Increased Water and Potassium Content in Erythrocytes in Essential Hypertension

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SUMMARY The water content in erythrocytes of subjects with borderline or established essential hypertension was measured by using gas-liquid chromatography and was found to be lower than that in normotensive controls (p<0.01). The water content in erythrocytes of normal controls (n = 14), borderline hypertensive subjects (n = 18), and established essential hypertensive subjects (n = 23) was (mean ± SE) 71.0 ± 0.2%, 69.9 ± 0.2%, and 69.3 ± 0.1% (vol/vol), respectively. A definite negative correlation was found between water content of erythrocytes and mean arterial pressure in normotensive and hypertensive subjects (n = 60, r = —0.59, p<0.001). Although there was no statistically significant between-group difference in the sodium content, the potassium content of erythrocytes from subjects with essential hypertension was significantly lower than that of normotensive controls (0.205 ± 0.003 vs 0.222 ± 0.004 mEq/L dry red blood cells; p<0.01). There was no between-group correlation of sodium and water content in erythrocytes, but the potassium content correlated with the water content (n = 46, r = 0.49, p<0.001). (Hypertension 8: 618–624, 1986)

KEY WORDS • membrane transport • borderline hypertension • gas-liquid chromatography

Several abnormalities of erythrocyte membrane transport in essential hypertension have been reported. The sodium concentration in erythrocytes has been found to be increased in many hypertensive subjects, and abnormalities of sodium transport mechanisms, such as the Na⁺-K⁺ pump, Na⁺-Li⁺ countertransport, and Na⁺-K⁺ cotransport, also have been reported, although these results have been inconsistent. Although the intracellular electrolyte composition can be readily and reliably measured without contamination by extracellular electrolytes, the water content in erythrocytes has been measured by conventional methods, such as the drying and weighing method or the indirect method calculated from the absorption of oxyhemoglobin. These methods have limited ability to detect small changes in the water content of erythrocytes. To elucidate the roles of various membrane transport systems in essential hypertension, we used a new method of gas-liquid chromatography that was superior to conventional methods in accuracy and reproducibility.

Subjects and Methods

Three groups of subjects were studied. The control group was composed of 14 normotensive subjects (11 men and 3 women), of which seven had a family history of hypertension and seven did not. Their average age was 47 ± 5 (SE) years, and the mean arterial pressure (MAP) was 89 ± 2 mm Hg. The second group was composed of 18 subjects (13 men and 5 women) with borderline essential hypertension according to World Health Organization (WHO) classification. Their average age was 49 ± 2 years, and the MAP was 108 ± 1 mm Hg. The third group was composed of 23 subjects (18 men and 5 women) with sustained essential hypertension (WHO Class I and II without nephropathy). Their mean age was 53 ± 2 years, and the MAP was 119 ± 2 mm Hg. None of the hypertensive subjects had any evidence of renal failure, heart fail-
ure, or severe retinopathy. Secondary forms of hypertension were excluded in all subjects based on the results of urinalysis and intravenous pyelography and normal values for serum creatinine, serum electrolytes, and urinary catecholamines. None of the hypertensive women were pregnant or taking oral contraceptives. All subjects were allowed unrestricted dietary salt (170 mEq of sodium per day). The water content in erythrocytes of five subjects with sustained essential hypertension was measured after 7 days of a low sodium diet (85-120 mEq/day). Subjects with sustained essential hypertension either were untreated or had stopped receiving antihypertensive therapy at least 2 weeks before the study.

Measurement of Water Content in Erythrocytes

Water content in erythrocytes was measured using a slight modification of a previously described method. In brief, after an overnight fast, venous blood samples (3 ml) were collected with a heparinized syringe from supine subjects and were centrifuged at 1600 g for 5 minutes to remove the buffy coat. Then, 1 ml of the blood sample, to which 20 μl of 20% sucrose had been added, was centrifuged in a hematocrit tube at 1600 g for 30 minutes. A 50-μl mixture of packed red blood cells (RBCs) and distilled water was then injected into sample bottles containing 2 ml of 96% methanol/4% n-butanol (internal standard), using a 50-μl microsyringe equipped with a Teflon plunger (Model B-110; Dynatech Precision Sampling, Baton Rouge, LA, USA). Plasma was injected into the bottle with a 50-μl microsyringe (Terumo K.K., Tokyo, Japan). The sample was shaken vigorously, and the aggregate of protein was centrifuged at 1600 g for 5 minutes. A 5-μl sample of the supernatant was then applied to the gas-liquid chromatograph for the measurement of water content (Figure 1A).

