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Interrelations among Na and K Content, Cell Volume, and Buoyant Density in Human Red Blood Cell Populations

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Summary. This study establishes a method for determining the concentration of Na and K in single red blood cells from electron probe microanalysis of a cell's Na and K content. To this end, red blood cells were separated into subpopulations according to their buoyant density by means of bovine serum density gradient centrifugation. Cell water and Na + K contents were then determined in each fraction by conventional analytic methods with cell volume estimated from measurements of hematocrits and cell number. It was found that an inverse relationship obtains between the mean cell volume and buoyant cell density since cells increased in size as density decreased. Although the amount of hemoglobin per cell was found to slightly increase as cell density decreased, hemoglobin concentration showed the inverse relationship, indicating that buoyant cell density differences are primarily the result of differences in hemoglobin concentration. In confirmation of Funder and Wieth (Funder, J., Wieth, J.O. 1966. *Scand. J. Lab. Invest.* **18**:167–180) cell water and cell volume was found to vary directly with the summed content of Na + K. Finally, by means of electron probe microanalysis of single cells, the cellular concentration of hemoglobin was found to vary inversely with the Na + K content, providing a quantitative basis for directly estimating cell volume, and thus ionic concentration, with this technique.

Key Words red blood cells · electron probe microanalysis · single cells · buoyant density · Na + K · cell volume

Introduction

The value of studying single red blood cells by electron probe microanalysis can be much improved by knowing the relationship between element content and cell water. This is because, where microanalysis provides an estimate of the amount of different elements present per cell, such as Na and K, the conversion of these values to concentrations requires a measure of cell volume. It has previously been suggested that the cellular sum of Na + K represents the primary determinant of cell water and therefore cell volume (Tosteson & Hoffman, 1960). Control of the cellular content of Na + K is a consequence of, and brought about by, the Na/K pump operating to offset any changes that occur by passive diffusion. Funder and Wieth (1966) found a direct correlation between the Na + K content and cell water both in red cell samples analyzed from different donors and in red cells separated by their density after centrifugation. In addition, there are a number of studies that indicate that the relative buoyant density of mammalian red cells is a function of their cation and water contents (*cf.* Keitel, Berman, Jones & Maclachian, 1955; Chalfin, 1956; Bernstein, 1959; Borun, 1963). If this is true then the variation in cell density should parallel changes in cell water, and hence volume, in a predictable manner. However, Leif and Vinograd (1964) found that the buoyant density of red cells separated after centrifugation in bovine serum albumin (BSA) gradients did not change as a result of changes in cell water. To clarify this situation we have reinvestigated the relationship between buoyant cell density, cell water, and the cell content of Na + K in BSA gradients in order to establish a dependable and quantitative basis for the conversion of the cellular content to concentration of elements determined by electron probe analysis of single red blood cells.

Materials and Methods

Preparation of Cells

Normal human blood (40 ml) was withdrawn by venipuncture into syringes containing sodium heparin (4 units, Upjohn). The blood was divided equally into two round-bottom polycarbonate tubes (50 ml) and spun for 5 min at 1500 × *g* in a refrigerated centrifuge (4°C, Sorvall RC-2B). After the plasma and white cells had been removed, the packed red cells were resuspended in 25 ml ice-cold (4°C) NaCl solution (155 mM) by covering the tubes with Parafilm® and gently inverting the tube several times. The

suspension was then centrifuged as before, repeating this washing procedure three times. These packed cells were used in the bovine serum albumin density gradient centrifugation separations as indicated below.

Preparation of Bovine Serum Albumin (BSA)

BSA solutions for forming density gradients were prepared by a procedure similar to that described by Leif and Vinograd (1964). BSA powder (Sigma, Fraction V, 18 g) was added to 200 g of distilled water with 50 g of Amberlite MB-3 resin (Mallinckrodt) in a 1000-ml beaker. This was placed in a cold room (4°C) and stirred overnight with a magnetic stirring bar to achieve complete solution of the BSA.

The solution was decanted from the resin into several polycarbonate tubes (50 ml) and centrifuged at 20,000 × *g* for 1 hr (Sorvall RC-2B, 4°C). The clear BSA solutions were pooled. The salt concentration was adjusted by the addition of each 100 g of the deionized BSA solution of the following amounts of salt: Na_2CO_3 (0.2391 g), NaCl (0.303 g), $MgCl_2 \cdot 6\,H_2O$ (0.1124 g), KCl (0.0296 g). The density of the resultant solution (33% wt/wt) was 1.105 g/ml. The pH was adjusted to 7.4 with $NaHCO_3$. A dilute BSA solution (22% wt/wt) was prepared by adding 33 g of a salt solution of the following composition to 67 g of the dense BSA solution (33% wt/wt): NaCl (7.472 g), $MgCl_2 \cdot 6\,H_2O$ (1.677 g) and KCl (0.427 g) in 1000 ml solution.

Preparation of Density Gradient and Fractionation

The density gradient was prepared by mixing 33 and 22% BSA solutions of 1.105 and 1.075 specific gravities carried out by means of an automatic gradient maker (ISCO, Model 579).

