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INFLUENCE OF FATTY ACID AND STEROL COMPOSITION ON THE LIPID PHASE TRANSITION AND ACTIVITY OF MEMBRANE-BOUND ENZYMES IN *ACHOLEPLASMA LAIDLAWII*

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SUMMARY

1. The temperature dependency of NADH-oxidase, *p*-nitrophenylphosphatase and Mg^{2+}-dependent ATPase was studied in *Acholeplasma laidlawii* cell membranes with varying fatty acid and sterol composition. In these membranes the gel→liquid crystalline phase transition of the membrane lipids was measured by differential scanning calorimetry.

2. The Arrhenius plots of the NADH oxidase and *p*-nitrophenylphosphatase activities showed no discontinuities although the membrane lipids underwent a phase transition in the temperature range studied.

3. Distinct breaks in the Arrhenius plot of the ATPase activity were observed and found to be dependent upon the fatty acid composition of the membrane lipids; these breaks occurred at the lower ends of the lipid phase transitions. We conclude from these results that at these temperatures the ATPase is associated with molecular lipid species with the lowest transition temperature.

4. Incorporation of cholesterol into the membrane decreased the temperature of the break in the ATPase activity and the temperature of the lower end of the lipid phase transition. This effect is due to the lipid–cholesterol interaction since (a) the effect is reversed by the polyene antibiotic filipin which complexes the cholesterol and (b) incorporation of epicholesterol which does not interact with other lipids did not influence the temperature of the break in the Arrhenius plot.

INTRODUCTION

Temperature induced phase transitions can occur in the lipid part of many model and biological membranes. At particular temperatures the fatty acid chains of the membrane lipids undergo a transition from an ordered crystalline or gel state to a more disordered liquid–crystalline state[1]. In the Arrhenius plot of the activity of several membrane bound enzymes of various organisms "breaks" occur (for a review see Lyons[2]). These breaks have often been attributed to phase changes in the lipid part of the membrane which would induce a conformational change in the enzyme. However, there is only limited direct experimental proof to substantiate this conclusion. Raison *et al.*[3] demonstrated with electron spin resonance (ESR)

that the temperature of the breaks in the Arrhenius plots of succinate dehydrogenase, succinate oxidase and cytochrome *c* oxidase in rat liver mitochondria coincide with the temperature of the break in the Arrhenius plot of the spin label mobility. Since the spin label mobility reflected the viscosity of the interior of the membrane this observation would suggest that a transition in the lipid phase could induce a break in the Arrhenius plot of enzymatic activity. However, there are some uncertainties in the interpretation of these results; (a) the spin probe will perturb the hydrocarbon chain packing to some extent and it is this specific area which is being monitored, (b) the spin probe might be non-randomly distributed in the membrane, (c) Blazyk and Steim[4] determined calorimetrically that the phase transition in rat liver mitochondria ranges from -20 up to $+30$ °C while the breaks in the Arrhenius plots of the various membrane bound mitochondrial enzymes cited above occur at 23–24 °C.

Esfahani *et al.*[5] demonstrated in *Escherichia coli* that the temperatures of the breaks in the Arrhenius plots of the succinate dehydrogenase activity and the proline uptake were dependent upon the fatty acid composition of the membrane lipids and coincided with the beginning of the phase transition in the membrane lipids as detected with X-ray diffraction. On the other hand, Steim demonstrated that the breaks in the Arrhenius plots of the rate of galactoside transport in *E. coli* did not correlate with any unique portion of the calorimeter peak of the lipid phase transition observed in these membranes[6]. Further direct proof for a correlation between breaks in the Arrhenius plots of membrane bound enzyme activities and lipid phase transition in bacteria is hampered by the complexity of this transition. This difficulty may in part be caused by the presence of the cell wall[6].

The absence of a cell wall and the possibility of varying the fatty acid composition of the membrane lipids in *A. laidlawii* make this organism very suitable for studies on lipid phase transition. Differential scanning calorimetry[6-9] and X-ray[10] studies demonstrate that the phase transition occurs over a 20–30 °C temperature range. Despite several studies on the phase transition in this membrane it is not known whether the lipid phase transition induces a change in the activation energy of membrane bound enzymes. We investigated the NADH oxidase, *p*-nitrophenylphosphatase and Mg^{2+}-dependent ATPase activity in the *A. laidlawii* cell membrane as a function of temperature and fatty acid composition of the membrane lipids. These enzymes were previously shown to be membrane bound[11,12].

