satisfied the following qualifications for validity: The plaques have been shown to be virus-associated by forming only in the presence of virus and by recovery of virus only from the plaque sites. Repeated plaque titrations with the same virus stock have given variation in plaque numbers of less than 15%. A linear relationship has been demonstrated between virus inoculum and plaque number. Specific immune sera have been shown to neutralize frog virus plaque formation (Lehane, unpublished).

Summary. A reliable plaque assay system has been developed for several recently isolated frog viruses. Cell lines from reptiles, amphibians, and fish were tested for their ability to be maintained under various solid overlay materials. Starch gel was found to be the most useful overlay material. The most sensitive assay system was shown to be *Terrapene* heart (TH-1) cell culture.

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Vitamin D-Induced Calcium Binding Factor in Rat Intestinal Mucosa.* (32011)

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It is now accepted that one of the chief functions of vitamin D is to enhance the intestinal absorption of calcium(1,2). However, the mechanism of action of vitamin D is still open to question, although several theories have been proposed(2). One of these is that the vitamin may stimulate the synthesis or operation of a carrier, possibly a protein, which facilitates the movement of calcium across the intestinal mucosa. There is some evidence indicating that vitamin D may act in a synthetic process since it has been shown that actinomycin D, an inhibitor of protein synthesis, prevents vitamin D-induced hypercalcemia in rats(3) and the vitamin D-stimulated absorption of calcium in rats(4) and chicks(5). Puromycin also inhibits the vitamin D-stimulated absorption of calcium in rats(6). In addition, Norman(7) has recently reported an increase in the synthesis of RNA, presumably mRNA, in chick intestinal mucosa after vitamin D_3 administration. However, Harrison & Harrison(8), on the basis of their studies, suggested that actinomycin D acts on the calcium active transport system *per se* and not specifically at the vitamin D-stimulated site.

Wasserman and Taylor have provided evidence suggesting that there is a substance present in the supernatant fraction of intestinal mucosal homogenates from vitamin D-treated rachitic chicks that depresses the uptake of Ca^{45} by the homogenate debris(9,

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10). Recently, the same authors reported that this substance, originally isolated from chick duodenal mucosa, is a distinct protein with the capacity to avidly bind calcium and has a soft tissue distribution that corresponds to known physiological sites of vitamin D action (11,12). It was speculated that this Ca-binding protein may be identical with or related to an intracellular carrier for calcium (12).

The studies described here were undertaken in an attempt to determine if a vitamin D induced calcium-binding factor could be found in the supernatant fraction of intestinal mucosal homogenates of another species, the albino rat. This was found to be the case.

Materials and methods. Male Sprague Dawley rats weighing 100 g were obtained from Blue Spruce Farms and placed on a rachitogenic diet according to the formula of Thomas *et al*(13) consisting of: 50% cornstarch, 20% dextrose, 15% alcohol extracted blood fibrin, 4% brewers yeast, 3% calcium carbonate, 7% vitamin A palmitate in corn oil (100 IU/ml) and 1% salt mixture. The salt mixture contained: 50% iodized sodium chloride, 44% potassium chloride, 2% ferric phosphate, 1.7% manganese sulfate (dried), 2% magnesium carbonate, and 0.3% cupric sulfate (anhydrous). The Ca and P concentrations of the diet were 1.17% and 0.07%, respectively. After 4 weeks on this diet, the animals demonstrated gross and histological evidence of rickets, and responded to vitamin D treatment with a rise in blood calcium levels, remission of rickets, and an increase in the intestinal absorption of calcium. Seventy-two hours before the experiment, 10 rats were given an oral dose of 500 IU of vitamin D_3 in one ml of a vegetable oil carrier by gavage, and another 10 animals were given 500 IU of vitamin D_2 in similar fashion. Ten control animals received only the vegetable oil carrier.

