Decreased bone carbonate content in response to metabolic, but not respiratory, acidosis

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Bushinsky, David A., Brian C. Lam, Rita Nespeca, Nelson E. Sessler, and Marc D. Grynpas. Decreased bone carbonate content in response to metabolic, but not respiratory, acidosis. *Am. J. Physiol.* 265 (*Renal Fluid Electrolyte Physiol.* 34): F530-F536, 1993.—In vitro cultured neonatal mouse calvariae release calcium and buffer the medium proton concentration in response to a decrease in the medium pH caused by a reduction in bicarbonate concentration ($[HCO_3^-]$), a model of metabolic acidosis, but not to an equivalent decrease in pH caused by an increase in the partial pressure of carbon dioxide (P_{CO_2}), a model of respiratory acidosis. We have postulated that the medium is in equilibrium with the carbonated apatite in bone. To determine whether bone carbonate is depleted during models of acidosis, we cultured calvariae in control medium (pH $\approx$ 7.4, $P_{CO_2} \approx 43$, $[HCO_3^-] \approx 26$) or in medium in which the pH was equivalently reduced by either a decrease in $[HCO_3^-]$ (metabolic acidosis, pH $\approx$ 7.1, $[HCO_3^-] \approx 13$) or an increase in P_{CO_2} (respiratory acidosis, pH $\approx$ 7.1, $P_{CO_2} \approx 86$) and determined net calcium flux (J_{Ca}) and bone carbonate content. We found that compared with control, after 3, 24, and 48 h there was a decrease in bone carbonate content during metabolic but not during respiratory acidosis. Compared with control, at 3 h J_{Ca} increased with both respiratory and metabolic acidosis; however, at 24 and 48 h J_{Ca} increased only with metabolic acidosis. J_{Ca} was correlated inversely with percent bone carbonate content in control and metabolic acidosis at all time periods studied ($r = -0.809$, $n = 23$, $P < 0.001$). Thus a model of metabolic acidosis appears to increase J_{Ca} from bone, perhaps due to the low $[HCO_3^-]$ inducing bone carbonate dissolution. However, a model of respiratory acidosis does not substantially alter J_{Ca}, perhaps because the elevated P_{CO_2} does not allow bone carbonate dissolution despite the reduced pH.

calvaria; calcium; bicarbonate; carbon dioxide; pH

ACUTE AND CHRONIC METABOLIC acidosis alter the bone mineral. On the basis of the reduction in systemic pH and bicarbonate concentration ($[HCO_3^-]$) observed after the administration of an acute acid load ~60% of the administered protons appear to be buffered outside of the extracellular fluid (36) by both soft tissues (28) and bone (5, 6, 10, 12). During chronic metabolic acidosis secondary to renal failure the systemic pH remains stable, although reduced, despite progressive proton retention, indicating that there are large stores of buffer, presumably contained within the bone mineral, being depleted (9). Experimental chronic metabolic acidosis leads to an increase in urine calcium excretion, without an increase in intestinal calcium absorption, leading to net negative calcium balance (27). Because the vast majority of body calcium is contained within the bone mineral (9), this suggests that bone is being depleted during acidosis, a hypothesis which has been supported by in vivo studies (1).

Respiratory acidosis appears to have less of an effect on the bone mineral. In some (16) but not all (26, 33, 34) studies there appears to be an increase in urinary calcium excretion, although any increase is far less than that during metabolic acidosis (16). Serum calcium may increase during in vivo respiratory acidosis (26, 32). The possible increase in urine calcium excretion and/or serum calcium concentration may indicate an increase in bone mineral dissolution.

Studies of cultured bone during in vitro models of metabolic and respiratory acidosis have led to a greater understanding of how protons, bicarbonate, and CO_2 affect the bone mineral (5–8, 10–15, 17, 25). We have previously shown that, during short- and long-term incubation of neonatal mouse calvariae in a reduced bicarbonate medium, a model of metabolic acidosis, there is greater calcium efflux than during incubation in similarly acidic medium produced by an increase in the partial pressure of carbon dioxide (P_{CO_2}), a model of respiratory acidosis (6, 7, 13–15, 17). In addition, during short-term (3 h) metabolic acidosis, but not respiratory acidosis, there is buffering of the additional protons by cultured bone (6). During these short-term incubations the calcium efflux during metabolic acidosis is due to an alteration in the physicochemical driving forces for mineral accretion and dissolution (8, 15), whereas over longer time frames there is stimulation of cell-mediated resorption as well (7, 25).

The mineral phase solubilized during acidosis has not been determined. In vivo studies have shown a loss of bone carbonate during metabolic acidosis (2, 4, 23). An early study of Irving and Chute (23) demonstrated a decrease in skeletal carbonate after several days of metabolic acidosis. Burnell (4) demonstrated that there was a loss of bone carbonate after 5–10 days of metabolic acidosis, and Bettice (2) determined that the decrease in bone carbonate during metabolic acidosis was correlated with the fall in extracellular $[HCO_3^-]$. However, these in vivo studies are difficult to perform and interpret. The environment of the bone in vivo cannot be precisely controlled. Hyponatremia occurs after an acid load, and hyponatremia has an independent effect on the store of bone carbonate (3). The respiratory response to metabolic acidosis lowers the P_{CO_2}, which also has an independent effect on bone carbonate content (30). In vitro we have shown that, when neonatal mouse calvariae are placed in organ culture, the medium appears to be in equilibrium with the calcium and carbonate within the mineral, probably in the form of carbonated apatite (11). However, the effect of metabolic and/or respiratory acidosis on bone carbonate content has not been determined in vitro.

