

The Concentration Dependence of Active K^+ Transport in the Turkey Erythrocyte

Hill Analysis and Evidence for Positive Cooperativity between Ion Binding Sites

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ABSTRACT A mathematical model is presented which describes the theoretical relationship between ligand concentration and physiological response for systems in which the response is dependent upon simultaneous occupancy of two receptor ligand-binding sites. The treatment considers both the possibility of intrinsic differences between the binding sites with regard to ligand affinity, as well as the possibility of mutually induced changes in affinity resulting from allosteric interactions. Unlike the Monod-Wyman-Changeux formulation for allosteric enzymes, the general model put forward here makes double occupancy an absolute requirement for enzymatic function. It is shown that such a model leads to the prediction of a curvilinear Hill plot from which one can obtain an explicit estimate of the degree of allosteric interaction between the two ligand binding sites as well as the Gibbs standard free energy change for the overall binding reaction. It is then shown that, in the specific instance of Na,K-ATPase-mediated K^+ transport by the turkey erythrocyte, the configuration of the Hill curve describing the rate of ouabain-sensitive K^+ transport as a function of external K^+ concentration conforms closely to that predicted by the model described above. The results are of particular interest because they indicate a strongly cooperative interaction between the two K^+ binding sites on the transport protein such that occupancy of one site results in an enhancement of the affinity of the other site for K^+ by a minimum of 15- to 20-fold. Finally, we consider in detail a model of the Monod-Wyman-Changeux type in which, by contrast, both singly and doubly occupied forms of the enzyme are assumed to be catalytically active, and which we analogously extend to allow for the possibility of asymmetry between the two ligand binding sites. Although it is shown that the two models can not be differentiated from each other in the present experimental system, they yield virtually identical estimates for the degree of positive cooperativity between the two K^+ binding sites.

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INTRODUCTION

Abundant evidence now indicates that the active transport of potassium ion effected by the Na,K-ATPase present in the plasma membrane of virtually all cells of higher organisms involves the binding of two external potassium ions at the outer cell surface as well as a phosphorylation and dephosphorylation cycle such that the two potassium ions are delivered at the interior of the cell (1-3). The evidence derives from observations of two types. First, studies on the stoichiometry of active monovalent cation transport, both in erythrocytes (4-7) as well as in systems derived from a variety of other tissues (8-13), have indicated that ATP hydrolysis is coupled to active potassium ion accumulation and sodium ion extrusion in a fixed ratio of one molecule of ATP to two potassium ions to three sodium ions. Second, detailed studies in erythrocytes dating back to the initial observations of Hoffman (14, 15) and of Sachs and Welt (16) have shown that the kinetics of K^+ transport are such that the dependence of the rate of Na,K-ATPase-mediated potassium accumulation upon external K^+ concentration in the presence of physiological external concentrations of sodium ion is well described by a model in which simultaneous occupancy of two K^+ binding sites is required for ion translocation, and in which furthermore the rate of potassium ion transport is proportional to the standing concentration of the enzyme in a doubly occupied form (17-20). Additional kinetic data derived from studies of the K^+ dependence of ATP hydrolysis rate by broken-membrane preparations *in vitro* further confirm the existence of two potassium-ion binding sites per enzyme molecule (21), although, as emphasized by other authors (22-25), interpretation of the latter studies is necessarily more difficult because experimentally induced changes in ion concentrations result in bifacial changes in environment rather than the unifacial changes subject to examination in transport studies in intact cells.

In contrast to substantial evidence for the existence of two external potassium-ion binding sites on the Na,K-ATPase, there is considerably less information about the nature of these sites themselves. In particular, little is known about the symmetry or asymmetry of these sites (as defined by equality or nonequality of their microscopic binding constants for K^+), or, perhaps of more interest, about the degree of allosteric interaction, if any, between the two sites (see, e.g., the discussion in ref. 26). Although numerous studies have demonstrated a "sigmoidal" relationship between external K^+ concentration and active K^+ transport rate consistent with a "two-site" symmetric model (14-18, 27), we know of no attempt at more detailed analysis of such curves to determine the presence or absence of site-site interactions.

In the initial portion of this paper we derive a general mathematical expression to predict the relationship between ligand concentration and physiological response for receptor systems in which the response is exclusively and directly proportional to the concentration of doubly occupied receptor (enzyme), and in which a variable degree of allosteric interaction as well as the possibility of intrinsic asymmetry between the two ligand binding sites is permitted. It is shown that if the concentrations of intermediate singly occupied forms are not disregarded, a conventional Hill plot will give rise, not

to a line of fixed slope, but rather to a curve whose slope decreases continuously from a value of 2 at low ligand concentrations to a value of 1 at high ligand concentrations. Based upon this model, it is then shown that if the characteristics of a given system are such that the magnitude of the response can be determined in a region of the Hill plot where the slope, m , is sufficiently different from its extreme values of 2 and 1 to permit accurate estimation of the two quantities $(2 - m)$ and $(m - 1)$, one can directly obtain a minimal estimate for the degree of cooperativity between the two ligand binding sites. Finally, as an application of the foregoing analysis, a detailed study is made of the K⁺ concentration dependence of ouabain-sensitive potassium ion transport rate in the turkey erythrocyte. It is shown (a) that the Hill plot for the relation between active transport rate and external K⁺ concentration shows a variable slope conforming closely to the predicted relationship derived for an "obligate" two-site system in which double occupancy is required for the biological response; and (b) that the observed slope of the curve relative to the fractional response indicates strong positive cooperativity between the two K⁺ binding sites such that occupation of either site by ligand can be calculated to enhance the affinity of the opposite site for ligand by a factor of at least 15-fold. In the Appendix we additionally consider the predictions of the corresponding non-obligate two-site model of the type originally proposed by Monod, Wyman, and Changeux (28), but which we extend in analogy to the obligate model to allow for the possibility of intrinsic asymmetry between ligand binding sites. It is shown that although the two models cannot be differentiated from each other in the present experimental system, both yield virtually identical estimates for the degree of positive cooperativity between the two ligand binding sites.

MATERIALS AND METHODS

Materials

White female turkeys weighing 12–15 lbs were obtained from local sources (The Butcher, Bronx, NY). 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 octahydrate was purchased from the Sigma Chemical Co. (St. Louis, MO) and [⁴²K⁺]KCl (0.11–0.15 Ci/g K⁺) was obtained from the New England Nuclear Corp. (Boston, MA).

Preparation of Erythrocytes

Heparinized blood (generally an 8-ml sample) was obtained by syringe from a wing vein. After centrifugation at 400 *g* for 10 min, the plasma and buffy coat were removed and the erythrocytes were suspended in incubation buffer containing 150 mM NaCl, 11 mM glucose, and 10 mM Tris at pH 7.4. After resedimentation of the erythrocytes and two further washes, the packed cells were resuspended in incubation buffer and kept on ice until initiation of the transport studies shortly thereafter.

Measurement of ⁴²K⁺ Influx

Potassium influx was determined by a minor modification of the method of Gardner et al. (29). Aliquots of washed turkey erythrocytes were transferred to capped scintillation vials containing pre-warmed incubation medium to which [⁴²K⁺]KCl

had been added to the desired final potassium ion concentration either in the absence or presence of a maximally inhibitory concentration (1 mM) of ouabain. The resulting erythrocyte suspensions, at final hematocrits ranging between 1.6 and 3.3%, were then incubated in a shaking water bath at 37°C. At specified times, 100- μ l aliquots of the incubation mixtures were transferred to plastic centrifuge tubes containing 300 μ l of chilled nonradioactive incubation medium, and the erythrocytes were immediately centrifuged down in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, CA). After aspiration of the supernatant fluid, the erythrocytes were resuspended in 300 μ l of fresh cold medium and were resedimented. After two additional identical washings, each centrifuge tube containing the final washed erythrocyte pellet was placed in a 12- \times 75-mm plastic tube and the radioactivity was determined directly in a Packard Auto-Gamma model 5266 scintillation spectrometer (Packard Instrument Co., Downers Grove, IL) with correction for isotopic decay during the period of counting. Additionally, the total radioactivity in 100- μ l samples of medium was measured directly to permit subsequent calculation of isotope specific activity. Potassium influx was shown to be linear with respect to time for at least 30 min; influx was therefore routinely determined by the difference between zero-time and 30-min samples in triplicate. The range of variation between triplicate samples by this method is extremely narrow, maximal variations rarely exceeding 5%. Radioactivity in zero-time samples was similarly low and generally <5% of that present after 30 min of incubation.

Potassium Determinations

Potassium ion concentrations in incubation media were checked in samples taken at both the beginning and the end of each incubation in an Instrumentation Laboratory (Boston, MA) model 143 flame photometer. The potassium concentration in the incubation medium rose by an average of <0.1 mM during the course of each 30-min incubation. In all instances the mean of the initial (0-min) and final (30-min) potassium ion concentrations was used for the calculation of influx.

Hemoglobin and Hematocrit Determinations

Hematocrits (percent packed red blood cell volumes) were measured in capillary tubes by means of an Adams microhematocrit centrifuge (Clay-Adams, Inc., Parsippany, NJ). Hemoglobin was measured by the cyanmethemoglobin method (30).

