On the Role of Membrane Phase in the Transmission Mechanism of Colicin E1†

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ABSTRACT: After colicin E1 is added to sensitive cells containing the lipophilic fluorescence probe *N*-phenyl-1-naphthylamine the probe fluorescence increases after a lag period. The lag period and the initial linear rate of the fluorescence increase are temperature dependent. Arrhenius plots of the rate of fluorescence increase are biphasic. The transition temperatures of the Arrhenius plots depend on the culture growth temperature and are at approximately 15, 18, and 20° for cultures grown at 20, 30, and 37° and harvested at midlogarithmic phase. The ratio of the slope of the Arrhenius plot in the low temperature region to that in the temperature region above the transition is approximately 3.4, 2.5, and 1.9 for cultures grown at 20, 30, and 37°. The fatty acid composition of the cultures grown under the conditions of these experiments shows an increase in the 16:0 acid and a decrease in the 16:1 and 18:1 acids as the temperature is increased, in agreement with results of others. The polarization of fluorescence of cell-bound *N*-phenyl-1-naphthylamine was also measured as a function of temperature. The polarization decreased in a 1–2° temperature range centered at approximately 14 and 17.5° for cells grown at 20 and 30°, respectively. The origin of the biphasic Arrhenius plots is thought to be most likely an order–disorder transition in the cell envelope involving the fatty acid chains. This implies that the transmission mechanism of colicin E1 involves movement of colicin and/or colicin-induced substances through fatty acid containing regions of the cell envelope. This movement would be facilitated when these regions are in a more disordered or fluid phase.

The ability of trypsin to reverse the lethal effects of adsorbed colicin E1 in the absence of metabolic inhibitors decays at a rate which is comparable or even faster than the rate of the decrease of intracellular ATP caused by colicin E1 (Phillips and Cramer, 1973). This implies that for this colicin the phenomenon of trypsin reversal cannot be used to support a model in which the colicin exerts its inhibitory effects from the outer trypsin accessible part of the cell surface (Nomura, 1964; Luria, 1964). One obvious interpretation of the relatively rapid rate of decay of trypsin reversibility of colicin E1 killing effects is that the colicin penetrates into the cell envelope or membrane before or during the time it exerts its inhibitory and lethal effects (Phillips and Cramer, 1973). The ribosomal nuclease activity associated with colicin E3 *in vitro* implies that the transmission mechanism of this colicin involves penetration through the cell envelope at least to the level of the cytoplasmic membrane (Boon, 1971, 1972; Bowman *et al.*, 1971).

If the mechanism for transmitting colicin inhibition and lethal effects involves penetration and movement through some part of the cell envelope, a major question is how proteins as large as the E colicins, with molecular weights of approximately 60,000 (Herschman and Helinski, 1967; Schwartz and Helinski, 1971), can move in the envelope or membrane. A precedent for the rapid movement of proteins in cell walls or membranes exists in a mammalian cell system (Frye and Edidin, 1970). One model for membrane structure which would allow for protein movement in a membrane is the fluid mosaic model documented by Singer and Nicolson (1972). The hydrophobic fatty acid chains of the membranes of a number of cell systems, including *Mycoplasma laidlawii* (Steim *et al.*, 1969; Melchior *et al.*, 1970; Engelman, 1970; Tourtellotte *et al.*, 1970; Rottem *et al.*, 1970), rabbit vagus nerve (Hubbell and McConnell, 1968) and the gram-positive bacterium *Micrococcus lysodeikticus* (Ashe and Steim, 1971), exist in a relatively fluid or disordered state at physiological temperatures. It has been pointed out by Singer and Nicolson (1972) that the characteristic times for the colicin E1 induced fluorescence probe response observed by Cramer and Phillips (1970) are much larger than would be expected in a model for cooperative changes in membranes based on the allosteric model for proteins (Changeux *et al.*, 1967), though these times are similar to the migration times observed by Frye and Edidin (1970).

