Conjugated Polyene Fatty Acids as Fluorescent Probes: Biosynthetic Incorporation of Parinaric Acid by *Escherichia coli* and Studies of Phase Transitions†

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ABSTRACT: The use of the fluorescent fatty acid, parinaric acid (9,11,13,15-octadecatetraenoic acid) (PnA), was studied in cells of an unsaturated fatty acid auxotroph of *Escherichia coli*. Growth conditions were found that permitted biosynthetic incorporation of PnA (up to 3%) into membrane phospholipids during growth on oleic or elaidic acid. Fluorescence measurements of incorporated PnA revealed phase transitions in cells, membranes, and phospholipids at temperatures that reflected the fatty acid composition of the sample. Transitions had a well-defined onset from high temperature, while the lower end point was less well defined. *cis*- and *trans*-PnA (*cis, trans, trans, cis,* and *all trans,* respectively) gave comparable results. Similar phase transitions were detected with PnA, which was not biosynthetically incorporated. Fluorescence of tryptophan was measured in *E. coli* membranes as a function of concentration of PnA. Significant quenching of tryptophan fluorescence by PnA was observed.

Recent work (Sklar et al., 1975) has led to the characterization of a naturally occurring conjugated polyenoic fatty acid, parinaric acid (PnA), as a probe of lipid and protein systems. Parinaric acid (9,11,13,15-octadecatetraneoic acid) is extracted from the seeds of *Parinari glabberimum*. The double bonds form a linear conjugated system, and render parinaric acid highly fluorescent. Parinaric acid (PnA[1]) can be prepared in two isomeric forms: *cis*-PnA has the double-bond configuration of cis-trans-trans-cis, while *trans*-PnA has the all-trans configuration. Both isomers have been used to detect thermal-phase transitions and lipid–protein interactions in model systems (Sklar et al., 1975, 1976, 1977a,b; Sklar, L. A., Hudson, B. S., and Simoni, R. D., manuscript in preparation).

PnA probes offer unique advantages to the study of biological membranes, as discussed (Sklar et al., 1975). The biological origin of the probe suggests the possibility of biosynthetic incorporation of PnA into membrane phospholipids. The phospholipid probe should have a well-defined orientation in the bilayer and behavior representative of the average membrane phospholipid. Although phospholipid forms of PnA can be prepared synthetically for use in model systems (Sklar et al., 1975, 1976, 1977b), phospholipid probes are not easily added to biological membranes.

This report describes the biosynthetic incorporation of PnA into phospholipids of an unsaturated fatty acid auxotroph of *Escherichia coli*. Previous investigations of phase transitions or lateral-phase separations in bacterial membranes (see, e.g., Thilo and Overath, 1976; Overath and Träuble, 1973; Träuble and Overath, 1973; Overath et al., 1975; Linden et al., 1973; Shechter et al., 1974; Linden and Fox, 1975; Morrisett et al., 1975; Ashe and Steim, 1971; Steim et al., 1969) provide a basis of comparison for establishing the use of PnA in a biological system. Our work confirms earlier studies and represents the first report of transitions detected in the cell by a fluorescent phospholipid of biosynthetic origin. We have (1) established conditions for biosynthetic incorporation of PnA, (2) used endogenous PnA to detect transitions in cells, membranes, and phospholipids, (3) demonstrated the correlation of transition parameters with cellular fatty acid composition, (4) compared the use of free fatty acid and phospholipid forms of PnA as probes in *E. coli*, and (5) demonstrated the use of free PnA to study lipid–protein interactions in *E. coli* membranes.

Materials and Methods

Preparation of Parinaric Acids. cis- and *trans*-PnA were prepared as described (Sklar et al., 1977b). Concentrations of PnA in stock solutions or in lipid extracts were determined from extinction coefficients at absorption maxima in ethanol or chloroform (Sklar et al., 1977a).

Growth of Cells. E. coli strain 30Ē βox^- was kindly supplied by Drs. C. Linden and C. F. Fox. This auxotroph is defective in β oxidation of fatty acids and in biosynthesis of unsaturated fatty acids (Linden et al., 1973). Oleic (9-*cis*-18:1) or elaidic (9-*trans*-18:1) acid was used as growth supplement at concentrations of 10–100 μg/mL or 20–200 μg/mL, respectively. PnA, when included in the growth medium (20–100 μg/mL), was added as an ethanol solution to 10% Triton X-100, warmed to 40 °C, and diluted with growth medium (35–40 °C) to give appropriate final concentrations of PnA and detergent. The final concentration of ethanol did not exceed 1%. The growth medium contained medium E, 0.5% Triton X-100, 1% casamino acids, and 5 μg/mL thiamine, essentially as described (Linden et al., 1973). Except where otherwise indicated,

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[1] Abbreviations used are: PnA, parinaric acid; *cis*-PnA, 9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid; *trans*-PnA, 9,11,13,15-*all-trans*-octadecatetraenoic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; 14:0, tetradecanoic acid; 16:0, hexadecanoic acid; 18:0, octadecanoic acid; 9-*cis*-18:1, 9-*cis*-octadecanoic acid (oleic acid); 9-*trans*-18:1, 9-*trans*-octadecanoic acid (elaidic acid).

