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Ion-transporting properties and ATPase activity of $(Na^+ + K^+)$-ATPase large subunit incorporated into bilayer lipid membranes

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A purified $(Na^+ + K^+)$-ATPase large subunit obtained from microsomes by water-alcohol extraction was incorporated into a bilayer lipid membrane. The protein formed in the membrane conductance channels which were sensitive to ouabain and selective for monovalent cations. ATP activated these channels in the presence of sodium and potassium ions. When sodium ions were eliminated ATP did not change the conductance of the modified membrane whereas *p*-nitrophenyl phosphate increased it. The $(Na^+ + K^+)$-ATPase large subunit incorporated into bilayer lipid membrane possessed an ATPase activity. The presence of a potential on the membrane was a necessary condition for the enzyme incorporated into a bilayer lipid membrane to show high ATPase activity. Increasing the potential above 100 mV resulted in the closing of conductance channels.

Introduction

$(Na^+ + K^+)$-ATPase discovered in 1957 by Skou [1] is responsible for active Na^+ and K^+ exchange through cellular membranes. The molecular mechanism of functioning of this enzyme is still unknown.

One of the approaches to study the mechanism of ion transport is the incorporation of the enzyme or its subunits into a bilayer lipid membrane and investigation of the ion-transporting properties of the incorporated proteins. In some studies, this approach was used to elucidate the $(Na^+ + K^+)$-ATPase functioning mechanism [2–6]. These investigations, however, left completely open the following questions: (1) which of the subunits is responsible for transmembrane cation transport, (2) whether cation transport is realized via a special channel or the enzyme functions as a carrier, (3) whether ouabain is bound to the ionophore part of the enzyme, (4) whether membrane potential has any effect on $(Na^+ + K^+)$-ATPase functioning, etc.

In this paper a new approach to reconstruct $(Na^+ + K^+)$-ATPase in bilayer lipid membrane is proposed. For the reconstruction, not the whole protein was taken but its catalytic subunit isolated by the method suggested by us earlier [7] which permitted isolating native cation-transporting proteins from the membranes. The advantage of this method is that the isolation procedure does not

Abbreviations: BLM, bilayer lipid membranes; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; pNPP, *p*-nitrophenylphosphate; P_i, inorganic phosphate.

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require detergents which not only disturb the nativity of protein but also make difficult the test for bilayer lipid membrane.

Materials and Methods

Isolation of microsomes, $(Na^+ + K^+)$-ATPase preparation and $(Na^+ + K^+)$-ATPase large subunit

Microsomes and the $(Na^+ + K^+)$-ATPase preparation were isolated from pig kidney medulla by Jørgensen's method [8] modified by Chetverin et al. [9]. The large $(Na^+ + K^+)$-ATPase subunit was obtained from a purified enzyme preparation isolated from pig kidney medulla after treatment with 3.3% SDS for 4 min at 100°C. The homogeneous preparation of large subunit was obtained by preparative electrophoresis in the gradient of 5% to 15% polyacrylamide gel with 0.1% SDS. To remove SDS, the protein was dialyzed against 5 mM Tris-HCl buffer (pH 7.5), in the presence of Dowex AG (1-X2) for three days at 4°C.

Isolation of ion-transporting subunit

The ion-transporting subunit was isolated from microsomes according to the technique developed for obtaining mitochondrial ion-transporting proteins [7]. Microsomes were treated with 96% ethanol at 4°C in the following proportion: 12 mg of microsomal protein in 6 ml of medium (20 mM Tris-HCl, 1 mM EDTA (pH 7.2)) per 90 ml of 96% ethanol. The extraction was performed at 4°C for 1 h under continuous stirring. The suspension was centrifuged at $5000 \times g$ for 15 min at 4°C. The sediment was dissolved in the equal volume of 50% ethanol and the above procedures of extraction and centrifugation were repeated. Supernatants I and II were combined and evaporated to dryness under vacuum at 30°C. The extract was dissolved in 6 ml of distilled water and defatted by adding 24 ml of chloroform/methanol mixture (2 : 1, v/v).

The $(Na^+ + K^+)$-ATPase preparation with a specific activity of 900–1000 μmol of P_i/mg of protein per 1 h was treated with ethanol at 4°C in the proportion: 2 ml of enzyme (0.5 mg protein/ml) per 16 ml of 96% ethanol. The extraction was performed for 0.5 h at 4°C under continuous stirring. Further extraction and removal of lipids were carried out by the above procedure used for isolation of the ion-transporting subunit from microsomes.

The $(Na^+ + K^+)$-ATPase large subunit preparation was treated with ethanol in the proportion 1 mg of protein (0.5 mg/ml) per 8 ml of 96% ethanol. The treatment was performed on a magnetic stirrer at 4°C for 30 min.

Purification of ion-transporting subunit

The extract obtained from 12 mg of microsome protein was applied on the Sephadex G-15 column (2.8×41 cm). Column equilibration and elution were performed with 5 mM Tris-HCl buffer (pH 7.2), at a rate of 60 ml/h under UV-analyzer control at 254 nm. Fractions of 10 ml were collected, lyophilized and their ion-transporting activity was tested using a bilayer lipid membrane. The ion-transporting fractions as well as the extracts obtained from preparations of $(Na^+ + K^+)$-ATPase and of its large subunit were purified of admixtures by electrophoresis in the polyacrylamide gel in the Davis system [10]. After electrophoresis the gels were stained by Coomassie brilliant blue. The protein zones were extracted from the gel with 5 mM Tris-HCl (pH 7.2) and concentrated. The extract was desalted by dialysis against 5 mM Tris-HCl buffer (pH 7.2) at 4°C for 3–4 h or on Sephadex G-25 column (1.5×43 cm). The elution medium was 5 mM Tris-HCl buffer (pH 7.2). All obtained proteins were tested for ability to transport Na^+ and K^+ through bilayer lipid membrane. The ion-transporting protein was tested for homogeneity and its molecular weight was determined by electrophoresis in 10% polyacrylamide gel in the presence of 0.1% SDS in Tris-acetate buffer (pH 7.4) [11].

