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Polarographic Studies of Membrane Particles Containing Na-K ATPase

D. Bach*, J. S. Britten, and M. Blank

Department of Physiology, Columbia University, College of Physicians and Surgeons, 630 W. 168 Street, New York, N.Y. 10032

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Summary. The properties of a suspension of membrane particles containing Na-K ATPase have been investigated with the aid of d–c and a–c polarography. In particular, we have studied the interaction of three cations, two very effective enzyme inhibitors and one activator, with the enzyme preparation. Ag^+ and Cu^{++}, which inhibit the enzyme at very low concentrations, bind very strongly. No binding could be found with the activating ion, Tl^+, however. Adsorption of a substance with an isoelectric point between pH 4 and pH 5.5 occurred at the electrode surface between −0.1 and −1.2 V at pH 7, and was associated with the random currents that appear during the measurements. The random currents arise when the membrane particles collide with the electrode and cause changes in the structure of the electrical double layer. (Added substances that adsorb more strongly at the mercury/water interface eliminate the random currents.) The adsorbed film impedes the flow of the free Ag^+ and Cu^{++} ions, and to a smaller extent, the flow of Tl^+ ions. The differences between the binding of inhibiting and activating ions are correlated with their effects on the ATPase enzyme activity.

The Na-K-activated ATPase, which is found in the membrane fractions of many tissues, is believed to be involved in the active transport of Na^+ and K^+ ions across cell membranes (Skou, 1965). The energy for the transport and enzymic processes comes from the hydrolysis of ATP, which proceeds by the phosphorylation of a membrane component (requiring the presence of Na^+ and Mg^{++}), followed by hydrolysis of the intermediate (requiring the presence of K^+). The way in which the Na^+ and K^+ ions activate the enzyme system, and are themselves transported as a result of the reaction, is unknown and has been the subject of many studies.

In a recent paper (Blank & Britten, 1970), we described some properties of membrane particles containing Na-K ATPase in contact with a mercury electrode surface. In the presence of these particles, one could detect random

* *Present address:* Polymer Department, The Weizmann Institute of Science, Rehovot, Israel.

currents that were independent of the presence of substrate, activating ions or enzyme activity. These currents could be eliminated by introducing drugs that interfere with electron transport and oxidative phosphorylation in mitochondria and also by the introduction of ouabain, but the mechanism of their appearance was unclear. We have attempted to obtain further information about the electrode properties of the membrane fragments, and with the same techniques to probe the role of cations in the action of the Na-K ATPase.

In this study we were particularly interested in the effect of ion binding on the properties of the enzyme. The problem of ion binding is difficult to approach experimentally because the enzyme is impure and present at low concentration; consequently, the binding of the activating ions, Na^+ and K^+, is difficult to detect. However, we have been able to obtain information indirectly by studying the binding of Tl^+ which can mimic K^+ in its ability to activate the enzyme (Britten & Blank, 1968), and also of two other ions that inhibit the enzyme at very low concentrations. We have done this with the aid of direct polarographic measurements using the dropping mercury electrode as the working electrode. This technique is well known as a very sensitive method for the quantitative analysis of dissolved substances and can be used for obtaining information on the binding of ions to macromolecules (Bach & Miller, 1967). The technique has also been applied to study the transport of ions through interfacial films (Miller, 1968). We have used polarography for determining the binding of ions by the enzyme preparation and the permeation of the same ions through the adsorbed films that form on the electrode surface. With the aid of a–c polarography we have also investigated the mechanism of the random currents appearing in the absence of depolarizing ions.

Materials and Methods

Membrane particles having Na-K-dependent ATPase activity were prepared from rabbit kidneys according to the method of Kinsolving, Post and Beaver (1963). The enzyme preparation was stored not more than 4 weeks in 0.02 N salt solutions containing either Tris NO_3 buffer (pH 7), Naac-Hac buffer (pH 7) or unbuffered $NaNO_3$. The activity, which was checked every two weeks, decreased at a rate of about 30 to 40% per week.

