The Response of Duck Erythrocytes to Nonhemolytic Hypotonic Media

Evidence for a volume-controlling mechanism

FLOYD M. KREGENOW

From the Laboratory of Kidney and Electrolyte Metabolism, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT Duck erythrocytes were incubated in hypotonic media at tonicities which do not produce hemolysis. The cells' response can be divided into two phases: an initial rapid phase of osmotic swelling and a second, more prolonged phase (volume regulatory phase) in which the cells shrink until they approach their initial isotonic volume. Shrinkage associated with the volume regulatory phase is the consequence of a nearly isosmotic loss of KCl and water from the cell. The potassium loss results from a transient increase in K efflux. There is also a small reduction in Na permeability. Changes in cell size during the volume regulatory phase are not altered by 10^-4 M ouabain although this concentration of ouabain does change the cellular cation content. The over-all response of duck erythrocytes is considered as an example of "isosmotic intracellular regulation," a term used to describe a form of volume regulation common to euryhaline invertebrates which is achieved by adjusting the number of effective intracellular osmotic particles. The volume regulatory phase is discussed as the product of a membrane mechanism which is sensitive to some parameter associated with cell volume and is capable of regulating the loss of potassium from the cell. This mechanism is able to regulate cell size when the Na-K exchange, ouabain-inhibitable pump mechanism is blocked.

INTRODUCTION

The results of perturbing erythrocytes by changing the organic and inorganic composition of an isotonic bathing medium have led to the concept that cell volume and the concentration gradients for Na and K between the cell and its surroundings are in large part regulated by a pump mechanism working against diffusion leaks. The pump is responsive to the concentration of extracellular K and intracellular Na and inhibited by ouabain. The rate coefficients for the Na and K leads are relatively insensitive to the cation composition of the external medium and uninfluenced by ouabain (for reviews see Tosteson, 1955 and Whittam, 1964). On the other hand, the results of
manipulating red cells in an anisotonic medium, more specifically in hypotonic solutions which do not produce hemolysis (nonhemolytic), have been less useful in clarifying the membrane events associated with monovalent cation transport. Davson (1937) has studied the "prelytic" loss of potassium in hypotonic media from the erythrocytes of several species. The cat erythrocyte, a low K cell, develops an increase in potassium permeability and a decrease in Na permeability as the tonicity of the bathing solution is lowered (Davson, 1940). Harris and Prankerd (1953) have shown that the rate constant for Na outflux in the human erythrocyte, a high K cell, diminishes in hypotonic solutions.

This paper is concerned with the response of duck erythrocytes to non-hemolytic hypotonic bathing media. The response of these cells to hypertonic solutions is reported in a subsequent paper. Duck red cells are nucleated elliptical erythrocytes which also gain Na and lose K in the presence of ouabain (Allen, 1967). Their cellular chloride content appears to be distributed passively as in the human erythrocyte (Tosteson and Robertson, 1956; Allen, 1967).

In hypotonic media, duck erythrocytes, after an initial phase of swelling, revert toward their original volume by virtue of a nearly isosmotic loss of cellular KCl and H2O. The K loss is a consequence of a temporary increase in K efflux. This example of volume regulation appears to require a membrane mechanism with receptor, transmitter, and effector properties which produces a temporary increase in the rate coefficient for the potassium leak. In contrast, the rate coefficient for the Na leak is either unchanged or decreased.

METHODS

General

The procedures for obtaining red blood cells from the common Muscovy duck, the preparation of experimental solutions before their use as either wash or incubation media, and the method for incubating and separating cells have been described previously (Riddick et al., 1971). The NaCl content of the standard synthetic solution (323 mosmols) was decreased to produce three hypotonic solutions, 280 mosmols, 234 mosmols, and 188 mosmols. These will be referred to as mildly, moderately, and maximally hypotonic solutions, respectively. High potassium media were prepared by isosmotic replacement of some of the sodium salts with potassium salts. Freshly drawn duck erythrocytes lose approximately 8 mM K and 2% of their cell water (w/w) during the first 90 min of incubation in the standard synthetic medium before stabilizing at a new lower steady-state level. Upon continued incubation, the cells remain in this lower steady state for at least an additional 90 min (Riddick et al., 1971). Only lower steady-state cells ([K]e \sim 110 \text{ mM/L}, [\text{Na}]e \sim 5 \text{ mM/L}, and [\text{Cl}]e \sim 51 \text{ mM/L}) \text{1 were employed in this study. They were obtained by preincubating}

\text{1 mM/L} = \text{mM/liter of red blood cells.}
freshly drawn cells for 90 min. At the end of the preincubation period, the cells were separated by centrifugation and added immediately to the experimental flasks (hematocrit [Hct.] either 1% or 5%). Hemolysis was always less than 0.2% (the resolution of the technique employed). The superantant and packed cells were analyzed after they had returned to room temperature. The pH of the supernatant fraction at the time of removal was 7.70 ± 0.02. In those samples analyzed at zero time for chloride, the pH values of the supernatant fraction were within 0.02 units of each other. A pH change of this magnitude will maximally produce a change in chloride content (Allen, 1967) of 0.8 mmole in those samples analyzed at zero time and 1.6 mmole in those samples analyzed serially in each of the hypotonic solutions. These possible errors are too small to effect significantly any of the net chloride changes reported in Table I or any of the conclusions derived from these observations. On the other hand, the chloride ratio, especially in the most hypotonic solution, will be significantly affected. Here the numerator of the fraction \( \frac{[\text{Cl}]_o}{[\text{Cl}]_i} \) is very small and an error of this magnitude will produce a large proportional change in the equally small denominator. For this reason the chloride ratios for the most hypotonic solution (188 mosmols) have been omitted from Table I. Possible errors are also present in the chloride ratios of both the mildly and moderately hypotonic solutions because of these possible pH differences. It is maximally 0.03 units in both solutions at zero time and 0.06 and 0.10 units, respectively, for any of the values analyzed serially in either the mildly or moderately hypotonic solutions. Errors of this magnitude, however, do not obscure the major point illustrated by these data (Table I), i.e. the chloride ratio falls initially in hypotonic media and then rises as the cells readjust their volume. A value of 1.3 ± 0.1%, using inulin-\(^{14}\text{C}\), was found to represent the percentage of medium trapped between the cells during centrifugation in the mildly and moderately hypotonic medium. In the maximally hypotonic medium the scatter in these values resulted in a much larger standard error of the mean, 1.3 ± 0.4. The reliability of the subtraction procedure used in the determination of Na influx and content of cells, which depends heavily on the constancy of trapped medium measurement, is, therefore, suspect in the maximally hypotonic medium. Accordingly, only the Na influx values and changes in electrolyte and water content of cells in mildly and moderately hypotonic bathing solutions are included in the tables and figures.

Analytical Techniques and Calculations

\(^{40}\text{K}\) and \(^{24}\text{Na}\) were obtained as the chloride from the International Chemical & Nuclear Corporation.\(^5\) Both isotopes were measured in a well-type automatic scintillation counter. Hemoglobin concentrations were estimated by measuring the optical density at 540 m\(\mu\) with a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The measurement of Na and K concentration, trapped medium, per cent cell \(\text{H}_2\text{O}\) (w/w), pH, osmolality, and the method for calculating concentrations per that number of cells which were initially present in 1 liter of lower steady-state cells (millimoles/\(L_{\text{oms}}\)) and per liter of cell \(\text{H}_2\text{O}\) (millimoles per liter cell \(\text{H}_2\text{O}\)) have been described previously (Riddick et al., 1971).

