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A Fluorescent Probe Study of the Lipid Mobility of Membranes Containing Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

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The temperature-activity relationship of a membrane ($Na^+ + K^+$)-ATPase preparation [Mg^{2+}-dependent, ouabain-sensitive, ($Na^+ + K^+$)-activated ATP phosphohydrolase, EC 3.6.1.3] obtained from sheep kidney cortex and medulla was determined and found to be very similar to that previously reported for preparations of this enzyme from either rabbit kidney or ox brain. These temperature-activity relationships can be shown as Arrhenius plots which characteristically are nonlinear and have transition temperatures near 22°. Two noncovalently bound fluorescent probes, 12-(9-anthroyl)stearic acid (12-AS) and *N*-phenyl-1-naphthylamine (NPN), were used to label the hydrophobic core of the partially purified membranes rich in ($Na^+ + K^+$)-ATPase. The fluorescence polarization of these probes was determined between 10° and 40°. The rotational relaxation times (ρ) for each probe were then calculated, and secondary plots of reciprocal relaxation time vs. reciprocal temperature were constructed. The plots for membranes labeled with 12-AS and NPN were nonlinear and showed transition temperatures near 22°, in good agreement with the transition temperature of the hydrolytic activity of the enzyme. A similar transition temperature was detected by right-angle light scattering of an unlabeled microsomal preparation of ($Na^+ + K^+$)-ATPase and of an aqueous suspension of liposomes made from a total lipid extract of the enzyme-containing membranes, thus excluding any direct effect of addition of the fluorescent probes to the membranes. The transition temperatures observed under all experimental conditions were very similar. We conclude that the nonlinear temperature-activity relationship of ($Na^+ + K^+$)-ATPase and the nonlinear fluorescence polarization–temperature profile both arise from a temperature-dependent change in the molecular mobility of the membrane lipids in the immediate environment of the probes and the "active center" of the ($Na^+ + K^+$)-ATPase subunits. These changes illustrate the strong cooperative effect between the physical state of the membrane lipids and the functional state of the enzyme protein in this particulate membrane enzyme system, and suggest a powerful modulating effect of membrane lipids in regulating enzyme activity, or drug-receptor interactions more generally.

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INTRODUCTION

The mechanism of energy transduction by biological membranes has been of inherent interest for many years, both mitochondrial and plasma membrane systems having been studied extensively (1). In the plasma membrane the majority of mechanisms which have been proposed to describe the transduction process at the molecular level involve conformational changes of membrane components. These changes are usually associated with the activity of membrane ATPases (2–5). Recent work in other systems has focused attention upon the cooperative effect of membrane lipids in modulating membrane protein activities (5, 6) and suggested that changes in the action of membrane-bound enzymes might well be accompanied by changes in the properties of the associated membrane lipids. The use of fluorescent probe techniques to detect changes in membrane lipids may have advantages over such other methods as electron spin resonance labeling, as they are frequently more sensitive (7–9). Vanderkooi (10) and Radda (11) have discussed in detail the use of fluorescent probes to examine changes in fluidity of membrane lipids, and very recently Bashford, Harrison, Radda, and Mehdi (12) used fluorescence polarization measurements to correlate the mobility of lipids in human thyroid membranes with other biological activities of their system. The latter technique is especially useful for detecting changes in the orientation of fluorescent molecules in both rigid and fluid environments (8, 9, 11, 13–15). When coupled with the use of fluorophors which are known to interact with specific areas of the membrane (15, 16) these techniques can be very suitable for detecting changes in fluidity in defined regions of biological membranes.

In the present study we utilized the fluorescent properties of two such compounds, 12-(9-anthroyl)stearic acid and *N*-phenyl-1-naphthylamine, to examine the lipid mobility of membrane preparations of $(Na^+ + K^+)$-ATPase [Mg^{2+}-dependent, ouabain-sensitive, $(Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3] prepared from the cortex and outer medulla of sheep kidney. These probes were used previously to examine lipid behavior in artificial and natural membrane systems, where they are noncovalently bound (10–12, 17). Binding to membranes results in a blue shift and enhancement of their fluorescence emission spectra (7, 10–12, 17). The location of the bound probes is thought to be deep within the hydrocarbon core of the membranes (16); hence they are potentially very suitable agents for detecting changes in the fluidity of the lipids within this region.

