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Influence of Dietary Ca, Mg, and P on Cyclic-AMP Excretion and Kidney Calcification in the Rat (39177)

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There is abundant evidence that one consequence of magnesium deficiency in animals is a disturbance of calcium metabolism (1, 2). Calcification of soft tissues (2, 3), alteration of serum calcium levels (4, 5), and elevated concentration of bone calcium (2) are frequently observed. Since parathyroid hormone (PTH) is a major factor controlling calcium metabolism, it is not surprising that hypotheses regarding alterations in parathyroid (PT) gland activity in magnesium deficiency have been proposed (5–7).

Most species of animals have been reported to develop hypocalcemia when fed a magnesium-deficient diet, although the data are not uniform (7, 8). Suggestions that Mg deficiency is accompanied by decreased PT activity are based on observations that serum Ca is decreased in Mg-deficient cattle (9), sheep (10), dogs (11), monkeys (7), man (12), and chickens (5). The rat may be unique in that most reports on this species indicate that Mg deficiency is associated with hypercalcemia, hypophosphatemia, and hyperphosphaturia, all changes that may be induced by PTH in excess (5, 6, 13). A number of studies have shown by tissue culture or *in situ* PT gland perfusion that PTH production is stimulated by low Mg as well as by low Ca, although it is agreed that the gland is more sensitive to the Ca concentration of the culture medium or perfusion fluid (14, 15). That the PT gland does play a role in the calcification of soft tissue seen in Mg deficiency is clear from the several experiments showing kidney calcification not to occur in parathyroidectomized rats (6, 17, 18).

The following series of experiments was conducted in an attempt to assess the effects of Ca deficiency, P excess, and Mg deficiency on PT activity in young rats. The major criterion for determining PT activity was the urinary excretion of cyclic adenosine monophosphate (cAMP). It would be desirable to determine circulating parathyroid hormone activity directly, but such an assay, applicable to the rat, was not available to us. It has been demonstrated that administration of exogenous PTH will activate renal cortical adenyl cyclase (19) and increase cAMP excretion briefly (16), but the effects of chronic differences in endogenous PT activity on cAMP excretion have not been previously reported.

Experimental procedures. In the several experiments reported here we employed male Sprague–Dawley rats weighing about 100 g at the start. The composition of the general basal diet is shown in Table I. Desired adjustments in Ca, Mg, and P content were made by appropriate changes in the salt mix.

The rats were housed singly in stainless steel cages in air-conditioned quarters, were fed *ad libitum*, and were given distilled water. Feed intake and weight gains were monitored, but the diets were so designed that large differences in these criteria did not usually occur. Exceptions are noted in description of individual experiments. Experiments were terminated by exsanguination of lightly etherized rats from the abdominal aorta.

Cation analyses were conducted by atomic absorption spectrophotometry. Phosphorus in serum was determined by the method of Fiske and Subbarow (20), and in other samples by the A.O.A.C. colorimetric procedure (21). Creatinine was determined by the alkaline picrate method (22). Urine samples to be analyzed for cAMP were collected over a 12-hr period. The collection tubes were immersed in an ice bath and the collected urine was frozen until analyzed. Each urine sample was diluted 20-

TABLE I. CONSTITUENTS OF THE CONTROL DIET.

Ingredient	%
Casein	15.0
DL-methionine	0.3
Corn oil	8.0
Cellulose	3.0
Vitamin mix[a]	5.0
Mineral mix[b]	3.8
Glucose monohydrate	64.9

[a] See (5).

[b] Complete mix of Jacob and Forbes, J. Nutr. **100,** 228 (1970).

fold with acetate buffer, pH 4, and analyzed for cAMP by the method of Gilman (23). Creatinine was determined on the same diluted sample, and the results were expressed as picomoles cAMP per milligram creatinine.

Statistical analyses were conducted by analysis of variance procedures followed by Tukey's *W* procedure for comparison of individual means where appropriate. Differences cited as significant or highly significant have *P* values of 0.05 or 0.01, respectively.

Experiment A was conducted to verify the effect of exogenous PTH on urinary cAMP excretion. Fifteen rats were fed the basal diet containing 700 ppm Mg, 0.6% Ca, and 0.6% P. After 3 days of adjustment to this diet the rats (average weight 130 g) were divided into three equal groups. The groups were given twice daily ip injections of saline, 45 units PTH (Eli Lilly Parathormone, 100 units/cc)[1], or 90 units PTH for 2 days. Two 12-hr urine collections were made from all rats, starting 12 and 36 hr after first injection. At the end of the experiment, blood samples and kidneys were removed; the serum was analyzed for Ca, Mg, and P, and the kidneys for Ca after obtaining dry weights and ashing with nitric acid followed by hydrogen peroxide (Table II).

