Kinetics and Thermodynamics of Ouabain Binding by Intact Turkey Erythrocytes

Effects of External Sodium Ion, Potassium Ion, and Temperature

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ABSTRACT The kinetics of association and dissociation for the ouabain-Na^+,K^+-dependent ATPase complex have been studied in intact turkey erythrocytes as a function of external Na^+ concentration, K^+ concentration, and temperature. At free ligand concentrations substantially exceeding the concentration of available binding sites, the association reaction exhibits pseudo-first-order kinetics with an association rate constant (k_1) that is conveniently determined over a wide range of temperatures (5–37°C). The dissociation reaction exhibits strict first-order kinetics with a dissociation rate constant (k_{-1}) that has the unusual property, in the turkey cell, of being sufficiently great to permit its direct determination even at temperatures as low as 5°C. Values for the equilibrium binding constant for the ouabain-ATPase complex (K_A) predicted from the ratio of the association and dissociation rate constants agree closely with independently measured values of K_A determined directly under conditions of equilibrium binding. K_A is a sensitive function of the composition of the external ionic environment, rising with increasing Na^+ concentration and falling with increasing K^+ concentration. These changes in K_A are shown to be quantitatively attributable to changes in the rate constant k_1, k_{-1} in contrast being unaffected at any given temperature by even very large changes in Na^+ or K^+ concentration. Arrhenius plots of k_1 and k_{-1} both yield straight lines over the entire temperature range corresponding to activation energies for association and dissociation of 29.5 and 24.2 kcal/mol, respectively. These observations have made it possible to calculate the following standard values for the ouabain binding reaction in the presence of 150 mM Na^+: $\Delta G^\circ = -9.8$ kcal/mol; $\Delta H^\circ = +5.3$ kcal/mol; $\Delta S^\circ = +48.7$ cal/degree/mol. The large positive value of ΔS° presumably reflects a highly ordered configuration of the ouabain-free ATPase molecule that is lost upon ouabain binding and that "drives" the reaction despite the positive value of ΔH°.

INTRODUCTION

Active sodium and potassium ion transport in erythrocytes is mediated by a Na^+,K^+-dependent ATPase that is specifically inhibitable by the cardiac

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glycoside ouabain (1–4). Erythrocytes bind ouabain with high affinity (5), the active transport of sodium and potassium ions is tightly coupled (6), and glycoside binding has been shown to be linearly related to inhibition of active potassium ion accumulation (5, 7–9). Examination of the characteristics of equilibrium binding in both human and turkey erythrocytes has demonstrated that the affinity with which glycoside is bound by intact cells is markedly influenced by ambient monovalent cation concentrations, affinity being enhanced by external sodium ion (5) and reduced by external potassium ion (5, 9).

The affinity of the human erythrocyte for tritiated ouabain is high ($\sim 3 \times 10^8\ M^{-1}$ in the absence of external potassium ion [5] and is in part attributable to the exceedingly slow rate at which ouabain dissociates once it has been bound. The latter rate for human cells is in fact so slow that accurate determination of the dissociation rate constant is difficult at 37°C and is impracticable at lower temperatures. A further consequence of this slow dissociation rate is that measurement of equilibrium binding of [^{3}H]ouabain at nonsaturating concentrations of ligand also becomes impracticable at lower temperatures because of the excessive time required to attain equilibrium between free and bound ligand. Uncertainty as to the temperature dependence of equilibrium binding has, in turn, precluded direct estimation of the standard enthalpy of binding ($\Delta H°$) in these cells.

The recent observation that, in contrast, erythrocytes from the domestic turkey bind [^{3}H]ouabain with somewhat lower affinity than do human cells, and, in particular, that the characteristics of this binding are such that association and dissociation rate constants for ligand binding are amenable to direct observation (9), now makes it possible to study directly the kinetics of ligand binding and dissociation as a function of temperature and, furthermore, to compare the equilibrium binding constant calculated from the ratio of these values with direct and independent measurements of equilibrium ligand binding as a function of both temperature and external ligand concentration. Examination of the effects of changes in external cation concentration on such measurements in intact erythrocytes (as contrasted with observations on broken-cell membrane preparations or partially purified preparations of the ATPase) moreover permits one to determine the effects of isolated "unifacial" changes in the ionic environment on both the kinetics and thermodynamics of glycoside binding. These observations form the basis of the present report.

With regard to the physiological significance of the binding examined in the studies that follow, it is important to emphasize that, as is known to be the case for the binding of glycoside to the human erythrocyte (5, 7, 8), specific binding of glycoside to the turkey cell—here defined as the binding of [^{3}H]-ouabain that is displaceable by a large excess of nonradioactive glycoside—is directly related to binding of the glycoside to Na^+,K^+-ATPase units: at all external concentrations of ouabain and at all extenal potassium ion concentrations at which [^{3}H]ouabain binding is directly measurable, fractional inhibition of ouabain-sensitive potassium influx and fractional occupancy of the ~4,500 ouabain binding sites per turkey erythrocyte have recently been shown to be identical (9).

MATERIALS AND METHODS

Materials

White female turkeys weighing 12–15 lb were obtained from local sources (The Butcher, Bronx, N. Y.). The birds were fed a regular poultry diet (Layena Complete Animal Feed, Ralston Purina Co., St. Louis, Mo.), and were provided with water *ad libitum.* Ouabain was obtained from Sigma Chemical Co., St. Louis, Mo. [^{3}H]Ouabain (12–20 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

Preparation of Washed Erythrocytes

Heparinized blood (generally a 5-ml sample) was obtained by syringe from a wing vein. After centrifugation at 400 *g* for 10 min, the plasma was removed and the erythrocytes were resuspended in incubation buffer containing 150 mM NaCl, 10 mM KCl, 11.1 mM glucose, and 10 mM Tris at pH 7.4. After resedimentation of the erythrocytes and two further washes, the cells were resuspended in incubation buffer and kept on ice unless otherwise indicated. In experiments in which effects of varying concentrations of sodium and potassium ion were subsequently to be studied, the concentrations of NaCl and KCl were reduced and replaced by equimolar concentrations of choline chloride.