Membrane preparations of $(Na^+ + K^+)$-ATPase have been shown to display nonlinear temperature-activity relationships, which might be explained by two different conformational forms of the enzyme above and below the transition temperature of the system. Both these forms of the enzyme would be capable of hydrolyzing substrate ATP in the presence of activating ligands but would have very different energies of activation (18–20). However, it is also well known that modification of the membrane lipids of this system will markedly alter the temperature-activity relationship of the $(Na^+ + K^+)$-ATPase (21, 22). In particular we have described how mild treatment with phospholipase A can abolish the transition temperature previously observed in the system, and the restoration of this effect by subsequent treatment with phosphatidylserine (23, 24). Thus a direct effect of membrane lipids upon $(Na^+ + K^+)$-ATPase may be responsible for the large change in activation energy which occurs in this system, without the primary cause lying in a major conformational change in the enzyme protein. This paper describes experiments designed to study more directly than previously the lipid-protein interactions of $(Na^+ + K^+)$-ATPase by correlating the motional characteristics of the membrane lipids of the system with the temperature-activity relationship of ouabain-sensitive ATP hydrolysis by the enzyme.

METHODS

Preparation of enzyme. The enzyme was prepared from the cortex and outer medulla of sheep kidney by a simple modification of the general procedure of homogeni-

zation and differential centrifugation first described by Charnock and Post (25). Following the separation of both a nuclear and then a mitochondrial fraction, the crude "heavy microsomal" fraction was collected as a pellet by centrifuging the post-mitochondrial supernatant at 35,000 × *g* for 1 h. It was resuspended in 10 mM Tris–1 mM EDTA (pH 7.4) and then diluted with a solution containing 3 mM Na_2ATP, 2 mM EDTA, 50 mM imidazole, and 0.2% sodium deoxycholate (pH 7.4) until the protein to detergent ratio was 1:1. After standing overnight at 4° the enzyme preparation was collected as a pellet by centrifugation at 35,000 × *g* for 2 hr. It was washed by centrifugation through a medium free of both ATP and deoxycholate, collected as a pellet at 35,000 × *g* for 2 hr, and then resuspended in 10 mM Tris–1 mM EDTA (pH 7.4) as before. In some later experiments incubation with 0.1% sodium dodecyl sulfate (60 min at 37°) replaced the overnight treatment with sodium deoxycholate (26). Small aliquots were rapidly frozen in an acetone–Dry Ice mixture for storage at −20° before use. These preparations can not be regarded as quite as highly purified as others recently reported (26–28), but they had a specific activity of 3–10 units/mg of protein (i.e., micromoles of ATP hydrolyzed per minute at 37°). They were more than 80% sensitive to inhibition by 0.1 mM ouabain and could be stored frozen without loss of activity for at least 4 weeks. As such they proved suitable for the type of study reported here.

Temperature-activity relationship of $(Na^+ + K^+)$*-ATPase*. This was determined by the general procedure described previously (19, 20). The assay for phosphate release from ATP was carried out in a buffered medium containing 50 mM glycylglycine–30 mM imidazole, 0.5 mM EDTA, 100 mM NaCl, 10 mM KCl, 2.5 mM Na_2ATP, and 2.5 mM $MgCl_2$ at pH 7.4. When necessary 0.1 mM ouabain was added to the system. Enzyme activity was determined with variable aliquots of enzyme suspension (25–100 μl) for 5-min incubation periods at the required temperature between 5° and 40°. The linear rate of inorganic phosphate release was determined by the method of Hurst (29) over a 4-min period, using a Technicon AutoAnalyzer (30).