Measurement of ion-transporting activity of proteins

The ion-transporting activity of isolated proteins was measured using the bilayer lipid membrane technique [12]. A teflon cell having a partition with a circular hole ($0.975 \cdot 10^{-2}$ cm^2) was used. Effective stirring was carried out with a magnetic stirrer. The experimental cuvette contained 3 ml of 30 mM imidazole-HCl buffer (pH 7.0) both in the 'cis' and 'trans' compartments. The electrode for measuring potential has been placed in the 'cis' compartment. The aqueous solutions of proteins and salts were introduced to

the compartments in the sequence depicted in the figures. Lipids were isolated from bovine brain according to Hara and Radin [13]. The lipid solution (20 mg/ml) in *n*-decane was used for bilayer lipid membrane formation. The conductance of membrane without protein was $1 \cdot 10^{-11}$–$3 \cdot 10^{-11}$ S/mm^2. Bilayer lipid membrane electroconductivity was determined by the voltage clamp method. The ion-selective properties of protein were determined by the potential which arose when a 3-fold ionic gradient was formed. Current and potential were measured with an electrometric amplifier Y5-9. The output current of the amplifier was registered with an 'Endim-b.21.02' recorder. The experiments were conducted at 19 to 21°C.

Determination of ATPase activity

The ATPase activity of protein was judged from the appearance in the medium of inorganic phosphate in the presence of $(Na^+ + K^+)$-ATPase large subunit incorporated into bilayer lipid membrane. The phosphate content was estimated in aliquots (0.05–0.1 ml) from both the compartments of the cuvette. Each compartment contained 3.0 ml of buffer. The compositions of media surrounding the bilayer lipid membrane are given in Table I. The reactions were triggered by adding $ATP \cdot Mg^{2+}$ into the 'cis' side, and $MgCl_2$ to the 'trans' side. In some experiments $ATP \cdot Mg^{2+}$ was added to the both sides of bilayer lipid membrane. Simultaneously, control measurements of phosphate content were made in test tubes, i.e. without contact with bilayer lipid membrane, in solutions: (1) incubation medium + $ATP \cdot Mg^{2+}$ + protein and (2) incubation medium + $ATP \cdot Mg^{2+}$ + protein + 0.01 ml of bilayer lipid membrane-forming lipids. The phosphate content was determined by a highly sensitive method [14] 3 h after triggering the ATPase reaction.

The protein content was determined according to Lowry et al. [15] using a bovine serum albumin as a standard. All measurements were made at 19–21°C.

Results

Purification of ion-transporting protein

The defatted extract obtained from microsome after applying to the Sephadex G-15 column had an elution profile presented in Fig. 1. The void volume of the column was 105 ml, the total volume 285 ml. Fractions 2 and 5 showed a capacity to change the permeability of artificial lipid membrane in the presence of Na^+ and K^+.

The subsequent electrophoresis of active fraction 2 showed that it contained several proteins (Fig. 2, I). The protein band with $R_F = 0.22$ possessed an ion-transporting activity. Electrophoresis showed that fraction 5 had only one protein, with $R_F = 0.22$ as well. The same protein band was revealed by electrophoresis of the extract obtained from the preparations of $(Na^+ + K^+)$-ATPase and its large subunit. Based on the fact that all protein zones with $R_F = 0.22$ had the same electrophoretic mobility upon electrophoresis in the presence of SDS (Fig. 2, II) a conclusion was made that ion-transporting proteins isolated from different preparations were identical. The defatted ethanol extract obtained from 12 mg of microsome protein contained 500 μg of protein on the average: fraction 2 contained about 160 μg protein, 20 to 30 μg of which was the ion-transporting protein.

Identification of ion-transporting proteins

As seen from Fig. 2, proteins possessing ion-transporting activity exhibit the same electrophoretic mobility on gel electrophoresis in the presence of SDS, no matter what is the source of

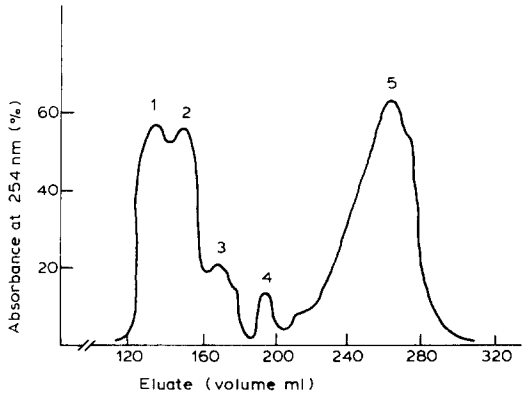

Fig. 1. Elution profile of microsome extract from Sephadex G-15 column.

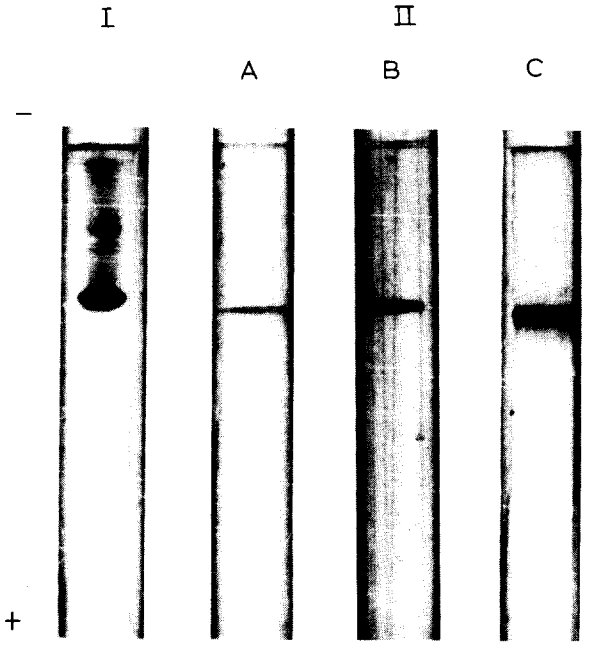

Fig. 2. Electrophoretograms of Fraction 2 in 7.5% polyacrylamide gel (I) and of ion-transporting proteins in 10% SDS-polyacrylamide gel (II) isolated from microsomes (A), $(Na^+ + K^+)$-ATPase preparation (B) and the large subunit of the enzyme (C).

protein. The use of standard proteins made it possible to calculate the molecular weight of the ion-transporting protein which appeared to be 90–95 kDa. As the molecular weight of large subunit is 96 kDa [16] a conclusion was made that the ion-transporting protein under study is the $(Na^+ + K^+)$-ATPase large subunit. As seen in Fig. 1, this protein was retained on the Sephadex G-15 column, which is probably due to adsorption of protein to the beads.

Cation-transporting properties of $(Na^+ + K^+)$-ATPase large subunit

An increase in membrane conductance was observed 1 to 5 min after introduction of 1 to 3 μg of protein under study to the Na^+- and K^+-containing medium surrounding the membrane. The same effect was also observed in the presence of only one of these two cations. The protein exhibited its ion transporting properties when added to one or both volumes separated by the membrane. The current changes in the modified membrane were discrete, which points to formation of conductance channels in bilayer lipid membrane (Fig. 3). The smallest steps are about 12 pS in 0.1 M NaCl.

