Phasic changes in bone CO_2 fractions, calcium, and phosphorus during chronic hypercapnia

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SCHAEFER, KARL E., STEPHEN PASQUALE, ARTHUR A. MESSIER, AND MICHAEL SHEA. *Phasic changes in bone CO_2 fractions, calcium, and phosphorus during chronic hypercapnia.* J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 48(5): 802–811, 1980.—The bone CO_2 buffering system and bone calcium and phosphorus were studied in guinea pigs exposed to 1% CO_2 for periods up to 8 wk and killed at weekly intervals together with control animals of the same age. Measurements were made of arterial CO_2 tension, pH, standard bicarbonate, and bone Ca and P. Heat-stabile bone CO_2 (carbonate) was determined as dry bone CO_2 and heat-labile bone CO_2 (bicarbonate) as Δ wet-dry bone CO_2. During the first 3–4 wk of exposure to 1% CO_2, a systemic acidosis was found as indicated in a lowered pH, increased arterial CO_2 tension, and decreased standard bicarbonate. The acidosis subsided during the last 4 wk of exposure. Phasic changes in bone bicarbonate were observed as shown in immediate rise lasting for 2 wk followed by a 2-wk decline and second rise after 6 and 8 wk. Bone carbonate exhibited the opposite change during the first 4 wk and thereafter remained stable at an elevated level. Bone Ca and P fell in association with increasing bone bicarbonate and rose with increasing bone carbonate.

acid-base balance

PROLONGED EXPOSURE OF MEN to 1.5% CO_2 for 42 days has been found to result in a phase of uncompensated respiratory acidosis lasting for about 3 wk followed by a compensatory respiratory acidosis (38). This long time period required to reach a compensation during exposure to 1.5% CO_2 is in marked contrast to the 4- to 5-day periods needed to accomplish maximal compensation in chronic hypercapnia induced by exposure to CO_2 concentrations of 3–15% CO_2. More recent studies of the effects of prolonged exposure to lower CO_2 concentrations (0.85–2% CO_2) have provided additional evidence showing longer time periods for maximal compensation of the CO_2-induced acidosis as judged by blood pH and bicarbonate measurements. During a 30-day exposure of men to 2% CO_2, Guillerm and Radziszewski (16) observed maximal compensation of the acidosis after 2 wk. In American and British submarine patrol studies in which the ambient CO_2 levels were kept between 0.8 and 1% CO_2, maximal compensation of the CO_2-induced respiratory acidosis was reached after 3–4 wk (22, 28, 37).

If one plots the time required for maximal compensation of CO_2-induced acidosis, which has been reported in the literature on prolonged exposure to different CO_2 concentrations, against arterial or venous CO_2 tensions measured, one obtains a graph that exhibits a systematic difference in response to levels of 3% CO_2 and above (time for compensation remains the same) compared with that at lower levels where the time periods required for compensation increase with decreased ambient CO_2 concentrations. Renal regulation (bicarbonate reabsorption) is fully active during exposure to higher CO_2 concentrations and becomes less and less effective during exposure to lower CO_2 concentrations. Under the latter conditions bone buffering, which has a slow time constant, becomes the dominant factor.

Evidence for an initial transient failure in the activation of renal bicarbonate reabsorption is indicated in findings showing that blood bicarbonate did not rise during the first 3 wk of exposure to 1.5 and 1% CO_2 (22, 38). However, during the subsequent period between 3 and 6 wk blood bicarbonate did increase. The reported phases in acid-base balance during chronic low-level hypercapnia were found to be reflected in calcium homeostasis, inasmuch as blood calcium levels mirrored the pH changes and urine calcium changes also exhibited related alterations (37, 38).

It has been suggested in an earlier study on calcium metabolism during exposure to 1.5% CO_2 that the initial 3-wk period of decline in blood calcium corresponding to the decrease in blood pH marks a period of deposition of CO_2 in bones and that bones play an important part in acid-base regulation during low-level chronic hypercapnia (38). Recent submarine studies have given further support to this hypothesis and demonstrated the need to carry out animal experiments with prolonged exposure to 1% CO_2, in which acid-base parameters, bone CO_2 exchange, and bone calcium and phosphorus could be measured simultaneously.

Bone comprises about 80% of the total CO_2 storage capacity of the body (13, 33) and is also known to be a major reservoir for electrolytes such as calcium, phosphorus, potassium, and sodium. Although the role of bone in the maintenance of mineral and acid-base homeostasis has been demonstrated (24, 27), little information is available in the literature about CO_2 exchange during hypercapnia and other acid-base balance changes. The earlier studies carried out under condition of chronic hypercapnia, such as those of Freeman and Fenn (13)

and Nichols (25), were limited to the measurement of dried bone CO_2 stores, which represent only fixed carbonates. However, it has been shown by Buchanan and Nakao (4), Neuman and Mulyran (23), and Poyart et al. (31) that the CO_2 stores in bone contain at least two major fractions: *1)* carbonate comprising approximately 60–70% of the total CO_2 content, probably located in the lattice of bone crystals, and *2)* bicarbonate assumed to be located in the hydration shell of the hydroxyapatite crystals. This fraction is considered to be easily exchangeable with blood CO_2.

Poyart et al. (32) determined in constant-infusion experiments using [^{14}C]bicarbonate that approximately 50% of the ^{14}C activity was lost upon heating. Based on their in vitro studies, Poyart et al. (31) concluded that this heat-labile CO_2 fraction may be considered as half of the bone bicarbonate pool, which accounts for 30% of the total bone CO_2 store.

The bone carbonate fraction does not seem to respond to acute hypercapnia. Freeman and Fenn (13) did not find any changes in rats exposed for 5–6 h at 10% CO_2. However, Bursaux and Poyart (5) demonstrated changes in total fresh bone CO_2 in response to acute hypocapnia and hypercapnia. We recently obtained CO_2-titration curves in rats during acute 1-h exposure to different CO_2 concentrations ranging from 1 to 15% CO_2. By use of a modified titration technique for the determination of the CO_2 content in fresh and dried paired bone samples, a linear relationship was found between arterial CO_2 tension (Pa_{CO_2}) and the increment in fresh bone CO_2 content (bicarbonate fraction); the dry bone CO_2 content (bone carbonate) did not change (26).

The responses of carbonate and bicarbonate CO_2 fractions to chronic hypercapnia have not been determined. It was one of the main purposes of the present investigation to clarify their role in adaptation to CO_2 and to document relationships of bone CO_2 uptake to calcium and phosphorus metabolism under conditions in which the established Pa_{CO_2}-dependent bone bicarbonate uptake could not play a significant role because of the minimal increase in Pa_{CO_2} during exposure to 1% CO_2.

METHODS

Mature male guinea pigs were exposed to 1% CO_2-21% O_2-balance N_2 in commercially built environmental control chambers with automatic temperature and humidity controls. The environmental temperature was kept at 25.6 ± 1.1°C and the humidity between 65 and 75%. The gas mixtures were prepared by mixing proportional amounts of CO_2 to air; O_2 was added from a high-pressure cylinder. The air within the chamber was recirculated 12 times/min. With this fast and large turnover of chamber air, mixing of CO_2 and air was nearly instantaneous. The CO_2 concentration in the chamber was continuously monitored with a Beckman O_2 analyzer. The CO_2 concentrations were kept at 1% (within the limits of ±0.1%) and the O_2 concentrations at 21% (±0.5%). Ammonia vapor was absorbed by boric acid placed in the chamber. The chamber was opened each morning for a period of 3–5 min to fill the water and food containers and to remove the urine and feces.

