Erythrocyte Phosphate Release in Essential Hypertension

UDO WALTER

SUMMARY In this study erythrocyte phosphate release depended on the intracellular hydrolysis of organic phosphate esters. Total phosphate release was increased in essential hypertension, which suggests an elevated phosphate ester metabolism. Ouabain-sensitive phosphate release was decreased, and the ratio of intracellular Na+/K+ concentrations was increased, a finding consistent with a diminished Na-K-ATPase activity. Furosemide in a concentration of 1.0 mmol/L inhibited erythrocyte phosphate release by half, probably owing to nonspecific membrane effects. The combination of ouabain and furosemide reduced phosphate transfer to a higher degree than did each substance individually. Because of the nonspecific alteration of erythrocyte membrane permeability by furosemide in a concentration of 1.0 mmol/L, ouabain-insensitive, furosemide-sensitive phosphate release and ouabain-insensitive, furosemide-sensitive Na+ efflux (Na-K cotransport) must not be regarded uncritically as specific transport systems. (Hypertension 7: 430-437, 1985)

KEY WORDS • essential hypertension • erythrocytes • phosphate release • intracellular phosphate • intracellular sodium • intracellular potassium • ouabain • furosemide

ABNORMAL Na+ and K+ transport across the plasma membrane might lead to a rise in intracellular Na+ concentrations in human essential hypertension and thus be the cause of an increase in peripheral vascular resistance.1 However, data on transport mechanisms resulting in net Na+ and K+ fluxes of various tissues and cells are controversial. In erythrocytes the Na-K pump activity has been shown to be decreased,2,3 normal,4,5 or elevated.6,7,8 In a recent study, a decreased pump activity was observed in high-renin and in normal-renin essential hypertension, whereas in low-renin essential hypertension ouabain-sensitive erythrocyte cation transport was increased.9 Passive red blood cell (RBC) permeability for Na+ was reported to be increased,3 this finding, however, could not be confirmed by others.2 Data on Na-K cotransport are inconsistent as well. Researchers from France6 reported that Na-K cotransport was decreased in essential hypertensive subjects, while German workers found it was normal.2,10 and groups from the United States found it was either increased11,12 or normal.13

Erythrocyte Na+ transport systems are apparently subject to considerable geographic variability. The main reason for the conflicting data on Na+ fluxes, however, may be the different methodologies applied. In some instances flux measurements were performed in freshly drawn erythrocytes,2 in other cases the maximal rate of erythrocyte Na+ efflux was measured after loading RBCs with Na+ by pretreatment with nystatin10 or with parachloromercuribenzene sulfonate (PCMB).6 Both compounds affect membrane permeability; moreover, mercuro-organic substances are effective inhibitors of the Na-K-adenosine triphosphatase (Na-K-ATPase) and consequently of the Na-K pump.11 It remains unsettled whether washing PCMB-treated erythrocytes restores Na-K-ATPase and membrane permeability completely, even when cysteine is present in the washing solution.9 Additionally, it cannot be excluded that erythrocytes of normotensive subjects and essential hypertensive subjects are susceptible to PCMB in a variable degree. Therefore, measurements of Na+ and K+ fluxes should be performed on erythrocytes not pretreated with mercuro-organic substances. On the other hand, in erythrocytes not loaded with Na+, the Na-K pump is not activated maximally. This might be of some disadvantage for flux measurements because a slight increase in intracellular Na+ results in a considerable increase in pump activity owing to activation of Na-K-ATPase by Na+ from inside the cell.15 The Na+ and K+ fluxes measured in freshly drawn erythrocytes represent those in

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physiological conditions, whereas data on Na\textsuperscript{+}-loaded cells do not allow any conclusions to be drawn concerning the intact cell in vivo conditions.

There is ample evidence indicating that the Na-K pump and Na-K cotransport are closely linked to organic phosphate ester metabolism. Therefore, in the present study RBC phosphate release and its sensitivity to ouabain and furosemide were measured in normotensive subjects and in essential hypertensive subjects. It has been demonstrated previously that ouabain sensitivity of erythrocyte phosphate release is due to Na-K-ATPase activity\textsuperscript{16, 17} and thus that ouabain-sensitive phosphate release of RBCs is a mirror image of the Na-K pump.

