Cellular Inhomogeneity in Dog Red Cells As Revealed by Sodium Flux

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ABSTRACT Unidirectional $^{22}$Na fluxes across the dog red blood cell membrane were measured. The kinetics were incompatible with a single time constant but could be accounted for in terms of a two-series compartment cell model, with approximately 1% of cell Na in the outer compartment. Dog red blood cells are known to be inhomogeneous in their Na and K permeabilities. Theoretical analysis showed that such cellular inhomogeneity in the Na permeability coefficient might in principle account for the flux data. In order to evaluate the inhomogeneity effect, a technique based on the differential response of cells suspended in isosmolar high K buffers was devised to measure the variations in Na permeability in the cell population. A variation in the Na permeability coefficient of approximately 30% was found. This inhomogeneity is insufficient to account for the flux data.

INTRODUCTION

Ion transport in the dog red cell is particularly interesting because an understanding of the ways in which it differs from that in the human erythrocyte should yield considerable information concerning general mechanisms. The dog red cell, unlike the human red cell, contains high Na and low K. The relationship between the K distribution between cell and plasma and the Cl distribution is close to expectations based on the Donnan equilibrium; however, there is a slight excess of Na in the plasma. There is no evidence for an ouabain-sensitive component in the Na flux. Parker and Hoffman (1965) have shown that the cation permeability characteristics of this cell are volume dependent. Na permeability is maximum in shrunken cells and minimum in swollen cells whereas K permeability is greatest in swollen cells and smallest in shrunken cells.

This study is concerned with $^{22}$Na flux across the dog red blood cell membrane. Na transport in this system is not governed by a single exponential process, and hence our observations are not compatible with a simple two-compartment system. Similar complexity in tracer flux data for Na and K in
the human red cell (Solomon and Gold, 1955; Solomon, 1952) has been interpreted in terms of cellular compartmentalization. A similar treatment of $^{24}$Na influx and efflux data in the dog red cell has led to the model shown in Fig. 1 in which the outer cellular compartment contains approximately 1% of the total cell Na.

Although this model accounts for the complexity of the data, other possible mechanisms which could give rise to such effects must be considered. In particular, Davson (1942) has shown that dog red cells are inhomogeneous in their permeability to cations. We have demonstrated by theoretical analysis that cellular inhomogeneity in Na permeability could lead to the behavior observed in the tracer experiments, and have described quantitatively the distributions which could account for the flux data. Consequently a direct measurement of the variations in permeability is essential in order to assess the importance of cellular inhomogeneity. A technique has been devised based on Davson's (1942) observation that if dog red blood cells are placed in isotonic KCl some of the cells swell and some of them shrink. These volume changes can be related to the variation of Na and K permeabilities in the cell population. Such individual variations make an important contribution to the overall kinetic picture but cannot account quantitatively for the time course of our data. These considerations, therefore, do not account for the kinetic behavior which has led us to postulate the existence of two intracellular compartments.

EXPERIMENTAL METHODS

Sodium Influx and Efflux

Blood was drawn from anesthetized (Nembutal) healthy male and female dogs and mixed with heparin (4000 USP units per liter blood). The blood was centrifuged at 2500 rpm for 15 min to remove the plasma which was retained for subsequent use. Theuffy coat was removed by suction and the cells were washed three times by alternate resuspension and centrifugation in buffer. The composition of the standard buffer was, in mM: NaCl, 150.0; KCl, 5.0; MgCl$_2$, 0.16; CaCl$_2$, 0.40; Na$_2$HPO$_4$, 5.2; NaH$_2$PO$_4$, 0.8; glucose, 10.0; pH, 7.4. This buffer was the same as that used by Sha'afi and Lieb (1967). $^{24}$Na flux was measured at 37.8°C. The incubation vessels were designed (Solomon, 1952) to keep the whole system under the surface of the
water bath. 5% CO₂–95% air, saturated with water at the bath temperature, was bubbled through the incubation vessels.

In order to determine whether the erythrocytes were in the steady state, the Na and K content of cells and plasma as well as the hematocrit was measured at intervals throughout the experiment on three occasions. Na and K concentrations were measured with an Instrumentation Laboratory model 143 flame photometer (Boston, Mass.). It was found that the steady state was maintained throughout the experiment to within 2%, the experimental error incurred in these measurements.

In order to reduce hemolysis, all glassware which came in contact with the blood was siliconized. It was found that by the addition of 0.1 ml plasma/ml packed RBC the degree of hemolysis estimated from the optical density at 540 mμ could be maintained at less than 0.5% for the duration of the experiment.

In influx experiments cells were suspended in standard buffer, and at zero time ⁴Na was added. At specified intervals thereafter 1 ml aliquots of suspension were removed and centrifuged at 2700 rpm for 5 min. (This centrifugation time was not added to the time of incubation as separation of the cells and buffer occurs extremely rapidly.) The erythrocytes were then resuspended and washed three times; each wash consisted of an alternate resuspension and 5 min centrifugation at 2700 rpm in standard buffer followed by removal of the supernatant by suction. Samples of packed erythrocytes were pipetted in vials with 2 ml water for counting. After counting, the hemoglobin (Hb) content of each vial was determined from the optical density at 540 mμ and used as a measure of red cell content (2% accuracy).

In two influx experiments the time course of radioactivity in the plasma was determined and found to be in good agreement with the time course as measured by red cell tracer uptake.

In efflux experiments the washed erythrocytes were preincubated at a hematocrit of 90% in standard buffer containing ⁴Na. After a time interval of either 1.0 or 2.5 hr had elapsed, unlabeled buffer was added to the blood to yield a hematocrit of approximately 46%. The blood was then incubated for a further period of several hours and 0.5 ml aliquots were removed at 0.5 or 1.0 hr intervals. The blood was centrifuged as in the influx experiments and the radioactive supernatant retained for counting. In both influx and efflux measurements the equilibrium specific activity was calculated from the measured radioactivity in a sample of suspension using the measured hematocrit and the Na content of cells and buffer.