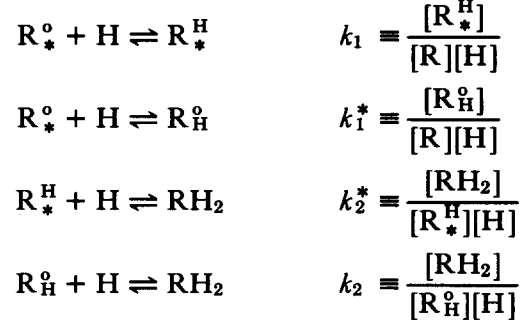
RESULTS

The results will be discussed under three separate headings: *Derivations*, *Observations*, and *Calculations*.

I. Derivations (Obligate Model)

THE MODEL; DEFINITION OF MICROSCOPIC BINDING CONSTANTS Consider an effector enzyme R (in this instance, the Na,K-ATPase) possessing two distinct binding sites for a ligand H (in this instance, external potassium ion), and for the purpose of illustration let these two sites be distinguished by a circle and an asterisk: R_{\circ}^* . For the sake of generality, assume that the intrinsic affinities of the two sites in the doubly unoccupied state of the enzyme may be different. Furthermore, let us introduce the possibility of an allosteric interaction between the two binding sites by assuming that the intrinsic affinity of either site may change upon occupancy of the other site by ligand. In this most

general model we can then define four microscopic equilibrium association constants as follows:



Note that the association constants bearing the asterisk refer to interactions of the ligand with the binding site bearing the asterisk, and, further, that the subscripts 1 and 2 refer to the interactions resulting in singly and doubly occupied forms of the enzyme, respectively. Note also that

$$k_1^* k_2 = \frac{[RH_2]}{[R][H]^2} = k_1 k_2^*. \quad (1)$$

Let R_t be the total concentration of empty, singly occupied, and doubly occupied species of enzyme. Then $R_t = [R] + [R_*^H] + [R_H^{\circ}] + [RH_2]$, and

$$R_t - [RH_2] = [R] + [R_*^H] + [R_H^{\circ}] = \frac{[RH_2]}{k_1 k_2^* [H]^2} + \frac{[RH_2]}{k_2^* [H]} + \frac{[RH_2]}{k_2 [H]}. \quad (2)$$

Let $\xi \equiv \frac{[RH_2]}{R_t - [RH_2]}$; then

$$\xi = \frac{1}{\frac{1}{k_1 k_2^* [H]^2} + \frac{1}{k_2^* [H]} + \frac{1}{k_2 [H]}} = \frac{k_1 k_2 k_2^* [H]^2}{k_2 + k_1 k_2 [H] + k_1 k_2^* [H]}, \quad (3)$$

and

$$\ln \xi = 2 \ln [H] + \ln k_1 k_2 k_2^* - \ln(k_2 + k_1 k_2 [H] + k_1 k_2^* [H]). \quad (4)$$

Note that Eq. 4 differs from the conventional Hill equation and reduces to the latter, yielding a log-log plot with a slope of 2 only under conditions where the concentrations of the singly occupied forms shown in Eq. 2 are both much lower than the concentration of the unoccupied form. Specifically, this will be the case only if $k_2 [H]$ and $k_2^* [H]$ are both $\gg k_1 k_2^* [H]^2$, i.e., (by Eq. 1) only if $k_1 [H]$, $k_1^* [H] \ll 1$.

DEFINITION OF ASYMMETRY AND COOPERATIVITY FACTORS; SIMPLIFICATION OF NOTATION Let us now define an *asymmetry factor*, a , describing the ratio of the intrinsic affinities of the two sites in the doubly unoccupied form of the enzyme such that $k_1^* = a k_1$ ($0 < a < \infty$). Let us, additionally, define *cooperativity factors*, c and c^* ($0 < c < \infty$; $0 < c^* < \infty$), describing possible changes in

intrinsic affinities of each of the two binding sites induced by occupancy of the other site, such that $k_2 \equiv ck_1$, and $k_2^* \equiv c^*k_1^*$. But, by Eq. 1, $k_1k_2^* = k_1^*k_2$; hence $k_1c^*k_1^* = k_1^*ck_1$, and $c^* = c$, the identity of c^* and c indicating that occupancy of either site by ligand induces a change in the affinity of the other site relative to its initial affinity that is proportionately identical. In conventional terminology, $c < 1$ corresponds to "negative cooperativity," $c = 1$ to "no cooperativity," and $c > 1$ to "positive cooperativity" between the two sites.

To permit eventual elimination of both subscripts and superscripts, let $k \equiv k_1$. Then:

$$\begin{aligned} k_1 &= k \\ k_1^* &= ak_1 = ak \\ k_2 &= ck_1 = ck \\ k_2^* &= c^*k_1^* = ck_1^* = cak. \end{aligned}$$

Thus Eq. 3 becomes

$$\xi = \frac{k_1k_2k_2^*[H]^2}{k_2 + k_1k_2[H] + k_1k_2^*[H]} = \frac{cak^2[H]^2}{1 + (a + 1)k[H]}; \quad (3')$$

hence:

$$\ln \xi = 2\ln[H] + \ln cak^2 - \ln[1 + (a + 1)k[H]], \quad (4')$$

and

$$m \equiv \frac{d\ln \xi}{d\ln[H]} = \frac{2 + (a + 1)k[H]}{1 + (a + 1)k[H]}. \quad (5)$$

Note again that, in contrast to a conventional Hill plot of constant slope 2 (obtained, as indicated above, by neglecting the concentrations of the singly occupied forms of the enzyme), Eq. 4' predicts a plot of $\ln \xi$ vs. $\ln[H]$ of changing slope, m . Specifically, it predicts that for all systems in which a measured response, T , is strictly proportional to the concentration of the

doubly occupied form, namely where $\xi = \frac{[RH_2]}{R_t - [RH_2]} = \frac{T}{T_{\max} - T}$, a plot of

$\ln \xi$ vs. $\ln[H]$ will yield a continuous curve of initial slope 2 [where $0 < (a + 1)k[H] \ll 1$] and final slope 1 [where $(a + 1)k[H] \gg 2$]. Further differentiation of Eq. 5 with respect to $\ln[H]$ in order to obtain the value of $[H]$ at which the slope of Eq. 4' is changing most rapidly with $\ln[H]$ shows that this occurs at

$[H] = \frac{1}{(a + 1)k}$, at which point $m = 3/2$. Fig. 1 shows different plots of Eq.

4' for representative pairs of values of the determining constants cak^2 and $(a + 1)k$. In each instance the result is a stereotyped, boomerang-shaped curve of identical form containing an 18° bend, and it can be shown that any one curve may be superimposed upon any other by movements restricted to translations parallel to the ordinate and to the abscissa. Although the general

form of the curve is thus the same whether or not asymmetry or allosteric interactions are present, it will be shown below that the relation between the slope of such a plot and the fractional response as a function of ligand concentration can be used to obtain a direct estimate of a minimal value for the cooperativity factor c .

PREDICTED RELATION BETWEEN LIGAND CONCENTRATION AND RATE OF ACTIVE POTASSIUM ION TRANSPORT; RELATION BETWEEN DEGREE OF COOPERATIVITY BETWEEN BINDING SITES AND SLOPE OF THE HILL PLOT AS A FUNCTION OF LIGAND CONCENTRATION If, in the present system, the rate of ouabain-sensitive potas-

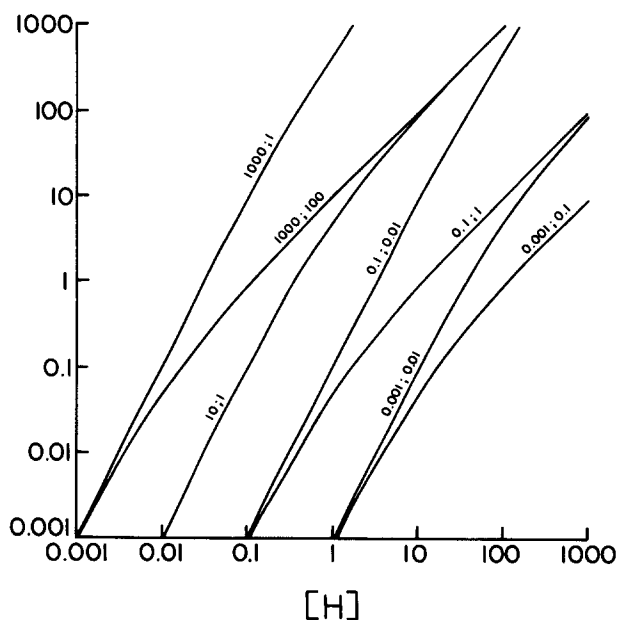


FIGURE 1. Plots of Eq. 4' for different pairs of values of the determining constants $ca k^2$ and $(a + 1)k$. Eq. 4' describes a family of curves of identical form of initial slope 2 and final slope 1 differing from each other only by their mutual degrees of displacement parallel to the ordinate and abscissa (see text). The pairs of values for $ca k^2$ (first number) and $(a + 1)k$ (second number) used to generate each of the curves are indicated in the figure.

sium influx, T , is proportional to the standing concentration of enzyme in the doubly occupied form $[RH_2]$ at external potassium ion concentration $[H]$ (16, 18, 22, 26), then

$$\frac{T}{T_{\max} - T} = \xi = \frac{ca k^2 [H]^2}{1 + (a + 1)k [H]}$$

If, further, H^0 is defined as the concentration of H at which ouabain-sensitive potassium ion transport is half-maximal,

then

$$\frac{T_{\max}/2}{T_{\max} - T_{\max}/2} \equiv 1 = \frac{cak^2H^{o2}}{1 + (a + 1)kH^o},$$

and

$$cak^2H^{o2} = 1 + (a + 1)kH^o. \quad (6)$$

Note that Eq. 6 expresses the relationship between k , the experimentally determined quantity H^o , and the asymmetry and cooperativity factors a and c .

