Interaction of External K, Na, and 
Cardioactive Steroids with the 
Na-K Pump of the Human Red Blood Cell

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ABSTRACT The interaction of extracellular Na \( (Na_o) \), K \( (K_o) \), and strophanthidin with the Na-K pump of the human red blood cell has been investigated. Inhibition by submaximal concentrations of strophanthidin rapidly reaches a level which does not increase further over a relatively long period of time. Under these circumstances, it is possible to apply a steady-state kinetic analysis to the interaction of Na\(_o\), K\(_o\), and strophanthidin with the pump. In Na-free solutions, strophanthidin increases the apparent K\(_1/2\) of the pump for K\(_o\), but does not change the form of the relation between the reciprocal of the active K influx \( (\frac{M}{K}^{-1}) \) and the reciprocal of \([K_o] (\frac{1}{[K_o]})\); the relation both in the presence and absence of strophanthidin is adequately described by a straight line. In solutions containing Na, strophanthidin changes the form of the curve describing the relation between \( tM_{K}^{-1} \) vs. \([K_o]^{-1}\); the curve becomes more parabolic in solutions containing strophanthidin. The rate of ouabain binding to K-free cells has also been measured; in the absence of K\(_o\), the rate of binding is unaffected by Na\(_o\). The data are considered in terms of a simple kinetic model.

The findings can be explained if it is supposed that at low external K the form of the pump combined with one Na\(_o\) is more likely to combine with strophanthidin than is the uncombined form of the pump. The uncombined form of the pump is more likely to combine with K even at very low K\(_o\) than with strophanthidin.

INTRODUCTION

It has been known for a number of years that the cardiac glycosides and aglycones are specific inhibitors of the Na-K pump (Schatzman, 1953; Glynn, 1964). The most commonly used of these substances, ouabain, has been shown to act only from the outside of the squid axon (Caldwell and Keynes, 1959) and the aglycone strophanthidin acts only from the outside of reconstituted red blood cell ghosts (Hoffman, 1966). The interaction of cardioactive steroids with the pump is inhibited by extracellular K \( (K_o) \) (Schatzman,
1953; Glynn, 1957; Hoffman, 1966; Baker and Willis, 1970) and is promoted by extracellular Na (Na\textsubscript{o}) (Schatzman, 1965; Baker and Manil, 1968; Beauge and Adragna, 1971; Baker and Willis, 1972).

The effect of Na\textsubscript{o} can be accounted for by either of two mechanisms: (a) Na\textsubscript{o} competes with K\textsubscript{o} and so reduces the ability of K\textsubscript{o} to prevent inhibition of the pump by the cardioactive steroids (Beauge and Adragna, 1971) or (b) Na\textsubscript{o} reacts directly with the pump and so increases its susceptibility to inhibition independently of any competition between Na\textsubscript{o} and K\textsubscript{o} (Gardner and Conlon, 1972). The first mechanism seems reasonable since it is known that Na\textsubscript{o} competes with K\textsubscript{o} at the outer aspect of the membrane and inhibits pump activation by K\textsubscript{o} (Post et al., 1960; Garrahan and Glynn, 1967; Sachs, 1967); this mechanism has been proposed to explain the effect of Na\textsubscript{o} on the kinetics of ouabain inhibition of the Na-K pump of the human red blood cell (Beauge and Adragna, 1971). However, in squid axons Na\textsubscript{o} promotes the effect of ouabain even though K\textsubscript{o} is not very effective as an inhibitor of the ouabain effect (Baker and Manil, 1968; Baker and Willis, 1972); it seems unlikely that the effect of Na\textsubscript{o} in this preparation can be accounted for by the first mechanism.

This paper reports the results of studies designed to explore the mechanism by which Na\textsubscript{o} produces its effect. It seemed possible to obtain some information about the mechanism by determining the kinetic characteristics of the Na-K pump in human red blood cells in the presence of submaximal concentrations of cardioactive steroids. However, it is difficult to interpret kinetic experiments involving ouabain since the inhibition of the Na-K pump of the human red blood cell produced by ouabain and the amount of [\textsuperscript{3}H]-ouabain bound when red cells were exposed to submaximal concentrations of the drug increase with time, and the pump inhibition and ouabain binding are only very slowly reversible (Hoffman, 1969; Dunham and Hoffman, 1971). This kind of behavior does not fulfill the assumptions underlying the steady-state treatment of kinetic data. In contrast, Hoffman (1966) has reported that the inhibition produced by strophanthidin can be removed by washing the drug from the cells. It was found that the inhibition of the pump produced when cells are exposed to submaximal concentrations of strophanthidin is reversible and rapidly reaches a constant level which does not change over long periods of time. It is possible therefore to apply a steady-state kinetic formulation to such an interaction and such a formulation is presented in this paper.

METHODS

Venous blood was obtained from healthy donors and heparin was used as an anticoagulant. The cells were separated from the plasma by centrifugation and the plasma and buffy coat removed by aspiration. The cells were then washed three times in unbuffered isosmotic (107 mM) MgCl\textsubscript{2} solution and used as indicated.
Alteration of intracellular cation concentrations was accomplished by a modification of the PCMBS (parachloromercuribenzenesulfonic acid) method described by Garrahan and Rega (1967). Washed cells were suspended at about 5% hematocrit in a solution containing (mM): PO$_4$ 3.4, Mg$^{2+}$ 1.0, PCMBS 0.1, Cl$^{-}$ 147, glucose 10, Na$^{+}$ 62, and either choline$^{+}$ or K$^{+}$ 88. Sucrose 13 mM was added to all solutions. The suspensions were incubated at 4°C for 36 h, and the solutions changed every 12 h. At 36 h the cells were separated from the solutions and resuspended in a solution identical to the PCMBS solution except that PCMBS was omitted and dithiothreitol 2 mM, adenine 3 mM, and inosine 2 mM were included. The cells were incubated in this solution for 1 h at 37°C, separated from the solution, washed three times in MgCl$_2$ solution (107 mM), and used for the determination of K influx.

