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ACTIVATION OF THE β-GALACTOSIDE TRANSPORT SYSTEM IN *ESCHERICHIA COLI* ML-308 BY *n*-ALKANOLS

MODIFICATION OF LIPID–PROTEIN INTERACTION BY A CHANGE IN BILAYER FLUIDITY

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SUMMARY

The net rate of transport of *o*-nitrophenyl-β-D-galactopyranoside by *Escherichia coli* ML-308 is increased at temperatures below the apparent phase transition of the lipid bilayer in the presence of *n*-alkanols. The degree of activation produced is determined both by the concentration and chain length of the *n*-alkanol used. At low concentrations *n*-alkanols "activate" transport, but do not cause either cell lysis or denaturation of β-galactosidase.

Arrhenius plots of the kinetic constants for transport indicate the K_m shows discontinuity with increasing temperature, while the slope for V changes only gradually. The presence of 10.5 mM *n*-hexanol increases the value of both K_m and V at low temperature. A comparison of these data to those obtained at a single substrate concentration (1.85 mM *o*-nitrophenyl-β-D-galactopyranoside) suggests the biphasic behavior of Arrhenius plots at that concentration may be attributed to changes in the K_m for the transport process.

INTRODUCTION

A wide variety of functions associated with biomembranes are generally attributed to specific protein components present in the lipid bilayer [1]; the β-galactoside "permease" of *Escherichia coli* is one such example [2]. The involvement of the lipid matrix in the transport process has been demonstrated; the evidence indicates the fatty acid composition of membrane lipids regulates the temperature characteristics of the transport of β-galactosides [3–6]. The rate of β-galactoside entry and exit also shows an abrupt decrease upon cooling to 0 °C, as does the non-permease mediated flow of galactosides across the membrane [7]. These observations indicate the biochemical functions of membrane-bound proteins may be modulated by the physical state of the lipids in the bilayer, such that below the transition temperature

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Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TDG, D-galactopyranosyl-β-thiogalactopyranoside.

transport would be limited by diffusion. The function of these proteins would be affected, then, by an increase in the "fluidity" of the bilayer caused by local perturbation or long-range lateral cooperativity [8]. Such an increase in the fluidity of a phospholipid bilayer can be induced through addition of *n*-alkanols [9–11]. The change in the packing order of the lipids may however be transmitted directly to a catalytic or binding site by altering the nature of a lipid–protein interaction, or indirectly by modifying the polar region of the membrane. These possibilities may be more important at temperatures below the transition temperature, for above the thermal phase transition the lipid environment may not be regulatory or rate limiting. Instead, the increased activity observed at high temperatures may be accounted for by a change in the rate-limiting step for the transport process, or by a thermally induced reversible change in protein structure [12–15].

In this communication we report the results of an in vivo examination of the effects of various *n*-alkanols on the net rate of facilitated transport of *o*-nitrophenyl-β-galactopyranoside (ONPG) across the limiting membrane of *E. coli* ML-308. The results show *n*-alkanols increase the rate of uptake of ONPG at low temperatures and modify the biphasic behavior of Arrhenius plots. The extent of the effects observed depends both upon the concentration and chain length of the activating *n*-alkanol. We have also examined the effect of temperature as well as temperature plus *n*-hexanol on the kinetic constants for transport (K_m and V). The data show the thermal phase transition in membrane lipids is reflected in a dramatic change in the apparent K_m rather than V for the transport process.

MATERIALS AND METHODS

The substrate, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), was obtained from Calbiochem. Analytical reagent grade *n*-hexanol was obtained from Cole-Matheson; all other *n*-alkanols were purchased from Fisher Scientific Co. (Certified Reagent Grade). The *n*-alkanols were fractionally redistilled before use (0.5 °C range cut taken).

Cultures of *E. coli* ML-308 ($i^-y^+z^+a^+$) and ML-35 ($i^-y^-z^+a^+$) were grown on medium M9 plus 0.2% succinate at 37 °C with aeration. Cells in the exponential phase of growth were harvested by centrifugation and resuspended in medium M9 plus 50 μg/ml chloramphenicol. Stock solutions of *n*-alkanols were prepared by dilution into medium M9 plus 50 μg/ml chloramphenicol. The rate of hydrolysis of ONPG to *o*-nitrophenol at pH 7.0 in the temperature range 14–36 °C was measured as previously described [7].