One ml of packed red cells was mixed with 19 ml of dense BSA (33%); this together with an equal volume of light BSA (22%) was used to form a linear gradient. Generally two to four 50-ml gradient tubes were prepared in a cold room at 4°C. The tubes containing the gradient were placed in a swinging bucket rotor (Sorvall H-B) and centrifuged at 4°C for 1 hr at 20,000 × *g*. This was found to be sufficient to bring the cells into density equilibrium within the albumin gradient. The density column was then fractionated into 12 parts with the aid of a fraction unloader (Buchler Auto Densi-Flow II). Specific gravities of the fractions were determined by weighing a volume of each fraction and comparing its weight with that of an equal volume of water in the same 500 μl Carlsberg pipette. Centrifugation and weighing was done on the separated fractions stored at 4°C.

Cellular Volume Estimated by Coulter Counter

In a repetition of the experiments performed by Leif and Vinograd (1964) samples of red cells from different buoyant density fractions were diluted with Isoton (modified Eagle's solution obtained from Coulter Electronics) to between 20,000 to 40,000 cells per 0.5 ml solution and the distributions of cell volume in the population was plotted by utilizing a 100-channel analyzer (Coulter Electronics, Model ZBI). The mean cell volume of the sampled population was computed by integrating the area of the plotted distribution of cell size. Calibration of the analyzer was carried out by using (a) red cells of known mean volume, determined by the hematocrit/cell number method was described below and (b) latex particles of known size.

Determination of Volume by Hematocrit Method

This method involves measurement of the hematocrit of a cell suspension and determining the proportionate number of cells in that suspension by electronic counting. Even though the accuracy of volume sizing by an electronic counter (Coulter Electronics) may be questionable, the counting accuracy appears to be reliable. The mean cell volume is obtained by dividing the hematocrit value by the cell count. Hematocrit measurements were carried out in 22% BSA solution or in $MgCl_2$ (120 mM) containing 10% glycyglycine buffer (3.4 g $MgCO_3$, 5.1 g glycyglycine in 100 ml H_2O, pH 7.4). When the hematocrit was determined with cells suspended in the $MgCl_2$ solution, the suspensions were loaded into microhematocrit tubes (ID 1.1–1.2 mm, length 75 mm, Clay-Adams), sealed by Seal-Ease (Clay-Adams) at one end and centrifuged for 10 min at 10,000 rpm in a microcentrifuge (Clay-Adams).

To measure the hematocrit of cells suspended in 22% BSA a computed volume of diluting salt solution (*see* above composition) was added to dilute the BSA in each denser BSA fraction to 22%. This suspension after appropriate dilution was centrifuged at 10,000 rpm for 5 min, the supernatant solution removed, and the remaining cells were resuspended in 22% BSA to give a final hematocrit of approximately 30–50%, determined, as before, with microhematocrits.

An aliquot of $^{22}NaCl$ (5 μl) was added to each suspension in order to obtain a measure of the volume of extracellular medium trapped in the packed cell column. The cell suspensions were carefully loaded into microhematocrit tubes (1.0 mm OD, length 32 mm, Clay-Adams) and sealed at one end with Seal-Ease (Clay-Adams). These tubes were then fitted into larger microhematocrit tubes (1.1 mm ID, length 75 mm) and centrifuged for 2 hr at 10,000 rpm in a cold room (4°C). Hematocrit readings were performed with the aid of a precision clipper, and the packed cell column of the hematocrit was corrected for trapped volume by measuring the radioactivity of ^{22}Na in the packed cells and that in a known volume of the suspending albumin solution. To obtain the radioactivity of ^{22}Na in the packed cell column, the glass tube was cut after the length of the column had been measured. Then the cut tube section with its packed cells were placed into 10-ml counting tubes containing 5 ml of distilled water. These were counted simultaneously with samples from the supernatant albumin solution that was obtained by centrifuging the remaining cell suspension. Aliquots of 10 μl of these supernatant solutions were added to 5 ml of distilled water and measured for ^{22}Na in an autogamma scintillation counter. The mean trapped medium volume was found to be 8.7 + 0.19% (SEM, n = 10).

Cell Hemoglobin Determination

Hemoglobin measurements were carried out by diluting a portion of each separated density fraction with Drabkin's reagent and measuring the absorbance at 540 nM. The mean cell hemoglobin content was obtained from the measurement of the hemoglobin concentration and cell number for the cells contained in each separated fraction.

Na and K Determination

Sodium and K concentrations were measured in cells washed free of BSA and external Na and K with the $MgCl_2$ solution (*see* above). After the densities of the BSA fractions had been determined the cells were washed 4 × with ice-cold 120 mM $MgCl_2$ (10% glycylglycine buffer, pH 7.4) by repeated centrifugation and resuspension. The hematocrit, hemoglobin content, and cell count of these suspensions were determined. At the same time 50 or 100 μl of each of these suspensions were added to a solution of 15 mM Li solution (final concentration, internal standard) and used to determine K and Na content by means of a flame photometer.