The temperatures at which the order–disorder transition of fatty acids in phospholipid or membranes will occur depend upon the fatty acid chain length and degree of unsaturation (Chapman and Wallach, 1968). The shorter the average chain length and the larger the degree of unsaturation, the lower the melting or transition temperature. It is known that the fatty acid composition of a wide variety of organisms including bacteria (Marr and Ingraham, 1962), fungi (Pearson and Raper, 1927), and insect larvae (Frankel and Hopf, 1940) shifts toward a greater degree of unsaturation at lower environmental temperatures. This phenomenon has been most completely documented in the case of *Escherichia coli*, where it appears that the regulation is at the level of the acyl transferase (Sinensky, 1971). The explanation of the dependence of fatty acid composition on growth temperature would seem to be at least partly that the organism tends to conserve a certain degree of membrane mobility.

A simple way to investigate the effect of membrane mobility on the efficiency of the colicin response is to vary the

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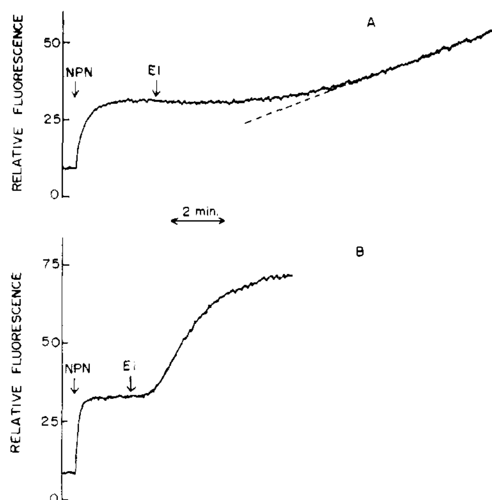

FIGURE 1: Colicin E1 induced fluorescence increase at 11.8 (A) and 26.2° (B). *E. coli* B/1,5 was grown at 30° and concentrated; *N*-phenyl-1-naphthylamine concentration, 1.75 μM; 1.25 and 0.9 μg/ml of colicin E1 added in A and B, which gave maximum rates of fluorescence change. Survival levels assayed at the end of the fluorescence runs shown above were 1.6×10^{-4} (A) and 1.7×10^{-5} (B).

growth and incubation temperatures of a strain normally sensitive to colicin. It is expected that the phospholipid-rich membrane of *E. coli* harvested and incubated at temperatures well below that at which they were grown will be in a relatively rigid or ordered phase. Conversely, the membrane population of a culture incubated at temperatures well above the growth temperature will be relatively fluid or disordered. As a simple way to measure the rate of the colicin response we have used the rate of increase of fluorescence of the probe *N*-phenyl-1-naphthylamine (Radda, 1971), which reflects colicin E1 induced structural changes in the cell envelope thought to be closely related to those structural or conformation changes responsible for the decrease in intracellular potassium and ATP levels (Phillips and Cramer, 1973).

Methods

Bacteria and Media. Escherichia coli strain B/1,5, obtained from Dr. Simon Silver, was grown in M9 medium containing (grams/liter): NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; glucose, 1.0. Cultures were grown from inocula small enough to allow at least five generations of growth to a titer of approximately 4×10^8 cells/ml.

Fluorimetry. Measurements were made as described previously (Phillips and Cramer, 1973) except that a Corning 3-73 filter was used in conjunction with the sodium nitrite filter as a blocking filter combination in the determination of the Arrhenius plots (Figure 2). Temperature measurements of thermostated stirred suspensions were made with a 12-in. -20 to $+100°$ laboratory thermometer whose accuracy was estimated to be $\pm 0.2°$ and a United Systems Corp. digital thermometer which could be read with an accuracy of 0.02°F. Polarization measurements were made in a 1×1 cm mechanically stirred thermostated cuvet. The excitation light for the polarization experiments was defined by the monochromator, a 363-nm interference filter, and a Glan-Taylor prism polarizer (Karl Lambrect Corp., GTYA 12). The polarizer was set to transmit vertically polarized light. The right angle emission was defined by a blocking filter combination of a Corning 3-75 filter, the sodium nitrite filter, and an analyzer made of a glass enclosed polaroid sheet. The analyzer filter was set to measure light polarized horizontally and thus perpendicularly ($I_{\perp}$) to the polarization vector of the incident beam, or light polarized vertically and parallel ($I_{\parallel}$) to incident polarization. The calculated polarization parameter was $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$. With the geometry used to measure polarization from concentrated *E. coli* suspensions the polarization parameter for a 3 μM rhodamine B solution with excitation at 547 nm was 0.37.