In the temperature range 0–13 °C water baths or ice baths were set up in a 2 °C cold room. Centrifuge tubes containing medium M9 plus chloramphenicol, with or without *n*-alkanol, were allowed to equilibrate to the proper temperature. The substrate for the reaction (ONPG) was then added along with any inhibitors (D-galactopyranosyl-β-thiogalactopyranoside (TDG) or formaldehyde). An aliquot of a concentrated suspension of bacteria was then added to each, and the times recorded. At set time intervals the samples were centrifuged in the cold, the supernatant decanted, and the absorbance due to the *o*-nitrophenol anion measured at 420 nm in the Cary spectrophotometer. The initial rates of hydrolysis of ONPG could thus be calculated from the timed samples, the formaldehyde controls subtracted, and the net rate determined.

The kinetic parameters K_m and V were calculated according to the statistical method described by Cleland [16]. The error associated with the calculated values of V was less than 10% in all the cases described. The error associated with the calculated values of K_m are generally larger and are as indicated in Fig. 4a. All values are expressed in terms of the value obtained $\pm$ S.D.

Finally, the V data, as well as those data which describe the rate of transport at a fixed substrate concentration (1.85 mM ONPG), were analysed via a computer program designed by Janice Rushton. The program calculates the best fit straight line, second, third, and fourth order polynomials. In addition, the program also tests all possible pairs of straight lines; one member of each pair being the best fit to a portion of the data, and the other to the remainder of the data. It then discards all but the best fitting pair of straight lines. The program computes the coefficients for the various lines and curves, as well as the intersection of the best fitting two straight lines in each case. It also calculates the variation about the intersection (transition point) within a 95% confidence limit. Of the five possibilities the overall best fit to the data is decided on the basis of F-tests which compare the regression sum of squares minus the residual sum of squares to the residual sum of squares. The significance level used was $\alpha = 0.001$ (or $P < 0.001$). Taking into account the degrees of freedom and the value of F obtained in each case, as well as the tabulated values of F at $\alpha = 0.001$, the more highly significant value of F was determined.

β-Galactosidase assay

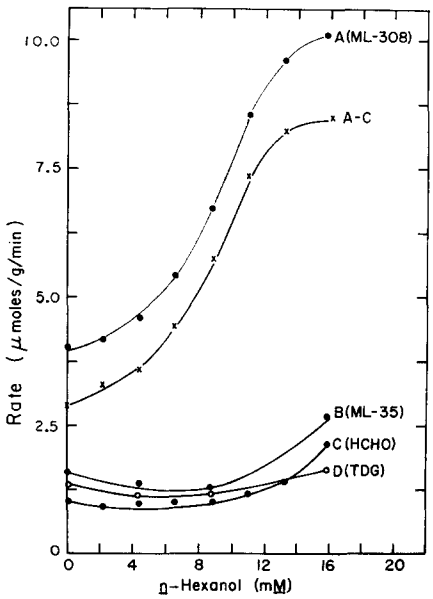
An aliquot of exponentially growing cells (ML-308), with 100 μg/ml chloramphenicol present, was placed in an ice bath. To 0.5-ml samples of the above, 10 μl of a 1 : 1 : 1 : 5 mixture of the following components was added: 10% sodium dodecylsulfate, 0.02 M $MnSO_4$, toluene, 2-mercaptoethanol [17]. The tubes were vigorously mixed and then incubated at 28 °C for 30 min. An aliquot of the lysate (0.1 ml) together with medium M9 (with or without *n*-hexanol) was placed in a cuvette. The substrate for the reaction (ONPG) was then added and the rate determined as described above.

Samples of β-galactosidase were also prepared by sonicating a suspension of ML-308 in the cold. The sonicate was centrifuged, the supernatant decanted, and the enzyme preparation diluted into cold medium M9. The in vitro rates of hydrolysis at 0 °C were determined by the timed samples method; the reactions were terminated by dilution into a 10-fold excess of cold medium M9.

