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THE INTERRELATIONSHIP OF MEMBRANE AND PROTEIN STRUCTURE IN THE FUNCTIONING OF THE ($Na^+ + K^+$)-ACTIVATED ATPase

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SUMMARY

The ($Na^+ + K^+$)-ATPase is irreversibly denatured by ethanol, *tert*-butanol, guanidine hydrochloride, and sodium dodecyl sulfate. Denaturation by the alcohols proceeds by disruption of the phospholipid bilayer immediate to the ATPase with no major disruption of protein structure. Denaturation by guanidine hydrochloride disrupts protein structure with little effect on phospholipid structure. Sodium dodecyl sulfate disrupts neither protein nor phospholipid structure at concentrations which irreversibly abolish ATPase activity. The phospholipid bilayer immediate to the ATPase is more ordered than the overall membrane phospholipid bilayer as measured by its ability to orient the long axis of 3-(4',4'-dimethyloxazolidinyl-*N*-oxyl)-5α-androstan-17β-ol perpendicular to the bilayer surface. However, the interior of the bilayer immediate to the ATPase is more fluid than for the overall membrane.

INTRODUCTION

The role of lipids in the functioning of the ($Na^+ + K^+$)-activated ATPase is not well understood. The ATPase is found in plasma membranes of considerably different lipid composition[1] although the properties of the ATPase obtained from various tissues are quite similar[2,3]. ATPase solubilized with sodium deoxycholate requires addition of phospholipids for activity[4,5] as does ATPase inactivated by treatment with phospholipase C[6]. Extraction of freeze–dried red blood cell ghosts with ether has no effect on ($Na^+ + K^+$)-activated ATPase activity although all the sterols and 23 % of the phospholipids are removed[7]. On the other hand, extraction of lipids from rat brains at −75 °C with chloroform–methanol inactivates the ATPase, with the cholesterol fraction, not the phospholipid fractions, restoring activity[8]. In this paper we present evidence that an intact phospholipid bilayer surrounding the ($Na^+ + K^+$)-ATPase is necessary for activity.

MATERIALS AND METHODS

Frozen, unwashed sheep kidneys and rabbit muscle were obtained from Pel-Freeze Biologicals and stored at −20 °C until used. Rabbit muscle lactic dehydro-

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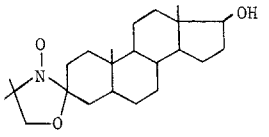
genase was obtained from Sigma Chemical Co. Rabbit muscle pyruvate kinase was prepared by the method of Tietz and Ochoa[9]. Synthesis of 3-(4',4'-dimethyloxazolidinyl-*N*-oxyl)-5α-androstan-17β-ol (I) was according to the procedure of Keana *et al.*[10]. 12-(4',4'-Dimethyloxazolidinyl-*N*-oxyl) stearic acid (II) and methyl 12-(4',4'-dimethyloxazolidinyl-*N*-oxyl) stearate (III) were prepared by the method of Waggoner *et al.*[11] and N-(2,2,6,6-tetramethyl-1-oxyl-piperidinyl) maleimide (IV) was prepared by the method of Griffith and McConnell[12].



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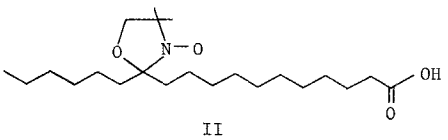

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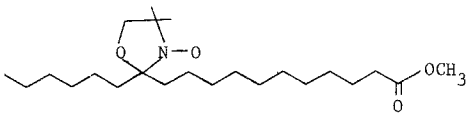

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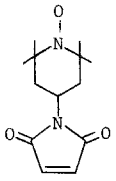


IV

Enzyme preparation

Two frozen lamb kidneys were thawed overnight at 0–4 °C in 0.32 M sucrose and 1 mM EDTA (pH 7.0). All operations were performed at 0–4 °C. The kidneys were dissected on a wooden block covered with filter paper saturated with 0.32 M sucrose and 1 mM EDTA (pH 7.0). The dark red outer medulla was removed and minced and was immediately homogenized in a Waring Blendor in 9 vol. of sucrose-EDTA. The homogenate was then fractionated according to the procedure of Schwartz *et al.*[13]. The microsomal fraction was resuspended to a protein concentration of 10 mg/ml and immediately frozen at −20 °C. Protein was determined by the method of Lowry *et al.*[14]. Two kidneys (120 g) usually yielded about 300 mg of microsomal protein. The specific activity of the ($Na^+ + K^+$)-activated ATPase at this stage was 0.30–0.35 μmole ATP hydrolyzed/mg protein per min. Henceforth this preparation will be called the microsomal enzyme.

