lower (5–15%) than expected from the fatty acid determination. Lipopolysaccharide was determined by analysis of the 2-keto-3-desoxyoctonic acid content (Weissbach and Hurwitz, 1959). A standard sample of 2-keto-3-desoxyoctonic acid was kindly provided by Dr. P. Mühlradt. One milligram of lipopolysaccharide contains 0.45 μmol of 2-keto-3-desoxyoctonic acid (Eidels and Osborn, 1971). According to these estimates the membranes contained 65–69% protein, 25–29% lipid, and 5–6% lipopolysaccharide. The lipopolysaccharide content of our preparations indicates a contamination by outer membrane components in the order of 10–15%. They have a composition similar to the inner membrane fraction recently isolated by Osborn *et al.* (1972)

from *Salmonella typhimurium*, which contains 5.3% lipopolysaccharide.

*Measurement of Phase Transitions.* LIPID DISPERSIONS (usually $2 \times 10^{-3}$ M in distilled water) were prepared by sonication with a Branson Sonifier for 3 min at 42° under a stream of nitrogen. After a 10- to 20-fold dilution these samples were used directly for 90° light-scattering measurements. Methanolic solutions of PhNap[1] (Kodak Co.) at final concentrations of $10^{-5}$–$10^{-6}$ M were added to the lipid dispersions (lipid concentration $2 \times 10^{-4}$ M). The methanol content was kept below 3% (v/v). This methanol concentration did not influence the lipid transition. The samples were subsequently incubated for 15 min at 42° and stored at room temperature. For comparison, some of the measurements were performed in CR buffer. The ANS measurements were made in samples containing $5 \times 10^{-5}$ M lipid, $5 \times 10^{-5}$ M ANS (Pierce Chemical Co.), and 1 M NaCl.

The *membrane suspensions* (final lipid concentration $2 \times 10^{-4}$ or $5 \times 10^{-5}$ M for the PhNap or ANS measurements, respectively) were mixed with PhNap or ANS and NaCl as described above, sonicated for 10 sec at 0° and incubated for 10 min at 37° (40° for *trans*-18:1-containing membranes). *Suspensions of freshly harvested cells* (1.9 mg of protein/ml) in CR buffer were measured directly after addition of the PhNap solution.

An Aminco-Bowman or Hitachi Perkin-Elmer Model MPF-3 spectrofluorimeter was used for the fluorescence and 90° light-scattering experiments. Excitation wavelengths of 360 and 350 nm and emission wavelengths of 480 and 420 nm were used for ANS and PhNap, respectively; 90° light scattering was measured at 350 or 400 nm for both excitation and emission. Continuous recording of the light intensity *vs.* temperature was accomplished by using a Hewlett-Packard X–Y recorder. A thermophile was dipped directly into the cuvet and connected to the *X* axis of the recorder while the change in light intensity was recorded on the *Y* axis. The temperature in the cuvet was changed at a rate of about 1°/min.

## Results

*Transport in Fatty Acid Auxotrophs.* The effect of membrane fatty acid substitution in *E. coli* fatty acid auxotrophs on a variety of carrier-mediated transport processes has been investigated in several laboratories. It is significant that in all these experiments, Arrhenius plots of the rate of transport show the general features first observed for the uptake and efflux of thiomethyl galactoside (Schairer and Overath, 1969; Overath *et al.*, 1970) and for *in vivo* NphGal hydrolysis (Wilson *et al.*, 1970). The relevant properties can be seen from Figure 1 which shows the temperature characteristic of NphGal transport in the fatty acid auxotroph K1062 (Overath *et al.*, 1971a) grown with *cis*-18:1, *trans*-16:1, and *trans*-18:1 as fatty acid supplements. (a) The plots are biphasic, the intersection of the two straight lines defines a transition temperature, $T_t$. (b) From the slopes apparent activation energies of 15–20 kcal/mol above and 40–60 kcal/mol below the transition can be calculated, *i.e.*, there is a change by a factor of

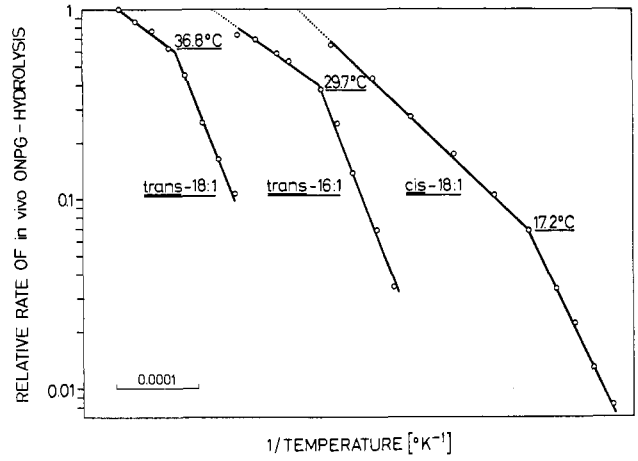


FIGURE 1: Temperature characteristic of *in vivo* NphGal hydrolysis. Strain K1062 was grown as described in Materials and Methods in the presence of the three fatty acids indicated to an optical density of 4–4.5 at 420 mμ. β-Galactosidase and the β-galactoside transport protein were then induced with 0.5 mM isopropyl β-thiogalactoside during a further increase in optical density of 0.5–0.7. The rate of NphGal hydrolysis was determined as described previously (Overath *et al.*, 1971a). The rate were normalized to a temperature of 43°. The transition temperature, $T_t$, given in °C for each curve is defined as the point of intersection of two straight lines. The scale of the abscissa in $°K^{-1}$ is given relative to this point by the bar in the lower left corner of the figure.