Experiment B was a preliminary experiment designed to detemine the effect of Mg deficiency and of low Ca, high P diets on the excretion of cAMP. Three groups of 10 rats each were employed. They were fed the experimental diets containing the Ca, P, and Mg levels shown in Table III for 14 days. After Day 13, urines were collected on ice overnight, then frozen and stored until analyzed for cAMP. Twenty-four hours later, the rats were killed and blood and kidneys taken for mineral analysis. Rats had access to feed until 9 AM, the time of killing.

Experiment C was designed to investigate the effects of various degrees of dietary Ca deficiency and P excess on urinary excretion of cAMP. All diets contained 900 ppm Mg with Ca and P levels as shown in Table IV. The experiment was run in two periods employing 24 rats in four groups. In each period, one group received the normal Ca and P diet as a control. The Ca and P levels were chosen to provide Ca-deficient and normal diets with normal or excess P, and a high Ca, normal P diet, which we expected to depress PT activity.

The rats were initially given 12 g of diet; this was increased daily to a limit set by animals consuming the least amount of food. At the end of 2 weeks they were fasted overnight, during which time a urine collection was made on ice for cAMP analysis. Blood, kidneys, and tibias were removed for analysis. The tibias were extracted successively with alcohol and Skelly F and the fat-free dry weights obtained, then dry-ashed at 600° and the ash weights determined.

Experiment D was conducted to extend the observations of Experiment B, which suggested that Mg deficiency, in contrast to Ca deficiency, might lower rather than raise urinary cAMP. Twenty-five rats were assigned in groups of five to diets as shown in Table V, providing four levels of Mg deficiency (16–75 ppm) and a Mg-adequate control (660 ppm). After 10 days the animals were fasted overnight while urine was being collected for cAMP analysis. The 12-hr fasted animals were then killed, and blood and kidneys removed for analysis.

Experiment E investigated the effects of Ca, Mg, and their combination on cAMP excretion. Twenty-four rats were assigned in groups of six to diets as shown in Table VI. After 10 days on these diets, the animals were treated as in the previous experiment. Blood, kidneys, and tibias were removed for analysis as before.

[1] Generously donated by Dr. Herbert Brown, Eli Lilly and Co.

TABLE II. EFFECT OF PTH ON SERUM MINERALS[1] AND KIDNEY Ca[2].

	Ca	P	Mg	Kidney Ca
Saline	11.7 ± 0.2[a]	11.7 ± 0.5[a]	2.59 ± 0.05[a]	0.34 ± 0.01[a]
45 IU	12.7 ± 0.3[b]	9.0 ± 0.4[b]	2.46 ± 0.08[a]	0.37 ± 0.03[a]
90 IU	13.5 ± 0.2[b]	9.7 ± 0.9[b]	2.83 ± 0.08[b]	26.9 ± 10.9[b]

[1] In mg/100 ml. Mean values ± SEM.
[2] In mg/g dry wt. Mean values ± SEM.
[3] Means in the same column bearing different superscripts are different at the 5% level of significance.

TABLE III. INDICES OF PARATHYROID ACTIVITY IN EXPERIMENT B.[1]

Diet (%)			Kidney Ca (mg/g dry wt)	Serum (mg/100 ml)			cAMP (pmoles/μg creatinine)
Ca	P	Mg		Ca	P	Mg	
0.74	0.64	0.086	0.36 ± 0.02	11.2 ± 0.2	10.5 ± 0.3	2.29 ± 0.06	21.2 ± 0.91[a]
0.73	0.64	0.009	9.2 ± 2.6	11.5 ± 0.1	9.2 ± 0.3	0.93 ± 0.06	19.3 ± 1.4[a]
0.004	1.8	0.086	5.8 ± 0.9	9.3 ± 0.2	10.7 ± 0.6	2.72 ± 0.09	29.9 ± 1.1[b]

[1]Mean ± SEM. Means in a given column bearing different superscripts are different at the 5% level of significance.