In order to determine whether all the cell Na was exchangeable, the cells were suspended in standard buffer containing 100 mμ sucrose and ⁴Na. Na flux was sufficiently large in these shrunken cells for equilibrium to be reached within 4 hr. By measuring radioactivity and Na content in samples of whole blood and buffer it was established that the specific activities in the cells and buffer agreed to within 1.0% at the end of 3.5 hr.

In some experiments influx and efflux were measured simultaneously. To this end the cells to be used for influx determinations were incubated in standard buffer for the period of time in which the cells to be used in the efflux measurements were incubated in standard buffer containing ⁴Na. The influx curve obtained after such an incubation period was compared with that measured using freshly drawn cells and found to agree within experimental error.
Hematocrits were measured by centrifuging the suspension to constant packed cell volume at 6,700 g for 50 min in the special centrifuge tubes described previously (Schultz and Solomon, 1961). Trapped volume was determined with $^{131}$I-labeled albumin using the method of Gold and Solomon (1955). The $^{131}$I human serum albumin (RISA-131-H, Abbott Laboratories Radiopharmaceuticals, North Chicago, Ill.) was dialyzed for 48 hr at 4°C against 0.167 M KI before use (Gold and Solomon, 1955). $^{44}$Na was obtained at specific activities 1000–1300 mci/g Na from Cambridge Nuclear Corporation, Cambridge, Mass. Half-life determinations were made over a period of 6 half-lives. The measured half-life (14.9 hr) agreed with the value in the literature.

**Determination of Na Permeability Variations**

In addition to the standard buffer described above, two other buffers having the same osmolarity but different amounts of Na and K were used in these experiments. These buffers, henceforth to be referred to as nonequilibrium buffers, were identical with the standard buffer except that in one instance the 150 mM NaCl was replaced entirely by 150 mM KCl to make high K buffer and in another instance it was replaced partially by sucrose to make low Na buffer containing 30 mM NaCl.

Dog red blood cells were suspended at low hematocrit (15 %) in standard buffer at 37.8°C and $^{44}$Na was added. After a 1.0–2.0 hr incubation period the suspension was centrifuged, the radioactive supernatant removed by suction, and the cells washed once with approximately 10 volumes of ice-cold nonequilibrium buffer. The cells were then resuspended in 5 volumes of the same nonequilibrium buffer at room temperature for time intervals of either 0.5 or 1.0 hr after which the suspension was centrifuged, and the cells washed once with 10 volumes of ice-cold nonequilibrium buffer. The cells were then resuspended in 5 volumes of the same nonequilibrium buffer at room temperature for time intervals of either 0.5 or 1.0 hr after which the suspension was centrifuged, and the cells washed once with 10 volumes of ice-cold nonequilibrium buffer. After most of the supernatant was removed, the blood was mixed and placed in a small centrifuge tube (12 X 100 mm). The tube was spun for 0.5 hr at 2700 rpm. The supernatant was removed and the contents of the tube divided into eight layers of approximately equal Hb content. Due to the increasing closeness of packing of the cells from the top of the tube down, the layers were of decreasing width down the tube. Samples from each layer were pipetted into counting vials and the radioactivity determined. Using the same pipette the Na content of parallel samples from each layer was determined by flame photometry. The error due to trapped Na was less than 0.1% due to the final KCl wash.

We were interested in the specific activity of cells at different layers; i.e., the ratio of radioactivity to Na content of the cells. Due to the packed condition of the cells, pipetted volumes were not very accurate. However, the ratio of two measurements involving the same pipette could be determined to a greater accuracy. Several specific activity determinations were made on blood from a single layer and the agreement was found to be within 1%.

The efficiency of the separation technique was assessed by labeling red cells by the rapid, irreversible uptake of $^{51}$Cr (Gray and Sterling, 1950). Initially the cells were layered as described above except that the preincubation with $^{44}$Na and the final wash in KCl were eliminated. After the final centrifugation, a given layer was incubated in the same nonequilibrium buffer used for the initial separation with the addi-
tion of $^{60}$Cr. The remainder of the column of packed cells was incubated in the same buffer without $^{60}$Cr during this part of the experiment. After 6 min the labeled cells were centrifuged, the radioactive supernatant removed, and the cells washed three times, each wash consisting of approximately 8 volumes of unlabeled buffer followed by a 3 min spin. The labeled and unlabeled cells were well-mixed, a second layering centrifugation performed, and samples of cells from each layer counted to determine the $^{60}$Cr content.

In the measurement of cell volumes at different layers, samples of packed cells from each layer were dried at 97 ± 3°C from 24 to 36 hr until no further weight loss occurred. Trapped interstitial fluid was determined using albumin.$^{131}$I. The density of the dried material was determined from a sample of whole blood to which albumin-$^{131}$I had been added. The densities of blood and plasma were determined by weighing known volumes. A sample of the blood was dried to constant weight. Using albumin-$^{131}$I content to correct for trapped space, the density of the dried material was computed from the blood and plasma densities and the weight of dried material. Cell volumes were then determined from the dry weight, the density of the dried material, and the water content of the cells.

The radioactivity of either $^{24}$Na, $^{51}$Cr, or $^{131}$I was measured with a Nuclear Chicago Automatic Gamma Counting System Model 4222 (Des Plaines, Ill.). Only a single isotope was used in any single experiment.

**RESULTS**

**Compartmental Analysis**

The results of a typical influx and efflux experiment are shown in Fig. 2 representing one of six experiments in which influx and efflux were measured simultaneously on blood from the same animal. The finite value of the initial buffer specific activity in efflux is due to the fact that the cells were not washed after preincubation with $^{24}$Na. In this way there was no uncertainty as to the initial conditions needed to carry out the fitting procedures described below. It is apparent from the form of the influx and efflux data that the system cannot be described in terms of a single permeability barrier. The slope of the straight line fit to the efflux data points from 2.0 to 5.0 hr by the method of least mean squares is found to be 1.5 times greater than the slope of the straight line similarly fit to the influx data. Moreover, although the influx may be well-fit by a single straight line, this line does not pass through the origin. The slight curvature found in the efflux points was always observed and is significant.

