

# Interaction of HK and LK Goat Red Blood Cells with Ouabain

JOHN R. SACHS, PHILIP B. DUNHAM, DONNA L. KROPP,  
J. CLIVE ELLORY, and JOSEPH F. HOFFMAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Dunham's present address is the Department of Biology, Syracuse University, Syracuse, New York 13210. Dr. Ellory's present address is the A. R. C. Institute of Animal Physiology, Babraham, Cambridge, England.

**ABSTRACT** The characteristics of the interaction of Na-K pumps of high potassium (HK) and low potassium (LK) goat red blood cells with ouabain have been determined. The rate of inhibition by ouabain of the pump of HK cells is greater than the rate of inhibition of the pumps of LK cells. Treatment of LK cells with an antibody (anti-L) raised in HK sheep by injecting LK sheep red cells increases the rate of inhibition of the LK pumps by ouabain to that characteristic of HK pumps; reduction of intracellular K ( $K_e$ ) in LK cells increases the rate at which ouabain inhibits their pumps and exposure of these low  $K_e$  cells to anti-L does not affect the rate of inhibition. There is considerable heterogeneity in the pumps of both HK and LK cells in the rate at which they interact with ouabain or the rate at which they pump or both. LK pumps which are sensitive to stimulation by anti-L bind ouabain less rapidly than the remainder of the LK pumps and exposure to antibody increases the rate at which ouabain binds to the sensitive pumps; the difference between the two types of pumps disappears if intracellular K is very low. The calculated number of ouabain molecules bound at 100% inhibition of the pump is about the same for HK and LK cells. Although exposure to anti-L increases the apparent number of ouabain binding sites in LK cells at normal  $K_e$ , it does not alter the apparent number of sites in LK cells when  $K_e$  has been reduced.

## INTRODUCTION

Sheep (Evans, 1954), goats (Evans and Phillipson, 1957), and some other species of mammals are unusual in that they can be divided into populations whose red cells contain either high (HK) or low (LK) concentrations of potassium. The potassium concentrations of the cells are correlated with their Na:K pump activities; the pump rate of HK cells is considerably higher than that of LK cells (Tosteson and Hoffman, 1960; Ellory and Tucker, 1970). By injecting LK sheep red cells into HK sheep an antibody (anti-L) is raised

which, when absorbed on LK sheep or goat red cells markedly stimulates the activity of the Na:K pump (Ellory and Tucker, 1969; Ellory and Tucker, 1970). It has been shown (Sachs et al., 1974) that anti-L does not increase the pump rate of LK goat cells with very low intracellular K ( $K_e$ ) and high intracellular Na ( $Na_e$ ) concentrations. Since this failure to stimulate the pump rate was not due to a failure of the antibody to bind to cells with very low intracellular K, the results were interpreted as indicating that the main effect of the antibody in these cells is to reduce the affinity of the pump for  $K_e$  which competitively inhibits the activation of the pump by  $Na_e$ .

Although anti-L did not further increase the pump rate of LK cells with low intracellular K concentrations, the maximal pump rate of LK cells at optimal concentrations of  $Na_e$  and  $K_e$  was considerably less than the comparable values for HK cells. One of the possible explanations for this difference is the presence of a greater number of pumps in the HK cells than in the LK cells. The relation between the number of [ $^3H$ ]ouabain molecules bound per cell and the resultant inhibition of the pump has been determined and used to estimate the number of Na:K pump sites per cell in human red cells (Hoffman, 1969) and in sheep red cells (Dunham and Hoffman, 1971). Using fresh sheep red cells in which intracellular cation contents were unaltered, it was found that LK cells have on the average 7.6 and HK cells 42.4 pump sites per cell; these numbers were proportional to the simultaneously measured active K influx in the same cells.

This paper reports the results of experiments designed to investigate the characteristics of the interaction of HK and LK goat red cells with ouabain and the effect of anti-L on this interaction. In addition, the number of ouabain binding sites in HK and LK goat red cells has been assessed, and the effect of anti-L on the number of ouabain molecules bound by LK cells evaluated.

#### METHODS

The methods for the preparation of the antibody, preparation of cells, alteration of intracellular cation concentration, exposure to anti-L antiserum, and determination of the K influx have been described (Sachs et al., 1974). When fresh cells were used they were first incubated for 1 h at 37°C in a solution which contained (mM): Na 170, Mg 1,  $PO_4$  27, Cl 128, adenosine 10, glucose 5, pH 7.4 in order to raise intracellular ATP concentrations.