RESULTS

The effect of n-hexanol on the rate of ONPG transport at 0 °C

The rate of steady-state uptake and hydrolysis of ONPG increases with increasing *n*-hexanol concentration as shown in Fig. 1. For *E. coli* ML-308 the in vivo rate of uptake is increased 2.5-fold as the *n*-hexanol concentration is increased from zero to about 16 mM. Controls were simultaneously performed using both the permease negative strain, *E. coli* ML-35, and samples of *E. coli* ML-308 inhibited with respect to the transport of β-galactosides by the presence of formaldehyde or TDG. As illustrated in Fig. 1, very little increase in the rate of ONPG uptake is observed in the cryptic cells or in the samples of ML-308 inhibited by formaldehyde or TDG, indicating the cell membrane remains intact under the conditions employed.



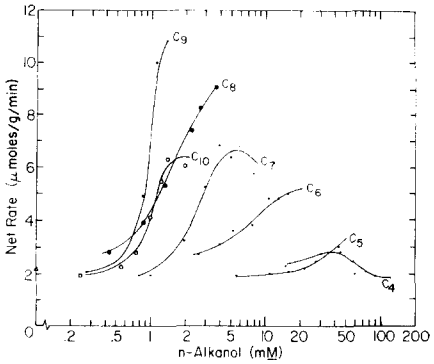


Fig. 1. Activation of the rate of transport of ONPG by *E. coli* ML-308 in the presence of *n*-hexanol at 0 °C. Hexanol dissolved in medium M9 was added in appropriate volumes to the incubation mixture. All tubes contained 1.85 mM ONPG. Formaldehyde (final concentration 4.51 mM) or TDG (final concentration 1.11 mM) were added to control runs where indicated; *E. coli* ML-35 served as an additional control. Each initial rate point was obtained from four samples incubated for varying periods of time. The net rate of uptake of ONPG was obtained by subtracting the rate of hydrolysis of ONPG in those cells treated with formaldehyde from the linear rate of hydrolysis of ONPG by samples of *E. coli* ML-308 in the presence of *n*-hexanol.

Fig. 2. Activation of the net rate of transport of ONPG by *E. coli* ML-308 in the presence of varying concentrations of several *n*-alkanols at 0 °C. C_4, *n*-butanol; C_5, *n*-pentanol; C_6, *n*-hexanol; C_7, *n*-heptanol; C_8, *n*-octanol; C_9, *n*-nonanol; C_{10}, *n*-decanol. Experimental conditions are as described in Materials and Methods. Initial and net rates of transport were determined as described in the legend to Fig. 1.

The effect of various n-alkanols on the rate of ONPG transport at 0 °C

The initial rate of transport of ONPG by *E. coli* ML-308 was studied as a function of the concentration of several *n*-alkanols at 0 °C (Fig. 2). Separate controls containing formaldehyde were performed at alternate concentrations of *n*-alkanol. The following features may be noted. First, the optimal activating concentrations for the alcohols decrease with increasing chain length. Second, the extent of activation increases with the chain length up to C_9 but then decreases for C_{10}. Third, the maximal slopes are different for different alcohols.

The rate of transport at 1.85 mM ONPG illustrated in Fig. 2 is characteristic of the maximal rate, V, at 0 °C, since the K_m in the presence of 10.5 mM *n*-hexanol is 0.3174 mM, and in the presence of 1.25 mM *n*-decanol the K_m is less than 0.6 mM. Control experiments at 0 °C indicate *n*-alkanols have no deleterious effect on β-galactosidase from sonicated cells.

Agents which have their site of action at a membrane or which pass through a membrane to reach their target site usually show a linear correlation on a log-log

plot between their *n*-octanol/water partition coefficients (P_{oct}) and their concentrations eliciting a particular response [18]. The *n*-alkanols used in this study follow such a relationship, with a slope suggesting the environment in which the alcohol is localized for its activating effect has the same hydrophobic properties as the bilayer region of biomembranes.

Effect of temperature and n-hexanol on the rate of transport at 1.85 mM ONPG

Arrhenius plots of the logarithm of the net rate of transport at 1.85 mM ONPG versus $1000/T$ for cells of *E. coli* ML-308, both with and without 10.5 mM *n*-hexanol, are presented in Fig. 3. The best fitting pair of two straight regression lines in each case extrapolates to an intersection at a unique transition temperature. The point of intersection for the slopes describing the untreated cells corresponds to a transition temperature of 21.4 °C; while the addition of 10.5 mM *n*-hexanol to samples of ML-308 shifts the transition temperature to 11.8 °C. For both the treated and untreated cells a data fit of two straight lines was highly significant ($P < 0.001$). At a 95% confidence limit the value of the transition temperature is known to within 4.8 °C in each case.

