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THE ORDER OF RELEASE OF SODIUM AND ADDITION OF POTASSIUM IN THE SODIUM–POTASSIUM PUMP REACTION MECHANISM

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SUMMARY

1. The characteristics of oligomycin inhibition of the Na–K pump of human red cell membranes was investigated. Oligomycin inhibition of the pump was found to be reversible.

2. Inhibition of Na–K ATPase activity was uncompetitive with respect to ATP in broken membrane preparations. In intact cells inhibition was uncompetitive with respect to both intracellular and extracellular Na.

3. Oligomycin did not significantly inhibit the K–K exchange if the cells were Na-free, but if the cells contained a small amount of Na, oligomycin inhibition of the K–K exchange became significant. Taken together with the findings described above, this indicates that oligomycin combines with an E_1P-Na conformation of the pump and not with any E_2 conformation.

4. When measurements are made in solutions high in Na, oligomycin is a non-competitive inhibitor with respect to external K, but in Na-free solutions, oligomycin inhibition is uncompetitive with respect to external K.

5. These findings indicate that, in the normal operation of the pump, Na is released to the outside before K adds.

INTRODUCTION

Bisubstrate enzyme reactions proceed by either of two general mechanisms (Cleland, 1963*a*). In the ping-pong mechanism the first substrate adds and the first product is released before the second substrate adds. After addition of the second substrate and release of the second product, a second cycle is begun by a new addition of the first substrate. The mechanism can be represented as:

$$\begin{array}{cccc} A & P & B & Q \\ \downarrow & \uparrow & \downarrow & \uparrow \end{array}$$

where A and B are substrates and P and Q products. Ping-pong mechanisms have several distinguishing characteristics. In general there are two stable enzyme forms, one of which combines with substrate A and the other with substrate B, and it is possible to demonstrate an isotope exchange between substrate A and product P in the absence of B and Q and between substrate B and product Q in the absence of A

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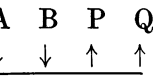
and P. The second mechanism, the simultaneous mechanism (called sequential by Cleland (1963*a*)) assumes that both substrates add before any products are released:



Simultaneous mechanisms may be ordered or random; the diagram represents an ordered simultaneous mechanism. The two mechanisms can usually be distinguished by steady-state kinetic measurements. The kinetic parameters apparent V_m and $K_{\frac{1}{2}}$ for one of the substrates is measured at several fixed concentrations of the other substrate in the absence of products of the reaction. By calculating how the ratio apparent V_m/apparent $K_{\frac{1}{2}}$ for the variable substrate varies with the concentration of the fixed substrate, one can choose between the two mechanisms.

If the concentrations of Mg and ATP are held constant, the Na–K pump can be considered as a bisubstrate reaction with inside Na and outside K as substrates and outside Na and inside K as products. There is an extensive body of evidence that the Na pump can exist in two major conformations, one of which combines preferentially with Na (E_1) and the other with K (E_2) (Post, Sen & Rosenthal, 1965; Post, Kume, Tobin, Orcutt & Sen, 1969; Bannerjee, Wong, Khanna & Sen, 1972; Bannerjee, Wong & Sen, 1972; Jorgensen, 1975). In addition, it is clear that the pump is capable of catalysing an exchange of inside for outside Na (Garrahan & Glynn, 1967*a*) and an exchange of inside for outside K (Simons, 1974). For some time, therefore, it seemed that the pump mechanism must be ping-pong.

Baker & Stone (1966) suggested that the reaction mechanism of the Na pump might be clarified by measuring the way in which the steady-state kinetic parameters vary with the concentrations of the two substrates. With the report by Garrahan & Rega (1967) of a convenient and reliable way of altering the internal ion concentrations of intact red cells it became possible to apply the steady-state kinetic tests for distinguishing between the two possible reaction mechanisms for the Na pump. Several reports of such measurements have been made (Hoffman & Tosteson, 1971; Garay & Garrahan, 1973; Chipperfield & Whittam, 1976; Sachs, 1977*b*) and, although differing in experimental procedure, in the details of the experimental findings, and in the theoretical approach, each report concluded that the Na pump reaction mechanism could be described by the simultaneous model but not by the ping-pong model.

If the reaction mechanism is indeed simultaneous, then at some point in each transport cycle both Na and K must be bound to the pump at the same time. Either external K must bind before Na is released to the outside or internal Na must bind before K is released to the inside. This paper reports the results of some experiments on the characteristics of oligomycin inhibition of the Na pump which indicate that K can add to the pump at the outside after Na is released, i.e. it is not necessary that K add to the pump before Na is released to the outside.

METHODS

Venous blood was collected from healthy donors with heparin as anticoagulant. Cells were separated from the plasma by centrifugation and the plasma and buffy coat removed by aspiration. When the cells were to be used for flux measurements they were first washed three times with unbuffered isosmotic (107 mM) $MgCl_2$ solution and their intracellular cation content was

then altered by a modification of the PCMBS (parachloromercuriphenylsulphonic acid) method described by Garrahan & Rega (1967); details of the procedure have been described (Sachs 1977*a*; Kropp & Sachs, 1977).

Methods used for the measurement of the unidirectional outflux of ^{22}Na or ^{42}K have been described (Kropp & Sachs, 1977). Cells were loaded with ^{22}Na, ^{42}K, or both isotopes while being exposed to the PCMBS solution. When ^{42}K outflux was measured the cells were first suspended at about a 20% hematocrit in a Tris-PO_4 solution (295 m-osmole/kg H_2O, pH 7·4 at 37 °C) and incubated at 37 °C for 0·5 hr. The cells were then separated from the solution and washed three times with isosmotic $MgCl_2$ solution. The isotope labelled cells were suspended in the appropriate solution at about 2% haematocrit and incubated at 37 °C. Samples were taken at measured intervals and the supernatants saved and counted. Outflux rate constants were calculated as previously described (Sachs & Welt, 1967). The solutions in which the flux measurements were made were similar to those previously described (Sachs, 1977*b*). Measurements were always made with and without 10^{-4} M-ouabain and the difference taken as the pump flux. Oligomycin (15% oligomycin A and 85% oligomycin B) was added as an ethanol solution (2 mg/ml.) and equal volumes of ethanol were added to the control solutions.

Broken membranes were prepared by osmotic lysis. Red cells were washed 3 times with an isosmotic (160 mM) NaCl solution and the packed cells rapidly squirted with stirring into 10 volumes of an ice cold solution containing 16 mM-NaCl and 2·5 mM-Tris Cl, pH 7·4 at 0 °C. The membranes were then collected and washed 5 times with the lysing solution and twice with a solution containing Tris Cl 1·0 mM and Tris-EDTA 0·5 mM, pH 7·4 at 37 °C. The washed membranes were resuspended in a solution containing Tris Cl 2·0 mM and Tris-EDTA 0·01 mM, pH 7·4 at 0 °C, frozen, and stored.

Ouabain-sensitive ATPase activity was estimated by measuring the amount of ADP produced using an assay coupled to the oxidation of NADH. The reaction mixture contained Tris Cl 21·8 mM, phosphoenolpyruvate 1·75 mM, EDTA 0·91 mM, dithiothreitol (DTT) 0·94 mM, pyruvate kinase 1·7 u./ml., $MgCl_2$ 0·91 mM greater than the sum of the concentrations of EDTA and ATP, and the concentrations of Na, K and ATP recorded in the figure legends. The pH of the solution was adjusted to 7·4 at 37 °C. About 1 mg broken membranes was added per ml. reaction mixture and the suspension was incubated at 37 °C for 1 hr. The reaction was stopped by immersing the tubes in an ice bath and the ghosts were separated from the suspension by centrifugation. One ml. of the supernatant was added to 2 ml. of a solution containing 8·6 u./ml. of lactate dehydrogenase and an appropriate amount of NADH. The mixture was incubated at room temperature for 30 min and the absorbance measured at 340 nm. Measurements were made with and without 10^{-4} M-ouabain.

Intracellular Na and K concentrations were estimated as previously described (Sachs & Welt, 1967).

Determinations were made in quadruplicate. The curves were fitted to the data points by a non-linear least-squares programme using a Hewlet–Packard 98–20 calculator. Points were weighted by using their variances. In the Figures each point is the mean of the quadruplicate determinations and the S.E. of mean is indicated.

