On the Mechanism of Stimulation of the Na/K Pump of LK Sheep Erythrocytes by Anti-L Antibody

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ABSTRACT Studies were undertaken to explore the mechanism of stimulation of the Na/K pump in LK sheep erythrocytes by anti-L antibody. First, the numbers of functioning pump sites were determined by correlating [3H]ouabain binding with levels of inhibition of the pump. Untreated (control) cells had ~41 pumps per cell, and anti-L treatment caused an increase in the number of functioning pumps to ~85 per cell. Reducing the intracellular K concentration, [K], near zero caused an increase in the number of pumps in control cells, but not in anti-L cells, such that the numbers of pumps per cell were about the same in the two cell types. These results led to the prediction that K is a noncompetitive inhibitor of the pump in control cells, and that anti-L stimulates the pump and increases number of functioning pumps by reducing noncompetitive inhibition by K. Kinetic studies were undertaken to test this prediction: activation of the pump by increasing [Na] was measured at three fixed levels of [K]. In control cells, the apparent maximum velocity of the pump (J' max) was reduced approximately threefold by raising [K], from 0.2 to 9 mmol/liter cells, demonstrating noncompetitive inhibition by K. In anti-L cells, J' max did not vary with [K], which shows that, as predicted, anti-L abolishes the noncompetitive inhibition by K. The modification of the kinetic properties of the pumps by the antibody is highly specific in that affinities for Na and K as substrates are unaffected. However, the effect of the antibody on noncompetitive inhibition by K does not explain the stimulation of the pump fully since there is significant stimulation at near-zero [K].

INTRODUCTION

An antigen-antibody interaction on erythrocytes from sheep of a particular phenotype results in severalfold stimulation in the rate of active transport through the Na/K pump. The basis for this unusual type of activation is controversial and is for the most part unexplained (see Ellory, 1977, and Lauf, 1983, for reviews). At the root of the phenomenon are two related polymorphisms of sheep erythrocytes. The first involves the regulation of cellular Na
and K concentrations. Cells from the HK (high-K) phenotype have high K and low Na concentrations, while LK (low-K) sheep have red cells with low K and high Na concentrations. The causes for this difference in composition are higher active Na and K fluxes and lower passive fluxes in HK than in LK cells (Tosteson and Hoffman, 1960), and a relatively greater number of Na/K pumps per HK cell (Dunham and Hoffman, 1971). In addition, the pumps of the two phenotypes differ in their affinity for one or more ligands.

The second polymorphism is a blood group antigen system, M/L, which is associated with the HK/LK polymorphism in that HK cells have only M antigen and L antigen is only on LK cells. (The LK allele is dominant; red cells from heterozygous LK sheep have both M and L antigens. Only the red cells differ between sheep of the HK/LK and M/L phenotypes.)

Ellory and Tucker (1969) discovered the stimulation of the pump by the antibody: brief incubation of LK cells with alloimmune anti-L antiserum (raised in an HK sheep immunized with LK cells) increased the activity of the Na/K pump. Only LK cells (and not HK) are subject to stimulation by alloimmune antiserum. The two extreme possible explanations for the stimulation are, on the one hand, an increase in maximal velocity ($J_{\text{max}}$) and, on the other hand, an alteration in the affinity of the existing pumps for one or more ligands. $J_{\text{max}}$ could be increased by raising the number of functioning pumps or by increasing the molecular activity of existing pumps. Ligands whose affinities might be modified are cellular Na ($Na_c$) and external K ($K_o$) as substrates, or cellular K ($K_c$) and external Na ($Na_o$) as inhibitors.

In the first investigation of the basis for anti-L's stimulation, Lauf et al. (1970) found an increase in the number of pumps per cell from ~50 to ~90 (the number of pumps was estimated using $[^{3}H]$ouabain, a highly specific inhibitor of the pump). More recently, Joiner and Lauf (1978a) found no increase in the number of pumps per cell after stimulation by anti-L, but did observe that anti-L caused an increase in the rate of ouabain binding.

If there is no increase in the numbers of functioning pumps, then stimulation must be ascribable either to an increase in molecular activity of the pumps or to changes in the affinity of ligands for the pump. Lauf et al. (1970) provided evidence for no increase in the apparent affinity, $K_{\text{inh}}$, for $K_o$, but did suggest an alteration in the relative affinities for $K_c$ and $Na_o$. Glynn and Ellory (1972) drew the surprising conclusion that the pumps of LK cells have a higher affinity for $K_c$ as a dead-end inhibitor than for $Na_o$ as a substrate. Kinetic studies indicated that stimulation by anti-L was a consequence of altering the relative affinities for $K_c$ and $Na_o$, perhaps by raising the affinity for $Na_o$. The conclusions were based on an analysis of the data of Lauf et al. (1970) and on new results on the Na,K-ATPase activity of LK goat red cells, cells whose pumps are also stimulated by sheep anti-L.

We have re-examined the basis for the stimulation of the Na/K pump of LK sheep cells by alloimmune anti-L. We have demonstrated, at physiological [K], a twofold increase in the number of functioning pumps per cell, i.e., the number of ouabain molecules bound per cell necessary for 100% inhibition of the pump. We confirmed that $K_c$ is an inhibitor of the pump with two types of action: the
expected competitive inhibition with Na, and also an unexpected noncompetitive inhibition (noncompetitive inhibition is used here in the “classic” sense, i.e., a reduction in maximum velocity with no effect on the apparent substrate affinity). We were able to demonstrate, by kinetic studies, that a part of the stimulatory action of anti-L is the elimination of the noncompetitive inhibition of the pump by K. The increase in the number of functioning pumps is probably a direct effect of this relief of noncompetitive inhibition. In contrast, competitive inhibition by K and the affinity for Na as a substrate appear to be unaffected by anti-L. Some of these results have been published in an abstract (Anderson and Dunham, 1984).

**MATERIALS AND METHODS**

**Cells**

Blood was drawn into heparin from Suffolk breed sheep maintained at Krutulis Laboratories, Inc., Bridgeport, NY. Within 1 h, the erythrocytes were washed by centrifugation and resuspension three times in an isotonic medium (290 mosmol/kg; Advanced Laboratories [Needham Heights, MA] osmometer), usually containing 150 mM NaCl, 5 mM glucose, and 10 mM Tris-HCl, pH 7.5. The genotypes of LK sheep (heterozygous, LM, or homozygous, LL) were determined with alloimmune anti-M antiserum as described elsewhere (Dunham et al., 1984).

**Antisera**

Alloimmune anti-L antisera were raised in HK Suffolk sheep immunized with LK cells by the staff at Krutulis Laboratories as described before (Dunham 1976b). Complement was inactivated at 56°C for 20 min. Cells were sensitized in undiluted antiserum at 5–10% hematocrit for 20 min at 37°C.

