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A Study of Sodium Release in the Course of ATP Hydrolysis by Membrane ATPase

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Summary. With the aid of sodium-sensitive glass electrodes, changes in sodium ion activity were studied in the course of subsequent additions of components required for ATP hydrolysis provided by Na^+-K^+-dependent membrane ATPase. Membrane ATPase was obtained from guinea pig kidney cortex. In the presence of ATP, Mg^{++} and Na^+ in media, the addition of K^+ caused an increase in Na^+ activity. The omission of ATP or its substitution by ADP as well as the addition of Ca^{++} to the media eliminated the above-mentioned increase of Na^+ activity. Quabain did not affect Na^+ release caused by the addition of K^+, although it significantly inhibited ATPase activity of the preparation. The data obtained were considered to be a direct indication of ion exchange during the course of membrane ATPase reaction. This ion-exchange stage of the reaction is not inhibited by ouabain. The ratio of sodium ions released per one inorganic phosphate formed in the course of the reaction was found to be much higher than that established for transporting membranes of intact cells. A possible cause of this difference is discussed.

The ability of the microsomal fraction isolated from a number of tissues to behave as Na^+-K^+-dependent ATPase has been reported by several authors [3, 14, 25]. This ATPase is supposed to be a part of the Na^+ transporting system because of similar changes caused in the enzyme activity and Na^+ transport-inhibiting and -activating agents [19]. A transient Na^+ binding postulated as a stage of active transport mechanism should also be the property of microsomal material, provided the latter is in fact a part of the transporting system. The binding of ^{22}Na by the microsomal fraction obtained from guinea pig kidney cortex dependent on the presence of ATP in the media has been demonstrated by Jarnefelt and Stedingk [8, 10] and by Charnock and Post [6]. The amount of ^{22}Na found in the pellet when K^+ was present in incubating media was about 5% less than that for the media containing no K^+ according to data obtained by Jarnefelt [8] and 47% less

according to the results of Charnock and Post [6]. These inconsistent data are of great importance because of some indirect evidence for the existence of a Na^+-for-K^+-exchange reaction in the course of ATP hydrolysis caused by membrane ATPase. For more direct evidence of Na^+-for-K^+ exchange, a release of bound Na^+ resulting from the addition of K^+ into media should be established.

The tracer methods used in the papers mentioned above [6, 8] seem not very suitable for detecting changes in ion binding *during the course* of ATPase reaction because of the necessity of sedimentation of microsomal material before radioactivity measurements. Another limitation of the tracer method is its inability to distinguish the position of bound or accumulated ions in cases where there are several compartments in microsomal preparation. When establishing a correlation between inorganic phosphorus production and ion-binding capacity, Jarnefelt and Stedingk [8] found the amount of ^{22}Na incorporated into the enzyme system to be much greater than that of incorporated ^{32}P. The ratio of 160:1 established in their work is puzzling if compared with stoichiometry of 3 or 2 Na^+ transported per 1 inorganic phosphate. Therefore the authors were compelled to suggest an accumulation of ^{22}Na inside small vesicles often found on electron micrographs of microsomal suspensions [12]. Thus, as an alternative to Na^+ binding by microsomal membranes, a redistribution of Na^+ between compartments as a result of ion transport has been suggested. This important question is not easy to solve using ordinary tracer techniques. Electrochemical determination of Na^+ activity in the media of microsomal suspension by means of Na-sensitive glass electrodes is a more convenient method for the observation of changes in the state of Na^+ in the course of ATPase reaction. Owing to the definite position of electrodes outside proposed vesicles, this method is especially suitable for the solution of the above-mentioned problem concerning the possibility of Na^+ redistribution.

Materials and Methods

A Na-$^+$K$^+$-dependent ATPase system was isolated from guinea pig kidney cortex according to the method of Post and Sen [20] with some previously reported modifications [18]. Imidazole buffer at pH 7, practically free from Na^+ and K^+, was used as a suspending media in all experiments. Protein content of the suspension varied from 2 to 3 mg/ml. ATPase activity of the preparations measured by the release of inorganic phosphate (P_i) split off from ATP under optimal conditions (concentrations of Na^+ and K^+ in the media were 25 and 4 meq, respectively; incubation at 44 °C) was about 100 μmoles P_i/ml of suspension per hr. The part of ATPase activity dependent on the presence of specific membrane Na^+-K^+-ATPase estimated as a ratio of ouabain-inhibited portion to the total activity for optimal ionic conditions was between 80 and 90%. A

5-ml sample of suspension placed in a small cuvette with a magnetic stirrer was used for each experiment. Na^+ activity was measured by means of Na-specific electrodes made of NABS 25-05-09 glass [4, 5, 16] immersed in the suspension together with a calomel reference electrode provided by a salt bridge with practically no leakage of KCl. Potential difference was measured by an electrometer of Vibron type (input resistance above 10^{13} Ω) connected with a recorder and an automatic device for correction of any possible drift of zero level of the measuring system. The accuracy of potential measurements was better than ± 0.5 mV.

