# Flexibility of an Active Center in Sodium-Plus-Potassium Adenosine Triphosphatase

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ABSTRACT In plasma membranes of intact cells an enzymatic pump actively transports sodium ions inward and potassium ions outward. In preparations of broken membranes it appears as an adenosine triphosphatase dependent on magnesium, sodium, and potassium ions together. In this adenosine triphosphatase a phosphorylated intermediate is formed from adenosine triphosphate in the presence of sodium ions and is hydrolyzed with the addition of potassium ions. The normal intermediate was not split by adenosine diphosphate. However, selective poisoning by *N*-ethylmaleimide or partial inhibition by a low magnesium ion concentration yielded an intermediate split by adenosine diphosphate and insensitive to potassium ions. Pulse experiments on the native enzyme supported further a hypothesis of a sequence of phosphorylated forms, the first being made reversibly from adenosine triphosphate in the presence of sodium ion and the second being made irreversibly from the first and hydrolyzed in the presence of potassium ion. The cardioactive steriod inhibitor, ouabain, appeared to combine preferentially with the second form. Phosphorylation was at the same active site according to electrophoretic patterns of proteolytic phosphorylated fragments of both reactive forms. It is concluded that there is a conformational change in the active center for phosphorylation during the normal reaction sequence. This change may be linked to one required theoretically for active translocation of ions across the cell membrane.

The basic idea in this paper is a change in the shape of the sodium and potassium pump during its reaction cycle. This change is not necessarily that assumed for translocation (1), but rather a change in the conformation of an active center. The evidence is indirect. It is assumed that the reactivity of a phosphoryl group attached covalently to an active site in an active center depends on the conformation of the active center surrounding the site. Thus a change in reactivity implies a change in conformation. It should be enough to show that this group at a single active site changes its reactivity in the course of the normal reaction sequence. The phosphoryl group is attached to sodium-potassium-dependent adenosine triphosphatase, an aspect of the pump.

At first, it will be helpful to review the pump (2–5). In the human erythrocyte the stoichiometry and localization are as follows. Per cycle three sodium ions move outward across the membrane, two potassium ions move inward and the terminal phosphate bond of one molecule of intracellular ATP is split, with catalysis by intracellular magnesium ion. The pump is inhibited by cardioactive steroids such as ouabain, digoxin, strophanthin, and scillaren (Fig. 1). When the plasma membranes are broken, pump activity remains as an ATPase activity requiring $Na^+$, $K^+$, and $Mg^{++}$ together and inhibited by cardioactive steroids, even though translocation can no longer accumulate cation gradients because of the holes in the membrane. Membrane preparations with particularly high ($Na^+$ + $K^+$)-ATPase activity can be obtained from tissues which pump much sodium ion, either electrically excitable tissues,

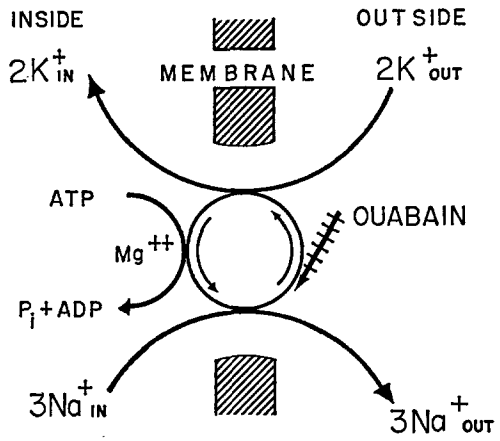


FIGURE 1. Stoichiometry and localization of the sodium and potassium ion pump in human erythrocytes. *Figure reprinted by permission from Regulatory Functions of Biological Membranes, Elsevier Publishing Co., Amsterdam, 1968.*

such as the electroplax of the electric eel, brain, nerve, heart, or secretory tissues, such as the salt gland of waterfowl, salivary gland or kidney. ($Na^+$ + $K^+$)-ATPase preparations from kidney, electroplax, and brain easily show the participation of a phosphorylated intermediate. The basic reaction sequence of the intermediate is the following. In the first step the terminal phosphate group of ATP is transfered to the enzyme with a requirement for $Mg^{++}$ and acceleration by $Na^+$; ADP is released. In a second step hydrolysis of the enzyme-phosphate bond is accelerated by $K^+$. The combined steps make up a ($Na^+$ + $K^+$)-ATPase activity (Fig. 2).

In the experiments reported here, to estimate the amount of the phosphorylated intermediate the reaction mixture contained membrane preparations made by differential centrifugation of sucrose homogenates of kidneys from guinea pigs or rabbits (6), ATP heavily labeled with $^{32}P$ in the terminal phosphate position, $Mg^{++}$ and monovalent cations at neutral pH. For transient

kinetic experiments it was best to work at 0°C in order to slow the turnover of the intermediate. The reaction was stopped with cold acid and the denatured precipitated membranes were washed free of ($^{32}$P)-ATP and counted. The radioactive phosphorus was related to the protein or to the total (phospholipid) phosphorus in the precipitate (Table I). Making the reaction mixture with individual inorganic monovalent cations showed that sodium ion

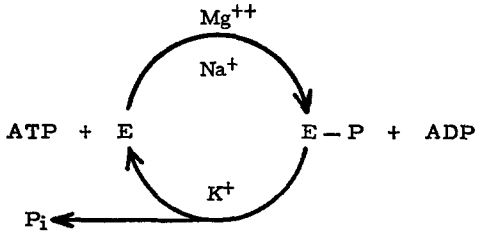


FIGURE 2. A partial reaction sequence for formation and breakdown of a phosphorylated intermediate in ($Na^+$ + $K^+$)-ATPase. *Figure reprinted by permission from Regulatory Functions of Biological Membranes, Elsevier Publishing Co., Amsterdam, 1968.*

TABLE I

REACTION MIXTURE FOR ESTIMATION OF PHOSPHORYLATED ($Na^+$ + $K^+$)-ATPase

| | |
|---|---|
| Membrane protein, *mg* | 0.5 |
| Imidazole glycylglycine, pH 7.6, *μmoles* | 10.0 |
| $MgCl_2$, *μmole* | 1.0 |
| $(Tris)_4$ ($\gamma$-$^{32}$P)-ATP, *μmoles* | 0.04 |
| NaCl or KCl, *μmoles* | 16.0 |
| Final volume, *ml* | 1.0 |
| Incubation at 0°C, *sec* | 1–60 |
| Trichloroacetic acid, 0.25 M containing 0.6 mM ATP and 15 mM $H_3PO_4$ at 0°C, *ml* | 35 |

