

The Effects of Sodium and Potassium on Ouabain Binding by Human Erythrocytes

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ABSTRACT Ouabain binding by the human erythrocyte membrane is reversible, exhibits a high degree of chemical specificity, and can be detected at ouabain concentrations as low as 1×10^{-10} M. The relation between ouabain binding and ouabain concentration can be described by a rectangular hyperbola permitting determination of the maximal binding (B_{\max}) and the ouabain concentration at which ouabain binding is half-maximal (K_B). Reducing the external sodium concentration increased K_B , while reducing the external potassium concentration decreased K_B . Neither cation altered B_{\max} . The reciprocal of K_B was a linear function of the sodium concentration at sodium concentrations ranging from 0 to 150 mM. Conversely, the relation between the reciprocal of K_B and the external potassium concentration was nonlinear, and raising the potassium concentration above 4 mM produced no further increase in K_B . These results are compatible with a model which postulates that the erythrocyte membrane contains a finite number of receptors each composed of a glycoside-binding site and a cation-binding site. When sodium occupies the cation-binding site, the affinity of the glycoside site for ouabain is increased; when potassium occupies the cation-binding site the affinity of the glycoside site for ouabain is decreased.

INTRODUCTION

Cardiac glycosides act at the outer surface of the erythrocyte membrane to inhibit the transport of sodium and potassium (1, 2). In effecting this inhibition, these agents act in relatively low concentrations and show a high degree of chemical specificity (2). Furthermore, sodium and potassium are each capable of modifying the effects of cardiac glycosides on cation transport. Glynn (3) observed that the inhibition of potassium influx by glycosides was reduced as the concentration of external potassium was increased, and concluded that glycoside molecules and potassium ions competed with each other for the potassium transport sites. On the other hand, Hoffman (4) concluded that glycosides were noncompetitive inhibitors of cation transport because cesium could substitute for potassium in the cation transport process, but cesium could not replace potassium in altering the effects of cardiac glycosides

on cation transport. Beauge and Adragna (5) found that the inhibition of rubidium influx by ouabain depended on the extracellular concentrations of ouabain, sodium, and rubidium, and concluded that ouabain and sodium cooperated to favor inhibition while rubidium antagonized inhibition. Finally, ouabain has been demonstrated to bind to intact erythrocytes as well as to erythrocyte ghosts, and this binding was abolished by increasing the extracellular potassium concentration (6, 7). Binding of ouabain by erythrocyte ghosts was reduced by 21% when the sodium in the incubation solution was replaced with choline (7).

In the present study we have further characterized the relation between the extracellular concentrations of sodium and potassium and binding of ouabain by human erythrocytes, and have developed a model to describe the interactions among these three agents.

METHODS

Erythrocytes obtained from 12 normal male and female volunteers (18–32 yr of age) were washed three times in isosmotic choline chloride.

To determine ouabain binding erythrocytes were added to incubation solutions (prewarmed to 37°C unless otherwise specified) containing ouabain-³H. The hematocrit of the incubation mixture was 5–10%. After mixing thoroughly, triplicate 100 μl samples were taken at appropriate times, placed in polyethylene micro test tubes (Beckman Instruments Inc., Fullerton, Calif.), and washed five times with 300 μl of isosmotic choline chloride by alternate centrifugation and resuspension. Centrifugation was performed using a Microfuge (Beckman Instruments, Inc.) at 10,000 *g* for 15 sec. After the final wash, each sample was treated with 100 μl of 10% perchloric acid, agitated, and centrifuged for 30 sec. The tube and its contents were inverted and placed in a vial containing 20 ml of liquid scintillation solution. When the vial was capped and shaken the supernatant passed from the sample tube into the counting solution and the precipitate remained in the tip of the sample tube.

Using mannitol-³H or polyethylene glycol-¹⁴C as a marker for extracellular fluid, the amount of incubation medium remaining at the end of the final wash was less than 1×10^{-5} of that originally present.

At some time during the incubation triplicate 100-μl samples of the incubation mixture were added to 100 μl of 10% perchloric acid, agitated, centrifuged, inverted, and placed in a vial containing liquid scintillation solution. The volume of cells counted was calculated from the hemoglobin concentration of the incubation mixture and the hemoglobin concentration and hematocrit determined on a separate tube containing the incubation solution and erythrocytes at an hematocrit of approximately 25%. The hematocrit was measured using a Drummond microhematocrit centrifuge (Drummond Scientific Co., Broomall, Pa.) and hemoglobin concentration was measured using the cyanmethemoglobin method (8).

The amount of ouabain bound was calculated from the counts per milliliter of cells and the specific activity of ouabain in the incubation medium. Binding of radioactive impurities or entrapment of ouabain-³H was determined from the number of counts

bound in the presence of 10,000-fold molar excess of nonradioactive ouabain. The concentration of ouabain in the ouabain- ^3H supplied by the manufacturer was determined by measuring the binding of radioactivity in the presence of constant ouabain- ^3H and varying concentrations of nonradioactive ouabain (Fig. 1).

To verify that the cell-associated radioactivity which we have detected is, in fact,

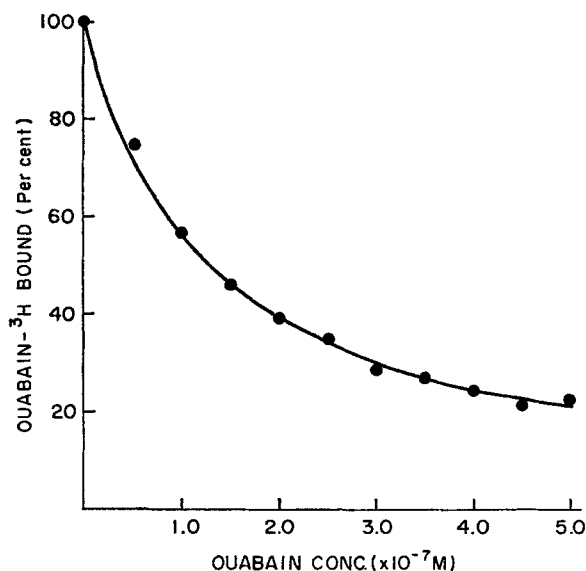


FIGURE 1. Determination of the ouabain concentration in ouabain- ^3H (New England Nuclear Corp., Lot No. 184-263). To each of a series of tubes containing erythrocytes and concentrations of nonradioactive ouabain ranging from 0 to 5×10^{-7} M was added an amount of ouabain- ^3H sufficient to produce maximal binding of radioactivity in the absence of added nonradioactive ouabain. Binding of radioactive impurities or entrapment of radioactivity was determined from the number of counts bound in the presence of 10^{-8} M nonradioactive ouabain. Ouabain- ^3H bound is expressed as the per cent of the counts bound in the absence of nonradioactive ouabain and each value has been corrected for the binding which occurred in the presence of 10^{-8} M nonradioactive ouabain. Each point was determined in quadruplicate. From the data presented here the ouabain- ^3H concentration in the incubation medium was $1.33 (\pm 0.09) \times 10^{-7}$ M. This is in close agreement with the value of 1.47×10^{-7} M which we calculated from the concentration of ouabain- ^3H given by the commercial supplier.

bound to the cell membrane, erythrocytes were incubated with ouabain- ^3H (1.13×10^{-7} M) at 37°C for 5 hr. The cells were washed five times with isosmotic saline and portions were taken for determination of radioactivity. The cells were hemolyzed by adding 20 vol of distilled water and centrifuged at 20,000 g for 20 min. Measurement of radioactivity in the sediment and supernatant indicated that less than 2% of the total ouabain- ^3H was present in the supernatant. In addition, at least 96% of the radioactivity in the sediment was extracted by 10% perchloric acid.

