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 ($^iM_K^{P-1}$) and the reciprocal of $[K_o]$ ($[K_o]^{-1}$); 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 $^iM_K^{P-1}$ 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, 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_o) (Schatzman, 1965; Baker and Manil, 1968; Beauge and Adragna, 1971; Baker and Willis, 1972).

The effect of Na_o can be accounted for by either of two mechanisms: (*a*) Na_o competes with K_o and so reduces the ability of K_o to prevent inhibition of the pump by the cardioactive steroids (Beauge and Adragna, 1971) or (*b*) Na_o reacts directly with the pump and so increases its susceptibility to inhibition independently of any competition between Na_o and K_o (Gardner and Conlon, 1972). The first mechanism seems reasonable since it is known that Na_o competes with K_o at the outer aspect of the membrane and inhibits pump activation by K_o (Post et al., 1960; Garrahan and Glynn, 1967; Sachs, 1967); this mechanism has been proposed to explain the effect of Na_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_o promotes the effect of ouabain even though K_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_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_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 steroid. 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 [^{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) $MgCl_2$ 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^{++} 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_c) was reduced to low levels in order to reduce variations in K_o over the course of the influx measurements since approximately constant K_o simplifies the calculation of the data. Intracellular Na (Na_c) was maintained at concentrations higher than the apparent $K_{1/2}$ of the pump for Na_c in order to minimize the effect of variations in the concentration of Na_c 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	${}^{i}M_K \pm$ SEM, $n = 4$ Preincubation solution	
	Control	Strophanthidin 10^{-6} M
	(*mM 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 $MgCl_2$ 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 ${}^{42}KCl$ and the other half of each batch was suspended in the same solution containing 10^{-6} M strophanthidin. ${}^{i}M_K$ was determined over a 1-h period; K_o during the determination of the K influx was 0.58 mM. Na_c was 9.3 mM RBC and K_c 104.5 mM RBC. Δ is the difference between ${}^{i}M_K$ in the control (strophanthidin-free) solution and that in the solution containing 10^{-6} M strophanthidin.

batch was preincubated in a solution containing 10^{-6} 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^{-6} M strophanthidin and K influx (${}^{i}M_K$) was measured. There was no difference between ${}^{i}M_K$ 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 ${}^{i}M_K$ 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

Control cells			Strophanthidin exposed cells		
Influx solution	${}^iM_K \pm$ SEM ($n = 4$)	% Inhibition	Influx solution	${}^iM_K \pm$ SEM ($n = 4$)	% Inhibition
	(*mM RBC·h*)			(*mM RBC·h*)	
K_o 0.21 mM					
Control	1.878±0.024				
Strophanthidin 1.5×10^{-7} M	0.804±0.004	57	K_o 0.23 mM Strophanthidin 1.5×10^{-7} M	0.892±0.020	53
K_o 16.6 mM					
Control	5.300±0.196				
Strophanthidin 1.5×10^{-7} M	4.308±0.306	19	K_o 16.3 mM Strophanthidin 1.5×10^{-7} M	4.118±0.116	22

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 ${}^iM_K$ 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 1.5×10^{-7} M and incubated at 37° for 0.5 h. At the end of this period, K was added to the suspension at the concentrations indicated and ${}^iM_K$ determined; samples were taken 0.5 and 1.0 h after the addition of K. % inhibition was calculated as: (${}^iM_K$ control cells without strophanthidin − ${}^iM_K$ strophanthidin inhibited cells) × 100/${}^iM_K$ control cells without strophanthidin. Na_c 30.7 mM RBC, K_c 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 1.5×10^{-7} M strophanthidin. At the end of this period the cells were separated into two lots, K was added at the concentrations shown, and ${}^iM_K$ 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°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