**Altering Cell Cation Concentrations**

Parachloromercuribenzenesulfonic acid (PCMBS) was used in a modification of a method described before (Sachs et al., 1974b) to raise cation permeability, and dithiothreitol was used to restore it. PCMBS solutions contained [Na] and [K] to give the desired cellular concentrations. In order to vary [Na], and [K], independently, choline was included as an inert substitute cation. At all cation compositions, cells were shown to have the same cell volumes as fresh cells (determined from hematocrits and hemoglobin concentrations). Incubations with PCMBS solutions were usually for 48 h at 4°C, 10% hematocrit, with three changes of solutions. PCMBS solutions with Na, K, and choline concentrations chosen to give the desired cellular concentrations were otherwise of the same composition as given before (Sachs et al., 1974b), except that sucrose was 60 mM throughout. The dithiothreitol solutions for washing and incubating the cells after PCMBS were also as described before. Cation concentrations of cell lysates were measured and calculated as described before (Dunham and Ellory, 1980).

**Influxes**

Unidirectional K influxes were measured and calculated using 86Rb as a tracer as described before (Sachs et al., 1974b). The incubations with tracer were for 30 min; the fluxes were measured in triplicate. Pump fluxes were taken as the difference between fluxes in aliquots of cells pretreated with ouabain (0.1 mM, 5 min) and untreated aliquots. The fluxes were measured in the isotonic medium described above, but with 5 mM K substituted for Na.
Ouabain Binding

The number of ouabain molecules bound per cell was determined using $[^{3}H]$ouabain by a modification of the method of Dunham and Ellory (1980). The specific activity of the purchased $[^{3}H]$ouabain was reduced ~10-fold to 1.4 Ci/mmol by addition of unlabeled ouabain. Cells were incubated in medium containing 0.3 mM $[^{3}H]$ouabain for three time intervals selected to cause 30–90% inhibition of the pump. Samples were taken and divided in two, one for determination of percent inhibition of the pump, and the other for $[^{3}H]$ouabain binding. Binding of tritiated contaminants was determined in each experiment from binding to cells incubated in medium with the same $[^{3}H]$ouabain concentration, but with the specific activity reduced 100-fold by addition of unlabeled ouabain. The correction for tritiated contaminants never exceeded 10%. Fluxes measured on these samples gave the ouabain-insensitive flux, i.e., the flux at 100% inhibition.

The samples taken for determination of bound $[^{3}H]$ouabain were washed three times and lysed with Triton X-100 (0.1% vol/vol, final concentration). Total volumes of cells were calculated from measurements of hemoglobin concentrations and hematocrits. Before counting the $[^{3}H]$, hemoglobin was precipitated by addition of trichloroacetic acid (5%, final concentration).

In calculating numbers of ouabain molecules per cell, the radioactivities of cell extracts were compared with the radioactivities of samples of suspending medium with known ouabain concentrations and with appropriate amounts of trichloroacetic acid and Triton X-100 added to adjust the quenching of the apparent radioactivity to that of the cell extracts.

Percent inhibition of the pump was given by comparison of fluxes in cells exposed to $[^{3}H]$ouabain with fluxes in cells with the pump inhibited 100% (cells exposed to a saturating ouabain concentration; see above) and fluxes in cells not treated with ouabain. Thus, in each experiment the mean was obtained for the ratio of (ouabain molecules per cell)/(percent pump inhibition) × 100, giving the number of ouabain molecules per cell necessary for 100% inhibition of the pump, or the number of functioning pumps. (As in earlier studies [Dunham and Hoffman, 1971; Joiner and Lauf, 1978a], there was a fairly good linear relationship between percent inhibition and ouabain molecules per cell, which suggests that there was little nonspecific binding and that one ouabain molecule was sufficient to inhibit each pump.)

It should be emphasized that this method measures the numbers of functioning pumps. Any pumps not functioning or pumping relatively slowly, and binding ouabain correspondingly slowly (Bodemann and Hoffman, 1976), will contribute to a minor extent to the estimation of the numbers of pumps.

ATP Analysis

Cellular ATP concentrations were determined in extracts of washed cells by the diprophosphoglycerate kinase method (Jaworek et al., 1974). Cellular concentrations were calculated from the hemoglobin concentrations determined for the same cell suspensions.

Analysis of Kinetics

The kinetic experiments were designed to measure active K influx at varying values of [Na], the substrate concentration, S, at selected, fixed values of [K], and the inhibitor concentration, I. The curves for activation of the pump by Na, were sigmoidal, as they are for Na/K pumps in general. There are two conventional approaches for the analysis of such data. In one, proposed by Garay and Garrahan (1973), equations of the following form are used: $J = J_{\text{max}}/[1 + (K/S)[1 + (I/K_{i})]]^{n}$, where J and $J_{\text{max}}$ are pump flux and maximal pump flux, respectively, $K_{S}$ is the "Michaelis constant" for substrate, $S$, $K_{i}$ is the inhibitory constant for inhibitor I, and $n$ is the number of ions of S binding per pump.
cycle. This equation has provided reasonable fits to data from human red cells and other systems. The other conventional approach to analyzing sigmoidal activation curves is an equation of the form: \( J = \frac{J_{\text{max}}}{1 + (K_i/S)^n[1 + (I/K_c)]^n} \). This is a form of the classic Hill equation and of the equation used by Albers et al. (1973) in analyzing the kinetics of inhibition of Na,K-ATPase. In this equation, \( n \) is related to the number of binding sites of the substrate ion and also the strength of the interaction between the binding sites.

There are reasonable and unreasonable assumptions underlying both of these equations (see Karlish and Stein, 1985, for a discussion). We used both of them in analyzing our results on the kinetics of activation of the pump by Na\(_i\) and inhibition of it by K\(_c\). In using the Garay-Garrahane equation, we set \( n = 3 \), the conclusion from many studies of the number of Na ions required to activate the pump. In using the Hill-Albers equation, the best value of \( n \) was determined (in this equation, \( n \) is not the same as the number of Na sites). The two equations yielded somewhat different estimates of the kinetic constants, as expected. Nevertheless, the curves fitting the data and the conclusions were exactly the same, as will be shown. Most of the results will be shown after analysis with the Hill-Albers equation, the complete form of which is:

\[
J = \frac{J_{\text{max}}}{1 + (K_i/S)^n[1 + (I/K_c)]^n},
\]

where \( J \) is the pump influx (millimoles per liter cells per hour); \( J_{\text{max}} \) is maximum \( J \); \( I \) is the inhibitor concentration, \( [K]_\text{i} \); \( K_{\text{IN}} \) is the inhibitory constant for \( K_c \) due to classic noncompetitive inhibition; \( K_{\text{IC}} \) is the inhibitory constant for \( K_c \) due to competitive inhibition; \( S \) is the substrate concentration, \( [\text{Na}]_\text{i} \); \( K_i \) is the “Michaelis constant” for Na\(_i\) (i.e., \( K_{\text{IN}}, K_{\text{IC}}, \) and \( K_s \) are all in millimoles per liter cells); and \( n \) is the “Hill coefficient,” a term related to the number of interacting sites and also the coefficients of their interaction, and is assumed to be the same for K as a competitive inhibitor and for Na as a substrate. Evidence will be presented that \( n = 1 \) for K acting as a noncompetitive inhibitor. When the Hill-Albers equation was employed in many of the analyses, it was for empirical convenience and because the alternative is not necessarily better conceptually or practically (cf. Karlish and Stein, 1985). There is no intention to imply that all of the assumptions on which the Hill-Albers equation is based apply. The kinetic constants were estimated from \( J, S, \) and \( I \) using iterative computer programs (both APL and SAS) for nonlinear least-squares fits to the data. Statistical analyses were carried out using the asymptotic standard errors for the kinetic constants so generated.