Fig. 1 shows the simplest series model which accounts for the data. The cell is assumed to consist of two series compartments. $S_1$, $S_2$, and $S_3$ are the steady-state amounts of Na in compartments 1, 2, and 3, respectively. $b_{ij}$ is the flux in milliequivalents Na/liter RBC hour from compartment $i$ into compartment $j$. Since, as has been shown, the system was in the steady state for the duration of the experiment, $\Phi_{12} = \Phi_{21}$ and $\Phi_{13} = \Phi_{31}$. The total cell
Na contents as well as those of the buffer constituting the plasma compart-
ment, were both known, so the three adjustable parameters in the model
consisted of two fluxes and the distribution of Na between the two cell com-
partments.

On the basis of the usual assumptions (Solomon, 1960) the equations de-
scribing the movement of isotope in the system are:

\[
S_1 \frac{dp_1^*}{dt} = \Phi_{12}(p_2^* - p_1^*) \tag{1a}
\]

\[
S_2 \frac{dp_2^*}{dt} = \Phi_{21}(p_1^* - p_2^*) + \Phi_{32}(p_3^* - p_2^*) \tag{1b}
\]

\[
S_3 \frac{dp_3^*}{dt} = \Phi_{32}(p_2^* - p_3^*) \tag{1c}
\]

in which \(p_1^*\), \(p_2^*\), \(p_3^*\) are the specific activities at time \(t\) in compartments 1, 2, and 3, respectively.

The solution to equations (1) is of the form,

\[
p_1^*(t) = A_1 e^{\lambda_1 t} + A_2 e^{\lambda_2 t} + A_3 \tag{2}
\]

in which \(\lambda_1\) and \(\lambda_2\) are functions of ratios of \(\Phi\)'s to \(S\)'s but not of the boundary
conditions and \(A_1\), \(A_2\), \(A_3\) are functions of these ratios as well as of the boundary conditions.

The solution of equations (1) was obtained using an analogue computer
(EAI model 680, Electronic Associates Inc., West Long Branch, N. J.). In

![Figure 2. Results of a typical influx experiment with simultaneously measured efflux. The straight lines are least mean square fits to influx and efflux from 2.0 to 5.0 hr.](image-url)
this computer five experimental points could be displayed together with the
time course of the radioactivity in each compartment. In order to facilitate
the fitting procedure, the influx data, which measured the uptake of radio-
activity by the cells, were translated into a corresponding decay of radio-
activity in the buffer. The influx data points were displayed on the screen and
the three parameters $\Phi_{11}, \Phi_{21},$ and $S_{2}$ were adjusted until a good fit was ob-
tained using the boundary condition that all the radioactivity was in the
first compartment at zero time. This constraint was such that a considerable
range of the parameters could be found to give a good fit to the data.

A much more stringent constraint could be obtained by using the efflux data
determined simultaneously with the influx data. For a given set of parameters,

\begin{table}
\centering
\caption{THREE-COMPARTMENT MODEL PARAMETERS
FROM ANALOGUE COMPUTER FIT}
\begin{tabular}{llll}
\hline
Experiment No. & Dog No. & $\Phi_{11}$ & $\Phi_{21}$ & $S_{2}$
\hline
 & & meq Na/liter RBC & meq Na/liter RBC & meq Na/liter RBC
\hline
1 & 1 & 25.0 & 12.0 & 0.006
 & & 20.0 & 14.0 & 0.012
2 & 2 & 25.0 & 8.5 & 0.014
 & & 14.0 & 10.6 & 0.028
3 & 1 & 18.4 & 14.7 & 0.006
 & & 18.0 & 11.3 & 0.012
\hline
\text{Average}* & & 20.3 & 12.2 & 0.012
\hline
\end{tabular}
\end{table}

* The average is obtained by summing the upper and lower limits in each case and dividing by 6.

using the appropriate hematocrit, the amounts of radioactivity in compart-
ments 2 and 3 at the end of a time interval equal to that of the efflux incuba-
tion period were read off the screen. These were the initial conditions in these
compartments at the beginning of the efflux experiment since the cells were
not washed between incubation and efflux. The initial condition in compart-
ment 1 was determined experimentally. The values of the parameters obtained
from the influx fit which also fit the efflux data, were then determined. The
range of parameters which fit both the influx and efflux data was considerably
smaller than the range which fit the influx data alone but the parameters
were still not uniquely specified.

For this reason, two efflux experiments of different preincubation times were
performed simultaneously with an influx experiment. The analysis of these
experiments yielded a much more restricted set of parameters describing the
system. The results are shown in Table I. The two values of the parameters
given for each experiment are the upper and lower limits obtained from a
simultaneous fit to influx and two effluxes of different preincubation times. It can be seen that the greatest percentage variation is in \( S_2 / (S_2 + S_3) \) but nonetheless the absolute excursion is between 0.6 and 2.8% of cell Na. The analogue computer fit to a typical set of experiments is shown in Fig. 3.

**INHOMOGENEITY THEORY**

The first step toward understanding the significance of the role played by cellular inhomogeneity is to determine whether inhomogeneity alone could account for the observed time course in the influx-efflux experiments. In order to explore this question it is consistent to assume that there is no cell compartmentalization and to attempt an explanation of the experiments on the basis of inhomogeneity. Such an analysis is carried out in this section and compartmentalization is neglected throughout.

A single cell in the population is described by two parameters, the volume of cell water, \( v \), and the membrane permeability coefficient, \( k \). The more useful variables are, however, \( v \) and \( K \), the membrane permeability coefficient.
per unit volume,

\[ K = \frac{k}{v} \]

We define a distribution function \( \rho(K, v) \) normalized so that

\[
\int dK dv \rho(K, v) = 1 \tag{3}
\]

If the total number of cells is \( N \), then \( N\rho(K', v') dK' dv' \) is the number of cells whose \( K \) value is \( K' \) within \( dK' \) and volume, \( v' \), within \( dv' \).