When cells are grown in media in the presence or absence of sterols, membranes can be obtained which contain either no sterol or about 9% sterol (wt % of total lipid[8,13]). This enabled us to study the possible effects of sterols upon the temperature dependence of the membrane bound enzymes. The lipid phase transition in the various membranes was determined by differential scanning calorimetry.

MATERIALS AND METHODS

Materials

Reduced β-nicotinamide-adenine dinucleotide (NADH) was purchased from Boehringer (Mannheim, Germany). ATP and *p*-nitrophenylphosphate were obtained from Sigma chemicals (St. Louis, U.S.A.) and Merck (Darmstadt, Germany), respectively. Cholest-5-en-3β-ol (cholesterol) and cholest-5-en-3α-ol (epicholesterol)

were supplied by Fluka (Buchs, Switzerland) and Mann Research Laboratories (New York, U.S.A.).

Methods

Organism. A. laidlawii strain B cells were cultured in a lipid-poor tryptose medium supplemented with various fatty acids (0.06 mM) and sterols (0.06 mM) as described before[8]. If not otherwise indicated, cells were isolated in the logarithmic growth phase by centrifugation at $14.600 \times g$ at 0 °C for 15 min.

Preparation cell membranes. Membranes were prepared as described by Van Golde *et al.*[14]. The final membrane pellet was suspended homogeneously in distilled water. After the determination of protein concentration the membrane suspensions were frozen and stored at −20 °C.

Enzyme assays

NADH oxidase. The NADH oxidase activty was measured according to Pollack *et al.*[11]. The rate of NADH oxidation was continuously monitored in a Perkin–Elmer Model 356 spectrophotometer equipped with a thermostated cuvette operating in in the dual mode with λ_1 at 339 nm and the reference wavelength λ_2 at 450 nm. The cuvette contained 1.2 ml 150 mM NaCl, 10 μl buffer (23.4 μmoles NaCl, 7.5 μmoles Tris–HCl buffer (pH 7.4) and 1.5 μmoles 2-mercaptoethanol) and 10 μl membrane suspension (0.2–2.0 μg protein). The reaction was started by the addition of 10 μl NADH solution (15 mg/ml). The NADH oxidation was linear in time for at least 15 min. The NADH oxidase activity was expressed as nmoles NADH oxidized per min per mg protein. We observed that freezing of the membranes caused an approximate 2-fold increase in the specific activity of the NADH oxidase when compared to unfrozen, freshly prepared membranes.

p-Nitrophenylphosphatase. The *p*-nitrophenylphosphatase activity was determined as described by Ne'eman *et al.*[12]. The reaction mixture consisted of 0.40 ml buffer (100 μmoles Tris–HCl buffer (pH 7.6), 5 μmoles $MgCl_2$) and 0.10 ml *p*-nitrophenylphosphate (10 μmoles). The reaction was started by the addition of 0.50 ml membrane suspension (300–400 μg protein). The reaction mixture was incubated at the desired temperature for 30 min with constant stirring by a magnetic bar. The reaction was stopped by the addition of 2.0 ml ice-cold 0.5 M Tris in 5% trichloroacetic acid (not neutralised). The protein precipitate was removed by centrifugation and the amount of liberated *p*-nitrophenol was measured in the supernatant at 420 nm. The *p*-nitrophenylphosphatase activity was expressed as nmoles *p*-nitrophenol liberated per min per mg protein. Freezing and storage of the membranes at −20 °C had almost no effect on the specific activity of *p*-nitrophenylphosphatase.

ATPase. The ATPase activity was measured according to Pollack *et al.*[11]. The reaction mixture consisted of 0.25 ml of a solution containing 1.25 μmoles $MgCl_2$, 1.10 μmoles NaCl, 50.0 μmoles Tris–HCl buffer (pH 8.0) and 0.60 μmole 2-mercaptoethanol. To this buffer 0.10 ml ATP (1.25 μmoles) was added and the reaction was started by the addition of 0.65 ml membrane suspension containing 50–300 μg membrane protein. The reaction mixture was incubated at the desired temperature with constant stirring using a magnetic bar. The reaction was stopped after 30 min by the addition of 1 ml ice-cold 10% trichloroacetic acid solution. The protein precipitate was removed by centrifugation and the amount of free phosphate in the

supernatant was determined according to Chen *et al.*[15]. The ATPase activity was expressed as nmoles phosphate liberated per min per mg of protein. Freezing storage of the membranes at −20 °C had almost no effect upon the specific activity of the ATPase. Furthermore the temperature at which the break in the Arrhenius plot occurred was not affected by freezing and storage of the membranes at −20 °C. The experimental variation in the determination of the temperature of the break in the Arrhenius plot of the ATPase activity was never more than ±0.5 °C.