**Sources of Materials**

\(^{86}\)Rb (Cl salt, aqueous solution) and \[^{3}H\]ouabain (in benzene/ethanol, taken to dryness and dissolved in an aqueous medium just before use) were from New England Nuclear, Boston, MA; choline Cl was from Agri Business Corp., Springfield, MO (further purified by recrystallization from hot ethanol); and ouabain, PCMS, and dithiothreitol were from Sigma Chemical Co., St. Louis, MO. For ATP analyses, nicotinamide-adenine dinucleotide (reduced, Na\(_2\)), phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN.

**RESULTS**

**Anti-L Increases the Number of Pumps**

Table I (upper row) shows the results of nine experiments (on cells from five LK sheep, both homozygous and heterozygous) in which the numbers of pumps per...
cell were determined in untreated fresh cells and fresh cells after brief exposure to anti-L. There was a highly significant twofold stimulation. This is the first part of the explanation for the stimulation of the pump by anti-L. The rest of this report is concerned with explaining how anti-L increases the number of functioning pumps, and determining whether there are additional aspects of the stimulation.

The estimate of the number of pumps on untreated or control cells, 41, is in the middle of the range reported by Joiner and Lauf (1978a), and is severalfold higher than our earlier estimate (Dunham and Hoffman, 1971), in error owing to the use in the calculations of an erroneous specific activity provided by the manufacturer of the [3H]ouabain.

The method used to obtain the numbers in Table I provides the numbers of functioning pumps; nonfunctioning pumps binding ouabain slowly would be counted after longer exposure, but in our method, such pumps would make a

| TABLE I |
| Numbers of Na/K Pumps per LK Sheep Red Cell at Physiological [K], Before (Control) and After Treatment with Anti-L Antibody |

| Cells ([K]c) | Control | Anti-L |
|-------------|---------|--------|
| Fresh (12 mmol/liter) | 41±8 (9) | 85±11 (9)* |
| PCMBS (10 mmol/liter) | 55±8 (7) | 85±12 (7)* |

The number of functioning pumps per cell was measured using [3H]ouabain as described in the text. PCMBS cells were prepared (see text) so as to have near-physiological [K]c. Both sets of experiments (with fresh and PCMBS cells) were carried out on cells from five different sheep, both homozygous and heterozygous for the LK allele. All experiments were carried out on paired samples (control and anti-L). Means ± SEM are shown (numbers of determinations are in parentheses). * Higher than control (P < 0.0001; t test for two means).

To investigate further the effect of anti-L on number of pumps, it was necessary to alter the cellular cation concentrations. The PCMBS method was chosen because it permits [K]c and [Na]c to vary independently by allowing entry of choline, an inert cation substitute, into the cells. With cells receiving the PCMBS treatment so as to yield physiological [K]c and [Na]c, the lower row of Table I shows that the same number of pumps was obtained as with fresh cells, both before and after anti-L, and the increase caused by anti-L was again highly significant.

Effect of Varying [K]c with and without Anti-L on the Numbers of Pumps

Cells were made with [K]c near the physiological concentration, and also at lower concentrations down to <1 mmol/liter cells. The numbers of functioning pumps per cell were then determined in cells not treated further (control) and after anti-L treatment. As shown by the experiment in Fig. 1, reducing [K]c in control...
cells led to an increase of ~70% in the number of pumps. At physiological [K]c, anti-L caused the usual increase. However, as [K]c was reduced, there was no further increase in the number of pumps on anti-L cells, and at near-zero [K]c, control and anti-L–treated cells had the same numbers of pumps.

One way to view this result is that Kc binding to a fraction of the pumps prevents them from binding ouabain, and, further, that anti-L relieves this inhibition by Kc of ouabain binding. As discussed above, Kc is a particularly potent inhibitor of the pump in LK sheep cells. If the Kc bound to pumps, which keeps ouabain off, also inhibits the pump, then it can be predicted that anti-L would reduce the inhibition of the pump by Kc. Since Kc reduces the number of functioning pump sites, and not simply the rate of ouabain binding, it can be predicted that the inhibition of the pump by Kc should be in part noncompetitive.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Number of Na/K pumps per LK sheep red cell with [K]c varied using the PCMBS method. Aliquots of cells were treated with anti-L antiserum. Results are also shown for control (untreated) cells. The highest [K]c is near physiological. Means ± SD are shown (n = 5). Similar results were obtained in three other experiments of the same design.

with Na, (this is a prediction, not a conclusion). The results of kinetic experiments in which these predictions were tested are presented in the next few sections.

**Kinetics of the Pump in Control Cells: Varying [Na]c at Fixed Values of [K]c.**

Before testing anti-L, it was necessary to characterize the kinetics of the pump in control cells. In previous kinetic studies on LK sheep cells, [K]c and [Na]c were varied reciprocally (Lauf et al., 1970), making analysis of the data cumbersome, and the constants obtained therefrom uncertain. Accordingly, cells were prepared with [Na]c values between 20 and 90 mmol/liter, and with three values of [K]c: near 10, near 4, and <1 mmol/liter. The results of 10 experiments on cells from four sheep are shown in Fig. 2. In each experiment, the pump fluxes were
FIGURE 2. Kinetics of activation of the Na/K pump in control LK sheep red cells by [Na], at three levels of [K],. The pump flux was the ouabain-inhibitable 86Rb influx; cell cation concentrations were altered using the PCMB method (see text). The fluxes in this experiment and most others were measured in an isotonic medium containing (mM): 145 NaCl, 5 KCl, 5 glucose, and 10 Tris-HCl, pH 7.5. In each experiment, the fluxes were normalized by setting at 1.0 the flux at the highest [Na], and the lowest [K], and expressing the other fluxes as the appropriate fractions. Results are shown for 10 experiments on cells from four different LK sheep, with 2–3 experiments on each. Symbols for fluxes show means ± SEM and the numbers of determinations. The means of the three levels of [K], (in millimoles per liter cells ± SEM) from all experiments are given at the right. In every experiment, there were four different values of [Na], at each [K],; within each experiment, each level of [Na], did not vary significantly among the cells at the three levels of [K],. However, the levels of [Na], did vary between experiments (e.g., the lowest [Na], in one experiment was similar to an intermediate [Na], in another). Six groups of [Na], levels were formed from the various experiments; they are indicated beneath the ordinate (means ± SEM, numbers of determinations). The curves were generated from the means of the normalized fluxes and the levels of [Na], using Eq. 2 as described in the text. The kinetic constants obtained from the computer fits are given in Table II.