Centrifuge, wash precipitate 3 X in cold trichloroacetic acid, dissolve in $CHCl_3$:MeOH:formic acid (4:2:1) (v:v:v) and analyze aliquots for radioactivity and protein or total (phospholipid) phosphorus. Estimate ratio of $^{32}$P to protein or total P. The ratio found in the presence of KCl corresponds to 0% of the phosphorylated enzyme. Subtract this ratio from that in the presence of NaCl to obtain the maximal value, 100%. (Modified from reference 7.)

uniquely produced a large increment in phosphorylation (Table II). The kinetics of the increment were as follows. In a control experiment with potassium ion, the membranes were phosphorylated at a slow, constant rate. Addition of a chase of unlabeled ATP had no effect. In the presence of sodium ion, on the other hand, the increment in phosphorylation appeared immediately (in less than 1 sec at 0°C in unpublished experiments) and remained constant with time. A chase of unlabeled ATP reduced the labeling. The label in the increment thus turned over rapidly (Fig. 3). In the increment the phosphate is attached to a carboxyl group (4); in the absence of $Na^+$ the phosphate is

attached to a hydroxyl group of serine (9). Thus, background phosphorylation appears to be different and unrelated to the pump. In further experiments the increment will be called the $^{32}$P-intermediate. Dephosphorylation of the inter-

TABLE II
MONOVALENT CATIONS ON PHOSPHORYLATION OF KIDNEY MEMBRANES

| Addition | Phosphorylation |
|---|---|
| | *μμmoles $^{32}$P/mg protein* |
| None | 26 |
| $Li^+$ | 20 |
| $Na^+$ | 97 |
| $K^+$ | 16 |
| $NH_4^+$ | 14 |
| $Rb^+$ | 18 |
| $Cs^+$ | 14 |
| $Tris^+$ | 19 |

120 μmoles of the chloride salts were substituted for the NaCl or KCl in Table I. The total phosphorylation is given. From reference 8.

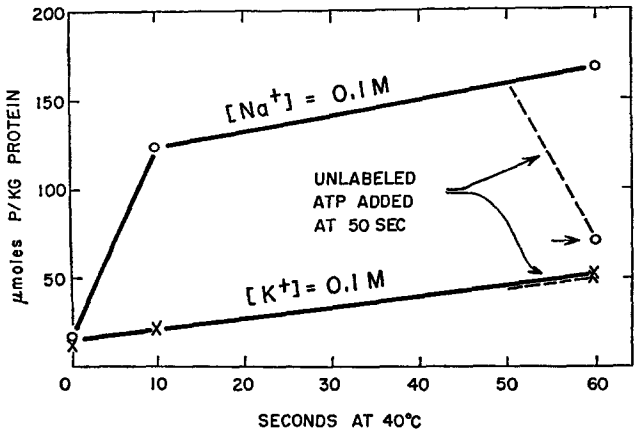


FIGURE 3. Kinetics of phosphorylation of kidney membranes by ($^{32}$P)-ATP. The horizontal arrow (→) indicates a reduction in the level of the $Na^+$-dependent increment calculated in proportion to the reduction of the specific activity of the ($^{32}$P)-ATP. *Figure reprinted by permission from J. Biol. Chem., 1965, 240: 1437.*

mediate by $K^+$ was best demonstrated during inhibition of formation. First the $^{32}$P-intermediate was formed and then at zero time excess EDTA was added to chelate the $Mg^{++}$ and block further formation of the intermediate. The intermediate disappeared with a time constant of about 16 sec. At 4 sec addition of $K^+$ split the intermediate within 4 more seconds even though the concentration of $K^+$ was only $\frac{1}{160}$th that of $Na^+$ (Fig. 4). In similar experiments (unpublished)

all the other inorganic monovalent cations in Table II, and thallous ion also, were similarly effective. The specificity is thus sodium ion for formation and all similar ions for breakdown. These similar ions might be called "not-sodium ion," where not-sodium ion means $Li^+$, $NH_4^+$, $K^+$, $Rb^+$, $Cs^+$, or $Tl^+$ (5, 8, 10).

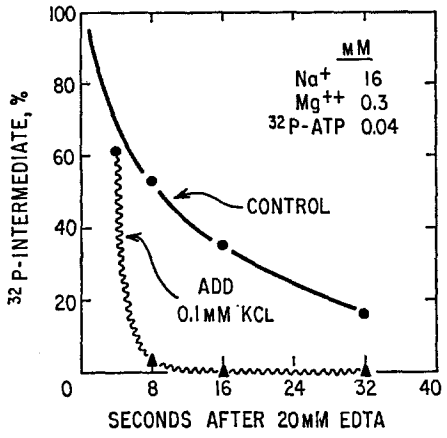


FIGURE 4. Dephosphorylation of the phosphorylated intermediate by $K^+$. The temperature was 0°C. *Figure reprinted by permission from Regulatory Functions of Biological Membranes, Elsevier Publishing Co., Amsterdam, 1968.*

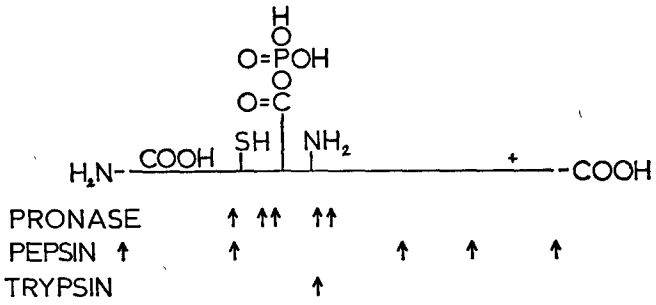


FIGURE 5. Sequence of reactive groups and bonds sensitive to proteolytic digestion in the primary structure of the phosphorylated protein of the phosphorylated intermediate from the native enzyme. The diagram indicates approximately the third peptic fragment (17); the intact protein is much larger (18). The spacings of the arrows correspond approximately to molecular weight calculated from the assumption that electrophoretic mobility varied inversely as the square root of the molecular weight. This fragment may contain perhaps 30 amino acid residues. From left to right the internal peptic cuts are the fourth, sixth, and fifth. Trypsin split the fourth, fifth, or sixth peptic fragments to a single fragment which moved faster toward the cathode than P6 at pH 2 and which stayed at the origin after performic acid treatment. (Unpublished experiments.)

In erythrocytes the pump shows a similar selectivity for sodium ion for outward transport and not-sodium ion for inward transport (11–13). Actually more sensitive experiments indicate weak substitution of $H^+$ for $Na^+$ and even of $Na^+$ for $K^+$ (14).