TABLE IV. RESPONSE TO VARIATION IN DIETARY CALCIUM AND PHOSPHORUS.[1]

Period	Diet		Urine cAMP (pmoles/μg creatinine)	Kidney Ca (mg/g dry wt)	Tibia ash, wt (g)			
	Ca(%)	P(%)				Ca	P	Mg
2	1.55	0.43	14.7[a] ± 0.8	0.33[a] ± 0.01	0.239 ± 0.006[a]	11.0 ± 0.1[a]	7.1 ± 0.4[a]	1.7 ± 0.1[a]
1	0.50	1.40	19.2[b] ± 0.8	41.6[b] ± 5.6	0.215 ± 0.003[a]	8.9 ± 0.1[ef]	7.8 ± 0.4[ab]	2.5 ± 0.1[bc]
1	0.50	0.43	22.3[bc] ± 1.2	0.31[a] ± 0.02	0.216 ± 0.004[a]	9.8 ± 0.2[cd]	9.8 ± 0.2[c]	2.5 ± 0.1[bc]
2	0.50	0.43	22.4[bc] ± 1.2	0.32[a] ± 0.01	0.224 ± 0.006[a]	10.3 ± 0.1[bc]	9.4 ± 0.2[c]	2.2 ± 0.04[b]
2	0.10	1.55	26.1[cd] ± 1.3	48.0[b] ± 12.1	0.176 ± 0.003[b]	8.7 ± 0.2[f]	7.0 ± 0.4[a]	2.6 ± 0.1[c]
1	0.02	0.43	27.3[d] ± 1.3	0.25[a] ± 0.01	0.152 ± 0.004[b]	8.6 ± 0.2[f]	9.9 ± 0.2[c]	2.5 ± 0.1[bc]
1	0.02	1.40	27.6[d] ± 1.5	20.0[b] ± 7.9	0.152 ± 0.002[b]	8.5 ± 0.2[f]	7.6 ± 0.3[ab]	2.5 ± 0.1[bc]
2	0.10	0.43	35.1[e] ± 1.9	0.29[a] ± 0.1	0.174 ± 0.009[b]	9.4 ± 0.1[de]	9.1 ± 0.3[bc]	2.3 ± 0.1[bc]

[1] Mean values ± SEM. Means in a given column bearing different superscripts differ at the 1% level of significance as judged by analysis of variance and Tukey's *W* test.

TABLE V. RESPONSES TO GRADED LEVELS OF DIETARY MAGNESIUM.[1]

Diet Mg (ppm)	Weight gain (g/10 days)	Urine cAMP (pmoles/μg creatinine)	Serum (mg/100 ml)		Kidney Ca (mg/g dry wt)
			Ca	Mg	
16	15 ± 1	10.0 ± 1.1[a]	12.7 ± 0.3[a]	1.1 ± 0.1[a]	24.9 ± 8.3
40	26 ± 2	14.9 ± 1.9[ab]	12.4 ± 0.2[a]	1.1 ± 0.1[a]	8.5 ± 2.6
60	30 ± 2	16.5 ± 2.4[ab]	12.6 ± 0.2[a]	1.1 ± 0.1[a]	4.6 ± 2.8
75	43 ± 4	16.0 ± 1.4[ab]	12.7 ± 0.1[a]	0.8 ± 0.1[a]	14.7 ± 7.9
660	51 ± 3	19.6 ± 1.5[b]	11.7 ± 0.2[b]	2.2 ± 0.2[b]	0.36 ± 0.01

[1] Mean ± SEM. Means in a given column bearing different superscripts are different at the 5% level of significance.

Results and discussion. The effect of injected PTH on the urinary excretion of cAMP (Experiment A) is shown in Fig. 1. Since the data obtained did not vary between periods as indicated by analysis of variance, they were pooled and demonstrate a nearly linear dose–response curve whose slope is highly significant ($P < 0.01$). Table II illustrates that the effects of PTH on serum Ca and P, and kidney Ca, occurred in the expected directions. Serum Ca level was significantly elevated by the smaller PTH dose, while the larger dose yielded an increase that was highly significant. Serum P was decreased significantly by the lower PTH dose, and serum Mg was

TABLE VI. RESPONSES TO DIETARY LOW MAGNESIUM, LOW CALCIUM, AND THEIR COMBINATION.[1]