The purpose of the current study is to determine whether in vitro models of metabolic and respiratory

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acidosis cause dissolution of bone carbonate. We found that 48 h of culture in medium modeling metabolic, but not respiratory, acidosis led to a decrease in the carbonate content of bone.

METHODS

Culture procedures. Neonatal (4–5 day old) CD-1 mice (Charles River, Wilmington, MA) were killed, their calvariae were removed by dissection, and adherent cartilaginous material was trimmed (5–8, 10–15, 17, 25). Exactly 2.8 ml of culture medium [Dulbecco's modified Eagle's medium with 4.5 g/l glucose containing heat-inactivated (1 h, 56°C) horse serum (15%), L-glutamine (2.8 mM), sodium heparin (10 U/ml), and potassium penicillin (100 U/ml)] were preincubated at a fixed chosen P_{CO_2}, 37°C for 3 h, in 35-mm Petri dishes. The two incubators used (both model 3158; Forma Scientific, Marietta, OH) maintained a constant temperature (±0.2°C) and a constant P_{CO_2} (±0.1%) at an ambient O_2 concentration of 21%. One milliliter of medium was then removed to determine preincubation pH, P_{CO_2}, and calcium concentration, and two calvariae were placed on a stainless steel wire grid in each dish. Experimental and control cultures were performed in parallel and in random order. Total bone content in each culture dish was controlled by using pups that were the same age and size, by using a standardized dissection procedure, and by placing two bones in each dish. The bones were incubated for 3, 24, or 48 h at 37°C, after which a second 1-ml sample was taken and analyzed for pH, P_{CO_2}, and total calcium. The calvariae were then removed from the incubation medium, rapidly washed with deionized distilled water three times, and then lyophilized until dry (at least 6 h) for determination of bone carbonate content.

Experimental groups. Calvariae were divided into three groups: control, metabolic acidosis and respiratory acidosis. In the control group calvariae were cultured in unaltered medium (pH ≈ 7.4) at a P_{CO_2} of 40 mmHg. In the metabolic acidosis group the medium pH was lowered to ≈7.1 by the addition of 0.010 ml of concentrated HCl to lower the medium [HCO_3^-]. In the respiratory acidosis group medium pH was lowered to ≈7.1 by increasing the P_{CO_2} of the incubator and thus the medium. In all cultures in which HCl was not added to the medium, 0.010 ml of deionized distilled H_2O was added in its place.

Viability of calvariae in chronic acidic culture. Neonatal mouse calvariae are routinely cultured for 96–168 h and remain viable and responsive to calcitropic agents, even in acidic medium (7, 20, 35). To confirm the viability of calvariae cultured under the conditions of this study, we determined the rate of protein synthesis measured as [^{3}H]proline incorporation in 20 calvariae during 45–48 h of culture.

Four groups of calvariae were dissected and cultured for 48 h. In the control group calvariae were cultured in unaltered medium (pH ≈ 7.4) at a P_{CO_2} of 40 mmHg. In the metabolic acidosis group the medium pH was lowered to ≈7.1 by the addition of 0.010 ml of concentrated HCl to lower the medium [HCO_3^-]. In the respiratory acidosis group medium pH was lowered to ≈7.1 by increasing the P_{CO_2} of the incubator and thus the medium. In the dead group the calvariae were killed with successive freeze-thaw cycles at *time 0* and then cultured for 48 h in unaltered control medium. A fifth group of calvariae was freshly dissected 3 h before the conclusion of the experiment and cultured at pH ≈ 7.4 for 3 h. In all cultures in which HCl was not added to the medium, 0.010 ml of deionized distilled H_2O was added in its place.

[^{3}H]proline (10 μCi) was added to each dish (2 calvariae in 1.8 ml) during the last 3 h of culture. At the conclusion of the 3-h pulse label, dishes were placed on ice. Each calvaria was then removed, washed in three successive 50-ml volumes of ice-cold phosphate-buffered saline, and homogenized in 2.5 ml of 10% trichloroacetic acid (TCA). Aliquots were removed for total counts and assay of total protein by the method of Lowry (29). The remaining homogenate was filtered on glass filters (GF/C; Whatman, Clifton, NJ) and washed three times with ice-cold 10% TCA and once with 95% ethanol. Filters were dried for 8 h and then counted in a liquid scintillation counter. TCA-precipitable counts were taken as a measure of radioactivity incorporated into protein. The difference between total counts and TCA-precipitable counts provided a measure of uptake of [^{3}H]-proline (24).

Incorporation of [^{3}H]proline into TCA-precipitable material was greater in fresh live calvariae than any of the other groups ($P < 0.05$ vs. each of the other groups; Fig. 1). There was no difference in incorporation in the calvariae in the control group compared with the metabolic or respiratory groups, which were not different from each other. Dead calvariae incorporated less [^{3}H]proline than any of the other four groups ($P < 0.01$ vs. each of the other groups). These results indicate that calvariae cultured under all conditions utilized in this study (control, metabolic, and respiratory acidosis) remain viable for the duration of the experiment.

