The Effect of Alterations in the Fluidity and Phase State of the Membrane Lipids on the Passive Permeation and Facilitated Diffusion of Glycerol in *Escherichia coli*

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(Received 15 August 1980; revised 12 November 1980)

The passive permeation and facilitated diffusion of glycerol into *Escherichia coli* K1060, an unsaturated fatty acid auxotroph, were studied as a function of temperature and membrane lipid fatty acid composition using a stopped-flow spectrophotometric assay of glycerol permeation. The relative rates of glycerol passive and mediated entry were both significantly influenced by the fluidity of the membrane lipids, increasing as the gel to liquid-crystalline phase transition midpoint temperature of the membrane lipids decreased. The rate of passive glycerol permeation, but not the rate of glycerol facilitated diffusion, decreased as the membrane lipids were converted to the gel state. The apparent activation energies for passive and facilitated diffusion of glycerol, measured in cells whose membrane lipids were in the liquid-crystalline state, were 15–16 and 10–11 kcal mol⁻¹, respectively, and neither value was significantly influenced by the fatty acid composition or fluidity of the membrane lipids. The mechanistic implications of these observations for the function of the glycerol facilitated diffusion system of *E. coli* are discussed.

INTRODUCTION

The relative rates at which glycerol and other polar non-electrolytes passively diffuse into the simple, cell wall-less prokaryote *Acholeplasma laidlawii* B have been shown to be markedly dependent on membrane lipid fatty acid and sterol composition (McElhaney *et al.*, 1970, 1973; Romijn *et al.*, 1972; de Kruijff *et al.*, 1973). Lipid compositional alterations which increased membrane lipid fluidity also increased permeation rates proportionally. In contrast, activation energies of permeation were not dependent on membrane lipid composition, reflecting instead the dehydration enthalpies of the permeant molecules. Similar results were previously reported for the permeation of glycerol and other non-electrolytes into liposomes prepared from a variety of synthetic and natural phospholipids (Demel *et al.*, 1968; de Gier *et al.*, 1968, 1971; McElhaney *et al.*, 1973). We concluded that glycerol dehydration and insertion into the hydrophobic core of the lipid bilayer was the rate-limiting and enthalpy-determining step in the overall permeation process, but that the rate of dehydration–insertion was increased by entropic factors in more disordered lipid membrane systems.

When *Escherichia coli* is grown in the presence of glucose, a catabolite repressor of the L-α-glycerophosphate regulon, glycerol enters cells primarily by passive diffusion; however, when *E. coli* is grown in the presence of glycerol or L-α-glycerophosphate as the sole carbon and energy source, a facilitated diffusion system for glycerol entry is induced (Sanno *et al.*, 1968; Richey & Lin, 1972; for review, see Lin, 1976). The objective of this study was to determine if the glycerol facilitated diffusion system of *E. coli* would exhibit a dependence on

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0022-1287/81/0000-9502 $02.00 © 1981 SGM
the fluidity and phase state of the membrane lipids similar to that which the passive diffusion of glycerol into this organism would be expected to exhibit, based on the previous studies of passive glycerol permeation reviewed above. To this end, the relative rates of glycerol entry by passive and facilitated diffusion into *E. coli* K1060, an unsaturated fatty acid auxotroph, were measured as a function of temperature and of membrane lipid fatty acid composition, in cells grown in the presence or in the absence of glycerol. The phase state of the membrane lipids at various temperatures was determined by differential thermal analysis. The effect of alterations in the fluidity and phase state of the membrane lipids on the entry of glycerol into *E. coli* by passive and facilitated diffusion was thus determined.

**METHODS**

Bacterial strain and growth conditions. *Escherichia coli* K1060 is an unsaturated fatty acid auxotroph unable to synthesize or degrade unsaturated fatty acids; it was originally constructed by Schairer & Overath (1969) and was the generous gift of David F. Silbert. The organism was grown in medium M63 (Miller, 1972) in the presence of an unsaturated fatty acid [75 μg ml⁻¹; solubilized with 0-12% (w/v) polyoxyethylene (20) cetyl ether (Brij 58)], thiamin (1 μg ml⁻¹), 0-3% (w/v) Casamino acids (Difco) and either 0-4% (w/v) glycerol or 0-23% (w/v) xylose or 0-54% (w/v) glucose as carbon and energy source. Organisms were first grown overnight in a small volume of complete growth medium with rapid shaking (approx. 170 rev. min⁻¹) at 37 °C (39 °C when elaidic acid-supplemented medium was used) in a New Brunswick Scientific Co. gyrotary shaker bath. The overnight culture was diluted 100-fold with fresh medium and shaking was continued until the mid-exponential growth phase was reached; the organisms were then harvested by centrifugation at 4 °C and 6000 g for 10 min in a Sorvall RC2-B centrifuge.