**Subjects and Methods**

The investigation was carried out in 14 white male essential hypertensive subjects. No patients had clinical or laboratory evidence of cardiac or renal failure, and none had received drug treatment for at least 4 weeks before the determinations were made. The mean age of the hypertensive patients was 33.0 ± 10.0 (± SD) years (range, 17–52 years). Mean sitting systolic blood pressure was 182.6 ± 21.2 mm Hg (range, 158–222 mm Hg), and mean diastolic blood pressure was 115.1 ± 17.0 mm Hg (range, 96–150 mm Hg). Relative weight was 118.0 ± 18.5% (range, 93.8–167.5%) of ideal body weight. Twenty-one male healthy normotensive volunteers with no family history of arterial hypertension were used as controls. They were of the same nationality with a similar socioeconomic status and similar health and lifestyle indices as the hypertensive subjects. The mean age of this group was 29.7 ± 6.2 years (range, 23–43 years). The sitting blood pressure was below 140/90 mm Hg in all subjects with a mean value of 128.3 ± 9.3/76.7 ± 5.8 mm Hg. Mean body weight of the normotensive subjects was 106.9 ± 9.2% (range, 87.3–127.2%) of ideal weight. Before blood sampling all subjects were on a free diet. For measurement of erythrocyte Na\textsuperscript{+}, K\textsuperscript{+}, inorganic phosphate (P\textsubscript{i}), and cell phosphate release, 50-ml blood samples were drawn at 0800 hours into heparinized syringes and cooled immediately in an ice-water bath. For determination of extracellular Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, and P\textsubscript{i} concentrations, blood was drawn without the addition of anticoagulants.

**Measurement of Erythrocyte Concentrations**

Erythrocyte Na\textsuperscript{+} and K\textsuperscript{+} concentrations were measured using 3.0 ml of freshly drawn heparinized blood. After three washes with cold isotonic magnesium chloride solution at 4°C (MSE Mistral 6 L, MSE, Great Britain), the RBCs were hemolyzed with distilled water in a final volume of 10.0 ml. The Na\textsuperscript{+} and K\textsuperscript{+} were measured by flame photometry (Zeiss Fl 6/7, Zeiss, Oberkochen, Germany). For K\textsuperscript{+} measurements a further dilution of the hemolysate by a factor of 10 was necessary. The results obtained were corrected for hematocrit determined by a radiosotope dilution procedure in which 131\textsuperscript{I}Na\textsuperscript{+} was used as previously reported.\textsuperscript{1} Immediately following the addition of 1.0 ml of freshly drawn heparinized blood to 0.1 ml of 32\textsuperscript{NaCl} dissolved in isotonic saline, the suspension was centrifuged. Hematocrit was calculated from the radioactivity of a 0.5-ml sample of the supernatant and the total radioactivity measured previously (Szintillationszähler Picker Pace I, Picker Bohrloch, Northford, CT). The erythrocyte water content was determined gravimetrically. The 0.4 ml of erythrocytes used, previously packed for 30 minutes at 6000 g, were dried initially at atmospheric pressure at room temperature and then for 3 days in a vacuum over phosphorous pentoxide.

Erythrocyte P\textsubscript{i} concentration was determined by a modification of the Fiske and Subbarow method\textsuperscript{18} using ascorbic acid as a reducing agent.\textsuperscript{19, 20} For the calibration curve 0.1 ml of sodium dodecyl sulfate (13.2%), 1.0 ml of trichloroacetic acid (TCA, 12.8%), 0.6 ml of molybdate solution (357.0 ml of 5N H\textsubscript{2}SO\textsubscript{4}, 107.0 ml of 4% (NH\textsubscript{4})\textsubscript{2}Mo\textsubscript{6}O\textsubscript{19} 4 H\textsubscript{2}O, 36.0 ml of 0.27% K\textsubscript{2}S\textsubscript{2}O\textsubscript{7}, 5 H\textsubscript{2}O, filled up to 1000 ml), and 0.6 ml of ascorbic acid (0.2 g/dl) were added to 1.0-ml samples of a phosphate solution with an increasing concentration of P\textsubscript{i}. All solutions were ice cold except for the sodium dodecyl sulfate. The final volume was 3.3 ml. The samples were mixed, and the molybdenum blue complex was developed within 20 minutes at 25.0°C in a water bath. Extinctions were read at 758 nm in 1.0-cm cuvettes (Photometer Eppendorf, Netheler und Hinz GmbH, Hamburg, Germany).

