The EMBO Journal vol.4 no.12 pp.3079–3085, 1985

Pump currents generated by the purified Na^+K^+-ATPase from kidney on black lipid membranes

K.Fendler, E.Grell, M.Haubs[1] and E.Bamberg

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, D6000 Frankfurt am Main, and [1]Organisch-Chemisches Institut, Universität Mainz, J.J. Becher Weg, D 6500 Mainz, FRG

Communicated by D.Oesterhelt

The transport activity of purified Na^+K^+-ATPase was investigated by measuring the electrical pump current induced on black lipid membranes. Discs containing purified Na^+K^+-ATPase from pig kidney were attached to planar lipid bilayers in a sandwich-like structure. After the addition of only μM concentrations of an inactive photolabile ATP derivative [P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate, caged ATP] ATP was released after illumination with u.v.-light, which led to a transient current in the system. The transient photoresponse indicates that the discs and the underlying membrane are capacitatively coupled. Stationary pump currents were obtained after the addition of the H^+, Na^+ exchanging agent monensin together with valinomycin to the membrane system, which increased the permeability of the black lipid membrane for the pumped ions. In the absence of ADP and P_i the half saturation for the maximal photoeffect was obtained at 6.5 μM released ATP. The addition of ADP decreased the pump activity. Pump activity was obtained only in the presence of Mg^{2+} together with Na^+ and Na^+ and K^+. No pump current was obtained in the presence of Mg^{2+} together with K^+. The electrical response was blocked completely by the Na^+K^+-ATPase-specific inhibitors vanadate and ouabain. No pump currents were observed with a chemically modified protein, which was labelled on the ATP binding site with fluoresceine isothiocyanate. The method described offers the possibility of investigating by direct electrical measurements ion transport of Na^+K^+-ATPase with a large variety of different parameters.

Key words: sodium potassium ATPase/pump currents/black lipid membranes

Introduction

Na^+K^+ transporting ATPase (EC 3.6.1.3), the Na^+K^+-ATPase, from mammalian cells, is an electrogenic pump where three Na^+ ions are exchanged for two K^+ ions in the presence of Mg^{2+}. The energy for the pump is obtained from hydrolysis of ATP. Electric pump currents of Na^+K^+-ATPase have been observed in intact cells (Abercrombie and De Weer, 1978; Lederer and Nelson, 1984; Gadsby *et al.*, 1985). The transport activity of Na^+K^+ATPase has been studied in red cells (Glynn and Karlish, 1978; Hoffmann *et al.*, 1979) as well as in reconstituted vesicles (Goldin and Tong, 1974; Brotherus *et al.*, 1983; Forbush III, 1984; Cornelius and Skou, 1984; Apell *et al.*, 1985). Time-resolved studies with a photolabile ATP derivative (P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate, caged ATP) have been reported on red cells and on reconstituted proteoliposomes (Forbush III, 1984; Kaplan *et al.*, 1978). For reviews see Glynn and Karlish (1975), Schuurmans-Stekhoven and Bonting (1981) and Jørgensen (1982).

ATP-driven electrical pump currents in reconstituted systems have not been observed until now. Reconstitution of microsomes or reconstituted vesicles on planar lipid bilayers showed a channel-like activity, which was blocked by ouabain, but could not be induced by ATP (Last *et al.*, 1983; Reinhardt *et al.*, 1984). These experiments suggested that the Na^+K^+-ATPase functions partially as an ion channel.

Here we describe how to induce and measure ATP-driven pump currents arising from the Na^+K^+-ATPase on black lipid membranes. Purified Na^+K^+-ATPase membranes in the form of cup-shaped discs (0.1 – 0.6 μm diameter) were associated in a sandwich-like structure with a planar lipid bilayer. The pump was activated by photolysis of caged ATP by a u.v.-flash, leading to a concentration jump of ATP of ~ 10 μM. The resulting pump current was measured by the capacitative coupling between the discs and the supporting lipid bilayer.

Results

Development of the pump current and the effect of specific inhibitors

Purified Na^+K^+-ATPase membrane discs were added with caged ATP to one side of the positively charged lipid bilayer. The aqueous solution contained 3 mM $MgCl_2$, 20 mM KCl, 130 mM NaCl, 25 mM imidazole buffer. The pH was adjusted to 7.5. After 15 min a transient current induced by a u.v.-light pulse was obtained. A record of the short-circuit current obtained 30 min after the addition of the protein and caged ATP to the lipid bilayer is shown in Figure 1, trace b. After the u.v.-flash the signal rises within 100 ms, followed by a decay, which can be described by two exponentials ($\tau_1 \sim 0.1$ s, $\tau_2 \sim 1.0$ s). The observed current is transient, because the discs form a sandwich-like structure by adsorption to the underlying planar lipid bilayer (Figure 2).

The sign of the current in all experiments was the same. Under identical conditions, different experiments gave current amplitudes with a variation of 50%. During the same experiment on the same membrane, however, the current amplitude was reproducible with an accuracy of 5%. The direction of the current corresponds to a movement of positive charges to the protein-free side of the membrane system, indicating that the periplasmic side of the ion-transporting discs is faced with the lipid bilayer, whereas the cytoplasmic side is directed to the electrolyte of the *cis*-chamber of the cuvette.

The discs are apparently not integrated into the black film, because in the case of catalytic (continuous) pump activity of the Na^+K^+-ATPase stationary pump currents should result in a short-circuit experiment, as long as liberated ATP is available for the enzyme. That the Na^+K^+-ATPase acts on the combined disc-bilayer system as a pump was demonstrated by stationary pump currents. The underlying lipid bilayer membrane was made permeable to Na^+ and K^+ as has been performed for the light-

