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ALTERATIONS IN PHOSPHOLIPID-DEPENDENT $(Na^+ + K^+)$-ATPase ACTIVITY DUE TO LIPID FLUIDITY

EFFECTS OF CHOLESTEROL AND Mg^{2+} *

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SUMMARY

The $(Na^+ + K^+)$-activated, Mg^{2+}-dependent ATPase from rabbit kidney outer medulla was prepared in a partially inactivated, soluble form depleted of endogenous phospholipids, using deoxycholate. This preparation was reactivated 10 to 50-fold by sonicated liposomes of phosphatidylserine, but not by non-sonicated phosphatidylserine liposomes or sonicated phosphatidylcholine liposomes. The reconstituted enzyme resembled native membrane preparations of $(Na^+ + K^+)$-ATPase in its pH optimum being around 7.0, showing optimal activity at Mg^{2+}: ATP mol ratios of approximately 1 and a K_m value for ATP of 0.4 mM.

Arrhenius plots of this reactivated activity at a constant pH of 7.0 and an Mg^{2+}: ATP mol ratio of 1:1 showed a discontinuity (sharp change of slope) at 17 °C, with activation energy (E_a) values of 13–15 kcal/mol above this temperature and 30–35 kcal below it. A further discontinuity was also found at 8.0 °C and the E_a below this was very high (> 100 kcal/mol).

Increased Mg^{2+} concentrations at Mg^{2+} : ATP ratios in excess of 1 : 1 inhibited the $(Na^+ + K^+)$-ATPase activity and also abolished the discontinuities in the Arrhenius plots.

The addition of cholesterol to phosphatidylserine at a 1.1 mol ratio partially inhibited $(Na^+ + K^+)$-ATPase reactivation. Arrhenius plots under these conditions showed a single discontinuity at 20 °C and E_a values of 22 and 68 kcal/mol above and below this temperature respectively. The ouabain-insensitive Mg^{2+}-ATPase normally showed a linear Arrhenius plot with an E_a of 8 kcal/mol. The cholesterol-phosphatidylserine mixed liposomes stimulated the Mg^{2+}-ATPase activity, which now also showed a discontinuity at 20 °C with, however, an increased value of 14 kcal/mol above this temperature and 6 kcal/mol below. Kinetic studies showed that cholesterol had no significant effect on the K_m values for ATP.

Since both cholesterol and Mg^{2+} are known to alter the effects of temperature on the fluidity of phospholipids, the above results are discussed in this context

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INTRODUCTION

Phospholipid fluidity seems to markedly affect the activity of a number of membrane-bound enzymes, including the $(Na^+ + K^+)$-activated Mg^{2+}-dependent ATPase (EC 3.6 1 3) [1, 2] Wilson et al. [3] and Overath et al [4], using *E coli* auxotrophs, showed that alterations in the saturation of fatty acid supplements affected the membrane transport properties and enzyme activity of these auxotrophs Arrhenius plots of these activities showed discontinuities or changes in slopes, and therefore activation energies (E_a), at characteristic temperatures which depended on the chain length and degree of saturation of the fatty acids. A large number of studies on *E. coli* [5, 6], *Mycoplasma* [7, 8], mitochondrial [9–13], microsomal [14] and sarcoplasmic reticulum [15] enzymes, and plasma membrane enzymes such as the $(Na^+ + K^+)$-ATPase [16–21] and adenyl cyclase [22, 23] have also shown discontinuities when the temperature dependence of their activities was plotted as Arrhenius plots

The fact that such discontinuities appear to reflect the phase transitions of membrane lipids has been demonstrated by studies which have shown a correlation between the discontinuity temperatures and independently measured phase transition temperatures of the intact membranes and the extracted lipids [2, 4, 7, 8, 14, 15, 24, 25]. The lipid phase transitions of cell membranes are generally quite broad, even in the absence of cholesterol [26] Recent studies have also indicated that Arrhenius plots of membrane enzyme activity often show at least two discontinuities which can be correlated with the limits of the broad phase transition or phase separation [24, 25].