The standard incubation solution had the following composition (millimoles/liter):

NaCl, 150; KCl, 10; tris(hydroxymethyl)aminomethane (Tris) buffer (pH = 7.4), 10; glucose, 11.1. Whenever the concentration of sodium or potassium was decreased, an equimolar amount of choline was substituted.

Liquid scintillation counting was performed using 20 ml of a solution composed of 15 parts toluene (J. T. Baker Chemical Co., Phillipsburg, N. J.), 5 parts Triton X-100 (New England Nuclear Corp., Boston, Mass.) and 1 part Liquifluor (New England Nuclear Corp.). The observed counts were usually such that their standard deviation was less than 2%. Variation in quenching was monitored by using the ratio of counts in two channels produced by an automatic external standard; however, since in all cases the maximum range was less than 1.5%, no quench correction was made.

To explore the relation between ouabain binding and cation transport, potassium influx was measured on cells which had been incubated with or without ouabain-³H. At appropriate times triplicate samples were taken for determination of ouabain binding and an additional sample was removed and washed three times with 30 vol of cold (4°C) isosmotic choline chloride. To determine potassium influx these washed cells were added to incubation solutions (37°C) containing ⁴²K. The hematocrit was 4% or less. After mixing thoroughly, duplicate 100- μ l samples were placed in polyethylene micro test tubes and washed four times with 300 μ l of cold, isosmotic sodium chloride. After the final wash each sample was treated with 100 μ l of 10% perchloric acid, agitated, centrifuged, inverted, and placed in 20 ml of liquid scintillation fluid for counting. At some time during the incubation, triplicate 100- μ l samples of the incubation mixture (i.e. cells plus medium) were added to a micro test tube containing 100 μ l 10% perchloric acid, agitated, centrifuged, inverted, and placed in liquid scintillation solution. The volume of cells counted was calculated from the hemoglobin concentration in the incubation mixture and the previously measured hemoglobin content per volume of cells. Erythrocyte sodium and potassium concentrations were determined as described previously (9). The incubation solution used to determine potassium influx had the following composition (millimoles/liter): NaCl, 150; KCl, 15; Tris buffer (pH = 7.4), 10; glucose, 11.1. The sodium and potassium concentrations of the incubation solutions were also determined at the end of the incubation period. Initially the uptake of ⁴²K was determined at 0, 15, 30, and 45 min; however, since the uptake was observed to be constant over this period, potassium influx was calculated from samples taken at zero and 40 min. Potassium influx was calculated using the method described by Sachs and Welt (10) and the average of the potassium concentrations in the incubation solutions at 0 and 40 min. All counts were corrected for decay. Sodium and potassium concentrations were measured with an Instrumentation Laboratory model 143 flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass.).

Digoxin was kindly supplied in crystalline form by Dr. Stanley T. Bloomfield, Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y. All other reagents were of the highest grade of purity obtainable. Ouabain-³H (Lot No. 184-263, specific activity 11.7 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass., and radiochemical purity was greater than 97% by the supplier's radiochromatographic and reverse isotope dilution criteria. ⁴²K was obtained as the chloride from International Chemical and Nuclear Corporation, (ICN), Burbank, Calif.

RESULTS

Fig. 2 illustrates the binding of ouabain- ^3H to human erythrocytes in the presence of 150 mM sodium as a function of time and temperature. Decreasing the temperature from 37° to 25°C reduced the rate but not the steady-state value of the binding reaction. In the presence of 1×10^{-7} M ouabain, the relation between ouabain binding and hematocrit was linear for hematocrit values ranging from 4 to 30%. Adding a 1000-fold molar excess of nonradioactive ouabain to cells which had been incubated in the presence of ouabain- ^3H for 1 hr demonstrates the reversibility of the binding reaction (Fig. 3).

Fig. 4 illustrates the effects of altering the external sodium and potassium

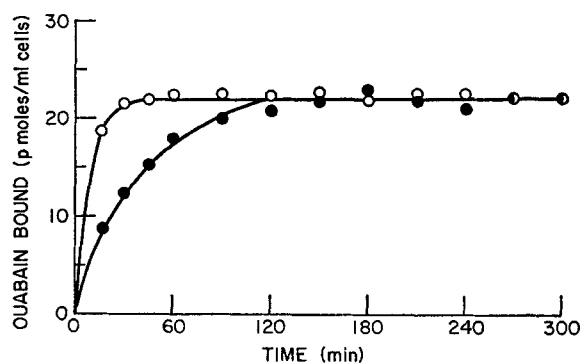


FIGURE 2. Time-course of ouabain binding by human erythrocytes. The incubation solution contained sodium, 150 mM and ouabain, 1.7×10^{-7} M. Open circles represent binding at 37°C; closed circles represent binding at 25°C. Each point represents the mean of four experiments.

concentrations on the binding of ouabain to human erythrocytes as a function of time. Replacing the external sodium with choline reduced both rate of the binding reaction and the steady-state value. Addition of 10 mM potassium to the choline medium produced a further decrease in both the rate of the binding reaction and the steady-state value. Unless otherwise specified all subsequent incubations were for at least 3 hr.

