THE AMELIORATION OF HYPERVITAMINOSIS D IN RATS WITH VITAMIN A*

By IRWIN CLARK, Ph.D., and C. ANDREW L. BASSETT, M.D.

(From the Orthopaedic Research Laboratories, Departments of Orthopaedic Surgery and Biochemistry, Columbia University, College of Physicians and Surgeons, New York)

Plates 16 to 19

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Two outstanding characteristics of hypervitaminosis D in experimental animals are skeletal abnormalities and soft tissue calcification, both of which occur in growing and adult animals (1–7). Although hypervitaminosis A causes rarefaction and fragility of the bones of growing animals (8, 9), soft tissue calcification is never found. On the contrary, vitamin A deficiency may cause renal calcification (10). Isotopic studies have shown that a single massive dose of vitamin D causes a marked increase in urinary radiocalcium derived from bone, whereas a single toxic dose of vitamin A increases urinary radiophosphorus (11). Since vitamins A and D appear to affect different constituents of bone, the following experiments were designed to investigate the effects of combined hypervitaminosis A and D in rats. Unexpectedly, it was found that the severity of the symptoms of hypervitaminosis D was greatly decreased and, in some cases, no toxicity was observed if sufficiently large amounts of vitamin A were administered simultaneously with vitamin D. A partial report of these observations has been published (12).

Experimental Procedure

General.—Albino male rats of the Holtzman strain were used in all experiments. The stock laboratory diet contained adequate amounts of vitamins A and D for normal growth and maintenance. The vitamin A palmitate (Merck) used in these experiments was generously supplied by the Merck, Sharp and Dohme Research Laboratories. Whenever necessary, it was diluted with purified sesame oil. The crystalline vitamin D_2 (calciferol) was kindly donated by Roxan-Philips Company. It was dissolved in a minimum of alcohol before diluting with purified sesame oil. The vitamins were administered daily by stomach tube on a body weight basis unless otherwise noted.

Specimens for histologic study were fixed in buffered neutral formalin. After suitable fixation, bones were decalcified with formic acid—sodium formate solution. The decalcified

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bones and soft tissues were embedded in paraffin and sections were stained with hematoxylin and eosin. In addition, the soft tissues were stained with alizarin red S to detect calcium deposits.

Specific.—

Experiment A-1: Five groups, each consisting of eight 120 to 150 gm rats, were used. Group I, control rats; Group II, 60,000 international units of vitamin D; Group III, 30,000 international units of vitamin A; Group IV, 60,000 units of vitamin D plus 15,000 units of vitamin A; Group V, 60,000 units of vitamin D plus 30,000 units of vitamin A. These animals were maintained for 5 weeks on this regimen until death or the time of sacrifice.

Experiment Q-2: Four groups, each consisting of fifteen 100 to 150 gm rats, were used. Group 1, control rats; Group II, 60,000 units of vitamin D; Group III, 30,000 units of vitamin A; Group IV, 60,000 units of vitamin D plus 30,000 units of vitamin A. The surviving animals were sacrificed at the end of 15 days. Vitamins were not administered to the rats over 2 week-ends.

Experiment C-3: Five groups, each consisting of six 85 to 100 gm rats, were used. Group I, control rats; Group II, 18,000 units of vitamin D; Group III, 18,000 units of vitamin D plus 300 units of vitamin A; Group IV, 18,000 units of vitamin D plus 3,000 units of vitamin A; Group V, 18,000 units of vitamin D plus 30,000 units of vitamin A. The experiment continued for 60 days at which time the animals were sacrificed.

RESULTS

The Effect of 60,000 Units of Vitamin D with and without Vitamin A on Weight Gain, Mortality, and Histology.—It may be seen in Table I that with the exception of one rat all animals which had received 60,000 units of vitamin D per 100 gm of body weight were dead within 3 weeks. The animals which had been given 15,000 units of vitamin A along with 60,000 units of D faired slightly better, only three were dead at the end of 3 weeks and at the end of 4 weeks seven were dead. The life span, however, of the rats which had received 60,000 units of vitamin D plus 30,000 units of vitamin A was significantly prolonged; only four animals out of eight were dead at the end of 5 weeks. Similar findings were observed in experiment Q-2 (Table II). At the time the animals were sacrificed (after 11 days of vitamin administration) eight of fifteen rats which had received vitamin D alone had died, whereas all rats that had been given 30,000 units of vitamin A along with 60,000 units of vitamin D were alive. Although the concomitant administration of vitamin A increased the survival time of the hypervitaminotic D rats, it did not counteract the loss in body weight. Rats which had been given 30,000 units of vitamin A alone did not gain as well as the controls.