It has been suggested [27] that the discontinuities seen for $(Na^+ + K^+)$-ATPase activity may be due to uncorrected temperature effects on the pH of the reaction media, although this study also involved variations in Mg^{2+} concentration. Divalent cations [2, 28, 29] and cholesterol [7, 8, 30, 31] have been shown to alter the fluidity of phospholipids, both in model systems and cell membranes, and this report is concerned with the effects of cholesterol and Mg^{2+} on the activity of reconstituted $(Na^+ + K^+)$-ATPase activity at varying temperatures and constant, optimal pH

METHODS AND MATERIALS

Preparation of $(Na^+ + K^+)$-ATPase $(Na^+ + K^+)$-ATPase was purified and delipidated using the microsomal fraction from homogenized, frozen rabbit kidney outer medulla as starting material. (Pel-Freez Biologicals, Inc., Rogers, Arkansas) This was done by deoxycholate treatment and subsequent ammonium sulfate fractionation exactly as previously described [2]. The deoxycholate was removed by dialysis at approximately 4 °C against 100 vols of the final suspending medium for the first 24 h, and 25 vols for the following 48 h. A subsequent 24-h dialysis showed some inhibition of activity (see Table I). It has been shown that this treatment removes at least 95 % of the lipid phosphorus present in the microsomal fraction [1].

Preparation of lipids and liposomes. Phosphatidylserine was prepared from bovine brain and phosphatidylcholine from egg yolks, as previously described [32, 33]

TABLE I

EFFECT OF INCREASING DIALYSIS TIME ON ENZYME ACTIVITY

The final $(NH_4)_2SO_4$ precipitate was suspended in 10 ml of 50 mM Tris/acetate, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA and 20 % (v/v) glycerol and dialyzed against the indicated volumes of the same buffer at ≈ 4 °C. The activity was measured at 37 °C as described under Materials and Methods in the presence of sonicated phosphatidylserine liposomes The total volume was 1 5 ml and 0.13 mg of enzyme protein was added.

Time of dialysis (h)	Volume of buffer (ml)	μmol P_i/mg protein/h	
		$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase
24	1000	15 1	15 02
72	250	28.5	16 52
96	250	19 94	17 70

It was chromatographically pure as determined by thin-layer chromatography on silica gel H with a solvent of chloroform/methanol/7M ammonia (230:90:15, v/v/v). It was stored at −40 to −50 °C as small aliquots (in sealed vials flushed with nitrogen) at a concentration of 10 μmol/ml. The μmol of phospholipid were determined by inorganic phosphate estimates after perchloric acid digestion. Each vial was used only once. Cholesterol was purchased from Sigma and recrystallized twice from methanol [33].

Sonicated or non-sonicated liposomes were prepared under a nitrogen atmosphere as previously described [2, 33]. In summary, the method was as follows; chloroform solutions of phosphatidylserine and cholesterol were first mixed to the appropriate concentrations in the sonicating tube, the chloroform evaporated, the tube flushed with nitrogen and buffer added. The closed tube containing a nitrogen atmosphere was then shaken for 10 min using a mixer When desired it was then sonicated in a bath-type sonicator at ≈ 20 °C for 1 h.

Assay of ATPase activity. ATPase activity was assayed in the following basic medium: 100 mM NaCl, 10 mM KCl, 50 mM histidine · HCl, 3 mM $MgCl_2$, 0.1 mM

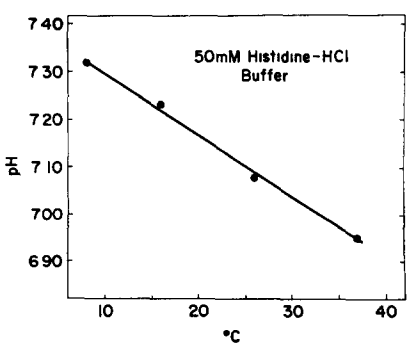

Fig. 1 Effect of temperature on pH of histidine·HCl buffer The composition of the medium was: 100 mM NaCl, 10 mM KCl, 50 mM histidine HCl, 3 mM $MgCl_2$ and 0.1 mM EDTA. The pH was adjusted to 7 08 with 1 M HCl at 26 °C and the pH measured at other values as indicated

