Induction of Aortic and Coronary Athero-arteriosclerosis in Rats Fed a Hypervitaminosis D, Cholesterol-Containing Diet[1] (36030)

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Although dietary cholesterol has been demonstrated to be atherogenic in a number of animal species including the rabbit (1–3), chick (4, 5), pig (6–8), monkey (9–12), and others, the rat, when fed ordinary cholesterol-containing diets, has failed to develop convincing atherosclerotic lesions. Wissler *et al.* (13) and later Fillios *et al.* (14) found that suppressing thyroid function by administering thiouracil caused atherosclerosis in rats fed a diet enriched with cholesterol and cholic acid. Hypophysectomy was also effective in producing atherosclerosis in cholesterol-fed rats, an effect apparently due to the resulting impairment in thyroid function (15). Atherosclerosis has also been induced in rats by subjecting them to various stressor agents and feeding them a cholesterol-containing diet. Thus exposure to X-irradiation has been shown to increase susceptibility to atherosclerosis in cholesterol-fed rats (16–18). Wilgram (19) observed that the administration of massive doses of vitamin D_2 to rats resulted in severe damage and calcification of the arterial media. When such treated animals were rendered hyperlipemic on a cholesterol–cholic acid thiouracil diet, marked atherosclerotic changes occurred in their coronary arteries. Constantinides (20) found that when arterial injury was induced in rats by vitamin D_2 administration (350,000 IU/kg by stomach tube on 4 successive days), coronary atherosclerosis occurred within 6 weeks in rats fed a diet containing 1.5% cholesterol, 0.5% cholic acid, and 0.2% propylthiouracil. Atherosclerosis was not observed at this time in any of the rats fed a similar diet but not administered vitamin D_2 nor did it occur in vitamin D_2-treated rats fed a cholesterol-free diet. In the present communication, data are presented indicating that massive doses of vitamin D_2 will induce atherosclerotic lesions in the aorta and coronary arteries of cholesterol-fed rats without the concurrent administration of antithyroid drugs.

Materials and Methods. The basal ration used in this study was a highly purified diet consisting of sucrose, 61% casein,[2] 24%; cottonseed oil, 10%; salt mixture,[3] 5%; and the following vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 1 mg; folic acid, 10 mg; para-aminobenzoic acid, 200 mg; inositol, 400 mg; vitamin B_{12}, 150 μg; 2-methyl-1,4-naphthoquinone, 5 mg; choline chloride, 2 g; vitamin A, 5000 USP units; vitamin D_2, 500 USP units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Forty-eight male rats of the Long-Evans strain (av 156 g in body wt; range 149–168 g), were selected for the present experiment. Animals were divided into 4 comparable groups of 12 animals each. Group I was fed the basal ration indicated above; group II was fed the basal ration + 1.5% cholesterol + 0.5% cholic acid; group III was fed the basal ration + 1.25 million USP units of vitamin D_2[4]/kg of diet; and group IV was fed

[1] This investigation was supported in part by Grant No. HE-11069 from the National Heart Institute, NIH, U.S. Public Health Service.

[2] Vitamin-Free Test Casein, General Biochemicals, Chagrin Falls, OH.

[3] Hubbell *et al.* (31) Salt Mixture, General Biochemicals, Chagrin Falls, OH.

[4] Viosterol (Irradiated Ergosterol) (1,000,000 USP units/g in oil), General Biochemicals, Chagrin Falls, OH.

the basal ration + 1.5% cholesterol + 0.5% cholic acid + 1.25 million USP units of vitamin D_2/kg of diet.[5] The test supplements were incorporated in the basal ration in place of an equal amount of sucrose. Animals were kept in metal cages with raised screen bottoms (3 rats/cage) and were provided the various test diets and water *ad libitum*. The animals were fed daily and all food not consumed 24 hr after feeding was discarded. The rats were weighed weekly during the course of the experiment.