Determination of relative rates of glycerol permeation. The relative rates of glycerol permeation in *E. coli* grown on glycerol, glucose or xylose were determined by the stopped-flow spectrophotometric technique of Alemohammad & Knowles (1974) as modified by Eze & McElhaney (1978). This technique involves placing cells in a hypertonic glycerol solution and following the rate of osmotically driven changes in cell volume with time. Initially, water moves very rapidly out of the cell due to a high osmotic gradient, the plasma membrane pulls away from the cell wall, and the cell volume is reduced (plasmolysis). However, as glycerol more slowly enters the cell moving down its concentration gradient, the osmolarity of the cell cytoplasm increases and water re-enters, causing the cell volume to increase to its original value. The rate of return to the original volume, which is proportional to the change of reciprocal absorbance at 550 nm, is a measure of the relative rate of glycerol entry. In the present experiments a glycerol concentration of 400 mM was used. The preparation of *E. coli* for the swelling rate assays, the assays themselves, the calculation of reciprocal relaxation times (1/τ (s⁻¹)), which are equivalent to the first-order rate constants (k (s⁻¹)), for glycerol entry, and the equipment utilized in these measurements have been described in detail previously (Eze & McElhaney, 1978).

Lipid extraction, purification and analysis. *Escherichia coli* inner membrane lipids were extracted by the method of Bligh & Dyer (1959) as modified by Saito & McElhaney (1977). The extracted lipid was purified by silicic acid column chromatography and membrane lipid fatty acyl groups were converted to the corresponding methyl esters and analysed by gas-liquid chromatography as described previously by Saito & McElhaney (1977).

Differential thermal analysis. The thermotropic phase behaviour of the extracted and purified total lipid fraction from either plasma membranes or whole cells was monitored on a DuPont 900 Thermal Analyzer as described previously (McElhaney *et al.*, 1973), except that the membrane lipids were dispersed in ethylene glycol/water (1:1, v/v) to avoid freezing and thawing of the aqueous phase at 0 °C during cooling and heating scans.

**RESULTS AND DISCUSSION**

Unsaturated fatty acid incorporation by *E. coli* K1060

Much less exogenous unsaturated fatty acid was incorporated by cells grown on glucose than by those grown on glycerol as the sole carbon and energy source (data not presented). Thus, a comparison of the relative rates of glycerol entry into glucose- and glycerol-grown cells was not feasible, since their fatty acid compositions (and hence probably the rates of passive glycerol entry) would not be comparable. Therefore, a number of other carbon and energy sources were examined in order to find one which would provide a fatty acid composition comparable to that of glycerol-grown cells yet would also act as a catabolite repressor of the *glp* regulon. Xylose proved to be a suitable carbon and energy source, since:
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Table 1. Fatty acid compositions of the total membrane lipids of E. coli K1060 grown in the presence of glycerol plus various exogenous unsaturated fatty acids

*Escherichia coli K1060 was grown in M63 medium containing 0.4% (w/v) glycerol and 75 μg ml⁻¹ of the unsaturated fatty acid indicated solubilized in 0.12% (w/v) Brij 58. The cultures were harvested in the mid-exponential growth phase and the fatty acid compositions of the total membrane lipids were analysed as described in Methods.