2–4. (c) The transition occurs in a relatively narrow temperature range of 2–4°. (d) The transition temperatures obtained are characteristic for the type of fatty acid incorporated and are reproducible within a range of 2–3° in independent experiments. Taking into account that various investigators used strains with different genetic background, there is also a quite remarkable agreement in the $T_t$ values observed with sugar and amino acid transport systems (Esfahani *et al.*, 1971; Rosen and Hackette, 1972). Thus, $T_t$ appears to be characteristic for the lipid composition and not for the transport system investigated. The problem is, how the properties listed above can be explained in molecular terms, *i.e.*, as a result of the crystalline–liquid crystalline phase transition in the membrane.

*Fatty Acid Composition.* The fatty acid composition of the isolated phospholipids and *E. coli* membranes is shown in Table I. The amount of incorporation of the three fatty acids supplied to the medium is similar to previously published values (Overath *et al.*, 1969, 1970). As expected (Kanemasa *et al.*, 1967), the fatty acid distribution in the various phospholipid classes is almost the same. The phospholipid extract contains phosphatidylethanolamine, cardiolipin, and phosphatidylglycerol in a molar ratio of 8.2:1.1:0.7.

*Phase Transitions in Total Phospholipids.* DILATOMETRIC MEASUREMENTS. An increase in the lipid volume is expected as the result of the formation of rotational isomers and the simultaneous increase in mobility of the lipid molecules at the phase transition (Träuble and Haynes, 1971). Like the change in specific heat (calorimetry), the volume increase is an integral expression of the conformational change. The volume change at the phase transition is revealed clearly after subtraction of the water expansion as seen for the two *trans*-fatty acid containing lipids in Figure 2. Transition temperatures, $T_t$, of 39.2 and 27.5° and transition ranges, $\Delta T$, of 5 and 10° are obtained for the *trans*-18:1- and *trans*-16:1-containing lipids, respectively. The transition involves a change in volume, $\Delta V/V$, of 1.02% for the *trans*-18:1 and 1.93% for the

[1] Abbreviations used are: PhNap, *N*-phenyl-1-naphthylamine; ANS, 8-anilinonaphthalene-1-sulfonate; *cis*-18:1, *cis*-$\Delta^9$-octadecenoic acid; *trans*-$\Delta^9$-octadecenoic acid; *trans*-16:1, *trans*-$\Delta^9$-hexadecenoic acid; NphGal, *o*-nitrophenyl β-D-galactopyranoside; sMeGal, thiomethyl β-D-galactopyranoside; I (12,3), I (5,10), and I (1,14) refer to stearic acid molecules carrying the paramagnetic *N*-oxyl-4′,4′-dimethyloxazolidine ring at carbon atoms 5, 12, and 16, respectively.

TABLE I: Fatty Acid Composition (%) of Isolated and Membranous Phospholipids.[a]

| | *trans*-18:1 | | | | | |
|---|---|---|---|---|---|---|
| | Total Phospholipids | Phosphatidylethanolamine | Phosphatidylglycerol | Cardiolipin | Total Phospholipids *trans*-16:1 | Total Phospholipids *cis*-18:1 |
| 12:0 | 1.6 (4.1) | 3.0 | 3.3 | 1.8 | (2) | (2.5) |
| 14:0 | 14.0 (8.1) | 16.6 | 14.6 | 10.9 | 4.3 (5.6) | 7.1 (9.3) |
| 16:0 | 13.5 (9.7) | 12.0 | 13.9 | 15.5 | 12.8 (12.7) | 27.2 (25.6) |
| *trans*-16:1 | 2.6 (–) | 2.7 | 2.0 | 1.1 | 82.8 (79.6) | |
| *trans*-18:1 | 68.3 (78) | 65.1 | 66.2 | 70.6 | | |
| *cis*-18:1 | | | | | | 54.4 (57) |
| 19Δ | | | | | | 11.6 (5.6) |

[a] The isolation of total phospholipids, their separation into the three polar lipid classes, and the preparation of membranes is described in Materials and Methods. Fatty acid analysis was performed as described previously (Schairer and Overath, 1969). The numbers in parentheses refer to the phospholipid fatty acid composition of *E. coli* membranes: 12:0, dodecanoic acid; 14:0, tetradecanoic acid; 16:0, hexadecanoic acid; 19Δ, 9,10-methyleneoctadecanoic acid.

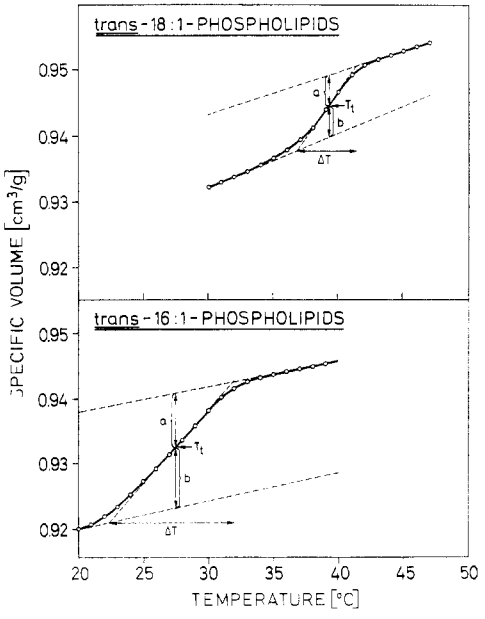


FIGURE 2: Dilatometric measurement of the phase transition. The temperature dependence of the volume of the lipid–water systems was measured in a simple volumetric dilatometer as described before (Träuble and Haynes, 1971). The dilatometer (2.506-ml total capacity) was calibrated against double-distilled water. The experiments were performed at a lipid concentration of 18.5% (v/v). The figure shows the change in specific volume of the lipid at increasing temperature after subtraction of the water expansion. $T_t$ is defined as the temperature where *a* equals *b*. The volume change at the phase transition, $\Delta V/V$, given in the text refers to the lipid volume, $V$, before the onset of the transition at the lower end of the curves.