The erythrocyte orthophosphate concentration was determined in a 1.0-ml sample of freshly prepared hemolysate designated for measurement of intracellular electrolytes. After precipitation of proteins by the addition of 2.0 ml of cold TCA and subsequent centrifugation, P\textsubscript{i} was measured in 2.0 ml of the supernatant. By measuring the optical density at intervals and extrapolating back to zero time, the true amount of P\textsubscript{i} was determined as previously reported.\textsuperscript{20}

**Erythrocyte Phosphate Transfer**

Erythrocyte phosphate transfer was measured as previously reported.\textsuperscript{20, 21} The erythrocytes of 30.0 ml of freshly drawn heparinized blood were washed three times in a cold solution containing 136.0 mM NaCl, 4.5 mM KCl, 4.4 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 33.0 mM glucose, 10.0 mM maleic acid, and 10.0 mM trishydroxymethylaminomethane, adjusted to pH 7.40 at 37.0°C. Subsequently, the cells were packed for 30 minutes at 6000 g. The buffy coat and the supernatant were discarded.

To measure phosphate transfer, 0.25, 0.5, 0.75, and 1.0 ml of erythrocytes were made up to 10.0 ml each with the buffer solution, preheated to 37°C. In a second, third, and fourth experiment, ouabain (0.5 mM), furosemide (1.0 mM), or a combination of both was present. At the start of the reaction and after 5, 15, 30, 45, and 60 minutes, 1.4-ml samples were taken and cooled immediately in an ice-water bath. After centrifugation 2.0 ml of TCA was added to 1.0-ml samples of the supernatant and mixed, and the sample was centrifuged again. The concentration of P\textsubscript{i} was measured as just described.
determined by the molybdenum blue method in a 2.0-
ml sample of the clear supernatant.

For evaluation of phosphate transfer, optical den-
sities of molybdenum blue were plotted against time of
incubation and regression lines were calculated for
each volume of erythrocytes. To determine absolute
phosphate elimination by erythrocytes, the coefficients
of regression, corrected for erythrocyte volume, were
plotted against volume of incubated cells. A linear
function was obtained, the slope of which is propor-
tional to phosphate transfer per milliliter of incubated
erythrocytes per minute of incubation time. The final
calculation was performed by the calibration curve
for $P_i$.

Measurement of Serum Concentrations

The levels of serum Na$^+$ and K$^+$ were determined
by flame photometry; serum Ca$^{2+}$ levels were obtained
by atomic absorption (Zeiss FL 6/7, Zeiss, Oberko-
chen, Germany). For measurement of serum ortho-
phosphate levels, 0.9 ml of water and 2.0 ml of TCA
were added to 0.1 ml of serum. After centrifugation $P_i$
levels were measured in a 2.0-ml sample of the super-
natant.

Statistical Analysis

The data were analyzed by the Student's $t$ test. The
values are means ± SD.

Results

Serum Electrolyte Concentrations

Serum concentrations of electrolytes are summa-
rized in Table 1. There were no significant differences
between groups for Na$^+$, K$^+$, or Ca$^{2+}$. The concentra-
tion of $P_i$ was significantly increased ($p < 0.05) in the
serum of essential hypertensive subjects as compared
with that of normotensive controls.

Erythrocyte Electrolyte Concentrations and
Water Content

Erythrocyte concentrations of Na$^+$, K$^+$, $P_i$, and red
cell water content are shown in Figure 1. In essential
hypertensive subjects intracellular Na$^+$ levels were
slightly increased and K$^+$ levels were decreased; the
differences between groups were not significant. After
correction for erythrocyte cell water content, which
was almost the same in both groups (Table 1), signifi-
cant differences in intracellular Na$^+$ and K$^+$ concen-
trations could not be demonstrated either. The ratio of
intracellular Na/K concentrations, however, was in-
creased in essential hypertensive subjects (0.06867 ±
0.00849) as compared with normotensive controls
(0.06436 ± 0.00624, $p < 0.05)$. Intracellular concentra-
tions of $P_i$ did not differ between groups. After cor-
rection for erythrocyte water content, mean intracellu-
lar concentration of $P_i$, per liter of erythrocyte water
was slightly higher (0.93 ± 0.24 mmol/L of RBC
water) than serum phosphate concentration in the nor-
motensive subjects (0.77 ± 0.12 mmol/L), whereas in

