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Histological Study of Hypervitaminosis D

2438

The Relative Toxicity of the Vitamin D of Irradiated Ergosterol and Tuna Liver Oil

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PFANNENSTIEL (1) is credited with the observation that irradiated ergosterol is toxic when given in large doses, a finding which has been repeatedly confirmed. Duguid, Duggan and Gough (2) have described the histological picture of hypervitaminosis D as affecting the kidneys, aorta, heart and stomach. Because of the heterogeneous nature and the relatively low Vitamin D potency of fish liver oils, little has been published on the toxicity of the natural, fish oil type, of Vitamin D. Martinelli (3) was unable to produce typical lesions by feeding a total of 64,375 rat units

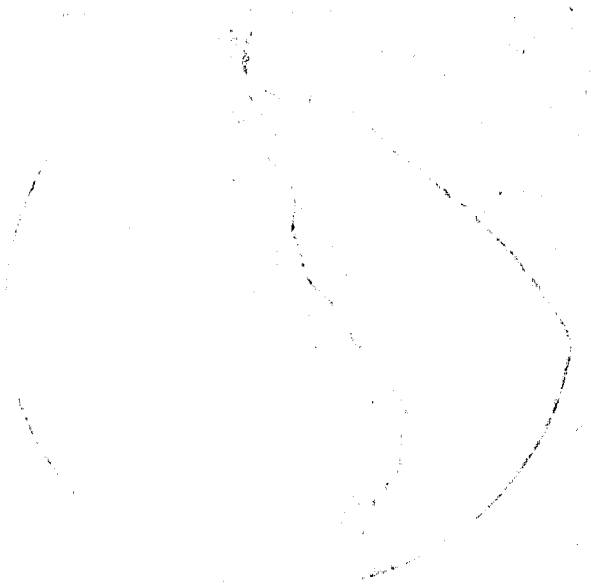

Fig. 1. Intense calcification of the stomach of Rat No. 17010 after receiving 50,000 USP units of Vitamin D as tuna liver oil daily for eighteen days.

(International Units?) of cod liver oil to each rat during fifty days. But we know now that this feeding level (1285 units daily) does not usually produce histologically detectable abnormalities. Harris and Moore (4) interfered with the reproductive capacity of rats by feeding relatively large amounts of cod liver oil, but it is hardly likely that Vitamin D was responsible, for cod liver oil devoid of Vitamin D also exhibited this effect.

In this study an attempt has been made to determine histologically the pathological effects of large quantities of Vitamin D in the form of irradiated ergosterol or tuna liver oil, and to compare the relative toxicity of these two types of Vitamin D.

Month old Wistar strain albino rats were used throughout this study. They were housed separately in galvanized wire cages with raised bottoms. Water was supplied ad libitum. In every instance vitamin supplement was mixed in the diet (Taylor (5)) and accurate records were kept of the intake. Each rat did not consume each day his daily portion of supplement, but by limiting the diet, the total vitamin supplement was assured.

HISTOLOGICAL PROCEDURE

Each animal was dissected on the day of death and the tissues prepared for histological examination. After remaining in 4 per cent formaldehyde (pH 6.8) for twenty-four hours, the tissues were transferred successively to 40 per cent, 60 per cent, 70 per cent, 80 per cent and 95 per cent solutions of ethyl alcohol, to absolute methyl alcohol, to absolute methyl alcohol and ether (1:1), to 7 per cent celloidin, and to 14 per cent celloidin, remaining in each solution for twelve to twenty-four hours. The tissues were then placed in wooden blocks, hardened by immersion in chloroform for thirty minutes and kept in 80 per cent alcohol until sectioned (15-30 microns) by a sliding microtome. The tissues were stained by the Carleton (1926) modification of the von Kossa method. The section was immersed in 1.7 per cent aqueous silver nitrate, irradiated under a strong light for thirty minutes, transferred to distilled water (two to three minutes), counter-stained with safranin (two minutes), transferred to a microscopic slide, washed with 70 per cent ethyl alcohol (ten seconds), washed with 95 per cent ethyl alcohol (until washings were colorless), cleared in oil of origanum, and mounted in dammar.

EXPERIMENTAL

Series 1: One group of five animals (No. 16340-44) was fed an equivalent of 16,000 USPXI units of Vitamin D in the form of tuna liver oil (potency 80,800 USP units per gram) per rat daily for twenty days. A second group of five animals (No. 16345-49) was fed 16,000 USPXI units in the form of irradiated ergosterol (7,273,000 USP units per gram) per rat daily for twenty days. In both cases the supplement was mixed in the diet.

There was no histological evidence of hypervitaminosis. Animals showed normal growth rate and no detectable calcification of the kidney, aortic arch, de-

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2438

Fig. 2. Calcification of the heart, showing calcification of blood vessels of Rat No. 17941 after receiving 80,000 USP units of Vitamin D as irradiated ergosterol daily for seven days.

scending aorta, heart, lung, spleen, liver and adrenal.

Series 2: A group of five rats (No. 17610, 17613, 17614, 17617, 17618, 17619) was fed an equivalent of 50,000 USP units of Vitamin D daily, until death, in the form of tuna liver oil (same oil as in series 1). A second group (No. 17620, 17621, 17623, 17624, 17627, 17628) was fed 50,000 USP units in the form of irradiated ergosterol (solution potency: 1,000,000 USPXI units per gram) daily until death. In both cases the supplement was mixed in the diet.

These animals all showed weight loss and died between the seventeenth and thirty-first day in the supplement feeding period. There was histological evidence of calcification of the kidney, aorta, stomach, lung and heart. The spleen, liver and adrenal body were not abnormal. Each of these organs was definitely more affected by the daily feeding of 50,000 units of irradiated ergosterol than by the feeding of an equivalent amount of tuna liver oil vitamin.

Series 3: A group of five rats (No. 17935-39) was fed 50,000 units of tuna liver oil concentrate (Nopco,* 3,000,000 USP units per gram) daily until death. A second group (No. 17945-49) was fed 50,000 units of irradiated ergosterol daily until death. In both cases the supplement was diluted to a convenient volume with ethyl alcohol and 1 cc. was delivered each day onto the diet.

All animals in this series lost weight and died within about thirteen days. The calcification of the heart, lung, stomach, and aorta was extremely mild in the tuna concentrate group as compared with the ergosterol group. The calcification of the kidney was more severe than that of other organs in both groups, but still the ergosterol-fed group showed heavier deposits.

Series 4: A group of five rats (No. 17930-34) was fed 80,000 USP units of tuna oil concentrate (Nopco,* 3,000,000 USP units per gram daily until death. A second group (No. 17940-44) was fed 80,000 USP units of irradiated ergosterol (1,000,000 USP units per gram) daily until death.

These animals lost more weight and died sooner than the animals of group 2. In both groups of this series there was evidence of calcification of the kidney, stomach and heart. Only the ergosterol-fed group showed calcification of the aorta and lung. In each organ the calcification was more severe in the ergosterol-fed group than in the group fed oil.

SUMMARY

It has been possible to show by this series of tests that the ingestion by the rat of large amounts of Vitamin D in the form of tuna liver oil or tuna liver oil concentrate will cause calcification of certain tissues.

The Vitamin D of irradiated ergosterol is definitely more tissue-calcifying than the Vitamin D of tuna liver oil. The calcification produced by feeding 50,000 units of irradiated ergosterol daily was more severe than that resulting from the feeding of 80,000 units of tuna liver oil concentrate daily.

Similar experiments are now being made, using a chemical, rather than a histological, technique to measure the hypervitaminic effect.

CONCLUSIONS

(1) The oral administration of 50,000 USP units of Vitamin D daily to rats, either in the form of irradiated ergosterol or tuna liver oil, produced histologically detectable tissue changes in the kidney, stomach, heart, aorta and lung.

(2) The Vitamin D of irradiated ergosterol is definitely more toxic than the Vitamin D of tuna liver oil, as measured by tissue pathology.

(3) The site of tissue calcification produced by the two vitamin sources is apparently the same.

(4) The kidney, stomach, aorta, heart and lung are more readily calcified than the liver, spleen and adrenals.

(5) The hypervitaminosis pathologica herein re-

* This concentrate was donated by National Oil Products Co., Harrison, N. J.

Fig. 3. Very intense calcification of the [illegible] No. 17627 after receiving 50,000 USP units [illegible] irradiated ergosterol daily for eighteen days.

period require the daily administration of at least 5,000 times the therapeutic dosage for rats.

(6) There is a difference in the severity of physiological effects in addition to the recognized chemical difference between the "animal" Vitamin D_3 and the "plant" Vitamin D_2 (calciferol).

REFERENCES

1. Pfannenstiel, W.: The Effects of Irradiated Ergosterol in Animal Experiments. *Munch. Med. Wochschr.*, 75:131, 1928.
2. Dugan, J. B., Dugan, M. M. and Gough: The Toxicity of Irradiated Ergosterol. *J. Path. and Bact.*, 41:383, 1930.
3. Martinelli, A.: Experimental Studies in Hypervitaminosis D. *Problema alimentaire*, 4:1, 1934.
4. Harris, L. J. and Moore, T.: Hypervitaminosis and Vitamin Balance. *Biochem. Jour.*, 22:1401, 1928.
5. Russell, W. C.: The Addition of Raw Beef or Meat Scrap to a Wheat-Milk Diet. *J. Nutrition*, 5:317, 1932.

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CHRONIC MODERATE HYPERVITAMINOSIS D IN YOUNG DOGS[1]

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Much experimental work beginning with the observations of Kreitmaier and Moll (1) has demonstrated that excessive amounts of vitamin D produce toxic effects. Some of the questions which have been incompletely answered concern the relative toxicity of the various forms of the vitamin, the protective value of other nutrients, particularly other vitamins, and the reversibility of the tissue changes produced by the hypervitaminosis. In view of the recent introduction of massive dose therapy for the prevention and cure of rickets further light on these problems appears desirable and necessary.

This study was undertaken to obtain new information on these questions and in the hope of unravelling further the normal mode of action of vitamin D through observation of its effects when administered in excess of the usual dosage and along with a diet of the calcium content characteristic of infant diets.

The study is reported in some detail because it included control of several factors not previously controlled in similar experiments. The dogs used were purebred cocker spaniels of uniform origin and produced under exact control of diet and care of their mothers during pregnancy and lactation. They were kept on the same purified diet throughout the experiment which extended over ten months. Since they were kept in separate metabolism cages and never left the laboratory except for occasional exercise on an adjacent concrete runway, they were not exposed to infections or parasites.

The conditions which affect the kind and severity of hypervitaminotic symptoms have been well reviewed by Reed, Struck and Steck (2). The importance of these seems to be in the following order: (a) the amount of excess and the period of administration, (b) the character of the diet, particularly its Ca and P content, (c) the age and size of the subject, (d) the efficiency of the renal apparatus, (e) the species. In addition, some evidence has also been found, (f) that the vitamin A status of the subject may affect the toxicity (3, 4), and (g) that the form of vitamin D used (5) may control the speed of appearance and disappearance of the toxic changes. These latter 2 factors were the chief variables considered in the study here reported.

Experimental procedure. Purebred cocker spaniels, born and reared in the laboratory colony, were used as subjects. When five to eight weeks of age, these dogs were placed on the experimental diet made up of casein 45.8, sucrose 27.8, corn starch 10.4, hydrogenated cottonseed oil 10.0, salts no. 5[2] 2.4, $CaCO_3$ 1.5. As source of the B vitamins, 1 gram dry brewery yeast and 1 gram wheat germ per kgm. per day were given separately. Three litters, 13 dogs in all, were placed on this diet at weaning. Previous experience with this diet supplemented with vitamins A and D, had shown that on it young dogs grew well and that reproduction was successful. The diet contained 0.9 to 1.0 per cent calcium and 0.7 to 0.8 per cent phosphorus and this composition was maintained consistently throughout the experiment. The dogs were kept in separate metabolism cages. In the first three or four months the dogs consumed about 50 grams per kgm. per day of the basal diet, but this amount later decreased to about 20, as the weight curves reached a plateau. The calcium and phosphorus intakes per kilogram per day were therefore approximately 0.48 and 0.37 gram at first, decreasing to 0.19 and 0.15.

This concentration of calcium and phosphorus was chosen since it represented a fair approximation to that of milk solids, the usual diet of the infants treated by single or repeated massive doses of vitamin D.

The vitamin D sources were irradiated ergosterol,[3] halibut liver oil[4], tuna liver oil[5], and dekasterol[6], and the vitamin A sources, the same fish liver oils, shark liver oil[7], and crystalline carotene[8]. Since the halibut liver oil had a high A:D ratio, 13 to 1 or 11 to 1, and the tuna liver oil a low ratio, 0.8 to 1, the former was used chiefly as source of vitamin A and the latter only for the group given excessive amounts of both vitamins. The arrangement of the experimental groups is shown in table 1.

The vitamin doses were made up of the calculated amounts of liver oils, irradiated ergosterol in oil, dekasterol or carotene, diluted with cottonseed oil to an appropriate volume and fed to the dogs by capsule daily. For example, the dose for one group was made up of 1.1 gram halibut liver oil (202 I.U. vitamin A and 61 I.U. vitamin D per mgm.) diluted to 300 grams with cottonseed oil and fed daily in the amount of 1 gram per kgm. to dogs 1 and 5, to provide 800 I.U. vitamin A and 72 I.U. vitamin D. Each of the liver oils, the irradiated ergosterol solution and the dekasterol were examined by biological assay for vitamin D and the liver oils by both biological and the Carr-Price chemical method for vitamin A.

For convenience, the vitamin D of irradiated ergosterol is designated as vita-

[1] This study was supported in part by a grant from the Nutrition Foundation, Inc.

[2] Salts No. 5 had the following composition:

$CaHPO_4$	29.2	Fe citrate	2.2
$CaCO_3$	27.3	K I	0.005
$MgSO_4$	1.9	Na F	0.055
NaCl	22.7	$MnSO_4$	0.021
Citrate	18.7	$Al_2(SO_4)_3$	0.026

[3] The irradiated ergosterol in oil was supplied by Mead, Johnson and Company of Evansville, Indiana. It contained 200,000 I.U. vitamin D per gram, no toxisterols, but presumably some lumisterol.

[4] The halibut liver oil was supplied by E.R. Squibb and Sons of New York and consisted of two lots, one with 202,000 I.U. vitamin A and 1,530 I.U. vitamin D per gram and the other with 1[illegible] I.U. vitamin A and 1,360 I.U. vitamin D per gram.

[5] The tuna liver oil, supplied by Mead, Johnson and Company had 47,000 I.U. vitamin A and 61,000 I.U. vitamin D per gram.

[6] The dekasterol which contained 300,000 I.U. vitamin D per gram was supplied by Dr. James Waddell of E. I. duPont de Nemours and Company, New Brunswick, N. J.

[7] The shark liver oil, supplied by Mr. T. Stanford of F. W. Booth Company, Berkeley, California, contained 130,000 I.U. vitamin A and 85 I.U. vitamin D per gram.

[8] Crystalline carotene, 90 per cent beta and 10 per cent alpha, supplied by General Biochemicals, Inc., Chagrin Falls, Ohio.

25477

min D_2 and that of fish liver oils and irradiated animal sterols (dehydrosterol) as vitamin D_3. There is no certainty, however, that all the vitamin D present in tuna liver oil and halibut liver oil is identical with irradiated 7-dehydrocholesterol, designated vitamin D_3, and presumably the potent substance of dehydrosterol.