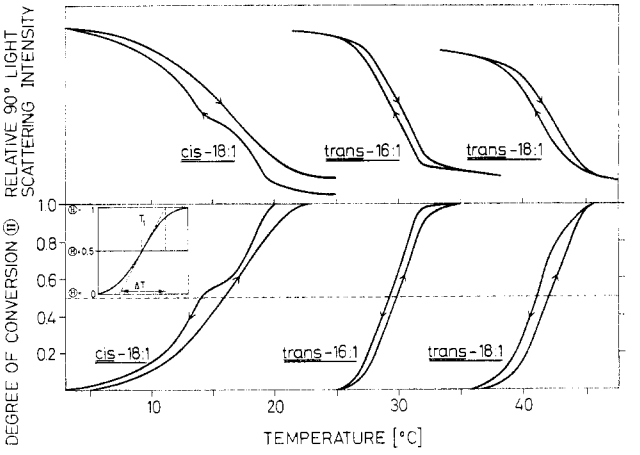


FIGURE 3: 90° light scattering of phospholipid dispersions. The upper part shows the original tracings of the total phospholipids measured as described in Materials and Methods. In the lower part of the figure the curves were normalized by calculating the degree of conversion, Θ, in the following way. The linear parts of the curves above and below the transition were extrapolated. Θ was defined as the ratio of the change in light-scattering intensity at a given temperature (vertical distance between the extrapolated line and the curve) and the total change in intensity at the phase transition for the same temperature (vertical distance of the extapolated lines). The inset shows the definition of $T_t$ and $\Delta T$.

*trans*-16:1 lipid. Thus, the volume change of *E. coli* lipids is of the same order of magnitude as that found for dipalmitoyllecithin ($\Delta V/V$ = 1.4%). The relevance of these data for the type(s) of rotational isomers (kinks) formed in the hydrocarbon chains at the phase transition has been discussed in detail by Träuble and Haynes (1971).

90° LIGHT SCATTERING.[2] Figure 3 and Table II summarize experiments using 90° light scattering (Träuble, 1972; Sackmann and Träuble, 1972) for revealing the phase transition. The upper part of Figure 3 shows the original tracings while in the lower part the degree of conversion is plotted *vs.* temperature. Virtually no hysteresis is observed in these experiments. $\Delta T$, as defined in the inset to Figure 3, is a measure of the width of the transition. The transitions are remarkably sharp for the *trans*-fatty acid containing lipids ($\Delta T$ = 3–7°) and much broader for the *cis*-18:1-containing lipid ($\Delta T$ = 9–12°). In the pH range from 5 to 9 the transition temperature of the *trans*-18:1 lipid is essentially constant. This result is not unexpected because the degree of dissociation of the zwitterionic phosphatidylethanolamine and the negatively charged cardiolipin remains essentially unaltered in this pH range. In accordance with these measurements, dispersions of synthetic dimyristoylphosphatidylethanolamine have a constant $T_t$ value in this pH region; a sharp decrease is observed at pH 9.5 (H. Träuble and H. J. Eibl, to be published). An increase in

[2] Recently, Fischer (1973) showed that the decrease in 90° light scattering at the phase transition is paralleled by a decrease in the refractive index of the lipid dispersion. This suggests that the change in 90° light scattering is a result of the change in specific volume of the lipid hydrocarbon region (*cf.* Figure 2, dilatometric measurements).

TABLE II: Influence of pH and Ionic Strength on the Phase Transition of Total Phospholipids.[a]

| Phospholipids Isolated from Cells Grown in the Presence of | Conditions | °C $T_t$ → | $\Delta T$ | $T_t$ ← | $\Delta T$ |
|---|---|---|---|---|---|
| *trans*-18:1 | $H_2O$ (pH 6.5) | 42 | 5 | 41 | 5 |
| | $H_2O$ (pH 5.2) | 42 | 3 | 42 | 2 |
| | | | | 39 | 2 |
| | $H_2O$ (pH 9.1) | 42 | 6 | 42 | 1 |
| | | | | 39 | 3 |
| | CR buffer | 38 | 4 | 37 | 2 |
| | | | | 34 | 2 |
| *trans*-16:1 | $H_2O$ | 30 | 5 | 29 | 5 |
| | CR buffer | 27 | 7 | 26 | 8 |
| *cis*-18:1 | $H_2O$ | 16 | 11 | 18 | 4 |
| | | | | 12 | 5 |
| | CR buffer | 13 | 12 | 13 | 12 |

[a] The thermotropic transition was recorded by 90° light scattering as reported in Materials and Methods. The pH in the aqueous dispersions was adjusted with dilute HCl or NaOH. $T_t$ and $\Delta T$ were calculated after normalization of the original tracings as described in the legend to Figure 3. The arrows indicate increasing (→) or decreasing (←) temperature.