<table>
<thead>
<tr>
<th>Exogenous unsaturated fatty acid</th>
<th>Unsaturated fatty acids</th>
<th>Cyclopropane fatty acids</th>
<th>Saturated fatty acids</th>
<th>Total unsaturated and cyclopropane fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elaidic (18:1&lt;sub&gt;t&lt;/sub&gt;)</td>
<td>16:1 18:1 18:2</td>
<td>17:0&lt;sub&gt;c&lt;/sub&gt; 19:0&lt;sub&gt;c&lt;/sub&gt;</td>
<td>12:0 14:0 16:0 18:0</td>
<td>68.7</td>
</tr>
<tr>
<td>Oleic (18:1&lt;sub&gt;c&lt;/sub&gt;)</td>
<td>0.1 68.6</td>
<td>—</td>
<td>1.9 19.7 7.8 1.9</td>
<td>61.3</td>
</tr>
<tr>
<td>Palmitoleic (16:1&lt;sub&gt;c&lt;/sub&gt;)</td>
<td>43.7 0.2</td>
<td>9.5 —</td>
<td>0.4 10.9 36.7 0.3</td>
<td>53.4</td>
</tr>
<tr>
<td>Linoleic (18:2&lt;sub&gt;c,c&lt;/sub&gt;)</td>
<td>1.1 1.3 48.4 1.0</td>
<td>—</td>
<td>0.3</td>
<td>51.8</td>
</tr>
</tbody>
</table>

* Fatty acids are designated by the number of carbon atoms present followed by the number of double bonds; the subscripts c and t refer to the cis- and trans-configurations, respectively, of the double bonds and the subscript cp denotes the presence of a cyclopropane ring system.

(i) the membrane lipid fatty acid compositions of cells grown on xylose and glycerol were comparable; (ii) aerobic glycerol-3-phosphate dehydrogenase activity was absent from the membranes of both glucose- and xylose-grown cells, but was present in the membranes of glycerol-grown cells (Eze & McElhaney, 1978), suggesting that the gil regulon was repressed by growth on xylose; and (iii) the addition of 0.05 μM-CuCl₂, a potent inhibitor of the glycerol facilitated diffusion system in erythrocytes (Stein, 1958), to cells grown on glucose or xylose did not inhibit glycerol entry into these cells, but did significantly reduce the rate of glycerol entry into glycerol-grown cells, again indicating that the glycerol transport system was absent from xylose-grown cells.

The fatty acid compositions of the total membrane lipids from E. coli K1060 grown in the presence of glycerol, as sole carbon and energy source, plus various single exogenous unsaturated fatty acids, are shown in Table 1. Of the unsaturated fatty acids tested, elaidic acid was incorporated to the greatest extent, representing over two-thirds of the total membrane lipid fatty acids. This trans-monounsaturated octadecenoic acid was not converted to the corresponding cyclopropane acid by the cells, in contrast to the two cis-monounsaturated fatty acids tested. Oleic and palmitoleic acids, plus their corresponding cyclopropane fatty acid derivatives, were directly incorporated to a lesser extent than was elaidate, although exogenous oleic and palmitoleic acid (and their cyclopropane derivatives) still made up well over half of the total membrane lipid fatty acids. Exogenous linoleic acid was incorporated to a slightly lesser extent than the other exogenous unsaturated fatty acids and was not converted to the corresponding cyclopropane acid. However, when the small amounts of the cis-monounsaturated and cyclopropane acids present in cells grown with linoleic acid were included, the total ‘fluidizing’ fatty acids in the membrane lipids still exceeded half of the total fatty acyl groups present. Although phospholipids containing cis-cyclopropane fatty acyl chains to undergo a gel to liquid-crystalline phase transition at somewhat higher temperatures than do phospholipids containing the corresponding cis-monounsaturated chains, these cyclopropane fatty acids more closely resemble unsaturated than saturated fatty acids in their thermotropic phase behaviour (Silvius & McElhaney, 1979). Therefore, the cyclopropane fatty acids are included, along with the unsaturated fatty acids, as ‘fluidizing’ fatty acids in the final column of Table 1.

The spectrum of endogenous saturated fatty acids present in the E. coli K1060 membrane lipids changed in response to the incorporation of the various exogenous unsaturated fatty
acids. Cells grown with elaidic acid exhibited a 14:0/16:0 ratio of more than 2.5, oleic acid-grown cells exhibited a 14:0/16:0 ratio of less than 0.5 and the 14:0/16:0 ratio in palmitoleic and linoleic acid-grown cells was about 0.2. A similar variation in the average chain length of the endogenous saturated fatty acids in response to the incorporation of exogenous fatty acids of differing physical properties has been reported for A. laidlawii B (Saito et al., 1977; Silvius et al., 1977). Such a response to exogenous fatty acid incorporation appears to be an example of metabolic compensation in order to maintain the fluidity of the membrane lipids as nearly constant as possible. The apparent inverse relationship between the melting temperature of the exogenous unsaturated fatty acids and the average chain length of the endogenous saturated fatty acids would provide a mechanism to antagonize the effect of the incorporation of the exogenous fatty acid on the overall physical properties of the membrane lipids, since the melting behaviour of the endogenous saturated fatty acids are markedly chain-length dependent. However, as will be seen below, this compensatory mechanism was only partially effective, and marked variations in the fluidity and phase state of the E. coli K1060 membrane lipids were induced by enrichment with exogenous fatty acids of differing physical properties, as has previously been reported for other E. coli unsaturated fatty acid auxotrophs (for review, see Cronan & Gelmann, 1975).

