# Sodium Transport in Turtle Erythrocytes

## *Apparent stimulation of exchange diffusion by anaerobiosis*

SAULO KLAHR, ALLEN B. SHAW, KUO HWA HWANG, and C. LINDSEY MILLER

From the Renal Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT Studies were performed on Na and K transport by red blood cells of the freshwater turtle under anaerobic and aerobic conditions. Although it had previously been assumed that cation transport in turtle red blood cells was dependent on respiration, the present data show greater Na efflux rates in $N_2$ than in $O_2$ . However, ouabain inhibited Na transport by the same amount quantitatively in $O_2$ and $N_2$ gas phases. Thus there was no difference in ouabain-sensitive or "pump" Na transport rates. Na influx rates were higher in nitrogen than in air and potassium influx rates were not significantly different under aerobic and anaerobic conditions. Moreover in the absence of sodium in the bathing medium no difference between air and nitrogen could be discovered. Finally with ethacrynic acid plus ouabain there was an additional decrease in Na efflux but there was a persisting difference between air and nitrogen. These studies do not rule out the existence of a ouabain-insensitive ethacrynic acid–inhibitable flux; however, they suggest that at least part of the activation of Na efflux observed in $N_2$ was due to increased exchange diffusion.

### INTRODUCTION

The present studies were undertaken to examine some aspects of sodium transport in the red blood cell of the freshwater turtle (*Pseudemys scripta elegans*). This reptile is uniquely equipped to tolerate prolonged oxygen deprivation and previous studies utilizing the isolated urinary bladder of the turtle have shown that sodium transport in this organ is well-maintained under anaerobic conditions (1, 2). In the past few years, we have studied various aspects of this anaerobic transport, including the coupling between ion transport and anaerobic metabolism (2, 3). The present studies represent an attempt to study cation transport by another structure from the turtle, the red blood cell. The experiments were designed to compare anaerobic with

aerobic transport patterns. Previous studies by Maizels on the African tortoise suggested that ion transport in the turtle erythrocyte is dependent upon respiration (4). However, the present data do not support this conclusion. No decrease in cation transport was demonstrable under anaerobic conditions. Indeed Na efflux rates were consistently greater during anaerobiosis. The present paper describes this phenomenon and presents a series of studies designed to explore its possible mechanisms.

## MATERIAL AND METHODS

Adult, freshwater turtles of the genus *Pseudemys*, weighing between 1–3 kg, were employed in these studies. The animals were obtained from The Snake Farm, La Place, La. They were kept in shallow freshwater at room temperature (approximately 22°C) prior to study. At the time of study blood was obtained by exsanguination of the decapitated turtle, heparin being used as the anticoagulant. 50–100 ml of whole blood could be obtained from the majority of the animals, with hematocrit values ranging from 15 to 25 volumes %. Samples were centrifuged for 15 min at 2000 rpm in a refrigerated centrifuge (4°C) and thereafter the plasma and buffy coat were removed. Cells were then washed twice with cold (4°C) Ringer solution having the following composition: $Na^+$ = 115 mEq/liter; $K^+$ = 3 mEq/liter; $Ca^{++}$ = 3.0 mEq/liter; $Mg^{++}$ = 4.0 mEq/liter; $Cl^-$ = 120 mEq/liter; $PO_4^=$ = 5 mEq/liter. The pH of the solution was 7.4 ± 0.1.

### *Sodium Efflux*

In the experiments designed to measure sodium efflux, approximately 20 ml of packed red blood cells were incubated in 50 ml of the turtle Ringer solution containing 80–120 μc of $^{22}NaCl$ (Abbott Corp., Chicago, Ill.) and incubation was carried out either at 4°C overnight or at room temperature (about 22°C) for 4–6 hr. At the end of the incubation, the red blood cell–Ringer mixture was transferred into six 50 ml centrifuge tubes. Centrifugation was carried out at 2000 rpm, the supernatant was discarded, and the cells were resuspended in nonradioactive cold turtle Ringer. Centrifugation and washing were repeated six times. A final cell pack was obtained and a portion of these cells was transferred to preweighed hematocrit tubes for determination of hematocrit, wet and dry weight. Hematocrits were measured after centrifugation at 2000 rpm for 30 min. Dry weight was determined by reweighing the tubes after 24 hr incubation in an oven at 105°C. The rest of the cells were added to a beaker containing turtle Ringer with 10 mM glucose in a volume calculated to provide a final hematocrit of approximately 5 volumes %. 15 or 20 ml samples of the well-mixed cell suspension were then transferred to 50 ml Erlenmeyer flasks, using a constant delivery automatic syringe. The contents of two of the Erlenmeyer flasks were used for control (zero time) determinations and processed immediately in the manner to be described below for the incubated cells. The remainder of the Erlenmeyer flasks were incubated at 25°C in a Dubnoff metabolic shaker. One set of flasks, comprised of one-half of the flasks, was incubated under aerobic conditions; the other set was gassed with 100% nitrogen, which was passed through an oxygen trap before it entered the flasks (5). Incubations were carried out for 1½ and 3 hr. In all the sequences (1½ or 3 hr incubations, aerobic

or anaerobic conditions) sodium efflux measurements were made with and without the presence of ouabain ($5 \times 10^{-5}$ M) in the media. In some of the experiments an additional set of flasks containing both ouabain and ethacrynic acid ($5 \times 10^{-4}$ M) was incubated aerobically and anaerobically.