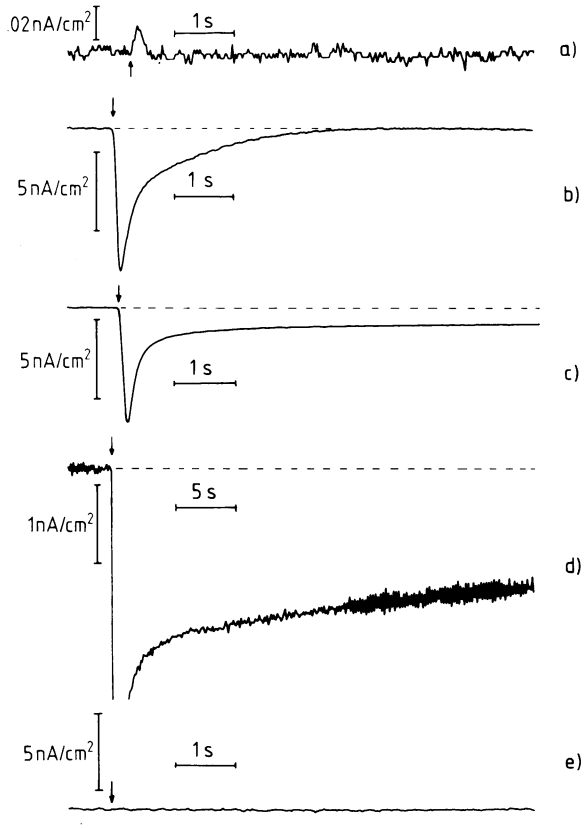

Fig. 1. Short circuit currents on the lipid bilayer system under different experimental conditions. The membrane bathing solution contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 25 mM imidazole buffer at pH 7.5, 35 μM caged ATP. Light intensity 3.7 W/cm², flash duration 0.125 s. The arrows indicate opening of the shutter. Trace **a**: u.v.-light artifact before the protein was added. Trace **b**: transient current after the addition of 50 μl purified Na^+K^+-ATPase, 2.5 mg protein/ml. Traces **c** and **d**: demonstration of stationary pump currents after addition of 1 μM valinomycin and 10 μM monensin. Note the different time scale of trace **d**. Trace **e**: Blocking of the pump current by 1 mM vanadate.

driven proton pump bacteriorhodopsin with protonophores or for the light-driven chloride pump halorhodopsin with a chloride-transporting system (Hermann and Rayfield, 1978; Bamberg *et al.*, 1979, 1984).

In the case of Na^+K^+-ATPase the H^+/Na^+, K^+ exchanging agent monensin was added to the system with the K^+-carrier valinomycin. As can be seen from Figure 1c, the current induced by a u.v.-flash became nearly stationary (in the 10-s range) after a transient component and disappeared after minutes (not shown), indicating that all liberated ATP is hydrolysed by the enzyme (which is also present in suspension in the rear compartment). The pump activity of the Na^+K^+-ATPase was demonstrated by use of the ion carriers monensin and valinomycin. For most experiments, however, only the transient currents in absence of the ionophores were considered, to avoid eventual interference of carriers with the enzyme activity.

The addition of 1 mM vanadate abolished the electrical pump current (Figure 1c). Ouabain did not block the pump if it was added to the lipid bilayer system at a concentration of 1 μM. If, however, the Na^+K^+-ATPase was pre-incubated for 10 min with 4 mM ouabain and then added to the lipid bilayer, no electrical response was obtained. It can be concluded that the binding site for ouabain is not accessible to those of the attached discs, which contribute to the pump current. The orientation of the attached discs is discussed below. Similar behaviour was found for the blocking agent erytrophlein, which is assumed to bind to the same site as ouabain (Goldin, 1977). The fluoresceine isothiocyanate-modified Na^+K^+-ATPase under similar experimental conditions did not show any electrical activity, which can be explained by the blocking of the ATP binding site by the label (Karlish *et al.*, 1979).

Figure 3 shows the action spectrum of the pump current with the absorption spectrum of caged ATP. The deviation of the photoresponse at longer wavelengths is due to the absorption of the protecting group of the caged ATP in this part of the spectrum. The asymmetry of the spectrum at shorter wavelengths arises from the absorption of imidazole buffer at 230 nm. It should be noted that the application of a second flash within a few seconds did not give more than 1/10 of the original signal. After 10 min, however, the signal was restored completely. Stirring lowered the recovery time to 1 min. The time course of the restoration of the electrical signal can be explained as follows. First, the caged ATP is bleached within the light beam. Then, after consumption of the ATP, new caged ATP has to be bound or it reaches the Na^+K^+-ATPase by diffusion.

Temperature dependence of the pump current

Since the activity of Na^+K^+-ATPase shows a marked temperature dependence, the pump current was measured at different temperatures (Figure 4). The hysteresis effect can be explained by inactivation of the protein and by thermolysis of the caged ATP. An evaluation of the temperature dependence yielded an activation energy of 55 kJ/mol, for increasing as well as for falling temperatures (Figure 4). The smaller value for the activation energy compared with previous results (Post *et al.*, 1965; Apell *et al.*, 1985) is presumably due to inactivation of the enzyme by warming-up in the cuvette during the experiment.

Ion specificity of the pump current

The membrane system presented above is attractive for studying the influence of the different ions on the pump current, because the ion composition can be changed on the same membrane simply by adding different ions successively or simultaneously. Figure 5 summarizes the results obtained with different electrolyte compositions. Trace a shows the electrical signal under normal conditions (3 mM Mg^{2+}, 20 mM K^+, 130 mM Na^+). Removing Mg^{2+} by addition of 10 mM EDTA abolished the pump current (trace b), showing the well-known requirement of Mg^{2+} for the Na^+K^+ ATPase activity. In another set of experiments only either Na^+ or K^+ were present, together with 3 mM Mg^{2+}. Trace c shows that K^+ does not give any electrical response, while in the presence of Na^+, an ATP-dependent electrical signal was measured (trace d). The signal amplitude at various Na^+ concentrations was studied in the absence and presence of 20 mM K^+ (Figure 6). The half-saturation for Na^+ was 7 mM in the absence of K^+ and 140 mM when K^+ was present. The electrical signal in the presence of Mg^{2+} and Na^+ was inhibited by the addition of 1 mM vanadate.

The effect of ATP, ADP and P_i on the electrical activity

The pump activity was measured at different concentrations of released ATP (Figure 7). The peak current was half-maximal at 6.5 μM in the presence of 130 mM Na^+, 20 mM K^+ and 3 mM Mg^{2+}. As shown previously, ADP inhibited the enzymatic activity of the pump by competition with ATP (Hegyvary and Post, 1971). Figure 8a shows that additional ADP reduced the electrical response of the pump. The reduction by ADP is more pronounced (by a factor of 2) in the absence of K^+. Similar behaviour was observed following the addition of P_i (Figure 8b).