TABLE 1

Amounts and sources of vitamins D and A fed young dogs

GROUP	SEX	VITAMIN D PER KGM. PER DAY	VITAMIN A PER KGM. PER DAY	TOTAL PERIOD ON DIET	NUMBER OF VITAMIN D DOSES GIVEN	TOTAL VITAMIN D GIVEN
		I. U.	*I. U.*	*days*		*I.U. × 1000*
1. Optimum vitamin D and vitamin A	1♂	72, tuna liver oil	500, tuna liver oil and halibut liver oil	302		161
	5♂			296		153
	9♀	72, irradiated ergosterol	500, halibut liver oil	290		110
2. Optimum vitamin D and excess vitamin A	2♂	72, halibut liver oil	10,000, halibut liver oil	302		161
	6♂			296		153
	8♀	72, irradiated ergosterol	10,000, carotene in oil	290		110
3. Excess vitamin D and excess vitamin A	11♀	10,000, tuna liver oil	10,000, tuna liver oil and halibut liver oil	350	236	15,500
	3♀	10,000, irradiated ergosterol	10,000, halibut liver oil	312	236	11,400
	7♂			296	236	13,900
	13♂*	10,000, irradiated ergosterol	10,000, shark liver oil	360	117 in 158 days	12,000
	12♂*	10,000, dehydrosterol	10,000, shark liver oil	360	127 in 188 days	9,500
4. Excess vitamin D and optimum vitamin A	4♀	10,000, irradiated ergosterol	500, halibut liver oil	312	236	15,500
	10♀			296	236	12,000

* These dogs were relieved of medication and allowed a recovery period of 140 days.

Serum calcium was determined at intervals, but blood inorganic phosphate was determined only once, just before the animals were sacrificed. The calcium method of Lesser and Greenberg (6) and the phosphorus method of Fiske and Subbarow (7) were used.

At the end of the experiment all dogs of the hypervitaminotic and two of the control dogs were killed and the femurs examined chemically and histologically. The femurs, jaws and teeth were examined by roentgenograph and histologically. Vitamin A was determined chemically in the livers and kidneys and ascorbic acid in the adrenals.

Litter A, consisting of four dogs, nos. 1, 2, 3 and 4, was placed on the diet earlier than the others, at 30 days of age. From the 52nd to the 60th day of age dogs 3 and 4 were given daily 20,000 I. U. per kgm. vitamin D_2 (as irradiated ergosterol) and either 10,000 or 100 I.U. vitamin A per kgm. These animals immediately became ill, refused to eat, had diarrhea, vomited and lost weight. This dosage was obviously dangerously excessive, and since a chronic, rather than an acute hypervitaminosis was sought, the vitamin D was withdrawn. According to Stock, Deutsch, Reed and Shrank (8) this level of vitamin D medication is safe for both adult dogs and human subjects, but on this diet and for these young dogs it was dangerously excessive. After 20 days of recovery during which the dogs improved rapidly, the dosing was resumed but at the level of 10,000 I.U. vitamin D_2 per kgm. per day.

One of the females of this litter, no. 3, was evidently a premature, since she was considerably smaller than the other members of the litter at birth; gained only 1.1 kgm. during the suckling period as compared with the litter average of 1.3 and lost 0.2 kgm. during the first two weeks on the diet. The other female, no. 4, subjected to the same treatment was able to maintain her weight during this period. The symptoms induced by the first doses of vitamin D were more severe in no. 3 than in any of the other animals and her recovery during rest periods was delayed and incomplete.

At this time litter B, consisting of seven dogs, nos. 5 to 11, which had been placed on the diet at 25 days of age, was 65 days old. Three of these animals, nos. 7, 10 and 11, were given 10,000 I.U. vitamin D per kgm. per day along with the two dogs from litter A. After six days of the dosing again there were serious signs of toxicity and after eight days the medication was discontinued. After 41 days' interval the vitamin D was again given at the 10,000 I.U. per kgm. per day level and was continued for 220 days. When this last period was begun the dogs were nearly four months old, and the normal males weighed 9 to 11 kgm., the females 8.0 kgm. The diarrhea disappeared in the experimental dogs, constipation appeared, the appetites were irregular, vomiting occurred occasionally, growth ceased, and in some cases stiff and painful gait developed.

Litter C consisted of two male dogs, nos. 12 and 13, weaned to the diet at 35 days of age. Administration of 10,000 I.U. vitamin D_3 per kgm. per day as dehydrosterol to no. 12 and of vitamin D_2 as irradiated ergosterol to no. 13, was begun when they were 60 days old. Both dogs received 10,000 I.U. vitamin A per kgm. per day in shark liver oil. It was at once apparent that dog 12 was the more severely affected. After 18 days the medication was withdrawn and after 21 days in the case of no. 13 also. Thereafter in 161 to 170 days, dog 12 received the excess dosage on 106 days and dog 13 on 123 days. For 140 days, from the 9th to the 13th month of their age, they were free of the medication. The dog which had received dehydrosterol made a more rapid functional recovery than did the dog which had received irradiated ergosterol. Gain in appetite, weight,

height and activity, and improvement in coat quality were all more striking in dog 12 than in dog 13.

Growth. The hypervitaminotic animals were stunted in both height and weight. Weight curves of litters A and B are shown in figure 1. In both litters the normal animals which received the excess vitamin A were larger at nearly all ages than those which received the smaller amount of vitamin A. This latter allowance, 220 I.U. per kgm. per day, is in excess of the amount usually considered adequate (9). The growth rate of the hypervitaminotic dog 11 given tuna liver oil, was also greater than that of the comparable animal 10 which was given the same amount of vitamin D but the lower amount of vitamin A, but dog 3, similarly comparable as to diet with dog 4, remained somewhat smaller throughout the latter part of the experiment than no. 4.

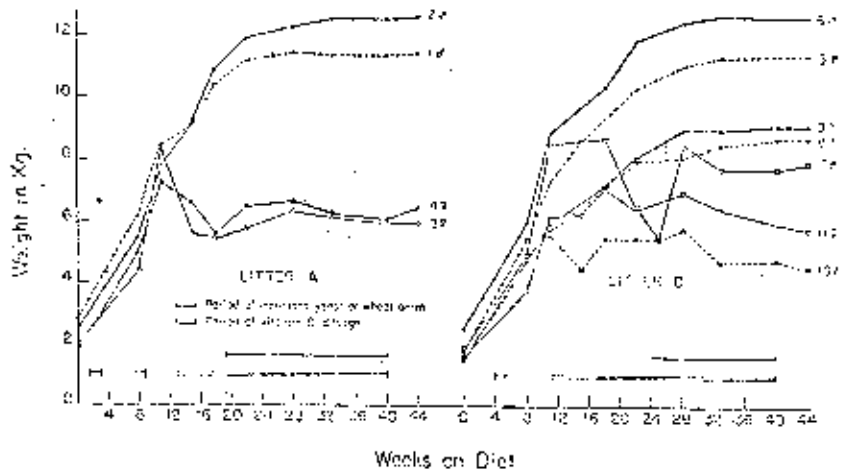

Fig. 1. Growth of young dogs as affected by excess vitamin D and optimum or excess vitamin A intakes. Dogs 1, 2, 5, 6, 8 and 9 were normal. 4 and 10 had excess vitamin D and optimum vitamin A. 3, 7 and 11 excess vitamin D and excess vitamin A.

Serum calcium. As was expected, the serum calcium level was raised in all the dogs given excess vitamin D (fig. 2). The highest levels were reached during periods of temporarily increased food intake and these periods were usually followed by altered loss of appetite, fall in body weight and reduced serum calcium. This occurred notably in the case of no. 7, but also in three other hypervitaminotic dogs of litters A and B when after the 24th week (litter B) or the 40th week (litter A) 4 grams per kgm. per day additional wheat germ or yeast was given them. This was done in the hope of improving the appetites which were poor in all the hypervitaminotic dogs. Wheat germ was given to nos. 7 and 4, yeast to nos. 3 and 10. There was an immediate increase in food intake and body weight in all four dogs. This continued for several weeks after which an abrupt fall in appetite and weight occurred with continued fluctuation for three to four thereafter. Given the calcium and phosphorus intake, except for the small amounts present in the wheat germ and yeast, was dependent

on the amount of food eaten, the rise in serum calcium following increased intake might be taken to be in response to the increased calcium ingested. But the high serum level thus stimulated in turn may have depressed other normal functions with resulting loss of weight, decreased intake and falling serum calcium level.

Under similar circumstances the dogs, nos. 3 and 7, given the excess vitamin D_2 as irradiated ergosterol maintained higher serum calcium levels than did no. 11 given vitamin D_3 in tuna liver oil.

Inorganic phosphorus, determined just before the dogs were sacrificed, was found to vary but little. There was no apparent relation between the P and

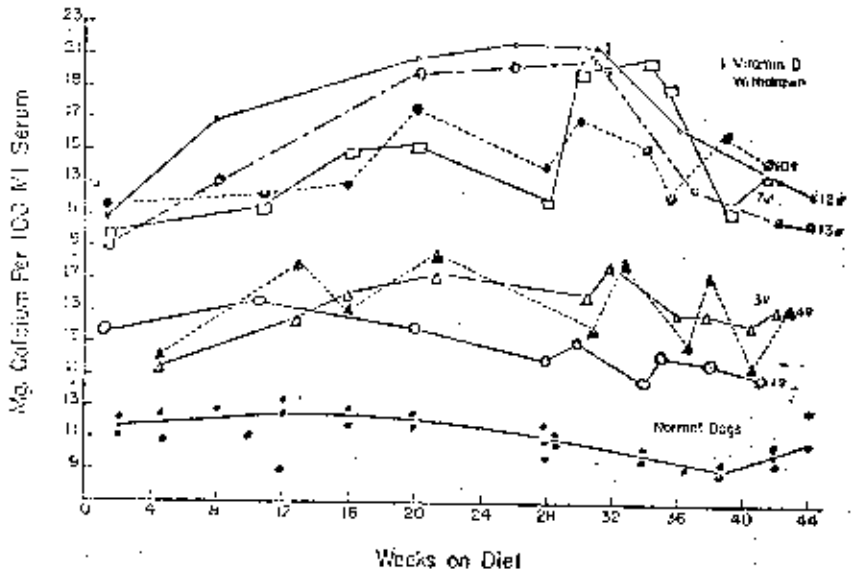

Fig. 2. Serum calcium of young dogs as affected by excess vitamin D and optimum or excess vitamin A intakes. Dogs 4 and 10 had optimum, the others excess, vitamin A intakes.

Ca serum levels. Control dog 6 had 8.7 mgm. per cent Ca and 4.5 mgm. per cent inorganic P, no. 11 had 11.5 and 6.0, no. 3, 15.5 and 5.2, no. 7, 13.9 and 4.1, no. 4, 15.4 and 4.7, no. 10, 13.5 and 4.4.

Condition of tissues. The two dogs given optimum amounts of vitamin D, nos. 1 and 6, at autopsy were found to be normal in all respects. The four controls which were not sacrificed were also in excellent condition and three were maintained in the colony as breeding stock for several years. The other male control, no. 5, which had been given the lower amount of vitamin A developed an infection after a few months and was sacrificed.

All of the hypervitaminotic animals presented abnormally calcified soft tissues in greater or less degrees.

The three dogs most affected by the treatment, nos. 3, 4 and 10, had extensive

and has gland-like deposits in the heart, muscles, stomach wall, lungs and kidneys. Hard white stones were seen on the surface of the cardiac papillary muscles, the chordae tendineae were thick and hard, and bicuspid valves thickened and fibrotic. Even the smaller blood vessels exhibited calcification. No stones were found in the kidneys but the renal tissue was tough and gritty, the cortico-medullary junction indefinite, streaked and mottled, the cortex reduced in width.

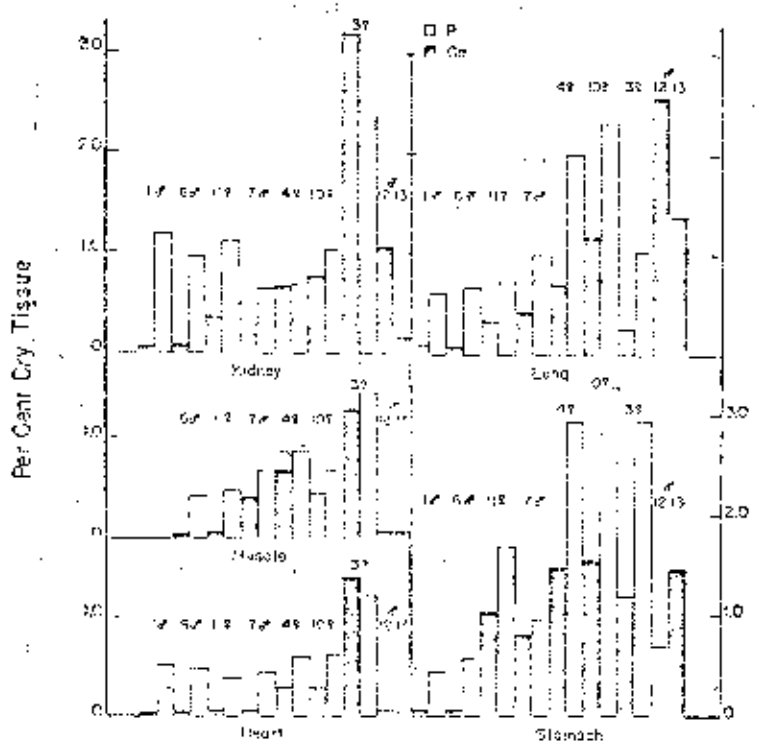

Fig. 3. Calcium and phosphorus content of soft tissues of young dogs as affected by excess vitamin D and optimum or excess vitamin A intakes. Dogs 1 and 6 were normal, 4 and 10 had optimum vitamin A and excess vitamin D, the others excess vitamin A and excess vitamin D.

Granular salt deposits were found adjacent to the bone extending along the tendons, in the muscle sheaths and connective tissue and laid down beside the ribs and between the vertebrae. These deposits looked as though a solution had seeped from the joints and deposited crystals in the adjoining tissues. Yet these bones were over-mineralized and the ash content of the femurs was somewhat greater, 62 per cent of dry extracted bone, than that of the normal dogs, 58 per cent. The long bones were extremely hard with irregular reduced marrow spaces and greatly enlarged within the shafts.

In dogs 7 and 11 calcareous deposits in the soft tissues were rare and the long

bones were more normal than in dogs 3, 4 and 10. Dog 11 which received tuna liver oil was most nearly normal of all.

Jaws and teeth. The normal control dogs 1 and 6 had large well-formed teeth, but the experimental dogs had small, poorly placed teeth with deformities of the roots in some cases, pulp stones and some degree of malocclusion. These faults were most striking in 3, 4 and 10, less obvious in 7 and least in 11. The jaw bones were sclerotic and the cementum pathologically calcified. A full description of the dental conditions has been published (10).

Dogs 12 and 13 in spite of nearly five months' recovery period, had enamel hypoplasia, pulp stones, root deformities, severe osteoporosis, atrophy of alveolar crests, heavy tartar formation and typical paradentosis. The changes produced in jaws and teeth appeared to be irreversible (11).

Kidney function in recovery. Blood urea was determined in dogs 12 and 13 as an index of renal damage. The values were 13.0 and 10.5 mgm. per cent blood before the vitamin D was given and gradually increased during the dosing period to 56 and 34 at the time the vitamin administration was discontinued. At this time no. 12 exhibited severe albuminuria. After a month of recovery no. 12 had lower blood urea than no. 13 and at the end of the five months' recovery the value for the former was 14 and for the latter 15 mgm. per cent. The kidney of no. 12 exhibited degenerate and calcified glomeruli; necrotic debris in both proximal and distal tubules and some cloudy swelling. There were no calcium deposits in the glomeruli in the case of no. 13, but some degenerated glomeruli and casts and debris in some of the tubules. Thus, renal function appeared to be more rapidly regained by no. 12 but anatomical repair was less complete than in no. 13.

Analysis of tissues. Calcium and phosphorus were determined in the soft tissues of all the dogs sacrificed, as shown in figure 3.[9] The calcium content of kidney, lung, heart, stomach and femoral muscle of dogs 3, 4 and 10 was excessive. Kidney, lung and stomach of no. 12 and lung and stomach of no. 13 were likewise highly calcified. The latter two animals which had been allowed to recover from the medication for 146 days had nearly normal heart and muscle calcium. In these cases apparently the decalcification which may have occurred proceeded most rapidly in the muscle tissue and persisted longest in the lungs. Dogs 7 and 11 had decidedly less serious lesions than any of the other hypervitaminotic animals except in the stomachs which were nearly as much calcified as were those of 3, 4 and 10.

