Intestinal Calcium-Binding Protein and Rickets[1] (35305)

EDWARD L. KRAWITT AND ARTHUR S. KUNIN
(Introduced by B. Forsyth)
Department of Medicine, College of Medicine, University of Vermont, Burlington, Vermont 05401

Calcium homeostasis is a complex process being in part a function of the calcium:phosphorus dietary ratio and in part a reflection of parathyroid function and the effects of vitamin D on bone, kidney, and intestine. In the rat, unlike man, rickets is not produced by vitamin D deficiency alone if the dietary Ca:P ratio is optimal (1, 2) but it can be produced by a dietary phosphate deficiency. When experimental rickets is present, glycolysis, as measured by lactate production and glycolytic enzyme activity, is increased in epiphyseal cartilage (3, 4). Several transport proteins have recently been isolated from bacterial and mammalian organisms (5), including calcium-binding proteins which are thought to have a role in calcium translocation. A vitamin D-induced intestinal calcium-binding protein was originally isolated from chicks by Wasserman and Taylor (6). A similar protein has subsequently been induced by vitamin D administration in rats (7–9).

These experiments were undertaken to determine the effect of phosphate deficiency and rickets on the induction of rat intestinal calcium-binding protein by vitamin D and to ascertain whether dietary phosphate supplementation, which prevents rickets, affects the level of calcium-binding protein.

Methods. Male weanling rats of the Sprague-Dawley strain were divided into 2 groups based on dietary regimen. One group was fed a rachitogenic diet which was vitamin D free, high in calcium and low in phosphorus (1.2% calcium, 0.1% phosphate) (10). A second group was fed the same basic ration modified, however, by the addition of NaH_2PO_4 to produce a calcium:phosphorus ratio of 1.4:1. The diets were prepared in this laboratory from basic ingredients obtained from Nutritional Biochemical and General Biochemical Companies. Free access to food and deionized, distilled water was permitted during 3 weeks of feeding. Animals were housed in metabolic cages in a windowless, constant temperature room at 22° under shielded incandescent lighting. In those animals given vitamin D, 500 IU of vitamin D_2 or D_3 was administered intragastrically 40 hr before sacrifice.

After the rats were killed by cervical dislocation, the proximal 10 cm of small intestine were removed through a midline abdominal incision and both proximal tibial heads were dissected rapidly from adjacent soft tissue.

Sagittal tissue slices of tibial epiphyseal cartilage, 0.3–0.5 mm in thickness were cut parallel to the axis of the bone shaft. (Gross and histological examination of the tibial epiphyses revealed florid rickets in the low phosphate, vitamin D-free group and somewhat thinner than normal epiphyseal plates in the group maintained on the phosphate supplemented diet.) The clearly defined horizontal plates of growth cartilage were carefully dissected from the adjacent bone in 8–12 animals and pooled in 0.85% NaCl at 2°, for metabolic studies. Prior to incubation, the cartilage slices were gently blotted and weighed on a torsion balance, approximately 1 hr elapsing between the time of sacrifice and incubation. Cartilage tissue incubations were carried out for 60 min as previously described (3). Five μmoles of glucose were added to each flask containing 3.0 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (adjusted by equilibration with 5% CO_2–95% O_2). Lactate was assayed from an aliquot of the incubation media utilizing lactate dehy-

[1] Supported in part by Research Grant AM 09632-05 from the National Institutes of Health, Bethesda, Maryland.

TABLE I. Composition and Lactate Production of Epiphyseal Cartilage.[a]

Dietary regimen	DNA (μg/100 mg of wet tissue) D−	D+	Lactate (μmoles/mg of DNA) D−	D+
Rachitogenic	38.1 ± 1.1 (6)	49.4 ± 3.4 (5)	50.7 ± 1.5 (8)	32.7 ± 1.6 (6)
Rachitogenic + NaH_2PO_4	75.4 ± 4.3 (7)	69.5 ± 4.5 (5)	26.3 ± 1.2 (6)	17.2 ± 1.1 (8)

[a] Values expressed as mean ± SE. Numbers in parentheses refer to number of tissue pools (each derived from 8–12 animals) upon which determinations were made. D—, no vitamin D administered; D+, 500 IU vitamin D_2 administered intragastrically 40 hr before sacrifice.

drogenase (EC 1.1.1.27) at 30° in 0.5 *M* glycine–0.4 *M* hydrazine, pH 8.0 (11). Tissue DNA concentrations were determined by Burton's modification (12) of the diphenylamine reaction employing deoxyribose (Calbiochem) as the standard.