The polarographic studies were done using a Metrohm polarograph with an attached a–c modulator. The dropping mercury electrode was used either with free dropping under hydrostatic pressure with a drop time of about 5 sec, or with a knocking device for rapid polarography, giving a drop time of 0.5 sec. The very short drop times minimize the influence of adsorption and the correction for the residual current is easier to make as there is little decay of the charging current at short times.

The reference electrode was Ag/AgCl with a mixed bridge (0.1 N NaCl-agar plug, 0.1 N $NaNO_3$) to prevent the entry of Cl^- into the solution and the formation of un-

dissociated anionic complexes with the cations studied. The binding of the cations to the membrane particles and their permeation through the adsorbed interfacial films were studied by d−c polarography. The adsorption on the mercury/water interface in the presence of the enzyme suspension and the properties of the random currents were studied with a−c polarography by application of 10 mV a−c on top of the d−c potential.

The cations studied were Tl^+, Ag^+ and Cu^{++}. $TlNO_3$, purchased from K & K Laboratories, Plainview, N.Y., was used without further purification or drying. The Ag^+ solution was prepared from dried (110 °C−2 hr) $AgNO_3$. The Cu^{++} solution was obtained by dissolving copper foil (Alfa Inorganics, Beverly, Mass.) in concentrated HNO_3 and diluting this solution. All the reagents were of analytical grade.

The cations were studied at concentrations of 10^{-4} and 5×10^{-5} N. The experiments with Tl^+ were done in different supporting electrolytes: Tris NO_3, Naac-Hac buffer and $NaNO_3$ in concentrations of 10^{-3} and 10^{-1} N. The Cu^{++} and Ag^+ experiments were performed in Naac-Hac and $NaNO_3$ (10^{-3} and 10^{-1} N), because of binding of Cu^{++} to the Tris ion (Hanlon, Watt & Whitehead, 1966). The pH of $NaNO_3$ with the cations was set to around 6, and was measured on the part of the stock solution that was not used for further experiments, because of the Cl^- that entered the solution from the calomel electrode. (No higher pH could be used because of the low solubility of $Cu(OH)_2$.)

A polarogram of the solution containing the cation was run. Then the "enzyme", in concentrations not higher than 0.4 mg/ml (final concentration), was added to the cation solution and a polarogram was rerun. In every case the current values obtained from the polarograms were corrected for the residual current determined by running the polarograms of the supporting electrolyte or of the supporting electrolyte plus enzyme alone. (The residual current correction was high compared to the total current in this system.) In other cases the insoluble particulate "enzyme" was mixed with the cation solution, centrifuged for 30 min at 14,000 rpm in a Lourdes refrigerated centrifuge, precipitated as a pellet on the bottom of the tube and the polarograms were run on the supernatant. The purpose of this procedure was to eliminate or decrease the adsorption of organic material on the electrode. The experiments were done with active (showing Na-K ATPase activity) native enzyme, heat-inactivated enzyme (heated for 5 min in water at 100 °C and cooled in ice), or on active enzyme dialyzed against 10^{-1} N Naac-Hac. All the experiments were done at 25 °C.

Results and Discussion

Adsorption on the Electrode

The adsorption of material from the ATPase preparation at the polarized mercury/water interface was studied by a−c polarography in the presence of 0.1 N $NaNO_3$. Around pH 7 the adsorption range is −0.1 to −1.2 V (*vs.* Ag/AgCl 0.1 N NaCl), as indicated by the appearance of adsorption and desorption peaks (*see* Fig. 1). At low enzyme concentration (0.1 mg/ml protein), desorption is complete above −1.2 V as the current for the salt in the presence and absence of the "enzyme" is the same. For high "enzyme" concentrations (0.5 mg/ml protein) at the very negative polarizations the desorption is probably not complete as the values of the current do not coincide.