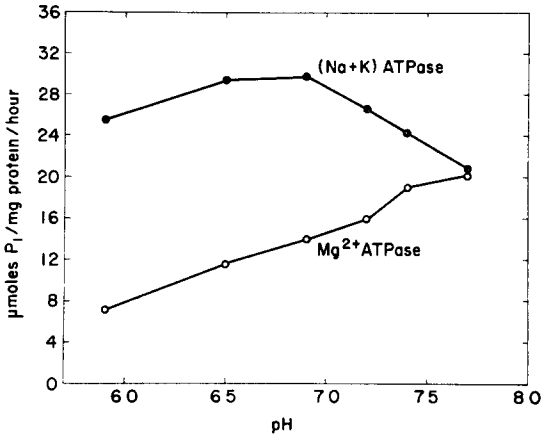

Fig 2 Effect of pH on activity of phosphatidylserine-activated ATPase Activity was assayed at 37 °C, as described in Materials and Methods, with 0 13 mg of enzyme protein in a total volume of 1 5 ml plus 0 5 μmol sonicated phosphatidylserine liposomes The reaction time was 20 min, and was initiated by adding 5 μmol ATP to a concentration of 3 3 mM

EDTA Other details are as described previously [1, 2] For certain experiments the $MgCl_2$ concentration was varied up to 10 mM $MgCl_2$ The $(Na^+ + K^+)$-ATPase activity was the activity sensitive to inhibition by 1 mM ouabain. The pH of the histidine-buffered medium varied by about 0.3 units for a 30 °C temperature change (see Fig 1). The pH in temperature studies was corrected for this, although an alteration in pH from the optimum activity at 7 0 at 8 °C to 7.3 at 37 °C would only decrease the activity by approx. 10 % (see Fig 2). The enzyme was equilibrated with the liposomes in the complete reaction mixture, except for substrate, at the reaction temperature for 15 min. The amount of enzyme protein present in the 1.5 ml final volume was 0.13 mg, except in the case of the experiments shown in Fig. 7 and some of the experiments shown in Fig. 6 when 0.076 mg protein was added The reaction was then initiated by adding 5 μmol ATP and measured for 10 or 20 min, although the reaction was linear up to at least 40 min.

Chemicals Ammonium sulfate (ultra pure) was from Schwarz/Mann, Orangeburg, N Y Sodium deoxycholate (M A) was from Mann Research Laboratories, Inc , New York, N Y ATP-(Na_2), ouabain octahydrate and L-histidine were from Sigma Chemical Co , St Louis, Mo. All other chemicals were of reagent, analyzed quality Water was double distilled, the final distillation being from glass.

RESULTS

Effect of sonication on reactivation

The effectiveness of phosphatidylserine in reactivating delipated $(Na^+ + K^+)$-ATPase is dependent on whether the phospholipid has been sonicated or not (see Fig. 3) We sonicated under conditions which have been shown to result in 90 % of the lipid phosphorus appearing as a homogeneous population of single-walled vesicles of 250–500 Å diameter [33, 34] As shown in Fig 3, sonicated phosphatidylserine liposomes activate $(Na^+ + K^+)$-ATPase from an average control value of 0.6

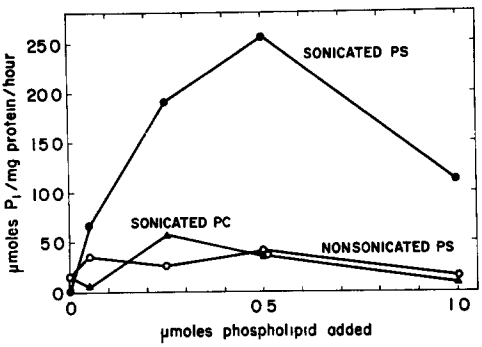

Fig 3. Effect of sonication of liposomes on reactivation of $(Na^+ + K^+)$-ATPase activity. The activity of the $(Na^+ + K^+)$-ATPase only is plotted The activity was assayed as described under Materials and Methods The liposomes were added at the various concentrations indicated to the reaction mixture at 37 °C containing 0 13 mg enzyme protein, and allowed to equilibrate for 15 min before initiating the reaction by adding ATP PS, phosphatidylserine; PC, phosphatidylcholine

μmol P_i/mg protein/h to a maximal value of 27.6 at 0.5 μmol phosphatidylserine. The original microsomal fraction had a specific activity of 14.5 μmol P_i/mg protein/h. Additions of higher concentrations of phosphatidylserine result in inhibition. In contrast, non-sonicated phosphatidylserine liposomes showed only minimal activation, as did sonicated phosphatidylcholine liposomes.

Reactivation and increasing Mg^{2+} concentration

Maximum $(Na^+ + K^+)$-ATPase activity is generally found at a mol ratio of around 1:1 Mg^{2+}:ATP, at ATP concentrations of 3 mM [35, 36]. This is also true for the phosphatidylserine-activated $(Na^+ + K^+)$-ATPase, as shown in Fig. 4.

