Evidence for a Vitamin D_3-Induced Calcium-Binding Protein in New World Primates[1] (35185)

R. H. WASSERMAN AND A. N. TAYLOR

Department of Physical Biology, New York State Veterinary College, Cornell University, Ithaca, New York 14850

Vitamin D_3, when administered to a rachitic chick (1), rat (2–4) or dog (5), induces the formation of an intestinal calcium-binding protein (CaBP). In the chick, CaBP is found in all segments of the intestinal tract including the colon (6), and the kidney (6, 7); in the laying hen, CaBP also occurs in the uterine shell gland (8). The chick CaBP has been isolated in high purity (mol wt, 24–28,000; Ca association constant, $2.5 \times 10^5\ M^{-1}$) (9) and, in the duodenum, has been localized in the brush border region and goblet cells by immunofluorescent antibody techniques (6). A considerable body of evidence, recently reviewed (10), has been accumulated which indicates that there is a high correlation between the presence of CaBP in the intestinal tissue and the capacity of the intestine to absorb calcium, suggesting that CaBP is intimately involved in the vitamin D-dependent absorptive process. The possible relevance of CaBP to the defective absorption of calcium in uremic rats was given in the recent report by Avioli *et al.* (11); the calcium absorptive defect could be partly restored by 25-hydroxycholecalciferol (25-HCC) but not by vitamin D_3 and, of these, only 25-HCC was capable of increasing the calcium-binding activity of the intestine.

Through the cooperation of the New England Regional Primate Research Center, intestinal tissue from squirrel and cebus monkeys was made available at autopsy for the purpose of determining its calcium-binding characteristics and for obtaining evidence for the presence of CaBP therein. With the same or similar monkeys as those used in this study, Hunt *et al.* (12, 13) showed that vitamin D_3 was more active than vitamin D_2 in promoting calcium absorption and causing remission of osteodystrophia fiobrosa in monkeys maintained on a vitamin D_3-free diet (with or without vitamin D_2). The present report provides evidence for the presence of a vitamin D_3-dependent calcium-binding protein in the primate.

Methods. Two species of monkeys were studied, these being the squirrel (*Saimiri sciureus*) and the cebus (*Cebus albifrons*). They were managed and maintained at the New England Regional Primate Research Center under the direction of Dr. R. D. Hunt, and made available to us through the kind cooperation of Dr. Hunt and Dr. D. M. Hegsted, Harvard School of Public Health. The eight squirrel monkeys were maintained on a commercial diet containing about 2000 IU of vitamin D_2/kg, which was rachitogenic for this species. Several weeks before sacrifice, half of the monkeys were given 500 to 1500 IU of vitamin D_3 (per os)/day. The two cebus monkeys were maintained on a vitamin D_3-free diet for about 1 year; at 9 days before sacrifice, one of the monkeys was given 500 IU of vitamin D_3 (per os)/day.

The animals were killed by injecting excess sodium pentobarbitol, the intestine was then taken and chilled immediately by placing in cold isotonic saline. Each segment was slit lengthwise; rinsed with cold saline; the mucosal layer was separated from the underlying muscle layers with a glass slide; and weighed. All of the subsequent steps were carried out at 4°. The mucosa was then homogenized in a Tris–NaCl buffer mixture (20%, w/v), using a Potter-Elvejem homogenizer with Teflon pestle. The composition of buffer mixture was: Tris–HCl ($1.37 \times$

[1] This investigation was supported by NIH Grant AM-04652 and U.S.A.E.C. Contract AT(30-1)-4039.

TABLE I. Effect of Vitamin D_3 on the Calcium-Binding Activity of Supernatant Fluid of Homogenates of Intestinal Mucosa from Two Species of New World Monkeys.

		Calcium-binding activity[a]			
		(% ^{45}Ca/ml)		(% ^{45}Ca/mg of protein)	
Species	Intestinal segment	$-D_3$	$+D_3$	$-D_3$	$+D_3$
Squirrel	Duodenum	8.9 ± 0.7 (4)[b]	19.1 ± 1.7 (4)	1.9 ± 0.2	3.7 ± 0.6
	Jejunum	7.1 ± 0.6 (4)	12.9 ± 2.8 (4)	1.3 ± 0.2	2.3 ± 0.5
	Ileum	8.7 ± 1.4 (4)	9.8 ± 0.9 (4)	1.5 ± 0.3	1.5 ± 0.1
Cebus	Duodenum	5.3 (1)	17.4 (1)	0.7	2.0
	Jejunum	5.4 (1)	12.7 (1)	0.6	2.0
	Ileum	4.2 (1)	5.2 (1)	0.5	0.8

[a] Calcium-binding activity was measured by the ion-exchange procedure described previously (1). In the assay, the soluble binding protein competes with the insoluble ion-exchange resin for added tracer ^{45}Ca. The amount of ^{45}Ca retained in the soluble phase is proportional to the concentration of soluble binder therein.