Time to start of influx measurement...	0.5 h		2.5 h	
	${}^iM_K \pm$ SEM, $n = 4$	% Inhibition	${}^iM_K \pm$ SEM, $n = 4$	% Inhibition
	(*mM RBC·h*)		(*mM RBC·h*)	
Experiment I: Na_o 12.8, Na_c 83.5 mM RBC, K_c 14.0 mM RBC				
K_o 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_o 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
Experiment II: Na_o 0, Na_c 72.7 mM RBC, K_c 12.4 mM RBC				
K_o 0.20 mM				
Control	1.84±0.08		1.70±0.04	
Strophanthidin 1.5×10^{-7} M	0.88±0.03	52	0.89±0.01	48
K_o 15.5 mM				
Control	4.62±0.08		4.32±0.08	
Strophanthidin 1.5×10^{-7} M	3.93±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_o 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 ^{42}KCl added for the determination of ${}^iM_K$; ${}^iM_K$ was measured over a 1-h period. The K_o during the measurement of ${}^iM_K$ is recorded in the table. % inhibition was calculated as: (${}^iM_K$ control $-$ ${}^iM_K$ strophanthidin) $\times$ 100/${}^iM_K$ control. Na_c is intracellular Na, K_c intracellular K, and ${}^iM_K$ 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. ${}^iM_K$ 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_o, K_o, and Strophanthidin with Pump

Possible reactions between Na_o, K_o, strophanthidin, and the external aspect of the pump are listed as Eqs. 1–8 in Table IV. K_o 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_o 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

TABLE IV

INTERACTION OF Na_o, K_o, AND STROPHANTHIDIN WITH THE Na-K PUMP

$$X + K_o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} XK \quad (1)$$

$$XK + K_o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} XKK \quad (2)$$

$$X + Na_o \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} XNa \quad (3)$$

$$XK + Na_o \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} XKNa \quad (4)$$

$$X + S \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} XS \quad (5)$$

$$XNa + S \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} XNa\,S \quad (6)$$

$$XK + S \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} XK\,S \quad (7)$$

$$XKK \xrightarrow{k_8} X + 2\,K_c \quad (8)$$

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

K_o is extracellular K, Na_o extracellular Na, S strophanthidin, and K_c 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 Na_o, K_o, and S with X.

influx in the experiments reported here permits the assumption of irreversibility. It is known that Na_o 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 Na_o and K_o 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 K_o, 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 K_o. 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 (^iM_K) or the strophanthidin-sensitive K influx ($^iM_K^P$) and the concentration of extracellular Na([Na_o]), K([K_o]), 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 $[Na_o]$, [S], or both equal to zero. Eq. 12 results from setting both $[Na_o]$ and [S] equal to zero in Eq. 10; Eq. 14 results if [S] alone is set equal to zero; and Eq. 16 arises if $[Na_o]$ 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

$$ {}^iM_K^P = \frac{k_8 X_T}{\phi_1} \quad (10) \qquad \frac{1}{{}^iM_K^P} = \frac{\phi_1}{k_8 X_T} \quad (11) $$

where

$$ \phi_1 = \left(\frac{K_1 A}{[K_o]^2} + \frac{k_8}{k_1[K_o]}\right)\left(1 + \frac{[S]}{K_5} + \frac{[Na_o]}{K_3}\left[1 + \frac{[S]}{K_6}\right]\right) + \frac{A}{[K_o]}\left(1 + \frac{[Na_o]}{K_4} + \frac{[S]}{K_7}\right) + 1. $$

${}^iM_K^P$ is the active K influx. $K_1 = \frac{k_{-1}}{k_1}$, $K_2 = \frac{k_{-2}}{k_2}$, etc. $A = K_2 + \frac{k_8}{k_2}$.

TABLE VI

Setting $[Na_o]$ and [S] = 0 in Eq. 10:

$$ {}^iM_K^P = \frac{k_8 X_T}{\phi_2} \quad (12) \qquad \frac{1}{{}^iM_K^P} = \frac{\phi_2}{k_8 X_T} \quad (13), $$

where

$$ \phi_2 = \frac{K_1 A}{[K_o]^2} + \left(\frac{k_8}{k_1} + A\right)\frac{1}{[K_o]} + 1. $$