The calculated energies of activation are collected in Table I. The observed E_{act} below and above the transition temperature in each case is essentially independent of *n*-hexanol (+30.82 kcal/mole as compared to +33.79 kcal/mole, below; and +20.39 kcal/mole as compared to +20.36 kcal/mole, above); while the values of the apparent

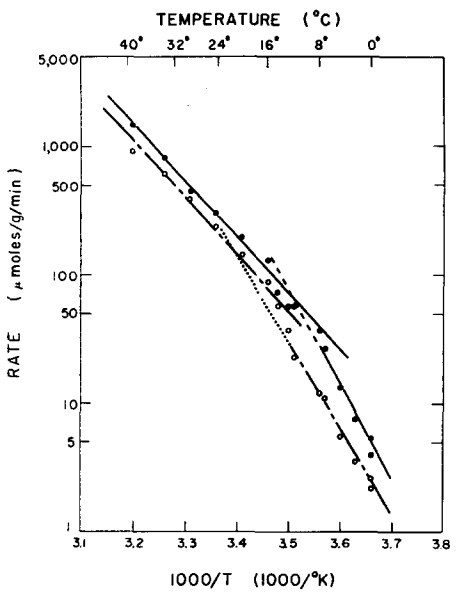


Fig. 3. Arrhenius plots for the net rate of transport of 1.85 mM ONPG by *E. coli* ML-308 in the absence and presence of 10.5 mM *n*-hexanol. The experimental conditions are described in the Materials and Methods. The symbols used are as follows: for the Arrhenius plot of the net rate of transport of 1.85 mM ONPG by *E. coli* ML-308 in the absence of 10.5 mM *n*-hexanol (○--○), and in the presence of 10.5 mM *n*-hexanol (●–●); the extrapolation of a line beyond the data points described by that line is indicated by either - - - or · · ·.

TABLE I

ENERGIES OF ACTIVATION FOR THE RATE OF TRANSPORT OF ONPG IN *E. COLI* ML-308

Description	Temperature range (°C)	Slope**	E_{act} (kcal/mole)
Rate of transport of 1.85 mM ONPG	0 –21.4	−6.7285	+30.82
	21.4–39.1	−4.4518	+20.39
Rate of transport of 1.85 mM ONPG + 10.5 mM *n*-hexanol	0 –11.8	−7.3779	+33.79
	11.8–39.1	−4.4463	+20.36
Maximal rate of transport of ONPG (*V*)*	0 – 8	−8.3278	+38.14
	14 –23	−5.7892	+26.52
	28 –35	−3.1584	+14.46
Maximal rate of transport of ONPG + 10.5 mM *n*-hexanol (*V* + *n*-hexanol)*	0 – 8	−7.8611	+36.00
	12 –20	−5.3887	+24.68
	28 –35	−2.550	+11.70

* The values represented are those obtained by drawing tangents to the curve within the given temperature ranges. The values are approximate, and are presented here only to indicate the general trend in the energies of activation for the *V* data obtained from Fig. 4b.

** The slope is calculated as the $\Delta \log_{10}$ rate versus $\Delta 1000/T$.

transition temperatures are themselves significantly different (21.4 as compared to 11.8 °C). It would appear that the presence of 10.5 mM *n*-hexanol shifts the apparent transition temperature of the lipid bilayer without radically changing the E_{act} above or below the transition point. A similar decrease in the phase transition temperature for dipalmitoyllecithin, due to the presence of *n*-hexanol, has been observed using the method of differential scanning calorimetry (Wray and Jain, unpublished).