At the completion of the incubations, the contents of each flask were centrifuged for 10 min at 2000 rpm at 4°C. The turtle-Ringer was then removed as completely as possible and aliquots were used for the measurement of $^{22}Na$. In eight instances the degree of hemolysis was estimated by measuring the hemoglobin concentration in the supernatant fluid. The values obtained were small and no correction was necessary. The packed red blood cells were washed once rapidly with cold isosmotic (75 mM) $MgCl_2$ to dilute the $^{22}Na$ and $^{23}Na$ in the trapped Ringer. After removing the $MgCl_2$ most of the packed cells in each tube were transferred to a 10 ml volumetric flask, and hemolyzed with distilled water and a few drops of a noncationic detergent Acationox (Scientific Products, Evanston, Ill.). Chemical Na concentration and $^{22}Na$ were determined in the hemolysate and these values were used in the calculation of the specific activities. Hemoglobin concentration of the hemolysate was measured by the cyanmethemoglobin method and the hemoglobin concentration values of a weighed aliquot of the original cell pack were also determined. In this manner the amount of cells in each volumetric flask could be calculated. The remaining portion of the packed cells was transferred to preweighed hematocrit tubes for determination of hematocrit, wet and dry weight. From these values the cell water content was calculated.

*Potassium Influx*

For the measurement of potassium influx, approximately 50 ml of washed turtle red blood cells were diluted to a final hematocrit of approximately 15–20 volumes % with turtle-Ringer containing 10 mM glucose. Thereafter 50–100 μc of $^{42}KCl$ (Cambridge Nuclear Corp., Cambridge, Mass.) were added, and after complete mixing 15–20 ml samples were transferred to 50 ml Erlenmeyer flasks, as described above. Four of these flasks contained ouabain ($5 \times 10^{-5}$ M). The contents of two of the Erlenmeyer flasks without ouabain were used for control determinations and processed immediately. Half of the remaining flasks were incubated at 25°C in a Dubnoff metabolic shaker for 1 hr. The other half was incubated for 2 hr. As in the sodium efflux measurements paired flasks were incubated aerobically and anaerobically. At the completion of incubation, the flasks were processed essentially as described for the experiments on Na efflux except that two $MgCl_2$ washes were employed. After the second wash, most of the cells were transferred to preweighed 10 ml volumetric flasks; the flasks were reweighed to determine the quantity of cells added and the cells were hemolyzed as described above. Chemical potassium and $^{42}K$ were measured in aliquots of the hemolysate, the figures for radioactive potassium being used for the calculation of intracellular isotopic accumulation. Potassium specific activity was calculated from the values of $^{42}K$ activity in the Ringer and the chemical K concentration in the Ringer.

*Sodium Influx*

Except for the use of $^{22}NaCl$ instead of $^{42}KCl$, the procedures employed were identical to those described for the measurement of potassium influx. 200–250 μc of $^{22}NaCl$ were added to a total volume of 250 ml of diluted cells.

$^{22}Na$ and $^{42}K$ were determined in an Autogamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Chemical sodium and potassium were determined by flame photometry. Na efflux was calculated in the following manner. The counts in 2 ml of supernatant at time $t$ (0, 90, or 180 min) were divided by the counts in a similar volume of cell suspension at time 0. The ratio of supernatant to cell counts was subtracted from 1 and the values were plotted against time on a semilogarithmic scale and the half-time ($t_{1/2}$) was calculated. The rate constant ($K$) was derived from the equation $K = 0.693/t_{1/2}$. The flux values were computed from the mean chemical sodium concentrations in the cells at 0–1½ hr or 1½–3 hr times the rate constant. Potassium influx was calculated as described by Sachs and Welt (6). The following formula was used:

$$^{i}M_K = \frac{70\,^{42}K_c - 10\,^{42}K_c}{\frac{10 \quad 70}{^{42}K_s}} \times 10[K_s]70$$

where $^{i}M_K$ is the influx of K in micromoles per gram of red blood cells, hour; $10\ ^{42}K_c$ is the activity of the cells in counts per minute per milliliter of cells at 10 min, and $70\ ^{42}K_c$ is the same quantity at 70 min; $\frac{10 \quad 70}{^{42}K_s}$ is the average of the activity in counts per minute per milliliter of the supernatant at 10 and 70 min; and $10\ [K_s]\ 70$ is the average of the K concentration in micromoles per milliliter of the supernatant at 10 and 70 min. Similar calculations were used for the second period (70–130 min). Na influx was calculated in the same manner as K influx. Flux values are expressed as micromoles of Na or K per gram RBC's per hour. "Pump" efflux and pump influx values for Na and K represent the difference between the total flux rates and the flux rates obtained in the presence of ouabain.

### *Lactate Formation and ATP Concentrations*

In some studies, lactate formation and ATP concentrations were determined in cells incubated at 25°C for 2 hr under aerobic and anaerobic conditions. The cells were processed as described previously (7). Lactate was determined enzymatically measuring the appearance of DPNH from DPN in the presence of beef heart LDH. The reaction was carried on in 0.2 M carbonate buffer at a pH of 9.7 and with 50 mM of hydrazine hydrate. ATP was measured by determining the rate of conversion of TPN to TPNH in the presence of glucose, hexokinase, glucose-6-phosphate dehydrogenase, and $MgCl_2$ in a 0.1 M Tris-Cl buffer solution at pH 7.5.[1]

## RESULTS

### *Sodium Efflux*

Fig. 1 shows a representative plot of the loss of $^{22}Na$ from the cells into the Ringer as a function of time. The data are expressed as $^{22}Na$ released from the

[1] DPN, diphosphopyridine nucleotide
DPNH, reduced diphosphopyridine nucleotide
LDH, lactic dehydrogenase
ATP, adenosine triphosphate
TPN, triphosphopyridine nucleotide
TPNH, reduced triphosphopyridine nucleotide

cells as a per cent of initial cell counts present. The rate of entry of $^{22}Na$ into the Ringer per unit weight of cells was somewhat higher under anaerobic than under aerobic conditions. In the presence of ouabain there was a marked decrease in the entry of $^{22}Na$ into the Ringer; however, the rate of entry remained higher in the anaerobic studies.