\(^5\) International Chemical & Nuclear Corporation, Irvine, Calif.
FLOYD M. KREGENOW  *Response of Duck Erythrocytes to Hypotonic Media* 375

**Influx Experiments**

Tracer quantities of $^{24}\text{Na}$ and $^{42}\text{K}$ were introduced to the experimental flasks before adding the cells (Hct. 5%). A zero time sample was obtained 1 min after exposure of the cells to tracer. Additional samples were removed at appropriate intervals thereafter. The samples were centrifuged to separate the cells and bathing medium. The cells were analyzed for Na, K, radioactivity, and cell water (w/w) and the bathing medium for Na, K, and radioactivity. A 250 µliter sample of the cell suspension was obtained at the end of the experiment and added to a 50 ml volumetric flask. This sample was used to determine the cation content and radioactivity of the whole suspension. The radioactivity of the cells, bathing medium, and cell suspension was obtained by counting a diluted 5 cc sample of each. The Na and K concentration of the packed cells were corrected for medium trapped between the cells during centrifugation using the previously determined value of 1.3%. Radioactivity measurements of the cells were corrected by subtracting the value of the zero time sample from the value at subsequent times. In all instances the counts in the zero time sample were in excess of that expected from a fractional volume distribution of 1.3% and may include part of a small, rapidly exchanging compartment similar to that described in the human red cell (Maizels and Remington, 1959).

**Outflux Experiments**

Cells were loaded with $^{24}\text{Na}$ during the last 30 min of the preincubation period or with $^{42}\text{K}$ during the entire preincubation period. Isotope was therefore incorporated while the cells were approaching the lower steady state. After the preincubation period, they were washed twice with 30 vol of ice-cold nonradioactive standard synthetic medium. After the second wash a sample of cells was added to 20 cc of experimental medium (Hct. 5%) and immediately reisolated by centrifugation. These cells were analyzed for Na and K, specific gravity, and cell H$_2$O and the values were used to calculate the intracellular cation concentration at zero time. The rest of the washed radioactive cells were added to the experimental flasks (Hct. 1%) and a zero time sample was obtained by pouring and centrifuging a sample of the cell suspension. The time between first introducing the cells and the initial centrifugation was approximately 1 min. Additional fractions were centrifuged at timed intervals thereafter. A 5 cc sample of the supernatant was analyzed for radioactivity. At the conclusion of the experiment, a portion of the cell suspension was pipetted for measurement of the total radioactivity of the suspension. This sample was processed in the same manner as a hemolyzed sample of packed cells, after hemolyzing the cells by adding distilled water and a drop each of Cutscum® and NH$_4$OH. A 5 cc sample of the supernatant was then removed for counting. The radioactivity in the zero time sample was subtracted from that of subsequent samples and the radioactivity of the total suspension before calculating the per cent $^{24}\text{Na}$ or $^{42}\text{K}$ released by the cells with time.

**Calculations from the Data**

A measurement of the inward movement of K or Na was obtained by determining

1 Fisher Scientific Company, Fair Lawn, N. J.
the amount of tracer entering the cells during a timed interval \((t - t_o)\). An influx
was obtained from this value by dividing by the mean specific activity of K or Na in
the medium during the same period. The influx measurements were corrected for
volume changes, present initially and during the experiment, by multiplying the
amount of tracer which entered the cell in the timed interval by an appropriate cor-
rection factor. This factor was similar to the one employed in the calculation of cation
concentration per original number of cells.

\[ iM = \frac{R_e}{X_m} \]

where \(iM\) = unidirectional influx (millimoles of Na or K that enter the same number
of cells present in 1 liter of lower steady-state cells [control] by time \(t\)). \(R_e\) = corrected
radioactivity of cells (counts per minute of \(^{24}\)Na or \(^{40}\)K that have entered the number
of cells equivalent to the number present in 1 liter of control cells by time \(t\)); \(X_m\) = mean specific activity of medium (counts per minute per millimole of Na or K during
the interval \(t - t_o\)).

Equation 1 applies to a two-compartment steady-state system in which the rate of
cellular isotope uptake is constant. The equation is most applicable when \(X_e\), the
specific activity of cells (counts per minute per millimole of cellular Na or K), is a
small fraction of \(X_m\). Since most of the experiments reported in this paper were
performed on cells not in the steady state and under conditions where the rate of
cellular isotope uptake was not constant, the procedure for performing influx measure-
ments was modified to minimize the errors introduced by using equation 1. This was
accomplished by performing the influx measurements over a sufficiently short time
interval so that for all practical purposes the cells were in the steady state and the
rate of cellular isotope uptake was constant. In practice then, the isotope was intro-
duced just before a time interval in which volume changes were relatively small, the
intracellular cation concentration remained nearly constant, the rate of isotope up-
take was nearly linear, and \(X_m\), the specific activity of the medium, was large and
invariant. All Na influx values were calculated by using equation 1.

The \(^{40}\)K specific activity of duck erythrocytes at isotopic equilibrium, \(X_{eq}\), is
equivalent to the \(^{40}\)K specific activity of the hemolyzed cell suspension (Tosteson
and Robertson, 1956). If, during the experimental period, a semilogarithmic plot of
cell \(^{40}\)K relative specific activity \((1 - X_e/X_{eq})\) as a function of time yielded a straight
line, then the K influx was calculated by using the unsteady-state method, equation
2, first described by Tosteson and Robertson (1956) and derived from the two-com-
partment non–steady-state model of isotope exchange kinetics developed originally
by Sheppard and Martin (1950):

\[ iM_K = \frac{K_e \cdot dx_e}{dt} \cdot \frac{1}{X_m - X_e}. \]

This equation gives the apparent unidirectional potassium influx for the original
number of cells at the point in time used for the computation of \(dx_e/dt\), which is
obtained graphically. Cell cation and radioisotope concentrations, used in the equation, were obtained at the same point in time and are expressed as millimoles or counts per original number of cells. The method is most accurate when $X_o$ is a small fraction of $X_m$ and transfer of isotope from cells to medium is small. Under these conditions, if $K_e$ changes only slightly and the rate at which $X_e$ varies with time remains constant, then equation 2 can be simplified and will yield equation 1.

The human red blood cell, with its biconcave shape, can vary in volume over a wide range without an increase in surface area. However, the surface area of the duck erythrocyte, an elliptical cell, may increase as the cell swells. The cells remain elliptical after placement in all three hypotonic media. The cells remain elliptical after placement in all three hypotonic media. The major gross anatomical change, most apparent when the cells are initially introduced to the maximally hypotonic medium, is an increase in the convexity of the cell's profile. The cell profile assumes its normal appearance as the cell readjusts its volume. An increase in the membrane surface area would decrease the calculated flux value. Since the influx has been calculated for the surface area associated with the number of cells in 1 liter of control cells, the same number of swollen cells, with a larger surface area, would have a smaller flux per unit area of membrane. No attempt has been made to adjust for this possible error. The following reasoning is used as justification. Even in the extreme case, when cell volume is increased 30%, there would be only approximately a 20% increase in the surface area of the duck erythrocyte. This would result in a decrease of slightly less than 20% in the calculated value for K and Na influx. An error of this size in K influx would not significantly affect the more pertinent change in the K efflux calculation which is several orders of magnitude greater. Using the same reasoning, the experimental decrease in Na influx, found when cells are incubated in hypotonic media, would be even more significant.