Effect of n-hexanol on the kinetic parameters of β-galactosidase and β-galactoside permease activities

The rate of transport in intact *E. coli* ML-308, as well as the β-galactosidase activity in lysed cells, were measured at several concentrations (8–10) of ONPG. Formaldehyde controls of the rate of transport in intact cells were run simultaneously. The values of the apparent affinity constants (K_m values) and maximal rates (V values) are collected in Table II. The results clearly show that at 0 °C the K_m for the transport process in intact cells increases significantly in the presence of 10.5 mM *n*-hexanol, from 0.2241 to 0.3174 mM. The K_m at 28 °C is only slightly decreased by the addition of *n*-hexanol. The V for transport at 0 °C in intact cells is also altered substantially from 4.06 μmoles/g per min to 7.67 μmoles/g per min in the presence of an activating concentration of *n*-hexanol (10.5 mM). However, only a slight change is observed at the higher temperature.

In contrast to these results, the addition of 10.5 mM *n*-hexanol to the reaction mixture does not affect either K_m or V for β-galactosidase activity in lysed cells.

Effect of temperature and n-hexanol on the K_m, V and k_{app} for the transport process

Semilogarithmic plots of K_m and V versus the reciprocal of the absolute

TABLE II

KINETIC CONSTANTS FOR β-GALACTOSIDASE AND ONPG TRANSPORT ACTIVITIES IN *E. COLI* ML-308

The values for the transport activity in vivo were determined by assaying the same batch culture of *E. coli* ML-308 at 8 different ONPG concentrations in the presence and absence of 10.5 mM *n*-hexanol, at 0 and 28 °C. The β-galactosidase activity was determined with samples obtained by lysis from the same batch culture of *E. coli* ML-308 at 10 different ONPG concentrations, in the absence and presence of 10.5 mM *n*-hexanol at 28 °C. The collected values of K_m and V are expressed as the values ±S.D.

System	Temperature (°C)	K_m (mM)	V (μmoles/g per min)
		Transport activity	
E. coli	0	0.2241 ±0.0208	4.0574± 0.0868
	28	1.4117 ±0.1289	724.355 ± 26.184
E. coli+10.5 mM	0	0.3174 ±0.0400	7.6664± 0.2416
n-hexanol	28	1.1916 ±0.0897	777.945 ± 21.790
		β-Galactosidase activity	
Lysed *E. coli*	28	0.18655±0.0141	9732 ±150
Lysed *E. coli*+10.5 mM *n*-hexanol	28	0.2102 ±0.0174	9734 ±168