RESULTS

The argument presented in this paper depends on the type of inhibition which oligomycin produces with respect to the substrates of the Na pump – inside Na, outside K and ATP. A reversible inhibitor may be competitive, uncompetitive or non-competitive with a substrate depending on which enzyme forms combine with the inhibitor. If the inhibitor combines only with the free enzyme form:

$$\begin{array}{l} E + S \rightleftharpoons ES \\ + \\ I \\ \downarrow \\ EI \end{array}$$

inhibition is competitive with respect to S; the inhibitor increases the apparent Michaelis constant (K_m) without altering the maximal velocity (V_m) so that in a reciprocal plot ($1/v$ *vs.* 1/S) the slope (K_m/V_m) is increased but the intercept at the $1/v$ axis ($1/V_m$) is not altered. Dixon plots ($1/v$ *vs.* inhibitor concentration) are linear and plots at different substrate concentrations intersect to the left of the $1/v$ axis and above the I axis. If the inhibitor binds only to the ES complex:

$$\begin{array}{ccc} E+S & \rightleftharpoons & ES \\ & & + \\ & & I \\ & & \downarrow \\ & & ESI \end{array}$$

inhibition is uncompetitive; both K_m and V_m are reduced by the same proportion so that in a reciprocal plot the $1/v$ axis intercept is increased but the slope is not altered. Plots at different I concentrations are parallel. Dixon plots at different S concentrations are also parallel. If the inhibitor binds to both the free enzyme and the ES complex with equal affinity:

$$\begin{array}{ccc} E+S & \rightleftharpoons & ES \\ + & & + \\ I & & I \\ \downarrow & & \downarrow \\ EI & & ESI \end{array}$$

inhibition is noncompetitive; V_m is reduced but K_m is unchanged. Both the slope and the $1/v$ intercept of reciprocal plots are increased and Dixon plots intersect on the I axis. In multisubstrate enzymes a reversible inhibitor may be competitive, uncompetitive or noncompetitive with respect to a particular substrate depending on the relation between the enzyme form to which the inhibitor adds and the form to which the substrate adds; Cleland (1963*b*) has proposed rules which allow one to predict the type of inhibition to be expected. If both the inhibitor and substrate add to the same enzyme form, inhibition is competitive. If the inhibitor combines with an enzyme form which occurs before the addition of the substrate, and the two enzyme forms are connected by reversible steps, inhibition is non-competitive (in this case, an irreversible step is one in which a product is released and the product is absent or a substrate adds and the substrate is saturating). If the two enzyme forms are separated by an irreversible step, or if the inhibitor adds after the addition of the substrate, inhibition is uncompetitive.

The reversibility of oligomycin inhibition was demonstrated by the experiment summarized in Table 1. Membranes were preincubated for an hour with and without oligomycin in a solution containing ATP, Na, K and Mg. The membranes were then diluted by transferring aliquots of the suspension to a solution whose composition was similar to that in which the preincubation took place and oligomycin was added to result in the final concentrations listed in the Table. ATPase activity was immediately assayed over a 15 min period. The results show that ghosts exposed to a concentration of oligomycin sufficient to result in significant inhibition of ouabain-sensitive ATPase activity recover their activity almost completely when the oligomycin

TABLE 1. Reversibility of oligomycin inhibition

Oligomycin (μg/ml.)		ATPase activity
Preincubation solution	Assay solution	(n-mole/mg ghosts per hr)
0	0	168·2 ± 5·7
0	4·7	48·7 ± 7·9
0	0·2	155·3 ± 4·4
4·5	4·7	62·9 ± 10·1
4·5	0·2	153·5 ± 7·2

Ghosts were preincubated for 1 hr at 37 °C in a solution containing the amounts of oligomycin indicated and (mM): ATP 1·87, Na 87, K 22, Mg 1·95 pH 7·4 at 37 °C. Aliquots (0·1 ml.) of the suspension were then distributed to 2·1 ml. of a solution which contained (mM) ATP 1·88, Na 91, K 23, Tris 22, EDTA 0·90, DTT 0·93 and Mg 2·97, pH 7·4 at 37 °C. Oligomycin was added to result in the final concentrations listed in the second column. The suspensions were incubated for 15 min at 37 °C and the amount of ADP produced estimated. The mean and S.E. of the mean are given (n = 6).

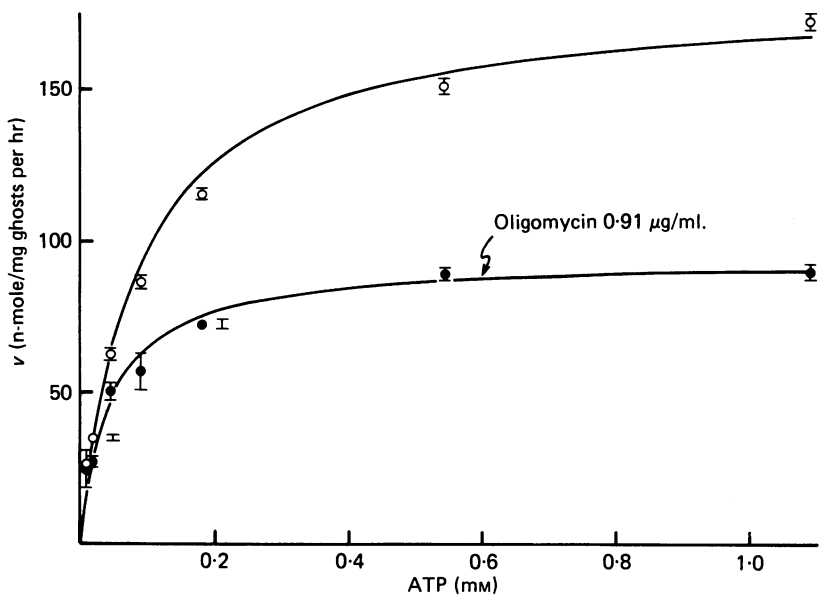


Fig. 1. Ouabain-sensitive ATPase activity (n-mole/mg ghosts per hr) *vs.* ATP concentration. The solutions contained (mM) Na 87, K 22, Tris 22, EDTA 0·91, DTT 0·94 and Mg 1·23 mM greater than the sum of the EDTA and ATP concentrations. The measurements were made in solutions containing 0·91 μg/ml. oligomycin and in oligomycin-free solutions. The curves are drawn to a Michaelis–Menten equation, $v = V_m S/(K_m + S)$, with V_m 180·5 n-mole/mg ghosts per hr and K_m 0·087 mM for the measurements made in inhibitor-free solutions and V_m 94·3 and K_m 0·046 for the measurements made in the presence of oligomycin. The mean and S.E. of the mean are given (n = 4).

concentration is rapidly reduced by dilution. Oligomycin inhibition is apparently readily reversible.

Oligomycin combines preferentially with a form E_1P-Na

It has long been known that oligomycin inhibits ATPase activity without inhibiting either the Na-dependent phosphorylation (Whittam, Wheeler & Blake, 1964) or the Na dependent ATP–ADP exchange reaction catalysed by the enzyme (Fahn, Koval & Albers, 1966). It has, therefore, been proposed that oligomycin stabilizes

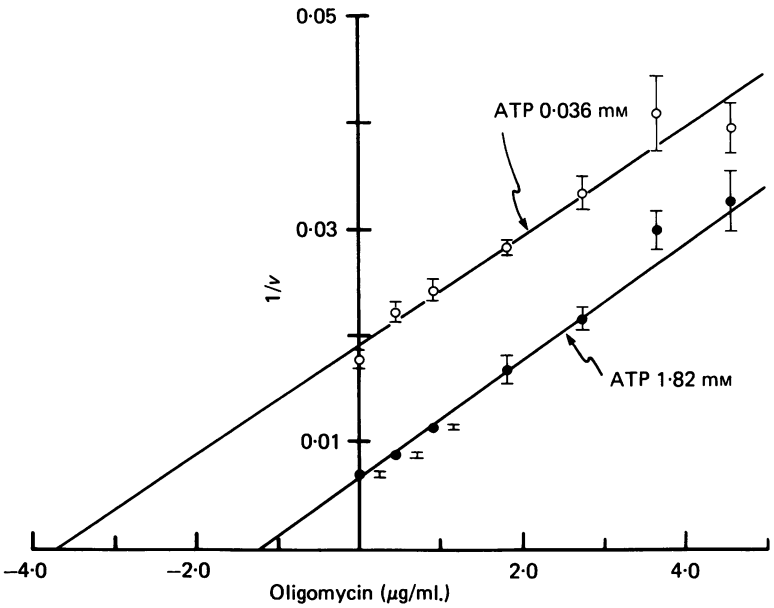


Fig. 2. Reciprocal of the ouabain-sensitive K influx *vs.* oligomycin concentration (μg/ml.). The solutions contained (mM) Na 87, K 22, Tris 22, EDTA 0·91, DTT 0·94 and Mg 1·31 mM greater than the sum of the EDTA and ATP concentrations. The measurements were made at the concentrations of ATP recorded. The lines are: $1/v = 0{\cdot}019 + 5{\cdot}14$ (oligomycin) for the measurements at low ATP concentration and $1/v = 0{\cdot}0066 + 5{\cdot}50$ (oligomycin) for the experiment at high ATP concentration. The mean and S.E. of the mean are given ($n = 4$).