Potassium efflux was calculated by using the expression:

$$oM_k = iM_k - \Delta K_e,$$

where $oM_k$ and $iM_k$ are unidirectional potassium outflux and influx, respectively, by time $t$ (millimoles K/L on); $\Delta K_e$ = net change in cellular potassium between time $t$ and $t_e$ (millimoles K/L on).

An estimate of unidirectional Na or K loss was obtained by following the loss of $^{24}$Na and $^{42}$K from prelabeled cells and plotting ln(1-per cent $^{42}$K or $^{24}$Na released) as a function of time. The slope of this line gives the transfer coefficient at any time for the prelabeled cellular potassium compartment when the loss of tracer is into an infinite bath. This assumes that a single cellular compartment has been prelabeled.

In Fig. 1, the theoretical cell volume was obtained by multiplying the isotonic per cent cell water (v/v) by the ratio $\pi / \pi$ and adding this value to the isotonic volume of the cell solids. This calculation assumes that all of the measured cell water is able to participate in the osmotic adjustment and that the volume of cell solids remains unchanged as the cells are placed in hypotonic media. In the same figure, the experimental cell volume was obtained by multiplying the isotonic cell volume (100%) by the same factors used to calculate concentrations in terms of original number of cells.
RESULTS

When duck erythrocytes are placed in solutions of decreasing osmolality, they behave as osmometers, swelling more the lower the tonicity of the bathing medium. As with other erythrocytes, the increase in cell volume at any tonicity is less than one would expect if the cells behaved as "ideal osmometers" (Lucké and McCutcheon, 1932; Fig. 1). The experimental increase in cell volume is approximately 2/3 of the calculated theoretical increase in all three of the experimental hypotonic solutions. It should be pointed out that hemolysis did not occur in the experiments reported in this paper. Hemolysis in duck erythrocytes begins when the tonicity of the medium is reduced so that \( \pi_0/\pi \) is equal to 1.8 (Lewis and Ferguson, 1966). The tonicity of the most dilute solution used in these studies yields a value of 1.7 when expressed in this manner.

Fig. 2 demonstrates that duck red cells, initially swollen in hypotonic media, subsequently lose water and revert toward the volume existent in isotonic media. The readjustment is not perfect; the mean 90 min per cent...
cell water (w/w) value falls between the upper steady-state and the lower steady-state values (control in Fig. 2) with sufficient scatter in the actual values to negate an analysis of which of these two values is approached, if either. The volume adjustment is more prolonged and more rapid initially the lower the osmolality of the bathing medium.

The over-all response of duck erythrocytes to nonhemolytic hypotonic media can therefore be divided into two phases: an initial rapid osmotic phase (Fig. 1), and a second more prolonged phase (Fig. 2), to be referred to as the "volume regulatory phase."

Table I demonstrates the net electrolyte changes associated with the movement of water during both phases in two typical experiments. The electrolyte alterations at zero time are associated with the gain in cell water indicated in Fig. 1 (osmotic phase). The net electrolyte changes associated with the water loss illustrated in Fig. 2 (volume regulatory phase) can be obtained by subtracting the zero time sample from the 30, 60, and 90 min values.

During the osmotic phase, there is a significant loss of cation only in the most hypotonic medium (188 mosmols) (4 ± 1.14 mMK/L ono). However there is an anion loss, chloride, in all three media. There is a growing consensus that all or part of the apparent inability of erythrocytes to behave as "ideal osmometers" stems from a failure to consider the changing osmotic coefficient and the gain and loss of all cell solutes as the osmolality of the bathing medium is changed. The change in the osmotic coefficient of hemoglobin as it is either concentrated or diluted is well known (Adair, 1929; McConaghey and Maizels, 1961). Recently Cook (1967) and Gary-Bobo and Solomon (1968) have presented evidence that Cl movement occurs under these conditions as well. The loss in cell chloride demonstrated here, if accompanied by a loss of cell H2O sufficient to maintain osmotic equilibrium, would explain only part (approximately 1/3) of the discrepancy between the initial experimental increase in cell volume and that of an "ideal osmometer."

During the volume regulatory phase, potassium is the major cation and chloride the major anion lost from the cell. In the mildly and moderately hypotonic media, the measured loss of chloride, if accompanied by an additional expected passive loss of HCO3−, is sufficient to maintain electroneutrality within the cell. In the most hypotonic solution, the measured and expected anion loss is insufficient, so that it is necessary in this instance to evoke the loss of other negatively charged solutes, a change in the electrical potential across the membrane, or both. In the mildly and moderately hypotonic media, if the potassium loss is associated with an osmotically equivalent quantity of anions, and the water movement across the membrane accompanies the loss of osmotically active solutes so that osmotic equilibrium is main-

*Standard error (SE) of the mean, where the number of experiments (N) = 10.
| Exp. | Medium osmolality | \(\Delta K\) in mm/L-cell | Time (min) | \(\Delta Na\) in mm/L-cell | Time (min) | \(\Delta Cl\) in mm/L-cell | Time (min) | \([Cl]_i/[Cl]_o\) | Time (min) |
|------|------------------|--------------------------|-------------|--------------------------|-------------|--------------------------|-------------|-----------------|-------------|
|      |                  | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 |
| 1    | 280 mOsm         | -1.0 | -9.0 | -11 | -12 | 0 | -0.6 | -0.6 | -0.4 | -4 | -9 | -12 | -12 |
|      | 234 mOsm         | -3 | -21 | -21 | -22 | 0 | -0.4 | -1.2 | -0.8 | -8 | -24 | -25 | -25 |
|      | 188 mOsm         | -5 | -33 | -43 | -47 | -16 | -32 | -36 | -35 | 1.49 | 1.65 | 1.70 | 1.69 |
| 2    | 280 mOsm         | -1 | -9 | -11 | -12 | -0.3 | -0.8 | -0.8 | -0.7 | -4 | -11 | -12 | -13 |
|      | 234 mOsm         | -1 | -25 | -29 | -29 | -0.4 | -1.1 | -1.0 | -1.5 | -8 | -23 | -27 | -26 |
|      | 188 mOsm         | -5 | -28 | -42 | -49 | -13 | -31 | -34 | -34 | 1.58 | 1.77 | 1.79 | 1.78 |

The experimental protocol was identical to the one described in Fig. 2. Control cells incubated in standard synthetic medium remained in the steady state during the duration of the experiment. \(\Delta\) values were obtained by calculating the difference between the value for cells in the hypotonic medium and the control value at each time. The control steady-state chloride ratio \([Cl]_i/[Cl]_o\) for Exp. 1 was 1.66, and 1.67 for Exp. 2.
tained, then the potassium loss is sufficient to explain the water loss. Utilizing these assumptions the lost cell water is apparently isosmotic and has a calculated osmolality of 274 ± 35 mosmols when the medium osmolality is 280 mosmols and 249 ± 34 mosmols when the medium osmolality is 234 mosmols. However, in the most hypotonic solution the calculated osmolality (216 ± 16 mosmols) exceeds the osmolality of the medium (188 mosmols). The chloride ratio [Cl]o/[Cl]i decreases initially in hypotonic media and then increases as the cells readjust their volume, indicating a change in the membrane potential.