After 6 weeks of feeding, the rats were anesthetized with sodium pentobarbital, blood was withdrawn from the heart into a heparinized syringe, and plasma total cholesterol was determined by the method of Searcy and Berquist (21). The rats were sacrificed at this time, and the hearts and aortas were fixed in 10% buffered formalin. The hearts were divided into 3 parts consisting of the apex, middle, and basal portion; and the aortas were cut transversely at the arch and the midthoracic level. Frozen sections of the above were prepared, cut at 16–20 μ in thickness and stained with Oil Red O and hematoxylin. Ten cross sections were prepared through each part of the heart and through the arch and and thoracic portions of the aorta. The tissues remaining after preparation of the frozen sections were prepared for paraffin embedding in the routine manner, sectioned at 6 μ in thickness, and consecutive sections were stained with hematoxylin and eosin, Von Kossa stain, Masson's trichrome stain, and Weigert's elastic tissue stain. Three sections stained with each of the above stains were prepared of each portion of the heart and aorta of each rat.

Results. Body weight. The final body weight of rats in the various groups is indicated in Table I. Rats fed the rations containing 1.25 million USP units of vitamin D_2/kg of diet (groups III and IV) gained significantly less weight than did those fed the basal ration supplemented with cholesterol and cholic acid alone (group II). Whereas rats in groups I and II showed a continuous weight increment during the course of the experiment, those in groups III and IV gained weight during the first week, lost weight during the subsequent 2 to 3 weeks and with the exception of approximately one fourth of the animals in each of these groups which continued to lose weight (several of which succumbed) thereafter resumed their increment in weight and by the end of week 6 of feeding had surpassed the weight at which they started the experiment. No significant differences in weight increment were observed between rats in groups III and IV.

Plasma cholesterol levels. The plasma total cholesterol levels of rats in the various groups is indicated in Table I. The lowest average cholesterol level occurred in rats fed the basal ration (group I). A highly significant increment in plasma cholesterol levels over that of rats in group I occurred in rats fed the basal ration + 1.5% cholesterol + 0.5% cholic acid + 1.25 million USP units of vitamin D_2/kg of diet (group IV). Intermediate levels were observed in rats in groups II and III.[6]

[5] Preliminary studies were conducted with rats fed the basal ration + 1.5% cholesterol + 0.5% cholic acid + graded levels of vitamin D_2, *i.e.*, 1, 1.25, 1.5, and 2 million USP units of vitamin D_2/kg of diet. Forty-eight male rats of the Long-Evans strain (av body wt of 148 g) were divided into 4 comparable groups of 12 animals each, which were fed the above diets. Extensive athero-arteriosclerotic lesions of the aorta and coronary arteries similar to those described in the present report were observed in all rats which survived the experimental period of 6 weeks and which were fed diets containing 1.25, 1.5, and 2 million USP units of vitamin D_2/kg of diet. These lesions were particularly marked in rats fed the highest level of vitamin D_2. Mortality increased with increasing levels of vitamin D_2. Thus only 4 rats survived in the group fed the diet containing 2 million USP units of vitamin D_2/kg of diet, 7 rats in the group fed 1.5 million USP units of vitamin D_2/kg of diet, and 11 rats in the group fed 1.25 million USP units of vitamin D_2/kg of diet. All 12 rats survived in the group fed 1 million USP units of vitamin D_2/kg of diet but the percentage of rats exhibiting atherosclerotic lesions (*i.e.* stainable lipid material in the areas of arterial injury) and the severity of such lesions were significantly less in this group than in those fed the higher levels of vitamin D_2 supplementation.

[6] These findings are in agreement with those previously reported by Hass *et al.* (29) that administration of toxic doses of vitamin D_2 to cholesterol-fed rabbits resulted in higher serum cholesterol levels than occurred in animals administered either vitamin D_2 or cholesterol alone.