The curves were generated from the data by a simplified form of Eq. 1:

\[ J = \frac{J_{\text{max}}'}{1 + \left( \frac{K'}{S} \right)^*} \]  

(normalized as described in the figure legend.

\[ J = \frac{J_{\text{max}}'}{1 + \left( \frac{K'}{S} \right)^*} \]
with the terms for inhibition omitted, giving estimates of $J_{\text{max}}'$ and $K_e'$ (apparent $J_{\text{max}}$ and $K_e$).

All three curves are sigmoid ($n > 1$), and $K_e$ is inhibitory. The most striking and surprising feature of the results in Fig. 2 is that the apparent $J_{\text{max}}$ appears to be reduced by $K_e$, which indicates noncompetitive inhibition of the pump by $K_e$.

Table II (A) shows the kinetic constants estimated using Eq. 2 from the data in Fig. 2. $J_{\text{max}}'$ at 3.6 mmol/liter [K], was 55% of that at 0.2 mmol/liter [K]. Raising [K] to 9.3 mmol/liter reduced $J_{\text{max}}'$ further, to 30% of its highest value. Using the asymptotic standard errors generated by the computer fits, the two lower $J_{\text{max}}'$ values were significantly reduced by raising [K]. The same statistical test did not indicate a significant difference between $J_{\text{max}}$ at 3.6 and 9.3 mmol/liter, though by inspection they appear different (there was considerable experimental error; the signal-to-noise ratio, pump/leak, was 1:1 at its best and 1:20 at its worst). Nevertheless, the results in Fig. 2 and their analysis in Table II (A) show noncompetitive inhibition of the pump by $K_e$ in LK cells, as predicted—an unusual effect of $K_e$ on the pump.

In the simplest model, $n$ would not vary with [K] and $K_e'$ would be positively correlated with [K] (because of competitive inhibition by $K_e$). However, the standard errors for $K_e'$ and $n$ estimated with Eq. 2 were too large to permit such conclusions to be drawn. Another approach will be taken below to evaluate $n$ and $K_e'$ vs. [K], or $I$, as it is symbolized.

**TABLE II**

Kinetic Constants for Activation of the Pump by Na, in Control LK Cells at Three Values of [K], Determined from the Data in Fig. 2

| [K], mmol/liter | $J_{\text{max}}'$ | $K_e'$ | $n$ |
|----------------|-----------------|--------|-----|
| 0.2            | 0.99±0.03       | 27     | 2.5 |
| 3.6            | 0.54±0.10*      | 35     | 2.9 |
| 9.3            | 0.29±0.06*      | 38     | 3.2 |

For A, curves had been fitted to the three sets of data individually using Eq. 2. For B, the same data were fitted at the same time using Eq. 1, as described in the text. $J_{\text{max}}'$ is the apparent maximum pump flux (normalized data); $K_e'$ and $K_e$ are the apparent and true Michaelis constants for Na as substrate; $K_{eC}$ is the constant for $K_e$ as competitive inhibitor. [K], $K_e'$, and $K_{eC}$ are all in millimoles per liter cells. Asymptotic standard errors from the computer fit are given (except for $K_e'$ and $n$ in A, which were obtained but not used in the analysis).

* Significantly different from $J_{\text{max}}'$ at [K] = 0.2 mmol/liter.

$K_{eC}$ 8.0±1.0

(All differences: 95% or larger confidence intervals.)
The results in Fig. 2 were also analyzed using a form of the Garay-Garrahane (1973) equation: \( \frac{J}{J_{\text{max}}} = \frac{1}{1 + (K_c/S)} \). For \([K_c]\) values of 0.2, 3.6, and 9.1 mmol/liter, the normalized \( J_{\text{max}} \) values were 1.39, 0.73, and 0.50, respectively. The relative \( J_{\text{max}} \) values were almost the same as those obtained using Eq. 2 (see Table II), and the fit of the curves with the Garay-Garrahane equation to the data was the same, but not as good statistically as with Eq. 2, as judged from the asymptotic standard errors.

The next step in the analyses of the results in Fig. 2, noncompetitive inhibition by \( K_c \) having been inferred, was to attempt to estimate \( K_{\text{IN}} \), the constant for noncompetitive inhibition by \( K_c \) (see Eq. 1). A replot of \( J_{\text{max}} \) and \( I \) (from Table II, A) was used:

\[
\frac{1}{J_{\text{max}}} = \frac{1}{J_{\text{max}} + (I/K_{\text{IN}})/J_{\text{max}}}. \tag{3}
\]

(This is the reciprocal of Eq. 1 with \( S \gg K_c \), and therefore \( J = J_{\text{max}} \).) There were three values of \( K_c \) and therefore three pairs of values; a good fit to a straight line was obtained (correlation coefficient = 0.999), and the estimated value of \( K_{\text{IN}} \) was 3.43 mmol/liter cells. Since the replot according to Eq. 3 yielded a straight line, we conclude that \( K_c \) acts as a noncompetitive inhibitor without any cooperative interaction between sites, and no Hill coefficient for the noncompetitive term need be included in Eq. 1. The estimation of two constants for inhibition by \( K_c \) assumes two separate sites of inhibitory action. The validity of this assumption will be shown below.

The third and last step in the analysis of the results in Fig. 2 was to fit all three sets of data (for all three values of \([K_c]\)) to Eq. 1, with two goals: first, to compare the curves obtained with those generated using Eq. 2 (which required three separate calculations, since Eq. 2 lacks terms for inhibition), and second, to attempt to estimate \( K_{\text{IC}} \), the constant for competitive inhibition by \( K_c \). In using Eq. 1, the value of \( K_{\text{IN}} \) estimated from Eq. 3 (3.43 mmol/liter) was used, and values for \( K_{\text{IC}}, K_c, \) and \( n \) were estimated. Fig. 3 shows the curves from Fig. 2 (dashed lines), generated using Eq. 2, and also the curves generated using Eq. 1 (solid lines). The two sets of curves are similar, and the data are fitted reasonably well by Eq. 1.

More important are the constants estimated using Eq. 1, shown in Table II (B) along with the asymptotic standard errors. \( K_{\text{IC}} \) is less than infinity, so there is, not surprisingly, inhibition by \( K_c \) competitive with \( Na_+ \). \( K_c \) is significantly greater than \( K_{\text{IC}} \) (and probably greater than \( K_{\text{IN}} \) also), which confirms the conclusion of Glynn and Ellory (1972) that LK pumps have a higher affinity for \( K_c \) as an inhibitor than for \( Na_+ \) as a substrate.