Fatty Acid Analysis. E. coli cells were pelleted by centrifugation and extracted for 4 hr with 20 vol of chloroform–methanol (2:1, v/v). Insoluble material was harvested by centrifugation and reextracted with 15 vol of chloroform–methanol (1:1, v/v). Combined extracts were evaporated *in vacuo* at 35°. Methyl esters were prepared by saponification and subsequent esterification with boron trifluoride–methanol (Metcalf *et al.*, 1966). Esters were separated on a $^1/_8$ in. $\times$ 10 ft stainless steel coiled column packed with 10% diethylene glycol succinate on 80–100 mesh Chromosorb W (acid washed) which was operated isothermally at 170° in a Varian-Aerograph Model 1200 gas chromatograph equipped with a flame ionization detector. The identity of methyl esters was ascertained by comparison of their retention times with those of authentic reference esters. The identity of unsaturated acids was verified by analysis before and after hydrogenation (Keenan *et al.*, 1970). Cyclopropane acids were identified by reference to lipid extracts from *E. coli* maintained in a stationary culture for an extended period. It is known that the 16:1 and 18:1 acids are converted to the respective cyclopropane derivatives under these conditions (Cronan, 1968). Quantitation was achieved by triangulation of chromatographic peaks. Analysis of a quantitative reference mixture (RM-6, Supelco, Inc., Bellefonte, Pa.) under these conditions revealed that the major components (>5%) were being analyzed with a relative error of less than 5%.

Results

After a saturating amount of colicin E1 is added to a cell suspension in the presence of *N*-phenyl-1-naphthylamine, there is a lag and then an increase in fluorescence which is very closely linear in time over at least half of the fluorescence rise (Figure 1). Both the lag and the rate of the linear increase are dependent on the temperature at which the cells are incubated during the fluorescence measurement. The culture was grown at 30° and the data of Figure 1A and B were obtained with cell suspensions equilibrated at 11.8 and 26.2°, respectively. The linear part of the fluorescence increase at 11.8° is shown extrapolated by a dashed line in Figure 1A. The amounts of colicin added in Figures 1A and B were saturating in that they caused maximum killing and a maximum rate of fluorescence change at the respective incubation temperatures. The minimum amount of colicin required to saturate was smaller, and the extent of the maximum cell killing always larger, at higher temperatures of equilibration. The general temperature dependence of the lag and initial rate of colicin response have been previously observed by Wendt (1970) in measurements of the colicin K induced potassium efflux, and thus the fluorescence probe response is again very similar to the biochemical response caused by colicins E1 or K.

The initial linear rate of the increase in fluorescence of the bound probe *N*-phenyl-1-naphthylamine shown in Figure 1 was quantitatively determined as a function of incubation temperature during measurement of the fluorescence change.

TABLE I: Fatty Acid Composition of *E. coli* B/1,5 Grown at Different Temperatures.[a]

Acid	20°	30°	37°	43.8°
14:0	4.3	3.0	3.8	3.4
16:0	30.9	34.5	40.0	41.8
16:1	26.7	26.0	22.4	21.8
Cyclo-16	2.0	4.5	8.6	8.1
18:1	36.1	32.1	24.1	23.3
Cyclo-18	Trace	Trace	1.1	1.6
(16.1 + 18.1 + cyclo-16) / (14:0 + 16:0)	1.84	1.67	1.26	1.18

[a] Half-liter cultures in 2-l. flasks or fourth-liter cultures in 1-l. flasks were grown in M9 medium with 0.1% glucose to a titer of 3–4 × 10^8 cells/ml on rotary shakers in warm rooms set at the different temperatures. The inocula were made from fresh stationary phase cultures and were small enough to allow at least five generations of growth. Data are percentages by weight of the total fatty acids.