The changes found in the skeletal system of hypervitaminotic D rats were the same as those described by earlier investigators (2, 6, 13). The tables of the skull were much thinner than the controls (Fig. 1) and showed many resorption cavities containing vascular elements. Subperiosteal new bone formation was seen on the tables. Medullary areas were filled with basophilic, reactive new bone. There were few multinucleated osteoclasts in the skull. Supplementation

of 60,000 units of vitamin D with 30,000 units of vitamin A altered this picture considerably (Fig. 2). The tables of this membranous bone were largely intact. Medullary areas contained only occasional resorption cavities which had fewer vascular elements than similar areas in the skulls of the animals which had received only vitamin D. Thin seams of basophilic osteoid were found on a few trabeculae, but none were seen in the subperiosteal areas. Skulls of the heaviest animals in this group appeared almost indistinguishable from those of the controls.

A similar beneficial of vitamin A was observed in the tibiae of these animals. The widths of the epiphyses of the tibiae of the animals which received only

TABLE I

Effect of 60,000 Units of Vitamin D with and without Vitamin A on Body Weight and Rat Mortality

Treatment	No. of animals	Weeks on treatment						No. dead at 5 weeks
		0	1	2	3	4	5	
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	
Control	8	137 (0)*	177 (0)	217 (0)	254 (0)	275 (0)	303‡ (0)	0
60,000 D	8	138 (0)	107 (1)	98 (3)	90 (3)	90‡		7
30,000 A	8	137 (0)	166 (0)	194 (0)	219 (0)	252 (1)	286‡ (0)	1
60,000 D + 15,000 A	8	138 (0)	118 (0)	100 (0)	90 (3)	90‡ (4)		7
60,000 D + 30,000 A	8	138 (0)	121 (0)	101 (0)	91 (0)	83 (1)	81* (3)	4

* The numbers in parentheses refer to the number of rats which died between weighing periods; the other numbers refer to the average body weight.

‡ Sacrificed for histology.

vitamin D were narrowed approximately 50 per cent as compared with those of the control animals (Fig. 3). Columns of hypertrophic cartilage cells frequently were disrupted and were staggered horizontally in a random fashion. Numerous sinusoids filled the metaphyseal area adjacent to the zone of provisional calcification. More distally in the metaphyses, the medullary areas and subperiosteal regions contained masses of basophilic osteoid. The diaphyseal cortex was honeycombed with resorption cavities which contained many multinucleated osteoclasts.

The organization and widths of the epiphyses of the tibiae of the rats that received 30,000 units of vitamin A simultaneously with vitamin D (Fig. 4) appeared almost normal, especially in the heavier animals. In this group, less basophilic osteoid was found in the medullary regions of the metaphyses. There was a marked reduction in the amount of subperiosteal new bone formation and in the number of resorption cavities in the diaphyseal cortex.

Changes in the kidneys of hypervitaminotic D rats in certain respects were similar to those reported by others (14). There was extensive cortical calcification (Fig. 5). Both convoluted and collecting tubules contained intraluminal casts. Little or no inflammatory reaction to the interstitial calcific deposits was found. In the medulla, occasional collecting tubules contained intraluminal calcific casts. Large arteries in hilar areas showed heavy deposits of calcium in the intima and in the deep layers of the media.

The administration of 30,000 units of vitamin A improved considerably the histologic picture. There was remarkably little calcification in cortical areas and in the hilar vessels of all animals (Fig. 6). Calcific casts were found in the medulla of some specimens. The kidneys of one of the heaviest animals in this group contained no calcific deposits.

TABLE II

Effect of 60,000 Units of Vitamin D with and without 30,000 Units of Vitamin A on Body Weight and Rat Mortality

Treatment	No. of animals	Days on treatment				
		0	4	7	11	15
		gm	*gm*	*gm*	*gm*	*gm*
Controls	15	133	142	158	179	191
60,000 D	15	129	111	109 (3)*	98 (2)*	96 (3)*
30,000 A	15	131	139	152	162	171
60,000 D + 30,000 A	15	133	110	110	101	101

* The numbers in parentheses refer to the number of rats which died between weighing periods; the other numbers refer to the average body weight.