Setting [S] = 0 in Eq. 10:

$$ {}^iM_K^P = \frac{k_8 X_T}{\phi_3} \quad (14) \qquad \frac{1}{{}^iM_K^P} = \frac{\phi_3}{k_8 X_T} \quad (15), $$

where

$$ \phi_3 = \left(\frac{K_1 A}{[K_o]^2} + \frac{k_8}{k_1[K_o]}\right)\left(1 + \frac{[Na_o]}{K_3}\right) + \frac{A}{[K_o]}\left(1 + \frac{[Na_o]}{K_4}\right) + 1. $$

Setting $[Na_o]$ = 0 in Eq. 10:

$$ {}^iM_K^P = \frac{k_8 X_T}{\phi_4} \quad (16) \qquad \frac{1}{{}^iM_K^P} = \frac{\phi_4}{k_8 X_T} \quad (17), $$

where

$$ \phi_4 = \left(\frac{K_1 A}{[K_o]^2} + \frac{k_8}{k_1[K_o]}\right)\left(1 + \frac{[S]}{K_5}\right) + \frac{A}{[K_o]}\left(1 + \frac{[S]}{K_7}\right) + 1. $$

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_5 and k_6. In this experiment the binding of ouabain to cells in a totally K-free system ($K_o = 0$ and $K_o \simeq 0$) 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 strophanthitin 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 strophanthidin 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 ${}^iM_K$ 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 ${}^iM_K^P$ 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_c CELLS

Preincubation solution	Na_o 154 mM		Na_o 0 mM	
	${}^iM_K^P \pm$ SEM	% inhibition	${}^iM_K^P \pm$ SEM	% inhibition
	(*mM RBC·h*)		(*mM RBC·h*)	
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_c 21.1 mM RBC, K_c 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 (${}^iM_K^P$) was determined in a glycylglycine buffered choline Cl solution containing K_o 4.9 mM. % inhibition is calculated as: (${}^iM_K^P$ in control cells − ${}^iM_K^P$ in cells exposed to ouabain) × 100/${}^iM_K^P$ in control cells.

straight lines. The experiment in Fig. 1 was performed using cells with low K_c and high Na_c; 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