Determination of Cell Water

Cell water was determined from wet and dry weight measurement of packed cells. After the BSA had been removed from each separated density fraction by repeated washing in 120 mM $MgCl_2$, the cell suspensions were transferred to polyethylene microcentrifuge tubes (0.5 ml volume) and centrifuged in a cold room (4°C) for 30 min at 10,000 rpm in a microcentrifuge (Beckman Instruments Co.). The supernatant solution and part of the top layer cells were removed by aspiration. The remaining packed cells were used for determination of wet and dry weights as well as K and Na content. An aliquot of the packed cells (about 100 μl) was weighed in a small boat made of aluminum foil and dried in an oven at 105°C to constant weight.

Electron Probe Microanalysis of K, Na and Fe

Fresh human red cells were used for these determinations. The washing procedure was the same as described previously except that the medium used contained sucrose instead of NaCl. This sucrose medium contained (in mM): 285, sucrose; 10, $MgCl_2$; 5, Tris-HCl (pH = 7.4). Cells were suspended in this medium to approximately 10% hematocrit and smeared onto pyrolytic graphite discs preheated to 70°C on a hot plate. Smearing was carried out by holding the disc against a rotating cotton swab, which had previously been dipped into the cell suspension. The swab was rotated at 100 rpm with a motor-driven hand drill. The sample was returned to the hot plate (70°C) for 5 min to allow complete sample drying and was stored in a vacuum desiccator until analysis. On the average, the dried cells were separated on the disc by at least one cell diameter, which minimized contamination from neighboring cells.

An electron probe (ETEC Corporation, Autoscan) equipped with two adjustable wavelength spectrometers was used to analyze individual cells. The cells were analyzed using 15 kV accelerating voltage and 200 nA beam current and an 8 × 12 μm raster. Calibration curves were established by measuring mean internal Na and K concentrations using flame photometry and X-ray microanalysis. The details of the procedures used have been previously described by Kirk, Crenshaw and Tosteson (1974).

Results

Leif and Vinograd (1964) separated red blood cells according to their buoyant density and found that the mean cell volumes of cells in the less dense fractions were relatively constant and did not change with change in density. We could repeat the observations (Fig. 1, inset) of Leif and Vinograd (1964) when we reproduced their conditions and used their methods which involved BSA density gradient centrifugation and determination of cell volumes with a Coulter counter. We found as they did that the measured values of cellular volume (V) and density (σ) deviated from those predicted by their equation,

$$V = [(\sigma - \sigma_o)/(\sigma_w - \sigma)]V_o + V_o \quad (1)$$

where σ_o and V_o are the original cell density and volume, respectively, and σ_w is the density of water. This equation assumes that the change in volume and therefore density is the sole result of variations in cell water content. Leif and Vinogard (1964) found, at both high as well as at low values of density, discrepancies between the experimental and theoretical volumes as shown in Fig. 1, inset. Because hemoglobin content per cell was also said to be the same for the whole population, these results, if correct, raise the problem of what the physical determinants of cell density are, since they do not seem to be due to differences in cell water.

This apparent paradox is resolved when the estimate of cell volume is assessed by employing methods different than used by Lief and Vinograd (1964). By determining the cellular volume from the hematocrit/cell number method instead of the Coulter counter method, we found (Fig. 1*A*) that the volume-buoyant density relationship of red cells exhibits, unlike the inset data (*see* Fig. 1*B*, a reasonably good fit to the theoretical line (Eq. 1) as shown in Fig. 1*C*. For the results presented in Fig. 1*A*, the buoyant density of the red cells was measured in BSA solutions while the determination of mean cell volume, by the hematocrit/cell number method, was carried out in the buffered 120 mM $MgCl_2$ solution. The volumes the cells assume in the latter solutions may not necessarily be the same as the volumes these cells had when they were in the BSA gradient in which the buoyant density was measured. To examine the possible errors arising from the use of these two different solutions we have had to adopt a compromise situation in order to evaluate density and volume relationships in suspending media of nearly the same composition. This is because it is not possible to measure cell volumes by the hematocrit technique when the cells are in an isodense BSA solution, since the cells cannot be packed by centrifugation. Therefore, the BSA concentration in each different buoyant density fraction, which ranged from 33 to 25% was adjusted to 22% BSA