Changes in the K influx and efflux of duck erythrocytes during the volume regulatory phase were examined to determine their role in affecting the net potassium loss. The efflux was calculated by subtracting the net change at each time from the corresponding measured 4K influx. Table II shows that the net potassium loss is almost entirely the result of an increase in K efflux.

The transient nature of the increase in K efflux is more clearly demonstrated by using radioactive tracer (Fig. 3). 4K-loaded cells were incubated in an isotonic or hypotonic medium and the appearance of label in the medium followed with time. In all three hypotonic bathing media, the isotope loss from cells is initially more rapid than the loss from control cells; the rate of loss, however, appears to subside with time. The possibility that the subsidence in tracer loss does not represent a decrease in K permeability at the rate limiting barrier but rather a back-flux of tracer from the medium to the cells was made unlikely, at least in the mildly hypotonic medium, by the following experiment. Cell 4K loss was followed in two flasks, containing the mildly hypotonic medium. The flasks were identical except for the hematocrit, which was 0.3% in the one and 3% in the other. Significant backflux, if present, should have been greater in the latter. The rate of tracer loss in the two flasks, however, was identical; both decreased simultaneously after the first 10–20 min.

An elevated K efflux, large enough to explain the net potassium loss, is also obtained when the apparent rate constant, determined from Fig. 3, is utilized in the flux calculation. Flux values determined at 30, 60, and 90 min by using these rate constants and the initial intracellular potassium concentration (millimoles/Lone), are presented in Table III (column 1). The additional K efflux which develops when cells are incubated in a hypotonic bathing medium was determined by subtracting the K efflux of control cells in an isotonic medium from these values. These calculated flux increases (column 2, Table III) were sufficient to account for the net potassium loss (column 3, Table III) when one considers that on a comparative basis K influx remains essentially unchanged under these circumstances (see Table II). Identical efflux measurements by two different procedures seems

\[^{±}\] of the mean, \(N = 7\).
**TABLE II**

CHANGES IN K INFLUX AND EFLUX ASSOCIATED WITH THE VOLUME REGULATORY PHASE

| Medium tonicity (mosmols) | Influx in μM/L<sub>one</sub> (Time (min)) | Net change in μM/L<sub>one</sub> (Time (min)) | Calculated efflux in μM/L<sub>one</sub> (Time (min)) |
|---------------------------|------------------------------------------|-----------------------------------------------|--------------------------------------------------|
|                           | 30 | 60 | 90  | 30 | 60 | 90 | 30 | 60 | 90 |                      |
| 323 (control)             | 4.3| 9.0| 14.0| -1 | -2 | -2 | 5.3| 11.0| 16.0|                      |
| 280                       | 3.8| 7.2| 12.2| -8 | -11| -11| 11.8| 18.2| 23.2|                      |
| 234                       | 6.5| 10.5| 14.6| -20| -24| -26| 26.5| 34.5| 40.6|                      |
| 188                       | 8.0| 14.8| 19.4| -28| -38| -42| 36.0| 52.8| 61.4|                      |

Duck erythrocytes were incubated in either the standard isotonic or hypotonic bathing media. The influx of the control cells was calculated by using equation 2; all of the other influx values were calculated by using equation 1. The 30 min influx value in all three hypotonic media is the sum of two measurements; one between 0 and 15 min and the other between 15 and 30 min. Separate measurements were also performed to determine the 30-60 and 60-90 min influx values in all three hypotonic media. A tracer quantity of $^{40}$K was introduced into a tracer-free sample of cell suspension at the beginning of each measurement. The values from the measurements at the earlier time periods were added to the values from the later time periods to arrive at a cumulative influx value at 60 and 90 min. The experiment described here is representative of five others.

**Figure 3.** Transitory nature of the increase in $^{40}$K loss during the volume regulatory phase. Duck erythrocytes, preloaded with $^{40}$K, were incubated in either the standard isotonic or hypotonic media (Hct. 5%). The experiment presented here is typical of four others. The intracellular potassium concentrations were obtained for cells in each medium at the beginning of the experiment and were used in calculating the efflux values presented in column 1 of Table III. Net changes were obtained in the same experiment from simultaneous measurements of the K concentration, specific gravity, and per cent cell water (w/w) of cells. A flux value has not been presented for the 90 min sample for cells incubated in the maximally hypotonic medium because of the large amount of scatter in this measurement.
to exclude the other possible explanation for the nonlinearity of the curves in Fig. 3, i.e., tracer loss from more than one cellular compartment. Calculating an efflux by the first method is, in this instance, independent of the number of cellular compartments. The possibility does exist, however, that the two measurements are identical by coincidence.

The initial rapid rate at which $^{42}$K is released from duck erythrocytes in hypotonic media was examined more closely in Fig. 4. Cells in a mildly

\begin{table}[h]
\centering
\caption{Table III}
\begin{tabular}{l c c c c}
\hline
Medium tonicity & Time & Calculated efflux in mEq/L & Calculated increase in efflux above control values & Net change in mEq/L \\
mosmols & min & & & \\
188 & 30 & 23.7 & 17.9 & $-18.1$ \\
 & 60 & 40.9 & 30.5 & $-30.9$ \\
 & 90 & $-$ & $-$ & $-38.5$ \\
234 & 30 & 22.4 & 16.6 & $-16.7$ \\
 & 60 & 34.3 & 23.9 & $-21.2$ \\
 & 90 & 42.5 & 27.7 & $-24.6$ \\
280 & 30 & 12.3 & 6.5 & $-7.0$ \\
 & 60 & 19.3 & 8.9 & $-8.2$ \\
 & 90 & 23.3 & 8.5 & $-10.3$ \\
323 (control) & 30 & 5.8 & $-$ & 0 \\
 & 60 & 10.4 & $-$ & 0 \\
 & 90 & 14.8 & $-$ & 0 \\
\hline
\end{tabular}
\end{table}

For discussion of Table III, see legend for Fig. 3.

hypotonic solution (280 mosmols) release $^{42}$K at a rate which is between that of control cells and the accelerated loss of cells in a moderately hypotonic medium (234 mosmols). This response has at least two possible explanations. First, all of the erythrocytes in the cell population may lose K concurrently and equally when placed in a hypotonic medium. The rate of $^{42}$K loss from all of the cells would increase as the osmolality of the medium decreases to 234 mosmols when a further reduction in tonicity no longer alters the initial rapid rate of release. Second, the cell response to hypotonicity could be an "all or none" effect. The response of cells in slightly hypotonic medium would then be a composite, some cells in the population losing K maximally while others remain in the steady state.