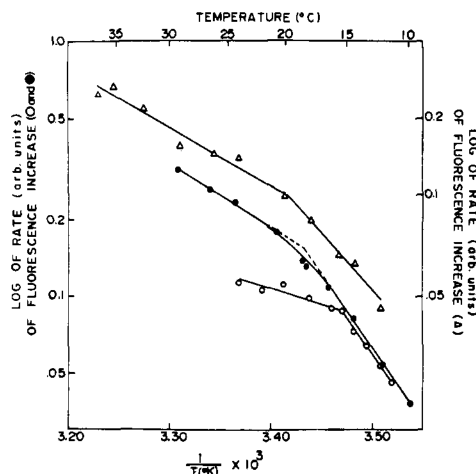

FIGURE 2: Arrhenius plots of the initial rate of the colicin-induced fluorescence increase for cells grown at 20, 30, and 37°. Conditions are as in Figure 1. For the 20° culture (open circles), a saturating amount (1.5 μg/ml) of colicin was added; the survival levels are: 11.1°, 1 × 10^{-4}; 12.7°, 3 × 10^{-4}; 13.2°, 1 × 10^{-5}; 14.2°, 6 × 10^{-5}; 15.5°, 5 × 10^{-5}; 16.0°, 5 × 10^{-5}; 17.9°, 5 × 10^{-5}; 19.9°, 4 × 10^{-5}; 21.8°, 1 × 10^{-5}; 23.9°, <1 × 10^{-6}. For the 30° culture (closed circles), with a saturating amount (0.9–1.25 μg/ml) of colicin added, survival levels are: 9.7°, 2 × 10^{-4}; 11.7°, 1.6 × 10^{-4}; 14.1°, 8.6 × 10^{-5}; 16.2°, 1.75 × 10^{-4}; 18.0°, 4.3 × 10^{-5}; 18.3°, 6.2 × 10^{-5}; 20.6°, 5.0 × 10^{-5}; 24.1°, 3.0 × 10^{-5}; 26.2°, 1.7 × 10^{-5}, 29.0°, 2.7 × 10^{-6}. For the 37° culture (open triangles), with a saturating amount (0.9–1.25 g/ml) of colicin added, survival levels are: 12.0°, 1.1 × 10^{-4}; 14.5°, 2.0 × 10^{-4}; 15.5°, 4.6 × 10^{-5}; 17.7°, 1.5 × 10^{-5}; 20.0°, 1.5 × 10^{-5}; 23.8°, 2.0 × 10^{-5}; 26.0°, 8.5 × 10^{-6}; 29.0°, 1.8 × 10^{-5}; 32.0°, 2.3 × 10^{-6}; 35.0°, 1.2 × 10^{-6}.

Arrhenius plots for the dependence of this rate on incubation temperature are shown in Figure 2 for cultures grown at 20, 30, and 37°. All of the Arrhenius plots are biphasic, with a defined slope above and below a certain temperature or narrow range of temperatures. The absolute slopes of the Arrhenius plots on both sides of the transition temperature were found to be variable, but the ratio of the slope below the transition temperature to that above was generally around 3.0 for cultures grown at 20 and 30°. The ratio of the slope below to that above the transition temperature for the culture grown at 20° (open circles) in Figure 2 is approximately 3.4. The transition temperature in this experiment is 15°. From six different experiments the experimental uncertainty in the transition temperature is estimated to be +1°,−2°. In all cases the transition appears to occur in a narrow temperature range. For cells grown at 30° (closed circles) the data of Figure 2 are suggestive of a more gradual transition between high- and low-temperature regions. The slope ratio in Figure 2 is approximately 2.5 for the cells grown at 30°, and the linear extrapolations intersect at approximately 18°. The transition temperatures found in three experiments range between 17 and 18°. The slope ratio and transition temperature for a culture grown at 37° are, respectively, 1.9 and approximately 20° (open triangles in Figure 4). In one experiment with a culture grown at 43° the slope ratio was found to be 1.3 and the transition temperature approximately 21.5° (data now shown). It should be emphasized that control experiments similar to those shown in Figures 6 and 7 of the preceding paper (Phillips and Cramer, 1973) show that the temperature transition observed in these Arrhenius plots does not affect receptor function and colicin adsorption, as the colicin is essentially completely adsorbed very early in the lag part of the fluorescence response.