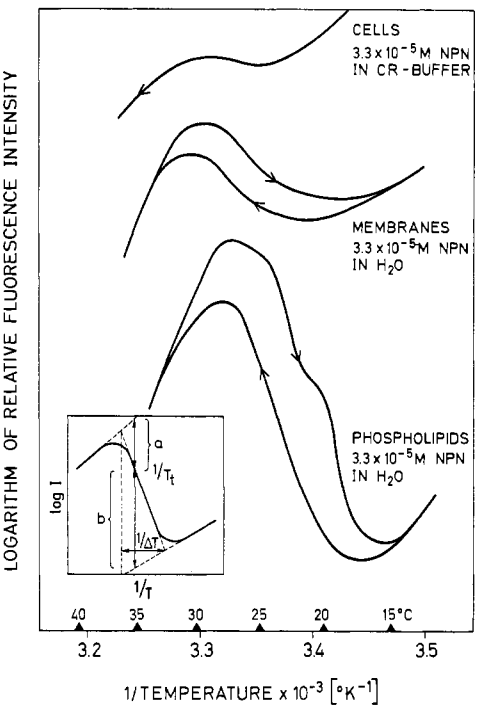


FIGURE 4: Phase transitions in *trans*-16:1-containing cells, membranes, and phospholipids using PhNap as a probe. Phospholipid and membrane phospholipid concentrations were $2 \times 10^{-4}$ M. Cells were measured at a protein concentration of 1.9 mg/ml. Other conditions are indicated in the figure. The original tracings were converted to a plot of log $I$ *vs.* $1/T$ because this allows a linear extrapolation of the curves above and below the phase transition as shown in the inset. $1/T_t$ is defined as the temperature where *numerus a* equals *numerus b*. The tangent through $1/T_t$ bisects the two extrapolated lines, thus defining $1/\Delta T$.

ionic strength at pH 7.3 (addition of CR buffer, ionic strength 0.28) lowers the transition temperature by 3–4°. As will be shown in a forthcoming publication, an increase in ionic strength is expected to reduce the transition temperature due to the decrease in electrostatic repulsion between the negatively charged lipid molecules (cardiolipin and phosphatidylglycerol). Throughout this paper, the buffer of the growth medium (CR) is used in the hope that it approximates the unknown ionic environment of the membrane *in vivo*.

It is apparent from Figure 3 and Table II that at increasing temperature the transition occurs in one step in all cases. The descending curves may exhibit a more complex behavior. The transition may be shifted to lower temperatures (hysteresis) and may occur in several more or less clearly discernible steps (compare also Figures 4 and 7). These phenomena are very sensitive to the pretreatment of the samples and the ionic environment. They are not easily reproducible and were not studied systematically in this work. This behavior was only observed with the isolated lipids but not with membranes or whole cells (Figures 4, 5, and 7). We consider this to be a significant difference in the behavior of the isolated and membraneous phospholipids. Model experiments using mixed distearoyllecithin–dimyristoyllecithin or dimyristoyllecithin–dimyristoylphosphatidylethanolamine dispersions or dispersions of highly charged lipids (*e.g.*, phosphatidic acid) show that the following two factors play a role in this behavior. Firstly, a phase separation of components with different hydrocarbon chains and/or polar groups; secondly, electrostatic interactions between the polar head groups especially in the presence of multivalent metal counterions. Since the *E. coli* lipids are heterogeneous with regard to the polar head groups as well as the hydrocarbon chain distribution to the 1 and 2 position of the glycerol moiety (Silbert, 1970) both factors may be involved in metastable states.

FLOURESCENT PROBES. ANS has been used by Träuble (1971) and Sackmann and Träuble (1972) as a probe for detecting the phase transition in dispersions of dipalmitoyllecithin. This amphiphilic dye carries a negative charge whereas its derivative, PhNap, is uncharged and sparingly soluble in water. Therefore, ANS interacts with lipid bilayers in the region of the polar head groups, the sulfonate group facing the aqueous phase while the aromatic ring system is buried in the hydrocarbon chain region. In contrast, PhNap is expected to penetrate into the hydrophobic core of the membrane. These interpretations are supported by the quantum yields of ANS ($Q = 0.16 \pm 0.04$ corresponding to a solvent dielectric constant $\epsilon \approx 35$) and PhNap ($Q = 0.33 \pm 0.03$ corresponding to an $\epsilon \lesssim 10$) bound to dipalmitoyllecithin in aqueous dispersions (Träuble and Overath, 1973). Further information on the properties and the location of these dyes in membranes may be obtained from papers by Gulik-Krzwicki *et al.* (1970), Lesslauer *et al.* (1971), Colley and Metcalfe (1972), Träuble and Overath (1973), and the reviews by Radda (1971a,b). Both PhNap and ANS can be used to detect the lipid phase transition in *E. coli* phospholipids and membranes by measuring changes in fluorescent intensity. However, for the indication of the phase transitions in membranes we have used preferentially PhNap since complications due to dye–protein interaction are much smaller than for ANS.

The lower tracings of Figures 4 and 5 show the phase transitions for the *trans*-16:1 and *trans*-18:1 lipids using PhNap as a label. Table III gives the values for $T_t$ and $\Delta T$ for the lipids at low PhNap concentrations for increasing temperatures. The transition temperatures are 1–4° lower compared

TABLE III: Phase Transitions Using PhNap as a Probe.[a]

| | | *trans*-$\Delta^9$-$C_{16:1}$ | | | | *trans*-$\Delta^9$-$C_{18:1}$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $H_2O$ | | CR Buffer | | $H_2O$ | | CR Buffer | |
| | PhNap Concn (M) | $T_t$ (°C) | $\Delta T$ (°C) | $T_t$ (°C) | $\Delta T$ (°C) | $T_t$ (°C) | $\Delta T$ (°C) | $T_t$ (°C) | $\Delta T$ (°C) |
| Lipids | $3.3 \times 10^{-6}$ → | 27 | 6 | 25 | 4 | 40 | 3 | 34 | 5 |
| | $1 \times 10^{-5}$ → | 26 | 6 | 24 | 4 | 39 | 4 | 33 | 6 |
| Membranes | $3.3 \times 10^{-6}$ → | 29 | 8 | 26 | 7 | 40 | 4 | 38 | 5 |
| | $3.3 \times 10^{-6}$ ← | 29 | 8 | 25 | 8 | 39 | 4 | 38 | 4 |
| | $1.0 \times 10^{-5}$ → | 27 | 7 | 24 | 8 | 40 | 4 | 38 | 6 |
| | $1.0 \times 10^{-5}$ ← | 26 | 8 | 23 | 9 | 39 | 4 | 37 | 5 |

[a] The experiments were performed at lipid or membrane lipid concentrations of $2 \times 10^{-4}$ M. Arrows indicate increasing (→) or decreasing (←) temperature.

to the light-scattering data (Table II). Both methods yield about the same values for $\Delta T$ (3–6°). *cis*-18:1 lipid has a transition around 11° ($\Delta T$ = 11°, $3 \times 10^{-5}$ M PhNap in $H_2O$). In accordance with the light scattering data, addition of CR phosphate buffer shifts the transition by 2–6° to lower temperatures. Hysteresis is moderate (2°) in the absence, but severe (>10°) in the presence of the phosphate buffer (data not shown).

