

Correction. In the article "Intracellular pH in human and experimental hypertension" by Lawrence M. Resnick, Raj K. Gupta, R. Ernest Sosa, Mary L. Corbett, and John H. Laragh, which appeared in number 21, November 1987, of *Proc. Natl. Acad. Sci. USA* (84, 7663-7667), the authors request that the following correction be noted. In the *Discussion*, in the paragraph beginning "Weak relationships were observed . . ." (page 7666, righthand column), the second sentence should read "A $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump has been identified in erythrocytes (17), and, although its physiological relevance is still uncertain, its operation, exchanging intracellular calcium for extracellular H^{+} , is consistent with primary increases in intracellular calcium reported in hypertension (3), and with lowering of intracellular pH, as observed here."

Intracellular pH in human and experimental hypertension

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ABSTRACT ^{31}P NMR spectroscopy was utilized to evaluate intracellular pH in erythrocytes from normotensive ($n = 15$) and from untreated ($n = 16$) and treated ($n = 24$) human essential hypertensive individuals. Intracellular erythrocyte pH was also measured in normotensive rats on different dietary calcium intakes as well as in volume-dependent deoxycorticosterone/saline and renin-dependent, 2 kidney, 1 clip (2K-1C) Goldblatt hypertensive rat models. Untreated essential hypertensives had significantly lower intracellular pH values compared with normotensive subjects [7.17 ± 0.02 vs. 7.28 ± 0.02 (mean \pm SEM), significance level = 0.01]. Treated hypertensives had intracellular pH values indistinguishable from normotensives [7.27 ± 0.02 (mean \pm SEM)]. Similarly, pH values for each rat model varied inversely with blood pressure, regardless of whether increased dietary calcium intake lowered pressure (normotensive and deoxycorticosterone/saline hypertensive rats) or elevated it (2K-1C Goldblatt hypertensive rats). These results demonstrate that lower intracellular pH values are commonly observed in various hypertensive states and suggest that they may contribute to the pathophysiology of the hypertensive process. Alterations in intracellular pH may also underlie the clinically observed linkage of hypertension with other disease syndromes, such as diabetes mellitus and obesity.

Numerous alterations of cellular mineral metabolism have been described in hypertension. Abnormal membrane transport of sodium, potassium, and calcium has been reported (1) as have altered cytosolic concentrations of sodium, calcium, and magnesium (2–4). The relevance of intracellular pH to these ionic events has not been well defined. It was believed that a constant intracellular pH was maintained under a wide variety of metabolic circumstances, perhaps related to the previous lack of reproducible, accurate methodology (5). Only recently, therefore, has the importance of intracellular pH as a control element in the regulation of intracellular events been emphasized (6).

Our group has recently demonstrated the value of NMR spectroscopy in evaluating intracellular mineral metabolism (7), and we have utilized ^{31}P NMR to assess abnormalities of intracellular free magnesium levels in human and experimental hypertension (4, 8). Since the chemical shift of a variety of phosphorylated compounds is highly pH-dependent, ^{31}P NMR spectroscopy has also been used to noninvasively evaluate intracellular pH (9). We therefore investigated erythrocyte intracellular pH levels in human and experimental hypertension and have found that a significant, consistent relationship exists between intracellular pH and blood pressure. We hypothesize that alterations of pH in hypertension may provide a basis for better understanding the linkage of hypertension with other clinical syndromes, such as diabetes mellitus.

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METHODS

Human Studies. Normotensive ($n = 15$) and essential hypertensive subjects, either untreated ($n = 16$) or on antihypertensive therapy ($n = 24$), were studied during the morning at The Cardiovascular Center of The New York Hospital–Cornell University Medical Center. All subjects had fasted overnight, had blood pressures measured by their physician, and then had peripheral venous blood drawn in the seated position for analysis of serum ionized calcium, erythrocyte intracellular free magnesium, and intracellular pH levels. Blood for serum ionized calcium was collected and processed anaerobically and was analyzed the same day using a calcium-specific ion electrode (Orion SS-20), where the normal range in our laboratory is 2.35–2.65 meq/liter. Ten milliliters of heparinized venous blood was collected and used for analysis of intracellular pH and intracellular free magnesium. Blood was spun at 2000 rpm for 10 min, and the plasma was discarded. The remaining erythrocyte fraction equilibrated with atmospheric oxygen was decanted into a 10-mm (o.d.) NMR tube, and subsequently was analyzed by using ^{31}P NMR spectra. NMR spectra were recorded at 81 MHz with a Varian XL-200 NMR spectrometer in the Fourier transform mode with wide-band proton noise decoupling. Typical sample volumes were ≈ 4 ml, and the temperature of the probe was maintained at 37°C by equilibration with purified air preheated to the appropriate temperature. Each erythrocyte spectrum was obtained after time-averaging for ≈ 30 min.