At any time \( t \), the number of \(^{23}\)Na ions inside the cells characterized by values \( K' \) and \( v' \) (within \( dK' \) and \( dv' \)) is defined as \( dK' dv' R(K', v', t) \). The integral of this quantity is therefore the number of \(^{23}\)Na ions inside the total cell population at time \( t \).

Let \( V \) be the buffer volume and \( P(t) \) the number of tracer ions in the buffer at time \( t \). The assumption that the rate of tracer movement across the membrane of each cell is proportional to the value of \( k \) for that cell and to the concentration gradient across the cell membrane leads to a diffusion equation for each cell of the form,

\[
\frac{dn(t)}{dt} = k \left[ \frac{P(t)}{V} - \frac{n(t)}{v} \right] \tag{4}
\]

in which \( n(t) \) is the number of \(^{23}\)Na ions in the cell.

The cells with the same \( K \) and \( v \) (within \( dK \) and \( dv \)) can be grouped together mathematically and in terms of the functions \( R(K, v, t) \) and \( \rho(K, v) \) equation (4) takes the form

\[
\dot{R}(K, v, t) = N\rho(K, v)K \frac{v}{V} P(t) - KR(K, v, t) \tag{5}
\]

Since tracer ions are conserved, those gained by the cells are lost to the plasma and

\[
\dot{P}(t) = - \int dK dv \dot{R}(K, v, t) \tag{6}
\]

Let

\[
R(K, t) = \int dv R(K, v, t)
\]

and

\[
\rho(K) = \int dv \frac{v}{\bar{v}} \rho(K, v)
\]

in which \( \bar{v} \) is the average cell volume.
Integrating equations (5) and (6) with respect to volume we obtain,

\[ \dot{R}(K, t) = N_p(K) \frac{\partial}{\partial K} KP(t) - KR(K, t) \tag{7} \]

\[ \dot{P}(t) = -\int dK \dot{R}(K, t) \tag{8} \]

The solution of equations (7) and (8) for different forms of the permeability coefficient distribution function \( \rho(K) \) was obtained by digital computer.\(^1\) Rather than use a continuous distribution of permeabilities, for computational facility, we divided the cell population into \( m \) groups or channels and replaced the integration in equation (8) by a summation. Each channel has two parameters associated with it: the fraction of the total cell population it contains and the permeability coefficient of this fraction. The channel permeability coefficients are assigned \( m \) discrete values uniformly spaced along an interval so that the distribution shape is entirely determined by the channel fractions. By making \( m \) large, any continuous distribution may be described with considerable precision. (We used 20 channels in describing the most continuous distribution studied. Increasing this number to 40 did not result in any significant change in the output.)

The computer program was designed to analyze both influx and efflux situations. For a given distribution, the influx curve was obtained and an incubation period simulated at the appropriate hematocrit value. This defined the initial conditions in the different channels for efflux. As the cells were not washed between incubation and efflux, the value of the buffer specific activity at the onset of efflux was completely determined by the specific activities in the different cells. Because the incubation hematocrit was large, a small error in the measurement of this hematocrit resulted in large deviation in the predicted value of the plasma specific activity at the onset of efflux. This specific activity was always determined experimentally so the program was designed to include a search, within limits set by the 2% experimental error, for the incubation hematocrit which matched the predicted initial plasma specific activity to that observed experimentally. With the use of this set of initial conditions the efflux curve was obtained.

Clearly all possible distributions could not be analyzed in practice but by carrying out a systematic search it was possible to determine which aspects of a given distribution give rise to particular manifestations in influx-efflux curves.

\(^1\) Equations related to equations (7) and (8) have been analyzed by Sheppard and Householder (1951) for influx and by Lange and Lange\(^3\) for both influx and efflux. The analytic solutions are instructive, but a computer solution to the equations proves to be the simplest method to obtain results suitable for comparison with experiment.
As could be expected from the analogue computer work discussed in the previous section, a large variety of distributions was found to give a good fit to the influx data and the requirement of an efflux fit therefore played a crucial role.

All the efflux experiments performed had two salient characteristics: (a) There was always a slight but significant curvature in the data over the first 2 hr; (b) From 2 to 5 hr the data could be fit by a straight line whose slope was about 1.5 times greater than the slope of the straight line fit to the influx data.

The following classes of distributions which include features of most general types of realistic distributions have been analyzed.

1. **Flat Distributions**

   In this case all the channel fractions were made equal. The width of the distribution was varied. Flat distributions could be found which gave the required difference between influx and efflux slopes. For these distributions the ratio of the maximum to the minimum permeability was approximately 1.5. However, the correct curvature was not obtainable.

2. **Shaped Distributions**

   This category included bimodal distributions using only one channel per peak and varying the channel fractions and the width. Studies were also made of distributions in which most of the cells were characterized by a single permeability coefficient and the remainder were distributed in a large number of channels each containing a relatively small number of cells.

   With the use of shaped distributions the correct efflux slope could be generated for various distributions. When most of the cells were assigned a single permeability coefficient and the remainder a higher permeability coefficient, such that the ratio of maximum to minimum coefficients was approximately 2, the fit to both experimental slopes was excellent. A width could always be found for shaped distributions, regardless of the choice of channel fractions, such that the experimental efflux slope was reproduced. However, the correct curvature was still not obtainable.

The three-compartment model which we have found to fit the data has a mathematical equivalent in which the two cellular compartments are in parallel instead of in series. Such a system belongs in the class of shaped distributions described above. By analyzing the differential equations which describe diffusion in a three-compartment system it is possible to obtain the correspondence between the parameters for the series and the parallel cases. This analysis shows that the distribution equivalent to the three-compartment
series model is bimodal and extremely wide with a very high percentage of the cells with lower permeability coefficients.\(^3\)

In accordance with this analysis, such special bimodal distributions were found to fit the efflux data, reproducing both the slope and the curvature correctly. In this special equivalent distribution approximately 99\% of the cells were characterized by a single permeability coefficient and the remaining cells had a coefficient two orders of magnitude greater.