At the active site for phosphorylation of $(Na^+ + K^+)$-ATPase, the sequence of amino acids is not known. Nevertheless the neighborhood of the site can be characterized by reactive groups and by bonds susceptible to specific proteolytic enzymes. The radioactive fragments released by proteolysis are charac-

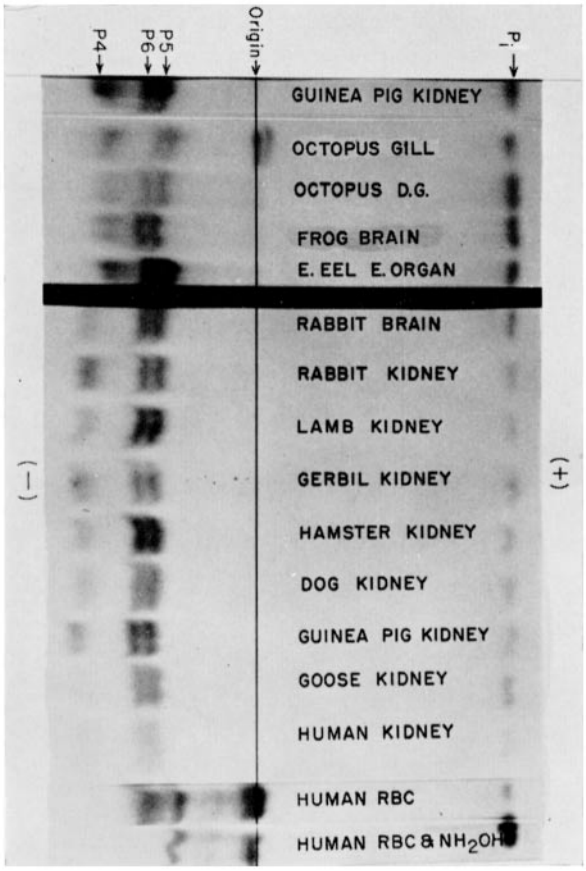


FIGURE 6. Comparison of the active site of $(Na^+ + K^+)$-ATPase from various sources Phosphorylated, denatured membrane preparations were digested with pepsin. The soluble radioactive fragments were electrophoresed at pH 2 and radioautographed. The symbols "P4," "P5," and "P6" indicate the fourth, fifth, and sixth fragments respectively. In the case of human erythrocyte membranes part of the solubilized material was treated with hydroxylamine before electrophoresis to distinguish the fragments with an acyl phosphate bond from background material. *Figure reprinted by permission from Biochim. Biophys. Acta, 1968, 150: 41.*

terized by their electrophoretic mobility. The radioactive acyl phosphate group can be identified by its sensitivity to splitting by hydroxylamine (4). The amino acid at this site is discussed in the companion paper by Dr. Hokin. An amino group of lysine (15) contributes an extra positive charge to all the

fragments so that they move toward the cathode at pH 2. Its charge can be neutralized by acetylation (16). A sulfhydryl group carries no change at pH 2 but acquires a negative charge after oxidation with performic acid (17). After digestion of the denatured phosphorylated enzyme with pepsin, six peptide fragments were separated by electrophoresis and detected by radioautography (17). Of these six the last three were used for comparison of intermediates. After digestion with pronase four fragments were obtained; of these only the first had a sulfhydryl group (Fig. 5). These peptides were also used for comparison.

Peptic fragments were used to compare the active sites of $(Na^+ + K^+)$-ATPase from various sources. Denatured phosphorylated membranes were digested with pepsin and the soluble radioactive fragments were applied to

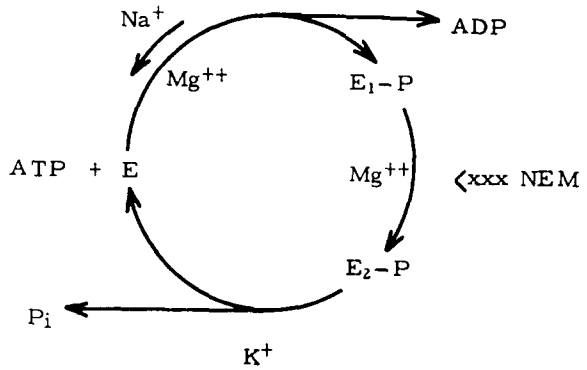


FIGURE 7. A partial reaction sequence for $(Na^+ + K^+)$-ATPase as proposed by Albers and coworkers. "E" indicates the enzyme and "$E_1$-P" and "$E_2$-P" indicate two phosphorylated intermediates. "NEM" indicates a step which can be selectively inhibited by *N*-ethylmaleimide or by oligomycin. *Figure reprinted by permission from J. Biol. Chem., 1966, 241: 1882.*

paper for electrophoresis at pH 2 with subsequent radioautography. The peptic fragments appear to be the same in a variety of tissues and species (Fig. 6). The "pronatic" peptides obtained by partial hydrolysis with pronase were also similar (19).

With these features in mind it is appropriate to consider further the reactivity of the phosphorylated intermediate. According to the hypothesis of Albers and coworkers (20, 21) the E-P of Fig. 2 is subdivided into a sequence of $E_1$-P followed by $E_2$-P. The conversion of $E_1$-P to $E_2$-P requires $Mg^{++}$ and is effectively irreversible. $E_1$-P reacts with ADP reversibly to resynthesize ATP, whereas $E_2$-P reacts with water irreversibly in the presence of potassium ion to yield inorganic phosphate (Fig. 7). The hypothesis is based on experiments with the $(Na^+ + K^+)$-ATPase from the electroplax of the electric eel. These workers observed first an exchange reaction between radioactive ADP and the

ADP group of ATP. The exchange required $Na^+$ and $Mg^{++}$, but was inhibited by $Mg^{++}$ at high concentrations. At these concentrations the phosphorylated intermediate persists. They required, therefore, one phosphorylated intermediate for the exchange reaction at low $Mg^{++}$ concentrations and another