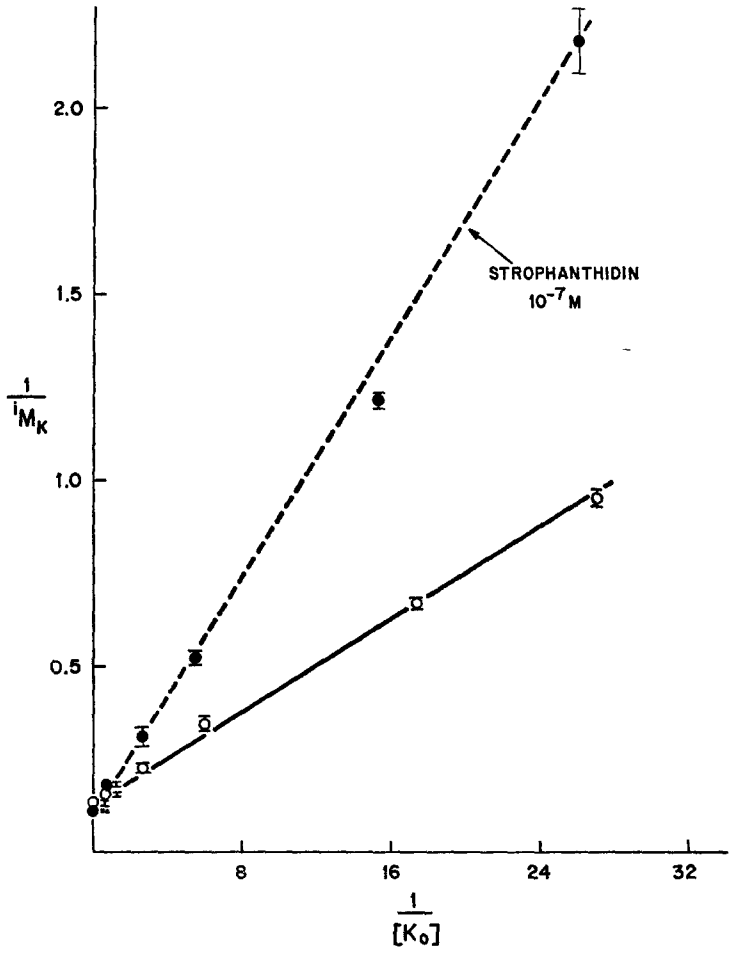

FIGURE 1. $1/{}^{i}M_K$ (millimoles/liter RBC·h)$^{-1}$ vs. $1/[K_o]$ (mM)$^{-1}$. The cells used in the experiment contained Na_c 44.5 and K_c 1.1 mM RBC. The major extracellular cation was choline, and K_o was replaced by choline. The line for the experiment in the absence of strophanthidin is $1/{}^{i}M_K = 0.132 + 0.0308\ 1/[K_o]$ and that for the experiment in the presence of strophanthidin $1/{}^{i}M_K = 0.095 + 0.0803\ 1/[K_o]$. In this and in succeeding figures each point is the mean of four determinations ± SEM.

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

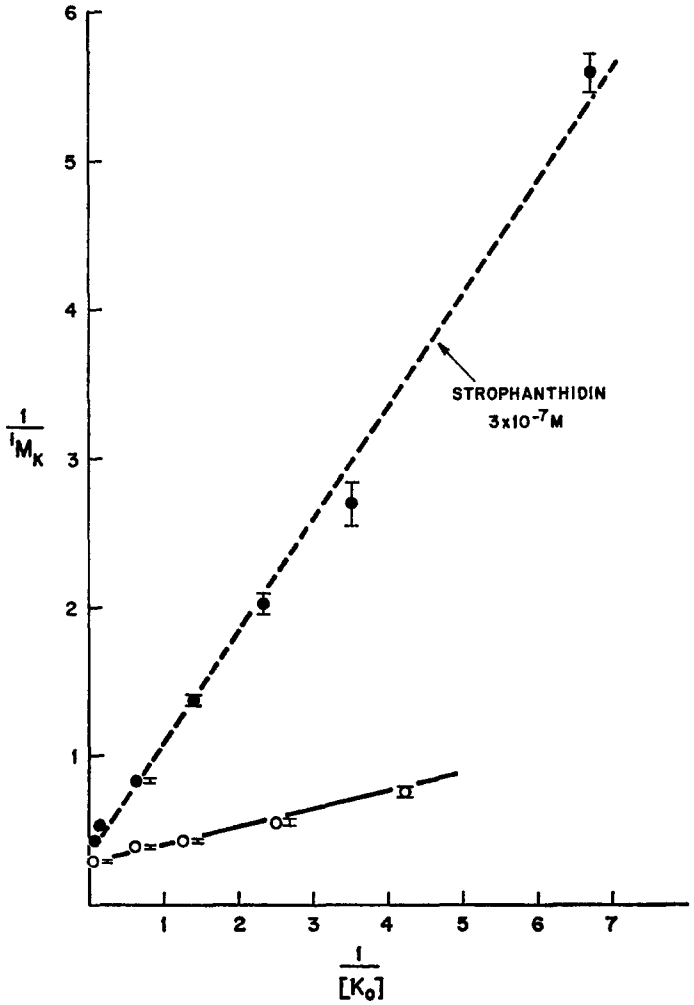

FIGURE. 2. $1/{}^{i}M_K$ (millimoles/liter RBC · h)$^{-1}$ vs. $1/[K_o]$ (mM)$^{-1}$. The cells used in the experiment contained Na_c 63.4 and K_c 57.8 mM RBC. The major extracellular cation was choline and K_o was replaced by choline. The line for the experiment in the absence of strophanthidin is $1/{}^{i}M_K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

(Sachs and Welt, 1967):

$$ {}^{i}M_K = \frac{a}{1 + \frac{b}{[K_o]} + \frac{c}{[K_o]^2}}, \tag{18} $$

in which a, b, and c are constants.