Carbonate content. Bone carbonate content was determined using an automated CO_2 coulometer (model 5012; UIC, Coulometrics) (19). In the acidification module, 2 N perchloric acid was added to the calvaria samples, and carbonate was released as CO_2. A stream of air, passed through a potassium hydroxide scrubber to remove atmospheric CO_2, carried the evolved CO_2 into the coulometer at a flow rate of 100 cm^3/min. The coulometer cell contained a solution of monoethanolamine (MEA) and thymolphthalein indicator (UIC, Coulometrics), a platinum cathode, and a silver anode. The cell is positioned between a light source and a photodetector. The CO_2 in the carrier gas reacted with the MEA to form hydroxyethylcarbonic acid, indicated by a color change. The titration current was automatically activated to electrochemically generate base until the solution was neutralized, as observed by photodetection. The

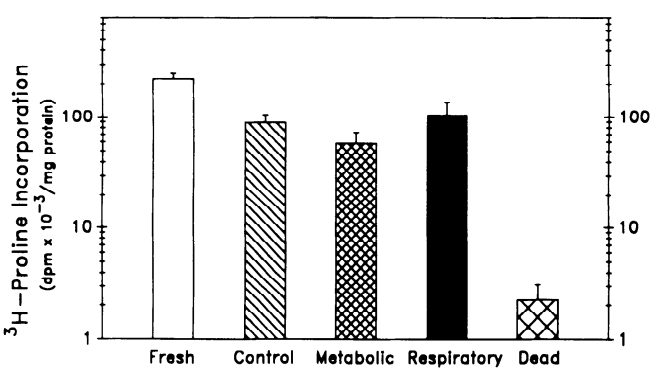

Fig. 1. Effects of culture in control and metabolic and respiratory acidoses on [^{3}H]proline incorporation in neonatal mouse calvariae. Fresh calvariae were dissected 3 h before the conclusion of experiment and incubated at pH ≈ 7.4 for 3 h; control calvariae were cultured for 48 h in unaltered medium (pH ≈ 7.4) at a partial pressure of carbon dioxide (P_{CO_2}) of 40 mmHg; metabolic acidosis calvariae were cultured for 48 h in medium in which pH was lowered to ≈7.1 by addition of 0.010 ml of concentrated HCl to lower medium bicarbonate concentration [HCO_3^-]; respiratory acidosis calvariae were cultured for 48 h in medium in which pH was lowered to ≈7.1 by increasing the P_{CO_2} of the incubator and thus the medium; dead calvariae were killed with successive freeze-thaw cycles at *time 0* and then cultured for 48 h in unaltered control medium. Incorporation of [^{3}H]proline into trichloroacetic acid (TCA)-precipitable material was greater in fresh live calvariae than any of the other groups ($P < 0.05$ vs. each of the other groups). There was no difference in incorporation in the calvariae in control group compared with metabolic or respiratory groups, which were not different from each other. Dead calvariae incorporated less [^{3}H]proline than any of the other 4 groups ($P < 0.01$ vs. each of the other groups).

Table 1. *Initial and final medium conditions*

	n	Initial				Final		
		Medium pH	P_{CO_2}, mmHg	$[HCO_3^-]$, meq/l	[Ca], mM	Medium pH	P_{CO_2}, mmHg	$[HCO_3^-]$, meq/l
3-h Incubation								
Control	8	7.404±0.005	42.9±0.9	26.4±0.3	1.87±0.01	7.392±0.004	43.4±0.7	26.0±0.2
Metabolic	8	7.115±0.008*	42.1±0.9	12.9±0.1*	1.86±0.01	7.115±0.006*	43.3±0.7	13.3±0.1*
Respiratory	8	7.101±0.002*	86.3±0.5*†	25.6±0.2†	1.86±0.01	7.092±0.007*†	86.8±1.3*†	25.2±0.3*†
24-h Incubation								
Control	8	7.407±0.006	41.8±0.7	26.0±0.2	1.85±0.02	7.267±0.005	43.6±0.5	19.4±0.3
Metabolic	8	7.110±0.008*	41.5±0.6	12.6±0.1*	1.85±0.01	7.005±0.009*	42.3±0.6	10.0±0.2*
Respiratory	8	7.098±0.001*	86.9±0.4*†	25.6±0.2†	1.85±0.01	7.020±0.005*	86.9±0.7*†	21.2±0.3*†
48-h Incubation								
Control	11	7.423±0.003	40.4±0.4	26.1±0.1	1.87±0.01	7.097±0.010	41.2±0.6	12.2±0.3
Metabolic	11	7.117±0.005*	40.0±0.4	12.3±0.2*	1.86±0.02	6.857±0.009*	40.4±0.6	6.6±0.1*
Respiratory	11	7.103±0.001*	84.1±0.9*†	25.1±0.2*†	1.87±0.01	6.917±0.005*†	85.1±0.5*†	16.2±0.2*†

Values are means ± SE; *n*, no. of pairs of calvariae in each group. Initial values were obtained before incubation; final values were obtained at conclusion of incubation; P_{CO_2}, partial pressure of carbon dioxide; $[HCO_3^-]$, medium bicarbonate concentration; [Ca], medium calcium concentration; control, calvariae cultured in unaltered medium; metabolic, calvariae cultured in medium acidified by a decrease in $[HCO_3^-]$; respiratory, calvariae cultured in medium acidified by an increase in P_{CO_2}. * Different from control same time period, $P < 0.05$. † Different from metabolic same time period, $P < 0.05$.

coulometer determines carbon content based on Faraday's law; each faraday of electricity expended is equivalent to 1 gram equivalent weight of CO_2 titrated. The carbon content was expressed as percent carbonate by weight.

