Active Cation Transport and Ouabain Binding in High Potassium and Low Potassium Red Blood Cells of Sheep

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ABSTRACT Red cells from high K sheep contained 82 mM K/liter cells and had a pump flux of 0.86 mM K/liter cells × hr; similarly, LK cells had 16.5 mM K/liter cells and a pump flux of 0.12 mM K/liter cells × hr. Using [^{3}H]-ouabain, the relation between the number of ouabain molecules bound per cell and the concomitant per cent inhibition of the pump was found to be approximately linear for both HK and LK cells. The number of glycoside molecules necessary for 100% inhibition of the pump was 42 for HK cells and 7.6 for LK cells, after correction for six nonspecific binding sites for each type of cell. The ratio of ouabain molecules/cell at 100% inhibition was 5.5, HK to LK, and the ratio of the normal K pump fluxes was 7.2, HK to LK. The similarity of these ratios suggests that an important difference between HK and LK cells, determining the difference in pump fluxes, is the number of pump sites. The turnover times (ions/site × min) are 6000 and 4800 for HK and LK cells, respectively. The results also indicate a high specificity of binding of ouabain to pump sites.

INTRODUCTION

In populations of sheep, the red blood cells of some individuals contain a K concentration typical for animal cells (about 80 mM/liter cells), while the majority of individuals have red cells with a low K concentration (about 13 mM/liter cells) (Evans, 1968; Evans et al., 1958). The Na concentrations in the two cell types are complementary to the K concentrations so that the sums of the Na and K concentrations are the same. Chloride concentrations, water content, and cell volumes are also the same (Tosteson and Hoffman, 1960). Progeny tests have shown that these two phenotypes are determined by a single genetic locus with two alleles, and that the allele for low K (LK) is

dominant over the allele for high K (HK) (Evans and King, 1955; Evans et al., 1956).

The cation transport characteristics of HK and LK red cells have been described previously by Tosteson and Hoffman (1960). The HK and LK cells were found to have qualitatively similar active cation transport systems. Both cell types were shown to transport K inward coupled one to one to outward Na transport. The different steady-state cation compositions of HK and LK cells could be ascribed to quantitative differences in transport rates and permeabilities. Under the conditions of their experiments, the rate of Na and K transport was about four times higher in HK than in LK cells. LK cells were about three times more permeable to K than HK cells while the Na permeabilities were more nearly the same.

Ouabain and related cardiotonic steroids are known to be specific inhibitors of active cation transport in red cells (Schatzmann, 1953) and in many other types of cells (Glynn, 1964). With the availability of tritiated glycosides, like [^{3}H]-ouabain, it has become possible to study in greater detail their interaction with cation transport mechanisms. There are reports of the binding of glycosides to microsomal preparations containing Na, K-ATPase activity isolated from *Electrophorus* electric organ (Albers et al., 1968), calf cardiac muscle (Matsui and Schwartz, 1968; Schwartz et al., 1968), or to human red cells (Hoffman and Ingram, 1968) and their ghosts (Hoffman, 1969). In all three systems, cardiac glycosides were shown to bind with high affinity. Binding was found to be promoted by Mg, by ATP and related compounds, and by Na under some conditions. The binding was prevented by K (Hoffman, 1966), accounting for the mechanism by which K reduces the inhibitory effect of glycosides on transport (Glynn, 1957).

In the present study we determined the minimum number of ouabain molecules bound per HK and LK red cell which will completely inhibit active transport. The similarity of the ratio of the pump rates, HK to LK, and the ratio of ouabain molecules per cell at 100% inhibition of transport is consistent with a high specificity of ouabain binding to transport sites in sheep red cells. The results make possible reliable estimates of the number of transport sites per cell and the turnover time per site. At the same time the results support the suggestion that an important difference between HK and LK cells is the number of transport sites per cell. Preliminary reports of some of the results have been published (Dunham and Hoffman, 1969; Hoffman, 1969).

MATERIALS AND METHODS

1. *General*

The sheep used were adults from a flock of Dorsets maintained by Yale University in Bethany, Connecticut. The blood (250–350 ml) was drawn into heparin from the

jugular vein, always on the day of its use in an experiment. 6 wk or more lapsed before blood was drawn again from a sheep.

The general experimental approach was to expose washed cells to [^{3}H]-ouabain at various times and concentrations. Then the cells were washed again and divided into two aliquots. On one aliquot, the amount of bound [^{3}H]-ouabain was determined. On the other aliquot, the rate of K influx was determined, and the extent of inhibition was calculated after comparison with the rate of transport in cells not exposed to ouabain. The details of these and other procedures are given below.

In order to prepare washed, packed cells for use in each experiment, the freshly drawn blood was first centrifuged at 12,000 *g* for 5 min at 0–4°C in 50 ml polycarbonate centrifuge tubes. The cells were resuspended in about five volumes of an isotonic NaCl-Tris medium [153 mM NaCl, 17 mM tris(hydroxymethyl)aminomethane chloride, and 200 mg% glucose at pH 7.5] and centrifuged again. This procedure was repeated three additional times, taking care to remove by aspiration the buffy coat from the surface of the packed cells at the end of each wash. After the final wash the supernatant was removed and the packed cells (hematocrit greater than 95%) were resuspended for incubation under various conditions specified below.

For all analyses of cells (e.g. for determination of bound [^{3}H]-ouabain or for the measurement of K influx) the cell suspensions were centrifuged at 12,000 *g* for 10 min in one of two kinds of special Lucite centrifuge tubes. These tubes were designed so that either 1.5 or 4 ml of cells could be packed at the bottom in a column about 3 cm in length. The tubes held about 15 ml of suspension. The interior was shaped so that the large upper part of the tube tapered in such a manner that the cells during centrifugation funneled into the smaller bore column at the bottom. The outside dimensions of the tubes were the same as standard 50 ml polycarbonate centrifuge tubes. After removal of the supernatant, the packed cells were pipetted from the bottom of the tube by calibrated syringe for the desired set of analyses (e.g. radioactivity, Na, K, hemoglobin). The hematocrit of the packed cells was approximately 97.5%

The concentrations of Na and K in hemolysates of packed cells, supernatants, and media were measured using an Instrumentation Laboratory (Instrumentation Laboratory, Inc., Boston, Mass.) flame photometer. Hemoglobin concentrations were determined spectrophotometrically at 540 nm. ^{42}KCl and ^{24}NaCl were obtained from Cambridge Nulcear Corp. and were counted in a well-type scintillation counter to ± 1% accuracy. The osmolality of all solutions used was approximately 310 milliosmolal determined with an Advanced Instrument osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.).

2. *Determination of Bound Ouabain*

Portions of the washed packed cells were incubated at 37°C in a reciprocating water bath, in media containing [^{3}H]-ouabain usually between 1×10^{-8} and 4×10^{-8} M. The incubation medium had the same composition as the washing solution, or with various concentrations of CsCl substituted for NaCl. Each flask contained 15 ml of cells in 45 ml of medium.

As a part of each experiment cells were also incubated without any ouabain. The subsequent measurement of K influx on these cells served as the control or uninhibited

flux. These cells were also used in background samples and internal standard samples in the determination of [^{3}H]-ouabain. Another flask in each experiment contained, in addition to the added [^{3}H]-ouabain, a high concentration of nonradioactive ouabain, usually 10^{-4} M, to reduce the specific activity of the [^{3}H]-ouabain. From the tritium content of cells incubated in the low specific activity [^{3}H]-ouabain, a correction could be made for bound tritiated contaminants. The same cells were also used later (see below) for determination of ouabain-insensitive K influx. All determinations of [^{3}H]-ouabain were carried out in duplicate on cells and supernatants obtained from each flask.