Experimental Hypertension. Four male Wistar rat models were studied, described elsewhere in more detail (10): uninephrectomized, saline-loaded (UNx-NaCl) controls ($n = 22$), two series of UNx-NaCl rats injected with 10 mg of deoxycorticosterone pivalate (deoxycorticosterone/NaCl, $n = 20$ in each group) per week, and renin-dependent, 2 kidney, 1 clip (2K-1C) hypertensive rats ($n = 20$). Each group was further divided to receive diets containing either 0.2% (low calcium) or 1.2% (moderate calcium) vs. 1.8% (high calcium) calcium content by weight (Table 2). Weights and systolic cuff-tail blood pressures were measured weekly. Blood was drawn by means of heparin-lock prepared polyethylene (PE-50) tubing inserted into the right carotid artery and tunneled subcutaneously to the dorsum of the neck 12 hr before sampling.

Intracellular free magnesium concentration was determined according to the formula (11) $[\text{Mg}^{2+}]_{\text{free}} = K_d^{\text{MgATP}}(\Phi - 1)$, where K_d^{MgATP} is the apparent dissociation constant for the reaction $\text{MgATP} \rightleftharpoons \text{Mg}^{2+} + \text{ATP} = 3.8 \pm 0.4 \times 10^{-5}$ M under physiologic ionic conditions (37°C, pH 7.2), and $\Phi = [\text{ATP}]_{\text{free}}/[\text{ATP}]_{\text{total}}$ is the fraction of ATP free, determined

Abbreviations: P-glycerate, 2,3-bisphosphoglycerate; NL, normotensive subject(s); Hi BP, untreated hypertensive subject(s); Hi BP-Rx, Hi BP on drug therapy; 2K-1C, 2 kidney, 1 clip; UNx-NaCl, uninephrectomized, saline-loaded.

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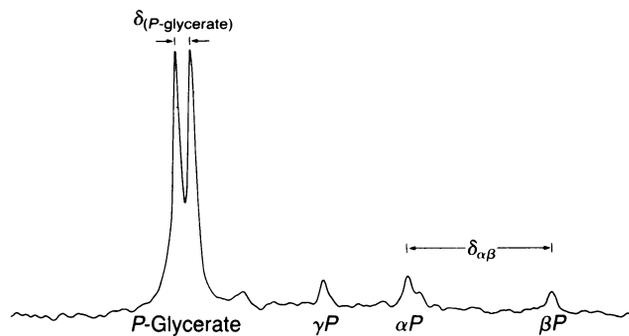


FIG. 1. Fourier-transform ^{31}P NMR spectrum of erythrocytes from a normotensive rat. Heparinized blood was analyzed at 37°C and 81 MHz. αP , βP , γP = α , β , and γ -phosphate resonances of ATP; $\delta_{(P\text{-glycerate})}$, chemical shift difference of 2- and 3-phosphate resonances of *P*-glycerate.

from the relative separation between the α - and β -phosphate group resonances of ATP (chemical shift difference, $\delta_{\alpha\beta}$) in the ^{31}P NMR spectra (Fig. 1). Since the apparent pK_a of MgATP under physiological ionic conditions is 6.4, a variation of 0.1 pH unit would alter the apparent dissociation constant K_d^{MgATP} , and thus the free Mg^{2+} value, by only 3%. The effects of small variations in intracellular pH were therefore neglected in our free Mg^{2+} calculations.