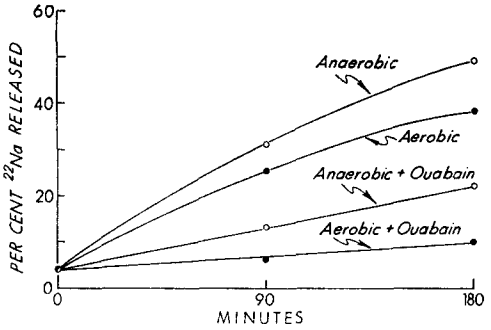


FIGURE 1. The number of $^{22}Na$ counts released into the Ringer solution are expressed as a per cent of the initial $^{22}Na$ counts present in the cells. This plot corresponds to the experiment described in detail in Table I.

TABLE I

$^{22}Na$ EFFLUX UNDER AEROBIC AND ANAEROBIC CONDITIONS

| Conditions | $^{22}Na$ Initial cell counts | $^{22}Na$ in Ringer | $\frac{^{22}Na \text{ in Ringer}}{^{22}Na \text{ initial cell counts}}$ | $1 - \frac{^{22}Na \text{ in Ringer}}{^{22}Na \text{ in cells}}$ | $t_{1/2}$ | $K\left(\frac{0.693}{t_{1/2}}\right)$ |
|---|---|---|---|---|---|---|
| | | | | | *hr* | |
| Control | 8725 | 385 | 0.04 | 0.96 | | |
| Aerobic conditions | | | | | | |
| 1½ hr | | 2188 | 0.25 | 0.75 | | |
| 3 hr | | 3335 | 0.38 | 0.62 | 4.5 | 0.154 |
| 1½ hr ouabain | | 546 | 0.06 | 0.94 | | |
| 3 hr ouabain | | 913 | 0.10 | 0.90 | 25.7 | 0.027 |
| Anaerobic conditions | | | | | | |
| 1½ hr | | 2715 | 0.31 | 0.69 | | |
| 3 hr | | 4240 | 0.49 | 0.51 | 3.0 | 0.231 |
| 1½ hr ouabain | | 1120 | 0.13 | 0.87 | | |
| 3 hr ouabain | | 1945 | 0.22 | 0.78 | 7.75 | 0.09 |

Control refers to initial values before incubation. See Material and Methods for a detailed description. The chemical sodium concentration in this experiment was 8.5 $\mu$Eq/ml of cell water.

Table I presents the detailed results of the experiment plotted in Fig. 1. The control measurements refer to the $^{22}Na$ counts in the cells and in the Ringer after 4 hr of isotopic loading without substrate, and after the washing procedures, but before incubation. The table includes the ratio of $^{22}Na$ counts in the Ringer to initial counts in the cells. Also given are the average values of the half-times for the loss of $^{22}Na$ under the different conditions and the

values for the rate constant ($K$). Sodium efflux values were calculated by multiplying the rate constant by the sodium content of the cells.

Table II summarizes the data from 12 sodium efflux experiments. Total Na efflux was greater in anaerobiosis than in aerobiosis in every experiment. The mean values were 1.95 $\mu$moles/g RBC per hr aerobically and 2.59 $\mu$moles/g RBC per hr anaerobically. These values are statistically different ($p < 0.01$). The variation in efflux values observed from experiment to experiment is probably due to variations in intracellular sodium concentrations. Thus, there was a tendency for Na efflux values to increase with increasing in-

TABLE II

$^{22}$Na EFFLUX UNDER AEROBIC AND ANAEROBIC CONDITIONS AND THE EFFECTS OF OUABAIN ($5 \times 10^{-5}$ M)