The electrode system was calibrated in pure NaCl solution before and after measurement of Na^+ activity in microsomal suspension. The matching of two calibration curves served as evidence of stability of electrode properties. Since the time constant of the electrode system was about 1 to 2 min, it took 3 to 4 min to attain a steady potential level at every measurement. After the initial activity of Na^+ in microsomal suspension had been determined, the effects of subsequent additions to the suspension of $MgCl_2$, Na_2ATP and KCl were studied. The standard procedure of the experiments is given in Table 1. In parallel with Na^+ activity measurements, ATPase activity of the preparation was determined in the course of the same experiment. It was shown that at final ionic concentrations shown in Table 1, Na^+-K^+-dependent ATPase activity becomes about 60% as compared with the optimal conditions mentioned above. The alteration of the optimal ionic conditions was inevitable in our experiments since slight changes of Na^+ activity could be detected only at low basic Na^+ concentration.

As a control for the above experiments, the effect of the addition of the same components to the suspension media with no ATPase was studied. In this series of experiments, some NaCl was preliminarily added to imidazole buffer to have the initial Na^+ activity similar to that of the microsomal suspension.

Due to a comparatively low sodium in respect to potassium specificity of NABS 25-05-09 glass (specificity constant $K_{NaK} = 0.2 - 0.6$)[1], there was a systematic error in determination of Na^+ activity changes when KCl was added to the microsomal suspen-

Table 1. *Standard procedure of addition of components required for ATP hydrolysis caused by Na^+-K^+-dependent membrane ATPase*

Sequence of addition	Components	Vol. added (ml)	Final concentration
1	Suspension of Na^+-K^+-ATPase	5.0	2 – 3 mg protein/ml
2	$MgCl_2$	0.1	2.0 mM
3	Na_2ATP	0.1	2.5 mM
4	KCl	0.1	2.0 mM

1 Since the dependence of the potential of NABS 25-05-09 glass electrodes on the log of K^+ activity at pH 7 is not linear [13], the ability of these electrodes to discriminate between Na^+ and K^+ depends on the absolute values and ratio of concentrations of these ions. At millimolar ionic concentrations, the discrimination is small. The specificity for NAS 11 – 18 glass is higher than that for NABS 25-05-09, but the above-mentioned unlinearity is also more pronounced. The two-electrode method, when besides the Na^+-specific a K^+-specific electrode is used, failed to give the necessary accuracy of measurements at low ionic concentrations.

sion. But this error might be easily accounted for when the effect in microsomal suspension was compared with the control experiments without ATPase in the media.

Ten samples of Na^+-K^+-ATPase obtained at various times were used for the experiments. The results of each experimental series underwent statistical treatment.

Results

The mean values of Na^+ activity changes in the course of adding reaction components (Table 1) to the microsomal suspension and to the suspension media (control) are shown in Fig. 1. The addition of equal amounts of Na_2ATP to control and to ATPase containing media caused a greater increase of Na^+ activity in the control but because of the rather wide range in basic Na^+ activity level, this difference is not statistically significant. The addition of KCl to a final concentration of 2 mM in the control series produced a nonspecific effect due to the influence of K^+ on the Na^+-sensitive glass electrode equivalent to the increase of Na^+ activity of 0.68 ± 0.10 mM (average value and standard deviation). When the same amount of KCl is added to ATPase-containing media, the measured increase of Na^+ activity is much more pronounced and reaches 1.11 ± 0.11 mM. The difference between the mean value of Na^+ activity increase for these two series of 0.43 mM Na^+ is statistically reliable (Table 2, variants 1 & 2). This difference in the increase of Na^+ activity may be considered as a release of Na^+ into the media from microsomal material caused by KCl. Such a release of Na^+ may be the result of an ion-exchange process associated with the properties of microsomal materials, independent of its ATPase activity. If so, the release of Na^+ should be obtained even with no ATP in the system. A series of nine experiments where Na_2ATP was substituted by the equivalent amount of NaCl revealed no differences from the control series (Table 2, variant 3). Thus the release of Na^+ may be obtained if both microsomal material and ATP are present in the media.