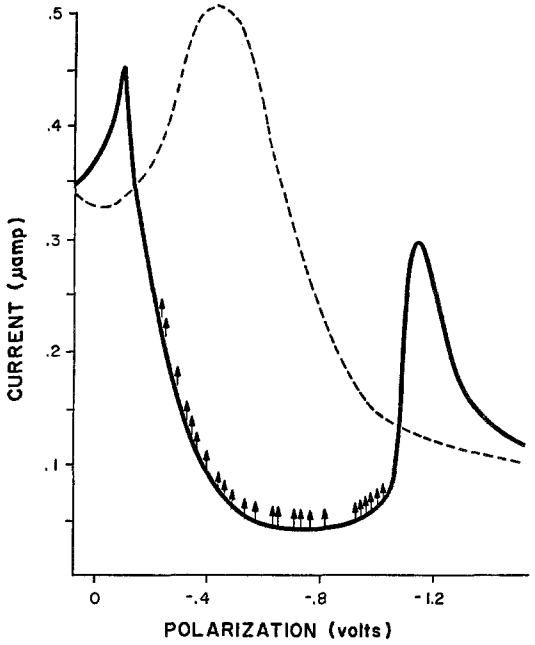

Fig. 1. The envelope of an a−c polarogram obtained in 0.1 N $NaNO_3$ (----) and in 0.1 N $NaNO_3$ containing enzyme at a concentration of 0.1 mg protein/ml (−). In both cases the a−c potential was 10 mV and the pH of the solution was 7. The arrows on the curve indicate the points at which the random currents (of various magnitudes up to 0.2 μamp and all in an upward direction) appeared in the presence of an enzyme preparation

Between pH 6 and pH 9, the adsorption has essentially the same characteristics as shown in Fig. 1. However, below pH 4 the range of adsorption is smaller. The adsorption peak appears around −0.5 V (with no adsorption in the range where Hg is positively charged), indicating that the adsorbing material is probably positively charged. From adsorption studies at various pH's it appears that the isoelectric point of the adsorbing material is between pH 4 and pH 5.5.

In the preparation of the enzyme, the membrane particles are rinsed and centrifuged at least four times, so the material that adsorbs on the electrode surface must be associated with the particles.

Random Currents

The polarographic results of Fig. 1 as well as other experiments involving the membrane fragments all indicate the random currents that have been reported earlier by Blank and Britten (1970). These are apparently due to the presence of particles in the solution since they do not appear in the

preparations where the particles are removed by centrifugation and they have been reported in suspensions of surfactants, phospholipids (Blank & Britten, 1970), and inorganic substances (Micka, 1965). The a–c polarograms show that the random currents appear in the region where adsorption occurs in the presence of the "enzyme". In our earlier report, we showed that the currents could be eliminated when various biologically active substances are added to the suspension of the membrane particles. From a–c polarography it was found that these substances are adsorbed at the mercury/water interface in the same region and usually more strongly than the "enzyme". These results indicate that the material adsorbed from the enzyme preparation would tend to be displaced from the electrode surface (in part or completely) when the other surface-active substances are added, and this can account for the elimination of the random currents by these substances.

The random currents are related to the changes in the electrical double layer at the mercury/water interface. From Fig. 1 we see that the capacity current decreases in the presence of the enzyme due to adsorption, and when the random currents appear, they also indicate changes in adsorption and in capacitance C. The charge on a surface $Q=CV$, where V=potential relative to the electrocapillary maximum (ECM), and the current required to charge the surface at constant V,

$$i=\frac{dQ}{dt}=V\frac{dC}{dt}.$$

The sign of i depends on the value of the polarization, and one would expect a change in direction of current at the ECM. The gradual change in the frequency of the random currents follows from the fact that V increases as one goes away from the ECM on either side, and the capacitance change on collision varies with the magnitude of V. The magnitude of the observed random currents, $\sim 10^{-9}$ amps, is also compatible with a change in double-layer capacitance.