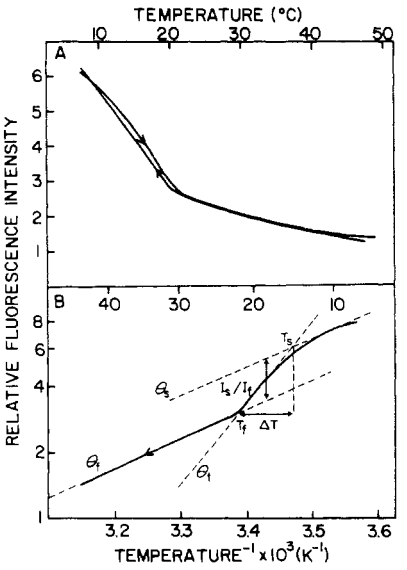

FIGURE 1: Data collection and data analysis. (A) X–Y recorder plot of fluorescence intensity of *trans*-PnA in phospholipids of strain 30E βox$^-$ grown on oleic acid, as a function of temperature (°C). Heating and cooling traces are indicated by arrows. A small amount of background fluorescence observed in phospholipid samples without PnA (not shown) was subtracted prior to further data analysis. (B) Heating trace plotted as fluorescence intensity (on a logarithmic scale) vs. $1/T$ (K^{-1}). Indicated parameters are obtained as described under Methods. Details of this experiment are described in the legend to Figure 6B.

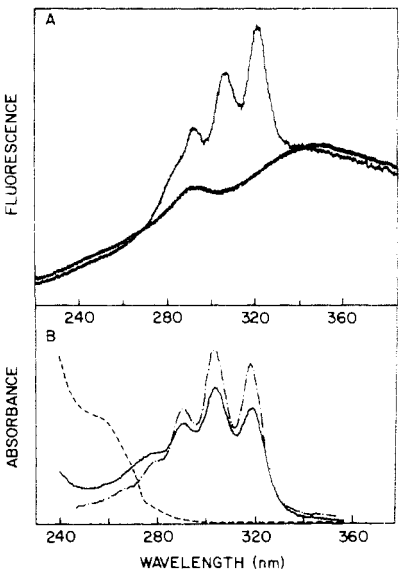

FIGURE 2: Excitation and absorption spectra of biosynthetically incorporated parinaric acid. (A) The uncorrected excitation spectrum of parinaric acid (upper curve) in cells grown on 40 μg/mL elaidic acid and 60 μg/mL *trans*-PnA and the excitation spectrum of cells grown on elaidic acid alone (lower curve). Washed cells were suspended at 2×10^8 cells/mL in medium E. Spectra were recorded at 10 °C using emission at 500 nm (slit 40 nm) and an excitation slit of 2 nm. A scattering peak at 250 nm was omitted in preparation of the figure. (B) The absorption spectra of the free fatty acid (- - -) and phospholipid (—) fractions purified from cells in A, compared to *trans*-PnA (- • -), all in chloroform. The phospholipids contained 0.4 mol % *trans*-PnA. Solutions of lipids in chloroform from control cells were used as reference samples.

growth was carried out at 35 °C with oleic acid, or at 39 °C with elaidic acid, and followed turbidimetrically for a tenfold increase in optical density. Typically, 250-mL cultures were maintained in 2-L flasks on a rotary platform incubator at 250 rpm. Cells were harvested by centrifugation at 37 °C, and washed three times with medium E containing 0.5% Triton, and twice with medium E. Cells were washed at 37 °C to facilitate removal of residual PnA. Washed cells were resuspended with medium E and kept on ice.

Preparation of Membranes. Membranes were prepared by sonication of cells (approximately 10^{10} cells/mL) for 4 min on ice in 5-mL batches, using the small probe of a Branson sonifier (Model 140W). Remaining whole cells were removed by centrifugation for 5 min at 3000*g*. Membrane fragments were pelleted at 100 000*g* for 1 h, resuspended to 5 mL with medium E, and kept on ice. Protein was measured spectrophotometrically with the Folin reagent (Lowry et al., 1951) using delipidated bovine serum albumin as a standard.

Lipid Analysis. Total lipids were extracted according to the method of Folch et al. (1957). As a precaution against oxidation of PnA, the chloroform–methanol reagent contained 10 μg/mL BHT. Phospholipids were purified by silicic acid chromatography. Phosphorus in phospholipid was determined by the method of Bartlett (1959) using dipalmitoylphosphatidylcholine as a standard. Phospholipid was calculated on the basis of 750 g of phospholipid per mole of phosphorus. Concentration of PnA, determined from molar extinction coefficients, was expressed as mol % of phospholipid. The fatty acid composition of phospholipid fractions was determined using a Hewlett Packard, Model 7610A, gas chromatograph. Methyl esters were prepared from phospholipids by acid methanolysis (2% H_2SO_4 in methanol) at 70 °C for 1 h. Identical results were obtained when methyl esters were prepared by saponification of samples and subsequent treatment with diazomethane. The relative composition of fatty acid species was calculated from peak areas, and expressed to the nearest percent. Silica Gel S/UV$_{254}$ (Macherey, Nagel & Co., Germany) was used for thin-layer chromatography with chloroform–methanol–water solvent (70:20:2).