Fig. 5 illustrates the correlation between the time-course of ouabain binding and that of the change in potassium influx observed for cells incubated in the presence of ouabain- ^3H . To explore further the relation between ouabain binding and changes in cation transport, potassium influx and the amount of ouabain bound were determined using cells which were incubated for 3 hr in the presence of different concentrations of ouabain- ^3H , and using cells which were incubated with one concentration of ouabain- ^3H for different periods of time. Fig. 6 illustrates the progressive decrease in potassium influx observed with increasing amounts of ouabain bound. Furthermore, the relation between ouabain bound and potassium influx in cells incubated

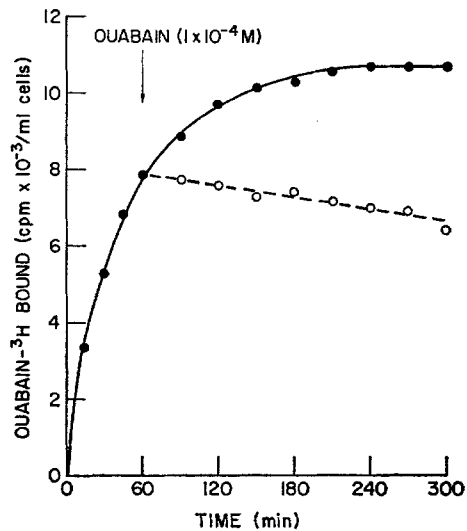


FIGURE 3. Effect of addition of nonradioactive ouabain on ouabain- ^3H binding by human erythrocytes. The incubation solution contained choline, 150 mM and ouabain- ^3H , 9.6×10^{-8} M. After 1 hr nonradioactive ouabain (1×10^{-4} M) was added to one of two incubation tubes and the amount of bound radioactivity was measured over the subsequent 4 hr (open circles). Closed circles represent binding of ouabain- ^3H in solutions to which nonradioactive ouabain was not added. Each point is the mean of three separate experiments.

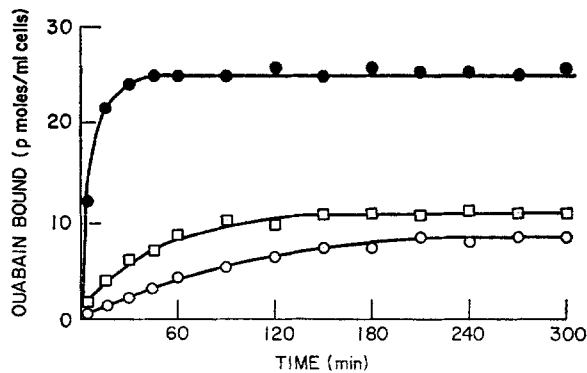


FIGURE 4. Effects of altering the cation composition of the incubation medium on the time-course of ouabain binding. The composition of the incubation media was sodium, 150 mM (closed circles); choline, 150 mM (boxes); or choline, 140 mM plus potassium, 10 mM (open circles). The ouabain concentration in all media was 1.7×10^{-7} M. Each point represents the mean of five experiments.

for 3 hr in different ouabain concentrations was similar to that for cells incubated for different times in the presence of one concentration of ouabain- ^3H .

There was a nonlinear relation between ouabain binding and ouabain

concentration (Fig. 7) and ouabain binding showed a tendency to saturate at higher concentrations. A double reciprocal plot of these data (Fig. 8) gave a straight line with a nonzero intercept. Ouabain binding can be related to

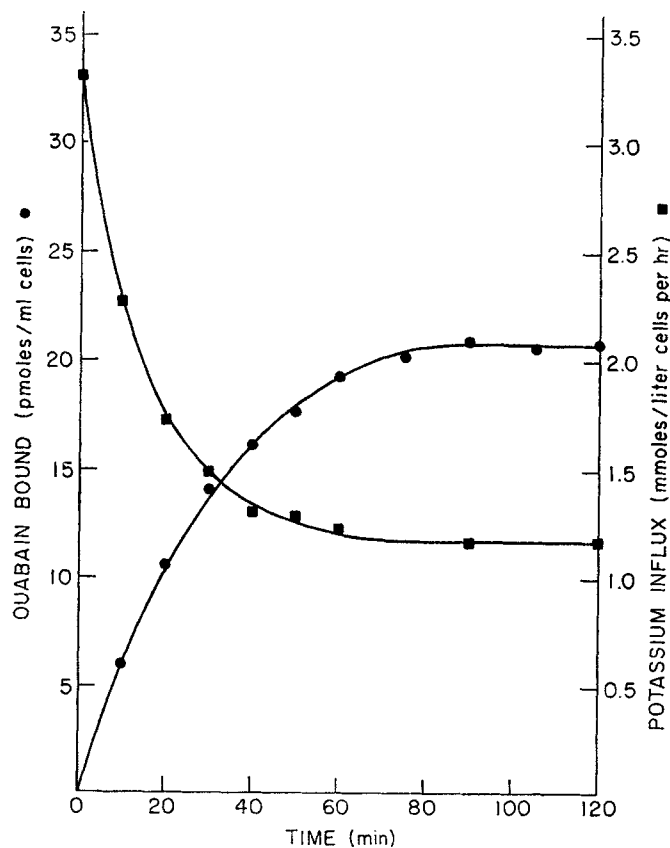


FIGURE 5. Time-course of ouabain binding and of potassium influx in human erythrocytes. Erythrocytes were preincubated (Hct = 3%) for 1 hr in a "potassium-free" solution containing sodium, 150 mM. At the end of this period ouabain- ^3H (final concentration 6.9×10^{-8} M) was added to the incubation mixture. At the indicated times samples were taken for determination of ouabain binding (circles) and potassium influx (boxes). The potassium concentration in the incubation solution used to determine potassium influx was 15.4 mM. The erythrocyte sodium and potassium concentrations (mmoles/liter cells) at the end of the preincubation period were 9.4 and 82.3, respectively. This experiment was representative of three others.

ouabain concentration by the equation

$$A_B = \frac{B_{\max}[A]}{K_B + [A]}, \quad (1)$$

where A_B is the amount of ouabain bound, B_{\max} is maximum ouabain bind-

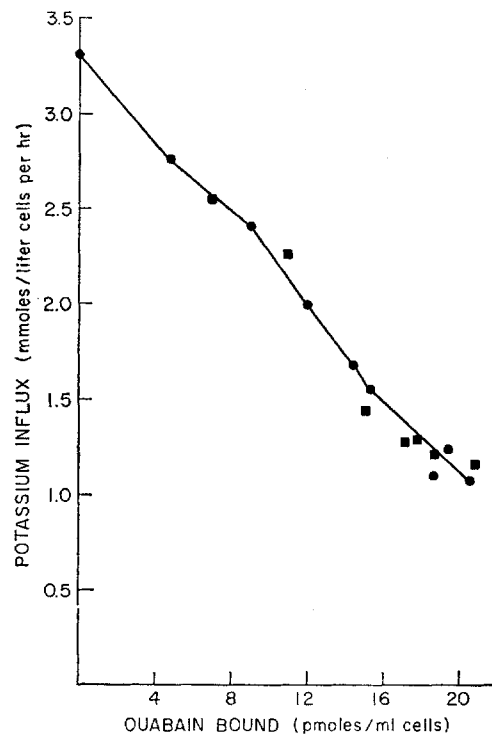


FIGURE 6. Potassium influx as a function of ouabain bound for human erythrocytes. Circles represent values determined on cells which had been incubated for 3 hr in "potassium-free" solutions containing sodium, 150 mM, and various concentrations of ouabain-³H. Boxes represent values determined on cells which had been incubated for varying periods of time in a "potassium-free" solution containing sodium, 150 mM, and ouabain-³H, 8.4×10^{-8} M. The potassium concentration in the incubation solution used to determine potassium influx was 15.6 mM. This experiment was representative of two others.