| Variable | Normotensive | Hypertensive | $p$
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<tbody>
<tr>
<td>[Ca$^{2+}$]$_i$ (mmol/L)</td>
<td>2.55 ± 0.09</td>
<td>2.46 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>[Na$^+$]$_i$ (mmol/L)</td>
<td>141.4 ± 4.8</td>
<td>141.2 ± 3.7</td>
<td>NS</td>
</tr>
<tr>
<td>[K$^+$]$_i$ (mmol/L)</td>
<td>4.24 ± 0.47</td>
<td>4.43 ± 0.46</td>
<td>NS</td>
</tr>
<tr>
<td>[Na$^+$]$_i$ (mmol/L of RBC)</td>
<td>5.99 ± 0.70</td>
<td>6.24 ± 0.78</td>
<td>NS</td>
</tr>
<tr>
<td>[K$^+$]$_i$ (mmol/L of RBC)</td>
<td>92.97 ± 4.52</td>
<td>90.82 ± 3.76</td>
<td>NS</td>
</tr>
<tr>
<td>[P$_i$]$_i$ (mmol/L)</td>
<td>0.77 ± 0.12</td>
<td>0.90 ± 0.20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>[P$_i$]$_i$ (mmol/L of RBC)</td>
<td>0.59 ± 0.16</td>
<td>0.57 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.06 ± 0.14</td>
<td>1.06 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Erythrocyte water (％ of wet weight)</td>
<td>63.97 ± 1.29</td>
<td>63.93 ± 0.84</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD.
NS = not significant.
ERYTHROCYTE PHOSPHATE RELEASE IN ESSENTIAL HYPERTENSION/Walter

FIGURE 2. Erythrocyte phosphate transfer and sensitivity to ouabain (0.5 mmol/L) and furosemide (1.0 mmol/L) in normotensive subjects (•) and in untreated patients with essential hypertension (□) (mean values ± SD).

essential hypertensive subjects erythrocyte water phosphate concentration (0.89 ± 0.21 mmol/L of RBC water) was almost the same as in serum (0.90 ± 0.20 mmol/L).

**Erythrocyte Phosphate Transfer**

Data on erythrocyte phosphate transfer are shown in Figure 2 and in Table 2. Phosphate elimination was linear throughout the incubation period of 1 hour. In the absence of any effectors, in 1 hour 0.981 ± 0.142 mmol of P_1_ was released into the extracellular compartment by 1.0 L of erythrocytes in the normotensive subjects. In the patients with essential hypertension, phosphate release was increased (1.063 ± 0.082 mmol/L of RBC/hour, p < 0.05). Intracellular concentration of P_1_ was 0.59 ± 0.16 mmol/L of RBC in the normotensive subjects and 0.57 ± 0.13 mmol/L of RBC in the hypertensive subjects. Thus, erythrocyte phosphate transfer per hour was almost twice that of RBC content of P_1_ in all subjects. Ouabain in a concentration of 0.5 mmol/L inhibited phosphate transfer by 11.8 ± 2.2% in the normotensive subjects and by 7.5 ± 2.5% in the hypertensive subjects (p < 0.001). Higher concentrations of ouabain had no further inhibitory effect. In the hypertensive subjects, ouabain-sensitive erythrocyte phosphate release was decreased (p < 0.001) and ouabain-insensitive phosphate release was increased (p < 0.01).

Furosemide in a concentration of 1.0 mmol/L reduced phosphate transfer by 50.1 ± 7.2% in the normotensive controls and by 50.2 ± 5.1% in the hypertensive subjects (furosemide-sensitive phosphate transfer), the residual 49.9 ± 8.9% and 49.8 ± 3.1% was furosemide insensitive. The percentage of inhibition of phosphate release by furosemide was not different between groups; however, absolute furosemide-sensitive phosphate transfer was slightly increased in subjects with essential hypertension (p < 0.05), whereas furosemide-insensitive phosphate transfer was not different between groups.