Fluorescence polarization. When polarized light falls onto a group of molecules, those molecules with their axes parallel to the plane of polarized light will be preferentially excited. If the excited molecules do not move out of the plane of polarization, the maximum fluorescence will pass through a second polarizer oriented in the same plane. Conversely, if all the excited molecules move out of the plane of polarization, no fluorescence will reach the detector. Hence changes in the intensity of polarized fluorescence can reflect motion of the molecules during their fluorescence lifetime (13). Thus fluorescence polarization is a convenient parameter for following changes in the fluidity of the environment of a fluorophor within a lipid medium. This principle, first proposed by Weber (14, 31), was employed in the construction of a suitable instrument to measure fluorescence polarization by Barratt, Badley, Leslie, Morgan, and Radda (15). Their instrument uses vertically polarized exciting light and dual photomultipliers mounted at right angles to the sample chamber. This arrangement allows the simultaneous measurement of fluorescence at right angles to the direction of excitation, and through a pair of polarizers oriented both vertically (I_{11}) and horizontally (I_1) with respect to the plane of excitation. Continuous recording of the components of the fluorescence emission is thus possible, permitting the automatic computation of the fluorescence polarization p, which is defined (8, 13, 32) as

$$p = \frac{I_{11} - I_1}{I_{11} + I_1}$$

The instrument is also fitted with a temperature-controlled sample compartment, which permits measurement of the fluorescence polarization between 10° and 40°. For each enzyme preparation the measurements were determined in at least quadruplicate. Values for the rotational relaxation time ρ were calculated using the Perrin equation (33) previously described by Weber (14, 31) and more recently used by Bashfold *et al.* (12):

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right) \times \left(1 + \frac{3\tau}{\rho}\right)$$

where p is the measured polarization, p_0 is the limiting polarization of a rigidly held, randomly oriented array of fluorophors, and τ is the fluorescence lifetime. The values for p_0 were obtained from the literature (34, 35) while those for τ were measured on an ORTEC nanosecond pulsed fluorometer (12). The latter parameter was assumed to have the same temperature dependence as the fluorescence intensity (I), which was measured directly (32, 36). Preliminary fluorescence intensity and fluorescence spectra measurements were determined on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer. The fluorescent probes 12-AS and NPN[3] were stored as 1 mM stock solutions in methanol in the cold. Sufficient probe to give a workable signal was added to about 100 μg of enzyme protein suspension at room temperature and allowed to equilibrate until the maximum fluorescence enhancement was observed. The final concentration of added probe was usually less than 1 μmol/50 μg of protein (37). The concentration of methanol in the experimental system did not exceed 0.3% (v/v).

As had been found before, the absolute value of ρ can vary slightly between experiments, but the internal consistency of any set of experimental values obtained with a given enzyme preparation is better than 98% (15).

Light scattering. Right-angle light scattering observations were made with an Aminco-Bowman spectrophotofluorometer by setting both the excitation and emission controls at 300 nm, and recording the effect of variable temperature (T) upon relative intensity according to the method described by Bangham, Hill, and Miller (38).

A total lipid extract of our enzyme-enriched membranes was obtained by the solvent extraction method of Nelson (39), and an aqueous preparation of liposomes was prepared according to the procedures of Bangham and his associates (38, 40).

[3] The abbreviations used are: 12-AS, 12-(9-anthroyl)stearic acid; NPN, *N*-phenyl-1-naphthylamine.

RESULTS

The temperature-activity relationship of ouabain-sensitive $(Na^+ + K^+)$-ATPase of sheep kidney is shown as an Arrhenius plot in Fig. 1. There is a sharp discontinuity in the rate of hydrolysis of ATP by this system between 21° and 22°. The apparent energy of activation above this transition temperature (T) is 15.7 kcal/mole, and is significantly less than that determined below this temperature (34.4 kcal/mole). Although these values for the apparent energies of activation above and below the tran-