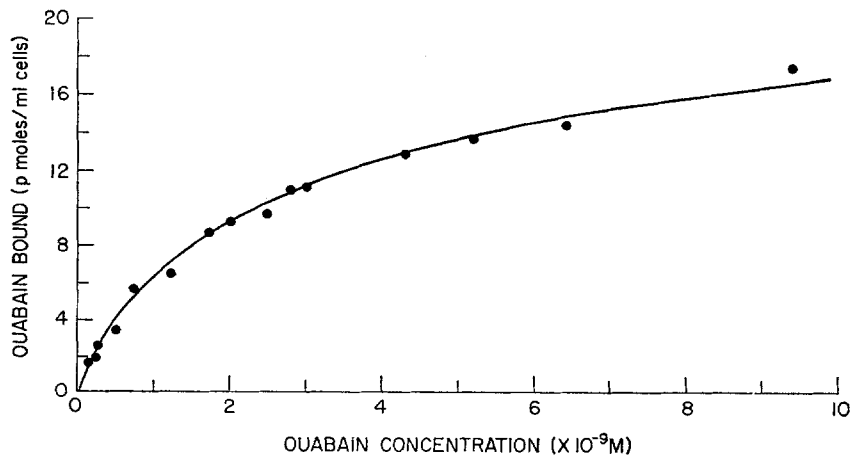


FIGURE 7. Ouabain binding as a function of ouabain concentration. The incubation solution contained sodium, 150 mM; each point represents the mean of five experiments.

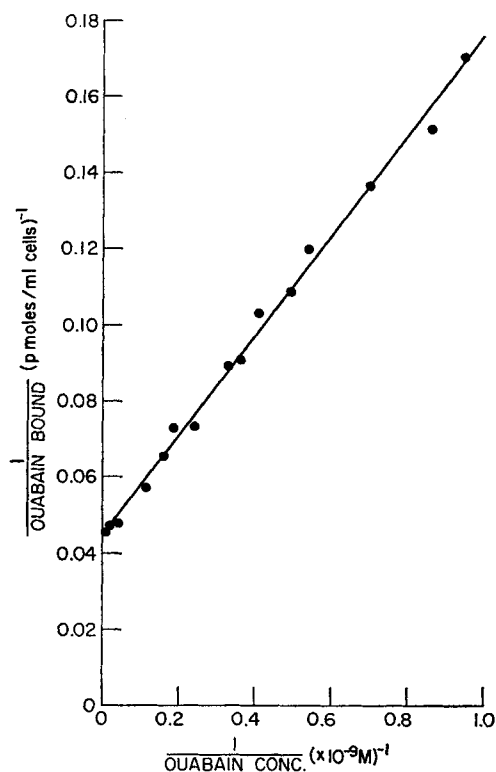


FIGURE 8. Double reciprocal plot of ouabain binding as a function of ouabain concentration. The incubation solution contained sodium, 150 mM; each point represents the mean of five experiments.

ing, $[A]$ is the ouabain concentration in the incubation medium, and K_B is the ouabain concentration at which binding is half maximal. Alternative methods (11) of plotting the data in Fig. 8 did not result in significantly different values for K_B and B_{max} . Furthermore, ouabain binding at ouabain concentrations ranging from 1×10^{-10} to 1×10^{-5} M indicated that only one class of binding sites is involved in this reaction.

Table I indicates that the ouabain binding site also has an affinity for digitoxin and its aglycone, but not for other structurally similar steroids or digitoxose. Fig. 9 illustrates the inhibition of ouabain-³H binding produced by ouabain, digoxin, and digitoxin. The inhibition constant, K_I , for ouabain was $2.96 (\pm 0.49) \times 10^{-9}$ M, SD and was in good agreement with the value calculated from the data illustrated in Fig. 8. The K_I values for digoxin and digitoxin were $3.46 (\pm 0.65)$ and $4.94 (\pm 0.91) \times 10^{-9}$ M, respectively, and indicate that these glycosides have a lower affinity than ouabain for the erythrocyte membrane (12).

To explore the effect of sodium, ouabain binding was measured at different

TABLE I
SPECIFICITY OF OUABAIN BINDING BY HUMAN
ERYTHROCYTES

Incubation medium	Ouabain- ³ H bound*
	%
Control	100±8
+ Ouabain	-0.7±4.1‡
+ Digitoxin	0.9±3.2‡
+ Digitoxigenin	0.4±4.3‡
+ Digitoxose	104±0.3
+ Progesterone	107±8
+ Testosterone	98±6
+ 17β-Estradiol	103±5
+ Cortisone	94±9
+ Hydrocortisone	96±7
+ Aldosterone	101±8

Erythrocytes were incubated for 3 hr at 37°C in a K-free solution (Na = 150 mM) containing ouabain-³H, 1×10^{-9} M. All glycosides and steroids were dissolved in 95% ethanol, and the ethanol concentration in all incubation media was 0.5% (v/v).

All steroids and digitoxose were present at 1×10^{-5} M.

* Values expressed as per cent of ouabain-³H bound in control (counts per minute/milliliter cells) and represent the mean of four experiments ± 1 sd.

‡ Significantly different from control ($P < 0.01$).

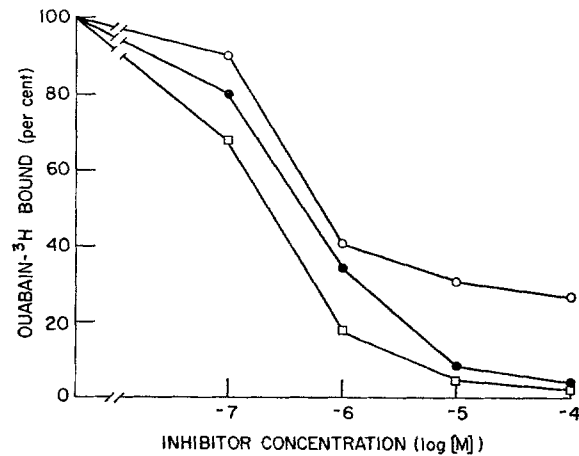


FIGURE 9. Effects of three cardiac glycosides on ouabain-³H binding by human erythrocytes. Ouabain-³H bound is expressed as the per cent of the counts bound in the absence of inhibitor. The incubation medium contained sodium, 150 mM and ouabain-³H 1.7×10^{-7} M. The inhibitors used were ouabain (boxes), digoxin (closed circles), and digitoxin (open circles). Each point represents the mean of four experiments.

ouabain and sodium concentrations in potassium-free media (potassium less than 0.4 mM). As is illustrated in Fig. 10, double reciprocal plots of ouabain bound as a function of ouabain concentration gave a straight line with a common nonzero intercept for each sodium concentration studied. As the sodium concentration in the incubation medium was increased, the K_B for ouabain binding decreased without a significant change in B_{\max} . Fig. 11 illustrates the direct, linear relation between the apparent affinity with which ouabain is bound ($1/K_B$) and the sodium concentration in the incubation medium.

