Experimental Hypervitaminosis D: Hypercalcemia, Hypermucoproteinemia, and Metastatic Calicification. (22971)

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Administration of massive doses of vit. D results in a disease, hypervitaminosis D, characterized by increased absorption of calcium and phosphorus, hypercalcemia, high urinary excretion of calcium, resorption of bone, and metastatic calcification(1). Engel(2) found that rats given large doses of parathyroid hormone showed a rise in blood levels of a mucoprotein called seromucoid (3) and an increase in concentration of soluble seromucoid in bone. He also observed alterations in staining characteristics of bone which suggested a depolymerization of bone matrix, and described deposition of calcium salts in renal tubular casts which contained polysaccharide. He suggested that parathyroid hormone induced a depolymerization of bone matrix so that mucoprotein components of the matrix leaked into the body fluids.

The present study was undertaken to determine whether hypervitaminosis D is associated with a change in seromucoid level of blood. The relationship of polysaccharide matrices to site of deposition of metastatic calcification was also studied.

Methods. Thirteen female white rats of the Wistar strain weighing 90-140 g were fed a diet of commercial Purina chow and given water *ad libitum*. The animals were injected intraperitoneally with 200,000 units of vit. D_2 in cottonseed oil (from Nutritional Biochemicals Co., Cleveland) daily for 8 days. Fourteen control animals were injected intraperitoneally daily with equal volumes of cottonseed oil. Untreated rats were also studied. Rats with hypervitaminosis D lost 15-30% of their body weight; therefore 8 untreated rats were starved for 5 days, during which they lost 40% of their body weight, and were examined as additional controls. Another group of 8 rats received 100,000 units of vit. D_2 for 3 days, with 8 animals receiving an equal volume of cottonseed oil as controls. At termination of dosage schedules, the rats were anesthetized with ether and exsanguinated by aortic puncture. Blood serum was separated and frozen in dry ice. Serum levels of calcium were determined by the method of Rehell(4). The levels of seromucoid were measured by the method of Winzler(3) and are expressed as mg of galactose-mannose/100 ml of serum. Seromucoid is the name given by Winzler to a mucoprotein which has been well characterized and found to be rich in galactose and mannose. The blood level of this compound increases in many conditions in which there is alteration of connective tissue, *e.g.*, infections and metastatic tumors(3), scurvy(5), and hypervitaminosis A(6). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-7 μ. Histologic sections were prepared with hematoxylin-eosin, with toluidine blue and with the periodic acid-Schiff method for polysaccharides before and after digestion with salivary amylase. Persistence of red color of periodic acid-Schiff reaction after digestion with amylase indicates that the color is not due to glycogen. Metachromasia in toluidine blue preparations demonstrates acid mucopolysaccharides. Some sections were colored by Giemsa's

TABLE I. Seromucoid Levels in Hypervitaminosis D.

	No. of animals	Mean	Stand. dev.	Probability
No treatment	18	9.8	2.3	.012 *
Starved 5 days	9	7.9	1.1	
Cottonseed oil, 8 days	14	13.0	6.35	.0019*
Vit. D, 200,000 units, 8 days	13	20.8	6.16	
Cottonseed oil, 3 days	8	11.3	2.31	.0071
Vit. D, 100,000 units, 3 days	8	14.9	2.81	

* Approximately.

method, by alizarin red, and by Masson's trichrome method. Alizarin red demonstrates calcium.

Results. Blood chemistry. Serum calcium rose from control levels of 9-11 mg% to levels of 14-18.5 mg% as measured on the third and eighth day of treatment with vit. D_2. Calcium levels did not change in animals receiving cottonseed oil. Seromucoid levels are tabulated in Table I together with a statistical analysis. One sided "t" tests were performed.