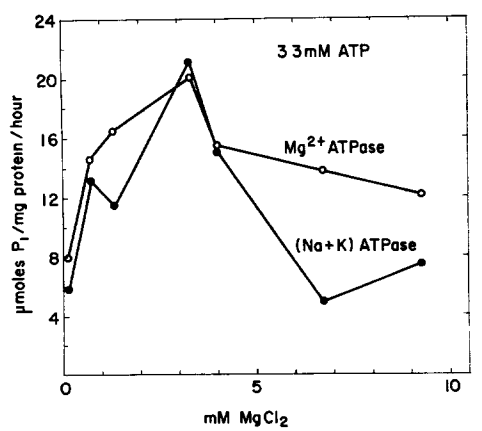

Fig. 4. Effect of increasing $MgCl_2$ concentration on ATPase activity. Activities were assayed at pH 7 08 for 20 min reaction times at 37 °C as described in Methods and Materials and Fig 2. For these experiments and the remainder of the figures, the activity is assayed in the presence of 0 5 μmol sonicated phosphatidylserine liposomes or 1 μmol 1 .1 mixed phosphatidylserine plus cholesterol liposomes

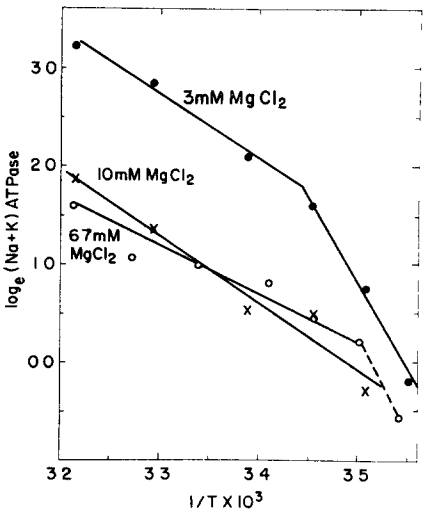

Fig 5 Effect of increased $MgCl_2$ on the Arrhenius plots of $(Na^+ + K^+)$-ATPase activity The activities were assayed as described previously, except that the $MgCl_2$ concentrations were increased as indicated

Inhibition is seen at concentrations higher than this, which for the reconstituted $(Na^+ + K^+)$-ATPase is somewhat greater than that usually seen for the $(Na^+ + K^+)$-ATPase [35, 36]

As mentioned in the introduction, Arrhenius plots of both native and reconstituted $(Na^+ + K^+)$-ATPase activity show discontinuities, which for the reconstituted enzyme are dependent on the type of lipid used [2]. Fig 5 shows that the $(Na^+ + K^+)$-ATPase reconstituted with phosphatidylserine has a discontinuity at around 17 7 °C when the ATP concentration is 3 3 mM and the Mg^{2+} 3.0 mM, in agreement with previous results [2] There is a 2.7-fold difference in activation energies; 13 0 kcal/mol at higher and 35 2 kcal/mol at lower temperatures, respectively When, however, the ATP concentration is kept at 3.3 mM but the Mg^{2+} concentration is increased, not only is the $(Na^+ + K^+)$-ATPase activity inhibited, as expected from the results shown in Fig 4, but the discontinuity is abolished.

Effects of cholesterol on Arrhenius plots

Since cholesterol has also been shown to inhibit the reactivation by phosphatidylserine of delipidated $(Na^+ + K^+)$-ATPase [2], it was of interest to investigate its effect on the temperature dependence of the $(Na^+ + K^+)$-ATPase activity As shown in Fig. 6, cholesterol at a 1 1 mol ratio to phosphatidylserine inhibits the $(Na^+ + K^+)$-ATPase activity throughout the temperature range It shifts the temperature of the discontinuity from 16 9 to 20 6 °C, and increases the activation energies (see Table II). In this series of experiments we included further points at 8.1 and 6.8 °C for the $(Na^+ + K^+)$-ATPase control curve and there appears to be a second discontinuity at around 8 1 °C. The two discontinuities may correspond to the lower and upper temperatures of the broad phase separation seen in the differential scanning calorimeter for brain phosphatidylserine [2, 29].