The excised portions of intestine were opened lengthwise and washed in 0.9% NaCl. A pool of mucosa from 4 to 10 rats was obtained by scraping the duodenum with a glass slide, and the tissue was prepared by the method of Kallfelz *et al.* (7). A 20% (w/v) homogenate was prepared in a buffer composed of 0.0137 *M* Tris–Cl, 0.12 *M* NaCl and 0.0047 *M* KCl, pH 7.4, using a Potter-Elvehjem homogenizer with a Teflon pestle. The supernatant obtained after the homogenate was spun for 20 min at 28,000*g* in a refrigerated centrifuge was heated at 60° for 10 min. After cooling, the preparation was again centrifuged at 38,000*g* for 20 min and the supernatant was removed for assay. The assay was based on the method of Kallfelz *et al.* (7). One ml of the heat-treated supernatant was mixed with 0.5 ml of Chelex-100 cation exchange resin, which had previously been equilibrated with Tris buffer and diluted to make a suspension in which the resin represented 50% of the total volume. To the mixture of resin and supernatant, 0.5 μCi of $^{45}CaCl_2$ was added and the mixture was agitated for 15 sec. After standing for 5 min, the mixture was again agitated for 15 sec and, after an additional 5 min, was spun at 1500*g* for 5 min. Aliquots of 0.2 ml of the supernatant thus obtained were added to 10 ml of alpha-naphthylphenyloxazole (ANPO) liquid scintillation fluid in a polyethylene vial for determination of radioactivity. Protein was determined by the method of Lowry *et al.* (13) and the calcium-binding activity was expressed as a percentage of the initial radioactivity added to the assay tube per milligram of protein, according to the method of Kallfelz *et al.* (7)

Results. Animal weights. The initial mean weight of the weanling rats was 50 g. After 3 weeks, the rats on a low phosphate, vitamin D-free rachitogenic diet averaged 90 g. During the 40-hr period after vitamin D administration there was a slight reduction in food and water intake and this group averaged 78 g in weight at time of sacrifice. In contrast, the animals on the rachitogenic diet supplemented with phosphate weighed an average of 123 g after 3 weeks of feeding. During the 40 hr following vitamin D administration they also lost weight and averaged 109 g at time of sacrifice.

Cartilage DNA and lactate production. DNA was used as an index of cellularity of the cartilage slices. Table I depicts DNA values for each group. Tissue from the rachitic group had the lowest concentration of DNA per 100 mg of wet tissue and the addition of vitamin D significantly increased tissue cellularity ($p<0.01$), most probably by decreasing tissue hydration and mucopolysaccharide content as noted in an earlier study (3). Rats fed the phosphate-supplemented rachitogenic diet for 3 weeks had the greatest index of cellularity but those of this group given vitamin D showed no significant change in DNA concentration per 100 mg of wet tissue.

Lactate formation from media glucose and

TABLE II. Intestinal Calcium-Binding Activity.[a]

Dietary regimen	% ^{45}Ca in supernatant/mg of protein			Ratio D+/D—
	D—	D+		
Rachitogenic (8)	2.6 ± 0.6	5.6 ± 0.2	$p < .01$	2.2 ± 0.2
Rachitogenic + NaH_2PO_4 (9)	2.9 ± 0.6	7.0 ± 1.1	$.01 < p < .02$	2.5 ± 0.3

[a] Values expressed as mean ± SE. Numbers in parentheses refer to numbers of tissue pools (each derived from 4–10 animals) upon which determinations were made. p values were calculated from Student's t test. D—, no vitamin D administered; D+, 500 IU of vitamin D administered 40 hr before sacrifice.

endogenous glycogen stores (14), interpreted on the basis of DNA content, is shown in Table I. As reported in earlier studies (3), the highest lactate levels were produced from cartilage from rachitic rats. Forty hr after vitamin D administration, lactate production was reduced approximately 40%. Lactate production by cartilage from rats on the phosphate supplemented diet was within normal limits (3), and was slightly but significantly ($p<.01$) below that of the rachitic rats given vitamin D. When the rats on the phosphate supplemented diet were given vitamin D, lactate production decreased to below normal levels.

Calcium-binding activity. Results of the experiments measuring calcium-binding activity in the intestinal mucosa are summarized in Table II. Animals raised on the rachitogenic diet and those on the phosphate supplements had similar levels of calcium-binding activity. The level of calcium-binding activity was increased by the administration of vitamin D in rats raised on both diets and comparison of levels before and after vitamin D treatment showed the increase to be significant in both groups. Comparison of the levels of calcium-binding activity before and after administration of vitamin D within each group, expressed as the ratio D+/D—, indicated that the increase in binding activity was of the same magnitude in both groups of rats.