Care was taken in these experiments to use cultures which had grown to a titer betwen 3 and 6 × 10^8 cells/ml. It was determined for a culture grown at 30° that the fatty acid composition did not change over this range of titers (data not shown). The principal fatty acids of cultures grown to middle logarithmic level over at least five generations of growth at the different temperatures used in Figure 2 are shown in Table I. The principal changes in fatty acid composition as the growth temperature is increased from 20 to 37° are a 30% increase in the 16:0 component (palmitic acid), a 33% decrease in the 18:1 component, and a significant increase in the cyclo-16 component. The trend in the ratio of the unsaturated and cyclopropane fatty acids to the saturated components is shown with the ratio decreasing by approximately 30% from 20 to 37°. The data obtained under our experimental conditions are in very good agreement with the data of Marr and Ingraham (1962). The only principal difference is that we do not find any abrupt increases in the trend at 43°. The data of Table I and Marr and Ingraham (1962) taken together with the existing knowledge on thermotropic transitions of phospholipids (Chapman and Wallach, 1968) imply that *E. coli* membranes, of which phospholipid is a dominant component, will undergo an order–disorder transition at higher temperatures as the growth temperature is raised. This suggests that a possible explanation for the biphasic Arrhenius plots of Figure 2 is that a region of the cell envelope involved in the transmission mechanism of colicin E1 undergoes a phase change in a temperature interval around the transition temperature of the Arrhenius plots and exists in a relatively fluid or disordered state above this temperature interval.

Discontinuities in the polarization of fluorescence of dyes bound to phospholipid micelles have been used to monitor the order–disorder transition caused by increasing temperature (Vanderkooi and Chance, 1972). An increase in the rates of sugar and amino acid uptake in isolated membrane vesicles above 20° has been correlated with a faster decline in the polarization of dansylphosphatidylethanolamine fluorescence above 20–25° (Shechter *et al.*, 1972). The ability of a fluorescence probe to sense the phase transition depends on the dye being bound in the region of the hydrocarbon fatty acid chains and on the fluorescence lifetime being comparable to

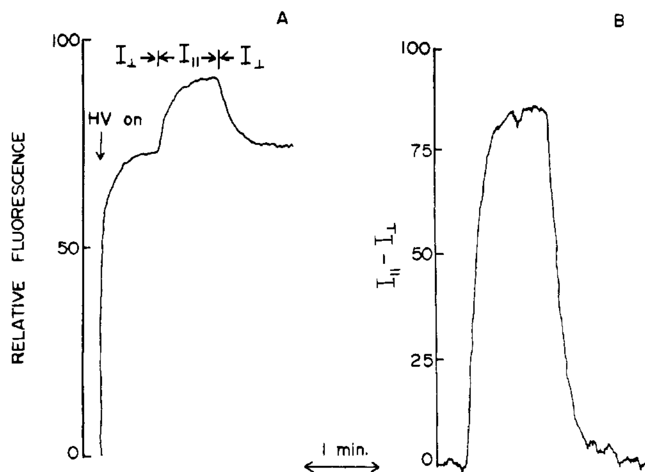

FIGURE 3: Measurement of polarization of fluorescence of cell-bound N-phenyl-1-naphthylamine. Cells were grown at 20° and concentrated. (A) Fluorescence levels with analyzer and polarizer perpendicular ($I_\perp$) and parallel ($I_\parallel$) after the high voltage (HV) is turned on. (B) The difference between $I_\parallel$ and $I_\perp$ of part A measured at a fivefold larger amplification; N-phenyl-1-naphthylamine concentration, 5 μM; temperature 13.3°; amplifier time constant, 10 sec.