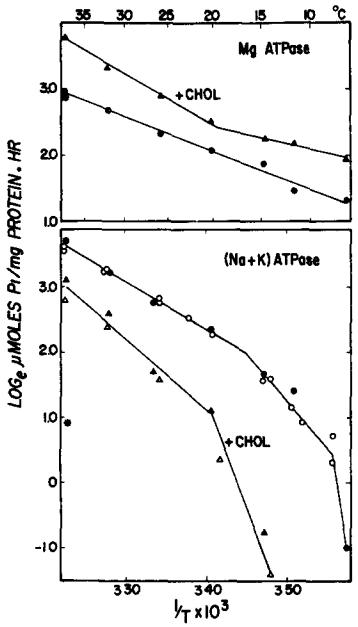

Fig. 6 Effects of cholesterol on Arrhenius plots of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activity The activities were assayed in the presence of 0 5 μmol sonicated phosphatidylserine and of sonicated mixed liposomes consisting of 0.5 μmol phosphatidylserine plus 0 5 μmol cholesterol. In the case of the results shown as closed symbols 0 076 mg enzyme protein was present. For the open symbols 0 132 mg enzyme protein was used as before. The pH of the media at the various temperatures was maintained within the pH range 6.91–7.07. The asterisks indicate the activities in the absence of added lipid

TABLE II

ACTIVATION ENERGIES AND DISCONTINUITY TEMPERATURES FROM ARRHENIUS PLOTS IN THE PRESENCE AND ABSENCE OF CHOLESTEROL

Values derived from plots shown in Fig. 6. Activity measured as described under Materials and Methods in the presence of sonicated phosphatidylserine or phosphatidylserine plus cholesterol liposomes.

	$(Na^+ + K^+)$-ATPase		Mg^{2+}-ATPase	
	Discontinuity temperatures (°C)	Activation energies (kcal/mol)	Discontinuity temperatures (°C)	Activation energies (cal/mol)
No cholesterol		> 100		
	7 9			
		30		
	16 9		—	8
		15		
+ cholesterol		68		6
	20.6		19.7	
		22		14

The actual values at which the discontinuities occur and the various activation energies derived from Fig 6 are shown in Table II. The activation energy values in Table II are shown on a line above or below the appropriate discontinuity temperature The temperature of the upper discontinuity shown in Fig. 6 of 16 9 °C for $(Na^+ + K^+)$-ATPase is very close to the value of 17 7 °C found in the separate experiment shown in Fig 5, and the value of 15 6 °C reported previously for phosphatidylserine-activated ATPase [2] The activation energies on either side of this discontinuity are also reasonably similar for Figs 5 and 6 The highest activation energy seen of greater than 100 kcal/mol for the lowest temperature part of the $(Na^+ + K^+)$-ATPase curve, is clearly very high and subject to considerable error in its calculation. The open and closed symbols shown in Fig. 6 are for two separate preparations and the results are from four different experiments.

In the presence of cholesterol the second discontinuity at lower temperatures is not seen, although this may be due to the activities being very low at these temperatures and, consequently, making accurate estimates of the activity difficult The lowest activity in the presence of cholesterol was a recorded absorbance difference of 0.005 absorbance units, which was about the smallest detectable difference we could measure.

The effect of cholesterol on the ouabain-insensitive Mg^{2+}-ATPase was opposite to that on the $(Na^+ + K^+)$-ATPase. It stimulates throughout the temperature, and in fact induces a reverse discontinuity at 19.7 °C, with is close to the temperature at which the discontinuity is seen for the $(Na^+ + K^+)$-ATPase The Mg^{2+}-ATPase in the absence of cholesterol seems perfectly linear with a low activation energy of 8 kcal/mol. The two activation energies in the presence of cholesterol are 14 and 6 kcal/mol. respectively

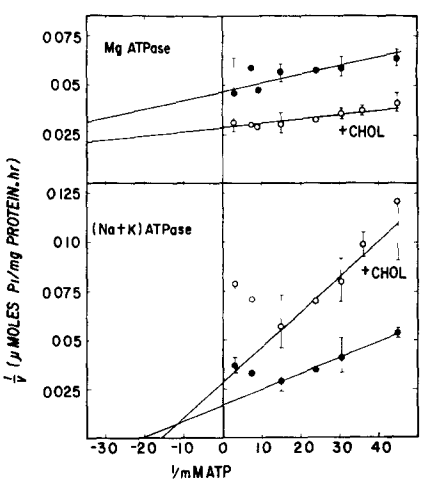

Fig 7 Effect of cholesterol on V and K_m of ATPase activity. The activities were assayed as described previously with sonicated phosphatidylserine or mixed phosphatidylserine-cholesterol liposomes (see legend to Fig. 6) The reaction media contained 0 076 mg enzyme protein and the rates were determined for a 10-min reaction time instead of the customary 20 min The calculations were done on a PDP 12 computer using a program based on ref 37 The Mg^{2+} ATP mol ratio was maintained at 1 1 throughout.