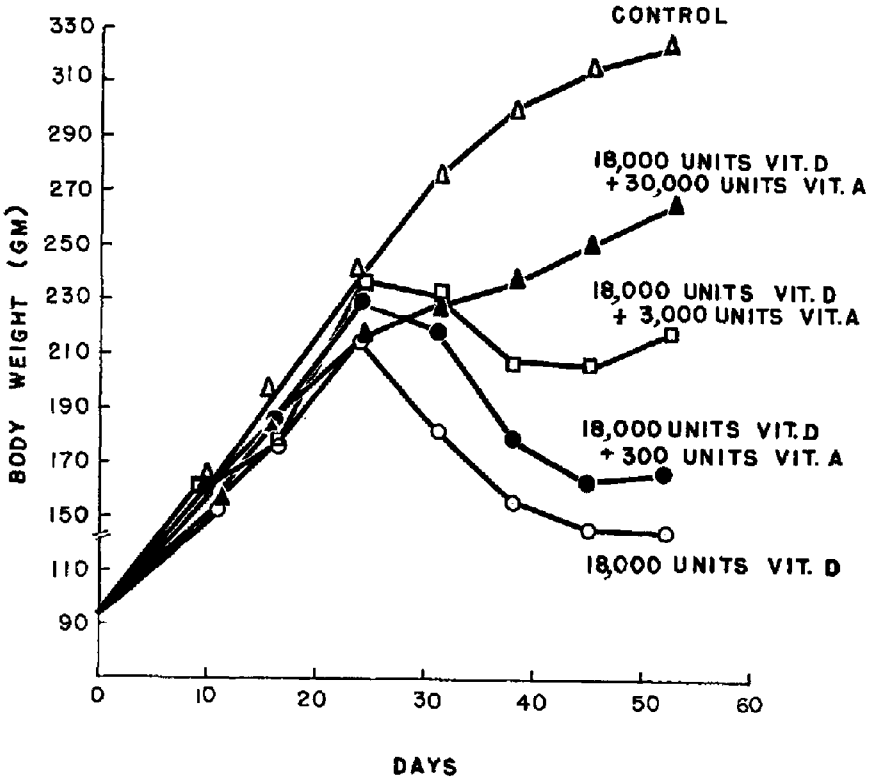
In the heart, vitamin D alone caused heavy calcification of some degenerating muscle fibers and of the intima of all vessels larger than small arterioles (Fig. 7). There was little or no evidence of an inflammatory response in areas where calcium was deposited. In none of the rats that received the vitamin A supplement was there significant myocardial damage (Fig. 8). Some specimens appeared normal while others had moderate calcification of the larger arterial walls. In this organ too the relative extent of arterial calcification paralleled the animal's weight at the time of sacrifice; the heavier the animal, the less the damage.

The bladders of all rats appeared normal, despite the severity of lesions elsewhere. Arterioles and even larger vessels of this organ showed no calcification.

The histologic picture seen in the animals receiving 15,000 units of vitamin A was intermediate between the one seen when vitamin D was used alone and that when 30,000 units of vitamin A was administered concomitantly with the vitamin D.

The rats which received only 30,000 units of vitamin A did not gain as much weight as the control rats (Tables I and II). Cement lines in the skull and tibial cortex seemed to be more prominent than they were in the normal controls. In some areas, the cement lines appeared in a mosaic pattern.

The Effect of Prolonged Administration of 18,000 Units of Vitamin D, with and without Varying Amounts of Vitamin A, on Weight Gain, Mortality, and Histology.—All groups gained weight for about 2 weeks. At this time the rats



TEXT-FIG. 1. Growth curves of rats which survived the 6 week period.

which received vitamin D alone or vitamin D supplemented with either 300 or 3,000 units of vitamin A began to lose weight rapidly (Text-fig. 1). The greater the supplement of vitamin A, the less was the weight loss. The rats which received 30,000 units of vitamin A together with the D gained weight throughout the experimental period, although not as well as the control animals. In this experiment also, the fewest pathologic changes were found in the heaviest animals.

Changes in the skulls and tibiae of the rats which had received 18,000 units

of vitamin D alone were essentially similar in pattern and degree to those seen in the animals which received 60,000 units of vitamin D alone (Figs. 1 and 3). Administration of 30,000 units of vitamin A daily simultaneously with the vitamin D almost completely prevented the appearance of pathologic changes in the skulls and tibiae of half the animals. In the other half, minimal amounts of porosity and basophilic osteoid were observed. Addition of 300 units of vitamin A did not alter significantly the changes seen when 18,000 units of vitamin D were given alone. The histologic appearance of the skulls and tibiae of the animals which had received 3,000 units of vitamin A was intermediate between that of the animals given vitamin D alone and that when 30,000 units of vitamin A was combined with the D.