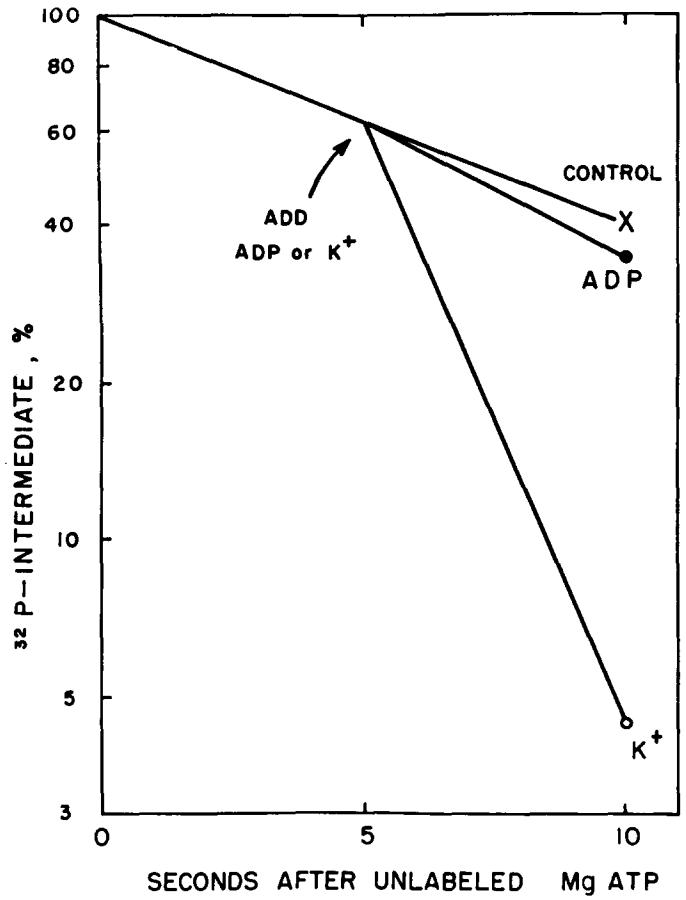


FIGURE 8. Sensitivity of the phosphorylated intermediate of the native enzyme to splitting in the presence of ADP or $K^+$. The intermediate was first formed from ($^{32}P$)-ATP as in Table I. At zero time the radioactive ATP was chased with a 100-fold excess of unlabeled ATP and additions were made after 5 sec as indicated. Qualitatively similar results were obtained also when formation was blocked with cyclohexyl-EDTA. (Unpublished experiment.)

for phosphorylation at high $Mg^{++}$ concentrations (22). In the second place they enhanced the exchange reaction by poisoning the enzyme with *N*-ethylmaleimide, NEM. This treatment abolished the ($Na^+$ + $K^+$)-ATPase activity but not phosphorylation (21, 23). Furthermore, the amount of the phosphorylated intermediate of the NEM-treated enzyme was not reduced in the presence

of $K^+$ (21). They concluded that treatment with NEM inhibited the conversion of $E_1$-P to $E_2$-P. Oligomycin had an effect similar to that of treatment with NEM. Thus, in principle, two forms of phosphorylated enzyme can be distinguished by their sensitivity to breakdown in the presence of ADP or $K^+$. $E_1$-P should be split only when ADP is added and $E_2$-P should be split only when $K^+$ is added. These tests were applied to native enzyme and to NEM-enzyme from guinea pig kidney. The intermediate of the native enzyme re-

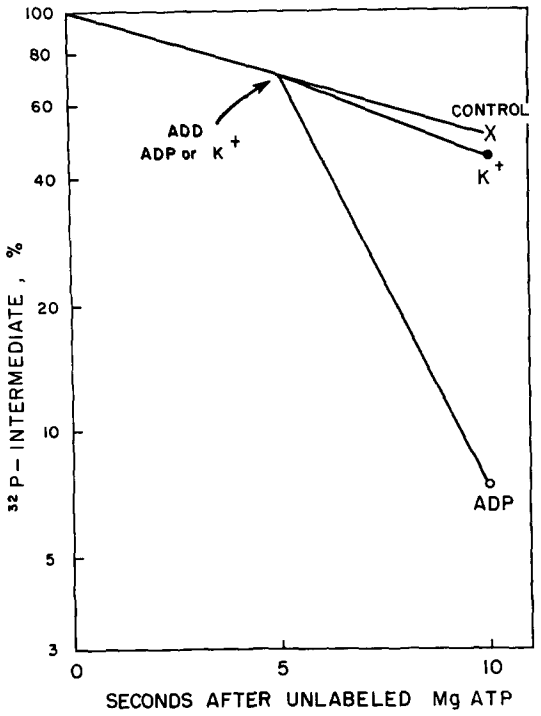


FIGURE 9. Sensitivity of the phosphorylated intermediate of an *N*-ethylmaleimide derivative of ($Na^+$ + $K^+$)-ATPase to splitting in the presence of ADP or $K^+$. The experimental conditions, except for the enzyme, were exactly the same as in Fig. 8. Qualitatively similar results were obtained also with cyclohexyl-EDTA in place of unlabeled ATP. (Unpublished experiment.)

sponded like $E_2$-P to $K^+$ but not ADP, and the intermediate of the NEM-enzyme responded like $E_1$-P to ADP but not $K^+$ (Figs. 8 and 9). Other experiments (unpublished) extended the experience of Albers and coworkers that the kinetics of formation of the intermediate in the native and poisoned enzyme were similar. The phosphoryl groups of the two intermediates had the same pH-stability profile and appeared to yield the same peptic fragments (Fig. 10). Of these fragments the fourth traveled at pH 2 toward the cathode faster than the fifth and sixth because of an extra positive charge. After per-

formic oxidation of the sulfhydryl group the fifth and sixth stayed at the origin and the fourth moved one-half as rapidly as before, now just a little slower than the fifth fragment before oxidation. *N*-ethylmaleimide treatment of the sulfhydryl group of the digested fragments protected it against performic oxidation (Fig. 10). Because the enzyme preparation, poisoned with NEM before phosphorylation, released the same peptic fragments after denaturation as the native enzyme, the reactive site for this type of poisoning by NEM is not the sulfhydryl group near the acyl phosphate. In addition, Dr. Albers kindly provided us with preparations of electroplax ($Na^+ + K^+$)-ATPase both native

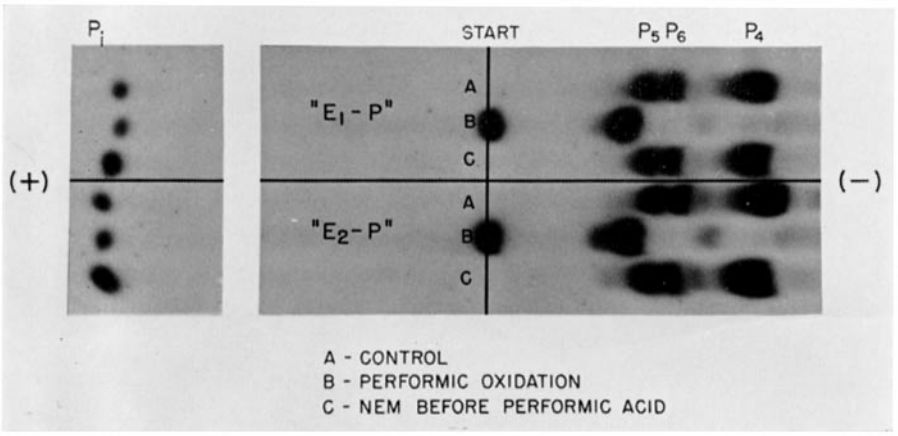


FIGURE 10. Comparison of the active site of $E_1$-P with that of $E_2$-P by electrophoresis of peptic fragments. $E_1$-P was prepared by phosphorylating enzyme previously treated with *N*-ethylmaleimide and adding $K^+$ with excess unlabeled ATP 5 sec before denaturation with acid. $E_2$-P was prepared by phosphorylating native enzyme and adding ADP with excess unlabled ATP 5 sec before denaturation with acid. The solubilized fragments were treated with A, nothing; B, performic acid; or C, *N*-ethylmaleimide as indicated. Electrophoresis and radioautography were done as in Fig. 6. (Unpublished experiment.)

