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Effect of Hypervitaminosis D on Serum Factors in the Rabbit.* (27242)

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Hypervitaminosis D is usually associated with changes in the levels of calcium or phosphate in the blood serum. It is often tacitly assumed that abnormal levels of these ions in the blood are responsible for the widespread soft tissue calcification associated with this disease state. A study of the calcific lesions in rabbits with hypervitaminosis D, however, revealed that there are characteristic patterns of distribution of these lesions which cannot be explained by this assumption alone and indicated that the lesions were related to local variations in susceptibility of tissue to the effects of excessive Vit. D(1). To clarify the matter, it seemed desirable to study the state of the calcium and phosphate in the serum of these animals. An electrophoretic study of the serum was also done to determine if there was a change in the serum protein pattern. Inasmuch as elevations of serum muco-proteins have been described in rat hypervitaminosis D(2), hexosamine levels were also determined.

Materials and methods. Three groups of animals were studied. In the first 2, male New Zealand white rabbits weighing between 5 and 6½ lb were fed a stock diet (Rockland rabbit ration containing 0.82 g calcium, 0.35 g phosphorus, and 21 units of Vit. D per 100 g of feed) and given water *ad libitum*. Control blood samples were drawn by cardiac puncture twice at weekly intervals. Twenty-five animals (Group A) were then injected intramuscularly with 100,000 units of Vit. D_2 (Viosterol, U.S.P., 1,000,000 units per ml in corn oil) twice weekly for 4 weeks with blood samples taken at weekly intervals. As additional controls, 16 rabbits (Group B) were treated identically, except that they were injected with equal volumes of corn oil. It was difficult to withdraw enough blood to do all chemical determinations on a single blood sample, so that not all determinations were done on each animal. Nine rabbits of similar size and sex (Group C) were injected with equal doses of Vit. D, but at more widely spaced intervals (once or twice every 4 weeks over a period of several months). These animals were on a mildly hypercholesterolemic dietary regime (stock diet plus 0.6% cholesterol in corn oil)(3). Calcium was measured by a flame photometric method, ultrafiltrable calcium by a modification of the method of Toribara *et al.*(4), inorganic phosphorus by the method of Fiske and Subbarow(5), hexosamine by a method described by Winzler (6) and citric acid by the method of Beutler and Yeh(7), the samples being treated with sulfuric acid and bromine water to remove interfering substances. Paper electrophoresis was done with a Spinco apparatus using 2 buffers, one a veronal buffer, pH 8.6, and the other the veronal buffer with 400 mg of $CaCl_2$ added per liter. Quantitation was done with a Spinco Analytrol recording densitometer.

Results. The normal control levels of total calcium and citric acid were high and variable in rabbit serum, with values ranging between 12.0 and 17.3 mg % for total calcium and between 5.08 and 14.05 mg % for citric acid.

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TABLE I. A Statistical Comparison of Sera from Group A before and during Vit. D Administration.

	No. of animals (n)	Mean difference (x)	S.E. of means (Sx)	t value	Significant at .05
Calcium	9	.1	.307	.322	—
Ultrafiltrable calcium	5	.47	.367	1.28	—
Inorganic phosphorus	7	2.88	.471	6.114	+
Citric acid	6	.29	.3895	.7445	—
β-2 globulin	8	1.57	.348	4.51	+
Hexosamine	7	3.55	2.319	1.530	—

$$Sx = \sqrt{\frac{(x - x)^2}{n(n-1)}} \qquad t = \frac{x}{Sx}$$

As shown in the statistical table (Table I), there was no consistent change in the level of total calcium, ultrafiltrable calcium, citric acid or hexosamine in the animals of Group A. The level of inorganic phosphorus began to rise during the second week after beginning injections of Vit. D. During this period, renal damage with calcification began to appear. When Vit. D was injected at more widely spaced intervals (Group C), but still in sufficient amount to induce widespread calcification, there was no change in serum calcium or phosphorus level. These animals had less severely damaged kidneys than those in Group A.

There was no change in the serum electrophoretic pattern when veronal buffer without $CaCl_2$ was used. Electrophoresis using a veronal buffer with added $CaCl_2$ showed a double beta globulin peak. The second or slower component, termed beta-2 globulin, though detectable in control sera, was consistently elevated in the hypervitaminotic animals of Group A (Fig. 1). There were no changes except a slight drop in hexosamine content in the serum level of any of these substances in the rabbits injected with corn oil (Group B).