TABLE I. Effects of Cholesterol + Cholic Acid, Massive Doses of Vitamin D_2 and Combined Supplements of Cholesterol + Cholic Acid + Massive Doses of Vitamin D_2 on the Weight Increment and Plasma Cholesterol Level of Rats (12 animals/group.)[a]

Dietary group		Body wt (g)		Terminal plasma total cholesterol, mg %
		Initial	Final	
I	Basal ration	156.0	368.3	74.5 ± 10.6[b]
Basal ration + following supplements:				
II	1.5% cholesterol + 0.5% cholic acid	156.2	381.4	126.4 ± 36.9
III	1.25 million USP units of vitamin D_2/kg of diet	156.4	166.6 (10)	96.3 ± 12.1
IV	1.5% cholesterol + 0.5% cholic acid + 1.25 million USP units of vitamin D_2/kg of diet	156.3	174.8 (11)	179.8 ± 40.4

[a] The values in parentheses indicate the number of animals which survived and on which data are based when this number was less than the initial number per group. Animals were sacrificed after 6 weeks of feeding.

[b] Standard deviation.

Histological findings. Aorta. In rats fed the basal ration (group I) and the basal ration + cholesterol + cholic acid (group II), the aorta was normal in microscopic appearance. In rats fed the basal ration + 1.25 million USP units of vitamin D_2 per kg of diet (group III), a number of pathologic changes were present. These occurred principally in the media and consisted primarily of degeneration, calcification (Fig. 1), plaque formation, metachromasia, and lysis and fragmentation of the elastic lamina. The above changes were present in all rats in this group. Three of the surviving 10 animals in this group also exhibited subintimal mesenchymal cell proliferation. In general, the intima of rats in group III remained intact and appeared normal in microscopic appearance. In several animals there was a rupture of the medial plaque through the intima accompanied by calcification of the plaque and the adjacent intima. All the changes indicated above were present to a comparable degree in rats fed the basal ration + 1.5% cholesterol + 0.5% cholic acid + 1.25 million USP units of vitamin D_2/kg of ration (group IV) (Fig. 2). The rats in the latter group, however, exhibited in addition extensive atherosclerotic lesions manifested by the presence of intracellular and extracellular stainable lipid material which was particularly marked in the areas of medial injury and plaque formation but was also present in the intima. Approximately one fourth of the animals in group IV also exhibited mesenchymal cell proliferation in the intima which was not observed in any of the rats in group III (Fig. 3).

Coronary arteries. No abnormalities were observed histologically in the coronary arteries of rats fed the basal ration (group I) and the basal ration + cholesterol + cholic acid (group II). In rats fed the basal ration + 1.25 million USP units of vitamin D_2/kg of diet (group III), a number of pathological changes were present. These occurred principally in the media and consisted primarily of degeneration, calcification (Fig. 4), metachromasia, and focal plaque formation. The medial changes were often so severe as to result in complete loss of cellular details and structure. Calcification of the media was massive and frequently extended throughout the entire wall of the artery (Fig. 4). The elastic membrane showed extensive fragmentation, duplication, and disorientation and was often absent over large areas. The intima was relatively normal in microscopic apeparance. The lumen of the coronary arteries was markedly dilated and often irregular in outline (Fig. 4). These changes were particularly marked in the main coronaries and their principal branches and were present throughout