But, from Eq. 5, $m = \frac{2 + (a + 1)k[H]}{1 + (a + 1)k[H]}$, which upon rearrangement yields

$$k = \left(\frac{2 - m}{m - 1}\right) \frac{1}{(a + 1)[H]}. \quad (5')$$

Note that in Eq. 5' k now appears as an explicit function of the experimentally determined quantities m and $[H]$ and the constant a . Note also that it is independent of the value of the constant c , and that for any given value of $[H]$ the corresponding value of m will be such that the quantity $[H] \frac{m - 1}{2 - m}$ will be invariant. Substitution of the value for k in Eq. 5' into Eq. 6 yields, after rearrangement of the terms:

$$c = \frac{(a + 1)^2}{a} \left[\left(\frac{m - 1}{2 - m}\right)^2 \left(\frac{[H]}{H^o}\right)^2 + \left(\frac{m - 1}{2 - m}\right) \frac{[H]}{H^o} \right]. \quad (7)$$

Now, define the following two dimensionless quantities:

$$\beta \equiv \left(\frac{m - 1}{2 - m}\right) \frac{[H]}{H^o} \quad (8)$$

$$\text{and } \gamma \equiv \beta(\beta + 1). \quad (9)$$

By means of these two substitutions it can be shown that Eq. 7 reduces to the simple expression

$$c = \frac{(a + 1)^2 \gamma}{a}. \quad (10)$$

¹ Note that the coefficient of γ in Eq. 10 has a special property, namely that $\frac{(a + 1)^2}{a}$ has identical values for $a = x$ and $a = 1/x$ for all values of x (i.e., for all pairs of reciprocal values of a). This derives from the flexibility of the original definition of a , above, in which, in considering the possibility of asymmetry between the two binding sites, and hence of non-identity of the two intrinsic binding constants k_1 and k_1^* , no arbitrary assumption was introduced as to which of the two sites had the higher (or lower) intrinsic affinity. Values of $a > 1$ correspond to those instances where the affinity of the asterisked site ($k_1^* = ak$) is higher than that of the non-asterisked site ($k_1 = k$), in which case k corresponds to the association constant for the site of lower affinity; conversely, values of $a < 1$ correspond to those instances in which

If, finally, we now define m^0 as the observed slope, m , at $[H] = H^0$ (namely, the value of m where $T = T_{\max}/2$, $\xi = 1$, and $\ln \xi = 0$), Eqs. 8 and 9 reduce, respectively, to

$$\beta = \frac{m^0 - 1}{2 - m^0}$$

and

$$\gamma = \frac{m^0 - 1}{(2 - m^0)^2},$$

and Eq. 10 becomes

$$c = \frac{(a + 1)^2}{a} \cdot \frac{m^0 - 1}{(2 - m^0)^2} \quad (10')$$

Since $\frac{(a + 1)^2}{a}$ achieves a minimal value of 4 when $a = 1$ (corresponding to identical intrinsic affinities for the two binding sites in the doubly unoccupied form), it follows that

$$c = 4 \frac{m^0 - 1}{(2 - m^0)^2} \quad (a = 1),$$

and

$$c > 4 \frac{m^0 - 1}{(2 - m^0)^2} \quad (a \neq 1).$$

The utility of this formulation is that Eq. 10' permits a direct estimate for a lower bound for c in those systems in which m^0 (i.e., the value of m at $[H] = H^0$) can be determined with sufficient precision to permit accurate evaluation of the ratio $\frac{m^0 - 1}{(2 - m^0)^2}$. Determination of the latter ratio will, in turn, be subject to least error in those instances in which the value of m^0 lies well away from either of the extreme values for m of 2 and 1; where this is the case, the concentrations of the intermediate, singly occupied forms omitted in the conventional Hill derivation are indeed not negligible, and the standard Hill plot will show a slope at $[H] = H^0$ that is comfortably intermediate between these two integral values. Under such circumstances, the cooperativity factor c can be calculated to have a minimal value of $\frac{4(m^0 - 1)}{(2 - m^0)^2}$, corresponding to

the affinity of the asterisked site is lower than that of the non-asterisked site, in which case k is the association constant for the site of higher affinity. The quantity $\frac{(a + 1)^2}{a}$ has a minimal value of 4 when $a = 1$.

² A more general expression for c can be obtained by combining Eqs. 3' and 5 to eliminate the quantity $k[H]$. By so doing it can be shown that $c = \frac{(a + 1)^2}{a} \cdot \frac{m - 1}{(2 - m)^2} \cdot \xi \geq 4 \cdot \frac{m - 1}{(2 - m)^2} \cdot \xi$. This reduces to Eq. 10' for $\xi = 1$ (i.e., where $m = m^0$).

a case in which there is no intrinsic difference in affinity between the two binding sites (i.e., where $a = 1$); the existence of any degree of intrinsic asymmetry between the binding sites will imply that c is *greater than* $\frac{4(m^0 - 1)}{(2 - m^0)^2}$.³

We shall show in the following sections that, for the turkey erythrocyte, the concentration dependence of ouabain-sensitive potassium ion transport is indeed such that a lower limit for c can be calculated with considerable precision, and that the value of this lower limit implies an impressively high degree of positive cooperativity between the two potassium-ion binding sites.

II. Observations

As noted in the Introduction, the rate of active potassium ion transport in the turkey erythrocyte is such that it can be determined with considerable precision over a wide range of external potassium ion concentrations. To determine the precise form of the concentration dependence of Na,K-ATPase-mediated active potassium ion accumulation, detailed measurements of the rate of potassium ion influx as a function of external potassium ion concentration were performed over a wide range of external potassium ion concentrations both in the absence and presence of a maximally inhibitory concentration of ouabain as described under Methods. Fig. 2A shows the results of a typical experiment. As observed in earlier studies (31, 32), the rate of potassium ion influx in the presence of ouabain is a steadily increasing function of external potassium ion concentration, whereas the rate of Na,K-ATPase-mediated active transport T (determined by the difference between the rate of potassium influx in the absence and presence of glycoside) shows rapid saturation with rising external potassium ion. A double-reciprocal plot of the ouabain-sensitive component of K^+ influx depicted by the solid line in Fig. 2A is shown in Fig. 2B and yields a value for T_{\max} of 9.1 meq/l cells/h.

³ For purposes of simplicity the case has been considered in some detail in which the measured slope is that at $[H] = H^0$. More generally, Eq. 7 shows that the slope at any point can be used with equal validity providing that it differs sufficiently from both 1 and 2 so that the quantity $\frac{m-1}{2-m}$ can be determined with precision. For example a different, but analogous, expression for c can be derived from Eq. 7 if the plot is used to determine the value of $[H]$ ($\equiv H^\dagger$) at which the curvature of the Hill plot is maximal and where the slope m is 3/2 (i.e., midway between its limiting values at low and high values of $[H]$). In such an instance it can be shown that

$$c = \frac{(a+1)^2}{a} \cdot \frac{H^\dagger}{H^0} \left(\frac{H^\dagger}{H^0} + 1 \right),$$

and hence that, the higher the value of H^\dagger relative to H^0 , the more "positive" the degree of cooperativity. Specifically, it can be shown in this latter instance that, for any case in which $\frac{H^\dagger}{H^0} \cong \frac{\sqrt{2}-1}{2} = \sim 0.2$, there will be positive cooperativity between the two ligand binding sites.

The two graphical methods are wholly analogous, the choice between the two being solely dependent upon the precision of the measurements obtainable in the concentration range of interest.