The measurements of the number of ouabain binding sites per cell were made by a modification of methods previously described (Dunham and Hoffman, 1971). The cells, after alteration of intracellular cation concentrations (*p*-chloromercuribenzenesulfonic acid [PCMBBS]-treated cells) or incubation in the high phosphate solution containing adenosine and glucose (fresh cells), were exposed to anti-L or to serum obtained from an HK nonimmune sheep. The cells were then washed and exposed to solutions containing [ $^3H$ ]ouabain  $10^{-6}$  M at 37°C. The reaction was stopped by rapidly cooling the suspensions to 4°C and the cells were separated from the suspen-

sions, washed four times with 10 vol of ice-cold isosmotic  $MgCl_2$  solution, and divided into two portions. Bound  $[^3H]$ ouabain was extracted from one portion for scintillation counting. This aliquot was used to calculate the number of ouabain molecules bound per cell from the counts per unit volume of packed cells, mean cell volume ( $16.7 \mu^3$  for goat cells), specific activity of the  $[^3H]$ ouabain, and the absolute counting efficiency. In each case the determination of the number of ouabain molecules bound per cell was made in duplicate; the average difference between duplicates was 1.5 sites/cell. The other portion of cells was used for the determination of K influx. The percent inhibition of the pump was calculated for cells exposed to  $[^3H]$ ouabain using fluxes measured in two other samples of cells, one not exposed to ouabain, giving total K influx, and the other exposed to  $10^{-4}$  M ouabain, giving the ouabain-insensitive influx. Percent inhibition was calculated as

$$\frac{M_K^p \text{ in control cells} - {}^iM_K^p \text{ in ouabain treated cells}}{{}^iM_K^p \text{ in control cells}} \times 100.$$

${}^iM_K^p$ , the pump influx of K, is the influx of K in the control cells or in the cells exposed to  $[^3H]$ ouabain minus the influx in the same cells exposed to  $10^{-4}$  M ouabain. In order to calculate the number of ouabain molecules per cell necessary for 100% inhibition of the pump, the number of ouabain molecules bound per cell was plotted as a function of the percent inhibition of the pump and the best straight line describing the data was calculated by the method of least squares and extrapolated to 100% inhibition. The number of ouabain molecules per cell necessary for 100% inhibition of the pump was taken as the number of Na:K pump sites per cell.

The  $[^3H]$ ouabain, obtained from New England Nuclear, Boston, Mass. was stated to have a specific activity of 11.7 Ci/mmol. Since in our experience the specific activity given by the manufacturer was not dependable, unlabeled ouabain (Sigma Chemical Co., St. Louis, Mo.) was added to reduce the specific activity to 1.0 Ci/mmol. Thus any uncertainty introduced into the results by errors in the specific activity reported by the manufacturer was reduced by an order of magnitude.

As shown previously cesium (25 mM or higher) included in the medium during the incubation with  $[^3H]$ ouabain decreased the number of ouabain molecules necessary for 100% inhibition of the pump for both human and sheep red cells (Hoffman, 1969; Dunham and Hoffman, 1971). It was concluded that Cs selectively inhibits binding of ouabain at sites other than pumps, called nonspecific binding. It has also been found (Ingram, 1970) that K at a relatively low concentration (5 mM) has the same effect as Cs on nonspecific binding. A large fraction of ouabain binding to goat red cells in K-free medium is nonspecific; therefore in most of the experiments reported here incubations with  $[^3H]$ ouabain were carried out in 5 mM K. K, of course, also inhibits binding of ouabain to pump sites. Therefore, the consequences of incubating cells with  $[^3H]$ ouabain in 5 mM K as compared to K-free medium are, first of all, fewer ouabain molecules per cell required for a particular level of inhibition, that is, less nonspecific binding, and second, a higher ouabain concentration or longer incubation time is necessary to achieve that level of inhibition with 5 mM K.

## RESULTS

*Effect of Anti-L on the Rate at Which Ouabain Inhibits the Pump*

In the course of the experiments measuring the number of ouabain binding sites it became apparent that on exposure to  $10^{-6}$  M ouabain the rate at which inhibition of the K influx developed in LK cells was less than the rate at which it developed in HK cells and that treatment of LK cells with anti-L increased the rate toward that found in HK cells. HK and LK cells, after alteration of intracellular ATP concentrations and exposure either to anti-L or to serum from a nonimmune HK sheep, were incubated for periods of 1–6 min in a solution containing  $10^{-6}$  M ouabain and 5 mM K. After exposure to ouabain the cells were washed and the ouabain-sensitive K influx determined. The ouabain-sensitive K influx in the ouabain-treated cells was compared with that in the appropriate ouabain-free control cells and the percent inhibition of the ouabain-sensitive K influx calculated. In Fig. 1 the percent inhibition of the pump is plotted against the duration of exposure to ouabain for HK cells and for LK cells from two animals. In the control LK cells the rate at which inhibition developed was less than that in the HK cells, but treatment of LK cells with anti-L increased the rate at which inhibition developed to that found in the HK cells.