In this experiment, calcification of the kidneys was confined largely to the medullary region. Few intraluminal casts were found in the cortex, even in the lightest animals. In half of the rats which had received 30,000 units of vitamin A in addition to vitamin D, kidneys were nearly normal; in the other half, only minimal amounts of calcification were observed. 300 units of vitamin A did not prevent the renal lesions, while the histologic picture of those rats given 3,000 units of vitamin A was intermediate between that seen with vitamin D alone and vitamin D plus 30,000 units of vitamin A.

DISCUSSION

The data presented in this report were derived from three of many experiments. In general, the findings have been consistent in young and adult rats. In these studies, a good correlation existed between the final body weight and the severity of bone, arterial, and renal lesions. The lighter the animal, the greater the damage. This was observed in the animals which had received vitamin D alone and also in those which had received vitamin A together with vitamin D. It is interesting to note that some tissues calcify heavily (*i.e.*, arterial walls) while others show no evidence of calcification (*i.e.*, bladder wall).

The pathologic findings in the kidneys of rats which had been acutely poisoned with massive doses of vitamin D (Experiments A-1 and Q-2) differed considerably from those of the animals chronically poisoned with smaller doses of the vitamin (Experiment C). In the former group, heavy calcification was found in the renal cortex and to a lesser extent in the medulla; while in the latter group, calcification was limited largely to the medulla. In acute experiments, there was rapid osteolysis which resulted in the release of large quantities of calcium from bone into the blood stream. Since the proximal convoluted tubules are believed to be the site of calcium reabsorption, it is not surprising that this added burden would cause extensive calcification in this area. On the other hand, in the chronic experiment where only 18,000 units of vitamin D were administered, the rate of bone resorption must have been less and, consequently, the proximal tubules were not affected as severely.

However, in this experiment there was still an appreciable amount of medullary calcification. Since the collecting tubules are responsible for reabsorbing water, it is not unreasonable to expect that the solubility products of calcium-phosphate salts might be exceeded and, consequently, these structures would become calcified in the more chronic form of hypervitaminosis D.

The skeletal changes in these studies are similar to those reported by Selye (6), Harris and Innes (2), and Follis (13). Similar changes also have been found in the rabbit (14). Fewer pathologic changes were seen in both long and flat bones when 30,000 units of vitamin A were given together with either 60,000 or 18,000 units of vitamin D. This amelioration of toxicity was more dramatic in the acute experiments (Experiments A-1 and Q-2). Severity of lesions seemed dependent on the amount of vitamin A administered with vitamin D. The more vitamin A given, the less was the damage to the tissues. Although the pathologic changes in the skeletons of hypervitaminotic D rats were greatly reduced by simultaneous administration of vitamin A, rarely were they abolished completely.

Following administration of excessive amounts of vitamin D, hypercalcemia and hypercalciuria are seen before histologic evidence of renal damage (2). Moreover, they occur on a calcium-free diet (2), suggesting that the first changes of hypervitaminosis D may be of skeletal origin and that the soft tissue changes are secondary to these. If this postulate is true, the beneficial effects of vitamin A in diminishing the soft tissue calcification of hypervitaminosis D could readily result from its action on skeletal tissues. Although the mechanism of the action of vitamin A in decreasing the toxicity of massive doses of vitamin D on bone is not known, at least two possibilities exist. First, vitamin A, when given in large amounts, may interfere with the osteolytic action of vitamin D. Evidence for this may be seen in Fig. 2, in which the combination of vitamin A with D resulted in a decreased number of resorption cavities in the osseous cortex. Another explanation for vitamin A action possibly may be found in its effect on normal bone. It is known that large amounts of this vitamin hasten both osseous maturation and remodeling (15). In hypervitaminosis D, large amounts of basophilic osteoid are produced. If vitamin A increases the rate of maturation of this osteoid, more mineral would be utilized and less spilled into the extracellular fluids. Either of these two possibilities or a combination might explain the beneficial action of this vitamin on hypervitaminosis D.

An interesting question raised by this investigation is whether the physiological or biochemical function of vitamin D is dependent on the tissue level of vitamin A or its metabolites. In these studies, it was noted that as the amount of vitamin A was increased in hypervitaminotic D animals, there were fewer pathologic changes, better weight gain and longer life span. Gross-Selbeck (16) in 1935 reported that large amounts of vitamin A concentrates decreased the

lethal toxicity of massive amounts of vitamin D. Thoenes (17) in an interesting paper speculated that vitamin A was an essential factor for the action of vitamin D and that administration of large amounts of vitamin D created a "relative avitaminosis A." This present study tends to support the concept that some of the damage of hypervitaminosis D may be in part due to a relative avitaminosis A.