Three days after vitamin D_3 or vitamin D_2 administration, the animals were killed by decapitation. The duodenum was immediately excised, cooled to 4°C, slit open, rinsed with cold 0.12 M NaCl, and blotted. The mucosal tissue was scraped from the underlying muscle layers with a glass slide and the pooled harvest from each group of animals was homogenized in Tris buffer (20% wet weight), using a Potter-Elvehjem homogenizer with a Teflon pestle. The Tris buffer contained 1.37×10^{-2} M Tris HCl, 0.119 M NaCl, 4.74×10^{-3} M KCl, 9.85×10^{-5} M glucose; the pH was adjusted to 7.4. The homogenates were spun at $38,000 \times g$ in a refrigerated centrifuge for 20 minutes, and the supernatant fractions were recovered. These supernatants were then heated to 60°C for 10 minutes; after heating, the solutions were cooled and recentrifuged at $38,000 \times g$ for 20 minutes. The supernatant fractions were again recovered from the proteins denatured by heating. Heat treatment decreased the protein content without greatly affecting Ca-binding activity. This procedure is the same as that used by Wasserman and Taylor(11,12) in studies with the chick calcium-binding protein.

The calcium-binding capacity of the supernatant fractions was determined by the use of a method similar to that of Briggs and Fleischman(14) as modified by Wasserman and Taylor(12). The procedure depends upon the competition between a cation exchange resin and a soluble Ca-binding substance for added radiocalcium. In the presence of a chelator or a soluble complexing factor, less Ca^{45} is sequestered by the resin. The resin, Chelex-100, was first washed in Tris buffer and then diluted in Tris to a resin concentration of 0.1 ml resin per 0.2 ml of suspension. With the resin maintained in suspension by a magnetic stirrer, 0.2 ml was pipetted into a small test tube containing 1 ml of either heat-treated rachitic, vitamin D_2, or vitamin D_3 supernatant, or Tris buffer alone. This was followed by 0.1 ml of the Ca^{45} solution (approx. 0.5 μc Ca^{45} and 0.7 μg stable calcium). The tube was then mixed thoroughly for 15 seconds and centrifuged. A 0.2 ml aliquot of the supernatant was then transferred to a liquid scintillation counting vial; 5 ml of Bray's solution(15) was added to each vial and Ca^{45} radioactivity was counted by the use of a liquid scintillation counter (Nuclear-Chicago).

The concentration of protein was determined by the analytical procedure of Lowry *et al*(16).

TABLE I. Binding of Radiocalcium by the Heat-Treated Supernatant Fraction of Duodenal Mucosal Homogenates from Rachitic, Vitamin D_2- and Vitamin D_3-Treated Rachitic Rats.

Values	Supernatant fractions		
	Rachitic	Vit. D_2-treated	Vit. D_3-treated
Protein conc. (mg/ml)	6.6	5.1	5.5
Ca^{45} in supernatant (%)	12.3	36.1	46.0
Ca^{45} in supernatant per mg protein (%)	1.9	7.0	8.4

Animals were given 500 IU vit. D_2, 500 IU vit. D_3, or only the vegetable oil carrier 72 hr before the experiment. Each value represents data from pooled samples of 10 rats per group. Calcium binding determined by competition between Chelex-100 resin and soluble Ca-binding substances for added Ca^{45} (see text). A duplicate experiment gave the same relative data.

After determining Ca-binding activity of the heat-treated supernatants, the supernatants were percolated through a Sephadex G-25 chromatographic column. The eluting solution was Tris buffer at pH 7.4, and 2 ml fractions were collected by the use of an automatic fraction collector. The first ultraviolet absorbing fractions of the mucosal supernatants, which contained primarily protein (m.w. 5,000 or greater), were pooled and concentrated by lyophilization. After reconstitution to a standard volume (15 ml), the supernatant protein fractions were again assayed for calcium-binding activity and protein concentration. The pooled protein-containing fractions were then further separated on a Sephadex G-100 column in order to affect a separation of the various proteins in the solution according to molecular size. Again, the eluting agent was Tris buffer at pH 7.4, and about 200-two ml fractions were collected.