Calculation of Intracellular pH. ^{31}P NMR spectra of erythrocytes demonstrate well-defined 3- and 2-phosphate group resonances of 2,3-bisphosphoglycerate (*P*-glycerate) (Fig. 1), which are dependent on the ambient intracellular pH (9). Thus, measurement of the relative separation between these phosphate resonances [$\delta_{(P\text{-glycerate})}$] at different known absolute pH levels allows for a determination of intracellular pH values in experimental samples.

Packed erythrocytes were lysed by repeated freezing in liquid nitrogen and thawing, and after centrifugation aliquots of the hemolysate were titrated to pH levels between 7.0 and 7.4, with HCl or NaOH at atmospheric oxygen levels. Cell resealing did not appear to be a problem; the residual centrifuged pellet was not perturbed by pipetting/decanting of the hemolysate, and linewidths of *P*-glycerate resonances were not noticeably altered when pH was varied. ^{31}P NMR spectra were obtained at each pH value and the relative separation of the 3- and 2-phosphate resonances of *P*-glycerate were plotted against the pH value at which that spectrum was obtained. This titration curve was essentially linear within the pH range tested and was utilized to determine the intracellular pH values of unknowns. Scatter for a given sample was ≤ 1 Hz, corresponding to ≤ 0.02 pH unit.

The data were analyzed by one-way analysis of variance with a subsequent modified *t* test (Bonferroni) for the level of significance. Pearson correlation coefficients and student *t* tests were used for linear regression analysis and for paired analysis of experimental rat data. All results are expressed as means \pm SEM.

Table 1. Clinical and laboratory data

Group	<i>n</i>	BP, mm of Hg	Age, yr	δ/f	Ca^{2+} , meq/liter	$\delta_{\alpha\beta}^{\text{cell}}$, Hz	Free $\text{Mg}^{2+}_{\text{intracellular}}$, μM	$\delta_{(P\text{-glycerate})}$, Hz	$\text{pH}_{\text{intracellular}}$
NL	15	$131 \pm 5/78 \pm 3$	53 ± 4	10/5	2.44 ± 0.02	700.0 ± 0.9	257 ± 6.6	69.45 ± 0.51	7.28 ± 0.02
Hi BP	16	$174 \pm 7/106 \pm 3^*$	57 ± 4	7/9	2.49 ± 0.04	$706.5 \pm 1.2^\dagger$	$198 \pm 9.2^\dagger$	$73.81 \pm 0.87^\ddagger$	$7.17 \pm 0.02^\ddagger$
Hi BP-Rx	24	$152 \pm 5^\dagger/89 \pm 3^\dagger$	56 ± 2	12/12	2.47 ± 0.02	703.6 ± 0.9	221 ± 8.3	69.67 ± 0.71	7.27 ± 0.02

BP, blood pressure; NL, normotensive subjects; Hi BP, untreated hypertensive subjects; Hi BP-Rx, hypertensive subjects on drug therapy.

† Bonferroni significance level of 0.05 vs. NL.

‡ Bonferroni significance level of 0.01 vs. NL.

*Bonferroni significance level of 0.001 vs. NL.