Fluorimetric Measurements. Sample Preparation. Fluorimetric measurements were made using samples with incorporated PnA or with free PnA (fatty acid) added after growth. Suspensions of washed bacterial cells were diluted with medium E to a concentration of $2\text{–}7 \times 10^8$ cells/mL. In experiments using free fatty acid probes, *cis*- or *trans*-PnA was added in a small volume of ethanol. During fluorescence experiments, cells remained in suspension without stirring. Details of PnA addition appear in figure legends. Membrane samples were used at 25 to 70 μg of phospholipid/mL for studies of phase transitions. The PnA concentration is reported in the figure legend to each experiment. Quenching studies were carried out with dilute membrane samples (3–4 μg/mL phospholipid). Dry phospholipids were dispersed by vortexing in 0.05 M phosphate buffer, pH 7.5, at 40 °C. Final concentration of phospholipid was 25 to 50 μg/mL. Free PnA was added to control samples at probe/phospholipid ratios typical of those obtained in incorporation experiments (10^{-3} to 10^{-2}). In order to minimize probe degradation, samples were deoxygenated by bubbling with argon. Measurements were made in quartz cuvettes (2- or 4-mL capacity) covered with tight fitting Teflon stoppers. Probe degradation during fluorimetric measurements was insignificant.

Spectroscopy. Fluorescence data were recorded using an Hitachi-Perkin-Elmer MPF-2A fluorimeter equipped with a Moseley 7030A X–Y recorder, or a Baird-Atomic Fluorispec fluorescence spectrophotometer, Model SF-100, equipped with a Houston Instrument Omnigraphic 2000 X–Y recorder. A circulating water bath was used to control sample temperature. Excitation and emission were recorded for samples containing

TABLE I: Conditions for Biosynthetic Incorporation of Parinaric Acid.[a]

Supplement (μg/mL)	PnA (μg/mL)	Growth Temp (°C)	mol % PnA in Phospholipid
15 (oleic)	30 (trans)	34	0.7
50 (oleic)	100 (cis)	35	0.2
40 (elaidic)	40 (cis)	39	1.3
40 (elaidic)	60 (trans)	39	0.4

[a] 30E βox⁻ cells, propagated as described under Methods with 100 μg/mL oleic acid at 37 °C, were washed and allowed to grow for three to four generations under the indicated conditions. PnA incorporation was determined spectrophotometrically in phospholipid extracts, and expressed to the nearest 0.1%. In these experiments, the limit of detection of PnA was 0.02 mol % of phospholipid.

PnA and compared to control spectra. Contribution of cellular chromophores to fluorescence emission varied with samples of cells, membranes, and phospholipids. Emission was monitored at a wavelength (410–500 nm) chosen to maximize the ratio of PnA to control fluorescence. The wavelength of excitation was 319 to 323 nm, as reported in each experiment. Fluorescence intensity was recorded as a function of temperature during heating and cooling of the sample at 1°C/minute. Energy-transfer studies measured the intensity of tryptophan fluorescence in dilute membrane suspensions as a function of PnA concentration in the sample. Details are given in the figure legend.

Analysis of Transitions. Fluorimeter output (Figure 1A) was corrected for background fluorescence, digitized, and plotted as log I vs. reciprocal temperature (K^{-1}). In logarithmic plots (Figure 1B), a nearly linear region was observed at higher temperatures, characteristic of PnA fluorescence in fluid lipid. The slope of this line, designated θ_f, describes the temperature range (K^{-1}) over which fluorescence intensity doubles (Sklar et al., 1975). With decreasing temperature, the slope of log I showed an abrupt increase. A new slope, θ_t, was drawn through this transition region. At lower temperatures, the slope of log I decreased, deviating from θ_t. In most experiments with the *E. coli* system, log I was not a linear function of temperature below the transition region. A line, θ_s, parallel to θ_f, was drawn tangent to the curve at low temperature.[2] The intersection of θ_f with θ_t is defined as T_f, and the intersection of θ_t with θ_s is defined as T_s. Temperatures are expressed to the nearest degree Celsius. The width of the transition, ΔT, is the difference $T_f - T_s$. I_s/I_f is the ratio of fluorescence intensity below and above the transition. Both heating and cooling records were used to determine transition parameters for each sample. θ_f was averaged for both curves and then each curve was treated individually as described above. Parameters are presented as mean values, with the amount of hysteresis indicated in parentheses for the parameters T_f and T_s.

Results and Discussion

Biosynthetic Incorporation of PnA. Neither *cis*-PnA nor *trans*-PnA supported growth of the strain 30E βox⁻. However, certain conditions of growth on oleic or elaidic acids were found to permit incorporation of sufficient PnA for fluorescence spectroscopy (0.2–2.0 mol % of phospholipid) (Figure 2). Incorporation required a two-step growth procedure. The strain

[2] Occasionally, the shape of the curve did not permit construction of θ_s parallel to θ_f. Exceptions are indicated in the text.

