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CHOLESTEROL IN MYCOPLASMA MEMBRANES

CORRELATION OF ENZYMIC AND TRANSPORT ACTIVITIES WITH PHYSICAL STATE OF LIPIDS IN MEMBRANES OF *MYCOPLASMA MYCOIDES* VAR. *CAPRI* ADAPTED TO GROW WITH LOW CHOLESTEROL CONCENTRATIONS

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SUMMARY

1. Membranes of a *Mycoplasma mycoides* var. *capri* strain adapted to grow with very low concentrations of cholesterol undergo a reversible phase transition detectable by differential-scanning calorimetry and fluorescence measurements. No phase transition could be detected in membranes of the cholesterol-containing native strain.

2. EPR spectrometry, as well as fluorescence measurements, demonstrated that at temperatures above phase transition the membranes of the adapted strain were more fluid than membranes of the native strain, although the former contained a higher percentage of saturated fatty acids.

3. Arrhenius plots of the ATPase activity of the adapted strain membranes showed breaks at temperatures corresponding to those of the phase transition of membrane lipids. The temperature of the break depended on the fatty acid composition of membrane lipids and on the age of the culture. No break could be detected in Arrhenius plots of the ATPase activity of the native strain.

4. No break could be demonstrated in the Arrhenius plot of α-methylglucoside uptake by the adapted strain. Yet the activation energy of the uptake process by the adapted strain was much higher than that of the native strain. On the other hand, activation energies of α-methylglucoside phosphorylation were the same for membranes of both strains. However, Arrhenius plots of α-methylglucoside efflux from cells of the adapted strain showed breaks at temperatures corresponding to those of the lipid phase transition.

5. It is concluded that cholesterol, by preventing the crystallization of membrane lipids maintains them in a state of fluidity essential for the optimal manifestation of several key activities of the membrane.

INTRODUCTION

The interrelationship between the physical state of membrane lipids and trans-

port activities was the subject of several recent publications employing an unsaturated fatty acid auxotroph of *Escherichia coli*[1–6]. These studies demonstrated breaks in the Arrhenius plots of the transport processes at temperatures which were found to depend on the fatty acid composition of membrane lipids. That the breaks are due to alterations in the physical state of the membrane lipid core has recently been indicated by Overath and Trauble[6] who showed the temperatures of the breaks to correspond to those of the crystalline–liquid crystalline phase transition of the membrane polar lipids.

The effects of cholesterol on the physical state of model membrane systems made of phospholipids was extensively investigated[7]. Cholesterol was shown to have a condensing or rigidifying effect on the hydrocarbon chains of the phospholipids at temperatures above their phase transition, and a fluidizing effect at temperatures below the phase transition. The phase transition could actually be eliminated on increasing the ratio of cholesterol to phospholipid to a certain level[7,8]. The membrane of *E. coli*, like that of other bacteria, does not contain cholesterol. Hence, experiments with the unsaturated fatty acid auxotroph of *E. coli* are unsuitable for studying the role of cholesterol in biomembranes. The sterol-requiring *Mycoplasma mycoides* var. *capri* has the advantage of requiring both the long-chain fatty acids and cholesterol for growth, allowing dramatic, but controlled, modifications in the composition of the membrane lipids[9,10]. The accompanying paper[9] describes the adaptation of *M. mycoides* var *capri* to grow with very low cholesterol concentrations. The effects of cholesterol depletion on the physical state of membrane lipids and on some enzymic and transport activities located in the membrane are presented in this communication.

MATERIALS AND METHODS

Organism and growth conditions

The native and adapted *Mycoplasma mycoides* var. *capri* strains[9] were grown in 100–200-ml volumes of a modified Edward medium[11] in which the PPLO serum fraction had been replaced by 0.5% fatty-acid-poor bovine serum albumin (Calbiochem, San Diego, Calif.), palmitic acid (1–5 μg/ml) and oleic or elaidic acid (5–10 μg/ml). The medium was supplemented with 10 μg cholesterol per ml for growth of the native strain and with 0.12 μg cholesterol per ml for growth of the adapted strain. The organisms were harvested after incubation at 37 °C for 16–20 h, treated with DNAase (100 μg/ml) for 15 min at 37 °C to remove small amounts of DNA released from some cells and washed once in 0.25 M NaCl.