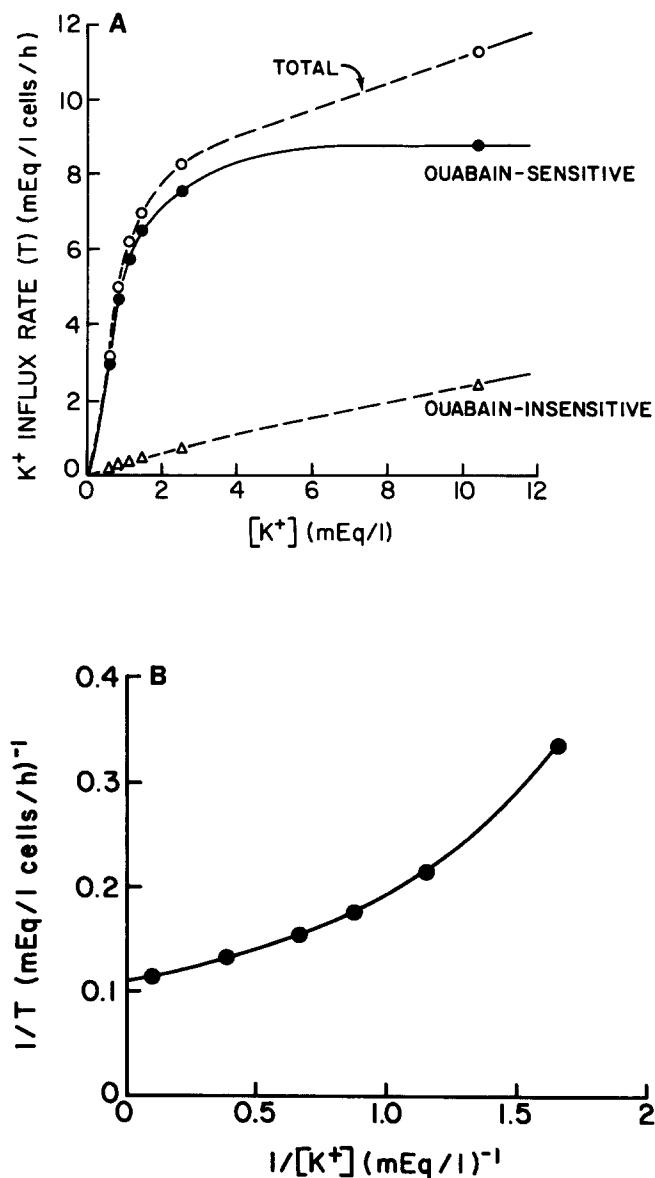


FIGURE 2. A. Rate of potassium influx as a function of external potassium ion concentration. Cells were incubated in the presence of various concentrations of external potassium ion either in the presence (Δ) or absence (\circ) of ouabain as described under Methods. The ouabain-sensitive influx rate (\bullet) is calculated as the difference in influx rate in the absence and presence of a maximally inhibitory concentration (1 mM) of ouabain. B. Double-reciprocal plot of the ouabain-sensitive component of potassium ion influx shown in A. At high values of $1/[K^+]$ (i.e., at low concentrations of K^+) the curve shows increasing upward convexity. Extrapolation to infinite $[K^+]$ yields a y -intercept corresponding to $1/T_{\max}$ of ~ 0.11 (meq/liter cells/h)⁻¹, or a T_{\max} of ~ 9.1 meq/liter cells/h.

In the curve shown in Fig. 2A, the rate of ATPase-mediated transport is seen to rise rapidly with increasing external potassium ion concentration, becoming half-maximal at an external potassium ion concentration of ~ 0.8 mM and reaching near-maximal values soon thereafter.

To test the conformity of the results illustrated in Fig. 2 with the predictions of Eq. 4', Fig. 3 shows the same data plotted in Hill form, namely a plot of $\ln \frac{T}{T_{\max} - T}$ vs. $\ln[K^+]$. Consistent with Eq. 4' for those systems in which

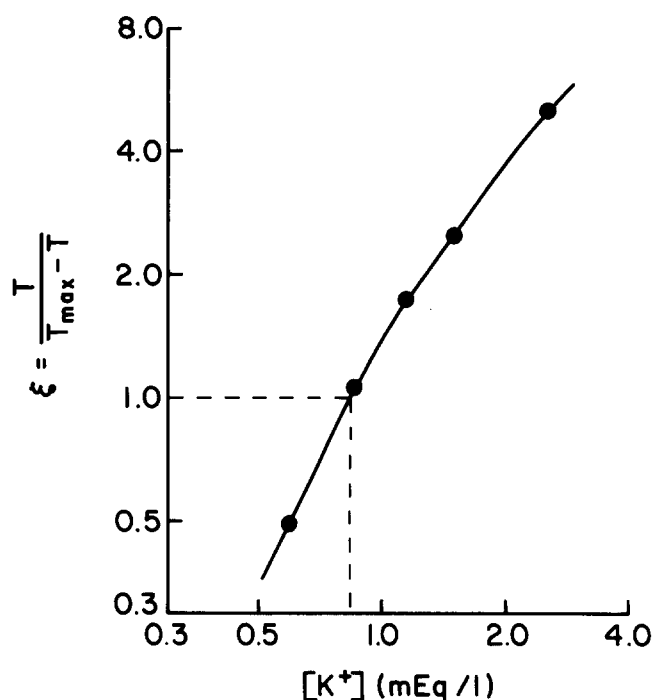


FIGURE 3. Hill plot of ouabain-sensitive K^+ influx rate for the same experiment shown in Fig. 2. In the experiment illustrated here, $\xi = 1$ (corresponding to a half-maximal active influx rate) at an external potassium ion concentration of 0.84 mM. See Fig. 4 for a composite plot of the individual data points obtained in all six experiments.

measurements are made at values of $[H]$ where the concentrations of the singly occupied forms R_0^H and R_H^* cannot be neglected, the result is not a line of constant slope, but rather a curve of steadily decreasing slope intermediate between the integral values of 2 and 1. Although the experimental results thus conform to Eq. 4' and hence to the underlying model, it will be shown that they cannot be used to distinguish it in the present instance from an otherwise analogous but less stringent non-obligate two-site model (see Appendix).

The precision of the measurements obtainable in the turkey erythrocyte

(where Na⁺ and K⁺ flux rates are considerably greater than in the human erythrocyte [4, 16, 27, 31, 32]) is such that multiple determinations of the rate of ouabain-sensitive potassium influx as a function of external potassium ion concentration yielded curves of the type illustrated in Fig. 2 from which maximal transport rate as well as the concentration of potassium ion at which ouabain-sensitive transport was half-maximal could be determined with great

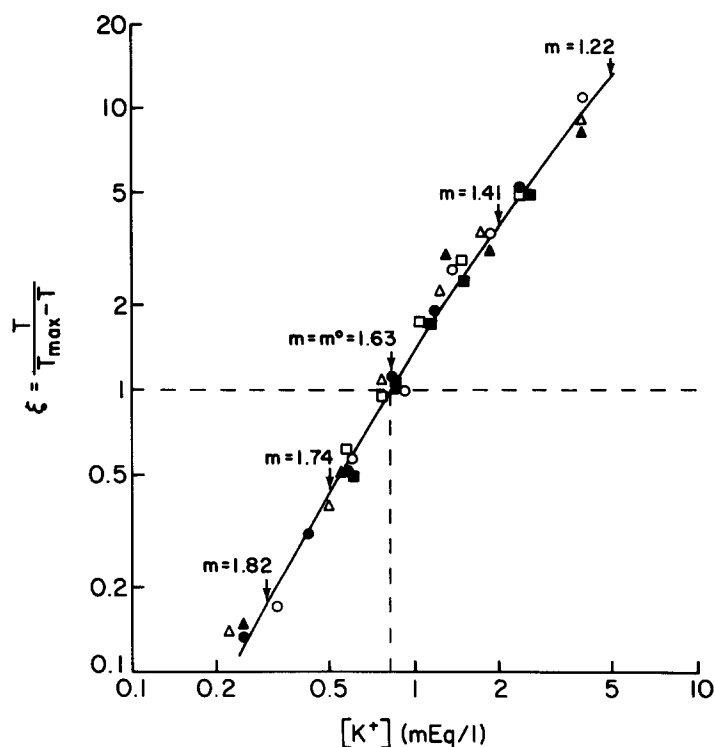


FIGURE 4. Composite of Hill plots of individual data points for six separate experiments. Calculation of the mean values for H° and for m° based upon separate plots of the data from each of the individual experiments yielded means (± 1 SEM) of 0.83 ± 0.03 mM and 1.63 ± 0.04 , respectively. The solid line shows a plot of the predicted form of the response curve for a two-binding-site system described by Eq. 4' where H° and m° correspond to the experimentally determined mean values given above (see text). Arrows indicate the slope of this theoretical curve at representative values of $[K^+]$ ($[H]$).

reproducibility. Fig. 4 shows a Hill plot of the type illustrated in Fig. 3 but now representing a composite of individual data points from six separate experiments, all performed on different days and using erythrocytes from several different animals. Calculation of the mean values for H° (the concentration of potassium ion at which the rate of active transport is half-maximal, corresponding to the value of $[H]$ at which the curve intersects the abscissa at

$\xi = 1$) and for the slope of the curve, m° , at $[H] = H^\circ$, based upon separate plots of the data from each of the six individual experiments, yielded a mean of 0.83 ± 0.03 mM for H° and 1.63 ± 0.04 for m° (means ± 1 SEM). For comparison, the solid line in Fig. 4 shows a plot of the predicted form of the theoretical response curve for a two-binding-site system described by Eq. 4' in which the response is exclusively dependent upon the concentration of doubly occupied forms and where H° and m° correspond to the experimentally determined mean values given above.⁴ Although the correspondence between the theoretical curve and the experimentally derived data (including the quantitative prediction of the rate of change of slope with changing external ligand concentration) is seen to be extremely close, the fit is equally close for the non-obligate model (see, again, Appendix).

III. Calculations (Obligate Model)

As indicated in the first section, Eq. 4' predicts that for all systems possessing two ligand binding sites and in which the biologic response is proportional solely to the concentration of doubly occupied receptor forms, Hill plots will not result in lines of constant slope, but rather will generate curves of stereotyped form having an initial slope at low ligand concentrations of 2 and a final slope at high ligand concentrations of 1. Although it may rarely prove feasible in a given system to perform precise measurements of biologic response over a sufficiently wide range of ligand concentrations to permit delineation of the entire curve,⁵ the foregoing derivation shows that for those systems in which the response curve can be accurately defined in the region of rapidly changing slope (in particular in a region where m lies comfortably intermediate between its limiting values of 2 and 1), such measurements can be used to obtain an estimate of the degree of cooperativity between the two ligand binding sites. The versatility of this approach, as applied instead in the instance of the corresponding non-obligate model (where the limiting values of m at both extremes of the Hill curve are 1) is illustrated in the Appendix.

