

Effects of Hypervitaminosis A and D on Skeletal Metabolism*

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The feeding of excessive amounts of vitamin D to animals causes pathological changes in the skeleton and calcification of soft tissues (1-4). Hypervitaminosis A causes bone fragility. Spontaneous fractures occur in growing rats, whereas in the adult rat only rarefaction develops (5-8). Although excesses of each vitamin cause specific pathological changes, the concomitant feeding of toxic amounts of both vitamins prevents to a large extent soft tissue calcification and the skeletal changes that accompany hypervitaminosis D (9-10). The present investigation was undertaken to determine how vitamin A exerts its beneficial action in offsetting some of the pathological changes observed in hypervitaminosis D. A preliminary account of these studies has been reported (11).

EXPERIMENTAL PROCEDURE

Male albino rats of the Holtzman strain were used in all experiments except one (Table V), and in this experiment, female rats were used. During metabolic studies, animals were housed individually in metabolism cages designed to separate urine and feces. In Experiments IX (Table I), AJ (Tables II and III), and Q (Table IV), urines were collected for 24 hours, and immediately thereafter the animals were killed; their gastrointestinal tracts were removed and combined with their feces. In Experiments I (Table V) and W (Table VI), urines and feces were collected every 48 hours. Suitable aliquots of urine were plated out in stainless steel planchets for radioactive determinations. Feces and gastrointestinal tracts were ashed, dissolved in concentrated HNO₃, and diluted to suitable volumes, and aliquots were plated out in stainless steel planchets. The radioactivity of all samples was determined with a proportional gas flow counter containing a Micromil window, and the counting period was sufficient to ensure a statistical accuracy of at least 1%. Whenever necessary, corrections for background and decay were made. Phosphorus was determined by the method of King (12); calcium by the method of Berger (13); hydroxyproline by the method of Stegemann (14) and hexosamine by the method of Belcher, Nutten, and Sambrook (15). The marrow was removed from the long bones after the shaft was carefully split. The bones were then extracted three times with chloroform-methanol (2:1) to remove the lipids. Each extraction period was for 24 hours, and the bones were shaken frequently. The bones were dried in an oven at 100-105°. Ashing of samples was done in a muffle furnace at 650°. The loss of weight of the bones after ashing is expressed as the organic fraction of bone. Bone samples were

hydrolyzed with 4 N HCl for 18 hours in sealed tubes at 110° before the estimation of hydroxyproline and hexosamine.

The vitamin A palmitate (Merck) used in these experiments was generously supplied by Merck, Sharp and Dohme Research Laboratories. The crystalline vitamin D₂ (calciferol) was kindly donated by Philips-Roxane Company. Purified sesame oil was used to dilute the vitamins and was fed to all control rats. The calciferol was dissolved in a minimum of ethyl alcohol before dilution. The vitamins were administered daily on a body weight basis by stomach tube. An analysis of variance was performed on each experiment.

RESULTS

Calcium and Phosphorus Absorption and Excretion in Adult and Young Rats—The smaller gain in weight observed in the adult rats fed 10,000 units of vitamin A per 100 g of body weight (Table I) indicates that this amount of vitamin A is slightly toxic. The same quantity of vitamin D is more toxic and caused a marked loss of weight. The administration of vitamins A and D together appears less toxic than vitamin D alone, since the animals fed the combination lost less weight than did those fed just vitamin D.

Comparison of the amounts of ⁴⁵Ca in the alimentary tract and feces of the control rats and those fed vitamin D (Table I) clearly shows the enhancement of calcium absorption in hypervitaminosis D; an effect which has been observed by others (16-18). Vitamin A had no effect on calcium absorption, and the combination of A and D did not alter the enhancing action of vitamin D on intestinal absorption of calcium. These results on absorption correlate well with the urinary data (Table I) which indicate greater spillage after increased absorption. Neither vitamin A nor vitamin D affected the absorption of phosphate from the gastrointestinal tract, although vitamin A did increase the urinary ³²P in this experiment.

In the rapidly growing young rat (Table II), vitamin A also diminished the toxicity of excessive amounts of vitamin D as measured by gains in body weight. Although the older animals lost weight when fed toxic amounts of vitamin D or the combination of vitamins A and D (Table I), these young animals continued to gain weight, although very slowly (Table II). Absorption of calcium or phosphorus was similar to that seen with the older rats in the previous experiment (Table I).