The proportion of excess phosphorus to excess calcium was in the neighborhood of 2 to 1 in the kidneys, hearts and femoral muscles of the three severely affected animals but was about 0.5 to 1 in the lungs and stomachs. The phosphorus of the tissues was not determined in the cases of dogs 12 and 13, in which calcium deposited in lungs and stomachs apparently persisted through the recovery period.

If the theory of Hofmeister (12) be accepted, that calcification occurs in alkaline tissues, then most serious and persistent deposits might be expected in

[9] Our thanks are due Nobuko Shimotori for many of these analyses.

gastric mucosa, lymph and gonad tubular cheese acid is neutral or excreted in these areas, leaving the cells relatively alkaline. The production of $CaCO_3$ to $Ca_3(CO_3)_2$ deposited in large and abnormal masses of these dogs must have been greater than in the other tissues, as indicated by Ca:P ratios of these deposits. The deposits in skeletal muscle and heart had ratios close to that of tertiary calcium phosphate, indicating a different mechanism of precipitation.

In the partially recovered animals, nos. 12 and 13, the Ca content of the lung was greater than in any of the acute cases, but that of hearts and muscles was normal, stomachs and kidneys intermediate. The removal of the accretions from areas of lower to higher alkalinity may be indicated.

The changes in calcium and phosphorus of the livers were not significant and were not included in figure 3. The normal dogs had 0.010 and 0.006 Ca per 100 grams liver solids, the recovered animals 0.024 and 0.027, and the 5 hypervitaminotic 0.010 to 0.027. The phosphorus was 0.90 and 0.71 per 100 grams liver solids in the 2 normal dogs and 0.61 to 0.76 in the 5 hypervitamotic animals. Thus, slight increases in calcium and larger decreases in phosphorus were noted in the livers of the affected animals. There were no decreases in phosphorus of any of the other tissues of the severely hypervitaminotic animals as compared with the values found in the normal dogs.

The variable and slight calcification of tissues found by Reed, Dillman, Thacker and Klein (13) in hypervitaminotic dogs is not comparable with the data here presented since they used adult animals of varied origins, varied doses of viosterol and short periods of observation. Since their normal controls were likewise adults of varied history the variability of composition of their tissues was much greater than in the present study. Although the calcium and phosphorus content of the diet used by these investigators was not stated the calcium was noted in one chart as about 70 mgm. per kgm. per day (14). This is well below the approximate 300 mgm. Ca per kgm. received daily by the young dogs in the present study.

Vitamin A deposition. The vitamin A content of the livers and kidneys of 7 of the dogs was determined by the method of Davies (15). As shown in table 2 the dogs which received 800 I.U. vitamin A per kgm. per day had about one-tenth as much of the vitamin in the kidneys and only one-hundredth as much in the livers as did the 4 dogs which had received 10,000 I.U. vitamin A per kgm. per day. The 5 dogs which had received excessive amounts of vitamin D apparently stored about the same amounts of vitamin A as did the 2 normal control animals. The relations in the 2 groups were in fact strikingly consistent. The dogs which received the smaller daily dose of vitamin A stored 3 to 8 per cent of the total intake and those which received the larger intake stored 30 to 44 per cent. This might be taken to indicate that the lower or "optimum" daily dose of 800 I.U. per kgm. was very little in excess of the actual needs of these young dogs.

Adrenal ascorbic acid. The three dogs which received the larger amount of vitamin A had smaller adrenal glands and higher ascorbic acid than did

those which received the smaller dose of vitamin A, regardless of vitamin D intake. Dogs 1, 10 and 4, of the latter group, had adrenals weighing 0.41, 0.55 and 0.61 gram and containing 0.16, 0.27 and 0.30 mgm. ascorbic acid. Dogs 7, 3 and 11, of the former group, had adrenals which weighed 0.20, 0.35 and 0.28 gram and containing 0.35, 0.33 and 0.45 mgm. ascorbic acid. The concentration of ascorbic acid in the glands of the 1 liver group was about four times that of the former. The vitamin A of the adrenals was not determined. The possible antagonism of excess vitamin A or a toxic substance accompanying vitamin A in fish liver oils by ascorbic acid was suggested by Vedder and Rosenberg (16). The amount of excess used by 0 the authors with rats was 200 times greater than was used in the present study of dogs, and the relationship which they suggested is not wholly consistent with the findings on the sea dogs. They found that excess vitamin D (added to decrease the toxicity produced by feeding jewish liver oil in sufficient amount to provide 100,000 I.U. vitamin A per 50 gram rat per day.

TABLE 2

Liver and kidney stores of vitamin A of dogs as affected by intake and by vitamin D medication

Dog	Vitamin D intake	Weight of kidneys	Vitamin A per gm. kidney	Total kidney vitamin	Weight of liver	Vitamin A per gm. liver	Total vitamin A liver	Total vitamin A: Intake	Total vitamin A: Stored	Total vitamin A: Stored
		gms.	I.U.	I.U. × 1000	gms.	I.U.	I.U. × 1000	I.U. × 1000	I.U. × 1000	per cent of intake
1	Normal	57	596	31	256	390	111	1,710	145	8.2
10	Excess	35	275	9	143	150	21	965	30	3.1
4	Excess	19	555	10	391	225	88	1,212	53	4.2
6	Normal	57	2,520	143	213	31,030	7,323	20,030	7,201	36.8
3	Excess	49	3,400	147	210	20,500	6,195	11,000	6,342	41.0
7	Excess	55	5,690	324	197	37,050	7,237	18,500	7,711	40.8
11	Excess	55	2,890	154	135	41,000	5,555	15,500	5,656	36.7

This is similar to the finding, discussed later in this report, of some protective effect of vitamin A excess against vitamin D overdosage effects.

Hemoglobin. When the animals were sacrificed, hemoglobin determinations were made by the Newcomer method (17). Dogs 1, 10 and 4, the low vitamin A group, had 16.0, 16.7 and 16.9 grams hemoglobin per 100 grams blood. Dogs 6, 3, 7 and 11, the high vitamin A group, had 15.8, 15.8, 14.0 and 15.6 grams per 100 grams blood. This difference, about 1 gram, in favor of the former may not be significant. It was not due to dehydration however since the hematocrits were comparable.

The cause of the calcemia. The degree of calcemia did not alone govern the calcification of tissues, the gross symptoms nor the growth of these dogs. Dog 7 which maintained the highest serum calcium in his group throughout the latter half of the experiment had less tissue calcification than nos. 3, 4, and 10 which had lower serum calcium. When no. 7 was induced to increase his food intake by addition of 20 grams of wheat germ daily to the diet, both his weight and serum calcium increased markedly (fig. 1 and 2). When his appetite decreased,

[illegible] rate of growth and bone calcification declined. This was taken to mean that [illegible] food intake was the cause of the rise in serum calcium and that perhaps partly as a result of this rise, eventually the toxic symptoms recurred. Similar but less definite changes were seen in no. 1, after given wheat germ, and in nos. 3 and 10 given dried brewer's yeast. This is in accord with the experience of many investigators, who have noted the effects of excessive vitamin D compensated by increase in calcium and phosphorus intake. Thus, the source of circulating serum and tissue calcium in these dogs may well have been the food rather than the skeleton, as has been suggested by the work of Morgan et al. (15) with vitamin D and parathyroid extract. Taylor and Weed (19) on the contrary believed the extra calcium to be withdrawn from the skeleton when excess vitamin D was given. But the amounts of dietary calcium used by the later investigators were small, varying from 1 to 141 mgm. per dog per day. There was evidence in the present study of recalcification of the long bones, other than of decalcification and the calcium intake was 120 to 420 mgm. per kgm. per day.

Toxicity of the various forms of vitamin D. The moderate degree of toxicity produced by the excess tuna liver oil in dog 11 illustrates the difficulty involved in comparing pharmacological effects of vitamin D in terms of antirachitic units. At low levels, tested on rats, the tuna liver oil had effects which were comparable with those of the irradiated ergosterol, the other fish liver oils and the irradiated animal sterols (dehydrosterol), but at the excess levels were much less pronounced than those of either the dehydrosterol or the irradiated ergosterol.

The dehydrosterol, presumably containing largely 5,6-dihydrocholesterol, held for more rapid and severely toxic effect on dog 12 than did the equal dosage of vitamin D_3, purity at least D_3, administered in tuna liver oil to no. 11. The same difference was noted by Morgan, Shimotori and Hendricks, (17), in rats and was ascribed by them to the more rapid and complete absorption and utilization of the potent principle of dehydrosterol. Dogs 7 and 13 which received irradiated ergosterol were less severely affected than no. 12 but more severely than dog 11. Earlier work with rats (4) had indicated similarly exaggerated effects of the same mixture, in terms of antirachitic activity, of irradiated ergosterol as compared with tuna liver oil. Harris, Ross and Dunker (20) reported similar observations. Jung (21), using crystalline vitamins D_2 and D_3 and carefully irradiated ergosterol, found no difference in their toxicity for rats but greater toxicity was obtained from similar doses of irradiated 7-dehydrocholesterol. Essentially the same conclusions as to the effects of vitamins D_2 and D_3 were reached by McChesney and Meyer (22) in dehydroprovitamin in dogs, and McChesney (23) as to toxicity for rats.

Our experience with these three forms of vitamin D would indicate that for young dogs excess dosage of the vitamin of tuna liver oil was least toxic, irradiated ergosterol intermediate and dehydrosterol (irradiated animal sterols) was most toxic.

The effects of excess vitamin A. There were no adverse effects due to the continuous feeding of 10,000 I.U. vitamin A per kgm. per day in any dog. In all of the three pairs of normal litter mates, nos. 1 and 2, 5 and 6, 8 and 9, the animal which received the excess vitamin A grew to a larger adult size (fig. 1). Of the seven dogs given excess vitamin A, the five which received equal amounts of vitamin D_2 may be compared. Three of these, nos. 3, 7 and 13 received the excess dose of vitamin A, 10,000 I.U. per kgm. per day, and two, nos. 4 and 10 received the lower dose of vitamin A, 400 I.U. per kgm. per day. The two males, nos. 7 and 13, were quite comparable as to growth, appetite, and calcification of tissues, and in all these respects were more nearly normal than the females 4 and 10. The female 3 proved an exception in that her condition was possibly the worst of all, even though she had received the larger amount of vitamin A. As noted previously, this animal was a premature and reacted to the treatment most unfavorably at all times.

The two remaining dogs, nos. 11 and 12, were assigned to receive the same treatment, that is, 10,000 units vitamin D_2 and 10,000 units vitamin A per kgm. per day. It is obvious, however, that their reactions were quite different, dog 11 on tuna liver oil showing little disturbance in growth, blood and tissue composition, and dog 12 on dehydrosterol and shark liver oil, serious symptoms of anorexia, nausea, diarrhea and collapse. Even after 146 days' recovery in the case of the latter, extensive calcification of kidney, lungs and stomach remained. His litermate, no. 13, treated similarly but given vitamin D_3, exhibited far smaller calcified residues in kidneys and lungs but a larger amount in the stomach.

These differences in the toxicity of the forms of vitamin D make it difficult to assess the effects of the excess vitamin A fed these dogs. The comparison must be confined therefore to no. 3 vs. no. 4 and no. 7 vs. no. 10. Dog 13 confirmed the findings in no. 7 but cannot be included in this group because of the recovery period allowed him. Again, since no. 3 was a premature, and apparently unusually susceptible to the vitamin D treatment, her response was unfavorable. Her condition was no better than that of no. 4. Dog 7 was in much better condition at all times than was no. 10. There can be only a tentative conclusion that the excess vitamin A may have had some protective effect against the excess vitamin D.

Comparison with single massive dose treatment of rickets. The total excess dosage given five of these dogs over 253 days was 2,500,000 I.U. vitamin D per kgm. or about four times the amount usually recommended (24) for the single dose treatment or for prophylaxis of infantile rickets. Two of the dogs were given 1,270,000 and 1,170,000 I.U. per kgm. over 360 days, yet the condition of their tissues was not much better than that of the former group. The dosage advocated for infants, 600,000 I.U. or 15 mgm., represents usually 100,000 to 200,000 I.U. per kgm. body weight but is given in one dose, as compared with the 6 to 24 times this amount given these young dogs over 253 days. The cumulative effect of the repeated moderately excessive dosage was on the whole not as severe as was the effect of one massive dose, 500,000 I.U. given another group of similar dogs (25) nor was recovery any more complete after 241 days in the 5 animals of the latter group than in the two allowed 146 days' recovery in the former group.

Some question as to the unusual susceptibility of premature infants to hy-

[illegible] D as well as to rickets may be raised. Zucker (26) treated 46 premature infants [illegible] D injections orally and parenterally and reported that protection from rickets resulted. However only 17 infants were followed for 1 year and only one for more than 6 months, and of them for only 30 days. Medical symptoms of late toxicity were not mentioned in cases of long duration. Certainly the animal judged to be premature in Chicago was very adversely affected by the treatment.

The more early appearance of toxicity of the rachitogenic component of vitamin D used in this investigation compared with that of similar in earlier reports may probably be attributed to the greater calcium content of the diet, the youth of the animals used and the longer period of medication.

SUMMARY

Thirty-two puppies of the mongrel breed in the laboratory colony were placed at weaning on a purified diet containing 1.0 per cent calcium and 0.73 per cent phosphorus. Seven were given 10,000 I.U. vitamin D per kgm. per day as irradiated ergosterol, dehydrosterol or tuna liver oil and six were given 72 I.U. Some of each group had 500 and some had 40,000 I.U. vitamin A per kgm. per day.

The treatment was continued for 8 to 10 months when the animals were sacrificed and [illegible] removed. Two of the dogs after three or four months were allowed to recover for 146 days without further medication.

All dogs which received the excess dosage exhibited some symptoms of toxicity but those which received the tuna liver oil had less, and the one which received dehydrosterol had more, than the others which received irradiated ergosterol. Of the latter group, two of which were given the larger amount of vitamin A had less exhibited soft tissues, better growth and less deformity of jaws and teeth than the two which received the smaller amount of vitamin A, but the fifth animal, apparently a premature, which received the larger dosage of vitamin A, showed the most abnormality of all.

The storage of vitamin A in kidneys and livers corresponded with the intakes, but was not affected by the excess vitamin D treatment. The ascorbic acid content of the adrenal glands but not the weight of these glands was greater in the dogs given the excess vitamin A than in those on the lower vitamin A intake, regardless of excess vitamin D intake.

The two dogs which were relieved of the medication exhibited good functional recovery as to appetite and growth but some signs of damage to teeth and jaws was noted. The animals showed no evidence of gross calcification of the hearts and muscles when sacrificed, but stomachs, lungs, and kidneys retained excessive amounts of calcium.

The growth of the dogs given excess vitamin D was inferior, nearly all the soft tissues were obviously calcified, the long bones were excessively mineralized and the shafts far wider in thickness. The teeth were small, the roots deformed, fractures were common and the gums thickened.

The serum calcium level was raised in all the hypervitaminotic animals, but was variable and apparently independent of the calcium intake.

It was evident that under the conditions of the [illegible] D of tuna liver oil had

less toxic effect than that of irradiated ergosterol, but dehydrosterol (irradiated animal sterol) had greater toxicity than either of the other two forms. Some evidence of potentiation of the hypervitaminosis D by intake of excess vitamin A was found.

