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A Vitamin D-Dependent Serum Factor Promoting Calcium Uptake by Bone (32856)

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In 1924, Shipley (1) reported that rachitic rat bone would not exhibit normal calcification when incubated in serum from rachitic rats, but would calcify in normal rat serum. Similar results were obtained with human tissue (2) and fowl tissue (3). However, such experiments were apparently discontinued after Nicolaysen and Jensen (4) reported that in vitamin D-deficient rats, the density of bone calcification was solely related to the amounts of calcium and phosphorus in the blood.

Recent experiments with vitamins D (5,6) and K (7) suggest that these vitamins exert their effects on calcium transport in the intestine and blood coagulation, respectively, by mediating the syntheses of specific proteins. In view of these results, we have repeated Shipley's experiment and have found that normal rat serum contains a vitamin D-dependent factor that stimulates the *in vitro* uptake of calcium by bone.

Materials and Methods. Weanling female rats[1] were kept in the dark, were given distilled water to drink, and were fed *ad libitum* the vitamin D-free, purified diet described by Steenbock and Herting (8). This diet contains normal concentrations of calcium (0.47%, as $CaCO_3$) and phosphorus (0.30%, as NaH_2PO_4) and has a Ca:P ratio of 1.2. The rats were maintained on this diet for 6–8 weeks, even though the concentration of serum calcium was decreased after 3–4 weeks.

There were two different groups of control animals. One control group consisted of normal, female, chow-fed[2] rats having the same body weights as the vitamin D-deficient animals. The second control group consisted of vitamin D-deficient animals which had been fed the purified diet containing vitamin D_2 (0.5 μg (20 IU)/gm of diet) for the last 5 days of the feeding period.

The animals were decapitated, and the blood was collected into 40-ml centrifuge tubes kept at 0°C. The clotted blood was centrifuged at 2°C and sera from similarly treated animals were pooled. Whole femurs, free from adhering tissue, were removed from both deficient and control animals as soon as possible after the death of the animal, and were kept at 0°C on filter paper moistened with saline. The bones were split longitudinally with a scalpel blade prior to incubation.

Calcium in the pooled sera was determined by a fluorometric procedure,[3] inorganic phosphorus was determined by the procedure of Kolb *et al.* (9), and alkaline phosphatase was measured by Phosphatabs.[4] The concentrations of total calcium and inorganic phosphorus in the pooled serum from the deficient animals were adjusted to the concentrations in the normal serum by adding a standard calcium chloride solution (3.0 mg Ca/ml) and

[1] Holtzman Rat Co., Madison, Wisconsin.

[2] Ralston-Purina Co., St. Louis, Missouri.

[3] Manual of Fluorometric Clinical Procedures, G. K. Turner Associates, Inc., 2524 Pulgas Avenue, Palo Alto, California.

[4] Warner-Chilcott, Morris Plains, New Jersey.

TABLE I. Effects of Vitamin D-Deficient Diet in Rats Maintained in Absence of Light.

Animal	Body wt. gain (gm/rat per day)	Serum constituents[a] Calcium (mg/100 ml)	Inorganic phosphorus (mg/100 ml)	Alkaline phosphatase (Bodansky units/100 ml)
Deficient	1.1	6.0	6.9	155
Vitamin D fed[b]	5.2	10.8	8.5	95
Normal controls		11.0	9.0	75

[a] Concentrations of pooled sera.

[b] These animals were fed the deficient diet to which crystalline calciferol was added (20 IU per gm of diet) for the last 5 days of the 7-weeks feeding period. Weight gain is that observed while calciferol was being fed.

a dry mixture of K_2HPO_4 and KH_2PO_4 (molar ratio 4:1).

The bone samples were placed into 3.0 ml of serum or Krebs–Ringer solution (10), and 0.50 ml of a saline solution of ^{45}calcium (specific activity, 2.1 $\mu C/\mu g$)[5] was added. The mixtures were incubated for 1.0 hour at 37°C in a shaking water bath. The bone samples were removed and rinsed with five 10-ml portions of distilled water; the washings of each bone sample were combined. After the calcium in the postincubation media had been determined fluorometrically, the media were combined with the corresponding bone washings and the mixtures were diluted to 100.0 ml.