Analyses of 24-hour urine samples, collected at the end of the experiment, indicate (Table III) that in these young animals, vitamin D increased calcium excretion, whereas vitamin A had no effect. The combination of vitamins A and D tended to lower the values observed after vitamin D alone, but the differences were not significant.

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TABLE I
Calcium and phosphorus absorption by adult rats with hypervitaminosis A and D

Experiment IX: Each group contained 12 rats. The animals received 10,000 units of vitamin A, or 10,000 units of vitamin D, or a combination of 10,000 units of vitamin A and 10,000 units of vitamin D per 100 g of body weight. The animals were maintained on this regimen for 30 days. Each animal received 10 μ c of ^{45}Ca or ^{32}P in 0.5 ml of milk *per os* 24 hours before being killed.

Treatment	Body weight		^{45}Ca			^{32}P		
	Initial	Final	Urine	Gastrointestinal tract and feces	Sum	Urine	Gastrointestinal tract and feces	Sum
	g		% dose			% dose		
Control.....	275	332	0.49	28.9	29.1	4.3	12.0	16.3
Vitamin A.....	275	297*	0.46	35.2	35.7	6.7*	20.2	26.9
Vitamin D.....	275	232*	10.6*	3.3*	13.9*	4.1	10.4	14.5
Vitamins A and D.....	275	255*	6.6*	6.1*	12.7*	5.0	20.0	25.0

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE II
Calcium and phosphorus absorption by young rats with hypervitaminosis A and D

Experiment AJ: Each group contained 16 rats. The animals received 10,000 units of vitamin A, or 15,000 units of vitamin D, or a combination of 10,000 units of vitamin A, and 15,000 units of vitamin D per 100 g of body weight. The animals were maintained on this regimen for 24 days. Each animal received 10 μ c of ^{45}Ca or ^{32}P in 0.5 ml of milk *per os* 24 hours before being killed.

Treatment	Body weight		^{45}Ca			^{32}P		
	Initial	Final	Urine	Gastrointestinal tract and feces	Sum	Urine	Gastrointestinal tract and feces	Sum
	g		% dose			% dose		
Control.....	85	190	0.88	31.2	32.1	10.9	11.5	22.4
Vitamin A.....	85	178*	1.06	36.1	37.2	12.3	9.5	21.8
Vitamin D.....	85	97*	7.9*	1.0*	8.9*	12.5	7.2	19.9
Vitamins A and D.....	85	120*	10.6*	1.4*	12.0*	13.4	7.0	20.4

* Statistically significant ($p < 0.01$) as compared to control values.

Vitamin D decreased phosphate excretion and vitamin A had no effect. When vitamin A was given with vitamin D, urinary phosphate values approached those of the controls. Although the administration of vitamin D increased calcium excretion, it decreased phosphate excretion, regardless of the method of calculation. Similar findings have been observed in acute experiments (19).

Increasing the amount of vitamin D to 60,000 units and of vitamin A to 30,000 units per 100 g of body weight caused a significant loss of weight in young growing rats, and the concomitant administration of vitamin A with this amount of vitamin D did not decrease significantly the loss in weight (Table IV), although it did decrease the mortality rate (10). In this experiment, the isotopes were administered parenterally and urinary labeled and unlabeled calcium again were significantly increased in the animals fed vitamin D or the combination of vitamin A and D. When calculated on a body weight basis, vitamin D alone or in combination with vitamin A increased urinary isotopic and unlabeled phosphorus. Urinary calcium or phosphate values of the rats treated with vitamin A did not differ significantly from those of the control animals.

Calcium and Phosphorus Excretion after Varied Levels of Vitamin A Intake—Vitamin A, 10,000 units per 100 g of body weight, again was toxic in these mature female rats, as evidenced by the greater loss in body weight (Table V). Despite this toxicity, the rats fed vitamin A excreted less unlabeled calcium and phosphate (urine and feces). Moreover, the decrease in unlabeled

TABLE III

Urinary values at 24 hours

Same legend as Table II.

	Calcium		Phosphorus	
	mg	mg/100 g body weight	mg	mg/100 g body weight
Control.....	0.47	0.23	11.4	6.0
Vitamin A.....	0.49	0.34	12.0	6.7
Vitamin D.....	3.76*	3.85*	4.5*	4.6*
Vitamins A and D.....	2.41*	1.98*	7.5*	6.3

* Statistically significant ($p < 0.01$) as compared to control values.

calcium excretion was accompanied by a significant increase in the elimination of radioactive calcium that had been deposited in the skeleton 120 days before.