the E_1P form of the enzyme. Oligomycin inhibition should then be uncompetitive with respect to both inside Na and ATP since the two substrates add before the appearance of E_1P and are necessary for its formation. Oligomycin inhibition has in fact been reported to be uncompetitive with respect to Na and ATP in microsomal preparations (Inturrisi & Titus, 1968; Robinson, 1971).

Fig. 1 is a plot of the ouabain-sensitive ATPase activity of broken membranes as a function of the ATP concentration; the measurements were made with and without oligomycin. The inhibitor reduced both V_m and K_m by about 50%. Fig. 2 is a Dixon plot of the reciprocal of the ouabain-sensitive ATPase activity *vs.* the concentration of oligomycin. The lines which describe the measurements at the two concentrations of ATP are parallel. Oligomycin inhibition is uncompetitive with ATP in human red cell membranes as in other ATPase preparations.

Fig. 3 is a plot of an experiment in which the ouabain-sensitive Na outflux was measured as a function of intracellular Na concentration in solutions with and without oligomycin 1 μg/ml. The curves are plots of

$$v = \frac{V_m}{\left(1+\frac{K_s}{Na_c}\right)^3 + \frac{I}{K_I}}, \quad (1)$$

which was derived by assuming that intracellular Na (Na_c) is at equilibrium with three identical and non-interacting sites each with a dissociation constant K_s, that

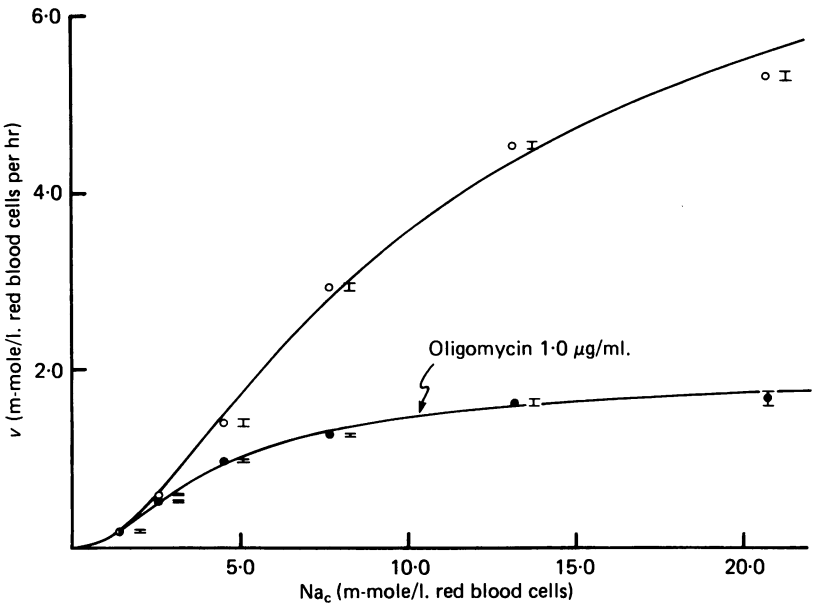


Fig. 3. Ouabain-sensitive Na outflux (m-mole/l. red blood cells per hr) *vs.* internal Na concentration (m-mole/red blood cells). The average internal K concentration was 50·0 m-mole/l. red blood cells, external K 16 mM and external Na 0. The curves are drawn to eqn. (1). The value of V_m was 9·16 m-mole/l. red blood cells per hr and K_s 3·72 m-mole/l. red blood cells for both curves, and K_I was 0·277 μg/ml. for the experiment in the presence of oligomycin, 1 μg/ml. The mean and S.E. of the mean are given (n = 4).

all three sites must be occupied before transport occurs, and that the inhibitor (I) combines with a dissociation constant K_I only with the form of the pump which has bound three Na ions (i.e. inhibition is uncompetitive with respect to Na_c). Fig. 4 is a Dixon plot at two intracellular Na concentrations; the lines do not intersect and, in fact, are approximately parallel. As expected, oligomycin inhibition is uncompetitive with respect to intracellular Na.

Oligomycin has been shown to inhibit the ouabain-sensitive Na–Na exchange of human red cells (Garrahan & Glynn, 1967*b*). Since outside Na is a substrate for the Na–Na exchange, we performed experiments to determine whether inhibition of the exchange by oligomycin is competitive, uncompetitive or non-competitive with respect to outside Na. Fig. 5 shows the results of an experiment in which the Na–Na exchange was measured as a function of the external Na concentration in solutions

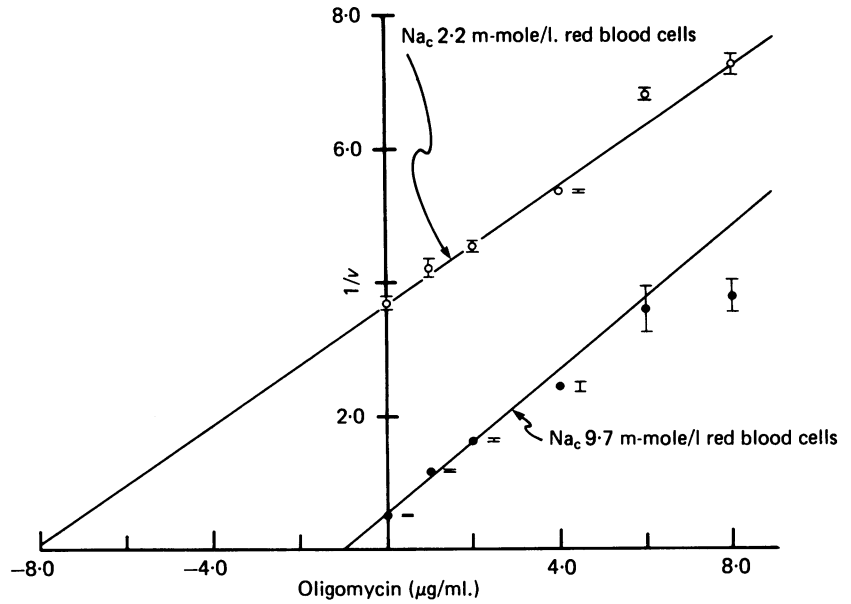


Fig. 4. Reciprocal of the ouabain-sensitive Na outflux *vs.* oligomycin concentration (μg/ml.) at the two intracellular Na concentrations indicated. The internal K concentration was 47·6 m-mole/l. red blood cells, external K was 16 mM and external Na 0. The lines are $1/v = 1{\cdot}82 + 223{\cdot}6$ (oligomycin) for the experiment at low internal Na and $1/v = 0{\cdot}27 + 269{\cdot}5$ (oligomycin) for the experiment at high internal Na. The mean and S.E. of the mean are given ($n = 4$).