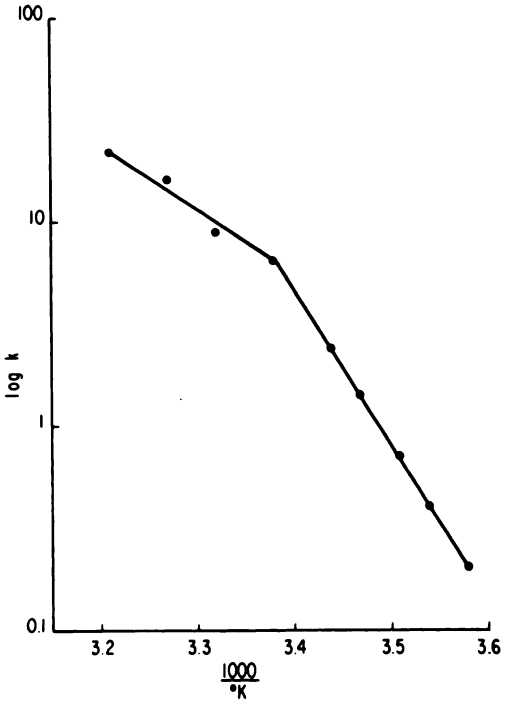

FIG. 1. *Arrhenius plot of ouabain-sensitive $(Na^+ + K^+)$-ATPase activity*

Enzyme preparation (25–100 μl; 0.91 mg of protein per milliliter) was incubated for 5 min at various temperatures in a buffered medium containing 100 mM NaCl, 10 mM KCl, and 2.5 mM MgATP with and without 0.1 mM ouabain. The rate of ATPase activity was obtained by continuously monitoring inorganic phosphate release from ATP at each temperature and then determining the slope obtained between the first and the fifth minute of reaction (30). The difference between the slopes obtained with and without 0.1 mM ouabain was calculated to give ouabain-sensitive $(Na^+ + K^+)$-ATPase activity. The transition temperature (T) is near 22°; the value for the apparent activation energy above this transition temperature is 15.7 kcal/mole, while the value below the transition temperature is 34.4 kcal/mole.

sition temperature are very similar to those reported previously for rabbit kidney and ox brain preparations (20, 23, 24), the transition temperature of 21–22° is slightly higher than that seen previously. Whether this reflects a significant species difference or is due to such environmental factors as season or diet of the experimental animals is not known at present.

The effect of temperature on the fluorescence polarization of (Na^+ + K^+)-ATPase-enriched membranes labeled with 12-AS is shown as mean reciprocal plots of rotational relaxation time (1000/ρ) vs. reciprocal temperature (1000/T) in Fig. 2. It is clear that there is a marked change in the value of ρ with temperature over the whole range examined. In addition there is a departure from linearity near 22° for

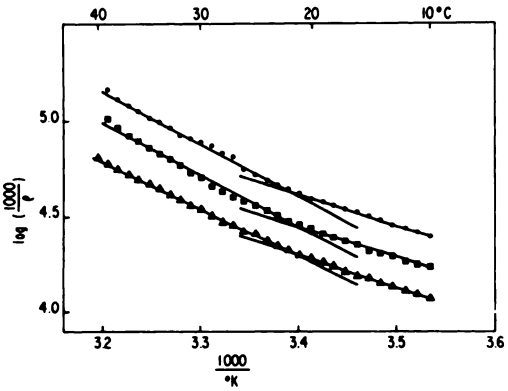

FIG. 2. *Effect of temperature on fluorescence polarization of (Na^+ + K^+)-ATPase labeled with 12-AS*

Either 3 or 10 μl of 1 mM 12-AS in methanol were incubated with varying amounts of enzyme protein suspension for 30 min at room temperature in 3 ml of buffer (10 mM Tris–1 mM EDTA, pH 7.4). The temperature dependence of fluorescence polarization was determined by the method of Bashford and Radda (12, 16) by alternately heating and cooling the sample between 10° and 40°. The fluorescence excitation was at 385 nm; emission, 437 nm (12). ●——●, enzyme treated with sodium deoxycholate, 137 μg of enzyme protein, 3 μl of probe; ■——■, enzyme treated with sodium deoxycholate, 117 μg of enzyme protein, 10 μl of probe; ▲——▲, enzyme treated with sodium dodecyl sulfate, 3.3 μg of enzyme protein, 10 μl of probe. Each point is the mean of at least four observations; the standard errors are small and lie within the experimental points. The slopes for all three experiments are very similar, and the transition temperatures are between 21° and 22°.

all three enzyme preparations. Although the values for ρ vary slightly from preparation to preparation, the data are the means of at least quadruplicate observations at a given temperature and the standard errors are sufficiently small to lie within the points shown. The internal consistency of any set of experiments is very high (15), as is evident from the slopes of the lines, which are very similar for all three enzyme preparations.