REFERENCES

(1) Kaufmann, H. and T. Moll. Münch. Med. Wchnschr. 75: 637, 1928.
(2) Reed, G. I., H. C. Sherman and F. E. Stock. Vitamin D: chemistry, physiology, pharmacology, pathology, experimental and clinical investigations. Univ. of Chicago Press, 1931.
(3) Gross-Lindner, G. Klin. Wchnschr. 14: 61, 1935.
(4) Morgan, A. F., L. Kramer and N. C. Hawkins. J. Biol. Chem. 120: 85, 1937.
(5) Morgan, A. F., N. Shafrroth and J. B. Hendricks. J. Biol. Chem. 154: 761, 1944.
(6) Larson, C. E. and D. M. Greenberg. J. Biol. Chem. 124: 193, 1938.
(7) Fisher, C. H. and V. Sherrow. J. Biol. Chem. 66: 375, 1925.
(8) Stock, I. E., H. Deutsch, C. J. Bunn and H. C. Sherman. Ann. Int. Med. 10: 951, 1937.
(9) Fromming, W. O. Proc. Soc. Exper. Biol. and Med. 33: 280, 1935.
(10) Becks, H. J. Am. Dent. Assoc. 29: 1917, 1942.
(11) Becks, H., D. A. Collins and R. M. Firstag. Am. J. Orthod. and Oral Surg. 32: 463, 1946.
(12) Hornemann, T. Ergebn. Physiol. 10: 429, 1910.
(13) Reed, G. I., L. M. Dillman, E. A. Tracker and R. J. Klein. J. Nutrition 6: 371, 1933.
(14) Reed, G. I., E. A. Tracker, L. M. Dillman and J. W. Welch. J. Nutrition 6: 355, 1933.
(15) Davies, A. W. Biochem. J. 27: 1770, 1933.
(16) Vilborg, E. B. and C. Rosenberg. J. Nutrition 16: 57, 1938.
(17) Wawersik, H. S. J. Biol. Chem. 37: 405, 1919.
(18) Morgan, A. F., E. A. Garrison, F. Gillam and M. J. Hills. This Journal 106: 621, 1932.
(19) Taylor, N. B. and C. B. Wilp. Brit. J. Exper. Path. 13: 109, 1932.
(20) Hanna, R. S., B. D. Ross and J. W. M. Bunker. Am. J. Digest. Dis. 6: 81, 1939.
(21) Jung, A. Schweiz. Med. Wchnschr. 73: 17, 1943.
(22) McCollum, E. V. and F. Meigs. This Journal 135: 577, 1942.
(23) McCollum, E. V. Proc. Soc. Exper. Biol. and Med. 47: 29, 1941.
(24) Wolf, J. J. J. Pediat. 23: 396, 1943.
(25) Morgan, A. F., H. T. Anderson and M. Gaddy. This Journal 149: 333, 1947.
(26) Zucker, C. J. Pediat. 17: 73, 1940.

2547

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October 10, 1973

Dr. Humphrey F. Sassoon
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Dear Dr. Sassoon:

The report in the JAMA is correct. The data reported are accurate.

Sincerely yours,

Robert I. Henkin, M.D., Ph.D.
Chief, Section on Neuroendocrinology
Experimental Therapeutics Branch

RIH/nss

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44

2550

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INTOXICATION WITH VITAMIN D

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INTRODUCTION

DESPITE a wide publicity in the medical literature on the dangers associated with the administration of large doses of preparations of vitamin D, (1–12) there are still many physicians who use these drugs without the necessary precautions which should be observed with substances potentially so toxic. During the past three years there have been recognized by us 11 instances of intoxication by such sterols. Ten of these patients had been given the drugs by practicing physicians who were not aware that the alarming symptoms exhibited were related to the therapy which they themselves were prescribing. The eleventh patient was one in whom we ourselves induced hypercalcemia with calciferol (crystalline vitamin D_2) in an extraordinarily short period of time (fourteen days). Analysis of the observations made on these 11 patients forms the subject of this report.

AGES OF PATIENTS, DOSAGE AND DURATION OF THERAPY PRIOR TO SYMPTOMS

The age of the 10 patients who had been given the drug as a therapeutic measure against arthritis varied from 33 to 68 years. Except for the youngest, all were more than 40 years old. Five of the group were males; five were females. Four had received "Ertron,"[1] two "Deltalin," two "Davitin," one "Dalsol" and one a combination of "Ertron" and "Darthroual."

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* Research Fellow, sponsored by Office of Naval Research.

[1] Ertron—capsules (Gelseal), Nutrition Research Laboratories, Chicago, Illinois.

Davitin—tablets (gelatin sealed), International Vitamin Corporation, New York City.

The highest daily dose received by any patient was 600,000 I.U.; the lowest dose was 150,000 I.U. Duration of therapy prior to onset of toxic symptoms was highly variable, ranging from two months to eighteen months. The patient who received the largest daily dose, 600,000 I.U., became ill earliest; but the patient who received the next highest dose, 500,000 I.U., did not manifest symptoms until he had taken the drug for eighteen months.

PRESENTING SYMPTOMS AND CLINICAL FINDINGS

Eight of the 10 patients had severe gastro-intestinal symptoms, namely, anorexia, nausea and vomiting. Weakness, fatigue and lassitude were prominent complaints in all ten. Weight loss was noted in 8, to a lesser degree in the patients who had lesser gastro-intestinal symptoms. Furthermore, in every instance weight loss and fatigue had been noted prior to anorexia and vomiting, and are, therefore, considered to be symptoms of intoxication with vitamin D per se. In none of the patients was vomiting of sufficient severity to produce alkalosis or to require intravenous therapy.

In 7 of the 10 patients polyuria, polydipsia or nocturia were present, having appeared more or less coincidently with the fatigue and malaise. Intense generalized pruritus was a prominent complaint in two patients.

Despite careful search only one of the patients in this group showed any of the more readily detectable evidences of gross vicarious calcification in periarticular, subcutaneous or renal tissues (4, 8, 9, 12, 13). In this patient they were so unusual as to warrant special mention (*see* following case report). Nine of the 10 patients, however, manifested the characteristic eye lesions described by Walsh and Howard (14). These lesions, seen as yet only in patients with hypercalcemia, are visible by slit-lamp examination, and the deposits beneath the conjunctival basement membranes are thought to be composed of calcium salts.

All ten patients, when first seen by us, were suffering from renal insufficiency (Table 1). Concentration of nonprotein nitrogen in the blood was elevated in all (46–106 mg. per 100 cc.). Concentration of calcium in the serum was elevated in every case, ranging from 12.4 to 15.1 mg. per 100 cc. Total protein of the serum was not abnormal in any case, and the A/G ratio was found normal in the 7 on whom this determination was carried

Dalsol—multivitamin capsules, U. S. Vitamin Corporation, New York City.

Deltalin—capsules (Gelsealed), Eli Lilly Co., Indianapolis, Indiana.

Darthroual—multivitamin capsules, J. B. Roerig Co., Chicago, Illinois.

All these preparations are alleged to contain 50,000 international units of vitamin D per capsule.

2793

TABLE 1

Case	Presenting Features		Serum values after cessation of medication: initial and follow-up								Initial Urinalysis		
			NPN mg./%	Ca mg./%	P mg./%	CO_2 mEq./L.	Cl mEq./L.	Total Prot. Gm./%	Alb. Gm./%	Glob. Gm./%	Alb.	RBC	Casts
Case 1. J.A.C. JHH #[illegible] W. M. [illegible]	Weight loss 20 lbs. in 6 mos. Anorexia, weakness and fatigue. Nausea and vomiting 2 wks. No genito-urinary complaints. No anemia. hgb. 13.1 Gm.	Initial	32	10.9	4.0			6.2	4.2	2.0	2+	0	0
		After 3 wks.	—	11.6	4.0	-	—	—	—	—	2+	0	
Case 2. J.S. JHH #[illegible] W. M. 51	Weight loss 40 lbs. in 6 mos. Anorexia, weakness and severe abdominal pain. Nausea and vomiting 3 wks. Nocturia 2-3X, for 4 mos. Anemia. hgb. 9.8 Gm. [illegible]	Initial	89	13.6	4.6	29.2	103.5	6.7	4.7	2.0	1+	0	Occ. hyal. & gran.
		After 2 wks.	27	11.1	3.1	27.0	100.0	6.5	—	—	1+	0	Occ. hyal. & gran.
		After 2 mos.	33	10.4	3.0	—	—	7.1	—	—	0	0	0
Case 3. W.C. JHH #[illegible] [redacted]	Weight loss 30 lbs. in 3 mos. Anorexia, weakness and fatigue 2-3 mos. Nausea and vomiting 2 mos. Nocturia 2-4X. Severe headache and joint pains 6 wks. Slight anemia. hgb. 12.0 Gm. RBC 4.4, Hct. 36. [illegible]	Initial	73	15.1	5.2	28.7	96.5	6.6	4.6	2.0	1+	0	Occ. hyal. cast
		After 3 wks.	61	12.7	3.6	—	-	7.0	—	—	0	0	
Case 4. A.W. JHH #[illegible] W. M. 59	Weight loss 40 lbs. in 1 yr. Weakness and fatigue. Nausea and vomiting (periodic) 1 yr. or. Hiccup periodic 3 mos. Polyuria, polydipsia, polyphagia and constipation. Anemia 3 mos. hgb. 8.6 Gm. RBC 3.2 Hct. 26 [illegible]	Initial	62	14.3	4.4	29.8	101.6	7.0	4.3	2.7	1+	0	Occ. gran. cast
		After 4 mos.	62	13.8	4.1	—	—	7.4	—	—	1+	0	0
		After 5 mos.	68	13.3	4.2	—	—	7.4	—	—	2+	0	0
		After 10 mos.	46	13.2	2.5	25.4	108.1	6.9	—	—	1+	0	0
		After 16 mos.	50	10.5	3.6	23.8	—	7.9	—	—	0	0	0
Case 5. C.H. JHH #[illegible] W. F. 10	Weight loss 25 lbs. in 6 mos. Weakness and fatigue. Nausea and vomiting 6 mos. No genito-urinary complaints. Anemia. hgb. 10.5 Gm. RBC 4.31, Hct. 34 [illegible]	Initial	45	12.6	3.9	25.4	107.0	7.1	4.5	2.6	0	0	0
		After 1 mos.	40	10.1	2.2	27.6	98.4	7.4	—	—			
Case 6. E.S. JHH #[illegible] W. F. 34	Slight weight loss 4 lbs. Marked indigestion, nausea and vomiting 11 months, weakness and fatigue. Nocturia 1-6X, 1 yr. Anemia. hgb. 9.8 Gm. RBC 3.4, Hct. 27. [illegible]	Initial	67	11.0	3.5	28.7	107.0	7.0	—	—	0	0	Occ. hyal. cast
		After 3 wks.	45	11.0	3.2	-	—	7.0	—	—			
		After 3 mos.	38	10.8	3.2	-	—	7.0	—	—			
		After 6 mos.	39	11.1	3.8	-	—	7.1	—	—			
Case 7. M.D. JHH #[illegible] W. F. 18	Gain weight 3 lbs. No G.I. complaints. Fatigue and weakness. General pruritus during past 12 months. Slight polyuria, left kidney removed 18 mos. previously and was polycythemic. Anemia. hgb. 9.2 Gm. RBC 3.34 (uremic and non-uremic)	Initial	53	13.6	3.8	—	—	6.3	4.0	1.9	Tr	0	0
		After 2 mos.	43	11.5	3.6	—	—	6.3	—	—	Tr	0	0
Case 8. J.H. Patient of Dr. [illegible] W. A. Baetjer W. M. 42	Weight loss 10 lbs. 6 mos. No [illegible] vomiting. Fatigue and weakness. Marked frequency, nocturia, polydipsia, nocturia 2 mos. Anemia. hgb. 11.1 Gm. RBC 4.2, Hct. 31. (uremic hypochromic)	Initial	75	14.0	3.6	-	—	6.5	—	—	0	Occ.	0
		After 10 days	56	13.2	3.6	—	—	6.8	—	-			
		After 4 mos.	55	12.2	2.1	-	—	6.9	—	-			
		After 11 mos.	40	10.7	2.5	--	—	6.6	3.0	2.7			
Case 9. W.K. Patient of Dr. C.H. Avery W. M. 62	No weight loss. Anorexia, fatigue and weakness. Intermittent nausea 2 wks. Nocturia and frequency. Anemia. hgb. 10.2, RBC 3.2 (uremic hypochromic)	Initial	106	12.4	3.0	-	—	--	—	—	Tr	1-3	0 -
		After 3 wks.	37	9.4	4.0	-	—	-	—	—	—	—	—
		After 4 mos.	30	8.9	3.8	-	—	7.6	4.2	3.4	—	—	—
		After 11 mos.	37	10.0	3.0		—	7.2	4.3	2.9	0	Occ.	Occ. gran.
Case 10. D.M. Patient of Dr. [illegible] [illegible]	No weight loss. Nausea and vomiting 2 [illegible] [illegible] 1 month. [illegible] hgb. 10.4 Gm. RBC [illegible] Hct. 30. [illegible]	Initial	51	13.7	5.0			6.2	4.8	1.4	Tr	0	0
		After 1 mos.		14.4	5.0	--	-	7.0		—	—		—
		After 3 mos.	—	15.5	4.6	-		7.0	--	—	Tr	0	0
		After 6 mos.		[illegible]	[illegible]		--	--	—	—	—	—	—

TABLE 1—continued

B.P. mm. Hg	Vit. D preparation and dosage	Length of time vit. D taken before symptoms appeared	Duration of symptoms when diagnosis established	Renal function: initial and follow-up	Metastatic calcification	Length of time for symptoms to subside following withdrawal of vit. D
135/95	150,000 units daily for 4 mos. "Ertron"	3½ mos.	10 days	Fishberg: 1.010 PSP: 28% Urea clearance: 38% & 20% No follow-up tests.	None found.	Improved considerably during hospital stay. No further follow-up.
140/90 In 2 wks. fell to 125/70	300,000 units daily for 8 mos. "Davitin"	4 mos.	2 mos.	Fishberg: 1.012 PSP: 15% After 2 wks.: PSP: 45% After 2 mos.: PSP: 61% Fishberg: 1.011. Urea clearance: 34% & 52%	Cornea & conjunctivae only. Recession of crystals in 1 mo. Sand keratitis less but persisting.	Practically complete return to well-being in 2 wks. Nausea persisted for 2 wks. After 2 mos. all symptoms gone; weight gain, return of alertness & strength. Moderate anemia persists.
158/88 In 3 wks. fell to 120/80	200,000 units daily for 18 mos. "Deltalin"	15 mos.	2 mos.	PSP: 15% Fishberg: 1.010 Urea clearance: 16% & 11% After 3 wks.: PSP: 22% Fishberg: 1.012	Cornea & conjunctivae only. No follow-up examination.	Marked symptomatic improvement during her 3-wk. hospitalization. Vomiting stopped immediately, backache disappeared; weight gain of 3 lbs. No further follow-up obtained.
140/70	500,000 units daily for 18 mos. stopped 4 mos. before admission "Ertron"	18 mos. Calcium deposits noticed in skin at 13 mos.	4 mos.	PSP: 10% Fishberg: 1.018 Urea clearance: 14% & 26% After 3 mos.: PSP: 35% After 10 mos.: PSP: 33% Urea clearance: 40% & 42% After 16 mos.: PSP: 18%	Cornea & conjunctivae, nail beds, lips, skin of hands and face. Recession of calcifications in 18 mos.	Symptoms minimal. Serum calcium normal in 20 mos. Renal insufficiency persists.
142/78	Dosage not accurately determined. Approx. 150,000 units daily for 12 mos. "Ertron" & "Darthronal"	12 mos.	6 mos.	PSP: 80% Fishberg: 1.026 After 4 mos.: PSP: 80%	Conjunctivae only. Recession of conjunctival crystals in 4 mos.	Improvement very definite after first admission. At 4 mos. follow-up there were no complaints.
170/85 In 3 wks. fell to 140/80	400,000 units daily for 12 mos. "Davitin"	10 mos.	6-8 wks.	PSP: 25% Fishberg: 1.014 After 3 wks.: PSP: 28% After 3 mos.: PSP: 55% After 6 mos.: PSP: 67% Fishberg: 1.022	Conjunctivae only. Recession of conjunctival crystals in 6 mos.	Improvement not immediate but by 3 mos. nocturia had lessened; gastro-intestinal symptoms were abated except for slight p.c. discomfort, anemia markedly improved. Exacerbation of arthritis 4 mos. after vit. D stopped.
125/76	600,000 units daily for 2 mos. "Delsol"	6 wks.	2 wks.	PSP: 5% Fishberg: 1.010 After 2 mos.: PSP: 20% Fishberg: 1.012	Cornea & conjunctivae only. No follow-up examination.	General well-being; anemia and pruritis all greatly improved 2 mos. after vit. D was stopped.
120/80	300,000 units daily (irreg.) for 15 mos. "Ertron"	12 mos.	6 mos.	PSP: 9% Fishberg: 1.010 After 2 wks.: PSP: 40% Fishberg: 1.011 After 11 mos.: PSP: 58% Fishberg: 1.018	Cornea & conjunctivae only. No follow-up examination.	In 2 wks. patient had marked return to well-being. Especially notable was return of strength. Arthritis remained quiescent. Anemia persisted for a while but was completely gone at 11 mos.
204/90 In 4 wks. fell to 130/70	300,000 units daily for 5 mos. "Deltalin"	4½ mos.	2 wks.	Fishberg: 1.011 Urea clearance: 21% & 16% After 11 mos.: PSP: 57%	None found.	In 4 days nausea had disappeared. In 1 wk. patient was asymptomatic. Anemia persisted but gradually improved and was gone at 11 mos. There was return of arthralgia and objective joint swelling within 10 days after vit. D stopped.
120/80	200,000 to 400,000 units daily for 4 yrs. "Ertron"	2 yrs. Soreness of eyes noted first	2 yrs.	No initial renal function test. After 2 mos.: PSP: 22% After 7 mos.: PSP: 25% After 9 mos.: PSP: 45%	Cornea & conjunctivae. Conjunctival involvement still present at 5 mos. No further follow-up.	Within 1 mo. dramatic improvement in well-being. Joints bothered patient less. Eyes much better with loss of soreness. Anemia gradually improved in 3 mos.