The current changes were identical for proteins from different sources (Fig. 4). With greater amounts of protein the membrane permeability reached 0.1–1 nS and the functioning of single channels became imperceptible (Fig. 5). A potentiometric study of the ionic specificity of modified bilayer lipid membrane showed that in this case the current fluctuations were caused by the selective permeability of the membrane to both Na^+ and K^+. The membrane potential arising at a 3-fold gradient of KCl or NaCl in the presence of protein is equal to 26–29 mV, i.e. is near to that theoretically calculated for monovalent cations. Ca^{2+} and Mg^{2+} ions are not transported through the channels.

The current-voltage characteristic of the membrane, when protein is added to one side of the membrane, is normally asymmetrical (Fig. 6). However, protein-modified membranes not always show asymmetrical current-voltage characteristics.

Inhibition of conductance of bilayer lipid membrane modified with $(Na^+ + K^+)$-ATPase large subunit

The specific inhibitor of the sodium pump, ouabain [17] at the concentration of $(1–5) \cdot 10^{-5}$M inhibited the conductance of bilayer lipid membrane modified with $(Na^+ + K^+)$-ATPase large subunit. The effect showed up as late as in 30 min,

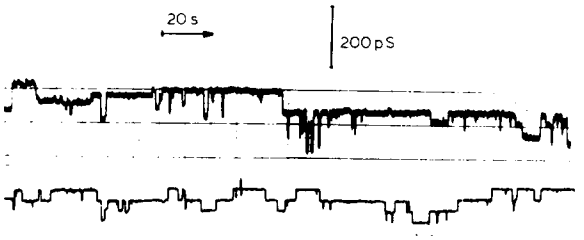

Fig. 3. Discrete conductance steps developed during interaction of bilayer lipid membrane with 1 μg/ml protein isolated from $(Na^+ + K^+)$-ATPase preparation. The aqueous solution contained: 20 mM Tris-HCl buffer (pH 7.5), 16 mM KCl and 80 mM NaCl. A potential of 50 mV was applied to the membrane. The protein was introduced on both sides of the membrane.

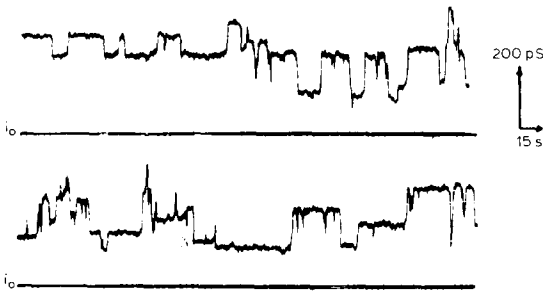

Fig. 4. Bilayer lipid membrane channels in the presence of 3 μg/ml of microsomal protein. The conditions are the same as in Fig. 3.

Fig. 5. Inhibition of the protein-induced current fluctuations in lipid bilayer by ouabain. The sample contained 5 μg protein/ml. The conditions are the same as in Fig. 3. Ouabain was added (arrow) to both sides of the membrane, final concentration $1 \cdot 10^{-4}$ M.

which corresponds to the literature data on the slow kinetics of binding of $(Na^+ + K^+)$-ATPase with ouabain [18]. Increase of ouabain concentration to $1 \cdot 10^{-4}$M resulted in a more rapid inhibition of conductance channels (Fig. 5).

It is known that K^+ is an antagonist of cardiac glycosides [19]. As seen from Fig. 7, addition of K^+ to the medium offsets the inhibitory effect of ouabain. The control experiments showed that 100

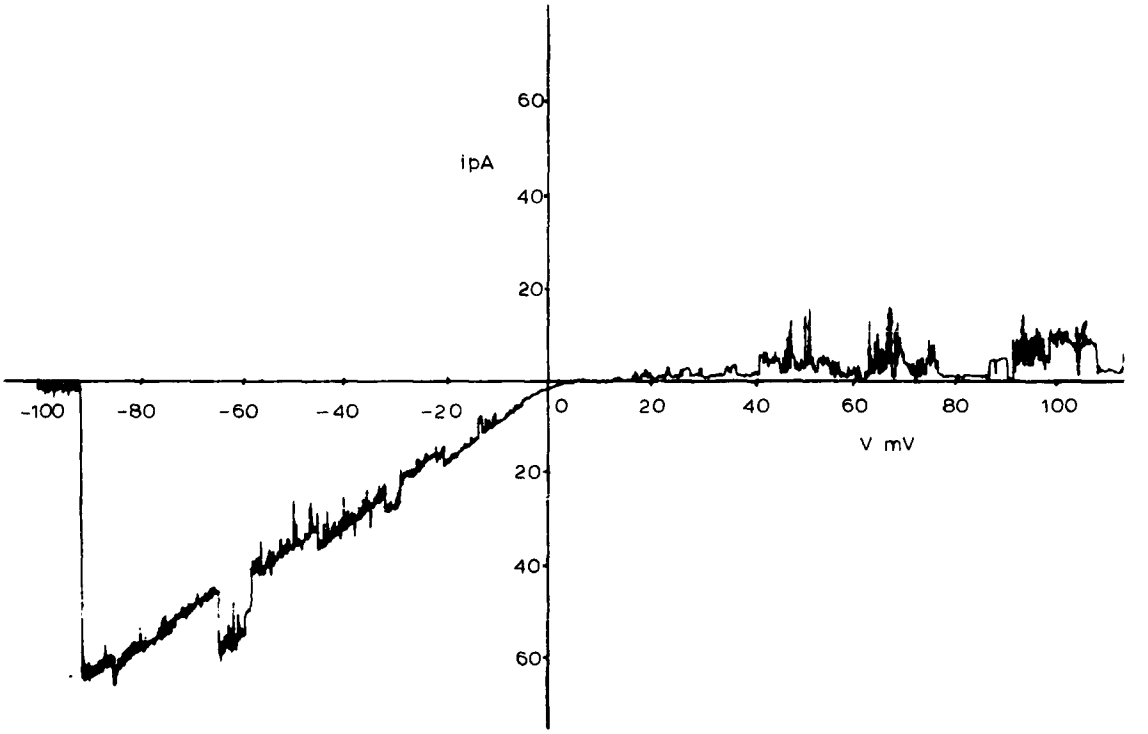

Fig. 6. Current-voltage relations of the conductance of a bilayer lipid membrane with incorporated $(Na^+ + K^+)$-ATPase large subunit. The aqueous medium contained: 30 mM imidazole-HCl buffer (pH 7.0), 10 mM KCl, 100 mM NaCl, protein at a concentration of 5 μg/ml was introduced to one side of the membrane; the sign of potential is denoted for the compartment containing protein; the rate of voltage change is 20 mV/min.