Unidirectional K influx was estimated as previously described (Sachs and Welt, 1967). Cells were suspended at about 5% hematocrit in solutions containing $^{42}$K. Samples were taken at 0.5 h and at either 1 h or 1.5 h after the start of the incubation, the cells separated from the solution, washed three times with ice-cold isosmotic MgCl$_2$ solution, hemolyzed in distilled water, and counted. The K influx was calculated from the amount of $^{42}$K taken up by the cells over the 0.5 or 1 h period and the specific activity of the solution.

The solutions in which the K influx was measured were basically glycylglycine buffered choline chloride solutions. All solutions were made up to an osmolality of 295 mosmol/kg water. Glycylglycine-Mg CO$_3$ buffer (glycylglycine 273 mM, MgCO$_3$ 54 mM, 295 mosmol/kg water, pH 7.4 at 37°C) comprised 10% by volume of all solutions, and bovine serum albumin was present at a concentration of 20 mg/100 ml solution. When NaCl or KCl was included in a solution, equal amounts of choline chloride were omitted. Strophanthidin was added as an ethanol solution, and equal amounts of ethanol were added to strophanthidin-free solutions. The maximum amount of ethanol present in any solution was 0.067 ml/100 ml. Purified choline chloride was obtained from Hoffman-Taff, Springfield, Mo. and was not further processed.

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

**Results and Discussion**

In most of the experiments reported in this paper, cells were used in which intracellular K ($K_i$) was reduced to low levels in order to reduce variations in $K_i$ over the course of the influx measurements since approximately constant $K_i$ simplifies the calculation of the data. Intracellular Na ($Na_i$) was maintained at concentrations higher than the apparent $K_{1/2}$ of the pump for Na$_e$ in order to minimize the effect of variations in the concentration of Na$_e$ over the course of the experiment. The remainder of the intracellular cation was made up of choline.

**Reversibility of Inhibition of Pump by Strophanthidin**

In order to treat a model of the interaction of strophanthidin with the Na-K pump by means of steady-state kinetics, it is necessary (a) that the interaction of the pump with strophanthidin is reversible and (b) that the inhibition of
the pump does not continuously increase with time of exposure to low concentrations of the steroid, but reaches some steady-state level at a finite time after exposure to the drug. Experiments were performed to determine whether these conditions are fulfilled.

If the interaction of strophanthidin with the pump is reversible, it should be possible to expose cells to the drug, wash the cells, and demonstrate that there is no residual inhibition of the pump after washing. Hoffman (1966) has previously reported that this is the case. An experiment designed to confirm this finding is summarized in Table I. Cells were separated into two batches; one

| TABLE I |
| REVERSIBILITY OF THE INHIBITION OF THE PUMP BY STROPHANTHIDIN |
| Influx solution | Preincubation solution |
|                 | Control | Strophanthidin 10^{-6} M |
| (nM RBC-h)       |         |                        |
| Control          | 0.391±0.010 | 0.383±0.006 |
| Strophanthidin 10^{-6} M | 0.109±0.001 | 0.110±0.001 |
| Δ                | 0.282±0.010 | 0.273±0.006 |

Cells were incubated at 37°C for 1 h in a glycylglycine buffered NaCl solution with and without 10^{-6} M strophanthidin (preincubation solution). At the end of the incubation, the cells were separated from the solution and washed seven times in isosmotic MgCl₂ solution; approximately 15 vol of wash solution were used for each volume of cells during each wash. The washing procedure was accomplished over a 1 h period. After the washing procedure half of each batch of cells was suspended at about 5% hematocrit in glycylglycine buffered NaCl solution containing 42KCl and the other half in the same solution containing 10^{-6} M strophanthidin. Δ is determined over a 1-h period; K⁺ during the determination of the K influx was 0.58 mM Na⁺ was 9.3 mM RBC and K⁺ 104.5 mM RBC. Δ is the difference between Δ in the control (strophanthidin-free) solution and in the solution containing 10^{-6} M strophanthidin.

batch was preincubated in a solution containing 10^{-4} M strophanthidin and the other batch was preincubated in a strophanthidin-free solution. After exposure to strophanthidin the cells were washed repeatedly in order to remove extracellular strophanthidin. Part of each batch was then suspended in the strophanthidin-free solution and part in the solution containing 10^{-4} M strophanthidin and K influx (ΔM_K) was measured. There was no difference between ΔM_K in the cells preincubated in the strophanthidin solution and that in the cells preincubated in the strophanthidin-free solution.

Table II contains the results of a second type of experiment designed to demonstrate the reversibility of the interaction of strophanthidin with the pump. Cells were separated into two batches; one batch (control) was used for the determination of ΔM_K under the circumstances recorded in the table. The inhibition produced by 1.5 × 10^{-7} M strophanthidin was considerably
### Table II

**Reversibility of the Inhibition of the Pump by Strophanthidin**

| Influx solution | $\Delta M_K \pm \text{SEM}$ | % Inhibition | Influx solution | $\Delta M_K \pm \text{SEM}$ | % Inhibition |
|-----------------|-------------------------------|--------------|-----------------|-------------------------------|--------------|
|                  | ($n = 4$)                      |              | ($n = 4$)       |                              |              |
| **Ko 0.21 mM**   |                               |              |                 |                              |              |
| Control          | 1.878±0.024                   |              |                 |                              |              |
| Strophanthidin   | 0.804±0.004                   | 57           | Strophanthidin   | 0.892±0.020                   | 53           |
| $1.5 \times 10^{-7}$M |                         |              | $1.5 \times 10^{-7}$M |                         |              |
| **Ko 16.6 mM**   |                               |              |                 |                              |              |
| Control          | 5.300±0.196                   |              |                 |                              |              |
| Strophanthidin   | 4.308±0.306                   | 19           | Strophanthidin   | 4.118±0.116                   | 22           |
| $1.5 \times 10^{-7}$M |                         |              | $1.5 \times 10^{-7}$M |                         |              |