Animals of the same age and weight as those exposed to CO_2 were kept in a second environmental chamber under identical environmental conditions except that the ambient air was free of CO_2. From the 1st to 4th wk of exposure, six experimental animals and three or four control animals were killed weekly. After 6 and 8 wk of exposure to 1% CO_2 and after an 8-week recovery period, the same procedure was used. The weight of both control and exposed animals corresponded closely. To avoid changes due to circadian cycles the animals were always killed between 9 and 10 A.M.

Prior to blood sampling, the animals received pentobarbital sodium, 40 mg/kg body wt intraperitoneally, and were returned to the CO_2 exposure chamber. The anesthesia was usually effective after 5 min; the animals were then taken out of the exposure chamber and immediately placed under a mask through which they continued to breathe the same CO_2 gas mixtures to which they had been exposed. Blood samples were drawn from the abdominal aorta. Blood pH and CO_2 partial pressure were determined with an Instrumentation Laboratory blood gas and pH analyzing system. For blood gas studies, usually eight control animals were used to obtain a significant number of arterial blood samples in which O_2 partial pressure was above 50 Torr, a criteria set in our laboratory for acceptable values in anesthetized animals. The femurs of both legs were removed and rapidly cleaned and stripped free of adhering tissues and bone marrow. Specimens of compact bone between 200 and 300 mg were kept on ice for determination of total CO_2 content and bone electrolytes. The time between procurement and analysis of the fresh samples did not exceed 2 h. Preliminary studies had shown that such a time delay did not produce significant changes in CO_2 measurements. Paired specimens were oven-dried to constant weight at 150°C for 18 h before analysis. Total CO_2 content of fresh and dry bone was determined by indirect titration with a modification of the method of Bursaux and Poyart (5), which resulted in a greater accuracy (26).

The dry bone CO_2 content or heat-stabile fraction is subsequently referred to as the carbonate CO_2 fraction. The difference between wet and dry bone CO_2 content represents the heat-labile CO_2 fraction. According to Poyart et al. (31) this bone CO_2 fraction corresponds to 50% of the rapidly exchangeable bicarbonate pool in the bone. Bicarbonate in the blood was calculated using the Henderson-Hasselbalch equation and a pK of 6.1. The volume of the extracellular fluid of the bone samples was assumed to be equal to the chloride space and calculated by the formula of Hastings and Eichelberger (17). A Donnan factor of 0.98 was used for the calculation of extracellular chloride concentration (21). The volume of intracellular water was obtained by subtracting the extracellular water from the total tissue water. The concentration of HCO_3 in extracellular water was calculated from the values of plasma water divided by a Donnan factor of 0.99 (21).

Bone and blood calcium were determined with an atomic absorption spectrometer. For calcium determination both bone samples were dry ashed at 650°C for 24 h. Approximate dilutions with 3 N hydrochloric acid were

used for analysis. Recovery was assessed by adding known amounts of calcium.

Measurement of phosphorus in bone samples was made in acid digests of bone. Bone samples were dissolved in 6 N hydrochloric acid, and appropriate dilutions were analyzed according to a modified method of Fiske and SubbaRow (12). Bone chloride was determined with the Atomic Absorption spectrometer. All statistical comparisons were done by Student's t test.

RESULTS

Data on body weight, arterial carbon dioxide tension (Pa_{CO_2}), arterial oxygen tension (Pa_{O_2}), pH, and standard bicarbonate of guinea pigs exposed to 1% CO_2 and control animals are presented in Table 1. CO_2 exposure did not affect the regular increase of body weight with time.

Pa_{CO_2} was found to be persistently elevated by 3–4 Torr in the exposed animals and statistically different from controls after 3 and 4 wk of exposure. Average Pa_{O_2} values ranged from 51 to 71 Torr. pH fell by about 0.03–0.04 pH units (statistically significant after 1, 2, and 4 wk). Standard bicarbonate was found to be 1–1.5 mM lower in the exposed animals (statistically significant after 2–3 wk of exposure).

Figure 1 shows the changes in CO_2 content of fresh bone and dry bone (heat-stabile CO_2 fraction), the difference between fresh and dry bone CO_2 content (heat-labile CO_2 fraction), and the water content of bone in exposed and control animals.

During the period of 8 wk, the CO_2 content of fresh bone increased in control animals by 21 mmol/kg. Since bone CO_2 content is known to increase with age, the observed rise in bone CO_2 of control animals is considered to represent the effect of age. The CO_2-exposed animals by contrast showed an increase of 111 mmol/kg. Subtracting the age-related increase in bone CO_2 content leaves 90 mmol/kg, which is associated with the very small increase in blood CO_2 tension.

After 8 wk of recovery following 8 wk of exposure, the fresh bone CO_2 content was still elevated by 40 mmol/kg when compared with the bone CO_2 content of the control animals. However, after 2 wk of recovery following 4 wk of exposure to 1% CO_2, bone CO_2 content was not statistically different from control levels.

The dry bone CO_2 content in control animals showed an initial rise that seemed to have become stabilized after 4 wk. During the first 2 wk of exposure to 1% CO_2, the dry bone CO_2 content declined below that of control animals; but it rose rather steeply during the 3rd and 4th wk and remained stable during the subsequent 4 wk of exposure. After 8 wk of recovery following 8 wk of exposure, the dry bone CO_2 values were still elevated above those of control animals.

The bone water increased significantly during the 2 wk of exposure, declined during the 4th wk when the heat-labile CO_2 content was at a low value, and remained during the last 4 wk of exposure below the level of control animals. After 8 wk of recovery, the bone water content returned to the control level.

Measurements of bone tissue water, bone chloride, serum water, and chloride are shown in Table 2. Levels of bone chloride were generally lower during exposure to 1% CO_2 as compared with control animals. There were no significant differences in serum water and serum chloride between controls and experimental animals. The extracellular water compartment of bone calculated on the basis of chloride space was found to be consistently less in animals exposed to 1% CO_2 as compared with control animals. The intracellular water increased during the first 3 wk and returned subsequently to control levels after 6 wk of exposure to 1% CO_2.

The bicarbonate levels in extracellular fluid and extracellular fluid compartment of the bone were calculated.