SUMMARY

The administration of relatively large amounts of vitamin A to hypervitaminotic D rats decreases the toxicity of this condition as evidenced by increased longevity, better weight gain, decreased soft tissue calcification and by considerable improvement in the histologic appearance of osseous structures.

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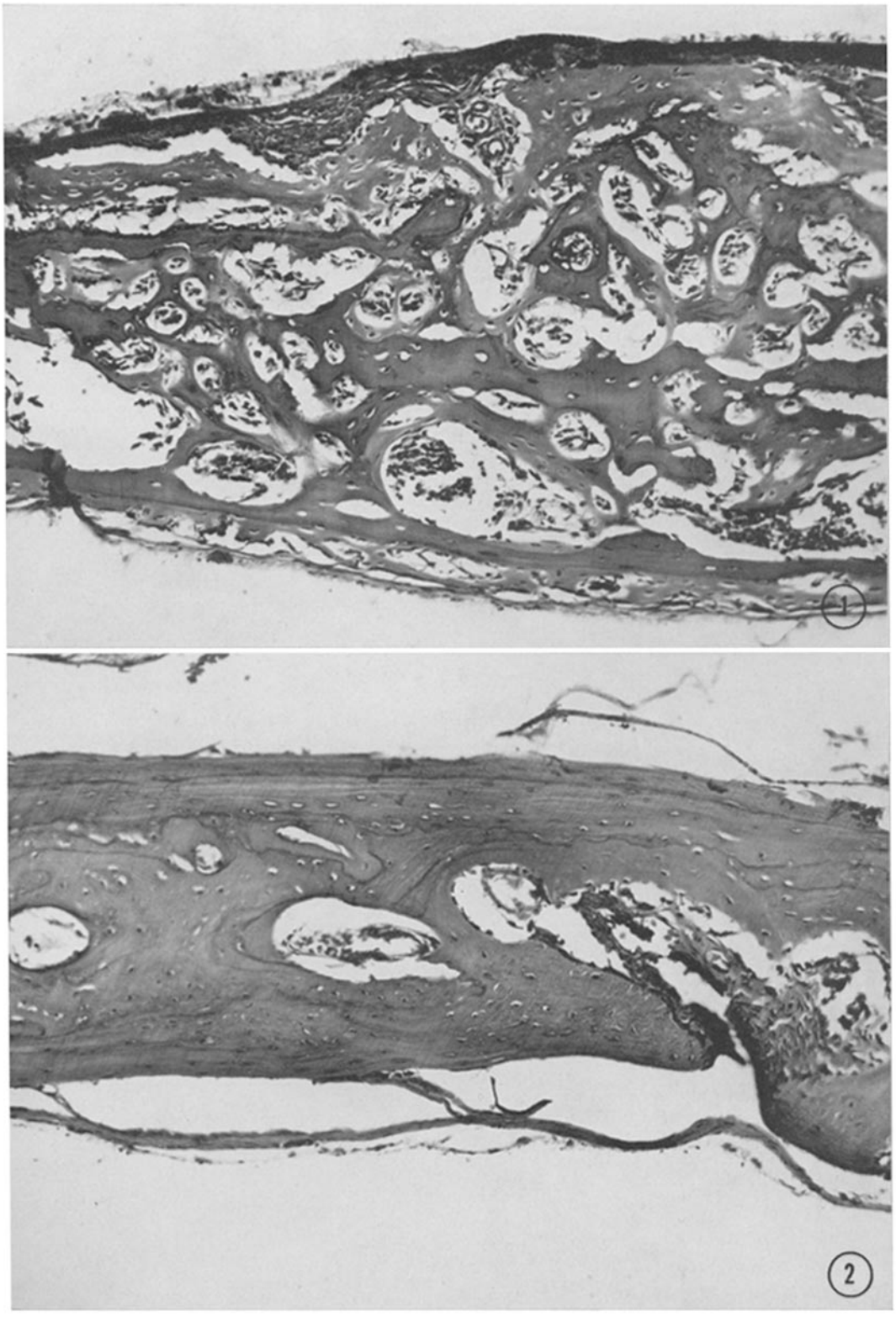
EXPLANATION OF PLATES

PLATE 16

FIG. 1. Skull, 60,000 units of vitamin D per 100 gm daily for 11 days. Hematoxylin and eosin. × 168. Cross-section just lateral to sagittal suture. Note rarefaction, vascularity, osteoid tissue in subperiosteal and medullary regions.