The [^{3}H]-ouabain was obtained from New England Nuclear Corp. The specific activities of the two preparations used were 515 and 620 mCi/mole. These values were given by the manufacturer and were used in the calculations to obtain the results reported in this paper.

At the end of the exposure to [^{3}H]-ouabain, the cells were washed twice by centrifugation in the cold with 10 volumes of NaCl-Tris medium (see above). The tritium content of the supernatant after two washes was always less than 20% above background. After washing, a portion of the cells was centrifuged 10 min at 12,000 *g* in the larger of the special Lucite tubes so that portions of the packed cells could be removed and analyzed directly for [^{3}H]-ouabain content. The remaining portion of the cells was used for determination of K influx (or Na outflux) as described below.

The [^{3}H]-ouabain bound to the cells was extracted for liquid scintillation counting in the following way: 1 ml of packed cells was transferred directly using a syringe into 18 ml of Bray's solution (Bray, 1960) contained in an ordinary counting vial (27.5 × 58 mm). The counting vial was immediately capped and stirred vigorously on a vortex mixer. Since very little lysis of cells occurred in Bray's solution, the cell mass could be sedimented by centrifuging the capped counting vial. The vial was centrifuged for 15 min at 3600 *g* in an RC-3 Sorvall centrifuge equipped with a swinging bucket rotor. The dark cell mass layered flat on the bottom of the vial below an almost colorless supernatant.

This extraction into Bray's solution removed virtually all the [^{3}H]-ouabain from the cells. This was shown by comparing the radioactivity of samples from the one batch of labeled cells prepared by extraction in Bray's solution, as described above, with cells solubilized in Nuclear Chicago Solubilizer (NCS). In order to reduce quenching after solubilization (0.070 ml cells in 1.5 ml NCS), the hemoglobin was partially bleached with 1.5 ml of a saturated solution of benzoyl peroxide in toluene. The scintillation fluid in this procedure was toluene containing the standard amounts of PPO and POPOP. The results obtained by this procedure were compared with the results obtained by extraction into Bray's solution, after determination of absolute counting efficiency (see below). In terms of disintegrations per minute per milliliter of cells, the results for cells dissolved in NCS were indistinguishable from those extracted into Bray's solution.

The background counts of the vials were increased by the presence of the cells, presumably due to fluorescence caused by a small amount of hemoglobin (or heme) in solution. Background samples were always prepared with unlabeled cells in Bray's solution. The background was reduced by storing the prepared vials in the cold for a day before counting.

Counting efficiency was determined in each experiment for every sample using both internal and external standards. Internal standards were made with 0.5, 1.0, and 1.5 ml of unlabeled cells in Bray's solution to which a known amount of [^{3}H]-toluene standard was added. The ratio of counts from an external standard in two channels of the Nuclear Chicago Mark I counter was determined for the internal standards. Absolute counting efficiency was plotted against the channels ratios for the external standard. The channels ratio for the external standard was also determined for each of the experimental samples. The absolute counting efficiency for each sample was then read from the plot of efficiency against channels ratio. The counting efficiency with 1 ml of cells in 18 ml of Bray's solution was near 7 %.

The number of ouabain molecules bound per cell was calculated using the expression:

$$G = \frac{C \cdot \frac{100}{E} \cdot N}{V \cdot S \cdot 222 \cdot 10^{10}}$$

where G = ouabain molecules per cell,
C = counts per min per ml cells,
E = per cent counting efficiency,
N = Avogadro's number,
V = number of cells per ml,
S = specific activity of [^{3}H]-ouabain in Ci/mole, and
$222 \cdot 10^{10}$ = disintegrations per min per Ci.

The determinations of number of cells per unit volume were made with a Celloscope counter (Particle Data, Inc., Elmhurst, Ill.) on dilutions of known volumes of packed cells. There were approximately 3.3×10^{10} cells/ml for both HK and LK cells determined on two sheep of each type. Thus, the mean cell volume was 30 μ^3.

3. *K Influx*

The influx of K was measured, using ^{42}K, in cells that had been washed after incubation either with or without [^{3}H]-ouabain as described above. This was done to determine the extent of inhibition caused by the bound [^{3}H]-ouabain. 6 ml of washed packed cells were added to each flask containing 30 ml of medium. The concentration of K in the influx medium was usually 5 or 10 mM. No additional ouabain was added for the influx measurement to cells which had previously been exposed to [^{3}H]-ouabain. The control (uninhibited) flux was measured on cells which had not been exposed to [^{3}H]-ouabain but which had been incubated and washed under the same conditions as the cells exposed to [^{3}H]-ouabain. The ouabain-insensitive flux was determined on control cells using 1×10^{-4} M ouabain.

After equilibrating the cell suspensions at 37°C for 10 min, 0.1 mCi of ^{42}K was added to each flask. Samples of cells (about 2 ml of packed cells) were collected for analysis in the small special Lucite centrifuge tubes at 15, 45, and 75 min (HK cells) or 30, 90, and 150 minutes (LK). Aliquots of 0.50 ml of packed cells and their supernatants were taken for analysis.

The unidirectional influx of K, ${}^iM_K$, in mM/liter cells × hour, was calculated from the following equation (see Sheppard, 1962), which assumes first-order kinetics in a closed, well-mixed, two-compartment system in steady state:

$$ {}^iM_K = ({}^ik_K + {}^ok_K) \frac{(K)_i(1 - H)(K)_o}{(H)(K)_i + (1 - H)(K)_o} $$

where H is the hematocrit, $(K)_i$ is the concentration of K in the cells in mM/liter cells' $(K)_o$ is the concentration of K in the medium in mM/liter, and $({}^ik_K + {}^ok_K)$ is the sum of the inward and outward rate constants in reciprocal hours. The hematocrit is obtained from the ratio of the concentration of hemoglobin in the cell suspension to that in the packed cells. $({}^ik_K + {}^ok_K)$ is evaluated by plotting the quantity ln $(1 - X_C/X_E)$ against time where X_C is the specific activity of the cells and X_E is the specific activity of the cell suspension at equilibrium.

4. *Na Outflux*

The unidirectional outflux of Na was measured along with [^{3}H]-ouabain binding in a few experiments on HK cells. The method, using ^{24}Na, as described by Hoffman (1962) for red cell ghosts, was adapted for use with intact cells. Cells were first washed four times by centrifugation at 12,000 *g* for 5 min with five volumes of the cold (0–4°C) NaCl-Tris medium at pH 7.5. The washed cells were divided into two portions, one of which was exposed to [^{3}H]-ouabain. The second portion of cells was incubated with [^{3}H]-ouabain under identical conditions but in addition was exposed to ^{24}Na. These cells were used for the determination of the inhibition of the Na pump by the measured extent of [^{3}H]-ouabain binding. From the control and ouabain-insensitive fluxes, the level of inhibition caused by the measured amount of ouabain could be calculated. All flasks were incubated at 37°C in a shaker bath for 3–5 hr at a hematocrit of 5%. Incubations with ^{24}Na were carried out for the entire 3–5 hr period. Exposure to [^{3}H]-ouabain was for various times up to 2 hr. Cells incubated without either ^{24}Na or [^{3}H]-ouabain served as background samples in conjunction with the determination of [^{3}H]-ouabain as described before. In addition, one pair of flasks incubated with and without ^{24}Na was also incubated in very low specific activity [^{3}H]-ouabain (less than 10^{-7} M [^{3}H]-ouabain + 10^{-4} M unlabeled ouabain). As described before, these cells served for the determination of the ouabain-insensitive flux and for the determination of any tritiated contaminants which might also be bound to the cells.