TABLE 1. *Effect of prolonged exposure of guinea pigs in an environmental chamber to 1% CO_2 on weight and acid-base parameters: comparison with control animals*

Time	Exposed to 1% CO_2					Control Animals				
	Wt, g	P_{CO_2}, Torr	P_{O_2}, Torr	pH, units	Std HCO_3^-, mM	Wt, g	P_{CO_2}, Torr	P_{O_2}, Torr	pH, units	Std HCO_3^-, mM
1 wk	521 ± 7 (8)	37.6 ± 1.3 (8)	64.6 ± 12.5 (8)	7.381 ± 0.009* (8)	20.6 ± 0.4 (8)	537 ± 8 (8)	34.0 ± 1.6 (8)	65.2 ± 4.4 (8)	7.429 ± 0.016 (8)	21.5 ± 0.7 (8)
2 wk	560 ± 4 (5)	37.2 ± 2.4 (5)	55.3 ± 10.0 (5)	7.342 ± 0.014* (5)	20.6 ± 0.4 (5)	568 ± 5 (7)	36.4 ± 1.2 (7)	66.1 ± 3.0 (7)	7.405 ± 0.008 (7)	22.3 ± 0.5 (7)
3 wk	577 ± 6 (5)	38.9 ± 0.4* (5)	56.6 ± 12.0 (5)	7.380 ± 0.013 (5)	19.5 ± 0.5* (5)	584 ± 3 (7)	33.6 ± 0.8 (7)	71.1 ± 4.3 (7)	7.422 ± 0.019 (7)	21.7 ± 0.5 (7)
4 wk	607 ± 5 (6)	40.9 ± 1.7* (6)	58.3 ± 8.3 (6)	7.358 ± 0.003* (6)	20.3 ± 0.3 (6)	602 ± 5 (7)	34.0 ± 1.5 (7)	61.3 ± 3.8 (7)	7.406 ± 0.017 (7)	20.9 ± 0.4 (7)
6 wk	655 ± 7 (5)	36.1 ± 1.0 (5)	71.3 ± 3.5 (5)	7.405 ± 0.004 (5)	21.1 ± 0.4 (5)	662 ± 5 (7)	34.2 ± 0.9 (7)	62.2 ± 4.6 (7)	7.424 ± 0.012 (7)	21.9 ± 0.3 (7)
8 wk	813 ± 12 (6)	39.2 ± 0.7 (6)	55.6 ± 0.5 (6)	7.377 ± 0.018 (6)	22.4 ± 0.7 (6)	819 ± 12 (6)	37.0 ± 0.8 (6)	52.0 ± 2.5 (6)	7.392 ± 0.013 (6)	22.0 ± 0.7 (6)
Recovery on air										
4 wk on 1% CO_2 + 2 wk on air	790 ± 10 (6)	34.0 ± 2.0 (6)	67.5 ± 4.0 (6)	7.371 ± 0.029 (6)	20.0 ± 1.1 (6)					
8 wk on 1% CO_2	913 ± 13 (6)	39.5 ± 1.7	52.6 ± 2.0	7.395 ± 0.013	21.7 ± 0.6	918 ± 11 (6)	38.2 ± 0.8 (6)	54.0 ± 3.0 (6)	7.396 ± 0.012 (6)	21.9 ± 0.5 (6)

Values are means ± SE; no. of animals given in parentheses. * Statistically significantly different from controls at the 0.5% level and better.

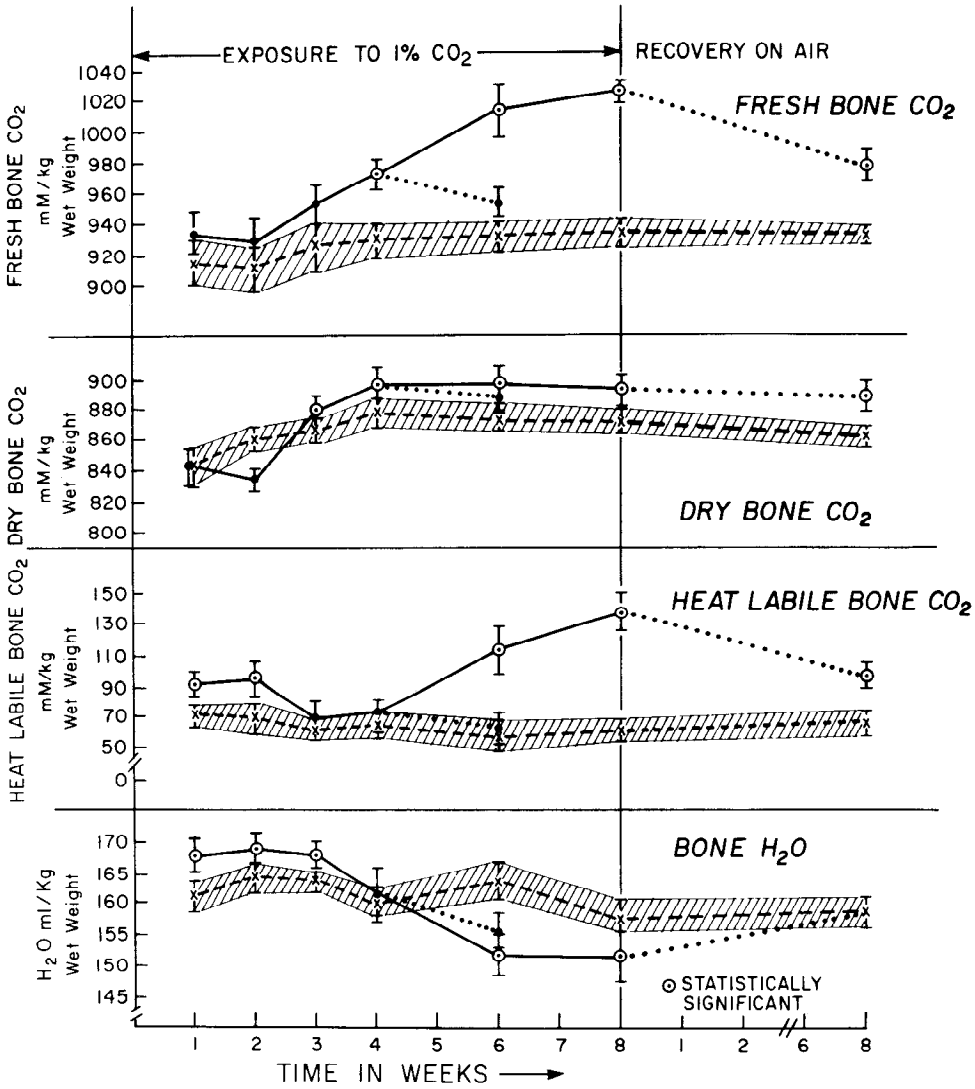

FIG. 1. Effect of prolonged exposure to 1% CO_2 on total bone CO_2 content of fresh bone, dry bone, difference between fresh bone and dry bone CO_2 content (heat-labile CO_2), and bone H_2O content. Data represents means and SE, in mmol/kg wet wt. Exposed animals ——; control animals -----; recovery following exposure ·····. Statistically significantly different from controls at 5% level and better. Each group of exposed animals consisted of 6 animals, each control group (littermates of the exposed guinea pigs) of 3–4 animals.

TABLE 2. *Effect of prolonged exposure to 1% CO_2 on extracellular and intracellular fluid compartments and extracellular bicarbonate*

Time	Bone H_2O, g/kg wet bone	Bone Cl, meq/kg wet bone	Serum H_2O, g/kg	Serum Cl, mmol/kg	Total $H_2O_{(e)}$, g extracellular fluid per kg wet bone	Total $H_2O_{(i)}$, g/ cellular H_2O per kg wet bone	$HCO_{3(e)}^-$, meq/kg extracellular H_2O	HCO_3^-, meq/kg extracellular bone H_2O
Controls (n = 4)								
1 wk	161 ± 2	16.6 ± 0.6	930.5 ± 19	102.8 ± 0.6	145 ± 4.5	15.0 ± 2.8	23.4	3.4
2 wk	163.0 ± 1.7	17.0 ± 0.4	915 ± 20	103.8 ± 1.2	149 ± 3.0	15.8 ± 1.4	22.4	3.3
3 wk	162 ± 1.8	17.7 ± 0.6	929 ± 16	109.5 ± 1.4	152.3 ± 5.0	11.0 ± 4.3	22.3	3.2
4 wk	161 ± 1.21	16.7 ± 0.4	928 ± 25	103.9 ± 1.6	145.8 ± 3.6	15.3 ± 4.1	22.5	3.4
6 wk	163 ± 3	17.1 ± 0.5	928 ± 28	102.3 ± 1.0	150 ± 5	12.0 ± 3.3	23.6	3.6
8 wk	159 ± 2	17.2 ± 0.6	929 ± 30	102.3 ± 0.4	153.5 ± 4	11.5 ± 3.0	23.7	3.9
Exposure to 1% CO_2 (n = 6)								
1 wk	168.8 ± 2*	14.66 ± 0.9	933 ± 28	104.0 ± 0.5	129.0 ± 7.9	41.6 ± 7.3*	23.5	3.0
2 wk	169.3 ± 1.8*	14.1 ± 0.8	932 ± 35	103.0 ± 0.8	124.0 ± 5.0*	44.0 ± 6.4*	21.1	3.0
3 wk	168.0 ± 1.5*	15.1 ± 0.5*	931 ± 21	102.0 ± 0.3	125.0 ± 4.7*	40.1 ± 5.8*	24.0	3.1
4 wk	162.8 ± 4.0	14.4 ± 0.5*	927 ± 19	101.8 ± 0.7	122.0 ± 4.1*	41.3 ± 4.2*	23.9	2.9
6 wk	151.5 ± 4*	15.1 ± 0.4*	927 ± 18	106.2 ± 0.3	128.0 ± 3.4*	23.0 ± 5.0	23.9	3.0
8 wk	152.1 ± 2*	15.1 ± 0.4*	926 ± 25	102.5 ± 0.5	133.0 ± 3.0*	18.3 ± 4.0	24.1	3.1

Values are means ± SE; *n*, no. of animals. * Statistically significantly different from controls at the 5% level and better.

As shown in Table 2, the extracellular bicarbonate concentration in bone is very small and does not play a role in the measured changes of the bone CO_2 fractions.

Calcium content of bone and blood in exposed and control animals is depicted in Fig. 2. After 1 and 6 wk of exposure, bone calcium content exhibited a sharp drop that corresponded with the rapid increase in CO_2 uptake of the heat-labile CO_2 fraction. During the rise of the heat-stabile CO_2 fraction, bone calcium content rose again. The bone calcium loss was reflected in a blood

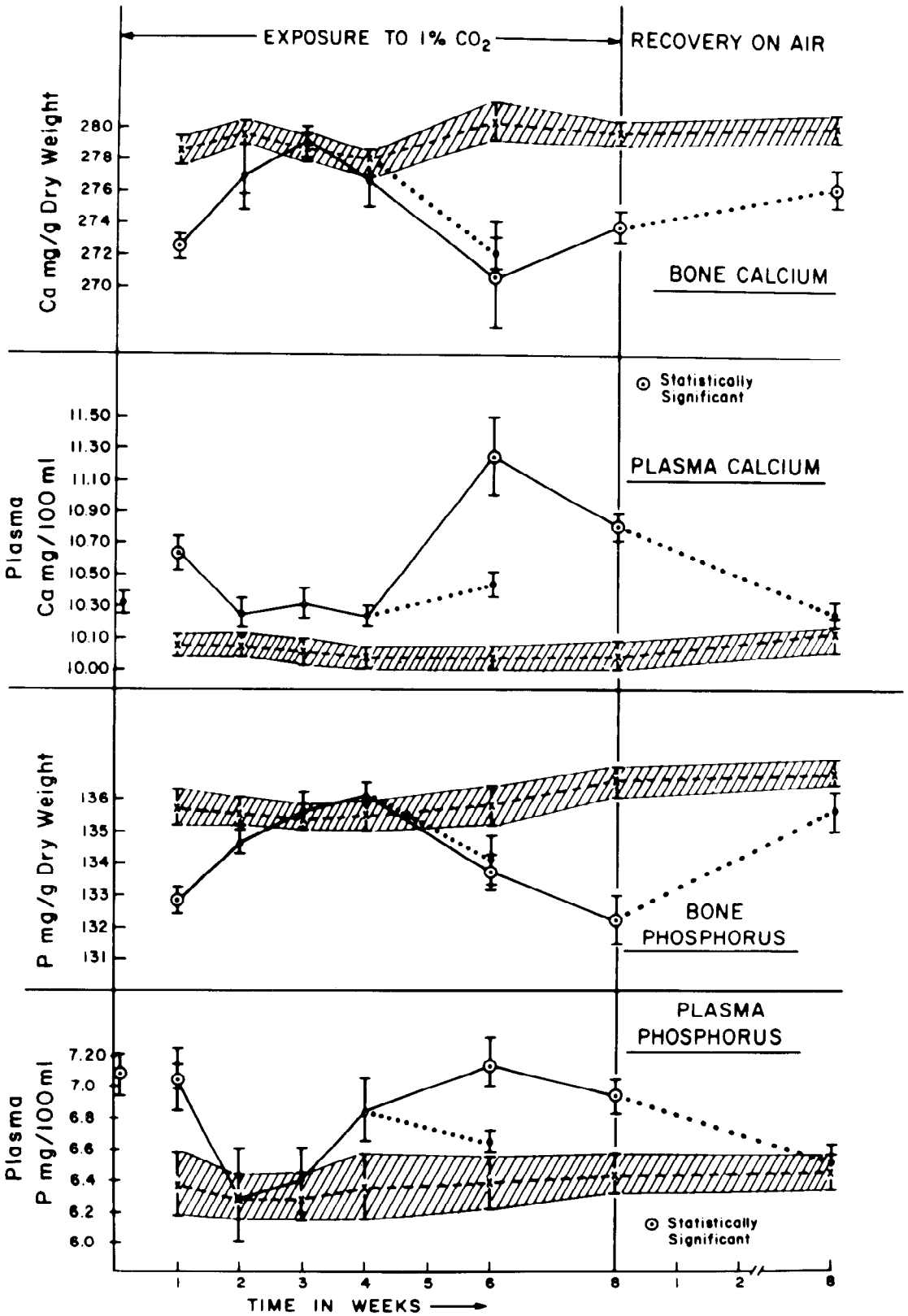

FIG. 2. Effect of prolonged exposure to 1% CO_2 on bone and blood calcium and bone and blood phosphorus. Data represent means and SE, in mg/g dry wt. Exposed animals ——; control animals -----; recovery following exposure ····· . Statistically significantly different from controls at 5% level and better. Each group of exposed animals consisted of 6 animals, each control group of 3–4 animals.

calcium tide. It should be noted that the blood level of exposed animals was at all times higher than those of control animals. At the end of 8 wk of exposure, bone calcium was significantly lower than that of control animals. After 8 wk of recovery on air, the bone calcium content was still statistically different from control levels.

Bone and blood phosphorus changes showed the same pattern as bone and blood calcium (Fig. 2).

To summarize the effects of prolonged exposure to 1% CO_2 on bone CO_2 fractions and calcium and phosphorus, the differences between control animals and experimental animals were calculated in milliequivalents per kilogram of wet bone and listed in Table 3.

The first 2 wk of exposure can be characterized as a phase of initial bone bicarbonate uptake and carbonate decline. During this period there is a maximum loss of calcium and phosphorus at 1 wk of exposure. During the 3rd and 4th wk bicarbonate uptake is reduced and carbonate uptake is increased. This phase exhibits a minimum change in bone calcium and phosphorus at 3 wk. During the period of 6 and 8 wk a large bicarbonate uptake occurs while the carbonate level remains stable. Increased losses in calcium and phosphorus are associated with this large bicarbonate uptake.

These data clearly demonstrate the existence of three different phases in the observed changes of bone CO_2 fractions and of bone calcium and phosphorus.

DISCUSSION

Throughout this experiment lasting for a total of 112 days, weights of control and experimental animals compared closely with each other indicating a lack of gross health effects attributable to CO_2.

During the first 4 wk of exposure to 1% CO_2 the Pa_{CO_2} level was found to be significantly elevated at 3 and 4 wk and the pH decreased at 1, 2, and 4 wk. Moreover, a slightly depressed standard bicarbonate level existed during this period, indicating that the kidney failed to increase bicarbonate reabsorption in response to this low-level hypercapnia.

During the subsequent period of 6 and 8 wk of exposure to CO_2, the acid-base parameters showed no significant differences from controls. Standard bicarbonate increased, reaching control levels. Based on these findings one can divide the acid-base status of the animals exposed to 1% CO_2 into two phases. First, an acidosis existed during the first 4 wk of exposure associated with a depressed renal bicarbonate reabsorption, which may be classified as metabolic acidosis. Second, a period (6 and 8 wk of exposure) in which the pH returned to near control levels and the kidney began to reabsorb bicarbonate at a limited scale. This second phase appears to be similar to a compensated respiratory acidosis, although it was not preceded by a typical respiratory acidosis. The data on fresh and dry bone CO_2 content we obtained in control animals are similar to those reported by Poyart et al. (31) on rats. Bone calcium content of 278.7 mg/g dry wt measured in guinea pigs is in close agreement with the average value of 270 mg/g dry wt reported by Easthoe and Easthoe (11) for this species. The results of bone chloride, phosphorus, and calcium determinations in rats made by Levitin et al. (19) are also similar to those observed in this experiment.

TABLE 3. *Phasic changes in bone calcium, phosphorus, carbonate, and bicarbonate produced by prolonged exposure of guinea pigs to 1% CO_2*

Exposure to 1% CO_2	ΔCa, meq/kg wet bone	ΔP, meq/kg wet bone	ΔCO_3^{2-}, meq/kg wet bone	ΔHeat-Labile CO_2, meq/kg wet bone	ΔHCO_3^-, meq/kg wet bone	Phases
1 wk	−390	−260	0	+21	+42	*I* (initial HCO_3^- uptake; CO_3^{2-} decline)
2 wk	−200	−30	−15	+25	+50	
3 wk	−30	−5	+13	+10	+20	*II* (CHO_3^- uptake reduced; CO_3^{2-} uptake increased)
4 wk	−196	−50	+32	+9	+18	
6 wk	−320	−100	+23	+62	+124	*III* (large HCO_3^- uptake; CO_3^{2-} remains stable)
8 wk	−148	−180	+30	+66	+132	

Differences between experimental animals and littermate controls.

Bone CO_2 Fractions

Total bone CO_2 content in fresh tissue was slightly above control values during the first 3 wk of exposure and rose significantly and continuously at 4, 6, and 8 wk of exposure (Fig. 1). This late rise in bone CO_2 is surprising. With such a small increase of Pa_{CO_2}, consisting of a few Torr, one would expect that an equilibrium of bone CO_2 uptake would have been reached at an early stage, e.g., after 1-2 wk, if the bone CO_2 exchange would be solely dependent on Pa_{CO_2}. These findings suggest that different mechanisms must be involved in the CO_2 accumulation during the 4- to 8-wk period of exposure to 1% CO_2, be it incorporation of CO_2 into the osseous tissue structure or chemical substitution. Similar observations were made by Reichart et al. (35), who measured CO_2 uptake in the whole body and in different organs such as liver, muscle, and bone in rats exposed to 8% CO_2 for 2, 4, 6, and 8 wk. They observed that whole-body CO_2 content and bone CO_2 content were still increasing after 8 wk of exposure, whereas the CO_2 content of liver and muscle had reached an equilibrium. The authors could not explain the disparity in the CO_2 uptake of different organs. Brown and Michel (3), who studied whole-body CO_2 exchange in rats during chronic hypercapnia (10% CO_2), also found that the CO_2 uptake did not reach an equilibrium within 6 wk of exposure. To our knowledge there is no information in the literature that would provide an explanation for the observed continuous rise of whole-body CO_2 and bone CO_2 content in chronic hypercapnia.

The major rise in total bone CO_2 content during the period of 4-8 wk of exposure to 1% CO_2 is due to the increase in the bicarbonate fraction since the carbonate fraction remains stable from 4 wk on through the remainder of the exposure period. Inasmuch as extracellular bicarbonate did not change during exposure to 1% CO_2 (Table 2), it could not have contributed to the large accumulation of total bone CO_2 in form of bicarbonate observed during the later periods of 6 and 8 wk of exposure.

Extracellular water was found to be reduced throughout the whole 8-wk exposure period. The observed shrinkage of the extracellular bone water compartment might have contributed to limit the role of extracellular bicarbonate in bone CO_2 uptake. Intracellular water was found increased during the first 4 wk of exposure to 1% CO_2 and returned to control levels at 6 and 8 wk of exposure. The movement of intracellular water marks two periods with different forms of bone CO_2 uptake. It is concluded from our experiments with 1% CO_2 that the large increase in bone bicarbonate after 6 and 8 wk of exposure must be based on an accumulation of bone bicarbonate not in equilibrium with the extracellular space and not dependent on Pa_{CO_2}. Similar observations were made by Reichart et al. (35).

Possible Mechanisms Involved in Phases of CO_2 Exchange in Chronic Hypercapnia

Three phases of CO_2 exchange in bone have been demonstrated (Table 3).

Phase I. Bone CO_2 uptake during the first 2 wk of exposure was limited to the bicarbonate fraction and was associated with an influx of water. At the same time, the carbonate fraction declined simultaneously with a loss in bone calcium and phosphorus. A decrease in bone carbonate was also observed by Nichols (25) in rats during the first 5 h of exposure to 24% CO_2. Findings of this first phase of CO_2 uptake in the bone provided confirming evidence for the theory proposed by Poyart et al. (31) that gaseous CO_2 hydrates with bone water to form carbonic acid, which dissociates into one HCO_3^- and one H^+ ion as follows

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^- + H^+ \qquad (1)$$

The H^+ ion is most probably located at the surface of the bone crystal structure to be able to participate rapidly in bicarbonate formation. It is probably taken up by the available carbonate fraction resulting in a fall in bone calcium and bone phosphorus.

$$H^+ + CaCO_3 \rightarrow HCO_3^- \rightarrow Ca^{2+} \qquad (2)$$

The influx of H_2O into the bone also fits this scheme well.

Phase II. During the subsequent period of 3 and 4 wk of exposure (*phase II*), a reversal takes place. The carbonate fraction increases in association with a rise in bone calcium and bone phosphorus. Conversely, the bicarbonate fraction decreases in conjunction with a decrease in bone water.

It has been pointed out that the process of CO_2 binding into the bone is dependent on Pa_{CO_2} and time, which involves a saturation mechanism (31). Under 1% CO_2, the saturation of the rapidly exchangeable pool of bone CO_2 is apparently accomplished within 2 wk, after which dehydration and loss of heat-labile CO_2 occurs. Heat-labile CO_2 fell by 15 mmol/kg during this period, corresponding to 30 mmol/kg reduction of bicarbonate. If the CO_2 exchange followed the reaction given in *Eq. 3* and 15 mmol/kg appeared in the form of carbonate as measured, then the other 15 mmol/kg must have been released as gaseous CO_2 into the extracellular space and blood.

$$2HCO_3^- \rightarrow CO_2 + CO_3^{2-} + H_2O \qquad (3)$$

Bone carbonate attains an equilibrium after 3–4 wk of exposure following transformation of part of the originally accumulated bicarbonate stores into carbonate. The increase of 22 mmol/kg corresponds to about 2.6% of the total carbonate pool. This process seems to be associated with the release of CO_2 in gaseous form as indicated in a rise in Pa_{CO_2} concomitant with the outflow of excess bone water accumulated during the first 2 wk of CO_2 loading. The release of CO_2 from the bone could by itself not have caused an increase in Pa_{CO_2}, unless other factors came into play at the same time. It has been theorized, that CO_2 released from the bones could trigger an activation of the renal bicarbonate reabsorption mechanism leading to an increase in blood bicarbonate and consequently to a compensation of the acidosis (37). Moreover, a reduction in the CO_2-induced hyperventilation has been found during the compensatory phase of respiratory acidosis in chronic hypercapnia induced in human subjects during prolonged exposure to 0.8–2% CO_2 (37). Of the three factors described, the first one consisting of the bone CO_2 exchange process underlying CO_2 release from the bone has been demonstrated in this experiment. The activation of renal bicarbonate reabsorption begins at 4 wk of exposure to 1% CO_2, since standard bicarbonate is increased from this time on for the rest of the exposure period. Ventilation was not measured in these animals, but it can be assumed that it follows the pattern observed in human studies.

Phase III. The second rise of the bone bicarbonate fraction after 6 and 8 wk of exposure to 1% CO_2 (*phase III*) was apparently based on a process that differs from the first increase in bone bicarbonate (*phase I*). Bone water does not change, nor does the carbonate fraction. After 8 wk of exposure, the heat-labile form of CO_2 has increased by 66 mmol/kg, corresponding to 132 mmol/kg, or 92% of the total bicarbonate pool originally present. This was a significantly larger increase than that observed during the first 2 wk and is out of proportion to any Pa_{CO_2}-dependent mechanism. It would seem to require a membrane and an active pump mechanism to accumulate so much CO_2 in the form of bicarbonate. Further investigations are necessary to clarify the nature of this process. The process of CO_2 binding under these circumstances probably involved deeper layers in the bone. It seems to correspond with the increased CO_2 binding found to occur during aging (31, 32, 42). This aging effect is also associated with loss of bone water. Poyart et al. (31) have suggested that the increased CO_2 binding in aging may be related to bicarbonate formation from a CO_2 fraction located deeper in the apatite crystals. The lowered levels of bone calcium and phosphorus found after 6 and 8 wk of exposure to 1% CO_2 support the argument that the calcium-phosphate-carbonate complex in the bone gives up carbonate for the formation of bicarbonate, and calcium and phosphorus are released as a consequence. Bicarbonate formed under these circumstances may not belong to a rapidly exchangeable CO_2 fraction of the bone, since 8 wk of recovery on air following 8 wk of exposure was not sufficient to let either the carbonate fraction or the bicarbonate fraction return to control levels. Therefore, the loss of bone calcium and phosphorus still existed at this time.

These findings suggest the existence of two carbonate

fractions involved in fast and slow exchangeable CO_2 pools in the bone as well as the presence of two bicarbonate fractions.

Application of Animal Study Results to Acid-Base Balance Studies of Chronic Hypercapnia in Men

The phasic changes in bone CO_2 uptake and their effects on acid-base balance in guinea pigs during prolonged exposure to 1% CO_2 can give a clue to the understanding of the cyclic changes in acid-base balance found in human subjects exposed to 0.7–1% CO_2 for prolonged periods in submarine patrols (14, 15, 22, 28, 36, 37). The dominant feature of the acid-base changes under these conditions was a metabolic acidosis during the first 3 wk of exposure, indicated by either no change or a decrease in blood bicarbonate commensurate with a slight increase in Pa_{CO_2} and H^+. During the 4th and 5th wk, however, blood bicarbonate and Pa_{CO_2} increased. During the second period (4–6 wk) acid-base status appeared as a compensated respiratory acidosis. The well-known renal response to hypercapnia observed during exposure to higher CO_2 concentrations consists of an increased acid secretion and renal bicarbonate reabsorption resulting in an increased blood bicarbonate (2, 7, 29, 34, 43). This response does not occur during the first 3 wk but develops during the subsequent 4th and 5th wk. The theory has been advanced that the stimulus for activation of renal bicarbonate reabsorption occurring after 3 wk of exposure to low levels of CO_2 on submarines may come from a larger acid load involving the release of CO_2 from the bone after the capacity of the rapidly exchangeable CO_2 stores has been reached (37). The results of this study supply evidence in support of this theory by showing that the fast exchangeable pool of bone CO_2 is indeed saturated during the first 2 wk, and that a decrease of the bicarbonate fraction with a release of CO_2 in the blood occurs during the subsequent period.

To bring this important point out more clearly a diagram depicting the phasic changes in bone carbonate and bicarbonate and their relationship to Pa_{CO_2} caused by prolonged exposure to 1% CO_2 is presented in Fig. 3. The differences between experimental and control animals have been averaged for 2-wk periods. Individual data points have been presented in Tables 1 and 3 and Figs. 1 and 2.

Bone Calcium and Phosphorus

A significant loss in bone calcium and phosphorus was found in association with the increase of the bicarbonate bone CO_2 fraction after 1 and 6 wk of exposure to 1% CO_2. Bone calcium and phosphorus showed an increase commensurate with the rise in the bone carbonate fraction. These results demonstrate that phasic alterations in bone calcium and phosphorus are related to specific phases of CO_2 binding in the bone involving the bicarbonate and carbonate CO_2 fractions. Loss of bone calcium and phosphorus has also been observed in chronic hypercapnia in rats exposed to 8% CO_2 for periods of 2, 4, and 6 wk (8). The decrease in calcium observed under these conditions was more pronounced after 6 wk of exposure as compared to 4 wk and 2 wk of exposure; this finding is in line with our observations. The sampling

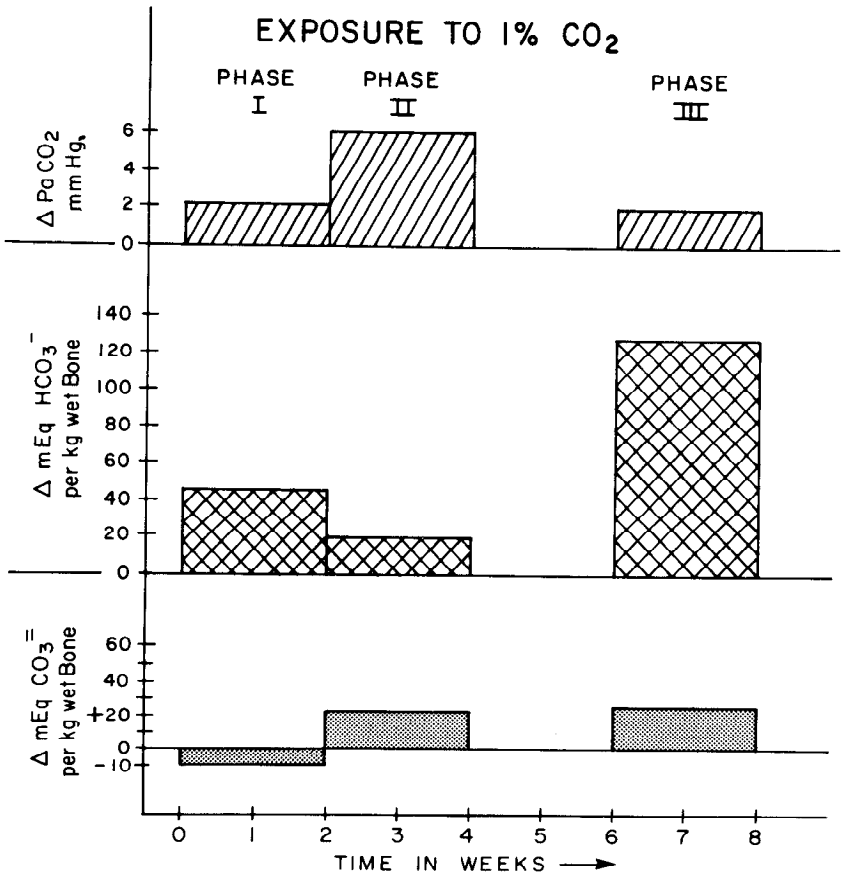

FIG. 3. Diagram showing differences in Pa_{CO_2}, bone HCO_3^-, and CO_3^{2-} between animals exposed to 1% CO_2 and control animals. Data are averaged for 2-wk periods.

periods, however, were not frequent enough to detect phases in bone calcium similar to those observed in our study. Leon (18) exposed rats to 1% CO_2 for 28 days and found small decreases in bone carbonate and bone calcium, which were not statistically significant.

Based on our findings of phases in bone calcium and phosphorus exchange it is concluded that the length of the exposure period to CO_2 will determine whether bone calcium and phosphorus is decreased or increased. Levitin et al. (19) observed, in rats exposed for 24 h to 8% CO_2, a statistically significant fall in bone phosphorus and a slight decline of bone calcium. Rats exposed to 10% CO_2 for 11 days showed a slight increase in bone calcium, which was, however, not statistically significant (6).

The Role of Parathyroid Gland

The cyclic changes in blood calcium and phosphorus are of such a nature that they can be expected to influence the endocrine balance of parathyroid hormone and calcitonin. In addition, bone calcium mobilization and resultant hypercalcemia (Fig. 2) was also found to be associated with the kidney calcification reported elsewhere (40). Kidney calcification was also demonstrated histologically in guinea pigs and rats exposed to 1.5% CO_2 for extended periods of time (40).

Administration of parathyroid hormone has been found to cause kidney calcification but did not change the calcium content in the heart and liver (9). Analysis of the calcium content in heart tissue of guinea pigs exposed from 1 to 4 wk to 1% CO_2 did not show an increase in calcium. These findings suggest that the kidney calcification seen during exposure to 1% CO_2 may involve parathyroid hormone (PTH). The fact that plasma calcium in animals exposed to 1% CO_2 remains continuously elevated independent of the two episodes of calcium flux out of the bone at 1 and 6 wk also seems to support the argument that a stimulation of the parathyroid gland existed during exposure to 1% CO_2.

Recent studies on PTH and CO_2 effects on kidney calcium metabolism further strengthen the argument for a CO_2-induced increase in PTH activity. Borle et al. (1) found that PTH produced intramitochondrial calcification in kidney cells. CO_2 alone appears to have the opposite effect of PTH. In isolated rat kidney cells exposure to 20% CO_2 depressed all cellular calcium pools and total cell calcium (41). However, CO_2 has been shown to increase PTH effects as measured by ^{45}Ca release from fetal bones (6, 20).

Parathyroid hormone stimulation may have been the cause of the acidosis observed during prolonged exposure to 1% CO_2. The decrease in standard bicarbonate indicates a failure of the kidney to reabsorb bicarbonate. PTH has been found to decrease bicarbonate reabsorption and to produce a systemic acidosis (10). Crumb et al. (10) also reported that hypercalcemia caused an increased bicarbonate reabsorption, but this effect could be suppressed by increased levels of PTH. Although PTH activity was not measured in this study, there is indirect evidence suggesting that PTH activity was indeed increased during exposure to 1% CO_2. The elevated blood calcium found in the study failed to produce an increase in bicarbonate reabsorption during the first 4 wk of exposure; this suggests that it was associated with increased PTH activity.

This study produced provocative findings showing that bone calcium and phosphorus were significantly lower than control values after 8 wk of exposure to 1% CO_2, indicating a demineralization of bone, which in the case of bone calcium was not alleviated by a recovery period on air for 8 wk. Such a demineralization caused by a small increase of Pa_{CO_2} points also to a CO_2-induced hyperparathyroid state.

These findings may also stimulate some research into the clinical significance of slightly increased Pa_{CO_2} levels in hyperparathyroid states.

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REFERENCES

1. BORLE, A. B., AND T. USCHIKAWA. Effects of parathyroid hormone on the distribution of calcium in cultured kidney cells. *Endocrinology* 102: 1725–1732, 1978.
2. BRACKETT, N. C., JR., J. J. COHEN, AND W. B. SCHWARTZ. Carbon dioxide titration curve of normal man. *N. Engl. J. Med.* 272: 6–12, 1965.
3. BROWN, E. B., JR., AND C. C. MICHEL. Whole body CO_2 exchange. *Int. Congr. Physiol. Sci. 24th Washington DC, 1968* p. 185–186.
4. BUCHANAN, D. L., AND A. NAKAO. Turnover of bone carbonate. *J. Biol. Chem.* 198: 245–257, 1952.
5. BURSAUX, D., AND C. POYART. Bone carbon dioxide stores and acid-base regulation. In: *Carbon Dioxide and Metabolic Regulations*, edited by G. Nahas and K. E. Schaefer. New York: Springer-Verlag, 1974, p. 189–195.
6. CARTER, N. W., D. W. SELDIN, AND H. C. TENG. Tissue and renal response to chronic respiratory acidosis. *J. Clin. Invest.* 38: 949–960, 1959.
7. CLARK, J. M., R. B. SINCLAIR, AND B. E. WELCH. Rate of acclimatization to chronic hypercapnia in man. In: *Proceedings of the Fourth Symposium on Underwater Physiology*, edited by C. J. Lambertsen. 1971, p. 399–417.
8. CLAUDON, F., E. REICHART, J. F. BOLOT, S. BERSTEIN, AND S. SABLIERE. Bone electrolytes in experimental chronic hypercapnia. *Bull. Physio-Pathol. Respir.* 12: 55–67, 1976.
9. COHN, D. V., R. BOWDON, AND G. J. ELLER. The effect of parathyroid hormone in vivo on the accumulation of calcium and phosphate by kidney mitochondrial function. *J. Biol. Chem.* 242: 1253–1255, 1967.
10. CRUMB, CH. K., M. MARTINER-MULDONADO, G. EKNOYAN, AND N. SUKI. Effect of volume expansion, purified parathyroid extract and calcium on renal bicarbonate absorption in the dog. *J. Clin. Invest.* 54: 1287–1294, 1974.
11. EASTHOE, J. E., AND B. EASTHOE. The organic constituents of mammalian compact bone. *Biochem. J.* 57: 453–459, 1954.
12. FISKE, C. H., AND H. SUBBAROW. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66: 375, 1955.
13. FREEMAN, F. H., AND W. O. FENN. Changes in carbon dioxide stores of rats due to atmospheres low in oxygen or high in carbon dioxide. *Am. J. Physiol.* 174: 422–430, 1953.
14. GRAY, S. P., R. J. W. LAMBERT, AND T. E. MORRIS. Calcium, magnesium, and phosphate metabolism during prolonged exposure to carbon dioxide. *J. R. Nav. Med. Serv.* 55: 238–243, 1969.
15. GRAY, S. P., J. E. W. MORRIS, AND C. J. BROOKS. Renal handling of calcium, magnesium, inorganic phosphate and hydrogen ions

during prolonged exposure to elevated carbon dioxide concentrations. *Clin. Sci. Mol. Med.* 45: 751–764, 1973.
16. GUILLERM, R., AND R. RADZISZEWSKI. Effects on man of 30-day exposure to $P_{I_{CO_2}}$ = 14 torr (2%) application to exposure limits. *Undersea Biomed. Res. Sub. Suppl.* 6: 91–114, 1979.
17. HASTINGS, A. B., AND L. EICHELBERGER. The exchange of salt and water between muscle and blood. *J. Biol. Chem.* 117: 73–93, 1937.
18. LEON, H. A. CO_2-aspirin interaction during chronic exposure and administration. *Proc. Aerosp. Med. Assoc.* 114–115, 1973.
19. LEVITIN, H., C. J. AMICK, AND F. H. EPSTEIN. Response of tissue electrolytes to respiratory acidosis. *Am. J. Physiol.* 200: 1151–1154, 1961.
20. MAHGOUB, A., AND P. STERN. Carbon dioxide and the effect of parathyroid hormone on bone in vitro. *Am. J. Physiol.* 226: 1272–1275, 1974.
21. MANERY, J. F. Water and electrolyte metabolism. *Physiol. Rev.* 34: 334–417, 1954.
22. MESSIER, A., E. HEYDER, W. R. BRAITHWAITE, C. MCCLUGGAGE, A. PECK, AND K. E. SCHAEFER. Calcium, magnesium, and phosphorus metabolism and parathyroid calcitonin function during prolonged exposure to elevated CO_2 concentration in submarines. *Undersea Biomed. Res. Suppl.* 6: 357–370, 1979.
23. NEUMAN, W. F., AND B. J. MULRYAN. Synthetic hydroxyapatite crystals. III. The carbonate system. *Calcif. Tissue Res.* 1: 92, 1967.
24. NEWMAN, W. F., AND M. F. NEUMAN. *The Chemical Dynamics of Bone Mineral.* Chicago, IL: Chicago Univ. Press, 1958.
25. NICHOLS, G., JR. Serial changes in tissue carbon dioxide content during acute respiratory acidosis. *J. Clin. Invest.* 37: 1111–1122, 1958.
26. PASQUALE, S., A. A. MESSIER, M. L. SHEA, AND K. E. SCHAEFER. Bone CO_2-titration curves in acute hypercapnia obtained with a modified titration technique. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 48: 197–201, 1980.
27. PELLEGRINO, E. D., AND R. M. BILTZ. The composition of human bone in uremia. *Medicine Baltimore* 44: 397–417, 1965.
28. PINGREE, B. J. W. Acid-base and respiratory changes after prolonged exposure to 1% carbon dioxide. *Clin. Sci. Mol. Med.* 52: 67–74, 1977.
29. PITTS, R. F. *Physiology of the Kindey and Body Fluids* (2nd ed.). Chicago, IL: Year Book, 1968.
30. POSNER, A. A. Crystal chemistry of bone mineral. *Physiol. Rev.* 49: 760–792, 1969.
31. POYART, C. F., E. BURSAUX, AND A. FREMINET. The bone CO_2 compartment: evidence for a bicarbonate pool. *Respir. Physiol.* 25: 89–99, 1975.
32. POYART, C. F., A. FREMINET, AND E. BURSAUX. The exchange of bone CO_2 in vivo. *Respir. Physiol.* 25: 101–107, 1975.
33. RAHN, H. The gas stores in the body with particular reference to carbon dioxide. In: *Man's Dependence on the Earthly Atmosphere*, edited by K. E. Schaefer. New York: Macmillan, 1962, p. 297–304.
34. RECTOR, F. C., JR. Carbon dioxide and the kidney. In: *Carbon Dioxide and Metabolic Regulations*, edited by G. Nahas and K. E. Schaefer. New York: Springer-Verlag, 1974, p. 217–225.
35. REICHART, E., F. CLAUDON, AND S. SABLIERE. CO_2 storage in various organs during chronic experimental hypercapnia. *Bull. Physio-Pathol. Respir.* 12: 19–23, 1976.
36. SCHAEFER, K. E. Effect of chronic hypercapnia on calcium metabolism. *Bull. Physio-Pathol. Respir.* 12: 51–53, 1976.
37. SCHAEFER, K. E. Physiological stresses related to hypercapnia during patrols on submarines. *Undersea Biomed. Res. Suppl.* 6: 15–47, 1979.
38. SCHAEFER, K. E., G. NICHOLS, JR., AND C. R. CAREY. Calcium phosphorus metabolism in man during acclimatization to carbon dioxide. *J. Appl. Physiol.* 18: 1079–1084, 1963.
39. SCHAEFER, K. E., N. NIEMOELLER, A. MESSIER, E. HEYDER, AND J. SPENCER. Chronic CO_2 toxicity: species difference in physiological and histopathological effects. *Nov. Submarine Med. Res. Lab. Rep.* 656, 1971.
40. SCHAEFER, K. E., S. PASQUALE, A. A. MESSIER, AND H. NIEMOELLER. CO_2 induced kidney calcification. *Undersea Biomed. Res. Suppl.* 6: 143–153, 1979.
41. STUDER, R. K., AND A. B. BORLE. Effect of pH on the calcium metabolism of isolated rat kidney cells. *J. Membr. Biol.* 48: 325–341, 1979.
42. TRIFFIT, J., T. A. TENZPKA, AND W. F. NEUMAN. A comparative study of the exchange *in vivo* of major constituents of bone mineral. *Calcif. Tissue Res.* 2: 165–176, 1968.
43. VAN YPERSELE DE STRIHOU, C. L. BRASSEUR, AND J. DECONINCOK. The carbon dioxide response curve for chronic hypercapnia in man. *N. Engl. J. Med.* 275: 117, 1966.