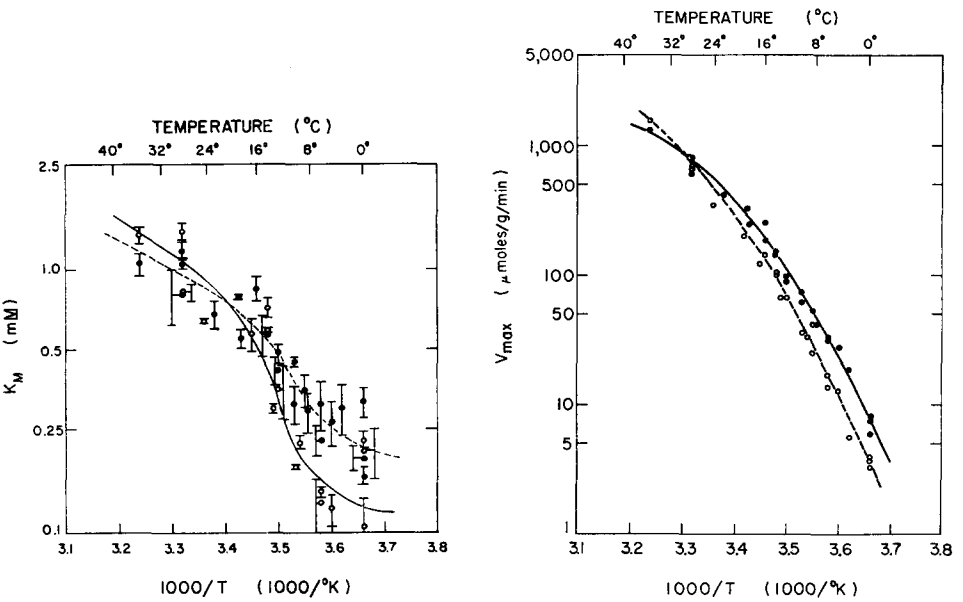


Fig. 4. a. Van 't Hoff plots for the K_m of the transport process in *E. coli* ML-308 in the absence and presence of 10.5 mM *n*-hexanol. The experimental conditions and the method of calculation are described in Materials and Methods. The symbols used are as follows: for the Van 't Hoff plot of the K_m of the transport process in *E. coli* ML-308 in the absence of 10.5 mM *n*-hexanol (○–○), and in the presence of 10.5 mM *n*-hexanol (●- - -●). b. Arrhenius plots of the maximal net rates (V_{max}) for the transport of ONPG by *E. coli* ML-308 in the absence and presence of 10.5 mM *n*-hexanol. The experimental conditions and method of calculation are as described in Materials and Methods. The symbols used are as follows: for the Arrhenius plot of V in the absence of *n*-hexanol (○- - ○), and in the presence of 10.5 mM *n*-hexanol (●–●).

temperature, both with and without *n*-hexanol, are shown in Figs 4a and b. The temperature profiles for K_m (Fig. 4a) in the absence and presence of 10.5 mM *n*-hexanol show a nearly discontinuous increase in the value of K_m with increasing temperature in the range 0–28 °C. In contrast to these observations, the temperature profiles for V, with and without 10.5 mM *n*-hexanol, show no sharp transitions (Fig. 4b). Rather, the best fit for each set of data presented in Fig. 4b is a second order polynomial. These curvilinear relationships indicate a gradual change in the energy of activation of the V process over the entire temperature range studied. The values of the energies of activation for the V data, obtained by drawing tangents to the curves, are collected in Table I. The apparent values of the E_{act} for V in the presence of *n*-hexanol being somewhat lower than for V measured in the absence of *n*-hexanol.

Since V and K_m for any kinetic mechanism are complicated functions of a number of rate constants for individual steps, a series of experiments were conducted to study the effect of temperature, in the absence and presence of *n*-hexanol, on another kinetic parameter, the apparent pseudo-first order rate constant k_{app}. Mathematically, k_{app} is the quotient of V and K_m. Experimentally, it is the quotient of the rate of hydrolysis at very low substrate concentration divided by that concentration. The results for a number of experimental runs conducted with different batch cultures of bacteria are collected in Table III. The value of k_{app} increases with increasing temperature, being some 11-fold greater at 28 than at 0 °C in the absence of *n*-hexanol, and some 17-fold greater at 28 than at 0 °C in the presence of 10.5 mM

TABLE III

THE EFFECT OF 10.5 mM *n*-HEXANOL ON THE APPARENT PSEUDO-FIRST ORDER RATE CONSTANT k_{app}

$k_{app} = v/[S]$, where [S] = 92.5 μmoles/l ONPG. The mean value of k_{app} under a particular set of conditions is expressed as the mean ±S.E.

k_{app} (ml/g per min)			k_{app} with 10.5 mM *n*-hexanol (ml/g per min)		Ratio of the mean value of k_{app} – *n*-hexanol to the mean k_{app} without *n*-hexanol (ratio ±S.E. of the ratio)
Temperature (°C)	Values	Mean ±S.E.	Values	Mean ±S.E.	
0	16.77 15.16 15.97	15.97± 0.46 (22.74± 0.66)★	28.45 24.52 27.56	26.84± 1.19 (34.88± 1.55)★	1.68±0.089 (1.53±0.081)★
7.9	59.50 87.38 78.46 55.51 49.53	66.06± 7.21 (102.46±11.16)★	84.54 83.11 98.02 93.08 88.55	89.46± 2.76 (117.32± 3.61)★	1.35±0.153 (1.14±0.129)★
28	269.91 201.95 239.66	237.17±19.66 (253.12±20.98)★	509.34 519.80 639.71	556.28±41.82 (600.61±45.15)★	2.34±0.262 (2.37±0.265)★

★ These values are obtained by extraprolating to zero substrate concentration.

n-hexanol. Also, the addition of 10.5 mM *n*-hexanol significantly increases the value of k_{app}. These data suggest the association of substrate to the β-galactoside permease system may take place in a lipid environment.