and treated with NEM. Their phosphorylated intermediates were sensitive to ADP or $K^+$ in the same way as those of the enzyme from guinea pig kidney and their radioactive "pronatic" fragments had the same electrophoretic mobility at pH 2 (unpublished experiment with Dr. Bader).

Assignment of specificity to an enzyme inhibitor for a particular step in a reaction sequence requires justification in the kinetics of the native enzyme. According to Fig. 7, in the native enzyme the simplest experiment should be a reduction of the $Mg^{++}$ concentration to simulate the conditions for $Na^+$-dependent ADP-ATP exchange (22). A control experiment at a relatively high $Mg^{++}$ concentration of 1 mM showed the reactivity of $E_2$-P (Fig. 11). After the intermediate was formed, further phosphorylation was prevented

after zero time by addition of cyclohexylene-diamine-tetraacetic acid, cyclohexyl-EDTA (24). Addition of ADP at 5 sec had no effect. Addition of a low concentration of $K^+$ produced a rapid loss (Fig. 11). Repetition of this experiment with formation partially limited by EDTA produced a partial reactivity like that of $E_1$-P (Fig. 12). 3 mM EDTA chelated some of the free $Mg^{++}$ and reduced the initial amount of the intermediate to 60 % of maximal. This material showed an increased sensitivity to ADP and a reduced sensitivity to $K^+$ in comparison with the control (Fig. 12). An increase in the proportion of

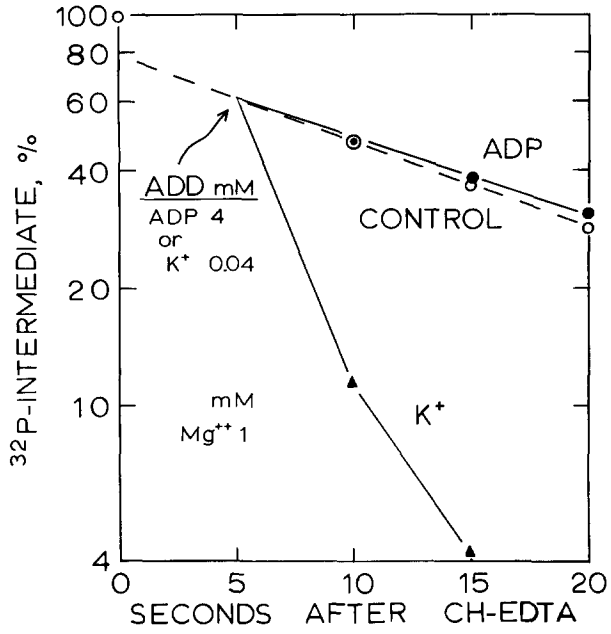


FIGURE 11. Comparison of ADP and $K^+$ sensitivities in the native enzyme with excess $Mg^{++}$. At zero time, formation of the intermediate was stopped with 10 mM cyclohexyl-EDTA, which chelated all the free $Mg^{++}$. Cyclohexyl-EDTA, (1,2-cyclohexylene-dinitrilo)-tetraacetate, is a derivative of EDTA and a stronger chelator (24). The $K^+$ concentration was low in order to allow small changes in sensitivity to appear more easily. (Unpublished experiment.)

$E_1$-P to $E_2$-P at a rate-limiting $Mg^{++}$ concentration is consistent with a requirement for $Mg^{++}$ for the irreversible conversion of $E_1$-P to $E_2$-P.

It was also possible to find other evidence in the native enzyme for this sequence of phosphorylated forms. This evidence came from pulse experiments. Reference to Fig. 7 will clarify the design and interpretation of the experiments. At first ($^{32}$P)-ATP was incubated with the enzyme in the absence of $Na^+$ and/or $Mg^{++}$. These conditions allowed ATP to become bound to the enzyme without transphosphorylation. At zero time the ($^{32}$P)-ATP was chased with unlabeled ATP so that no further significant quantity of ($^{32}$P)-

ATP could enter the reaction sequence from the medium. Simultaneously transphosphorylation was accelerated by supplementation of the missing $Mg^{++}$ and/or $Na^+$. The result was a pulse of radioactivity in the phosphorylated intermediate with disappearance kinetics similar to those of an intermediate which had been fully formed initially. The advantage of a pulse experiment is the opportunity to observe formation and breakdown as separate steps in a single experiment and to exclude effects due simply to competition for the initial binding site of ATP. According to Fig. 7 a sequence from E·

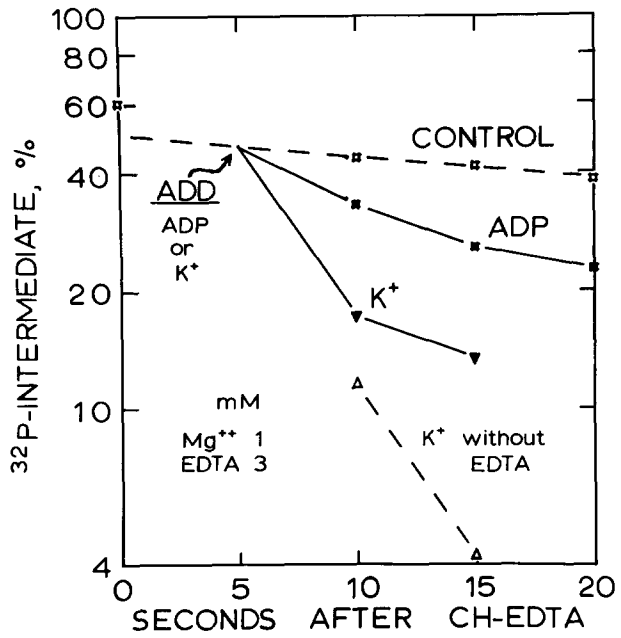


FIGURE 12. Comparison of ADP and $K^+$ sensitivities in the native enzyme with a rate-limiting concentration of free $Mg^{++}$. The experiment is identical to that in Fig. 11 except that 3 mM EDTA was present. The dotted line and open triangles, △------△, show data from Fig. 11 replotted for easy comparison. (Unpublished experiment.)