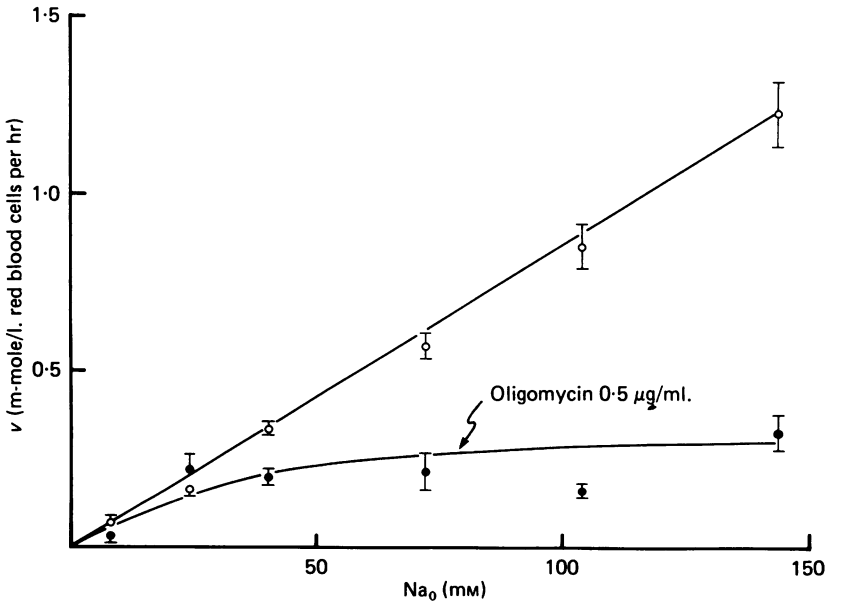


Fig. 5. Ouabain-sensitive Na outflux (m-mole/l. red blood cells per hr) *vs.* external Na concentration (mM). Internal Na concentration was 20·1 m-mole/l. red blood cells, internal K 0·36 m-mole/l. red blood cells and external K 0. The curves were drawn by eye. The mean and S.E. of the mean are given.

with and without oligomycin. Without oligomycin there is no indication of either the V_m or $K_{\frac{1}{2}}$ for outside Na; Garay & Garrahan (1973) have shown that the $K_{\frac{1}{2}}$ for external Na as a substrate for the Na–Na exchange is about 120 mM. In solutions with oligomycin the curve relating Na–Na exchange to outside Na is now saturable, and therefore oligomycin must reduce both V_m and $K_{\frac{1}{2}}$. Fig. 6 shows the results of an experiment in which the ouabain-sensitive Na outflux was measured in high Na

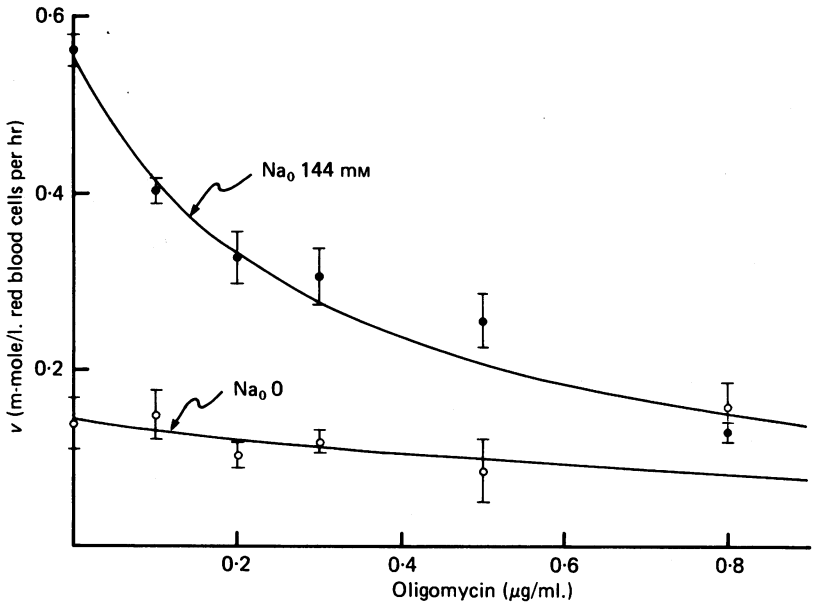
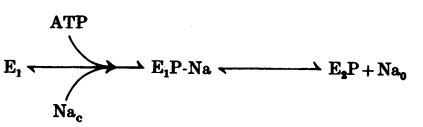


Fig. 6. Ouabain-sensitive Na outflux (m-mole/l. red blood cells per hr) *vs.* oligomycin concentration (μg/ml.). Internal Na was 18·1 m-mole/l. red blood cells, internal K 0·35 m-mole/l. red blood cells, external K 0, and external Na as recorded. The curve for the experiment at high Na concentration is $v = (1{\cdot}80 + 6032\ (\text{oligomycin}))^{-1}$ and the curve at zero outside Na is $v = (6{\cdot}89 + 6503\ (\text{oligomycin}))^{-1}$. The mean and S.E. of the mean are given.

solutions and in Na-free solutions at varying concentrations of oligomycin. The initial slope of the curve is much steeper when the measurements were made at high external Na than when they were made in Na-free solutions which is characteristic of uncompetitive inhibition. Oligomycin inhibition of the Na–Na exchange is therefore at least partially uncompetitive with respect to outside Na. Oligomycin inhibition is uncompetitive with inside Na and ATP, and at least partially uncompetitive with respect to outside Na; therefore some intermediate whose steady state level is increased by the three substrates preferentially binds the inhibitor. In the sequence



where Na_c is inside Na and Na_0 outside Na we conclude that oligomycin combines preferentially with the form E_1P-Na which occurs after the addition of Na_c and ATP to E_1 and after the addition of Na_0 to E_2P. In addition to fitting the kinetic data

such a hypothesis allows one to understand how oligomycin inhibits the Na–Na exchange while at the same time stimulating the Na-dependent ATP–ADP exchange. By combining with E_1P-Na oligomycin prevents the conversion of a form which is in the direct pathway of Na–Na exchange but not in the direct pathway of the ATP–ADP exchange which involves intermediates to the left of the form E_1P-Na.

TABLE 2. Oligomycin inhibition of the K–K exchange

Intracellular cation concentration (m-mole/l. red blood cells)	Ouabain-sensitive K outflux (m-mole/l. red blood cells per hr)	
		+ Oligomycin
Na 0·86, K 12·1	0·525 ± 0·073	0·500 ± 0·076
Na 3·7, K 12·8	0·530 ± 0·027	0·108 ± 0·051

Cells were prepared containing the intracellular Na and K concentrations indicated, and the ouabain sensitive K outflux was measured in solutions containing 16 mM-K and no Na. Oligomycin, when present, was 20 μg/ml. The mean and S.E. of the mean are given (n = 4).

The experiment summarized in Table 2 indicates that oligomycin does not significantly combine with E_2P-K. The experiment was designed to determine the oligomycin sensitivity of the K–K exchange which almost certainly involves the E_2P form of the enzyme (Simons, 1974). In Na-free cells oligomycin at a concentration much greater than that necessary to maximally inhibit the Na–K exchange has almost no effect on the K–K exchange; apparently oligomycin does not combine with E_2P-K. Under other circumstances oligomycin is capable of inhibiting the K–K exchange; if the cells contain a small amount of Na (an amount which, by itself, does not inhibit the exchange) oligomycin produces marked inhibition. The small amount of Na present within the cells permits the formation of E_1P-Na which is then stabilized by combination with oligomycin; as a result the amount of E_2P available for the K–K exchange is markedly reduced. A somewhat similar observation has been reported by Askari & Koyal (1968) who studied the K-stimulated *p*-nitrophenylphosphatase activity demonstrated by ATPase preparations. In the absence of Na and ATP oligomycin had little effect on the phosphatase activity; when Na and ATP were present together the phosphatase activity was stimulated and oligomycin caused significant inhibition.

The findings so far presented support the hypothesis that oligomycin preferentially combines with a form E_1P-Na and not with E_2P-K.

Oligomycin inhibition and external K

Oligomycin inhibition has been reported to be non-competitive with K in microsomal preparations (Inturrisi & Titus, 1968; Robinson, 1971). We have found that oligomycin inhibition is non-competitive with respect to outside K_0 in high Na solutions and mostly uncompetitive in Na-free solutions.