Cells were separated into two batches. One batch (control cells) was suspended in glycylglycine buffered choline chloride solutions containing the concentrations of K and strophanthidin indicated, and $\Delta M_K$ determined. Samples for the determination were taken 0.5 and 1.0 h after suspension of the cells in the solution. The second batch (strophanthidin exposed cells) was suspended in a K-free glycylglycine buffered choline chloride solution containing strophanthidin $1.5 \times 10^{-7}$M and incubated at $37^\circ$C for 0.5 h. At the end of this period, K was added to the suspension at the concentrations indicated and $\Delta M_K$ determined; samples were taken 0.5 and 1.0 h after the addition of K. % inhibition was calculated as: $\left(\Delta M_K \text{ control cells without strophanthidin} - \Delta M_K \text{ strophanthidin inhibited cells}\right) \times 100 / \Delta M_K \text{ control cells without strophanthidin}$. Na was 0.7 mM RBC, K was 0.21 mM RBC.

Greater in the low K solutions than in the solutions containing 16 mM K; this reflects the ability of K to reduce inhibition of the pump by strophanthidin. The second batch was incubated for 0.6 h in a K-free solution containing $1.5 \times 10^{-7}$M strophanthidin. At the end of this period the cells were separated into two lots, K was added at the concentrations shown, and $\Delta M_K$ was measured. Since, as shown in the control cells, 0.5-h incubation was sufficient to allow strophanthidin inhibition to develop, it would be expected that if the inhibition of the pump by strophanthidin is irreversible preincubation in K-free solutions would allow combination of the steroid with the pump and the subsequently measured inhibition would be the same at high and low K concentrations. The inhibition was, however, much less when measured at high K concentrations than when measured at low concentrations; the ability of K to reduce the amount of inhibition demonstrates that the interaction of strophanthidin with the pump is reversible.

The results of an experiment designed to determine whether the inhibition of the pump produced by low concentrations of strophanthidin reaches a constant level are in Table III. Cells were exposed at $37^\circ$C either to a strophanthidin-free solution or to a solution containing strophanthidin at a concentration which produces a submaximal inhibition of the pump. The
TABLE III
CHANGE IN PUMP INHIBITION BY STROPHANTHIDIN WITH TIME

| Experiment | Na\(_a\) | K\(_e\) | (mM RBC-h) | (mM RBC-h) |
|------------|---------|---------|------------|------------|
| I: Nao 12.8, Na\(_e\) 83.5 mM RBC, K\(_e\) 14.0 mM RBC | | | | |
| \(K_e\) 0.19 mM | Control | 0.157±0.005 | 0.153±0.002 | | |
| | Strophanthidin \(10^{-7}\) M | 0.055±0.006 | 65 | 0.054±0.001 | 65 |
| \(K_e\) 15.5 mM | Control | 4.84±0.37 | 8.47±0.45 | | |
| | Strophanthidin \(10^{-7}\) M | 3.40±0.05 | 30 | 6.54±0.69 | 23 |
| II: Na\(_a\) 0, Na\(_e\) 72.7 mM RBC, K\(_e\) 12.4 mM RBC | | | | |
| \(K_e\) 0.20 mM | Control | 1.84±0.08 | 1.70±0.04 | | |
| | Strophanthidin \(1.5 \times 10^{-7}\) M | 0.88±0.03 | 52 | 0.89±0.01 | 48 |
| \(K_e\) 15.5 mM | Control | 4.62±0.08 | 4.32±0.08 | | |
| | Strophanthidin \(1.5 \times 10^{-7}\) M | 3.95±0.07 | 15 | 3.50±0.26 | 19 |

After alteration of intracellular cation content, cells were washed and added at about 5% hematocrit to glycylglycine-MgCO\(_3\) buffered solutions containing either choline 128 mM (Experiment I) or Na 128 mM (Experiment II). Half of each set were exposed to strophanthidin at the concentrations indicated and the suspensions were incubated at 37°C; during this period \(K_e\) was either 0 or 15 mM. After 0.5-h and after 2.5-h exposure to strophanthidin part of each batch of cells was removed and \(\Delta K\) added for the determination of \(\Delta M_K\); \(\Delta M_K\) was measured over a 1-h period. The \(K_e\) during the measurement of \(\Delta M_K\) is recorded in the table. % inhibition was calculated as: \((\Delta M_K \text{ control} - \Delta M_K \text{ strophanthidin}) \times 100/\Delta M_K \text{ control}\). Na\(_a\) is intracellular Na, K\(_e\) intracellular K, and \(\Delta M_K\) is the K influx.

exposure was carried out at either a very low or a relatively high concentration of K and at either a high concentration of Na or in Na-free solutions. \(\Delta M_K\) was determined over a 1-h period beginning at either 0.5 or 2.5 h after exposure to strophanthidin. Under each circumstance the inhibition produced by strophanthidin was the same after 2.5-h exposure as it was after 0.5-h exposure. Strophanthidin inhibition does not increase with time after 0.5 h but reaches a steady-state level.