The method for further purification of the ATPase is similar to that of Jorgensen and Skou[15]. Typically, 16 ml of the microsomal enzyme was thawed and diluted to 160 ml in 0.12 M sucrose, 25 mM imidazole (pH 7.0), 2 mM EDTA and 0.6 mg/ml sodium deoxycholate and left to stand at 20–25 °C for 30 min. The suspension was then centrifuged at 0–4 °C at 33000 rev./min (87000 × g_{av}) in a Beckman 50.1 rotor using polycarbonate screwcap tubes. The supernatant was discarded and the pellet resuspended in 16 ml of the deoxycholate–imidazole buffer and left to stand at 20–25 °C for 30 min. 3- ml portions were then layered onto 35-ml sucrose gradients which were 16.5–40.0 % (w/w) and centrifuged at 27000 rev./min (95000 × g_{av}) in a Beckman SW27 rotor for 4 h at 0–4 °C. Fractions were obtained from the sucrose gradients from a puncture in the bottom of the centrifuge tubes. The fractions con-

taining the peak enzyme activity were pooled, diluted 3-fold with 50 mM imidazole (pH 7.0) and centrifuged at 33000 rev./min for 2 h in a Beckman 50.1 rotor. The pellets were resuspended in imidazole buffer to a protein concentration of 10 mg/ml and immediately frozen at −20 °C. Two kidneys generally yielded 10–13 mg of protein with a specific activity of 14–18 μmoles ATP hydrolyzed/mg protein per min with 99 % of the ATPase activity being abolished by the cardiac glycoside ouabain. This preparation will henceforth be called the gradient enzyme. The detailed properties of this preparation will be described elsewhere.

The ATPase was assayed by the continuous method[16] with care being taken to insure that the coupling enzymes lactic dehydrogenase and pyruvate kinase were in at least a 100-fold excess.

Inhibition studies

Both the microsomal and gradient enzymes were incubated with various concentrations of ethanol, *tert*-butanol, guanidine hydrochloride and sodium dodecyl sulfate. Aliquots were taken and diluted 3000-fold into the assay mixture. It was determined independently that the various denaturants had no effect on enzyme activity at this dilution.

Spin labeling studies

Labeling of microsomal and gradient enzyme membranes with the androstanol (I), the stearate (II) and the methyl stearate labels (III) was accomplished by evaporation under a stream of nitrogen of a 1 mM solution of label in ethanol in an 0.5 ml test tube. The enzyme suspension (about 10 μl) was then added and "buzzed" for 1 min using a vortex mixer. The final concentrations were generally 0.1 mM label and 10 mg/ml protein. Labeling with the maleimide spin label (IV) was accomplished by diluting a 10 mM solution of the maleimide label in acetonitrile to 0.2 mM with a 10 mg/ml enzyme preparation, followed by incubation at 37 °C for 30 min. The suspension was then centrifuged at 40000 rev./min (106000 × g_{av}) in a Beckman 40 rotor for 1 h. The pellet was resuspended in 50 mM imidazole buffer (pH 7.0) and washed three times by the same procedure. The final pellet was suspended to 10 mg/ml. After labeling of the microsomal and gradient enzymes with the three labels, the enzyme preparations were fully active.

The labeled enzymes were suspended in various concentrations of the denaturants described above. After incubating the enzyme–denaturant mixtures for 30 min at 20–25 °C, EPR spectra were taken with a Varian E-3 spectrometer.

RESULTS

Inhibition studies

The data for denaturation of the $(Na^+ + K^+)$-ATPase by various denaturants are shown in Fig. 1. The concentrations necessary to give 50 % inhibition are shown in Table I. The concentration range in which denaturation occurs is quite narrow. The data in Fig. 2 give the time course for denaturation in the narrow transition region. There is an initial rapid denaturation followed by a much slower process in which little further denaturation occurs. The data in Fig. 1 represent the extent of denaturation after an incubation long enough to insure that the plateau phase