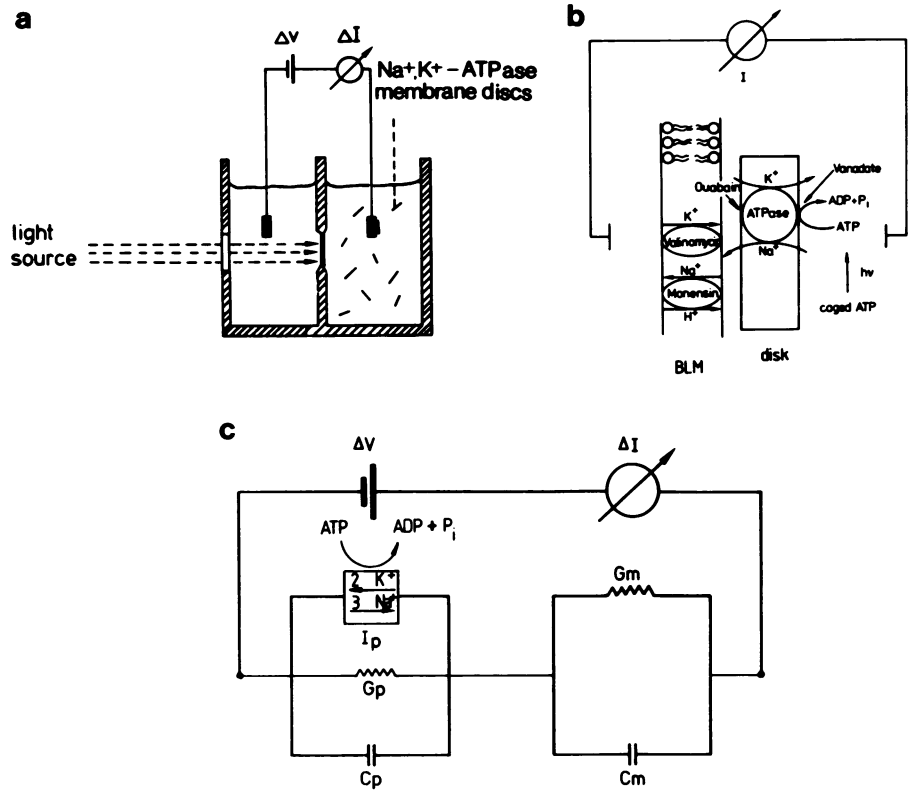

Fig. 2. Schematic representation of the bilayer setup. (i) Teflon chamber with bilayer and adsorbed Na^+K^+-ATPase membrane discs. (ii) Proposed sandwich-like arrangement of discs and underlying lipid membrane. The two membranes are capacitatively coupled. (iii) Equivalent circuit diagram of the two membranes in series. G_m, G_p conductance of the black lipid membrane and the disc membrane, respectively. C_m, C_p capacitance of the black lipid membrane and the disc membrane, respectively. I_p pump current generator. For small values of G_m only transient currents are observable, whereas for large values of G_m, produced by valinomycin and monensin, stationary pump currents were obtained.

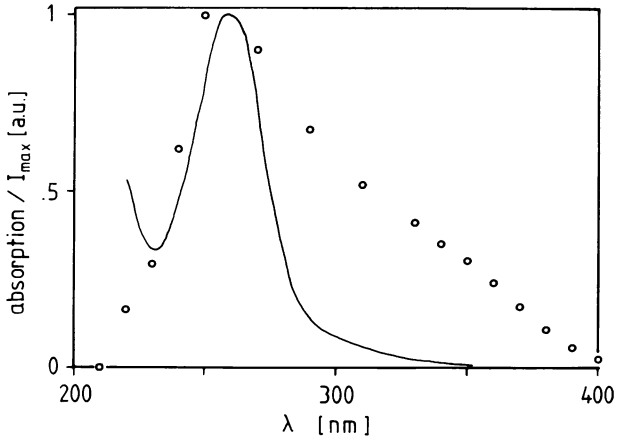

Fig. 3. Action spectrum of the peak currents (I_{max}) generated by the caged ATP/Na^+K^+-ATPase system normalized to equal quantum flux density (0). Conditions as in Figure 1, trace b. For comparison, the absorption spectrum of the caged ATP is included (−).

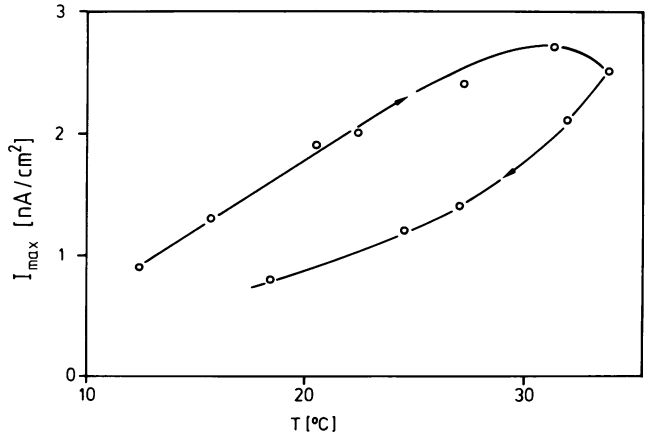

Fig. 4. Temperature dependence of the Na^+K^+-ATPase activity. The peak current I_{max} is plotted *versus* temperature. The sequence of measurements is indicated by the arrows. Time interval between two successive measurements 15 min. Conditions as in Figure 1, trace b.

At concentrations of 15 mM P_i the original signal was reduced by a factor of 2, in agreement with inhibition of the enzymatic activity as described previously (Robinson *et al.*, 1978). It should be noted that at high concentrations (1 mM) of free ATP in the solution the light-induced pump current also disappeared, because of competition between the liberated and free ATP.

Discussion

Detailed studies of light-driven ion pumps such as the H^+ pump bacteriorhodopsin and the Cl^- pump halorhodopsin on black lipid membranes have been described (Hermann and Rayfield, 1978; Bamberg *et al.*, 1979, 1984). These pumps are chromoproteins with the chromophore retinal. On the black lipid membrane system these pumps from the salt-loving bacteria *Halobacterium halobium* can be activated directly by light, whereas the Na^+K^+-ATPase has to be activated by a light-induced concentration jump of the substrate ATP. The liberation time for the caged ATP initiated by a u.v.-flash at pH 7.5 is ~ 10 ms (McCray *et al.*, 1980).