The results in this section show that cellular inhomogeneity would have to be of an extreme nature to account for the observations completely. Nonetheless a knowledge of the inhomogeneities present in the experimental system is necessary to assess the contribution of such inhomogeneity to the form of the data. The extreme class of distributions required to fit the data is unlikely to exist in red cell populations. Nonetheless, inhomogeneity could modify the three-series compartment model parameters so that an experimental study of the inhomogeneity in Na permeability present in the system was desirable.

**Permeability Distribution Measurements**

It is difficult to separate red cells cleanly according to changes in permeability properties. Two separate techniques were devised, both of which derived from Davson’s observation that volume changes occur when dog red cells are suspended in isosmotic KCl, some cells shrinking and others swelling.\(^4\) He interpreted this effect as being due to the fact that a certain proportion of the cells are more permeable to K than to Na while other cells are more permeable to Na than to K. Cells in the former class will tend to swell in KCl because K enters the cell faster than Na leaves while cells in the latter class will shrink because Na leaves at a greater rate than K enters. Centrifugation of cells which have been incubated in a high K medium causes a physical separation of cells according to the relative Na and K permeabilities of the cells.

Since such volume changes are the result of differences in the relative permeabilities to Na and K, a separation based on this criterion alone does not provide a true separation according to the Na permeability coefficient, which is the parameter of greatest interest to us. A separation according to the Na permeabilities alone may be achieved if the cells are incubated in a buffer in which K is in equilibrium with the cell interior while Na is not. If the medium Na is low, intracellular Na and hence water are lost and the cells shrink at a rate determined primarily by their Na permeability, so that these cells may also be separated by centrifugation.

The red cell water content was measured under both experimental condi-

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\(^3\) Lange, R. V., and Y. Lange. 1970. The diffusive behavior of an inhomogeneous collection of multi-compartment systems. To be published.

\(^4\) We should like to express our gratitude to Dr. J. F. Hoffman for pointing out the relevance of this observation.
tions, and found to be closely correlated with the position of the red cells in the centrifuge tube, as discussed below. This confirms that the separation procedure does indeed reflect a change in the physical volume of the cells.

In order to determine the Na permeability of the cells separated by either method they were incubated at equilibrium with $^{24}$Na prior to separation. In view of the marked volume dependence of both the Na and the K permeabilities of the dog red blood cell it is important in the interpretation of the experiments discussed in this section to bear in mind that the cell uptake of $^{24}$Na occurs at equilibrium. All subsequent nonequilibrium processes involving cell volume changes serve merely to separate the cells.

If $\Delta t$, the incubation time in $^{24}$Na, is short, conditions may be chosen such that buffer specific activity, $p^*$, remains essentially constant and much greater than cell specific activity, $s^*$. The cell uptake is then given by

$$\frac{ds^*}{dt} \approx k_{Na}p^*$$

and

$$s^* \approx k_{Na}p^*\Delta t$$

Thus the specific activity of the cells is proportional to their Na permeability coefficient. If the incubation is carried out at a sufficiently low hematocrit, cells of different Na permeability coefficient will have specific activities which reflect their individual permeabilities. Since the Na concentration gradient is high (130 mmos) and directed outwards, cell Na specific activity can be assumed to remain unchanged throughout the subsequent separation procedure. Hence, after the final centrifugation, the specific activities at different layers of the centrifuge tube reflect the Na permeability coefficient of the cells in these layers. Typical results of these experiments are shown in Table II. Separations in buffer having high K–low Na, or low Na–equilibrium K, each yield permeability coefficient differences between the top and bottom layers of approximately 28%. From Table II it can be seen that the specific activity varied smoothly down the column of packed cells.

When the separation was made in low Na buffer the cells most permeable to Na were found at the bottom of the centrifuge tube, as these cells shrank rapidly in this buffer. In the case of a high K–low Na separation buffer the cells with the greatest Na permeability coefficient were found at the top of the tube. It follows that the cells which are most permeable to Na are even more permeable to K and hence tend to swell in this buffer.

The permeability associated with a single layer represents an average for a large number of cells. Moreover, cells of very different volume and hence very different permeability could be found in the same layer due to the ran-
domness inherent in the centrifugation process. It follows from these considerations that the true permeability distribution could be considerably wider than the one observed.

The underestimation in the observed width can be assessed by performing two separations in series on a sample of cells. The extent to which the separation process is repeatable gives a measure of how good the first separation is. A satisfactory label for a given cell layer is $^{51}$Cr which is taken up rapidly and irreversibly by red cells (Gray and Sterling, 1950). When red cells are incubated in buffer containing $^{51}$Cr, a readily measurable uptake occurs in the first 5 min and the $^{51}$Cr is not removed by subsequent washing. After an

| Layer | Specific activity, cpm/meq Na |
|-------|-----------------------------|
|       | High K buffer | Low Na buffer |
|       | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 | Experiment 5 | Experiment 6 |
| 1     | 10.3          | 11.1          | 11.7          | 12.0          | 11.5          | 10.2          |
| 2     | 10.7          | 11.9          | 10.5          | 11.9          | 10.5          |               |
| 3     | 10.6          | 11.4          | 12.7          | 10.9          |               |               |
| 4     | 10.2          |              | 13.2          | 11.4          |               |               |
| 5     | 7.3           | 9.9           | 14.2          | 11.3          |               |               |
| 6     | 8.9           | 9.7           | 15.9          | 15.1          | 12.0          |               |
| 7     | 8.8           |              |               |               |               |               |
| 8     | 7.0           | 8.3           | 8.9           |               |               |               |

The absolute values of the specific activities in these experiments are not directly comparable due to the fact that different amounts of isotope were used in the preliminary incubation with $^{51}$Na.

initial separation it was therefore possible to remove a given layer from the centrifuge tube and rapidly label it with $^{51}$Cr. These cells were then mixed with the cells from the remaining seven layers, and the centrifugation and division into layers repeated. The distribution of $^{51}$Cr-labeled cells among the layers was then measured. By preparing several centrifuge tubes of packed cells in a preliminary separation it was possible to remove several different layers in the same experiment and measure the amount of return of each layer in a second separation. The distribution of $^{51}$Cr-labeled cells among the layers of the second separation is a measure of the efficiency of the initial separation.