TABLE III

K_m AND V VALUES FOR PHOSPHATIDYLSERINE ACTIVATED ATPase IN THE PRESENCE AND ABSENCE OF CHOLESTEROL

Values ±S.E M. derived from the plots shown in Fig 7 according to the method of Bliss and James [37] excluding the values at the two highest substrate concentrations for $(Na^+ + K^+)$-ATPase Assay conditions as in Table II

	V (μmol P_i/mg protein per h)		K_m (mM ATP)	
	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase
No cholesterol	21 5±1.6	56 2±15 6	0 095±0.047	0 44±0 25
+ cholesterol	34.9±1 9	34 2±10 7	0 075±0 031	0 62±0.34

Effect of cholesterol on V and K_m values

Fig. 7 shows the influence of cholesterol on the kinetics of ATP hydrolysis. Reciprocals of the initial rates over the first 10 min are plotted against reciprocals of the ATP concentration. The ouabain-sensitive $(Na^+ + K^+)$-ATPase is shown in the lower and the ouabain-insensitive Mg^{2+}-ATPase in the upper panel. The points shown on the graph are means ± standard deviation. The lines are the calculated best fit for all the data points according to the method of Bliss and James [37]. The points at the two highest substrate concentrations for the $(Na^+ + K^+)$-ATPase lines were, however, excluded as they are beginning to show substrate inhibition. The derived values of V and K_m are shown in Table III, ± standard error of the mean. As can be seen, the V values differ significantly in the presence and absence of cholesterol for the Mg^{2+}-ATPase, but the standard errors overlap in the case of the V values for the $(Na^+ + K)^+$-ATPase. Cholesterol inhibits the mean V of the $(Na^+ + K^+)$-ATPase 49 %, and stimulates the Mg^{2+}-ATPase V 62 %. The K_m values for both ATPase activities are not significantly different

DISCUSSION

Comparison of reconstituted and membrane preparations of $(Na^+ + K^+)$-ATPase

Studies on the reconstitution of delipidated membrane enzymes with lipids provides a convenient way of studying the effects of variations in lipid composition or physical properties on enzyme activity. It provides a method of investigating what may be the lipid requirements of the enzymes in situ, and the details of the interaction of the enzyme with its membrane environment at the molecular level. This approach has been used extensively to investigate the lipid requirements of the $(Na^+ + K^+)$-ATPase (see recent review, ref. 38) and more recently to examine the effects of variations in lipid fluidity [1, 2].

A critical premise in such an approach is that the properties of reconstituted enzymes significantly resemble the enzyme in its natural state. The present preparation reconstituted with brain phosphatidylserine resembles membrane preparations of $(Na^+ + K^+)$-ATPase in several basic properties. Thus, the optimal Mg^{2+}: ATP mol ratio of 1·1 is identical to that found for the natural membrane preparation, as well as the inhibition at high Mg^{2+} levels [35, 36, 39]. The pH optimum of 7.0 for the $(Na^+ + K^+)$-ATPase and a more alkaline pH optimum for the Mg^{2+}-ATPase is similar to that reported for ATPase activities from a variety of tissues [39].

The apparent K_m value for ATP for the $(Na^+ + K^+)$-ATPase of 0 44 to 0.62 mM is within the range of published values of 0 25–1.0 mM for rat brain $(Na^+ + K^+)$-ATPase [40, 41] and 0.30 mM for the $(Na^+ + K^+)$-ATPase activity of the rectal gland of the dogfish [42] Lower K_m values for the Mg^{2+}-ATPase was also seen for rat brain microsomal ATPase [40] Substrate inhibition is not generally seen for the $(Na^+ + K^+)$-ATPase, although in most cases previous authors did not show similarly high concentrations of ATP However, it may be a feature of the reconstituted enzyme. Since we did not see substrate inhibition in the case of the Mg^{2+}-ATPase, it is unlikely to be due to the presence of a non-specific inhibitor of ATPase activity. It could be due to product inhibition by ADP during the course of the reaction, although we found less than 5 % of inorganic phosphate in our ATP control samples and at the highest concentration of ATP added (5 μmol) only about 10 % of the ATP was hydrolyzed in 10 min. The inhibition by cholesterol is quite marked at the highest ATP concentration of 3 3 mM (5 μmol in 1 5 ml), although it is seen throughout the concentration range. All our assays were done at this concentration to conform with previous work and the main body of published work [39].