The washed bone samples were dried overnight at 110°C and then heated at 600–700° for 8 hours or until the ash was completely white. The calcium contents of the bone samples were estimated by multiplying the ash weights by 0.366 (11); calcium in the samples of bone ash was determined by atomic absorption and agreed closely with the estimated amounts of calcium.

The ^{45}calcium contents of the preincubation media, postincubation media plus bone washings, and bone ash solutions were determined using a thin window, gas-flow counter. In this procedure, carrier calcium chloride solution was added, as necessary, to suitable volumes of these solutions to standardize the calcium content, and the calcium was precipitated as calcium oxalate. After reprecipitation, the calcium oxalate precipitates were collected on filter paper discs in layers which were uniform in thickness, area, and weight. Results of counting such duplicate plates agreed within 2%.

Results. The vitamin D-deficient diet retarded growth, decreased the concentrations of serum calcium and inorganic phosphorus, and increased the level of serum alkaline phosphatase activity (Table I). The concentrations of these serum constituents were nearly normal after the deficient animals had been fed vitamin D_2 for 5 days.

The results obtained by incubating the possible combinations of serum and bone from vitamin D-deficient and normal control animals and the two kinds of bone in Krebs–Ringer phosphate solution containing glucose are shown in Table II. The calcium uptakes of the normal and vitamin D-deficient bones were identical in each incubation medium. However, the calcium uptake by bone was significantly greater ($p<0.01$) in normal serum than in vitamin D-deficient serum. Calcium uptakes by bone were essentially the same in the Krebs–Ringer phosphate solution and in serum from vitamin D-deficient animals. Serum from deficient animals which had been fed vitamin D_2 for 5 days supported calcium uptake by bone to the same extent as the serum from normal animals (Table III).

In similar experiments, calcium uptake by the ends of femurs incubated in normal control serum was greater than the shafts (Table IV). The addition of 0.50 ml of normal rat serum to 2.5 ml of the Krebs–Ringer phosphate solution increased the calcium uptake by bone. Normal human serum did not en-

[5] The amount of ^{45}calcium/incubation vessel varied from 0.05 μc to 1.0 μc in these experiments.

TABLE II. Influence of Serum on the *in Vitro* Deposition of Calcium in Bones from Vitamin D-Deficient and Normal Rats.

Incubation mixture	Uptake[a] of calcium by bone as indicated by: Decrease in Ca concentration of incubation medium (mg/100 ml)	Uptake[a] of calcium by bone as indicated by: ^{45}Ca in bone (cpm)
Deficient serum + deficient bone	3.81 ± 0.209 (11)[b]	66,720 ± 6760 (9)
Deficient serum + control bone[d]	4.27 ± 0.290 (9)	79,020 ± 8620 (8)
Control serum[d] + deficient bone	6.38 ± 0.245[c] (10)	109,760 ± 8320[c] (8)
Control serum + control bone	6.25 ± 0.146[c] (11)	117,240 ± 8940[c] (12)
Krebs–Ringer–phosphate–glucose + deficient bone	2.59 ± 0.096 (5)	64,340 ± 5080 (8)
Krebs–Ringer–phosphate–glucose + control bone	3.03 ± 0.455 (5)	69,520 ± 7400 (8)

[a] Values are expressed on the basis of equal concentrations of calcium in the incubation mixtures and a weight of bone equivalent to 20.0 mg of bone calcium.

[b] Mean ± SE of the mean; the number of samples is in parentheses.

[c] Values are significantly different from values obtained with deficient serum; $p \leq 0.01$, Fisher's t test.

[d] Serum and femurs were obtained from normal female rats that had the same body wt. as the deficient rats.