FIG. 2. Skull, 60,000 units of vitamin D per 100 gm and 30,000 units of vitamin A per 100 gm daily for 11 days. Hematoxylin and eosin. × 168. Cross-section just lateral to sagittal suture. This picture does not differ significantly from that seen in the normal control and should be compared with Fig. 1.

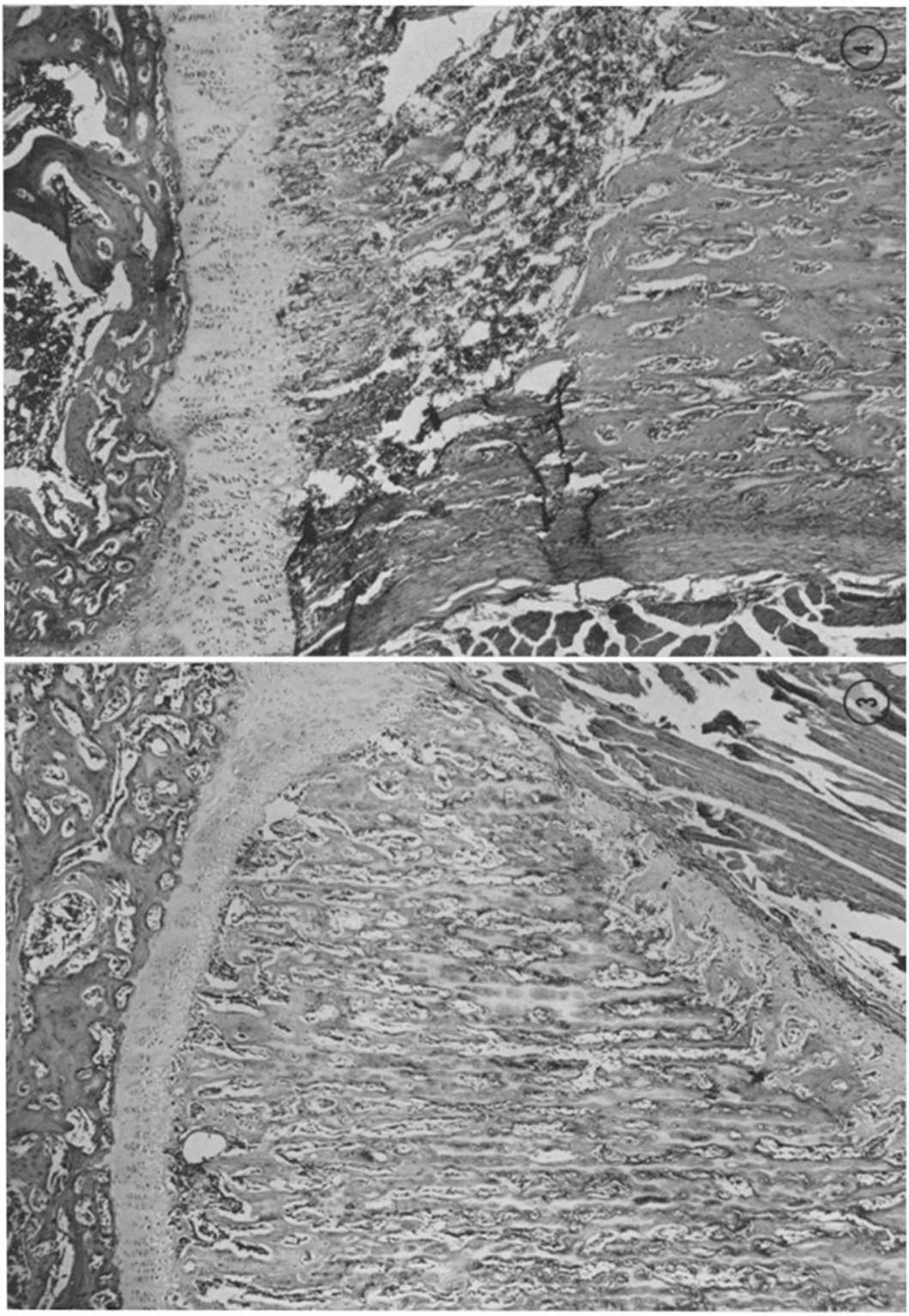


(Clark and Bassett: Amelioration of hypervitaminosis D)

PLATE 17

FIG. 3. Upper tibia, same treatment as Fig. 1. Hematoxylin and eosin. × 65. Longitudinal section. Note thin epiphyseal plate, masses of endosteal and subperiosteal osteoid tissue.