| Experiment No. | $^{23}$Na | Aerobiosis | | | | Anaerobiosis | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | $O_M P_{Na}$ | Ouabain | Decrease | Control | $O_M P_{Na}$ | Ouabain | Decrease |
| | $\mu Eq/ml$ cell $H_2O$ | *Na $\mu$M/g RBC per hr* | | | % | *Na $\mu$M/g RBC per hr* | | | % |
| 1 | 8.5 | 1.31 | 1.07 | 0.24 | 81.7 | 1.96 | 1.08 | 0.88 | 55.1 |
| 2 | 7.8 | 1.31 | 1.09 | 0.22 | 83.2 | 1.78 | 1.01 | 0.77 | 56.7 |
| 3 | 5.7 | 1.09 | 0.94 | 0.15 | 86.2 | 1.63 | 1.05 | 0.58 | 64.4 |
| 4 | 9.2 | 1.15 | 0.93 | 0.22 | 80.9 | 1.82 | 1.13 | 0.69 | 62.1 |
| 5 | 11.8 | 1.51 | 1.21 | 0.30 | 80.1 | 1.90 | 1.18 | 0.72 | 62.1 |
| 6 | 12.2 | 1.88 | 1.72 | 0.16 | 91.5 | 2.75 | 1.65 | 1.10 | 60.0 |
| 7 | 13.9 | 1.99 | 1.36 | 0.63 | 68.3 | 2.90 | 1.68 | 1.22 | 57.9 |
| 8 | 19.3 | 1.74 | 1.24 | 0.50 | 71.3 | 1.96 | 1.06 | 0.90 | 54.1 |
| 9 | 26.7 | 2.42 | 1.11 | 1.31 | 45.9 | 3.00 | 1.10 | 1.90 | 36.7 |
| 10 | 28.2 | 2.95 | 1.43 | 1.52 | 48.5 | 3.70 | 1.76 | 1.94 | 52.4 |
| 11 | 27.9 | 2.96 | 1.95 | 1.01 | 65.9 | 3.83 | 2.11 | 1.72 | 55.1 |
| 12 | 29.5 | 3.04 | 1.98 | 1.06 | 65.1 | 3.86 | 2.00 | 1.86 | 51.8 |
| Mean | | 1.95 | 1.34 | 0.61 | 72.4 | 2.59 | 1.40 | 1.19 | 55.7 |

Experiments were calculated as described in Material and Methods. $O_M P_{Na}$ represents pump efflux calculated as a difference between the control and ouabain-inhibited flux rates. Statistical differences between the different conditions in aerobiosis vs. anaerobiosis are given in the text.

tracellular Na concentrations. Ouabain produced a marked inhibition in sodium efflux in both air and nitrogen. The mean decrease was 72.4% in air and 55.7% in nitrogen. Values for the ouabain-sensitive component of Na efflux (i.e. so-called pump efflux) were closely comparable aerobically and anaerobically. The mean values were 1.34 $\mu$M/g RBC per hr in air and 1.40 $\mu$M/g RBC per hr in nitrogen ($p > 0.05$). On the other hand, the mean values for the ouabain-insensitive Na efflux (i.e. the Na efflux persisting in the presence of ouabain) were almost twice as great in nitrogen as in air (0.61 $\mu$M/g RBC per hr under aerobic conditions vs. 1.19 $\mu$M/g RBC per hr under anaerobic conditions ($p < 0.005$). Thus, most of the increase in Na efflux observed during anaerobiosis could be accounted for by a ouabain-insensitive component.

*Potassium Influx*

Table III presents the detailed results of a representative study of $^{42}K$ influx. Data are included for chemical and isotopic potassium concentrations both in cell water and media. Chemical K concentrations in cell water ranged from 95 to 104 mEq/liter and no difference was detectable between aerobic and anaerobic conditions; however, values were lower in the presence of ouabain both aerobically and anaerobically. The potassium concentration in the Ringer was 3 mEq/liter. In contrast to sodium efflux $^{42}K$ accumulation per

TABLE III

$^{42}K$ INFLUX UNDER AEROBIC AND ANAEROBIC CONDITIONS

| Conditions | RBC | $^{39}K$ | | Total cpm per g cells | Total cpm per g cells per hr | $^{42}K$, *cpm/ml Ringer* | | | $^{42}K$ cells / $^{42}K$ Ringer | K influx |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cpm per ml | Mean value 10–70 min | Mean value 70–130 min | | |
| | *% $H_2O$* | *μEq/ml cell $H_2O$* | *μEq/ml Ringer* | | | | | | | *μM/g cells per hr* |
| Control | 70.9 | 104 | 3 | 2,246 | | 23,397 | | | | |
| Aerobic conditions | | | | | | | | | | |
| 1 hr | 71.2 | 100 | 3 | 14,437 | 12,191 | 20,877 | 22,137 | | 0.55 | 1.65 |
| 2 hr | 71.3 | 100 | 3 | 24,298 | 9,861 | 18,050 | | 19,464 | 0.51 | 1.53 |
| 1 hr ouabain | 71.9 | 97 | 3 | 6,435 | 4,189 | 21,902 | 22,650 | | 0.18 | 0.54 |
| 2 hr ouabain | 72.2 | 96 | 3 | 11,051 | 4,616 | 19,803 | | 20,853 | 0.22 | 0.66 |
| Anaerobic conditions | | | | | | | | | | |
| 1 hr | 71.1 | 101 | 3 | 14,458 | 12,221 | 20,934 | 22,166 | | 0.55 | 1.65 |
| 2 hr | 71.4 | 99 | 3 | 22,929 | 8,471 | 18,675 | | 19,805 | 0.43 | 1.29 |
| 1 hr ouabain | 72.0 | 96 | 3 | 6,325 | 4,079 | 21,782 | 22,590 | | 0.18 | 0.54 |
| 2 hr ouabain | 71.9 | 95 | 3 | 9,513 | 3,188 | 20,020 | | 20,901 | 0.15 | 0.45 |

The control sample represents the influx of $^{42}K$ in the initial 10 min. This control has been subtracted from the determinations of $^{42}K$ present in the erythrocytes at 70 min and 130 min after the incubation was started. Values for K influx (last column) are given in μM/g of cells per hr. Experiments were calculated as described in Material and Methods.

gram of cells per hour was closely comparable under aerobic and anaerobic conditions. Mean potassium influx rates averaged 1.59 μM/g RBC per hr under aerobic conditions and 1.47 μM/g RBC per hr under anaerobic conditions. In the presence of ouabain potassium influx rates averaged 0.60 μM/g RBC per hr aerobically and 0.50 μM/g RBC per hr anaerobically. Thus anaerobiosis did not affect either total K influx or ouabain-insensitive potassium influx.