A similar result was found when NPN was used as the fluorescent probe. Although some hysteretic effect was found between heating and cooling with this agent, both temperature plots again showed a departure from linearity near 22°, with the value obtained on heating being about 2° higher than that seen on cooling. A double-reciprocal plot of the mean rotational relaxation time (1000/ρ) vs. temperature (1000/T) is shown for two enzyme preparations in Fig. 3. The data are the means of at least quadruplicate observations, and the standard errors again lie within the points shown. The mean transition temperature is very similar to that found in the hydrolysis of substrate by these preparations and in the fluorescence polarization–temperature profile when the membranes were labeled with 12-AS.

It is significant that these effects of temperature are similar to those previously observed in both human and bacterial membranes labeled with fluorescent probes (12, 41) and in some plant and animal systems labeled with paramagnetic probes (5, 6, 42). However, as the introduction of extrinsic probe molecules into the (Na^+ + K^+)-ATPase system must inevitably perturb it to some extent, we sought to confirm our findings by the use of the less sensitive but nonperturbing light scattering method recently described by Bangham *et al.* (38).

Figure 4 shows the results of an experiment again using a membrane preparation of (Na^+ + K^+)-ATPase obtained from sheep kidney. When a double-reciprocal plot of relative intensity (RI) vs. absolute temperature (T) is constructed from the mean data of three experiments it is clear that the resultant plot is nonlinear and

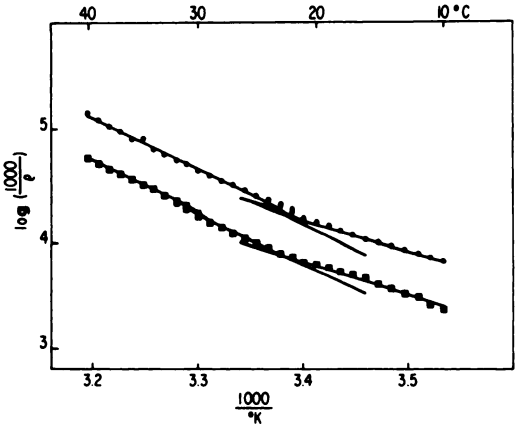

FIG. 3. *Effect of temperature on fluorescence polarization of (Na⁺ + K⁺)-ATPase labeled with NPN*

Either 6 or 10 μl of 1 mM NPN in methanol were incubated with varying amounts of enzyme protein suspension at room temperature for 5 min in 3 ml of buffer (10 mM Tris–1 mM EDTA, pH 7.4). The temperature dependence of fluorescence polarization was determined by the method of Bashford and Radda (12, 16) by alternately heating and cooling the sample between 10° and 40°. The fluorescence excitation was at 340 nm; emission, 407 nm (12). ●——●, enzyme treated with sodium deoxycholate, 137 μg of enzyme protein, 6 μl of probe; ■——■, enzyme treated with sodium deoxycholate, 117 μg of enzyme protein, 10 μl of probe. Each point is the mean of at least four observations; the standard errors are small and lie within the experimental points. The slopes for both experiments are very similar, and the transition temperatures are between 21° and 22°.