It is known that alkali ions may form complexes with ATP [23]. Possible substitution of Na^+ by K^+ in such complexes was taken into account by means of a control experimental series (with no ATPase). But still there is a possibility that in the presence of the microsomal material this nonspecific complex formation is somehow increased, providing the increase in Na^+ release in response to the addition of K^+ to the media. It is known that ATP is a specific source of energy for the Na^+ pump [11] as well as a very specific substrate for Na^+-K^+-ATPase [19]. ADP is much less effective as a substrate for ATPase, but its ability for complex formation should not differ much from that of ATP.

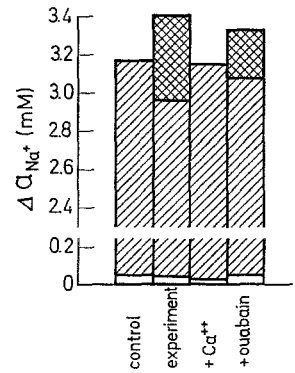

Fig. 1. The effect of adding 2 mM $MgCl_2$ (open part of columns), 2.5 mM Na_2ATP (hatched part), and 2 mM KCl (cross-hatched part) to microsomal suspension on its Na^+ activity. The nonspecific effect simulating increase of Na^+ activity due to the influence of K^+ on Na^+-sensitive electrode (measured as an equivalent increase in Na^+ activity of 0.68 mM after addition of 2 mM KCl in control series) was subtracted from results obtained in all series of experiments. The vertical axis gives the increase of sodium activity of preparations in mM

Table 2. *Changes of Na^+ activity caused by the addition of 2.0* mM *KCl to the media and ATPase activity of the preparation at the same conditions*

Experimental variants	Omission (−) or addition (+)[a]	Increase of Na^+ activity			ATPase activity (%)
		No. of exp	Mean and SD (mM)	P_{diff} (in respect to variant 1)	
1	None	30	1.11 ± 0.11	–	100
2	−ATPase (control)	24	0.68 ± 0.10[b]	<0.01	0
3	$-Na_2ATP$; $+NaCl$ (5 mM)	9	0.67 ± 0.14	<0.01	0
4	$-Na_2ATP$; $+Na_2ADP$ (2.5 mM)	4	0.65 ± 0.08	<0.01	43
5	$+CaCl_2$ (2.0 mM)	8	0.67 ± 0.15	<0.02	27
6	+ouabain (10^{-4} to 10^{-3} M)	14	0.92 ± 0.12	>0.2	65

[a] Omissions and additions are given with respect to standard procedure (*see* Table 1).
[b] Nonspecific effect due to the influence of K^+ on Na^+-sensitive glass electrode.

In a special series of experiments, Na_2ATP was substituted by Na_2ADP. The enzymatic activity of microsomal preparation in these experiments measured by inorganic phosphate formation decreased by 57% as compared with the tests when ATP was present. The effect of KCl addition for this series was the same as for the control (Table 2, variant 4). It shows that Na^+ release is specifically associated with ATPase activity of the preparation.

To demonstrate the correctness of the last statement, a series of experiments with inhibitors of ATPase activity was carried out. Ca^{++} is a well-known inhibitor of the active transport of Na^+ and Na^+-K^+-ATPase activity [9]. It is suggested that Ca^{++} replaces Na^+ at ion-binding sites of the enzyme [22], and hence it should effect Na^+-for-K^+ exchange and decrease the established release of Na^+.

In one series of experiments, 2 mM $CaCl_2$ was added to the microsomal suspension just before the addition of Na_2ATP. This resulted in a sharp decrease of ATPase activity and abolished Na release caused by KCl (Table 2, variant 5) if compared with the control.

The most specific inhibitor of Na^+ transport and Na^+-K^+-ATPase is ouabain (Strophatidin G). As has been established by Kinsolving, Post and Beaver [12], 10^{-5} M ouabain completely represses the activity of Na^+-K^+-ATPase obtained from guinea pig kidney cortex without affecting Mg^{++}-activated ATPase also present in such preparations. To have a reliable effect, we used ten-times-greater concentrations of ouabain (1×10^{-4} M) introduced simultaneously with Na_2ATP, but no changes in Na^+ release were revealed in response to the addition of KCl if compared with the standard procedure without ouabain. In the following experiments, ouabain concentration was increased to 1×10^{-3} M and, taking into consideration the time dependence of ouabain action [22], the enzyme was preincubated 10 to 15 min with ouabain before addition of ATP. However, even with such a severe treatment, we failed to obtain depression of Na^+ release (Table 2, variant 6). At the same time, it was shown that ouabain at both concentrations used caused inhibition of ATPase activity of about 35%. When sufficiently high ouabain concentrations are used, one would expect much higher inhibition of ATPase activity by about 80%. The remaining 20% is generally believed to depend on the presence of ouabain-insensitive Mg^{++}-activated ATPase. But, as mentioned before, with the ionic conditions used, Na^+-K^+-ATPase activity is depressed up to 60%, whereas for Mg^{++}-activated ATPase, the ionic conditions are not far from the optimal ones. Thus a part of Mg^{++}-activated ATPase in the total enzymatic activity becomes much greater and hence ouabain inhibition less prominent.