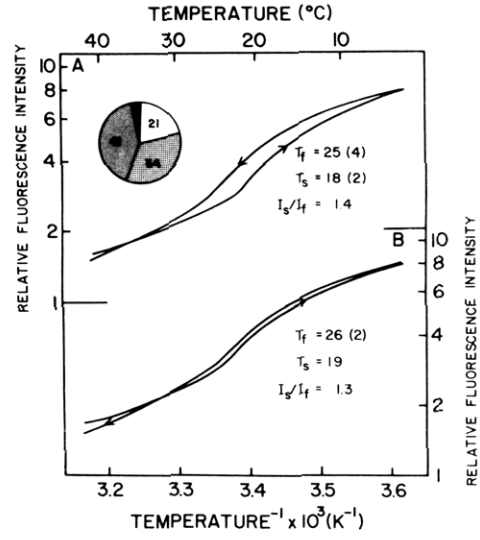

FIGURE 3: Phase transition detected in cells (A) and phospholipid (B) by *trans*-PnA incorporated during growth on oleic acid. *trans*-PnA, 0.7% (mol % of phospholipid), was incorporated by 30E βox⁻ cells grown for three to four generations at 34 °C in 15 μg/mL oleic acid and 30 μg/mL *trans*-PnA. The circular graph indicates (clockwise) the percentages of myristic (clear), palmitic (stippled), oleic (thatched), and all other fatty acids (black) present in phospholipid. Determination of T_s, T_f, and I_s/I_f is described under Methods. (A) Cells suspended in medium E at 2 × 10⁸/mL. Excitation and emission wavelengths were 319 and 500 nm, with slit widths of 2 and 40 nm, respectively. (B) Extracted phospholipid dispersed in 0.05 M phosphate buffer, pH 7.4, at 25 μg/mL. Excitation and emission wavelengths were 319 and 410 nm, with slit widths 1.5 and 20 nm, respectively.

was propagated at 37 °C in 100 μg/mL oleic acid, but, in the presence of PnA, supplement concentration was reduced. Many combinations of growth temperature, PnA, and supplement concentrations led to incorporation of PnA, but not always to cells enriched in exogenously supplied unsaturated fatty acid. Consistent results were obtained with (1) growth at 35 °C in 25 μg/mL oleic acid and 50 μg/mL PnA (2–3 doublings) or (2) growth at 39 °C in 40 μg/mL elaidic acid and 40–60 μg/mL PnA (3–5 doublings). Under these conditions, *cis*-PnA was incorporated more extensively than *trans*-PnA. Details are given in each experiment. Thin-layer chromatography of phospholipids containing PnA showed comigration of fluorescent moieties with the three phospholipid species present in *E. coli* membranes: phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

In the course of these studies, data has been obtained from cells grown under different conditions. Table I describes growth conditions for cells which met the following criteria: (1) PnA was biosynthetically incorporated by cells during growth on 18:1 supplement; (2) free PnA was negligible in the free fatty acid fraction of lipid extracts; (3) the 18:1 supplement was incorporated as the predominant fatty acid species; and (4) intact cells exhibited thermally induced lipid transitions. Data appear in Figures 3 through 5.

Lipid Transitions Detected by Incorporated PnA. The fluorescence of *cis*- and *trans*-PnA, incorporated by strain 30E βox⁻ during growth, was found to be highly sensitive to the physical state of cellular phospholipids. The phospholipid probes detected well-defined, reversible, phase transitions at temperatures characteristic of cells enriched in oleic or elaidic acid. Figures 3 through 5 illustrate transitions observed in cells grown under conditions described in Table I. In each experiment, fatty acid composition is indicated in a circular graph; values for the end points (T_f and T_s) and magnitude (I_s/I_f) of

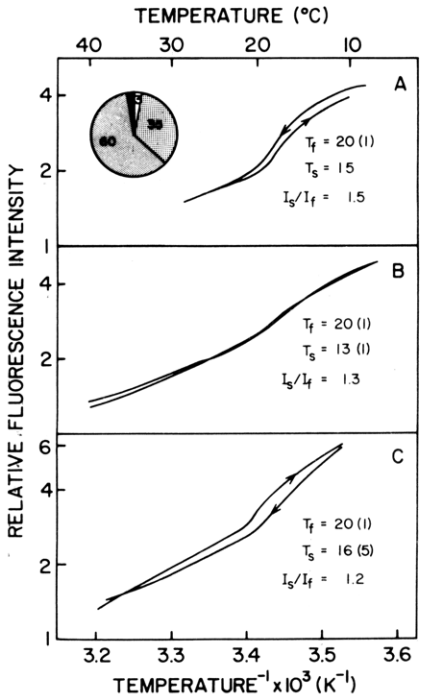

FIGURE 4: Phase transitions detected in cell membranes and phospholipid by *cis*-PnA incorporated during growth on oleic acid. 30E βox$^-$ cells were grown for three generations at 35 °C in 50 μg/mL oleic acid and 100 μg/mL *cis*-PnA; 0.2 mol % *cis*-PnA was incorporated into phospholipid. Fatty acid composition is indicated in the circular graph (see legend to Figure 3); parameters T_s, T_f, and I_s/I_f are described (see Methods). (A) Cells suspended at 2×10^8/mL of medium E. Wavelengths for excitation and emission were 321 (slit 1.5) and 430 (slit 21) nm, respectively. (B) Membranes suspended at 25 μg of phospholipid/mL of medium E. Excitation and emission wavelengths were 323 (slit 1.5) and 410 (slit 21) nm, respectively. (C) Phospholipid dispersed in 0.05 M phosphate buffer, pH 7.5, at 50 μg/mL. Excitation and emission wavelengths as in B.