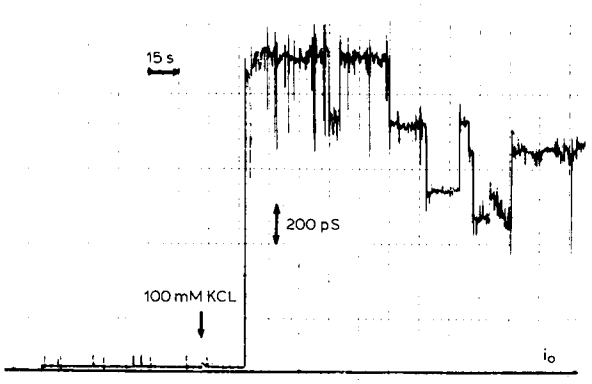

Fig. 7. Elimination of the ouabain effect by potassium ions. The conditions are the same as in Fig. 5. Ouabain concentration is $1 \cdot 10^{-4}$ M.

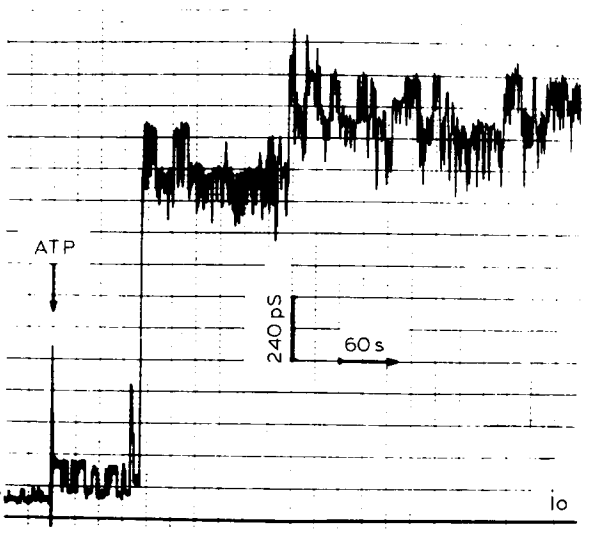

Fig. 8. The activating effect of ATP on the conductance of a membrane modified with protein (1 μg/ml $(Na^+ + K^+)$-ATPase large subunit). The aqueous medium contained: 30 mM imidazole-HCl buffer (pH 7.0), 100 mM NaCl; 10 mM KCl. 5 mM ATP·$MgCl_2$ was added to the 'cis' side of the membrane to which a potential of −50 mV was applied, simultaneously 5 mM $MgCl_2$ was introduced to the 'trans' side.

mM KCl added to a protein free membrane under analogous conditions did not produce any marked increase in membrane conductance.

The activity of $(Na^+ + K^+)$-ATPase is also inhibited by the agents that bind sulfhydryl groups of protein [20]. It is probably due to the fact that the large subunit of the enzyme contains sulfhydryl groups [16]. For the study, a sulfhydryl reagent, fluorescein mercuric acetate (FMA), was chosen which easily penetrates into the hydrophobic region of protein [21]. It was revealed that fluorescein mercuric acetate at a concentration of $2 \cdot 10^{-5}$ M inhibits the conductance channels formed in the membrane by the $(Na^+ + K^+)$-ATPase large subunit.

Effect of ATP and p-nitrophenyl phosphate on the conductance of bilayer lipid membrane modified with $(Na^+ + K^+)$-ATPase large subunit

The experiment of this and subsequent series were conducted on the large subunit isolated from microsomes in the absence of detergent by the above technique.

When at the 'cis' side of the membrane ATP in complex with Mg^{2+} is added in the presence of sodium and potassium ions, the conductance of bilayer lipid membrane is increased by 0.5 to 1.5 orders (Fig. 8). Ouabain added at the 'trans' side of the membrane closes ATP-activated channels. Removal of Mg^{2+} from the incubation medium prevents the ATP effect. When K^+ was removed from the medium the activating effect of ATP · Mg^{2+} persisted.

If in the incubation medium only potassium ions are available, ATP · Mg^{2+} does not increase the permeability of the modified membrane (Fig. 9). As seen from the figure, under these conditions the addition of *p*-nitrophenyl phosphate first elongates the life-time of the open state of the channels and then increases the number of these channels. The formed channels are selective for monovalent cations.

It was shown in the control experiment that the used *p*-nitrophenyl phosphate concentrations have no effect on bilayer lipid membrane conductance in the absence of protein.

ATPase activity of $(Na^+ + K^+)$-ATPase large subunit incorporated into bilayer lipid membrane

The large subunit incorporated into bilayer lipid membrane possesses ATPase activity (Table I). The tabulated data show that the protein exhibits a significant ATPase activity only when incorporated into bilayer lipid membrane. The phosphate

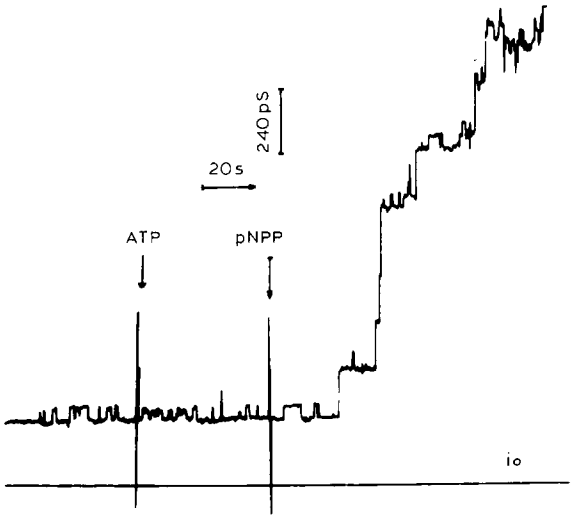

Fig. 9. Effect of ATP and *p*-nitrophenyl phosphate on the conductance of bilayer lipid membrane modified with ($Na^+ + K^+$)-ATPase large subunit. The aqueous medium contained: 30 mM imidazole-HCl buffer (pH 7.0), 100 mM KCl. Additions: 1 μg/ml protein, 5 mM ATP·$MgCl_2$, 3 mM *p*-nitrophenyl phosphate were introduced to the 'cis' side of the membrane. A potential of −50 mV was applied to the 'cis' side where additions were made.

was estimated in the aliquots taken from the 'cis' side of the membrane. When during the experiment the membranes were replaced some small amount of phosphate was found on the 'trans' side of the membrane as well, this is very likely explained by mixing of solutions from both sides. When Mg^{2+} is removed from the surrounding medium, ATP hydrolysis is not practically observed. Preincubation of protein with ouabain 0.5 h before ATP addition results in inhibition of both ATPase activity (Table I(5)) and the ion conductance induced in BLM by this protein.