For mathematical analysis of the separation process, we define a probability matrix, $P$, whose element $P_{ij}$ is the probability that a cell in layer $i$ after the first separation goes to layer $j$ after a second separation. The $^{51}$Cr-labeling experiments described above provide a measure of these matrix
elements. Clearly if the separation were perfect, all cells would return to their initial layers after the second separation and all the $^{61}$Cr-labeled cells would be in a single layer. This corresponds to a diagonal $P$ matrix.

Table III shows the results of these measurements. The values given are the average of three different experiments. The first row of this matrix gives the distribution of $^{61}$Cr among the layers of the second separation when the first layer in the initial separation had been removed and labeled. Most of the labeled cells returned to the first three layers with fewer in the third layer and almost none in the lower layers. Approximately 83% of the cells were found in the diagonal element and in the elements closest to it in both the first and the seventh rows of the matrix. However, in the fourth row only 64% of the cells were in the diagonal and adjacent elements.

The qualitative structure of the $P$ matrix can be used to estimate a correction to the observed permeability width. A mathematical analysis given in the Appendix yields a correction of approximately 8%. This analysis suggests that the techniques we have used to measure the permeability coefficient width do not lead to serious underestimation.

As a further check on the assumptions used in the separation method, the cell volumes at different layers of the packed column of cells were determined after separation in nonequilibrium buffer. Cell water content, determined by drying the cells to constant weight, was taken as a measure of relative cell volume.

The results of a typical experiment are given in Table IV. In the case of the high K buffer separation some of the cells were swollen while some of the

| P MATRIX |
|----------------|
| FRACTION OF CELLS RETURNED TO ORIGINAL LAYER AFTER LABELING WITH $^{61}$CR |
|---|---|---|---|---|---|---|---|
| 0.33 | 0.30 | 0.20 | 0.08 | 0.03 | 0.02 | 0.02 | 0.02 |
| 0.13 | 0.15 | 0.22 | 0.26 | 0.16 | 0.03 | 0.02 | 0.02 |
| 0.01 | 0.01 | 0.01 | 0.02 | 0.10 | 0.25 | 0.29 | 0.30 |
cells were shrunken and in the case of the low Na separation all the cells were shrunken. The unperturbed cell volume used as a reference was determined from cells incubated in isotonic standard buffer. These results show that our centrifugation techniques give good separation according to cell volume and confirm that the variations in cell permeability give rise to differences in cell volume when cells are suspended in nonequilibrium buffers.

Joyce (1958) has shown that there is a variation of Na and K permeability as well as of cellular Na and K content in human erythrocytes. His experimental methods were similar to ours; however, he observed variations in cells packed by centrifugation after suspension in isosmolar buffer containing

| TABLE IV |
|-----------|
| **CELL VOLUMES AT DIFFERENT LAYERS OF CENTRIFUGE TUBE AFTER SEPARATION IN NONEQUILIBRIUM BUFFERS** |
| Layer measured from top of tube | High K buffer | Low Na buffer |
| --- | --- | --- |
| 1 | 76.3 | 66.2 |
| 2 | 73.7 | 66.6 |
| 3 | 71.4 | 65.0 |
| 4 | 69.9 | 63.2 |
| 5 | 67.4 | 62.9 |
| 6 | 66.3 | 62.2 |
| 7 | 65.2 | 62.1 |
| 8 | 64.1 | 61.9 |
| 9 | 64.7 | 61.6 |
| 10 | 63.6 | 61.5 |

Unperturbed cell volume = 70.0%

$^{24}$Na. In this case the separation depends only on density differences in cells in the absence of osmotic pressure gradient. In his experiments cells at the top of the tube were found to have higher $^{24}$Na, Na, and K content than those at the bottom. Joyce interprets his results as a measure of red cell age, increasing age leading to increasing cell density. Other investigators have previously shown that red cell density is related to age both for human beings (Borun, Figueroa, and Perry, 1957; Pranker, 1958) and for the rabbit (Chalfin, 1954). Joyce suggests that the Na permeability coefficient differences in human red blood cells are due to age differences in the population, the younger cells being the most permeable to Na.

We have measured the cell Na and $^{24}$Na content of different portions of a column packed by centrifugation from standard Na buffer containing $^{24}$Na. Typical results of these measurements are shown in Table V. The variation in Na content of the cells down the column was found to be 13%, averaged over
three experiments. This variation is smaller than the 18% observed by Joyce in human erythrocytes but is in the same direction. Unlike Joyce, we observed no consistent variation in $^{23}Na$ content down the column and our results suggest that the gross variations in permeability observed by nonequilibrium separation methods are not related to cell age. However, the sensitivity of our methods is not sufficient to exclude a small contribution resulting from cell aging.

The variation in Na content among the cells raises the possibility that our distribution of specific activities is due to differences in Na content rather than to differences in cell permeability coefficients. The specific activity differences between the top and bottom layers in the case of separation in a high K buffer (see Table II) could be accounted for if the distribution of cells among the layers after centrifugation was such that those cells characterized by a naturally high Na content were to be at the bottom of the tube while the cells with low Na content were to be at the top of the tube.

This question was investigated using the $^{51}$Cr cell-labeling technique. Cells were packed by centrifugation from standard buffer and a portion removed from the top of the column was labeled with $^{51}$Cr. These are the cells with the highest equilibrium Na content (see Table V). The washed labeled cells were mixed with the remainder of the cells and incubated in high K buffer for 1.0 hr. The suspension was centrifuged for 0.5 hr and the distribution of $^{51}$Cr in the column of packed cells measured. It was found that most of the labeled high Na cells were concentrated in the upper portion of the tube. It follows that the specific activity difference between the top and bottom layers observed after separation in high K buffer results from differences in cell permeability, rather than differences in cell Na content.