Kinetic Model of Interaction of Na\(_a\), K\(_e\), and Strophanthidin with Pump

Possible reactions between Na\(_a\), K\(_e\), strophanthidin, and the external aspect of the pump are listed as Eqs. 1–8 in Table IV. K\(_e\) apparently must interact with the pump at two sites before transport occurs (Sachs and Welt, 1967) and this is represented by Eqs. 1, 2, and 8. The combination of K\(_e\) with the pump is represented as reversible, and the translocation step (Eq. 8) is represented as irreversible. Although it is likely that the translocation step is, in fact, reversible (Glynn et al., 1970), the measurement of unidirectional K
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**TABLE IV**

INTERACTION OF Na₀, K₀, AND STROPHANTHIDIN WITH THE Na-K PUMP

| Reaction                                      | Rate Equation |
|-----------------------------------------------|---------------|
| \(X + K₀ \xrightarrow{k₁ \_K \_} XK⁰\)          | (1)           |
| \(X + S \xrightarrow{k₃ \_K \_} XS\)           | (5)           |
| \(XK + K₀ \xrightarrow{k₂ \_K \_} XKK\)        | (2)           |
| \(XNa + S \xrightarrow{k₆ \_K \_} XNa S\)      | (6)           |
| \(X + Na₀ \xrightarrow{k₃ \_Na \_} XNa\)       | (3)           |
| \(XK + S \xrightarrow{k₇ \_K \_} XK S\)        | (7)           |
| \(XK + Na₀ \xrightarrow{k₄ \_Na \_} XKNa\)     | (4)           |
| \(XK \xrightarrow{k₈ \_K \_} X + 2 K₀\)        | (8)           |

\(X_T = XK + XKK + XNa + XKNa + XS + XNa S + XKS + X\) (9)

\(K₀\) is extracellular K, \(Na₀\) extracellular Na, S strophanthidin, and \(Kᵦ\) intracellular K. \(X\) is the pump which is assumed to have two binding sites for K both of which must be filled before transport occurs. \(X_T\) is the total amount of pump at the external aspect of the cell and is the sum of all the possible combinations of \(Na₀\), \(Kᵦ\), and S with \(X\).

Influx in the experiments reported here permits the assumption of irreversibility. It is known that \(Naᵦ\) competitively inhibits the active K influx (Post et al., 1960; Garrahan and Glynn, 1967; Sachs, 1967); this is represented in Eqs. 3 and 4. Although it seems likely that the competition between \(Naᵦ\) and \(Kᵦ\) is directly at the K binding sites, the equations written do not exclude an allosteric competition. Eqs. 5–7 represent the interaction of strophanthidin with the pump. It is assumed that strophanthidin can bind to the pump when it is not combined with any ion (Eq. 5) or when it is combined with one Na or one K (Eqs. 6 and 7). Since it is known (Glynn, 1957; Hoffman, 1966) that inhibition of the pump by low concentrations of cardiotonic steroids can be completely prevented by a high enough concentration of \(Kᵦ\), combination of strophanthidin with the form of the pump with two bound K ions is not represented. If combination of strophanthidin with the form XKK in the model of Table IV occurred, strophanthidin would inhibit the pump to some extent at all concentrations of \(Kᵦ\). All the reactions of Na and strophanthidin with the pump are represented as reversible.

Assuming that the transport system is in the steady state, the kinetic equations of Table IV result in the rate equation of Table V (Eq. 10) (Alberty, 1953). The rate equation is complex and it is unrealistic to expect to obtain values for the various rate coefficients and equilibrium constants. However, by determining the form of the relationship between either the total K influx \(\langle M_K \rangle\) or the strophanthidin-sensitive K influx \(\langle M_K^S \rangle\) and the concentration of extracellular Na([Na₀]), K([Kᵦ]), and strophanthidin ([S]), it should be possible to decide whether all of the Eqs. 1–8 are necessary to describe the system. This is the strategy adopted in this paper.
Table VI lists modifications of the rate equation (Eq. 10) obtained by setting the variable \([Nao]\), \([S]\), or both equal to zero. Eq. 12 results from setting both \([Nao]\) and \([S]\) equal to zero in Eq. 10; Eq. 14 results if \([S]\) alone is set equal to zero; and Eq. 16 arises if \([Nao]\) alone is zero. Two types of experiments were performed in order to test the predictions of these equations. In one experiment \(iM_k\) was measured as a function of \([K_o]\) in Na-free solutions both with and without strophanthidin; the relation between \(iM_k\) and \([K_o]\) under these circumstances should be described by Eqs. 12 and 16 if the model adequately describes the transport system. A second experiment involved

**Table V**

**RATE EQUATION RESULTING FROM THE STEADY-STATE SOLUTION OF THE KINETIC MODEL OF TABLE IV**

\[
\begin{align*}
\frac{iM_k^p}{\phi_1} &= \frac{k_xX_T}{\phi_1} \quad (10) \\
\frac{1}{iM_k^p} &= \frac{1}{k_xX_T} \quad (11)
\end{align*}
\]

where

\[
\phi_1 = \left( \frac{K_1A}{[K_o]^2} + \frac{k_8}{k_4} \right) \left( 1 + \frac{[S]}{K_6} + \frac{[Na_0]}{K_5} \left( 1 + \frac{[S]}{K_6} \right) \right) + \frac{A}{K_a} \left( 1 + \frac{[Na_0]}{K_4} + \frac{[S]}{K_5} \right) + 1.
\]

\(iM_k^p\) is the active K influx. \(K_1 = \frac{k_1}{k_4}, K_2 = \frac{k_2}{k_5}, \text{etc. } A = K_2 + \frac{k_8}{k_5}.