It should be noted that Table I summarizes the data obtained with the proteins that increase the conductance of bilayer lipid membrane up to 800–1200 pS. If the conductance of the protein-modified membrane was below 800 pS or above 1200 pS the increment of inorganic phosphate was below or above 150 nmol/h (Fig. 10), respectively. Thus, there is a direct correlation between the averaged membrane conductance for monovalent cations and the increment of inorganic phosphate in the medium surrounding the modified membrane.

In Fig. 10 data obtained in several experiments are summarized. The conditions under which protein was incorporated into bilayer lipid membrane (the ionic composition of medium and the poten-

TABLE I

ATPase ACTIVITY OF THE CHANNEL-FORMING ($Na^+ + K^+$)-ATPase PROTEIN

The potential was −50 mV from the membrane side where ATP was added. Phosphate increment is given per 3 ml of medium. BLM, bilayer lipid membrane.

No,	Addition [a]	Phosphate increment (nmol/h)
1	5 mM ATP·$MgCl_2$ + 2 μg/ml protein	5.3
2	5 mM ATP·$MgCl_2$ + 2 μg/ml protein + lipid	6.4
3	5 mM ATP·$MgCl_2$ + 2 μg/ml protein incorporated into BLM	155.6
4	5 mM ATP + 2 μg/ml protein incorporated into BLM (Mg^{2+} is removed from incubation medium)	11.7
5	5 mM ATP $MgCl_2$ + 2 μg/ml protein incorporated into BLM + $1 \cdot 10^{-4}$ M ouabain	12.8

[a] The surrounding medium contained 100 mM NaCl, 10 mM KCl, 10 mM Tris-HCl (pH 7.5).

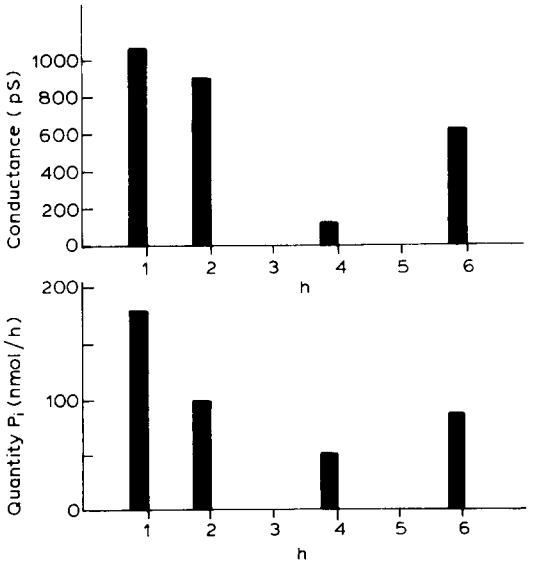

Fig. 10. Relationship between the ATPase- and ion-transporting activity of the ($Na^+ + K^+$)-ATPase large subunit incorporated into bilayer lipid membrane.

tial on the membrane) were the same. The above correlation may be followed in one experiment as well (Fig. 11). To do this, membranes with different areas of black film were formed. Thus varying the number of protein molecules that could be incorporated in bilayer lipid membrane and, respectively, the membrane conductance. The replacing of the membranes was made after 1 or 2 h of functioning. Prior to membrane replacing, aliquots were taken from the surrounding medium to determine phosphate increment. These studies showed a direct correlation between the rate of ATP hydrolysis and the conductance of protein-modified membrane (Fig. 11).

There was also correlation between the ATPase activity of the large subunit incorporated into bilayer lipid membrane and that of the initial microsome preparation the large subunit was obtained from. As seen from Fig. 12, the ATPase activity of the protein obtained from freshly isolated microsomes is substantially higher than that of microsomes stored for one year.

In case the microsome $(Na^+ + K^+)$-ATPase preparation loses its ATPase activity the isolated protein possesses neither ion-transporting nor ATPase activity.

The isolated protein when stored dry at 4°C retains its ATPase activity essentially unchanged during 1 to 2 weeks. In solution (at 4°C) the protein was less stable, its activity decreased substantially after one week storage (Fig. 13).

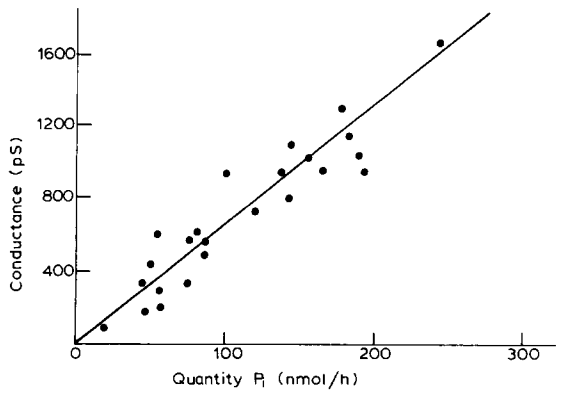

Fig. 11. Correlation between the rate of ATP hydrolysis and the conductance of the protein-modified membrane.

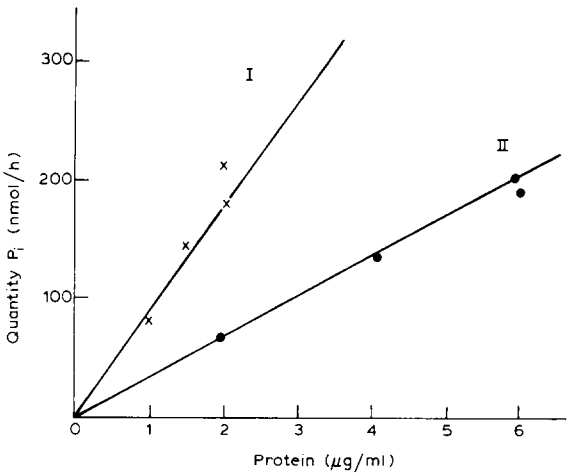

Fig. 12. Dependence of the ATPase activity of the $(Na^+ + K^+)$-ATPase large subunit on the nativity of microsomes. The preparation was obtained from: I, freshly isolated microsomes; II, microsomes stored at −20°C for one year, whose ATPase activity was decreased more than 5-times.

Effect of potential on the function of the $(Na^+ + K^+)$-ATPase large subunit incorporated in bilayer lipid membrane

The ATPase activity of $(Na^+ + K^+)$-ATPase large subunit incorporated in bilayer lipid membrane can be determined only when a potential is applied to the bilayer lipid membrane.