The second kind of experiment is shown in Fig. 3. The reciprocal of ${}^{i}M_K$ is plotted as a function of the reciprocal of $[K_o]$; the measurements were made

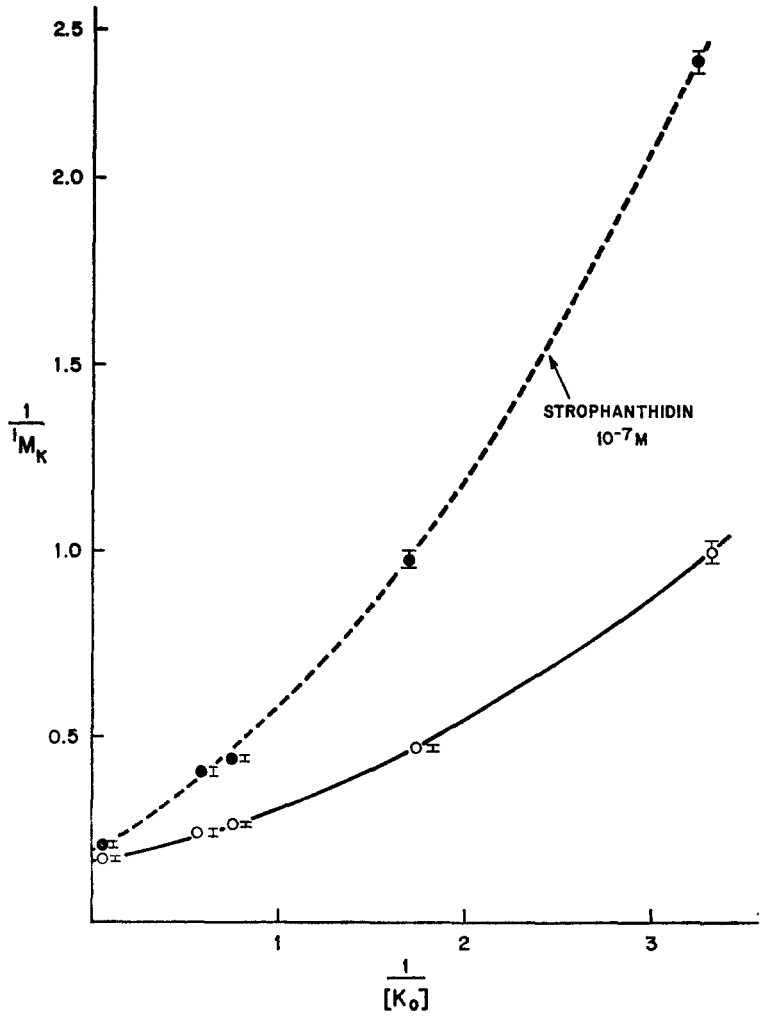

FIGURE 3. $1/{}^{i}M_K$ (millimoles/liter RBC·h)$^{-1}$ vs. $1/[K_o]$ (mM)$^{-1}$. The cells used in the experiment contained Na_c 50.4 and K_c 1.0 mM RBC. The solution contained Na_o 32 mM and the major cation was choline; K_o was replaced by choline. The curve for the experiment in the absence of strophanthidin is $1/{}^{i}M_K = 0.169 + 0.088\ 1/[K_o] + 0.049\ 1/[K_o]^2$ and that in the presence of strophanthidin $1/{}^{i}M_K = 0.196 + 0.253\ 1/[K_o] + 0.124\ 1/[K_o]^2$.

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:

$$\frac{1}{{}^{i}M_K^P} = \frac{1}{a} + \frac{b}{a}\frac{1}{[K_o]} + \frac{c}{a}\frac{1}{[K_o]^2}, \tag{19}$$

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 Na_o (129 mM) and using cells containing normal Na_c and K_c; 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 $1/{}^iM_K^P$ and $1/[K_o]$ must contain a term in $1/[K_o]^2$, and the coefficient of $1/[K_o]$ is greater than when the measurements were made in Na-free solutions. Strophanthidin increases both the coefficient of $1/[K_o]$ and $1/[K_o]^2$ when the measurements are made in solutions containing Na.