Since it has been shown (Sachs et al., 1974) that anti-L has little effect on the active K influx in LK cells in which  $K_o$  is very low, an experiment was performed to determine whether alteration of  $K_o$  changes the rate at which

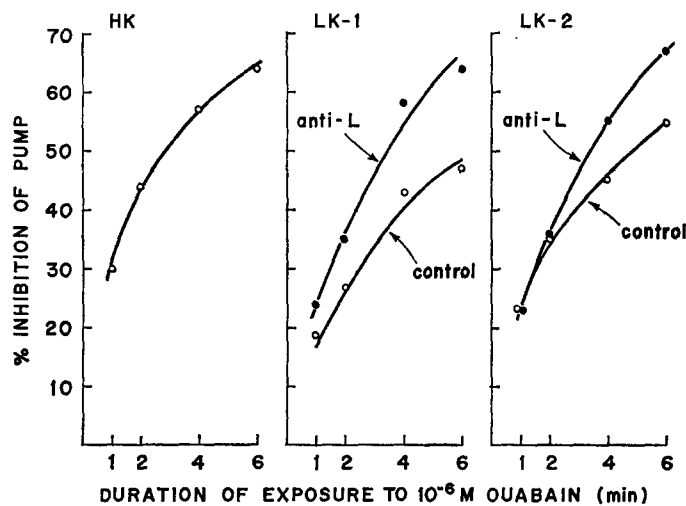


FIGURE 1. Percent inhibition of the pump vs. time of exposure to  $10^{-6}$  M ouabain. The experimental procedure is described in the text.

inhibition of the pump by ouabain develops and whether anti-L affects this process in cells with very low K concentrations. The results of an experiment are presented in Fig. 2; the experimental procedure was similar to that described above, except that intracellular K concentrations were altered by exposure of the cells to PCMBS. In cells with high potassium concentrations, exposure to anti-L increased the rate at which inhibition by ouabain developed as it does in fresh LK cells. In the LK cells with low intracellular K, treatment of the cells with anti-L had little effect on the rate of development

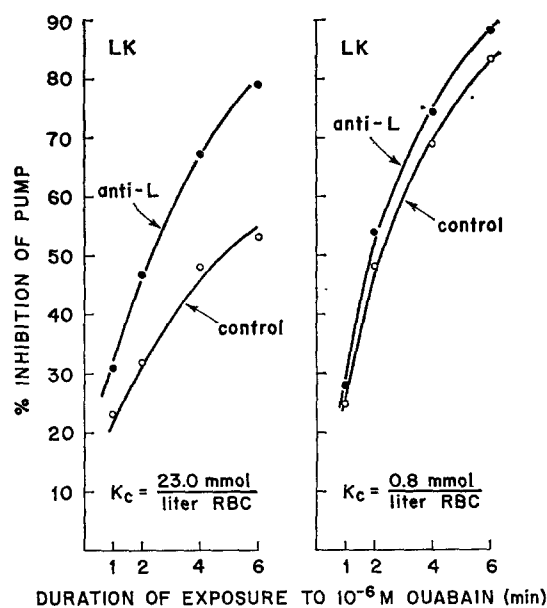


FIGURE 2. Percent inhibition of the pump vs. time of exposure to  $10^{-6}$  M ouabain using LK cells in which  $\text{Na}_e$  and  $\text{K}_e$  were altered.

of inhibition, and this rate was greater than that found in the anti-L-treated cells with high  $\text{K}_e$ . Anti-L does not, therefore, influence the rate at which pumps are inhibited by ouabain if the intracellular K concentration is very low.

The rate at which inhibition develops on exposure to a fixed concentration of ouabain can be taken as a measure of the forward reaction of the pump with ouabain if the interaction with ouabain is irreversible. In order to determine whether the interaction of LK goat cells with ouabain can be considered irreversible under the conditions of these experiments, cells were exposed to a maximally inhibitory concentration of ouabain, washed free of extracellular ouabain, and the ouabain-sensitive K influx measured in ouabain-free solutions under circumstances similar to those used in obtaining the data in Figs. 1 and 2. The results, which are in Table I, show that the

TABLE I  
IRREVERSIBILITY OF OUABAIN INHIBITION OF ACTIVE K INFLUX

Cells		Inhibition of pump
Na <sub>c</sub>	K <sub>c</sub>	
<i>mmol/liter RBC</i>		%
66.4	22.4	Control 96
		Anti-L Treated 99
65.2	0.8	Control 98
		Anti-L Treated 98

Intracellular cation concentrations of LK goat red cells were altered by treatment with PCMBMS and the cells were exposed to anti-L or to HK nonimmune serum (control). The cells were then exposed to  $10^{-4}$  M ouabain for 0.5 h at 37°C; in order to calculate percent inhibition of the pump, another sample of each batch of cells was incubated in a ouabain-free solution. The cells were washed and the ouabain-sensitive K influx determined.

pump is virtually completely inhibited and that neither alteration of K<sub>c</sub> nor treatment with anti-L changes the amount of inhibition. The combination of the pump with ouabain is, therefore, essentially irreversible and the rate at which the pump is inhibited by ouabain can be taken as a measure of the forward reaction of the pump with ouabain.

The data of Figs. 1 and 2 indicate that the rate at which HK pumps bind ouabain is greater than that of LK pumps and exposure of LK cells to anti-L increases the rate at which the LK pumps bind ouabain to that of HK pumps. The lower rate of binding of LK pumps depends on intracellular K since it is increased by reducing K<sub>c</sub>. The rate of ouabain binding, therefore, represents another difference in the properties of HK and LK pumps which, like the difference in pump rates, appears to be due to the increased sensitivity of LK pumps to the inhibitory effect of intracellular K. Anti-L apparently modifies the rate of ouabain binding by altering the affinity of LK cells for K<sub>c</sub>.