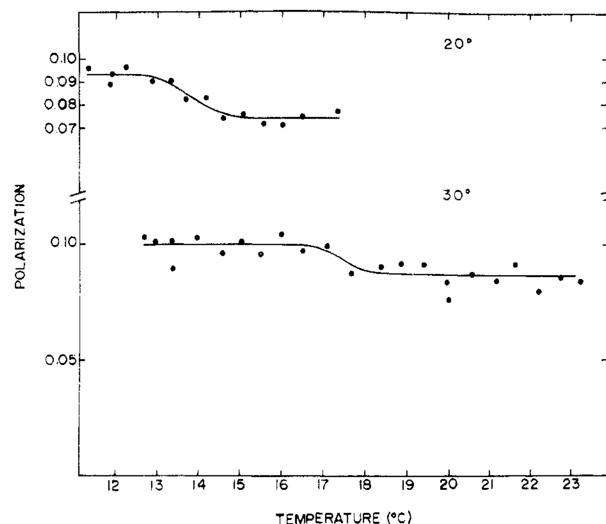

FIGURE 4: Polarization ($I_\parallel - I_\perp$) of N-phenyl-1-naphthylamine fluorescence as a function of temperature. Cells grown at 20° (upper curve) and 30° (lower curve). Conditions as in Figure 3.

the rotational diffusion times in the region of the micelle or membrane to which the dye is bound. A decrease in fluorescence polarization under these circumstances is indicative of increasing motion and disorder in the micelle or membrane. Figure 3 shows the raw data for computation of the fluorescence parameter $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ for cells grown at 20° and incubated at 13.3° during the measurement of fluorescence with the probe N-phenyl-1-naphthylamine. The fluorescence signal increases as the photomultiplier tube high voltage (HV) is turned on at an amplifier time constant of 10 sec, with the analyzer perpendicular ($I_\perp$) to the vertical polarization of the incident light (Figure 3A). When the analyzer is turned so that the E vector of the emitted light is parallel to that of the incident light ($I_\parallel$), there is an increase of approximately 20% in the fluorescence level ($I_\parallel$ in Figure 3A). The difference between $I_\parallel$ and $I_\perp$ is more sensitively measured after $I_\perp$ is set to zero and the signal level amplified by a factor of five (Figure 3B). For the purpose of calculating $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ as a function of temperature, the numerator was obtained from data like that in Figure 3B and the denominator from Figure 3A. The value of the polarization parameter as a function of the incubation temperature during the measurement of fluorescence is shown in cultures grown at 20 and 30° in Figure 4. The absolute values of polarization measured here are probably somewhat low since with the geometry employed the polarization of rhodamine B was 0.37 (Methods) instead of 0.44 (Weber, 1956). There is also probably some depolarization due to scattering by the cell suspension, even with the emission detected from the front face of the cuvet. The values of the polarization are very similar to those observed by Vanderkooi and Chance (1972) using the probes 12-(9-anthroyl)stearic acid and 8-anilino-1-naphthalenesulfonate with artificial membranes and mitochondria. Discontinuities in the polarization with increasing temperature could not be seen with mitochondria. The polarization of fluorescence of 5 μM N-phenyl-1-naphthylamine bound to *E. coli* in the absence of colicin shows a discontinuity as a function of incubation temperature (Figure 4). There is a decrease in polarization with increasing temperature in an interval around 14 and 17.5° for cells grown at 20 and 30°, respectively. The temperature interval in which the polarization changes take place is between 1 and 2°. The difference in the polarization at high and low temperatures appears to be smaller for the culture grown at 30°. The experimental uncertainty in a given point due to noise is approximately ±0.003. Although the change at 30° is small, the existence of a transition at approximately 17.5° seems certain from the fact that all 12 experimental points above this temperature are at lower values than the ten values measured below 17.5°, the exception being one point at 13.5°. There is very little, if any, drift in the level of polarization outside the transition region. The transition temperatures of 14 and 17.5° observed in the polarization measurements of Figure 4 are, within experimental error, the same as the transition temperatures of the Arrhenius plots of Figure 2 for cells grown at 20 and 30°.