| Layer measured from top of tube | Na content, mg Na/liter RBC | $^{23}Na$ content, pmol/mg Hb |
|-------------------------------|-----------------------------|-----------------------------|
|                               | Experiment 7 | Experiment 8 | Experiment 9 | Experiment 7 | Experiment 8 | Experiment 9 |
| 1                             | 125.3        | 110.4        | 115.5        | 28.8        | 25.0        | 31.0        |
| 2                             | 107.2        | 109.5        | 109.5        | 25.4        | 29.6        |
| 3                             | 103.7        | 109.5        | 25.7         | 29.9        |
| 4                             | 115.1        | 100.2        | 108.0        | 30.1        | 25.0        | 30.2        |
| 5                             | 102.1        | 107.3        | 26.0         | 30.3        |
| 6                             | 99.2         | 105.5        | 25.9         | 30.0        |
| 7                             | 100.2        | 105.5        | 26.0         | 29.9        |
| 8                             | 105.0        | 97.9         | 103.7        | 31.0        | 25.8        | 29.9        |
DISCUSSION

In all the experiments performed involving a separation of cells having different permeabilities a smooth variation of permeabilities was observed. The specific activities in the cell columns packed from nonequilibrium buffers varied in a linear way down the column. The cell volumes down these columns also varied monotonically.

The separation techniques we have used measure average quantities associated with a large number of cells and might therefore not be sensitive to an anomalous component of the population. However, both \(^{24}\text{Na}\) specific activities and cell volume vary smoothly and continuously among 8–10 layers, each containing 13–10% of the cells. This suggests that an anomalous component of 5% of the cells, i.e. approximately one-half of a layer would be detectable whereas one containing 1% of the cells; i.e., approximately one-tenth of a layer could not be seen. However, the existence of a 1% component seems unlikely and we therefore assume that the distribution is smooth and near the corrected width observed. Fig. 4 gives the predicted influx and efflux curves resulting from the Na permeability distribution given in Table II, corrected by about 8% as discussed in the previous section.

The predicted efflux curve closely approximates a straight line. When this line is fitted to the initial measured point, it does not fit the rest of the experimental points although the slope is close to that of the points between 2.0 and 5.0 hr. This limiting slope is 1.5 times greater than the influx slope. The analogue computer fit described earlier was made on the assumption that the system analyzed consisted of a homogeneous population of compartmented cells. From Fig. 4 it can be seen that an inhomogeneous population of un-
compartmented cells having the observed distribution in permeabilities would lead to influx-efflux slope differences of the kind we have observed. It therefore appears that the presence of the measured distribution in cell permeability has a definite effect on the tracer experiments but does not completely account for the complexity observed in the present experiments. In consequence the simplest satisfactory model is a superposition of the three-compartment model previously discussed and the distribution arising from cellular inhomogeneity.

Inhomogeneity cannot give rise to the short time curvature which must therefore be a manifestation of cell compartments. On the other hand, the difference between the influx slope and the long time efflux slope can be largely accounted for by inhomogeneity. It is therefore possible to remove artificially those aspects of the data which are caused by the inhomogeneity and fit the data obtained in this way to the three-compartment model using the analogue computer. To do this with complete rigor is a formidable task and a simplified procedure has been followed. Since the new parameters obtained in this manner are qualitatively the same as those obtained earlier, the present corrected fit appears to be a satisfactory approximation.

From the considerations outlined above it is clear that the parameters which take inhomogeneities into account should be chosen to leave the early part of the efflux curve the same and change the long time slope. An upper bound on the changes which would occur in the parameters if inhomogeneity were rigorously taken into consideration may be obtained by assuming that all the long time slope difference between the influx and efflux data is due to inhomogeneity. This assumption was used to obtain the "corrected" efflux data points marked with X's in Fig. 5. The long time slope of the corrected

![Figure 5. Effect of inhomogeneity on three-compartment parameters.](image-url)
efflux curve is the same as the slope of the influx curve. The difference between the dotted curve and the dashed curve represents the effect of the correction. The data points (open circles) used are those of experiment 2 (Table I).

Starting with the parameters previously determined (labeled “fit neglecting inhomogeneity”) it was found that if $S_2$ were increased, $F_{32}$ decreased, and $F_{12}$ left unchanged, the long time efflux curve could be given the required slope without affecting the short time fit, giving the curve labeled “fit to corrected data points.” $S_2$ increased by approximately 10% of its previous value and the new value of $F_{32}$ was approximately 15% smaller than the previous one. These parameters are compared in Table VI with those previously obtained, given in Table I. Since the changes in the parameters are small the qualitative features of the model remain unchanged, thus validating the approximation involved in the inhomogeneity correction.

The present experiments on dog red cell Na transport strongly suggest the existence of a small outer compartment in the cell, although all possible alternative explanations for the observed effects in influx-efflux experiments have not been rigorously excluded. The compartment contains approximately 1% of the cell Na, which is approximately 1.0 meq per liter of cells. If this component were located in the cellular membrane, there would be three Na sites/1000 $A^2$ of membrane surface.

The separation of the cell population into two fractions, one swollen and the other shrunken, which occurs when dog red blood cells are incubated in isosmolar high K buffer is a phenomenon which has not been investigated since Davson’s (1942) initial observations. An understanding of the underlying mechanisms responsible for the different behavior of the two cell fractions should yield new information about Na and K transport in this system.

|                 | $\Phi_{32}$ | $\Phi_{12}$ | $S_2 / S_2 + S_1$ |
|-----------------|------------|------------|------------------|
| **Previous value** | 25.0       | 8.5        | 0.014            |
| **Corrected value** | 25.0       | 8.0        | 0.016            |

It has been shown (Sheppard, Martin, and Beyl, 1951; Frazier, Sicular, and Solomon, 1954) that K transport in dog red cells is described by a single exponential process. This finding, together with our study both of the kinetics of $^4$Na movement and of the nature of the heterogeneities present in the cells, provides a basis for further experiment on this interesting system.
The $N \times N$ matrix, $P_{ij}$, is defined as the probability that a cell appearing in layer $i$ after a separation will be found in layer $j$ after mixing the cells and repeating the separation. In this Appendix we develop a method which enables us to estimate the effect on observables of the imperfection of the separation technique.