Diet				Serum			
Mg (ppm)	Ca (%)	Weight gain (g/10 days)	Urine cAMP (pmoles/μg creatinine)	Ca (mg/100 ml)	Mg (mg/100 ml)	Kidney Ca (mg/g dry wt)	Tibia ash (g)
600	0.6	56 ± 9	24.4 ± 1.4[b]	9.2 ± 0.2[a]	2.4 ± 0.1[a]	0.36 ± 0.08[a]	0.204[a]
50	0.6	37 ± 2	16.9 ± 2.6[a]	9.6 ± 0.1[a]	1.0 ± 0.1[c]	18.2 ± 5.9[b]	0.207[a]
600	0.01	49 ± 7	36.0 ± 1.7[c]	7.7 ± 0.4[b]	2.6 ± 0.1[a]	0.32 ± 0.01[a]	0.141[b]
50	0.01	39 ± 9	33.1 ± 1.7[c]	7.2 ± 0.1[b]	1.6 ± 0.1[b]	9.6 ± 6.6[b]	0.136[b]

[1] Mean values ± SEM. Means in a given column bearing different superscripts are different at the 5% level of significance.

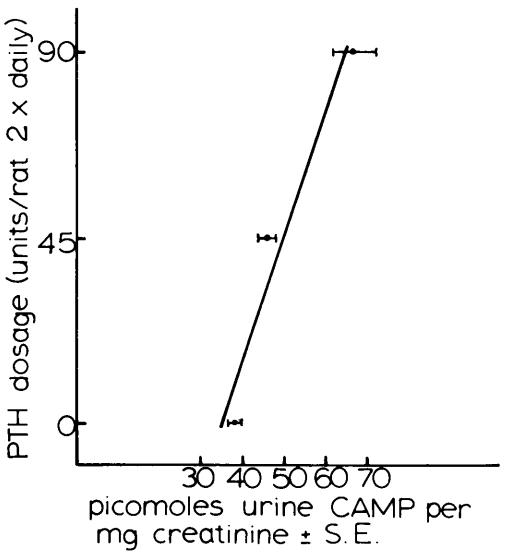

FIG. 1. Response of urinary cyclic-AMP to twice daily intraperitoneal injection of parathyroid hormone. Five rats per treatment group. Overnight collection ending 24 and 48 hr after initial injection. Data for the two periods are pooled.

raised significantly by the higher PTH treatment. Only the higher dose yielded a significant degree of calcium deposition in the kidneys.

Chase and Aurbach (16) obtained a striking, although brief, increase in cAMP excretion in parathyroidectomized rats in response to injected PTH. This experiment has shown that one may detect an increase in the urinary excretion of cAMP in response to exogenous PTH in a 12-hr collection in intact rats receiving a normal diet.

It is generally agreed that diets low in Ca or excessive in P content result in an increase in PT activity. Experiment B was conducted to determine if the endogenously altered PTH resulting from such treatment could be correlated with increased cAMP excretion and also to determine the effect of Mg deficiency on cAMP excretion. The data obtained are shown in Table III. It can be seen that the low Ca, high P diet did indeed significantly increase cAMP excretion but that Mg deficiency failed to do so. Both of the experimental diets resulted in kidney calcification.

Experiment C was conducted to assess the effects of varying separately the Ca and P contents of the diets. The results are given in Table IV. Diets containing 0.5 and 0.43% of Ca and P, respectively, were the control diets. As a result of the controlled feed intake, body weight gains averaged 2.8 g daily and did not vary significantly between groups. The excretion of cAMP was depressed to 65% of the control value by increasing Ca threefold ($P < 0.01$) but was not affected by increasing dietary P about threefold. On the other hand, decreasing dietary Ca increased cAMP excretion by 17 to 57% without a clear relationship to dietary P. Since an initial objective of our studies has been an investigation of a possible relationship between kidney calcification and PT activity, it is of importance to note that calcification occurred only in those animals receiving high phosphorus diets, irrespective of calcium level or of PT activity as judged by cAMP excretion. Increasing dietary P level decreased the fasting values of serum P at normal or low calcium intake levels and also decreased serum Ca except at the lowest Ca intake. Data showing the same directional trend have been reported by Clark (24) and by Sie *et al.* (25) for Ca. The reduction in fasting serum P might be a result of reduced P

reabsorption in the kidney associated with accelerated PT activity. Sie *et al.* (25) have interpreted the reduced fasting serum Ca accompanying increased dietary P as a result of stimulated PT activity. It is also possible that this effect is a reflection of slow adjustment or overcompensation at the kidney tubule level to the necessity of excreting the excess P during the period it was actively being absorbed from the intestine. A similar reduction in serum P was not observed in Experiment B (Table III) in which blood was drawn from nonfasted animals. In any event, the lack of cAMP response to high P intake and the increase of cAMP excretion in Ca deficiency, both situations accepted as increasing PTH activity, cannot be explained at present. It may be that the high P diet decreases extrarenal cAMP production as much as the induced hyperparathyroidism increases renal cAMP production, whereas this does not occur in Ca deficiency.