In Table IV, a summary is presented of potassium influx values for five experiments performed aerobically and anaerobically with and without

ouabain. In each experiment the 0–1 hr values (A) are closely comparable to the 1–2 hr values (B). Mean K influx in air was 1.98 $\mu$M/g RBC per hr as compared with 1.89 $\mu$M/g RBC per hr in nitrogen. Mean values for ouabain-sensitive influx were closely comparable in air (1.40 $\mu$M/g RBC per hr) and nitrogen (1.42 $\mu$M/g RBC per hr). The mean per cent decrease with ouabain was 70.4% in air and 74.1% in nitrogen. Thus, for potassium, the difference

TABLE IV

$^{42}$K INFLUX UNDER AEROBIC AND ANAEROBIC CONDITIONS AND THE EFFECTS OF OUABAIN ($5 \times 10^{-5}$M)

| Experiment No | Aerobiosis | | | | Anaerobiosis | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | $I_M P_K$ | Ouabain | Decrease | Control | $I_M P_K$ | Ouabain | Decrease |
| | | | | % | | | | % |
| 1 A | 1.90 | 1.35 | 0.55 | 71.1 | 1.84 | 1.40 | 0.44 | 76.1 |
| B | 1.80 | 1.14 | 0.66 | 63.3 | 1.38 | 0.94 | 0.44 | 68.1 |
| 2 A | 1.65 | 1.11 | 0.54 | 67.3 | 1.65 | 1.11 | 0.54 | 67.3 |
| B | 1.53 | 0.87 | 0.66 | 56.9 | 1.29 | 0.84 | 0.45 | 65.1 |
| 3 A | 2.50 | 1.87 | 0.63 | 74.8 | 2.30 | 1.91 | 0.39 | 83.0 |
| B | 2.01 | 1.61 | 0.40 | 80.1 | 1.95 | 1.56 | 0.39 | 80.0 |
| 4 A | 2.30 | 1.77 | 0.53 | 77.0 | 2.24 | 1.83 | 0.41 | 81.7 |
| B | 1.84 | 1.49 | 0.35 | 81.0 | 1.74 | 1.38 | 0.36 | 79.3 |
| 5 A | 2.49 | 1.59 | 0.90 | 63.9 | 2.42 | 1.71 | 0.71 | 70.7 |
| B | 1.80 | 1.24 | 0.56 | 68.9 | 2.10 | 1.47 | 0.63 | 70.0 |
| Mean | 1.98 | 1.40 | 0.58 | 70.4 | 1.89 | 1.42 | 0.48 | 74.1 |

Experiments were performed as detailed in Table III. $I_M P_K$ represents pump influx calculated as a difference between the control and ouabain-inhibited flux rates.

in ouabain-insensitive transport between aerobiosis and anaerobiosis was minimal.

*Sodium Efflux in the Absence of External Sodium*

Table V presents data for aerobic and anaerobic Na efflux in the absence of external Na (115 mM choline chloride Ringer). Control values averaged 1.48 and 1.51 in comparison to 1.95 and 2.59 in Na Ringer (Table II). Ouabain produced a marked decrease in Na efflux values in both air and nitrogen. Of considerable interest is the fact that values for Na efflux were no greater under anaerobic than under aerobic conditions when sodium was absent from the external media.

### *Effects of Ethacrynic Acid on Ouabain-Insensitive Na Efflux*

Table VI presents the effects of ethacrynic acid on the ouabain-insensitive portion of Na efflux both aerobically and anaerobically in six experiments. As described above, Na efflux was greater during anaerobiosis than during

TABLE V

$^{22}$Na EFFLUX IN THE ABSENCE OF EXTERNAL Na UNDER AEROBIC AND ANAEROBIC CONDITIONS AND THE EFFECTS OF OUABAIN ($5 \times 10^{-5}$ M)

| Experiment No. | Aerobiosis | | | Anaerobiosis | | |
|---|---|---|---|---|---|---|
| | Control | Ouabain | $O_M P_{Na}$ | Control | Ouabain | $O_M P_{Na}$ |
| | *μM Na/g RBC per hr* | | | *μM Na/g RBC per hr* | | |
| 1 | 1.41 | 0.37 | 1.04 | 1.39 | 0.35 | 1.04 |
| 2 | 1.45 | 0.30 | 1.15 | 1.42 | 0.37 | 1.05 |
| 3 | 1.61 | 0.36 | 1.25 | 1.73 | 0.36 | 1.37 |
| 4 | 1.46 | 0.34 | 1.12 | 1.51 | 0.38 | 1.13 |
| Mean | 1.48 | 0.34 | 1.14 | 1.51 | 0.36 | 1.15 |

Experiments were calculated as described in Material and Methods. External sodium was replaced by equimolar amounts of choline chloride.