Davson's (1937) findings that some erythrocytes (rabbit > ox > horse > guinea pig > pig > human) lose potassium when incubated at 40°C in hypotonic media enhances the significance of the latter possibility. The pre-
lytic K loss demonstrated by Davson also decreased with time and increased as the tonicity of the medium was lowered. He was impressed with the similarity of the sigmoid-shaped curve relating hypotonic hemolysis and tonicity and the curve relating K loss and tonicity and suggested "that there is a critical volume for each cell which it must obtain before its permeability constant to potassium attains significant dimensions." This critical volume was felt to be less than the hemolytic volume but otherwise analogous. The increase in potassium loss with each stepwise reduction in tonicity was interpreted as the consequence of more cells in the population exceeding the critical volume. However, the following experiment using the mildly hypotonic medium indicates that the first explanation presented is probably correct. The red cell population, preloaded with $^{42}$K, from a single duck was centrifugally separated into four equal fractions. To demonstrate that the population of duck erythrocytes can be separated by this procedure, the following experiment was performed. Ducks were injected intravenously with $^{59}$Fe citrate and a centrifuged column of cells was analyzed for radioactivity 2 days later. Most of the radioactivity is located in the top fraction of the centrifuged column as it is with human erythrocytes (Borun et al., 1957), indicating that the younger cells are located here. The time-course of $^{42}$K loss was nearly identical in all four fractions. None of the fractions demonstrated a K loss similar to the maximal response.

In four out of six experiments an apparent lag of 1–2 min in the onset of increased $^{42}$K loss was present in all three hypotonic bathing media. The experiment presented here illustrates this point.
Although the salient response of duck erythrocytes during the volume regulatory phase is a transient increase in K permeability, changes in Na permeability also occur. Table IV contains the Na influx values and corresponding rate constants for the first 5 min following incubation of cells in either hypotonic or isotonic media. Both the Na influx and corresponding rate constant are less when the cells are in hypotonic media. If the entry of Na into cells is predominantly a passive process, then, in a hypotonic medium, Na influx should be reduced by both the decrease in the chemical (removal of Na from the medium) and electrical (dilution of the total number of fixed negative charges) gradient for Na across the membrane. However, the experimental reductions, demonstrated in Table IV, do not establish that Na entry is passive, or, if this assumption is correct, that the reductions can be entirely explained by a diminution in the electrochemical gradient for sodium.

The outward permeability characteristics of the duck erythrocyte membrane to sodium are also altered during the volume regulatory phase. Duck red cells, preloaded with $^{24}$Na, were incubated in isotonic or hypotonic media and the appearance of the label in the bathing solution followed with time (Fig. 5). In hypotonic media, cells lose a smaller percentage of their $^{24}$Na with time, largely because of a decrease in an ouabain ($10^{-4}$ M)–insensitive component.

In paired experiments, the Na content of cells incubated for 30 min in

| Exp. | Medium osmolality | Na influx in mm/L·c for 5 min | Corresponding rate constant |
|------|------------------|-----------------------------|---------------------------|
| Na   | mosmols          |                             |                           |
| 1    | 323              | 0.61                        | 0.41                      |
| 2    | 280              | 0.40                        | 0.31                      |
|      | 234              | 0.33                        | 0.32                      |
| 2    | 323              | 0.60                        | 0.40                      |
|      | 280              | 0.44                        | 0.33                      |
|      | 234              | 0.29                        | 0.29                      |
| 3    | 323              | 0.54                        | 0.35                      |
|      | 280              | 0.49                        | 0.38                      |
|      | 234              | 0.23                        | 0.22                      |

Duck erythrocytes were incubated in either the standard isotonic or hypotonic ($\tau = 280$ and 234 mosmols) media. The rate constant in the moderately hypotonic medium and the Na influx in both the mildly and moderately hypotonic media were statistically different from the control paired isotonic values ($P < 0.01; N = 7$).
mildly or moderately hypotonic bathing medium was 0.30 ± 0.31 \textsuperscript{e} and 0.60 ± 0.61 \textsuperscript{e} mm/L (P for both < 0.05) less, respectively, than the sodium content of control cells incubated identically in an isotonic medium. Because of this marginal level of statistical significance, one can say only that if changes in Na content do occur in the volume regulatory phase they are minimal when compared to the changes in potassium content.

When the cells swell initially in hypotonic media, there is a concomitant fall in the cellular concentration (millimoles per liter cell H\textsubscript{2}O) of the total (Na + K) monovalent cations. From a value of 173 ± 1.5 \textsuperscript{e} for control cells in an isotonic medium, the value falls to 155 ± 2.1 \textsuperscript{e}, 134 ± 1.7 \textsuperscript{e}, and 108 ± 2.2 \textsuperscript{e}, respectively, for cells in the mildly, moderately, and maximally hypotonic media. Once established at this lower level, however, there is no further change in the mildly and moderately hypotonic media as the cells undergo the volume regulatory phase. Since the concentration value for cellular Na never varies by more than 2 mm/liter cell H\textsubscript{2}O and often less under these circumstances, the individual Na and K concentration values also remain relatively constant. In the maximally hypotonic medium, on the other hand, the total (Na + K) cellular concentration value does decrease further during the volume regulatory phase. In this instance, the calculated 90 min value is 5 ± 2.8 \textsuperscript{e} mm/liter cell H\textsubscript{2}O less than the initial value (P < 0.01).

\textsuperscript{e} se of the mean, N = 8.
Since the essential cellular change during the volume regulatory phase appears to be a modification in the cation content of cells, cells were incubated with the cardiac glycoside, ouabain, an agent known to inhibit the Na-K exchange pump mechanism in erythrocytes. $10^{-4}$ M ouabain does not affect the changes in cell volume as duck erythrocytes regulate their volume in moderately hypotonic medium (Fig. 6). The gain in sodium and loss of potassium, a change usually attributed to inhibition of the Na-K exchange pump mechanism, is, however, similar to that found when control cells (isotonic medium) are incubated with this agent (Table V). A larger concentration of ouabain ($10^{-3}$ M) produces an identical result. Therefore the changes in cell volume during the volume regulatory phase are not altered by inhibiting the Na-K exchange pump mechanism. A small amount of Na appears to replace an equivalent amount of K as the osmotically active intracellular cation as cells undergo the volume regulatory phase in the presence of ouabain.

In order to determine the influence that alterations in the extracellular medium, other than variations in osmolality, have on the volume regulatory phase, the experimental protocol was modified. A comparison was made of the time-course of K influx, efflux (as indicated by a plot of $\ln [1$-per cent $^{40}$K released] versus time), and net potassium loss in duck erythrocytes under two different circumstances. In one case, cells were incubated in the standard isotonic or mildly hypotonic (280 mosmols) medium to obtain control values. The control values were then compared to the response of cells incubated in media of identical osmolality to the control bathing solutions but otherwise modified depending upon the experimental factor under investigation. The removal of Ca and Mg from the bathing solution or the addition of $10^{-4}$ M propranolol does not influence the volume regulatory phase.