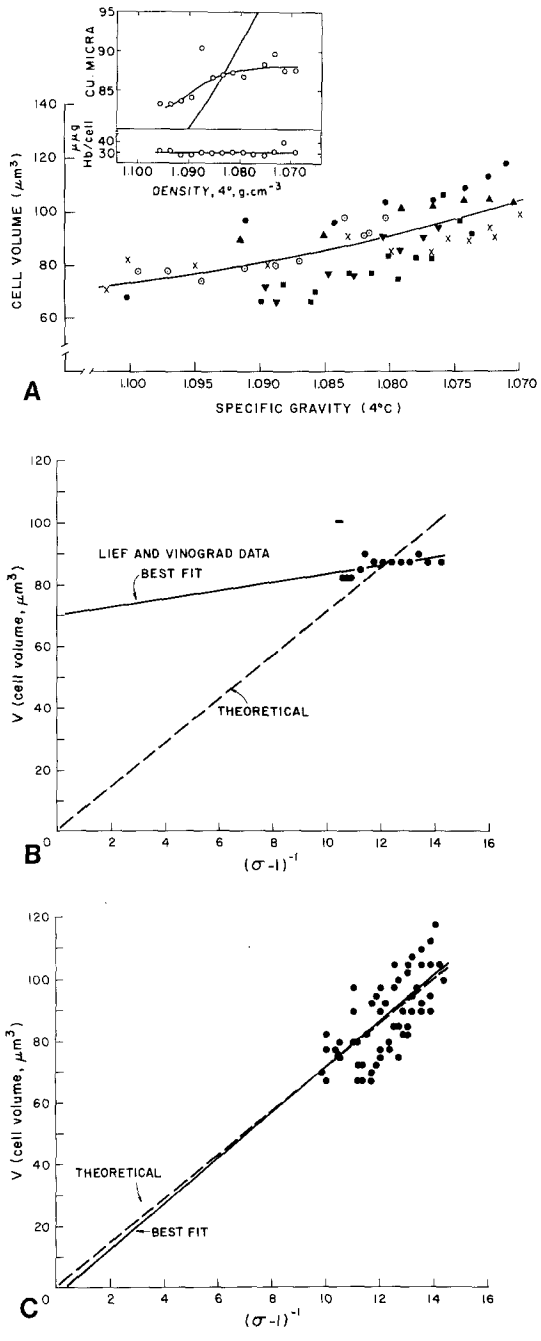

Fig. 1. (*A*): The relationship between buoyant density and volume in human red blood cells. Cells of different densities were obtained by bovine serum albumin (BSA) density gradient centrifugation. Each point represents the mean value for all the cells that appear in each separated fraction. Densities of the cells were determined by weighing 500 μl of the fractions and comparing it with the weight of an equal volume of distilled water at the same temperature. The mean cellular volume of the cells in each fraction was determined by the hematocrit/cell number method, after having removed the cells from their original BSA solution in the fraction and resuspending them in either 22% BSA (⊙, ●) or in 120 mM $MgCl_2$ solution (▲, ×, ■, ▼), as described in Materials and Methods. The theoretical line was calculated from Eq. (1) based on the assumption that changes in density are due to changes in water. The inset shows the original results (their Fig. 8) reported in Leif and Vinograd (1964), where cell volumes were obtained from electronic sizing with a Coulter counter. The theoretical line for the data of Leif and Vinograd (1964) shown in the inset is the one going from the top of the bottom of the graph passing through only one point. (*B* and *C*): The data in *A* have been replotted according to the relation $V = \alpha\beta$, which is obtained by rearrangement of Eq. (1), where $\alpha = V_o(\sigma_o - 1)$ and $\beta = 1/(\sigma - 1)$. If this relation holds then the data should conform to a simple linear regression through the origin. (*B*): The inset data of Leif and Vinograd (1964) has been replotted yielding an intercept on the ordinate of 69.7 ± 5.7 (SEM), significantly different from zero. (*C*): This graph is a replot of our data presented in *A*. The best fit yields an intercept on the ordinate of −4.5 ± 13 (SEM), which is not on Eq. (1)

before the hematocrit determination. This 22% BSA concentration is low enough to permit sedimentation of cells and high enough to approximate the concentration of BSA in the original fractions. Cellular volumes were also determined by suspending the cells (at 50–60% hematocrit) from each fraction in 120 mM $MgCl_2$ solutions and measuring the hematocrit and the cell number as described in Materials and Methods. In both cases the volume changes follow the density variations as shown in Fig. 1. These results indicate that the volume determinations in the BSA and $MgCl_2$ solutions agree well with values predicted by Eq. (1).

To further evaluate the effect of BSA on red cell volume as well as K and Na composition, we have exposed cells for 4 hr at 4°C to 22 and 33% BSA, which are the two limiting BSA solutions used for preparing the density gradients. Because, as mentioned before, direct assessment of cell volume is hampered by inaccuracies of measurement of the hematocrit in BSA solutions, the cells were resuspended in $MgCl_2$ before cell volume was determined as is described in the legend to the Table. Thus the Table shows that the relative cell volume is not appreciably affected by exposure to the different solutions and that the Na and K content did not change. In addition, we have also examined the cells from the pooled fractions of the density gradient and likewise found that exposure on the gradient did not appreciably alter the cation composition and cellular volume as evaluated after resuspension in $MgCl_2$.