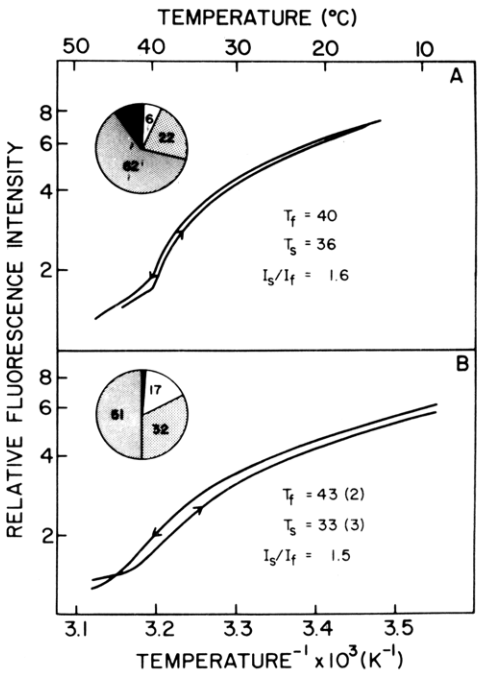

FIGURE 5: Phase transitions detected in cells by PnA incorporated during growth on elaidic acid. 30E βox$^-$ cells were grown for three to four generations at 35 °C in 40 μg/mL elaidic acid and either 60 μg/mL *trans*-PnA (A) or 40 μg/mL *cis*-PnA (B). Fatty acid composition of each culture is indicated by a circular graph as described in the legend to Figure 3, except that the thatched area represents elaidic acid. Parameters T_s, T_f and I_s/I_f are described under Methods. Excitation and emission wavelengths were 321 (slit 2) and 500 (slit 40) nm, respectively. (A) Cells (5×10^8/mL of medium E) containing 0.4 mol % *trans*-PnA incorporated into phospholipid; (B) cells (2×10^8/mL of medium E) containing 1.3 mol % *cis*-PnA incorporated into phospholipid.

the transition are included for each pair of heating and cooling curves.

Data obtained from cells grown on oleic acid and *trans*-PnA are shown in Figure 3. The upper pair of curves (A) shows the transition measured in cells, the lower curves (B) in phospholipid dispersed in buffer. The striking similarity of A and B indicates that the ability of the phospholipid probe to monitor the physical state of host lipid is largely unaffected by the presence of protein (approximately 60% by weight of *E. coli* membranes) or by structural and metabolic integrity of the cell.

Figure 4 shows data from a culture grown on oleic acid and *cis*-PnA. Transitions in cells (A), membranes (B), and phospholipids (C) are compared. with less than 1 °C variation, T_f was detected near 20 °C in each preparation. Other parameters compare well. Results shown in Figures 3 and 4 indicate that either isomer of PnA can be biosynthetically incorporated for detection of phase transitions in vivo.

It should be pointed out that the broad transition attributed to a high percentage of oleic acid was observed between 19 and 25 °C in Figure 3, and between 14 and 20 °C in Figure 4. Such differences between experiments were observed frequently, and reflect differences in fatty acid composition. Increased incorporation of saturated species has the effect of raising the transition temperature (Thilo and Overath, 1976).

Data presented in Figure 5 demonstrate transitions in cells grown on elaidic acid. Data were obtained in two experiments with cells grown on elaidic acid and *trans*-PnA (A) or *cis*-PnA (B). Transitions, distinct from those seen in cells grown on oleic acid, are observed near the growth temperature (39 °C). As in the case of cultures grown with oleic acid, increased synthesis of saturated fatty acid (B) has the effect of increasing the temperature of the observed transition. It is interesting to note that cells of this composition may not be completely fluid at the growth temperature. We have observed that the strain grows poorly on elaidic acid below 39 °C and that a finite number of doublings are obtained when cells are maintained in elaidic acid.

Lipid Transitions Detected by Free PnA. Because relatively few biological systems are easily manipulated in terms of probe incorporation, it was of interest to compare the use of free and biosynthetically incorporated probes in the *E. coli* system. Experiments were carried out using free PnA added directly to samples in probe/phospholipid ratios similar to those obtained during incorporation experiments.

Figures 6 and 7 illustrate the ability of free *trans*-PnA to detect transitions in samples enriched in oleic or elaidic acid, respectively. In these examples, striking transitions are observed at temperatures which are correlated with the fatty acid composition of the culture; data are similar with cells and extracted phospholipids.

On the basis of numerous experiments similar to those shown in Figures 6 and 7, it appears that both free *trans*-PnA and esterified PnA can be used to detect phase transitions with essentially equivalent results. These comparisons justify the use of free *trans*-PnA to detect phase transitions in biological membranes. Advantages of the free probe include experimental convenience and access to information in organisms which cannot be made to incorporate PnA into phospholipid.

Comparison of cis-PnA and trans-PnA as Probes in E. coli.