Requirement for sonicated liposomes

The absolute requirement for sonicated phosphatidylserine liposomes shown in Fig 3 is quite dramatic. It is undoubtedly a reflection of the large increase in surface area in going from multilamellar liposomes of 1–10 μm diameter, to bi- or unilamellar liposomes of 200–500 Å diameter [32]. There is also some evidence that sonicated liposomes show phase transitions at somewhat lower temperatures [2, 31], indicating a more fluid bilayer

Arrhenius plots and fluidity

Although some reports have indicated linear Arrhenius plots for $(Na^+ + K^+)$-ATPase activities, these were for limited temperature ranges that did not go below 20 °C [40] or for extrapolated V values at pH 8 0, which might have had some effect [41]

As outlined in the discussion, a large number of studies do show discontinuities for $(Na^+ + K^+)$-ATPase activity. It has recently been suggested, however, that these discontinuities were due to uncorrected, temperature-induced pH changes in the buffer [27]. In these studies, however, the pH-corrected experiments which gave linear Arrhenius plots were also performed at increased Mg^{2+} concentrations, which, as we have shown here, can eliminate these discontinuities and decrease the E_a values We do not know, however, whether the present results can be extrapolated to natural membrane preparations of $(Na^+ + K^+)$-ATPase [27].

As mentioned in the introduction, the discontinuities or sharp changes in slope seen in Arrhenius plots have been correlated with the phospholipid phase transitions of reconstituted or natural membranes for several enzyme activities. Recently, at least two discontinuities have been detected for several enzyme activities in *E coli* [24] and mouse LM cells [25], which have been suggested to represent the lower (t_1) and upper (t_h) temperature limits of a broad-phase separation [24]. The present results appear to be consistent with this (see Fig. 6), with a T_h at 17 °C and a T_1 at 8 °C. It is of interest that two discontinuities at 20 and 6 °C were also reported for rat brain ATPase, although this was found for both total and Mg^{2+}-ATPase [16].

These values are close to the values of 5 and 20 °C reported as the limits for the phase transitions of sonicated brain phosphatidylserine liposomes in 0.1 M NaCl at pH 7.4 as measured in the differential scanning calorimeter [2], and in the same range as the values of 0 and 12 °C also reported [29] for the same phospholipid. The amount of free Mg^{2+} available should be very small under the ATPase reaction conditions, with an equilibrium constant for Mg^{2+}-ATP of 10^{-5}–10^{-6} [43]. The absence of a discontinuity for the Mg^{2+}-ATPase further supports the concept that the discontinuities reflect phase transitions or separations of the phospholipids, since the Mg^{2+}-ATPase has a relatively low E_a value and the phospholipids have no stimulating effect on it (see Fig. 6). It was stimulated, however, by the presence of cholesterol which appears to be due to the decreased fluidity, since saturated phospholipids also stimulate [2].

Effects of cholesterol

The effect of cholesterol on the shape of the Arrhenius plot is consistent with its known effects of decreasing the fluidity of fluid phospholipid membranes and progressively decreasing the enthalpy, but not the temperature, of the phase transition [30]. Thus, we find that the position of the discontinuity is shifted only slightly from 16 9 to 20 6 °C (Table II). This could remain the upper limit of the phase transition, the lower temperature being undetectable because the activity is too low to be detected. The increases in activation energy are then consistent with a decreased fluidity of the membrane. However, cholesterol at a 1.1 mol ratio abolishes the phase transitions of a variety of phospholipid liposomes as detected by several techniques which measure overall bulk properties [30, 31]. Thus it is necessary to postulate a modification of this effect of cholesterol in the vicinity of the $(Na^+ + K^+)$-ATPase. There is some evidence for such microenvironments for several membrane enzymes including the $(Na^+ + K^+)$-ATPase [20]. Indeed, the fact that so many enzymes located in membranes which contain high levels of cholesterol appear to respond to the occurrence of temperature-induced phase transitions in membrane phospholipids implies the existence of cholesterol-poor microenvironments around such enzymes.