At low concentrations, PhNap does not influence the transition temperature, at higher concentrations a decrease in $T_t$ by about 7° is observed. This effect is shown in Figure 6 for the *trans*-18:1 lipid as an example. Presumably, intercalation of PhNap into the bilayer interferes with the orderly packing of the hydrocarbon chains leading to a decrease in hydrophobic and van der Waals interaction.

ANS can alternatively serve as an indicator of the transition. As can be seen from the ascending branches of the lower curves in Figure 7, *trans*-16:1 ($T_t$ = 26°, $\Delta T$ = 8°) and *trans*-18:1-containing lipids ($T_t$ = 37°, $\Delta T$ = 7°) give transition temperatures very similar to the PhNap and light-scattering experiments.

MONOLAYERS AT AN AIR–WATER OR WATER–ORGANIC INTERFACE. A correlation between lipid dispersions (bilayers) and monolayers could be demonstrated in two ways. On the one hand, we have repeated previous experiments (Overath *et al.*, 1970) with the *trans*-18:1 lipid used in the present study. Figure 8 demonstrates the behavior of monomolecular films at a nitrogen–water interface at various temperatures. The temperature ($T_t \geq$ 41–42°) at which a transition to the condensed state disappears, agrees with the $T_t$ values of the lipid dispersions in water (see Table V for comparison). Alternatively, lipid monolayer vesicles carrying an inner organic solvent phase were obtained by sonicating the phospholipids and dibutylether in water (Träuble and Grell, 1971; Sackmann and Träuble, 1972). Using ANS as an indicator, the same transition temperatures were observed in these monolayer vesicles

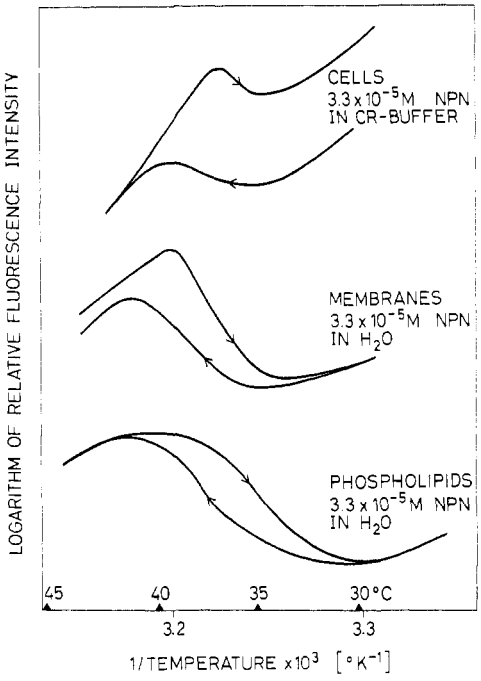


FIGURE 5: Phase transitions in the *trans*-18:1-containing cells, membranes and phospholipids using PhNap as a probe. The same conditions were used as in Figure 4.

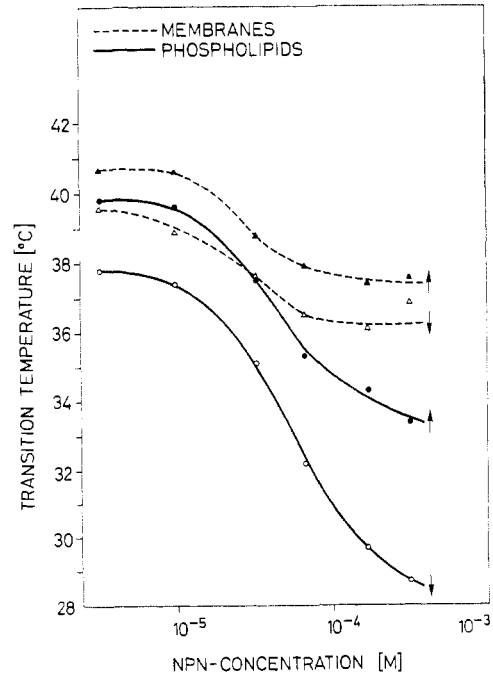


FIGURE 6: Influence of PhNap on the phase transition. The phase transition was measured with *trans*-18:1-containing phospholipids and membranes ($2 \times 10^{-4}$ M final lipid concentration) in water at various PhNap concentrations as described in Figures 4 and 5. The $T_t$ values for the ascending (↑) and descending (↓) branches are plotted *vs.* the total PhNap concentration.