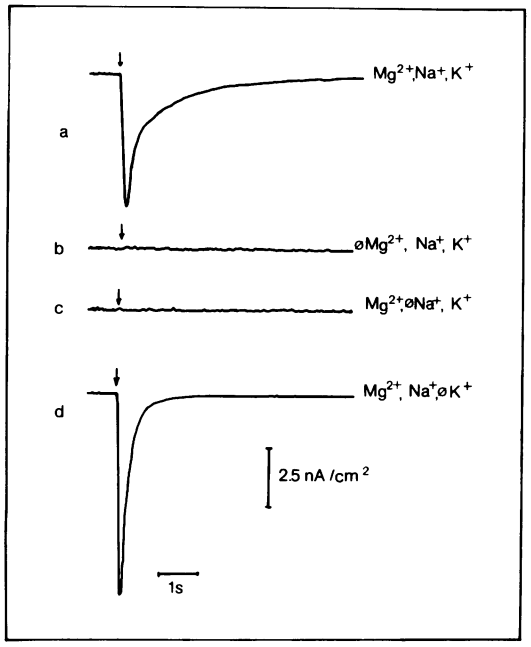

Fig. 5. Ion specificity of the electrical response of the Na^+K^+-ATPase. Conditions as in Figure 1, trace b, except the ion composition of the electrolyte. Trace **a**: 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl. Trace **b**: 0 $MgCl_2$, 10 mM EDTA, 130 mM NaCl, 20 mM KCl. Trace **c**: 3 mM $MgCl_2$, 0 NaCl, 20 mM KCl. Trace **d**: 3 mM $MgCl_2$, 130 mM NaCl, 0 KCl. The arrows indicate the opening of the shutter, flash duration 0.125 s.

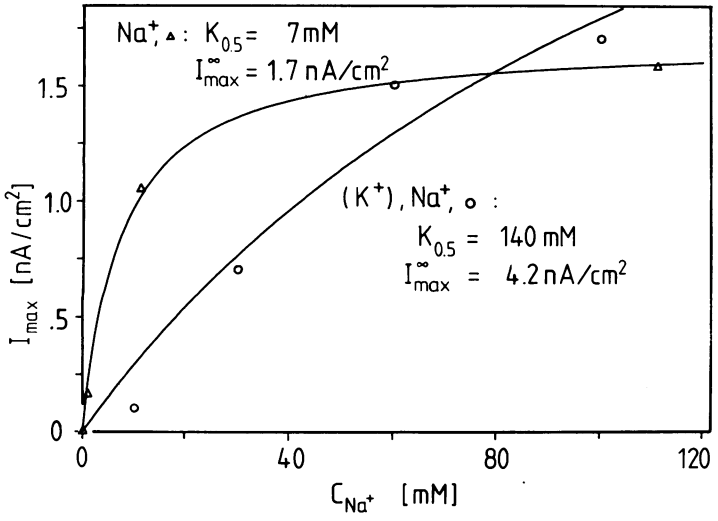

Fig. 6. Dependence of the peak currents on Na^+ concentration with 20 mM K^+ (○) and without K^+ (△). The line drawn represents the fit according a Michaelis Menten formalism. Other conditions as in Figure 1, trace b.

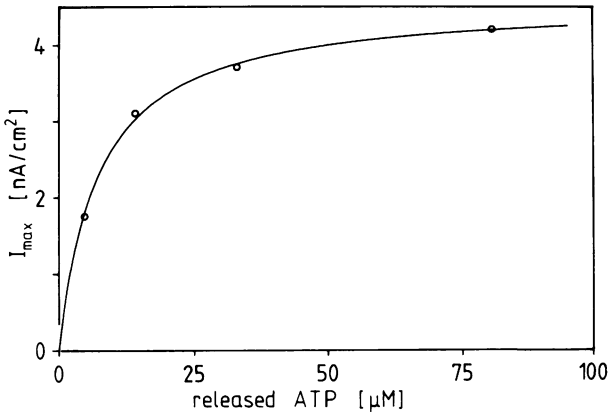

Fig. 7. Peak current dependency on concentration of ATP released per 0.125-s light flash. The concentration of the ATP released per light flash was measured under stirring with the luciferin/luciferase assay. For the concentrations given in this figure it has been taken into account that ATP is released only within the light beam, which includes only 1% of the whole volume of the cuvette. The line drawn represents the fit according a Michaelis Menten formalism. Other conditions as in Figure 1, trace b.

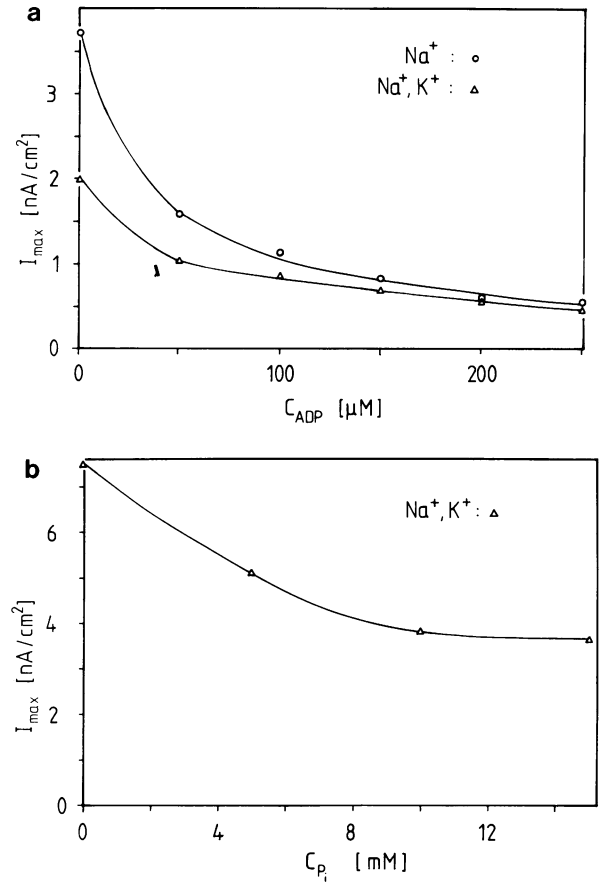

Fig. 8. Inhibition of the Na^+K^+ -ATPase activity by additional ADP and P_i. **(a)** Inhibition by ADP in presence (△) and absence (○) of K^+. **(b)** Inhibition by P_i in presence of Na^+ and K^+. The straight line is a guide to the eye. Other conditions as in Figure 1, trace b.