Fig. 5 shows a family of curves representing Eq. 4' drawn to intersect the abscissa at the experimentally determined point $[H] = H^\circ = 0.83$ mM.⁶ The

⁴ Eq. 4' is unambiguously defined when the values of the two parameters ca^2k^2 and $(a + 1)k$ are known. Both parameters, in turn, can be determined directly by Eqs. 5' and 6 from the experimentally determined quantities H° and m° :

$$ca^2k^2 = \frac{1}{(m^\circ - 1)H^{\circ 2}}; \quad (a + 1)k = \left(\frac{2 - m^\circ}{m^\circ - 1} \right) \cdot \frac{1}{H^\circ}.$$

⁵ It can be shown, for example, that an 81-fold increase in $[H]$ is required for a decrease in m from 1.9 to 1.1; similarly, a 16-fold increase in $[H]$ is necessary for a fall in m from 1.8 to 1.2.

⁶ The family of curves is constructed by re-writing Eq. 4' in terms of the two parameters m° and H° and setting H° equal to its experimentally determined value of 0.83 mM. Substitution of the values for k and c given by Eqs. 5' and 10', respectively, into Eq. 4' yields

$$\ln \xi = \ln \left[\frac{1}{m^\circ - 1} \left(\frac{[H]}{H^\circ} \right)^2 \right] - \ln \left[1 + \frac{2 - m^\circ}{m^\circ - 1} \cdot \frac{[H]}{H^\circ} \right]. \quad (4'')$$

Fig. 5 shows plots of Eq. 4'' for values of m° ranging between 1 and 2 for $H^\circ = 0.83$ mM.

result is a pencil of curves with slopes m° at H° varying between 1 and 2, corresponding to minimal values of the cooperativity factor c (for $a = 1$) ranging between 0 (“infinite negative cooperativity”) and ∞ (“infinite positive cooperativity”), respectively. Solution of Eq. 10' for the case where both a and c are 1 yields a value for m° of 1.17. Hence, if the binding sites have identical

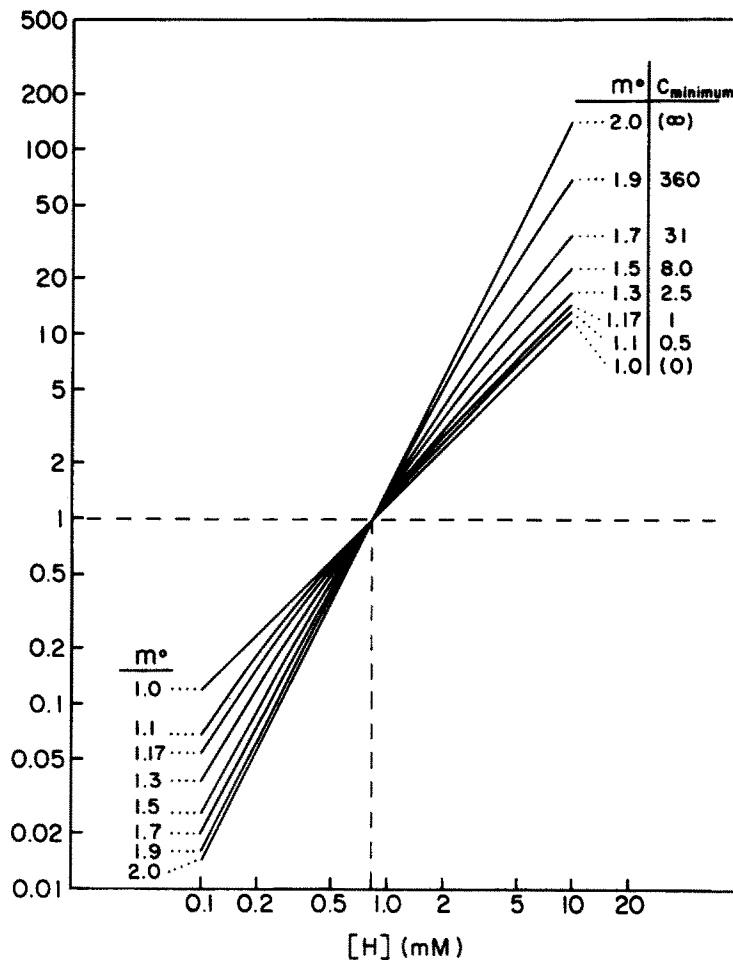


FIGURE 5. Plots of Eq. 4'' corresponding to different values for m° for $H^\circ = 0.83$ mM (see footnote 5). For each curve characterized by a different value of m° there is indicated a minimal value for the associated cooperativity factor c , corresponding to the case where $a = 1$.

intrinsic affinities for ligand, a slope of 1.17 at $[H] = H^\circ$ will correspond to the case where there is no interaction between the two binding sites (i.e., where $c = 1$ and there is “no cooperativity”), and any observed slope $m^\circ > 1.17$ will imply positive cooperativity of a magnitude equal to $\frac{(a + 1)^2}{a} \times$

$\frac{m^{\circ} - 1}{(2 - m^{\circ})^2} = 4 \frac{m^{\circ} - 1}{(2 - m^{\circ})^2}$. For instances where, in contrast, there is asymmetry

(i.e., where $a \neq 1$), the factor $\frac{(a + 1)^2}{a}$ will have a value >4 (see footnote 1),

and the calculated value for c will be correspondingly greater than that calculated from m° on the assumption of symmetry. Table I shows limiting values of m° determining the presence or absence of cooperativity, calculated from Eq. 10' for different values of a . Note that, the larger the value of a , the lower the value of m° corresponding to the absence of cooperative interaction between the two sites.

The conclusion from the above analysis is that any observed slope $m^{\circ} > 1.17$ implies, whatever the degree of asymmetry a , the existence of positive cooperativity between the two sites. Fig. 5 shows that such is indeed the case in the present system, in which the experimentally determined slope at [H]

TABLE I
LIMITING VALUES OF m° CORRESPONDING TO $c = 1$ FOR
DIFFERENT DEGREES OF ASYMMETRY, a

$a, 1/a$	$\frac{(a + 1)^2}{a}$	m° if $c = 1$ ($m_c^{\circ} - 1$)
1	4.0	1.17
2	4.5	1.16
5	7.2	1.11
10	12.1	1.07
20	22.1	1.04
50	52.0	1.02
100	102	1.01

For any given value of a , an observed value of m° greater than that shown above for $c = 1$ ($m_c^{\circ} - 1 < m^{\circ} < 2$) implies the existence of positive cooperativity between the two binding sites; for there to be negative cooperativity, the observed values of m° must be less than those shown above ($1 < m^{\circ} < m_c^{\circ} - 1$).

$= H^{\circ}$ was found to be 1.63 ± 0.04 . Substituting the value $m^{\circ} = 1.63$ into Eq. 10' yields

$$c = \frac{(a + 1)^2}{a} \cdot \frac{m^{\circ} - 1}{(2 - m^{\circ})^2} = \frac{(a + 1)^2}{a} \cdot 4.6.$$

Since, as noted above, the quantity $(a + 1)^2/a$ has a minimal value of 4 in the instance where $a = 1$ (corresponding to identical intrinsic binding affinities for the two sites), one can calculate in the instance of the obligate double-occupancy model a minimal value for c of 4×4.6 , or ~ 18 (if instead one considers a range of values for m° of 1.59–1.67, corresponding to its mean value ± 1 SEM, this leads to a minimal estimate of c ranging between 14 and 25). This high value for c —representing an increase of at least an order of magnitude in the affinity of each binding site for potassium induced by occupation of the opposite site—is particularly impressive when it is noted

that it represents a minimal estimate and that any intrinsic asymmetry between the two individual binding sites will lead to even higher calculated values.

The values of the individual intrinsic affinity constants, k and ak , can be obtained from Eq. 5':

$$k = \left(\frac{2-m}{m-1} \right) \frac{1}{(a+1)[H]}, \quad \text{which, at } [H] = H^{\circ}, \text{ gives}$$

$$k = \left(\frac{2-m^{\circ}}{m^{\circ}-1} \right) \frac{1}{(a+1)H^{\circ}} = \left(\frac{2-1.63}{1.63-1} \right) \frac{1}{(a+1)0.83} = \frac{0.71}{(a+1)} \text{ mM}^{-1}.$$

TABLE II
CALCULATED INDIVIDUAL K⁺ AFFINITY CONSTANTS FOR
DIFFERENT DEGREES OF ASYMMETRY BASED UPON AN
OBSERVED SLOPE m° OF 1.63 AT $[H] = H^{\circ} = 0.83$ mM

<i>a</i>	<i>c</i>	Site of lower intrinsic affinity		Site of higher intrinsic affinity	
		<i>k</i>	<i>ck</i>	<i>ak</i>	<i>cak</i>
		(opposite site unoccupied)	(opposite site occupied)	(opposite site unoccupied)	(opposite site occupied)
		mM^{-1}	mM^{-1}	mM^{-1}	mM^{-1}
1	18	0.36	6.5	0.36	6.5
2	21	0.24	5.0	0.48	10.1
5	33	0.12	4.0	0.60	19.8
10	56	0.065	3.6	0.65	36
20	102	0.034	3.5	0.68	69
50	239	0.014	3.3	0.70	167
100	469	0.007	3.3	0.70	328

Similarly,

$$ak = \frac{0.71a}{(a+1)} \text{ mM}^{-1}.$$

As indicated above (see footnote 1), if $a > 1$, k gives the affinity of the site of intrinsically lower affinity. In the case where $a = 1$ (i.e., in the instance of the sites having equal intrinsic affinities),

$$k = \frac{0.71}{2} = 0.36 \text{ mM}^{-1},$$

$$\text{and } ck = 18 \times 0.36 = 6.5 \text{ mM}^{-1}.$$

These values, in turn, correspond to dissociation constants of 2.8×10^{-3} M and 1.5×10^{-4} M for the binding site-ligand complex in the instances where the opposite site is simultaneously either unoccupied or occupied, respectively. Table II shows similar calculations for representative values of $a \neq 1$ (i.e., for cases where various degrees of asymmetry are assumed to be present).