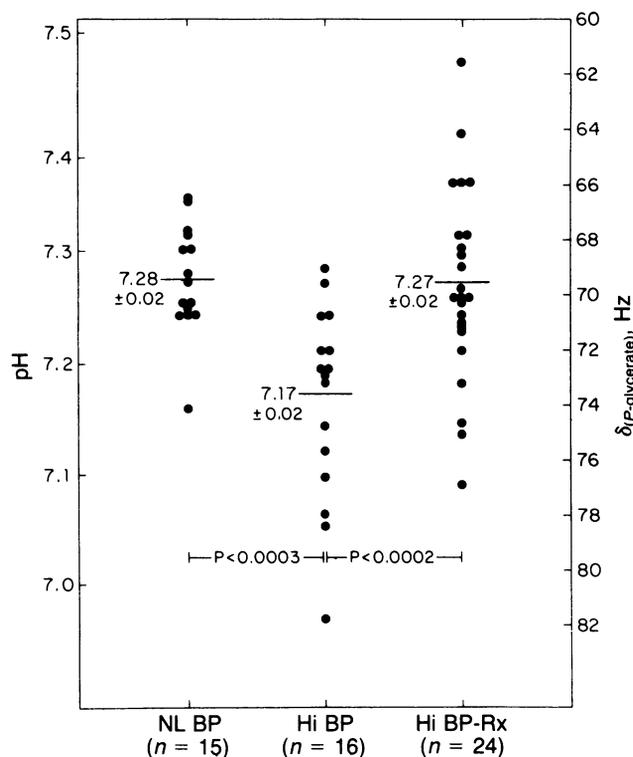


FIG. 2. Intracellular pH and $\delta_{(P\text{-glycerate})}$ values among patient groups. Mean values \pm SEM for each group are indicated. *P* values are based on pooled variance *t* tests modified for multiple comparisons (Bonferroni).

RESULTS

Clinical Studies. Clinical and laboratory data for human normotensive and hypertensive subjects are listed in Table 1. There were no significant age differences among the groups, and males and females were equally distributed among hypertensive groups (7/9 and 12/12), whereas there were more males among normotensive subjects (10/5). Systolic and diastolic blood pressures differed in each group from each other: normotensives, $131 \pm 5/78 \pm 3$ mm of Hg; untreated hypertensives, $174 \pm 7/106 \pm 3$ mm of Hg (significance level = 0.001/0.001 vs. normotensives); hypertensives on therapy, $152 \pm 5/89 \pm 3$ (significance level = 0.05/0.05 vs. normotensives; 0.05/0.001 vs. untreated hypertensives).

Relative chemical shift values for the α - and β -phosphate resonances of ATP ($\delta_{\alpha\beta}$) and for the 2- and 3-phosphate resonances of *P*-glycerate [$\delta_{(P\text{-glycerate})}$] and their derived quantities, intracellular free magnesium and intracellular pH, respectively, are also listed in Table 1. As in our smaller initial series (4), intracellular free magnesium values were clearly suppressed in untreated hypertensives compared with normotensive control subjects (198 ± 9.2 vs. 257 ± 6.6 μM ,

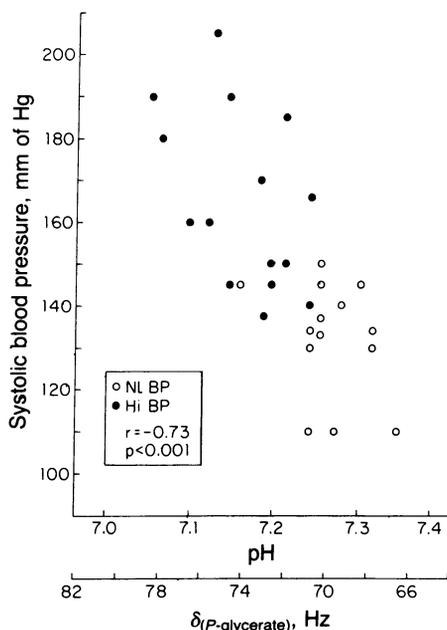


FIG. 3. Relationship of intracellular pH and $\delta_{(P\text{-glycerate})}$ values to systolic blood pressure in untreated NL and Hi BP. Regression analysis used the Pearson correlation coefficient and Student *t* test for level of significance.

$P < 0.005$). Similarly, continuous inverse relationships were observed between intracellular free magnesium and diastolic ($r = -0.73$, $P < 0.001$) and systolic ($r = -0.55$, $P < 0.01$) blood pressures.

As is shown in Fig. 2, intracellular pH values for normotensive control subjects averaged 7.28 ± 0.02 , a value similar to those reported utilizing other measurement techniques (12, 13). The average intracellular pH for untreated essential hypertensive subjects (7.17 ± 0.02) was significantly lower than either for normotensive control subjects (significance = 0.01) or for hypertensive subjects on antihypertensive therapy (7.27 ± 0.02 , significance = 0.01 vs. untreated hypertensives). Additionally, for all untreated individuals, strong continuous relationships were observed between intracellular pH and diastolic blood pressure ($r = -0.69$, $P < 0.001$) as well as between intracellular pH and systolic blood pressure ($r = -0.73$, $P < 0.001$) (Fig. 3). Intracellular pH was weakly and inversely related to serum ionized calcium ($r = -0.39$, $P < 0.05$) and was weakly and directly related to intracellular free magnesium ($r = 0.35$, $P < 0.05$).