Let $x$ be the cell property to be measured and let the range of $x$ be divided into $N$ intervals so that there are an equal number of cells in each interval. The cells in the $i$th interval will be considered to have the value $x_i$ for the property $x$.

We define the matrix $Q$ whose element $Q_{ij}$ is the probability that a cell in the $i$th interval will be in the $j$th layer after the separation procedure has been carried out. If two identical, independent separations can be made successively with complete remixing of the cells between the separations, then the probability that a cell from the $k$th interval will appear in the $j$th layer in the first separation and in the $i$th layer in the second separation is $Q_{kj}Q_{ki}$. The probability of a cell appearing first in the $j$th layer and then in the $i$th layer, regardless of the interval from which it comes, is the sum over such products so that $P_{ii}$ is given by

$$P_{ii} = P_{ij} = \sum_k Q_{kj}Q_{ki} \quad A 1$$

Since both $P$ and $Q$ are probability matrices, the sum of the elements in any row or column of either matrix must be unity. $P$ is symmetric, however $Q$ need not be. All the elements of both matrices are positive, real, and less than unity.

Let $a_i$ denote the value of the property $x$, observed in layer $i$ after separation. Then,

$$a_i = \sum_j x_j Q_{ji} \quad A 2$$

and

$$a_i^2 = \sum_{jk} x_j x_k Q_{ji} Q_{ki} \quad A 3$$

Since the matrix $Q$ is not directly observable it is necessary to find a function of the observables $a_i$ which is related to $P$ rather than to $Q$. This cannot be done in general but if we assume that $Q$ is symmetric then,

$$\frac{1}{N} \sum_i a_i^2 = \frac{1}{N} \sum_{jk} x_j x_k \sum_i Q_{ji}Q_{ki} \quad A 4$$

$$= \frac{1}{N} \sum_{jk} x_j x_k \sum_i Q_{ij}Q_{ik} \quad A 5$$

$$= \frac{1}{N} \sum_{jk} x_j x_k P_{jk} \quad A 6$$

and thus the mean square of the observed values of $x$ in the layers will be related to...
the true values $x_i$ through the $P$ matrix. The assumption that $Q$ is symmetric which was necessary to obtain equation A 6 is unfortunate since it is possible to construct nonsymmetric $Q$ matrices which, if used in equations A 4 and A 5, yield significantly different numerical relationships between the quantities $x_i$ and $a_i$. It is also true that asymmetry in $Q$ can turn out to have no numerical significance if the $x$ values happen to vary in an appropriate fashion. We will proceed with equation A 6 with the understanding that the numerical estimates of the relationship between observed and real width can be distorted by this assumption but not necessarily. Note that

$$\hat{a} = \frac{1}{N} \sum_i a_i = \frac{1}{N} \sum_i x_i = \bar{x}$$  \hspace{1cm} (A 7)

and for further analysis assume that both the true values $x_i$ and the observed values $a_i$ each take on a set of evenly spaced values about their respective means. The $x_i$ values and $a_i$ values will be assumed to spread over a range of width of $\gamma$ and $\lambda$, respectively. That is

$$a_i = \hat{a} + \frac{\lambda}{2} \left( \frac{2i - 1 - N}{N - 1} \right)$$  \hspace{1cm} (A 8)

and

$$x_i = \bar{x} + \frac{\gamma}{2} \left( \frac{2i - 1 - N}{N - 1} \right)$$  \hspace{1cm} (A 9)

Combining equations A 6, A 7, A 8, and A 9 we find,

$$\sum_i \left[ \frac{\lambda}{2} \left( \frac{2i - 1 - N}{N - 1} \right) \right]^2 = \sum_{jk} \gamma^2 \left( \frac{2j - 1 - N}{N - 1} \right) \left( \frac{2k - 1 - N}{N - 1} \right) P_{jk}$$  \hspace{1cm} (A 10)

so that the underlying real width, $\gamma$, can be expressed in terms of the observed width, $\lambda$, by

$$\gamma^2 = \lambda^2 \left[ \frac{\sum_i \left( \frac{2i - 1 - N}{N - 1} \right)^2}{\sum_{jk} P_{jk} \left( \frac{2j - 1 - N}{N - 1} \right) \left( \frac{2k - 1 - N}{N - 1} \right)} \right]$$  \hspace{1cm} (A 11)

It is interesting to note how two special cases affect equation A 11. In the first case, for $P_{ij} = \delta_{ij}$, we get $\gamma^2 = \lambda^2$. That is, if the separation is perfectly repeatable the correction factor for the width is unity. On the other hand, if the separation has no repeatability at all, so that the $P_{ij} = \frac{1}{N}$, independent of $i$ and $j$, we get $\frac{\lambda^2}{\gamma^2} = 0$, so that there could be no observed width $\lambda$ unless the underlying width $\gamma$ were infinite.

In our experiments we did not measure the entire $P$ matrix. However, Table III gives us sufficient elements such that, using $P_{ij} = P_{ji}$ and $\sum_i P_{ij} = \sum_j P_{ij} = 1$
we can construct an approximate $P$ matrix embodying the essential structure shown in Table III.

We have constructed several $P$ matrices to test the $\gamma - \lambda$ relationship. An example of a simple one which reflects the essential structure shown in Table III is given in Table VII.

With this choice of $P$ using equation A 11 it is found that

$$\gamma = 1.08\lambda$$

Slight changes in the $P$ matrix do not affect this factor appreciably.

| TABLE VII | CONSTRUCTED $P$ MATRIX |
|-----------|------------------------|
| 0.37      | 0.32 0.23 0.08 0 0 0 0 |
| 0.32      | 0.33 0.23 0.12 0 0 0 0 |
| 0.23      | 0.23 0.26 0.24 0.04 0 0 0 |
| 0.08      | 0.12 0.24 0.30 0.22 0.04 0 0 |
| 0         | 0 0.04 0.22 0.30 0.24 0.12 0.08 |
| 0         | 0 0 0.04 0.24 0.25 0.23 0.23 |
| 0         | 0 0 0 0.12 0.23 0.33 0.32 |
| 0         | 0 0 0 0 0.08 0.23 0.32 0.37 |

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