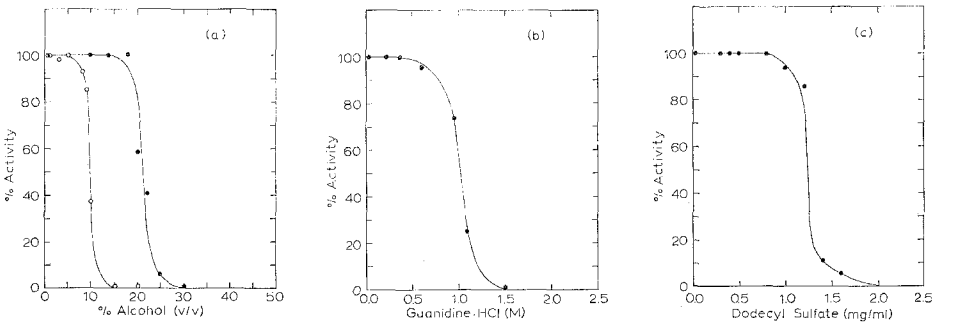


Fig. 1. Effect of various denaturants on $(Na^+ + K^+)$-ATPase activity. The gradient enzyme was incubated for 30 min in 50 mM imidazole (pH 7.0) at 25 °C with the indicated concentrations of denaturants and then diluted 3000-fold into the assay mixture. The protein concentration was 10 mg/ml for the sodium dodecyl sulfate incubation. In (a) the denaturants are ethanol (●—●) and *tert* butanol (○—○).

TABLE I

EFFECTS OF SEVERAL DENATURANTS ON THE $(Na^+ + K^+)$-ACTIVATED ATPase

The gradient or microsomal enzyme was incubated with various concentrations of denaturants as described in Figs 1–3 and assayed as described in Materials and Methods.

Denaturant	*Concentration for 50% irreversible denaturation*	
	Gradient enzyme	*Microsomal enzyme*
Ethanol	22%	22%
tert-Butanol	10%	10%
Guanidine·HCl	1.1 M	—
Dodecyl sulfate	1.2 mg/ml*	—

* Protein, 5 mg/ml.

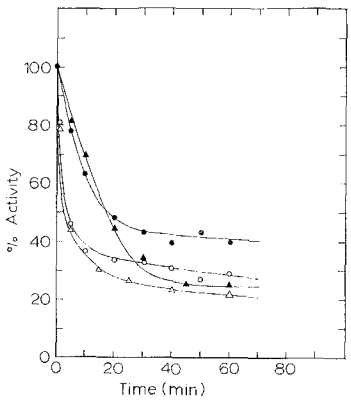


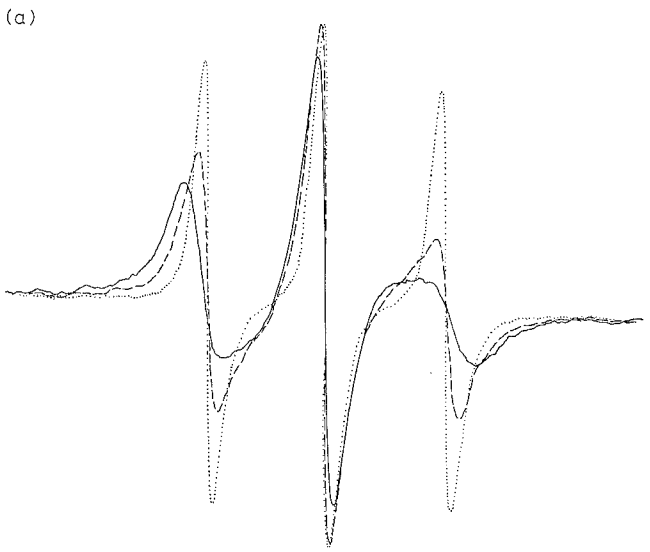
Fig. 2. Time course for denaturation. Aliquots of the incubation mixtures were taken at various times and assayed as for Fig. 1, for 22% (v/v) ethanol (●—●); 10% (v/v) *tert*-butanol (○—○); 1.1 M guanidine hydrochloride (▲—▲); and 1.2 mg/ml sodium dodecyl sulfate (△—△).

represented in Fig. 2 had been reached. The EPR experiments to be described were also performed after an equivalent incubation period with the denaturants. It should be noted that the results presented here represent irreversible effects on the enzyme activity and that the denaturants are diluted to a non-inhibitory concentration before assaying the enzyme. Reversible inhibition occurs at much lower concentrations.

Spin labeling studies

Some typical EPR spectra of the microsomal and gradient enzymes labeled with Compounds I, III, and IV are shown in Fig. 3. The spectra obtained by labeling with Compound II were essentially identical to those obtained from III. However the membrane labeling technique we used, which was necessitated by the size of the enzyme samples, resulted in irreproducible amounts of micelle formation in addition to membrane labeling, making interpretation of the data difficult.