To investigate the extent to which these differences in

intracellular pH might be determined by differences in extracellular pH, we measured pH in venous plasma of another series of untreated normotensive ($n = 20$) and essential hypertensive ($n = 26$) subjects. Samples were processed in a fashion identical to NMR-analyzed samples. Values obtained for extracellular pH in both groups were indistinguishable (NL = 7.478 ± 0.014 vs. Hi BP = 7.452 ± 0.010 , $P =$ not significant).

Experimental Hypertension. Table 2 displays blood pressure and ^{31}P NMR-derived intracellular pH values in normotensive UNx-NaCl rats, two series of volume-dependent hypertensive rats (deoxycorticosterone/NaCl), and renin-dependent, 2K-1C Goldblatt hypertensive rats. For each model, differing calcium intakes altered blood pressure, but not in a uniform manner (Table 2). Increased calcium intake lowered blood pressure in volume-loaded normotensive and hypertensive animals but exacerbated pressure in renin-dependent rats. Interestingly, there appears to be a close correspondence between the alterations of blood pressure and associated changes in intracellular pH. Though different absolute blood pressures were observed at similar pH levels among different rat models, for a given series of paired rat models, higher blood pressures were always associated with lower pH values, as depicted in Fig. 4.

DISCUSSION

This study utilized ^{31}P NMR spectroscopy to noninvasively assess intracellular pH in erythrocytes of nonhypertensive and hypertensive human subjects before and after drug therapy and in experimental rat models under different dietary salt and calcium intakes. The main findings were that (i) under the conditions utilized, NMR-derived intracellular pH values were remarkably similar to published values in the literature for erythrocytes, utilizing different methods of analysis; (ii) essential hypertensive subjects had significantly lower intracellular pH values compared with control NL; (iii) antihypertensive drug therapy sufficient to significantly lower blood pressure in hypertensive individuals also normalized intracellular pH; (iv) in salt-loaded normotensive and deoxycorticosterone/NaCl hypertensive rats, increased dietary calcium intake lowered blood pressure and elevated intracellular pH; and (v) in renin-dependent, 2K-1C Goldblatt hypertension, higher dietary calcium intake elevated blood pressure and lowered intracellular pH. Hence, under all of these conditions, blood pressure and intracellular pH were inversely related; the higher the pressure, the lower the intracellular pH. Altogether, these results suggest a linkage between the control of intracellular pH and the cellular pathophysiology of hypertension and may also, we believe, be relevant to understanding

Table 2. Blood pressure and laboratory data in experimental hypertension

Group	<i>n</i> *	Calcium diet†	Systolic BP, mm of Hg	$\delta_{(P\text{-glycerate})}$, Hz	pH _{intracellular}
UNx-NaCl	11	Low	133 ± 2.9‡	74.45 ± 0.56‡	7.15 ± 0.02‡
	11	High	124 ± 2.4	72.81 ± 0.52	7.19 ± 0.01
Deoxycorticosterone/NaCl§					
1	8	Low	186 ± 6‡	75.8 ± 0.8‡	7.12 ± 0.02‡
	7	High	156 ± 3	72.7 ± 0.75	7.20 ± 0.02
2	6	Moderate	227 ± 10‡	74.5 ± 0.5‡	7.15 ± 0.01‡
	9	High	205 ± 7.5	72.5 ± 1.25	7.20 ± 0.03
2K-1C¶	10	Low	146 ± 14‡	72.7 ± 0.75‡	7.20 ± 0.02‡
	8	High	178 ± 8	76.5 ± 0.7	7.10 ± 0.02

BP, blood pressure.

*Number of animals surviving at time of analysis.

†0.2% (low), 1.2% (moderate), or 1.8% (high) calcium by weight.

‡Significantly different ($P < 0.05$) vs. high calcium diet for each rat model.

¶2K-1C Goldblatt hypertension.

§Numbers 1 and 2 indicate two series of deoxycorticosterone/NaCl hypertensive rats.