After the above-described incubations with ^{24}Na and [^{3}H]-ouabain the cells were washed again four times to remove any remaining extracellular radioactivity. For the determination of the Na outflux, 0.5 ml of packed, washed cells was added to 30 ml of NaCl-Tris medium containing 10 mM KCl. Samples of about 7 ml of the suspension were removed immediately and centrifuged at 12,000 *g* for 5 min in 12 ml polycarbonate tubes. 4 ml of the supernatant was set aside for determination of the initial concentration of ^{24}Na in the medium (zero time sample). Subsequent samples of the medium were taken in the same manner at 30 and 60 min. The equilibrium distribution of ^{24}Na was obtained from the radioactivity contained in a 4 ml sample of the whole cell suspension. Samples of packed, washed cells were also taken for determination of intracellular Na and K concentrations by the method described above.

The outward rate constant, ${}^{o}k_{Na}$, in units of reciprocal hours, was calculated for the 0–30 and 0–60 min intervals using the following expression:

$$ {}^{o}k_{Na} = -\frac{1}{t} \ln \left[1 - \frac{R_t - R_o}{R_{eq} - R_o} \right] $$

where R_t = cpm of ^{24}Na in the supernatant at time t,
R_o = cpm of ^{24}Na in the zero time supernatant,
R_{eq} = cpm of ^{24}Na of the cell suspension, and
t = the time interval between R_o and R_t.
The ${}^{o}M_{Na}$ is given by the product of the mean ${}^{o}k_{Na}$ and $(Na)_i$.

5. *Determination of Genotype*

The genotype of sheep at the Ka locus (the notation is that of Rasmusen and Hall, 1966), the determinant of cation composition, can be evaluated by progeny tests, or more conveniently, by determining the phenotype of the individuals with respect to the presence or absence of the antigenic factor M. M-positive red cells are hemolyzed by an ovine antiserum and M-negative cells are not. The M-positive allele is dominant. Rasmusen and Hall (1966) found that, of 115 sheep, all M-negative sheep (22) were also LK. All the sheep known from progeny tests to be heterozygous at the Ka locus ($Ka^{L}ka^{h}$) were M-positive. These results suggested that all M-negative sheep (mm) are also homozygous LK ($Ka^{L}Ka^{L}$), and further that the alleles m and Ka^{L} and the alleles M and ka^{h} are associated. It is not yet known whether the alleles are closely linked or identical. No instances of apparent crossing-over are known. Since M and Ka^{L} are dominant, tests of red cells from a sheep for both cation content and antigen M will give the genotype at the Ka locus. LK, M-negative = $Ka^{L}Ka^{L}$ (homozygous LK); LK, M-positive = $Ka^{L}ka^{h}$ (heterozygous LK); HK, M-positive = $ka^{h}ka^{h}$ (homozygous HK). We are indebted to Dr. P. K. Lauf of Duke University for determining the M-antigen phenotypes.

RESULTS

1. *Sheep Population*

Red cells from 31 adult rams and ewes were analyzed for Na and K. The frequency distribution of the intracellular K concentrations (Fig. 1) shows the discontinuous distribution of the phenotypes. $(K)_i + (Na)_i$ was about the same in all individuals. The mean $(K)_i$ in freshly drawn cells in the 11 HK and 20 LK sheep were 82 and 16.5 mM/liter cells, respectively. The frequency distribution of red cell cation concentrations in our sheep is similar to that of some other breeds (see Evans, 1968).

The genotype of each sheep at the Ka locus was determined from the phenotypes with respect to cation composition and the closely linked antigenic factor M. Four of the LK sheep were M-negative, and therefore were homozygous at the Ka locus ($Ka^{L}Ka^{L}$). The remaining 16 LK sheep were M-positive, and therefore were heterozygous at the Ka locus ($Ka^{L}ka^{h}$). All the HK

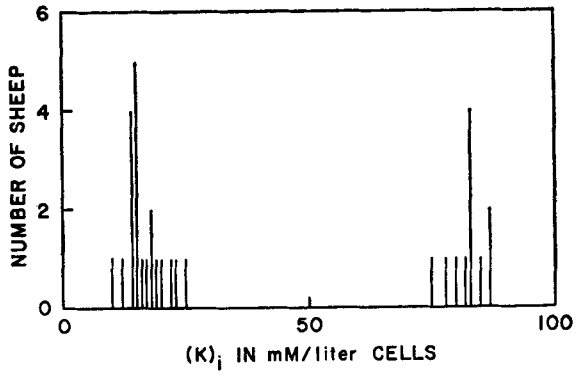

FIGURE 1. Frequency distribution of potassium concentrations, $(K)_i$, in red blood cells from Dorset sheep.

sheep were M-positive and homozygous recessive at the Ka locus (ka^hka^h), as expected. As will be shown below, the red cells from LK homozygotes had somewhat lower $(K)_i$ and pump rates than did heterozygous LK cells. It is interesting in this connection that Evans et al. (1956) suggested that the Ka^L allele was not completely dominant. The observed frequencies of the genotypes conformed closely to the distribution expected for a randomly breeding population with a dimorphism controlled by two alleles at a single locus, as given by the Hardy-Weinberg law.

2. *Potassium Influx*

The unidirectional influxes of K were determined on HK and LK red cells as a function of the K concentration in the external medium. Figs. 2 *a* and 2 *b* show in HK and LK sheep the pattern of K activation of K influx together with the effect of ouabain.

It is apparent in both HK and LK type cells that maximum activation of the ouabain-sensitive flux is achieved between 10 and 20 mM $(K)_o$. The passive or ouabain-insensitive (leak) component of K influx ($^iM_K^L$) apparently increases linearly with increasing $(K)_o$ in HK cells but shows partial saturation in LK cells. The same results presented in Fig. 2 were obtained in separate experiments on another sheep of each type.

Since the pump flux of K ($^iM_K^P$) represented by the ouabain-sensitive component (see Tosteson and Hoffman, 1960) conforms approximately to Michaelis-Menten kinetics, the data in Fig. 2 were analyzed accordingly. From a double reciprocal plot of $^iM_K^P$ against $(K)_o$ for HK cells in Fig. 2 *a*, the maximum pump flux was found to be about 0.90 mM K/liter cells × hour, and the external K concentration which gives half-maximal activation was about 3 mM. The pump rate at 10 mM $(K)_o$, the concentration used in most experiments on HK cells, was 86% of the maximum. Similarly, $^iM_K^P$ in