Differential scanning calorimetry

Phase transitions in *A. laidlawii* cell membranes were detected with a Perkin–Elmer D.S.C.-2B calorimeter as described in detail previously[8,16]. In all cases the sample pan contained about 0.35 mg membrane protein.

Analytical methods

Protein was determined according to Lowry *et al.*[17] using bovine serum albumin as a standard. The fatty acid pattern of the total lipids extracted from the *A. laidlawii* membranes according to the procedure of Bligh and Dyer[18] was determined as described before[8]. The amount of fatty acid was determined by gas–liquid chromatography by reference to a C_{20} standard.

RESULTS

Influence of fatty acid composition upon the NADH oxidase, p-nitrophenylphosphatase and ATPase activity in the A. laidlawii cell membrane

Fig. 1 shows the Arrhenius plots of the NADH oxidase activity in membranes of *A. laidlawii* cells grown in the presence of oleic (18:1c), elaidic (18:1t) and stearic acid (18:0). Over the temperature range of 5–35 °C the activation energy is constant for all the membranes. Below 5 °C a possible increase in the activation energy occurs. Since the lipid phase transition occurs (in part) in the temperature

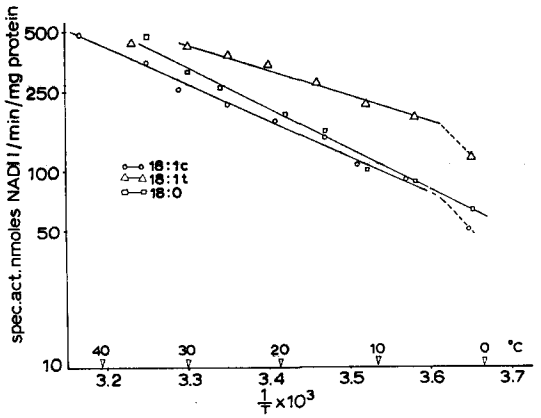

Fig. 1. Arrhenius plots of the NADH oxidase activity in membranes obtained from *A. laidlawii* cells grown in media supplemented with various fatty acids. Cells were grown on the fatty acids indicated in the figure.

traject investigated for membranes prepared from *A. laidlawii* cells grown in 18:1t and 18:0 (ref. 8 and Figs 4 and 5), we can conclude that the lipid phase transition does not influence the activation energy of NADH oxidase. The differences in activity shown in Fig. 1 for cells grown on 18:1c, 18:1t and 18:0 are not significant. In other experiments the order of the NADH oxidase activities of cells grown on 18:1c, 18:1t and 18:0 was completely reversed. Despite these variations in absolute activity we never observed a break in the Arrhenius plot of the NADH oxidase activity in any of the membranes tested.

The Arrhenius plots of the *p*-nitrophenylphosphatase activity in the membranes of cells grown on palmitic acid (16:0), 18:1t and 18:0 are shown in Fig. 2. No discontinuities are noted in these Arrhenius plots although the membrane lipids