[b] The no. in parentheses = no. of animals per group.

10^{-2} *M*); NaCl (0.119 *M*); KCl (4.74×10^{-3} *M*), pH 7.4. The homogenate was centrifuged at 38,000*g* for 20 min and the supernatant fluid heated at 60° for 10 min. This step was previously found to yield a two- to threefold purification with respect to chick CaBP (1). The heat-treated supernatant was recentrifuged at 38,000*g* for 20 min. Calcium-binding activity of the final supernatant fluid was measured by the Chelex-100 ion-exchange assay procedure, described in detail elsewhere (1).

Pooled duodenal supernatant fluids from both the $-D_3$ and $+D_3$ squirrel monkey groups were placed, in turn, on a Sephadex G-100 column and the effluent fractions were

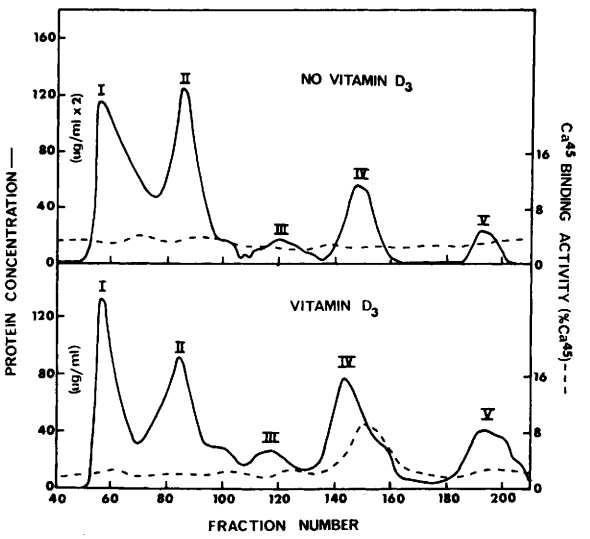

FIG. 1. Distribution of proteins and calcium-binding activity in fractions of supernatant fluid of homogenates of duodenal mucosa separated by dextran gel filtration (Sephadex 100). The applied samples were from either untreated (upper); or vitamin D_3-supplemented squirrel monkeys (lower). The only significant binding activity above background was associated with peak IV in fluid derived from the vitamin D_3-supplemented group. Calcium-binding activity was determined on 1-ml samples of the effluent by the Chelex 100 procedure (1).

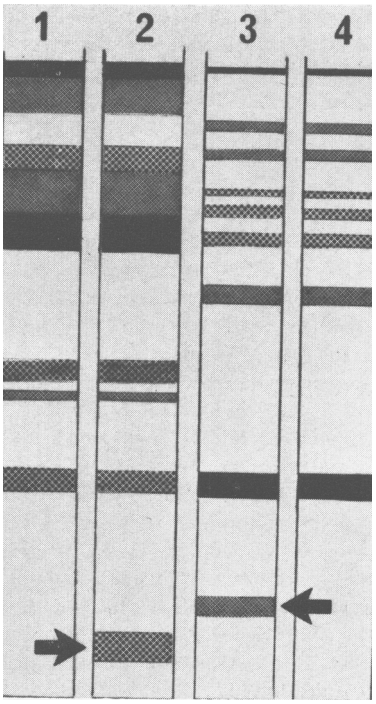

FIG. 2. Acrylamide gel electrophoresis (pH 8.3) of supernatant fluid from homogenates of duodenal mucosa from (1) rachitic chicks, (2) vitamin D_3-treated rachitic chicks, (3) vitamin D_3-supplemented squirrel monkeys, and (4) untreated squirrel monkeys. All samples were separated on a single gel slab. The arrow designates the bands that appeared only after vitamin D_3 treatment. Thirty-μl samples (about 5 mg/ml) were placed in the sample slots of the gel slab (top) and 200 V was applied for 3 hr. Migration was toward the anode (bottom).

measured for both protein concentration and calcium-binding activity. Also, proteins in aliquots of the supernatant fluids were separated electrophoretically on acrylamide gel by the slab gel technique (E. C. Apparatus, Philadelphia), as described before (7). For comparative purposes, samples of supernatant fluid of mucosal homogenates from rachitic and vitamin D_3-treated chicks, prepared as before (1), were placed on the same gel.