The theoretical prediction of cellular volume changes by Eq. (1) assumes that the hemoglobin content per cell (mean corpuscular hemoglobin) remains constant for the cells contained in the various density fractions. However, the results shown in Fig. 2 indicate that a small systematic variation in hemoglobin content does occur. The cells with

Table. Cell volume and K and Na content of human red blood cells exposed to bovine serum albumin (BSA)

Samples	Volume $(\mu m)^3$	Na (10^{-16} moles/cell)	K	Na + K
0.9% NaCl	93.3 ±3.4(6)	9.3 ±.4(6)	85.9 ±5.4(6)	95.3 ±7.0(6)
22% BSA	92.7 ±2.9(6)	8.6 ±.9(6)	86.3 ±6.6(6)	94.9 ±7.1(6)
33% BSA	93.1 ±3.0(6)	8.6 ±1.0(6)	84.5 ±8.4(6)	93.1 ±8.7(6)
Pooled	93.0 ±3.2(6)	8.8 ±.6(6)	86.0 ±5.8(6)	94.9 ±6.4(6)

One ml of packed human red cells was mixed with 19 ml of either 0.9% NaCl, 22% BSA, or 33% BSA media. The suspensions were kept at 4°C for 4 hr before the cells were centrifuged and washed four times with 120 mM $MgCl_2$ (buffered with glycylglycine, pH = 7.4). Mean volumes of the cells were determined by measuring the hematocrit and cell number of the final suspension in $MgCl_2$, and K and Na were measured by flame photometry. The samples denoted "pooled" indicate that these cells have been processed through the BSA density gradient centrifugation and separation procedure and then mixed together before analysis. The results represent the mean values of six experiments ±(SD).

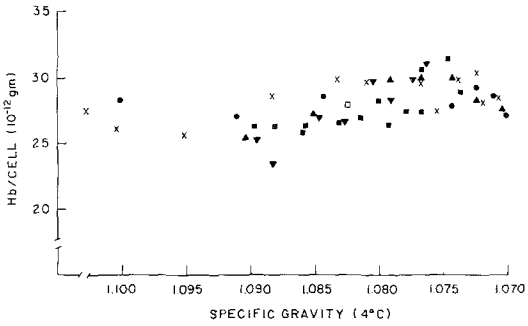

Fig. 2. The relation between the hemoglobin content (mean corpuscular hemoglobin, MCH), in 10^{-12} g/cell, and buoyant cell density. Each symbol represents the results obtained from different experiments. Each point represents the mean value calculated from analysis of all the cells in each separated buoyant density fraction

lower densities tend to have higher hemoglobin content. This increment in the amount of hemoglobin per cell will tend to increase the cellular density if volume remains constant. However, the cells with larger amounts of hemoglobin were found to have lower densities, apparently due to the larger volume of water present in these lower density cells. This is shown in Fig. 3 where the concentration (MCHC) rather than the amount of hemoglobin per cell has been plotted as a function of the density. Comparison of Figs. 2 and 3 indicate that relative cell density is due more to variations in water content than to the amount of hemoglobin present in any normal population of red cells. This underlies the inverse relationship that exists between cell volume and buoyant cell density (Fig. 1).

The foregoing discussion then emphasizes the basic role water content has in determining cell volume and density. Since it is known that human red cells are in osmotic equilibrium with their environment, that essentially all of the cell water is available for the solution of ions such as K and Cl and that Na and K are the principal cations within the cell (*see* Dunham & Hoffman, 1978), it could be expected that the water content would be a direct function of the cell's Na + K content (Tosteson & Hoffman, 1960; Funder & Wieth, 1966). Evidence has previously been presented that a cell's Na + K content, and therefore its volume, is regulated by the balance between the active transport and passive diffusion of Na and K across its membrane (Tosteson & Hoffman, 1960). In addition, Funder and Wieth (1966) found that there was a direct relationship between the Na + K and water contents of red cells obtained from 128 different donors. Funder and Wieth (1966) also showed that this relationship held for a packed column of red cells separated into upper and lower halves following centrifugation. The results presented in Fig. 4*A* and *B* extend these latter observations and show that under our conditions for cells separated from the different BSA density fractions, that Na + K content is directly correlated with cell water (4*A*) and cell volume (4*B*). These relationships not only help to establish the basis for the density differences among cells as already discussed, but, important for the purposes of this paper, also provide an index for cell water for the computation of concentration from measurements of Na + K content.

The relationship between hemoglobin and Na + K in single red cells can be determined by means of electron probe microanalysis. The Fe content in a single cell may be used as an index of the hemoglobin content since essentially all of the Fe present in normal cells is contained in hemoglobin (Noyes et al., 1964; Ganzoni, 1969). Random analysis of single cells in a whole population shows (Fig. 5*A*) that Fe content increases slightly with Na + K content, consistent with the results shown in Fig. 2. On the other hand, since, as shown in Fig. 4*A*, Na + K content is proportional to water content, the data presented in Fig. 5*A* have been replotted (Fig. 5*B*), indicating that the quantity Fe/(Na + K) decreases with increasing Na + K content of single cells. Since in this situation the ratio, Fe/(Na + K), represents the relative concentration of hemoglobin (Kirk, Bronner, Barba & Tosteson, 1978), these results are con-

sistent with those reported before (Fig. 3) where hemoglobin concentration in cells obtained from the separated BSA fractions was found to vary directly with cell density. Note that the upper abscissa in Figs. 5*A* and *B* are expressed in terms of cell volume. This is possible because of the relation between Na + K content and cell water as discussed before in connection with the data presented in Figs. 4*A* and *B*.