On the other hand, elevating the $[K]_e$ distorts the volume regulatory phase (Fig. 7). The volume of cells (as indicated by the per cent cell water [w/w]) incubated in mildly hypotonic media with an elevated $[K]_e$ is larger after 90 min than the volume of control cells incubated simultaneously in either the standard hypotonic medium ($[K]_e = 2.5$ mM) or isotonic medium with an equivalent increase in $[K]_e$. When the $[K]_e$ is 43 mM in the mildly hypotonic medium, the cells shrink less during the volume regulatory phase than when the $[K]_e$ is 2.5 mM; while at a $[K]_e$ of 75 mM they remain near their initial swollen volume. The net potassium changes are proportional to the cell water changes under these circumstances (Table VI). Cells incubated in the mildly hypotonic bathing solution with a $[K]_e$ of 43 mM lose less potassium than when the $[K]_e$ is 2.5 mM. When the $[K]_e$ is 75 mM very little potassium is lost. Table VI also shows that these differences in cellular potassium content develop in large part because of an increase in K influx. However, there is also a $[K]_e$-dependent alteration in the calculated efflux at least in
the 30 min value. At this time, the calculated efflux is greater when the [K]_o
is 43 or 75 mM than when it is 2.5 mM (hypotonic control). These
differences in K efflux are due in large part to the length of time the initial rapid rate of
K loss persists. In cells incubated in the high potassium hypotonic media the
rate of ^4K loss fails to decrease as much during the latter part of the 30 min
incubation period (Fig. 8). As a result the [K]_o-dependent change in K efflux

\[ \text{Mean difference} \pm \text{SE.} \]

is more apparent at 30 min than at 15 min. At 15 min the [K]_o-dependent
change in the calculated K efflux (+0.6 ± 1.4) is not significant (P > 0.05,
N = 5). However, the areas under the different curves in Fig. 8 between zero
time and 15 min suggest that a small portion of K efflux may increase as the
[K]_o is elevated. In any case, during this interval more than 2/3 of the
increase in K influx, as one raises the [K]_o, is not associated with a possible
corresponding [K]_o-dependent change in K efflux.

**Figure 6**

The effect of ouabain on the changes in cell volume during the volume
regulatory phase in the moderately hypotonic medium. Duck erythrocytes were incu-
bated in the isotonic or moderately hypotonic medium. Ouabain (10^-4 M) was added
to the appropriate flask just before adding the cells. The experiment was performed in
duplicate and is representative of a total of four experiments. The points represent the
average values from the duplicate experiments; the range at each point is ±0.1 or less.

**Figure 7**

Effect of increasing [K]_o on the volume regulatory phase of duck erythro-
cytes. Duck erythrocytes were incubated in either the standard or modified isotonic
(σ = 323 mosmols) or mildly hypotonic (σ = 280 mosmols) medium. The [K]_o was
increased by an isosmotic replacement of extracellular NaCl with KCl.
TABLE V
OUABAIN'S EFFECT ON THE Na AND K CONTENT OF DUCK ERythROCYTES DURING THE VOLUME REGULATORY PHASE IN THE MODERATELY HYPOTONIC MEDIUM

| Exp. | Medium osmolarity | Ouabain $10^{-4}$ M | $\Delta K_e$ in mm/Lcell 90 min | $\Delta Na_e$ in mm/Lcell 90 min | $\Delta$ Ouabain in mm/Lcell 90 min |
|------|-------------------|----------------------|-------------------------------|-------------------------------|----------------------------------|
| A    |                   |                      |                               |                               |                                  |
| 323  | -                 | +0.8                 | +0.3                          | (-6.2                        | +6.3                             |
| 323  | +                 | -6.8                 | +6.6                          |                               |                                  |
| 234  | -                 | -28.2                | +0.1                          |                               | (-6.1                        | +6.7                             |
| 234  | +                 | -34.3                | +6.8                          |                               |                                  |
| B    |                   |                      |                               |                               |                                  |
| 323  | -                 | -0.7                 | +0.3                          | (-5.1                        | +5.7                             |
| 323  | +                 | -5.8                 | +6.0                          |                               |                                  |
| 234  | -                 | -36.1                | -0.4                          |                               | (-4.1                        | +5.6                             |
| 234  | +                 | -40.2                | +5.2                          |                               |                                  |

The experimental procedure was identical to the one described in Fig. 6. The values for $\Delta K_e$ and $\Delta Na_e$ were obtained by subtracting the value at zero time from those at 90 min. The values for $\Delta$ Ouabain are the differences in the Na and K content of cells in each medium after a 90 min incubation with ouabain and are obtained from the columns labeled $\Delta K_e$ and $\Delta Na_e$.

TABLE VI
NET POTASSIUM CHANGES, POTASSIUM INFLUX, AND EFFLUX ASSOCIATED WITH THE RESPONSE OF DUCK ERythROCYTES TO AN INCREASE IN THE $[K]$ DURING THE VOLUME REGULATORY PHASE

| Exp. | $[K]_0$ (mM) | Medium osmolarity | K influx ($\mu$mol/100 g) 15 min | Net K change ($\mu$mol/100 g) 15 min | Calculated K efflux ($\mu$mol/100 g) 15 min |
|------|--------------|--------------------|-----------------------------------|--------------------------------------|-------------------------------------------|
| I    | 2.8          | 323                | 2.1                               | 0.0                                  | 2.1                                       |
|      |              | 280                | 2.0                               | -8.2                                 | -10.0                                     |
|      |              | 43.0               | 2.6                               | -0.2                                 | 0.0                                       |
|      |              | 280                | 8.3                               | -2.0                                 | -5.0                                      |
|      | 75.0         | 323                | 2.8                               | +1.1                                 | 2.8                                       |
|      |              | 280                | 11.8                              | +0.8                                 | -0.4                                      |
| II   | 2.9          | 323                | 1.9                               | 0.0                                  | 1.9                                       |
|      |              | 280                | 2.2                               | -8.7                                 | -11.8                                     |
|      |              | 43.0               | 2.5                               | +0.1                                 | +0.3                                      |
|      |              | 280                | 8.5                               | -2.9                                 | -5.1                                      |
|      | 75.0         | 323                | 2.8                               | +0.8                                 | +1.2                                      |
|      |              | 280                | 11.6                              | -0.2                                 | -1.4                                      |

Duck erythrocytes were incubated in either the standard or modified isotonic or mildly hypotonic medium. Equation 1 was used to calculate cell influx values. The 30 min influx value is the sum of two measurements, one between 0 and 15 min and the other between 15 and 30 min. A tracer quantity of $^{42}$K was introduced into a tracer-free sample of cell suspension at the beginning of each of these measurements.
When the [K]o is increased, the resultant alterations in potassium content and influx during the first 15 min are consistent with the view that an increase in the "leak" pathway occurs (see Discussion). If the primary event were an increase in the K "leak," elevating the [K]o on one side of the membrane (thereby increasing the driving force for K movement into the cell) should produce a large unilateral increase in K influx. The increased inward potassium movement would then compensate for the expected net loss of potassium so that the loss in K content would be less. As the [K]o is raised further,

![Figure 8](image)

**Figure 8.** The effect of increasing the [K]o of a hypotonic medium on 42K loss from duck erythrocytes during the volume regulatory phase. Duck erythrocytes, preloaded with 42K, were incubated in mildly hypotonic (280 mosmols) media with an elevated [K]o or in the standard isotonic (π = 323 mosmols) or mildly hypotonic (π = 290 mosmols) medium.