Isolation of cell membranes

Cell membranes were isolated by osmotic shock of the organisms[12]. The membranes were collected by centrifugation at $34000 \times g$ for 30 min, washed twice in deionized water and resuspended in β-buffer[13] diluted 1:20 with deionized water (dilute β-buffer).

Determination of ATPase activity

ATP phosphohydrolase (ATPase) (EC 3.6.1.3) activity was measured by the release of P_i from ATP in reaction mixtures incubated at various temperatures for 5–40 min. The reaction mixtures (1.0 ml) contained 0.1–0.3 mg membrane protein,

50 μmoles Tris–HCl (pH 7.4), 5 μmoles $MgCl_2$ and 2 μmoles ATP. The reaction was stopped by the addition of 1.0 ml cold 10% trichloroacetic acid to each test tube. The test tubes were immediately centrifuged at $3000 \times g$ for 10 min and the amount of P_i in the deproteinized supernatant fluid was determined according to Fiske and SubbaRow[14]. Since the rate of ATP hydrolysis by the membranes was constant during the first 10–20 min of incubation the initial rate of ATPase activity was expressed as nmoles of P_i released per mg membrane protein in 5 min.

Determination of phosphotransferase activity

Phosphotransferase activity of *M. mycoides* var. *capri* membrane preparations was determined as described before[15]. The phosphorylated sugar was separated from the unphosphorylated sugar by transfer through a Bio-Rad AG 1-X2 (50–100 mesh) anion-exchange resin column[16] or by the precipitation of the phosphorylated sugar as the barium salt. For the precipitation, 2 ml of 1 M $BaCl_2$, 1 ml of 1 M Na_2HPO_4 and 0.25 ml of 10 M NaOH were added to a test tube containing 1 ml of sample. The precipitate which was immediately formed was separated from the supernatant fluid by centrifugation.

Measurement of α-methylglucoside uptake and efflux

α-Methyl-D-glucopyranoside (α-methylglucoside) uptake was measured as previously described[17] in an uptake medium (0.15 M NaCl, 0.01 M $MgCl_2$, 0.05 M Tris–HCl buffer, pH 8.0) containing $5 \cdot 10^{-6}$ M uniformly labeled α-[^{14}C]methylglucoside (0.03 μCi/ml). Samples (0.2 ml) were removed from the incubation mixture at various intervals, filtered and washed on 0.45-μm Millipore filters (HA-45) and counted in 10 ml of a dioxane–toluene scintillation liquor[12] in a Packard Tri-Carb liquid scintillation spectrometer. Efflux of α-[^{14}C]methylglucoside from *M. mycoides* var. *capri* was determined with cells preloaded with the radioactive sugar. The cells were loaded in a mixture containing washed cells (1 mg cell protein per ml), 0.05 M Tris–HCl (pH 8.0), 0.15 M NaCl, 0.01 M $MgCl_2$ and 0.05 μCi of α-[^{14}C]methylglucoside ($1.3 \cdot 10^{-5}$ M) in a total volume of 1 ml. After 10 min of incubation at 37 °C, 10 ml of the suspension was centrifuged and the cell pellet was resuspended in 0.6 ml of a solution containing the above ingredients except for the α-methylglucoside and the cells. Samples (100 μl) of the cell suspension were then added to 20 ml of the same solution prewarmed to the desired temperature; 4-ml samples were withdrawn at various time intervals and filtered through a 0.45-μm Millipore filter. The filters were washed with 10 ml of 0.25 M NaCl containing 0.01 M $MgCl_2$, dried and their radioactivity was determined.

Analytical methods

Lipids were extracted by chloroform–methanol (2:1, v/v) and washed according to Folch *et al.*[18]. Separation of neutral from polar lipids and gas–liquid chromatography of the fatty acid methyl esters of membrane polar lipids were performed as described before[9]. Protein was determined according to Lowry *et al.*[19] and cholesterol by the $FeCl_3$ method[20].