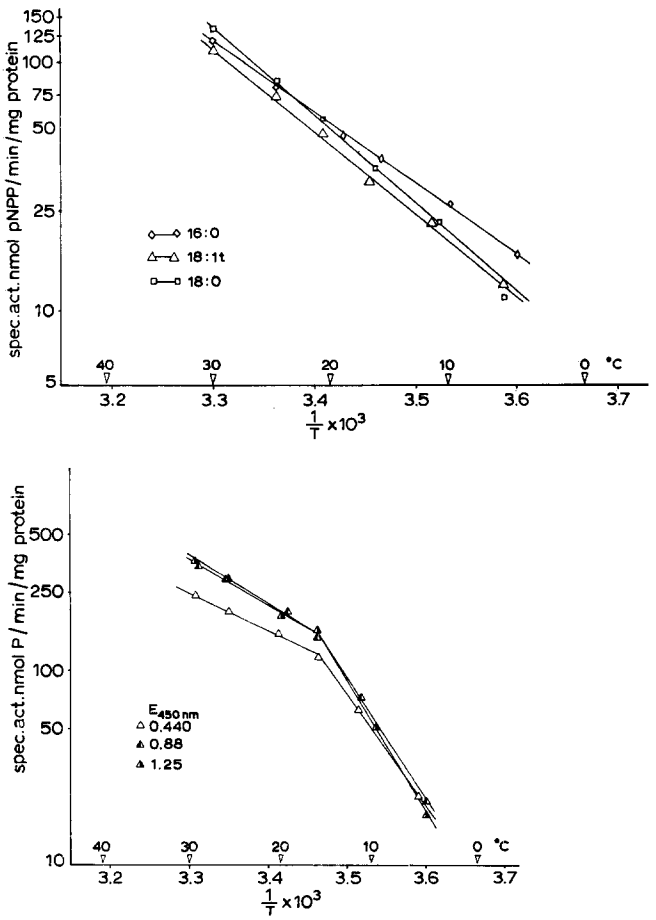

Fig. 2. Arrhenius plots of the *p*-nitrophenylphosphatase activity in membranes isolated from *A. laidlawii* cells grown in media supplemented with various fatty acids. Cells were grown on the fatty acids indicated in the figure. PNPP, p-nitrophenylphosphate.

Fig. 3. Arrhenius plots of the ATPase activity in membranes from *A. laidlawii* cells grown on 18:1t harvested at different growth phases. Cells were harvested at the absorbance values indicated in the figure.

undergo a phase transition in the temperature range investigated (see Figs. 4–6). Furthermore the *p*-nitrophenylphosphatase activity was not very much dependent upon the fatty acid composition of the membrane lipids (Fig. 2).

In the Arrhenius plot of the ATPase activity in the *A. laidlawii* cell membrane, breaks were observed. This is shown in Fig. 3 for cells grown on 18:1t and harvested at different phases of growth. Unrelated to the growth phase a sudden change in activation energy occurs at about 16 °C. A comparison of Fig. 2 and Fig. 3 reveals that the *p*-nitrophenylphosphatase and ATPase activity must be the expression of different enzymes since no break in the Arrhenius plot of the *p*-nitrophenylphosphatase activity was observed in these same membranes.

In order to determine whether the break in the Arrhenius plot of the ATPase activity was related to the fatty acid composition and the lipid phase transition of the membrane, we measured the temperature dependence of the ATPase activity in membranes of cells grown on 18:1t, 18:0, 16:0, 18:1c and linoleic acid (18:2c). The fatty acid composition of the membrane lipids (Table I) and the lipid phase transi-

TABLE I

FATTY ACID COMPOSITION OF *A. LAIDLAWII* MEMBRANE LIPIDS OF CELLS GROWN IN MEDIA SUPPLEMENTED WITH 0.06 mM OF VARIOUS FATTY ACIDS

Fatty acid supplemented in the growth medium	*Fatty acid composition of the membrane lipids (mole %)*										
	12:0	*13:0*	*14:0*	*15:0*	*16:0*	*17:0*	*18:0*	*18:1c*	*18:1t*	*18:2*	*Unknown*
18:1t	4.9	0.2	3.0	0.9	14.0	—	1.8	—	69.8	1.1	4.3
18:0	20.1	2.2	21.2	1.4	16.4	0.4	30.9	1.0	—	1.1	5.3
16:0	7.6	1.2	20.2	0.9	62.7	—	1.7	2.8	—	1.5	1.5
18:1c	5.8	1.1	6.7	1.5	26.4	0.3	6.5	45.1	—	1.4	5.2
18:2	3.5	Trace	7.9	Trace	45.7	Trace	1.9	3.6	—	33.2	4.2

tion was determined on aliquots of the same membrane preparation as used for the ATPase assay. The results of these experiments are given in Figs. 4–7. It is obvious that the temperature of the break in the Arrhenius plot of the ATPase activity is dependent upon the fatty acid composition of the membrane lipids. For cells grown on 18:1t, 18:0 and 16:0 the breaks occurs at 15, 18.5 and 18.0 °C, respectively. The calorimetric scan of the membranes of 18:1t cells shows a broad endothermic transition in the temperature range 13–35 °C in agreement with previous studies[8]. The break in the Arrhenius plot of the ATPase activity occurs at the temperature of the beginning of the phase transition (Fig. 4). The same can be concluded for cells grown on 18:0 and 16:0 (Figs 5 and 6). Although the upper end the phase transition in the membranes of cells grown on 18:0 is difficult to determine, these membranes also show a broad endothermic transition. The "break" in the Arrhenius plot of the ATPase activity occurs at or somewhat below the temperature at the beginning of the lipid phase transition. The starting temperature of the lipid phase transition in membranes of cells grown on 16:0 apparently lies a few degrees above the temperature of the break in the Arrhenius plot of the ATPase activity. However, with these