As seen from Table II, without applying a potential to the membrane, the protein incorpo-

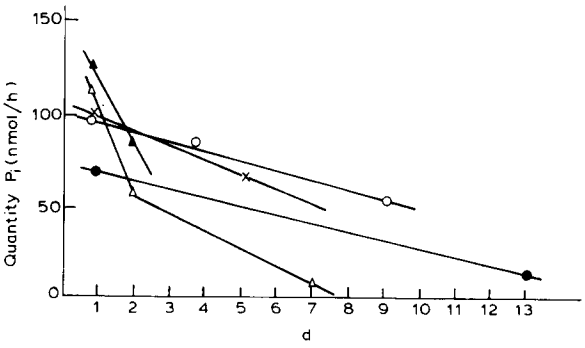

Fig. 13. Dependence of the ATPase activity of the bilayer lipid membrane bound protein on its storage time in solution at 4°C. This dependence was obtained by studying protein preparations of different isolations (△——△, ▲——▲, ○——○, ●——●, ×——×).

TABLE II

DEPENDENCE OF ATPase ACTIVITY OF $(Na^+ + K^+)$-ATPase LARGE SUBUNIT ON THE POTENTIAL APPLIED TO THE MODIFIED MEMBRANE

Conditions are the same as in Table I(3), $n = 7$

Membrane potential (mV)	Phosphate increment (nmol/h)
0	8.5
50	169.3

rated into bilayer lipid membrane does not decompose any substantial quantity of ATP.

Two series of experiments were conducted in order to study the effect of potential on the ATPase activity and conductance of bilayer lipid membrane modified with cation-transporting protein.

In the first series, the potential was removed from the membrane at the moment of ATP addition. The phosphate increment was measured three hours later. Both phosphate increment and membrane conductance were insignificant (Table II). Then a potential was applied to the membrane. In this case the membrane conductance increased by 1.5 to 2 orders. The phosphate content increased substantially,

In the second series, a potential was applied to the modified membrane at the moment of ATP addition. The membrane was functioning three hours after which aliquots for phosphate determination were taken. The potential was removed from the membrane and it was further functioning for 3 h. The estimation of phosphate content showed that its increment took place only when a potential was almost no increment during the 3 hour-long functioning of the membrane in the absence of potential. Therefore, not only ATP and Mg^{2+} but also a transmembrane potential are necessary for the functioning of the $(Na^+ + K^+)$-ATPase subunit in bilayer lipid membrane.

Taking into account the importance of this conclusion, two series of control experiments were made. In the first series the protein was removed from the medium surrounding bilayer lipid membrane and a potential was applied to the mem-

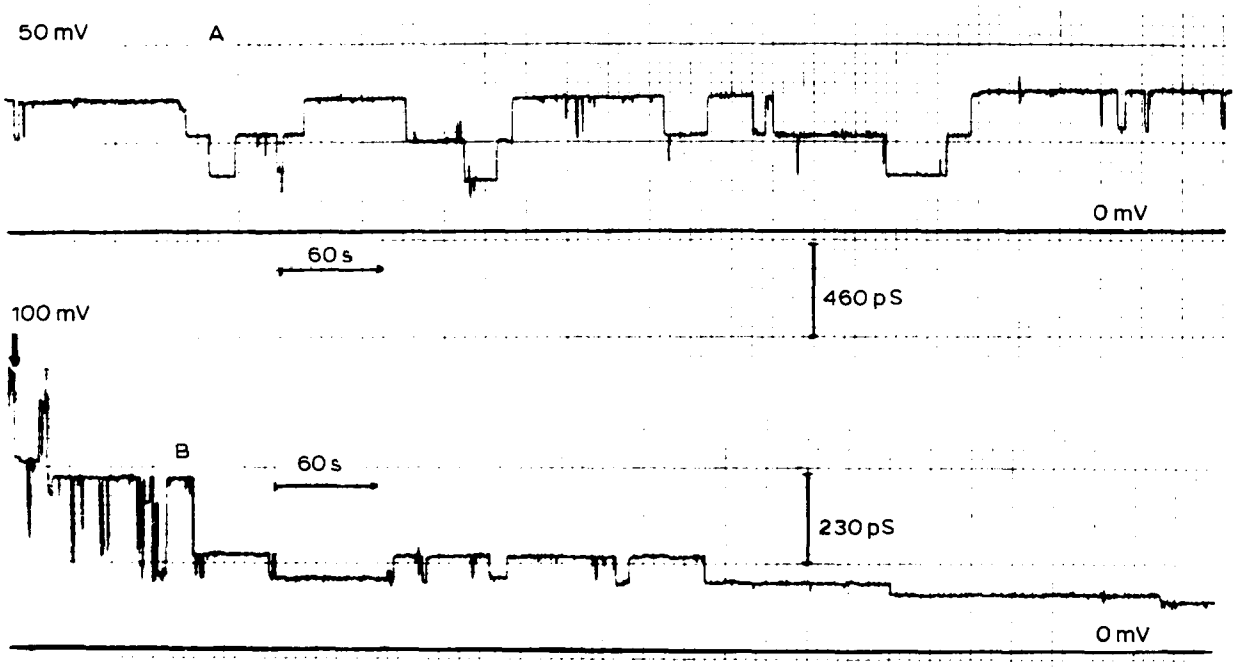

Fig. 14. Effect of potential on the conductance of bilayer lipid membrane modified with $(Na^+ + K^+)$-ATPase large subunit (3 μg/ml). The conditions are the same as in Fig. 3. B is the continuation of A.

brane in the presence of ATP. The membrane was functioning during 3–5 h, neither conductance increase nor phosphate increment being observed. In the second series of control experiments the ion-transporting protein was replaced with one of the following compounds: (1) protein isolated from microsomes during the purification of the large subunit, ($R_F = 0.51$) (Fig. 2.I) which did not show ion transporting properties even at a concentration of 60 to 100 μg/ml; (2) mitochondrial K^+-transporting protein [22] which at a concentration of 10 μg/ml produced an increase in membrane conductance by three orders under the conditions described; (3) gramicidin derivative (gramicidin-CH_2-gramicidin) which increased the bilayer lipid membrane conductance by three orders at a concentration of $8 \cdot 10^{-9}$M in the conditions described.

The experiments showed that the modification of bilayer lipid membrane with any of the above substances under the conditions of a potential applied to the membrane caused neither ATP hydrolysis nor conductance increase under the effect of $ATP \cdot Mg^{2+}$.