a point should be reached where intra- and extracellular potassium are in electrochemical equilibrium. At this [K]o, the rates of potassium movement in and out of the cell would both be increased but equivalent and the cells should remain swollen. Intracellular potassium appears to be at electrochemical equilibrium when the [K]o is 75 mM. However, for this line of reasoning to be applied precisely, K movement across the membrane must occur only through diffusional pathways. In this regard the measured K influx in Table VI does not only represent the passive entry of potassium. Besides the possibility that K exchange diffusion exists (see next paragraph), the 30 min K influx value of cells incubated in the hypotonic medium with a [K]o of 43 mM is inhibited slightly by $10^{-4}$ M ouabain or replacing the extracellular NaCl with an isosmotic quantity of MgCl₂ (15% and 25% inhibition, respectively) but not at all by $10^{-4}$ M propranolol.
After 15 min the persistent increase in K influx and efflux when the [K]₀ is elevated could be either a continuation of K movement through diffusional pathways or an exchange diffusion process, since now both K influx and efflux are significantly affected by changes in the [K]₀. If a large portion of K movement now represents an exchange diffusion process then a major alteration in the method for translocating K across the membrane develops during the response (from a process involving the net transfer of K to one involving no net change). The increase in influx and efflux persists beyond the usual 90 min incubation period when the [K]₀ is 75 mM. When the [K]₀ is 43 mM, the influx and efflux values remain elevated for almost 60 min. Besides the possibility that these persistent flux measurements result from a direct effect of an increased [K]₀ on the membrane in hypotonic media, it is possible that this phenomenon has its origin in the prolonged increase in cell volume.

The effect of raising [K]₀ from 2.5 mM to 4.5 mM in a hypotonic medium does not appreciably influence potassium influx, efflux, or net change during the volume regulatory phase. This is mentioned because with a hematocrit of 5%, the [K]₀ increases slightly during the response as the cells lose potassium. The maximum increase occurs in the most hypotonic medium (188 mosmols) where the [K]₀ increases from 2.5 mM to 4.5 mM after 90 min.

**DISCUSSION**

The major findings in this paper are (a) the demonstration that duck erythrocytes are capable of readjusting their volume in hypotonic media after first swelling, and (b) the characterization of the cellular process responsible for volume regulation under these circumstances. As demonstrated in the Results, it is possible to divide the cell's response empirically into two processes. The first appears upon initially placing cells in a hypotonic solution and is characterized by a smaller increase in volume than would be expected if the cells behaved as "ideal osmometers." This faculty is present in other cells besides erythrocytes, and it has been suggested previously that the phenomenon may function physiologically to minimize changes in cell hydration (Dick, 1966). The initial net cell chloride loss suggests that in the duck erythrocyte as in the human erythrocyte (Cook, 1967; Gary-Boo and Solomon, 1968) chloride shifts may be responsible for part of the "nonideal behavior."

The second process, the volume regulatory phase, produces the largest alteration in cell volume and is responsible for the final volume adjustment. An intrinsic part of the latter mechanism is a means for producing the controlled loss of osmotic particles from the cell (effector) as well as a macromolecular device, a "volumestat," sensitive to some parameter associated with cell volume which in turn can control the effector. The postulated
volumestat must have receptor and transmitter properties to explain the onset and later the decrease in the excessive outflow of osmotic particles once cell hydration has returned to its original level.

The potassium loss during the volume regulatory phase is in the same direction as the electrochemical gradient for potassium. Although the increase in K efflux could result from the active transport of potassium down this gradient, it would be more economical for the cell to execute the cation loss by transiently increasing the K⁺ leak. Since a net loss occurs along with a unilateral increase in a flux, an exchange diffusion process is excluded by definition. When the [K]ᵢ is increased the alterations that develop in net cell potassium, cell H₂O, and K influx during the first 15 min of the volume regulatory phase are consistent with this view that an increase in the diffusional pathway has occurred. To the extent that the hydrostatic pressure difference across the duck erythrocyte membrane is similar to the pressure differences that develop across the human erythrocyte membrane in hypotonic media (Rand and Burton, 1964), it is unlikely that the K and H₂O loss could result from a uniformly distributed mechanical force in the membrane capable of squeezing out electrolyte and water.

The small changes in the concentration of potassium or the total monovalent cations (millimoles per liter cell H₂O) that develop during the volume regulatory phase make it unlikely that these factors could serve as the volume sensitive indicator. As the cell contents are diluted there are numerous intracellular substances that could function in this capacity. One parameter, though, membrane elasticity, is an engaging possibility since it is theoretically capable of large relative changes as the cell swells. This possibility is enhanced by the finding of a fibrous system in the human erythrocyte membrane which appears to have some relationship to the elastic properties of the membrane and which when isolated is associated with Ca⁺⁺-sensitive adenine triphosphatase (ATPase) activity (Kregenow and Rosenthal, in preparation). Changes in membrane elasticity, if involved, would then develop in localized areas of the membrane and somehow increase K leak permeability.

An increase in the K leak, if present, must coincide with essentially no change in the Na leak in view of the absence of any gain in cell sodium and the decrease in Na permeability that develops during the volume regulatory phase. This relationship, a large selective increase in the apparent K leak but associated with a possible decrease in Na permeability, is characteristic of a phenomenon studied extensively in the human erythrocyte called the “Gardos effect” after the investigator who first demonstrated the relationship between

---

Kregenow, F. M., and A. S. Rosenthal. 1971. The isolation of a group of proteins from the human erythrocyte membrane with a fibrous appearance and ouabain insensitive ATPase activity. Manuscript in preparation.
the change in membrane permeability and extracellular Ca\(^{++}\) (Gardos, 1958). The human red cell, when incubated with certain metabolic poisons or depleted of its endogenous substrates and then subsequently exposed to extracellular Ca\(^{++}\), develops a large selective increase in K leak permeability at a time when Na permeability is unchanged or decreased (Passow, 1964; Kregenow and Hoffman, 1962). One of the important questions has been whether the Gardos effect and several related phenomena (see Passow, 1964) represent an alternation in a physiological mechanism or are simply an experimental curiosity. The former has been suggested by Kregenow and Hoffman (1962) on the basis of the response of depleted human red cell to Ca\(^{++}\).

In both the Gardos effect and the K loss in hypotonic media reported here, the already accentuated rate of \(^{42}\)K loss increases further when there is an additional decrease in the tonicity of the bathing medium (Kregenow and Hoffman, in preparation). The two phenomena differ, however, in that the presence or absence of extracellular Ca has no effect on the potassium loss reported in this paper.

Since the cells return to their original hydration state, the over-all response can be considered an example of "isosmotic intracellular regulation." The term isosmotic intracellular regulation was first proposed to define the cellular adaptation that reverts a cell's hydration and size to its original value by adjusting the number of intracellular osmotic particles when alterations occur in the osmolality of the bathing medium (Jeuniaux et al., 1961). It is considered a primitive adaption (Florkin and Schoffeniels, 1969) and has been readily demonstrated in euryhaline invertebrates (see Potts and Parry 1964).

In invertebrates, the organic constituents of the cell account for the majority of the intracellular osmotic substances altered during isosmotic intracellular regulation. The major changes occur in the low molecular weight non-essential amino acids and in some instances in taurine and trimethylamine oxide as well. Changes in the inorganic constituents also occur. The magnitude of these changes is much less, however, and varies from one species to another (Florkin and Schoffeniels, 1969).