ATP to $E_2$-P should pass through $E_1$-P, which is a sensitive to ADP but not $K^+$. Therefore $K^+$ should not affect formation of $E_2$-P but should accelerate breakdown, whereas ADP should impair formation without affecting breakdown.

The effect of a low concentration of $K^+$ was tested by putting it into the initial reaction mixture (Fig. 13). This arrangement gave it an opportunity to influence formation without regard to transient kinetics initiated by the chasing solution. $Na^+$ was present initially and the pulse was started with $Mg^{++}$ and unlabeled ATP. The presence of $K^+$ accelerated breakdown six-fold without any effect on formation as estimated by extrapolation to zero

time (Fig. 13). The effect of ADP was tested by putting it into the chasing solution in order to keep it from displacing bound ($^{32}$P)-ATP before the pulse was started (Fig. 14). $Na^+$ was omitted from the initial reaction and added with the unlabeled ATP in the chasing solution. ADP inhibited formation but did not affect breakdown kinetics, as is indicated by the reduction in the amount of intermediate with increasing ADP concentration and the similarity of the slopes of the lines of disappearance (Fig. 14). ADP was more effective than unlabeled ATP in preventing formation. It could not have driven off

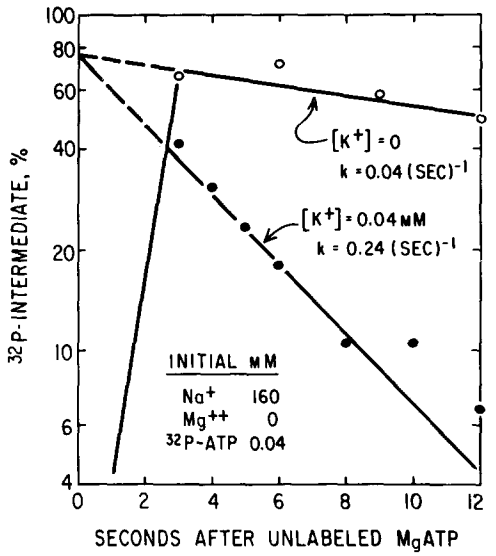


FIGURE 13. The action of $K^+$ on formation and breakdown of the phosphorylated intermediate of the native enzyme in a pulse experiment. In addition to the components shown and the enzyme, the initial reaction mixture of 0.9 ml contained 10 μmoles of $(Tris)_4$ EDTA to trap traces of $Mg^{++}$ in the enzyme preparation. The chasing solution contained 4 μmoles of unlabeled $(Tris)_4$ ATP and 16 μmoles of $MgCl_2$ in 0.1 ml. The reaction mixture was stirred continually; the chasing solution was injected by hand and acid was poured in at times read from a stopwatch. When present, $K^+$ was in the initial reaction mixture. (Unpublished experiment.)

bound ATP except by combination at a second site with a peculiar allosteric effect. It might have been a product inhibitor. In this case it must have come off the enzyme as product in the absence of added ADP in order to go back on as inhibitor when excess ADP was added. If ADP came off before $E_2$-P was formed, there must have been an antecedent $E_1$-P. This experiment thus shows supporting evidence for a sequence of two phosphorylated intermediates in the native enzyme.

At this stage, the argument begins to escalate. The next point is that ouabain knows the difference between $E_1$-P and $E_2$-P, reacting preferentially with

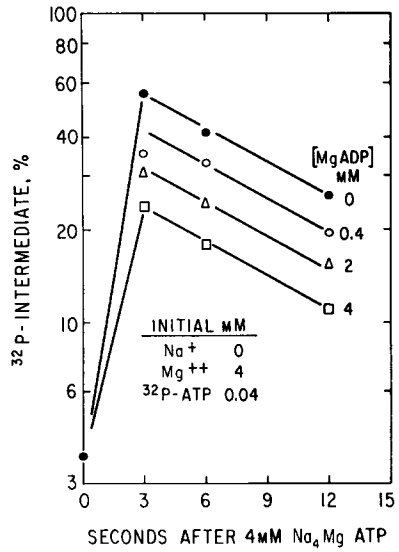


FIGURE 14. The action of ADP on formation and breakdown of the phosphorylated intermediate in the native enzyme in a pulse experiment. The reaction system was similar to that in Fig. 13 except that EDTA was absent and the enzyme was washed free of $Na^+$ immediately before use. The ADP was added in the chasing solution. The concentrations given in the figure are those of the final reaction mixture. The scale of the vertical axis is logarithmic so that exponential disappearance is represented by a straight line. The rate constant is proportional to the slope of the line. (Unpublished experiment.)

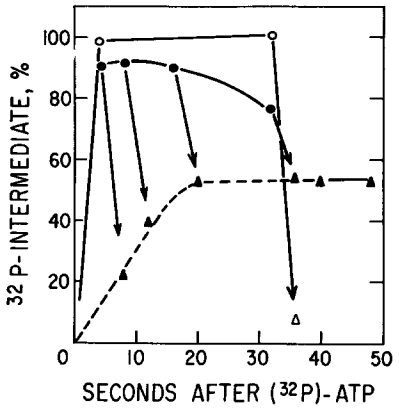


FIGURE 15. Reaction between $E_2$-P and ouabain in the native enzyme. The reaction mixture contained the enzyme, 1 μmole of $MgCl_2$ and 16 μmoles of $Na_2SO_4$. At zero time 0.04 μmoles of ($^{32}$P)-ATP were added and the reaction was stopped with acid at the times shown (○, ●). To test the stability of the intermediate at the times indicated by the tails of the descending arrows (↓), 16 μmoles of KCl and 1 μmole of unlabeled ATP were added with acid 4 sec later (△, ▲). In the control experiment there was no ouabain (○, △); in the test 0.25 μmoles of ouabain were present (●, ▲). The dashed line and solid triangles show the formation of a relatively stable ouabain derivative of the phosphorylated intermediate (▲------▲). (Unpublished experiment.)