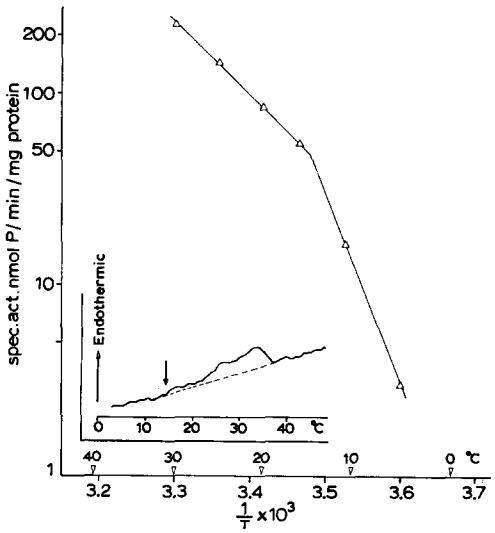

Fig. 4. Arrhenius plots of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 18:1t. The lipid phase transition was recorded at a range of 0.5. The arrow in the calorimetric scan indicates the temperature of the break in the Arrhenius plot of the ATPase activity.

membranes we had to record the lipid phase transition at range 2 setting of the calorimeter while the other membranes were recorded at higher sensitivities. Because of this lower sensitivity the starting temperature of the lipid phase transition is more difficult to determine.

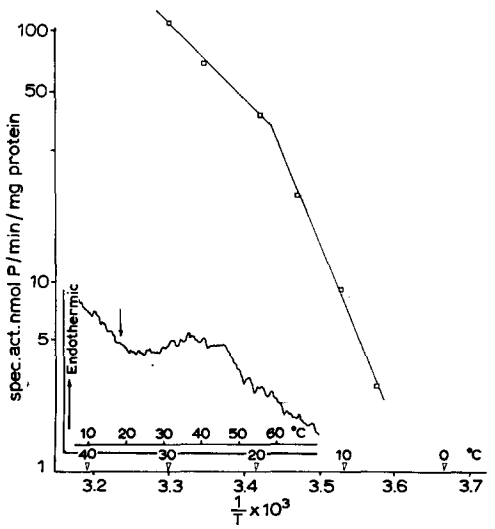

Fig. 5. Arrhenius plot of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 18:0. The lipid phase transition was recorded at a range of 0.2.

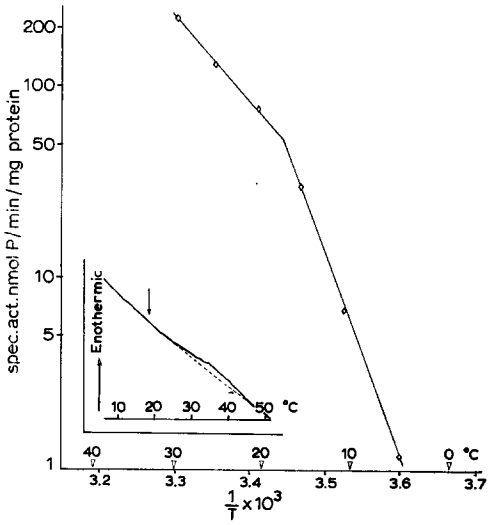

Fig. 6. Arrhenius plot of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 16:0. The lipid phase transition was recorded at a range of 2.

Membranes from cells grown on 18:1c and 18:2c show no breaks in the Arrhenius plot of the ATPase activity in the temperature range 5–30 °C. In this temperature range, no lipid phase transition could be detected (Fig. 7). Steim *et al.*[7] found that the lipid phase transition in membranes of *A. laidlawii* cells grown on 18:1c was centered around −15 °C which is in agreement with our observation. From these results we conclude that the break in the Arrhenius plot of the ATPase activity is associated with the lipid phase transition and occurs at the temperature where (almost) all the lipids attain the gel state.

Influence of cholesterol and epicholesterol incorporation upon the NADH oxidase and ATPase activity in the A. laidlawii cell membrane

Cholesterol incorporation in the *A. laidlawii* cell membrane (9 wt % of total lipid) did not significantly influence the NADH oxidase activity (data not shown). Furthermore, the activation energy of the NADH oxidase activity remained constant from 5 to 35 °C as was described in Fig. 1.

Cholesterol influences the lipid phase transition in the *A. laidlawii* cell membrane causing a reduction in the energy content of the transition and a shift of the starting point of the transition to a somewhat lower temperature[8]. The effect of cholesterol incorporation in the *A. laidlawii* cell membrane upon the ATPase activity is shown in Figs 8 and 9. In Fig. 8 cells were grown on 18:1t and cholesterol and in Fig. 9 the cells were grown on 18:0 and cholesterol. The fatty acid composition of the membrane lipids is not influenced by the incorporation of cholesterol in the membrane[8]. It is obvious that in both membranes the incorporation of cholesterol decreases the temperature of the break in the Arrhenius plot of ATPase activity about 6 °C. This shift also is observed in the calorimetric scans of these membranes. Cholesterol incorporation decreased the starting temperature of the lipid phase transition.