Labels I and II are known to intercalate into bilayer regions of artifical and natural membranes, and have proven to be useful probes of membrane structure[17–21]. Based on the similarity of the EPR spectra of membranes labeled with II and III, it can be safely concluded that the two labels are experiencing similar environments. The maleimide label (IV) should react principally with SH groups and so serve as a probe of major structural changes in the protein. Data obtained from the EPR spectra are shown in Figs 4–9. One of the characteristics of the androstanol label in natural membranes and phospholipid bilayers is a very rapid rotation around an axis roughly parallel to the long axis of the steroid with restricted motion of the axis itself (Fig. 10)[17]. Furthermore it has been shown that the long axis of the steroid goes into natural membranes perpendicular to the surface. The angle θ is the time average deviation of the long axis of the steroid from the rotational axis. A value of 0° would correspond to the steroid being held rigidly perpendicular to the membrane, while a value of 55° would correspond to completely isotropic motion. The spectrum shown in Fig. 3 for no added ethanol is characteristic of membranes and phospholipid



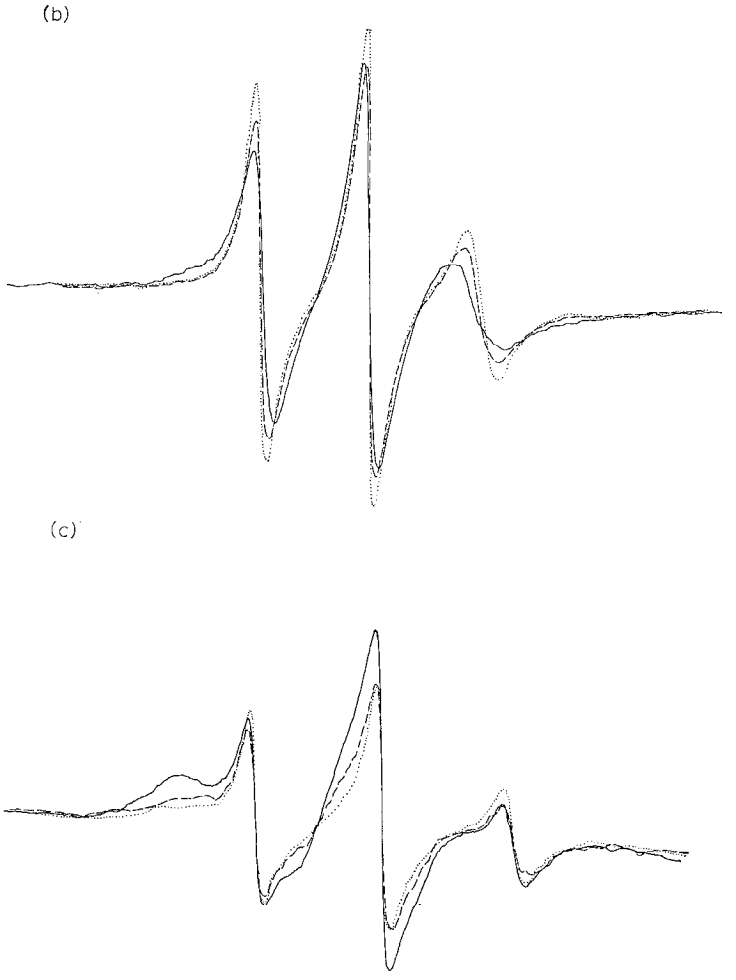


Fig. 3. EPR spectra of the spin-labeled gradient enzyme. The labeled enzyme was incubated in 50 mM imidazole for 30 min at 25 °C with the indicated concentrations of denaturants before the EPR spectra were taken. Label I was used in (a) with no denaturant (———); 22% ethanol (– – –); and 40% ethanol (· · · · ·). Label III in (b) for no denaturant (———); 12% *tert*-butanol (– – – –); and 20% butanol (· · · · ·); and Label III in (c) for no denaturant (———); 1.1 M guanidine hydrochloride (– – – –); and 2 M guanidine hydrochloride (· · · · ·).