The question arises, regarding the experiment in Fig. 2, as to the choice of 5 mM \([K_c]\), and whether it is a saturating concentration, particularly as intracellular concentrations are varied. The choice was made because the pump/leak ratio declines as \([K_c]\) is increased, and the signal-to-noise ratio was a critical factor in these experiments. Therefore, the \( K_{1/2} \) for \( K_c \) was determined, permitting estimation of the fraction that \( J \) was of \( J_{\text{max}} \) at 5 mM \([K_c]\), and the experiment was carried out at two values of \([K_c]\): 1.0 and 12 mmol/liter. The flux at 5 mM \([K_c]\) was 83% of \( J_{\text{max}} \) in 12 mmol/liter \([K_c]\), cells and 87% in 1.0 mmol/liter \([K_c]\), cells
(constants were estimated using Eq. 2). The $K_v$ values for $[K]_o$ were 2.9 and 2.8 mM, respectively. Therefore, the use of fluxes measured at 5 mM $[K]_o$ did not lead to erroneous conclusions.

**Kinetics of the Pump in Anti-L–treated Cells**

Before testing the prediction that anti-L abolishes noncompetitive inhibition by $K_v$, we attempted to confirm the observation of Lauf et al. (1970) that stimulation by anti-L is not accompanied by a change in $K_v$ for $K_o$. The results of an experiment on fresh cells (Fig. 4) show a threefold stimulation of $J_{\text{max}}$, but no change (within 10%) in $K_v$ for $[K]_o$.

![Figure 3. Curves for activation of the Na/K pump by Na, in control LK sheep cells generated from the data in Fig. 2 by two different strategies. The dashed lines are the curves from Fig. 2, obtained using Eq. 2. The solid lines were calculated using Eq. 1, with a value for $K_{\text{IN}}$, the constant for K as a noncompetitive inhibitor, obtained using Eq. 3. $J$ is the normalized pump influx (see legend of Fig. 2 for normalization procedure); $I$ is the inhibitor concentration, $[K]_i$; and $S$ is the substrate concentration, $[Na]_c$ (both in millimoles per liter cells). The kinetic constants obtained in generating the curves are given in Table II.](image-url)

Fig. 5 shows the results of a single experiment like those in Fig. 2, designed to determine the effect of anti-L on noncompetitive inhibition by $K_v$. Pump influx was measured with varying $[Na]_c$ at two values of $[K]_i$ but with cells treated with anti-L, as well as control cells. Absolute, not normalized, fluxes are shown. With control cells, $J_{\text{max}}$ appears to decrease with increasing $[K]_i$, as shown in Fig. 2 and Table II (this part of the experiment in Fig. 5 was included in Fig. 2). Anti-L seemed to have two effects: first, $J_{\text{max}}$ was increased, and second, the two curves appear to converge at high $[Na]_c$, the prediction if anti-L abolishes
noncompetitive inhibition by $K_c$. The enormous stimulation by anti-L at low $[K_c]$ and low $[Na]_o$ (25 mmol/liter) was unusual; stimulation under these conditions was normally about twofold (see Fig. 8).

To be certain that the true $J_{\text{max}}$ was increased by anti-L, it was calculated using Eq. 2 from three experiments like those in Fig. 5 for normalized fluxes at $[K_c] < 2$ mmol/liter. The $J_{\text{max}}$ values were 0.62 ± 0.10 for control cells and 0.99 ± 0.02 for anti-L cells. (These are normalized fluxes, with the fluxes in control cells normalized to the flux in anti-L cells at the highest $[Na]_o$.) The true $J_{\text{max}}$ (1.08) was <10% higher than $J'_{\text{max}}$ (0.99) at $[K_c] = 0.2$ mmol/liter in control cells (Table II). It is likely that this relationship between $J_{\text{max}}$ and $J'_{\text{max}}$ also obtains for anti-L–treated cells and therefore that anti-L stimulates the true $J_{\text{max}}$ by ~60%.

The central question about the effect of anti-L on noncompetitive inhibition by $K_c$ is whether or not the $J'_{\text{max}}$ values are the same at all values of $[K_c]$. Table III shows $J'_{\text{max}}$ values from several experiments, calculated using Eq. 2. The errors are large because the fluxes are absolute; normalized fluxes cannot be used in judging whether $J'_{\text{max}}$ is a function of $[K_c]$ since these numbers were from separate experiments. The three $J'_{\text{max}}$ values are all about the same.

The question was also approached with kinetic experiments like those in Fig. 2, carried out with anti-L–treated cells with varying $[Na]_o$ at three fixed values of $[K_c]$; the results are shown in Fig. 6. The curves were generated using Eq. 2, except that $n$ was set at 2.5, as found for control cells (Table II). (This was done because the data in Fig. 6 did not permit a reliable estimate of $n$, mainly because
no flux values were obtained at [Na], values much below 25 mmol/liter.) A reasonably good fit was obtained assuming \( n = 2.5 \), which indicates that anti-L treatment did not modify the Hill coefficient. At 2.5, \( n \) is slightly higher than reported in similar experiments on human red cells (Garay and Garrahan, 1973), but it is consistent with there being three interacting Na-loading sites on each pump, which is the general view of the Na/K pump (Glynn and Karlish, 1975; Robinson and Flashner, 1979).

The kinetic constants generated by the three computer fits using Eq. 2 are shown in part A of Table IV. The three apparent maximum velocities are about the same (within \( \sim 8\% \)), which is consistent with the conclusion from the results in Table III, that anti-L abolishes noncompetitive inhibition by \( K_c \).

![Graph showing kinetic activation of the Na/K pump in LK sheep red cells in one experiment in cells pretreated with anti-L and in control cells. [Na] was varied at two levels of [K], (as indicated) by the PCBMS method. The curves were fitted by eye.](image)

**Figure 5.** Kinetics of activation of the Na/K pump in LK sheep red cells in one experiment in cells pretreated with anti-L and in control cells. [Na], was varied at two levels of [K], (as indicated) by the PCBMS method. The curves were fitted by eye. Summaries of a number of such experiments are shown in Fig. 2 for control cells and in Fig. 6 for anti-L cells.

The apparent \( K_s \) values obtained from the three separate fits using Eq. 2 appeared to increase as \([K]_c \) was increased, which is consistent with competitive inhibition by \( K_c \). To test this, all of the values of Fig. 6 were simultaneously fitted using Eq. 1 (with \( n = 2.5 \) and \( K_{IN} = \infty \), i.e., no noncompetitive inhibition); the kinetic constants generated are shown in part B of Table IV. \( J_{max} \) 0.98, was close to the mean \( J_{max} \) 0.97, obtained from the three separate fits (part A). Good estimates were also obtained for \( K_s \), the Michaelis constant for \( Na_c \), the substrate, and \( K_{IC} \), the constant for \( K_c \) as a competitive inhibitor. An important conclusion can be drawn by comparing \( K_s \) and \( K_{IC} \) for control cells (Table II) and anti-L cells (Table IV). Both are similar for the two types of cells (\( K_s \): 28.6 and 23.3; \( K_{IC} \): 8.0 and 9.3; control and anti-L, respectively), which indicates that anti-L...
did not alter the interaction of Na\textsubscript{c} with the pumps, or the interaction of K\textsubscript{c} as a competitive inhibitor at the Na-loading sites.