It is interesting that opposite effects of cholesterol on E_a values are seen for the Mg^{2+}-ATPase. It is unclear whether this Mg^{2+}-ATPase is also derived from the plasma membrane. It could partly represent mitochondrial ATPase present as an impurity in the microsomal fraction. However, the mitochondrial ATPase is lipid dependent and shows discontinuities in Arrhenius plots of its activity [9, 11, 12]. Increasing membrane sterol levels leads to decreased discontinuity temperatures with no marked changes in E_a values in the case of *Acholeplasma* ATPase [8] and yeast mitochondrial ATPase [44].

The results from the kinetic studies of Fig. 7 show that the only significant effect of cholesterol is to increase the V of the Mg^{2+}-ATPase. It may decrease the V of the $(Na^+ + K^+)$-ATPase but the present data is insufficiently precise for a definite statement to be made on this. It is clear that there is no affect on the affinity of the two ATPases for ATP. These results are of interest since it implies that effects on the V of the enzyme need not indicate only a change in the amount of active enzyme, but might also be due to alterations in the state of its membrane environment. Effects of alterations in membrane lipids on the V but not the K_m of the ATPase reaction have been reported by others [45, 46].

Effects of $MgCl_2$

Increased Mg^{2+} is known to inhibit the $(Na^+ + K^+)$-ATPase [35, 36] and other ATPases [43] at concentrations in excess of the ATP concentration. These effects may be partially due to an effect on the phospholipids essential for enzyme activity, although a direct inhibitory effect due to increased free Mg^{2+} or decreased free ATP has also been postulated [36]. Several studies have indicated that Mg^{2+} can increase the temperature of the phase transition of acidic phospholipids [2, 28, 29, 47, 48], although Mg^{2+} is considerably less effective than Ca^{2+} [29] Mg^{2+} at 3–5 mM causes shifts in the temperature of the phase transition of phosphatidylserine liposomes of around 10–15 °C [2, 29] Since in the experiment shown in Fig. 5 we have a net Mg^{2+} concentration of 3 3 and 6 7 mM, the discontinuity could increase from about 17 °C in this experiment to around 37 °C, which is the upper limit of the temperature range. We might expect, however, if the phospholipids were in the solid state below 37 °C that the slope of the Arrhenius plot would be parallel to the lower temperature part of the control curve, whereas it is parallel to the upper temperature part with activation energies of 10 and 14 kcal/mol This is somewhat anomalous but resembles the effect of Ca^{2+} in decreasing the fluidity but increasing the permeability of liposomes [29] While this manuscript was in preparation an inhibitory effect of Mg^{2+} on the activity of the reconstituted Ca^{2+}-ATPase from sarcoplasmic reticulum was reported [49] Mg^{2+} inhibited the temperature activation of the enzyme and was effective only when the enzyme was reconstituted with acidic phospholipids and not with neutral lecithins, strongly supporting the idea that the inhibitory effect of Mg^{2+} was due to its interaction with acidic phospholipids. Since the $(Na^+ + K^+)$-ATPase is ineffective with neutral lipids (see Fig 3 and ref 1), we were unable to perform a similar experiment. No Arrhenius plots were reported in this study [49], so that the effect of Mg^{2+} on E_a values cannot be compared.

The present studies show that the ability of cholesterol to inhibit $(Na^+ + K^+)$-ATPase activity is consistent with its alteration of the properties of the phospholipids required for $(Na^+ + K^+)$-ATPase activity, and therefore might be a reflection of this effect The possibility that it could have this function in a normal or pathological state has been suggested [50] but not established However, because of the widespread occurrence of cholesterol in membranes it might function in this way, possibly as a relatively long-term modulator of $(Na^+ + K^+)$-ATPase activity

The role of Mg^{2+} in $(Na^+ + K^+)$-ATPase activity has not been clearly delineated. This report suggests that some of its effects, especially its inhibitory effect at high concentrations, could be via the membrane phospholipids In contrast to cholesterol, it could serve as a rapidly acting modulator of ATPase activity Similarly, the activation and inhibitory effects of Ca^{2+} on membrane ATPase enzymes might also be due to its effects on membrane phospholipids

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