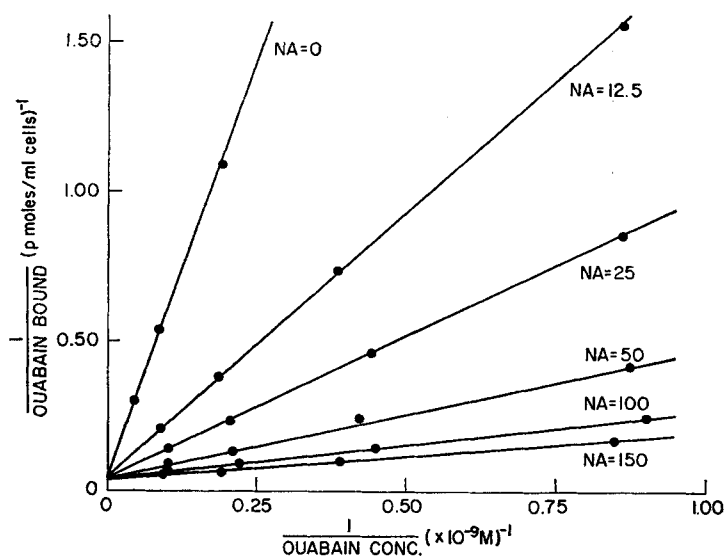


FIGURE 10. Double reciprocal plots of ouabain bound as a function of ouabain concentration at different sodium concentrations. Each point represents the mean of three experiments.

To explore the effect of potassium, ouabain binding was measured at different ouabain and potassium concentrations in sodium-free media (sodium less than 0.9 mM). As is illustrated in Fig. 12, double reciprocal plots of ouabain bound as a function of ouabain concentration gave a straight line with a common nonzero intercept for each potassium concentration studied. As the potassium concentration in the incubation medium was increased to 4 mM, the K_B for ouabain binding increased without a significant change in B_{\max} . At external potassium concentrations greater than 4 mM, there was no significant change in K_B . Fig. 13 illustrates the inverse, non-linear relation between the apparent affinity with which ouabain is bound ($1/K_B$) and the potassium concentration in the incubation medium. These

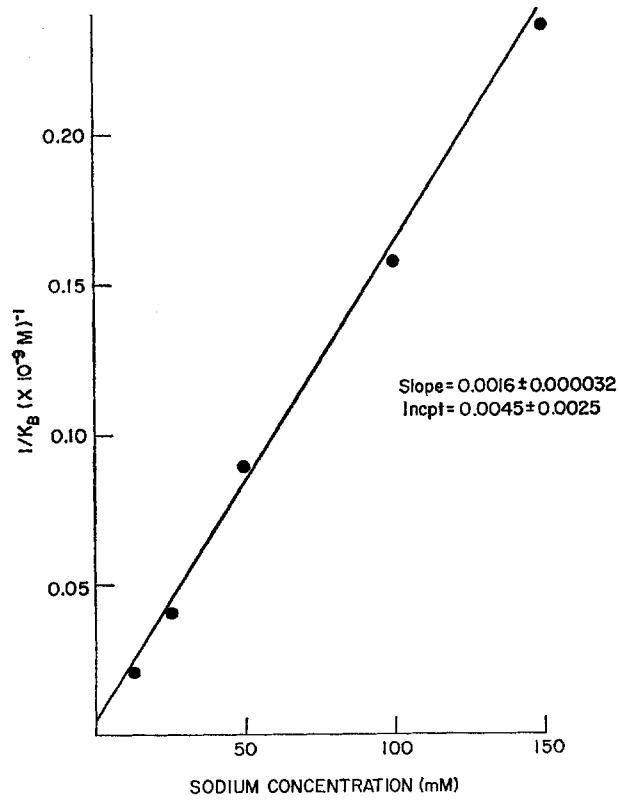


FIGURE 11. Effect of sodium concentration on the apparent affinity ($1/K_B$) with which ouabain is bound. The values were calculated from the data in Fig. 8.

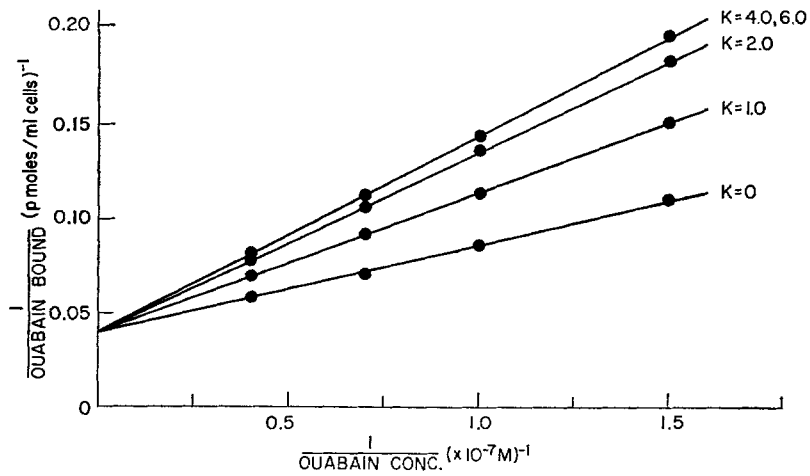


FIGURE 12. Double reciprocal plots of ouabain bound as a function of ouabain concentration at different potassium concentrations. Each point represents the mean of six experiments.