The curves fitted to the data in Fig. 6 by the two strategies (Eqs. 1 and 2) are plotted together in Fig. 7. The good correspondence shows that the results in Table III

| [K] \textsubscript{c} (mmol/liter cells) | J\textsubscript{max} (mmol/liter cells \cdot h) |
|-------------------------------|---------------------|
| 0.2                           | 0.33±0.10 (6)       |
| 5                             | 0.38±0.13 (7)       |
| 13                            | 0.30±0.10 (3)       |

Values for J\textsubscript{max} were calculated from absolute fluxes (not normalized data) using Eq. 2. The highest values for [Na\textsubscript{c}] were 76–80 mmol/liter cells. Means ± SEM are shown (numbers of experiments are in parentheses).

Figure 6. Kinetics of activation of the Na/K pump by Na\textsubscript{c} in anti-L–treated LK sheep red cells at three levels of [K\textsubscript{c}]. Alteration of cell cation composition, measurement of K influx, and normalization of the fluxes were carried out as described for the experiments in Fig. 2. Results are shown for four experiments on cells from three sheep. The mean [K\textsubscript{c}] values from all experiments are shown (millimoles per liter cells; n = 16). The curves were calculated from the mean normalized fluxes and mean [Na\textsubscript{c}] levels using Eq. 2, except that n = 2.5, as found for control cells (Table II). The kinetic constants obtained from the computer fits are given in Table IV, part A. The mean [Na\textsubscript{c}] levels differed slightly for the three [K\textsubscript{c}] levels, as shown. To reduce confusion, the errors for the mean [Na\textsubscript{c}] levels are not shown; none exceeded 3.2 mmol/liter.
TABLE IV

Kinetic Constants for Activation of the Pump in Anti-L-treated LK Cells by Na, at Three Values of [K], Determined from the Data in Fig. 6

(A) From Eq. 2 with \( n = 2.5 \)

| \([K]_e\) | \( J_{\text{max}} \) | \( K_c \) |
|---|---|---|
| 2.3 | 0.98±0.08 | 23.0±4.6 |
| 5.9 | 1.01±0.03 | 27.8±1.7 |
| 13.0 | 0.95±0.06 | 34.7±3.8 |

(B) From Eq. 1 with \( K_{\text{IN}} = \infty \) and \( n = 2.5 \)

\[ J_{\text{max}} = 0.98±0.03 \]
\[ K_c = 23.3±2.1 \]
\[ K_{\text{IN}} = 9.3±1.4 \]

For A, curves had been fitted to the three sets of data individually using Eq. 2 with \( n = 2.5 \) as calculated for control cells (Table II). For B, the same data were fitted in a single series of iterations using Eq. 1 with \( K_{\text{IN}} = \infty \) (no noncompetitive inhibition). See Table II for meaning of abbreviations and errors.

Fig. 6 are consistent with a single model (Eq. 1). Good correspondence could be expected since no term was included in the model for noncompetitive inhibition and since \( n \) was assumed, not estimated.

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Curves for activation of the Na/K pump by Na, in anti-L-treated cells generated from the data in Fig. 6 by two different strategies. The dashed lines are the curves from Fig. 6, obtained using Eq. 2. The solid lines were calculated using Eq. 1, \( n = 2.5 \) as for control cells; Table II), and \( K_{\text{IN}} = \infty \) (no noncompetitive inhibition; see Table III). \( J \) is the normalized pump influx; \( I \) is the inhibitor concentration, \([K]_e\); and \( S \) is the substrate concentration, \([\text{Na}]_e\) (both in millimoles per liter cells). The kinetic constants obtained in generating the curves are in Table IV, part B.
It may be useful to compare in the same graph the calculated relationships between fluxes and intracellular ion concentrations for control and anti-L cells. Fig. 8A shows pump fluxes, calculated using Eq. 1 from the experimentally estimated kinetic constants (except $n$ for anti-L cells, which was assumed), as a function of $[\text{Na}]_c$ ($S$) at two values of $[\text{K}]_c$ ($I$), zero and near-physiological. Fig. 8B shows fluxes calculated as a function of $I$ at a single high $S$ (90 mmol/liter). The relationships in Fig. 8A are similar to those in Fig. 5, results from a single experiment. In Fig. 8B, $S$ is $\sim 4 \times K_s$, and stimulation ranges from 60% at zero $[\text{K}]_c$ to 13-fold at 20 mmol/liter $[\text{K}]_c$.

**ATP in Anti-L Cells**

Fig. 6 contains no values near zero $[\text{K}]_c$ (unlike Fig. 2). Under the condition of expected maximum pump flux, i.e., anti-L cells with high $[\text{Na}]_c$ and zero $[\text{K}]_c$, the pump flux was often lower than in cells with 2 mmol/liter $[\text{K}]_c$. This is probably attributable to the activation of pyruvate kinase by $K$ (Kachmar and Boyer, 1953; Bashan et al., 1975), and the inability of glycolysis to synthesize ATP at a sufficient rate in anti-L--treated cells with zero $[\text{K}]_c$ (apparently the rate of ATP synthesis is sufficient in the more slowly pumping control cells with zero $[\text{K}]_c$).

$[\text{ATP}]$ in cells with near-zero $[\text{K}]_c$ was measured at the end of the normal 1-h incubation after PCMBS (with dithiothreitol, inosine, adenine, and orthophosphate in the medium). It ranged from 0.15 to 0.28 mmol/liter ($n = 8$). After an additional hour (in the same medium, but lacking dithiothreitol), $[\text{ATP}]$ increased, but never exceeded 0.48 mmol/liter. The medium for these cells contained no $K$, so the pump was consuming no ATP. Therefore, the rate of ATP synthesis was $\sim 0.26$ mmol/liter cells·h. With 2 mol of $K$ pumped per mole of ATP hydrolyzed, a pump flux of 0.5 mmol/liter cells·h could be sustained at this rate of synthesis, a flux far higher than the maximum in control cells, but less than the fluxes often observed in anti-L cells. The problem with obtaining good kinetic data in near-zero $[\text{K}]_c$, anti-L cells is therefore attributable to an insufficient rate of ATP synthesis in the absence of $K_c$.

**Maximum Pump Turnover**

The increase in the number of functioning pumps at physiological $[\text{K}]_c$, and the reduction of noncompetitive inhibition do not explain all of the stimulation by anti-L: at near-zero $[\text{K}]_c$, the numbers of functioning pumps are the same in control and anti-L cells (Fig. 1), and $I_{\text{max}}$ is $\sim 60\%$ higher in anti-L than control cells. Therefore, the turnover number of the pumps is higher in anti-L cells.