While for the retinal-binding proteins the time resolution is limited by the measuring system for photocurrents to ~1 μs (Fahr *et al.*, 1981), the reaction rate for photolysis of the caged ATP is limiting for the Na^+K^+-ATPase. In addition, as discussed below, effects produced by the photoproducts and diffusion phenomena are probably important for the time course of the observed currents.

All results obtained above can be explained by the assumption that the discs with the purified Na^+K^+-ATPase are adsorbed to the lipid bilayer and not integrated into it. The disadvantage of the method described consists of the sandwich-like structure on which the interfacial area between the two membranes is not easily accessible for ions and substrates. Stationary pump currents can only be obtained with the help of the ionophores. On the other hand, the adsorption of membrane fragments to the underlying lipid membrane is advantageous, because the in-

vestigated protein must not be transferred into a new lipid environment. Therefore the data obtained from other studies using completely different methods can be compared meaningfully, without objections, with the electrical measurements related to the lipid environment and solvent contents of the lipid bilayer. The different dependencies of the electrical pump activity on ATP, ion composition of the electrolyte and Na^+K^+-ATPase specific inhibitors give strong evidence for the functional reconstitution. Since the methods to measure the pump current in the sandwich-like systems have been described and discussed previously for bacteriorhodopsin and halorhodopsin (Bamberg *et al.*, 1979, 1984), this discussion is focused on Na^+K^+-ATPase specific points of the bilayer system.

Sidedness of the adsorbed discs

As described above, the Na^+K^+- ATPase was blocked differently on the black lipid membrane by vanadate, ouabain and erytrophlein. It is important to note that the binding site of the Na^+K^+-ATPase for vanadate is intracellular, whereas ouabain and erytrophlein bind at the extracellular site (Figure 2). While vanadate inhibited the electrical activity within a minute after addition to the membrane, ouabain had no effect and erytrophlein blocked the system by 50% one hour after the addition. However, discs pre-incubated wih ouabain and erytrophlein added to the black lipid membrane, did not show any pump current. This, together with the fact that the sign of the pump current was always same for all experiments, can be explained in two ways: (i) The discs adsorb with populations oppositely oriented to the lipid bilayer. Both populations are accessible on the interface between the disc and lipid bilayer membrane for the caged ATP as well as ouabain (erytrophlein). Therefore only one population facing the extracellular side of the lipid bilayer can be activated by ATP, and consequently cannot show any ouabain effect. (ii) If, however, the caged ATP has access to both sides of the discs, the membrane fragments have to be adsorbed with a high degree of orientation. Otherwise the application of ouabain or erytrophlein should lead to an increase of the pump current because the contribution of the oppositely oriented pump should be blocked.

Shape of the electrical signal

After a u.v. flash of 0.125 s the current rises linearly within 100 ms and decays within seconds to zero (Figure 1, trace b). The increase is not limited by the measuring system (the shape of the signal is unchanged for rise times as low as 3 ms), but rather by the production rate of active ATP during the u.v.-light flash. This is shown in Figure 9, giving the rise of the current at different light intensities. Considering the geometry of the light beam and the ATP production measured with a luciferin-luciferase assay, with every flash of 0.125 s (3.7 W/cm²) ~10 μM ATP were liberated within the light beam after application of 35 μM caged ATP. Correspondingly less ATP was liberated at lower light intensities. The decay of the signal is due to the capacitative coupling between the two membranes and the depletion of ATP by consumption of the enzyme and the related diffusion phenomena near the disc membrane.

Valinomycin with monensin yielded, in addition to the capacitative current, a stationary component. As shown in Figure 1c and d, the current decays within minutes due to the consumption of liberated ATP. Preliminary experiments with u.v.-laser as light source (10-ns pulse duration, 100 mJ/cm²) decreased the rise time of the signal to 10 ms, which is close to the rate of photolysis of the caged ATP at pH 7.5. The time resolution can

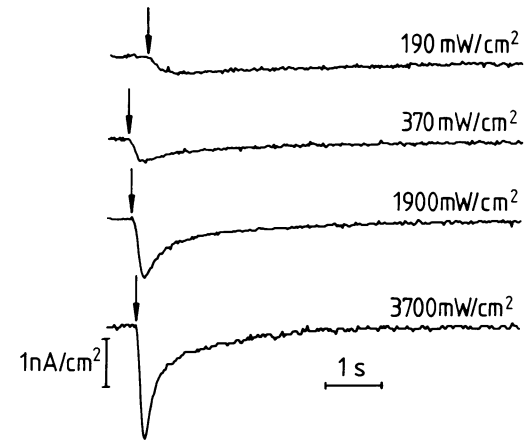

Fig. 9. Time course of the short circuit currents at different light intensities. The arrows indicate the opening of the shutter, flash duration 0.125 s. Other conditions as in Figure 1, trace b.

be enhanced to ~1 ms by lowering the pH to 6.2 – 6.5 (McCray *et al.*, 1980).

Inactivation of enzyme activity by u.v.-light and the photoproducts

Inhibition of the enzymatic activity of Na^+K^+-ATPase by the photoproduct orthonitroacetophenone was reported by Kaplan *et al.* (1978). These experiments were carried out at 1.8 mM caged ATP. In the present study, however, only 35 μM caged ATP was used, so that this effect should be negligible. In addition, the application of glutathione which removed the inhibition by the photoproduct (Kaplan *et al.*, 1978), did not influence the electrical signal of the lipid bilayer system, indicating no measurable interference by the photoproduct with the electrical activity of the enzyme.

The enzyme was not inhibited by the applied u.v.-light flash (0.125 s, 3.7 W/cm²). This was proven by application of 15 flashes every 15 min under the same conditions on the same membrane. All 15 experiments resulted in almost the same current amplitude, showing that the enzyme was not damaged and the pool of caged ATP was not exhausted. An illumination time of 5 min led to the inactivation of the system due to complete photolysis of the caged ATP and to partial damage of the enzyme by u.v.-light. The addition of further caged ATP restored the electrical activity to ~30% of the original value. In addition, the use of a 320 nm cut-off filter did not change the signal at a flash duration of 0.125 s. Therefore under these light conditions no damage to the protein was detectable.