Calibration of the water content was performed using the standard sample, which was injected with 50 μl of water into the sample bottle. All measurements were done in triplicate, and the value was obtained from the mean readings. The gas chromatograph was equipped with a thermal conductivity detector (Model GC-4BPTF; Shimadzu Seisakusho, Kyoto, Japan). A ratio of peak area of water to n-butanol was automatically printed by an integrator (Shimadzu 1-IA) connected to the gas chromatograph. From the ratio of standard sample (100% H₂O), the water content in the plasma and the packed cells was obtained. A small amount of water contained originally in the methanol and n-butanol was subtracted from the value of samples. The column temperature was kept at 110°C, and the helium flow was maintained at 60 ml/min. The trapped water content was measured by using sucrose, which did not permeate RBC membranes but dispersed uniformly in plasma and was easily quantitated by using gas-liquid chromatography with a flame ionization detector (Shimadzu GC-OA). The supernatant in the sample bottle was transferred to a conical tube and washed with n-hexane to remove the lipids. The solvents in the conical tube were removed through vacuum distillation, and then the residual sucrose was trimethylsilylated by adding 30 μl of pyridine containing 0.05% of arachidonic methyl (internal standard), 20 μl of N-(trimethylsilyl)-imidazole, and 10 μl of trimethylchlorosilane, according to the method described by Sennello. Then, 1.5 μl of the derivatized sample was chromatographed (Figure 1B). The quantitative determination of sucrose was made by comparison with sucrose solution samples of known concentrations. The column temperature was kept at 240°C, and the nitrogen flow rate was maintained at 50 ml/min.

Calculation of Water Content in Erythrocytes

The water content in the volume (V₁) of plasma and packed cells was calculated from the equation W₁ or W₂ = V₁X, where W₁ and W₂ denote the water content.
(in microliters) in the $V_o$ (in microliters) of plasma and packed cells, respectively, and $V_X$ is the ratio of sample divided by the ratio of standard sample (in microliters). The volume of extracellular space ($V_T$, in microliters) and trapped water content ($W_T$, in microliters) were calculated as follows:

$$V_T = \frac{S_2}{S_1} \times V_o \quad (1)$$
$$W_T = \frac{S_2}{S_1} \times W_1 \quad (2)$$

where $S_1$ and $S_2$ denote the ratio of sucrose concentration of plasma and packed cells, respectively.

$$W_{RBC} = W_2 - W_T \quad (3)$$

where $W_{RBC}$ ($\mu l$) is water in RBCs after correction.

$$V_{RBC} = V_o - V_T \quad (4)$$

where $V_{RBC}$ ($\mu l$) is the volume of RBCs after correction.

Accordingly, water content in erythrocytes was obtained from the equation $(W_{RBC}/V_{RBC}) \times 100$ (%). There was no difference in trapped extracellular water content between normotensive and hypertensive subjects. Trapped extracellular water was 2.6%.

### Measurement of Sodium and Potassium in Erythrocytes

For the measurement of sodium and potassium in erythrocytes, 0.3 ml of freshly drawn heparinized blood was used. The blood sample was washed three times with cold 0.1 M magnesium chloride solution, which does not affect RBC electrolyte concentrations.

The blood samples were thoroughly dried in a dessicator at 40°C until a constant weight was obtained (at least 1 month) and then weighed with an electronic analytical balance. The dried cell pellet was extracted in 1.0 M nitric acid, and the sodium and potassium content were determined by an atomic absorption spectrophotometer (Shimadzu AA-625-01).

### Statistics

Data were analyzed statistically using Duncan's new multiple-range test for multiple comparisons preceded by one-way analysis of variance (SAS program). Correlations between different variables were sought using linear regression analysis. Values are means ± SE.

### Results

Repeated measurement on an identical sample from a normotensive control indicated an interassay coefficient of variation ([SD/mean] × 100) of 0.4% (n = 12).

The water content of fresh erythrocytes from normotensive (n = 14), borderline hypertensive (n = 18), and essential hypertensive subjects (n = 23) was 71.0 ± 0.2%, 69.9 ± 0.2%, and 69.3 ± 0.1%, respectively (Figure 2). It was significantly decreased in borderline and essential hypertensive subjects as compared with normotensive subjects ($p < 0.01$ and $p < 0.01$, respectively). Erythrocyte water content was also decreased significantly in essential hypertensive subjects as compared with borderline hypertensive subjects ($p < 0.05$). There was no statistical difference in erythrocyte water content between normotensive subjects with (n = 7; 71.0 ± 0.3%) and without a family history of hypertension (n = 7; 70.9 ± 0.3%). As shown in Figure 3, a significant correlation was found between MAP and water content in erythrocytes of normotensive and hypertensive subjects, including borderline hypertensive subjects (n = 60, $r = -0.59$, $p < 0.001$).