The explanation may be that anti-L alters the affinity for a ligand other than the ones tested, which were $K_o$, $\text{Na}_o$, and $K_c$. Perhaps there is an increase in affinity for $Mg$ or $\text{ATP}$, or a decrease in affinity for $\text{Na}_o$, $\text{ADP}$, or orthophosphate as inhibitors. Since $K_o$ at 5 mM is nearly saturating, an effect on $\text{Na}_o$ as an inhibitor is likely only if $\text{Na}_o$ is a noncompetitive inhibitor; this is unlikely, but has not been tested.

We tested the possibility that $Mg_c$ is limiting, and that anti-L raises the pumps' affinity for it. As shown in Table V, setting $[\text{Mg}]_c$ at a saturating concentration
FIGURE 8. Curves for the activation of the Na/K pump in control and anti-L-treated cells. The curves were calculated using Eq. 1 from the constants in Tables II and IV and in the text. (A) pump flux ($J$) as a function of [Na]$_c$ ($S$) at two [K], levels (I). (B) $J$ as a function of $I$ at $S = 90$ mmol/liter. See Table II for the measurements and units of the other symbols. The constants are given below.

| Cells  | $J_{\text{max}}$ | $K_a$ | $K_{\text{mNa}}$ | $K_{\text{mC}}$ | $n$ |
|--------|------------------|-------|------------------|-----------------|----|
| Control| 0.63             | 28.6  | 3.43             | 8.0             | 2.5 |
| Anti-L | 1.00             | 23.3  | $\infty$         | 9.3             | 2.5 |
with 1 mM Mg and 10 μM A23187 in the medium (Robinson and Flashner, 1979) had no effect on stimulation of the pump by anti-L. The inhibition of the pump by A23187 without Mg is presumably due to a reduction in [Mg]_; on the other hand, an increase in cellular Ca cannot be ruled out, even though the cells had been washed in a medium containing EGTA. Anti-L cells were inhibited less with A23187 than were control cells. The meaning of this is not clear, and the effect was not always observed.

With the difficulties described above in setting and maintaining the desired cellular ATP concentrations, the intriguing possibility that anti-L raises the turnover number of the pumps by raising their affinity for ATP was not tested. On the other hand, an increase in “intrinsic” turnover number of the pump is conceivable, i.e., an increase in the maximum rate of pump function with no limiting ligand (activator or inhibitor).

**TABLE V**

| K pump influx | Control    | Solvent-control | A23187 | A23187 + Mg |
|---------------|------------|-----------------|--------|------------|
| mmol/liter cells.h | 0.34±0.04  | 0.36±0.02       | 0.02±0.08 | 0.23±0.05 |
| Anti-L-treated | 0.71±0.03  | 0.77±0.02       | 0.41±0.07 | 0.65±0.07 |

Cells were washed in the standard solution with EGTA added (2 mM) to reduce Ca. After treatment with anti-L, fluxes were measured (with or without ouabain) as described in the text under the conditions indicated. Stock A23187 solutions (25 mM) were in ethanol. The Mg concentration was 1 mM. Final A23187 concentrations were 10 μM. The solvent-control contained ethanol at 0.4% vol/vol. Means are shown ± SD (n = 4) from one experiment. Similar results were obtained in six other experiments on cells from this sheep and four others.

**DISCUSSION**

We have studied the mechanism by which an antibody specific for a blood group antigen on red cells from sheep of the LK phenotype stimulates the Na/K pump in these cells. We employed two experimental approaches: (a) measurement of the number of functioning Na/K pumps by correlating ouabain binding with inhibition of the pump; and (b) kinetic studies of activation of the pump by Na at selected concentrations of Kc. We made the following four observations. (a) Anti-L causes a twofold increase in the number of functioning pumps at physiological [K]c; lowering [K]c in control cells also increases the number of functioning pumps, but not in anti-L cells. (b) As predicted from these observations on the numbers of pumps, Kc was shown to be a noncompetitive inhibitor of the Na/K pump, an unusual interaction of Kc with the pump. (c) As predicted further from the experiments on the numbers of pumps, anti-L abolishes noncompetitive inhibition by Kc, thereby increasing both pump fluxes and the number of functioning pumps at physiological [K]c. (d) Finally, there is an additional stimulatory effect of anti-L on the LK pumps, either owing to an alteration in affinity for a ligand other than Na and K, or perhaps owing to an increase in “intrinsic” turnover number.
Our first observation, that anti-L promoted a twofold increase in the number of functioning pumps in fresh LK sheep red cells, is controversial; Joiner and Lauf (1978b) reported no increase. The discrepancy is difficult to resolve completely, but perhaps some helpful points can be made. We measured the numbers of functioning pumps by extrapolation through a plot of percent inhibition of the pump vs. the number of ouabain molecules bound per cell. In most experiments, the maximum inhibition was 80%, so the technique minimized the counting of slowly binding, inactive pumps.

In contrast, Joiner and Lauf, in many of their experiments, took as the number of pumps the number of ouabain molecules bound per cell near saturation binding. Therefore, Joiner and Lauf may have included in the number of pumps some inactive ones with low ouabain affinity. On the other hand, our number of functioning pumps (41) is in the middle of Joiner and Lauf’s range (30–50). It is worth noting that in an earlier study from Lauf’s laboratory, in which stimulation of a number of pumps by anti-L was reported (Lauf et al., 1970), the experimental approach was the same as ours used here.

We also found in the present study that decreasing $[K]_c$ caused an increase in the number of functioning pumps in control, but not anti-L cells, and furthermore that the numbers for the two cell types was the same at near-zero $[K]_c$ (Fig. 1). Joiner and Lauf (1978b) carried out a similar experiment (Fig. 6 of their paper). At low $[K]_c$, they also found no difference between anti-L and control cells (though again ouabain binding was taken to saturation and not correlated with transport). It is of interest that at $[K]_c$ slightly above physiological (25 mmol/liter cell water) in their experiment, the extrapolation of the curve for anti-L cells was considerably higher than for control cells, a result that is consistent with ours, but on which they do not comment.

We made the following prediction from the increase in number of pumps with decreasing $[K]_c$ in control cells and the lack of increase in anti-L cells: $K_c$, in controlling the number of ouabain-binding sites (not the rate of ouabain binding, which it also does), is acting as a noncompetitive inhibitor of the pump. (If $K_c$ controlled the rate of binding, but not the number of ouabain molecules required for 100% inhibition, competitive inhibition by $K_c$ would be a sufficient explanation of the result.)

This prediction was tested by determining whether $K_c$ reduced the apparent maximum velocity of the pump, $J_{\text{max}}$. $K_c$ as a competitive inhibitor would reduce the apparent affinity for Na, the substrate, but not $J_{\text{max}}$. The results of the experiments (Fig. 2) and their analysis (Table II) show that $K_c$ is indeed a noncompetitive inhibitor of the pump in LK sheep cells; $J_{\text{max}}$ at near-physiological $[K]_c$, 9.3 mmol/liter cells·h, was threefold lower than that at a $[K]_c$ of 0.2 mmol/liter.