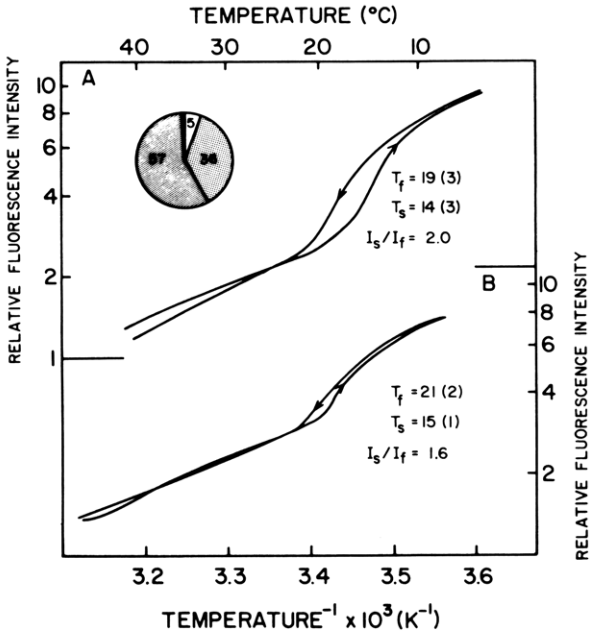

FIGURE 6: Transition detected by free *trans*-PnA in cells and phospholipid-enriched in oleic acid. 30E βox$^-$ cells were grown for approximately four generations at 30 °C in 20 μg/mL oleic acid. Circular graph and transition parameters are described in the legend to Figure 5 and Methods, respectively. (A) Cells (7 × 10^8/mL of medium E) containing free *trans*-PnA. PnA was added (0.25 μg/mL) at 4 °C; unbound PnA was removed by centrifugation of cells. Excitation and emission wavelengths were 321 (slit 1.5) and 500 (slit 40) nm, respectively. (B) Extracted phospholipid (50 μg/mL in 0.05 M phosphate buffer, pH 7.5) containing free *trans*-PnA (0.25 μg/mL). Excitation and emission wavelengths were 319 (slit 1.5) and 410 (slit 20) nm, respectively.

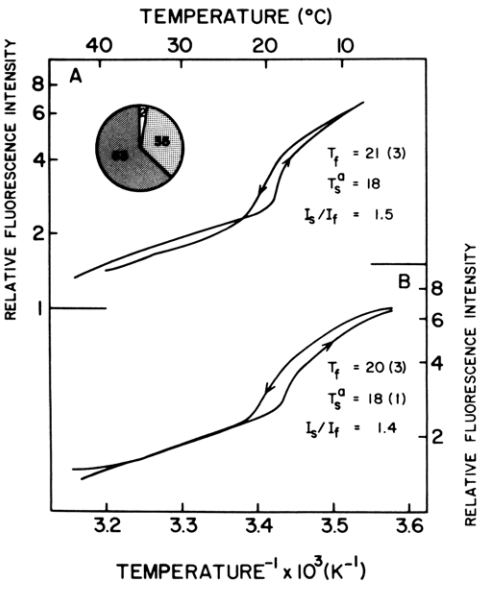

FIGURE 8: Comparison of *cis*- and *trans*-PnA in detecting *E. coli* transitions in identical samples. Phospholipid dispersions (50 μg/mL in 0.05 M phosphate buffer, pH 7.5) were prepared from cells grown for three to four generations at 35 °C in 50 μg/mL oleic acid. PnA was added at 0.05 μg/mL. Fatty acid composition is described in the circular graph (see legend to Figure 5). Transition parameters are determined as described (Methods), except that θ_s was not parallel to θ_f, and thus T_s^a is not strictly analogous to T_s reported in Figures 5–8. I_s/I_f is the ratio of intensities of θ_s and θ_f at the midpoint of T_s^a and T_f. (A) Transition detected with *trans*-PnA. Excitation and emission wavelengths were 319 (slit 2) and 410 (slit 40) nm, respectively. (B) Transition detected with *cis*-PnA. Excitation and emission wavelengths were 321 (slit 2) and 410 (slit 40) nm, respectively.

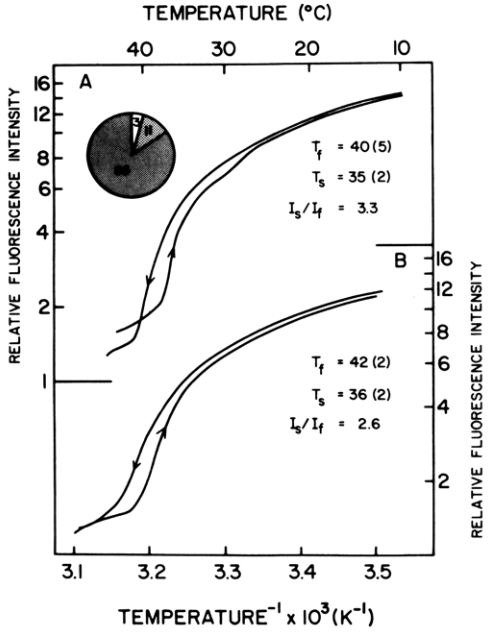

FIGURE 7: Transitions detected by free *trans*-PnA in cells and phospholipid-enriched in elaidic acid. 30E βox$^-$ cells were grown for three to four generations at 39 °C in 200 μg/mL elaidic acid. Description of circular graph and transition parameters appear in legend to Figure 5 and Methods, respectively. (A) Transition in cells (2 × 10^8/mL of medium E) containing free *trans*-PnA. PnA was added (0.6 μg/mL) at 39 °C, cells were incubated at 39 °C for 10 min, and unbound PnA was removed by centrifugation of cells. Excitation and emission wavelengths were 321 (slit 2) and 500 (slit 40) nm, respectively. (B) Transition in extracted phospholipid (50 μg/mL in 0.05 M phosphate buffer, pH 7.5) containing free *trans*-PnA (0.05 μg/mL). Excitation and emission wavelengths were 320 (slit 2) and 410 (slit 40) nm, respectively.