**Table VI**

**Setting \([Na_0]\) and \([S]\) = 0 in Eq. 10:**

\[
\begin{align*}
\frac{iM_k^p}{\phi_2} &= \frac{k_xX_T}{\phi_2} \quad (12) \\
\frac{1}{iM_k^p} &= \frac{1}{k_xX_T} \quad (13),
\end{align*}
\]

where

\[
\phi_2 = \frac{K_1A}{[K_o]^2} + \frac{k_8}{k_4} + \frac{k_8}{k_4} + 1.
\]

**Setting \([S]\) = 0 in Eq. 10:**

\[
\begin{align*}
\frac{iM_k^p}{\phi_3} &= \frac{k_xX_T}{\phi_3} \quad (14) \\
\frac{1}{iM_k^p} &= \frac{1}{k_xX_T} \quad (15),
\end{align*}
\]

where

\[
\phi_3 = \left( \frac{K_1A}{[K_o]^2} + \frac{k_8}{k_4} \right) \left( 1 + \frac{[Na_0]}{K_5} \right) + \frac{A}{K_a} \left( 1 + \frac{[Na_0]}{K_4} + \frac{[S]}{K_5} \right) + 1.
\]

**Setting \([Na_0]\) = 0 in Eq. 10:**

\[
\begin{align*}
\frac{iM_k^p}{\phi_4} &= \frac{k_xX_T}{\phi_4} \quad (16) \\
\frac{1}{iM_k^p} &= \frac{1}{k_xX_T} \quad (17),
\end{align*}
\]

where

\[
\phi_4 = \left( \frac{K_1A}{[K_o]^2} + \frac{k_8}{k_4} \right) \left( 1 + \frac{[S]}{K_6} \right) + \frac{A}{K_a} \left( 1 + \frac{[S]}{K_5} \right) + 1.
\]
measuring \( iM_K \) as a function of \([K_o]\) in solutions containing Na with and without strophanthidin; Eqs. 10 and 14 describe the behavior of the model under these conditions.

A third type of experiment was performed in order to obtain some estimate of the relative rates of the forward reactions 5 and 6, i.e., the relative magnitudes of the rate constants \( k_6 \) and \( k_7 \). In this experiment the binding of ouabain to cells in a totally K-free system (\( K_o = 0 \) and \( K_s \approx 0 \)) was estimated in Na solutions and in choline solutions. The binding of ouabain is only very slowly reversible so that the rate of ouabain binding may be taken as a measure of the magnitude of the forward rate constants. Since ouabain and strophanthin apparently bind to the same receptor (Hoffman, 1969), it is not unreasonable to assume that the relative rate of binding of ouabain in high Na and Na-free solutions will be similar to the relative rate of binding of strophanthin under the same circumstances.

It is convenient to describe the experimental results first; the agreement of the experimental results with the predictions of the model will then be examined.

**Experimental Results**

The data upon which the analysis will be based are presented in Figs. 1–3 and Table VII. Fig. 1 shows the results of an experiment of the first type. The reciprocal of \( iM_W \) is plotted as a function of the reciprocal of \([K_o]\); the solution was Na-free. Since under these circumstances the strophanthidin-insensitive K influx is a very small fraction of the total K influx (less than 3%), \( iM_K \) is very little different from \( iM_W \) and can be used in its place. The result of the experiment performed either with or without strophanthidin falls on

| TABLE VII |
| EFFECT OF Na\(_o\) ON THE RATE OF OUABAIN BINDING TO LOW K\(_o\) CELLS |
| --- |
| **Na\(_o\)**  | Preincubation solution | \( iM_K \) ± SEM | % inhibition | \( iM_K \) ± SEM | % inhibition |
| --- | --- | --- | --- | --- | --- |
| 154 mM | No ouabain | 2.179±0.009 | — | 2.278±0.023 | — |
| | Ouabain 10\(^{-8}\) M | 1.076±0.018 | 51 | 1.105±0.011 | 51 |
| | Ouabain 3 × 10\(^{-8}\) M | 0.680±0.042 | 69 | 0.632±0.005 | 72 |

Cells prepared by exposure to PCMBS (Na\(_o\) 21.1 mM RBC, K\(_o\) 1.4 mM RBC) were exposed for 1 h at 37°C to low concentrations of ouabain in glycylglycine buffered NaCl solutions (Na\(_o\) 154 mM) or glycylglycine buffered choline Cl solutions (Na\(_o\) 0 mM); cells were also exposed to the same solutions free of ouabain (control cells). After the 1-h exposure, the cells were removed from the solutions and washed three times in 30 vol of MgCl\(_2\) solution. Using these cells, ouabain-sensitive K influx (\( iM_K \)) was determined in a glycylglycine buffered choline Cl solution containing K\(_o\) 4.9 mM. % inhibition is calculated as: (\( iM_K \) in control cells − \( iM_K \) in cells exposed to ouabain) × 100/(\( iM_K \) in control cells)
straight lines. The experiment in Fig. 1 was performed using cells with low
$K_\text{s}$ and high $Na_\text{s}$; in Fig. 2 are the results of an experiment of the same design
using cells with higher intracellular K concentrations. Again the results are
described by straight lines. The linear relation between the reciprocal of

\[ \frac{1}{iMK} \text{ (millimoles/liter RBC.h)} = 0.132 + 0.0308 \frac{1}{[K_\text{o}]} \]

and the reciprocal of $[K_\text{o}]$ results in a direct plot of $iMK$ vs. $1/[K_\text{o}]$ (mM)$^{-1}$. The cells used in
the experiment contained $Na_\text{s}$ 44.5 and $K_\text{s}$ 1.1 mM RBC. The major extracellular cation
was choline, and $K_\text{o}$ was replaced by choline. The line for the experiment in the absence
of strophanthidin is $1/iMK = 0.132 + 0.0308 \frac{1}{[K_\text{o}]}$ and that for the experiment in
the presence of strophanthidin $1/iMK = 0.095 + 0.0803 \frac{1}{[K_\text{o}]}$. In this and in succeeding
figures each point is the mean of four determinations $\pm$ SEM.