bilayers labeled with the androstanol label. The time average angular deviation of the long axis of the steroid can be calculated from Eqn1 where $2T_{\perp}'$ is the maximum splitting observed in the EPR spectrum[22]. The mean angular deviation of the steroid

$$\cos^2 \theta = \frac{4T_{\perp}' - 48.2}{25.0} \qquad (1)$$

label in the microsomal membrane is considerably larger than in the gradient enzyme preparation (Fig. 5), indicating the phospholipids are more ordered in the gradient enzyme than in the overall microsomal membrane. The sodium deoxycholate treat-

ment involved in preparing the gradient enzyme is not responsible for a tightening up of the phospholipid structure, because the pooled membrane fragments after deoxycholate treatment have an EPR spectrum Label I which is indistinguishable from that of the original microsomes. As organic solvents are added the phospholipid structure loosens up as is indicated by the increase in θ. As γ approaches 1.0, the EPR spectra approach that for a "weakly immobilized" label with isotropic motion. From Fig. 4 it can be seen that a transition occurs in the environment that the label experiences at $\gamma = 1.0$. Near $\gamma = 1.0$ θ becomes greater than 40° (Fig. 5) indicating the

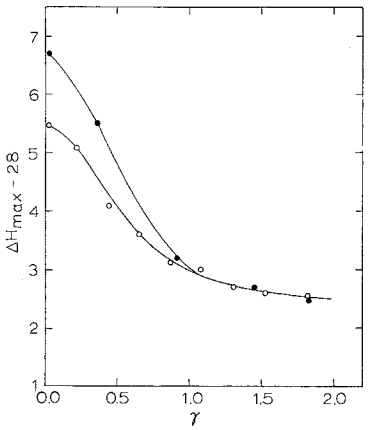


Fig. 4. Effect of ethanol on the EPR spectra of the ATPase labeled with 3-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)-5α-androstan-17β-ol. The spectral data were obtained as for Fig. 3, where ΔH_{max} is the maximal splitting in the EPR spectrum and γ is defined to be the ratio of the actual denaturant concentration to the concentration necessary to give 50% denaturation, for the gradient enzyme (●—●) and the microsomal enzyme (○—○).

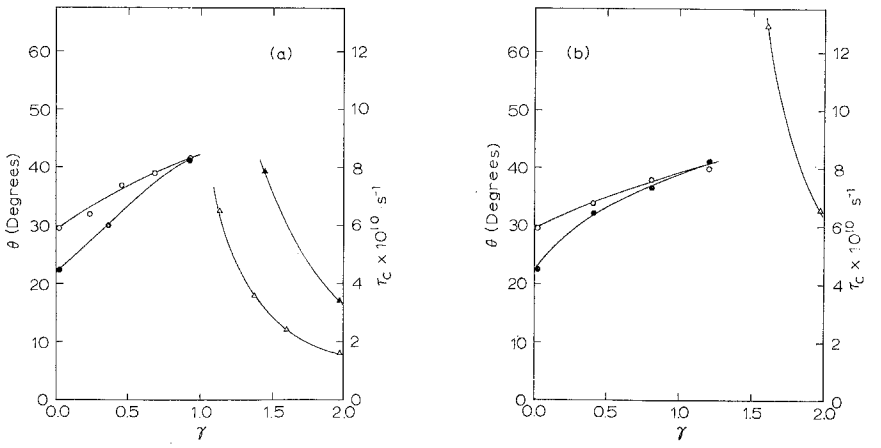


Fig. 5. The effect of alcohols on 3-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)-5α-androstan-17β-ol in the ATPase membrane. The angle θ is calculated from Eqn 1 (see Fig. 10) for the gradient enzyme (●—●) and the microsomal enzyme (○—○). The rotational correlation time τ_c is calculated from Eqn 2 for the gradient enzyme (▼—▼) and the microsomal enzyme (▽—▽). γ is defined in Fig. 4. In (a) ethanol is used and in (b) *tert*-butanol.

label is no longer experiencing a well defined lamellar structure. For $\gamma > 1$ θ can no longer be calculated from the EPR spectra. However, a rotational correlation time τ_c can be calculated[23] using Eqns 2 and 3, where $\Delta H(0)$ is the line width in gauss of the central line of the spectrum and the $h(i)$ are the derivative amplitudes of the lines corresponding to $\Delta m = i$ ($i = -1,0,1$). The correlation time is of the order of 10^{-9}

$$\tau_c = 7.14 \cdot 10^{-10}(R - 2)\Delta H(0) \tag{2}$$

$$R = \sqrt{\frac{h(0)}{h(1)}} + \sqrt{\frac{h(0)}{h(-1)}} \tag{3}$$

to 10^{-10} s for $\gamma > 1$ (Fig. 5) showing the steroid is in a very fluid environment. The hyperfine splitting for Label I at $\gamma = 2$ is 14.1 gauss, requiring that the label be in a region of very low dielectric constant and not in the aqueous phase[24], and so the increased mobility of the label is not due to its being washed out of the membrane.