The literature contains several examples of isosmotic intracellular regulation in vertebrate erythrocytes when the latter have been placed in hypotonic media. Lange and Fugelli (1965; Fugelli, 1967) have shown that the erythrocytes of the flounder, Pleuronectus flesus, can readjust their volume both in vivo and in vitro after there has been a 20% reduction in plasma osmolality. An attempt was made to account for the volume adjustment in terms of a reduction in the intracellular concentration of free ninhydrin-positive sub-

---

* Kregenow, F. M., and J. F. Hoffman. 1971. Some characteristics of the "Gardos" phenomenon. Manuscript in preparation.
stances (N.P.S.). However, only 1/8 of the expected decreases in the intra-
cellular osmotically active solutes can be attributed to the decrease in cellular
N.P.S. if the latter are unionized.

In the cat erythrocyte, a cell with a high intracellular Na, the permeability
to K and Na increase and decrease respectively in hypotonic media (Davson,
1940). Davson and Rainer (1942) were aware that these membrane altera-
tions would tend to reduce the intracellular cation content in hypotonic
plasma and therefore reduce cell swelling. Parker and Hoffman (1965) have
studied these permeability changes using tracers in dog erythrocytes, also
a high Na cell. It should be noted that the direction of the permeability
changes for Na and K are similar in the duck erythrocyte, a high K cell, in
hypotonic media. The findings of Robinson and Hegnauer (1936) suggest
that the rabbit erythrocyte may also be capable of isosmotic intracellular
regulation.

The author wishes to express his gratitude to Dr. Jack Orloff for his helpful criticism of the manu-
script.

This work was performed with the technical assistance of Mary Frances Spears.

Received for publication 18 January 1971.

REFERENCES

ADAIR, G. S. 1929. Thermodynamic analysis of the observed osmotic pressures of protein salts
in solutions of finite concentration. Proc. Roy. Soc. Ser. A. Math. Phys. Sci. 126:16. ;

ALLEN, D. W. 1967. Cation transport in duck erythrocytes. Ph.D. Thesis, Duke University,
Durham, N. C.

BORUN, E. R., W. G. FIGUEROA, and S. M. PERRY. 1957. The distribution of 69Fe tagged human
erthrocytes in centrifuged specimens as a function of cell age. J. Clin. Invest. 36:576.

COOK, J. S. 1967. Nonsolvent water in human erythrocytes. J. Gen. Physiol. 50:1311.

DAVSON, H. 1937. The loss of potassium from the erythrocyte in hypotonic saline. J. Cell
Comp. Physiol. 10:247.

DAVSON, H. 1940. Ionic permeability. The comparative effects of environmental changes on
the permeability of the cat erythrocyte membrane to sodium and potassium. J. Cell. Comp.
Physiol. 15:317.

DAVSON, H., and J. M. RAINE. 1942. Ionic permeability. An enzyme-like factor concerned in
the migration of sodium through the cat erythrocyte membrane. J. Cell Comp. Physiol. 20:325.

DICK, D. A. T. 1966. Cell Water. Molecular Biology and Medicine Series. E. Edward Bittar,
editor. Butterworth Inc., Washington, D.C.

FLORKIN, M., and E. SCHOFFENIELS. 1969. Molecular Approaches to Ecology. Academic
Press, Inc., New York. 89.

FUGELLI, K. 1967. Regulation of cell volume in flounder (Pleuronectes flesus) erythrocytes
accompanying a decrease in plasma osmolarity. Comp. Biochem. Physiol. 22:253.

GARDOS, G. 1958. Effect of ethylenediaminetetraacetate on the permeability of human
erthrocytes. Acta Physiol. Acad. Sci. Hung. 14:1.

GARY-BORO, C. M., and A. K. SOLOMON. 1968. Properties of hemoglobin solutions in red cells.
J. Gen. Physiol. 52:825.

HARRIS, E. J., and T. A. J. FRANKERD. 1953. The rate of sodium extrusions from human
erthrocytes. J. Physiol. (London). 121:470.

JEUNIAUX, CH., S. BRICETEUX-GREGORIE, and M. FLORKIN. 1961. Contribution des acides
aminés libres à la régulation osmotique intracellulaire chez deux crustacés euryhalins
Leander serratus F. et Leander squilla L. Cah. Biol. Mar. 2:373.
KREGENOW, F. M., and J. F. HOFFMAN. 1962. Biophys. Soc. Annu. Meet. Abstr.
LANGE, R., and K. FUGELLI. 1965. The osmotic adjustment in the euryhaline teleosts, the
flounder, Pleuronectus flesus L. and the three-spined stickleback, Gasterostius aculeatus L.
Comp. Biochem. Physiol. 15:283.
LEWIS, J. H., and E. E. FERGUSON. 1966. Osmotic fragility of premammalian erythrocytes.
Comp. Biochem. Physiol. 18:589.
LÜCKÉ, B., and M. McCUTCHEON. 1932. The living cell as an osmotic system and its perme-
ability to water. Physiol. Rev. 12:68.
MAIZELS, M., and M. REMINGTON. 1959. Percentage of intracellular medium in human
erthrocytes centrifuged from albumin and other media. J. Physiol. (London). 145:658.
McCONAHEY, P. D., and M. MAIZELS. 1961. The osmotic coefficients of haemoglobin in red
cells under varying conditions. J. Physiol. (London). 155:28.
PARKER, J. C., and J. F. HOFFMAN. 1965. Interdependence of cation permeability, cell volume
and metabolism in dog red cells. Fed. Proc. 24:589.
PASSOW, H. 1964. Ion and water permeability of the red blood cell. In: The Red Blood Cell.
C. Bishop and D. M. Surgenor, editors. Academic Press, Inc., New York. 71.
POTTS, W. T. W., and G. PARRY. 1964. Osmotic and ionic regulation in animals. Int. Ser.
Monogr. Pure Appl. Biol. Div. Zool.
RAND, R. P., and A. C. BURTON. 1964. Mechanical properties of the red cell membranes. I.
Membrane stiffness and intracellular pressure. Biophys. J. 4:115.
RIDDICK, D. H., F. M. KREGENOW, and J. ORLOFF. 1971. The effect of norepinephrine and
dibutyryl cyclic adenosine monophosphate on cation transport in duck erythrocytes. J. Gen.
Physiol. 57:732.
ROBINSON, E. J., and A. H. HEGNAUER. 1936. The water and electrolyte distribution between
plasma and red blood cells following intraperitoneal injections of isotonic glucose. J. Biol.
Chem. 116:779.
SHEPPARD, C. W., and W. R. MARTIN. 1950. Cation exchange between cells and plasma of
mammalian blood. I. Methods and application to K exchange in human blood. J. Gen.
Physiol. 33:703.
TOSTESON, D. C. 1955. Electrolytes Biol. Syst. Symp. 123.
TOSTESON, D. C., and J. S. ROBERTSON. 1956. Potassium transport in duck red cells. J. Cell.
Comp. Physiol. 47:147.
WHITTAM, R. 1964. Transport and Diffusion in Red Blood Cells. Monographs of The Physio-
logical Society. The Williams and Wilkins Co., Baltimore, Md.