Increasing the level of vitamin A 10-fold (Table VI) resulted in an enhanced excretion of both labeled and unlabeled calcium and phosphorus.

Bone Composition of Hypervitaminotic A and D Rats—Analyses of two representative types of bones (Table VII), *i.e.* tibia and calvaria of the animals described in Table II, showed that the ash content was considerably reduced in the animals fed vitamin D. This loss of mineral was prevented to a large extent by the simultaneous administration of vitamin A. Vitamin A alone had no effect on the ash content of the bone.

TABLE IV

Urinary calcium and phosphorus values at hours after parenteral administration of nuclides

Experiment Q: Each group contained 7 rats. The animals received 30,000 units of vitamin A, 60,000 units of vitamin D, or a combination of 30,000 units of vitamin A and 60,000 units of vitamin D per 100 g of body weight. The animals were maintained on this regimen for 15 days before being killed. ^{45}Ca was administered as calcium acetate intraperitoneally and ^{32}P as Na_2HPO_4 subcutaneously.

Treatment	Body weight		Calcium			Phosphorus		
	Initial	Final	^{45}Ca	^{40}Ca	^{40}Ca	^{32}P	^{31}P	^{31}P
	g		total counts $\times 10^4$	mg	mg/100 g body weight	total counts $\times 10^4$	mg	mg/100 g body weight
Control.....	135	202	5.5	1.3	0.67	8.5	12.5	5.7
Vitamin A.....	135	167*	7.9	1.8	1.10	7.2	8.6*	5.2
Vitamin D.....	135	91*	40.8*	8.9*	9.9*	14.8*	8.9*	9.9*
Vitamins A and D.....	135	101*	36.4*	8.4*	8.5*	13.0*	9.2*	9.2*

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE V

Total excretion values (12 days)

Experiment I: Each group consisted of 6 rats. The daily dosage of vitamin A was 10,000 units/100 g of body weight. Each rat received 100 μC of ^{45}Ca intraperitoneally and 200 μC of ^{32}P subcutaneously 120 days before the collection period. The amount of ^{32}P excreted after 120 days was too little to measure with any degree of accuracy.

Treatment	Body weight		Urine			Feces		
	Initial	Final	^{40}Ca	^{45}Ca	^{31}P	^{40}Ca	^{45}Ca	^{31}P
	g		mg	total counts	mg	mg	total counts	mg
Control.....	223	213	14.4	9,600	77.1	1,400	176,000	722
Vitamin A.....	223	189*	13.3	21,900*	57.3*	950*	354,000*	539*

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE VI

Total excretion values (12 days)

Experiment W: Each group consisted of 8 rats. The daily dosage of vitamin A was 100,000 units/100 g of body weight. During the collection period and for 14 days before the animals were fed a diet low in calcium and phosphorus. Isotopes were administered intravenously 48 days before collection period.

Treatment	Body weight		^{40}Ca		$^{45}\text{Ca}^*$		^{31}P		$^{32}\text{P}^*$	
	Initial	Final	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
	g		mg		total count $\times 10^4$		mg		total count $\times 10^4$	
Control.....	212	213	7.1	21.1	2.0	21.6	210	57.5	12.2	0.7
Vitamin A.....	214	185*	19.3*	43.2*	22.7*	145.0*	205	112.7*	15.2*	1.3*

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE VII

Bone analyses

Same legend as Table II.

Treatment	Dry, defatted weight of tibia	Ash		Hydroxyproline				Hexosamine			
		Calvaria	Tibia	Calvaria		Tibia		Calvaria		Tibia	
				Dry weight	Organic weight	Dry weight	Organic weight	Dry weight	Organic weight	Dry weight	Organic weight
	mg	%		%		%		%		%	
Control.....	212	60.7	59.4	2.93	7.2	2.63	6.48	0.20	0.54	0.47	1.14
Vitamin A.....	191	61.9	59.7	3.03	7.9	2.73	6.76	0.18	0.47	0.41	1.02
Vitamin D.....	146*	49.8*	47.9*	4.04*	7.7	3.71*	7.17*	0.27*	0.50	0.59*	1.13
Vitamins A and D.....	144	55.9*	54.0*	3.20	7.2	3.10	6.76	0.23	0.51	0.48	1.05

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE VIII
Bone analyses

Same legend as Table IV.