Our initial studies of *E. coli* transitions focused on *trans*-PnA. Growth experiments indicated that *cis*-PnA offered several advantages, namely, greater solubility in growth medium, more complete removal from cells, and more extensive biosynthetic incorporation.

Data presented in Figures 4 and 5 show that *cis*-PnA, esterified to phospholipid, detects *E. coli* lipid transitions. The transition temperatures reflect the fatty acid composition of the sample. A direct comparison of *cis*- and *trans*-PnA phospholipid probes required samples of identical fatty acid composition, which were not obtained in these experiments. We have directly compared the behavior of *cis*- and *trans*-PnA added as free fatty acids to identical samples of membranes or lipids (Figure 8). In phospholipids containing 63% oleic acid, transitions detected by *cis*- and *trans*-PnA were quite similar. Parameters T_f and T_s show isomer differences of only a degree or so. The width (ΔT), magnitude (I_s/I_f), slope, and hysteresis of the transition regions compare well.

In recent experiments, free *trans*-PnA has been used to detect transitions in membranes of a wild-type strain of *E. coli*. These membranes show no transition with free *cis*-PnA (data not shown). Results indicate that *trans*-PnA is sensitive to the appearance of small amounts of solid lipid, while *cis*-PnA continues to sample bulk fluid lipid. In studies of partitioning of free PnA in defined lipids (see preceding paper in this issue, Sklar et al., 1977b), it was observed that *trans*-PnA preferentially samples solid lipid, in systems where solid and fluid lipid coexist. Movement of free *trans*-PnA to regions of solid-lipid formation may permit detection of transitions in biological systems where disordered lipid persists over the physiological-temperature range.

Use of PnA in Studies of Lipid–Protein Interactions. There

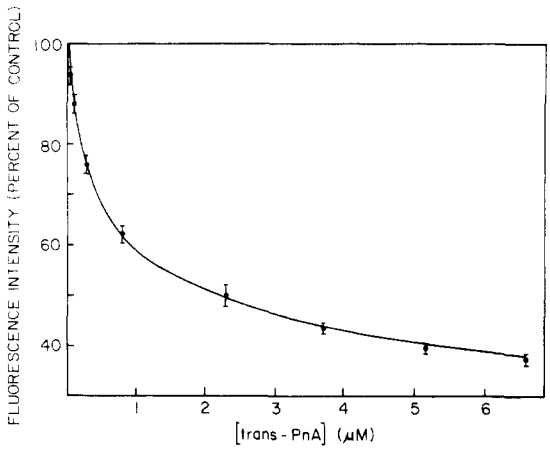

FIGURE 9: Decrease of tryptophan fluorescence in 30E βox⁻ membranes with sequential addition of *trans*-PnA. Membrane sample (4 mL) contained 20 nmol of phospholipid in medium E. PnA (2–5-μL aliquots) was added from stock solutions in ethanol, and the sample was mixed after each addition by bubbling with argon. External access to the sample compartment through a microliter-syringe assembly permitted PnA additions without interruption of fluorescence measurements. Scans measured 30 s and 5 min after PnA addition were essentially identical. Excitation and emission wavelengths were 235 and 345 nm, respectively, using 20-nm slits. Quenching was measured at 41 °C.

is considerable evidence that phase transitions in *E. coli* membranes involve gross reorganization of lipid and protein constituents. Freeze-fracture studies by Kleeman and McConnell (1974) and Shechter et al. (1974) used fatty acid auxotrophs of *E. coli* to demonstrate the appearance of large patches of particle-free inner-membrane lipid when samples were rapidly frozen from below the transition temperature. Samples frozen rapidly from above the transition temperature showed a regular distribution of particles (presumably protein). Additional freeze-fracture evidence for lateral-phase separation has been obtained in a variety of reconstituted systems (Grant and McConnell, 1974; Hubbell, 1975; Kleeman and McConnell, 1976), showing protein to be excluded from domains of ordered lipid. Recently developed nuclear magnetic resonance techniques allow detection of lipid–protein interactions in situ, and provide supporting evidence for freeze-fracture studies in model systems (Brûlet and McConnell, 1976).

Fluorescence energy transfer is a sensitive means to monitor intermolecular distance, and potentially a powerful technique for study of lipid–protein interactions in cell membranes. As described previously (Sklar et al., 1975, 1976), PnA absorbs in the region of tryptophan emission. The intermolecular distance for 50% energy transfer was calculated as 30–35 Å, and use of tryptophan-PnA energy transfer to study lipid binding to bovine serum albumin was demonstrated (Sklar et al., 1976; manuscript in preparation. We have used the *E. coli* system to investigate the possibility of measuring energy transfer in a heterogeneous system, toward the goal of studying lipid–protein interactions in cell membranes.