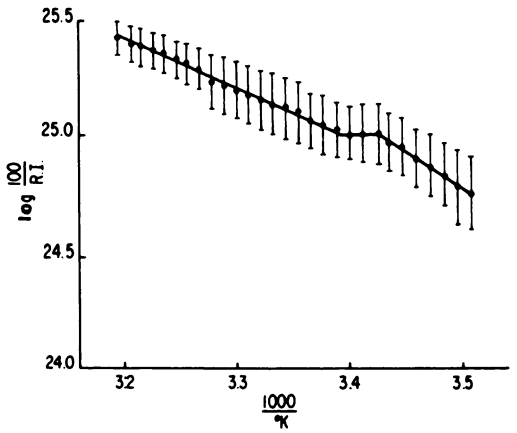

FIG. 4. *Effect of temperature on right-angle light scattering of (Na⁺ + K⁺)-ATPase-enriched membranes*

A sodium deoxycholate-treated membrane suspension (400 μl; 380 μg of protein per milliliter) was diluted to 2 ml in 10 mM Tris–1 mM EDTA buffer at pH 7.4. The percentage relative intensity (R.I.) was measured at 300 nm through an ascending temperature gradient from 10° to 38°. The points shown are the means of three sets of observations. A transition occurs between 18° and 22°. The rates of change in relative intensity are similar above and below the transition.

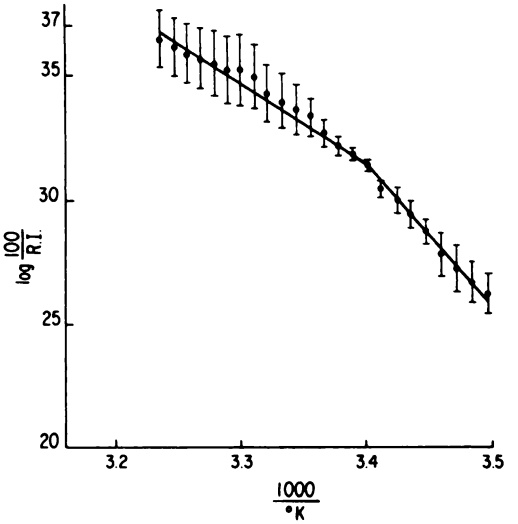

FIG. 5. *Effect of temperature on right-angle light scattering of a liposome preparation from (Na⁺ + K⁺)-ATPase-enriched membranes*

Membrane lipids (2.75 mg) were resuspended in 6 ml of water distilled from alkaline $KMnO_4$. The percentage relative intensity (R.I.) was measured at 300 nm through an ascending temperature gradient from 12° to 36°. The points shown are the means of five observations. The transition temperature is near 21°.

that a temperature-dependent transition occurs between 18° and 22°. The position of this transition is in good agreement with the finding of a similar transition temperature by means of the fluorescent probes and the hydrolytic activity of the enzyme. We believe that this excludes a direct effect of probe addition to membrane lipids as the cause of the temperature transitions observed. If the transition in fluorescence polarization and in light scattering is a reflection of a change in the properties of the membrane lipids per se, it should also be possible to demonstrate this phenomenon in preparations of the isolated lipids. We therefore examined the effect of temperature on the right-angle light scattering of a liposome preparation made from a total lipid extract of our (Na⁺ + K⁺)-ATPase-enriched membrane system (38, 40). The mean data from five experiments are

given in Fig. 5 as a double-reciprocal plot of relative intensity (RI) vs. absolute temperature *(T)*. The data obtained are in excellent agreement with our previous experiments (Figs. 1–4), as there is again a nonlinear response to temperature with a transition point near 21°.

Thus the nonlinear temperature dependence of the hydrolytic activity of (Na^+ + K^+)-ATPase can also be seen in the rotational relaxation time of two fluorescent probes incorporated into the lipids of the supporting membranes, as well as in the light scattering measurements made both in the intact membrane and in liposome preparations derived therefrom.

DISCUSSION

Several previous studies reported from this and other laboratories have demonstrated that untreated membrane preparations of (Na^+ + K^+)-ATPase, prepared from both rabbit kidney and ox brain, possess nonlinear temperature-activity relationships which are characteristically reflected in discontinuous Arrhenius plots. These plots demonstrate transition temperatures close to 20°, and values for the apparent energy of activation for the hydrolysis of substrate ATP are about 3 times greater below the transition temperature than above it (19–22, 43).