**Differential thermal analysis of membrane lipids**

Due to the substantial amounts of water associated with our plasma membrane preparations from E. coli K1060 and because the membranes themselves were predominantly protein, we were unable to obtain good differential thermal analytical curves with our relatively low-sensitivity instrument. Instead, the total lipids from plasma membranes were extracted and purified and then dispersed in a minimal amount of ethylene glycol/water (1:1, v/v). The phase transitions observed in isolated E. coli membrane lipids are essentially identical to the lipid transitions observed in the membranes from which the lipids were derived (Overath & Trauble, 1973; Sackmann et al., 1973; Trauble & Overath, 1973; Baldassare et al., 1976), and ethylene glycol does not significantly perturb the phase behaviour of E. coli membranes or isolated lipids relative to pure water dispersions (Baldassare et al., 1976); thus, we believe that the differential thermal analysis results reported below accurately reflect the phase behaviour of the membrane lipids in situ.

Temperature-based differential thermal analysis thermograms of ethylene glycol/water dispersions of the total membrane lipids from E. coli K1060 grown in the presence of various unsaturated fatty acids are presented in Fig. 1. The phase transition lower boundary ($T_s$), upper boundary ($T_l$) and midpoint temperature ($T_m$) are indicated. All membrane lipid dispersions exhibited thermotropic gel to liquid-crystalline phase transitions corresponding to the cooperative hydrocarbon chain melting of the phospholipid molecules arranged in bilayer form. However, the degree of cooperativity and the position of the phase transition varied considerably depending on the nature and degree of incorporation of the exogenous unsaturated fatty acid. The cooperativity of the transition, as measured by the temperature range over which the phase transition occurred, decreased in the order $18:1_r > 18:1_s > 16:1_c > 18:2_{c,c}$, which was also the order of decreasing phase transition midpoint temperatures. Membrane lipids enriched in $18:2_{c,c}$ exhibited a quite broad endotherm extending over a range of about 38 °C and showing two peaks, one centred near −7 °C and another near +17 °C. The existence of two overlapping peaks, which was not noted in the other lipid dispersions, implies a partial immiscibility of the membrane lipids enriched in $18:2_{c,c}$ in the gel state. The order of decreasing cooperativity noted above also corresponds to the order of decreasing enrichment of the unsaturated fatty acid supplement (and its cyclopropane derivative, if present) in the membrane lipid (see Table 1). Thus, one reason for the observed increase in the broadness of the phase transition may relate to the decreasing
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Fig. 1. Temperature-based thermograms of isolated lipids from membranes of *E. coli* K1060 grown in the presence of glycerol plus various unsaturated fatty acids. The temperature differentials between the lipid dispersions in ethylene glycol/water (1:1, v/v) and the inert reference material are plotted as a function of the temperature of the reference, using a heating rate of 5 °C min⁻¹. Cooling thermograms were identical except that they were exothermic and shifted 2 to 3 °C toward lower temperatures. The lower and upper boundaries of the lipid phase transitions are indicated by *Tₜₚ* and *Tₜₘ*, respectively, and the temperature at which the transition is half complete, as measured by the area under the endothermic peaks, is designated *Tₚₘ*.