FIG. 4. Tibia, same treatment as Fig. 2. Hematoxylin and eosin. × 65. Longitudinal section. Note normal appearance of epiphyseal plate, lack of subperiosteal new bone and relatively little osteoid in medullary areas. Compare with Fig. 3.

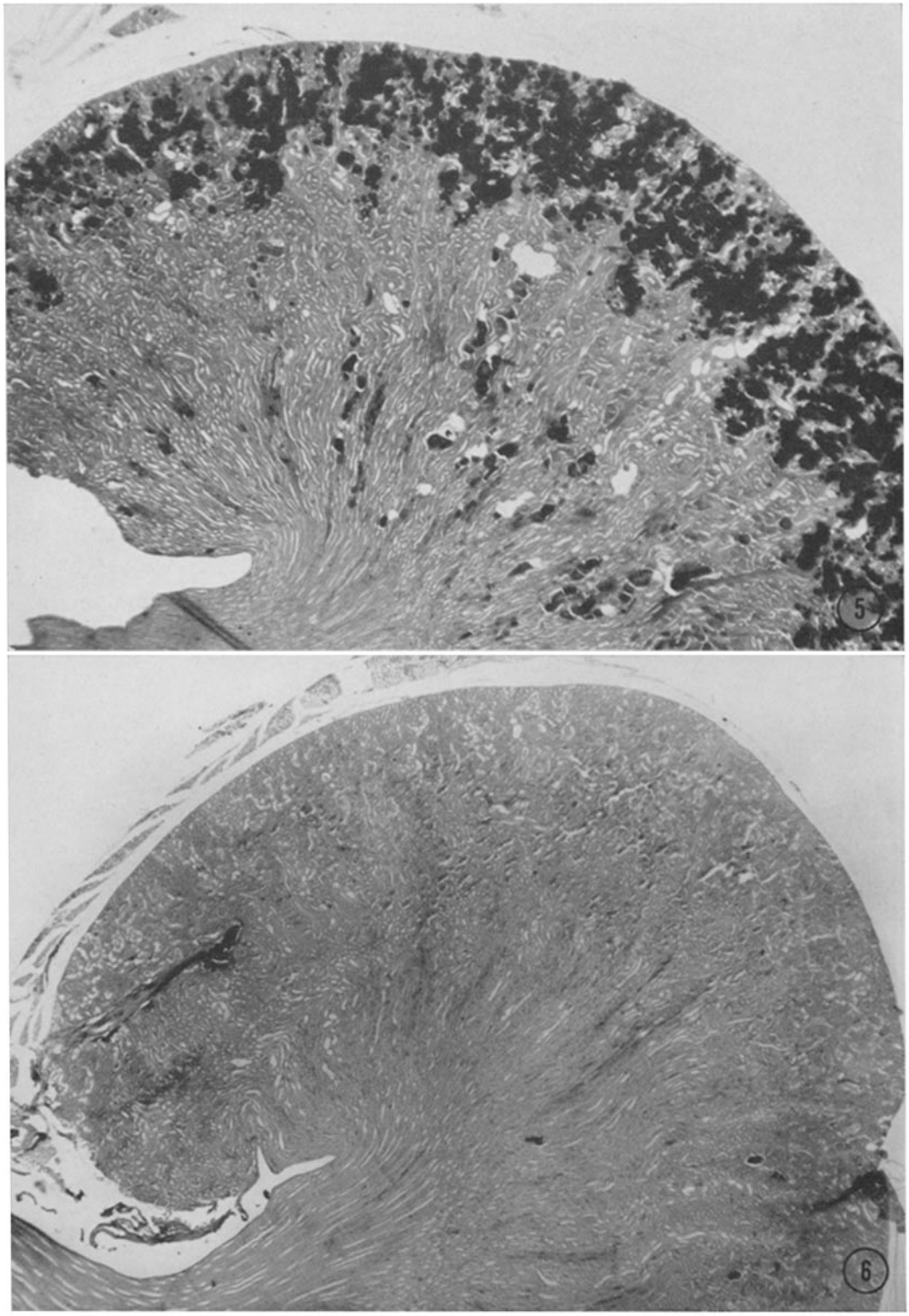


(Clark and Bassett: Amelioration of hypervitaminosis D)

PLATE 18

FIG. 5. Kidney, same treatment as Fig. 1. Alizarin red S. × 19. Sagittal section. Areas of calcification appear black. Note relative difference in the extent of cortical and medullary involvement.