Fluorescence polarization measurements

Microsviscosity in the lipid phase was determined by the fluorescence polari-

zation technique[21,22]. The membrane preparations were labeled with the fluorescent probe diphenylhexatriene. A solution of $2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran was diluted 1000-fold in a 0.25 M NaCl solution. The clear aqueous diphenylhexatriene dispersion formed was then diluted 1:1 with membrane suspensions in 0.25 M NaCl containing 1 mg membrane protein per ml, and left at room temperature for 1 h for equilibration. The degree of fluorescence polarization and the excited state life time of the diphenylhexatriene labeled membranes were simultaneously determined at a series of temperature in the range of 0–45 °C. The values obtained were then applied to a calibration curve from which the corresponding microviscosities, $\bar{\eta}$, were derived.

Differential-scanning calorimetry

A Perkin-Elmer DSC-2B differential calorimeter operating at a scan rate of 5 or 10 °C per min was used for calorimetric measurements. Samples (15 μl) of a thick membrane suspension, containing 0.6–0.9 mg membrane protein in dilute β-buffer, were sealed in the sample pan and scanned at least 5 times from 0–50 °C. For the determination of the base line, 15 μl of deionized water were scanned under the same conditions. The energy and temperature calibration of the calorimeter was done with benzoic acid, naphthalene, cyclohexane and water as standards.

Paramagnetic resonance spectroscopy

Membranes were spin-labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid (Syva, Palo Alto, Calif.) by exchange from bovine serum albumin[23]. EPR spectra of the spin-labeled membranes were obtained by a Varian E-4 spectrometer. Results were expressed as the hyperfine splitting $2T$ maximum ($2T_m$) of the spectra, previously shown to be related to the freedom of motion of the nitroxide radical[24], greater freedom of motion being associated with smaller values of $2T_m$. $2T_m$ was measured to within ± 0.5 G.

RESULTS

Effects of the adaptation on the physical state and phase transition of the membrane lipid core

Membranes of the *M. mycoides* var. *capri* strain adapted to grow in the cholesterol-poor medium showed by fluorescence polarization and differential-scanning calorimetry a thermal phase transition which could not be detected in membranes of the native strain.

Fluorescence polarization. Fig. 1 shows the changes in microviscosity of the membrane with temperature based on fluorescence measurements of the fluorescent probe diphenylhexatriene incorporated into the membranes. The Arrhenius plots obtained with membranes of the adapted strain clearly demonstrated a transition at 24 °C between two phases characterized by fusion activation energies of $\Delta E = 3.7$ kcal/mole below and 10.5 kcal/mole above the transition temperature. Membranes of the native strain showed no transition point, within the temperature range of -10–45 °C, but a constant phase with an activation energy of $\Delta E = 4.5$ kcal/mole.

Differential-scanning calorimetry. Thermograms of membranes of the native and adapted strains are presented in Fig. 2. The scanning of membranes of the