<table>
<thead>
<tr>
<th>Phosphate transfer (mmol/L of RBC/hr)</th>
<th>Normotensive</th>
<th>Hypertensive</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.981±0.142</td>
<td>1.063±0.082</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ouabain-sensitive</td>
<td>0.116±0.022</td>
<td>0.080±0.026</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ouabain-insensitive</td>
<td>0.865±0.131</td>
<td>0.983±0.066</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Furosemide-sensitive</td>
<td>0.491±0.071</td>
<td>0.534±0.054</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Furosemide-insensitive</td>
<td>0.490±0.087</td>
<td>0.529±0.033</td>
<td>NS</td>
</tr>
<tr>
<td>(Ouabain + furosemide)-sensitive</td>
<td>0.541±0.077</td>
<td>0.587±0.055</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ouabain-insensitive, furosemide-insensitive</td>
<td>0.440±0.080</td>
<td>0.476±0.039</td>
<td>NS</td>
</tr>
<tr>
<td>Ouabain-insensitive, furosemide-sensitive</td>
<td>0.425±0.066</td>
<td>0.507±0.037</td>
<td>&lt;0.001</td>
</tr>
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Values are mean ± SD.
NS = not significant.

The combination of both effectors—(ouabain and furosemide)-sensitive phosphate transfer—influenced erythrocyte phosphate transfer to a higher degree than did each drug alone. Phosphate release of 0.541 ± 0.077 mmol/L of RBC/hour in the normotensive subjects and of 0.587 ± 0.055 mmol/L of RBC/hour in the hypertensive subjects (p < 0.05) was inhibitable. In contrast to absolute values, the percentage of inhibition was not different between groups and amounted to 55.1 ± 7.8% in the controls and to 55.2 ± 5.2% in the patients.

The residual phosphate transfer (ouabain-insensitive, furosemide-insensitive) was not different be-
between groups and could be inhibited by neither ouabain nor furosemide in the concentrations applied. Ouabain-insensitive, furosemide-sensitive phosphate transfer, being the fraction of phosphate release sensitive solely to furosemide and not to ouabain, was higher in the hypertensive subjects ($p < 0.001$) than in the normotensive controls.

There was an inverse relationship between the intracellular Na/K ratios and ouabain-sensitive erythrocyte phosphate transfer for both normotensive subjects ($y = -1.31x + 0.20; r = 0.4475, n = 21, p < 0.05$) and hypertensive subjects ($y = -1.99x + 0.22; r = 0.7213, n = 14, p < 0.01$) as well as for the two groups together ($y = -2.06x + 0.24; r = 0.6028, n = 35, p < 0.001$). Correlations between levels of intracellular Na$^+$ and intracellular P, between intracellular and extracellular levels of P, and between levels of intracellular P (intracellular Na$^+$) and any pathway of erythrocyte phosphate release could not be demonstrated. There was also no correlation between relative body weight and any parameter measured.

**Discussion**

To determine the true amount of P$_i$ present in erythrocytes, optical densities of the molybdenum blue complex were measured at intervals and extrapolated back to time zero. Thus, phosphate formation by molybdenum-catalyzed hydrolysis of organic phosphate esters deriving from within the RBCs was eliminated.

In control subjects and in patients with essential hypertension, erythrocyte phosphate concentration was in the region of 0.55 mmol/L of RBC, which is consistent with previous data on normotensive subjects. Erythrocyte phosphate release was linear for more than 1 hour in both groups studied. Within this period total phosphate transfer (i.e., phosphate transfer in the absence of ouabain and furosemide) exceeded the intracellular content of P$_i$ in all subjects by a factor of almost two. Thus, to maintain constant phosphate elimination, organic phosphate esters must have been hydrolyzed within the cell, which indicates that the rate-limiting step for erythrocyte phosphate transfer might be the intracellular hydrolysis of organic phosphate esters rather than the permeation of orthophosphate through the cell membrane, as previously suggested. It cannot be stated definitely which phosphate esters had been predominantly cleaved, but hydrolysis of adenosine triphosphate (ATP) by Na-K-ATPase (and by ouabain-insensitive ATPase as well) might be involved, because part of the phosphate transfer was ouabain sensitive. Hydrolysis of ATP alone presumably would not maintain the linearity of phosphate elimination for more than 1 hour, as the erythrocyte ATP concentration in healthy subjects has been reported to be between 1.2 and 2.0 mmol/L of RBC. Therefore, the adenosine diphosphate (ADP) generated by the hydrolysis of ATP must have been regenerated by other organic phosphate esters and/or other non-ATP esters must have been cleaved as well, due to either enzymatic or spontaneous hydrolysis.

Total phosphate transfer was slightly increased in erythrocytes of patients with essential hypertension as compared with normotensive controls, whereas erythrocyte phosphate content was decreased; the latter difference was not significant, however. These data suggest an increased phosphate ester metabolism in RBCs of essential hypertensive subjects.