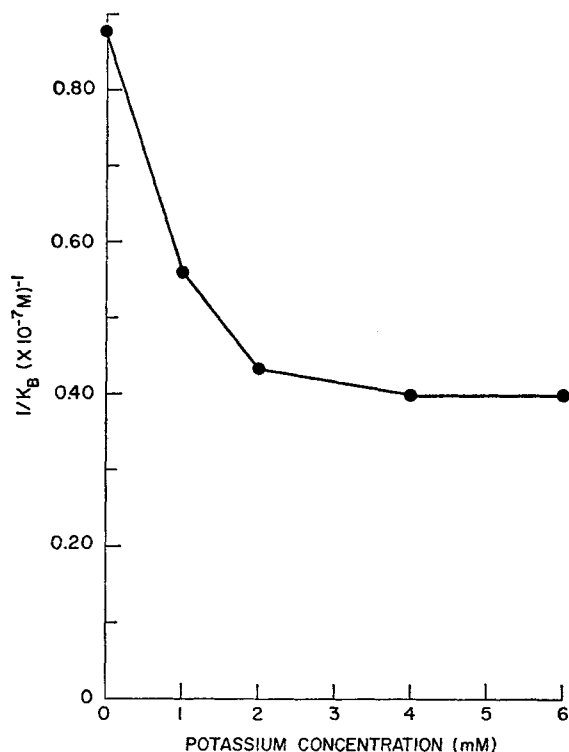


FIGURE 13. Effect of potassium concentration on the apparent affinity ($1/K_B$) with which ouabain is bound. The values were calculated from the data in Fig. 10.

data demonstrate that there is an external potassium concentration above which there is no further decrease in the affinity with which ouabain is bound to the erythrocyte membrane.

To explore further the interactions among sodium, potassium, and ouabain, ouabain binding was measured at two different ouabain concentrations and four different concentrations of sodium and potassium (Fig. 14). At each of the two ouabain concentrations studied, the stimulation of ouabain binding produced by sodium was reduced in the presence of potassium. Furthermore, the higher the potassium concentration, the greater the reduction of the sodium effect and at a ouabain concentration of 1×10^{-8} M, 50 mM potassium abolished the stimulation of ouabain binding produced by adding 100 mM sodium to the medium. At a ouabain concentration of 3.4×10^{-7} M, the inhibition of ouabain binding produced by external potassium decreased with increasing sodium concentrations. In contrast, at a ouabain concentration of 1×10^{-8} M, the inhibition of ouabain binding produced by potassium increased with increasing sodium concentrations. These data indicate that at relatively low concentrations of sodium and ouabain the inhibition of ouabain

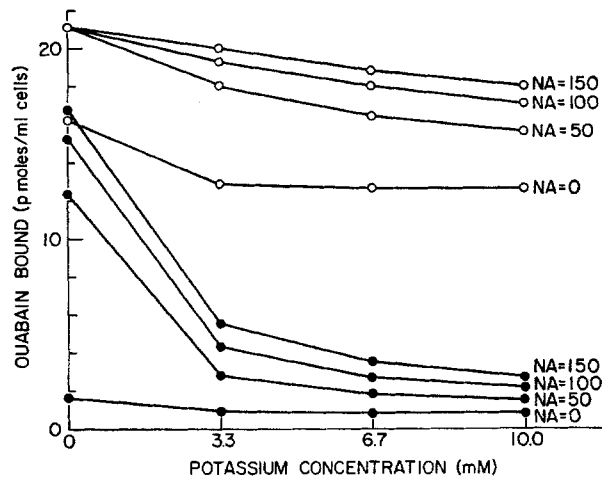


FIGURE 14. Effects of altering the sodium and potassium concentrations on ouabain binding at two different ouabain concentrations. The ouabain concentrations were 3.4×10^{-7} M (open circles) and 1×10^{-8} M (closed circles). Each point represents the mean of five experiments.

binding produced by potassium will initially increase with increasing concentrations of either ouabain or sodium, but as the concentration of either ouabain or sodium is increased further the effect of potassium will reach a maximum and begin to diminish. At a ouabain concentration of 5×10^{-7} M, 150 mM sodium abolished the inhibition of ouabain binding produced by adding 2 mM potassium to the incubation medium.

DISCUSSION

Ouabain binding to the membrane of human erythrocytes can be detected at ouabain concentrations as low as 1×10^{-10} M and this binding is reversible and exhibits a high degree of chemical specificity. Others have demonstrated that similar features characterize the effect of ouabain on erythrocyte cation transport (3, 4). The rate of binding, but not the amount bound at the steady state, is reduced by decreasing the incubation temperature from 37° to 25° C. On the other hand both the rate of ouabain binding and the amount bound at the steady state are increased by adding sodium to the incubation medium and both are decreased by adding potassium to the incubation medium.

Some of our observations differ from those published previously by others. In contrast to the finding of Hoffman and Ingram (6) that ouabain binding could be abolished by raising the external potassium concentration to 30 mM, we found that raising external potassium decreased but did not abolish ouabain binding. The specific activity of the ouabain- 3 H used in the studies

of Hoffman and Ingram (6) was approximately 5% of that used in the present studies (0.54 Ci/mmmole versus 11.7 Ci/mmmole). These authors also used a relatively low ouabain concentration (10^{-9} M) and measured binding to erythrocytes by the disappearance of radioactivity from the supernatant. Thus, the method may not have been sufficiently sensitive to detect ouabain binding in the presence of potassium. Furthermore, we can exclude the possibility that radioactivity bound to erythrocytes in the presence of 10 mM potassium represents the binding of radioactive impurities since this binding is abolished in the presence of a 10,000-fold molar excess of nonradioactive ouabain.

Hoffman and Ingram (6) also stated that ouabain molecules continued to be bound to the erythrocyte after the "sodium-potassium pump" was inhibited completely. These ouabain molecules which were bound after potassium influx was maximally inhibited were thought to be bound "non-specifically" in the sense that they were not associated with further inhibition of potassium influx. Conversely, we found good agreement between the time-course of ouabain binding and that of the change in potassium influx (Fig. 5). Furthermore, we observed a similar relation between potassium influx and ouabain binding whether cells were incubated for 3 hr in different ouabain concentrations or incubated for different times in the presence of one concentration of ouabain (Fig. 6).

Using the criterion of complete inhibition of the sodium-potassium pump, Hoffman has calculated the number of ouabain binding sites per cell to be approximately 250 (6, 7); however, assuming an erythrocyte volume of $87 \mu^3$ (13) our data (Table II, Figs. 8, 10, and 12) indicate that there are approximately 1200 ouabain binding sites per cell and this latter value agrees with the indirect estimation of Glynn (3). Assuming that the value of 250 reported

TABLE II
PARAMETERS FOR MODEL DESCRIBING EFFECTS OF
SODIUM AND POTASSIUM ON OUABAIN BINDING
BY HUMAN ERYTHROCYTES

K_1	$1.38 (\pm 0.35) \times 10^{-7}$ M
K_2	5.00×10^{-10} M*
K_3	$2.55 (\pm 0.48) \times 10^{-7}$ M
K_4	$918 (\pm 217) \times 10^{-8}$ M
K_5	$2.81 (\pm 0.47) \times 10^{-8}$ M
K_6	$0.276 (\pm 0.167) \times 10^{-3}$ M
K_7	$0.616 (\pm 0.276) \times 10^{-3}$ M
B_{max}	21.6 ± 2.83 pmoles/ml cells

Each value represents the mean of six separate experiments \pm SD.