Binding of [^{3}H]Ouabain

Suspensions of washed erythrocytes were added to glass scintillation vials containing incubation medium and [^{3}H]ouabain either in the presence or absence of an additional displacing concentration of nonradioactive ouabain as indicated below. Stock [^{3}H]-ouabain in 9:1 ethanol:benzene was evaporated to dryness under nitrogen and redissolved in buffer before use, and a known concentration of nonradioactive ouabain was added to reduce the specific activity of the radioligand to a range of 1.5–3.5 Ci/mmol. The vials were then capped, and the resulting suspensions, at a final hematocrit ranging between 3 and 5%, were incubated in a shaking water bath at the desired temperature. Ouabain binding to erythrocyte membranes was determined by transferring duplicate 100-μl aliquots of incubation mixture to 5 ml of distilled water at 0°C, vortexing the mixture, and trapping the hemolyzed erythrocyte membranes on Gelman A/E glass fiber filters (Gelman Instrument Co., Ann Arbor, Mich.). The filters were washed four times with additional 5-ml aliquots of iced distilled water and transferred to glass scintillation vials, and radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) after the addition of 10 ml of Bray's solution (10). Under such conditions, specific binding, defined as the difference in radioligand binding in the absence and presence of a displacing concentration (0.5–1.0 mM) of added nonradioactive ouabain, is linear with erythrocyte concentration, and agreement between duplicate samples is excellent, differences rarely exceeding 5%.[1] At free ligand concentrations markedly exceeding the concentration of total binding sites, the concentration of free ligand remains essentially constant during the binding reaction, and initial rates of binding exhibit strict pseudo-first-order kinetics (Table I). All experiments

[1] Control experiments in which the hemolyzed erythrocyte membranes were instead trapped by filtration over Millipore mixed-cellulose-ester filters (1.2-μm pore size; Millipore Corp., Bedford, Mass.), or in which binding was determined by dilution and subsequent centrifugation of intact cells in a Beckman Microfuge (Beckman Instruments, Palo Alto, Calif.) followed by three washes with nonradioactive incubation medium, yielded results identical to those obtained by means of the Gelman filters. The latter filters were chosen for standard use because of the convenience of the filtration method and the rapid filtration rates obtainable.

included Scatchard (11) analyses performed in parallel to determine the total concentration of binding sites present.

Unless otherwise noted, dissociation kinetics were examined after the addition of a large excess of nonradioactive ouabain (1 mM) to suspensions of erythrocytes that had been incubated with a near-saturating concentration of [^{3}H]ouabain (0.8–0.9 μM) for 30 min at 37°C. Where indicated, parallel studies were performed in which the dissociation of [^{3}H]ouabain was induced instead by dilution of the radioligand either in the presence or absence of added nonradioactive ouabain; in such studies, after preincubation with [^{3}H]ouabain, erythrocytes were chilled to 4°C, sedimented, and washed five times with choline buffer before being resuspended in incubation medium at the desired temperature.

Hemoglobin and Hematrocrit Determinations

Hematocrits (percent packed red blood cell volumes) were measured in capillary tubes by means of an Adams microhematocrit centrifuge (Clay-Adams, Inc., New York, N. Y.). Hemoglobin was measured by the cyanmethemoglobin method (12).

TABLE I

KINETICS OF [^{3}H]OUABAIN BINDING AS A FUNCTION OF FREE RADIOLIGAND CONCENTRATION

[Ou]	$t_{1/2}$	$k_1 = \frac{\ln 2}{t_{1/2}\,[Ou]}$
M	*min*	$min^{-1}\,M^{-1}$
8.1×10^{-8}	5.1	1.7×10^{6}
1.7×10^{-7}	2.2	1.9×10^{6}
3.2×10^{-7}	1.2	1.8×10^{6}
8.1×10^{-7}	0.49	1.7×10^{6}

Incubations were performed in the presence of 150 mM Na^+ and 0.11–0.12 mM K^+ at 37°C, and the binding of [^{3}H]ouabain was determined as a function of time as described under Methods. At free ligand concentrations markedly exceeding the concentration of total binding sites, the initial rate of binding at a given temperature and erythrocyte concentration is strictly proportional to free radioligand concentration. [Ou], [^{3}H]ouabain concentration; $t_{1/2}$, observed half-time for disappearance of empty binding sites; k_1, pseudo-first-order association rate constant.

Potassium concentrations in incubation media were checked at the end of each incubation in an Instrumentation Laboratory (Boston, Mass.) Model 143 flame photometer.

RESULTS

Kinetics of Association and Dissociation; Equilibrium Binding

Fig. 1 illustrates the kinetics of ouabain binding by turkey erythrocytes at 11°, 20°, and 37°C in the presence of 150 mM Na^+, 0.5 mM K^+, and 0.85 μM [^{3}H]ouabain. Binding is rapid, and, at 20°C or higher, equilibrium between free and bound radioligand is attained within 15 min. Fig. 2 *A* and Table I show that at all three temperatures the initial rate of binding exhibits pseudo-first-order kinetics, the absolute and net rates of association being equal and closely proportional to free ligand concentration and to the number of

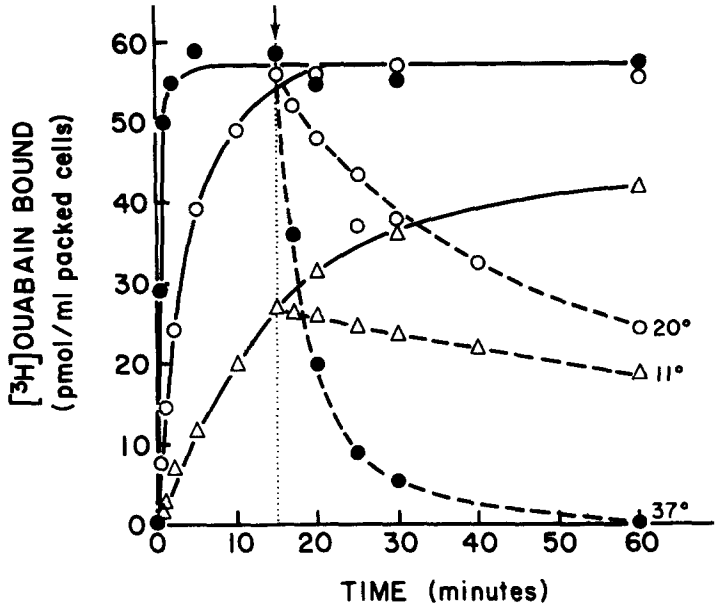

FIGURE 1. Binding and release of [^{3}H]ouabain from turkey erythrocytes as a function of temperature and time. Erythrocytes were incubated in the presence of 150 mM Na^+, 0.5 mM K^+, and 8.5×10^{-7} M [^{3}H]ouabain as described under Methods. After 15 min of incubation, nonradioactive ouabain (1 mM) was added to one set of samples at each temperature, and the rate of loss of previously bound radioactive ouabain was then followed as a function of time. The ordinate shows the amount of specifically bound [^{3}H]ouabain per milliliter of packed erythrocytes.