Flux calculations. Net calcium flux (J_{Ca}) was calculated as $V_m \times ([Ca]_{final} - [Ca]_{initial})$ where V_m is the medium volume (1.8 ml), and $[Ca]_{final}$ and $[Ca]_{initial}$ are the final and initial medium calcium concentrations, respectively (5–8, 10–15, 17, 25). A positive value indicates movement of the ion from the bone into the medium, and a negative value indicates movement from the medium into the bone. Values are expressed as nanomoles per bone per 24 h.

Conventional measurements. pH and P_{CO_2} were measured with a blood-gas analyzer (model ABL 30; Radiometer, Copenhagen, Denmark). Medium $[HCO_3^-]$ was calculated from the pH and P_{CO_2} using the Henderson-Hasselbalch equation as we have described previously (5–7, 10–12). Total medium calcium concentration was measured by automatic fluorometric titration, which we have shown to give results similar to those obtained using atomic absorption spectroscopy (8).

Statistics. All tests of significance were calculated using analysis of variance with Bonferroni correction for multiple comparisons and linear regression by groups (BMDP; University of California at Los Angeles, CA) on an IBM Personal System/2 computer (model 90 XP 486; IBM, Armonk, NY). Values are means ± SE; $P < 0.05$ was considered significant.

RESULTS

Three-hour incubations. Compared with control, with a model of metabolic acidosis, the lower initial medium $[HCO_3^-]$, and with a model of respiratory acidosis, the increased initial P_{CO_2}, each resulted in a decrease in the initial medium pH (Table 1). There was no difference in initial medium pH between metabolic and respiratory acidosis.

Compared with control, J_{Ca} increased with both metabolic and respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis (Fig. 2). J_{Ca} was correlated inversely with initial medium pH in control and metabolic acidosis ($J_{Ca} = -350.0 \times$ initial medium pH + 2,584; $r = -0.953$, $n = 16$, $P < 0.001$) and in control and respiratory acidosis ($J_{Ca} = -252.4 \times$ initial medium pH + 1,862; $r = -0.894$, $n = 16$, $P < 0.001$) (Fig. 3, *top*).

Compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was lower in metabolic than in respiratory acidosis (Fig. 4). Percent bone carbonate was correlated directly with initial medium pH in control and metabolic acidosis (percent bone carbonate = $0.839 \times$ initial medium pH − 3.67; $r = 0.822$, $n = 8$, $P = 0.012$) but

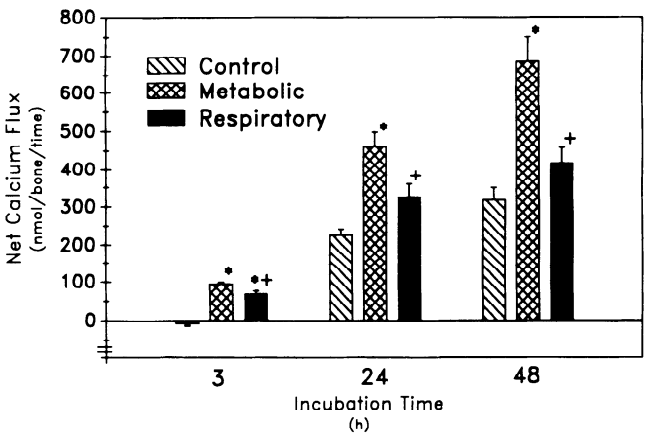

Fig. 2. Net calcium flux (J_{Ca}, nmol·bone^{-1}·time^{-1}) in relation to cultured neonatal mouse calvariae at 3 different incubation periods. Values are means ± SE; see Table 1 for corresponding number of experiments for each group. A positive value indicates movement of calcium from bone into medium, and a negative value indicates the opposite. Control calvariae were cultured in unaltered medium; metabolic acidosis calvariae were incubated in medium in which initial medium pH was lowered to ≈ 7.1 by addition of concentrated HCl to lower initial medium $[HCO_3^-]$; respiratory acidosis calvariae were cultured in medium in which initial medium pH was lowered to ≈7.1 by increasing the P_{CO_2} of the incubator and thus the medium. * Different from control same time period, $P < 0.05$. † Different from metabolic acidosis same time period, $P < 0.05$. Over 3 h, compared with control, J_{Ca} increased with metabolic and with respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis. Over 24 h, compared with control, J_{Ca} increased with metabolic but not with respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis. Over 48 h, compared with control, J_{Ca} increased with metabolic but not with respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis.