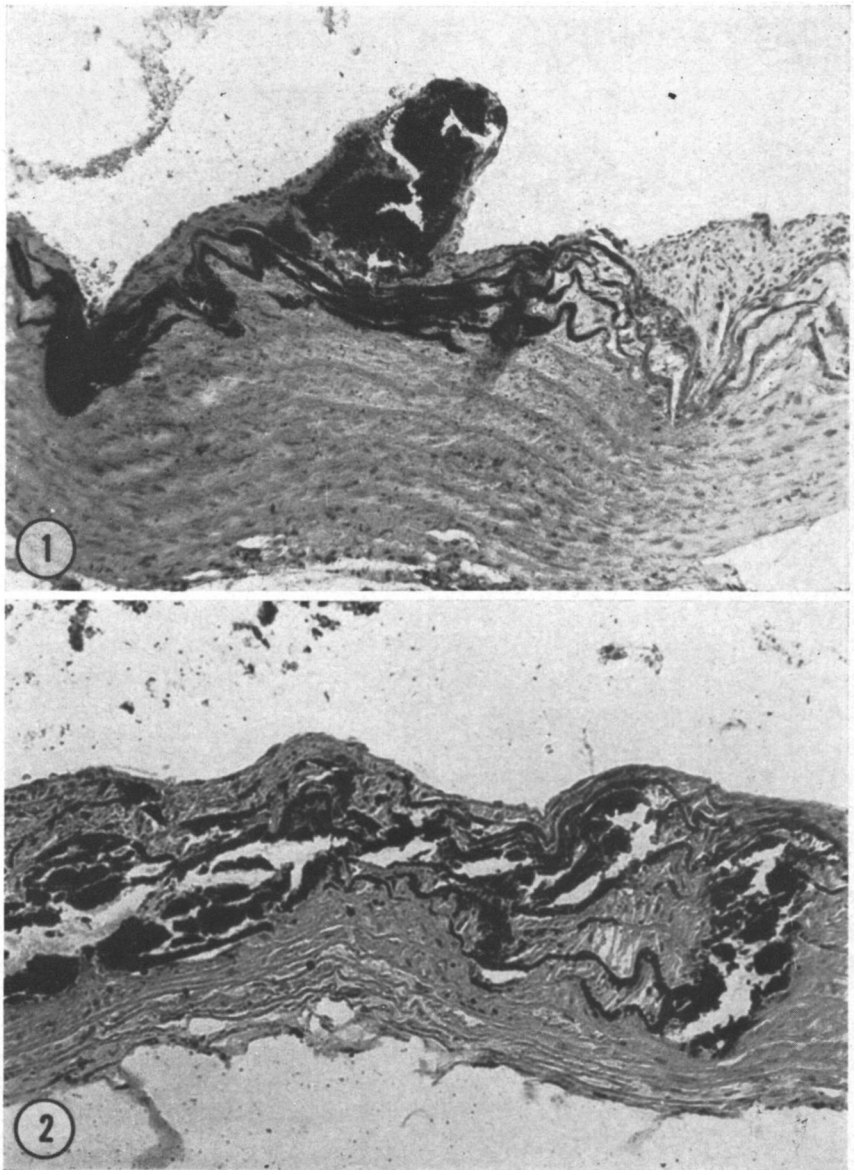


FIG. 1. Section of the aorta from a rat in group III: Note the massive calcification of the plaque and the degenerated media. The calcification is typically distributed along the damaged elastic lamina. Von Kossa stain, 48×.

FIG. 2. Section of the aorta from a rat in group IV: Note the marked calcification in the damaged areas of the media. Von Kossa stain, 48×.

the course of these arteries. The above changes were present in all rats in this group. Three of the 10 surviving animals of this group also exhibited subintimal proliferation of mesenchymal cells. The adventitia exhibited infiltration of inflammatory cells and fibroblastic proliferation. Although the above changes were noted in all parts of the heart, they were particularly severe in the proximal portion. All the changes indicated above were also present in rats fed the basal ration + 1.5% cholesterol + 0.5% cholic acid +

1.25 million USP units of vitamin D_2/kg of diet (group IV); but the degree of medial metachromasia and calcification (Fig. 5) and the extent of lumen dilatation was considerably less in rats of group IV than in those of group III. Rats in group IV, however, did exhibit atherosclerotic lesions manifested by the presence of intracellular and extracel-