#### *Number of Pump Sites in HK and LK Goat Red Cells*

To estimate the number of pump sites by [<sup>3</sup>H]ouabain according to the procedure described above requires (a) that there be very little ouabain binding to sites other than active pump sites (nonspecific binding sites), and (b) that the pumps be relatively homogeneous in their turnover numbers and in the rate at which they bind ouabain. Neither of these conditions is entirely fulfilled for HK or LK goat red cells; nevertheless, by correcting for nonspecific binding it is possible to compare the number of pump sites in HK and LK cells even though the absolute numbers may be a little uncertain.

Fig. 3 is a plot of the number of ouabain molecules bound to HK goat red cells as a function of time of exposure to ouabain. The experiment was performed both in solutions containing 5 mM K ( $10^{-6}$  M ouabain) and in K-free solutions ( $10^{-7}$  M ouabain). In each case it can be seen that, even after a

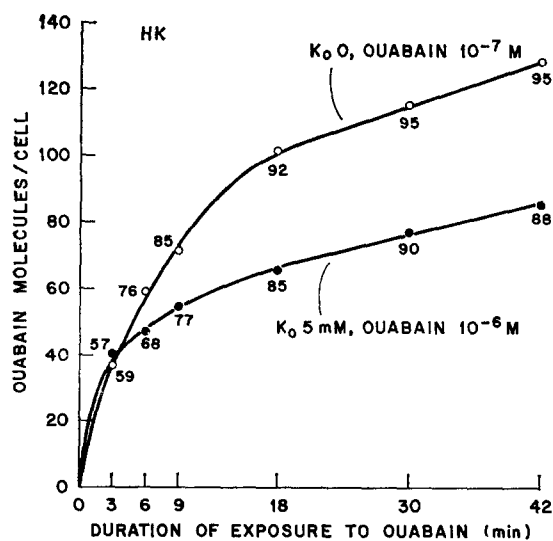


FIGURE 3. Ouabain molecules bound per cell in HK cells vs. duration of exposure to solutions containing ouabain. The number next to each point is the simultaneously determined percent inhibition of the ouabain-sensitive K influx.

maximal inhibition is attained, the number of ouabain molecules bound continues to increase linearly with time; the slope of the line connecting the last three points (for which the percent inhibition does not change) is greater (1.14 sites/min) when the measurement is made in K-free solutions than when it is made in solutions containing K (0.82 sites/min) even though the ouabain concentration was 10 times higher in the latter case.

A correction for the linear portion of the nonspecific binding can be made by estimating the slopes of the portions of the curves of Fig. 3 in which the inhibition is constant, calculating the nonspecific binding which would occur at each time point if the process were linear, and subtracting the calculated nonspecific binding from the experimentally determined total binding. Fig. 4 shows the number of sites calculated in this way from the data of Fig. 3 plotted against the simultaneously determined percent inhibition of the pump. The number of ouabain molecules bound at 100% inhibition of the pump calculated from the least squares plot is 86 molecules/cell for the experiment performed in the absence of external K and 57 molecules/cell for the experiment performed in the presence of external K; external K apparently reduces binding to a greater extent than can be accounted for from the correction derived from the linear part of Fig. 3. In each case there is a significant nonzero intercept at 0% inhibition, positive for the experiment performed in solutions containing K and negative for the experiment performed in K-free solutions. The nonzero intercept might be explained by assuming that there is considerable inhomogeneity of the pumps in these cells both in

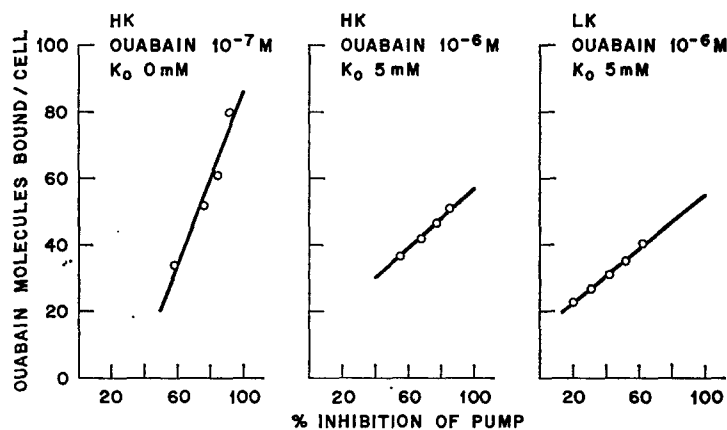


FIGURE 4. Ouabain molecules bound per cell vs. percent inhibition of the pump calculated as described in the text; the calculations for HK cells were made from the data of Fig. 3. The line for the experiment performed at  $10^{-7}$  M ouabain using HK cells is  $Y = 45.8 + 1.32 X$ ; that for the experiment performed at  $10^{-6}$  M ouabain using HK cells is  $Y = 12.4 + 0.45 X$ ; and the line for the experiment using LK cells is  $Y = 14.4 + 0.41 X$  where  $Y$  is the number of ouabain molecules bound per cell and  $X$  is the percent inhibition of the pump.

regard to their turnover number and their rate of ouabain binding. If the rate of ouabain binding is faster for pumps with low turnover numbers when the experiment is performed in the presence of  $K$ , a positive zero intercept would be expected; conversely, if ouabain preferentially binds to pumps with high turnover numbers when the experiment is performed without  $K$ , a negative intercept would be expected. That there is considerable inhomogeneity of the pumps as far as their interaction with ouabain is concerned can be seen in Fig. 5 in which the logarithm of residual pump rate is plotted as a function of the duration of exposure to ouabain. The plot is not a straight line as one would expect if the pumps each had the same turnover number and bound ouabain with a single rate constant. Similar shaped curves were obtained using LK cells.