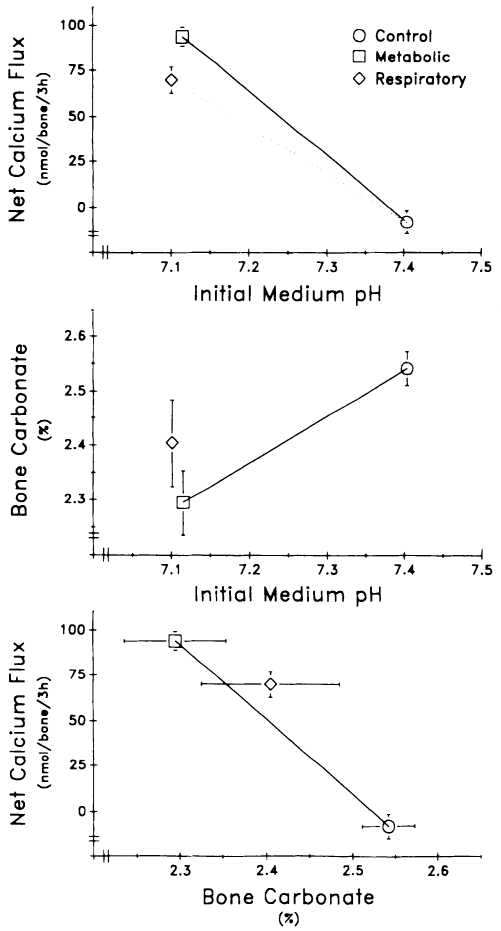

Fig. 3. *Top*: J_{Ca} (nmol·bone^{-1}·3 h^{-1}) as a function of initial medium pH for neonatal mouse calvariae incubated for 3 h in neutral pH medium (control, open circles) or acidic pH medium produced by a reduction in initial medium [HCO_3^-] (metabolic acidosis, open squares) or by an increase in initial medium P_{CO_2} (respiratory acidosis, open diamonds). Values are means ± SE (SE for some values are less than size of symbol) for 8 pairs of calvariae in each group. J_{Ca} was correlated inversely with initial medium pH in control and metabolic acidosis ($r = -0.953$, $n = 16$, $P < 0.001$) and in control and respiratory acidosis ($r = -0.894$, $n = 16$, $P < 0.001$). *Middle*: bone carbonate (%) as a function of initial medium pH for neonatal mouse calvariae incubated for 3 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was correlated directly with initial medium pH in control and in metabolic acidosis ($r = 0.822$, $n = 8$, $P = 0.012$) but not in control and respiratory acidosis ($r = 0.586$, $n = 8$, $P > 0.05$). *Bottom*: J_{Ca} (nmol·bone^{-1}·3 h^{-1}) as a function of bone carbonate (%) for neonatal mouse calvariae incubated for 3 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was correlated inversely with J_{Ca} in control and metabolic acidosis ($r = -0.821$, $n = 8$, $P = 0.012$) but not in control and respiratory acidosis ($r = -0.632$, $n = 8$, $P > 0.05$).

not in control and respiratory acidosis ($r = 0.586$, $n = 8$, $P > 0.05$) (Fig. 3, *middle*).

Percent bone carbonate was correlated inversely with J_{Ca} in control and metabolic acidosis (percent bone carbonate = $-294 \times J_{Ca} + 7.60$; $r = -0.821$, $n = 8$, $P = 0.012$) but not in control and respiratory acidosis ($r = -0.632$, $n = 8$, $P > 0.05$) (Fig. 3, *bottom*).

Twenty-four-hour incubations. Compared with control, with metabolic acidosis, the lower initial medium [HCO_3^-], and with respiratory acidosis, the increased initial medium P_{CO_2}, each resulted in a decrease in initial medium pH (Table 1). There was no difference in initial medium pH between metabolic and respiratory acidosis.

Compared with control, J_{Ca} increased with metabolic but not with respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis (Fig. 2). J_{Ca} was correlated inversely with initial medium pH in control and metabolic acidosis ($J_{Ca} = -798 \times$ initial medium pH + 6,135; $r = -0.856$, $n = 16$, $P < 0.001$) and in control and respiratory acidosis ($J_{Ca} = -327 \times$ initial medium pH + 2,645; $r = -0.591$, $n = 16$, $P = 0.016$) (Fig. 5, *top*).

Compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was less in metabolic than in respiratory acidosis (Fig. 4). Percent bone carbonate was correlated directly with initial medium pH in control and metabolic acidosis (percent bone carbonate = $1.25 \times$ initial medium pH − 6.85; $r = 0.818$, $n = 7$, $P = 0.024$) but not in control and respiratory acidosis ($r = -0.476$, $n = 8$, $P > 0.05$) (Fig. 5, *middle*).

Percent bone carbonate was not correlated with J_{Ca} in control and metabolic acidosis ($r = -0.681$, $n = 7$, $P \geq 0.05$) nor in control and respiratory acidosis ($r = 0.588$, $n = 8$, $P > 0.05$) (Fig. 5, *bottom*).

Forty-eight-hour incubations. Compared with control, with metabolic acidosis, the lower initial medium [HCO_3^-], and with respiratory acidosis, the increased initial medium P_{CO_2}, each resulted in a decrease in initial medium pH (Table 1). There was no difference in initial medium pH between metabolic and respiratory acidosis.