Ion selectivity

The pump current was dependent on the composition of the electrolyte. Under normal conditions, i.e., at 3 mM Mg^{2+}, 130 mM Na^+ and 20 mM K^+. A transient current, and with valinomycin and monensin a stationary current, was obtained. For any electrical activity the presence of Mg^{2+} was required (Figure 5b). No signal, however, was obtained in the absence of Na^+ (Figure 5c), which is consistent with an electro-neutral K^+,K^+ exchange (Simons, 1974). An interesting result is the appearance of electrical currents in the presence of 3 mM Mg^{2+} at different concentrations of Na^+. The half-saturation in the K^+-free medium was 7 mM compared with 140 mM in the presence of 20 mM K^+ (Figure 6). $K_{0.5}$ values for the binding of Na^+ have been determined by measuring the enzymatic activity *via* the splitting of ATP. At 0 K^+ a $K_{0.5}$ of 1.3 mM was found, whereas the

presence of K^+ the value increased to 14 mM (Robinson, 1977). Taking the experimental data directly without curve fitting an apparent half saturation of 35 mM for Na^+ in the presence of K^+ is obtained. The difference from the electrical measurements is probably due to the different ATPase preparation (the ATPase was taken from brain). To decide whether Na^+ currents in the absence of K^+ are due to a single turnover or to the continuous pump activity, the lipid bilayer must be made permeable for Na^+ ions to obtain stationary currents. Unfortunately, neither the Na^+ carrier system monensin plus carbonyl cyanide p (trifluoromethoxy)phenylhydrazone (FCCP) nor the Na^+-transporting channel gramicidin A can be used, because of a marked u.v.-sensitivity of FCCP and gramicidin A, so that this question remains open. To prove whether the interface between the disc membrane and the underlying lipid bilayer is Na^+-depleted or enriched with Na^+, the electrical response of the enzyme was tested with and without the electrically silent Na^+, H^+ carrier monensin. No difference between the two experiments was found. Therefore the successive addition of Na^+ to the sandwich-like system yields symmetrical conditions for both sides of the disc, or possible asymmetries did not affect the result. In the literature (Garrahan and Glynn, 1967; Garay and Garrahan, 1973) two mechanisms for Na^+ transport in the absence of K^+ outside the cell are reported. (i) The Na^+, Na^+ exchange, which requires K^+ inside the cell and high Na^+ outside and the presence of very small concentrations of ATP. (ii) The uncoupled Na^+ flux, which requires high inside Na^+ concentrations and the presence of K^+ (Glynn and Karlish, 1978). Neither of these conditions, in one way or the other, are fulfilled for the Na^+-dependent activity presented, because no K^+ is present in our experiments.

Since no K^+ was present, Na^+ transport is possibly due to Na^+, Na^+ exchange and/or uncoupled Na^+ flux. Na^+, Na^+ exchange is electro-neutral but may contribute to an electrical signal, if the back and forth transport are kinetically different. Another interpretation of our experiments is that Na^+ is released as an early electrogenic event, before K^+ is bound, as suggested in Forbush III (1984). The result is difficult to understand in the light of the current models, where Na^+ can only be transported simultaneously with K^+. It should be noted, however, that the sodium solutions made from p.a. reagents (Merck, Darmstadt) contained 0.01% K^+, which resulted in a K^+ concentration at 100 mM Na^+ of ~ 10 μM.

Even at low concentrations of ATP ($K_{0.5}$ = 6.5 μM) a transient current with only Na^+ and with Na^+ and K^+ was observed, which is consistent with the high affinity binding of ATP to the enzyme. The decay of the signal, (Figure 5, trace a, d) is delayed in the presence of K^+ compared with the Na^+ pump signal, perhaps reflecting the continuous pump activity. It is important to note that the amplitude of the transient current is essentially insensitive to the presence of K^+.

This surprising result implies that the method used here allows differentiation between a K^+-independent single step in the transport cycle due to the transient current, and a Na^+- as well as K^+-dependent transport cycle, which can be attributed to the stationary current, reflecting the continuous function of the pump. The simplest possible molecular interpretation of the transient signal is tentatively related to a resulting net transport of Na^+ across the membrane discs to the interfacial space, according the following reaction scheme.

$$\mathrm{ATP + E_1 + n\ Na^+ \leftrightharpoons E_1(ATP) + n\ Na^+ \overset{Mg^{2+}}{\leftrightharpoons} E_1 - P(Na^+)_n + ADP \rightarrow E_2 - P + ADP + n\ Na^+}_{\text{interfacial}}$$

This partial transport sequence represents the initial K^+-independent reaction steps of the overall transport cycle (Albers, 1976) and is also assumed to be involved in the 1:1 Na^+ exchange (Garrahan and Glynn, 1967; Garray and Garrahan, 1973). E_1 and E_2 represent the main conformational states of the enzyme, which are relevant for the transport activity. n is a stoichiometric coefficient. Since ATP is also bound to the enzyme in the absence of Mg^{2+} (Jensen and Norby, 1971), the contribution of Mg^{2+} is assigned to a subsequent reaction step. Addition of K^+ to the Na^+-containing medium would lead to the formation of the K^+ complex and thus would lead to a reduction of the concentration of E_1 due to an equilibrium shift. Therefore, a diminished apparent affinity of Na^+ to the enzyme must result, which indeed has been experimentally observed (Figure 6).

The results gave no evidence for the expectation that the addition of K^+ to the Na^+-containing medium should drastically alter the affinity of ATP to the enzyme as far as the amplitude of the transient current is concerned. Stationary currents were obtained at μM concentrations of ATP. With respect to the stationary current the question arises whether the processes can be assigned to the overall pumping process. We are now trying to clarify this point.