Ion Binding and Enzyme Activity

The polarographic current of an ion undergoing an electrode reaction is proportional to the square root of its diffusion coefficient in the vicinity of the electrode. If an ion is bound to material having lower diffusion coefficient, this causes a decrease in polarographic current. Depending on the magnitude of the association constant between the ion and the slower substance, the diffusion current of the ion will be given by the average diffusion coefficient of the bound and the free ion (when there is a low association constant), or by the diffusion coefficient of the bound ion

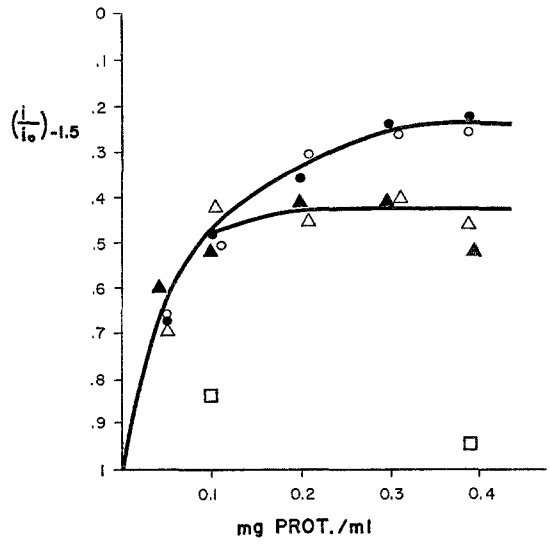

Fig. 2. The variation of (i/i_0) at -1.5 V when native or denatured enzyme preparations (in mg protein/ml) are added to a solution containing 0.1 N $NaNO_3$ and 5×10^{-5} N cation. The cations are: ○ Ag^+, △ Cu^{++} and □ Tl^+. The open symbols correspond to the experiments with native enzyme while the full symbols are for denatured enzyme

(Kacena & Matousek, 1963). In both cases, a decrease in current will be observed that is independent of applied polarization.

However, since an adsorbed film can also result in lower currents, it is necessary to perform the binding experiments at polarizations where there is no adsorption; i.e., above -1.2 V according to Fig. 1. Therefore, the binding of Tl^+, Cu^{++} and Ag^+ to the enzyme preparation was studied at -1.5 V. The supporting electrolyte was either Naac-Hac buffer or $NaNO_3$ at concentrations of 10^{-3} and 10^{-1} N, and the preparations were either native or heat-denatured enzyme.

Figs. 2 and 3 present i/i_0, the ratio of the current in the presence of the "enzyme" to the current of pure ion as a function of the added "enzyme" at different salt concentrations. From these data it appears that the binding of Tl^+ is undetectable in this system, and that there is a strong binding of Cu^{++} and Ag^+, with Cu^{++} binding being a bit lower than that of Ag^+. There is also a negligible influence of the change of salt concentration. We can calculate the average weight of the protein per binding site as about 5,000, indicating that there is probably a large amount of binding to sites unrelated to the enzyme activity. This is in line with our observation that there is no difference between the binding to the native and denatured enzymes. (The results of Figs. 2 and 3 were obtained on the supernatant solution after removing the membrane particles, and essentially the same