Compared with control, J_{Ca} increased with metabolic but not with respiratory acidosis, and J_{Ca} was greater in metabolic than in respiratory acidosis (Fig. 2). J_{Ca} was

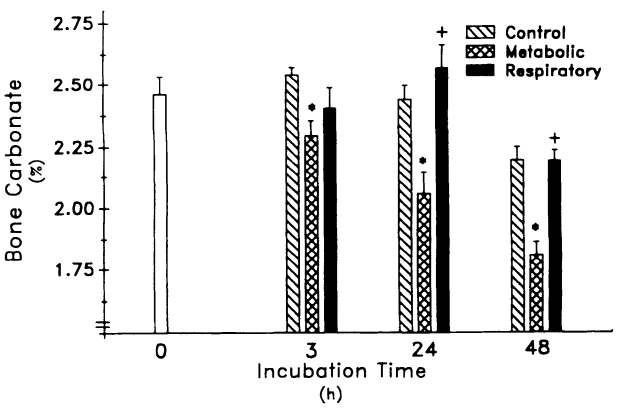

Fig. 4. Bone carbonate (%) of neonatal mouse calvariae at conclusion of incubation at 3 different time periods. Values are means ± SE; see Table 1 for corresponding number of experiments for each group. Control calvariae were cultured in unaltered medium; metabolic acidosis calvariae were incubated in medium in which initial medium pH was lowered to ≈7.1 by addition of concentrated HCl to lower initial medium [HCO_3^-]; respiratory acidosis calvariae were cultured in medium in which initial medium pH was lowered to ≈7.1 by increasing the incubator and thus the initial medium P_{CO_2}. * Different from control same time period, $P < 0.05$. † Different from metabolic acidosis same time period, $P < 0.05$. At conclusion of the 3-h time period, compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was lower in metabolic than in respiratory acidosis. At conclusion of the 24-h time period, compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was less in metabolic than respiratory acidosis. At conclusion of the 48-h time period, compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was less in metabolic than in respiratory acidosis.

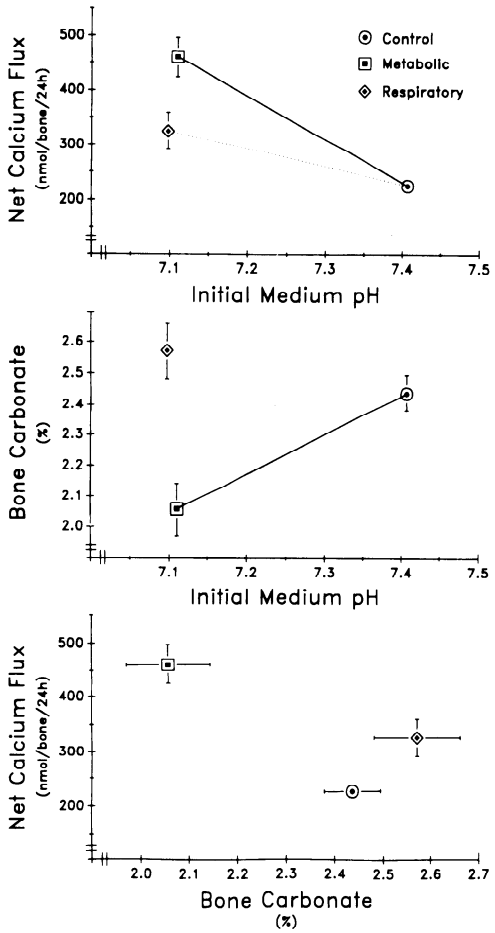

Fig. 5. *Top*: J_{Ca} (nmol·bone^{-1}·24 h^{-1}) as a function of initial medium pH for neonatal mouse calvariae incubated for 24 h in neutral pH medium (control, circles) or acidic pH medium produced by a reduction in initial medium [HCO_3^-] (metabolic acidosis, squares) or by an increase in initial medium P_{CO_2} (respiratory acidosis, diamonds). Values are means ± SE (SE for some values are less than size of symbol) for 8 pairs of calvariae in each group. J_{Ca} was correlated inversely with initial medium pH in control and metabolic acidosis ($r = -0.856$, $n = 16$, $P < 0.001$) and in control and respiratory acidosis ($r = -0.591$, $n = 16$, $P = 0.016$). *Middle*: bone carbonate (%) as a function of initial medium pH for neonatal mouse calvariae incubated for 24 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was correlated directly with initial medium pH in control and metabolic acidosis ($r = 0.818$, $n = 7$, $P = 0.024$) but not in control and respiratory acidosis ($r = -0.476$, $n = 8$, $P > 0.05$). *Bottom*: J_{Ca} (nmol·bone^{-1}·24 h^{-1}) as a function of bone carbonate (%) for neonatal mouse calvariae incubated for 3 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was not correlated with J_{Ca} in control and metabolic acidosis ($r = -0.681$, $n = 7$, $P \geq 0.05$) or in control and respiratory acidosis ($r = 0.588$, $n = 8$, $P > 0.05$).

correlated inversely with initial medium pH in control and metabolic acidosis ($J_{Ca} = -1{,}211 \times$ initial medium pH + 9,305; $r = -0.760$, $n = 22$, $P < 0.001$) and in control and respiratory acidosis ($J_{Ca} - 329 \times$ initial medium pH + 2,754; $r = -0.422$, $n = 22$, $P = 0.05$) (Fig. 6, *top*).