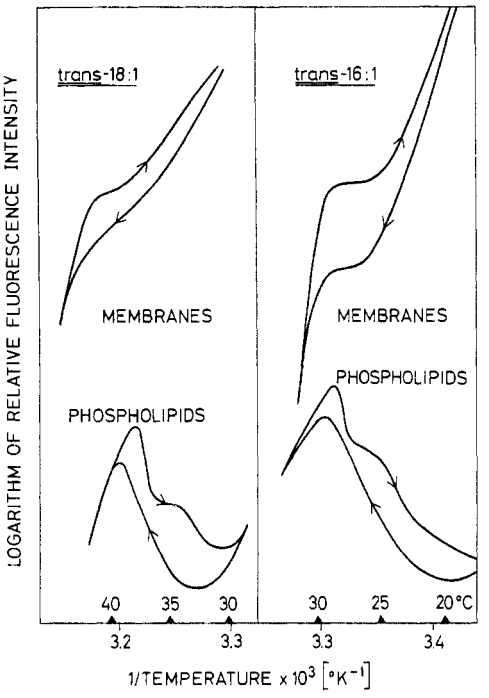


FIGURE 7: Phase transitions in phospholipids and membranes using ANS as a probe. The samples contained $5 \times 10^{-5}$ M phospholipid or $5 \times 10^{-5}$ M membrane phospholipid, $5 \times 10^{-5}$ M ANS, and 1 M NaCl. Values for $T_t$ and $\Delta T$ were obtained as described in the legend to Figure 4.

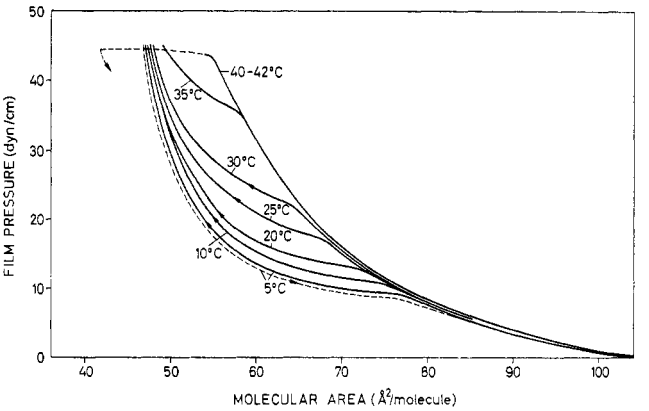


FIGURE 8: Force–area isotherms of *trans*-18:1-containing phospholipids. A chloroform solution of phospholipids was applied to a covered Langmuir trough (MGW Lauda, Lauda/Tauber, Germany) using bidistilled water as the subphase and $N_2$ as the gaseous phase. After evaporation of the solvent the film was compressed at a rate of 5 Å²/min. Compression was interrupted shortly before the collapse point in order to ensure reversible expansion. The same film was used at temperatures ranging from 5 to 40°.

as in lipid dispersions. In summary, it appears that the behavior of a monolayer is not greatly altered when either a gas or an organic solvent or another lipid monolayer forms the adjacent phase.

*Phase Transitions in Phospholipid Classes.* The total phospholipids of *E. coli* were separated into the three main phospholipid classes by column chromatography and subsequently analyzed by 90° light scattering (Table IV). The main phospholipid, phosphatidylethanolamine (82 mol %), gives a well-defined and essentially reversible transition at 38°, which is not affected by an increase in ionic strength. In contrast, the negatively charged cardiolipin, a minor component (11 mol %), gives a rather high transition temperature ($T_t$ = 53°, increasing temperature) which is 20° lower in the presence of CR buffer. The data shown for phosphatidylglycerol, a minor component under our growth conditions (7 mol %), can only be taken as preliminary because of the small amount of pure substance available. A reconstituted sample behaved similarly as the total phospholipid mixture. In the present context, the conclusion from Table IV is that the properties of the total phospholipids are essentially determined by its major component, phosphatidylethanolamine. The lowering of the $T_t$ by an increase in ionic strength (compare also Tables II and III) is caused by the shielding of negative charges of cardiolipin and phosphatidylglycerol.

*Phase Transitions in Membranes and Whole Cells.* In *E. coli* membranes, phase transitions were not detectable by 90° light scattering. However, they could be measured using the fluorescent dyes PhNap and ANS, and by spin-label probes. The spin-label data are discussed in more detail in a subsequent publication (Sackmann *et al.*, 1973). Figures 4 and 5 and Table III summarize the experiments using PhNap as a probe. Clearly, the membranes and whole cells (upper tracings in Figures 4 and 5) behave very similarly to the isolated phospholipids. The transition temperature and the range of the transition are essentially the same, as is the influence of ionic strength. As mentioned above, no or only little hysteresis was observed with the membranes even in the presence of CR buffer.

Another parallelism between the behavior of lipids and membranes concerns the influence of the PhNap concentration on the transition point. According to Figure 6 high concentrations of PhNap lower the transition point in the membranes by about 4° compared to a 7° decrease in phospholipid dispersions. This "fluidization" of the membrane matrix also occurs in intact cells. PhNap increases the rate of passive diffusion of NphGal.

Using PhNap at a concentration of $0.33\text{–}1 \times 10^{-5}$ M and a membrane lipid concentration of $2 \times 10^{-4}$ M, the phase transition in *cis*-18:1-containing membranes was detected by a deviation of the temperature dependence of the fluorescence intensity, characteristic for $T \gg T_t$. When cooling the sample,

TABLE IV: Transition Temperatures of *E. coli* Lipid Classes.[a]

| | $H_2O$ | | | | CR Buffer | | | |
|---|---|---|---|---|---|---|---|---|
| | $T_{t\rightarrow}$ | $\Delta T$ | $T_{t\leftarrow}$ | $\Delta T$ | $T_{t\rightarrow}$ | $\Delta T$ | $T_{t\leftarrow}$ | $\Delta T$ |
| Total phospholipids | 43 | 6 | 42 | 6 | 39 | 5 | 38 | 3 |
| | | | | | | | 35 | 2 |
| Phosphatidyl-ethanolamine | 39 | 4 | 37 | 5 | 38 | 5 | 37 | 5 |
| Cardiolipin | 53 | 10 | 46 | 4 | 33 | 9 | 29 | 5 |
| Phosphatidylglycerol | 29 | 7 | 19 | 9 | 24 | 8 | 19 | 8 |
| Phosphatidyl-ethanolamine + cardiolipin (4:1, w/w) | 42 | 5 | 42 | 4 | 39 | 7 | 38 | 6 |

[a] The phospholipids were isolated from cells grown in the presence of *trans*-$\Delta^9$-$C_{18:1}$. The phase transition was measured by 90° light scattering. For reconstitution, phosphatidylethanolamine and cardiolipin were first dissolved in benzene and the mixture taken to dryness. The lipids were then dispersed as described in Methods. The arrows indicate increasing (→) or decreasing (←) temperature.