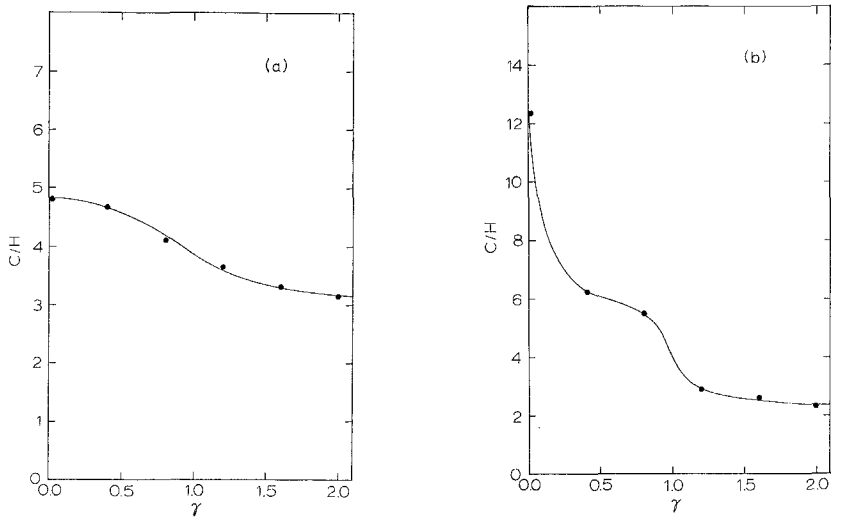


Fig. 6. Effect of *tert*-butanol on methyl 12-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)stearate in the ATPase membrane. In (a) is shown the effect of *tert*-butanol on the label in the gradient enzyme membrane and in (b) for the microsomal enzyme, where γ is defined in Fig. 4, and C/H is the ratio of intensities of the middle and high field peaks of the EPR spectrum (see Fig. 3b).

Alcohols also increase the mobility of the methyl stearate label* (Fig. 6). A transition in the freedom of motion of the methyl stearate label occurs at $\gamma = 1.0$. The microsomal enzyme also shows a dramatic increase in mobility for $\gamma < 0.2$ which does not occur for the gradient enzyme. Furthermore, with no added alcohol, Label III has greater mobility in the gradient enzyme than in the microsomes. Since the 4′,4′-oxazolidinyl-*N*-oxyl group is near the end of the hydrocarbon chain, it should serve as a reporter of the mobility of the interior of the membrane, and so with respect

* The ratio of the intensities of the center and high field peaks (C/H) is 1.0 for rapid, isotropic motion and is about 10 for a nitroxide in a rigid glass. Therefore, a decrease in C/H indicates greater mobility for the label.

to the regions in which the label is situated, the interior of the membrane immediate to the ATPase is more fluid than the interior of the overall microsomal membrane. The estimated concentration of the label in the gradient and microsomal membranes is 10^{-2}–10^{-3} M, which is the lower limit of the concentration range for which spin-spin interactions between the labels can occur. Since the EPR spectra show no evidence of spin–spin broadening, it is quite unlikely that the label is situated in small, local regions of the membranes, but that it is fairly widely distributed in the membrane; otherwise appreciable spin-spin broadening would occur. This makes it probable that the label spectra reflect overall membrane fluidity rather than only that for small local regions.

If the gradient enzyme is treated with guanidine hydrochloride, a dramatic transition occurs in the EPR spectrum of the maleimide label (IV) at $\gamma = 1.0$ (Fig. 7). On the other hand, little if any changes occur for the lipid labels (Fig. 8), so structural changes leading to denaturation occur principally in the protein*. Conversely, alcohols produce no transition in the EPR spectrum of IV (Fig.7).