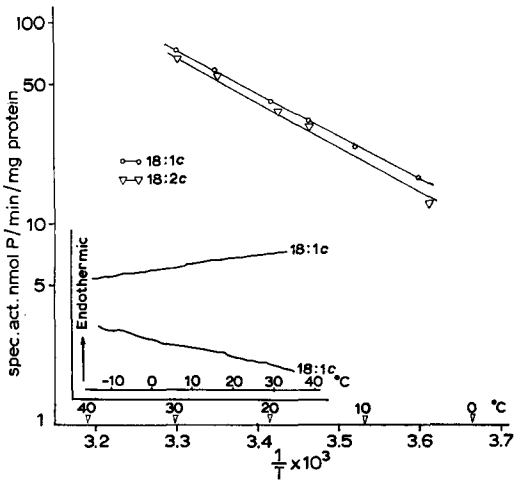

Fig. 7. Arrhenius plot of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 18:1c and 18:2c. The calorimetric scans were recorded at a range of 1.

Recently Cobon and Haslam[19] observed that an increase in the ergosterol content of yeast cells produced a decrease in the break in the Arrhenius plot of the yeast mitochondrial ATPase.

Since it is known that the polyene antibiotics can complex cholesterol in the

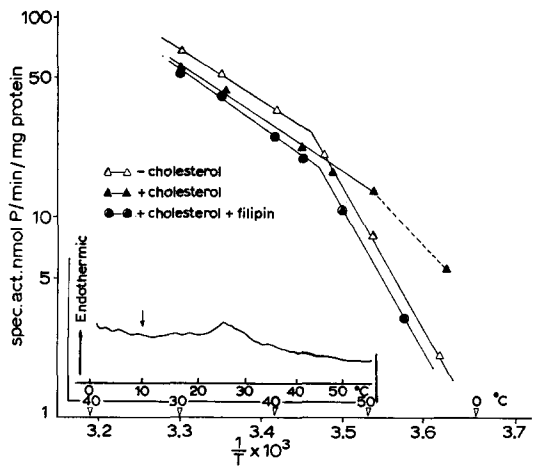

Fig. 8. Arrhenius plot of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 18:1t with or without cholesterol. The calorimetric scan of the cholesterol containing membranes was recorded at a range of 0.5. The effect of filipin upon the break in the Arrhenius plot of the ATPase activity in the cholesterol containing membranes was determined as follows. 10 ml membrane suspension (containing 1 mg protein) was incubated at 25 °C for 30 min with 1 mg filipin (10 mg/ml dimethylformamide). Immediately after the incubation 0.65-ml aliquots were taken for the determination of the ATPase activity at various temperatures as described in Materials and Methods. In control experiments it was found that the dimethylformamide did not influence the activity of the ATPase at the various temperatures.

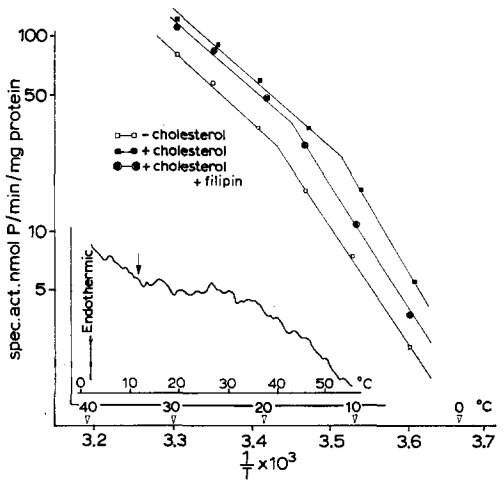

Fig. 9. Arrhenius plots of the ATPase activity and lipid phase transition in membranes from *A. laidlawii* cells grown on 18:0 with or without cholesterol. The calorimetric scan of the cholesterol containing membranes was recorded at a range of 0.2. For the effect of filipin, see the legend of Fig. 8.

A. laidlawii cell membrane[16,20] we also investigated the effect of filipin upon the break in the Arrhenius plot of the ATPase activity in membranes of *A. laidlawii* cells grown on cholesterol. Figs 8 and 9 clearly demonstrate that the addition of filipin to the cholesterol-containing membranes increases the temperature of the break in the ATPase activity up to the value observed in the cholesterol free membranes. Filipin withdraws cholesterol from its interaction with other lipids and we now observe the same temperature dependency of the ATPase activity as in the cholesterol-free membranes.