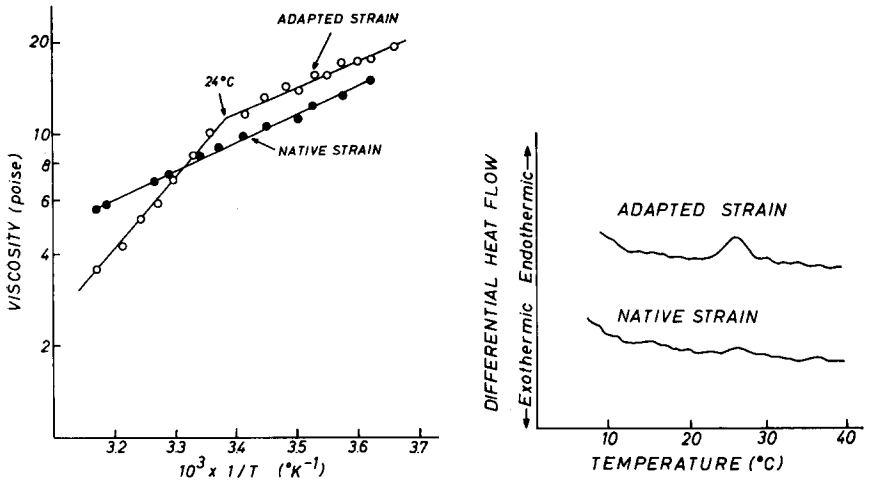

Fig. 1. The effect of temperature on the microviscosity of membrane lipids of the native and adapted *M. mycoides* var. *capri* strains as determined by fluorescence measurements of diphenylhexatriene incorporated into the membranes. The growth medium was supplemented with palmitic and oleic acids (5 μg/ml of each) and with 10 μg cholesterol per ml for the native strain and 0.12 μg cholesterol per ml for the adapted strain.

Fig. 2. Thermograms of *M. mycoides* var. *capri* membranes in dilute β-buffer scanned by heating at a rate of 10 °C per min. The suspension of membranes of the adapted strain contained 0.61 mg membrane protein per ml and that of membranes of the native strain contained 0.87 mg membrane protein per ml. The fatty acid and cholesterol supplements to the growth medium were the same as in Fig. 1.

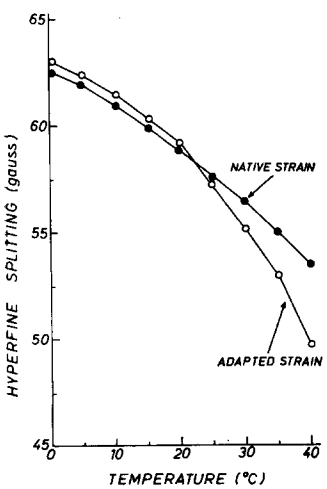

Fig. 3. The effect of temperature on the hyperfine splitting ($2T_m$) of *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid in membranes of the native and adapted *M. mycoides* var. *capri* strains.

adapted strain show a reversible endothermic transition between 22 and 29 °C, centered at about 25 °C, whereas in membranes of the native strain the transition is absent or much smaller. The energy content of the transitions in all samples of membranes of the adapted strain was, however, very low as compared to that of *Acholeplasma laidlawii* membranes scanned under the same conditions.

Paramagnetic resonance spectrometry. Fig. 3 shows that above 25 °C the freedom of motion of the spin-labeled fatty acid incorporated into membranes of the native strain was lower in membranes of the adapted strain suggesting a more viscous lipid core in the cell membrane of the native strain.

Effects of the adaptation on some enzymic and transport activities of the membranes

ATPase activity. Both native and adapted *M. mycoides* var. *capri* strains possessed an active Mg^{2+}-dependent ATPase localized exclusively in their cell membrane. Arrhenius plots of the ATPase activity in membrane preparations of the native and adapted strains are shown in Figs 4–6. The curves obtained with membranes of the native strain (Fig. 4) were always linear and showed the same slope throughout the temperature range tested (10–40 °C). However, the curves obtained with membranes of the adapted strain were biphasic and two straight lines intersected at a certain point, defining a transsition temperature.

The transition temperature was found to depend on the fatty acid composition of the membrane lipids. Thus, by growing the adapted strain with 5 μg/ml of the *trans* isomer of Δ^9-octadecenoic acid (elaidic acid) instead of the *cis* isomer (oleic acid), the transition temperature shifted from 18–22 to 30–32 °C (Fig. 5). Elaidic