$E_2$-P. Disregarding the details at first, comparison of Fig. 15 ($E_2$-P) with Fig. 16 ($E_1$-P) shows that ouabain obviously affected the former but not the latter. Fig. 15 shows a reaction between $E_2$-P and ouabain. In the control experiment

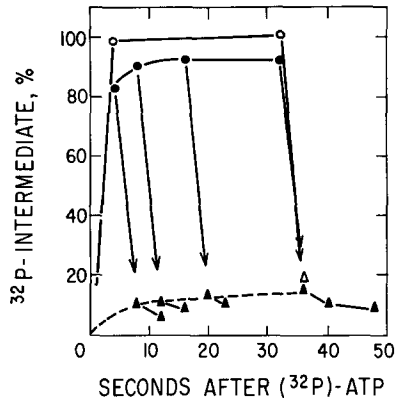


FIGURE 16. Lack of reaction between $E_1$-P and ouabain in enzyme treated with *N*-ethylmaleimide. The reaction mixture and additions were the same as in Fig. 15 except that the enzyme was treated with *N*-ethylmaleimide beforehand and that 2 μmoles of $(Tris)_3ADP$ and 3 μmoles of $MgCl_2$ were substituted in place of 16 μmoles of KCl. The symbols also are the same. (Unpublished experiment.)

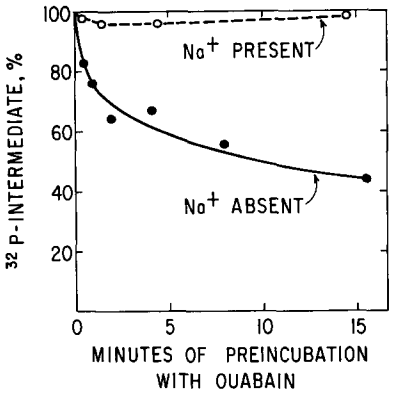


FIGURE 17. Inhibition of the dephospho-enzyme by ouabain and protection against this inhibition by sodium ion. The reaction mixture contained 1 μmole of $MgCl_2$. Preincubation was started with 0.25 μmoles of ouabain. At the times indicated for the solid circles and line, ●——●, 0.04 μmoles of ($^{32}$P)-ATP with 16 μmoles of NaCl were added and acid was added 3 sec later. In the case of the open circles and dashed line, O------O, the NaCl was present before the ouabain was added. (Unpublished experiment.)

the $^{32}$P-intermediate was formed, remained stable, and then split rapidly and completely after addition of $K^+$ and unlabeled ATP. In the presence of ouabain, addition of $K^+$ and unlabeled ATP at progressively later intervals revealed a developing resistance to splitting with formation of an obviously

more stable phosphorylated derivative (Fig. 15). In contrast to $E_1$-P this derivative was resistant also to ADP (unpublished experiment). 2 or 3 min later this derivative had spontaneously lost its phosphate group so that the inhibited enzyme neither retained nor accepted again the terminal phosphate group of ATP. To compare the action of ouabain on $E_1$-P, an analogous experiment was designed with substitution of NEM-treated enzyme for native enzyme and ADP for $K^+$ (Fig. 16). No effect of ouabain was observed. The ability of ouabain to distinguish between $E_1$-P and $E_2$-P depends on experimental conditions; in a different type of experiment at 23°C, inhibition of NEM-treated enzyme was observed.

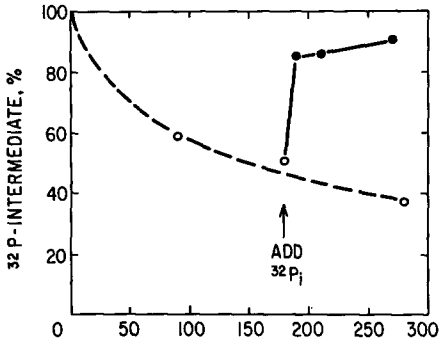


FIGURE 18. Phosphorylation by ($^{32}$P)-inorganic phosphate of dephospho-enzyme inhibited with ouabain. The reaction mixture contained 0.2 μmoles of $MgCl_2$. Preincubation was started with 0.25 μmoles of ouabain. At the times in sec indicated by the open circles and dashed line, O-----O, ($^{32}$P)-ATP was added with $MgCl_2$ and NaCl as in Fig. 17 and acid was added 10 sec later. In the case of the solid circles and solid line, ●——●, 0.4 μmoles of ($^{32}$P)-$(Tris)_3PO_4$ were added at 180 sec as indicated. The specific activity of the ($^{32}$P)-$(Tris)_3PO_4$ was the same as that of the ($^{32}$P)-ATP. (Unpublished experiment.)

The ability of ouabain to distinguish between $E_1$-P and $E_2$-P may extend to a distinction between two corresponding forms of the dephospho-enzyme, $E_1$ and $E_2$. Postulation of such forms is attractive from a consideration of symmetry (20). Cardioactive steroids appear to bind most easily to the phospho-enzyme (25) but binding to dephospho-enzyme in the presence of $Mg^{++}$ with $P_i$ has also been found (26, 27). In our own experiments with dephospho-enzyme, ouabain slowly made it resistant to phosphorylation by ($^{32}$P)-ATP. To observe this resistance ouabain was preincubated with the enzyme for several minutes before ($^{32}$P)-ATP and $Na^+$ were added. 5 sec later the reaction was stopped with acid. This inhibition of phosphorylation required $Mg^{++}$ and low ionic strength. It was accelerated by $P_i$. The inhibition was prevented when $Na^+$ was added before the ouabain (Fig. 17). One might suppose that $Mg^{++}$ facilitated and that $Na^+$ hindered the binding of ouabain by some sort of direct action at its binding site, but an experiment (unpub-

lished) on the reactivity of ouabain with $E_2$-P indicated no direct requirement for $Mg^{++}$ when $Mg^{++}$ was removed from $E_2$-P before ouabain was added. In another experiment (unpublished) a high concentration of $Na^+$ actually accelerated the reaction of $E_2$-P with ouabain. The effects of $Mg^{++}$ and $Na^+$ in favoring and opposing respectively inhibition of the dephospho-enzyme by ouabain could therefore be due to an indirect conformational change, assuming that the binding site for ouabain is the same in the phospho- and dephospho-enzyme. It might be supposed that $Na^+$ stabilizes an $E_1$ conformation