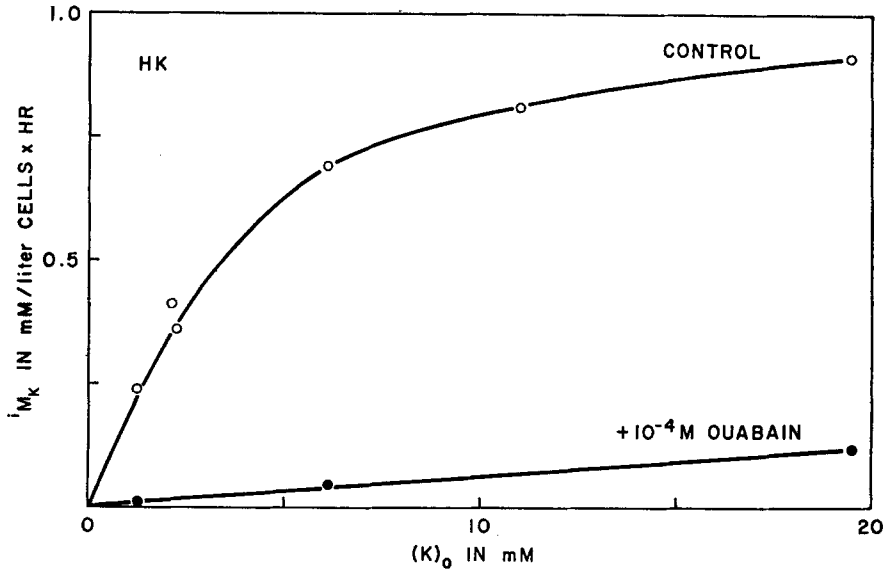

FIGURE 2 *a*

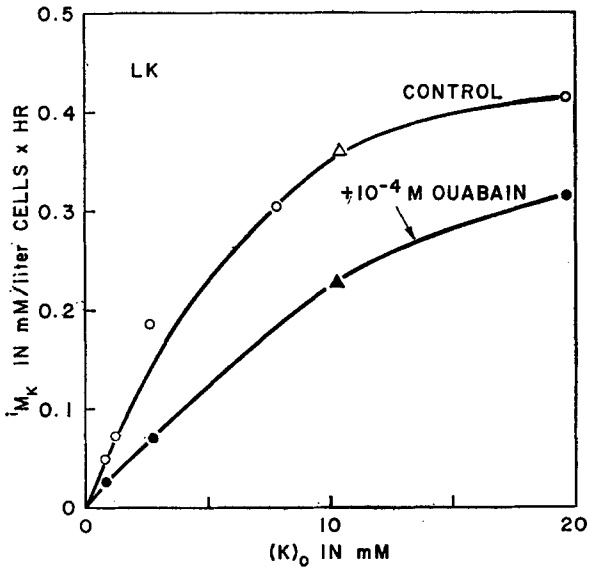

FIGURE 2 *b*

FIGURE 2. Unidirectional K influx ($^{i}M_K$) in HK (Fig. 2 *a*) and LK (Fig. 2 *b*) red cells plotted as a function of external K concentration, $(K)_o$, with and without ouabain. The triangles in the LK curves indicate determinations made on a different day but on the same sheep.

LK cells was 80% of the maximum at 5 mM $(K)_o$ and the external K concentration which gives half-maximal activation was also about 3 mM.

Summarized in Table I are the mean values for the pump and leak components of K influx for HK cells and heterozygous and homozygous LK cells. Also shown are the mean $(Na)_i$ and $(K)_i$ for samples taken during the flux determination. $(K)_i$ is lower and $(Na)_i$ higher than in fresh cells because of the several hours of incubation before taking the samples.

TABLE I

Intracellular $(K)_i$ and $(Na)_i$ concentrations and active, ${}^iM_K^P$, and passive, ${}^iM_K^L$, components of K influxes in HK and LK sheep red cells. The concentrations and fluxes are means of one mean value per sheep. Variance is given as the standard deviation. The total range is given for the LK homozygous sheep. Fluxes for HK cells were measured at 10 mM $(K)_o$. Fluxes on LK cells were measured at 5 and 10 mM $(K)_o$. Active fluxes in LK cells were nearly the same at 5 and 10 mM $(K)_o$ and ${}^iM_K^L$ was approximately proportional to $(K)_o$ up to 10 mM (Fig. 2 *b*). Accordingly, the means and variances of ${}^iM_K^L$ for LK cells were calculated for 10 mM $(K)_o$ after doubling the values measured at 5 mM $(K)_o$.

Cell type	No. of sheep	$(K)_i$	$(Na)_i$	${}^iM_K^P$	${}^iM_K^L$
		mM/liter cells		*mM/liter cells × hr*	
HK	5	76.1±5.2	27.2±5.0	0.86±0.10	0.060±0.012
LK (heterozygous)	7	14.2±2.2	93.5±5.1	0.12±0.022	0.23±0.060
LK (homozygous)	3	11.5 (8.8–13.4)	95.5 (92.5–101)	0.083 (0.07–0.10)	0.31 (0.24–0.42)

3. *Sodium Outflux*

Fig. 3 shows the unidirectional outflux, ${}^oM_{Na}$, from HK cells as a function of $(K)_o$ with the ouabain-sensitive and ouabain-insensitive components indicated. Similar results were obtained for three other HK sheep. The pump flux, ${}^oM_{Na}^P$, is near maximal at 10 mM $(K)_o$. The Na fluxes in Fig. 3 were determined on the same HK sheep as the K fluxes shown in Fig. 2. The ratio of the maximum Na to K pump rates is between 1:1 and 3:2. It is apparent that the passive Na outflux is much greater than the inward leak of K for a comparable driving force, but much of the passive unidirectional Na outflux is exchange diffusion (Tosteson and Hoffman, 1960).

The pump flux of Na in LK cells was too small a fraction of the total outflux of Na to measure accurately even in the absence of external Na.

4. *Ouabain Binding and Inhibition of the Na:K Pump*

Figs. 4 and 5 show the time courses of simultaneous measurements of binding of ouabain (molecules per cell) and inhibition of active transport in HK and LK cells. Fig. 4 shows inhibition of ${}^iM_K^P$ and ouabain binding during relatively short exposures to [^{3}H]-ouabain. The time course of inhibition is similar

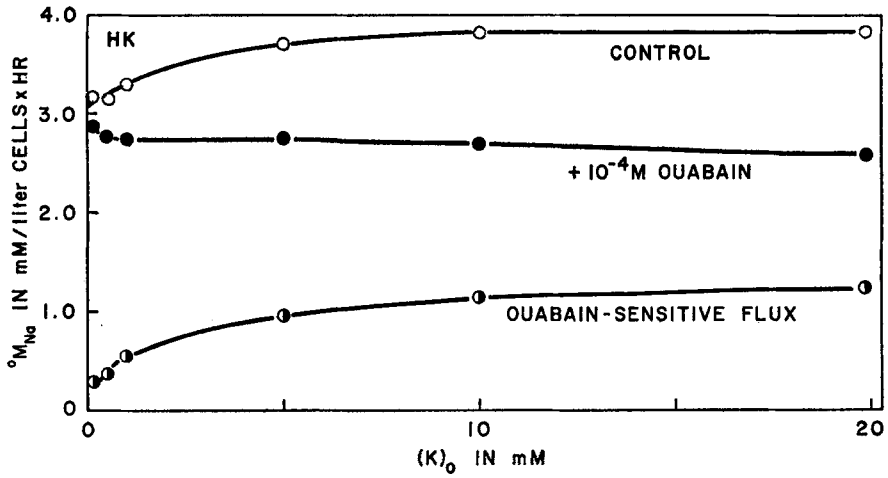

FIGURE 3. Unidirectional Na outflux ($^{o}M_{Na}$) in HK red cells as a function of external K concentration, with and without ouabain. The ouabain-sensitive flux was obtained by subtracting the ouabain-insensitive flux from the control flux.

for HK and LK cells, but fewer ouabain molecules per cell need be bound on LK than on HK for comparable levels of inhibition.