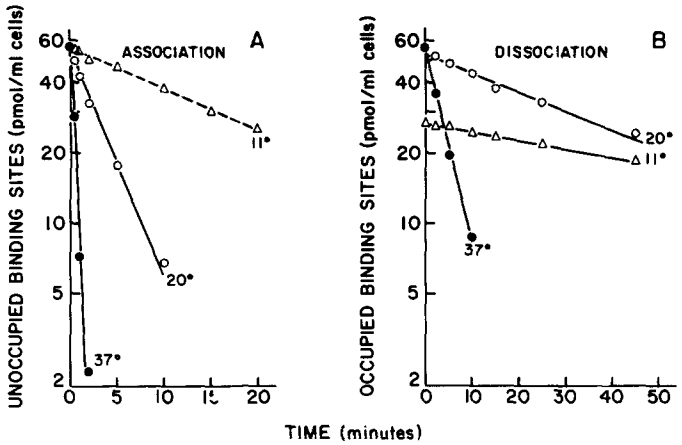

FIGURE 2. (*A*) Semilogarithmic plot of the binding of [^{3}H]ouabain as a function of time and temperature in the presence of 8.5×10^{-7} M radioligand (data from the experiment shown in Fig. 1). The ordinate shows the number of residual unoccupied binding sites as a function of time after exposure to the radioligand. (*B*) Semilogarithmic plot of the loss of [^{3}H]ouabain binding after the addition of excess (1 mM) nonradioactive ouabain.

unoccupied sites until the number of occupied sites (and, hence, the absolute rate of dissociation) is no longer negligible. The subsequent addition of a large excess of nonradioactive ouabain results in a prompt loss of radioligand binding that obeys strict first-order kinetics (Fig. 2 *B*). If dissociation of radioligand is induced instead by dilution of free radioligand, the rate of dissociation is found to be identical both in the presence and absence of a large excess (1 mM) of added nonradioactive ouabain (see below).

It is of interest to compare the rate constants for the association and dissociation reactions for turkey erythrocytes with the corresponding values previously observed for human red cells. At 37°C, the values for k_1 and k_{-1} in Fig. 2 are 7.7×10^5 min^{-1} M^{-1} and 0.16 min^{-1}, respectively, whereas the

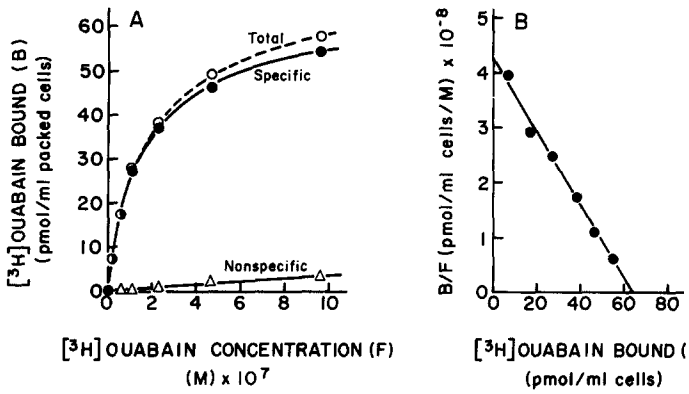

FIGURE 3. (*A*) Equilibrium binding of [^{3}H]ouabain by turkey erythrocytes as a function of radioactive ligand concentration in the presence of 150 mM Na^+ and 0.5 mM K^+ at 37°C. Specific binding (●) was defined as the difference between radioligand binding in the absence (○) and presence (△) of a displacing concentration of added nonradioactive ouabain (5×10^{-4} M). (*B*) Scatchard analysis (11) of the data in *A*. The linear plot indicates a single class of ouabain binding sites with an affinity (K_A) of 5.6×10^6 M^{-1} for ouabain ($K_D = 1.8 \times 10^{-7}$ M). Extrapolation of the line to infinite ouabain concentration indicates a total of 64 pmol of binding sites per milliliter of packed erythrocytes; the estimated number of sites per cell is 4,500 ± 200 (9). *B*, picomoles of [^{3}H]-ouabain bound per milliliter of packed erythrocytes; *F*, free [3H]ouabain concentration (M).

corresponding values for ouabain binding by human erythrocytes under similar conditions can be estimated to be 5.3×10^5 min^{-1} M^{-1} (5) and ~0.001 min^{-1} (5, 7). The corresponding difference of approximately two orders of magnitude in ouabain binding affinity between turkey and human erythrocytes predicted from the ratio k_1/k_{-1} in these kinetic experiments is borne out directly by equilibrium binding studies performed at various free ouabain concentrations: the dissociation constant (K_D) for the ouabain-erythrocyte complex calculated directly from the Scatchard analysis shown in Fig. 3 is 1.8×10^{-7} M (for comparison, the ratio k_{-1}/k_1 in Fig. 2 is 2.1×10^{-7} M), whereas the corresponding value for human erythrocytes under similar con-

ditions of temperature and extracellular electrolyte concentrations can be calculated to be 3.5×10^{-9} M (5). Although k_1 is slightly greater for turkey than for human erythrocytes, k_{-1} is greater by a factor of ~150, and it is the latter difference that largely accounts for the strikingly higher equilibrium dissociation constant for the ouabain-ATPase complex in turkey cells.