TABLE VI

EFFECTS OF ETHACRYNIC ACID ON OUABAIN-INSENSITIVE Na EFFLUX

| Experiment No. | Aerobiosis | | | Anaerobiosis | | |
|---|---|---|---|---|---|---|
| | Control | Ouabain | Etha + ouabain | Control | Ouabain | Etha + ouabain |
| | *Na μM/g RBC* per hr | | | *Na μM/g RBC* per hr | | |
| 1 | 1.31 | 0.24 | 0.10 | 1.96 | 0.88 | 0.33 |
| 2 | 1.74 | 0.50 | 0.31 | 1.96 | 0.90 | 0.49 |
| 3 | 1.99 | 0.63 | 0.21 | 2.90 | 1.22 | 0.52 |
| 4 | 2.71 | 0.49 | 0.21 | 2.96 | 0.70 | 0.41 |
| 5 | 2.95 | 1.52 | 0.79 | 3.70 | 1.94 | 0.96 |
| 6 | 1.88 | 0.16 | 0.03 | 2.75 | 1.10 | 0.41 |
| Mean | 2.10 | 0.59 | 0.28 | 2.71 | 1.12 | 0.52 |

Ethacrynic acid was used in a final concentration of $5 \times 10^{-4}$ M. The column labeled Etha + ouabain represents the residual Na efflux persisting in the presence of both substances.

aerobiosis and with both gas phases ouabain produced a marked inhibition in Na efflux. The addition of ethacrynic acid together with ouabain resulted in further inhibition of Na efflux both aerobically and anaerobically. The mean decrease in the ouabain-insensitive sodium efflux induced by ethacrynic acid was 0.31 μM/g RBC per hr aerobically and 0.60 μM/g RBC per hr anaerobi-

cally. However, the values for Na efflux which persisted in the presence of both ouabain and ethacrynic acid were greater under anaerobic conditions than under aerobic conditions (0.52 $\mu$M/g RBC per hr in $N_2$ vs. 0.28 $\mu$M/g RBC per hr in air).

TABLE VII

Na INFLUX UNDER AEROBIC AND ANAEROBIC CONDITIONS

| Experiment No. | Aerobiosis | | Anaerobiosis | |
|---|---|---|---|---|
| | Control | Ouabain | Control | Ouabain |
| | *Na $\mu$M/g/RBC per hr* | | *Na $\mu$M/g/RBC per hr* | |
| 1 | 1.32 | 1.47 | 1.81 | 2.14 |
| 2 | 1.78 | 2.28 | 2.03 | 2.15 |
| 3 | 1.54 | 1.60 | 2.06 | 2.40 |
| 4 | 1.93 | 2.47 | 2.80 | 3.48 |
| 5 | 1.56 | 1.57 | 2.12 | 1.99 |
| 6 | 1.17 | 1.20 | 1.35 | 1.59 |
| Mean | 1.55 | 1.77 | 2.03 | 2.29 |

The differences in Na influx values between aerobiosis and anaerobiosis are statistically significant ($p < 0.01$).

TABLE VIII

LACTATE FORMATION AND ATP CONCENTRATION IN TURTLE RED BLOOD CELLS UNDER AEROBIC AND ANAEROBIC CONDITIONS

| Experiment No. | Lactate | | ATP | |
|---|---|---|---|---|
| | Aerobiosis | Anaerobiosis | Aerobiosis | Anaerobiosis |
| | *$\mu$ moles/kg cells per hr* | | *mmoles/kg cells* | |
| 1 | 590 | 970 | 1.43 | 1.39 |
| 2 | 480 | 870 | 1.08 | 1.02 |
| 3 | 760 | 1040 | 0.89 | 0.84 |
| 4 | 820 | 1240 | 0.74 | 0.76 |
| 5 | 640 | 960 | 1.02 | 0.94 |
| 6 | 750 | 1030 | — | — |
| Mean | 673 | 1018 | 1.03 | 0.99 |

The difference between the mean values for aerobic and anaerobic lactate formation is statistically significant ($p < 0.01$). The mean values for ATP concentration are not significantly different.

*Sodium Influx*

Table VII summarizes the results of Na influx studies. The values with $N_2$ as the gas phase exceeded those observed under aerobic conditions consistently. Ouabain did not decrease Na influx values. Indeed, values tended to be somewhat higher in the presence of ouabain. Thus, the differences in influx

values observed in anaerobic vs. aerobic experiments were not abolished by ouabain; the anaerobic values averaged approximatley 0.5 μM/g RBC per hr, more than the aerobic values both in the presence and absence of ouabain.

*Lactate Formation and ATP Concentrations*

In Table VIII values are shown for aerobic and anaerobic rates of glycolysis for turtle red blood cells. The mean value for lactate production under aerobic conditions was 673 μmoles/kg RBC per hr. Lactate formation increased markedly in the anaerobic flasks. The mean value was 1018 μmoles/kg per hr. The increase in lactate production under anaerobic conditions varied from 36.8% to 81.3%.

Values for ATP levels are also shown in Table VIII. The mean value for cells incubated under aerobic conditions was 1.03 μmoles/kg and the value for the cells incubated in nitrogen averaged 0.99 μmole/kg RBC. The two values are not significantly different from each other.

## DISCUSSION

The freshwater turtle is a reptile. Its erythrocytes, though nucleated and presumably capable of respiring (4), are similar to those of many mammals (e.g. man, rabbit, horse) in that they contain a high concentration of potassium and a low concentration of sodium although they normally exist in plasma with low K and high Na concentrations.

In order to maintain these characteristic intracellular Na and K concentrations, Na must be extruded and K taken up against chemical and presumably, at least for Na, electrochemical gradients. Available information favors the view that active transport of potassium inward and active transport of Na outward are responsible for this distribution of cations. It is generally believed that this active component of Na and K transport in red cells is linked and characteristically inhibited by cardiac glycosides (ouabain).