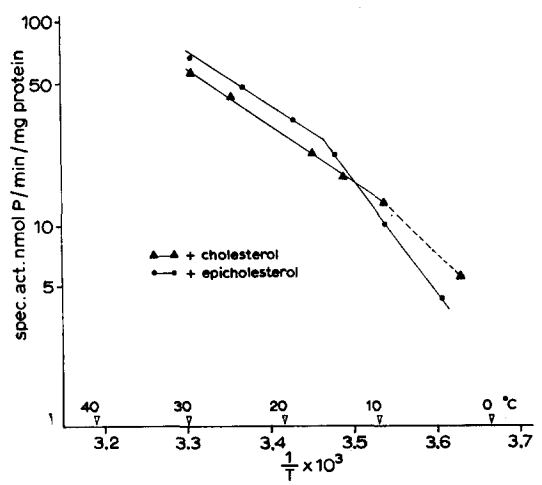

Fig. 10. Comparison of cholesterol and epicholesterol incorporation in decreasing the temperature of the break in the ATPase activity in membranes from *A. laidlawii* cells grown on 18:1t.

Epicholesterol, the 3α-OH isomer of cholesterol can also be incorporated into the *A. laidlawii* cell membrane up to 9 wt % of total lipid[8,13]. It was demonstrated that this sterol caused virtually no change in the lipid phase transition[8] because its interaction with other membrane lipids is much less than cholesterol[8,13]. This is also evident in the temperature dependence of the ATPase activity. Fig. 10 shows that the incorporation of epicholesterol in the *A. laidlawii* cell membrane does not decrease the temperature of the break in the Arrhenius plot of the ATPase activity as is observed with cholesterol. These experiments strongly support the conclusions reached above that the break in the Arrhenius plot of the ATPase activity is associated with the temperature of the lower end of the lipid phase transition.

Our results agree well with the data obtained by Kimelberg and Papahadjopoulos[21] on the reactivation by lipids of a lipid depleted $(Na^+ + K^+)$-ATPase preparation of rabbit kidney cortex. They observed reactivation only with lipids where the fatty acid chains were in the liquid crystalline state. Furthermore, in the Arrhenius plot in the activity of the activated $(Na^+ + K^+)$-ATPase, a break occurred at the temperature of the gel→liquid–crystalline transition of the phospholipid used for activation.

DISCUSSION

The Arrhenius plots of the NADH oxidase and *p*-nitrophenylphosphatase activity in the *A. laidlawii* cell membrane showed no discontinuities although the fatty acid chains of the membrane lipids underwent a gel→liquid–crystalline phase transition in the temperature range studied. In the solubilization studies of Ne'eman *et al.*[22] it was observed that the NADH oxidase and *p*-nitrophenylphosphatase activity but not the ATPase activity of the *A. laidlawii* cell membranes, could be separated from the membrane lipids. Both findings indicate that the former enzymes, although membrane bound, do not depend on membrane lipids for activity.

In the Arrhenius plot of the ATPase activity in the *A. laidlawii* cell membrane, discontinuities were observed. Similar non-linear Arrhenius plots of membrane bound ATPases have been described in various organisms[2,19,21]. In this paper we demonstrate that the break in the Arrhenius plot of the ATPase activity is associated with the broad lipid phase transition occurring in the *A. laidlawii* cell membrane. This broad transition must be caused by a gradual melting of the molecular species of the various lipids present in the membrane. First the molecular species with the lowest transition temperature will "melt" and as the temperature is further increased the molecular species with higher transition temperatures will "melt". By varying the fatty acid composition of the membrane lipids and thus varying the temperature of the lipid phase transition, we observed that the temperature of the break in the Arrhenius plot occurs at the temperature of the lower end of the lipid phase transition (Figs 4–7). From these results we conclude that at the temperature of the discontinuity in the Arrhenius plot the ATPase is associated with these molecular species of lipids which have the lowest transition temperature.