A particularly useful consequence of the rapid dissociation rate of the turkey erythrocyte-ouabain complex is that the dissociation rate constant, in contrast to that for the human erythrocyte-ouabain complex, is amenable to direct measurement over a wide range of temperatures. Thus, k_1 and k_{-1} can both be determined directly, and corresponding values of K_D can be calculated from the ratio k_{-1}/k_1, even at low temperatures where direct determination of K_D by equilibrium binding measurements at low free ligand concentrations is no longer feasible. In addition to permitting the calculation of a variety of thermodynamic parameters of interest, such measurements, performed under conditions of various cation concentrations, permit one to determine the effects of unifacial changes in ambient monovalent cation concentration on membrane-ligand association rates and dissociation rates, and, hence, to investigate independently the relative contributions of changes in k_1 and k_{-1} in accounting for the recognized dependence of K_D upon external sodium and potassium ion concentrations (5, 9).

Association Kinetics: Effects of External Sodium and Potassium Ion

Earlier studies have shown that the equilibrium dissociation constant for the human erythrocyte-ouabain complex is a strong function of both external sodium and potassium ion concentrations, binding affinity being enhanced by the former, and reduced by the latter, cation (5). The dissociation constant for the turkey erythrocyte-ouabain complex is similarly markedly influenced by changes in external sodium and potassium ion concentrations. Fig. 4 *A* shows that, in the presence of 150 mM sodium ion, an increase in external potassium ion concentration from 0.5 to 5.5 mM results in a 15–20-fold increase in K_D from 1.3×10^{-7} to 2.1×10^{-6} M; conversely, Fig. 4 *B* shows that, in the presence of a fixed low concentration of potassium ion, an increase in external sodium ion concentration from 0.2 to 150 mM results in a 20-fold decrease in K_D from 3.6×10^{-6} to 1.2×10^{-7} M.

To determine to what extent these changes in dissociation constant defined under conditions of equilibrium binding might be specifically attributable to changes in the formation rate for the ouabain-ATPase complex, association kinetics were examined at varying ambient cation concentrations. Fig. 5 *A* and *B* shows that association kinetics are indeed markedly affected, and Fig. 6 *A* and *B* presents simultaneous plots of independently determined values for K_A ($= 1/K_D$) and k_1 as functions of external potassium ion and sodium ion concentrations, respectively. Fig. 6 *A* shows that a rise in external potassium ion concentration results in a marked decrease in the association rate constant k_1, and, furthermore, that this decrease is strictly parallel to the fall in K_A itself as determined independently under equilibrium conditions at various external radioligand concentrations. Fig. 6 *B* shows, conversely, that k_1 rises

with rising external sodium ion. The rise is again parallel with that of K_A, and, as reported for human erythrocytes by Gardner and Conlon (5), K_A is seen to be linear with external sodium ion concentration.

Dissociation Kinetics: Independence of Ambient Sodium and Potassium Ion Concentrations

The preceding observation that changes in k_1 induced by changes in external sodium and potassium ion concentrations are precisely paralleled over a range of at least 20-fold by quantitatively identical changes in K_A itself suggested that the modifications in K_A resulting from changes in the external ionic

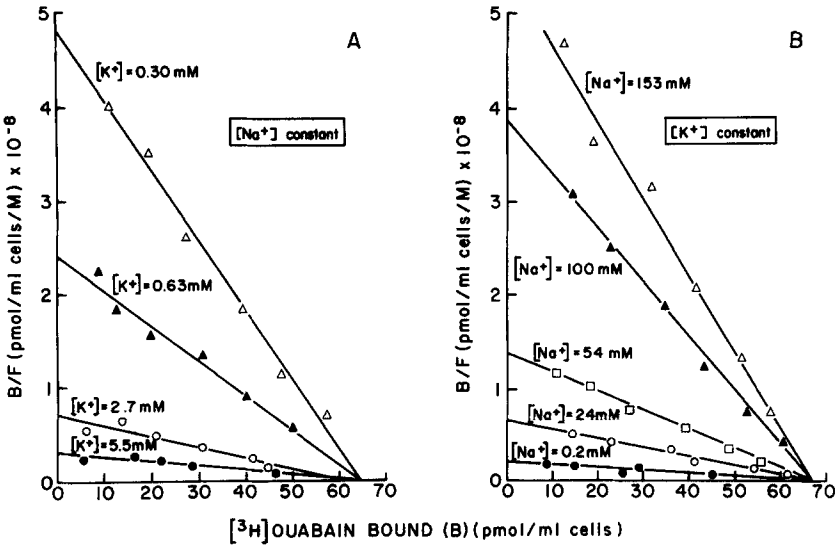

FIGURE 4. Equilibrium binding of [3H]ouabain by turkey erythrocytes at 37°C as a function of external ligand concentration and varying external concentrations of sodium and potassium ion. (*A*) $[Na^+]$ constant (150 mM): As $[K^+]$ is increased from 0.3 to 5.5 mM, K_D increases from 1.3×10^{-7} to 2.1×10^{-6} M; (*B*) $[K^+]$ constant (0.2–0.3 mM): As $[Na^+]$ is increased from 0.2 to 153 mM, K_D decreases from 3.6×10^{-6} to 1.2×10^{-7} M. *B*, picomoles of $[^3H]$ouabain bound per milliliter of packed erythrocytes; *F*, free $[^3H]$ouabain concentration (M).

environment are mediated exclusively by changes in association rate. Direct confirmation of this conclusion is provided in Fig. 7, which shows that the kinetics of dissociation for the membrane-ouabain complex are entirely unaffected by even enormous changes in external sodium and potassium ion concentrations. Despite the marked changes in k_1 induced by even small changes in the external ionic environment, k_{-1} is seeen to remain invariant, at a given temperature, over a wide range of independent changes in concentration (from ~0 to 150 mM) for both sodium and potassium ion. Fig. 7 also indicates that the dissociation rate after dilution of radioligand is independent of whether or not a large excess (1 mM) of nonradioactive ouabain is present.