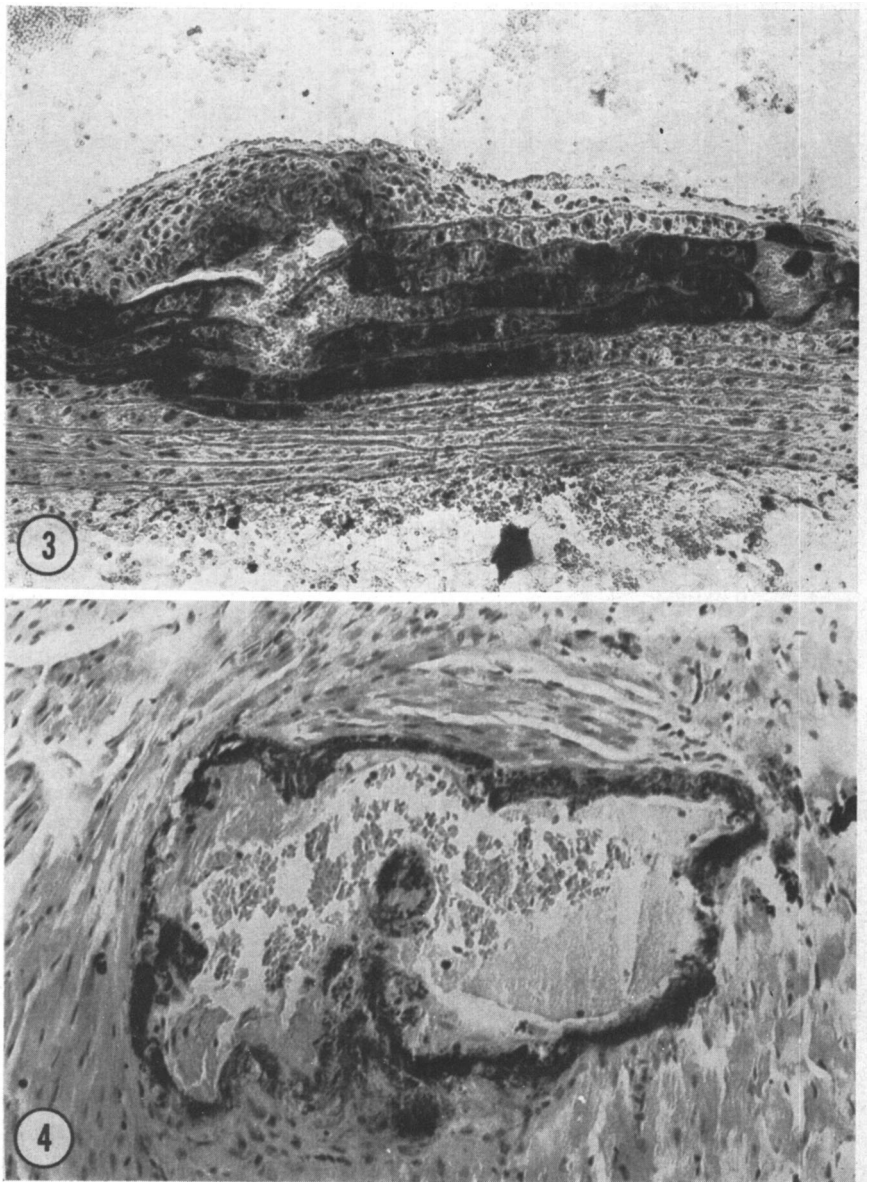
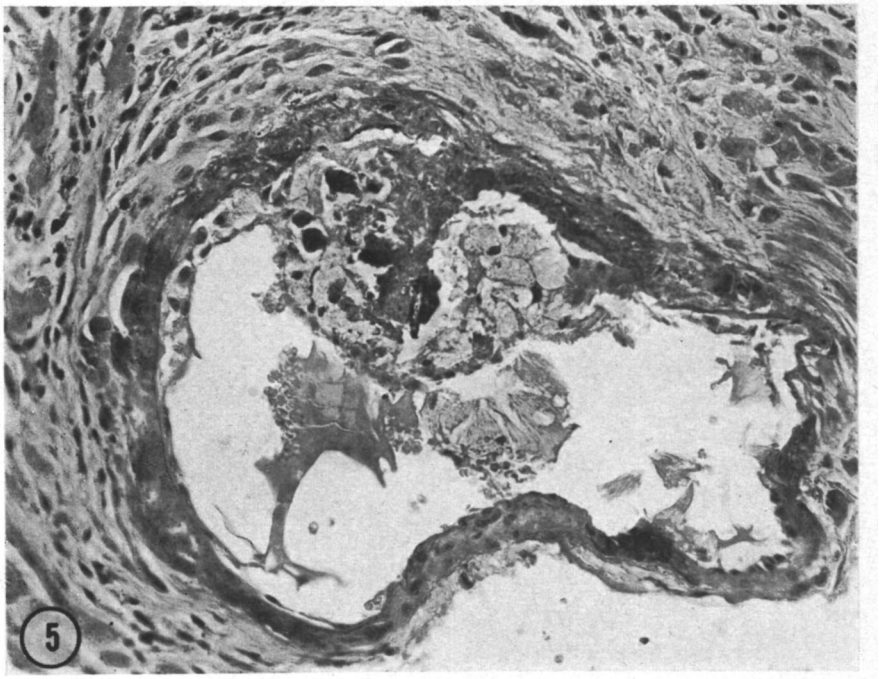