homogeneity of the membrane lipid fatty acyl groups as one proceeds from the 18:1<sub>c</sub> to the 18:2<sub>c,c</sub>-enriched preparation. Another factor which may contribute to differences in the cooperativity relates to the mixing properties of the fatty acyl moieties of the predominant phospholipid species present. Thus, the 1-16:0, 2-18:1, and 1-18:1<sub>c</sub>, 2-18:1, molecular species present in 18:1<sub>c</sub>-enriched lipid dispersions, because of their relatively more similar physical properties, would be expected to exhibit a more nearly ideal phase mixing behaviour than would the 1-16:0, 2-14:0 and 1-16:0, 2-18:2<sub>c,c</sub> species present in 18:2<sub>c,c</sub>-enriched membranes. The variation in the phase transition midpoint temperatures with unsaturated fatty acid supplementation is exactly that expected from the known dependence of the gel to liquid-crystalline phase transition temperatures of synthetic phospholipid bilayer and biological membranes on the number and geometrical configurations of double bonds present and on the relative lengths of the hydrocarbon chains (for reviews, see Cronan & Gelmann, 1975; Melchior & Steim, 1976). Since, in general, membrane lipid fluidity, measured at some given temperature above the phase transition range, is inversely proportional to the gel to liquid-crystalline lipid phase transition temperature (for review, see Shinitzky & Barenholz, 1978), one would expect that the fluidity of the various *E. coli* membranes at, say, 40 °C would increase in the order 18:1<sub>c</sub> < 18:1<sub>c</sub> < 16:1<sub>c</sub> < 18:2<sub>c,c</sub>. However, differential thermal analysis is a thermodynamic technique for measuring phase state changes and does not provide a direct measure of membrane lipid 'fluidity'.
Fig. 2. (a) Temperature dependence of the rate of passive glycerol permeation into E. coli K1060 grown with xylose plus various unsaturated fatty acids. The rates of passive glycerol entry were measured as the reciprocal relaxation times (1/τ) of cell swelling in hypertonic glycerol. Unsaturated fatty acids: ●, linoleic acid (18:2ω6); ■, palmitoleic acid (16:1ω7); ○, oleic acid (18:1ω9); □, elaidic acid (18:1ω9).
(b) Arrhenius plots of the data in (a).

Effect of fatty acid composition and temperature on the rate of glycerol passive diffusion

The relative rates of passive glycerol entry, measured as the reciprocal relaxation times for cell swelling in hypertonic glycerol, are plotted against temperature in Fig. 2(a) for E. coli K1060 grown in the presence of xylose and various unsaturated fatty acids. In all four types of cells there were marked increases in the rates of glycerol permeation with increasing temperature. In addition, the relative rates of glycerol passive entry were significantly dependent on the unsaturated fatty acid supplement employed. Glycerol permeability decreased in the order 18:2ω6 > 16:1ω7 > 18:1ω9 > 18:1ω9. The ratios of the passive glycerol permeation rates at 40 °C, where the membrane lipids in all cells tested should be entirely in the liquid-crystalline state, were approximately 2.9 : 2.1 : 1.9 : 1.0 for 18:2ω6, 16:1ω7, 18:1ω9 and 18:1ω9-enriched cells, respectively. The relative permeabilities to glycerol of these four cell types were inversely related to the gel to liquid-crystalline phase transition midpoint temperatures of their membrane lipids, suggesting that the rate of glycerol passive diffusion increases with increasing membrane lipid fluidity.

Arrhenius plots of the data reported in Fig. 2(a) revealed regions of apparently linear relationship between the natural logarithm of the rate of glycerol permeation and the reciprocal of the absolute temperature (Fig. 2b), permitting the calculation of apparent activation energies for the overall passive glycerol permeation process for each cell type over the temperature ranges studied. For E. coli K1060 enriched with 18:2ω6 or 16:1ω7, the linear relationship was observed over the entire temperature range of 10 to 45 °C. The apparent activation energies, calculated from the slope of these lines, were about 15–15.5 kcal mol⁻¹ (1 cal = 4.184 J). However, for 18:1ω9 and 18:1ω9-enriched cells, a linear relationship was observed only at temperatures above about 17 °C and 40 °C, respectively; below these temperatures, the slopes abruptly increased, giving apparent activation energies of 30–35 kcal mol⁻¹, while above these characteristic temperatures similar activation energies (15–16 kcal
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Effect of fatty acid composition and temperature on the rate of glycerol facilitated diffusion