below normal figures, with restoration of normocalcemia. The patients with the slowest fall in serum calcium were cases 4, 8 and 10; the first mentioned still had hypercalcemia of 12.2 mg. per 100 cc. fourteen months after stopping the vitamin D preparation. At this time serum phosphorus was 2.5 mg. and nonprotein nitrogen was still elevated at 46 mg. per 100 cc. despite improvement in PSP excretion from 13 to 33 per cent in two hours. In case 8 the patient still had, four months after drug withdrawal, hypercalcemia of 12.9 mg. per 100 cc.; serum phosphorus was 2.1 mg.; nonprotein nitrogen had fallen from 75 to 35 mg.; PSP excretion had risen from 24 to only 40 per cent in two hours; and the patient still could not concentrate the urine above a specific gravity of 1.011.

CASE REPORTS

A brief outline of the development of the intoxication and the course of the disease in case 4 seems worthy of mention since it presents certain unusual features:

J.H.H. #194939. The patient was a farmer, born in 1887, who had always enjoyed exceptionally good health. His only injury had been a blow to the left eye in childhood. In 1938 he began to have progressive arthritic symptoms involving the ankles, knees, shoulders, wrists, elbows, fingers and hips, in that order. He was admitted to the Johns Hopkins Hospital in April, 1941 where the diagnosis of subacute rheumatoid arthritis was made by Dr. C. W. Wainwright. There was present, low grade fever, no anemia, WBC 11,000. Extensive studies revealed: no evidence of renal damage; blood pressure 135/80; and a normal concentration of the following substances in the serum—nonprotein nitrogen, albumin, globulin, uric acid, cholesterol, calcium, phosphorus. He was given a course of 12 injections of gold thiomalate, at weekly intervals, without the appearance of any renal irritation. He was symptomatically improved when discharged in July, 1941, weight and blood pressure unchanged. Thereafter he was followed in the Arthritic Clinic at intervals until July, 1944, always with normal urinary findings and without anemia. His weight was 158 pounds.

The patient was not seen again until December, 1946. Beginning in February, 1945, his family physician had prescribed vitamin D, 500,000 international units daily. There was symptomatic improvement in his joint symptoms coincident with this medication, and it was continued regularly until early in September, 1946—a period of eighteen months. In August, 1946 itching lesions appeared on the right arm and soon became general in distribution. In the latter part of this same month there was noticed a thin line of yellowish-white deposits along the outer borders of his lips and similar larger plaques appeared beneath the finger nails. The latter were somewhat tender and caused him to stop taking the vitamin D preparation. Since stopping the medication he had noticed several small whitish painless areas beneath the skin of the thenar eminences. Nocturia, present previously only when he drank large quantities of fluid before retiring, now was consistently present, four or five times per night. The patient thought this had begun in August. Slight nausea and anorexia had been experienced.

Physical examination revealed a 33 pound weight loss (from 158 to 125 pounds) and a blood pressure of 130/70. His arthritic status seemed unchanged. The color was good. The remainder of the general examination was not revealing. A dermatologic note by

FIG. 1. Lip lesions (Case 4).

Dr. L. W. Ketron: "Patient presents two conditions; over the trunk, extremities and face are split-pea sized, hyperkeratotic nodules, some excoriated, that resemble prurigo nodularis. The lesions of the lip are limited to the vermilion border. Here one sees little whitish implantations which are roundish, irregular or linear in shape and without any definite infiltration (Fig. 1). Some of these have a slightly yellowish tint. Nothing is seen

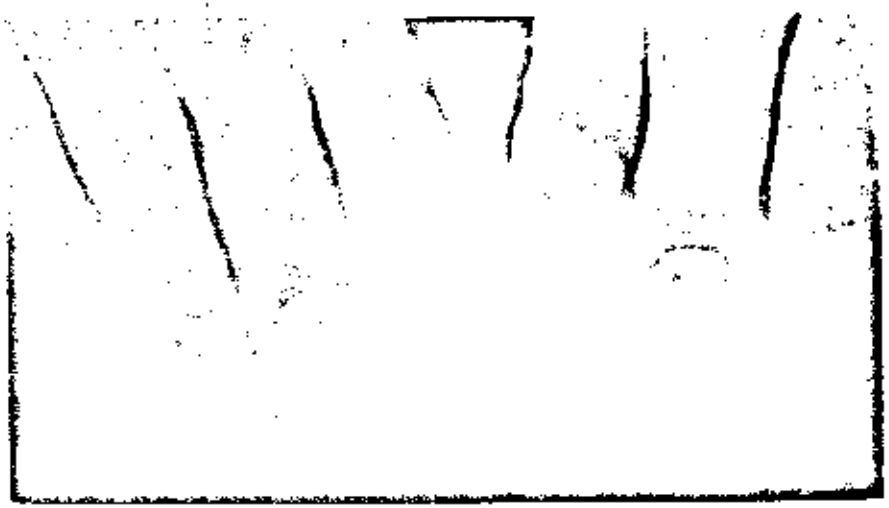

FIG. 2. The yellowish patches in the finger nail beds (Case 4).

27693

on the mucous membranes of the mouth or tongue. There are also yellowish-white patches in the finger nail beds (Fig. 2). The plate is apparently not affected. There are also some whitish striae at the base of the thumbs involving an area about the size of a 5-cent piece. These are not definitely developed or infiltrated. I do not believe the eruption over the body has anything to do with the nail or lip condition. Am not sure what those latter lesions are but think they are most likely some sort of a degenerative phenomenon, although some of the members of the staff feel they are xanthoma. A biopsy will be taken from a lesion on one of the thumbs." *Biopsy report:* considerable senile

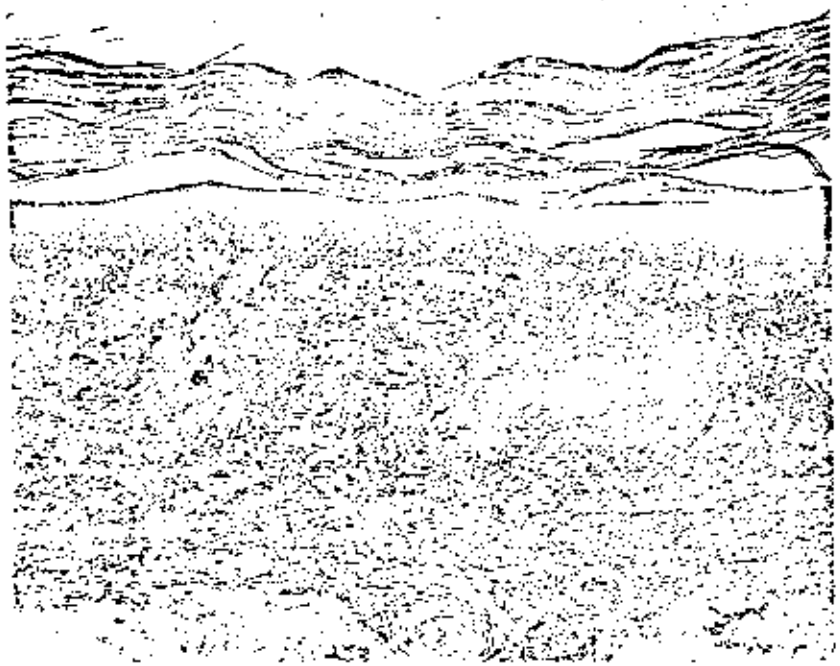

FIG. 3. Biopsy of skin showing senile elastosis with deposits of calcium in the mid-corium (Case 4). ×50 (approx.)

elastosis, with deposits apparently of calcium in a patchy way, in the mid-corium (Fig. 3).

The urine showed a heavy trace of albumin; no abnormal elements in the sediment; and a strongly positive test with Sulkowitch reagent; concentration did not exceed 1.018; PSP excretion was 2 per cent in fifteen minutes and 10 per cent in two hours. Blood non-protein nitrogen was 62, serum calcium 14.3 and phosphorus 4.6 mg. per 100 cc.; serum protein 7.0 Gm. per 100 cc.; and alkaline phosphatase activity, 3.0 Bodansky units. The hemoglobin was 8.6 Gm., RBC 3.2 millions, white blood cell and differential counts were normal, and the sedimentation rate was 16 mm. in one-half hour, corrected. Roentgenograms of the fingers revealed clearly the deposits beneath the nail beds (Fig. 4); but attempts to visualize the deposits in the lips by roentgen ray were unsuccessful. Slit-lamp examination of the eyes by Dr. Frank B. Walsh revealed both the conjunctival crystalline de-

posits and the band keratitis lesions (11). There was a cataract in the left eye, but this was the result of the old injury.

A diagnosis was made of poisoning with vitamin D. He was advised to eat a general diet but to take no milk, cheese or ice cream. Cream and butter were allowed for palata-

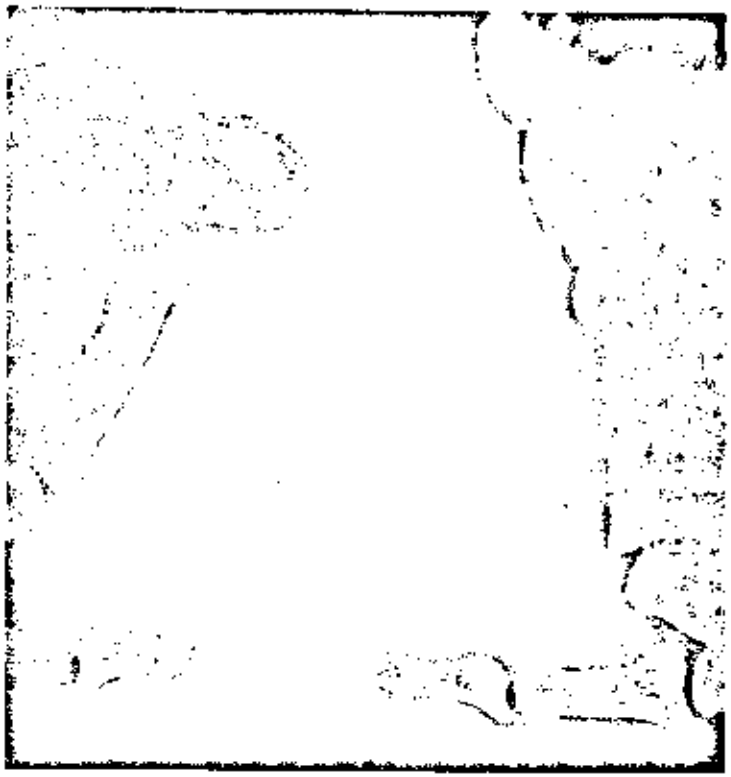

FIG. 4. Roentgenogram of the hands showing the plaques of calcium in the tissues beneath the finger nails (Case 4).

bility. He was urged to force the fluid intake to 4 liters daily. He was followed at intervals, and his general symptomatology changed but little. He was able to do moderate farm work, ate and slept well and had no complaints save for mild joint pains. Laboratory data obtained at these visits appear in the following table:

Date	Wt. lbs.	B.P.	Hgb. Gm.	NPN mg. %	Ca mg. %	P mg. %	Alk. phosphatase (unit)	Prot. Gm. %	Renal function: PSP test 2 hrs.	Urine	
										Albumin	Sulkowitch test
12/ 9/46	126	110/70	8.6	62	14.3	4.6	3.0	7.0	10%	+	+++
1/20/47	120	110/70	11.6	62	13.9	4.1	3.9	7.4	24%	+	++
4/ 1/47	125	110/80	12.5	68	13.3	4.2	5.0	7.4	16%	+	++
6/25/47	125	105/70	13.0	55	13.1	3.7	4.2	7.6	Not done	+	+++
10/28/47	130	120/80	Not done	46	12.2	2.5	Not done	8.9	33%	.	+
4/ 8/48	135	130/80	13.0	60	10.6	2.6	Not done	7.9	18%	0	+

2793

On 1/14/48 the following note was made: "The patient appears just about the same. Skin lesions, though he thinks they are smaller, seem not much changed. It is exceedingly interesting that at last the serum calcium has returned to normal concentration, and coincidently he has had increased arthritic discomfort. He continues to milk his six cows and do gardening."

Dr. Walsh's ophthalmological note: "The cornea is now practically free of any opacity. On the medial aspect of the right cornea is a slight opacity which might pass for a Vogt's white limbus girdle, but even it is very incomplete. The conjunctiva is now free of crystals except for a few small ones that are over the pterygium on the medial aspects of both corneae."

An example of extremely rapid development of intoxication by calciferol is the following:

J.H.H. (366121), a single white woman of 36, had all her life suffered with a complexity of medical difficulties, which in retrospect seemed likely to have all stemmed from unrecognized idiopathic hypoparathyroidism. Because of the main feature of interest here is the rapidity with which she developed hypercalcemia during vitamin D therapy, only the brief summary will be given. In childhood she suffered with convulsions and attacks of laryngeal stridor. At the age of 26 hysterectomy was performed because of menorrhagia. The following year, because of nervousness, tremor, weight loss and an elevated B.M.R., subtotal thyroidectomy was performed. Because this operation neither relieved the symptoms nor lowered the B.M.R., total thyroidectomy was carried out a few months later, still without benefit. Over the ensuing two years mild hypertension developed and bilateral lumbar sympathectomy was performed. In 1941, at the age of 32, the presence of hypocalcemia was recognized at the Massachusetts General Hospital and the patient was given 1.25 mg. of dihydrotachysterol daily, with partial symptomatic relief. Moderate skeletal rarefaction was discovered and a biopsy of bone disclosed the presence of wide osteoid seams along the bony trabeculae (17). The patient was seen here in 1945. Nutrition, blood pressure, blood morphology and urine were normal. On the routine ward diet, with 2 Gm. of calcium chloride after each meal and 1.25 mg. of dihydrotachysterol daily, the patient was free of tetany and the serum calcium, phosphorus and protein were in normal concentrations. An Ellsworth test (18) was performed; sensitivity to parathyroid extract was disclosed by a sharp rise in urinary excretion of phosphorus.