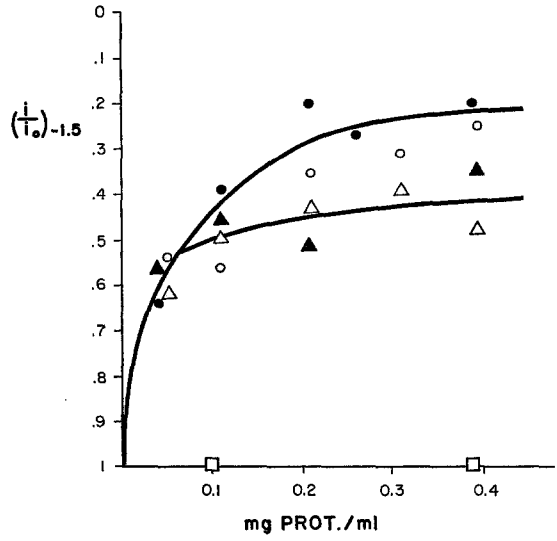

Fig. 3. The variation of (i/i_0) at -1.5 V when native or denatured enzyme preparations (in mg protein/ml) are added to a solution containing 0.001 N $NaNO_3$ and 5×10^{-5} N cation. The cations are: ○ Ag^+, △ Cu^{++} and □ Tl^+. The open symbols correspond to the experiments with native enzyme while the full symbols are for denatured enzyme

results were obtained with the particles present.) The shape of the curves in Figs. 2 and 3 points toward a low association constant, above 0.1 mg/ml protein. There is a small decrease in i/i_0 with increasing protein concentration and in all cases i/i_0 does not reach values lower than 0.2. The lack of a difference between the binding experiments done with and without centrifugation suggests that the binding is caused by some soluble component that does not sediment upon centrifugation.

Ag^+ and Cu^{++} ions interact strongly with the Na-K ATPase, and cause a loss of activity at a relatively low ionic concentration. Fig. 4 shows the effect of these ions on the enzyme activity as well as the inhibitory effect of Tl^+ which occurs at a much higher concentration. The inhibiting effect of Cu^{++} on $(Na^+ + K^+ + Mg^+)$ ATPase from rat brain microsomes and rat erythrocyte ghosts was also described by Bowler and Duncan (1970). [Tl^+ also has an activation effect (Britten & Blank, 1968) on the enzyme, and this can be seen from Fig. 4.] Comparing Fig. 4 to both Figs. 2 and 3 we see that the pattern of binding parallels the pattern of inhibition, with $Ag^+ > Cu^{++} \gg Tl^+ \sim 0$. However, the concentrations of Ag^+ and Cu^{++} studied were in excess of those required for complete inhibition. This evidence is a further indication that the ions bound to many sites in addition to those required for inhibition. (This is to be expected, especially in view of the impure nature of the enzyme preparation.)

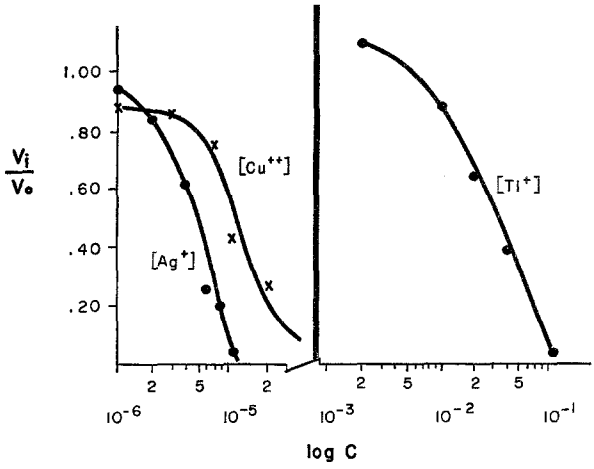

Fig. 4. The relative enzyme activity (V_i/V_0) as a function of the molar concentration of added ion. The Na-K-ATPase activity was determined in the usual way

These results suggest that detectable binding to the enzyme, as in the case of Ag^+ and Cu^{++} ions, destroys the activity, but that an activating ion, Tl^+, does not bind. This is compatible with the thought that an activating ion interacts with the enzyme to exert its effect, but that the "bond" is weak and easily split.

Adsorption and Interfacial Ion Transport

Since substances from the enzyme preparation are adsorbed in a wide range of potentials, it is possible to study the transport of cations across the adsorbed layer at many polarizations. From a–c polarography (as in Fig. 1) the capacity current remains constant above 0.2 mg/ml and there is a shallow minimum around −0.8 V, so this potential was chosen for comparing the transport of cations.