Fig. 7 is a plot of the ouabain-sensitive Na outflux *vs.* external K concentration in solutions with and without 0·4 μg/ml. oligomycin; the measurements were made in high Na solutions. The curves are drawn to the equation

$$v = \frac{V_m}{\left(1+\frac{K_s}{K_o}\right)^2\left(1+\frac{I}{K_I}\right)} \quad (2)$$

which was derived by assuming that outside K combines at two identical and non-interacting sites, that both sites must be filled before transport occurs, and that the inhibitor I combines with all forms of the enzyme (with and without bound K) with equal affinity and with dissociation constant K_I (i.e. inhibition by I is non-competi-

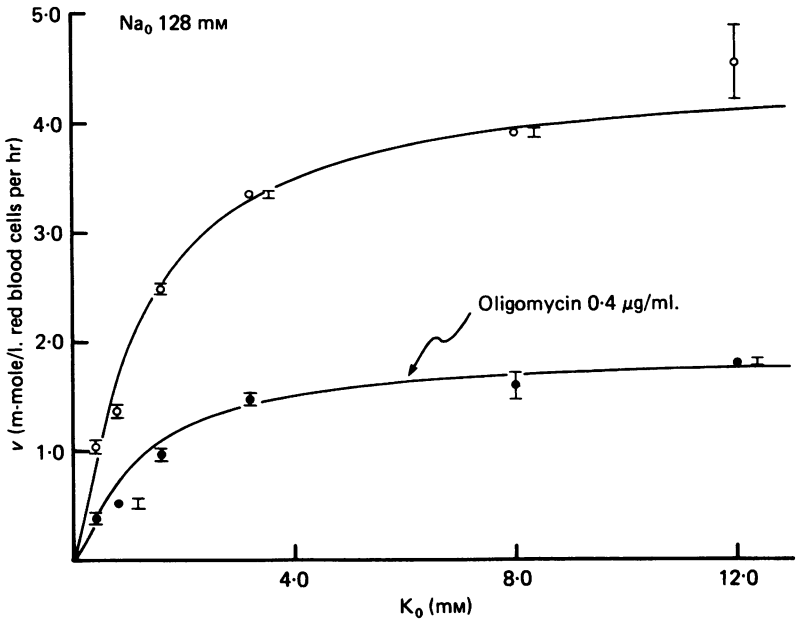


Fig. 7. Ouabain-sensitive Na outflux (m-mole/l. red blood cells per hr) *vs.* external K concentration (mM). The cells contained 19·6 m-mole/l. red blood cells Na and 0·29 m-mole/l. red blood cells K. External Na was 128 mM. The curves are drawn to eqn. (2). V_m 4·49 m-mole/l. red blood cells per hr and K_s 0·54 mM for both curves. K_I was 0·303 μg/ml. for the experiment with 0·4 μg/ml. oligomycin. The mean and S.E. of the mean are given (n = 4).

tive with respect to K_0). Fig. 8 is a Dixon plot at two external K concentrations in high Na solutions; the lines intersect on the I axis. Oligomycin inhibition is non-competitive with respect to outside K when measurements are made at high external Na in agreement with the findings using microsomal preparations.

The experiments were then repeated in solutions free of Na. Fig. 9 is a plot of the ouabain-sensitive Na outflux as a function of the external K concentration in Na-free solutions. In the absence of Na the relation between the pump rate and the external K concentration is adequately described by a Michaelis–Menten equation. Both V_m and K_m are reduced by oligomycin, V_m to a slightly greater extent than K_m. Fig. 10 is a Dixon plot at two external K concentrations in Na-free solutions. The lines are nearly parallel. In the absence of external Na, then, oligomycin inhibition becomes mostly uncompetitive with outside K.

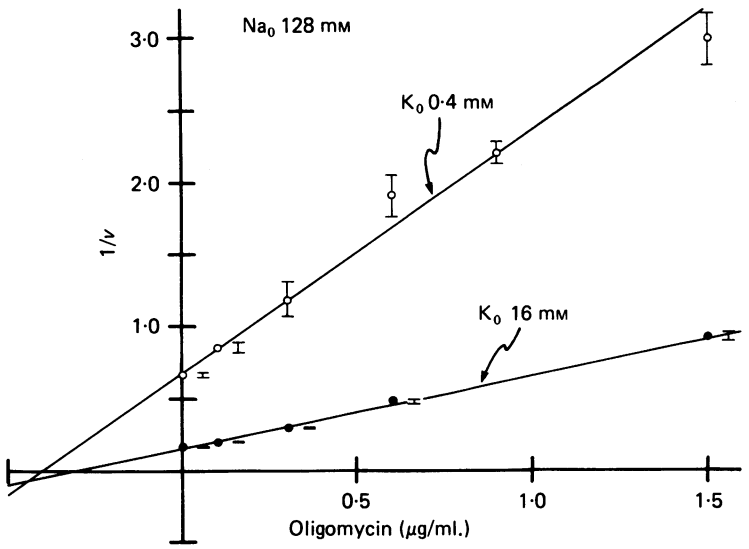


Fig. 8. Reciprocal of the ouabain-sensitive Na outflux *vs.* oligomycin concentration (μg/ml.) at two external K concentrations. The cells contained 19·8 m-mole/l. red blood cells Na and 0·68 m-mole/l. red blood cells K. External Na was 128 mM and external K was as recorded. The lines are $1/v = 0{\cdot}67 + 1689$ (oligomycin) for the experiment at low external K and $1/v = 0{\cdot}15 + 507$ (oligomycin) for the experiment at high external K. The mean and S.E. of the mean are given ($n = 4$).

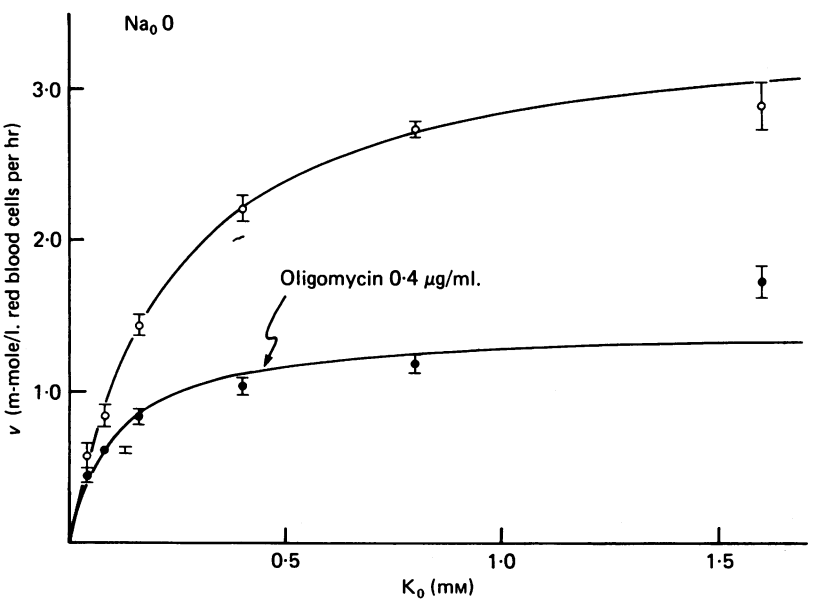


Fig. 9. Ouabain-sensitive Na outflux (m-mole/l. red blood cells per hr) *vs.* external K concentration (mM). Internal Na concentration was 29·7 m-mole/l. red blood cells and internal K concentration 0·25 m-mole/l. red blood cells. The solutions were Na-free. The curves are drawn to a Michaelis–Menten equation, $v = V_m S/(K_m + S)$, with V_m 3·47 m-mole/l. red blood cells per hr and K_m 0·228 mM for the experiment without inhibitor and V_m 1·42 m-mole/l. red blood cells per hr and K_m 0·106 mM for the experiment with oligomycin, 0·4 μg/ml. The mean and S.E. of the mean are given ($n = 4$).