The best estimate of the number of pump sites is, then, taken as the calculated number of ouabain molecules bound to the cell at 100% inhibition of the pump when the binding experiment is carried out in the presence of  $K$  and when correction is made for binding which occurs after inhibition of the pump is maximal. It is possible that this number is still too high; nevertheless it should be possible to compare the number of sites in HK cells with the number in LK cells when the measurements are carried out in the same way. In Fig. 4 the number of ouabain molecules bound to LK cells is plotted as a function of the simultaneously determined inhibition of the pump influx of  $K$ . Binding was performed in the presence of 5 mM  $K$  and the calculations including the correction for nonspecific binding were made as described above for HK cells.



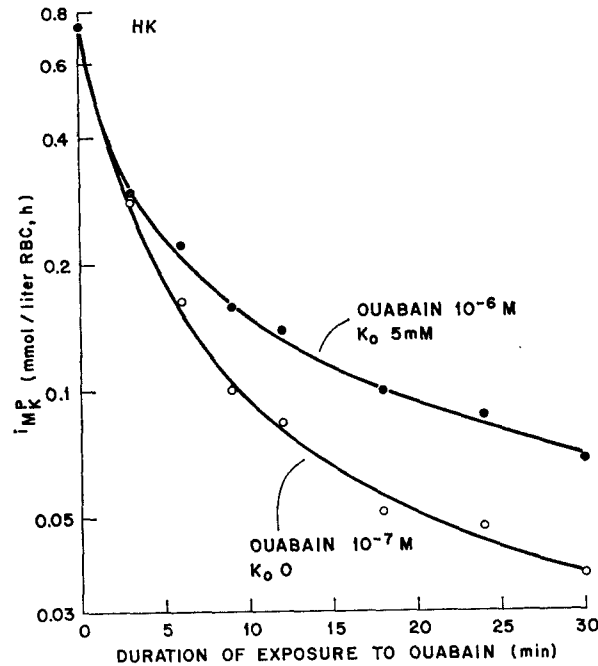


FIGURE 5. Logarithm of the residual pump influx of potassium ( $iM_K^P$ ) in HK cells vs. duration of exposure to ouabain.

The calculated number of binding sites at 100% inhibition of the pump for LK cells (55/cell) is the same as the number for HK cells (57/cell).

#### *Effect of Anti-L on the Number of Pump Sites in LK Cells*

One of the mechanisms by which anti-L might exert its effect is by activating pumps which pump and bind ouabain very slowly in the absence of the antibody. The evidence previously presented (Sachs et al., 1974) that the antibody has no effect on the pump rate of LK cells with very low intracellular K concentrations must mean that such inactive pumps are more sensitive to inhibition by intracellular K than are the remainder of the pumps. The increased sensitivity of the inactive pumps to inhibition by intracellular K might be manifested by a marked heterogeneity of the LK pump rates. The heterogeneity could arise from a heterogeneous population of pumps on each cell, or from a heterogeneous population of cells each with a homogeneous population of pumps, or both.

If the LK pumps are heterogeneous and if one effect of anti-L is to increase both the turnover number and the affinity for ouabain of pumps whose rate of ouabain binding and turnover number in the absence of anti-L are very small compared to the more active pumps, then it might be expected that exposure of LK cells to anti-L will increase the number of ouabain molecules

bound at 100% inhibition of the pump. (In the experiments described below, no correction was made for nonspecific binding since the purpose was to compare the number of ouabain molecules bound under similar circumstances; the absolute number of ouabain binding sites is not therefore a reliable measure of the number of pump sites.) Table II presents the results of experiments in which the number of ouabain binding sites was counted in fresh LK cells exposed to either anti-L or to a nonimmune serum; exposure to anti-L increased the number of ouabain binding sites at 100% inhibition of the pump. When the same experiment was performed using PCMBS-treated cells in which intracellular K was very low (and in which anti-L has no effect

TABLE II  
TOTAL OUABAIN BINDING SITES ( $^3\text{H}$ )OUABAIN MOLECULES BOUND PER CELL  
CALCULATED AT 100% INHIBITION OF THE PUMP) IN LK  
GOAT RED CELLS

	$\text{Na}_e$	$\text{K}_e$	Ouabain binding sites per cell	
			Control	Anti-L treated
	<i>mmol/liter RBC</i>			
<b>Fresh cells</b>				
LK-1	62	40	52	81
LK-2	55	31	44	58
<b>Cells with altered <math>\text{Na}_e</math> and <math>\text{K}_e</math></b>				
LK-2	65	23.0	44	62
LK-2	63	0.8	53	48

The ouabain binding sites at 100% inhibition were calculated as described in the Methods sections. Two other similar experiments were performed with similar results.

on the pump rate), there is almost no increase in the number of ouabain molecules bound in the anti-L-treated cells although anti-L still increased the number of binding sites in similarly treated cells with high intracellular K. The results are consistent with the hypothesis that LK pumps are heterogeneous in their rate of ouabain binding and pump rate and these activities are diminished by the inhibitory effect of intracellular K.