Treatment	Dry, defatted weight of femur	Ash		Hydroxyproline				Hexosamine			
		Calvaria	Femur	Calvaria		Femur		Calvaria		Femur	
				Dry weight	Organic weight	Dry weight	Organic weight	Dry weight	Organic weight	Dry weight	Organic weight
		mg	%	%		%		%		%	
Control.....	299	64.0	60.9	2.25	6.20	2.43	6.12	0.137	0.40	0.44	1.13
Vitamin A.....	230*	63.8	62.3	2.20	5.55	2.12	5.82*	0.141	0.39	0.38*	0.99*
Vitamin D.....	212*	50.4*	51.0*	3.1	6.65	3.49*	7.16*	0.241*	0.49*	0.49*	0.97
Vitamins A and D.....	220*	54.1*	54.5*	2.7	5.90	3.19*	6.96*	0.205*	0.43	0.45	0.98*

* Statistically significant ($p < 0.01$) as compared to control values.

TABLE IX
Bone analyses

Same legend as Table VI.

Treatment	Control	Vitamin A
Dry, defatted weight (mg)		
Femori.....	353	328*
Humeri.....	166	131*
Ash (%)		
Calvaria.....	63.9	62.2
Femori.....	61.3	60.0
Humeri.....	63.0	62.0
Hydroxyproline		
Calvaria		
Dry, defatted weight (%).....	2.94	2.84†
Organic weight (%).....	8.2	7.9†
Humeri		
Dry, defatted weight (%).....	2.57	2.31†
Organic weight (%).....	7.03	6.31*
Hexosamine		
Calvaria		
Dry, defatted weight (%).....	0.24	0.24
Organic weight (%).....	0.67	0.62*
Humeri		
Dry, defatted weight (%).....	0.55	0.53
Organic weight (%).....	1.08	0.83*

* Statistically significant ($p < 0.01$) as compared to control values.

† Statistically significant ($p < 0.05$) as compared to control values.

Hydroxyproline values, used as a measure of collagen content, were elevated significantly in hypervitaminotic D animals, when calculated as a percentage of the weight of dry, defatted bone. When calculated as a percentage of the organic fraction bone, the hydroxyproline values were usually higher than control values, although the results were not always statistically significant. Administration of 10,000 units of vitamin A, alone or in combination with vitamin D, had no significant effect on the hydroxyproline content of the bone in this 24-day experiment.

Hexosamine content, although not very specific, was used as an index of mucopolysaccharide content. In this experiment (Table VII), vitamin D caused a relative increase in hexosamine content when calculated as a percentage of the dry, defatted weight of bone, but this increase was not significant when cal-

culated as a percentage of the organic weight. Vitamin A tended to decrease the hexosamine content of bone and to prevent the increase observed with vitamin D alone regardless of the method of calculation.

When the dosages of vitamins A and D were increased 3- and 4-fold, respectively, essentially similar findings were observed (Table VIII). Vitamin D decreased significantly the ash content of both types of bones, while the combination of vitamins A and D prevented, to a large extent this loss of mineral. In this experiment, vitamin D increased the hydroxyproline content of femurs. The hydroxyproline values of the bones of the animals fed the combination of vitamins A and D were less, although not significantly different from those of the animals fed vitamin D alone.

Vitamin D increased the hexosamine content of the calvaria, regardless of the method of calculation, but the increase in hexosamine content of the femur was only relative. Vitamin A decreased significantly the hexosamine content of the femur but not of the calvaria. The combination of vitamin A and D again tended to prevent the increase caused by vitamin D alone.

Administration of 100,000 units of vitamin A for 12 days reduced the weight of the femurs and humeri significantly (Table IX). Despite this marked loss of osseous tissue, the percentage of ash was normal. The hydroxyproline content of the bones was significantly decreased regardless of the method of calculation. The hexosamine content was usually reduced in the animals fed the vitamin.

DISCUSSION

The administration of large amounts of vitamin A to rats causes increased excretion of calcium and phosphorus ((20, 21) and Table VI) and if continued long enough, extensive rarefaction of bone results ((5, 8) and Table IX). Despite these findings, the ash content of the bones appears to be normal ((8, 21) and Table IX). The present studies show that there is a loss of collagen and mucopolysaccharide from bone (Tables VIII and IX). Since the amounts of these substances in bone are small compared to the mineral content, small changes in their values would not be readily detected by gross measurement of ash content.

Tissue culture studies have shown that excessive amounts of vitamin A arrest the growth of bone and cause a disappearance of the matrix leaving behind apparently normal cartilage cells. In addition, resorption of bone occurred simultaneously with

normal soft tissue growth surrounding the explant (22, 23). The chemical data in these studies *in vivo* support the observations *in vitro*. Under the influence of large excesses of vitamin A, there is a decrease in the collagen content (as measured by hydroxyproline) and mucopolysaccharide content (as measured by hexosamine) of the skeleton (Tables VIII and IX).