* This value is the maximum value which K_2 can have without producing a significantly nonlinear relation ($P < 0.05$) between the extracellular sodium concentration and $1/K_B$ a potassium-free medium.

by Hoffman and Ingram (6) was calculated from data obtained using the method described by Dunham and Hoffman in their study of sheep erythrocytes (14) there are several potential sources for the discrepancy between the calculated values for the number of ouabain binding sites per cell. These sources appear to be attributable to differences in the method of extracting bound radioactivity from the cells, in the composition of the liquid scintillation mixture and the procedure used to correct for variable counting efficiency.

Dunham and Hoffman (14) used Bray's solution (15) to extract bound radioactivity from the cells, while we have used perchloric acid. Fig. 15

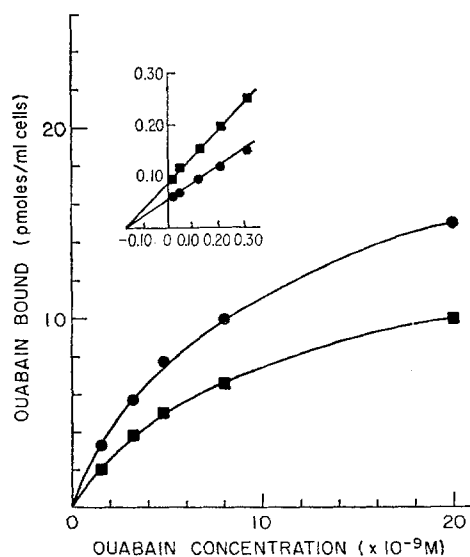


FIGURE 15. Ouabain binding as a function of ouabain concentration. The incubation solution contained sodium, 150 mM; each point represents the mean of three experiments. Circles represent values obtained using 10% perchloric acid; boxes represent values obtained using Bray's solution (15). *Insert:* Double-reciprocal plot of ouabain binding as a function of ouabain concentration. Units for ordinate and abscissa are the reciprocals of those given in the main figure.

depicts data obtained by incubating cells in the presence of various concentrations of ouabain- ^3H and extracting with Bray's solution or 10% perchloric acid. At the end of the incubation period three 100- μl samples were washed and processed as described in Methods. Three other samples taken from the same incubation mixture were processed in a similar fashion except 100 μl of Bray's solution was used instead of perchloric acid. Three samples of the incubation mixture were added to 100 μl of 10% perchloric acid and three other samples were added to 100 μl of Bray's solution.

The values obtained using Bray's solution were approximately 60% of those obtained using perchloric acid. Similar experiments performed using

300 μl of Bray's solution to extract the cells gave values which were not significantly different from those obtained using 100 μl of Bray's solution. The double-reciprocal plot in Fig. 15 illustrates that the effect of extracting with Bray's solution is to decrease the value for ouabain binding capacity from 19.6 pmoles/ml cells to 11.8 (or from 1026 molecules/cell to 618) without altering the ouabain concentration at which ouabain binding is half-maximal ($6.25 \times 10^{-9} \text{ M}$). We believe, however, that it is important to make the point that this discrepancy between our calculated value for the number of ouabain binding sites per cell and the value calculated by Hoffman and Ingram (6) does not affect the major results or conclusions of the present study.

In considering possible models to describe the effects of sodium and potassium on ouabain binding by human erythrocytes, several experimental observations must be taken into account. At the various concentrations of sodium and potassium studied, the relation between ouabain binding and ouabain concentration can be described by a rectangular hyperbola. Addition of sodium to the incubation medium increases the affinity ($1/K_B$) of ouabain binding while addition of potassium decreases the affinity. Neither cation alters the maximum binding capacity (B_{max}). There is a linear relation between the affinity ($1/K_B$) of ouabain binding and the external sodium concentration at sodium concentrations ranging from 0 to 150 mM. Conversely, the relation between the affinity and the external potassium concentration is nonlinear, and raising the potassium concentration above 4 mM produced no further increase in K_B . At a given ouabain concentration, the magnitude of the effect of sodium or potassium on ouabain binding varies depending on the concentration of the other cation and each can abolish the effect of the other. The effects of both sodium and potassium on ouabain binding can be abolished by using a sufficiently high concentration of ouabain in the incubation medium.

A model which will satisfy these requirements is illustrated in Fig. 16. We

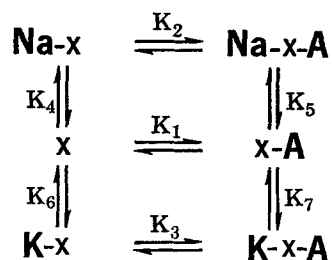


FIGURE 16. Model system describing the effects of sodium and potassium on ouabain binding by human erythrocytes. X, Na, K, and A represent receptor, sodium, potassium, and ouabain, respectively. K_i are the dissociation constants for the various substrate-receptor interactions.

have assumed that the erythrocyte membrane contains a finite number of receptors each composed of two sites: a glycoside-binding site and a cation-binding site. We have assumed that one receptor site can associate reversibly with one glycoside molecule and the other with one cation. In the absence of sodium or potassium in the incubation medium, ouabain combines reversibly with the glycoside-binding site. When sodium occupies the cation-binding site, the affinity of the glycoside site for ouabain increases; when potassium occupies the cation-binding site, the affinity of the glycoside site for ouabain decreases.

The relation between the amount of ouabain bound (A_B) and the extracellular ouabain concentration $[A]$ is given by equation 1 in the Results section. As is discussed in more detail in the Appendix, when sodium and/or potassium is present in the incubation medium, the ouabain concentration at which binding is half-maximal (K_B) is given by the expression

$$K_B = \frac{K_1 K_2 K_3 (K_6 [\text{Na}] + K_4 [\text{K}] + K_4 K_6)}{K_1 K_3 K_6 [\text{Na}] + K_1 K_2 K_4 [\text{K}] + K_2 K_3 K_4 K_6} \quad (2)$$

Table II summarizes the values for the parameters of the model illustrated in Fig. 16.

The possibility that potassium is acting as a competitive inhibitor of ouabain at the glycoside-binding site can be excluded by the observation that there is a potassium concentration above which there is no further inhibition of ouabain binding, i.e., potassium behaves as a partially competitive inhibitor (12). The possibility that sodium combines with the cation-binding site only if the glycoside site is occupied (i.e. K_2 and $K_4 = 0$) requires a linear relation between $[\text{Na}]$ and $1/K_B$; however, it is not compatible with the observation that sodium increases the rate of the ouabain binding reaction even as early as 3 min. The observed linear relation between $1/K_B$ and the external sodium concentration up to 150 mM can be attributed to a low affinity of the cation-binding site for sodium when the glycoside site is unoccupied.