Finally, from Eqs. 1, 5', and 7 it can be shown that the value of the equilibrium constant K for the overall reaction $R + 2H \rightleftharpoons RH_2$ is

$$K \equiv \frac{[RH_2]}{[R][H]^2} = k_1 k_2^* = cak^2 = \frac{1}{H^{o2}} \left[1 + \left(\frac{2-m}{m-1} \right) \frac{H^o}{[H]} \right] = \frac{1}{(m^o - 1)H^{o2}},$$

and hence that $\Delta G^o = -RT \ln K = RT \ln(m^o - 1)H^{o2}$, (11)

again quantities that can be calculated directly from the experimentally determined values of H^o and the slope of the Hill plot at that point, m^o . For

the present system, $K = \frac{1}{(1.63 - 1)(0.83)^2} = 2.3 \text{ mM}^{-2}$, corresponding to

$$\Delta G^o = -RT \ln K = -1.99 \times 310 \times \ln[2.3 \times 10^{-6}] = -9,040 \text{ cal/mol},$$

or approximately -9.0 kcal/mol for the overall reaction for the binding of both potassium ions, $R + 2H \rightarrow RH_2$.

DISCUSSION

The derivation of the predicted concentration-response relationship for the allosteric two-site transport model put forward here presents two aspects that deserve special emphasis. First, in contrast to the Monod-Wyman-Changeux model for allosteric enzymes, in which singly as well as multiply occupied species of enzyme are assumed to be catalytically active, and in which activity is directly proportional to the total number of sites occupied by ligand (28), the allosteric model advanced here makes double occupancy an absolute requirement for enzymatic function (see Appendix). Second, it is shown that the present model, by taking into account the existence of these singly occupied, though "catalytically inactive," forms, leads to the specific prediction of a curvilinear Hill plot with a slope varying continuously between the extremes of 2 and 1 from which a number of parameters of interest can be derived.

The results of these considerations, applied in the present instance to the most general obligate two-site model, are not only the prediction of a Hill plot of variable slope, but also the prediction that accurate determination of the slope of such a plot at any point should permit both a direct estimate of the degree of allosteric interaction between the two ligand-binding sites and calculation of the overall Gibbs standard free energy for the binding reaction. In the specific instance of the turkey erythrocyte it is shown, first, that the configuration of the curve describing ouabain-sensitive K^+ transport as a function of external potassium ion concentration conforms closely, over the experimentally observable range, to the theoretical model described above, and, more interestingly, that the slope of the curve at an external K^+ concentration resulting in a half-maximal rate of active K^+ transport is such that a lower bound can be obtained for the degree of cooperativity between the two K^+ binding sites on the enzyme. It is shown in the Appendix that although this model can not be differentiated from the corresponding non-obligate model in the present experimental system, the two models in this

instance yield virtually identical estimates for the degree of allosteric interaction between the two ligand-binding sites.

The results are of particular interest, for they indicate a strongly cooperative interaction between the two K⁺ binding sites on the enzyme such that occupation of one site results in an enhancement of the affinity of the other site for K⁺ by at least one to two orders of magnitude. Although a two-site model for K⁺ transport has been strongly suggested on the basis of kinetic data ever since the classical observations of Hoffman (14, 15) and of Sachs and Welt (16), and has been borne out by further observations of the concentration dependence of K⁺ transport in erythrocytes by other workers (17–20), as well as by direct studies of the stoichiometric relationship between K⁺ accumulation, Na⁺ extrusion, and transport-related ATP hydrolysis in erythrocytes (4–7) and in other cells (8–13), earlier transport studies, to our knowledge, have not examined in any quantitative sense the possibility of an allosteric interaction between the two sites. Although various forms of Hill analysis have previously been applied to the kinetics of both potassium accumulation and sodium extrusion in intact erythrocytes from other species, such analyses in general either have not explicitly considered the possibility of asymmetry or have assumed, for the purpose of mathematical simplicity, a symmetrical model without allosteric interactions (see, e.g., ref. 18). The present treatment shows that for an obligatory two-site model—whether or not there is asymmetry or cooperativity between the two binding sites—the expected Hill plot will in fact be of constant form. Varying degrees of asymmetry or cooperativity will affect only the relation of the slope of such a curve to the degree of saturation of the transport process, and it is precisely this experimentally determined relationship that permits one to assign a lower limit to the degree of cooperativity whatever asymmetry may exist between the intrinsic affinities of the two binding sites. It is of interest that although the slope of the Hill plot provides no information whatever about the presence or absence of asymmetry, it does permit one to calculate a lower limit for the degree of cooperativity between the two sites.

The turkey cell, by virtue of its relatively high transport rates, and the precision with which these rates can be determined, serves as a particularly useful model in which to examine in detail the external K⁺ dependence of Na,K-ATPase-mediated K⁺ transport rate. As is the case for other studies using intact cells (and in contrast to studies examining ATP hydrolysis rates in broken-membrane preparations *in vitro*), one has the advantage of examining selectively the effects of unifacial changes in ionic environment on enzyme turnover rate. It can be calculated that the maximal rate of ouabain-sensitive K⁺ transport of 9.2 ± 0.3 meq/liter cells/h (mean \pm 1 SEM) observed in the present study corresponds, for cells of mean volume $140 \mu\text{m}^3$ (33) and possessing $\sim 4,500$ ouabain binding sites per cell (32), to a turnover of ~ 49 potassium ions per site per second or (for a two-site model) a turnover number for the enzyme of $\sim 25 \text{ s}^{-1}$. This can be compared with a value of ~ 65 potassium ions per site per second calculated from corresponding data for the human erythrocyte (16, 34) and a turnover number for Na,K-ATPase in

membrane preparations from human erythrocytes in the range of 100–200 s⁻¹ at 37°C (35–37).

As indicated above, the estimate obtained for the degree of positive cooperativity between the two K⁺ binding sites is a minimal one corresponding to the instance where the intrinsic affinities of the two sites for potassium ion are identical, and any degree of intrinsic asymmetry will in fact only serve to increase this minimal estimate (see Table II). The impressive magnitude of the cooperative effect resulting from the attachment of so small a ligand as a potassium ion presumably reflects the contribution of strong electrostatic interactions in inducing the allosteric effect and in providing the strongly negative overall ΔG° of binding.

The approach described here should prove generally applicable to systems in which simultaneous occupancy of more than one binding site is required for a biological effect. Conformity of the observed concentration dependence to that predicted by the analysis presented here can be used, within the limitations explicitly noted in the Appendix, not only to test further the validity of such an approach, but, additionally, to obtain, by precise examination of the concentration–response relationship at a sensitive portion of the response curve, a quantitative measure of the direction and degree of allosteric interactions at ligand binding sites.

APPENDIX

Obligate vs. Non-Obligate Double Occupancy Models

The allosteric model developed in the body of this paper is one that makes simultaneous occupancy of both ligand binding sites an absolute requirement for enzymatic activity, or, in the instance of the Na,K-ATPase of the intact cell, for a “transport event” to take place. This model differs from the corresponding model proposed by Monod, Wyman, and Changeux (28) for allosteric multiple-site enzymes, where multiple occupancy, though permitted, is non-obligate in the sense that both singly and multiply occupied forms of the enzyme are assumed to be catalytically “active.” It is of interest to compare the predictions of these two models for a two-site enzyme and to consider the conditions under which the models can be differentiated from each other experimentally.

I. Analogous Non-Obligate Model: Influence of Asymmetry on the Predictions of the Monod-Wyman-Changeux Formulation

We begin by considering the predictions of a non-obligate two-site model of the type proposed by Monod, Wyman, and Changeux, but which we extend here (as in the instance of the obligate model) to allow for the possibility of asymmetry between the two binding sites. If, in the instance of the present system, both doubly and singly occupied forms were to participate in K⁺ translocation [namely, if the ouabain-sensitive transport rate were proportional to $(2[\text{RH}_2] + [\text{R}_\circ^{\text{H}}] + [\text{R}_{\text{H}}^*])$], then $\frac{T}{T_{\text{max}} - T} \equiv \xi'$ would be equal to

$$\begin{aligned} \frac{2[\text{RH}_2] + [\text{R}_o^{\text{H}}] + [\text{R}_{\text{H}}^*]}{2\text{R}_t - 2[\text{RH}_2] - [\text{R}_o^{\text{H}}] - [\text{R}_{\text{H}}^*]} &= \frac{2[\text{RH}_2] + [\text{R}_o^{\text{H}}] + [\text{R}_{\text{H}}^*]}{[\text{R}_o^{\text{H}}] + [\text{R}_{\text{H}}^*] + 2[\text{R}]} \\ &= \frac{2cak^2[\text{H}]^2 + k(a+1)[\text{H}]}{2 + k(a+1)[\text{H}]}, \end{aligned} \quad (\text{A1})$$

and the slope of the corresponding Hill plot would be given by

$$\begin{aligned} m &= \frac{d \ln \xi'}{d \ln [\text{H}]} \\ &= \frac{2cak^2(a+1)[\text{H}]^2 + 8cak[\text{H}] + 2(a+1)}{2cak^2(a+1)[\text{H}]^2 + [4cak + k(a+1)^2][\text{H}] + 2(a+1)}. \end{aligned} \quad (\text{A2})$$

It can be shown that an extremum for m in this latter instance will exist at

$[\text{H}] = \text{H}^\circ = \frac{1}{k} \sqrt{\frac{1}{ca}}$, at which point the value of m will be given by

$$m = m^\circ = \frac{a+1 + 2\sqrt{ca}}{a+1 + \sqrt{ca} + \frac{(a+1)^2}{4\sqrt{ca}}}. \quad (\text{A3})$$

This model (restricted, however, to instances where $a \equiv 1$) is discussed at some length in refs. 38–40.