Discussion. Although ionized calcium was not measured in these experiments, that part of the total calcium which is bound to proteins and citric acid accounts for virtually all non-ionized calcium in the body fluids(8). The high level of inorganic phosphate found on the higher dosage schedule (Group A) is presumably secondary to renal damage produced by the vitamin. Scarpelli *et al.*(9) have shown that in the rat histochemical evidence of renal damage in hypervitaminosis D preceded renal calcification. That the disseminated calcification in the rabbit is not necessarily due to hyperphosphatemia is evident from the observation that, with a lower dosage of the vitamin, calcification can occur with minimal renal damage and without hyperphosphatemia. The renal damage does not appear to be due to an increased level of ionized calcium in the serum because there was no demonstrated change in the serum total calcium, ultrafiltrable calcium or citric acid. There is evidence that the tissue damage induced by such agents as Vit. D is not necessarily due to hypercalcemia as judged by comparing the effects of injections of calcium gluconate and parathyroid hormone on

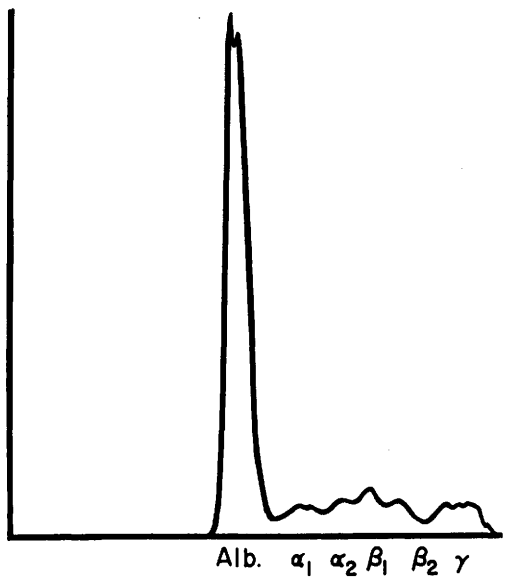

FIG. 1. Densitometric tracing of paper electrophoretic pattern of serum in a rabbit of Group A during Vit. D inj. to illustrate the prominent β-2 globulin peak.

mouse kidneys(10). Calcium gluconate induced calcification of basement membranes unaccompanied by tubular damage, while parathyroid extract induced striking tubular damage, thus indicating that calcium alone was not responsible for the tubular damage. In many species, including man, hypervitaminosis D is associated with increased levels of calcium, ultrafiltrable calcium and citric acid(11,12). The normal human serum levels of calcium and phosphate are so high as to be considered supersaturated(8). An increase in level of either should by physical-chemical principles favor calcification. But hypercalcemia does not appear to be required for metastatic calcification due to hypervitaminosis D in the rabbit. In calves, although hypervitaminosis D induces a more rapid turnover of calcium in the blood, there is no hypercalcemia(13). The reason for the absence of hypercalcemia in some species with hypervitaminosis D is not apparent, but may be related to variable dosage regimes used by different investigators or to some other factor such as renal clearance or bone accretion and resorption rates.

The increase in the beta-2 globulin level is at present unexplained. An increased binding capacity of serum proteins for calcium has been described in rats with hypervitaminosis D using a dialysis method(14). Also, an unusual globulin migrating in the beta area has been identified by an immunoelectrophoretic method in the serum of humans with hyperparathyroidism(15). These changes may be related to transport of the vitamin or hormone as has been suggested by the authors who described the changes in the hypervitaminotic rat and in man with hyperparathyroidism.

Summary. Hypervitaminosis D in the rabbit induced widespread calcification of many organ systems unaccompanied by changes in concentration of calcium, ultrafiltrable calcium, citric acid or hexosamine in the serum. With a high dosage level, there was a conspicuous increase in serum level of inorganic phosphate, presumably secondary to renal damage. At a low dosage level where there was minimal renal damage and no change in the serum inorganic phosphorus or calcium, widespread soft tissue calcification occurred. These data indicate that, in the rabbit with hypervitaminosis D, metastatic calcification is not necessarily due to hypercalcemia or hyperphosphatemia. The vitamin induced an unexplained rise in level of β-2 globulin, a fraction split from the β-globulin when $CaCl_2$ is added to the electrophoresis buffer.

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