The third type of experiment was designed to estimate the effect of Na_o 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_o competitively inhibits the activation of the pump by K_o (Garrahan and Glynn, 1967; Sachs, 1967), it might be expected that K_o would more effectively prevent ouabain binding at low Na concentrations than at high. The reported effect of Na_o 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_o itself has an effect on ouabain binding in the complete absence of K_o, 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_c high Na_o 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_o 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_6 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 $1/{}^iM_K^P$ 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 $1/{}^iM_K^P$ 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 $1/{}^iM_K^P$ 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 $1/{}^iM_K^P$ 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 $1/{}^iM_K^P$ 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 $[Na_o]/K_3$ 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 $1/{}^iM_K^P$ 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 $1/[K_o]$ 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 $1/[K_o]$ would be increased without increasing the coefficient of $1/[K_o]^2$. The model will therefore describe the observed data if, at low inhibitory concentrations of strophanthidin, the concentration of strophanthidin is greater than K_7, the equilibrium constant for reaction 7, but not much greater than K_5, the equilibrium constant for reaction 5.

When measurements are made in solutions containing Na (Fig. 3), strophanthidin increases the coefficient of both $1/[K_o]^2$ and $1/[K_o]$. 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 $1/[K_o]$ but not for the increase in the coefficient of $1/[K_o]^2$. The increase in the coefficient of $1/[K_o]^2$ must be attributable to either the magnitude of the term $[S]/K_5$ or that of $[S]/K_6$; if either term is significant the coefficient of $1/[K_o]^2$ would be increased. At this concentration of strophanthidin $[S]/K_6$ was not great enough to increase the coefficient of $1/[K_o]^2$ when the measurements were made in Na-free solutions (Fig. 1), and therefore it seems likely that the increase in the coefficient of $1/[K_o]^2$ produced by strophanthidin in solutions containing Na is due to the magnitude of the term $[S]/K_6$; i.e., the inhibitory concentration of strophanthidin is greater than the equilibrium constant for reaction 6. It is possible that the terms $[S]/K_5$ and $[S]/K_6$ are about the same, but the effect of $[S]/K_6$ on increasing the coefficient of $1/[K_o]^2$ is greater since it is multiplied by the term $[Na_o]/K_3$.

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, 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, 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_5) of strophanthidin with the uncombined pump (X) is similar to its rate of reaction (k_6) with the form XNa. In order that $[S]/K_6$ be greater than $[S]/K_5$, it is necessary that K_6 be less than K_5. Even if the values for the forward reaction are about the same, as is implied by the ouabain binding experiment (Table VII), K_6 would be less than K_5 if k_{-6} were less than k_{-5}, 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_6) of strophanthidin with XNa may be greater than the rate of its reaction (k_5) with X.

The observed effects of strophanthidin on the form of the relation between $1/{}^{i}M_{K}$ 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 $1/{}^{i}M_{K}$ 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:

$$\frac{1}{{}^{i}M_{K}^{P}} = C_1 + C_2\,[Na_o], \tag{20}$$

where C_1 and C_2 are constants: $1/{}^{i}M_{K}^{P}$ under these circumstances should be a linear function of Na_o. Fig. 4 is a plot of an experiment in which $1/{}^{i}M_{K}^{P}$ 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_c and high concentrations of Na_c. The results of a similar experiment in which $1/{}^{i}M_{K}^{P}$ was measured as a function of $[Na_o]$ in solutions with a low $[K_o]$ using cells with normal Na_c and K_c 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 $1/{}^{i}M_{K}^{P}$ vs. $[Na_o]$ to be parabolic with the concavity directed upwards).