Temperature-Dependence of Association and Dissociation Rate Constants; Energies of Activation; Relation between Calculated and Observed Equilibrium Constants; Calculation of Free Energy, Enthalpy, and Entropy of Ouabain Binding

As indicated above, the kinetics of ouabain association and dissociation are such in the turkey erythrocyte that both can be studied directly and with convenience over a wide range of temperatures. Typical data are illustrated in Fig. 8, which shows that over a temperature range of from 5 to 37°C both association and dissociation reactions display simple first-order (or, more

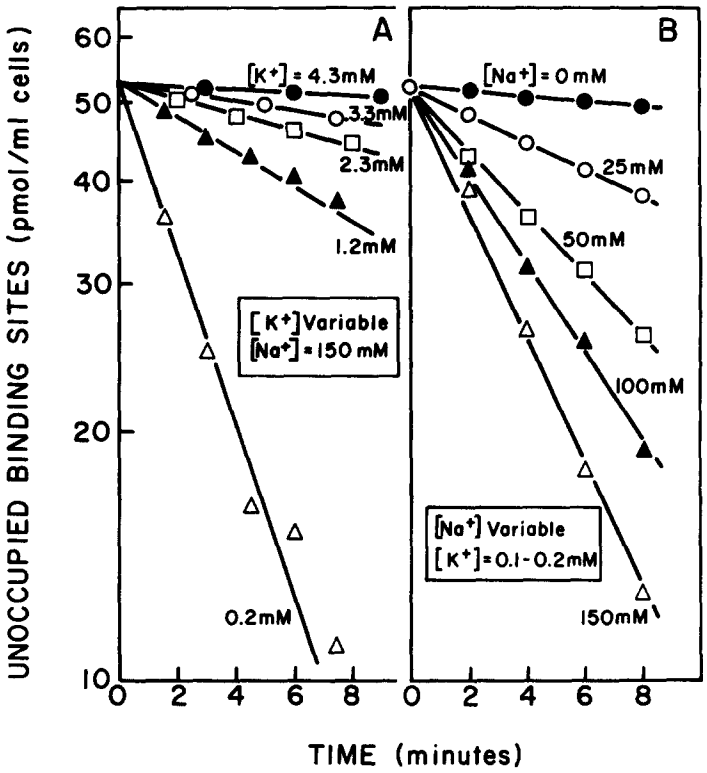

FIGURE 5. Association kinetics at 20°C and in the presence of 9.2×10^{-7} M [^{3}H]ouabain as a function of external sodium and potassium ion concentrations. Association rate is markedly decreased in the presence of potassium ion (*A*) and increased in the presence of sodium ion (*B*).

strictly, in the instance of association, pseudo-first-order) kinetics from which one can readily calculate k_1 and k_{-1}, respectively. Arrhenius plots of these latter values are shown in Fig. 9. Both plots yield single straight lines over the entire temperature range, without a detectable break in slope. Calculated activation energies for the association and dissociation reactions are 29.5 and 24.2 kcal/mol, respectively. The difference in the slopes of the two lines indicates that binding in fact becomes somewhat "tighter" at higher temperatures and leads to a calculated (positive) ΔH° of 5.3 kcal/mol. From this

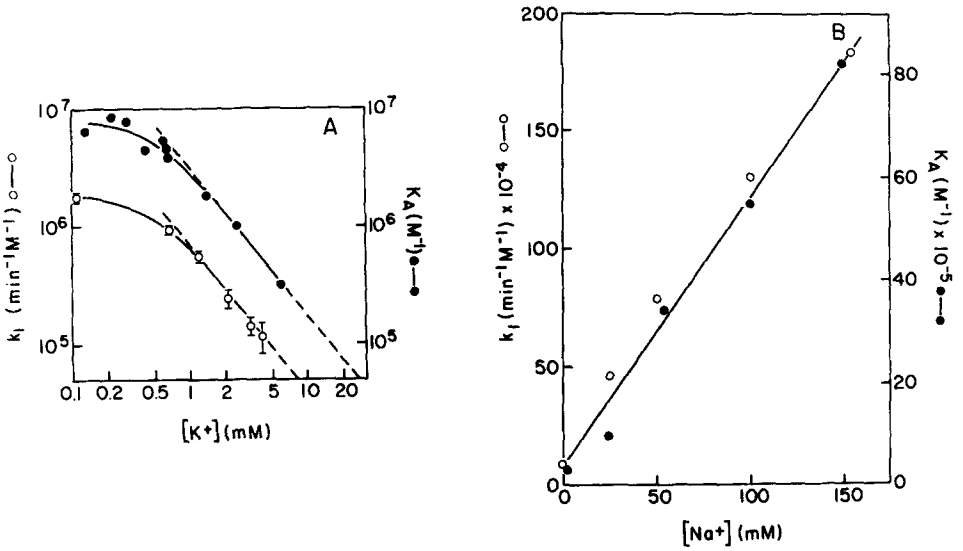

FIGURE 6. Simultaneous plots of independently determined values for K_A and k_1 as functions of external potassium ion and sodium ion concentrations at 37°C. (*A*) Double-logarithmic plot of K_A and k_1 against potassium ion concentration (sodium ion concentration constant at 150 mM). At all concentrations of potassium ion, the ratio of K_A to k_1 is constant; at high concentrations of potassium ion, both plots become linear and intersect the abscissa at an angle of 45° (i.e., have slopes of −1; see Discussion). (*B*) Plot of K_A and k_1 against sodium ion concentration (potassium ion concentration constant at 0.2–0.3 mM). Both K_A and k_1 increase linearly and in parallel with sodium ion over the entire concentration range examined (0–150 mM). Points with vertical bars indicate means ± 1 SEM; points without bars indicate the results of individual experiments.

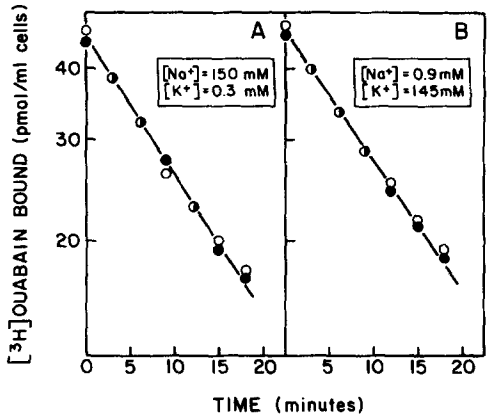

FIGURE 7. Kinetics of dissociation (30°C) induced by dilution of radioligand either in the presence (●) or absence (○) of added nonradioactive ouabain (1 mM). Note that the kinetics of dissociation are entirely uninfluenced by extreme variations in external cation concentrations (*A* vs. *B*) or by the presence of a large excess of nonradioactive ligand.

latter value, and a measured K_D of 1.3×10^{-7} M at 37°C, the $\Delta G°$ and $\Delta S°$ for the ouabain binding reaction in the presence of 150 mM sodium ion are calculated to be −9.8 kcal/mol and +48.7 cal/degree/mol, respectively. Agreement between K_D as directly measured by equilibrium binding (at temperatures where this is feasible) and as calculated indirectly by the ratio k_{-1}/k_1 is good, the two values never differing from each other by more than a factor of 2 (corresponding to differences of <0.5 kcal/mol in the estimation of $\Delta G°$).