$iMK$ and the reciprocal of $[K_\text{s}]$ results in a direct plot of $iMK$ vs. $[K_\text{s}]$ which is
described by a rectangular hyperbola. It has been shown that at very low
$[K_\text{s}]$, the curve formed by plotting $iMK$ as a function of $[K_\text{s}]$ is slightly sigmoid
(Garrahan and Glynn, 1967); such a curve is a plot of an equation of the form

\[ iMK = \frac{B}{[K_\text{s}]} + A \]
SACHS Interaction of K, Na, and Cardioactive Steroids with Na-K Pump

Figure 2. $1/iM_K$ (millimoles/liter RBC·h)$^{-1}$ vs. $1/[K_o]$ (mM)$^{-1}$. The cells used in the experiment contained Na, 63.4 and K, 57.8 mM RBC. The major extracellular cation was choline and K was replaced by choline. The line for the experiment in the absence of strophanthidin is $1/iM_K = 0.288 + 0.111 1/[K_o]$ and that for the experiment in the presence of strophanthidin $1/iM_K = 0.341 + 0.754 1/[K_o]$.

(Sachs and Welt, 1967):

$$iM_K = \frac{a}{1 + b[K_o] + c[K_o]^2}, \quad (18)$$

in which $a$, $b$, and $c$ are constants.

The second kind of experiment is shown in Fig. 3. The reciprocal of $iM_K$ is plotted as a function of the reciprocal of $[K_o]$; the measurements were made.
in solutions containing Na 32 mM and were made with and without strophanthidin. In this case both the curve produced in strophanthidin-free solutions and that produced in solutions containing strophanthidin are described by parabolas; the equations which fit the curves are of the form:

$$\frac{1}{iM_K} = \frac{a}{a} + \frac{b}{a[K_a]} + \frac{c}{a[K_a]^2},$$

(19)
where \(a\), \(b\), and \(c\) are constants (Eq. 19 is the reciprocal of Eq. 18). The constants \(b\) and \(c\) were greater when the measurements were made in solutions containing strophanthidin than when they were made in strophanthidin-free solutions. In a previous publication (Sachs and Welt, 1968) similar experiments were reported in which the measurements were made at higher \(N_{ao}\) (129 mM) and using cells containing normal \(N_{ao}\) and \(K_{ao}\); under those circumstances \(b\) was 0.72 and \(c\) 0.87 in strophanthidin-free solutions, and in solutions containing \(10^{-7}\) M strophanthidin \(b\) was 3.33, and \(c\) 8.67. When measurements are made in solutions containing \(Na\), therefore, curves describing the relation between \(1/M\), \(1/[K_{o}]\), and \(1/[K_{o}]^2\) must contain a term in \(1/[K_{o}]^3\), and the coefficient of \(1/[K_{o}]^3\) is greater than when the measurements were made in \(Na\)-free solutions. Strophanthidin increases both the coefficient of \(1/[K_{o}]^3\) and \(1/[K_{o}]^3\) when the measurements are made in solutions containing \(Na\).

The third type of experiment was designed to estimate the effect of \(N_{ao}\) on the rate of ouabain binding. It has been reported that ouabain binding occurs more rapidly in solutions containing \(Na\) than in \(Na\)-free solutions (Beauge and Adragna, 1971; Gardner and Conlon, 1972). Such experiments are complicated by the leakage of \(K\) from the cells into the solution over the course of the experiments so that the extracellular solution is only nominally \(K\)-free. Since \(Na\) competitively inhibits the activation of the pump by \(K\) (Garrahan and Glynn, 1967; Sachs, 1967), it might be expected that \(K\) would more effectively prevent ouabain binding at low \(Na\) concentrations than at high. The reported effect of \(Na\) in promoting ouabain binding might therefore result from its competition with \(K\) leaking from the cells. Some indirect evidence that this is the case has been reported (Beauge and Adragna, 1971). In order to determine whether \(Na\) itself has an effect on ouabain binding in the complete absence of \(K\), the rate of ouabain binding in \(Na\)-free solutions and in solutions containing \(Na\) was measured using cells which were virtually \(K\)-free. The rate of ouabain binding was estimated by measuring the residual ouabain-sensitive \(K\) influx in cells which had been preexposed to a low concentration of ouabain for a fixed time (Hoffman, 1966).

The results of the experiment are shown in Table VII. Low \(K\), high \(Na\) cells were incubated for 1 h in a high \(Na\) solution and in an \(Na\)-free solution at two low concentrations of ouabain and in the absence of ouabain. After the incubation the cells were removed from the solutions and the ouabain washed away. Using these cells the ouabain-sensitive \(K\) influx was then measured. It can be seen that inhibition of the \(K\) influx at each ouabain concentration is the same whether the cells were exposed to the drug in the high \(Na\) or the \(Na\)-free solution.

Since the binding of ouabain is only very slowly reversible, these results can be interpreted as indicating that the rate of ouabain binding is the same whether the pump is combined with \(Na\) or not. If it is assumed that stro-
phanthidin binding occurs at the same site and by the same mechanism as ouabain binding (Hoffman, 1969), it seems reasonable to conclude that the rate constants $k_5$ and $k_4$ of Eqs. 5 and 6 (Table IV) are about equal, that is, that the binding of strophanthidin to the form $X$ occurs at about the same rate as its binding to the form $XNa$.

**Evaluation of Kinetic Model**

The relation between $1/iM$ and $1/[K_o]$ is described by a straight line when the measurements are made in Na-free solutions (Fig. 1); strophanthidin changes the slope of the line but does not alter its shape. On the other hand, when the measurements are made in solutions containing Na (Fig. 3), the relation between $1/iM$ and $1/[K_o]$ is described by a parabola; under these circumstances strophanthidin alters the shape of the curve in that the curve becomes more parabolic. If the kinetic model adequately described the system, it must be able to account for these observations.

The prediction from the model for the relation between $1/iM$ and $1/[K_o]$ when the measurements are made in Na-free and strophanthidin-free solutions (Fig. 1) is represented by Eq. 13. Eq. 13 will describe straight line if the coefficient of $1/[K_o]^2$ is very small, i.e., if either $K_1$ or $A$ is small. The coefficient of $1/[K_o]^2$ cannot be zero since it has been reported (Garrahan and Glynn, 1967) that there is a slight nonlinearity in the relation between $1/iM$ and $1/[K_o]$ when the measurements are made at very low concentrations of $K_o$ (about 0.015 mM) in Na-free solutions.