Discussion

The data presented show that the increase in the activity of Na^+ subsequent to the addition of K^+ to media containing microsomal material is connected with the ATPase activity of this system. The presence of ATP and Mg^{++} is necessary for both the increase in Na^+ activity and the ATPase

reaction. The substitution of ATP for ADP and the addition of Ca^{++} to the media eliminated Na^+ release and significantly depressed ATPase activity of the preparation. The only discrepancy in a traditional set of proofs for the connection of the above two processes was a lack of ouabain effect with respect to Na^+ activity changes when its influence on the ATPase activity was obvious.

The increase of Na^+ activity in the media of microsomal suspension after K^+ has been added may be considered as evidence for the release of Na^+ previously bound at some earlier stage of ATPase reaction. Some indication of Na^+ binding may be found in the material presented when the increases in Na^+ activity subsequent to the addition of Na_2ATP to the control and to the ATPase-containing media are compared (*see* Fig. 1). In its simplest form, the hydrolysis of ATP caused by Na^+-K^+-ATPase may be regarded as a two-stage reaction [6, 17]:

$$\begin{aligned} &E+\mathrm{ATP}\xrightarrow{Mg^{++},\,Na^+}E\sim P+\mathrm{ADP}\\ &E\sim P\xrightarrow{K^+}E+P_i \end{aligned} \tag{1}$$

One may elaborate on this scheme to define the role of Na^+ and K^+:

$$\begin{aligned} &E+\mathrm{ATP}+\mathrm{Na}^+\xrightarrow{Mg^{++}}E\sim P-\mathrm{Na}+\mathrm{ADP}\\ &E\sim P-\mathrm{Na}+\mathrm{K}^+\longrightarrow E-P-\mathrm{K}+\mathrm{Na}^+\\ &E-P-\mathrm{K}\longrightarrow E+P_i+\mathrm{K}^+ \end{aligned} \tag{2}$$

If the above assumption is true, ATP and Mg^{++} which provide the formation of energy-rich enzyme-phosphate complex ($E\sim P$) are, at the same time, responsible for the formation of ion-exchange sites with high affinity for Na^+. Taking into consideration ionic conditions for Na^+-transporting membranes, a transition of cation specificity of these sites from predominantly Na^+ to predominantly K^+ should be postulated. Since ATP hydrolysis is accomplished on the inner side of the cell membrane when Na^+-for-K^+ exchange takes place at its outer side, the energy-poor $E-P$ complex should contain the K^+-specific sites rather than energy-rich $E\sim P$ complex. In this respect, the release of Na^+ in response to the addition of K^+ when the latter are present in media in lower concentration indicates the existence of K^+-specific sites. The lack of ATP prevents formation of these sites, thus abolishing the possibility for ion exchange. If Ca^{++} replaces Na^+ sites at enzyme phosphate complexes due to stronger affinity, both the Na^+ binding and subsequent Na^+ release after the addition of K^+ should be

eliminated. In fact, our experiments show that the addition of Ca^{++} to the media removes not only Na^+ release in response to KCl, but also the difference in increase of Na^+ activity when Na_2ATP is added to enzyme-containing media with respect to the control (*see* Fig. 1). As mentioned before, this difference may be regarded as representing Na^+ binding.

As a probable explanation for the mechanism of ouabain inhibition, a competition between ouabain and K^+ for K^+-specific sites was assumed [1, 22]. However, according to kinetic data obtained by Matsui and Schwartz [15] as well as by Ahmed, Judah and Scholefield [2], K^+ is not substituted by ouabain and there are different sites for ouabain and K^+ binding. In any case, according to the recent concept concerning the mechanism of ouabain action within the limits of the above scheme, ouabain should not prevent two first stages of reaction (2). Thus it seems natural that ouabain does not prevent either Na^+ binding or Na^+ release after the addition of KCl (*see* Fig. 1, Table 2, variant 6).