If the explanation for the results described above is indeed that anti-L increases the rate of ouabain binding to a group of sites which, in the unstimulated state, bind ouabain slowly and have a low turnover number, then it should be possible to demonstrate that, after exposure to ouabain for a period which results in submaximal inhibition of the pump, incubation with anti-L will result in cells in which the inhibition of the anti-L-stimulated pump rate is less than the inhibition of the control rate. Table III contains the results of such an experiment. LK cells were exposed to  $10^{-6}$  M ouabain in the presence of 5 mM K for the times indicated, washed free of the glycoside, exposed to anti-L or to nonimmune serum, and the ouabain-sensitive K influx

TABLE III  
EFFECT OF ANTI-L ON OUABAIN-SENSITIVE K INFLUX ( ${}^iM_K^P$ ) AFTER  
BRIEF EXPOSURE TO OUABAIN

Exposure time	Control		Anti-L treated		Anti-L stimulated flux (Anti-L treated—Control)	
	${}^iM_K^P \pm \text{SEM}$	<i>I</i>	${}^iM_K^P \pm \text{SEM}$	<i>I</i>	${}^iM_K^P \pm \text{SEM}$	<i>I</i>
<i>min</i>		%		%		%
0	0.260±0.005	—	0.597±0.012	—	0.337±0.013	—
1	0.228±0.016	12	0.552±0.006	7	0.324±0.017	4
4	0.157±0.005	40	0.423±0.005	29	0.266±0.007	21

LK cells were exposed at  $10^{-6}$  M ouabain in 5 mM K solutions for the times indicated. The cells were then washed and exposed to either anti-L or a nonimmune serum. The cells were again washed and the ouabain-sensitive K influx determined,  $n = 4$ .  ${}^iM_K^P$  is expressed in mmol/liter RBC, h.

determined. The K influx which is stimulated by anti-L is less sensitive to ouabain inhibition than is the control influx; this suggests that anti-L stimulates a group of LK pumps which, before stimulation by anti-L, are characterized by a low turnover rate and a slow rate of binding of ouabain.

The cells used in the experiment described above were also used for the determination of the time-course of ouabain binding; in Fig. 6 the number of ouabain molecules bound per cell is plotted as a function of the percent inhibition of the pump. As expected, the number of ouabain molecules bound to the cells at 100% inhibition is greater in the cells exposed to anti-L than in the cells exposed to the nonimmune serum; the difference is less in the cells preincubated with ouabain for 4 min, and in these cells the anti-L-stimulated pump influx was inhibited to some extent by preexposure to the glycoside (Table III). The positive zero intercept in the control cells is converted to a negative intercept in the cells preexposed to ouabain for the longest time interval; this may again reflect heterogeneity in the ouabain binding characteristics and turnover rates of the pumps.

Although the anti-L-stimulated pump influx is less sensitive to inhibition by ouabain than the control influx, the difference is relative; if the cells are exposed to a concentration of ouabain which results in 100% inhibition of the pump, subsequent exposure to anti-L no longer increases the K influx. In a preliminary experiment (Fig. 7) the effect of preexposure of LK cells for 0.5 h to varying concentrations of ouabain in K-free solutions on the magnitude of the ouabain-sensitive K influx was determined. Almost complete inhibition of the ouabain-sensitive K influx occurred at  $2.5 \times 10^{-7}$  M ouabain. LK cells were therefore exposed to ouabain at  $2.5$  and  $5.0 \times 10^{-7}$  M ouabain for 0.5 h, washed free of the inhibitor, and then exposed to either anti-L or to non-immune serum. After exposure to the serum the ouabain-sensitive K influx was measured; in addition a portion of the cells was exposed to  $10^{-6}$  M [ ${}^3\text{H}$ ]-

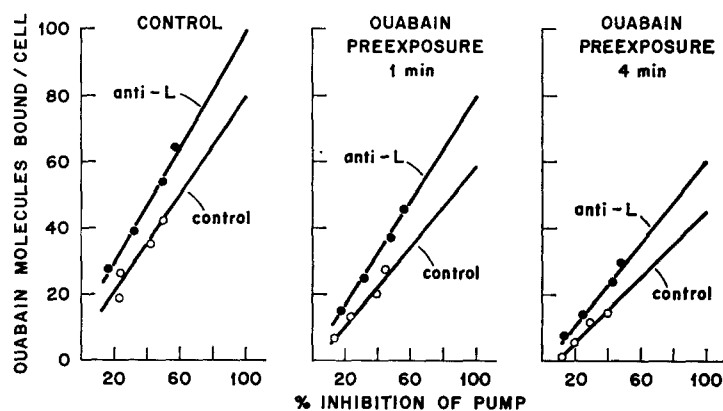


FIGURE 6. Number of ouabain molecules bound per cell vs. percent inhibition of the pump. The cells used for the experiment were the same as those described in Table III; after exposure to [ $^3\text{H}$ ]ouabain for varying time periods, the cells were washed and incubated either with anti-L or a nonimmune serum. The cells were again washed and the number of tritiated ouabain molecules bound per cell and ouabain-sensitive K influx determined.