Fig. 5 shows the time course of ouabain binding to HK and LK cells during a longer exposure to [^{3}H]-ouabain. Inhibition of $^{o}M^{p}_{Na}$ is shown for the HK cells. A sufficiently high concentration of [^{3}H]-ouabain was used in the experiments shown in Fig. 5 to give complete inhibition in 1 hr.

In the experiments shown in Fig. 4, as in most experiments, the ouabain concentrations and exposure times were selected to give less than 100% inhibition of the pump. This approach was used because ouabain binding continues, although at a reduced rate, after complete inhibition of the pump, as shown in Fig. 5. This type of observation suggests that there are nonspecific as well as specific membrane sites to which glycoside can bind. In this context, nonspecific sites are any binding sites not associated with transport, for example the binding in Fig. 5 that occurs after 100% inhibition of transport. Determinations of the number of pump sites based on ouabain binding after complete inhibition of the pump would be subject to a greater error due to nonspecific binding than would determinations made before inhibition is complete. On the other hand, since low levels of pump inhibition involve relatively large errors in their estimation, the accuracy in the determination of the number of binding sites increases with increasing levels of inhibition. Therefore an arbitrary level of 30% inhibition was chosen, below which no values were taken for calculation of total ouabain sites/cell. However, equal weight was given for all values of inhibition above 30%.

Even though LK cells bind fewer ouabain molecules per cell than do HK cells, the rates of binding after complete inhibition of the pump, i.e. the binding

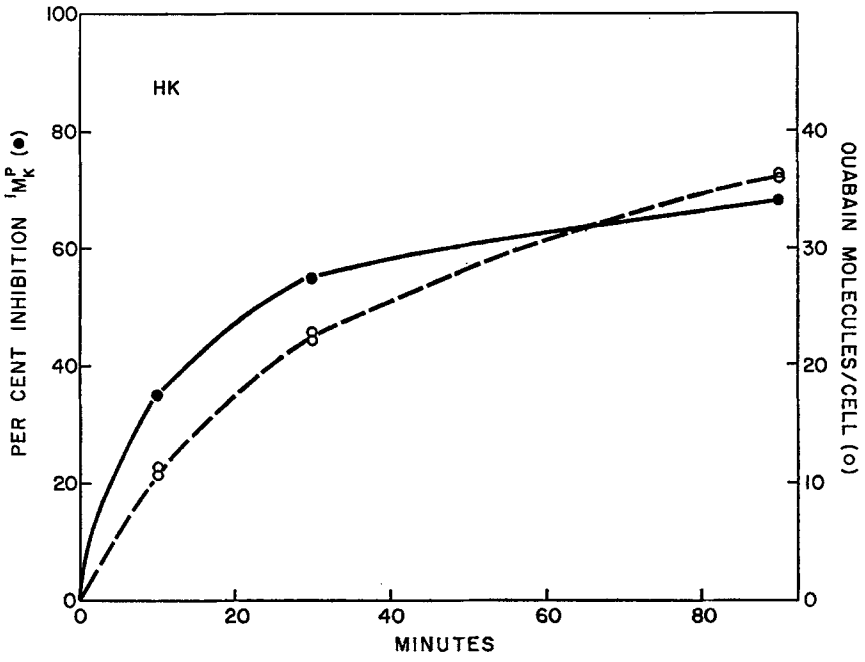

FIGURE 4 *a*

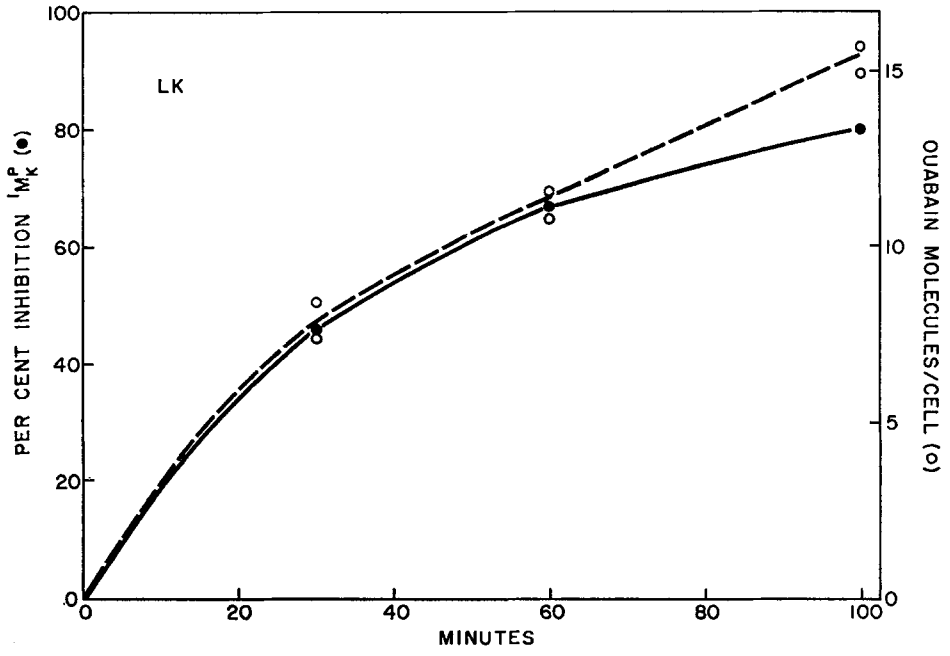

FIGURE 4 *b*

FIGURE 4. Time course of the inhibition of $^iM_K^P$ by ouabain and of the simultaneously measured binding of ouabain for HK (Fig. 4 *a*) and LK cells (Fig. 4 *b*). The [^{3}H]-ouabain concentration was approximately 1×10^{-8} M in both experiments. $(K)_o$ during incubation with [^{3}H]-ouabain was 0.3 mM for both HK and LK cells.

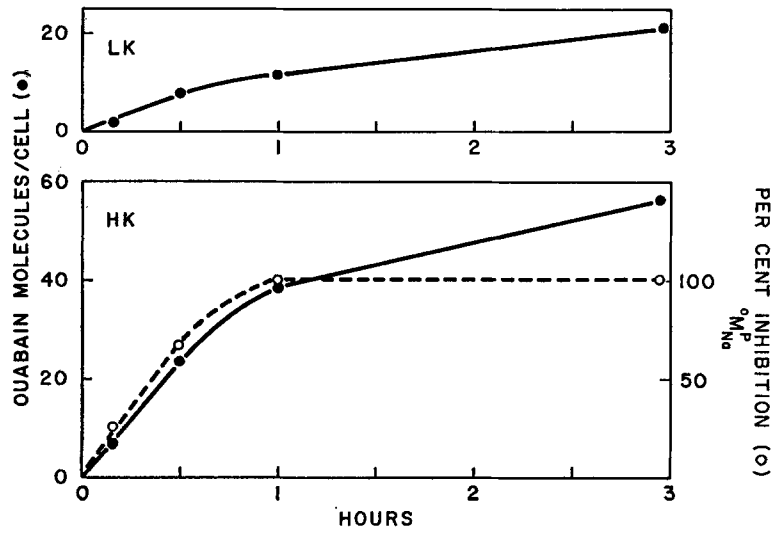

FIGURE 5. The time course of ouabain binding to HK and LK cells. The [^{3}H]-ouabain concentration was approximately 4×10^{-8} M in both experiments. Also shown are simultaneously measured levels of inhibition of ${}^{o}M^{P}_{Na}$ for the HK cells.

at nonspecific sites, are similar in the two cell types. This can be seen in Fig. 5 where the slopes of the curves change after 1 hr to a rate of binding between 5 and 10 molecules per cell per hour for both LK and HK cells.