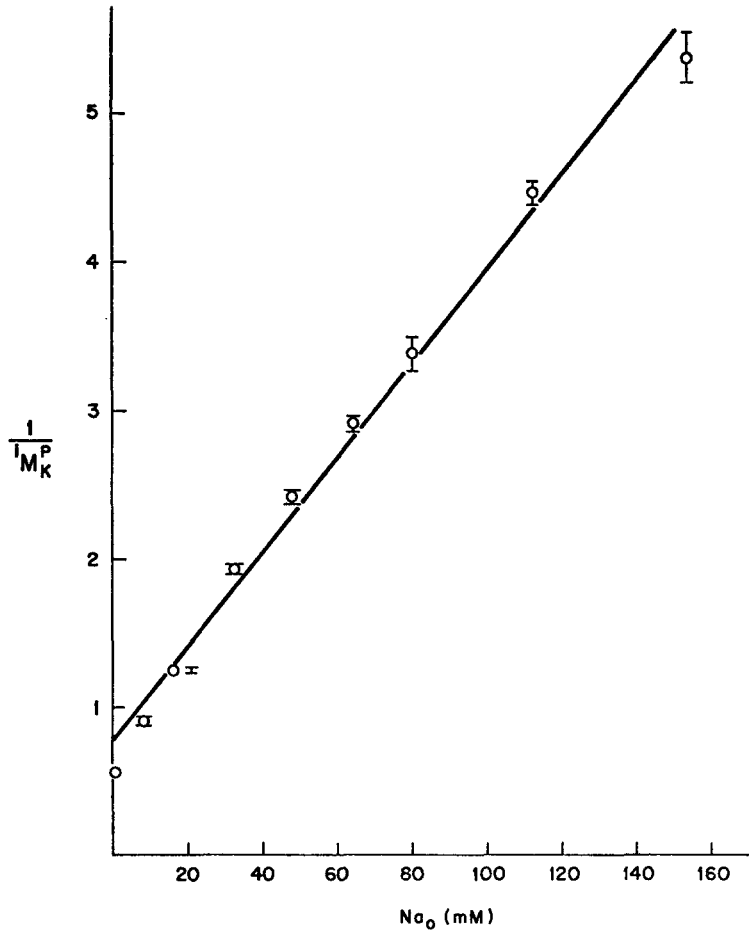

FIGURE 4. $1/{}^{i}M_K^P$ (millimoles/liter RBC·h)$^{-1}$ vs. $[Na_o]$ (mM). The cells used in the experiment contained Na_c 53.9 and K_c 6.8 mM RBC. NaCl was replaced by choline chloride; $[K_o]$ = 0.23 mM. ${}^{i}M_K^P$ was calculated as the difference between ${}^{i}M_K$ determined in strophanthidin-free solutions and ${}^{i}M_K$ determined under similar circumstances in solutions containing 10^{-4} M strophanthidin.

Concentration Dependence of Strophanthidin Inhibition

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

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

where C_1, C_2, C_3, and C_4 are constants. The equation predicts that, if $1/{}^{i}M_K^P$ 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 $1/{}^{i}M_{K}^{P}$ 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_o experiment, it is not possible to compare the two experiments since