When the measurements are made in solutions containing Na, the model predicts that the relation between $1/iM$ and $1/[K_o]$ will be described by Eq. 15. The experimental observation (Fig. 3) is that the curve under this circumstance is parabolic and the coefficients of $1/[K_o]^2$ and $1/[K_o]$ are greater than when the measurements are made in Na-free solutions. Comparing Eqs. 13 and 15, it can be seen that, in order to account for these observations, it is necessary only that the term $[Na_c]/K_3$ be greater than unity, i.e., that, at concentrations at which Na causes the curve to become parabolic, the concentration of Na is greater than the equilibrium constant for reaction 3. It is possible, but not necessary, that the concentration of Na is also greater than the equilibrium constant for reaction 4.

Eq. 17 describes the relation between $1/iMP$ and $1/[K_o]$ when the measurements are made in solutions free of Na but containing strophanthidin. The experimental observation (Fig. 1) is that strophanthidin increases the magnitude of the coefficient of $1/[K_o]$ without causing the curve to become parabolic. If the concentration of strophanthidin at which this effect is seen is greater than $K_5$, the equilibrium constant for reaction 5, it would be expected that the result would be similar to that found when Na is used, i.e., both the coefficient of $1/[K_o]^2$ and $1/[K_o]$ would be increased and the
curve would become parabolic. Since this is not the case, the increase in the coefficient of $1/[K_\alpha]$ must be due to another cause. If the term $[S]/K_7$, is greater than unity, the observed result would be obtained; the coefficient of $1/[K_\alpha]$ would be increased without increasing the coefficient of $1/[K_\beta]^3$. The model will therefore describe the observed data if, at low inhibitory concentrations of strophanthidin, the concentration of strophanthidin is greater than $K_\alpha$, the equilibrium constant for reaction 7, but not much greater than $K_\delta$, the equilibrium constant for reaction 5.

When measurements are made in solutions containing Na (Fig. 3), strophanthidin increases the coefficient of both $1/[K_\alpha]^3$ and $1/[K_\beta]$. The prediction of the model for this circumstance is contained in Eq. 11. If $[S]/K_7$ is greater than unity, this would account for the increase in the coefficient of $1/[K_\alpha]$ but not for the increase in the coefficient of $1/[K_\beta]^3$. The increase in the coefficient of $1/[K_\alpha]^3$ must be attributable to either the magnitude of the term $[S]/K_\delta$ or that of $[S]/K_\gamma$; if either term is significant the coefficient of $1/[K_\alpha]^3$ would be increased. At this concentration of strophanthidin $[S]/K_\delta$ was not great enough to increase the coefficient of $1/[K_\alpha]^3$ when the measurements were made in Na-free solutions (Fig. 1), and therefore it seems likely that the increase in the coefficient of $1/[K_\alpha]^3$ produced by strophanthidin in solutions containing Na is due to the magnitude of the term $[S]/K_\delta$; i.e., the inhibitory concentration of strophanthidin is greater than the equilibrium constant for reaction 6. It is possible that the terms $[S]/K_\delta$ and $[S]/K_\gamma$ are about the same, but the effect of $[S]/K_\delta$ on increasing the coefficient of $1/[K_\alpha]^3$ is greater since it is multiplied by the term $[Na_\alpha]/K_\delta$.

In order for the model to describe the experimental results it is therefore necessary to conclude that in Na-free solutions strophanthidin does not combine with the form of the pump ($X$) which is free of K (reaction 5), but does combine with the form which is associated with one K ion ($XK_\alpha$, reaction 7). On the other hand, in solutions containing Na strophanthidin is capable of combining with the form of the pump associated with Na ($XNa_\alpha$, reaction 6) in addition to the form associated with K. However, if the reaction of strophanthidin with the pump is similar to the reaction of ouabain with the pump (Table VII), it would be expected that the rate of reaction ($k_\delta$) of strophanthidin with the uncombined pump ($X$) is similar to its rate of reaction ($k_\delta$) with the form $XNa$. In order that $[S]/K_\delta$ be greater than $[S]/K_\gamma$, it is necessary that $K_\delta$ be less than $K_\gamma$. Even if the values for the forward reaction are about the same, as is implied by the ouabain binding experiment (Table VII), $K_\delta$ would be less than $K_\gamma$ if $k_\delta$ were less than $k_\gamma$, i.e., if the dissociation of the complex $XNaS$ occurred at a slower rate than the dissociation of the complex $XS$. It is, of course, also possible that the results of the ouabain binding experiment are not directly applicable to the kinetics of strophanthidin inhibition, and the rate of reaction ($k_\delta$) of strophanthidin with $XNa$ may be greater than the rate of its reaction ($k_\delta$) with $X$. 

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The observed effects of strophanthidin on the form of the relation between $1/i\frac{M}{K}$ and $1/[K_{o}]$ apparently arise from the relative affinities of the various forms of the pump for $Na_{o}$, $K_{o}$ and strophanthidin. Presumably the ratio of the affinity of $X$ for $K$ to the affinity of $X$ for strophanthidin is such that at the concentrations of strophanthidin used here, and even at very low concentrations of $K_{o}$, the form $X$ will bind with $K$ and almost none will combine with strophanthidin. On the other hand, the affinity of the form $XK$ for $K_{o}$ is more comparable to its affinity for strophanthidin and strophanthidin will combine with this form of the pump at low concentrations of $K_{o}$. When $Na$ is present, some $X$ will combine with $Na_{o}$ rather than $K_{o}$ and this form of the pump will combine with strophanthidin. As a result of these events the observed effect of strophanthidin on the shape and slope of the curve $1/i\frac{M}{K}$ vs. $1/[K_{o}]$ can be explained.