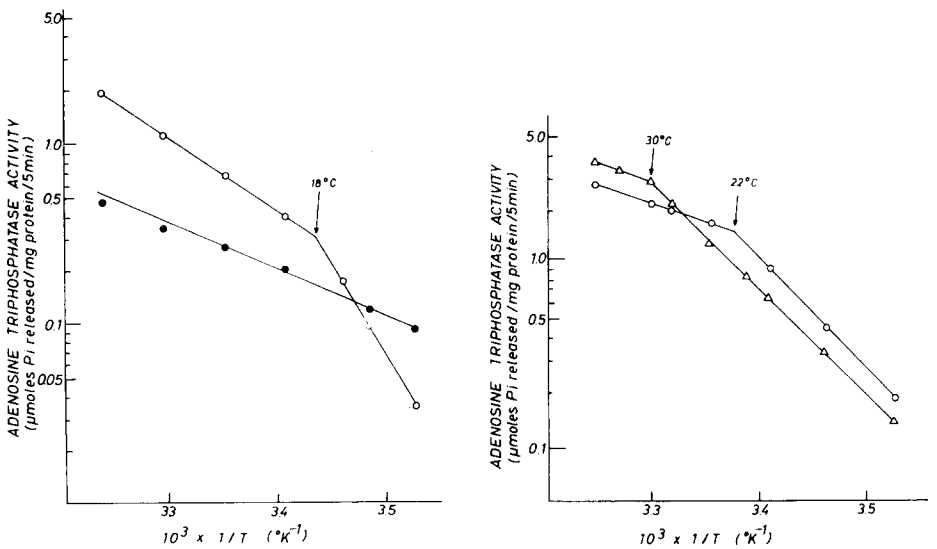

Fig. 4. Arrhenius plots of ATPase activity of membranes of the native and adapted *M. mycoides* var. *mycoides* cells grown in a modified Edward medium containing palmitic and oleic acids (5 μg/ml of each). ●—●, membranes of the native strain; ○—○, membranes of the adapted strain.

Fig. 5 Arrhenius plots of ATPase activity of membranes of the adapted *M. mycoides* var. *mycoides* cells grown in a modified Edward medium supplemented with 5 μg/ml of palmitic acid and 5 μg/ml of either one of the octadecenoic acid isomers: ○—○, oleic acid; △—△, elaidic acid.

acid was found to comprise about 50% of the total fatty acids in membrane polar lipids of the elaidate-grown cells, as compared to 42% of oleic acid in the polar lipid fraction of membranes from the oleate-grown cells. Nevertheless, the transition temperature of the ATPase activity of the adapted strain was found to vary somewhat with different batches of membranes from cells grown with the same fatty acid mixture. As may be seen in Fig. 6 the age of the culture from which the membranes were derived may be responsible for the variations. The transition temperature increased with age, apparently because of the increase in the saturation of membrane polar lipids found to occur upon aging (Table VII in accompanying paper).

α-Methylglucoside uptake. M. mycoides var. *capri* cells were shown to accumulate α-methylglucoside by the phosphoenolpyruvate-dependent phosphotransferase system[15]. The effect of cholesterol on the transport process was investigated by using the native and adapted *M. mycoides* var. *capri* cells grown with palmitic and oleic acids (5 μg/ml of each). Temperature characteristics of the α-methylglucoside transport by these strains are presented in Fig. 7. The uptake of α-methylglucoside by cells of the adapted strain was greatly affected by lowering the temperature, and no measurable transport could be demonstrated below 25 °C. The differences revealed between the native and adapted strains in the slopes of the Arrhenius display of α-methylglucoside uptake are most remarkable. From these slopes an apparent activation energy of 57.2 kcal/mole was calculated for cells of the adapted strain as against an activation energy of 36.5 kcal/mole for cells of the native strain. In the temperature range of 25–40 °C no breaks could be demonstrated in the Arrhenius