Discussion

The rate of change of N-phenyl-1-naphthylamine fluorescence caused by colicin E1 is similar to the rate of the decrease in intracellular ATP and potassium levels. The fluorescence probe response occurs with an unchanged rate in the presence of an inhibitor of the ATP decrease and in the presence of high potassium levels (Phillips and Cramer, 1973). It is assumed in the present work that the fluorescence probe response is closely associated with the transmission of the inhibitory effects of colicin E1 and can be used to monitor the transmission process.

The activation energy for the rate of transmission of the colicin response decreases above a characteristic transition temperature by a factor as large as 3.4 (Figure 2). During the measurement of these rates the colicin is completely adsorbed. The transition temperature increased by approximately 5° as the growth temperature was raised from 20 to 37°. The effect of changing growth temperature on the transition temperature of the Arrhenius plots is very similar to the effect of different fatty acid supplements on the transition temperatures

of Arrhenius plots of the rate of glucoside and galactoside transport by fatty acid auxotrophs of *E. coli* (Wilson *et al.*, 1970; Overath *et al.*, 1970). The activation energies associated with glucoside and galactoside transport decreased markedly above a temperature interval determined by the fatty acid supplement. Addition of relatively unsaturated fatty acids or fatty acids with a relatively low melting temperature caused a downward shift in the transition temperature. The system utilized in our experiments, cultures grown at different temperatures to induce altered fatty acid composition, may not be completely analogous to that of the fatty acid auxotrophs. Different growth temperatures may cause changes in other temperature-dependent envelope components besides the fatty acids. However, the fluorescence polarization experiments (Figures 3 and 4) imply that the transition temperatures of the Arrhenius plots are associated with a disordering or melting of part of the cell envelope. The fatty acid (Table I) and polarization data (Figure 4) imply that the decrease in activation energy for the colicin E1 transmission is associated with a disordering or melting of fatty acid chains in part of the cell envelope. Wendt (1970) has suggested that a similar effect may explain the temperature dependence of the lag period observed for potassium efflux.

The involvement of membrane phospholipid in the transmission mechanism of colicin E1 supports our previous interpretation of the rapid loss of trypsin reversibility, that this represented a decrease in accessibility to trypsin due to penetration of colicin E1 into the cell envelope (Phillips and Cramer, 1973).

If it is assumed that the decrease in activation energy for transmission of colicin E1 effects is due to disordering of the hydrocarbon fatty acid chains and creation of a relatively fluid region in the hydrophobic core of a membrane, it is possible that colicin E1 moves through this region by a diffusive process (Singer and Nicolson, 1972). It should be noted that a relative motion of the colicin in the membrane might occur if the colicin were stationary in the membrane but the phospholipid underwent lateral diffusive motion, although the lateral diffusion constant for phospholipid appears to be approximately 1000 times as great as that for protein movement in the Frye and Edidin (1970) experiment (Scandella *et al.*, 1972). Singer and Nicolson (1972) suggested further that colicin might trigger formation of protein aggregates in the membrane. Considering the recent data on intrinsic or induced degradative enzyme activity associated with colicins E3 and E2 (Boon, 1971, 1972; Bowman *et al.*, 1971; Almendinger and Hager, 1972) it seems more likely that colicin E1 perturbs the cell envelope and associated functions of membrane or envelope through an intrinsic or an induced enzyme activity associated with it as it diffuses through the membrane. It has previously been suggested that phospholipase activity is associated with the action of colicin E1 and K (Cavard *et al.*, 1968), although the maximal rate of the phospholipase action seems much slower than maximal rates of decay of intracellular ATP and potassium levels caused by colicin E1 (Phillips and Cramer, 1973).

Acknowledgment

We wish to thank Miss Lee Loh for her assistance in these experiments.

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