Clearly, interaction of Na at some external site must be responsible for changing oligomycin from an uncompetitive inhibitor with respect to outside K to a non-competitive inhibitor. There appear to be several sites at which outside Na can interact: a high affinity site (with a dissociation constant of less than 1 mM) at which outside Na inhibits the pump rate even in the presence of outside K (Cavieres & Ellory, 1975; Hobbs & Dunham, 1976; Sachs, 1977*a*, *b*); the site for outside K at which Na

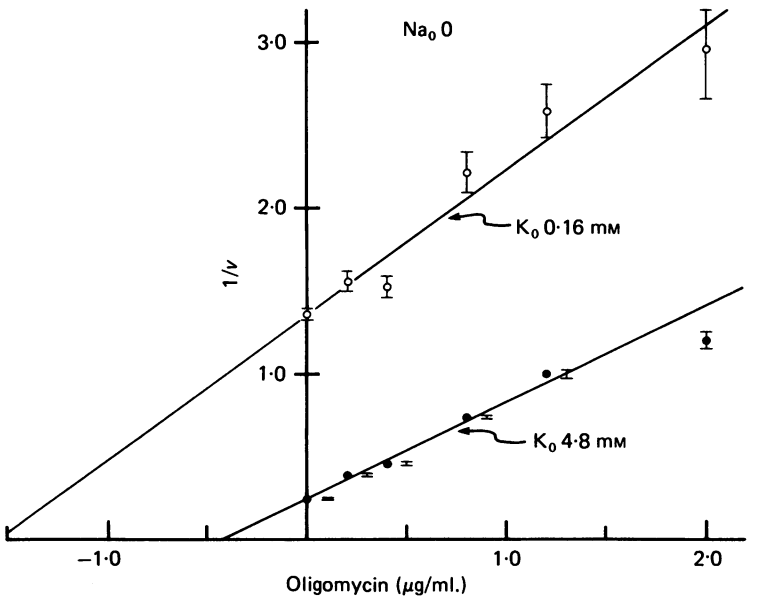


Fig. 10. Reciprocal of the ouabain-sensitive Na outflux *vs.* oligomycin concentration (μg/ml.). Internal Na concentration was 21·0 m-mole/l. red blood cells and internal K 0·36 m-mole/l. red blood cells. The solutions were Na-free. The lines are $1/v = 1{\cdot}35 + 876$ (oligomycin) for the experiment at low external K concentration and $1/v = 0{\cdot}25 + 588$ (oligomycin) for the experiment at high K. The mean and S.E. of the mean are given ($n = 4$).

competes with a dissociation constant of about 9 mM (Sachs, 1977*a*, *b*); and the Na discharge sites which have a dissociation constant for Na of about 30 mM as judged by the effect of outside Na on the Na–Na exchange (Garay & Garrahan, 1973). In order to determine whether Na interacting at either of the higher affinity sites is responsible for the alteration of oligomycin inhibition, the experiment recorded in Fig. 11 was performed. Dixon plots at two external K concentrations in solutions containing 9·6 mM-Na (sufficient to saturate the highest affinity sites for Na and to half saturate the sites at which Na competes with K) are shown. The lines are approximately parallel and in fact the ratio of the slopes of the lines in Fig. 11 is about the same as the ratio in Fig. 10; 9·6 mM-Na has no discernible effect on the type of inhibition with respect to K produced by oligomycin. It is not Na acting at either of the two higher affinity sites, but Na acting at low affinity sites, which alters the type of inhibition produced by oligomycin.

Oligomycin inhibition is non-competitive with respect to outside K when the measurements are made in solutions high in Na. Therefore, according to the rules

proposed by Cleland (1963*b*), the inhibitor must add to an enzyme form which occurs in the reaction sequence before the enzyme form to which K adds, and the two forms must be reversibly connected when outside Na is high. When external Na is removed, inhibition becomes uncompetitive with respect to outside K; removal of Na eliminates the reversible connection between the enzyme form to which the inhibitor adds and

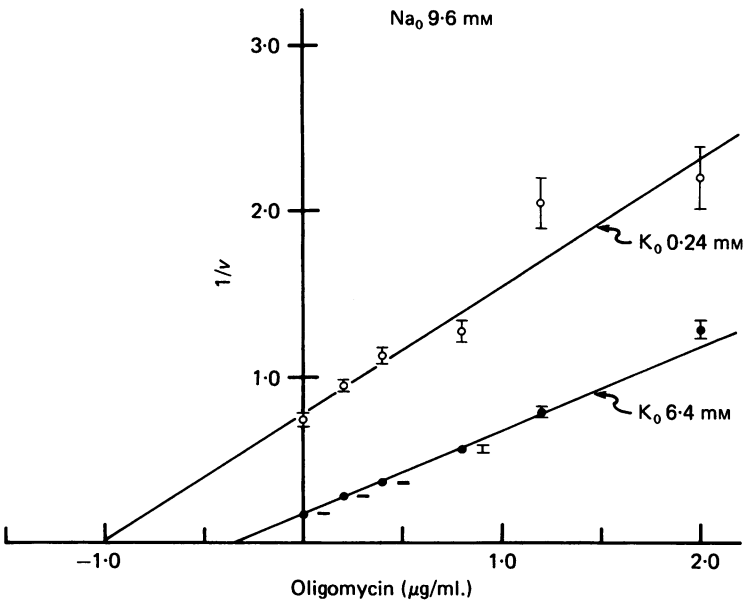
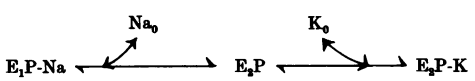


Fig. 11. Reciprocal of the ouabain-sensitive Na outflux *vs.* oligomycin concentration (μg/ml.). Internal Na concentration was 19·0 m-mole/l. red blood cells and internal K 0·58 m-mole/l. red blood cells. External Na was 9·6 mM. The lines are $1/v = 0{\cdot}78 + 769$ (oligomycin) for the experiment at low external K and $1/v = 0{\cdot}18 + 503$ (oligomycin) for the experiment at high K. The mean and S.E. of the mean are given.

the form to which K adds. In this case, irreversibility results from the reduction of the concentration of a product of the reaction to zero, and therefore it must be concluded that K combines after Na is released to the outside:



When outside Na is zero the step between E_1P-Na, to which oligomycin binds, and E_2P, to which outside K adds, becomes irreversible.

It is generally believed that when the pump is carrying out Na–K exchange K combines with the E_2P form of the pump and that it combines with the same form when the pump is carrying out K–K exchange. The experiment recorded in Fig. 12 demonstrates that the apparent K_m for external K for the two processes differs. In this experiment the ouabain-sensitive Na outflux and the ouabain-sensitive K outflux were simultaneously measured in the same batch of cells at varying external K concentrations. The apparent K_m for the activation of the Na outflux is less than a quarter of the apparent K_m for activation of the K outflux. The experiment does not

necessarily mean that the form with which K combines when it promotes K–K exchange differs from the form with which it combines when it promotes Na–K exchange (although the experiment does not exclude this). The apparent K_m probably is not a simple equilibrium binding constant for K in either case, but is more likely a composite constant which includes rate constants for steps in the pathways in addition to the steps which lead to the binding of K; since the pathways for the two

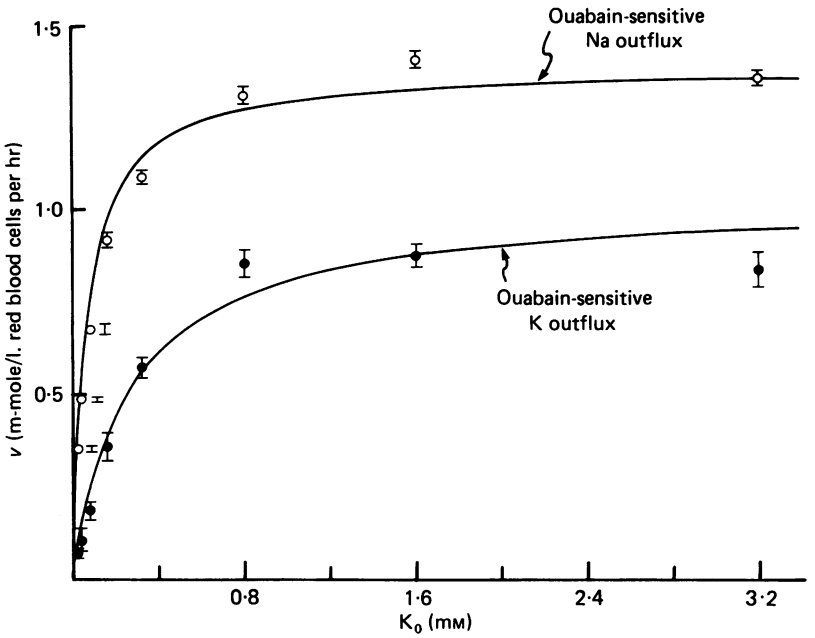


Fig. 12. Ouabain-sensitive Na outflux (O) and ouabain sensitive K outflux (●) (m-mole/l. red blood cells per hr) *vs.* the extracellular K concentration (mM). The cells contained 3·64 m-mole/l. red blood cells Na and 22·4 m-mole/l. red blood cells K. The solutions were Na-free. The curves were drawn to a Michaelis–Menten equation with V_m 1·3852 m-mole/l. red blood cells per hr and K_m 0·0704 mM for the Na outflux measurement and V_m 1·0297 and K_m 0·2785 for the K outflux experiment. The mean and S.E. of the mean are given (n = 4).