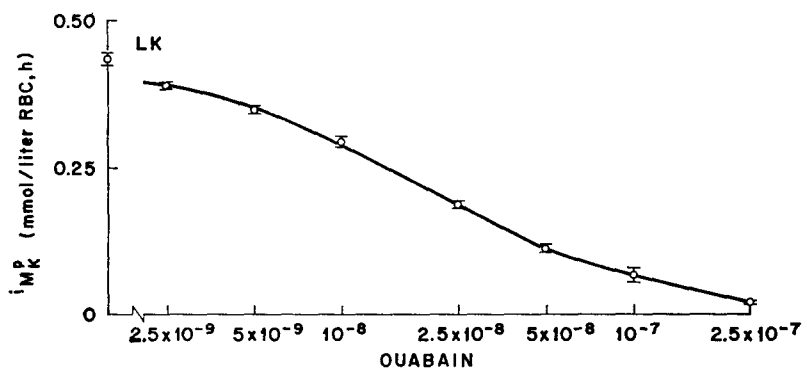


FIGURE 7. Ouabain-sensitive K influx ( $iM_K^P$ ) ( $\pm$ SEM,  $n = 4$ ) vs. ouabain concentration (logarithmic scale) to which the cells were preexposed. LK cells were incubated for 0.5 h at  $37^\circ\text{C}$  in K-free solutions containing the indicated concentrations of ouabain, separated from the solutions, washed four times, and the ouabain-sensitive K influx measured.  $K_o$  during the measurement of the influx was 2.4 mM.

ouabain in solutions containing 5 mM K for 6 min, the cells were washed, and the number of ouabain molecules bound estimated. The results are in Table IV. The percent inhibition of the pump is about the same for the control cells and the anti-L-treated cells, and exposure of the cells to anti-L does not increase the number of ouabain molecules bound during the 6-min exposure. When the exposure to ouabain proceeds to 100% inhibition of the pump, therefore, both the anti-L-stimulated flux and the control flux are completely

TABLE IV  
ANTI-L EFFECT ON OUABAIN-SENSITIVE K INFLUX AND OUABAIN BINDING  
AFTER PROLONGED EXPOSURE TO OUABAIN

Ouabain concentration during preincubation	Control			Anti-L treated		
	${}^3M_K^P \pm \text{SEM}$	<i>I</i>	Ouabain molecules bound in 6 min	${}^3M_K^P \pm \text{SEM}$	<i>I</i>	Ouabain molecules bound in 6 min
		%			%	
0	0.218±0.010	—	25	0.507±0.013	—	50
2.5×10 <sup>-7</sup> M	0.018±0.006	92	0	0.033±0.002	93	2
5.0×10 <sup>-7</sup> M	0.012±0.003	95	1	0.016±0.004	97	2

LK cells were exposed to the indicated concentrations of ouabain for 0.5 h in K-free solutions, washed, and exposed to either anti-L or a nonimmune serum. Part of the cells were then used for the determination of the ouabain-sensitive K influx ( $n = 4$ ) and another part were exposed to [<sup>3</sup>H]ouabain (10<sup>-6</sup> M in a solution containing 5 mM K) for 6 min, washed, and the number of ouabain molecules bound to the cells determined.

inhibited and, as expected, anti-L does not expose any new ouabain binding sites. Although the pumps sensitive to stimulation by anti-L bind ouabain less readily than the pumps which are not stimulated by anti-L, the difference is relative and can be overcome by a sufficiently high ouabain concentration.

#### DISCUSSION

One of the conclusions from the results presented in this paper is that the number of pumps in HK and LK goat red cells are about the same (Fig. 4). The previously reported observation (Sachs et al., 1974) that the pump rate in HK cells at optimal concentrations of intracellular Na and K is greater than the pump rate in LK cells under the same circumstances cannot therefore be due to a difference in the total number of pumps. Even with optimal intracellular concentrations of Na and K the average turnover number of HK pumps must be greater than that for LK pumps.

The results presented in Fig. 1 indicate that HK pumps bind ouabain more rapidly than LK pumps and that treatment of LK cells with anti-L increases the rate at which they bind ouabain to that characteristic of the HK pumps. This represents another difference in the operational characteristics of the transport system of HK and LK cells, and this difference also seems to be dependent on intracellular K. Thus the experiment shown in Fig. 2 indicates that the pumps of LK cells with very low  $K_o$  bind ouabain at a faster rate more comparable with HK cells or LK cells treated with anti-L. When  $K_o$  is very low, treatment of the LK cells with anti-L does not accelerate the rate at which their pumps bind ouabain. The circumstances under which the ouabain binding rate of the LK pumps is the same as that of the HK pumps (low  $K_o$  or anti-L-treated high  $K_o$  cells) are those under which the pump rate is greatest.