Our data on inhibition of Na^+ binding are in perfect agreement with the results of measurements of ^{22}Na incorporation in the same enzyme complex performed by Jarnefelt and Stedingk [8, 10]. In their experiments, the incorporation of ^{22}Na was strongly inhibited by Ca^{++} when not affected (or slightly increased) by ouabain. But their suggestion that ouabain inhibits Na^+ release contradicts the results of the present work.

The existence of certain stoichiometrical coupling between ATP hydrolysis and Na^+ transport was shown for a number of tissues and preparations. The ratio of 3 Na^+ transported per 1 inorganic phosphate released, established for human erythrocytes and reconstituted ghosts, seems to be acceptable for microsomal Na^+-K^+-ATPase as well [7, 24]. But the number of Na^+ transported across membranes must not necessarily be equal to the number of Na^+ bound and then released from the enzyme at some stage of the reaction. As already mentioned, Jarnefelt and Stedingk [10] were the first to attempt comparison of ^{22}Na binding and ^{32}P incorporation. The value of ^{32}P incorporated (P_{inc}) was assumed to be equal to the number of molecules of *P*-intermediate (P_{inc}). Their data show that the ratio of Na/P_{inc} was dependent on the incubation time and varied from 160 for a short incubation period to 11 for a longer one. Similar calculations may be carried out with respect to released Na^+ in our data if the concentration of *P*-intermediate is known. Fortunately for us, Post, Sen and Rosenthal [21], while studying ^{32}P incorporation, used the same kind of preparation of microsomes from guinea pig kidney cortex. The turnover number found by these authors was 20,000 per min at 44 °C and estimated to be about 3,000 per minute at room temperature. Knowing the turnover number, one must determine the

rate of inorganic phosphate (P_i) formation to calculate the concentration of P_{int}.

To estimate the rate of P_i formation for our working conditions, we used a typical plot of P_i production for the period longer than that of our experiment (Fig. 2). The curve of saturation type indicates a prominent deficiency of ATP after the first 5 min of the reaction. Because of this deficiency, only the very initial part of the curve may be used for estimation of the rate of P_i formation for the preparation. In our case the value of about 1 millimole/min per liter was considered to be a reasonable minimal rate of P_i formation. Using this value and the turnover number of 3,000 per min, we found P_{int} concentration to be 3.3×10^{-4} mM/liter. When the amount of Na^+ released is compared with the above concentration of P_{int}, the ratio of $1.3 \times 10^3 : 1$ can be found. If a ten-times-greater rate of P_i formation is assumed to be maximal, the ratio of Na^+ released to P_{int} should be about $10^2 : 1$. Thus the ratio established appeared to be of the same order as found in the work of Jarnefelt and Stedlingk [10].

The transporting vesicle hypothesis suggested by these authors as an explanation for such a large ratio is not applicable to our case. Since the glass electrode measuring Na^+-activity changes is undoubtedly placed outside submicroscopic vesicles, the increase in Na^+ activity should be considered as the result of sodium efflux. It means that the membranes of the vesicles in our case are normally oriented, whereas the Na^+-accumulating vesicles proposed by Jarnefelt and Stedingk ought to have membranes turned inside out. For the vesicles with normally orientated membranes, the interpretation of results involves some difficulty. First, in order to have a significant amount of Na^+ released into the outer media, a large concentration of Na^+ should have previously accumulated inside the vesicles; this does not seem reasonable. The membrane of the vesicles should be permeable to ATP and Ca^{++} because these agents may influence the ATPase activity only when interacting with the inner side of the transporting mem-

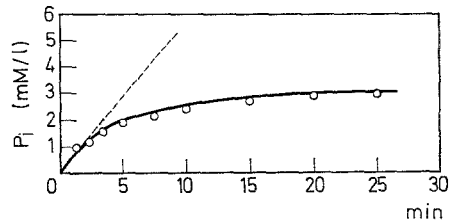

Fig. 2. Time curve of ATP hydrolysis by membrane ATPase in the conditions used (*see* Text). The dotted line corresponds to the mean rate of P_i formation for the first minute of the reaction

brane. Finally, the absence of ouabain suppression of Na^+ release when ATPase activity is significantly inhibited is direct evidence against the redistribution mechanism of Na^+-activity changes and, hence, against vesicle hypothesis. The latter, however, was regarded as the only explanation for the discrepancy of the ratio Na/P_i found in transport experiments on intact cells and for microsomal suspension. If not for the vesicle hypothesis given above, it should be considered that much greater numbers of ions are bound and then released when compared with those transported across the membrane.

The large ratio of Na^+ released per unit of P_i observed in this investigation may also reflect the idle-transport conditions existing in microsomal suspension as compared to the Na^+ transport in experiments with intact cells with high uphill electrochemical gradients.

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