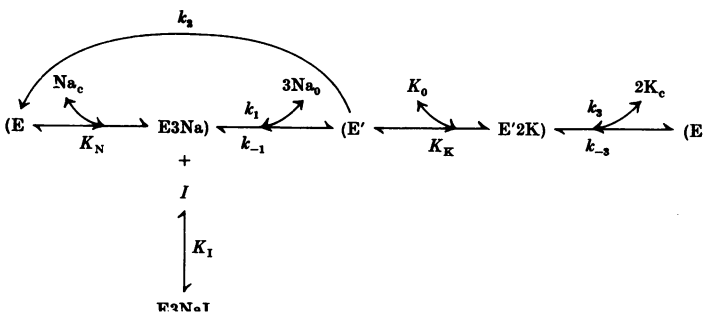
exchanges differ it is not surprising that the apparent K_m for K also differs. The experiment does demonstrate, however, that the form of the relation between pump activity and external K concentration is not invariant, but depends on the pathway by which activity is measured. It should be pointed out that Glynn, Lew & Lüthi (1970) reported that when measured in a solution with a low Na concentration the apparent K_m for K when it activated the K outflux was about the same as the apparent K_m for K when it activated the Na outflux (measured in separate experiments). The apparent agreement probably was fortuitous since the cells were likely to have contained a high concentration of Na which resulted in a high apparent K_m for K when the Na outflux was measured (Sachs, 1977*b*). In the present experiment the intracellular Na concentration was low so that the apparent K_m for K was low when the Na outflux was measured and the difference in the K_m for the two processes became apparent.

DISCUSSION

These experiments were performed in an attempt to clarify the mechanism of the Na pump as reflected in steady-state kinetics. Although the existence of two major conformations of the pump and the presence of an Na–Na exchange, a K–K exchange, an ATP–ADP exchange and a phosphate–water exchange lead to the conclusion that the pump must be ping-pong, steady-state kinetic evaluations are consistent with a simultaneous mechanism. If the pump mechanism is simultaneous, then at some point in the cycle both ions must be bound to the pump at the same time; either K must combine with the pump at the outside after Na has bound at the inside and before it is released to the outside, or Na must combine at the inside after K has bound at the outside and before it is released to the inside. The argument presented above that outside K can add after Na is released is based on Cleland's (1963*b*) rules which predict the type of inhibition which will be displayed with regard to a particular substrate based on the relation within the reaction sequence between the enzyme form with which the inhibitor combines and the form with which the substrate combines.

The experimental findings are that oligomycin combines preferentially with the enzyme form E_1P-Na and that oligomycin is a non-competitive inhibitor with respect to outside K when the solution contains Na (it does not affect the apparent $K_{\frac{1}{2}}$ for K but reduces the apparent V_m), but when the solutions are Na free oligomycin inhibition is uncompetitive with respect to K (it reduces both the apparent $K_{\frac{1}{2}}$ for K and the apparent V_m). The circumstances under which an inhibitor is uncompetitive with respect to a substrate can be seen intuitively as follows: the apparent $K_{\frac{1}{2}}$ for a substrate is decreased by an inhibitor if combination of the substrate with the enzyme is necessary for the appearance of the enzyme form which combines with the inhibitor; as the concentration of the substrate is increased the steady-state concentration of the enzyme form which combines with the inhibitor increases. Therefore inhibition increases as the concentration of substrate is increased and the apparent $K_{\frac{1}{2}}$ for the substrate decreases. In the absence of outside Na formation of E_1P-Na occurs only after the addition of outside K and therefore oligomycin inhibition is uncompetitive with outside K. On the other hand when the solution contains Na, E_1P-Na can be formed either after addition of K to E_2P or by addition of Na to E_2P. Formation of E_1P-Na, the form to which oligomycin binds, will therefore be independent of outside K and the inhibitor does not change the apparent $K_{\frac{1}{2}}$ for the substrate.

A ping-pong mechanism for the Na pump in which oligomycin (I) combines with the enzyme form which occurs after the addition of Na at the inside and before its release to the outside can be represented as:



which is similar to a ping-pong model which was discussed in an earlier publication (Sachs, 1977*b*) except for the pathway characterized by the rate constant k_2 the significance of which will be discussed below. The model assumes that inside Na (Na_c) rapidly equilibrates at three non-interacting sites each with the dissociation constant K_N; all three sites must be filled before Na crosses the membrane and is released at the outside with formation of E′. Outside K then rapidly equilibrates at two non-interacting sites on E′ each with a dissociation constant K_K. Both must be filled before K crosses the membrane and is released at the inside with formation of E and the beginning of a new cycle (this assumption is a simplification since there is kinetic evidence that the pump with a single bound K ion can transport the ion across the membrane (Sachs, 1977*a*; Kropp & Sachs, 1977), but the simplification does not alter the nature of the conclusions drawn below). The pathway from E′ to E with rate constant k_2 represents the movement of Na sites without bound Na from the outside to the inside and therefore accounts for the uncoupled Na outflux which occurs in solutions free of Na and K (Glynn & Karlish, 1976). E combines with Na at the inside, Na crosses to the outside and is released with formation of E′ and E′ then reverts to the form E which can again combine with inside Na. I is assumed to combine with E3Na which occurs after the combination of inside Na with the enzyme form E and before the release of Na to the outside.

Rate equations for this mechanism and for the mechanisms described below were derived by the method of Cha (1968). If Na_c is saturating and K_c, Na_0 and k_2 are zero, the mechanism yields the rate equation

$$v = \frac{V_m}{\frac{k_1}{k_3}\left(\frac{K_K}{K}+1\right)^2\left(1+\frac{I}{K_I}\right)}. \tag{3}$$

In the absence of external Na, the relation between the pump rate and the external K concentration is adequately described by a rectangular hyperbola so that eqn. (3) can be written

$$v = \frac{\text{app } V_m}{\frac{\text{app } K_m}{K}+1}$$

where app $V_m = V_m/(1+k_1/k_3+I/K_I)$ and app $K_K = (k_1/k_3)\,K_K/(1+k_1/k_3+I/K_I)$. As the concentration of I increases both app V_m and app K_K are reduced by the same proportion. The reciprocal of eqn. (3)

$$\frac{1}{v} = \frac{1}{V_m}\left(\frac{k_1}{k_3}\left(\frac{K_K}{K}+1\right)^2+\left(1+\frac{I}{K_I}\right)\right)$$

shows that a plot of the reciprocal of the pump rate against the concentration of inhibitor is independent of the external K concentration; Dixon plots at different outside K concentrations are parallel.

On the other hand, when the concentration of external Na is significant, the relation between the pump rate and the external K concentration can be written

$$v = \frac{V_m}{\frac{k_1}{k_3}\left(\frac{K_K}{K}+1\right)^2+\left(1+\frac{I}{K_I}\right)\left(\left(\frac{k_{-1}}{k_3}Na_0+\frac{k_2}{k_3}\right)\left(\frac{K_K}{K}\right)^2+1\right)}. \tag{4}$$

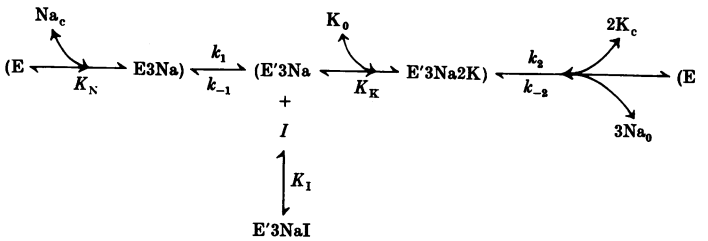
In this case the term containing I is multiplied by a term containing $(K_K/K)^2$ and is therefore similar to eqn. (2) which describes non-competitive inhibition. The reciprocal plot is

$$\frac{1}{v} = \frac{1}{V_m}\left(\frac{k_1}{k_3}\left(\frac{K_K}{K}+1\right)^2 + \left(1+\frac{I}{K_I}\right)\left(\left(\frac{k_{-1}}{k_3}Na_0 + \frac{k_2}{k_3}\right)\left(\frac{K_K}{K}\right)^2 + 1\right)\right)$$

which shows that the slope of a plot of the reciprocal of the pump rate against the inhibitor concentration will decrease as external K increases since the term containing I is multiplied by a term in $(K_K/K)^2$; Dixon plots at different external K concentrations will intersect to the left of the $1/v$ axis.