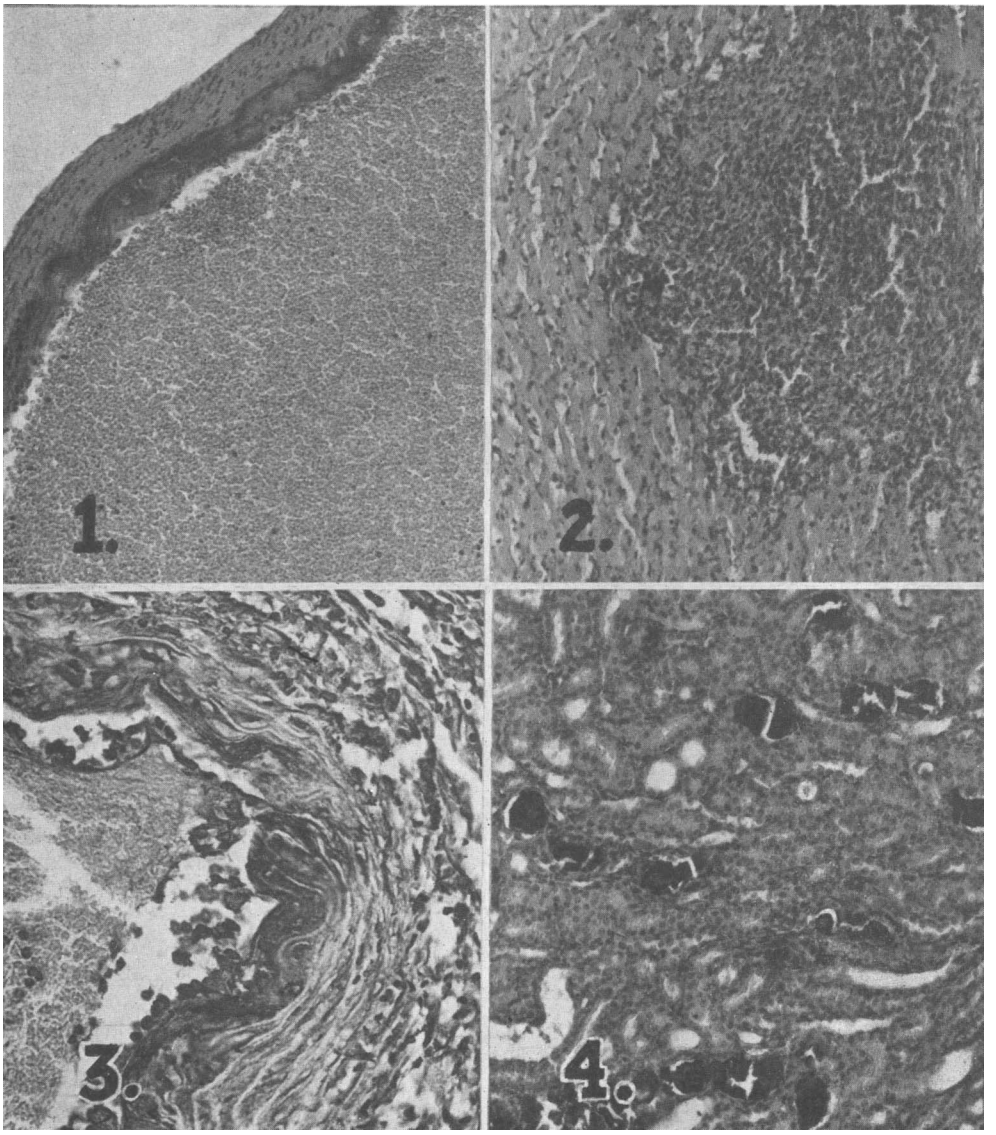

FIG. 1. Aorta of rat with hypervitaminosis D showing calcification of media. Hematoxylin-eosin stain ×140.

FIG. 2. Heart of rat with hypervitaminosis D showing myocarditis unassociated with calcific deposits. Hematoxylin-eosin stain ×140.

FIG. 3. Aorta of hypervitaminotic rat showing elevation of endothelium with collection of mononuclear cells beneath it, and accumulation of amorphous metachromatic material in interfibrillar spaces of the media. Toluidine blue stain ×400.

FIG. 4. Hypervitaminotic rat kidney with calcified casts in tubular lumina. Hematoxylin-eosin stain ×140.

It can be seen that seromucoid levels are higher in rats injected with vit. D_2 than in those injected with cottonseed oil.

The histological changes described below were found only in those animals which had received 200,000 units of vit. D_2 for 8 days, and were, in general, similar to those which have been described previously by Ham(7). No such changes were observed in animals injected with 100,000 units of vit. D_2 for 3 days or in those which had received only cottonseed oil.

Kidney. Tubular casts of 2 types were found; brightly eosinophilic casts and others which were calcified. Both types were bright red in periodic acid-Schiff preparations before and after digestion with salivary amylase. Calcified casts were metachromatic, while uncalcified casts were rarely found to be metachromatic.

Heart: A severe myocarditis was seen. It was characterized by destruction of cardiac muscle fibers, and an inflammatory exudate consisting primarily of large mononuclear cells, with some lymphocytes and Anitschkow myocytes. Calcareous deposits were present in many of the inflammatory foci, but many other foci were free of histologically identifiable calcium. In the areas of inflammation, there was an accumulation of extra-cellular material which was colored red in periodic acid-Schiff preparations and was metachromatic. Many branches of the coronary arteries showed calcific deposits in the media and accumulation of amorphous material in all coats of the vessels. Both calcific deposits and amorphous material were periodic acid-Schiff positive and metachromatic.