FIG. 3. Section of the aorta from a rat in group IV. Note the extensive medial degeneration, plaque formation, smooth muscle degeneration and subintimal and intimal mesenchymal cell proliferation, as well as the stainable intracellular and extracellular lipids in the thickened intima and damaged media. Oil Red O and hematoxylin stains, 60×.

FIG. 4. Section of the proximal coronary artery of a rat in group III: Note the massive calcification of the media along the entire circumference of the artery. Calcification of the adjacent myocardium is also present. Von Kossa stain, 120×.



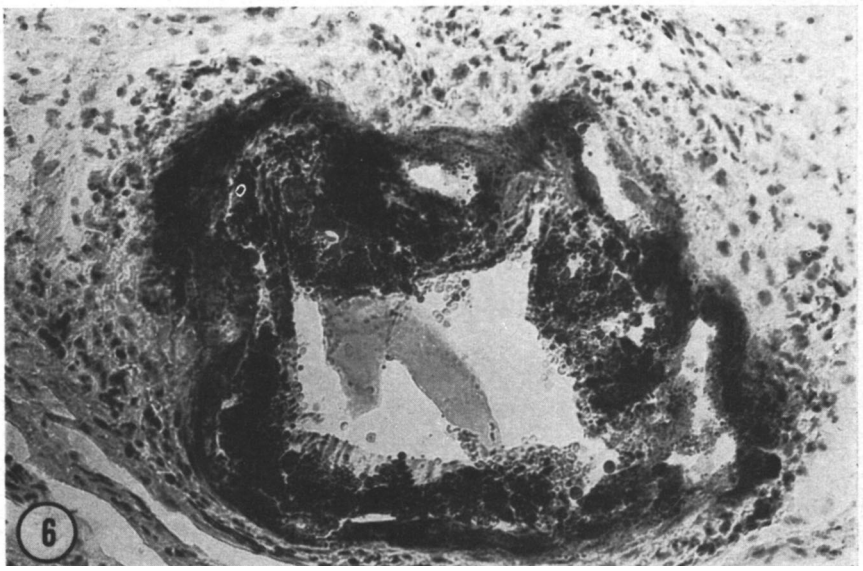


FIG. 5. Section of the proximal coronary artery of a rat in group IV: Note the atheromatous plaque consisting of foam cells and calcific deposits (dark staining) protruding into the arterial lumen. The degree of medial calcification was significantly reduced compared to that of rats in group III (Fig. 4). Von Kossa stain, 120×.

FIG. 6. Section of the proximal coronary artery of a rat in group IV: Note the advanced atherosclerosis involving the intima, subintima and media. The lipid material is dark staining and globular appearing and is present both intracellulary and extracellulary. Oil Red O and hematoxylin stains, 120×.

lular stainable lipid material which was not observed in any of the rats in groups I, II, and III. In some arteries the lipid was so extensive as to cause a significant narrowing of the arterial lumen (Fig. 6). The lipid was present primarily in the areas of the damaged media, plaque, and subintima. Atherosclerotic plaque formation also involved the intima and frequently protruded extensively into the arterial lumen.