DISCUSSION

The results show *n*-alkanols activate the rate of permease-mediated transport of ONPG below the apparent phase transition temperature, in the concentration range tested, without affecting the passive uptake of ONPG, and therefore the integrity of the membrane, to any significant extent (Figs 1 and 2). The stimulatory effect of various *n*-alkanols is correlated with their chain length (Fig. 2). As the chain length of the alcohol is increased beyond C_9 the activating effect of the alcohol decreases; perhaps because the longer chain *n*-alkanols are more like the normal membrane lipids, thus causing little or no perturbation of the bilayer. The decline in the activation profiles observed at high concentrations of C_4, C_7, and C_{10} may be attributed to the presence of greater than optimal concentrations of *n*-alkanol (Fig. 2). The increase in V for ONPG transport at 0 °C caused by 10.5 mM *n*-hexanol (Table II), may be the consequence of a change in membrane fluidity and lipid organization due to the presence of alcohol [8–11, 19].

The discontinuity in the Arrhenius plot for the rate of transport at 1.85 mM ONPG may be attributed to a sharp temperature-dependent change in K_m, which causes the substrate concentration to become less saturating at temperatures above the transition point. The Arrhenius plot for V does not exhibit any significant discontinuity, rather the slope, or energy of activation, for the V process changes gradually. These results suggest that a change in temperature induces a radical change in the apparent affinity of the permease for its substrate, rather than significantly affecting the rate at which the permease transports substrate across the membrane.

The apparent phase transition temperature measured at 1.85 mM ONPG is decreased in the presence of 10.5 mM *n*-hexanol. It also varies with the degree of unsaturation of the fatty acid supplement, being highest for saturated fatty acids and lowest for unsaturated fatty acids [4, 6, 20]. The sharp transitions in the Arrhenius plots for transport at 2.0 mM ONPG in fatty acid auxotrophs of *E. coli* have been correlated with the phase transitions observed both in isolated phospholipid monolayers at an air/water interface and in spin-labeled *E. coli* inner membranes [5, 21], but are not consistent with the broad transitions witnessed in X-ray diffraction studies [22]. These sharp transitions, as opposed to the gradual transition in the Arrhenius plot for V (Fig. 4b), may also be due to sharp temperature-dependent changes in K_m. However, a temperature profile of K_m in fatty acid auxotrophs of *E. coli* is not yet available. The broad transitions observed in X-ray diffraction studies, as opposed to the sharp transitions in spin-labeled *E. coli* inner membranes, may reflect the regions of the bilayer which each method explores.

The interpretation of the data may be further complicated if the lipid composition of the membrane is not uniform, particularly if lipids in the vicinity of transport components are different than those of the bulk lipids. In this case transitions in the temperature profiles for the rate of transport might not correspond to changes in the bulk lipids alone, but rather to a composite change characteristic of both the bulk

lipids and those in the vicinity of transport components. The thermal phase transition might, then, occur in stages. The gradual change in V might be the consequence of a cooperative melt of the lipid bilayer as different regions or "patches" become more "fluid" with increasing temperature. In addition if the lipid–protein interactions were strong, the conformation of the permease might be radically altered by a change in the "fluidity" of the lipids surrounding it. The sharp transition in the K_m for the transport process might be the result of such a conformational change (one which affects the active or binding site of the permease). The two phase transitions detected by spin-labeling studies of *E. coli* membrane vesicles are consistent with such an hypothesis [23].

The data for k_{app}, or the experimentally measured rate of binding at low substrate concentration, show this rate constant is increased by the addition of 10.5 mM *n*-hexanol and by an increase in temperature. Over the range 0–28 °C, k_{app} changes some 15-fold, while the presence of *n*-hexanol causes the rate of binding to increase yet another 1.35–2.34-fold. These data indicate binding takes place in a lipid environment which is affected by the presence of *n*-hexanol.

The evidence presented in this paper suggests the sharp discontinuities observed in Arrhenius plots for the rate of transport at 1.85 mM ONPG may be the consequence of changes in the K_m for the transport process. The data further show that the slope of the Arrhenius plot for V changes gradually, and that V is increased at low temperatures in the presence of 10.5 mM *n*-hexanol. The activating effect of low concentrations of *n*-alkanols evidenced at low temperature may be the consequence of the increased fluidity of the bilayer which these agents induce. In general, transport is not limited by carrier mobility alone, but may instead be regulated by a complex series of interactions.

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