Compared with control, percent bone carbonate fell with metabolic but not with respiratory acidosis, and percent bone carbonate was less in metabolic than respiratory acidosis (Fig. 4). Percent bone carbonate was correlated directly with initial medium pH in control and metabolic acidosis (percent bone carbonate = $1.32 \times$ initial medium pH − 7.57; $r = 0.896$, $n = 8$, $P = 0.003$) but not in control and respiratory acidosis ($r = 0.009$, $n = 8$, $P > 0.05$) (Fig. 6, *middle*).

Percent bone carbonate was correlated inversely with J_{Ca} in control and metabolic acidosis (percent bone carbonate = $-904 \times J_{Ca} + 2{,}343$; $r = -0.714$, $n = 8$, $P < 0.05$) but not in control and respiratory acidosis ($r = -0.023$, $n = 8$, $P > 0.05$) (Fig. 6, *bottom*).

All incubations. In all incubations combined percent bone carbonate was correlated inversely with J_{Ca} in control and metabolic acidosis (percent bone carbonate = $-822 \times J_{Ca} + 2{,}124$; $r = -0.809$, $n = 23$, $P < 0.001$) (Fig.

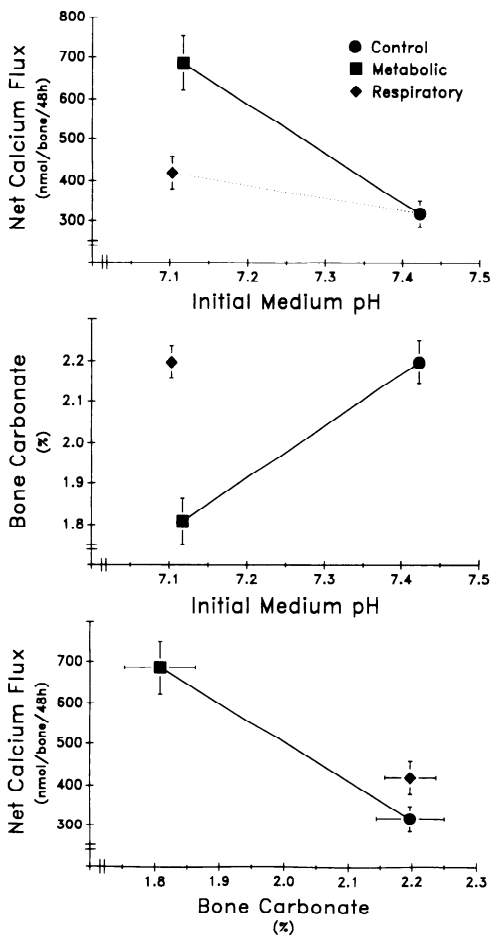

Fig. 6. *Top*: J_{Ca} (nmol·bone^{-1}·48 h^{-1}) as a function of initial medium pH for neonatal mouse calvariae incubated for 48 h in neutral pH medium (control, closed circles) and acidic pH medium produced by a reduction in initial medium [HCO_3^-] (metabolic acidosis, closed squares) or by an increase in the initial medium P_{CO_2} (respiratory acidosis, closed diamonds). Values are means ± SE (SE for some values are less than the size of the symbol) for 11 pairs of calvariae in each group. J_{Ca} was correlated inversely with initial medium pH in control and metabolic acidosis ($r = -0.760$, $n = 22$, $P < 0.001$) and in control and respiratory acidosis ($r = -0.422$, $n = 22$, $P = 0.05$). *Middle*: bone carbonate (%) as a function of initial medium pH for neonatal mouse calvariae incubated for 24 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was correlated directly with initial medium pH in control and metabolic acidosis ($r = 0.896$, $n = 8$, $P = 0.003$) but not in control and respiratory acidosis ($r = 0.009$, $n = 8$, $P > 0.05$). *Bottom*: net calcium flux J_{Ca} (nmol·bone^{-1}·48 h^{-1}) as a function of bone carbonate (%) for neonatal mouse calvariae incubated for 3 h in control and in metabolic and respiratory acidosis. Percent bone carbonate was correlated inversely with J_{Ca} in control and in metabolic acidosis ($r = -0.714$, $n = 8$, $P < 0.05$) but not in control and respiratory acidosis ($r = -0.023$, $n = 8$, $P > 0.05$).

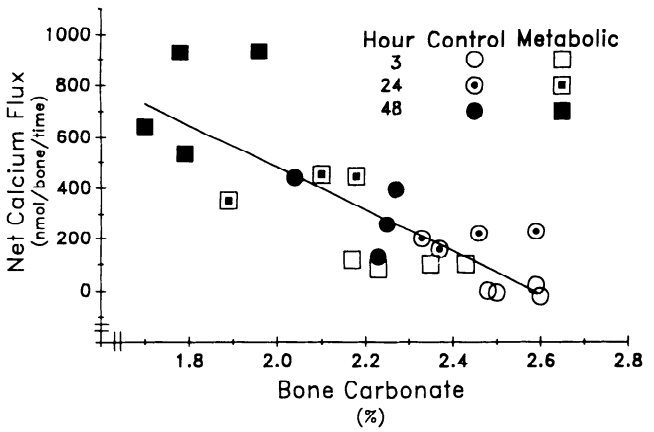

Fig. 7. J_{Ca} (nmol·bone^{-1}·time^{-1}) as a function of bone carbonate (%) for neonatal mouse calvariae incubated for 3, 24, or 48 h in control medium (circles, calvariae cultured in unaltered medium) or in metabolic acidosis (squares, calvariae incubated in medium in which initial medium pH was lowered to ≈7.1 by addition of concentrated HCl to lower initial medium [HCO_3^-]). Percent bone carbonate was correlated inversely with J_{Ca} in control and metabolic acidosis ($r = -0.809$, $n = 23$, $P < 0.001$).