Discussion. In the presence or absence of rickets, vitamin D deficiency in the rat produces a defect in intestinal calcium transport (15), which can be reversed by vitamin D administration. The intestinal vitamin D-induced calcium-binding protein originally isolated by Wasserman and co-workers has been shown to correlate with calcium absorption in response to manipulation of physiological and nutritional variables in chicks (16) and in rats (9), although the actual role of this protein in the physiological state has not yet been determined. Whether or not it is actually a carrier or in some other way associated with calcium translocation in intestine is not known but studies to date indicate that it is a binding protein synthesized in response to stimuli that affect intestinal calcium absorption.

The rat intestinal calcium-binding protein originally studied was induced in animals raised on a rachitogenic diet similar to ours (1.17% calcium, 0.07% phosphate) (7). If this calcium-binding protein is dependent only on the vitamin D status of the rat, then dietary phosphate and the absence or presence of rickets should have no influence upon its level. In our study, rickets was produced by a high calcium, low phosphate, vitamin D-free diet. Administration of supplemental phosphate had no effect upon the levels of calcium-binding activity, although its administration reduced epiphyseal cartilage lactate production indicating prevention of rickets. In addition, administration of vitamin D to both the rachitic and the nonrachitic vitamin D-deficient animals resulted in a comparable induction of intestinal calcium-binding activity.

Since the increase in epiphyseal glycolysis induced by the low phosphate, vitamin D-deficient diet could be reversed to normal either by dietary phosphorus or vitamin D, while calcium-binding activity responded only to vitamin D administration, the synthe-

sis of calcium-binding protein appears to be independent of dietary phosphate deficiency and of the presence of rickets.

Summary. To determine the influence of dietary phosphate deficiency and rickets on the level of intestinal calcium-binding protein, weanling rats were raised on 2 types of vitamin D-deficient diets. A group fed a rachitogenic diet, which was vitamin D-free, high in calcium, and low in phosphorus, developed rickets as measured by increased lactate formation after *in vitro* incubation of tibial epiphyseal cartilage slices. A second group, fed the same diet supplemented with phosphate, did not develop rickets. The level of intestinal calcium-binding activity was the same in animals raised on both dietary regimens and vitamin D administration induced an increase in binding activity of the same magnitude in both groups of rats. Thus the synthesis of calcium-binding protein appears to be independent of dietary phosphate deficiency and of the presence of rickets.

The authors gratefully acknowledge the assistance of Michael Giancola, Janice Bruns, Jennifer Pastore, and Anne Montague.

1. McCollum, E. V., and Simmonds, N., "The Newer Knowledge of Nutrition," 594 pp. Macmillan Co., New York (1929).

2. Follis, R. H., "Deficiency Disease," 577 pp. Thomas, Springfield, Ill. (1958).

3. Kunin, A. S., and Krane, S. M., Biochim. Biophys. Acta **107**, 203 (1965).

4. Meyer, W. L., and Kunin, A. S., Arch. Biochem. Biophys. **129**, 438 (1969).

5. Pardee, A. B., Science **162**, 632 (1968).

6. Wasserman, R. H., and Taylor, A. N., Science **152**, 791 (1966).

7. Kallfelz, F. A., Taylor, A. N., and Wasserman, R. H., Proc. Soc. Exp. Biol. Med. **125**, 54 (1967).

8. Harmeyer, J., and DeLuca, H. F., Arch. Biochem. Biophys. **133**, 247 (1969).

9. Schachter, D., *in* "Biological Membranes" (R. M. Dowben, ed.), p. 157. Little Brown, Boston (1969).

10. Harrison, H. E., and Harrison, H. C., J. Biol. Chem. **185**, 857 (1950).

11. Ellis, J. P., McCain, S. M., and Williams, E. W., "U.S. Air Force School of Aerospace Medicine Technical Documentary Report," No. SAM-TDR-63-49, June (1963).

12. Burton, K., Biochem. J. **62**, 315 (1956).

13. Lowry, O. H., Rosebrough, N. R., Farr, D. L., and Randall, R. J., J. Biol. Chem. **193**, 268 (1956).

14. Kunin, A. S., and Meyer, W. L., Arch. Biochem. Biophys. **129**, 421 (1969).

15. Dowdle, E. B., Schachter, D., and Schenker, H., Amer. J. Physiol. **198**, 269 (1960).

16. Taylor, A. N., and Wasserman, R. H., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **28**, 1834 (1969).

Received Sept. 23, 1970. P.S.E.B.M., 1971, Vol. 136.