It is well known that $K_c$ is a competitive inhibitor of the Na/K pump in many types of cells (Glynn and Karlish, 1975; Robinson and Flashner, 1979); we are not aware of another report of $K_c$ as a noncompetitive inhibitor. The interesting feature of the noncompetitive inhibition by $K_c$ in LK cells is that it can be abolished by binding of anti-L to the L antigen at the extracellular membrane surface (Tables III and IV and Fig. 6), as predicted from Fig. 1.
It was also demonstrated that anti-L treatment had little or no effect on the binding of Na\(_a\) as a substrate, or on the interference with that binding by K\(_c\), i.e., competitive inhibition (see legend to Fig. 8). One implication of this result is that anti-L, binding extracellularly, exerts a highly specific effect on the pump at its intracellular surface. A related implication is that the site at which K\(_c\) exerts its noncompetitive inhibitory effect is not closely associated with the sites at which Na\(_a\) and K\(_c\) compete.

It could also be predicted from Fig. 1 that the pumps on LK cells have a heterogeneous affinity for ouabain, and in raising the number of functioning pumps with K\(_c\) present, anti-L should not only increase the rate of ouabain binding, but should also have the greatest effect on the pumps with lowest affinity and lowest pump activity, thereby increasing not only the number of functioning pumps and the rate of binding, but also the homogeneity of the rate of binding. This was observed and reported some years ago (Dunham, 1976a).

The effect of anti-L on K\(_c\) binding leads to another prediction, namely that K\(_c\) affects antibody binding. We recently tested this hypothesis, and demonstrated that indeed K\(_c\) has such an effect. More specifically, K\(_c\) enhances the extent of anti-L binding (Farquharson and Dunham, 1986).

In a preliminary report of a recent study, kinetic constants were presented for the Na,K-ATPase activity of LK sheep red cell membranes (Ellory, 1985). In making these estimates, it was necessary to correct for activation by K at its extracellular site since sidedness is not possible in a broken membrane preparation. In control membranes, results similar to ours were reported: a K\(_f\) for Na\(_a\) of 32 mmol/liter, and an inhibitory constant for K\(_c\), K\(_i\), of 4 mmol/liter. Unlike our results, anti-L treatment seemed to raise both affinities, with K\(_f\) reduced to 5 mmol/liter and K\(_i\) to 1.7 mmol/liter. Another important difference from our results was that anti-L did not appear to increase f\(_{\text{max}}\). Finally, no distinction was made between competitive and noncompetitive effects of K\(_c\). It is difficult to resolve the differences between these two sets of results, ours on transport in intact cells, and Ellory's, obtained by a slightly less direct approach on ATPase in a broken membrane preparation. There are three possible explanations: (a) a difference in the effects of two different antibody preparations (cf. Dunham et al., 1984), (b) a consequence of differences in technique, and (c) a difference in red cells between breeds of sheep.

The stimulation of the pump by anti-L is explained only in part by our results on the action of K\(_c\). At near-zero [K\(_c\)], where the number of functioning pumps is the same in control and anti-L cells (Fig. 1), and K\(_c\) is insufficient to inhibit significantly (K\(_{\text{in}}\) > 3 mmol/liter), the pump flux in anti-L-treated cells exceeded that in control cells by >50%. Therefore, each pump has a higher molecular activity in anti-L-treated cells, which was the conclusion of Joiner and Lauf (1978b) about the sole effect of anti-L. We have tentatively ruled out an increase in affinity for Mg as being responsible for the stimulation (Table V); the affinities for ligands other than K\(_c\), K\(_i\), and Na\(_a\) have not been tested. It is possible that a change in the conformation of the pump caused by anti-L alters a limiting rate constant of one of the reactions of the pump without changing the affinity for a ligand. This conformational change may be related to the one resulting in reduced affinity for K\(_c\) as a noncompetitive inhibitor.
Goats possess an HK/LK polymorphism similar to that of sheep, and the Na/K pumps of LK goat red cells are stimulated by alloimmune sheep anti-L antiserum (Ellory and Tucker, 1970). Several studies have been undertaken to determine the basis for the stimulation in LK goat red cells. Sachs et al. (1974b) suggested, from results of kinetic studies, that stimulation was largely or entirely a consequence of the reduction of the affinity for $K_c$ as an inhibitor. More recently, Cavieres and Ellory (1977) presented results from kinetic studies of the Na,K-ATPase activity of broken membrane preparations of LK goat cells before and after anti-L treatment. Since the preparation lacked sidedness, it was necessary when $[K]$ was varied to correct for the activation by K at its extracellular loading site (as in Ellory's more recent study [1985] on sheep cell ATPase), a correction obviously not necessary in kinetic studies on intact cells when $[K]_c$ is varied. Nevertheless, the correction employed by Cavieres and Ellory (1977) appeared valid, and the results of their study indicated that stimulation in LK goat cells was due entirely to a reduction in the affinity of $K_c$ as a competitive inhibitor. Our conclusions on sheep cells are different: we found that $K_c$ is a noncompetitive inhibitor and that noncompetitive inhibition was abolished by anti-L. Cavieres and Ellory found no indication that $K_0$ could inhibit noncompetitively in goat cells. Despite the difference in experimental approach (pump fluxes in intact cells in our experiments: Na,K-ATPase activity of broken membranes on the goat cells) and source of antibody, a true species difference is a possible explanation for the difference in results.

In another study on LK goat cells, Dunham and Ellory (1980) found that destruction by trypsin of the L antigen resulted in stimulation of the pump entirely ascribable to reduced inhibition by $K_c$. $J_{\text{max}}^{\text{max}}$ at near-zero $[K]_0$ was the same in control and trypsinized cells, unlike in anti-L–treated sheep cells at near-zero $[K]_0$. These results on goat cells are therefore consistent with those of Cavieres and Ellory (1977). However, the experiments are not directly comparable to those reported here, since trypsinization of LK sheep cells destroys the L antigen but does not stimulate the pump (Lauf et al., 1971).

There has been one study on the effect of anti-L on the number of functioning pumps of LK goat cells. At the same time that the apparent effects of anti-L on affinity of the pumps for $K_c$ were shown (Sachs et al., 1974b), Sachs et al. (1974a) showed that anti-L increased the number of functioning pumps per cell by ~20%. The measurements were made in the same manner as in the present work: correlation of percent inhibition of the pumps with ouabain molecules bound per cell at levels of inhibition of 20–80%.

It is assumed, but has not been demonstrated, that the L antigen is a separate molecular entity from the pump. If it is, then the antigen is an inhibitor of the pump. A similar interaction may occur with the $F_1F_0$-ATPase of mitochondrial membranes and its peptide inhibitor (Pederson et al., 1981). Little is known of the mechanism of action of this inhibitor, but it may nevertheless be worth considering the L antigen in the broader context of membrane-associated ATPase inhibitors.

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