The experimental findings were that oligomycin inhibition is non-competitive with external K when the solutions were high in Na and almost uncompetitive in the absence of external Na. The fact that competition is not completely uncompetitive when Na is missing results from a non-zero k_2; even in the absence of outside Na there is a small term in $(K_K/K)^2$ multiplying the term containing I. The model fits the experimental findings in detail.

The simultaneous model may be represented as



The model differs from the ping-pong model in that it is assumed that outside K adds before Na is released to the outside. It is assumed that the inhibitor binds to the form E'3Na which represents the conformation which exists after Na has been transported to the outside and before K adds, but the argument is not altered if it is assumed that I adds to E3Na. The other assumptions made in deriving the rate law are the same as described above for the derivation of the ping-pong model. If K_c is zero and Na_c is saturating

$$v = \frac{V_m}{\frac{k_1}{k_2}\left(\frac{K_K}{K}+1\right)^2 + \left(1+\frac{I}{K_I}\frac{k_1}{k_{-1}K_K}\right)\frac{k_{-1}}{k_2}\left(\frac{K_K}{K}\right)^2 + 1}. \qquad (5)$$

Eqn. (5) does not contain a term in Na_0 and therefore it is apparent that outside Na will have no effect on the type of inhibition with respect to K which is observed; since the equation contains a term in which the inhibitor concentration is multiplied by $(K_K/K)^2$ inhibition will be non-competitive with respect to K whether or not the solutions contain Na. The reciprocal of eqn. (5)

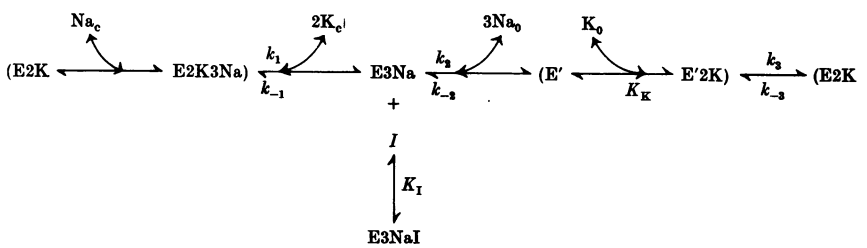
$$\frac{1}{v} = \frac{1}{V_m}\left(\frac{k_1}{k_2}\left(\frac{K_K}{K}+1\right)^2 + \left(1+\frac{I}{K_I}\frac{k_1}{k_{-1}K_K}\right)\frac{k_{-1}}{k_2}\left(\frac{K_K}{K}\right)^2 + 1\right.$$

shows that the slope of Dixon plots vary with the K concentration and outside Na is without effect. Since the simultaneous model does not predict that the type of

inhibition with respect to K depends on whether or not the solutions contain Na, it does not fit the experimental data.

The ping-pong model satisfactorily accounts for the experimental findings described in this paper. However, we previously measured the apparent $K_{\frac{1}{2}}$ and apparent V_m for Cs and K in cells with different fixed internal Na concentrations and found that the relation between the ratio app V_m/app $K_{\frac{1}{2}}$ and the internal Na concentration was adequately described by the simultaneous model but not by a ping-pong model (Sachs, 1977*b*). The ping-pong model which was tested differed from the one described above in that it did not contain the pathway characterized by the rate constant k_2, i.e. the pathway which accounts for the uncoupled Na outflux. Recently we refit the data previously reported to a rate equation derived from a ping-pong model similar to that described above in that it had the pathway for the uncoupled Na outflux (Sachs, 1979). The fit was excellent. When the parameters which were obtained from the curve fitting technique were used to calculate the magnitude of the uncoupled Na outflux which would occur in the absence of outside Na and K, the values obtained were somewhat higher than those found experimentally, but nevertheless satisfactory considering that the parameters were obtained from measurements of the Na–K exchange in solutions containing K. The modified ping-pong model (modified to include a pathway which accounts for the uncoupled Na outflux) adequately describes both the data described here and that reported in our previous publication.

It would be satisfying to end the discussion here, but unfortunately it must be admitted that it is also possible to define a simultaneous mechanism which fits both the data presented here and those reported in our previous publication. If it is assumed that inside Na adds before K is released to the inside, the following mechanism may be defined

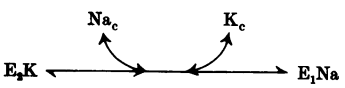


If it is further assumed that K_c is zero and Na_c is saturating, the model results in the equation

$$v = \frac{V_m}{\frac{k_1}{k_3}\left(\frac{K_K}{K}+1\right)^2 + \frac{k_1}{k_3}\left(1+\frac{I}{K_I}\right)\left(1+\frac{k_{-2}}{k_3}\left(\frac{K_K}{K}\right)^2 Na_0\right) + \frac{k_1}{k_3}}. \tag{6}$$

The term containing I is multiplied by a term in $(K_K/K)^2$ only if Na_0 is not zero. The assumptions made in formulating this model are similar to those discussed above except that it is assumed that internal Na combines with an enzyme form with K bound and that K is released to the inside only after the addition of Na. I adds to the form with Na bound and with the bound Na exchangeable with outside Na. The movement of K across the membrane takes place at the step in which E'2K is conver-

ted to E2K. This model predicts that oligomycin inhibition is non-competitive with respect to outside K if external Na is present but uncompetitive in Na free solutions. The reciprocal of eqn. (6) predicts that the slopes of Dixon plots decrease with increasing outside K if the solution contains Na but are independent of outside K in Na free solutions. The model fits the experimental findings and therefore represents a form of simultaneous model (since at one point in the cycle Na and K are bound to the pump at the same time) which conforms to the restriction that Na is released to the outside before K adds. Garrahan & Garay (1976) discussed a model similar to this which they attributed to R. L. Post. As they pointed out, the model seems to predict that either external or internal K must be present in order to demonstrate the Na–Na exchange and the uncoupled Na outflux; in fact, both of these fluxes are inhibited by outside K (Garrahan & Glynn, 1967*b*; Sachs, 1970) and are easily demonstrated in the absence of internal K (Garay & Garrahan, 1973; Glynn & Karlish, 1976). It is possible that this model describes the major pathway of Na–K exchange and that Na–Na exchange occurs by an alternate pathway in which addition of Na to the pump does not requite that K be bound. The model also suggests that addition of Na at the inside promotes the release of K. There is clear evidence for the existence of a form of the enzyme in which K stabilizes the E_2 conformation (E_2K) and from which K is only slowly released (Post, Hegyvary & Kume, 1972). Release of K seems to be promoted by addition of ATP but not Na when the pathway is $E_2P \rightarrow E_2PK \rightarrow E_2K$, but when the enzyme is preincubated with cations before the addition of ATP Na seems to promote phosphorylation by displacing the equilibrium



to the right (Mardh, 1975). This model also seems to be consistent with the observation that intracellular K stimulates the pump rate in human (Garay & Garrahan, 1973) and in goat (Sachs *et al.* 1974) red cells. At any rate it is not possible to select on the basis of the evidence presented here or in our previous publication (Sachs, 1977*b*) between this simultaneous model and the modified ping-pong model; an unambiguous selection may result from evaluation of the kinetic characteristics of the Na–Na and K–K exchanges.

One further point deserves discussion. Garrahan & Garay (1976) have pointed out that in experiments which they have reported (Garay & Garrahan, 1973) the apparent $K_{\frac{1}{2}}$ for either inside Na or outside K seems to be invariant with the concentration of ions on the opposite side of the membrane and with the type of flux which is measured. From this they conclude that the pump mechanism must be of a limited class of simultaneous mechanisms. Other investigators (Chipperfield & Whittam, 1976; Sachs, 1977*b*) have reported that the apparent $K_{\frac{1}{2}}$ for some of the fluxes appears to be a function of the concentration of ions on the opposite side of the membrane. Part of the discrepancy probably is due to the difficulty of the experiments and the variation in methods. The experiment of Fig. 12 therefore seems to be particularly important since it demonstrates that the apparent $K_{\frac{1}{2}}$ for outside K when the Na–K exchange is measured differs from the apparent $K_{\frac{1}{2}}$ when the K–K exchange is measured even when the measurements are made in the same cells at the same time. The apparent $K_{\frac{1}{2}}$ for K at least is clearly not invariant.

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