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

| | *cis*-$\Delta^9$-$C_{18:1}$ | | | | *trans*-$\Delta^9$-$C_{16:1}$ | | | | *trans*-$\Delta^9$-$C_{18:1}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $H_2O$ | | CR Buffer | | $H_2O$ | | CR Buffer or 1 M NaCl | | $H_2O$ | | CR Buffer or 1 M NaCl | |
| | $T_t$ | $\Delta T$ | $T_t$ | $\Delta T$ | $T_t$ | $\Delta T$ | $T_t$ | $\Delta T$ | $T_t$ | $\Delta T$ | $T_t$ | $\Delta T$ |
| Transport | | | | | | | | | | | | |
| [$^{14}$C]sMeGal efflux | | | 15 | | | | | | | | 38 | |
| *In vivo* NphGal hydrolysis | | | 16 | | | | 31 | | | | 37 | |
| Cells | | | | | | | | | | | | |
| PhNap fluorescence | | | | | | | 28 | 6 | | | 38 | 4 |
| Membranes | | | | | | | | | | | | |
| PhNap fluorescence | 15 | 13 | | | 29 | 8 | 26 | 7 | 40 | 4 | 38 | 5 |
| ANS fluorescence | | | | | | | 28 | 5 | | | 41 | 8 |
| Electron spin resonance | | | | | | | | | | | | |
| I (12,3) | | | 14 | 4 | | | 24 | 3 | | | 29 | 3 |
| I (5,10) | | | 17 | 4 | | | 28 | 3 | | | 37 | 3 |
| I (1,14) | | | | | | | 28 | 3 | | | 36 | 4 |
| Phospholipids | | | | | | | | | | | | |
| PhNap fluorescence | 11 | 11 | | | 27 | 6 | 25 | 4 | 40 | 3 | 34 | 5 |
| ANS fluorescence | | | | | | | 26 | 8 | | | 37 | 7 |
| Light scattering | 16 | 11 | 13 | 12 | 30 | 5 | 27 | 7 | 42 | 5 | 38 | 4 |
| Dilatometry | | | | | 28 | 10 | | | 39 | 5 | | |
| Monolayers | 15 | | | | | | | | 41 | | | |

[a] NaCl (1 M) was only used for the ANS measurements. The data for [$^{14}$C]sMeGal efflux are taken from Overath *et al.* (1970), the spin-label data from Sackmann *et al.* (1973). I (12,3), I (5,10), and I (1,14) refer to stearic acid molecules carrying the paramagnetic *N*-oxyl-4′,4′-dimethyloxazolidine ring at carbon atoms 5, 12, and 16, respectively. $T_t$ values for *in vivo* NphGal hydrolysis are the average of several experiments. The PhNap, ANS, and light-scattering data refer to ascending branches of the temperature scans. PhNap concentration used were $3.3 \times 10^{-6}$ M for lipids and membranes and $1.7 \times 10^{-5}$ M for cells. All data in the table are accurate within a range of ±1°.

this deviation was observed at a temperature of about 22°. The fluorescent intensity then remained constant down to 10° and occasionally showed a further slight increase for $T \lesssim 10°$. From these experiments we estimate $T_t \sim 15°$ and $\Delta T \sim 10$–15°.

The upper part of Figure 7 shows the temperature dependence of the fluorescence intensity of ANS in membrane suspensions. Unlike PhNap, ANS interacts strongly with proteins. The change in fluorescence intensity at the phase transition is therefore superimposed on the fluorescence of protein-bound ANS. Nevertheless, the transition can be clearly discerned with both the *trans*-16:1- ($T_t = 28°, \Delta T = 5°$) and the *trans*-18:1-containing membranes ($T_t = 41°, \Delta T = 8°$).

Table V presents a summary of the transition temperatures of the *in vivo* measurements of transport and of the physical transitions in phospholipid monolayers and dispersions, in membranes and in whole cells. The table also includes the spin-label data of Sackmann *et al.* (1973).

## Discussion

*Correlation of Transition Temperatures.* The results presented in this paper (Table V) clearly support the previous notion (Overath *et al.*, 1970) that the breaks in the temperature dependence of carrier-mediated transport processes are related to the crystalline–liquid crystalline phase transition in the membrane. (a) In *trans*-18:1-grown cells the transitions in transport occur at 36–38°. This agrees with the physical measurements. Depending on the method used, transition temperatures between 34 and 41° are found for the *trans*-18:1-containing phospholipids, membranes, and whole cells. An exception are the spin-label measurements using I (12,3) as a probe which yield a transition temperature 7–8° lower. This result is discussed in more detail in a subsequent publication (Sackmann *et al.*, 1973). (b) The transition of *in vivo* NphGal hydrolysis in *trans*-16:1-supplemented cells occurs in a temperature range of 29–32°. This is slightly higher than the $T_t$ values of the physical measurements, but within the upper part of the transition range. (c) Finally, there is a satisfactory agreement between the transition temperatures of the physical and the *in vivo* studies in the *cis*-18:1 system. We conclude that the breaks in the temperature characteristic of the *in vivo* parameters are the result of the crystalline–liquid crystalline transition in the lipid phase of the membrane.