Results and discussion. Table I summarizes the calcium-binding data and protein concentrations of the three heat-treated supernatant fractions. The data indicate that, although vitamin D treatment appeared to decrease slightly the concentration of protein in the supernatant fraction of duodenal mucosal homogenates, the binding of calcium by the homogenates, on a volume basis, was increased by vitamin D_2 and vitamin D_3 by factors of about 3 and 4, respectively. When the data are expressed as radiocalcium binding per mg of protein, the factorial increase due to vitamin D treatment was similar. A duplicate experiment gave essentially the same results.

The vitamin D-enhanced calcium-binding activity of the supernatant fractions was found to be associated with the initial, *i.e.*, the protein containing, peak from the Sephadex G-25 chromatographic separation (Table II). This evidence suggests that the vitamin D-induced calcium-binding factor of rat mucosal tissue is associated with a macromolecule, presumably a protein.

The various fractions from the Sephadex G-100 column were analyzed for protein concentration and calcium-binding activity. These data for the 3 mucosal homogenates are depicted in Fig. 1. It is seen that, in the rachitic supernatant (Fig. 1A), there was some calcium-binding activity associated with the first protein peak to be eluted from the column. This early protein peak had calcium-binding activity in the supernatant of the vitamin D_3-treated rats (Fig. 1C) but, inexplicably, was inactive in that of the vitamin D_2-treated animals (Fig. 1B). In the rachitic

TABLE II. Binding of Radiocalcium by the Sephadex G-25 Excluded Supernatant Fractions from Rachitic, Vitamin D_2- and Vitamin D_3-Treated Rats.

Values	G-25 protein fractions		
	Rachitic	Vit. D_2-treated	Vit. D_3-treated
Protein conc. (mg/ml)	3.0	3.4	3.3
Ca^{45} in supernatant (%)	13.5	35.8	33.4
Ca^{45} in supernatant per mg protein (%)	4.5	10.5	10.1

Protein fractions were isolated by passage of the heat-treated supernatants through a Sephadex G-25 column. Calcium binding determined by Chelex-100 resin assay (see text).

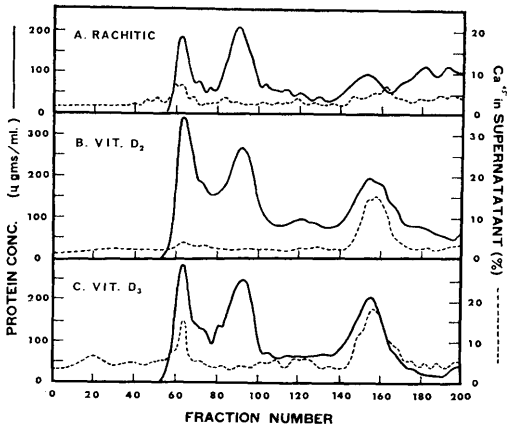

FIG. 1. Fractionation of proteins and Ca-binding activity in (A) rachitic, (B) vitamin D_2, and (C) vitamin D_3 supernatants by Sephadex G-100. The eluting solution was Tris Buffer (pH 7.4); 2.0 ml fractions were collected. Protein concentration was determined by the method of Lowry *et al.*(16) and the calcium-binding activity by the Chelex-100 assay (see text).

supernatant, this initial binding peak appeared to be the only protein fraction with significant calcium-binding activity. However, in the vitamin D_3- and vitamin D_2-treated animals, there was a very significant calcium-binding peak associated with the protein in the Sephadex G-100 fractions 150 to 160. This particular protein peak was less prominent in the protein spectrum of the supernatant fraction of the mucosal homogenates of rachitic animals. These results suggest that the vitamin D-dependent binding is due to the formation of a Ca-binding protein and that the calcium-binding activity induced by vitamin D_2 and vitamin D_3 appears to be associated with only certain proteins in the mucosal homogenate supernatant and is not a general property of all the proteins contained therein. In addition to the above results acrylamide gel electrophoresis (pH 9.1) of the intestinal mucosal homogenates demonstrated a protein band which was present in the supernatants of vitamin D_2 and vitamin D_3-treated rats but absent from those of the rachitic rats.