In addition to the movement of Na and K by an active transport or pump pathway, two other major possible pathways for cation transport across the red blood cell membrane have been considered. One of these is the "leak" which represents passive diffusion of the ion under consideration in the direction of its electrochemical gradient. This passive diffusion or leak will be directed from the bathing media to cell water for Na ions and from inside out for K ions. The other pathway is exchange diffusion which represents a special type of diffusion, not thought to require energy, that can be seen only with the use of radioactive tracers (8, 9). Exchange diffusion does not modify the total concentrations of ions in the cell since it represents the exchange of unlabeled ions for labeled ions of the same species. It has been suggested recently that in human red cells the phenomenon of exchange diffusion does not exist. Instead, a second active Na transport mechanism insensitive to ouabain

and dependent upon the presence of external Na has been postulated (10). This portion of the Na flux is inhibited by ethacrynic acid.

The data presented in this study are consistent with active Na efflux in the erythrocytes of the freshwater turtle. The mean value for sodium efflux in air was approximately 2.0 μM/g RBC per hr, a figure somewhat lower than that observed in human erythrocytes. Ouabain reduced sodium efflux by approximately 70% and the ouabain-insensitive component averaged 0.61 μM/g RBC per hr. Ouabain reduced anaerobic sodium efflux by approximately 55%. Thus the ouabain-sensitive component of Na efflux was closely comparable in air and $N_2$; but the ouabain-insensitive component was almost twice as great in $N_2$ (i.e. 1.19 μM/g RBC per hr). The pump rate for Na efflux therefore was closely comparable in air vs. nitrogen while the greater value for total Na efflux in the nitrogen atmosphere was due to a substantially higher ouabain-insensitive component. It is fair, therefore, to assume that the increase in Na efflux observed during anaerobiosis could be due to (*a*) an increased Na leak, (*b*) increased Na exchange diffusion, or (*c*) activation under anaerobic conditions of an ethacrynic acid–inhibitable flux.

In the experiments with choline chloride Ringer (Table V) the pump rate for Na was 75% of the rate observed with Na present in the external media; but the ouabain-insensitive component was only 56% in air and 30% in nitrogen of the values observed in sodium Ringer. Thus the ouabain-insensitive flux rate in the absence of external sodium was approximately 0.3 μM/g RBC per hr in both air and nitrogen as compared to values of 0.61 μM and 1.19 μM/g RBC per hr in air and nitrogen, respectively when Na was present in the external medium. In the absence of external Na oxygen deprivation did not lead to an increase in either total or ouabain-insensitive Na efflux. These data do not support the primacy of an increased Na leak under anaerobic conditions. Ethacrynic acid inhibited a portion of the ouabain-insensitive sodium efflux both aerobically and anaerobically. The inhibition was somewhat greater under anaerobic conditions than under aerobic conditions (0.60 μM/g RBC per hr vs. 0.31 μM/g RBC per hr). However, even in the presence of both ouabain and ethacrynic acid the values for Na efflux were greater anaerobically than aerobically (see Table VI).

Anaerobiosis produced approximately a 50% increase in Na influx (Table VII). Ouabain did not modify this difference but Na influx tended to be somewhat higher both aerobically and anaerobically in the presence of ouabain. Sodium influx rates were somewhat lower than efflux rates; however, the difference between these two Na flux values was not marked.

It is of interest that potassium influx values were closely comparable in air and nitrogen. Ouabain produced a marked but equal (approximately 70%) decrease in both aerobic and anaerobic potassium influx rates (Table IV). Thus ouabain-insensitive potassium influx was equal in air and nitrogen

averaging approximately 30% of total K influx. Consequently, the observed increase in Na efflux anaerobically is not coupled to potassium. The data therefore suggest that a pathway other than a closely coupled Na-K pump is responsible for the increase in sodium efflux observed in the anaerobic experiments.

The cumulative data support the existence of an activation of exchange diffusion for Na during anaerobiosis. The evidence is as follows: (*a*) there was a greater ouabain-insensitive component of Na efflux during anaerobiosis with no change in pump (ouabain-sensitive) efflux; (*b*) there was no change in ouabain-insensitive K influx during anaerobiosis; (*c*) there was no increase in ouabain-insensitive Na efflux during anaerobiosis in the absence of external sodium; (*d*) there was an increase in Na influx during anaerobiosis; (*e*) there was no measurable change in chemical Na concentrations in cell water in air vs. nitrogen; and (*f*) there was a greater ouabain–ethacrynic acid–insensitive sodium efflux anaerobically.

The present data, however, do not rule out the activation under anaerobiosis of a pump mechanism insensitive to ouabain. However, if a ouabain-insensitive, ethacrynic acid–inhibitable flux did contribute to the increase in total Na efflux rates observed in a deoxygenated system, it cannot account for the total increase. Thus in the presence of ouabain plus ethacrynic acid Na efflux rates were 86% greater in $N_2$ than in $O_2$. We believe therefore that these data point to the existence of exchange diffusion in the red blood cells of the freshwater turtle.

Increased potassium fluxes during anaerobiosis have been described previously by Tosteson and Robertson in duck red blood cells (11). They found that when duck red blood cells are incubated at 37°C in a medium containing 140 mM Na and 5 mM K per liter with glucose as the substrate and 5% $CO_2$ in $O_2$ as the gas phase, the approach to isotopic equilibrium indicated at least two intracellular K phases. When the gas phase consisted of 5% $CO_2$ in nitrogen all cellular K behaved as one phase. They also found that the steady-state exchange of K was higher in nitrogen than in oxygen. When the energetics of the system were considered, it was suggested that exchange diffusion was responsible for a considerable portion of the K flux (11, 12). These studies have been repeated and extended by McManus (13) and Allen and McManus (14) who sugeested that the increase in K influx and Na efflux observed in duck red cells during anaerobiosis was related to stimulation of active transport of both ions. This stimulation of active transport anaerobically was attributed to an increase in intracellular Na leading to stimulation of the Na pump and a subsequent increase in K influx due to coupling between the ion pumps.