Tl^+ transport was studied in three different electrolytes at different salt concentrations in the presence of adsorbed native and denatured "enzyme". The polarograms look completely normal as it is possible to see from Fig. 5. The fluctuations were random and independent of salt and enzyme concentrations, and in all cases the decrease of current was much smaller than 20%. Part of the decrease in current is due to the dilution associated with the addition of the enzyme, so the net effect is that the Tl^+ current is impeded to a very slight extent by the adsorbed film.

In the case of solutions containing Ag^+ and Cu^{++} ions there is a very big decrease of Ag^+ or Cu^{++} current amounting to about 80% at −0.8 V (*see* Fig. 6). The decrease is due to two factors: the binding of ions and the

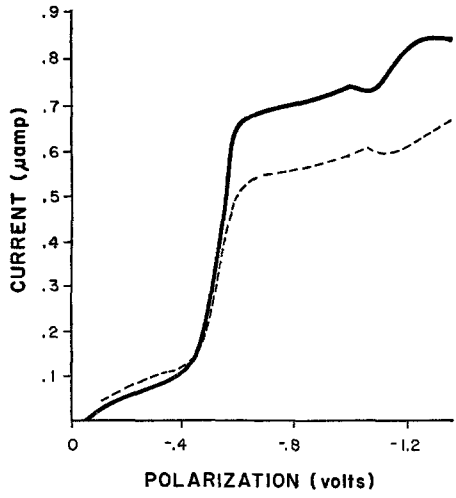

Fig. 5. A polarogram obtained for 5×10^{-5} N Tl^{+} in a 0.1 N $NaNO_3$ medium (—) and in the presence of added enzyme (----). (The random currents are not indicated)

interfacial resistance due to the adsorbed film. From Figs. 2 and 3 we can estimate that about 80% of the decrease is due to the binding and about 20% to the resistance of the interfacial film. The relatively low resistance of the adsorbed film to the flow of cations is expected from the observation that it has a net negative charge at neutral pH.

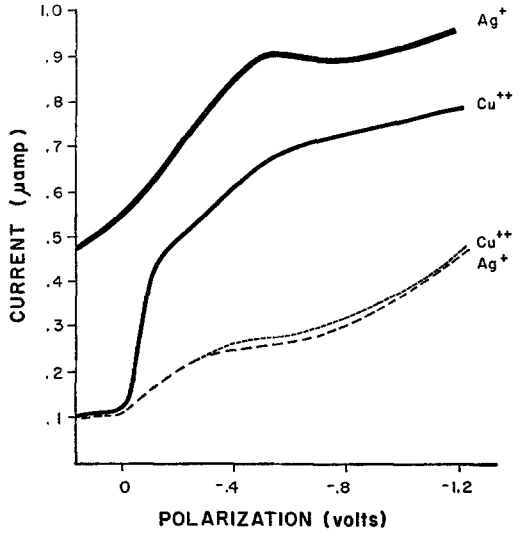

Fig. 6. A polarogram obtained for 5×10^{-5} N Ag^{+} or Cu^{++} (as indicated on the curves) in a 0.1 N $NaNO_3$ medium (—) and in the presence of added enzyme (----). (The random currents are not indicated)

Conclusion

The results of these studies indicate that there are clear differences between the physical properties of the two inhibiting ions and the one activating ion studied. The inhibiting ions bind strongly to the Na-K-ATPase preparation while the activating ion does not. While the studies indicate that there is a considerable amount of nonspecific interaction of the ions with the enzyme preparation, the qualitative differences between the binding of the inhibiting and the activating ions suggest that detectable binding to the ATPase system results in a loss of activity, but that an activating ion does not bind.

The random currents in particulate systems appear to be caused by the collision of the particles with the electrode and subsequent changes in the double-layer structure. Since the changes in the double layer indicate changes in adsorption, the random currents may be measuring the transfer of minute amounts of surface active material between an electrode and a particle.

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