The results of this experiment show that, in the rachitic rat, as in the chick(11,12), vitamin D increases the Ca-binding activity of the supernatant derived from homogenates of duodenal mucosal tissue. The gel filtration studies with Sephadex G-25 and G-100 further suggest that this increased activity is associated with a protein. Whether the elaboration of this calcium-binding protein represents an effect of vitamin D on *de novo* protein synthesis, a polymerization of existing proteins, a change in the binding characteristics of existing proteins, or the release of a factor from bound intracellular sites, remains to be determined.

Other studies from this laboratory have suggested that vitamin D enhances the diffusional permeability of the intestinal mucosa to calcium in the rachitic rat(17) and thus the movement of calcium in both directions across the intestine, *i.e.*, lumen to plasma and plasma to lumen, is increased by vitamin D treatment. In the intact animal, the calcium binding protein may possibly act as an intracellular carrier for calcium increasing the movement of calcium back and forth across the intestinal mucosal cell.

Summary. Oral administration of 500 IU of vitamin D_2 or vitamin D_3 to rachitic rats resulted in the elaboration of a Ca-binding factor which could be detected in the supernatant fraction of duodenal mucosal homogenates at 72 hours after administration of the vitamin. This factor was found to be associated with the initial or protein containing portion of the supernatants after percolation through Sephadex G-25; this indicates that the factor is a macromolecule, presumably a protein. Further separation of the supernatants by Sephadex G-100 gel filtration showed that the increased binding activity seen after vitamin D administration was mainly associated with only one of the protein peaks present in the Sephadex G-100 fractions. This particular peak was much less prominent in the fractionated supernatant of the mucosal homogenates from rachitic rats. The results suggest that the vitamin D-dependent binding activity is due to the presence of a specific calcium binding protein in the supernatant fraction of duodenal mucosal homogenates from vitamin D-treated rachitic rats.

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Incompetent Relationship Between Coxsackievirus A6 Ribonucleic Acid and Mouse Cells.* (32012)

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Previous work(1) established the paradoxical insusceptibility of primary and stable mouse cells *in vitro* to coxsackievirus A6, an enterovirus lethal for newborn mice *in vivo*. It was suggested that cultivation of mouse cells *in vitro* may alter viral receptors or disrupt intracellular mechanisms for synthesis of this virus.

Subsequently Came and Crowell(2), employing coxsackieviruses A13 and A18 capable of quantitation by plaque assay, demonstrated that the resistance of mouse fetal cells *in vitro* to these enteroviruses may be explained by loss of viral receptors. These investigators studied viral attachment to cultivated cells in order to elucidate this biologic paradox.

Kinetic studies of viral attachment to host cells or tissues necessitate precise quantitation with which strains of coxsackievirus A6 may be obtained only by newborn mouse bio-assay *in vivo*. In order to resolve the issue of altered viral receptors, primary mouse fetal cells were exposed to RNA extracted from the test virus, coxsackievirus A6, and a positive control virus, mengovirus, capable of multiplication and cytopathogenicity in cultivated mouse cells. The role of receptors in this host cell-virus relationship was further clarified by the study of coxsackievirus A6 replication in suspensions of non-trypsinized, minced, mouse fetal tissue. The biologic integrity of the preparations of viral RNA was assessed by intracerebral inoculation of susceptible newborn mice.

Materials and methods. Viruses. Coxsackievirus A6, strain C. G. (Gdula), was obtained from the American Type Culture Collection (#VR 165) as a 20% suspension of newborn mouse (NM) torso and brain and passed in-

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