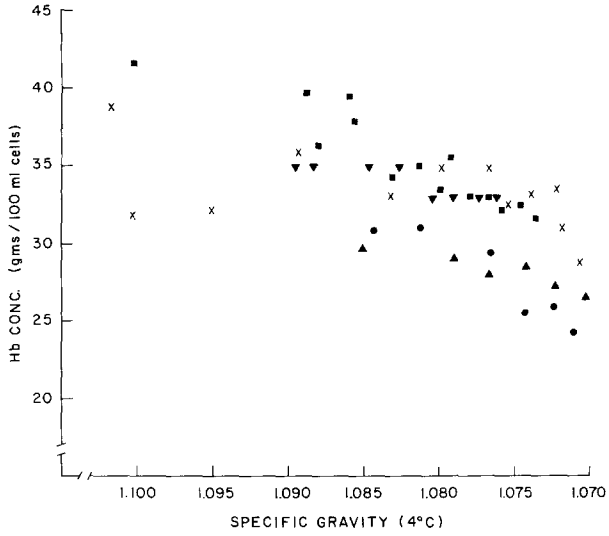

Fig. 3. The relation between the mean corpuscular hemoglobin concentration (MCHC), in grams hemoglobin/100 ml cells, and buoyant cell density. The results shown in this figure are derived from the same sets of experiments described in Fig. 2. Each symbol represents the results obtained in different experiments. Each point represents the mean value calculated from analysis of all the cells contained in each separated individual fraction

Discussion

The main objective of this work was to find a basis for converting content to concentration in single red blood cells, of different elements (e.g., Na, K, Fe) determined by the electron probe microanalytic technique. Our approach made use of the variations in buoyant cell density that can be predicted from changes in cell volume based solely on changes in cell water and Na + K content. Since density is defined by the mass-to-volume ratio and since the major components that contribute to mass and volume in a red cell are hemoglobin and water, i.e. Na + K content, any change in either parameter will result in a density change. The amount of hemoglobin per cell was found to be slightly higher the less dense the cells (Fig. 2). However, the hemoglobin concentration is lower in these less dense cells because of the larger amount of cellular water (Fig. 3). Therefore, the density is not the result of changes in the cellular content of hemoglobin since the denser cells have less hemoglobin, whereas the less dense cells have more. The increase in hemoglobin content could, because it would increase the intracellular net negative charge, increase the Na + K content in

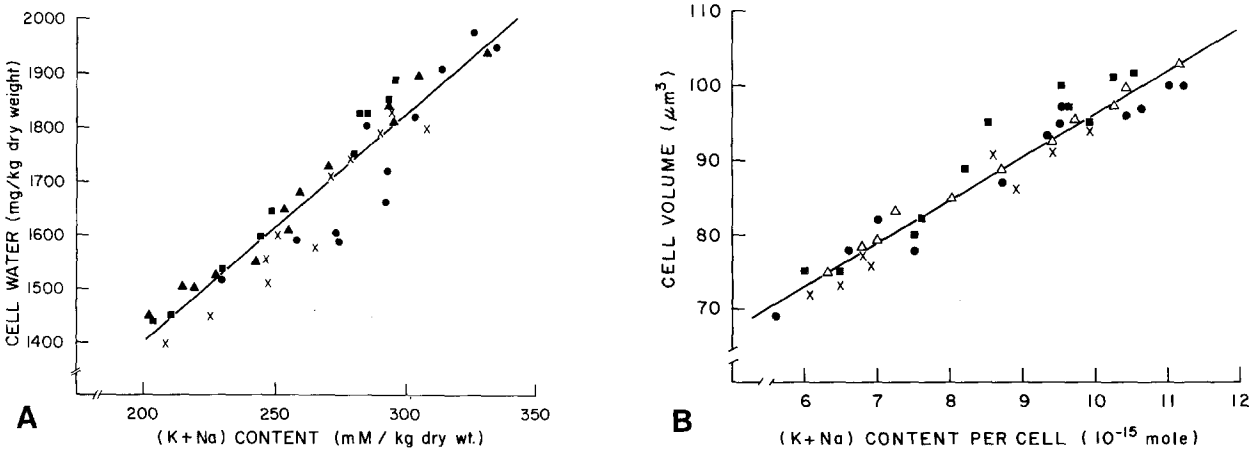

Fig. 4. (*A*): Relationship between the cell water (mg/kg dry wt) and the Na + K content (mmol/kg dry wt). Each symbol represents the results from different experiments, and each point represents the mean value of cells obtained from each density fraction. Na and K were measured by flame photometry and the cell water was determined from wet wt/dry wt of packed cells obtained from each density fraction. The regression equation for the results presented here is $y = 4.25x + 552$. The correlation coefficient, $r = 0.901$. (*B*): Relationship between cell volume and Na + K content in cell. The cell volume was determined from the hematocrit and the number of cells in each fraction. The Na + K content per cell was determined from flame photometric measurements. The regression equation for the results presented here is $y = 5.8x + 38.3$. The correlation coefficient, $r = 0.964$

order to maintain electroneutrality. If this occurred, it would cause an increase in water content with an overall effect of reducing the cell density. It should be emphasized that hemoglobin is not the sole determinant of the Na + K content in red blood cells. If this were so, cell water content would vary in direct proportion to the hemoglobin content and the mean corpuscular hemoglobin concentration would be expected to change in the opposite direction to that seen in Fig. 5*B*. It is obvious that other factors such as the pump-leak relationship of the cell must be critical in regulating the relative amounts of Na and K and therefore cell water (Tosteson & Hoffman, 1960). From electron probe analysis of Fe content (Fig. 5*A*) it is clear that the variation of hemoglobin content from cell to cell can be almost twofold. This is indicative of the type of adjustments that the pump and leak components of the membrane have to make in order to control cell volume. However, it is not clear to what extent variations in pump, leak and/or area, volume parameters contribute in defining the distribution of the different equilibrium states in a population of cells. It would also be of interest to know the range of variation in red cell volume present in each of separated BSA density fractions, but this requires further work.