Discussion. Available data indicate that the administration of massive doses of vitamin D is highly active in inducing atherosclerotic lesions in a number of animal species including the rabbit (22–24), rat (25), dog (26), and monkey (27). Injury to the cardiovascular system resulting from administration of vitamin D has also been observed in man (28). In the absence of hyperlipemia, lipid-stainable material (true atheromata) was not observed at the sites of arterial injury in animals fed toxic doses of vitamin D. In lipemic animals, however, atherogenesis is greatly increased and accelerated at the sites of vascular injury induced by massive doses of vitamin D. Thus Hass *et al.* (29) observed that rabbits fed viosterol in conjunction with a cholesterol-containing diet developed more severe atherosclerotic lesions than those in rabbits fed a similar diet with either the viosterol or cholesterol omitted. Similarly Constantinides (20) found that rabbits fed a cholesterol-free diet for 3 weeks had terminal cholesterol levels of 62 mg/100 ml and were free of aortic atherosclerotic lesions; whereas rabbits which were fed a similar diet but which received viosterol by stomach tube at a level of 200,000 IU/kg on 3 successive days had an average terminal cholesterol level of 145 mg/100 ml (the increment was probably due to the kidney damage or alterations in the hepatic and fecal output of cholesterol and bile acid caused by this steriod); these animals developed aortic atherosclerotic lesions within 3 weeks in 100% of the animals tested. In contrast to the latter group, rabbits fed a cholesterol-containing diet but not administered viosterol failed to develop lesions within a comparable period of feeding despite a terminal cholesterol concentration in excess of 5 times the above level. It would appear from these findings that arteries damaged by toxic doses of vitamin D will develop atherosclerosis at much lower lipemia levels than uninjured ones—within a given time. Of possible pertinence in this regard is the finding of Hess and Loustalot (30) that calciferol increases the rate of lipid accumulation in the rat aorta.

In the present experiment massive atherosclerotic lesions of the aorta and coronary arteries were induced in 100% of the rats surviving an experimental period of 6 weeks by feeding them a purified diet containing 1.5% cholesterol + 0.5% cholic acid + 1.25 million USP units of vitamin D_2/kg of diet without the concurrent administration of thiouracil or propylthiouracil. Rats fed a similar diet with the vitamin D_2 supplement omitted were free of such lesions and were indistinguishable in the microscopic appearance of their aorta and coronary arteries from rats fed a control ration without the cholesterol, cholic acid, and vitamin D_2 supplements. Rats fed the control ration supplemented with 1.25 million USP units of vitamin D_2/kg of diet showed extensive arteriosclerotic lesions but stainable lipids (true atheromata) were not observed at the sites of arterial injury. The occurrence of atherosclerotic lesions in rats fed the diet containing cholesterol, cholic acid, and the toxic vitamin D_2 supplement was correlated with terminal plasma cholesterol levels which exceeded those of rats fed the other diets tested but were far less than those which have been reported for rats fed cholesterol-containing diets with antithyroid drugs.

Summary. Massive athero-arteriosclerotic lesions of the aorta and coronary arteries were induced in 100% of the young adult male rats fed a purified basal ration supplemented with 1.5% cholesterol, 0.5% cholic acid, and 1.25 million USP units of vitamin D_2 (viosterol)/kg of diet for an experimental period of 6 weeks. Rats fed a similar diet with the cholesterol and cholic acid omitted showed extensive arteriosclerotic lesions but no lipid-stainable material (true atheromata) was present at the sites of arterial injury. No abnormal microscopic findings were observed in the aorta and coronary arteries of rats fed the basal ration alone or the basal ration plus cholesterol and cholic acid without the vita-

min D_2 supplement.

The authors are indebted to Dr. Roslyn B. Alfin-Slater and Mr. H. J. Hernandez of the School of Public Health, University of California at Los Angeles, for the Plasma Cholesterol determinations.

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Received June 3, 1971. P.S.E.B.M., 1971, Vol. 138.