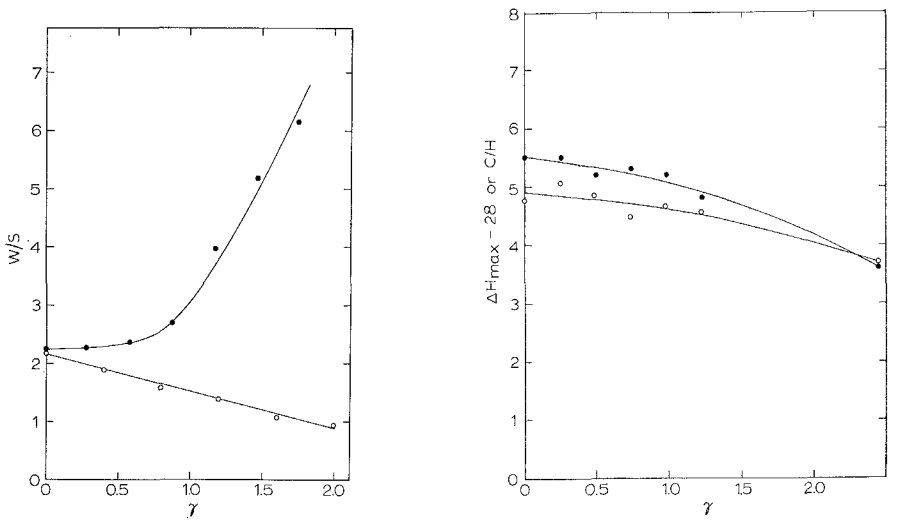


Fig. 7. Effect of *tert*-butanol and guanidine hydrochloride on the gradient enzyme labeled with *N*-(2,2,6,6-tetramethylpiperidinyl-1-oxyl) maleimide. The low field peak of the EPR spectrum of the ATPase labeled with IV shows a broad and a sharp component (Fig. 3c). The broad component is due to strongly immobilized and the sharp component to weakly immobilized label, and W/S is the ratio of the intensities of these components for guanidine hydrochloride (●—●) and *tert*-butanol (○—○) where γ is defined in Fig. 4.

Fig. 8. Effect of guanidine hydrochloride on the gradient enzyme membrane labeled with the androstanol and methyl stearate labels. The ratio C/H is as defined in Fig. 6 and ΔH_{max} and γ as defined in Fig. 4, with the androstanol label (●—●) and the methyl stearate label (○—○).

The striking element in the effect of denaturing concentrations of sodium dodecyl sulfate on the ATPase is the lack of any significant perturbation of the spectra of I, III, and IV (Fig. 9).

* When the guanidine·HCl concentration is increased to 3 M the phospholipid bilayer is destroyed, as is indicated by the observation that the EPR spectrum of I becomes characteristic of isotropic motion with a correlation time of about 10^{-9} s.

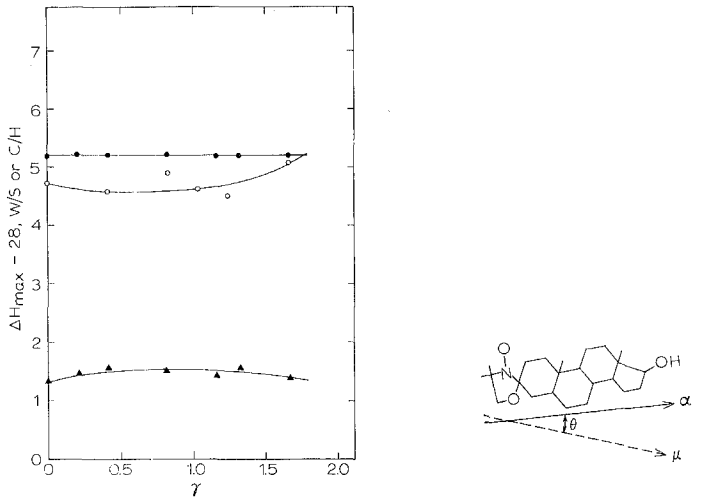


Fig. 9. The effect of sodium dodecyl sulfate on the spin labeled gradient enzyme. The ratio C/H is as defined in Fig. 6, ΔH_{max} and γ as defined in Fig. 4, and W/S as in Fig. 7 for the androstanol label (●—●), the methyl stearate label (○—○), and the maleimide label (▲—▲).

Fig. 10. 3-(4′,4′-Dimethyloxazolidinyl-*N*-oxyl)-5α-androstan-17β-ol. The μ is the rotational axis of the steroid in membranes (see the Results section), α is the long axis of the steroid, and θ the time average angular deviation of the rotational axis from the long axis of the steroid.