While the increase in density of red blood cells with age has been well documented (Hoffman 1958; Bernstein, 1959; Borun, 1963; Leif & Vinograd, 1964; Bishop & Prentice, 1967) there has been disagreement as to whether cation content or water content was the main determinant for the density change. Bernstein (1959) and Borun (1963) found that the cation and water content of cells decreased as the relative density increased and suggested that cell water variation was the reason for the density change. However, Leif and Vinograd (1964) separated human red blood cells in BSA gradient and found that the volume variation in the different density fractions was too small to account for the observed density changes. Coppersmith and Ingram (1968) also found it difficult to explain the variation

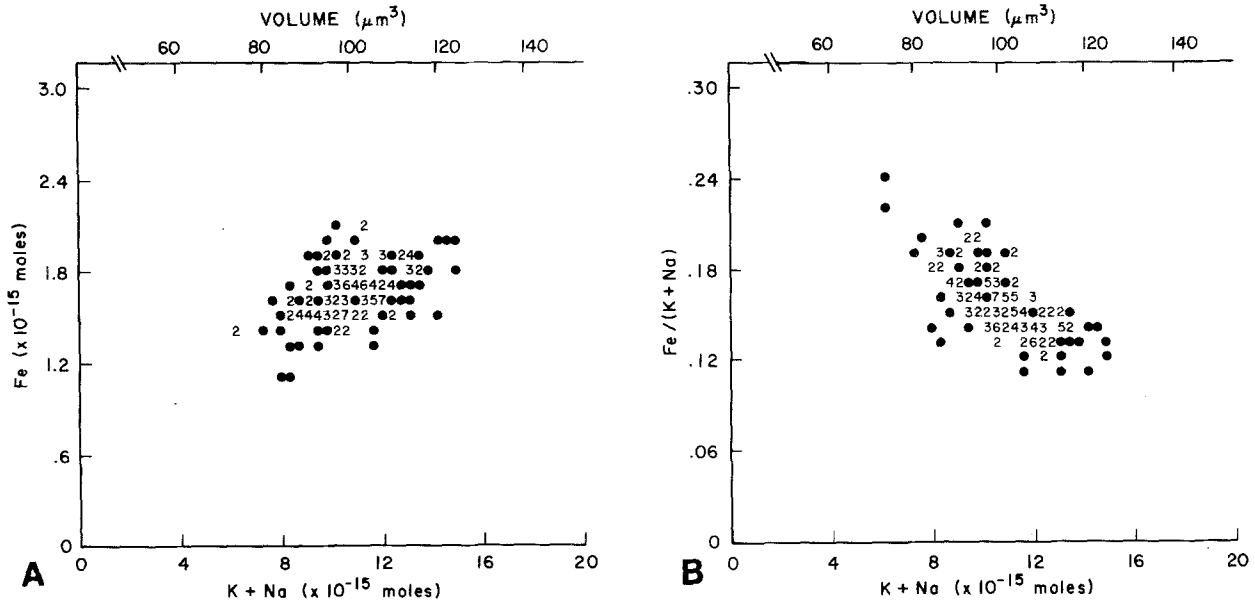

Fig. 5. Relationship between Fe content and the amounts of Na + K in randomly selected single human red cells measured by electron probe microanalysis. The relationship between Fe content and Na + K content is shown in *A*. In *B*, the quantity, Fe/(Na + K), which is equivalent to hemoglobin concentration, is plotted against Na + K content. Note that the cell volume is given on the upper abscissa in each graph. Each point represents the analyzed values of Fe, Na and K for a single cell. When two or more cells have the same value, the number of cells located at a given point is indicated by a number instead of a black dot. The cell Fe, Na and K contents were obtained from the intensity of the X-ray signals from individual cells. The calibration of the Fe X-ray signal was done by comparing the mean X-ray signal with the mean Fe content computed from the mean cell hemoglobin measured spectrophotometrically (each g of hemoglobin contains 3.34 mg of Fe). The calibrations of the Na and K X-ray signals were done by comparing the cell Na and K contents determined by flame photometry and the X-ray intensities. This method of calibration is possible since it has previously been shown that there is a linear relationship between X-ray intensity and cellular elemental content in red blood cells (Roinel & Passow, 1974; Kirk et al., 1978). The volume scale was obtained by converting the Na + K values shown on the abscissa to the equivalent values in volume by using the linear relationship between the volume and the Na + K content established in Fig. 4*B*. Thus, the equation used for the conversion was: cell volume = 5.8 (Na + K) + 38.3, where the units for cell volume is μm^3 and for cell content of Na + K is 10^{-15} mole. The regression equation for the results presented in *A* and *B* are Fe = 0.0524 (K + Na) + 1.1138 and Fe/(K + Na) = −0.0096 (K + Na) + 0.2598, respectively. The corresponding correlation coefficients are 0.473 and 0.680