Calcium chloride and dihydrotachysterol were discontinued and 300,000 I.U. of "Drisdol" were given daily, beginning 11/20/45. The course followed by the concentration of calcium and phosphorus during fasting was as follows:

Date 1945	Ca mg. %	P mg. %	Prot. Gm. %	
11/20	10.2	3.6	7.4	"Drisdol" begun
11/23	10.1	3.5	7.4	
11/28	10.9	4.5	7.1	
12/4	12.0	6.2	6.9	"Drisdol" discontinued
12/7	15.8	4.3		
12/11	13.8	4.2	7.5	
12/15	12.8	3.6	7.9	
12/17	12.0	3.3	7.4	

On 12/7/45 serum chloride concentration was found to be 90.4 mEq. per liter and serum CO_2, 33.0 mEq. Unfortunately, determination of these substances had not been carried out previously, and we are not in a position to attribute these suggestive evidences of alkalosis to the calciferol intoxication. There had been no vomiting. Nonprotein nitrogen in the blood and urinalysis remained normal. On 12/18/45 Dr. Walsh again examined the patient and found on examination with the slit lamp "in the conjunctivae of the palpebral fissure areas are definite changes. They appear like tiny cysts and deposits of grumous material near the corneal margins."

It was difficult to evaluate the presence or absence of any symptomatic change during the period of hypercalcemia induced by the calciferol. There was a multiplicity of complaints, many of which were felt to be on neurotic and hysterical bases. The patient insisted on discharge from the hospital on 12/19/45. We have the statement of Dr. Milton Little that when the patient arrived home in New England, the conjunctival deposits described above were plainly visible, but re-examination five months later showed that they had entirely disappeared.

DISCUSSION

Though there are many reports in the medical literature attesting the safety of large doses of sterols of this type, it seems clear that serious damage from their use is a frequent occurrence. One drug firm which advertises its product to the medical profession as "safe," attributes its lesser toxicity to the method of manufacture (Whittier process). Though there is reason to believe that this product is more widely used in the treatment of arthritis than other brands of vitamin D, nevertheless 5 of the 10 patients in this series had been intoxicated with this preparation.

The mechanisms by which these drugs may be injurious is an interesting field for speculation, and a rich one for further study. The outstanding symptoms of intoxication with these compounds are fatigue, weight loss, anorexia and vomiting. Impairment of renal function and degenerative lesions with vicarious calcification are the outstanding clinical signs. Since these symptoms and signs are also commonly seen in hyperparathyroidism (19, 20, 21, 22), one is tempted to place the blame on hypercalcemia, which is common to both conditions. The ophthalmic lesions, demonstrated in 10 of the 11 patients of this group, have been found by us only in patients who have had hypercalcemia (14). It seems reasonable to postulate that the hypercalcemia is responsible for similar depositions of lime salts in the other areas of vicarious calcification, and may be the prime cause of the renal insufficiency.

Intoxication with vitamin D, like hyperparathyroidism, seems to induce a situation wherein more than the normal amount of physiologically active calcium is carried in the serum. The older experiments of Jones and Rapoport (23) indicated that irradiated ergosterol greatly enhanced the intestinal absorption of calcium and phosphorus when these two elements were fed independently to the dog. Warkany showed the same in rabbits (24).

2798

Bauer, Marble and Claflin (25) found that in normal persons doses of 30 mg. of irradiated ergosterol lowered fecal calcium and phosphorus, while urinary calcium and phosphorus were increased. Their findings were interpreted to mean that absorption of calcium and phosphorus had been enhanced by the drug. Hypercalcemia would thus seem more likely to result in patients on high calcium diets than on low, especially if the high calcium intake is not accompanied by a high phosphorus intake.

However, Hess and his coworkers found that large doses of irradiated ergosterol produced hypercalcemia in infants and rats even when there were minimal quantities of calcium in the diet (26), and later found this to be true in dogs fed diets absolutely free of calcium (27). Calcium balance was negative in the dogs so treated, and the ash of the bones of rats receiving large doses of irradiated ergosterol was lower than that of control animals (28). Their conclusion was that "the source of the marked increase in serum calcium, induced by excessive amounts of irradiated ergosterol, is the tissues, more particularly the bones which are the great storehouses of calcium in the body." Whereas it might be said that the sterols used by these earlier workers were crude mixtures containing other sterols which might have been responsible for some of their findings, McLean has shown that pure preparations of vitamin D "in calcemic doses" produce resorption of bone and mobilization of bone mineral (29). A large experience with preparations of pure vitamin D over the past 10 years in patients suffering with hypoparathyroidism has convinced us of the correctness of McLean's view (29) that the fundamental precepts mentioned above hold good in patients treated with pure vitamin D_2 (calciferol). A rise in the total and physiologically active components of the serum calcium follows administration of large doses (100,000 to 400,000 units; 2.5 to 10 mg. daily) of calciferol. This rise may be obtained even though minimal quantities of calcium are presented in the diet, but can be greatly enhanced by administration of calcium salts by mouth (30).

The history in the present group of patients did not indicate that any had received high calcium, low phosphorus diets. One patient had received a quart of milk daily on the physician's advice, and another had been given calcium phosphate wafers coincident with the vitamin D therapy. All our patients were given diets very low in calcium immediately on recognition of their condition; yet hypercalcemia was slow to regress. It seems likely that the bones were the major source of the excess calcium in the serum.

A bone biopsy was carried out in only one of our patients. A small wedge-shaped piece of the ilium was removed, in which no abnormality could be detected by Dr. R. H. Follis. This patient had taken 400,000 units of vitamin D daily for a period of twelve months, and conjunctival deposits were readily detected by slit-lamp examination. A sternal biopsy specimen, obtained from a patient previously reported from this clinic, with acute toxicity resulting within twelve days of the administration of very large doses of vitamin D, likewise revealed normal bone (31).

Despite the frequency of digestive complaints, none of the patients in this series took sodium bicarbonate or other alkali. This may be the reason for the absence of more conspicuous vicarious calcification. Hess observed that injection of sodium bicarbonate into dogs poisoned with irradiated ergosterol lowered the serum calcium and brought about reduction of urinary and fecal calcium, but led to increased precipitation of calcium and phosphorus in the tissues of the body (27).

Reversibility of the damage resulting from vitamin D intoxication is of great interest. Others have observed massive deposits of lime salts to disappear after varying periods following withdrawal of the drug (4, 12).

All observers are agreed that the sooner manifestations of vitamin D intoxication are recognized and the drug withdrawn, the more likely will be complete recovery. However, in only two of the patients in our series have we felt recovery to be complete, and in only one of these was there any evidence of renal damage at the time the intoxication was recognized (case 6). In all the others, for the periods in which they have been followed, there have remained some evidences of renal damage, either inability to form highly concentrated urine or reduction in PSP excretion. But if the visible pathologic changes in the eyes are guides to regressive anatomic changes elsewhere, it may be that time will disclose all damage to have been repaired. This possibility seems unlikely, however, especially if the arteries have become calcific.

Using the criterion of continued hypercalcemia as an indicator of persistence of active drug intoxication, the duration of such activity, after the administration of the drug has ceased, seems to be highly variable. In patient No. 4, a period of twenty months elapsed before serum calcium returned to normal concentration. We were concerned lest some unrecognized concomitant process might be present, such as hyperparathyroidism; but there is no evidence that vitamin D poisoning stimulates parathyroid overfunction, and subsequent events have proved our fears to be groundless. Though the calcific deposits in the conjunctivae of this patient were seen to be diminishing gradually at each visit while the serum calcium remained at abnormal levels, it was only at the last visit in April, 1948, when serum calcium was found normal for the first time, that the conjunctival deposits had disappeared entirely. Thus despite his continued renal insufficiency, there may be still hope of further improvement in renal function.

Just as clinical evidence of improvement after drug withdrawal is highly variable as to time, so, too, the dose and duration of administration of the

2293

drugs prior to intoxication seems to rest on factors of individual susceptibility, as yet unknown (1). Many authors have given to patients doses as high or higher than those given to the patients here reported, and over far longer periods of time, without any apparent intoxication (2). Perhaps variations in intestinal conditions may render absorption better in some patients than in others, or there may be more cellular resistance of unknown nature to the action of the drug in some than in others. It has been stated (31) that the effectiveness of certain preparations of vitamin D are greatly enhanced when it is given in milk. None of our patients received it thus.

On the other hand, the great *rapidity* with which the patient with hypoparathyroidism became intoxicated is of interest. We have used preparations of vitamin D almost exclusively for the past 10 years in the treatment of severe cases of hypoparathyroidism. Usually the dose required to maintain normocalcemia in such patients has ranged between 100,000 and 300,000 international units (2.5 to 7.5 mg. calciferol) per day on normal diets without calcium supplements. It was astonishing, therefore, to meet with a rise in serum calcium to 15 mg. per 100 cc. and the rapid deposition of conjunctival deposits in a period of two weeks, using only 300,000 I.U. (3.75 mg.) of the drug daily.

One further curious and unexplained feature in these patients deserves comment. Seven of the 10 arthritic patients insisted that their joint symptoms were improved during the period of vitamin D administration. It is not known how long hypercalcemia had been present before *symptoms* of intoxication appeared, but usually the patients reported that the discomfort in their joints had decreased within two weeks after beginning to take the drug. Many authors have attested to symptomatic relief of arthritis with vitamin D preparations without the production of hypercalcemia (2). It seems unlikely, therefore, that the symptomatic effect on the joints is due to hypercalcemia. After withdrawal of the drug, several patients complained of a sharp increase in arthritic discomfort and this coincided fairly closely with return of the serum calcium to normal. This was especially conspicuous in patient No. 4, whose serum calcium remained elevated for twenty months after drug withdrawal. Coincident with the eventual return to normocalcemia, his arthritis, symptomatically quiescent since beginning the vitamin D therapy, began to give him increasing trouble.

SUMMARY AND CONCLUSIONS

Eleven cases of intoxication by preparations of vitamin D are reported. The symptomatology and pathologic physiology of this condition are discussed. The wide variation in susceptibility of patients to these drugs is emphasized. Ocular manifestations, subconjunctival deposits and lesions

of "band keratitis" may be visible on slit-lamp examination before symptoms of intoxication or signs of renal irritation appear. It is believed that the hypercalcemia is largely responsible for the symptoms and signs of vitamin D poisoning, but the mechanisms by which the hypercalcemia is induced remain obscure. Patients to whom these drugs are administered should be carefully followed by frequent slit-lamp examination, urinary analyses and determinations of the serum calcium.

REFERENCES

1. Abrams, N. R., and Bauer, W.: The treatment of rheumatoid arthritis with large doses of vitamin D; critical evaluation. *J.A.M.A.* **111**: 1632–1639 (Oct. 29) 1938.
2. Reed, C. I.; Struck, H. C., and Steck, I. E.: Vitamin D; Chemistry, Physiology, Pharmacology, Pathology. Experimental and Clinical Investigations, University of Chicago Press, 1939.
3. Tumulty, P. A., and Howard, J. E.: Irradiated ergosterol poisoning; report of 2 cases, *J.A.M.A.* **119**: 233–236 (May 16) 1942.
4. Danowski, T. S.; Winkler, A. W., and Peters, J. P.: Tissue calcification and renal failure produced by massive dose vitamin D therapy of arthritis, *Ann. Int. Med.* **23**: 22–29 (July) 1945.
5. Freeman, S.; Rhoads, P. S., and Yeager, L. B.: Toxic manifestations associated with prolonged ertron ingestion. *J.A.M.A.* **130**: 197–202 (Jan. 26) 1946.
6. Kingsbury, I. W.: Vitamin D poisoning; hypercalcemia—case report, *Connecticut M. J.* **10**: 25 (Jan.) 1946.
7. Hench, P. S.: Therapeutic "Information Please"; arthritis, *J.A.M.A.* **132**: 974–979 (Dec. 21) 1946.
8. Bauer, J. M., and Freyberg, R. H.: Vitamin D intoxication with metastatic calcification, *J.A.M.A.* **130**: 1208–1215 (April 27) 1946.
9. Kaufman, P.; Beck, R. D., and Wiseman, R. D.: Vitamin D ("Ertron") therapy in arthritis; treatment followed by massive metastatic calcification, renal damage and death, *J.A.M.A.* **134**: 688–690 (June 21) 1947. Correction **134**: 971 (July 12) 1947.
10. Frost, J. W.; Sunderman, F. W., and Leopold, I. S.: Prolonged hyperglycemia and metastatic calcification of the sclera following the use of vitamin D in the treatment of rheumatoid arthritis, *Am. J. M. Sc.* **214**: 639, 1947.
11. Schneider, R. W., and Kammer, H.: Hypervitaminosis D—report of 9 cases, *Cleveland Clin. Quart.* **15**: 82, 1948.
12. McLean, G., and Lebo, L. A.: Multiple calcinosis associated with hypervitaminosis D: report of a case, *South. M. J.* **41**: 389, 1948.
13. Bevans, M., and Taylor, H. K.: Lesions following the use of ertron in rheumatoid arthritis, *Am. J. Path.* **23**: 367–387 (May) 1947.
14. Walsh, F. B., and Howard, J. E.: Conjunctival and corneal lesions in hypercalcemia, *J. Clin. Endocrinol.* **7**: 644–652 (Sept.) 1947.
15. Barney, J. D., and Sulkowitch, H. W.: Progress in the management of urinary calculi, *J. Urol.* **37**: 746–762 (June) 1937.
16. Longcope, W. T., and Winkenwerder, W. L.: Clinical features of contracted kidney due to pyelonephritis, *Bull. Johns Hopkins Hosp.* **53**: 255–287 (Nov.) 1933.
17. Albright, F. Personal communication.

2203

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VITAMIN D INTOXICATION IN A CHILD WITH RHEUMATOID ARTHRITIS

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HARMFUL and lethal effects resulting from the prolonged administration of large doses of vitamin D used in the treatment of arthritis in adults are well established.[1] The administration of high potency vitamin D in the treatment of rheumatoid arthritis in a child resulting in soft tissue calcification and renal insufficiency, to our knowledge, has not been previously reported. These findings in a patient under our observation prompted this case report and discussion.

REPORT OF A CASE

G. G., a 12 year old white girl, was admitted to the Research and Educational Hospitals on May 25, 1949, because of painless abdominal enlargement of one month's duration.

The past history revealed that she had "scarlet fever" at 6 months of age, from which recovery was uneventful. When the child was 17 months of age, the mother noted that she began to limp and was unable to move the right knee through a full range of motion. At 18 months the child had a rash, which again was considered to be "scarlet fever." The right knee continued to be the only joint manifesting symptoms until she reached 4 years of age, when within several weeks pain, swelling and limitation of motion of the joints of the hands and feet developed. Pain and limitation of motion of the spine, particularly in the cervical region, developed. The appetite became poor, and gain in weight was slow. The joint deformities gradually became more pronounced, and weakness and easy fatigability developed. High potency vitamin D capsules[2] were given in doses of 2 to 3 capsules (100,000 to 150,000 U. S. P. units) orally each day from the development of multiple joint symptoms at 4 years of age to the time of her first admission to this hospital, on March 10, 1947. This was a total of five and one-half years of therapy.

Within a few days after the start of therapy, the mother noticed that the child had definitely increased thirst. She asked for a large glass of water at bedtime, a request which she had never made before. Soon she began to awaken once or twice during each night to urinate and to drink more water. Since then she has required about eight large glasses of water daily. Three weeks after vitamin D therapy was begun, she was relieved of much of her pain. Whereas previously she was flat on her back in bed with pain, she was then able to get up and go to the toilet by herself for the first time in months. At this time, anorexia developed, and she vomited several times a week. Appetite for sweet foods was lost. She never liked milk, but she drank about two glasses a day. She failed to gain weight while receiving vitamin D therapy, weighing about 33 pounds (15 Kg.) for five years. Her skin became dry and pruritic. Stupor was not noted except for delirium of two days' duration during an episode of pneumonia in March 1948. Sulfonamides were used sparingly because it was recognized that her kidneys had poor function. Attacks of pain, swelling and stiffness in joints continued to occur after infections of the upper respiratory tract.