DISCUSSION

Denaturation of the $(Na^+ + K^+)$-ATPase by alcohols, guanidine hydrochloride and sodium dodecyl sulfate produces quite different effects on the lipid and protein components of the ATPase. The alcohols denature by disrupting the phospholipid bilayer with much smaller effects on the protein, guanidine hydrochloride denatures by disrupting protein structure with little effect on phospholipid structure, while sodium dodecyl sulfate appears to produce no major structural changes in either protein or phospholipid structure at concentrations which irreversibly inhibit the ATPase.

The concentrations of ethanol and *tert*-butanol necessary to give irreversible inhibition of the ATPase correspond quite closely to the concentrations necessary to lyse red blood cells[7]. Since these concentrations also correspond to the point at which the motion of Label I becomes almost isotropic (Figs 4 and 5), it seems quite probable that the principal factor in denaturation of the ATPase (and in the lysis of red blood cells) by alcohols is disruption of the phospholipid lamellar structure. The motion of Label III also becomes less restricted for $\gamma > 1$ (Fig. 6). The protein Label IV undergoes changes on the addition of alcohols (Fig. 7). The effect is primarily to restrict the motion of the maleimide, indicating a tightening up of protein structure. It is important to note, however, that the changes in the environment of the maleimide label on addition of alcohols occur uniformly over the entire concentration range examined, with no abrupt transition near $\gamma = 1$. This suggests quite strongly that, although alcohols do produce changes in protein structure, these changes do not play an important role in the irreversible denaturation process. The lack of an observable transition in the maleimide label near $\gamma = 1$ cannot be due to insensitivity of the

label to major structural changes in the protein, since guanidine hydrochloride effects a pronounced change near $\gamma = 1$ (Fig. 7). Furthermore, the high specific activity of the $(Na^+ + K^+)$-ATPase used for these studies (14–18 μmoles ATP hydrolyzed/mg protein per min) is comparable to the activity of a preparation (13.2 μmoles ATP hydrolyzed/mg protein per min) which has been estimated to be 20–60 % pure[25]. Although there is almost certainly some labeling of proteins other than those of the ATPase it is likely that an appreciable fraction of the labeled protein is associated with the functioning of the ATPase.

For concentrations of guanidine hydrochloride less than 3 M the principal effects are in the protein (Figs 7 and 8). A major change occurs in the structure of the protein when denaturation occurs (Fig. 7). It is apparent that the ATPase can be denatured either by disrupting the phospholipid bilayer immediate to the protein without a major disruption of the protein, or by disrupting the protein without disrupting the phospholipid bilayer. It is clear that both the protein and the bilayer phospholipid structures must be intact for the functioning of the ATPase. This strongly suggests that it may not be possible to truly solubilize the ATPase, stripping it of the surrounding phospholipid bilayer, and still maintain enzymatic activity. With 2 mg/ml sodium dodecyl sulfate neither protein nor phospholipid structure is disrupted even though the solution visibly clears. The effect of the detergents in clearing the membrane suspension is probably a fragmenting of the membrane into smaller pieces rather than micellation of the lipids. Quite unlike the effects of alcohols and guanidine hydrochloride, lowering the protein concentration also lowered the detergent concentration necessary to produce denaturation. This suggests a fairly specific interaction between the ATPase and the detergent as the source of denaturation.

The mean angular deviation of the long axis of I from being perpendicular to the membrane surface is about 9° less for the gradient enzyme (Figs 4 and 10). It was shown that the sodium deoxycholate used in preparation of the gradient enzyme could not produce this effect, since pooling of all the membrane fractions and then labeling with I resulted in material with an EPR spectrum identical to the starting microsomes. This must mean that the regions in which Label I is situated are more rigid immediate to the ATPase than in the overall plasma membrane. As was the case with Label III, Label I is estimated to be 10^{-2}–10^{-3} M in the membrane. Since there is no evidence for spin-spin broadening, Label I cannot be localized in small regions of the membrane, and so the EPR spectra probably reflect membrane structure over extensive areas of the membrane. In contrast, Label III, which is probably reporting the interior of the membrane, experiences a more fluid environment in the lipids near the ATPase. A possible explanation for the greater fluidity of the interior of the membrane near the ATPase would be a greater fraction of unsaturated fatty ester chains in the phospholipids. The *cis* double bonds may tend to disrupt structure in the interior of the bilayer without affecting overall ordering of the membrane.

Both Labels I and III probably perturb the structure of the surrounding lipids, and so the EPR spectra reflect the perturbed structure rather than the native structure. However, it is reasonable to expect the perturbations of structure to be in the same direction in both the microsomes and the gradient enzyme, and so the differences observed between the two labeled preparations probably reflect differences in the native structures.

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