TABLE III. Enhancement of Calcium Deposition in Bone by Serum from Normal Rats.

Incubation medium[d]	Uptake[a] of calcium by bone as indicated by: Decrease in Ca concentration in incubation medium (mg/100 ml)	Uptake[a] of calcium by bone as indicated by: Decrease of ^{45}Ca content of incubation medium (cpm)	Uptake[a] of calcium by bone as indicated by: Total ^{45}Ca in bone (cpm)
Vitamin D-deficient serum	0.73 ± 0.083 (10)[b]	4660 ± 135 (10)	4420 ± 188 (11)
Vitamin D-refed serum	1.74 ± 0.119[c] (11)	5760 ± 111[c] (11)	5580 ± 326[c] (6)
Normal serum	1.49 ± 0.050[c] (10)	5700 ± 133[c] (12)	5660 ± 268[c] (11)
Krebs–Ringer–phosphate–glucose	0.81 ± 0.054 (10)	5020 ± 188 (5)	4320 ± 262 (4)

[abc] Notations are as in Table II.

[d] Bone samples from both vitamin D-deficient and untreated control animals were used. However, since both kinds of bones gave the same response in the same medium, all results with the same kind of serum have been combined.

TABLE IV. Calcium Deposition *in Vitro* in Ends and Shafts of Bones.

Incubation medium	Bone sample	Uptake[a] of calcium by bone as indicated by: Decrease in Ca concentration in incubation medium (mg/100 ml)	Uptake[a] of calcium by bone as indicated by: Decrease of ^{45}Ca content of incubation medium (cpm)
Deficient serum	Shafts and ends[d]	2.52 ± 0.196 (19)[b]	24,240 ± 1820 (12)
Normal serum	Ends	6.43 ± 0.416[c] (5)	42,760 ± 148[c] (8)
Normal serum	Shafts	4.65 ± 0.246[c] (10)	29,800 ± 1740[c] (9)

[abc] Notation as in Table II.

[d] The bones were sectioned into portions which were composed only of the shaft and the ends which were composed of the epiphysis and the epiphysial disk; these fragments were split longitudinally before use. The calcium uptakes by these two kinds of bone fragments were the same when incubated in deficient serum; the results were therefore combined.

hance calcium uptake by rat bone nor did the addition of 1.0 mg of diatomaceous earth coated with 30.0 μg (1,200 IU) of vitamin D_2 to vitamin D-deficient serum. This procedure of adding the vitamin D to serum was analogous to the procedure for adding cholesterol to serum in successful studies of cholesterol esterification (12).

Discussion. The retarded growth rate, the decreased concentrations of calcium and inorganic phosphorus and the increased concentration of alkaline phosphatase in the serum, and the ability of dietary vitamin D_2 to restore the concentrations of these constituents to normal values suggest that the experimental rats were indeed vitamin D-deficient.

In these experiments, the uptake of calcium by bone was measured by determining three quantities and employing two independent procedures: (a) the decrease of total calcium in the incubation media by a fluorometric procedure, (b) the decrease in the incubation media of ^{45}calcium, and (c) the appearance of ^{45}calcium in bone. Similar changes were observed by all of these procedures.

In a specific medium the bones from normal and vitamin D-deficient rats had identical uptakes of calcium. Therefore any differences in calcium uptake cannot be attributed to a difference in the bones. The increased calcium uptake in normal serum compared to vitamin D-deficient serum must be due to a factor in normal serum that is absent in vitamin D-deficient serum. Since the concentrations of calcium and phosphorus in the two sera were equal, these constituents cannot account for the increased calcium uptake. Protein per se cannot be responsible since the calcium uptake by bone in vitamin D-deficient serum was the same as the uptake in Krebs–Ringer phosphate solution containing glucose. Vitamin D_2 added to vitamin D-deficient serum, in such a manner that the vitamin would be expected to be available, did not enhance the uptake of calcium by the bone. Also normal human serum, which contains vitamin D and its metabolites, did not enhance the calcium uptake by rat femur. Therefore, vitamin D per se or its metabolic products are probably not responsible for the enhancement of calcium uptake by bone.