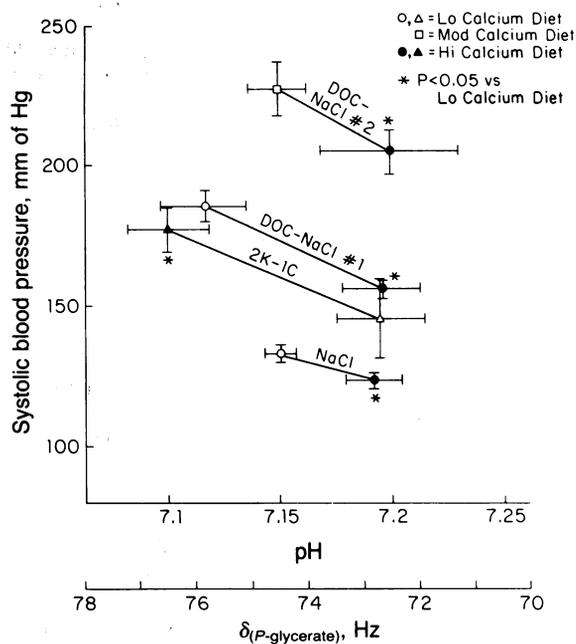


FIG. 4. Relationship of intracellular pH and $\delta_{(P\text{-glycerate})}$ values to systolic blood pressure in experimental hypertensive models on different dietary calcium intakes. DOC-NaCl #1 and #2, two series of deoxycorticosterone/NaCl hypertensive rats; 2K-1C, Goldblatt hypertensive rats; NaCl, UNx-NaCl rats.

the interaction of hypertension with abnormalities of intermediary metabolism, such as diabetes mellitus.

The measurement of intracellular pH has only recently provided reliable and reproducible values. Previous methods, employing direct pH measurements on cell lysates or introduction of pH indicator dyes, were insensitive and often not reproducible. However, other more recent techniques, such as radiolabeled weak acids (5,5-dimethyl 2,4-oxazolinedione) or weak bases (9-aminoacridine), pH microelectrodes, or ^{31}P NMR spectroscopy, provide values that are consistent and comparable with each other. Of these techniques, NMR methodology can detect small changes in pH, and it is the only technique that is noninvasive. In 1973, Moon and Richards (9) reported the use of NMR spectroscopy to measure intracellular pH in erythrocytes, basing their calculated pH values on pH-dependent differences in the chemical shifts of the phosphate resonances of *P*-glycerate. We have modified their procedure somewhat to make the results more biologically meaningful. We measured NMR spectra of experimental samples in heparinized blood at near physiologic pH (7.3–7.5), instead of at pH 6.7, in acid/citrate/dextrose blood as in their study, and without carbon monoxide treatment. Furthermore, we maintained oxygenation of hemoglobin with ambient air equilibration, which has been shown to result in $\delta_{(P\text{-glycerate})}$ values identical to those obtained with a physiologic gas mixture (12). To calculate intracellular pH values for a given $\delta_{(P\text{-glycerate})}$, we titrated erythrocyte hemolysates at different induced pH values rather than using solutions of pure *P*-glycerate, to preserve as much as possible the normal intracellular environment. As a result, the intracellular pH that we measured in normotensive humans, 7.28 ± 0.02 , is in close agreement with other NMR measurements (7.29 ± 0.08 ; ref. 12) and with pH values obtained using 5,5-dimethyl 2,4-oxazolinedione or $(\text{NH}_4)_2\text{SO}_4$ at the same external pH, where values of 7.27 and 7.25, respectively, were reported (13).