Tryptophan fluorescence was measured in membranes, prepared from 30E βox⁻ cells grown on elaidic acid, as aliquots of PnA were added to the sample. Figure 9 illustrates the extent of quenching (60%) obtained with *trans*-PnA in these experiments. Identical experiments with *cis*-PnA (data not shown) reveal 80% quenching of membrane fluorescence. These results indicate that tryptophan and PnA can serve as an efficient donor–acceptor pair in a system as heterogeneous as the *E. coli* membrane. In view of the evidence for segregation of lipid and protein during phase transitions in this system, it will be of interest to compare quenching at temperatures above and below the transition.

Interpretation of Lipid Transitions. These studies with incorporated PnA provide convincing evidence that phospholipids of *E. coli* undergo broad, reversible, structural transformations over a temperature range that reflects the fatty acid composition of phospholipids. Similar results have been reported by many investigators using a variety of physical and spectroscopic methods to study lipid transitions in auxotrophs of *E. coli* (Linden et al., 1973; Morrisett et al., 1975; Thilo and Overath, 1976; Overath et al., 1975; Overath and Träuble, 1973; Shechter et al., 1972, 1974; Ashe and Steim, 1971; Steim et al., 1969; for reviews of transitions in *E. coli*, see Cronan and Gelmann (1975) and Melchior and Steim (1976)).

Although these transitions are now well documented, the nature of the molecular events which constitute a transition in biological membranes has not been established. It is difficult to distinguish experimentally between two classes of conformational changes in bilayers of mixed head group and chain composition: (1) the acyl-chain melting of phospholipid molecules and (2) lateral segregation of immiscible phases into domains of different composition. A causal relationship may exist between the former and latter events, depending on properties of the system. Phase diagrams have been constructed for two-component model systems (Shimshick and McConnell, 1973) to describe the composition of fluid and solid domains at different temperatures, as a function of the composition of the mixture. Details of phase diagrams are unique for a given two-component system (Wu and McConnell, 1975). As discussed in the preceding section, evidence of lateral-phase separation has been obtained for *E. coli* membranes, as well as model systems.

The transitions observed in *E. coli* with PnA are asymmetric, being sharper at the high-temperature end. This asymmetry has also been observed in calorimetric measurements of *E. coli* lipids enriched in elaidic acid (Jackson, 1976). For a two-component system, the observed sense of the asymmetry corresponds to a phase diagram in which the fluidus curve bends downward sharply at a temperature near T_f, while the solidus curve is smooth through the temperature region from T_f to T_s. The effective number of thermodynamic components in this *E. coli* auxotroph system is unknown, but it is certainly greater than two, and it is therefore not surprising that the phase behavior is poorly understood.

The results of this and other studies do not yet lead to a complete picture of the molecular events which occur during phase transitions in vivo. Extensions of this study will utilize partitioning differences of free *cis*- and *trans*-PnA to address the question of formation of solid and fluid domains during transitions observed in *E. coli*.

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Analysis of the Defect Structure of Gel-Phase Lipid†

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ABSTRACT: The partitioning of the spin label 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) into phosphatidylcholine bilayers and the monomer–aggregate equilibrium for chlorophyll a incorporated into phosphatidylcholine bilayers have been interpreted in terms of the formation of defects in the gel-phase lipid, starting some 20 °C below the temperature of the main gel to liquid crystalline phase transition. By contrast, defects seem to be largely absent from bilayers of dipalmitoylphosphatidylethanolamine in the gel phase. The defect structure accounts for the continuous nature of the phase transition for phosphatidylcholines, and also for the increase in width of the transition caused by the addition of alcohols.

Biological membranes are often said to exist within the lipid-phase transition, in the sense that lipids are present both in the gel and in the liquid-crystalline phases (see Lee, 1975a; Cronan and Gelmann, 1975). Further, from the fatty acid compositions, determined especially for bacterial membranes (Cronan and Gelmann, 1975), it seems that at ambient temperatures many of the lipids will be within a few degrees of their respective phase-transition temperatures. It therefore becomes of importance to study the properties of lipids close to the phase transition, particularly since major changes in physical properties are expected in this region.

Ubbelohde (1965) has argued convincingly for the presence of premelting and prefreezing phenomena close to many solid–liquid transitions, where the premelting phenomena make the solid more "liquid-like" and the prefreezing phenomena make the liquid more "solid-like". Evidence has already been presented consistent with the presence of prefreezing phenomena in lipid bilayers, with the formation of quasicrystalline clusters of lipid present within a matrix of otherwise freely dispersed, liquid-crystalline, phase lipid (Lee et al., 1974b; Ting and Solomon, 1975; Bashford et al., 1976). Here, evidence is presented consistent with premelting phenomena in lipid bilayers in the crystalline or gel phase.

Of course, at all temperatures above absolute zero, a crystalline solid will contain defects, the number of defects increasing with increasing temperature, simply because the formation of defects is associated with an increase in entropy. The defects may simply be vacant sites in the otherwise regularly packed lattice, or they may adopt more complex arrangements. One such possibility is the formation of large-angle grain boundaries. A grain boundary is simply the boundary separating two crystals or grains that differ in orientation, and, as shown in Figure 1a, when two grains differ only slightly in orientation, packing at the grain boundary is not very different from bulk packing. However, when the difference in orientation of the two grains is more considerable

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