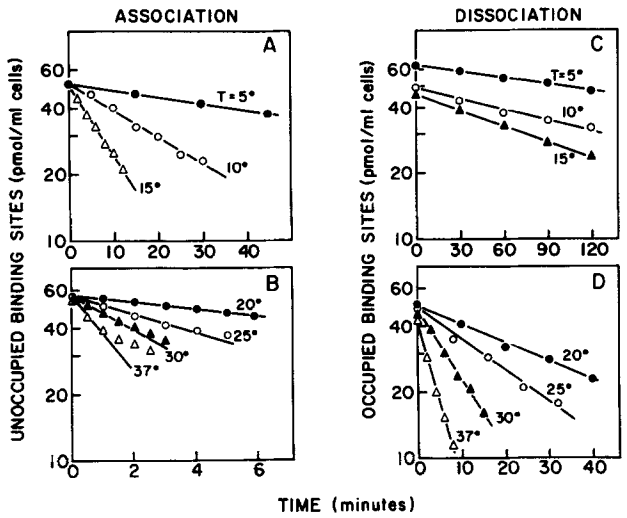

FIGURE 8. Kinetics of association (*A* and *B*) and dissociation (*C* and *D*) as a function of temperature in the presence of 150 mM Na^+ and 0.1–0.2 mM K^+. In *A* and *B* the concentration of free [^{3}H]ouabain was 8.9×10^{-7} M and 2.1×10^{-7} M, respectively. At the latter concentration (only slightly greater than K_D itself), departure from linearity becomes evident at relatively low levels of receptor saturation (cf. Fig. 2 *A*). In the dissociation experiments, occupation of binding sites was initially induced by incubation of cells in the presence of 8.5×10^{-7} M [^{3}H]ouabain at 37°C for 30 min; the reaction mixtures were then cooled to the desired temperatures, and the rate of dissociation of [^{3}H]ouabain followed, as a function of time, after the addition of a large (1 mM) excess of nonradioactive ouabain.

DISCUSSION

The observations reported here indicate that the turkey erythrocyte is a particularly convenient system in which to study the kinetics and thermodynamics of ouabain binding. The magnitudes of the association and dissociation rate constants are such that both can be studied independently over a wide range of temperatures, and the information thus obtained can be directly employed to calculate a number of kinetic and thermodynamic parameters of interest. In particular, this makes it possible to investigate the role of specific changes in association and dissociation rate constants in mediating the familiar

marked dependence of the equilibrium constant for ouabain binding upon the sodium and potassium ion concentrations in the external medium. A special advantage of such studies on intact cells (as compared with studies on fragmented or porous membrane preparations) is that one can examine the effects on ouabain binding of isolated (unifacial) changes in the external ionic environment. The importance of the "side-dependence" of effects of sodium and potassium ion on ouabain binding has recently been emphasized by Bodemann and Hoffman (13) in studies on reconstituted human red blood cell ghosts. That the observed effects of changes in external sodium and potassium ion concentrations on ouabain binding in the present system can be attributed directly to the changes in external environment per se (rather

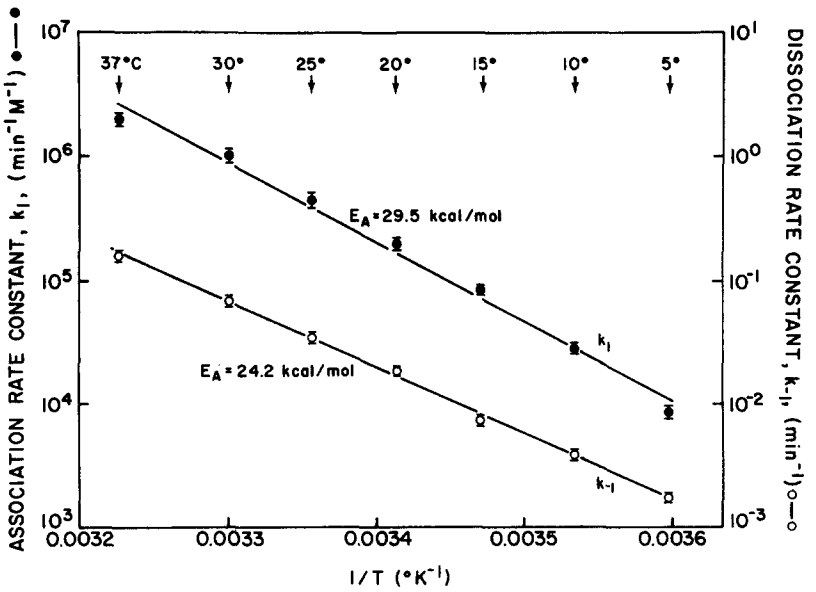

FIGURE 9. Arrhenius plots of association and dissociation rate constants (k_1 and k_{-1}, respectively) as a function of temperature. The slopes of the two lines provide a measure of the energy of activation (E_A) for the association and dissociation reactions. Points and bars indicate means ± 1 SEM.

than to some change in the intracellular milieu secondary to changes in the ambient environment) is supported by the fact that the value of k_{-1}/k_1 derived from the initial slopes of the semilogarithmic plots of the binding and dissociation reactions correlates extremely closely with the value of K_D measured independently after prolonged exposure of cells to different external ionic environments under conditions of equilibrium binding. Equilibrium binding studies in the presence of 150 mM sodium ion at 37°C indicate a value for K_D that extrapolates to $\sim 1.3 \times 10^{-7}$ M at the lowest concentrations of potassium ion, as compared with 3.5×10^{-9} M for the calculated corresponding value for human erythrocytes (5). The approximately 35-fold higher value for K_D in the turkey cells reflects a markedly higher dissociation rate

constant ($k_{-1} = 0.152 \pm 0.002$ min^{-1} vs. ~0.001 min^{-1} for human erythrocytes [5, 7]), which is accompanied by a much smaller concomitant increase in the association rate constant ($k_1 = 2.1 \pm 0.1 \times 10^6$ min^{-1} M^{-1} vs. a calculated value of $\sim 5.3 \times 10^5$ min^{-1} M^{-1} for human cells [5]).