Materials and methods

Preparation of caged ATP

1-(2-nitro)phenylethyl phosphate: 1-(2-nitro)phenylethanol (3 g, 18 mmol) in 10 ml acetone was added under stirring to an ice-cooled mixture of 8 g $POCl_3$ (52 mmol) and 4 g triethylamine (40 mmol) in 70 ml acetone. Stirring was continued for 30 min after removal of the ice bath. The reaction mixture was poured into ice water (100 ml) and the acetone removed by evaporation *in vacuo*. The resulting oily phase was separated by filtration and the filtrate was extracted four times with diethyl ether. After evaporation *in vacuo* of the combined ether extracts 3.5 g of an oily residue were obtained. The residue was dissolved in 15 ml acetone and neutralized with 2 N NaOH to pH 4.5. Upon addition of 60 ml acetone white crystals of the mono-sodium salt trihydrate separated, which were sucked off and washed with acetone (3.5 g). The crystals were dried for 12 h *in vacuo* at 65°C over P_2O_5 to give the semihydrate. Maximum u.v.-absorption was observed at 265 nm in water (ϵ = 5500), where ϵ is the molar extinction coefficient and the FD-mass spectrum exhibited a m/e value of 246 (100%); otherwise the properties corresponded to those reported previously (Kaplan *et al.*, 1978).

Caged ATP. The sodium 1-(2-nitro)phenylethyl phosphate semihydrate was reacted in an equimolar ratio with the 4-morpholine N,N'-dicyclohexylcarboxamidinium salt of ADP-morpholidate (Wehrli *et al.*, 1965) in dry dimethyl sulfoxide and separated as in Kaplan *et al.* (1978). The fractions containing the triethylammonium salt of caged ATP were evaporated *in vacuo* at 25°C. The FD-mass spectrum indicated a m/e value of 655 (100%); otherwise the properties were as reported by Kaplan *et al.* (1978).

Preparation of Na^+K^+-ATPase

The microsomal fraction was prepared from dissected tissue of the red outer medulla of pig kidneys according to Jørgensen (1974a). After a 30-min SDS incubation of the microsomal fraction (1.15 mg SDS/ml, 2.8 mg protein/ml) in the presence of 1 mM Na_2 EDTA and 1.5 mM Na_2ATP, membrane-bound Na^+K^+ ATPase was separated by a 16-h sucrose density-gradient centrifugation at 90 000 *g* and 4°C in a SW 28 swinging-bucket rotor (Beckman Instruments, Dreieich, FRG) in 25 mM imidazole-HCl pH 7.5 containing 1 mM Na_2EDTA (Jørgensen, 1974b). Fractions containing the pure enzyme were diluted with 25 mM imidazole-HCl pH 7.5 and centrifuged for 5 h at 70 000 *g* and 4°C. The pellet was taken up in the same buffer at a concentration of 3 mg protein/ml and stored in ice. Enzyme activity was determined on the basis of spectrophotometric P_i determinations (Jørgensen, 1974b) with 3 mM Tris ATP in 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl and 25 mM histidine-HCl pH 7.5 in the presence of $\sim$ 1 ng protein/ml. Less than 1% residual activity was found with 1 mM ouabain. The specific activities ranged between 30 and 40 μmol P_i $mg^{-1}min^{-1}$ at 37°C and between 15 and 20 μmol P_i $mg^{-1}min^{-1}$ at 25°C. Protein determinations were performed according to a modified procedure as reported by Lowry *et al.* (1951). The calibration was carried out relative to an enzyme reference sample (obtained from Dr. P.L.Jørgensen) which was characterized by amino acid analysis. The enzyme preparation exhibited only two protein components as shown by SDS-gel electrophoresis. Kinetic studies performed at 25°C indicated that 50% inhibition of ATP hydrolysis occurs in the test medium given above

with 1.5×10^{-5} M ouabain, 2×10^{-6} M erytrophlein sulphate and 2×10^{-7} M magnesium vanadate.

Fluorescein isothiocyanate (FITC)Na^+K^+-ATPase was prepared according to Hegyvary and Jørgensen (1981). The final pellet was suspended in 25 mM imidazole-HCl pH 7.5 with a protein concentration of ~2 mg/ml and stored in ice. Whereas the Na^+K^+ ATPase activity of the FITC-enzyme at 37°C in the test medium given above was < 1 μmol $mg^{-1}min^{-1}$, the 2,4-dinitrophenyl phosphatase activity as performed by Gache *et al.* (1979), with 1 mM lutidinium 2,4-dinitrophenyl phosphate in 20 mM $MgCl_2$, 20 mM KCl and 25 mM imidazole-HCl pH 7.5 at 37°C containing ~1 ng protein/ml reached values between 20 and 25 μmol $mg^{-1}min^{-1}$.

Lipid bilayer setup

Optically black lipid membranes with an area of $\sim 9 \times 10^{-3}$ cm^2 were formed in a thermostated Teflon cell with 7 ml of an appropriate electrolyte solution in each compartment (Bamberg *et al.*, 1979). The membrane-forming solution contained 1.5% (w/v) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel-de-Haen, Hannover, FRG) in n-decane (positively charged surface). The latter agent was used to get a higher adsorption of the negatively charged Na^+K^+-ATPase discs to the positively charged surface of the lipid bilayer membrane as has been shown previously for the adsorption of purple membranes from *Halobacterium halobium* (Dancshazy and Karvaly, 1976). The temperature was kept at 25°C unless otherwise indicated. The membrane was connected to an external measuring circuit *via* Ag/AgCl electrodes. To avoid artificial photoeffects, the electrodes were separated from the aqueous compartments of the cell by agar-agar saltbridges. To prevent light pipe effects, the saltbridges were made from polyethylene tubes. In addition, the agar contained black ink to avoid any light conduction to the electrodes.

To photolyse the caged ATP, light pulses from a 200-W Mercury-Xenon high-pressure lamp (Hannovia 901-B1) were focused on the lipid bilayer. For the action spectrum of the caged ATP, i.e., measured pump current *versus* wavelength of the exciting light, a xenon high-pressure lamp was used (Osram XBO 150-W-1). Light pulses of 0.125-s duration with an intensity of 3.7 W/cm^2 were applied, unless otherwise indicated. The light intensity at the membrane plane was measured with a pyrometer (Gentec ED 200). The action spectrum was measured with a grating monochromator (Schoeffel GM 100-1). The intensity of the u.v.-light was attenuated by neutral density filters (Melles-Griot, Netherlands).

Chemicals

Erytrophlein sulphate was kindly made available by E.Merck (Darmstadt, FRG). Sucrose was obtained from Bethesda Research Labs. (Gaithersburg, MD, USA); magnesium vanadate was from ICN Pharmaceuticals (Plainview, NY, USA); TrisATP and FITC isomer I were from Sigma (Taufkirchen, FRG). Lutidinium 2,4-dinitrophenylphosphate was synthesized according to Kirby and Varvoglis (1966). Valinomycin and ouabain were purchased from Serva, Heidelberg, FRG. Monensin was a gift from Dr. G.Szabo, Galveston, TX. All other chemicals used were of analytical grade.