When studying the effect of potential on the conductance of bilayer lipid membrane modified with $(Na^+ + K^+)$-ATPase large subunit it was revealed that the probability of the open state of conductance channels decreases with increasing potential (Fig. 14). A potential increase above 100 mV mostly results in a closing of channels (Fig. 6).

Discussion

As follows from the data presented, the $(Na^+ + K^+)$-ATPase large subunit isolated by ethanol forms conductance channels in bilayer lipid membrane which are selective for monovalent cations. The functional relationship of the protein that we have isolated to the transport enzyme is confirmed by the fact that the specific inhibitor of $(Na^+ + K^+)$-ATPase, ouabain, closes the protein-induced channels. This is also evidenced by the fact that the protein exhibits an ion-transporting activity only when isolated from microsome preparations possessing a $(Na^+ + K^+)$-ATPase activity.

Somewhat unexpected appeared the fact of the functioning of the ouabain-sensitive conductance channels in the absence of ATP, at the expense of the electric transmembrane potential. It is known, however, that the $(Na^+ + K^+)$-ATPase localized in the erythrocyte membrane is capable to transport K^+ along the ouabain-sensitive pathway under conditions of a substantially decreased ATP concentration or ATPase activity inhibition [23]. In this case K^+ transport takes place at the expense of an artificially induced membrane potential. Besides, it has been shown that purified $(Na^+ + K^+)$-ATPase incorporated into liposomes is also able to bring about the ouabain-sensitive K^+ transport in the absence of ATP [24]. ATP increases the rate of the ion transport 40 times. $(Na^+ + K^+)$-ATPase is assumed to function in the absence of ATP as an ouabain-sensitive K^+ channel [24].

Large subunit-induced conductance channels have an ion-gated mechanism, thus providing ions to be transported in a definite direction. This is evidenced by current-voltage characteristic for conductance channels taken when protein was added on one side of the membrane (Fig. 6). The fact that the asymmetry in current-voltage characteristic is observed not in all experiments suggests that the incorporation of protein into the membrane is not strictly vectorial.

The presented findings show that ATP activates ouabain-sensitive conductance channels formed in the membrane by $(Na^+ + K^+)$-ATPase large subunit (Fig. 8). The activation is observed in the presence of Na^+ and K^+ or Na^+ only. When only potassium ions are present in the surrounding medium, ATP does not induce any marked increase in the conductance of the modified membrane, which correlates with the evidence that the activity of $(Na^+ + K^+)$-ATPase depends on the ionic composition of the medium [1]. Addition of another substrate of $(Na^+ + K^+)$-ATPase, *p*-nitrophenyl phosphate, increases the membrane conductance (Fig. 9).

It is known that $(Na^+ + K^+)$-ATPase possesses a potassium-dependent phosphatase activity, i.e. an ability to hydrolyse *p*-nitrophenyl phosphate by the ouabain-sensitive pathway. According to modern viewpoints, *p*-nitrophenyl phosphate is involved at the stage of binding of K^+ to the enzyme with subsequent translocation of the ion into the cell [25]. In literature there are conflicting opinions concerning the effect of this substrate on

the transporting function of the enzyme [26,27]. We are inclined to agree with Garrahan and Rega [27] that specific conditions are needed for *p*-nitrophenyl phosphate to exhibit its ability to activate the transport of monovalent cations.

In our opinion, one of these conditions might be the presence of a potential on the membrane, which probably promotes K^+ transport into the cell. The $(Na^+ + K^+)$-ATPase large subunit along with the ion-transporting properties possesses also an ATPase activity. It is known that the catalytic center [28] as well as the centres to bind nucleotides and ouabain [29] are localized on this subunit. Recently, evidence has been obtained that the $(Na^+ + K^+)$-ATPase large subunit has an ATPase activity which is comparable with that of the whole enzyme [30] and that the antibodies to this protein inhibit this activity [31], whereas those for the small subunit do not influence it. All the above said provides support for the view that both catalytic and ion-transporting properties of the enzyme are conditioned by the large subunit of $(Na^+ + K^+)$-ATPase whereas the small subunit probably performs a regulatory function [32,33].

The evidence presented suggests that the ATPase activity of the large subunit in solution is much lower than that of the protein incorporated into bilayer lipid membrane (Table I). It could be assumed that one of the reasons for the increase in activity of the protein on its incorporation into the lipid membrane is its enrichment with lipids. According to the technique, one of the stages in purification of large subunit is the treatment with organic solvents which is known to result in the removal of functionally significant phospholipids and the decrease of $(Na^+ + K^+)$-ATPase activity [34]. It is assumed that the role of membrane lipids is to control the transport function of the enzyme [35]. In this connection, our evidence for the absence of activation of enzyme in solution on adding lipids of which bilayer lipid membrane are formed (Table I(2)) suggests that the lipid activates only the bilayer lipid membrane-incorporated fraction of the enzyme.

In our opinion, the most probable explanation for the increase in the activity of the enzyme during its incorporation into the lipid bilayer is the presence of a potential on the membrane. It follows from our evidence that the enzyme can exhibit a marked ATPase activity only if a potential is applied to the membrane (Table II).

The influence of a potential on the ATPase activity of the enzyme can be explained in two ways. First, the transmembrane potential can influence one of the stages of ion transport. The recent findings on the activation of ouabain-sensitive potassium transport in erythrocyte membranes by applying potentials confirm this assumption and suggest that the potential is responsible for the stage of potassium transfer [23]. It is highly probable that during functioning of the enzyme in vivo the negative charge occurring on the inner side of the cell membrane facilitates the transport of positively charged potassium into the cell.

The activating effect of the potential on the ATPase activity and ion-transporting behaviour of the $(Na^+ + K^+)$-ATPase large subunit may be associated with the ability of the potential to enhance the incorporation of the enzyme into bilayer lipid membrane. It was shown by special calculations (see Appendix) that the phosphate quantity estimated in the medium is characteristic of the tight packing of large subunit in the bilayer lipid membrane (as calculated in terms of the specific activity of this protein in solution). But such a packing is probable only theoretically, therefore the activity of the protein incorporated into bilayer lipid membrane is much higher than the specific activity of $(Na^+ + K^+)$-ATPase in solution due to a potential applied to the membrane. Since a potential is usually present on the cell membrane we suppose, that the in vivo activity of $(Na^+ + K^+)$-ATPase is also higher, i.e. the cell potential (along with ATP) regulates the work of the enzyme.

The increase of ouabain-sensitive K^+ transport under the action of potential has been revealed earlier in experiments on erythrocytes [26]. The high level of adenylate kinase in the cell, however, did not permit measuring ATPase activity in these experiments. Hence, the model experiments are advantageous in solving this problem.