As is depicted in Fig. 16, the proposed model predicts that when the glycoside site is occupied, the affinity of the cation site for sodium is increased and for potassium is decreased. We have not tested this prediction experimentally and cannot exclude the possibility that cations bind to the cation site only when the glycoside site is unoccupied (i.e., K_5 and $K_7 = 0$). However, as is discussed in the Appendix, the other five parameters can be evaluated independently of K_5 and K_7 and do not change if K_5 and K_7 are zero. That is, insofar as ouabain binding is concerned, the validity with which the proposed model describes experimental phenomena does not require evaluation of K_5 and K_7 . Another possibility which we cannot exclude from the present studies is that the effects of sodium and potassium are not exerted on the

erythrocyte membrane but on the ouabain molecules themselves. Sodium and potassium may alter the structural configuration of the ouabain molecule in such a way that its affinity for the erythrocyte membrane is altered.

The relation between the ouabain binding which we have characterized in the present study and the effects of ouabain on the transport of cations across the erythrocyte membrane remains to be determined. It is clear, however, that the three previously delineated, functionally distinct pathways for ouabain-sensitive sodium outflux (16) are not reflected in the characteristics of ouabain binding to the erythrocyte membrane. Presumably, the different components of ouabain-sensitive cation transport reflect inherent properties of the transport apparatus or the mechanism through which ouabain binding affects cation transport rather than properties of the ouabain binding system itself.

APPENDIX

The derivation of the equations describing the model depicted in Fig. 16 is based on the assumption that the erythrocyte membrane contains a finite number of receptors each composed of a glycoside-binding site and a cation-binding site which can associate reversibly with one glycoside molecule and one cation, respectively.

At the steady state, the concentrations of sodium, potassium, ouabain, free receptors, and occupied receptors are related by a series of dissociation constants.

$$\begin{aligned}
 K_1 &= \frac{[X][A]}{[XA]} & K_2 &= \frac{[NaX][A]}{[NaXA]} & K_3 &= \frac{[KX][A]}{[KXA]} \\
 K_4 &= \frac{[Na][X]}{[NaX]} & K_5 &= \frac{[Na][XA]}{[NaXA]} & K_6 &= \frac{[K][X]}{[KX]} \\
 K_7 &= \frac{[K][XA]}{[KXA]},
 \end{aligned}$$

where X = receptor; A = ouabain; Na = sodium; K = potassium.

Letting B_{max} represent the total concentration of receptors, the amount of ouabain bound (A_B) is given by the equation

$$A_B = pB_{max} \tag{1 a}$$

where

$$p = \frac{([XA] + [NaXA] + [KXA])}{([X] + [NaX] + [KX] + [XA] + [NaXA] + [KXA])}. \tag{2 a}$$

Substituting the relations given by the dissociation constants one obtains

$$A_B = \frac{B_{max}[A]}{[A] + K_B}, \tag{3 a}$$

where

$$K_B = \frac{K_1 K_2 K_3 (K_6 [\text{Na}] + K_4 [\text{K}] + K_4 K_6)}{K_1 K_3 K_6 [\text{Na}] + K_1 K_2 K_4 [\text{K}] + K_2 K_3 K_4 K_6}. \quad (4a)$$

When sodium is absent from the medium

$$K_B = \frac{K_1 K_3 ([\text{K}] + K_6)}{K_1 [\text{K}] + K_3 K_6}. \quad (5a)$$

When the potassium concentration is zero, $K_B = K_1$ and as the potassium concentration increases the value of K_B approaches K_3 .

Similarly, when potassium is absent from the medium

$$K_B = \frac{K_1 K_2 ([\text{Na}] + K_4)}{K_1 [\text{Na}] + K_2 K_4}. \quad (6a)$$

When the sodium concentration is zero, $K_B = K_1$ and as the sodium concentration is raised the value of K_B approaches K_2 . Furthermore, if $K_4 \gg [\text{Na}]$, there is a linear relation between $[\text{Na}]$ and $1/K_B$ since

$$1/K_B = \frac{[\text{Na}]}{K_2 K_4} + \frac{1}{K_1}. \quad (7a)$$

As is evident from the dissociation equations

$$K_1 K_5 = K_2 K_4 \quad (8a)$$

and

$$K_1 K_7 = K_3 K_6, \quad (9a)$$

therefore, any two of these seven constants can be calculated from the other five.

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REFERENCES

- SCHATZMANN, H. J. 1953. Herzglykoside als Hematoffe für den aktiven Kalium und Natrium Transport durch die Erythrocytenmembran. *Helv. Physiol. Pharmacol. Acta.* **11**:346.
- GLYNN, I. M. 1964. The action of cardiac glycosides on ion movements. *Pharmacol. Rev.* **16**:381.
- GLYNN, I. M. 1957. The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol. (Lond.)* **136**:148.
- HOFFMAN, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Am. J. Med.* **41**:666.
- BEAUGE, L. A., and N. ADRAGNA. 1971. The kinetics of ouabain inhibition and the partition of rubidium influx in human red blood cells. *J. Gen. Physiol.* **57**:576.
- HOFFMAN, J. F., and C. J. INGRAM. 1968. Cation transport and the binding of T-ouabain to intact human red blood cells. Proceedings of the First International Symposium on Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes. Vienna. 420.

7. HOFFMAN, J. F. 1969. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. Gen. Physiol.* **54**:343 s.
8. DAVIDSOHN, I., and B. B. WELLS. 1963. *Clinical Diagnosis by Laboratory Methods*. W. B. Saunders Company, Philadelphia. 73.
9. GARDNER, J. D., A. LAPEY, A. P. SIMOPOULOS, and E. L. BRAVO. 1971. Abnormal membrane sodium transport in Liddle's syndrome. *J. Clin. Invest.* **50**:2253.
10. SACHS, J. R., and L. G. WELT. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46**:65.
11. DOWD, J. E., and D. S. RIGGS. 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.* **240**:863.
12. DIXON, M., and E. C. WEBB. 1964. *Enzymes*. Academic Press, Inc., New York. 315.
13. DAVIDSOHN, I., and B. B. WELLS. 1963. *Clinical Diagnosis by Laboratory Methods*. W. B. Saunders Company, Philadelphia. 948.
14. DUNHAM, P. B., and J. F. HOFFMAN. 1971. Active cation transport and ouabain binding in high potassium and low potassium red blood cells of sheep. *J. Gen. Physiol.* **58**:94.
15. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279.
16. SACHS, J. R. 1970. Sodium movements in the human red blood cell. *J. Gen. Physiol.* **56**:322.