Recent reports (24-27) have suggested that vitamin A may act at the cellular or subcellular level to liberate a protease which in turn may hydrolyze tissue proteins. Proteolysis of the non-collagenous protein of bone by such a protease may disrupt the collagen fibrils in such a manner as to render them vulnerable to attack by proteolytic enzymes. Another possibility may be the liberation of a collagenase by the stimulus of increased metabolic activity of bone during hypervitaminosis A since this condition results in acceleration of remodeling processes (28) which must involve removal of collagen. Although no collagenase has been isolated from mammalian tissue, recent studies have shown the production and liberation of a collagenase by amphibian tissues undergoing rapid remodeling (29). The loss of collagen from the bones of the rats with hypervitaminosis A (Tables VIII and IX) could be explained by such an action of vitamin A.

The isotopic data (Table V) support the histological observations of Wolbach (28) that excess vitamin A accelerates the remodeling process. In this experiment, the animals had received ^{45}Ca 120 days before the administration of vitamin A. The animals fed vitamin A excreted less unlabeled calcium and phosphate than the controls but excreted twice as much isotope, indicating a more rapid turnover or remodeling of bone that had been formed 3 months previously. Since no balance studies were done in this experiment, it is possible that the decreased excretion of unlabeled calcium and phosphate by the rats fed vitamin A may have resulted from a decreased food intake, since these animals did not gain as much weight as the control rats and may not have eaten as much.

Vitamin D intoxication in rats causes a thinning of bones accompanied by an accelerated bone production in the medullary cavities of the long bones with large quantities of osteoid about the original trabeculae. There also is increased deposition of new matrix (4, 10). These histological observations implied less ash and more organic matter and this was confirmed by the chemical analyses (Tables VII and VIII). The bones of these animals had less ash and more collagen and mucopolysaccharide than those of the normal animals. Whether this increased amount of matrix is the result of increased synthesis or decreased breakdown or both is not known.

The feeding of large amounts of vitamin A with toxic amounts of vitamin D prevents to a large extent the pathological changes of hypervitaminosis D on the skeleton (9-11). Chemical analyses of the bones of the animals treated with the combination of vitamins A and D (Tables VII and VIII) show that the ash, collagen, and mucopolysaccharide content approach the control values and in several instances are significantly different from the values of the bones of the rats with hypervitaminosis D. This improvement in the composition of the bone has also been observed histologically (10). The role of vitamin A may be either to prevent the action of vitamin D in increasing collagen and mucopolysaccharide synthesis or more likely as in hypervitaminosis A, to increase the rate of remodeling (28) to remove the excess mucopolysaccharide and protein (24-27) resulting in a more normal bone composition and structure. It is clear that vitamin A does not exert its beneficial action by decreasing the

enhanced absorption of calcium (Tables I and II) which results from increased vitamin D intake (16-18). In these experiments, neither vitamin A nor vitamin D had any effect on the intestinal absorption of phosphate. Any beneficial effect of vitamin A on the renal handling of calcium and phosphate must also be discounted, since no differences in the elimination of parenterally administered ^{45}Ca and ^{32}P were observed between the vitamin D-treated rats and those treated with the combination of these vitamins.

It is conceivable that substances derived from bone as a result of the increased rate of remodeling seen in hypervitaminosis A may act to prevent soft tissue calcification (9, 10). The increased elimination of phosphorus caused by large amounts of vitamin A may in part tend to prevent renal calcification, since it has been shown that increasing phosphate in the diet tends to prevent kidney calculi (30). It is also possible that the increased breakdown products of mucopolysaccharide and collagen may prevent the precipitation of calcium-phosphate salts in the soft tissues.

SUMMARY

Increased bone mineral turnover has been observed with large amounts of vitamin A. Toxic amounts of vitamin A decrease mucopolysaccharide and collagen content of bone with no demonstrable changes in ash content. Toxic amounts of vitamin D decrease the ash while increasing the absolute amounts of mucopolysaccharide and collagen content. The combination of large amounts of vitamin A with vitamin D partially prevents the pathological changes observed in the skeleton in hypervitaminosis D. It is suggested that the beneficial effect of vitamin A results from increased mucopolysaccharide and collagen turnover. Vitamin A does not prevent the enhanced intestinal absorption of calcium resulting from an excessive intake of vitamin D.

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