FIG. 6. Kidney, same treatment as Fig. 2. Alizarin red S. × 19, sagittal section. No calcification is seen. Compare with Fig. 5.

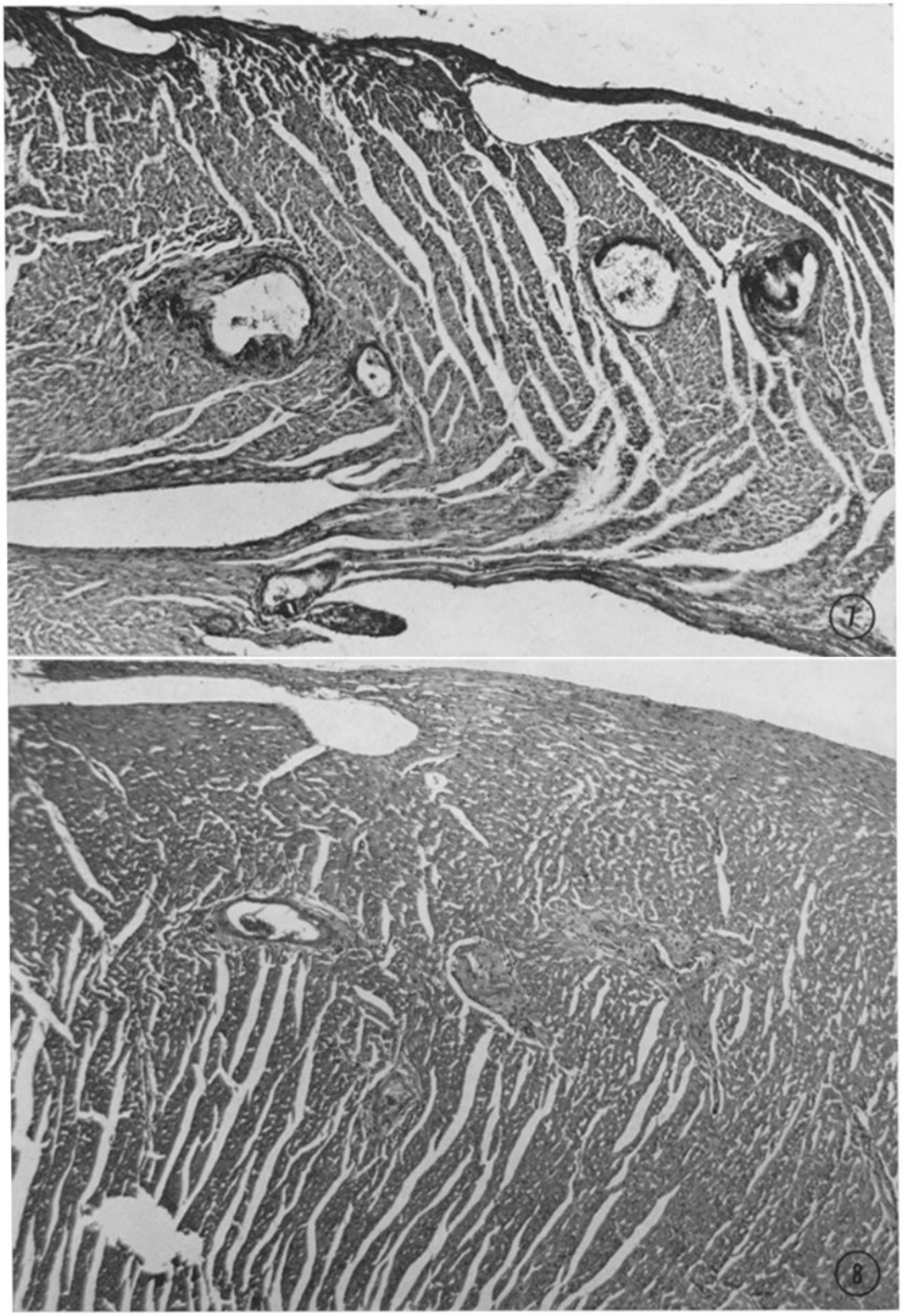


(Clark and Bassett: Amelioration of hypervitaminosis D)

PLATE 19

FIG. 7. Heart, same treatment as Fig. 1. Hematoxylin and eosin. × 83. Note black areas in intimal regions of vessels.

FIG. 8. Heart, same treatment as Fig. 2. Hematoxylin and eosin. × 83. No evidence of calcification of vessels. Compare with Fig. 7.



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