Fig. 6 shows simultaneously determined values for the number of ouabain molecules bound per cell as a function of the inhibition of ${}^{i}M^{P}_{K}$ in a number of HK and LK sheep with several determinations on each sheep. The different number of bound ouabain molecules required for comparable levels of inhibition in the two cell types is readily apparent. The slopes of the curves relating inhibition and binding appear to increase slightly at higher levels of inhibition for both HK and LK cells. This relationship is consistent with the view that the relative number of ouabain molecules bound at nonspecific sites increases with the increasing length of exposure required to obtain the higher levels of pump inhibition.

It is apparent from the foregoing discussion that the number of glycoside molecules needed to be bound for 100% inhibition of the pump can be calculated from the determination of the number of ouabain molecules bound per cell at incomplete levels of inhibition of transport. The mean numbers so calculated of ouabain molecules bound at 100% inhibition are presented in Table II.

In addition to the difference between the two types of sheep cells in their capacity for specific glycoside binding, it also appears that there is a difference between HK and LK cells in their affinity for ouabain. This is shown in Fig. 7 where the per cent inhibition of ${}^{i}M^{P}_{K}$ in HK and LK cells is plotted against

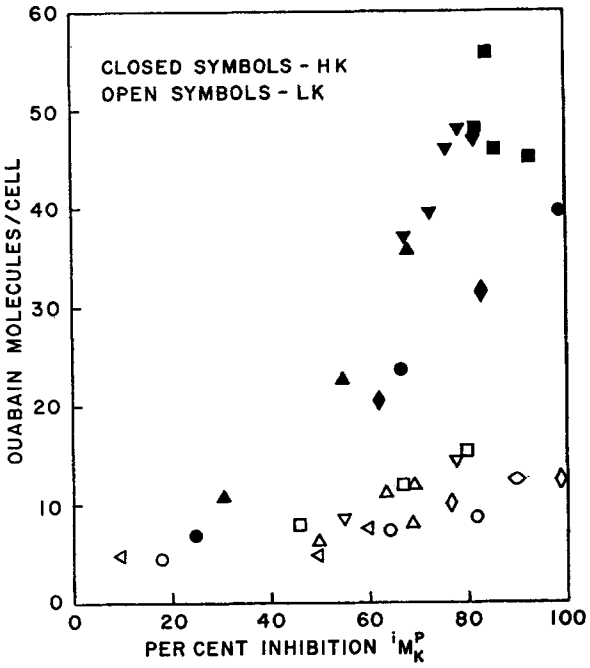

FIGURE 6. Ouabain molecules bound per cell and simultaneously measured levels of inhibition of $^{i}M_{K}^{P}$ for HK (closed symbols) and LK (open symbols) cells. Each symbol represents one set of determinations. All determinations on the same sheep are represented by the same symbol.

the product of the ouabain concentration and the length of the exposure to [^{3}H]-ouabain. Thus, to reach a particular level of inhibition, a higher ouabain concentration or a longer exposure time is necessary for LK than for HK cells. It should be said that during the exposure to [^{3}H]-ouabain the mean external concentrations of K were 0.83 mM for HK cells (7 experiments) and 0.31 mM for LK cells (11 experiments). Therefore, the apparent lower uptake rate of glycoside by LK compared to HK cells is not due to a higher $(K)_o$ (see Hoffman, 1966). On the other hand, it has been reported (P. G. Hoffman and Tosteson, 1969; P. G. Hoffman, 1969; Lauf et al., 1970) that there are qualitative differences between HK and LK cells in the kinetic parameters of the pumps, demonstrated by changing the Na and K concentrations on the two sides of the membrane. Conceivably, therefore, the difference in the ouabain uptake rates between HK and LK cells might be related to their different affinities for K, but there is no direct evidence bearing on this possibility.

5. *Effect of Cesium on Ouabain Binding*

When Cs was substituted for part of the Na in the [^{3}H]-ouabain incubation medium, both ouabain binding and inhibition of transport were reduced.

TABLE II

Total ouabain-binding sites (ouabain molecules bound per cell, calculated for 100% inhibition of ${}^{i}M_{K}^{P}$) for red cells from HK and LK sheep. The control (uninhibited) K pump flux for each sheep is given in millimoles/liter cells × hour. The correction of total sites for the Cs effect is the difference between total sites determined after the [^{3}H]-ouabain incubation with and without Cs. All LK sheep are heterozygous at the Ka locus except 475, which is homozygous. Total range and number of determinations (N) are given for each sheep.

Sheep		${}^{i}M_{K}^{P}$	Total sites (ouabain molecules/cell at 100% inhibition)		Correction of total sites for cesium effect	
			Mean	Range (N)	Mean	Range (N)
HK	149	0.74	42.7	32.4–50.4 (6)	—	—
	489	0.94	56.4	46.2–66.3 (8)	11.1	9.7–12.4 (10)
	287	0.93	35.7	32.0–39.0 (4)	2.9	0.5–4.5 (6)
	465	0.92	58.8	52.9–63.0 (10)	4.0	0.0–8.0 (2)
Mean		0.88	48.4		6.0	
LK	312	0.12	12.4	9.8–14.5 (8)	—	—
	11	0.10	11.1	8.8–14.5 (4)	—	—
	20	0.11	10.8	10.3–12.0 (4)	—	—
	307	0.14	17.7	16.1–19.4 (6)	6.0	4.3–8.7 (4)
	16	0.08	12.7	12.4–13.0 (2)	—	—
	308	0.11	16.9	15.5–19.5 (4)	3.7	2.9–6.5 (3)
	475	0.08	14.6	10.7–19.2 (8)	8.7	6.3–11.9 (4)
Mean		0.11	13.7		6.1	

However, the calculated number of ouabain molecules bound per cell at 100% inhibition of K transport was less in the presence than in the absence of Cs. The means of the Cs effect on LK and HK cells are included in Table II. These results provide additional evidence for two populations of glycoside-binding sites, with selective inhibition or reduction of the nonspecific binding by Cs. The mean effect of Cs was the same for HK and LK cells, even though there was considerable variability between sheep within both phenotypes. Although various concentrations of Cs between 25 and 75 mM were tried, the maximum reduction by Cs in nonspecific binding was obtained at 50 mM Cs with HK cells and at about 30 mM with LK cells.