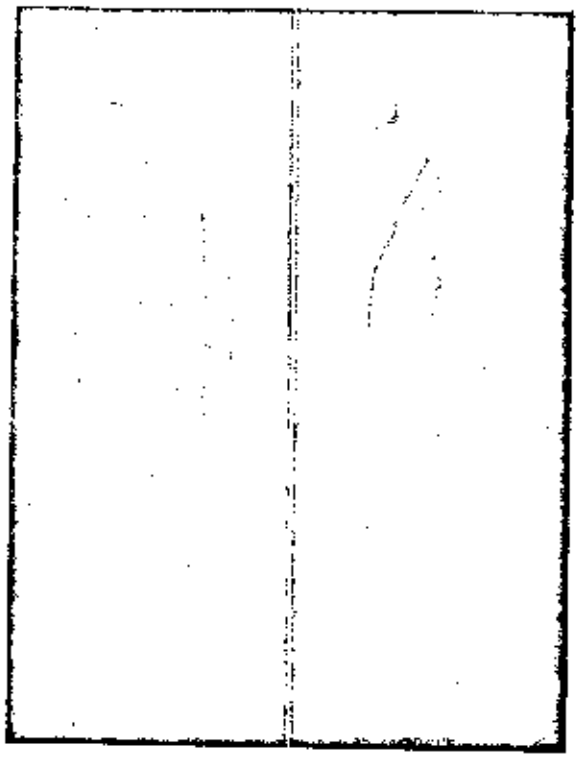

Fig. 1.– The patient at 9½ years of age, weighing 33 pounds (15.0 Kg.), height 45 inches (114.30 cm.). Note the muscular atrophy and multiple joint deformities.

From the Department of Pediatrics, University of Illinois College of Medicine.

1. (a) Donegan, C. K.; Messer, A. L., and Orgain, E. S.: Vitamin D Intoxication Due to Ertron: Report of 2 Cases, Ann. Int. Med. **30**:429-435, 1949. (b) Kaufman, P.; Beck, R. D., and Wiseman, R. D.: Vitamin D ("Ertron") Therapy in Arthritis. Treatment Followed by Massive, Metastatic Calcification, Renal Damage and Death, J. A. M. A. **134**:688-690 (June 21) 1947; correction, ibid. **134**:971 (July 12) 1947.

2. The preparation used was arkalyn,® manufactured by the Paul B. Elder Company, Bryan, Ohio. Active constituent is irradiated ergosterol in oil, biologically standardized according to USP XIII methods.

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The family history revealed that the father died of heart trouble in September 1946. The mother, aged 41, was living and well. There were three brothers, aged 13 years, 7 years and 4 months, all in good health. The patient had been immunized against diphtheria but had not had a vaccination for smallpox.

She was first seen by us when she was admitted to the hospital, March 10, 1947, because of pain, swelling and deformity of numerous joints. Physical examination of the child at that time revealed that she was thin and undersized, with multiple joint deformities (fig. 1). Her weight was 33 pounds, and her height was 45 inches (114.3 cm.); both measurements ranked her below the 3 percentile group for her age (Stuart-Meredith charts). The head tilted to the right but could be moved to the left. The cardiac and pulmonary findings were normal. Blood pressure was 88 diastolic and 70 systolic. The spleen was palpable 5 cm. below the left costal margin and was firm. The liver was not palpable. The extremities were very thin; marked muscular atrophy was observed. There was fusiform swelling of all the

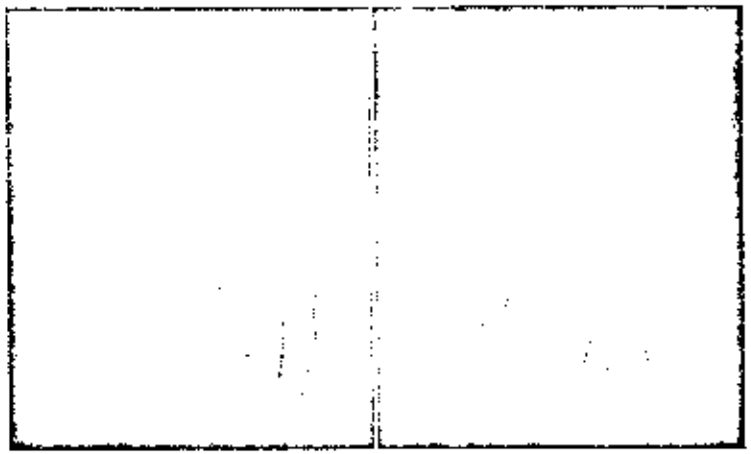

Fig. 2.—Fusiform swelling of the joints of the fingers and swelling of the wrists.

joints of the fingers (fig. 2); moderate swelling about the ankles, knees and wrists, with considerable limitation of motion of these joints, was observed. The patient walked with a shuffling gait consisting of very short steps. All the mandibular movements were possible with only slight restriction of range.

The relevant laboratory data are presented in the table.

The roentgenograms indicated narrowing of the joint spaces of the knees, with marked calcification of the popliteal arteries (fig. 3). The lateral aspects of the femoral and tibial epiphyses were irregular in contour, with some soft tissue calcification about the knee. There was moderate soft tissue swelling about the joints. The cervical portion of the spine demonstrated irregular calcification and contour. Roentgenograms of the hands and wrists revealed distortion of the distal portions of the ulna and radius, moderate destruction and deformity of the carpal bones, thinning and irregularity of the epiphyses of the proximal phalanges and marked loss of calcific density throughout all the bones of the hands and forearms. Laminograms of the temporomandibular joints revealed marked decrease in excur-

sion of the condyles, although the degree of opening appeared adequate.[3] The condylar heads were flattened and irregular, more on the left than on the right. The joint spaces appeared to be narrowed. Plain roentgenograms of the abdomen showed calcification of the aorta and of the iliac and femoral arteries. Intravenous pyelography disclosed marked delay in the appearance of the dye, with a suggestion of early calyectasis on the right. The roentgenograms of the chest showed marked increase in bronchovascular shadow on the right with pleural adhesions medially. The cardiac shadow was normal.

Laboratory Data

	March 1947	May 1948	June 1949
Blood pressure, mm./Hg	88/70	174/124	100/110
Erythrocytes	4,350,000	4,000,000	....
Hemoglobin, Gm./100 cc.	12.5	12.0	....
Leukocytes	7,100	6,000	4,000
Sedimentation rate, mm./hr. (Wintrobe)	30	14	....
Congo red, per cent disappeared from blood	..	20	....
Urine			
Albumin	1+	1+	3+
Microscopic appearance	Normal	Normal	Normal
Sulkowitch reaction	2+	Negative	Negative
Maximum specific gravity	1.018	..	1.016
Phenolsulfonphthalein, per cent excreted in two hours	15	..	25
Urea clearance,* per cent of normal	74	..	56
Inulin clearance,* per cent of normal	..	40	46
Paraaminohippurate clearance,* per cent of normal	..	31	27
Blood			
Nonprotein nitrogen, mg./100 cc.	41	38	87
Urea nitrogen, mg./100 cc.	14.5	19	80
Uric acid, mg./100 cc.	..	..	3.8
Creatinine, mg./100 cc.	..	..	1.6
Carbon dioxide content, mM./liter	..	..	23.7
Serum			
Calcium, mg./100 cc.	10.1	8.7	..
Phosphorus, mg./100 cc.	4.0	4.2	..
Alkaline phosphatase, Bodansky units	4.3	3.5	..
Sodium, mEq./liter	..	..	136
Potassium, mEq./liter	..	..	4.72
Chloride, mEq./liter	..	..	106
Proteins, Gm./100 cc.	8.0	6.9	..
Albumin, Gm./100 cc.	4.9	4.2	..
Globulin, Gm./100 cc.	3.1	2.7	..
Cephalin flocculation, 48 hours	1+	..	Negative
Thymol turbidity, units	..	..	9.5
Response to epinephrine			
Increase in uric acid-creatinine ratio, per cent	..	..	26.5
Reduction in eosinophils, per cent	..	..	56.0
Wassermann reaction	Negative	..	..
Mantoux reaction	Negative (1:10,000)	..	Negative (1:1,000)

* Corrected for a surface area of 1.79 meters.

Her progress in the hospital was marked by a recurrent low grade febrile course. The use of vitamin D was discontinued on admission. Physical therapy was given to increase motion of the joints. Transient swelling of the right temporomandibular joint occurred, and she complained of occasional pains in the heels. Excessive thirst and nocturia were noted. She weighed 35½ pounds (16.1 Kg.) on discharge, July 22, 1947.

3. More detailed description of the involvement of the temporomandibular joint of this patient has been presented elsewhere (Engel, M. B.; Richmond, J. B., and Brodie, A. G.: Mandibular Growth Disturbances in Rheumatoid Arthritis of Childhood, Am. J. Dis. Child. 78:728 [Nov.] 1949, case 5).

2832

After the patient's discharge, there was no recurrence of arthralgia. Stiffness of joints and irritability were present in the early morning. As the day progressed, the "joints would loosen up and she became her normal cheery self." She was unable to extend the arms completely or to stoop over. She continued to walk with a shuffling gait.

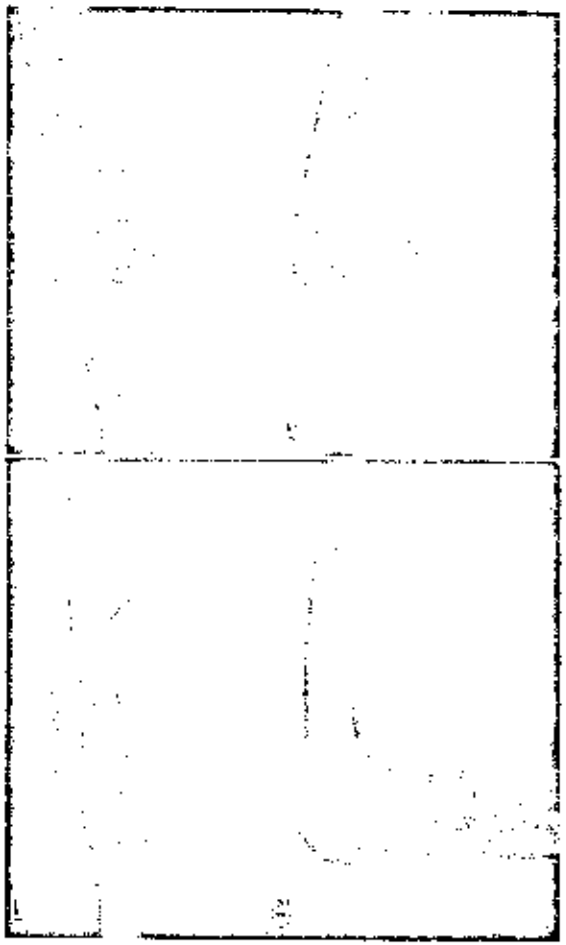

Fig. 3.—Roentgenograms demonstrating the narrowing of the joint spaces of the knees (*A*, right, and *B*, left) with marked vascular calcifications of the popliteal arteries. The lateral aspects of the epiphyses are irregular in contour. Soft tissue calcification is present about the knee.

During November 1947, she had pneumonia, which responded satisfactorily after eight days of penicillin therapy. She was seen at infrequent intervals by the family physician. In April 1949, a check-up examination revealed a 4+ reaction for albumin in the urine. Two weeks later, she complained of a sore throat and had a temperature of 102 F. for four days. During this time the abdomen began to

enlarge. The child's slacks were "let out" to adjust to her increasing abdominal circumference. She gained 3 pounds (1.3 Kg.) in two weeks. She had no edema of the extremities or eyelids.

Her appetite was fair. She did not have vitamin supplements after her return home. Excessive thirst and polyuria continued, and nocturia persisted despite the discontinuance of vitamin D therapy.

Physical examination on May 25, 1949 showed the continued presence of emaciation and multiple skeletal deformities in this 12 year old girl. She was cooperative and cheerful. Her temperature was 99.4 F., weight 46 pounds (20.9 Kg.) and height 52½ inches (133 cm.). Her blood pressure was 168 systolic and 114 diastolic. The skin was dry. There was mild generalized lymphadenopathy. Several carious teeth were noted. The heart and lungs were normal. Slight shifting dulness and a minimal fluid wave were noted in the moderately protuberant abdomen. The liver was palpable 2 cm. below the right costal margin. The spleen was felt 4 cm. below the left costal margin. The genitalia were those of an immature female, with scanty pubic hair.

There was full flexion at the elbows, but extension was limited to 160 degrees bilaterally. Flexion of the fingers was moderately restricted. The phalangeal, metacarpophalangeal and wrist joints presented knobby enlargement bilaterally. The knee joints had complete range of motion. There was bilateral hallux valgus with marked adduction of the great toes. No edema was detected. Reflexes were equal and hyperactive.

Ophthalmologic examination with the slit lamp revealed a whitish ring of opacification along the limbus of the cornea bilaterally. It appeared to lie superficially, mostly in Bowman's zone, and was limited to the interpalpebral area both temporally and nasally. There were numerous small calcareous deposits in the bulbar conjunctivas and also in the interpalpebral zones. Funduscopic study showed the margins of the disks to be mildly blurred and the vessels slightly arched over the margins. The vessels presented variation in the caliber of the arterioles with tortuosity and dipping, arteriovenous compression and disproportion (1:3). Vision was 20/17, bilateral and uncorrected. These findings were interpreted to be grade II hypertensive retinopathy and calcareous deposition in the corneas and conjunctivas, probably representing further evidence of hypervitaminosis D.

Roentgenograms of the cervical portion of the spine showed the vertebral bodies to be abnormally straightened; a roentgenogram of the chest revealed a moderate obstructive emphysema of the left lung for which bronchoscopy could demonstrate no definite cause, and arthritic changes of the shoulder joints were observed. A roentgenogram of the abdomen demonstrated enlargement of the spleen, calcification of the abdominal aorta, renal, iliac and femoral arteries and sclerosis of the acetabular margins with loss of joint space in both hips. Lateral views of the knees in flexion revealed calcified vascular shadows in the popliteal fossae. The bones were demineralized, the joint spaces decreased and the joint surfaces were irregular; views of the hands showed spotty demineralization throughout with loss of joint space and destruction of joint surfaces and bony substances in the region of the joints. A barium swallow revealed no esophageal varices.

The pertinent laboratory data at this time are recorded in the table.

Hospital Course.—The patient continued to be free of pain. Nocturia was especially notable. The abdominal enlargement remained unchanged, but peripheral edema was not observed. On repeated examinations her blood pressure was shown to range between 176 systolic and 114 diastolic and 190 systolic and 130 diastolic.

2832

The venous pressure was 110 mm. of water in the antecubital vein. The child remained afebrile; she was permitted unlimited activity and was discharged June 11, 1949.

Résumé.—This is a report on a 12 year old white girl who has had rheumatoid arthritis since infancy. She received an irradiated ergosterol preparation in large doses for five and one-half years, with the subsequent development of anorexia, vomiting, polydipsia, polyuria, nocturia, calcification of blood vessels, periarticular soft tissue, conjunctivas and corneas, hypertension, hypertensive retinopathy and renal insufficiency. After the discontinuance of vitamin D therapy, some amelioration of her symptoms was noted, but there was no significant improvement in renal function after two years of observation, indicating irreversible kidney damage.