A source of vitamin D is necessary for the occurrence of a substance in serum which promotes calcium deposition in the bone, since the substance was absent in the serum of vitamin D-deficient rats and present in serum from deficient rats which had been refed vitamin D_2 for 5 days.

Recent reports (5,6) have implied that a major, and possibly the only, role for vitamin D in the deposition of bone salts is its promotion of calcium transport across the intestinal mucosa. However, others (13) have suggested that there is a direct effect of vitamin D on bone.

The results of our experiments have no obvious relation to calcium transport in the intestine and suggest that normal bones and vitamin D-deficient bones can take up calcium equally. The results also suggest that vitamin D is necessary for the appearance of a substance in serum which enhances the calcium uptake by bone. This substance is absent from the serum of vitamin D-deficient rats and is present in the serum of normal rats and in the serum of vitamin D-deficient rats which have been refed vitamin D. Since the substance is apparently not vitamin D itself or a metabolic product of vitamin D, it may be concluded that vitamin D is necessary for the biosynthesis of this calcifying factor. This possibility is considered very likely because vitamin D does induce the synthesis of a protein required for calcium transport in the intestine (5,6,14).

Summary. Femurs from normal and vitamin D-deficient rats are equally capable of taking up calcium *in vitro*. However, normal rat serum contains a vitamin D-dependent substance that stimulates the calcium uptake by bone. This factor which is not present in the serum from vitamin D-deficient rats, is apparently not vitamin D itself or a metabolic product of vitamin D; however, vitamin D may regulate the synthesis of this factor.

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Pituitary and Plasma Growth Hormone-Like Activity after Administration of Highly Purified Pig Growth Hormone-Releasing Factor* (32857)

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Evidence for the existence of a growth hormone-releasing factor (GRF) in the hypothalami of animals of various species and humans has been reported using *in vitro* and *in vivo* methods by many laboratories (1–10). The *in vivo* studies were based on the depletion of pituitary growth hormone (GH) content in recipient rats following the administration of extracts from hypothalamic tissue. Since 30–60% depletion of the pituitary stores in GH in rats was induced by crude extracts (5) or purified GRF preparations (7) from pig or beef hypothalamic tissue, it was hypothesized that it would be possible to detect increased plasma levels of GH-like activity in the rat, even with the relatively insensitive tibial cartilage bioassay, if the "depletion" truly reflects "release." However, crude hypothalamic extracts are reported to contain substances other than GRF, such as vasopressin or adrenaline, which stimulate the release of GH from the pituitary in monkeys as measured by radioimmunoassays (11,12). Thus it is desirable to utilize more purified GRF preparations free from these contaminants. Recently, purification procedures for GRF in the hypothalami of domestic animals have been developed in our laboratory and a highly purified pig GRF preparation, which is free from vasopressin and other known releasing factors, has become available (13).

The purpose of the present study is to establish that the "depletion" of pituitary GH content in rats caused by injection of the highly purified GRF actually indicates the "release" of GH, by making simultaneous determination of pituitary and plasma GH-like activity with the "tibia test" method (14). The TSH activities of the pituitary and plasma were also determined, since it was reported that TSH can influence the "tibia test" (15) and also to verify the specificity of action of GRF.

Materials and Methods. Highly purified preparations of GRF were obtained from lyophilized, defatted pig hypothalami by extraction with 2 *N* acetic acid followed by heating, lyophilization, reextraction with glacial acetic acid, gel filtration on Sephadex G-25, free flow electrophoresis, and chromatography on CMC. The details of the procedures have been described elsewhere (13).

Test for GH-releasing activity of the GRF. Sprague-Dawley female rats (130–140 gm body wt.), obtained from Cheek-Jones Co., Houston, Texas, were used as the recipient animals of the test samples. The rats were given 0.5 ml of saline or 4 μg of GRF in 0.5

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