6. *Reversibility of Ouabain Binding*

In the simultaneous determinations of ouabain binding and transport, it should be recalled that the cells were first incubated in medium containing [^{3}H]-ouabain, then washed and divided into two portions. One portion was set aside immediately for determination of (^{3}H)-ouabain. The other portion was returned to the 37°C bath for determination of ${}^{i}M_{K}^{P}$. Given this procedure,

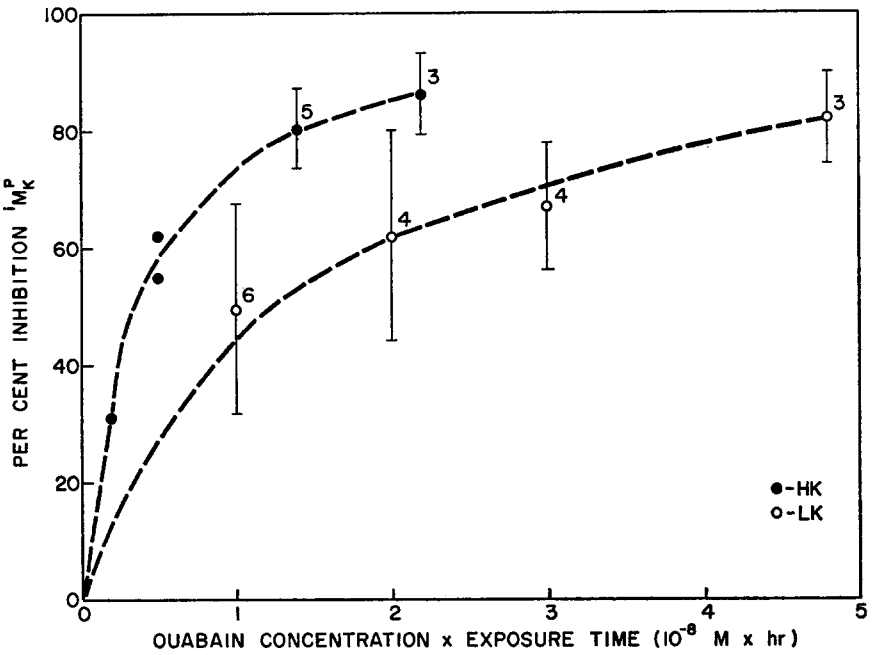

FIGURE 7. Per cent inhibition of $^iM_K^P$ in HK and LK cells as a function of the product of the ouabain concentration and the exposure time to the ouabain just prior to the determination of the flux. Numbers of determinations and one standard deviation are indicated. Points without vertical lines represent single determinations.

it is possible that the calculations of ouabain molecules bound per cell at 100% inhibition could be in error if the inhibition of transport were reduced by elution of some of the ouabain from the cells during the determination of transport. In order to estimate the magnitude of this error, HK cells were incubated with [^{3}H]-ouabain and washed in the usual manner. On one aliquot of cells, ^{42}K influx and [^{3}H]-ouabain binding were determined within 15 min. Other aliquots were returned to the bath for various lengths of time, and were washed again before the determination of [^{3}H]-ouabain and K flux. The results of this experiment are shown in Table III. In 6 hr there was a loss of about 20% of the ouabain which was bound to the cells after the first wash. Thus the loss of bound ouabain during the time required for measuring the fluxes in the experiments described above, and the consequent error in calculating ouabain molecules per cell at 100% inhibition were less than 10%. The reduction in inhibition of transport was proportional to the number of ouabain molecules removed, showing that the inactivation of the pump brought about by ouabain is reversed when ouabain desorbs from its binding sites. As a consequence, the calculated number of ouabain molecules bound per cell at 100% inhibition of $^iM_K^P$ remained constant. It should be mentioned that

TABLE III

The reversibility of ouabain binding and inhibition of ${}^{i}M_{K}^{P}$ in HK cells. The cells were incubated with [^{3}H]-ouabain, washed, and returned for continued incubation at 37°C. At various subsequent times (indicated by hours) samples were removed for the determinations of ${}^{i}M_{K}^{P}$ and glycoside bound. See text for further details.

Time	Inhibition of ${}^{i}M_{K}^{P}$	Ouabain molecules/cell	
		Measured	Calculated at 100% inhibition
hr	%		
0.25	82	47	58
2	76	46	60
4	73	39	55
6	68	37	55

$(K)_i$, $(Na)_i$, and the control pump flux changed minimally during the 6 hr incubation.

DISCUSSION

The main conclusion to be drawn from the experiments reported in this paper is that HK cells require approximately six times more ouabain molecules bound per cell than do LK cells for complete inhibition of the K pump. Since the ratio of the pump fluxes, HK to LK, is about seven, the results suggest that the major difference in the transport characteristics between the two cell types resides in the number of membrane pumps. The results also emphasize the high specificity with which glycoside binds to the membrane, with rather little being bound at sites other than at the locus of the Na:K pump.

Although specificity of binding was established by correlation of ouabain uptake with inhibition, it was possible to eliminate and correct for at least one kind of binding at nonpump-associated sites by measuring the rate of binding in the presence of Cs (Table II). Nonspecific binding was also apparent in uptake curves (Fig. 5) when binding continued at a reduced rate after all pump activity was stopped. Both types of estimates of nonspecific binding yielded approximately the same low values as well as showing that the amount of nonspecific binding was essentially the same in the two types of red cells. These results indicate that the affinity of the membrane for binding of ouabain to specific sites is higher than for nonspecific sites and supports the idea that the nonspecific sites are not involved in transport. Because there could be additional forms of nonspecific binding, the present data should be considered as representing maximal values of specific binding sites even though the number of nonspecific sites remaining must be low. It should be mentioned that the specificity of binding referred to above is also of importance when

identifying isolated components of the cation transport system labeled with glycoside (Dunham and Hoffman, 1970).

Given a measure of specific binding a question of considerable interest concerns the relationship between the number of pump sites and the number of glycoside-binding sites. While this question cannot be answered unequivocally, curves of the form shown in Fig. 6 can be useful in evaluating certain models of the glycoside to pump relationship. Models for pumps which require two or more glycosides per site before inactivation are thus eliminated provided no interaction takes place between the two glycoside-requiring sites. Such a model would require that the relationship in Fig. 6 be a parabolic one with a decreasing slope as inhibition increases. On the other hand, models involving either single sites with no interactions or sites showing cooperativity, e.g. a glycoside bound at one site enhancing the binding of a glycoside molecule at an adjacent site, are indistinguishable from each other and consistent with the data presented in Fig. 6. Therefore until further clarification is forthcoming, the most reasonable and useful approach is to assume a one to one correspondence between numbers of pump sites and glycoside-binding sites.

On the basis that one molecule of ouabain is bound per pump site, the number of sites per cell and the rate of K transport give directly the turnover time per site. The mean numbers of specific sites and therefore pumps per cell were 42.4 and 7.6 for HK and LK, and the mean pump rates for HK and LK cells were 0.86 and 0.12 mM K/liter cells × hour, respectively. Thus, the turnover times in ions pumped per site per minute are about 6000 for HK and 4800 for LK cells. These turnover times are probably indistinguishable from each other since six LK sites per cell would give 6000 ions pumped per site per minute. These turnover times are similar to those previously obtained using [^{3}H]-ouabain for human red cells (Hoffman, 1969; Hoffman and Ingram, 1968), but are somewhat higher than that found for rabbit nerve fibers (Landowne and Ritchie, 1970). In terms of pump density HK cells are about one-half that of human red cells in having about one pump per μ^2 of membrane surface. Obviously the site density in LK cells is considerably reduced.

The active transport rates reported in this paper for sheep red cells are not maximal rates since it is known that internal K and external Na may inhibit the pump and the concentrations of $(Na)_i$ and $(K)_o$ used were not saturating. Since the maximal specific binding capacities are evidently not influenced by the rate of pumping (Hoffman, 1969), the question arises as to what are the proper relative pump rates to use in the comparison of ratio of pump rates to ratio of binding sites. P. G. Hoffman (1969) measured $^tM^p_K$ in HK and LK red cells from Blackface sheep after altering intracellular cation composition by treatment of the cells with-*p*-chloromercuibenzene sulfonate (PCMBS), in such a manner as to maximize the pump flux in both cell types. After re-

versing the effect of PCMBS on permeability with dithiothreitol, $(K)_i$ was less than 1% of $(Na)_i + (K)_i$ in LK cells and less than 3% of $(Na)_i + (K)_i$ in HK cells. The flux, ${}^iM_K^p$, measured in Na-free medium, was increased more than threefold in both cell types, but the ratio of the pump rates, HK to LK, was approximately the same as in normal, unaltered cells. (It should be noted that the ratio of pump rates, HK to LK, was less than that found in the present study.) Thus it is probable that the ratio of the pump fluxes as given in the present paper approximates the ratio of maximum pump rates and provides, therefore, a reasonable basis for evaluating the ratio of ouabain-binding sites on the two cell types.