Both pumps of HK cells and those of LK cells display marked heterogeneity

as far as their rate of ouabain binding or their turnover number or both is concerned. The heterogeneity of the pumps of LK cells is reflected in the ability of anti-L to increase the number of ouabain molecules bound at 100% inhibition of the pump. Exposure to anti-L must increase both the turnover rate and the rate of ouabain binding of a group of LK pumps. Since removal of intracellular K produces LK cells in which exposure to anti-L does not increase the number of ouabain molecules bound at 100% inhibition, the low turnover rate and rate of ouabain binding of the anti-L-sensitive pumps reflects an effect of intracellular K on these characteristics and indicates that the pumps responsive to anti-L are more sensitive to this effect of intracellular K than are the remainder of the LK pumps or the HK pumps. The different rate of ouabain binding of the anti-L-sensitive sites is relative; when exposed to a high enough concentration of ouabain these pumps are inhibited and anti-L affects neither the pump rate nor the number of ouabain molecules bound during a subsequent exposure to the inhibitor. It is not clear whether the heterogeneity of the pumps is due to a heterogeneous population of pumps on each cell, a heterogeneous population of cells, or both.

The results reported here for goat cells differ from those previously reported for sheep red cells (Dunham and Hoffman, 1971) in that LK sheep cells have many fewer pump sites than HK sheep cells; thus fresh LK sheep cells were found to have on the average 7.6 and HK cells 42.4 pump sites per cell. It was suggested, however, that pumps of LK sheep cells have a lower affinity for ouabain than HK pumps. It has been reported that exposure of LK sheep red cells to anti-L increases the number of ouabain binding sites (Lauf et al., 1970), but the effect of lowering  $K_c$  on this number has not been reported. More recently, it has been reported (Joiner and Lauf, 1974) that the number of sites on HK sheep cells (120–140) was higher than the number on homozygous LK cells (50–70); that the rate of binding to HK cells was much more rapid than to LK cells; and that anti-L increased the rate of binding to LK cells toward that of HK cells although anti-L did not increase the number of binding sites on LK cells. The reasons for the differences between the two studies using sheep cells and the present using goat cells is not clear; it is possible that the abnormality of the pumps of LK goat cells may not be the same as the abnormality of LK sheep pumps, but methodological differences may also be partly responsible for the discrepancies.

This work was supported by grants from the National Institutes of Health AM-13981, AM-05644, AM-16196, and HE-09906, from the National Science Foundation GB-18924, and from the American Heart Association.

This work was done during Dr. Sachs' tenure of an Established Investigatorship of the American Heart Association.

Dr. Kropp was a postdoctoral fellow of the Division of General Medical Sciences of the National Institutes of Health.

*Received for publication 18 October 1972.*

## REFERENCES

- DUNHAM, P. B., and J. F. HOFFMAN. 1971. Active cation transport and ouabain binding in high potassium and low potassium red blood cells of sheep. *J. Gen. Physiol.* 58:94.
- ELLORY, J. C., and E. M. TUCKER. 1969. Stimulation of the potassium transport system in low potassium type sheep red cells by a specific antigen-antibody reaction. *Nature (Lond.)* 222:477.
- ELLORY, J. C., and E. M. TUCKER. 1970. Active potassium transport and the L and M antigens of sheep and goat red cells. *Biochim. Biophys. Acta.* 219:160.
- EVANS, J. V. 1954. Electrolyte concentrations in red blood cells of British breeds of sheep. *Nature (Lond.)* 174:931.
- EVANS, J. V., and A. T. PHILLIPSON. 1957. Electrolyte concentrations in the erythrocytes of the goat and ox. *J. Physiol. (Lond.)* 139:87.
- HOFFMAN, J. F. 1969. The interaction between tritiated ouabain and the Na:K pump in red blood cells. *J. Gen. Physiol.* 54:343s.
- INGRAM, C. J. 1970. The binding of ouabain to human red cells. Ph.D. Thesis. Yale University, New Haven, Conn.
- JOINER, C. H., and P. K. LAUF. 1974. <sup>3</sup>H-ouabain binding to HK and LK sheep red cells and the effect of anti-L. *Fed. Proc.* 33:265.
- LAUF, P. K., B. A. RASMUSEN, P. G. HOFFMAN, P. B. DUNHAM, P. COOK, M. L. PARMELEE, and D. C. TOSTESON. 1970. Stimulation of active potassium transport in LK sheep red cells by blood group-L-antiserum. *J. Membr. Biol.* 3:1.
- SACHS, J. R., J. C. ELLORY, D. L. KROPP, P. B. DUNHAM, and J. F. HOFFMAN. 1974. Kinetic characteristics of the Na:K pump in goat red blood cells. *J. Gen. Physiol.* 63:389.
- TOSTESON, D. C., and J. F. HOFFMAN. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* 44:169.