Ouabain inhibition of phosphate release was significantly reduced in erythrocytes of patients with essential hypertension. In 8 of the 14 hypertensive subjects ouabain-inhibitable phosphate transfer was lower than in any normotensive control. It was only higher than the mean value for the 21 controls in 2 patients. Thus, although a low ouabain-sensitive erythrocyte phosphate release was indicative of essential hypertension, a clear discrimination between hypertensive and normotensive subjects is not possible.

It generally has been recognized that digitalis glycosides are specific inhibitors of Na-K-ATPase affecting enzymatic activities exclusively from outside the cell membrane, because the binding site of Na-K-ATPase for digitalis glycosides is on the outside of the cell membrane, whereas the active site for ATP hydrolysis is located on the inside. Non-specific membrane effects of digitalis glycosides in the concentrations used in the present study have not been reported. Therefore, ouabain inhibition of erythrocyte phosphate release may be assumed to be due to inhibition of ATP hydrolysis by Na-K-ATPase. This assumption is supported by the positive correlation between ouabain-sensitive RBC phosphate transfer and ouabain-sensitive Na$^+$ efflux we previously have observed in normotensive controls and in patients with chronic uremia on hemodialysis.

Ouabain-sensitive phosphate transfer might be directly related to, but not identical to, Na-K-ATPase activity, because P$_i$ formed within the erythrocyte will partially be used for the resynthesis of organic phosphate esters and only the residual portion can be eliminated by the cell. This assumption is supported by the observation that P$_i$ ions are released from human erythrocytes by the splitting of intracellular ATP catalyzed by an ATPase in such a way that ADP remains within the cell, whereas P$_i$ is transferred to the extracellular compartment. Assuming a pump stoichiometry of 3 Na$^+$.2 K$^+$.1 ATP, by simultaneous measurements of ouabain-sensitive Na$^+$ efflux and ouabain-sensitive phosphate release, we have previously demonstrated in normotensive subjects that 21% of P$_i$ being generated by Na-K-ATPase activity was released in the incubation medium, whereas 79% of P$_i$ formed within the erythrocytes was recycled, provided glucose was available.

In our normotensive subjects ouabain-sensitive phosphate transfer was 0.116 mmol/L of RBC/hour, thus representing an intracellular hydrolysis of 0.552 mmol/L of RBC/hour of ATP by Na-K-ATPase (0.116 mmol/L of RBC/hour/0.21), whereas in hypertensive subjects ouabain-sensitive phosphate transfer was 0.080 mmol/L of RBC/hour, which is equivalent to an ATP cleavage of 0.381 mmol/L of RBC/hour. The
pump stoichiometry of 3 Na⁺:1 ATP suggests an active Na⁺ transport of 1.66 mmol/L of RBC/hour in normotensive subjects and of 1.14 mmol/L of RBC/hour in hypertensive subjects, which is consistent with previous data on ouabain-sensitive Na⁺ efflux from freshly drawn RBCs. Consequently, the data of the present study suggest a diminished Na-K-ATPase activity in intact erythrocytes of patients with essential hypertension. In accordance with this theory, intracellular Na⁺ concentration was slightly increased and intracellular K⁺ concentration was decreased. Unfortunately, the differences between the two groups were not significant; however, a significant increase could be demonstrated in the ratio of erythrocyte Na/K concentrations. Additionally, there was an inverse correlation between intracellular Na/K ratios and ouabain-inhibitable phosphate transfer, both in normotensive controls and in hypertensive subjects, as well as in the two groups together. These data suggest a diminished pump activity. This assumption is consistent with a decreased Na-K-ATPase activity of hemolyzed and dialyzed erythrocytes, which may not have been centrifuged following hemolysis, and with a reduced ouabain-sensitive rate constant for Na⁺ actively transported out of erythrocytes and leukocytes. Because Na-K-ATPase of hemoglobin-free erythrocyte membranes and ouabain-binding sites of intact erythrocytes have been demonstrated not to be different in normotensive controls and in untreated patients with essential hypertension, the diminished Na-K-ATPase activity of hemolyzed and dialyzed erythrocytes, the diminished rate constant for actively transported Na⁺, and the diminished ouabain-sensitive erythrocyte phosphate transfer observed in the present study apparently are due to a Na-K-ATPase inhibitor, which might either be tightly bound to the outside of the erythrocyte membrane or which might be present within the cell.