Concentration Dependence of Na Inhibition

If, in Eq. 15, $[K_{o}]$ is set equal to a constant, the equation reduces to one of the form:

$$\frac{1}{iM_{K}^{P}} = C_{1} + C_{2} [Na_{o}],$$

where $C_{1}$ and $C_{2}$ are constants; $1/iM_{K}^{P}$ under these circumstances should be a linear function of $Na_{o}$. Fig. 4 is a plot of an experiment in which $1/iM_{K}^{P}$ was measured at variable $Na_{o}$ concentrations and at a constant $[K_{o}]$; the $K_{o}$ concentration chosen was low since the affinity of the pump for $K_{o}$ is much greater than its affinity for $Na_{o}$. The straight line in the figure was drawn by eye. It is not clear that the points are accurately described by the straight line; they might be better fitted by a slightly curved line with its concavity directed downwards. The cells used in this experiment contained low concentrations of $K_{o}$ and high concentrations of $Na_{o}$. The results of a similar experiment in which $1/iM_{K}^{P}$ was measured as a function of $[Na_{o}]$ in solutions with a low $[K_{o}]$ using cells with normal $Na_{o}$ and $K_{o}$ have been reported (Sachs, 1967). The points more clearly fell on a straight line with no concavity either upward or downward.

The results of this experiment indicate that, in terms of the model and within the limits of the experimental procedures, it is sufficient to suppose that the pump is inhibited when it is combined at the outside with a single $Na$ ion. It is not necessary to assume that the inhibition produced when the pump is combined with two $Na$ ions is any greater than when it is combined with one (if this were the case one would expect the plot of $1/iM_{K}^{P}$ vs. $[Na_{o}]$ to be parabolic with the concavity directed upwards).
FIGURE 4. \(1/M_K^P\) (millimoles/liter RBC·h)\(^{-1}\) vs. [Na\(_a\)] (mM). The cells used in the experiment contained Na\(_a\) 53.9 and K\(_a\) 6.8 mM RBC. NaCl was replaced by choline chloride; [K\(_a\)] = 0.23 mM. \(M_K^P\) was calculated as the difference between \(M_K\) determined in strophanthidin-free solutions and \(M_K\) determined under similar circumstances in solutions containing 10\(^{-4}\) M strophanthidin.

Concentration Dependence of Strophanthidin Inhibition

By setting \((K_s)\) constant in Eq. 11, one can obtain an equation of the form:

\[
\frac{1}{M_K^P} = C_1(1 + C_2 [Na_a]) + C_3(1 + C_4 [Na_a]) [S] \tag{21}
\]

where \(C_1, C_2, C_3,\) and \(C_4\) are constants. The equation predicts that, if \(1/M_K^P\) is measured as a function of the strophanthidin concentration, the resulting curve should describe a straight line whether the measurements are made in
solutions containing Na or in Na-free solutions. The slope of the line should, however, be greater in the solution containing Na. Fig. 5 represents the results of an experiment in which $1/M_R^p$ was measured as a function of the strophanthidin concentration in Na-free solutions and Fig. 6 is from a similar experiment performed in high Na solutions. In both cases the curves are well described by straight lines. Although the slope of the curve is greater in the high Na experiment, it is not possible to compare the two experiments since

![Graph](attachment:graph.png)

**Figure 5.** $1/M_R^p$ (millimoles/liter RBC h$^{-1}$) vs. [Strophanthidin] (mM x $10^8$) in Na-free solutions. The cells contained Na, 51.4 and Kc 1.3 mM RBC. The major extracellular cation was choline and $[K_o] = 1.02$ mM.

the $[K_o]$ was higher in the Na-free experiment and this would be expected to lower the slope of the line.

The linear relation between $1/M_R^p$ and [S] indicates that it is sufficient to assume that a single strophanthidin molecule is necessary to inhibit each pump; if the combination of two strophanthidin molecules with the pump produced a greater inhibition than did the combination of one, the relation between $1/M_R^p$ and [S] would be parabolic. Although these data do not exclude the possibility that more than one strophanthidin molecule binds to the pump even though the binding of a single molecule is sufficient to
produce maximal inhibition, there is evidence from the interaction of ouabain with microsomal preparations that, in fact, only one steroid molecule interacts with each pump (Matsui and Schwartz, 1968; Barnett, 1970; Hansen et al., 1971).

**Effect of Na+ on Interaction of Strophanthidin with Pump**

On the basis of the kinetic data presented here, it seems likely that, because of the relative affinities of the pump for Na+, K+, and strophanthidin, the form of the pump combined with Na+ is more likely to interact with strophanthidin than is the uncombined form of the pump; the uncombined form of the pump is much more likely to combine with K+ even at very low K+ concentrations. The observed increase in inhibition of the red cell pump by cardioactive steroids produced by Na+ seems, therefore, to be due primarily to the competition between Na+ and K+ for the uncombined form of the pump. The
finding (Table VII) that in a K-free system ouabain binds as rapidly in an Na-free solution as in a solution containing Na indicates that, as far as the forward binding reaction is concerned, Na by itself does not promote binding. It is of course still possible that the affinity of the pump for cardioactive steroids is increased by Na since it is possible that the rate of dissociation is different in Na solutions than in Na-free solutions.

Using microsomal preparations (Albers et al., 1968; Sen et al., 1969; Allen et al., 1970, Akera and Brody, 1971), permeable red cell ghosts (Hoffman, 1966), and reconstituted impermeable ghosts (Bodemann and Hoffman, in preparation) it has been demonstrated that the requirements for [H]-ouabain binding are quite specific and it seems likely that ouabain binds specifically to some particular conformation of the pump which occurs during the transport cycle. The present results indicate that ionic conditions at the outer aspect of the membrane are also of importance in the formation of the conformation which combines with cardioactive steroids and provides a kinetic basis for the observation that Na increases the rate of ouabain interaction with the pump.

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