U.v. artifacts

Before the activity of the Na^+K^+-ATPase was determined, the influence of irradiation with u.v.-light was tested. (i) The pure bilayer did not show any effect after a u.v.-flash of 0.125 s duration and 3.7 W/cm^2 light intensity; (ii) the bilayer with adsorbed protein showed no effect; (iii) the bilayer in the presence of 35 μM caged ATP showed a small (< 10 pA/cm^2) photoeffect (Figure 1, trace a); (iv) after the addition of the ionophores valinomycin and monensin no further photoeffect was detectable.

Acknowledgements

The authors wish to thank Mrs. A.Hüby, Mrs. A.Ifftner and Mr. E.Lewitzki for excellent technical assistance; Dr. U.Schäfer for help on the purification of caged ATP and Drs. K.Hartung and Cl.Slayman for many helpful discussions during this work. The work was supported partially by the Deutsche Forschungsgemeinschaft (SFB 169).

References

Abercrombie,R. and De Weer,P. (1978) *Am. J. Physiol.*, **44**, 389-400.
Albers,R.W. (1976) in Martonosi,A.N. (ed.), *The Enzymes of Biological Membranes*, Vol. III, Plenum Publ. Corp., NY, pp. 283-301.
Apell,H.-J., Marcus,M.M., Anner,B.M., Oetliker,H., Oetliker,H. and Läuger,P. (1985) *J. Membr. Biol.*, **85**, 49-63.
Bamberg,E., Apell,H.-J., Dencher,N., Sperling,W., Stieve,H. and Läuger,P. (1979) *Biophys. Struct. Mech.*, **5**, 277-292.
Bamberg,E., Hegemann,P. and Oesterhelt,D. (1984) *Biochim. Biophys. Acta*, **773**, 53-60.
Brotherus,J.R., Jacobsen,L. and Jorgensen,P.C. (1983) *Biochim. Biophys. Acta*, **731**, 290-303.
Cornelius,F. and Skou,J.C. (1984) *Biochim. Biophys. Acta*, **772**, 357-373.
Dancshazy,Z. and Karvaly,B. (1976) *FEBS Lett.*, **72**, 136-138.
Fahr,A., Läuger,P. and Bamberg,E. (1981) *J. Membr. Biol.*, **60**, 51-62.
Forbush III,B., (1984) *Proc. Natl. Acad. Sci. USA*, **84**, 5310-5314.
Gache,C., Rossi,B., Leone,F.A. and Lazdunski,M. (1979) in Skou,J.C. and Norby,J.G. (eds.), *Na,K-ATPase Structure and Kinetics*, Academic Press, London, pp. 301-31.
Gadsby,D.C., Kimura,J. and Noma,A. (1985) *Nature*, **315**, 63-65.
Garay,R.D. and Garrahan,P.J. (1973) *J. Physiol. (Lond.)*, **231**, 297-325.
Garrahan,P.J. and Glynn,I.M. (1967) *J. Physiol. (Lond.)*, **192**, 181-216.
Glynn,I.M. and Karlish,S.J.D. (1975) *Ann. Rev. Physiol.*, **37**, 13-55.
Glynn,I.M. and Karlish,S.J.D. (1978) *J. Physiol. (Lond.)*, **256**, 465-496.
Goldin,S.M. (1977) *J. Biol. Chem.*, **252**, 5630-5642.
Goldin,S.M. and Tong,S.W. (1974) *J. Biol. Chem.*, **249**, 5907-5915.
Hegyvary,C. and Jørgensen,P.L. (1981) *J. Biol. Chem.*, **256**, 6296-6303.
Hegyvary,D. and Post,R.L. (1971) *J. Biol. Chem.*, **246**, 5234-5240.
Hermann,T.R. and Rayfield,G.W. (1978) *Biophys. J.*, **21**, 111-125.
Hoffmann,J.F., Kaplan,J.H. and Callahan,T.J. (1979) *Fed. Proc.*, **38**, 2440-2441.
Jensen,J. and Norby,J.G. (1971) *Biochim. Biophys. Acta*, **233**, 395.
Jørgensen,P.L. (1974a) *Biochim. Biophys. Acta*, **356**, 36-52.
Jørgensen,P.L. (1974b) in Fleischer,S. and Packer,L. (eds.), *Methods Enzymol.*, **32**, 277-290.
Jørgensen,P.L. (1982) *Biochim. Biophys. Acta*, **694**, 27-68.
Kaplan,J.H., Forbush III,B. and Hoffmann,J.F. (1978) *Biochemistry (Wash.)*, **17**, 1929-1935.
Karlish,S.J.D., Beauge,L.A. and Glynn,I.M. (1979) *Nature*, **282**, 333-335.
Kirby,A.C. and Varvoglis,A. (1966) *J. Am. Chem. Soc.*, **8**, 1823-1824.
Last,T.A., Gantzer,M.L. and Tyler,C.D. (1983) *J. Biol. Chem.*, **258**, 2392-2404.
Lederer,W.J. and Nelson,M.T. (1984) *J. Physiol. (Lond.)*, **348**, 665-667.
Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.
McCray,J.A., Herbette,L., Kihara,T. and Trentham,D.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7237-7241.
Post,R.L., Sen,A.K. and Rosenthal,A.S. (1965) *J. Biol. Chem.*, **240**, 1437-1445.
Reinhardt,R., Lindemann,B. and Anner,B. (1984) *Biochim. Biophys. Acta*, **774**, 147-150.
Robinson,J.D. (1977) *Biochim. Biophys. Acta*, **482**, 427-437.
Robinson,J.D., Flashner,M.S. and Marin,G.K. (1978) *Biochim. Biophys. Acta*, **509**, 419-428.
Schuurmans-Stekhoven,F. and Bonting,S.L. (1981) *Physiol. Rev.*, **61**, 1-76.
Simons,T.J.B. (1974) *J. Physiol. (Lond.)*, **237**, 123-155.
Wehrli,W.E., Verheyden,D.L.M. and Moffatt,J.G. (1965) *J. Am. Chem. Soc.*, **57**, 2265-2277.

Received on 5 August 1985