The tibia ash data clearly reflect the decreased bone mass in the Ca-deficient rats. The percent ash did not differ significantly between Ca levels. Within Ca levels, dietary P changes did not affect tibia ash weight. Some portion of the observed differences in tibia ash weight is doubtless a reflection of the inadequate intake of Ca for bone formation, although an increased resorption stimulated by increased PTH could also contribute to these differences. We cannot assess the relative contributions of these two factors.

Since the data of Experiment B did not support the concept of hyperparathyroidism in Mg deficiency as judged by cAMP excretion, a further examination (Experiment D) was undertaken, employing graded levels of dietary Mg, with results as shown in Table V. As in the previous experiment, the Mg-deficient animals had reduced feed intakes and consequently low weight gains. There was a significant trend towards a reduced cAMP excretion. The Mg-deficient animals showed a significant elevation of serum Ca, a highly significant lower serum Mg, and a definite accumulation of Ca in the kidneys, all indications of Mg deficiency. The failure of a severe Mg deficiency to increase cAMP excretion again illustrates a difference in response of the rat between Mg and Ca deficiencies and indicates that in the former deficiency PT activity is decreased rather than increased.

The possibility remained that Mg deficiency exerted a specific effect on the response of the kidney to PTH. If a low serum Mg prevented the activation of renal cortical adenyl cyclase by PTH, then hyperparathyroidism could be present without resulting in an increased excretion of cAMP. Experiment E, in which a deficiency of Ca and Mg occurred together, was designed to test this hypothesis. The results are shown in Table VI. We see that Mg deficiency itself reduced cAMP excretion 30%, while Ca deficiency either alone or in the presence of Mg deficiency increased it 41% in comparison to the control group. Thus, Mg deficiency per se does not prevent the cAMP response to Ca deficiency, and we presume that the PTH-adenyl cyclase system remains intact in the Mg-deficient rat. Anast *et al.* (12) have shown markedly depressed serum immunoreactive parathyroid hormone levels in a magnesium-deficient human. This was quickly responsive to intramuscular injection of $MgSO_4$.

The serum Ca values were predictably depressed in the Ca-deficient rats, as were the serum Mg values in the Mg-deficient animals. Those animals deficient in both minerals exhibited a less severe depression in seurm Mg than those deficient in Mg only. This was accompanied by less severe external signs (erythema and skin lesions) of Mg deficiency. Kidney calcification was evident in both the Mg-deficient and the (Mg and Ca)-deficient animals, and was therefore not associated with increased cAMP excretion. Ca-deficient animals had decreased bone mass, as shown by the tibia ash weights. Again, this may reflect a failure of bone formation due to inadequate Ca intake, or excessive bone resorption due to increased PTH secretion, or both.

We are left, then, with a paradox. On the one hand, the PT glands must somehow be involved in kidney calcification, since the kidneys of parathyroidectomized Mg-deficient rats fail to calcify (6, 17, 18). The cAMP excretion data, on the other hand, suggest that the Mg-deficient rat has, if any-

thing, less PTH output than the normal animals, and we have shown that this is not a simple failure of the ability to excrete cAMP. Clearly, more information is needed before the mechanism of kidney calcification can be determined.

Summary. A series of five experiments was conducted with young male albino rats to investigate effects of various levels of dietary Ca, P and Mg on urinary cAMP excretion and kidney calcification. Urinary cAMP excretion was shown to be directly correlated with injected parathyroid (PT) hormone dose level and to be inversely associated with dietary Ca intake. Thus, cAMP excretion may be presumed to reflect PT activity in the young rat. Magnesium deficiency tended to reduce cAMP excretion, while P excess did not affect it. Each treatment induced kidney calcification. Calcium deficiency increased cAMP excretion irrespective of Mg status, although nephrocalcinosis appeared only in the Mg-deficient animals. These data support the view that nephrocalcinosis of dietary origin in the rat is not mediated by increased PT activity.

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