Aorta: The arch of the aorta showed 3 types of histological alterations; elevation of endothelium with accumulation of mononuclear cells beneath it, calcification of media and elastic interna, and collection of amorphous interfibrillary material in the media. Calcium was encrusted upon the elastic fibers. Again, at sites of calcareous deposition, there was an accumulation of metachromatic, periodic acid-Schiff positive material. Amorphous interfibrillary material was also metachromatic and periodic acid-Schiff positive.

Interpretation of results: Since the work of Harris and Innes(1), there has been abundant evidence that administration of massive doses of vit. D results in dissolution of bone (10,11). In parathyroid hormone poisoning, there is an increase in blood level of seromucoid which apparently has its origin in the bone matrix(2). Our experiments have demonstrated 3 changes in rats with hypervitaminosis D: (1) a rise in level of serum calcium, (2) an increase in serum seromucoid, and (3) deposition of calcium in tissues in a matrix composed at least partially of an unidentified polysaccharide. It would seem reasonable, then, to suggest that administration of massive doses of vit. D_2 results in release of bone minerals and organic matrix into the general circulation with their subsequent deposition in the soft tissues.

Tissue stains in our experiments demonstrate only the presence of polysaccharides in general. The polysaccharides of connective tissue consist of a variety of molecular species(12). In dissolution of bone many of these must be degraded or released from their bound state in the tissue, so it would seem unlikely that seromucoid itself is directly involved in deposition of calcium. The calcium-binding components of the matrix may be other compounds not measured here, perhaps chondroitin sulfates.

Rubin and Howard(8) found that calcification of soft tissues in a variety of locations and pathologic states occurred in a matrix of chondroitin sulfate, as revealed by histochemical evidence. However, Hass(9) pointed out that pathologic calcification occurs in a variety of matrices, some of which seem to be free of chondroitin sulfate.

Summary. 1. Rats given massive doses of vit. D_2 show increased serum levels of calcium and seromucoid (a mucoprotein), calcareous deposition in the heart, kidneys, and blood vessels, and accumulation of polysaccharides in the same locations. Myocarditis, unassociated with deposition of calcium as assessed histologically, was observed. 2. Calcium deposits occur in a matrix containing acid mucopolysaccharides.

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Sodium Retaining Activity of 19-Nor-Steroids in Adrenalectomized Rats. (22972)

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Small changes in the structure of steroids cause large differences in sodium retaining activity. Aldosterone, for example, possesses about 25 times the potency of desoxycorticosterone(1-3). The 9α-halo-analogs(1,4-6) of cortisone and hydrocortisone and their 6-dehydro-modifications(7) are stronger sodium retainers than the parent compounds. Hydrocortisone is only weakly active, but its 2-methyl- and 2-methyl-9α-halo-derivatives (8,9) are highly potent. Large differences in sodium retention are also seen with removal of the methyl group at carbon 10 (the 19-nor-steroids).

The present report summarizes our results in adrenalectomized rats with a series of these steroids, synthesized by Colton and associates(10,11) of our laboratories.

Materials and methods. The method of bioassay has been described in detail(12). Solutions of steroids in corn oil are injected subcutaneously into adrenalectomized rats with a saline load. A 2-hour sample of urine is collected in the bladder and analyzed for sodium. Amounts of 2 to 12 μg of desoxycorticosterone acetate (DCA) caused by this method a progressive retention of sodium. To minimize the day-to-day variation, one group of rats receiving DCA was used as a standard. An active response was defined arbitrarily as retention of sodium which was at least equal to that of 6 μg DCA. The probability is about 0.05 that an inactive steroid will elicit an equivalent or greater effect. Two steroids, 19-nor-desoxycorticosterone acetate and 19-nor-Reichstein's Compound S acetate, were compared separately with DCA in 4-point bioassays after several preliminary tests. Sodium concentrations were determined on the Beckman flame photometer for calculation of total excretion.

Results. The effects of nine 19-nor-steroids in adrenalectomized rats are summarized in Table I, together with data from simultaneous tests of 6 μg DCA. Sodium excretion with no DCA was 116 μEq/rat in early tests with 111 rats.

With 19-nor-desoxycorticosterone acetate (I), doses of 1.5 to 100 μg elicited a marked retention of sodium. Results of a 4-point bioassay with 0.75 and 1.5 μg of the compound and 2 and 4 μg of DCA indicate that I is approximately 5.1 times as potent as DCA. The results of the test are illustrated in Fig. 1.

The data show that sodium retaining activity is decreased strongly by an hydroxyl group placed at carbon 11 or 17. Fig. 2