The observed alterations in intracellular pH reported here may also reflect altered cellular handling of other ions as well. For instance, increased Na^+/H^+ exchange has been reported

in hypertension (14). This is consistent with findings in cardiac cells where decreased intracellular pH stimulated Na^+/H^+ exchange (15, 16). Thus, changes in pH may help to explain the increased intracellular sodium reported in hypertensive cells (2). We have previously utilized ^{31}P NMR spectroscopy to study intracellular free magnesium levels in a smaller series of normotensive, untreated and treated essential hypertensive individuals and found a tight, inverse correlation between the height of the blood pressure and the free magnesium concentration. This was also apparent in the larger series of subjects reported here, confirming our earlier report and demonstrating that with changes of blood pressure higher intracellular free magnesium levels are associated with a higher intracellular pH. Similarly, in experimental hypertension, regardless of whether dietary calcium loading increased or decreased blood pressure, higher blood pressures were associated with suppression of intracellular free magnesium levels (8). Therefore, the lower intracellular pH found in experimental rat models with higher blood pressures also implies a linkage between cellular hydrogen ion and magnesium homeostasis. Clearly, however, factors other than pH must also be involved in determining blood pressure, considering the same pH levels observed at different blood pressures in different animal models (Fig. 4).

Weak relationships were observed between serum ionized calcium and intracellular pH in clinical hypertension. A $\text{Ca}^{2+}/\text{H}^+$ exchange pump has been identified in erythrocytes (17), and, although its physiological relevance is still uncertain, its operation, exchanging extracellular calcium for intracellular H^+ , would lead to intracellular calcium accumulation, as reported in hypertension (3), and to lowering of intracellular pH, as observed here. However, in renin-independent, Goldblatt hypertension, despite no significant change in serum ionized calcium on low vs. high dietary calcium intake (8), blood pressure and pH were still significantly and reciprocally related. On the other hand, the relation of cytosolic free calcium and other intracellular minerals, such as sodium and potassium, to pH regulation is still unclear and may be better defined in the future utilizing similar NMR techniques (18).

The question arises, What is the possible clinical significance of our reported findings? The concepts must be considered, (i) that the small changes in intracellular pH measured here may themselves influence peripheral vasoconstriction and thus blood pressure; (ii) that intracellular pH changes result from other primary changes in metabolism associated with abnormalities of blood pressure; or (iii) alternatively, both, that the control of intracellular pH and the control of vasoconstrictor smooth muscle tone are each linked. Indeed, lowering intracellular pH can increase constrictor tone, in association with simultaneous increases in cytosolic free calcium, as has been reported for barnacle and frog muscle fibers (19, 20). Conversely, induced elevations of cytosolic free calcium in sheep purkinje fibers and whole rat hearts are routinely associated with a decrease in intracellular pH (21, 22). Given the tight linkage of blood pressure and cytosolic free calcium (3), this apparent mutual interaction between intracellular pH and cytosolic free calcium would lead us to expect that changes in intracellular pH ought to routinely be present in clinical abnormalities of blood pressure control, as we have observed here. On the other hand, our data cannot help us decide whether the changes in intracellular pH measured in this study were primarily involved in causing the hypertension or were reflective of it. Even if the decreased intracellular pH were a consequence of the hypertension, it might still, in turn, contribute to the blood pressure. Thus, decreasing pH dramatically decreases calcium-calmodulin interactions, perhaps providing a brake on the increased vasoconstriction resulting from calcium-calmodulin activation of myosin-like chain kinase (23).

The hypothesis above, that blood pressure and the control of intracellular pH are mutually interdependent, may also help explain how hypertension relates at the subcellular level to other disease syndromes long appreciated clinically to occur concomitantly with hypertension. One such example for which our hypothesis may apply is the well-known increased incidence of diabetes mellitus in hypertensive individuals and of hypertension in already diabetic subjects. It is now appreciated that insulin directly raises intracellular pH (24). It is thus intriguing to wonder whether in diabetes a "primary" lowering of intracellular pH consequent to peripheral lack of insulin or of cellular insulin resistance might induce or at least predispose to increases in cytosolic free calcium and thereby to increased peripheral vasoconstriction and elevated blood pressure (25). Interestingly, in a well-studied large population, it was early in the clinical course of diabetes, when only chemically abnormal glucose metabolism was detected, that clearly increased blood pressures were observed (26). Conversely, in an already hypertensive individual, a "secondary" lowering of intracellular pH may predispose to or even be involved in the peripheral insulin resistance preceding overt diabetes mellitus, especially in the adult-onset, type II, form. A similar pattern of thought might also be used to help explain the relation of obesity to hypertension. These and other exciting possibilities raised by our observations should be the focus of future research.

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