COMMENT

Physiologic Action of Vitamin D.—A survey of the literature[4] reveals that no completely satisfactory explanation of the physiologic action of vitamin D in antirachitic or massive dosages has been formulated. The disorder resulting from the prolonged administration of huge doses of the vitamin has been considered by Tumulty and Howard[5] to be a hypervitaminosis. Some consider it to be poisoning, assuming that vitamin D in massive doses has toxic properties which cause necrosis and subsequent calcification of tissues. Albright and Reifenstein[6] ascribed the manifestations of hypervitaminosis D to an exaggeration of one or both of the known actions of vitamin D: (1) to increase absorption of calcium from the gastrointestinal tract and (2) to increase excretion of phosphorus in the urine. The first of the two properties probably plays the chief role when vitamin D is used in excess. Tissue necrosis according to these authors is not due to a toxic property of vitamin D but is secondary to the precipitation of calcium phosphate in the tissues. Reed[4b] expressed the opinion that tissue injury must occur first, however. The probable sequence of events is increased calcium absorption, increased serum calcium level, decreased parathyroid activity, decreased urinary phosphorus excretion, increased serum phosphorus level, supersaturation of the blood with respect to calcium phosphate and precipitation of the calcium at abnormal sites.

4. (*a*) Reed, C. I.; Struck, H. C., and Steck, I. E.: Vitamin D: Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations, Chicago, University of Chicago Press, 1939. (*b*) Reed, C. I.: Vitamin D and Tissue Calcification, Am. Pract. & Digest Treat. **1**:164, 1950.

5. Tumulty, P. A., and Howard, J. E.: Irradiated Ergosterol Poisoning: Report of 2 Cases, J. A. M. A. **119**:233-236 (May 16) 1942.

6. Albright, F., and Reifenstein, E. C., Jr.: The Parathyroid Glands and Metabolic Bone Disease: Selected Studies, Baltimore, Williams & Wilkins Company, 1948, p. 95.

Freeman, Rhoads and Yeager[7] studied the effect of 100,000 U. S. P. units of vitamin D (ertron*) daily on the calcium balance of a 9 year old boy with rheumatoid arthritis. There was an increased urinary excretion of calcium and decreased calcium retention on both high and low calcium diets. On the high calcium diet, albuminuria developed; this had never been present before, and it cleared up one week after administration of vitamin D was discontinued. The serum calcium was not elevated at the end of the metabolic study. These findings demonstrate that increased urinary calcium excretion and conditions favorable to the deposition of calcium in the kidney may precede, and need not be accompanied with, hypercalcemia. The toxicity of vitamin D can be explained by its effect on calcium and phosphorus metabolism and on renal function.

Clinical and Laboratory Findings.—Debré[8] reported the toxic effects of overdoses of vitamin D in 21 children, some of which were serious, causing death of 2 patients. Some of the children had received large amounts of vitamin D prescribed to accelerate the healing of tuberculosis. Typical symptoms observed were anorexia, nausea and vomiting, excessive thirst associated with polyuria and constipation alternating with diarrhea. Other features noted were headache and pain in the abdomen, back and extremities, associated with stiffness and muscular cramps. If use of the drug was not discontinued, the child soon became pale out of proportion to the anemia. There was loss of weight, the skin and mucous membranes became dry and weakness and irritability developed. At times the temperature was elevated. Finally, stupor and deep breathing occurred and death followed.

The most important group of symptoms is related to disturbances of renal function, notably the appearance of calcium in abnormal amounts and of calcium-containing casts in the urine, albuminuria and finally fixation of the specific gravity at low levels, together with signs of renal insufficiency. An early sign of danger is a persistently positive result of the Sulkowitch test.[9] The unfavorable prognosis of high blood pressure and of a high blood urea nitrogen level has been emphasized by Debré.

There is frequently a low erythrocyte count. The serum calcium is usually high, ranging between 12 and 21 mg. per hundred cubic centimeters. With uremia, the blood urea, sulfate, creatinine and phosphorus levels are elevated and the calcium levels decreased.

7. Freeman, S.; Rhoads, P. S., and Yeager, L. B.: Toxic Manifestations Associated with Prolonged Ertron Ingestion, J. A. M. A. **130**:197-202 (Jan. 26) 1946.

8. Debré, R.: Toxic Effects of Overdosage of Vitamin D_2 in Children, Am. J. Dis. Child. **75**:787-791 (June) 1948.

9. Barney, J. D., and Sulkowitch, H. W.: Progress in the Management of Urinary Calculi, J. Urol. **37**:746-762, 1937.

2883

Roentgenograms may show bony atrophy and destruction from hypervitaminosis D. Calcium deposits may be seen in the kidneys, bronchi and alveoli, gastric mucous membranes, blood vessels and periarticular tissues.

Walsh and Howard [10] and Fleischner and Shalek [11] have described calcium deposits in the superficial layers of the periphery of the cornea, most marked at the palpebral fissures (band keratitis). These may be seen under direct vision without magnification in patients with prolonged hypercalcemia. Biomicroscopy also may reveal crystalline calcium deposits in the deep conjunctivas of the palpebral fissure in patients with chronic hypercalcemia.

Factors Contributing to Toxicity.—The dose of irradiated ergosterol in this case for a 5½ year old child was between 6,000 and 10,000 U. S. P. units per kilogram of body weight per day. Gordon and Sevringhaus [12] have pointed out that toxicity does not depend entirely on the size of the dose. There is a wide range of threshold of toxicity in human beings. The result depends on multiple factors. According to these authors, the factors are (1) the dose per unit of body weight; (2) the duration of administration; (3) the composition of the diet in both mineral and organic matter; (4) the state of the alimentary tract; (5) individual susceptibility; (6) age; (7) preexisting pathologic conditions; (8) the vehicle in which the vitamin is administered; (9) the route of administration; (10) the functional state of the endocrine system; (11) the purity of the vitamin preparation, and (12) the source of the vitamin. Additional factors, yet unknown, are also suggested.

Differential Diagnosis.—Early hypervitaminosis D usually raises the serum calcium level. Donegan, Messer and Orgain [13] pointed out that in hyperparathyroidism with definite changes in the bone the serum alkaline phosphatase is elevated. The absence of this finding in hypervitaminosis D may be a helpful diagnostic point. These authors also indicated that intoxication from the administration of massive doses of vitamin D is more widespread than the reports in the literature would appear to indicate, for it may masquerade as renal disease. They presented 2 cases to demonstrate disturbances in calcium phosphorus metabolism with serious renal damage. The history of massive ingestion of vitamin D is most important in the differential diagnosis.

10. Walsh, F. B., and Howard, J. E.: Conjunctival and Corneal Lesions in Hypercalcemia, J. Clin. Endocrinol. 7:644-652, 1947.

11. Fleischner, F. G., and Shalek, S. R.: Conjunctival and Corneal Calcification in Hypercalcemia: Roentgenologic Findings, New England J. Med. **241**:863-865, 1949.

12. Gordon, E. S., and Sevringhaus, E. L.: Vitamin Therapy in General Practice, Chicago, The Year Book Publishers, Inc., 1940, p. 128.

Pathology of Vitamin D Intoxication.—Metastatic calcification has been defined [14] as a condition in which calcium salts are deposited in tissues previously normal but with a tendency toward an alkaline reaction. This term implies a transference of calcium from the skeleton to the soft tissues.

The net result of excessive doses of vitamin D is retention of calcium and phosphorus, so that the solubility constant of both ions is relatively easily exceeded and precipitation of both occurs in those soft tissues susceptible to metastatic calcification. Susceptible tissue exists where there is a reduction of tissue concentration of hydrogen ions, phosphatase activity and tissue injury due to the initial calcific changes, such as may be produced by the toxic action of irradiated ergosterol. Ham and Portuondo [14] suggested that, although the calcifications of hypervitaminosis D may be explained by the shift in the calcium from the blood to the tissues, it is possible that other features of the lesion, such as necrosis and infiltration by inflammatory cells, may be caused primarily by a disturbance in the calcium balance between the cells and tissue fluids; this balance conceivably is disturbed on the upswing of the serum calcium curve because of the withdrawal of calcium from the tissues. The cause of the calcification may not be identical with the cause of the necrosis and infiltration by inflammatory cells.

The calcium deposition may be found in any of the soft tissues; the blood vessels, kidneys and lungs are especially susceptible to calcification. Bauer and Freyberg [15] observed that the renal calcification seemed to be primarily in arterioles, the tubular and glomerular involvement being secondary. Microscopic study often reveals that the basement membrane of the cells of the distal tubules is necrotic and calcified. Desquamation occurs, giving rise to obstruction of the tubules, which contributes to the picture of uremia.

Abnormal matrix formation accompanying degenerative or inflammatory changes in tissues establishes sites of affinity for calcium in the presence of normal calcium levels in body fluids. This is observed commonly in hematoma and bursitis and may be a contributing factor when metastatic calcification is observed in rheumatoid arthritis.

Toxic effects may result from vitamin D or related sterols. The purest crystalline preparations of vitamin D may give rise to disturbances of calcium metabolism.[8]

13. Mulligan, R. M.: Metastatic Calcification, Arch. Path. **42**:177-230 (Feb.) 1947.

14. Ham, A., and Portuondo, B. C.: Relation of Serum Calcium to Pathologic Calcifications of Hypervitaminosis D, Arch. Path. **16**:1-14 (July) 1933.

15. Bauer, J. M., and Freyberg, R. H.: Vitamin D Intoxication with Metastatic Calcification, J. A. M. A. **130**:1208-1215 (April 27) 1946.

2832

Rosenberg, Baggenstoss and Hench[16] were unable to demonstrate lesions specifically diagnostic of rheumatoid arthritis in the kidneys. In a high percentage of their 30 cases, evidence of glomerular endothelial proliferation was present. Thus we may postulate that unrecognized subclinical glomerulitis may have enhanced the toxic effects of the drug in the patient under consideration. When vitamin D is used in large doses, the study of renal function before and after treatment should be carried out. With early detection of excess vitamin D the toxic effects are reversible if use of the drug is discontinued.[1a]

The symptomatic improvement which is related to the sedative effect of hypercalcemia probably warranted experimentation with massive dosage of vitamin D for rheumatoid arthritis in the past. The present knowledge of the toxic effects produced in this manner and the recent information concerning endocrine factors in rheumatoid arthritis should invalidate massive vitamin D therapy.

SUMMARY

The effect of large doses of vitamin D has been observed in a 12 year old girl with rheumatoid arthritis. Daily doses of 100,000 to 150,000 U. S. P. units of a proprietary irradiated ergosterol product were given for five and one-half years.

Toxic manifestations of renal insufficiency and metastatic calcification of the soft tissues were observed. The salient features of intoxication resulting from the prolonged use of large doses of vitamin D are discussed.

16. Rosenberg, E. F.; Baggenstoss, A. H., and Hench, P. S.: Causes of Death in 30 Cases of Rheumatoid Arthritis, Ann. Int. Med. 20:903-919, 1944.

Special Article

THE HUMAN REQUIREMENT OF VITAMIN D

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AND
GENEVIEVE STEARNS, PH.D.
IOWA CITY

This article and others recently published or to be published comprise a new series on the present status of our knowledge of the vitamins. They have been prepared under the general auspices of the Council on Pharmacy and Chemistry and the Council on Foods. The opinions expressed are those of the authors and not necessarily the opinions of either council. Reprints are not available but the articles will be published later in book form.—Ed.

For many persons some vitamin D in addition to that ordinarily obtained by exposure to sunshine is necessary for the most efficient utilization of calcium and phosphorus. The vitamin acts by increasing the amounts of these substances available for the mineralization of bones and teeth. Measurement of the requirement of vitamin D presupposes the ingestion of a diet adequate in all other respects and particularly containing ample amounts of calcium and phosphorus; it should include also recognition that the human requirement of vitamin D (or of any other nutritional factor) includes both the quantity necessary to prevent obvious clinical pathologic changes and an amount which promotes a condition of normal nutrition and health.

The requirements of vitamin D may be defined as those amounts which, with ample intakes of calcium and phosphorus and a diet otherwise adequate, insure sufficient retention of calcium and phosphorus to permit (*a*) normal growth and mineralization of the skeleton and teeth of infants and children, (*b*) maintenance of bony and dental structures during adult life and (*c*) a sufficient supply for mother and infant during pregnancy and lactation.

Unfortunately no one yet knows the normal rate of growth of children.[1] Instead, there are available only many average rates of growth under widely varying conditions of nutrition. It is known from studies of racial food habits that when the diet of a people is nutritionally good and includes a plethora of milk and ingested vitamin D or its equivalent in sunlight the people of that race are tall and well formed, with well calcified skeletons and freedom from dental caries.[2] On the other hand, peoples whose mineral or vitamin D intake is very low are small of stature and prone to be subject to rickets or osteomalacia.[3] Even in this country, children from better class homes are taller and heavier than children of poor families,[4] and children from better class homes of today are taller than those of the same class a generation or so ago.[5] One may postulate, therefore, that "average" rates of growth of today still represent suboptimal rather than optimal skeletal growth, so that, if linear growth is used as a criterion of adequacy of vitamin D intake, the rate should be at least "average."

During childhood and adolescence, not only does the skeleton grow in size but a part of its water content is replaced by inorganic salts. The rate of replacement is not known with certainty but is most rapid in early life. Because of the variability of standards of growth and mineralization of the skeleton, the optimal amounts of calcium and phosphorus to be retained at any age are not known. Various methods of calculating these requirements have appeared in the literature from time to time.[6] The required retentions estimated for calcium by the different investigators vary from 0.1 Gm. daily throughout childhood[6a] to a graded scale increasing from 0.3 Gm. daily at 1 year to 0.5 Gm. daily during adolescence.[6b] As improvement in nutrition has so far resulted in increased average stature, and as mineralization of the skeleton may lag far behind growth,[6] the higher figures are thought to be a safer guide.

The requirements of normal persons for only the ingested forms of vitamin D will be considered in the present discussion. The criteria commonly used are freedom from rickets or osteoporosis, good growth and development, normal values for serum calcium and phosphorus, ample retentions of these elements for growing individuals and "equilibrium" for those fully grown.

REQUIREMENT DURING INFANCY

Infants Fed Cow's Milk.—Studies of the vitamin D requirement during infancy may be divided into two groups. The larger group comprises studies planned to determine a minimal requirement using the prevention of rickets as the sole or chief criterion. Some of these include also the levels of serum calcium, phosphorus and phosphatase, and a few have taken into consideration the rates of growth. The smaller group comprises studies which include in addition to the preceding criteria quantitative determinations of the retention of calcium and phosphorus correlated with the rate of growth and the relative intakes of calcium and phosphorus. The emphasis in this group has been not on rickets prevention but on growth and development and on the fully adequate rather than the minimum requirement for vitamin D.

All these studies include periodic roentgen examination of the radius or tibia; in the first group of studies this constitutes the chief basis of judgment. Little agreement has been achieved in the interpretation of minor aberrations of growth at the epiphyseal line,[7] but much greater uniformity in the determination of rickets of "clinical significance." Use of only the latter criterion is necessary if comparisons are to be made between the various studies. Rickets prevention alone probably should not be considered as a criterion of an adequate vitamin D supply. It is at least reasonable to think that the optimum amount of any nutri-

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1. From the Department of Pediatrics, State University of Iowa College of Medicine.

2. Kellerman, R.: Problems of Nutrition in India, Nutrition Abstr. & Rev. 2:1 (July) 1932. Orr, J. B., and Gilks, J. L.: Physique and Health of Two African Tribes, Medical Research Council, Special Report Series, No. 155, London, His Majesty's Stationery Office, 1931. Bay, E.: Die Diät und Kreislaufbeing der medizinischen Lappen in Nordschweden, Acta med. Scandinav. 82:543 (Dec. V-VI) 1934.

3. Maxwell, J. P.: Osteomalacia and Diet, Nutrition Abstr. & Rev. 4:1 (July) 1934. [illegible]

4. Gilbert, H. T.: Physical Growth of Children from Birth to [illegible] Years, University of Iowa Studies, Studies in Child Welfare 1, No. 1, [illegible] Gray, Horace, and Fredericks, Frederick: Growth Standards: Heights and Weight for Private School Boys, Am. J. Dis. Child. 32:554 (Oct.) 1926.

5. Gray, Horace: Increase in Stature of American Boys in the Last [illegible] Years, J. A. M. A. 88:908 (March 19) 1927.

6. (a) White House Conference Report: Growth and Development of the Child: III. