Evidence for Skeletal Resistance to Parathyroid Hormone in Magnesium Deficiency: *STUDIES IN ISOLATED PERFUSED BONE*

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J Clin Invest. 1979;64(5):1238-1244. https://doi.org/10.1172/JCI109578.

Hypocalcemia during magnesium (Mg) depletion has been well described, but the precise mechanism(s) responsible for its occurrence is not yet fully understood. The hypocalcemia has been ascribed to decreased parathyroid hormone (PTH) secretion as well as skeletal resistance to PTH. Whereas the former is well established, controversy exists as to whether or not Mg depletion results in skeletal resistance to PTH. These studies examine the skeletal response to PTH in normal dogs and dogs fed a Mg-free diet for 4-6 mo. Isolated tibia from normal (serum Mg 1.83±0.1 mg/100 ml) and experimental dogs (serum Mg 1.34±0.15 mg/100 ml) were perfused with Krebs-Henseleit buffer during a constant infusion of 3 ng/ml of synthetic bovine PTH 1-34 (syn b-PTH 1-34). The arteriovenous (A-V) difference for immunoreactive PTH (iPTH) across seven normal bones was 37.5±3%. In contrast, the A-V difference for iPTH was markedly depressed to 10.1±1% across seven bones from Mg-depleted dogs. These findings correlated well with a biological effect (cyclic AMP [cAMP] production) of syn b-PTH 1-34 on bone. In control bones, cAMP production rose from a basal level of 5.8±0.2 to 17.5±0.7 pmol/min after syn b-PTH 1-34 infusion. In experimental bones, basal cAMP production was significantly lower than in controls, 4.5±0.1 pmol/min, and increased to only 7.1±0.4 pmol/min after syn b-PTH 1-34 infusion. Even when PTH concentrations were increased to […]

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STUDIES IN ISOLATED PERFUSED BONE

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ABSTRACT Hypocalcemia during magnesium (Mg) depletion has been well described, but the precise mechanism(s) responsible for its occurrence is not yet fully understood. The hypocalcemia has been ascribed to decreased parathyroid hormone (PTH) secretion as well as skeletal resistance to PTH. Whereas the former is well established, controversy exists as to whether or not Mg depletion results in skeletal resistance to PTH. These studies examine the skeletal response to PTH in normal dogs and dogs fed a Mg-free diet for 4–6 mo. Isolated tibia from normal (serum Mg 1.83±0.1 mg/100 ml) and experimental dogs (serum Mg 1.34±0.15 mg/100 ml) were perfused with Krebs-Henseleit buffer during a constant infusion of 3 ng/ml of synthetic bovine PTH 1–34 (syn b-PTH 1–34). The arteriovenous (A-V) difference for immunoreactive PTH (iPTH) across seven normal bones was 37.5±3%. In contrast, the A-V difference for iPTH was markedly depressed to 10.1±1% across seven bones from Mg-depleted dogs. These findings correlated well with a biological effect (cyclic AMP [cAMP] production) of syn b-PTH 1–34 on bone. In control bones, cAMP production rose from a basal level of 5.8±0.2 to 17.5±0.7 pmol/min after syn b-PTH 1–34 infusion. In experimental bones, basal cAMP production was significantly lower than in controls, 4.5±0.1 pmol/min, and increased to only 7.1±0.4 pmol/min after syn b-PTH 1–34 infusion. Even when PTH concentrations were increased to 20 ng/ml, cAMP production by experimental bones was lower than in control bones perfused with 3 ng/ml. Histological examination of bones from Mg-deficient dogs showed a picture compatible with skeletal inactivity. These studies demonstrate decreased uptake of iPTH and diminished cAMP production by bone, which indicates skeletal resistance to PTH in chronic Mg deficiency.

INTRODUCTION

Hypocalcemia as a direct consequence of magnesium depletion has been well described (1–12). The various possible pathogenetic mechanisms that may be responsible for the hypocalcemia associated with hypomagnesemia include: increased urinary calcium excretion, decreased gastrointestinal calcium absorption, and decreased calcium mobilization from bone (13). Evidence to date indicates that neither of the first two mechanisms is a significant etiologic factor in the hypocalcemia of magnesium depletion states (4, 13). The most likely explanation for magnesium depletion-induced hypocalcemia, therefore, is decreased calcium mobilization from bone. Several factors could be responsible for this decreased mobilization. Early studies by Buckle et al. (14) and Sherwood et al. (15) suggested that an inverse relationship exists between acute changes in magnesium concentration and the secretion of immunoreactive parathyroid hormone (PTH).[1] However, numerous subsequent studies have shown, in both the dog (9) and man (2–4, 11, 12), that chronic magnesium depletion is associated with normal to low serum immunoreactive PTH levels despite the concomitant

This work was presented in part at the American Federation for Clinical Research meeting, Chicago, Ill., November 1978.

Dr. Freitag is the recipient of a Fellowship from the National Kidney Foundation. Dr. Bellorin-Font is the recipient of a Fellowship from Consejo Nacional de Investigacione Cientifica y Tecnologicas of Venezuela.

Received for publication 19 March 1979 and in revised form 5 July 1979.

[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; PTH, parathyroid hormone; syn b-PTH 1–34, synthetic bovine PTH 1–34.

existence of hypocalcemia. The failure of serum PTH levels to increase in response to the hypocalcemia appears to be primarily a result of decreased secretion of PTH by the parathyroid glands because PTH biosynthesis does not appear to be affected by low magnesium concentrations in vitro (16, 17). These data are further supported by the data of Anast et al. (18) and Rude et al. (12), which show that serum immunoreactive PTH levels in hypomagnesemic subjects increase markedly within minutes after acute magnesium administration.

Studies by Neuman and Neuman (19) and MacManus and Heaton (20) have shown that the heteroionic exchange between the hydration shell of bone and the extracellular fluid is decreased in hypomagnesemic states and could be a factor contributing to the hypocalcemia. Skeletal resistance to PTH could also be a contributing factor to the hypocalcemia associated with magnesium restriction. Controversy, however, exists in the literature regarding whether the response of the skeleton to exogenous PTH during magnesium deficiency is normal (3, 4, 7, 18, 21, 22) or reduced (5, 6, 8–10, 12, 23). As noted by Connor et al. (10), many variables, including species difference, degree of magnesium depletion, dose and preparation of PTH used, age of the study subject, and degree of hypocalcemia, need to be considered in attempting to reconcile the differing results reported.

Our studies were designed to examine directly, with an isolated perfused bone preparation, the skeletal response to PTH in normal dogs and dogs fed a magnesium-free diet for 4–6 mo.

METHODS

Animals. Adult mongrel dogs that weighed 18–25 kg were used for all studies. Seven dogs were fed 400 g daily of magnesium-free diet (ICN Nutritional Biochemicals, Cleveland, Ohio), which contained 0.89% calcium and 0.46% phosphorus, for 4–6 mo. The relatively high calcium content of this test diet (3.8 g Ca^{++}/d) ensured the maintenance of the serum calcium within the normal range (9.3–10.5 mg/100 ml). Without this high calcium intake these dogs fed the magnesium-free diet would have become hypocalcemic as noted by Levi et al. (9). Supplemental dietary calcium, therefore, eliminated changes in serum calcium as a potential variable in the interpretation of the results. Tube feeding of animals was used when necessary to insure total daily consumption of food. The control animals consisted of seven dogs fed the same diet but with added magnesium (0.15%).

Experimental model. The experimental model used in these studies was an isolated perfused tibia preparation previously described (24). Briefly, through an anterolateral incision in each hind limb of the anesthetized (Pentobarbital [The Vitarine Co., Inc., Springfield Gardens, N. Y.] 30 mg/kv i.v.) dog, the vasculature was dissected to localize the nutrient artery to the tibia. After the administration of Heparin (The Vitarine Co., Inc.) (5,000 U i.v.), the bone was removed, stripped of its muscles, and the nutrient artery cannulated either via the main anterior tibial artery or directly using PE-30 polyethylene tubing (Becton, Dickinson & Co., Rutherford, N. J.). The tibia was then placed in a specially designed perfusion chamber that was heated to maintain an internal temperature of 37–39°C. The perfusate, a Krebs-Henseleit buffer, which contained 1% bovine serum albumin and 1 mM of magnesium at a pH of 7.4 when continuously gassed with 95% O_2–5% CO_2, was pumped into the bone via the nutrient artery at a pressure of 90–100 mm Hg. Flow rates ranged from 1.5 to 2.5 ml/min in the bones from normal or magnesium-deficient animals. The venous return that drained from the nutrient vein and oozed from the bone surface was collected for analysis. Preliminary angiographic studies indicated that after entering the bone the nutrient artery divides within the marrow space into multiple branches supplying the bone cortex. We attribute the effects of PTH described in these studies to effects on bone because we have been unable to demonstrate PTH-stimulated cyclic AMP (cAMP) production by leukocytes or erythrocytes. Although PTH-responsive adenylate cyclase has been described in fat cell ghosts, the doses required are many orders of magnitude higher than those employed in this study (25).

Study protocol. After perfusion was established, the tibia was allowed to equilibrate for 15 min. During the next 20 min, four 5-min venous (effluent) samples were collected for determination of basal cAMP output. After the equilibration period and collection of samples for basal cAMP, synthetic bovine PTH 1–34 (syn b-PTH 1–34) was added to the perfusate at a concentration of 3 ng/ml, and six 5-min simultaneous arterial and venous samples were collected in chilled tubes for determination of arteriovenous difference for immunoreactive PTH across the bone and for determination of PTH-stimulated cAMP production by the perfused bone.

Chemical analysis. Phosphorus was measured by the methods of Kraml (26) and Hurst (27) as adapted for the Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.). Total calcium and magnesium were determined by atomic adsorption spectroscopy (model 503, Perkin-Elmer Corp., Norwalk, Conn.). Ionized calcium was measured with a calcium-specific electrode (Orion Research Inc., Cambridge, Mass.).

Radioimmunoassay for PTH. Perfusate and effluent PTH concentrations during the infusion of syn b-PTH 1–34 were measured by radioimmunoassay with antiserum CH9N (specific for amino-terminal portion of PTH) as described previously in detail (27, 28). Serum immunoreactive PTH levels were measured from venous samples obtained at the time of study by the method previously described (28, 29) with antiserum CH9 (predominant affinity for the carboxy-terminal portion of PTH) and a hyperparathyroid dog serum as the standard.

Radioimmunoassay for cAMP. cAMP was measured by radioimmunoassay as described by Steiner et al. (30). The cAMP antiserum was a gift of Dr. Charles Parker (Washington University, St. Louis, Mo.). The tracer was ^{125}I-succinyl cAMP purchased from New England Nuclear, Boston, Mass. Preparation of samples for cAMP radioimmunoassay were as described previously (24) with the exception that the dried samples were reconstituted in 1 ml of sodium acetate buffer (50 mM, pH 6.2). Results are expressed as cAMP produced in picomoles per minute (venous cAMP minus arterial cAMP multiplied by perfusate flow rate).

Analysis of chemical content of bone. In six animals (three magnesium-deficient dogs and three normal dogs) both 10th ribs were obtained at the time of study and stripped of adhering tissues and periosteum. One rib was analyzed for bone mineral content. After sectioning of the rib into 1-cm pieces and removal of the marrow by force of air and water, the pieces were defatted overnight in a 3:1 ethanol:ether solution and subsequently dried for 24 h at 100°C to determine dry weight. The dried rib sections were then heated in porcelain

TABLE I
Serum Chemistries and Immunoreactive PTH in Normal Dogs and Dogs Fed a Magnesium-free Diet for 4–6 mo

	Calcium	Phosphorus	Magnesium	i-PTH*
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*μeq/ml*
Normal (n = 7)	10.3±0.2	4.0±0.2	1.83±0.1	85±7
Magnesium-deficient (n = 7)	9.5±0.3	3.1±0.2	1.34±0.15	34±11
P value	<0.05	<0.02	<0.01	<0.001

Values are mean±SEM. Statistical analysis by nonpaired t test.
* Immunoreactive PTH concentration.

crucibles to 800°C for 24 h, and the resultant ash was dissolved in 10 ml of concentrated hydrochloric acid, diluted 1:20 with distilled water, and assayed in triplicate for calcium, magnesium (Atomic Absorption Spectrophotometer 502, Perkin-Elmer Corp.), and phosphorus (Technicon AutoAnalyzer, as described above).

Bone histology. The other rib was fixed in neutral buffered formalin, subsequently embedded (nondecalcified) in methylmethacrylate, cut in sections 5-μm thick (model K Sledge Microtome, Jung Instruments, Heidelberg, West Germany), and subjected to histomorphometric analysis as previously described (31). At the time of the histologic examination, the pathologist was unaware of the identity of the sample.

Source of PTH. Syn b-PTH 1–34 (3,850 U/mg in rat renal adenylate cyclase system) was purchased from Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

RESULTS

Serum and bone chemistries. In Table I, the mean (±SEM) values for serum calcium, phosphorus, and magnesium levels (mg/100 ml) from seven dogs fed the magnesium-free diet for 4–6 mo are contrasted with those obtained from seven normal dogs. Although the serum calcium in the magnesium-restricted dogs was significantly lower than in the normals, the mean was still within the normal range for canine serum total calcium concentration (9.3–10.5 mg/100 ml). The maintenance of the serum calcium within the normal range can be attributed to the high calcium intake of the test animals (see Methods).

Serum immunoreactive PTH in the magnesium-restricted animals, 34 μleq/ml (a mean which excludes two undetectable values), was also significantly lower than in the controls (85±7 μleq/ml).

Table II contrasts the calcium, magnesium, and phosphorus content of bone obtained from dogs fed a normal diet with that from dogs fed the magnesium-free diet. The magnesium content of these latter bones (2.5 ±0.1 mg/g dry weight) was significantly lower (P < 0.001) than that of the normal bones (3.6±0.1 mg/g dry weight). No difference in calcium or phosphorus content of bone was found.

Uptake of syn b-PTH 1–34 by the isolated perfused bone. Fig. 1 contrasts the percent extraction of immunoreactive PTH (arterial-venous difference for immunoreactive PTH across the bone divided by the arterial immunoreactive PTH concentration) in seven normal bones with that obtained in seven bones from

TABLE II
Bone Chemistries in Normal Dogs and Dogs Fed a Low-Magnesium Diet for 4–6 mo

	Calcium	Phosphorus	Magnesium
	mg/g dry wt	*mg/g dry wt*	*mg/g dry wt*
Normal (n = 3)	227.2±6.2	109.6±2.8	3.6±0.1
Magnesium-deficient (n = 3)	217.2±1.6	108.3±1.3	2.5±0.1
P value	NS	NS	<0.001

Results are expressed as mean±SEM of triplicate determinations of bone mineral from three to five segments of rib from three animals in each group. Statistical analysis by nonpaired t test.

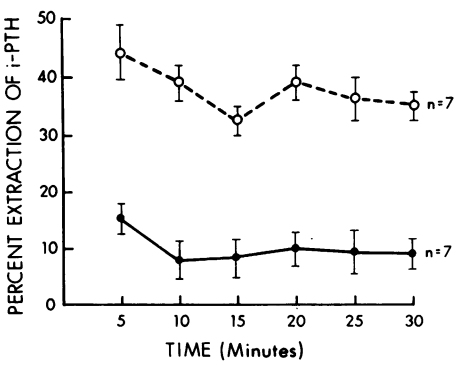

FIGURE 1 Mean percent extraction (±SEM) of amino-terminal immunoreactive PTH (i-PTH) by isolated perfused bones from seven normal (○) and seven hypomagnesemic (●) dogs during a constant infusion of syn b-PTH 1–34 at a concentration of 3 ng/ml.

hypomagnesemic dogs during a continuous infusion of syn b-PTH 1–34 at a concentration of 3 ng/ml. The mean percent extraction of immunoreactive PTH in the magnesium-deficient bones (10.1±1.2%) was markedly reduced ($P < 0.001$) when compared with the controls (37.5±3%).

Studies of PTH-stimulated cAMP production. The ability of the above infusion of syn b-PTH 1–34 to stimulate cAMP production by the perfused bone was used to assess its biologic effect. These results are shown in Fig 2. As a group, mean basal cAMP production by the bones from the magnesium-restricted dogs (4.5±0.1 pmol/min) was significantly less ($P < 0.01$) than that from the controls (5.8±0.2 pmol/min). During the continuous infusion of syn b-PTH 1–34, cAMP production by the control bones increased to a mean of 17.5±0.7 pmol/min for the next 30 min, whereas the cAMP response in magnesium-deficient bones was blunted, rising to a mean of only 7.1±0.4 pmol/min ($P < 0.001$). Even when perfusate syn b-PTH 1–34 concentration was increased to 20 ng/ml, cAMP production by the bones ($n = 4$) from the magnesium-restricted dogs was lower than that by the control bones perfused with syn b-PTH 1–34 at a concentration of 3 ng/ml (data not shown). Attempts to achieve acute magnesium repletion in vitro by perfusing the bones from magnesium-deficient animals with perfusate that contained 5 mM of Mg^{++} for 30–60 min did not reverse the blunted cAMP response to PTH (data not shown).

Bone histology. To determine if magnesium restriction resulted in morphological changes in bone, undecalcified sections of ribs were examined by histologic techniques and quantitative micromorphometry. Representative histologic appearance is shown in Fig. 3. The results of the quantitative micromorphometric analysis of ribs obtained from three normal dogs and from three magnesium-restricted dogs are shown in Fig. 4. Skeletal tissue from the magnesium-restricted animals is characterized by significant decrements ($P < 0.05$) in both the percentage of nonmineralized bone matrix (percent relative osteoid volume) and the percentage of trabecular bone surface covered by osteoid (percent total osteoid surface).

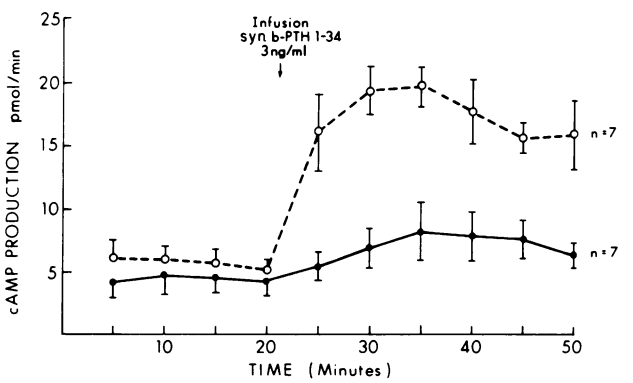

FIGURE 2 Mean cAMP production (±SEM) by isolated perfused bone from seven normal (○) and seven hypomagnesemic (●) dogs during infusion of syn b-PTH 1–34 at a concentration of 3 ng/ml.

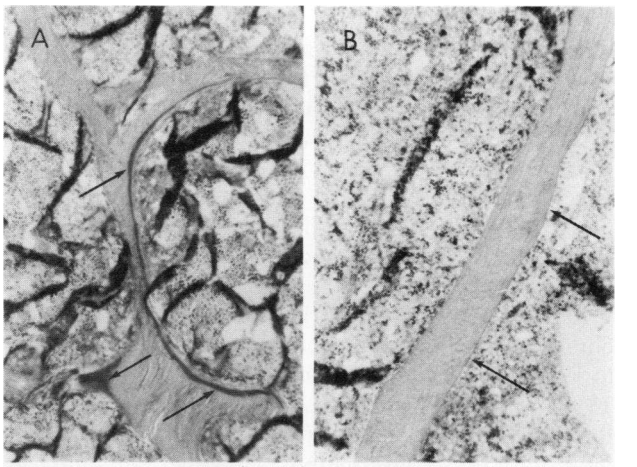

FIGURE 3 Representative bone histology from a normal dog is shown in panel A. The arrows indicate the presence of moderate amounts of dark-staining osteoid. In magnesium deficiency (panel B), osteoid is virtually absent.

DISCUSSION

These studies were designed to examine the skeletal response to PTH in magnesium deficiency. This was examined directly by perfusing bone from adult mongrel dogs in vitro with a low dose (0.7 nM) of the synthetic, biologically active, amino-terminal portion of bovine PTH, syn b-PTH 1–34. Hypomagnesemia and magnesium deficiency were induced by a prolonged dietary restriction of magnesium alone. Serum calcium was intentionally maintained within the normal range by ensuring adequate calcium intake by the dogs fed the magnesium-free diet (3.8 g Ca^{++}/400 g diet), thus minimizing a potential modification of the skeletal response to PTH secondary to variations in serum calcium.

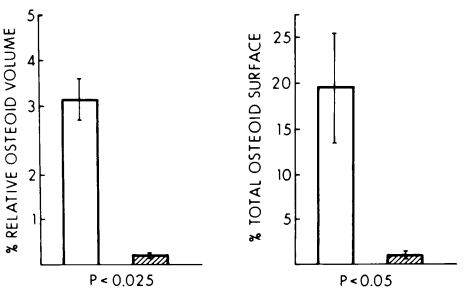

FIGURE 4 Quantitative bone histology (±SEM) from three normal (□) and three magnesium-depleted dogs (▨). Statistics calculated by nonpaired *t* test.

Without this excess supplemental calcium, the dogs would have become hypocalcemic on the magnesium-restricted diet analogous to the hypocalcemia observed with magnesium deficiency in man. Only syn b-PTH 1–34 was used in these studies because it had been shown previously (24) that the isolated perfused bone is relatively insensitive to exogenously administered intact b-PTH 1–84 (i.e., there is no extraction of the intact hormone nor a marked increase in cAMP by bone after its administration).

Many observations in the literature indicate that chronic magnesium deficiency is associated with normal to low levels of immunoreactive PTH (3, 4, 9, 11, 12, 18). The reduced serum immunoreactive PTH levels found in our magnesium-restricted dogs are in agreement with these observations. However, although the levels of immunoreactive PTH were low in the hypomagnesemic dogs as compared with normal, they still fell within the normal range for canine immunoreactive PTH. Therefore, "hypoparathyroidism" may not be the only cause of hypocalcemia in chronic magnesium depletion. Rather, these observations suggested that other factors are important in the induction of hypocalcemia associated with magnesium deficiency.

Our studies with isolated perfused bones demonstrate skeletal resistance to the action of PTH during magnesium deficiency. Both PTH uptake (extraction) and cAMP production in response to PTH were markedly reduced in bones from hypomagnesemic dogs. Because many studies, both in vivo (22, 32, 33) and in vitro (34–36), have suggested that cAMP production may be an indicator of PTH-stimulated bone resorption, the blunted PTH-stimulated cAMP release in magnesium-deficient bone (Fig. 2) very likely represents a blunted biological effect of the hormone on the skeleton. This reduced biological effect correlates well with the finding of a reduced arteriovenous difference for syn b-PTH 1–34 across the magnesium-deficient bones (Fig. 1).

The reason(s) for the reduced PTH extraction and blunted cAMP production by the magnesium-deficient bones is not known at this time. Because adenylate cyclase activity is magnesium dependent, it is possible that chronic magnesium deficiency may impair adenylate cyclase activity, resulting in reduced cAMP production (37). Although this is a tenable explanation for the observed impairment of PTH-stimulated cAMP production in these studies, it does not account for the reduction in the extraction of PTH across bones from the hypomagnesemic animals. A potential additional effect of magnesium deficiency may be suggested based on the recent studies of Williams et al. (38), who demonstrated that hormone binding to the adenylate cyclase-coupled receptor is impaired at low magnesium concentrations in vitro. Therefore, it is possible that in our studies the reduced skeletal uptake of PTH and the blunted cAMP response in the magnesium-deficient dogs represents impaired binding of PTH to its receptors in bone. Furthermore, this could explain why the inverse correlation between receptor number (and/or affinity) and circulating levels of hormone found in other endocrine systems (39) does not appear to be operative in these studies.

In addition to effects of magnesium deficiency itself on PTH extraction by bone, it is possible that structural alterations in the skeleton or changes in the microcirculation per se could account for the reduced PTH extraction and blunted cAMP response observed in these experiments. Previous studies by Reddy et al. (8) examined by microradiographic and histologic techniques epiphyseal bone of growing, magnesium-deficient chicks and demonstrated an increase in nonmineralized tissue. These authors suggested that the skeletal resistance to PTH may be secondary to this excess osteoid. Accordingly, we examined trabecular bone from normal and magnesium-deficient adult dogs by the sensitive method of quantitative micromorphometry. The results of these histologic studies suggest that the skeletal tissue is indeed altered in magnesium-restricted animals. The ribs from these animals, however, manifested a marked decrement in both percent relative osteoid volume and percent total osteoid surface. Because osteoid in normal animals represents sites of current bone formation (40), magnesium deficiency appears to markedly suppress skeletal synthesis and result in a histologic picture compatible with skeletal inactivity. This adynamic-appearing bone could also play a role in the reduced immunoreactive PTH excretion and blunted cAMP response observed in these magnesium-restricted dogs. However, it is also possible that the histologic picture observed in bone in magnesium deficiency is not the cause but the consequence of decreased bone turnover as a result of diminished extraction and action of PTH on the skeleton.

In summary, the dogs fed a magnesium-free diet for 4 mo manifested a decreased serum calcium, phosphorus, magnesium, and immunoreactive. PTH. Bones from these dogs had a reduced magnesium content and a histologic picture compatible with skeletal inactivity. Percent immunoreactive PTH extraction was significantly lower, and cAMP production was significantly blunted, both in the basal state and after PTH infusion, in the bones from the magnesium-restricted dogs. These data suggest that skeletal resistance to PTH is a feature of chronic magnesium depletion.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Henry Burke for his technical assistance, and Mrs. Mary Worsdell and Mrs. Pat Verplancke for their secretarial assistance.

This work was supported by U. S. Public Health Service National Institutes of Arthritis, Metabolism, and Digestive Diseases grants AM-09976, AM-07126, and AM-11674.

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