Very similar results for a preparation of (Na^+ + K^+)-ATPase from sheep kidney were obtained in the present study, although the transition temperature found here was consistently 1–2° higher than that seen with enzyme preparations examined previously. This small difference may be due to a different source of the enzyme, or perhaps to such factors as the dietary intake or environmental temperature of the experimental animals.

It has been suggested that the very significant increase in the apparent energy of activation of (Na^+ + K^+)-ATPase which occurs below the transition temperature could be brought about by a temperature-dependent conformation of the system which is much less favorably oriented toward the hydrolysis of substrate ATP than that which occurs above the transition temperature (18, 19). More recent work has strongly implicated the nature of the membrane lipids in influencing the rate of substrate utilization by this enzyme (21–24). However, little direct evidence of the physical state of the membrane lipids is presently available (44, 45). The recent use of fluorescent probes to examine the molecular mobility of membrane lipids in other systems (7–12, 16, 17, 46) suggested that this technique is suitable for a direct study of the role of lipids in influencing the temperature-activity relationship of (Na^+ + K^+)-ATPase.

12-AS and NPN are fluorescent probes which bind noncovalently to biological membranes and penetrate the hydrocarbon core of the membrane (11, 12, 16, 47). Therefore they are capable of reflecting changes in the mobility of the membrane lipids in this region. Because fluorescence polarization techniques are suitable for following the motion of fluorescent molecules in a variety of media (8, 9, 11, 13–15), we examined the fluorescence polarization vs. temperature profiles of (Na^+ + K^+)-ATPase-containing membranes labeled with either 12-AS or NPN. The temperature range was that previously used to examine the hydrolytic activity of the enzyme. The good correlation in transition temperature observed with fluorescence labeling and by measuring hydrolytic activity indicates that changes in the molecular mobility of the membrane lipids coincide with changes in the ability of the enzyme to hydrolyze its substrate. The changes in probe rotation which are seen here probably reflect fluidity changes in the hydrophobic region of the membrane, as these probes are unlikely to reveal alterations in the physical properties at the polar-apolar interface regions of the membrane. It therefore seems prudent to refer to the observed changes as *thermal transitions* rather than phase changes or phase separations in the membrane lipids.

In addition, simple right-angle light scattering observations demonstrated thermal transitions in both untreated membrane and liposome preparations. These thermal transitions probably reflected a temperature-dependent change in the refractive index, volume, and particularly in

the case of the microsomal membrane studies, the integrity of the systems.[4]

In all these studies the use of temperature perturbations reveals the correlation between the physical state of the membrane lipids and the hydrolytic activity of $(Na^+ + K^+)$-ATPase, which is a property of the enzyme dependent on the protein components of the system (48–51). Our experiments cannot preclude a direct effect of temperature upon the conformation of the "active center" of $(Na^+ + K^+)$-ATPase. Nevertheless, the observation that all the transition temperatures occurred near 22°, and under widely different experimental conditions, strongly suggests that the effects are due to a fundamental property of the membranes. Although these temperature-dependent transitions must reflect a cooperative interaction between the protein and the lipids of the system, we conclude that the primary effect is a marked increase in the fluidity of the lipids in the membrane adjacent to the active centers of the $(Na^+ + K^+)$-ATPase protein.

This conclusion strongly supports the concept that the lipids of biological membranes have a powerful modulating effect upon membrane enzyme activity in general (5, 6, 12), and upon the transport and hydrolytic function of $(Na^+ + K^+)$-ATPase in particular (23, 24, 52, 53). Finally, it seems reasonable to suggest that both drug-receptor and hormone-receptor interactions in biological membranes might also be modulated by similar changes in the properties of membrane lipids adjacent to macromolecular binding sites. Indeed, the recent work of Kenakin, Krueger, and Cook with H_1 and H_2 histamine receptors (54) is in direct accord with this hypothesis.

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[4] A. D. Bangham, personal communication.

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