One of the striking conclusions from the results of the kinetic studies is that the profound changes in K_D induced by changes in the external concentrations of sodium and potassium ion are entirely attributable to changes in association rate, and that, in contrast, the rate constant for dissociation remains invariant over a wide (~0–150 mM) range of external sodium and potassium ion concentrations. The marked influence of potassium ion upon association rate, resulting in directly proportional changes in binding affinity, can be regarded as reflecting either changes in the accessibility of the ouabain binding site to bombardment by ouabain or changes in the proportion of total binding sites present at any given moment that are capable of binding ouabain (these two models being formally indistinguishable). The absence of curvature in the Scatchard plots with invariance of the extrapolated binding of ouabain at infinite radioligand concentration (Fig. 4 *A*), the strict parallel between K_A and k_1, and the invariance of k_{-1} at all concentrations of potassium ion are consistent with simple competitive inhibition of ouabain binding by potassium ion and with a conclusion that ouabain and potassium ion compete for a common binding site (or partially overlapping sites) on the erythrocyte membrane. In particular, the absence of curvature in the Scatchard plots and the constancy of k_{-1}, despite changes in potassium ion concentration resulting in some 20-fold changes in both K_A and k_1, argue against an allosteric model in which simultaneous binding of both ouabain and potassium ion is possible and in which the attachment of potassium ion results in a reduction in affinity of the ouabain binding site for the glycoside. If potassium ion and ouabain exhibit simple competition for a common binding site, then K_A will be equal to $K_A^\circ D/(D + [K^+])$, where K_A is the apparent equilibrium constant for ouabain binding in the presence of potassium ion at concentration $[K^+]$, K_A° is the binding constant in the absence of potassium ion, and D is the dissociation constant for the potassium ion–ATPase complex. Because in this model the pseudo-first-order association rate constant observed at a given concentration of potassium ion will be proportional to the fraction of potassium-free sites, k_1 will similarly be equal to $k_1^\circ D/(D + [K^+])$, and K_A and k_1 will hence change in parallel as the concentration of potassium ion is altered. For a double-logarithmic plot of either K_A or k_1 vs. $[K^+]$:

$$\log K_A \text{ (or } k_1) = \log K_A^\circ \text{ (or } k_1^\circ) + \log \frac{D}{[K^+] + D}. \quad (1)$$

For $[K^+] \ll D$, K_A and k_1 approach K_A° and k_1° respectively, and the curves of log K_A and log k_1 vs. log $[K^+]$ will intersect the ordinate at right angles; conversely, for $[K^+] \gg D$, K_A and k_1 approach $K_A^\circ D/[K^+]$ and $k_1^\circ D/[K^+]$, respectively, and the curves of log K_A and log k_1 both become linear with log $[K^+]$, intersecting the abscissa at angles of 45° (i.e., with slopes of −1). A linear relationship between log K_D (= −log K_A) and log $[K^+]$ has been verified

experimentally for concentrations of potassium ion between ~0.5 and 6 mM (9), and these relationships are all further confirmed by the experimental curve illustrated in Fig. 6 *A*, whose form conforms precisely to that predicted by Eq. 1. Differentiation of Eq. 1 with respect to log $[K^+]$ yields

$$\frac{d\log K_A}{d\log [K^+]} \equiv \frac{d\log k_1}{d\log [K^+]} = \frac{-[K^+]}{[K^+] + D},$$

which describes the slope of both curves in Fig. 6 *A*, and which will have a value of −0.5 at $[K^+] = D$. The concentration of potassium ion at this point (where tangents to the curves will intersect the abscissa at an angle of −30°) is found to be ~0.4 mM, yielding by this model an estimate of D, the dissociation constant for the potassium ion–ATPase complex in the presence

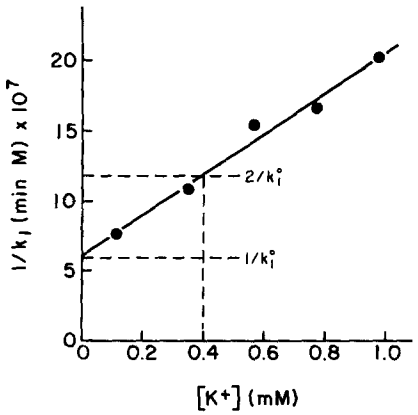

FIGURE 10. Reciprocal plot of k_1 vs. potassium ion concentration. k_1 was measured at 37°C and in the presence of 150 mM sodium ion as a function of potassium ion concentrations ranging between 0.1 and 1 mM. The y intercept corresponds to a value of 1.7×10^6 min^{-1} M^{-1} for k_1°; the potassium ion concentration at which $k_1 = k_1^\circ/2$ is 0.4 mM, yielding an estimate of D (see text).

of 150 mM sodium ion.[2] A similar value of 0.33 mM can be calculated from the formula of Gardner and Conlon for human erythrocytes (5). It is of interest that similar potassium ion affinities in these two different systems have been preserved in the course of evolution, whereas the ouabain affinities differ by one and one-half orders of magnitude. Previous experiments have shown that potassium influx rate in intact turkey erythrocytes is very nearly constant at external potassium ion concentrations between 2 and 10 mM, but that below

[2] The preceding graphical method permits the estimation of D without requiring extrapolation of either K_A or k_1 to zero potassium ion concentration. An alternate and more conventional approach is illustrated in Fig. 10, which presents a reciprocal plot of k_1 vs. $[K^+]$ over a concentration range of from ~ 0.1 to 1 mM. The value of D, in this instance determined from the slope of the resulting straight line and its intercept on the ordinate ($1/k_1^\circ$), is identical to that calculated above (0.4 mM).

2 mM the rate falls. The estimated value of 0.4 mM for the dissociation constant for the turkey-cell potassium-ATPase complex is similar to the external potassium ion concentration (~0.5 mM) at which the influx rate is observed to be half maximal (9, 14).