in density of young and old dog red cells by changes in water content. However, Leif and Vinograd (1964) and Coppersmith and Ingram (1968) used electronic sizing to estimate cell volume that for reasons already discussed were inadequate and misleading. Our measurements of cell volume by means of the hematocrit/cell number method show that the density behavior of the red cells is completely predictable from water content.

However, it should be kept in mind that cell separation in a density gradient may also result from factors other than simple density differences, such as pH and ionic gradients. The pH and ionic gradients in a BSA density gradient arise because of the low pK values (4.7) of the BSA solution. At neutral pH, the pH at which the gradients are normally made, the BSA molecules bear net negative charges. Since the BSA density gradient is prepared by mixing two BSA solutions of two different limiting concentrations (33% *vs.* 25%) in various proportions, the concentrations of BSA in different density fractions are not the same. This gives rise to a fixed charge gradient of external impermeant anions that results in a chloride gradient in the various BSA density fractions and that presumably would have its consequences in altering relative cell volume by forcing adjustments in the Donnan equilibrium ratios (that is, internal to external chloride concentration) along the gradient. With these types of gradients, the cells in the top fractions (with less concentrated BSA and a higher Donnan ratio) can be expected to be swollen compared to the cells in the bottom fractions (with more concentrated BSA and a lower Donnan ratio), which would be shrunken relative to the mean cell volume if the BSA or chloride gradient was uniform. This effect will cause some of the cells to change their density and shift from their original isodensity location to a new location. From the results shown in the Table, it is clear that if these shifts do occur they are not detectable in terms of altering cell volume or cation content. This conclusion is also supported by the single cell analysis where the inverse relation between hemoglobin concentration and Na + K content was found (Fig. 5*B*) in the absence of a gradient.

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References

Bernstein, R.E. 1959. Alternations in metabolic energetics and cation transport during ageing of red cells. *J. Clin. Invest.* **38**:1572–1585

Bishop, C., Prentice, T.C. 1967. Separation of rabbit red cells by density in a bovine serum albumin gradient and correlation of red cell density with cell age after *in vivo* labelling with ^{59}Fe. *J. Cell. Physiol.* **67**:197–208

Borun, E.R. 1963. Some differences in erythrocyte composition and uptake of radioactive potassium, sodium, chromate, and triiodothyronine associated with *in vivo* ageing. *J. Lab. Clin. Med.* **62**:263

Chalfin, D. 1956. Differences between young and mature rabbit erythrocytes. *J. Cell. Comp. Physiol.* **47**:215–239

Coppersmith, A.M., Ingram, M. 1968. Red cell volume and erythropoiesis: Age: density: volume relationship of normocytes. *Am. J. Physiol.* **215**:1276–1283

Dunham, P.B., Hoffman, J.F. 1978. Na and K transport in red blood cells. *In:* Physiology of Membrane Disorders. T.E. Andreoli, J.F. Hoffman, and D.D. Fanestil, editors. Plenum, New York

Funder, J., Wieth, J.O. 1966. Potassium, sodium and water in normal human red blood cells. *Scand. J. Clin. Lab. Invest.* **18**:167–180

Ganzoni, A.M. 1969. Iron uptake and heme synthesis in rat reticulocytes. *Helv. Med. Acta* **34**:416–427

Hoffman, J.F. 1958. On the relationship of certain erythrocyte characteristics to their physiological age. *J. Cell. Comp. Physiol.* **51**:415–423

Keitel, H.G., Berman, H., Jones, H., Maclachian, E. 1955. The chemical composition of normal human red blood cells, including variability among centrifuged cells. *Blood* **10**:370–376

Kirk, R.G., Bronner, C., Barba, W., Tosteson, D.C. 1978. Electron probe microanalysis of red blood cells: Methods and evaluation. *Am. J. Physiol.* **235**:C245–C250

Kirk, R.G., Crenshaw, M.A., Tosteson, D.C. 1974. Potassium content of single human red cells measured with an electron probe. *J. Cell. Physiol.* **84**:29–36

Leif, R.C., Vinograd, J. 1964. The distribution of buoyant density of human erythrocytes in bovine albumin solutions. *Proc. Natl. Acad. Sci. USA* **51**:520–528

Noyes, W.D., Hosain, F., Finch, C.A. 1964. Incorporation of radioiron into marrow heme. *J. Lab. Clin. Med.* **64**:574–580

Roinel, N., Passow, H. 1974. A study of the applicability of the electron microprobe to a quantitative analysis of K and Na in single human red blood cells. *FEBS Lett.* **41**:81–84

Tosteson, D.C., Hoffman, J.F. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44**:169–196

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