Although the transition temperatures obtained with a number of different methods agree quite well (Table V) the $T_t$ values observed by X-ray diffraction are about 5–10° higher than the breaks in the Arrhenius plots of transport parameters (Esfahani *et al.*, 1971; Shechter *et al.*, 1972; Dupont *et al.*, 1972). One possible explanation would be that the X-ray experiments are performed at insufficient hydration of the membrane lipids which would cause an increase in the transition temperature.

*Range of the Transition.* The next point which deserves discussion is the sharpness of the transition. In the transport studies, the change in slope at the transition occurs within 2–4° (Figure 1). In accordance with this, the spin-label data for all three membrane preparations indicate a sharp transition within 3–4° (Table V). The fluorescent probes give $\Delta T$ values of 4–8° for the *trans*-fatty acid containing cells, lipids,

and membranes and 11–13° for the *cis*-18:1-containing lipids and membranes. Thus, the width of the transition depends on the type of membrane preparation investigated and, less pronounced, on the physical parameter used for the indication. This result appears plausible for two reasons. Firstly, the sharpness of the transition is related to the cooperativity of the hydrocarbon chain interaction which is expected to be greater for lipids containing trans-unsaturated and saturated chains than for lipids containing cis-unsaturated and saturated chains. Secondly, the various physical methods used reveal different aspects of the transition process, *i.e.*, ANS probes the changes in the region of the polar head groups whereas PhNap directly indicates changes in the hydrocarbon chain region.

*Lipid Transition and the Functional State of the Membrane.* Above the phase transition about 80% of the lipids form a continuous phase in which lateral mobility favors a random arrangement of the lipid molecules (Träuble and Overath, 1973; Sackmann *et al.*, 1973). This does not preclude specific interaction of some lipid molecules with membrane proteins. A protein like the β-galactoside carrier may be pictured to be imbedded within the lipid matrix. In order to perform some kind of rotational motion connected to solute translocation across the membrane, the protein requires a fluid state of the surrounding lipid molecules. When the phase transition is induced by a decrease in temperature, the following mechanisms for the change in membrane conformation and/or function may be considered. (1) The lipid phase "solidifies" without any gross changes in the arrangement of the lipid as well as the protein molecules. This process may occur in two ways. (a) The crystallization starts at certain sites (nucleation) and spreads throughout the membrane when the temperature is lowered. The break in the transport process (Figure 1) would then indicate the onset of the phase transition. The steep decrease in rate for temperatures below the break could be interpreted as a gradual decrease of the number of protein molecules in a functional state. In this case, one would expect a second break at a somewhat lower temperature indicating the termination of the transition. Also, the temperature range between the two breaks should correlate with the observed width of the lipid phase transition. Since no second break was observed (compare also the [$^{14}$C]sMeGal efflux experiments described in Overath *et al.*, 1970), we have no evidence to support this mechanism. (b) There is a gradual decrease in mobility of the hydrocarbon chains (increase of viscosity of the lipid phase) in the course of the transition. In this case, the mobility (lateral and/or rotational diffusion) of the transport protein molecules would be affected by the increasing environmental constraint. A case in point is the ionophore valinomycin which cannot transport potassium across a "frozen" lipid bilayer (Krasne *et al.*, 1971; Stark *et al.*, 1972). (2) The phase transition causes a gross change in the spatial distribution of the membrane components (phase separation of different lipid classes, aggregation of proteins). This process is too complicated to allow speculations about the correlation of the breaks in transport and the width of the lipid transition.

At present, there is little experimental evidence to differentiate between possibilities 1b and 2. Aggregation of membrane proteins which may be caused by a phase separation of lipids has recently been observed by Verkleij *et al.* (1972) in freeze-etching electron micrographs of *trans*-18:1-supplemented *Mycoplasma* membranes. Aggregation is only observed when the membranes are quenched from a temperature below the phase transition. These authors also note that *E. coli* membranes exhibit a similar phenomenon.

*Lipid Phase Transitions as a Tool for Investigating Lipid–Protein Interaction.* Current views on membrane structure differentiate between integral and peripheral membrane proteins (see Singer, 1971, and Rothfield and Romeo, 1971, for reviews; Träuble and Overath, 1973). Analysis of lipid phase transitions in relation to protein-dependent functions allows a similar differentiation. (1) A first class of proteins requires a mobile state of the lipid phase for activity and therefore shows discontinuities in Arrhenius plots at the phase transition. The analysis of six different transport processes (Table V, Wilson *et al.*, 1970; Esfahani *et al.*, 1971; Rosen and Hackette, 1972) suggests that transport proteins belong to this group. (2) The *activity* of the second class of proteins is not directly affected by the phase transition. For example, three tightly membrane-bound enzymes involved in phospholipid synthesis in *E. coli* show linear Arrhenius plots throughout the phase transition (Mavis and Vagelos, 1971). For enzymatic reactions involving hydrophobic substrates it is difficult to differentiate between an effect of the lipid transition on the catalytic activity of the protein or, indirectly, on the mobility of the substrate dissolved in the lipid phase (*cf.* Sumper and Träuble, 1973). An interesting example provides the recent study by Esfahani *et al.* (1972); the breaks in the Arrhenius plots of succinic–coenzyme Q reductase observed by these authors may be caused by an impairment of the mobile redox-carrier function of coenzyme Q below the phase transition. In summary, lipid phase transitions appear to be a useful tool for investigating the relation of the physical state of the lipid phase to protein function; however, mechanistic details of this relationship will only be revealed by further experiments, for example, by analysis of reconstituted systems from purified components.

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