As indicated above, the inhibition of ouabain binding by potassium ion can equally be regarded as either simple competition between the two ligands for a common membrane binding site or as a homogeneously decreased accessibility of ouabain binding sites in the presence of external potassium ion. Such a decrease might, for example, result from changes in transmembrane potential or from a physical retreating of the ouabain binding site into the membrane induced by the binding of extraerythrocytic potassium at other sites. Although in the instance of the effects of potassium ion these two models are perhaps equally plausible and indeed formally indistinguishable, such would not appear to be the case for the observed enhancement of ouabain binding by sodium ion, which is demonstrable at external potassium ion concentrations considerably less than D and as low as 0.1 mM. Here, the enhancement of binding by sodium ion is linear with sodium concentration at all concentrations examined, suggesting a formal dissociation constant for the interaction of sodium ion with its effector site that is considerably in excess of the highest sodium concentration tested (150 mM). Such an observation, in conjunction with the strict linearity of the Scatchard plots for ouabain binding at all concentrations of sodium ion (Fig. 4 *B*), is difficult to reconcile with a sodium-induced shift within the receptor population resulting in an increased proportion of receptors in a sodium-containing, high-ouabain-affinity form and, rather, suggests a homogeneous shift in receptor conformation or receptor availability that enhances the rate of glycoside attachment. Such a change, again, might result from changes in transmembrane potential induced by changes in extracellular sodium ion or from changes induced by the binding of sodium to the erythrocyte membrane at sites removed from the ATPase itself.

The observation that the enhancement of ouabain binding with increasing sodium shows no departure from linearity, even at concentrations of 150 mM is in agreement with observations of Gardner and Conlon on intact human erythrocytes (5) but is in sharp contrast with the findings of Lindenmayer and Schwartz (15), who noted a half-maximal sodium effect on k_1 for their partially purified ox-brain ATPase at a concentration of only 13.7 mM. The discrepancy between the findings in intact cells and those in broken-membrane preparations may, at least in part, reflect the fact that, in the latter systems, modification of the composition of the medium results in bifacial changes in ionic exposure. The corresponding estimated dissociation constants for the interaction of potassium ion with the ATPase in the presence of 150 mM sodium ion are 0.4, 0.33, and 2.2 mM for the intact turkey erythrocyte, the intact human erythrocyte (5), and the partially purified ox-brain ATPase (15), respectively. In agreement with earlier qualitative observations on human erythrocytes (7) but in contrast to several reports based on studies on systems in which the ATPase has been partially purified or studied in fragmented

membrane preparations (16, 17), we have found no effect of even very high concentrations of potassium ion on k_{-1}. As noted above, our observation that k_{-1} remains invariant up to an external concentration of 150 mM potassium ion (corresponding to several hundred times the estimated dissociation constant for the potassium ion–ATPase complex) suggests but does not rigorously prove the identity (or physical overlap) of the potassium and ouabain binding sites on the enzyme.

The kinetics of association and dissociation in the present system are such that not only can they be examined as functions of the external cation concentrations, but both k_1 and k_{-1} can be studied directly over a wide range of temperatures. Arrhenius plots for k_1 and k_{-1} in both instances yielded straight lines with slightly differing slopes, indicating activation energies for the association and dissociation reactions of 29.5 and 24.2 kcal/mol, respectively (Fig. 9). The value for k_1 is similar to values of 21–23 kcal/mol reported by Wallick and Schwartz (18) for ouabain binding by Na^+,K^+-ATPase preparations isolated from ox-brain cortex microsomes. By contrast, comparative estimates for the activation energy of the dissociation reaction are unavailable in the literature, the dissociation rate constant being so much lower for most other systems that its value, even at 37°C—much less its temperature dependence at progressively lower temperatures—has been difficult to determine with any precision.

The absence of apparent curvature in either Arrhenius plot indicates activation energies for both association and dissociation that are constant over a temperature range of from 5 to 37°C. This finding is similar to earlier observations of Wallick and Schwartz (18) on k_1 for partially purified preparations of Na^+,K^+-ATPase from ox-brain microsomes and contrasts with the familiar sharp break in activation energy at 20°C that becomes evident when one examines the kinetics of the ATPase reaction itself as a function of temperature (19, 20). Recent evidence suggests that the latter break in activation energy is attributable to rather sharp changes in membrane fluidity occurring at about 20°C (21); the contrasting absence of such a break in the energies of activation for ouabain binding and dissociation is consistent with the fact that the ouabain binding site is external to the lipid matrix and directly exposed to the external aqueous environment (6). A similar distinction between surface-mediated functions and those buried more deeply in the lipid matrix of the cell membrane has recently been made by Brasitus et al. (22), who noted sharp breaks in the Arrhenius plots of a number of intestinal microvillus activities associated with ion and glucose transport, as opposed to single-slope plots in the instance of a variety of cell-surface-associated disaccharidase activities.

The ability to measure both k_1 and k_{-1} directly as functions of temperature, in addition to yielding the above information, has made it possible to derive the temperature dependence of the association constant for the ouabain-ATPase complex, which, because of the excessive time required for equilibrium at lower temperatures, is inaccessible to measurement by conventional methods employing equilibrium binding in the presence of nonsaturating concen-

trations of free radioligand. The steady, gentle divergence of the two Arrhenius plots in Fig. 9 indicates a temperature dependence that is slightly greater for k_1 than for k_{-1}. The difference in the two slopes indicates that the value of the association constant (K_A) actually increases at higher temperatures, and, hence, that $\Delta H°$ for the association reaction is positive (+5.3 kcal/mol). The linearity of both plots moreover indicates that $\Delta H°$ is constant over the entire temperature range studied. The ability to measure both $\Delta H°$ and $\Delta G°$ in this system in turn has made it possible to determine $\Delta S°$ for the binding reaction, and has shown that it is the large positive value of $\Delta S°$ that in fact "drives" the binding reaction despite the positive value of $\Delta H°$. Similar large positive values for $\Delta S°$ have recently been reported for other receptor systems in which the unoccupied membrane receptor is presumably in a highly oriented configuration, and in which combination with ligand similarly results in a large negative free energy change despite an "unfavorable" change in enthalpy (see, e.g., reference 23).

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