7), in control and respiratory acidosis (percent bone carbonate = $-423 \times J_{Ca} + 1{,}226$; $r = -0.428$, $n = 24$, $P = 0.037$), and in all groups combined (percent bone carbonate = $-647 \times J_{Ca} + 1{,}761$; $r = -0.669$, $n = 35$, $P < 0.001$).

DISCUSSION

During a model of metabolic acidosis, in which the culture medium is acidified by a decrease in the medium [HCO_3^-], there is J_{Ca} from bone and a decrease in the percent of bone carbonate. However, during a model of respiratory acidosis, in which the medium is acidified by an increase in P_{CO_2}, there is little J_{Ca} relative to bone and no significant change in the percent of bone carbonate. The release of bone calcium into the medium is strongly correlated with the decline of bone carbonate, suggesting that the calcium is being released as the carbonate is being depleted, perhaps because of the dissolution of bone carbonated apatite (21, 31).

The decline in the percent of bone carbonate during a model of metabolic, but not respiratory, acidosis is consistent with our previous findings that cultured bone buffers some of the additional protons during metabolic, but not respiratory, acidosis. We have previously shown that in acute 3-h cultures there is a net influx of protons into the bone when the medium is acidified, within the physiological range, by a decrease in [HCO_3^-] but not when the medium is acidified to the same extent by an increase in P_{CO_2} (6). Additionally, we have also shown that there is greater calcium efflux from bone at 3, 27, 48, and 96 h when the medium is acidified by a decline in [HCO_3^-] compared with an increase in P_{CO_2} (6, 7, 13–15, 17). In the context of our previous studies, the current results suggest that acidosis produced by a [HCO_3^-] leads to a loss of bone carbonate in addition to calcium, whereas acidosis produced by an increase in P_{CO_2} does not.

It is unclear from these studies whether the effect of acidosis on bone carbonate is due to physicochemical dissolution or cell-mediated resorption of the bone mineral. Early studies of bone described the mineral as simply apatite and then as a mixture of hydroxyapatite and amorphous calcium phosphate (18). However, more recently bone mineral has been better described as a highly substituted carbonated apatite also containing HPO_4^{2-} substitutions (21, 31). A low pH produced by a low [HCO_3^-] would tend to favor the physicochemical dissolution of carbonated apatite, whereas the high P_{CO_2} during respiratory acidosis would tend to favor the physicochemical accretion of carbonated apatite. Alternatively, the differential effect of models of metabolic and respiratory acidosis on bone carbonate could be a function of alterations in osteoclast function; the decreased [HCO_3^-] during metabolic acidosis and the increased P_{CO_2} during respiratory acidosis may have different effects on osteoclasts. Osteoclasts secrete protons into the microenvironment between the resorbing bone cell and the mineral. For every H^+ secreted, a cytosolic HCO_3^- is generated, which must exit the osteoclast via exchange with Cl^- to prevent intracellular alkalinity (22, 37). During metabolic acidosis the low [HCO_3^-] would promote osteoclastic proton secretion by favoring osteoclastic Cl^-/HCO_3^- exchange, whereas the normal [HCO_3^-] during respiratory acidosis would not. We have previously shown that a model metabolic acidosis stimulates osteoclastic function (25) and that chronic metabolic, but not respiratory, acidosis promotes cell-mediated resorption of bone (7). Further studies will be necessary to determine whether the loss of bone carbonate during metabolic acidosis is a physicochemical or cell-mediated process.

It is unclear from these studies whether medium [HCO_3^-] has an independent effect on the bone carbonate or whether the reduction in pH is necessary to induce the loss of bone carbonate. To determine whether [HCO_3^-] has an independent role in maintenance of bone carbonate content, in future studies we must vary medium [HCO_3^-] during neutral isohydric conditions by altering the medium P_{CO_2} (13).

Although the current study confirms the in vivo observations that metabolic acidosis causes a depletion of bone carbonate (2, 4, 23), it is difficult to compare our results obtained in vitro with these studies, as there are considerable differences between cultured bone and mammalian bone perfused by blood. Calvariae in culture respond to protons and calcium-regulating hormones, synthesize DNA and protein, and have functioning osteoblasts and osteoclasts as human bone does in vivo (35). In addition, we have shown that under the conditions of this study there is no difference in proline incorporation between the control and the two acidic groups. However, calvariae are neonatal woven bone, compared with mostly mature cortical bone in humans, and in culture calvariae are not perfused by blood. Additionally, proline incorporation is slightly, but significantly, lower after 48 h of culture than in freshly dissected bone (Fig. 1). Whether these differences alter the response of bone carbonate to protons is not known. Thus, although our results support in vivo observations, we must be cautious in applying these results obtained in culture to in vivo phenomena.

We thank Dr. John D. B. Featherstone, Eastman Dental Center, Rochester, NY, for helpful discussions about the mineral phase of bone.

This study was supported by National Institutes of Health Grant AR-39906 and by the Arthritis Society of Canada.

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Received 28 December 1992; accepted in final form 28 May 1993.

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