In contrast to the form of the Hill plot for the obligate model, which was shown to be fixed, the form of the Hill plot for the non-obligate model is seen to be variable (Fig. 6). Whereas the Hill curve for the obligate model is asymmetrical with a slope that decreases continuously from 2 to 1, that for the non-obligate model has both initial and final slopes of 1, possesses an extremum at $[\text{H}] = \text{H}^\circ$, and is symmetrical in that it is superimposable upon itself upon rotation through 180° at this point. Whereas for the obligate model it was seen that $c = \frac{(a+1)^2}{a} \cdot \frac{m^\circ - 1}{(2 - m^\circ)^2} \cong 4 \cdot \frac{m^\circ - 1}{(2 - m^\circ)^2}$, it can be shown that

for the otherwise analogous non-obligate model $c = \frac{(a+1)^2}{4a} \cdot \left(\frac{m^\circ}{2 - m^\circ}\right)^2 \cong \left(\frac{m^\circ}{2 - m^\circ}\right)^2$. In both models, therefore, a progressive degree of asymmetry will, for a given observed value of m° , imply an increasingly large value for c (see again Fig. 6).

The extension of the non-obligate two-site model to allow for the possibility of asymmetry introduces several features which deserve special attention. The allosteric model of the type proposed by Monod, Wyman, and Changeux was initially advanced to account for the catalytic properties of oligomeric enzymes composed of two or more identical catalytic subunits, and hence postulated the presence of symmetry as one of its fundamental features (28). In such a

model (namely, where $a \equiv 1$), an observed value of $m^{\circ} > 1$ necessarily implies the presence of positive, and a value of $m^{\circ} < 1$, of negative, cooperativity. Note, however, that when this model is extended as in the present treatment to allow for the possibility of intrinsic asymmetry between binding sites, the slope at H° no longer provides an unambiguous measure of the degree of allosteric interaction between the sites, but instead leads only to an estimate for the *minimal permissible* value for c . In the instance of the obligate model it

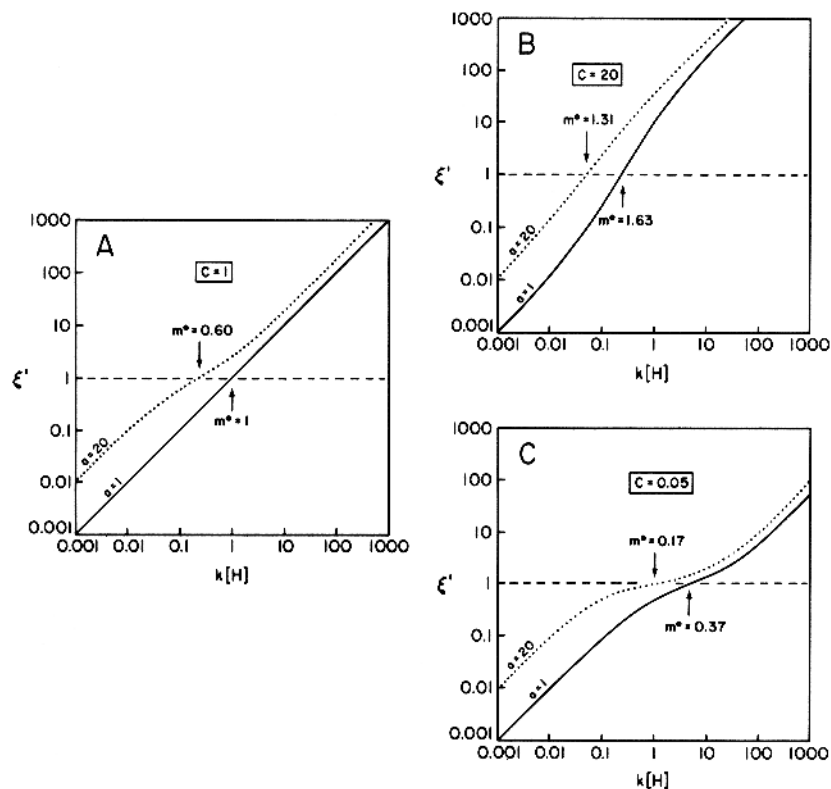


FIGURE 6. Effect of asymmetry on Hill plots for the non-obligate model. Each of the three panels shows double-logarithmic plots of ξ' vs. $k[H]$ for $a = 1$ (solid line) and for $a = 20$ (dotted line). Panel A: $c = 1$ ("no cooperativity"); panel B: $c = 20$ ("strong positive cooperativity"); panel C: $c = 0.05$ ("strong negative cooperativity"). In each instance the presence of asymmetry decreases the slope at m° .

was shown that an increasing degree of asymmetry will, for any fixed value of c , lead to a progressive decrease in the observed value of m° to a limiting value of 1. In the instance of the non-obligate model—whatever the value of c —a progressive increase in asymmetry will similarly cause m° to decrease to a limiting value, in this case to a value of zero (Eq. A3). For this reason, an observed slope (m°) of < 1 at $[H] = H^{\circ}$ does not necessarily imply the presence of negative cooperativity, but instead only sets a lower limit for the value of

c ; indeed, for values of a sufficiently large, an observed value of $m^{\circ} < 1$ may lead to a calculated value of $c > 1$ and thus reflect the presence of positive cooperativity. The point that asymmetry can mimic negative cooperativity in its effects upon the Hill curve has not, to our knowledge, been made before, and is of some practical importance in the interpretation of experimental data; conclusions about the presence or absence of cooperativity on the basis of a standard Hill plot may be erroneous if the tacit assumption that there is symmetry is unjustified.

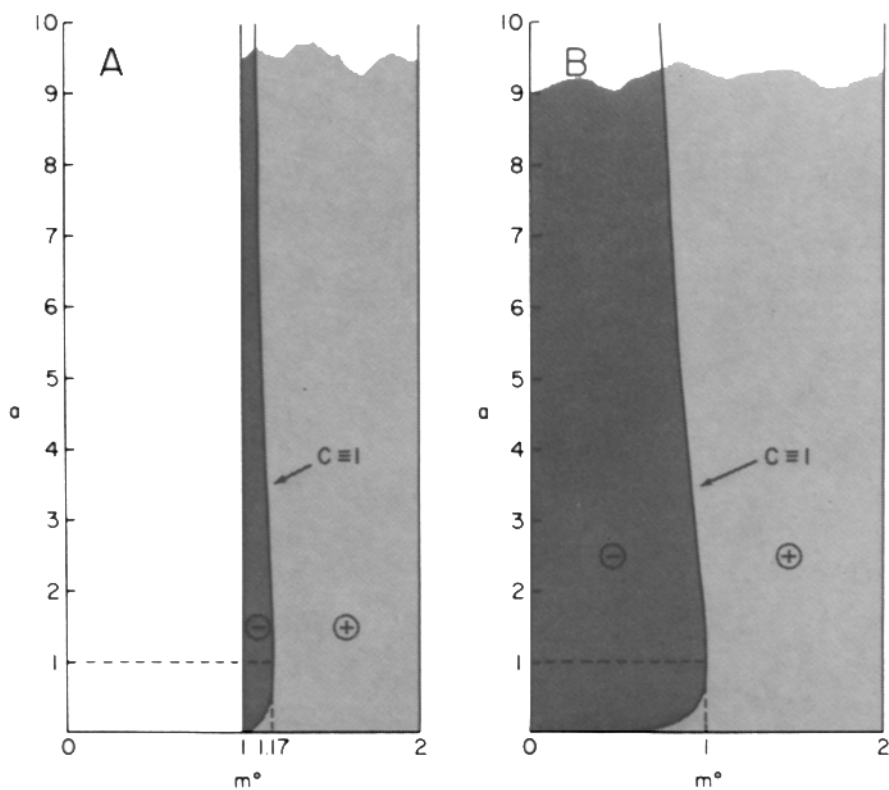


FIGURE 7. Graphical summary of relationships between a , c , and m° for the obligate (A) and non-obligate (B) two-site models. The domains corresponding to the presence of positive ($c > 1$) or negative ($0 < c < 1$) cooperativity are defined by the lines corresponding to $c \equiv 1$. Shaded areas indicate permissible ranges for m° .