The data obtained indicate that the level of ATPase activity is dependent on the quantity of the protein incorporated into bilayer lipid membrane. It is most clearly seen from the data presented in Fig. 11. The data suggest that the de-

crease in the amount of protein incorporated into the membrane as a result of reduced area of bilayer lipid membrane, leads to a corresponding decrease of phosphate increment in the medium. This is also supported by a direct correlation between the averaged ion conductance of protein-modified membrane and the ATPase activity of this protein (Fig. 10).

The analysis of current-voltage characteristic for the conductance channels induced on bilayer lipid membrane with the $(Na^+ + K^+)$-ATPase large subunit showed that at potentials above 100 mV the channels are mostly closed (Figs. 6, 14). Such behaviour of ion channels, might play an essential role in preventing membrane hyperpolarization. Recently, an assumption has been made in literature that the properties of the transport enzyme in the cell membrane and of that in solution must be different. Chapman et al. [36] and Slayman and co-workers [37] have developed a conception that one or more stages of sodium pump functioning can be dependent on the potential. It has been assumed that the potential substantially affects the stage of potassium translocation while changing the binding of the enzyme with ions and nucleotides [37,38]. In this connection the obtained evidence for the effect of potential on the ATPase activity is an experimental confirmation of the above assumptions.

Appendix

Calculation of tightness of packing of $(Na^+ + K^+)$-ATPase large subunit in bilayer lipid membrane under experimental conditions described in Table I

Based on the evidence that the diameter of monomer of the large and small subunits of $(Na^+ + K^+)$-ATPase is equal to 27 Å [39], the diameter of the large subunit was taken 18 Å, i.e. the protein was assumed to occupy an area of 300 $Å^2$.

The area of bilayer lipid membrane used was 1 mm^2, i.e. 10^{14} $Å^2$, hence, $3.6 \cdot 10^{11}$ molecules of the large subunit can be incorporated into it. This amounts to 0.06 μg of the protein, as its molecular weight is 100000. Hence, in the case of tight packing 0.06 μg of large subunit protein could be incorporated into bilayer lipid membrane. The specific activity of $(Na^+ + K^+)$-ATPase is equal to 2400 nmol P_i/μg protein per h [39], i.e. 0.06 μg of protein produce 144 nmol P_i/h. A nearly equal amount of P_i was determined experimentally (Table I).

Hence, the tight packing of large subunit in the bilayer lipid membrane is theoretically possible.

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References

1 Skou, J.C. (1957) Biochim. Biophys. Acta 23, 394–401
2 Jain, M.K., Strickholm, A. and Cordes, E.N. (1969) Nature 222, 871–872
3 Shamoo, A.E. and Ryan, F.E. (1975) Ann. New York Acad. Sci. 264, 98–111
4 Hyman, E.S. (1975) Biophys. J. 15, p.2 304a
5 Sorokina, Z.A. (1980) Fiziol. Zh. 26, 655–670
6 Last, T.A. Gantzer, M.L. and Tyler, C.D. (1983) J. Biol. Chem. 258, 2399–2404
7 Mironova, G.D., Sirota, T.V., Pronevich, L.A., Trofimenko, N.V., Mironov, G.P., Grigorjev, P.A. and Kondrashova, M.N. (1982) J. Bioenerg. Biomembranes 14, 213–215
8 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
9 Chetverin, A.B., Brazhnikov, E.V. and Chirgadze, Yu.N. (1979) Biokhimiya 44, 945–952
10 Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404–427
11 Fairbanks, G., Theodore, L., Steck, L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
12 Mueller, P., Rudin, D.O., Tien, H.T. and Wescott, W.C. (1963) J. Phys. Chem. 67, 534–535
13 Hara, A. and Radin, N. (1978) Anal. Biochem. 90, 420–426
14 Muzbek, L., Szabo, T. and Fesüs, L. (1977) Anal. Biochem. 77, 286–288
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Dzhandzhugazyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Bioorg. Khim. 7, 847–857
17 Bonting, S.L. (1970) in Membranes and Ion Transport (Bittar, E.E., ed.), Vol. 1, pp. 257–363
18 Rossi, B., Vuillenmer, P., Gacke, C., Balerna, M. and Lazdunski, M. (1980) J. Biol. Chem. 255, 9936–9941
19 Lishko, V.K. (1977) in, The Sodium Pump of Biological Membranes, Naukova Dumka, Kiev
20 Schoot, B.M., Schoots, A.F.M., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1977) Biochim. Biophys. Acta 483, 181–192
21 Mironov, G.P., Kaminski, Yu.G. and Kondrashova, M.N. (1971) Vopr. Med. Khim. 17, 83–88

22 Mironova, G.P., Fedotcheva, N.I., Makarov, P.R., Pronevich, L.A. and Mironov, G.P. (1981) Biofizika 26, 451–457
23 Serpersu, E.H. and Tsong, T.G. (1984) J. Biol. Chem. 259, 7155–7162
24 Anner, B.M. (1981) Biochem. Int. 2, 365–371
25 Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–75
26 Askari, A. and Rao, S.N. (1971) Biochim. Biophys. Acta 241, 75–88
27 Garrahan, P.J. and Rega, A.F. (1972) J. Physiol. London 223, 595–617
28 Jean, D.N. and Albers, R.W. (1976) Biochim. Biophys. Acta 552, 219–226
29 Haley, B.E. and Hoffman, S.F. (1974) Proc. Natl. Acad. Sci. USA 71, 3367–3371
30 Freytag, W. (1983) FEBS Lett. 159, 280–284
31 Ball, W.J., Collins, J.H., Lane, L.K. and Schwartz, A. (1983) Arch. Biochem. Biophys. 221, 371–380
32 O'Connell, M.A. (1982) Biochemistry 21, 5984–5991
33 Brotherus, J.R., Jacobsen, L. and Jørgensen, P.L. (1983) Biochim. Biophys. Acta 731, 290–303
34 Wheller, K.P. (1975) Biochem. J. 146, 729–738
35 Boldyrev, A.A. (1979) Biol. Nauk. 3, 6–17
36 Chapman, J.B. and Johnson, E. and Kootsey, J.M. (1983) J. Membrane Biol. 74, 139–153
37 Gradman, D., Hansen, U.P. and Slayman, C.L. (1981) Curr. Top. Membranes Transport 16, 257–276
38 Chapman, J.B. and Johnson, E.A. (1978) J. Gen. Physiol. 72, 403–408
39 Deguchi, N., Jørgensen, P.L. and Maunsbach, A.B. (1977) J. Cell Biol. 75, 619–634