The water content in erythrocytes of five hypertensive subjects was measured during a low sodium diet (85–120 mEq/day) and was increased in four of the five subjects as compared with that during their normal diet (170 mEq of sodium per day).

The sodium content in erythrocytes from normotensive (n = 14), borderline hypertensive (n = 16), and essential hypertensive subjects (n = 18) was not significantly different between groups (23.2 ± 0.9, 22.7 ± 0.9, 20.8 ± 0.8 nmol/mg of dry RBCs, respectively; Figure 4). The potassium content in erythrocytes was...
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Figure 3. Correlation of mean arterial pressure (MAP) and water content in red blood cells (RBCs) from normotensive, borderline hypertensive, and essential hypertensive subjects.

Cytes from the three groups was significantly different only between normotensive (n = 14) and essential hypertensive subjects (n = 18; 0.222 ± 0.004 vs 0.205 ± 0.003 μmol/mg of dry RBCs; p < 0.01; Figure 5). Similarly, there was no between-group correlation of sodium and water content; however, a significant correlation was found between potassium and water content in erythrocytes (n = 46, r = 0.49, p < 0.001; Figure 6).

Discussion
Numerous reports on the membrane abnormalities in essential hypertension have been published in recent years. Tobian and Binion found increased sodium and water concentrations in the renal artery of hypertensive subjects and proposed the hypothesis that "waterlogged" arterioles may play a role in the pathogenesis of hypertension. Increased sodium and water in erythrocytes of hypertensive subjects were reported by Losse et al. in 1960. Many subsequent studies indicated that intracellular sodium concentration in erythrocytes of hypertensive subjects is higher than that in normotensive subjects. These observations led to a general belief that the water content in vascular smooth muscle and endothelial cells of hypertensive subjects is increased because of intracellular sodium retention.

Published data on the water content in erythrocytes of hypertensive subjects, however, are inconsistent. Aderounmu and Salako reported increased water content in erythrocytes of hypertensive subjects, whereas Walter and Distler, and Canessa and colleagues observed no significantly increased water content. Since all these data on water content in erythrocytes were obtained by conventional methods, such as the drying and weighing method or the indirect method calculated from the absorbance of oxyhemoglobin, the different results may reflect the inaccuracy of the methods. Other factors, such as different pre-treatment conditions for the erythrocytes, may also affect the results.

We measured the water content in erythrocytes under physiological conditions with the use of gas-liquid chromatography, which is superior in accuracy and reproducibility. We found that the water content in erythrocytes of borderline and essential hypertensive subjects was significantly lower than that in normotensive subjects and that there was a definite negative correlation between water content and MAP. These

Figure 4. Sodium content in red blood cells (RBCs) from normotensive (N), borderline hypertensive (BH), and essential hypertensive (EH) subjects. Values are means ± SE. NS = not significant.
results suggest that the decreased water in erythrocytes of hypertensive subjects represents the results of changes in erythrocyte membrane transport caused by factors that affect these systems and possibly induce hypertension as a result.

The sodium content (mole per RBC dry weight) in erythrocytes of hypertensive subjects was not increased significantly. On the other hand, the intracellular potassium content in erythrocytes of established essential hypertensive subjects was significantly lower than that of normotensive subjects, and there was a significant between-group correlation of water and potassium content in erythrocytes. The fact that no significant correlation was found between sodium and water in erythrocytes from any group suggests that the decreased water content in erythrocytes of hypertensive subjects was induced mainly by the decrease in potassium.

Since we expressed the sodium and potassium content as mole per RBC dry weight, the values were not influenced by the change in water content. Our sodium and potassium data seem to be inconsistent with other published data in which the sodium concentration was increased. However, when we calculated (see Figure 7 for further explanation) the intracellular sodium and potassium concentrations (expressed as mole per liter of RBCs or per liter of cell water), the sodium concentration, in contrast to the sodium content (expressed as mole per dry RBC weight), was increased in essential hypertension by 5.9% per liter of RBCs or 8.5% per liter of cell water and the potassium concentration was only changed by -2.3% or +0.1%, respectively. These results are in accordance with previous reports and indicate that increased intracellular ion concentration (mole per liter of RBCs or per liter of cell water) does not necessarily imply increased ion content in cells when intracellular water is decreased.