An Na-K-ATPase-inhibiting factor has been observed repeatedly in the plasma of essential hypertensive patients. On the other hand, low concentrations of intracellular magnesium adenosine triphosphate (Mg-ATP), being the substrate for Na-K-ATPase, will also result in a diminished activity of this ATP-hydrolyzing enzyme. This might either be due to a decreased concentration of erythrocyte Mg²⁺, recently observed in essential hypertension or to an increased Ca²⁺ concentration or Ca²⁺ binding of the erythrocyte membrane, as reported by Postnov et al. In the latter case, high intracellular Ca²⁺ levels will compete with Mg²⁺ for the binding site of ATP, which results in the predominant formation of Ca-ATP and a decrease of Mg-ATP. Thus, Ca²⁺ inhibition of the Na-K-ATPase could be a consequence of Ca-ATP-Mg-ATP competition, Ca²⁺-Mg²⁺ competition, or a combination of both mechanisms. Additionally, sensitivity of Na-K-ATPase to a cytoplasmic temperature-labile inhibitor from within the RBC has been shown to be significantly increased with increasing Ca²⁺ concentrations.

Ouabain-insensitive erythrocyte phosphate release was lower in 9 of our 21 normotensive controls than in any of the hypertensive patients. For the residual individuals there was a complete overlap between both groups. Therefore, ouabain-insensitive phosphate transfer — although significantly elevated in essential hypertension — appears to be of no value for the diagnosis of this disease. In both groups, ouabain-insensitive phosphate transfer in mmol/L of RBC/hour was almost twice that of the intracellular P₃, which suggests an increase in mean Na-K-ATPase-independent erythrocyte phosphate ester metabolism in patients with essential hypertension. The exact significance of this observation has not yet been established. It might depend on a rise in Ca-ATPase activity due to an increased Ca²⁺ binding to erythrocyte membranes, which could in turn result in an activation of Ca-ATPase.

Furosemide, in a concentration of 1.0 mmol/L, reduced erythrocyte phosphate transfer by 50%, both in normotensive subjects and in essential hypertensive subjects. Data on furosemide inhibition of ATPases are controversial. Inhibition has been observed by some researchers and not at all by others; the inhibitory effect, whenever observed, was of a minor degree only. Thus, furosemide probably influences erythrocyte phosphate transfer by nonspecific membrane effects. Transformation of the biconcave shape of human erythrocytes into crenated spheres or crenat discs by furosemide in a concentration of 1.0 mmol/L has been reported. It was suggested that crenation was induced by an interaction of anionic drugs with cationic groups within the erythrocyte membrane. This interaction might result in a nonspecific alteration of the biochemical and permeability properties of the cell membrane. Furosemide in a concentration of 1.0 mmol/L has been shown by others to inhibit erythrocyte transfer of or P, by almost the same amount as it inhibited phosphate release in our studies. It can therefore be inferred that the inhibitory effect of furosemide on phosphate efflux is not primarily a specific one; it might depend predominantly on reduction of the permeability of the erythrocyte membrane for P₃. Inhibition of some phosphate ester hydrolyzing enzymatic activity, however, cannot be excluded as an additional possibility.

On the other hand, it may be assumed that crenation of erythrocytes will also affect permeability of cell membranes for cations. Hence, the inhibition of Na⁺ efflux by furosemide in a concentration of 1.0 mmol/L in the presence of ouabain must not be regarded uncritically as a specific mechanism caused by inhibition of Na-K cotransport. This transport system is subject to considerable variability, and in essential hypertensive subjects there are data on a decreased, normal, and increased Na-K cotransport activity. In some instances, Na-K cotransport was measured in freshly drawn erythrocytes; other studies used RBCs previously loaded with Na⁺ by pretreatment with nystatin or PCMBs, which affects the permeability
of the erythrocyte membrane for cations as well as certain enzymatic activities. It may therefore be assumed that these cropped-versal data are due to the different methodologies used in the measurement of Na-K cotransport.

In the present study, the ouabain-insensitive, furosemide-sensitive component of erythrocyte phosphate transfer (ouabain-insensitive, furosemide-sensitive Na+ efflux regarded to be Na-K cotransport) was considerably increased in essential hypertension. Although the significance of this observation remains unclear, it may be due to a nonspecific effect that might depend on a variable furosemide-dependent erythrocyte deformability in normotensive controls and in patients with essential hypertension.

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