It should be emphasized that the absolute number of ouabain-binding sites per cell as determined in the present study could be in error if the value for the specific activities of the [^{3}H]-ouabain used were in error. After the present study was completed, the ouabain-binding sites were determined again on cells from sheep 489 (see Table II) using a new batch of [^{3}H]-ouabain. The number of ouabain molecules per cell at 100% inhibition of the pump, previously found to be 56, was now 84, 1.5 times the earlier estimate. Thus, if this later value could be shown to be more reliable, the values for pump sites per cell given above would be 67% of the actual numbers. There have been two preliminary studies on ouabain binding to LK red cells. One gave a value of 8.4 per cell (Ellory and Tucker, 1970), in agreement with the present report. In another study, the numbers ranged from 27 to 97 sites per cell (Lauf et al., 1970). The breed of sheep, the techniques, and the batch of [^{3}H]-ouabain were different than those used in the present study. It is not known which factor accounts for the different results. Thus while the actual numbers of binding sites presented here might be changed, conclusions regarding the ratio of binding sites in the two types of sheep, and the specificity of binding, would remain unaltered.

In addition to the different number of Na:K pumps, there appear to be qualitative differences in the pump sites between HK and LK cells with regard to the relative effects of Na and K. For instance, P. G. Hoffman and Tosteson (1969) and P. G. Hoffman (1969) found that in Na-free media the concentration of external K giving half-maximal activation of ${}^iM_K^p$ was 0.2 mM in LK cells and 0.6 mM in HK cells. These differences were not seen in Na-containing media in which the $(K)_o$ giving half-maximal activation was 3 mM for both HK and LK cells, the same as found in the present study. In addition, they found a difference between HK and LK cells in the effects Na and K had at the inner surface of the membrane on the characteristics of the Na:K pump. They kept $(Na)_i + (K)_i$ constant and varied the ratio of $(Na)_i/(K)_i$ by treatment with PCMBS. In HK cells the K pump was in operation over the entire range of $(Na)_i$ and increased gradually from very low $(Na)_i$ to a maximum at 100 mM $(Na)_i$, the highest concentration used. In contrast, no

K pump influx was observed in LK cells when $(Na)_i$ was below 70 mM and the shapes of the curves relating ${}^iM_K^P$ to $(Na)_i$ were different in LK and HK cells. In the present study LK cells appeared to have a lower affinity for ouabain than HK cells (Fig. 7). Compared to HK cells, LK cells required a longer exposure time or a higher ouabain concentration to attain a particular level of inhibition of active transport. The difference could conceivably be related to the different affinities for K but there is no direct pertinent evidence.

Finally, it is of interest to consider another type of implication derived from the data presented in this paper. As we have discussed before, the single genetic locus that controls the membrane expression of the pump in HK and LK type cells also controls the leak in both cell types. If we assume that the total number of membrane cation sites, i.e. pumps + leaks, in LK cells is the same as in HK cells, then we can suppose that the gene functions to control the *ratio* of pumps to leaks in each cell type, carrying the implication that interconversion of HK and LK cells is accomplished by changing pumps to leaks or vice versa. This model can be quantitated as shown in Table IV once the pump and leak fluxes (Table I) and the number of transport sites (Table II) are known. It should be noted that this analysis also assumes that the turnover times of leak sites are the same in HK and LK cells and is based only on measurements of K influxes. The results show that the majority of the transport sites in HK cells are concerned with pumping in contrast to LK cells in which leak type pathways dominate. In addition, the turnover time at leak sites is lower than at pump sites. While this model presumably contains a number of weaknesses, it is nevertheless of interest since it emphasizes the fact that so few sites of either the pump or leak type are necessary to account in rather reasonable terms for the total ion transport capacity of the cell.

TABLE IV

Quantitative consequences of a hypothetical scheme for pump and leak flux sites in HK and LK red cells, assuming the total number of sites on the two cell types is the same. The asterisks indicate values calculated, according to this assumption, from the experimentally determined K fluxes (mM per liter × hr) and pump sites per cell (ouabain molecules per cell at 100% inhibition of ${}^iM_K^P$). The turnover time given for pump sites is the mean of the experimentally determined values for HK and LK cells (6000 and 4800, respectively).

		HK	LK	Turnover time
				ions/site × min
Pump	Flux	0.86	0.12	5400
	Sites	42.4	7.6	
Leak	Flux	0.06	0.23	1500*
	Sites	12*	46*	
Total sites		54*	54*	

It should be understood that a more general application of this model would have to take into account the permeability particularly with regard to the rather different concentration gradients which presumably provide the driving force for the leaks of both Na and K in the two cell types. Regardless of any conversion of sites in either HK or LK cells, the cell volume [and therefore $(Na)_i + (K_i)$] and ϕ, the membrane potential (defined by the Donnan ratio for chloride), must be maintained constant since the volume and chloride content are the same in the two cell types (Tosteson and Hoffman, 1960). If we assume that the sodium pump, M^p_{Na}, is balanced by an equal passive movement to which the constant field equation (Hodgkin and Katz, 1949) applies, then, as pointed out to us by Dr. W. K. Chandler, the equation:

$$(Na)_i + (K)_i = [(K)_o + (Na)_o]e^{-\phi} - \frac{1 - e^{-\phi}}{\phi}\left[1 - \frac{P_{Na}}{P_K}\right]\frac{M_{Na}}{P_{Na}}$$

would hold, where P_{Na} and P_K are the passive permeabilities to Na and K. Given the restrictions of constant volume $[(Na)_i + (K)_i]$ and constant membrane potential (ϕ), it is apparent that the quantity

$$\left[1 - \frac{P_{Na}}{P_K}\right]\frac{M_{Na}}{P_{Na}}$$

would also have to be maintained constant independent of any conversion in types of sites. But there are simply not enough data of the proper kind available to evaluate this aspect of the model.

Another aspect of this model concerns the changes that accompany the maturation of LK cells. Genotypically LK red cells newly released into the circulation in LK adult sheep contain high K and have pump characteristics like HK cells. Several weeks are required for these new cells to become phenotypically LK as shown by studies on circulating reticulocytes in adult sheep (Lee et al., 1966). As implied by our model this developmental process might utilize a mechanism based on the conversion of pump sites to leak sites. The Ka^L allele would determine a lability of the pump sites such that coupling of metabolic energy to the transport of ions and capacity to bind ouabain are lost. The difference in kinetic properties of the pump in HK and LK cells might be explained by a range of kinetic characteristics of pump sites on HK cells and LK reticulocytes, and selective inactivation of the sites with respect to their kinetic properties during maturation of LK cells.

Lastly, Ellory and Tucker (1969) found that the rate of active transport could be dramatically increased by treatment of LK cells with an antiserum prepared against LK cells. In preliminary experiments Lauf et al. (1970) found that this treatment of LK cells increased the number of transport sites, as measured by ouabain binding. There was some evidence that the K leak may be concomitantly decreased. The results on both LK reticulocytes and on

the effect of the antiserum are consistent with and would be predicted by the model but much more quantitative information is needed before a detailed evaluation can be made.

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