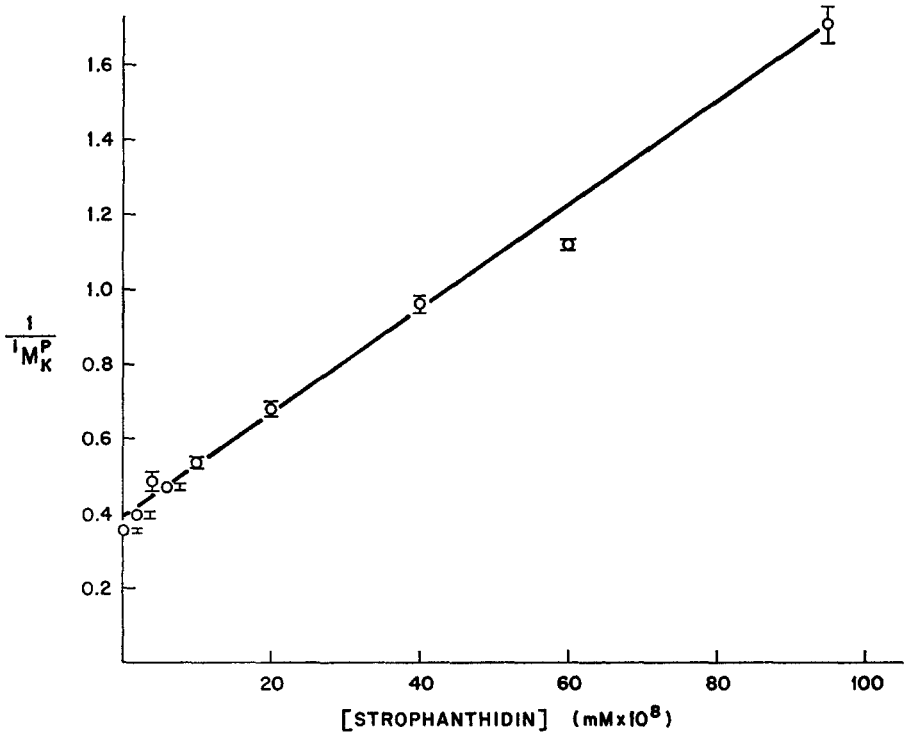

FIGURE 5. $1/{}^{i}M_{K}^{P}$ (millimoles/liter RBC·h)$^{-1}$ vs. [Strophanthidin] (mM $\times$ 10^8) in Na-free solutions. The cells contained Na_c 51.4 and K_c 1.3 mM RBC. The major extracellular cation was choline and $[K_o]$ = 1.02 mM.

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 $1/{}^{i}M_{K}^{P}$ 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 $1/{}^{i}M_{K}^{P}$ 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

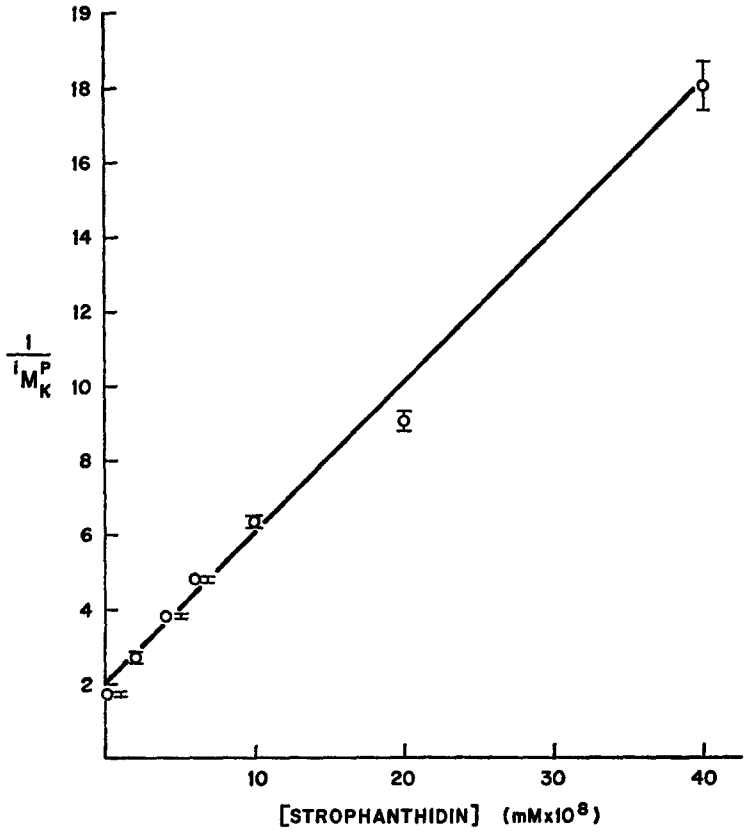

FIGURE 6. $1/{}^{i}M_K^P$ (millimoles/liter RBC·h)$^{-1}$ vs. [Strophanthidin] (mM $\times$ 10^8) in solutions containing Na. The cells contained Na_c 53.9 and K_c 1.35 mM RBC. $[Na_o]$ was 144 mM and $[K_o]$ 0.51 mM.

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_o 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_o seems, therefore, to be due primarily to the competition between Na_o and K_o 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_o 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_o 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 [^{3}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_o increases the rate of ouabain interaction with the pump.

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