In summary, an observed slope $m^{\circ} > 1$ in the non-obligate model necessarily implies positive cooperativity (whatever the degree of asymmetry), whereas values of m° between 0 and 1 ($0 < m^{\circ} \leq 1$) do not exclude the presence of positive cooperativity provided sufficient asymmetry is also present. The corresponding ranges for m° in the obligate model were shown above to be $m^{\circ} > 1.17$ and $1 < m^{\circ} \leq 1.17$, respectively (see Fig. 7).

II. Comparison of the Two Models

The preceding section indicated that there are important qualitative differences between the predictions of the obligate and non-obligate two-site models derived above. Despite these differences, however, the two models may in practice be difficult to distinguish experimentally. The difficulty derives from the fact that in the region of $[H] = H^0$, where in general the quantity $T/(T_{\max} - T)$ can be determined with the greatest precision, the quantitative differences in the predictions of the two models are quite small, whereas precise determination of the quantity $T/(T_{\max} - T)$ at much higher or much lower values of $[H]$ (i.e., where the two functions ξ and ξ' diverge) becomes progressively less practicable. At very low values of $[H]$, accurate determination of T [and hence of the quantity $T/(T_{\max} - T)$] may present major experimental difficulties; conversely, small fractional errors in the determination of either T_{\max} , or of T at very high values of $[H]$, may lead to very large fractional errors in the quantity $(T_{\max} - T)$ and hence in the determination of ξ and ξ' .

The problem is illustrated graphically in Fig. 8, which shows simultaneous plots of ξ and ξ' as a function of the ratio $[H]/H^0$ for a range of observed slopes compatible with both models, namely for $1 < m^0 < 2$.⁷ Under such conditions the Hill plots for ξ and ξ' are tangent to each other at $[H]/H^0 = 1$. Since, as noted earlier, the curvature of the plot for ξ' is zero at this point [where m has its extremum (m^0)], while the plot for ξ is always curved, it follows that the two curves will differ maximally from each other in the region about $[H] = H^0$ if the plot for ξ is maximally curved at this point. Fig. 8B shows superimposed Hill plots for ξ and ξ' in such a case, namely in the hypothetical instance where m^0 is observed to be 1.5. Even under such maximally favorable circumstances it is seen that at no point over a fivefold range of $[H]$ to either side of H^0 (corresponding to an overall concentration range of 25-fold in a region where precise determinations of $T/(T_{\max} - T)$ are generally most feasible) do the values of the two Hill functions differ from each other by a factor of >1.5 .

The conclusion from the above treatment is that experimental differentiation between the obligate and non-obligate models may be expected to be difficult in many, if not most, instances. This proves to be the case for the present system, where accurate determination of ouabain-sensitive K^+ influx rate at external K^+ concentrations sufficiently far from H^0 to distinguish between the predicted values of ξ and ξ' is not experimentally feasible. Although, in the present instance, the observed experimental data fit both models extremely closely and hence cannot be used to differentiate between the two, the ambiguity as to the choice of model does not affect the conclusion that there exists a high degree of positive cooperativity between the two potassium-ion binding sites. In the instance of the obligate model it was shown

⁷ The expression for $\ln \xi$ as a function of m^0 and the ratio $[H]/H^0$ is given in Eq. 4". The corresponding expression for the non-obligate model is

$$\ln \xi' = \ln \left[\left(\frac{[H]}{H^0} \right)^2 + \frac{2 - m^0}{m^0} \cdot \frac{[H]}{H^0} \right] - \ln \left[1 + \frac{2 - m^0}{m^0} \cdot \frac{[H]}{H^0} \right]. \quad (A1')$$

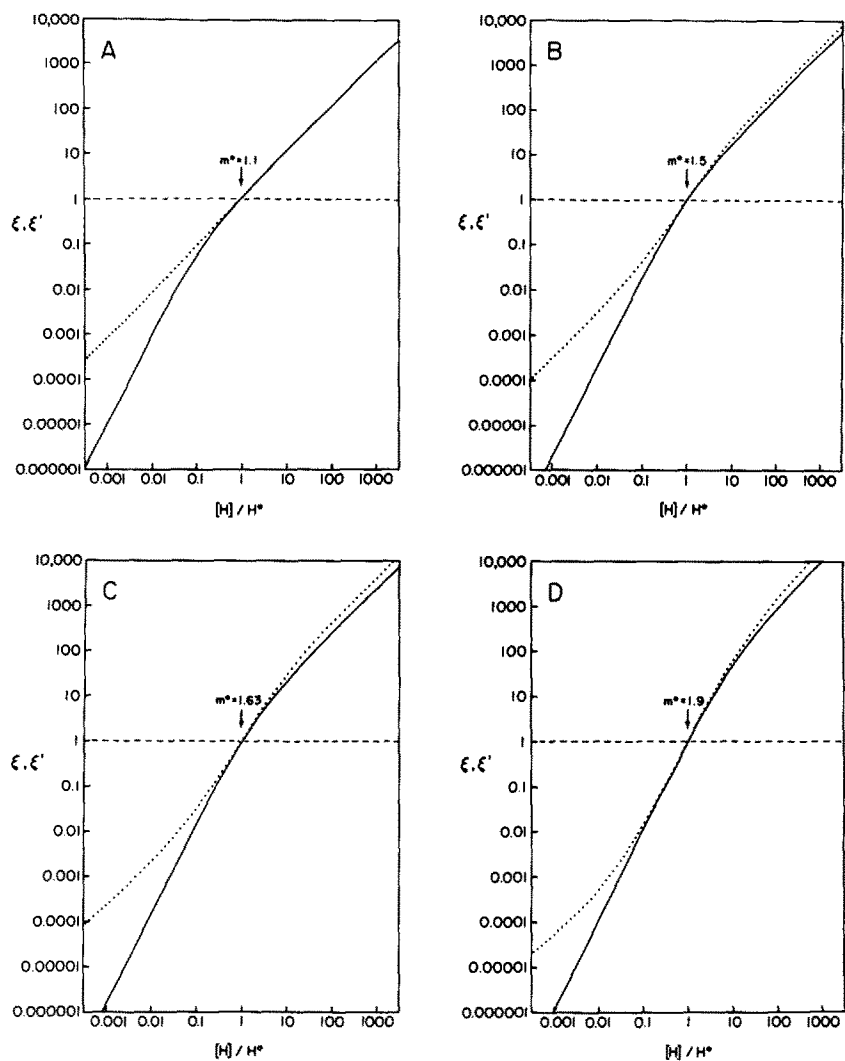


FIGURE 8. Double-logarithmic plots of ξ (solid line; Eq. 4'') and ξ' (dotted line; Eq. A1') as functions of $[H]/H^0$ and sharing common tangents (m^0) at $[H] = H^0$ (i.e., at $[H]/H^0 = 1$). A. $m^0 = 1.1$. B. $m^0 = 1.5$. C. $m^0 = 1.63$. D. $m^0 = 1.9$. It can be shown that as $[H]/H^0 \rightarrow \infty$, the ratio ξ'/ξ approaches a limiting value of m^0 ; as $[H]/H^0 \rightarrow 0$, the curves for ξ and ξ' diverge without limit. For intermediate values of $[H]/H^0$ in the range of unity, ξ and ξ' will differ maximally if $m^0 = 1.5$ (see text). Curves corresponding to $m^0 = 1.63$ (the experimentally determined value for m^0 in the present system) are shown in panel C.

that $c_{ob} = \frac{(a+1)^2}{a} \cdot \frac{m^o - 1}{(2 - m^o)^2}$, whereas for the non-obligate model it was shown that $c_{non-ob} = \frac{(a+1)^2}{4a} \cdot \left(\frac{m^o}{2 - m^o}\right)^2$. The ratio of the degrees of cooperativity calculated on the basis of these two different models is therefore $\frac{c_{non-ob}}{c_{ob}} = \frac{m^{o2}}{4(m^o - 1)}$. This ratio (always >1 for values of m^o consistent with both models) rapidly converges to 1 as $m^o \rightarrow 2$, and indeed differs from unity by $<25\%$ for all values of $m^o > 1.4$. In the present instance, where m^o is observed to be 1.63, the ratio differs from unity by only $\sim 5\%$, and both models hence yield virtually identical estimates for the minimal degree of positive cooperativity between the potassium-ion binding sites (i.e., for the degree of cooperativity if $a = 1$):

$$c_{ob} = \frac{(a+1)^2}{a} \cdot \frac{m^o - 1}{(2 - m^o)^2} \cong 4 \cdot \frac{m^o - 1}{(2 - m^o)^2} = 4 \cdot \frac{0.63}{(0.37)^2} = 18.4,$$

and

$$c_{non-ob} = \frac{(a+1)^2}{4a} \cdot \left(\frac{m^o}{2 - m^o}\right)^2 \cong \left(\frac{m^o}{2 - m^o}\right)^2 = \left(\frac{1.63}{0.37}\right)^2 = 19.4.$$

The overall ΔG^o of binding is given by $\Delta G^o = -RT \ln K = -RT \ln(cak^2)$, which, for the non-obligate model, is equal to $2RT \ln H^o$. In the present instance this yields a value for ΔG^o of -8.8 kcal/mol, which differs only slightly from the value of -9.0 kcal/mol calculated on the basis of the obligate model (Eq. 11). Note that the calculated ΔG^o for the non-obligate model will always be less negative by an increment of $-RT \ln(m^o - 1)$ than that for the obligate model, but that this difference will be trivial as long as m^o is appreciably >1 .

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