It has recently been suggested by Benesch et al. (15, 16) that 2,3-diphosphoglycerate in the case of mammalian erythrocytes and inositol hexaphosphate

in the case of avian erythrocytes are bound preferentially to the deoxygenated form of hemoglobin. As a result these organic phosphate esters would be removed to a certain extent by hemoglobin under anaerobic conditions. This binding of phosphate esters by deoxygenated hemoglobin may affect cation permeability. It should be pointed out that two recent observations in duck red blood cells lend some support to this hypothesis: (*a*) respiratory inhibitors do not reproduce the effects of anaerobiosis on ion transport (14) (under these conditions, hemoglobin would still be in the oxygenated state and would therefore not modify the levels of phosphate esters); (*b*) no increase in ion transport is observed when CO (100%) is used as the gas phase (14). Presumably carbon monoxyhemoglobin, like oxyhemoglobin, will not bind free organic phosphate under physiological conditions. One feature of turtle red cells, which is shared by avian red cells, is the presence of phytic acid (inositol hexaphosphate) in high concentrations. The fact that both bird and turtle erythrocytes possess this compound and the finding of increased ion movements anaerobically in the red blood cells of both birds and reptiles are intriguing.

According to Maizels (4), cation transport in the erythrocytes of the African tortoise, snapping turtle, and in various teleosts and elasmobranches is apparently dependent upon respiration. The present data indicate, that at least in the erythrocytes of the freshwater turtle, ion transport persists in the absence of molecular oxygen. Furthermore, the rate of both sodium and potassium transport is not decreased in anaerobiosis, indicating that energy derived from glycolysis is sufficient to maintain an adequate ion composition in these red cells. It is of note in this respect that in our studies glycolysis increased markedly during anaerobiosis and the levels of ATP were comparable in cells incubated for 2 hr anaerobically to the levels measured simultaneously in cells incubated aerobically for the same length of time.

We sincerely thank Dr. Neal S. Bricker for his critical review of this manuscript.
Part of this work was done during the tenure by Dr. Klahr of an Established Investigatorship of the American Heart Association.
This work was supported by United States Public Health Service Program Project Grant AM-09976, United States Public Health Service Training Grant T1-AM 05248, and the Office of Saline Water, Department of the Interior, Grant No. 14-01-001-686.

*Received for publication 29 January 1969.*

## BIBLIOGRAPHY

1. KLAHR, S., and N. S. BRICKER. 1964. Na transport by isolated turtle bladder during anaerobiosis and exposure to KCN. *Amer. J. Physiol.* **206**:1333.
2. KLAHR, S., and N. S. BRICKER. 1965. Energetics of anaerobic sodium transport by the fresh water turtle bladder. *J. Gen. Physiol.* **48**:571.
3. BRICKER, N. S., and S. KLAHR. 1966. Effects of dinitrophenol and oligomycin on the coupling between anaerobic metabolism and anaerobic sodium transport by the isolated turtle bladder. *J. Gen. Physiol.* **49**:483.

4. MAIZELS, M. 1956. Sodium transfer in tortoise erythrocytes. *J. Physiol. (London).* **132**:414.
5. UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER. 1959. Manometric Techniques. Burgess Publishing Co., Minneapolis. P. 69.
6. SACHS, J. R., and L. G. WELT. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46**:65.
7. BRICKER, N. S., L. GUERRA, S. KLAHR, W. BEAUMAN, and C. MARCHENA. 1968. Sodium transport and metabolism by erythrocytes of the dogfish shark. *Amer. J. Physiol.* **215**:383.
8. WHITTAM, R. 1964. Transport and Diffusion in Red Blood Cells. The Williams and Wilkins Co., Baltimore.
9. USSING, H. H. 1949. Transport of ions across cellular membranes. *Physiol. Rev.* **29**:127.
10. HOFFMAN, J. F., and F. M. KREGENOW. 1966. The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N.Y. Acad. Sci.* **137**:566.
11. TOSTESON, D. C., and J. S. ROBERTSON. Potassium transport in duck red cells. 1956. *J. Cell. Comp. Physiol.* **47**:147.
12. TOSTESON, D. C. 1955. Sodium and potassium transport in red blood cells. Symposium on Electrolytes in Biological Systems. American Physiological Society, Washington, D. C. P. 123.
13. MCMANUS, T. J. 1967. Comparative biology of red cells. *Fed. Proc.* **26**:1821.
14. ALLEN, D. W., and T. J. MCMANUS. 1968. A non-metabolic role for oxygen in the control of passive cation permeability in the duck red cell. *Biophys. J.* **8**:A125.
15. BENESCH, R., and R. E. BENESCH. 1968. Oxygenation and ion transport in red cells. *Science. (Washington).* **160**:83.
16. BENESCH, R., R. E. BENESCH, and C. I. YU. 1968. Reciprocal binding of oxygen and diphosphoglycerate by human hemoglobin. *Proc. Nat. Acad. Sci. U.S.A.* **59**:526.