The relative rates of mediated glycerol entry, measured as the reciprocal relaxation times for cell swelling in hypertonic glycerol and corrected for the passive diffusion background, are plotted against temperature in Fig. 3(a) for E. coli K1060 grown in the presence of glycerol and various unsaturated fatty acids. Although all cell types studied exhibited significant increases in the rate of glycerol facilitated diffusion with temperature, the temperature dependence of mediated glycerol permeation was considerably smaller than that observed for passive glycerol entry. The relative rates of mediated glycerol entry were again found to vary appreciably with the fatty acid composition of the membrane lipids, decreasing in the order 18:2<sub>c,c</sub> > 16:1<sub>c</sub> > 18:1<sub>c</sub> > 18:1<sub>r</sub>, as observed for passive glycerol permeation. At 40°C, the ratios of the rates of glycerol facilitated diffusion were 2.6:1.9:1.5:1.0 for 18:2<sub>c,c</sub>, 16:1<sub>c</sub>, 18:1<sub>c</sub> and 18:1<sub>r</sub>-enriched cells, respectively. These ratios were slightly lower but similar to those observed for the passive permeation process, indicating that protein-mediated glycerol transport is also sensitive to the membrane lipid composition. The inverse relation again observed between the relative rates of glycerol facilitated diffusion and the gel to liquid-crystalline membrane lipid phase transition temperatures suggests that the rate of glycerol facilitated diffusion also increases with increasing membrane lipid fluidity, as previously reported for glucose transport in A. laidlawii B (Read & McElhaney, 1975).

Arrhenius plots of the data reported in Fig. 3(a) revealed apparently linear relationships between the natural logarithm of the rate of glycerol facilitated diffusion and the reciprocal of the absolute temperature over the entire temperature range studied for all fatty acid compositions tested (Fig. 3b). In particular, no abrupt breaks in the slopes of the Arrhenius plots were observed with 18:1<sub>c</sub> and 18:1<sub>r</sub>-enriched cells near the boundaries of the membrane lipid phase transitions, as previously observed for passive glycerol entry, although subtle changes in the slopes of these plots might have been masked by an increased scatter of the experimental points. This result suggests that mediated glycerol permeation is not markedly sensitive to the phase state of the bulk membrane lipid. The apparent activation energies for glycerol facilitated diffusion varied little with membrane lipid fatty acid composition, ranging from 9.5 kcal mol<sup>-1</sup> for 18:1<sub>c</sub>-enriched cells to 11.3 kcal mol<sup>-1</sup> for 18:2<sub>c,c</sub>-enriched cells; similar behaviour was observed for glucose transport in A. laidlawii B.
Fig. 3. (a) Temperature dependence of the rate of entry of glycerol by facilitated diffusion into E. coli K1060 grown in the presence of glycerol plus various unsaturated fatty acids. The rates of glycerol facilitated diffusion were measured as the difference in reciprocal relaxation times (1/τ) of cell swelling between glycerol- and xylose-grown cells. Unsaturated fatty acids: ○, linoleic acid (18:2\(_{\text{cis}}\)); ■, palmitoleic acid (16:1\(_{\text{c}}\)); O, oleic acid (18:1\(_{\text{c}}\)); □, elaidic acid (18:1\(_{\text{c}}\)).

(b) Arrhenius plots of the data in (a).

(Read & McElhaney, 1975). These values are significantly lower than the apparent activation energies of 15–16 kcal mol\(^{-1}\) observed for glycerol passive permeation, as expected for a protein-mediated transport process (Bowyer, 1957). The apparent lack of an effect of the lipid phase transition on the function of the glycerol facilitator may indicate that this protein functions as a membrane channel, as has recently been suggested by Heller et al. (1980). However, the apparent influence of membrane lipid fluidity on the rates of mediated glycerol permeation would be difficult to explain if this were the case, unless the fatty acid composition influences the number of functional carriers in the E. coli membrane. Alternatively, the boundary lipid immediately adjacent to the glycerol carrier protein may remain in a fluid or semi-fluid state at temperatures where the bulk membrane lipid is solid. The ability of intrinsic membrane proteins to disorder gel state lipid (for review, see Chapman et al., 1979) and to retain some function below the phase transition range of the bulk membrane lipid (Silvius & McElhaney, 1980) has recently been documented.

We thank Mr Steve Cook and Mrs Nannette Mak for excellent technical assistance and Dr Joel Weiner for valuable discussions and advice. This research was supported by grant MT 4261 from the Medical Research Council of Canada.

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