Protein was measured by the method of Lowry *et al.* (14).

Results and Discussion. Table I shows that vitamin D_3 administration increased the relative calcium-binding activity of the duodenum and jejunum of both the squirrel and cebus monkeys. Little or no difference was noted in the calcium-binding activity of the material from the ileum. The comparative calcium-binding activity of material from the different segments followed, on the average, the sequence: duodenum>jejunum>ileum, which was similar to that observed in the vitamin D_3-replete chick (7). In the latter species, the capacity of each segment to absorb calcium followed the same relative order (10).

Figure 1 shows the gel filtration patterns of the $-D_3$ and $+D_3$ supernatant fluids from the homogenate of duodenal mucosa. The only fractions containing calcium-binding activity above background were those associated with protein peak IV of the $+D_3$ group. The elution pattern of the primate calcium-binding activity is similar to that of the chick, but speculation on the relative molecular sizes of two calcium-binding species is not yet warranted.

The acrylamide gel pattern (Fig. 2) showed that a protein band was present in the supernatant fluid of the $+D_3$ monkey intestine which was absent in that of $-D_3$ fluid. The vitamin D_3-dependent band in the squirrel monkey intestinal supernatant fluid migrated slightly slower than the chick CaBP band. As with the chick CaBP, the monkey protein moved more rapidly than any other protein in the mixture under the conditions used. The fact that the electrophoretical mobility of the monkey calcium-binding band is less than that of the chick, suggests that the former has a lower charge density and/or a greater molecular size.

The present data indicate that the intestinal mucosa of primates, like that of the chick, rat, and dog, is capable of synthesizing a calcium-binding factor in response to vitamin D_3. Although not conclusively demonstrated, this factor is most likely a protein because of its similarity to those of other species, its elution pattern from a gel filtration column and the presence of a unique vitamin D_3-dependent, protein-staining band on the acrylamide gel. Further, these charactertistics suggest that the same procedures used in the isolation of the chick CaBP (9) might be used successfully for the monkey protein, at which time its

physical and chemical properties could be more fully assessed. These results also make it more than likely that a vitamin D-induced calcium-binding protein occurs in human intestine.

Summary. The evidence presented indicates that two species of new world monkeys, the cebus and the squirrel, are capable of synthesizing an intestinal calcium-binding factor in response to vitamin D_3 supplementation. Vitamin D_3 increased the calcium-binding activity of supernatant fluid of intestinal homogenates. The binding activity was partially purified by gel filtration chromotography. Acrylamide gel electrophoresis also showed a unique protein-staining band in the fluid from vitamin D_3-treated monkeys which was not present in the untreated animals. The monkey binding factor appears analogous to the vitamin D-induced calcium-binding proteins identified in other species.

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

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

3. Schachter, D., Kowarski, S., and Reid, P., J. Clin. Invest. **46**, 1113 (1967).

4. Moriuchi, S., Ooizumi, K., and Hosoya, N., J. Vitaminol. (Kyoto) **15**, 178 (1969).

5. Taylor, A. N., Wasserman, R. H., and Jowsey, J., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **27**, 675 (1968).

6. Taylor, A. N., and Wasserman, R. H., J. Hisotchen. Cytochem. **18**, 107 (1970).

7. Taylor, A. N., and Wasserman, R. H., Arch. Biochem. Biophys. **119**, 536 (1967).

8. Corradino, R. A., Wasserman, R. H., Pubols, M. H., and Chang, S. I., Arch. Biochem. Biophys. **125**, 376 (1968).

9. Wasserman, R. H., Corradino, R. A., and Taylor, A. N., J. Biol. Chem. **243**, 3978 (1968).

10. Wasserman, R. H., and Taylor, A. N., *in* "Mineral Metabolism" (C. L. Comar and F. Bronner, eds.), Vol. 3, Chap. 5. Academic Press, New York (1969).

11. Avioli, L.V., Scott, S., Lee, S. W., and DeLuca, H. F., Science **166**, 1154 (1969).

12. Hunt, R., Garcia, F. G., Hegsted, D. M., and Kaplinsky, N., Science **157**, 943 (1967).

13. Hunt, R. D., Garcia, F. G., and Hegsted, D. M., Lab. Anim. Care **17**, 222 (1967).

14. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

Received July 24, 1970. P.S.E.B.M., 1971, Vol. 136.