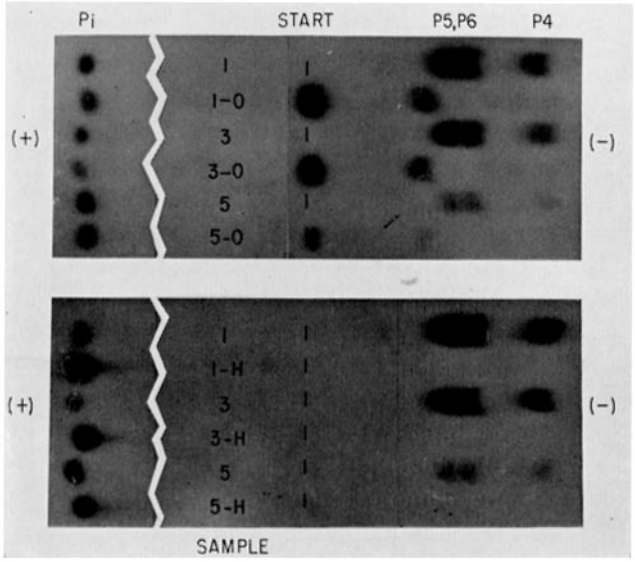


FIGURE 19. Electropherogram of radioactive fragments released by peptic digestion of native phospho-enzyme, sample 1; the stable ouabain derivative of native phospho-enzyme prepared as in Fig. 15, sample 3; and the enzyme-ouabain complex phosphorylated by $^{32}P_i$ as in Fig. 18 (without ($^{32}P$)-ATP), sample 5. Samples marked 1-O, 3-O, and 5-O were the same as those marked 1, 3, and 5 except for performic oxidation (16, 17) before electrophoresis. Samples marked 1-H, 3-H, and 5-H were treated with hydroxylamine before electrophoresis. The designation of the fragments is the same as in Figs. 6 and 10. (Unpublished experiment.)

and that $Mg^{++}$ stabilizes an $E_2$ conformation, consistent with its requirement for conversion of $E_1$-P to $E_2$-P (Fig. 7). The requirement of $Mg^{++}$ for phosphorylation of $E_1$-P would be separate from this conformational effect.

$P_i$ accelerated inhibition of the dephospho-enzyme by ouabain. Because of this, $^{32}P_i$ of the same specific activity as the ($^{32}P$)-ATP was added in an inhibition experiment like that of Fig. 17. $^{32}P_i$ rapidly phosphorylated the inhibited enzyme (Fig. 18) (compare also references 27 and 28). The peptic and "pronatic" peptides of the native $E_2$-P, $E_2$-P combined with ouabain, and ouabain-enzyme complex treated with $^{32}P_i$ were the same (Figs. 19 and 20).

Combination of the ouabain-enzyme complex with $^{32}P_i$ therefore seemed to yield the same product as combination of ouabain with $E_2$-P. This product appears to be a third form of the same active site. The overall proposed reaction sequence is illustrated in Fig. 21.

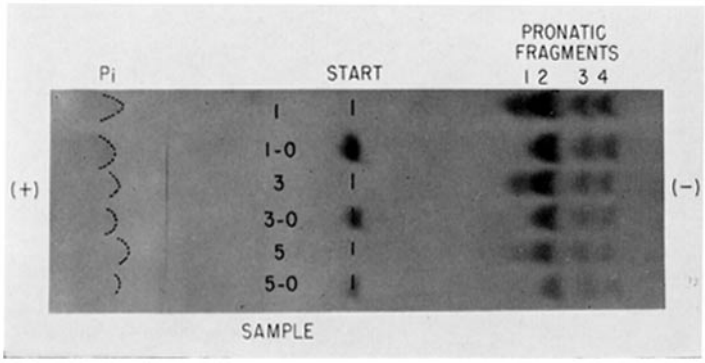


FIGURE 20. Electrophrogram of radioactive fragments released by digestion with pronase of the same phosphorylated enzyme preparations shown in Fig. 19. Samples marked 1-O, 3-O, and 5-O were treated with performic acid before electrophoresis. The numbers above the fragments indicate the sequence in which they appear and disappear during graded digestion. (Unpublished experiment.)

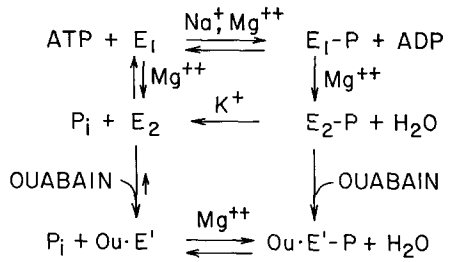


FIGURE 21. Tentative reaction sequence for the phosphorylation of $(Na^+ + K^+)$-ATPase and for its inhibition by ouabain. $E_1$ and $E_2$ indicate two forms of the enzyme in the normal sequence. E′ indicates the form of the enzyme when combined with and inhibited by ouabain. $E_1$-P, $E_2$-P, and E′-P indicate the corresponding phosphorylated forms. All three forms appear to be phosphorylated at the same active site. This scheme will probably prove to be an oversimplification.

In conclusion, the transport system appears to have at least two physiological conformations of an active center for phosphorylation and dephosphorylation, $E_1$ and $E_2$. It is tempting to suppose that these conformations are linked to conformations of an ion carrier in a translocation cycle (Fig. 22). $E_1$ would be linked to a conformation in which the carrier faces inward because presumably the $Na^+$ which catalyzes transphosphorylation of $E_1$ to $E_1$-P comes from the solution on the side of the membrane facing the intracellular

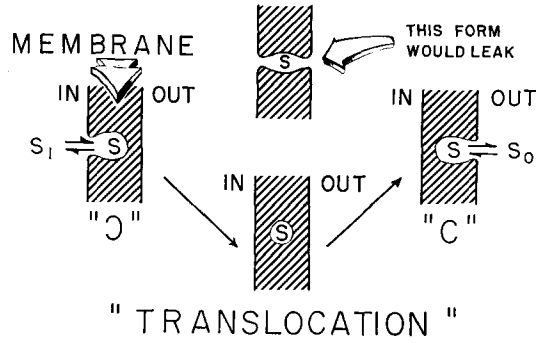


FIGURE 22. Active transport implies conformational change in a pump. The argument is that of Rosenberg and Wilbrandt (1). "S" represents a substrate for active transport, "$S_I$" is on the inside of the cell and "$S_0$" is on the outside. "C" and "C" represent an active site for translocation in alternating conformations required for loading or unloading the substrate from or to solutions on the inside and outside of the plasma membrane respectively. The conformations are mutually exclusive because otherwise the pump would leak. This analysis pertains only to reaction sequence and should apply to any molecular model, such as a classical lipid-soluble carrier (29), or gates on either side of a binding site in a protein (30, 31).

solution (or what was the intracellular solution before the membrane was broken) and is the same $Na^+$ which is translocated outward across intact membranes. $E_2$ would be linked to a conformation in which the carrier faces outward because presumably the $K^+$ which catalyzes hydrolysis of $E_2$-P comes from the solution on the side of the membrane facing the extracellular solution (or former extracellular solution) and is the same $K^+$ which is translocated inward across intact membranes. A linkage between conformational changes in a center for phosphorylation and one for translocation of ions may thus be a basic feature of the mechanism of the pump.

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