This preferential association might be caused by two possible mechanisms; (a) at higher temperatures, above the lipid phase transition the ATPase is specifically associated with those molecular species of lipids which have the lowest transition temperature. When the temperature is decreased to below the temperature of the

lipid phase transition of these molecular species of lipid the enzyme undergoes a conformational change which affects its activation energy. (b) Alternatively at temperatures above the lipid phase transition the enzyme may be randomly associated with all molecular species of lipids present in the membrane. There is a fast exchange between the liquid–crystalline species associated with the enzyme and the liquid–crystalline species present in the membrane. However, the association between the enzyme and a molecular species of a lipid in the liquid–crystalline state (EL_l) is more favourable than the association of the enzyme with a molecular species of a lipid in the gel state (EL_g) according to Eqn 1:

$$EL_g + L_l \rightarrow EL_l + L_g \quad (1)$$

As soon as a molecular species of lipid associated with the ATPase attains the gel state, it will be exchanged for a molecular species of lipid which still is in the liquid–crystalline state. This can proceed until the lower end of the phase transition is reached. When the molecular species of lipids with lowest transition temperature to which the enzyme is associated, also undergo a phase transition, a change in the conformation of the enzyme occurs which gives an increased activation energy. Another formulation of this second mechanism could be that the ATPase associates only with the lipids which are in the liquid–crystalline state because of the tendency of lipids in the gel state to segregrate in the membrane[1]. Support for this mechanism is found in the work of Verkley *et al.*[23]. They demonstrated that the particles observed by freeze–etch electron microscopy in the fracture face of the *A. laidlawii* cell membrane became aggregated at temperatures in or below the lipid phase transition. These particles which are believed to be of protein nature apparently tend to be excluded from those parts of the membrane where the lipids are in the gel state.

Cholesterol incorporation decreased the temperature of the break in the Arrhenius plot of the ATPase activity and the starting temperature of the lipid phase transition in the *A. laidlawii* cell membrane to the same extent. In our previous work we noticed that cholesterol incorporation in the *A. laidlawii* cell membrane decreased the temperature below which the cells become fragile[8,13].

Before trying to explain the temperature shift observed with cholesterol we will very briefly summarize the present state of knowledge about the lipid–cholesterol interaction and in particular the effect of cholesterol upon the lipid phase transition. From differential scanning calorimetry and NMR work the concept has been advanced that in lecithin bilayers containing less than 33 mole % cholesterol, two pools of lecithin molecules are present (see review by Phillips[1]). One pool is complexed by cholesterol and independently of temperature, the fatty acid chains are in a state of intermediate fluidity. The other pool consists of uncomplexed lecithin molecules with fatty acid chains in the liquid–crystalline (above the transition temperature) or in the gel state (below the transition temperature). The phase transition in the uncomplexed lecithin molecules is broadened because of loss of the cooperative movements, produced by the presence of cholesterol–lecithin complexes. Above 33 mole % cholesterol no phase transition can be detected with differential scanning calorimetry. Apparently only cholesterol–lecithin complexes are present. However, Raman spectroscopy studies indicated that in this system a 20 °C broad non-cooperative transition occurs[24], indicating that the lipids complexed by cholesterol may still undergo a phase transition. In the *A. laidlawii* cell membrane about 16

mole % cholesterol can be incorporated[8]. This incorporation decreased the energy content of the lipid phase transition because of the formation of lipid–cholesterol complexes[8]. However, a considerable transition of uncomplexed lipid molecules remains (ref. 8, see also Figs 8 and 9).

There are several possible mechanisms for explaining the cholesterol-induced decrease in the break in the Arrhenius plot of the ATPase activity observed in this study. First, if we assume that cholesterol randomly interacts with all molecular species of lipids present in the membrane a general broadening of the phase transition of the uncomplexed lipids will give a decreased starting temperature of the lipid phase transition and a decreased temperature of the break in the Arrhenius plot of the ATPase activity. The broadening of the lipid phase transition also must be observed as an increase in the temperature of the upper end of the phase transition. As yet we have no indication that cholesterol increases the upper end of the lipid phase transition in the *A. laidlawii* cell membrane. In some experiments we observed no effect of cholesterol upon the upper end of the phase transition (*cf.* Figs 4 and 8) and sometimes cholesterol even induces a small decrease of the temperature of the upper end of the phase transition[8].

Another possibility would be that cholesterol preferentially interacts with particular molecular species of the various lipids present in the membrane. In the liposomal system we have demonstrated with differential scanning calorimetry that cholesterol interacts specifically with lecithins in the liquid–crystalline state when both lecithins in the liquid–crystalline and gel state are present[25]. If we extrapolate this observation to the *A. laidlawii* cell membrane than we should expect to have at temperatures in the lipid phase transition a specific complexation of cholesterol with the molecular species of lipids which are still in the liquid–crystalline state. At the temperature of the lower end of the phase transition cholesterol will be preferentially associated with the molecular species of lipids with the lowest transition temperature. Since the ATPase also is associated with these molecular species, a broadening of the transition of these molecular species by the complex formation with cholesterol, would account for the observed decrease in the temperature of the break in the Arrhenius plot of the ATPase activity.

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