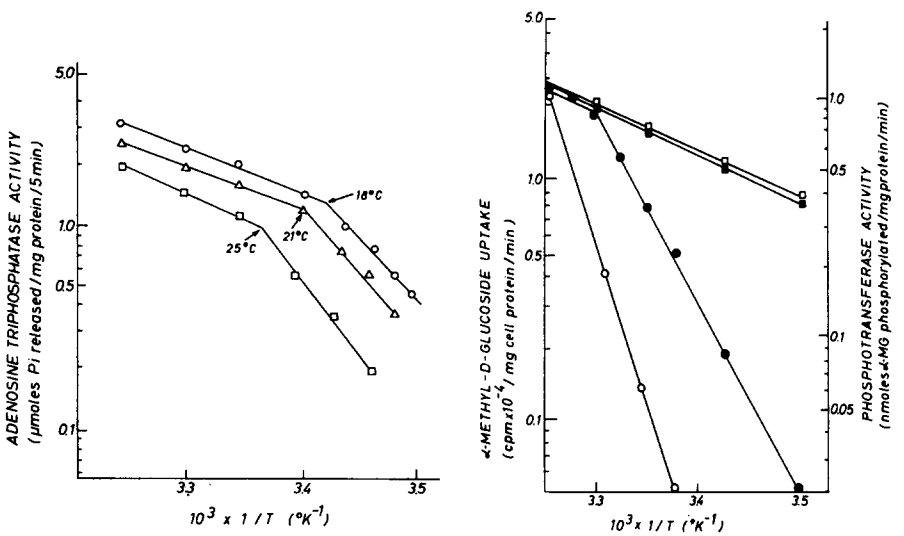

Fig. 6. Arrhenius plots of ATPase activity of membranes of the adapted *M. mycoides* var. *mycoides* cells harvested at different ages of culture. ○—○, cells grown for 18 h; △—△, cells grown for 21 h; □—□, cells grown for 25 h.

Fig. 7. Arrhenius plots of α-methylglucoside (α-MG) uptake and phosphotransferase activity of *M. mycoides* var. *capri* cells and membrane preparations. ●—●, uptake by cells of the native strain; ○—○, uptake by cells of the adapted strain. ■—■, phosphotransferase activity of membranes of the native strain; □—□, phosphotransferase activity of membranes of the adapted strain.

plots of α-methylglucoside uptake by cells of the adapted strain. However, the plots of the α-methylglucoside uptake by the native strain showed a break at 30 °C. Since alterations in the fatty acid composition of membrane polar lipids of the native strain had no effect on the temperature of this break, and because of the high cholesterol content (about 25%, w/w) of these membranes, it would appear that the break does not represent a transition temperature of membrane lipids.

No differences were found in the phosphorylation rate of α-methylglucoside by the phosphotransferase system of membrane preparations of the native and adapted strains. The Arrhenius plots of this activity formed a straight line throughout the temperature range tested with equal slopes for membranes of the native and adapted strains (Fig. 7).

Efflux of α-methylglucoside. The efflux of α-[^{14}C]methylglucoside from preloaded cells of the native and adapted *M. mycoides* var. *capri* strains was tested at various temperatures in a medium containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl and 10 mM $MgCl_2$. The rate of efflux from cells of either the native or the adapted strain suspended in this medium was very low regardless of the temperature used. However, the addition of 10^{-3} M D-mannose to the medium increased most dramatically the rate of α-methylglucoside efflux. Arrhenius plots of the data obtained with cells grown with palmitic and oleic acids (5 μg/ml of each) are presented in Fig. 8. Most remarkable is the break at 23 °C in the plot representing the efflux from the adapted strain. This break was reproducible within a range of 2–3 °C in independent experiments and did not appear in plots of efflux from cells of the native strain within the temperature range of 5–40 °C. As with the ATPase activity, the replacement of oleic acid in the growth medium by the *trans* isomer elaidic acid shifted

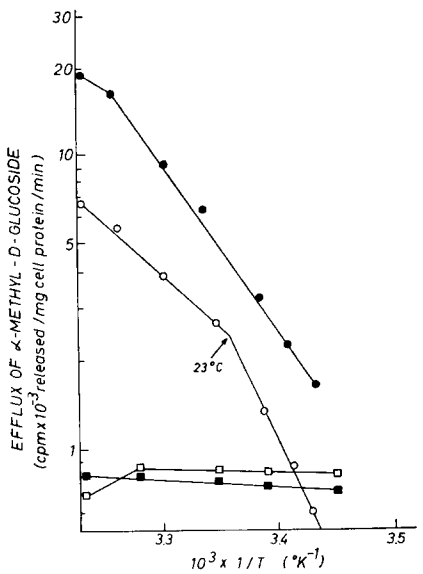

Fig. 8. Arrhenius plots of α-methylglucoside efflux from native and adapted *M. mycoides* var. *mycoides* cells in the presence or absence of D-mannose ($1 \cdot 10^{-3}$ M). ●—●, native strain with mannose; ■—■, native strain without mannose; ○—○, adapted strain with mannose; □—□, adapted strain without mannose.

the break from 23–33 °C. The enhancement by mannose of α-methylglucoside efflux may be associated with a dephosphorylation process, since, whereas in whole cells 85% of the radioactivity was recovered as a phosphorylated derivative of α-methylglucoside, as indicated by the precipitation as the barium salt by anion-exchange chromatography, the radioactivity released into the efflux medium was recovered mainly (83%) as free α-methylglucoside.