Many investigators have studied the cause of the increased sodium concentration in erythrocytes of hypertensive subjects in relation to various membrane transport systems. However, there are no consistent results in regard to sodium transport mechanisms such as the Na⁺-K⁺ pump, Na⁺-Li⁺ countertransport, and Na⁺-K⁺ cotransport. It is still uncertain how each transport system participates in controlling water, sodium, or potassium in erythrocytes. We found that water and potassium were decreased under physiologi-
FIGURE 7. Presumptive mean volume (V, V'), water volume (W, W'), sodium content (C\textsubscript{Na}, C'\textsubscript{Na}), and potassium content (C\textsubscript{K}, C'\textsubscript{K}) of an erythrocyte in normal controls and in essential hypertensive subjects, respectively. Shadowed area represents volume (P) of substances other than water. W/V = 0.71 in normal controls, and W'/V' = 0.693 in essential hypertensive subjects. Since substances other than water in erythrocytes are considered to be constant, the decrease in total volume of an erythrocyte is equal to the decrease in water volume (V - V' = W - W'). There was no significant difference in sodium content between normotensive and hypertensive subjects (C\textsubscript{Na} = C'\textsubscript{Na}), but potassium was lower in hypertensive subjects by 7.7% (C\textsubscript{K} = 0.923 C'\textsubscript{K}). With the use of these equations, the ratio of sodium concentration (per liter of red blood cells or per liter of cell water) can be expressed as (C'\textsubscript{Na}/W')(C\textsubscript{Na}/V) = 1.059 and (C'\textsubscript{Na}/W')(C\textsubscript{Na}/W) = 1.083, respectively. This means sodium concentration is increased in essential hypertension by 5.9% and 8.5%, respectively. The ratio of potassium concentration (per liter red blood cells or per liter of cell water) can be similarly obtained: (C'\textsubscript{K}/W')(C\textsubscript{K}/V) = 0.977 and (C'\textsubscript{K}/W')(C\textsubscript{K}/W) = 1.001, respectively. The difference of potassium concentration in established essential hypertension is only changed by -2.3% and +0.1%, respectively.

cal conditions, which permitted us to speculate about the participation of transport systems that induce membrane transport changes in erythrocytes of hypertensive subjects. Hamlyn et al.\textsuperscript{19} found a highly significant correlation between the amounts of a plasma inhibitor of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity and MAP in normotensive and hypertensive subjects. We also found a significant correlation between the water content and MAP in normotensive and hypertensive subjects. The decreased water content in erythrocytes may relate to the inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} pump by the plasma Na\textsuperscript{+}, K\textsuperscript{+}-ATPase inhibitor. However, because the Na\textsuperscript{+}-K\textsuperscript{+} pump exchanges Na\textsuperscript{+} and K\textsuperscript{+} in a ratio of 3:2, inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} pump would only lead to loss of K\textsuperscript{+} and retention of net water and Na\textsuperscript{+} in erythrocytes.

Recently, some reports on Na\textsuperscript{+}-K\textsuperscript{+} cotransport stated that Na\textsuperscript{+}-K\textsuperscript{+} cotransport activity was increased in essential hypertension\textsuperscript{13} and was positively correlated with MAP. Garay et al.\textsuperscript{17} showed that the increase in erythrocyte Na\textsuperscript{+} concentration led to stimulation of outward Na\textsuperscript{+}-K\textsuperscript{+} cotransport fluxes. Duhm and Go-bel\textsuperscript{18} demonstrated that the Na\textsuperscript{+}-K\textsuperscript{+} cotransport system was involved in the control of total cation content and cell volume of ouabain-poisoned human erythrocytes in vitro. We suggest that the increased Na\textsuperscript{+}-K\textsuperscript{+} cotransport activity may partially compensate for the sodium retention and further increase the loss of potassium. Consequently, the amount of potassium ions lost exceeds the amount of sodium ions retained, and a decrease in water content results. However, since different transport abnormalities may result in decreased water and potassium content, further study is needed to confirm that the plasma Na\textsuperscript{+}, K\textsuperscript{+}-ATPase inhibitor and the Na\textsuperscript{+}-K\textsuperscript{+} cotransport system play a major role in inducing the decrease of water and potassium in erythrocytes of hypertensive subjects.

Acknowledgments

We thank Prof. Kenjiro Yamamoto, M.D., Department of Pharmacology, Osaka City University Medical School, for his critical review of the manuscript. Dr. Woodrow G. Wilson, Medical Director of Nippon Wellcome K.K., helped to prepare the manuscript. A statistical review was done by Prof. Satoshi Kitabatake, Ph.D., Laboratory of Statistics, Osaka City University Medical School. We thank Assoc. Prof. Takatoshi Inoue, M.D., of the First Department of Internal Medicine, Osaka City University Medical School, for his help in performing this study.

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Hypertension. 1986;8:618-624
doi: 10.1161/01.HYP.8.7.618

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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