DISCUSSION

Fluorescence polarization and differential-scanning calorimetry measurements failed to detect any thermal phase transition in membranes of the native *M. mycoides* var. *capri* strain which contain appreciable quantities of cholesterol (about 20–25% of the total membrane lipid[9]). Phase transitions were, however, observed in membranes of the adapted *M. mycoides* var. *capri* strain, which contained much lower amounts of cholesterol (up to 3% of the total lipid[9]). This finding supports the observations of De Kruyff *et al.*[8] who showed that the incorporation of cholesterol into membranes of *A. laidlawii* (up to 12% of the total lipid) reduced the energy of the lipid phase transition by about two thirds. Extensive studies carried out on model membranes made of mixtures of phospholipids and cholesterol[7,25,26] have demonstrated that the steroid nucleus effectively prevents the hydrocarbon chains of the phospholipids from crystallizing at low temperatures.

The EPR spectra of spin-labeled fatty acids in membranes of the native and adapted *M. mycoides* var. *capri* strains indicated a more restricted chain mobility in the cholesterol-containing membranes of the native strain at temperatures above 25 °C. These findings are in accord with X-ray diffraction[27,28] and EPR studies[29,30] on model phospholipid membranes. The restriction in mobility is apparently another manifestation of the condensation effect of cholesterol, first described in monolayer studies[31]. The dual effect of cholesterol (the condensation of the hydrocarbon chains at high temperatures and the prevention of crystallization of the chains at low temperatures) are the properties which make cholesterol an excellent regulator of the fluidity of the membrane lipid core, keeping it at the optimum "intermediate fluid state" under varying growth temperatures or during changes in the fatty acid composition of membrane lipids[28].

Our results support the notion that the physical state of the membrane lipids may have a determinative influence on the activity of membrane-associated enzymes and transport systems. Thus, the Arrhenius plots of the ATPase activity of the adapted *M. mycoides* var. *capri* strain showed definite breaks at temperatures which depended on the fatty acid composition of membrane lipids, resembling in this respect several membrane-associated enzymic activities of the unsaturated fatty-acid auxotroph of *E. coli*[32,33]. Nevertheless, as was found for some membrane-bound enzymes of *E. coli*, the phosphorylation of α-methylglucoside by the membrane-associated phosphotransferase system of *M. mycoides* var. *capri* was not influenced by the physical state of membrane lipids, since this enzymic activity was not affected by the fatty acid composition nor by the cholesterol content of the membrane.

In spite of the finding that the α-methylglucoside phosphorylation step by itself was independent of the composition of membrane lipids, the uptake and accumulation of this sugar by the mycoplasma was markedly affected by changes in the com-

position of membrane lipids, as was indicated by the much higher activation energy of the uptake process by the adapted strain (Fig. 7). However, aside from the differences in the activation energies between the native and adapted strains, we were unable to demonstrate a break in the Arrhenius plot of the α-methylglucoside uptake by the adapted strain. This failure may be due to the fact that at and below the expected transition temperature (18–22 °C) the rate of α-methylglucoside uptake by the adapted strain was too low to be measured (Fig. 7). On the other hand, breaks in the Arrhenius plots of α-methylglucoside efflux could be clearly demonstrated with cells of the adapted strain, but not with cells of the native, cholesterol-containing strain.

Our results extend those obtained with the fatty-acid auxotroph of *E. coli* by the inclusion of cholesterol as an additional factor which influences the physical state of the lipid domain in biological membranes. Comparison of the data obtained with the native and adapted *M. mycoides* var. *capri* strains shows clearly that the presence of large quantities of cholesterol in the membrane eliminates both the phase transition of membrane lipids and the resulting discontinuities in the Arrhenius plots of the membrane-associated ATPase activity and α-methylglucoside efflux.

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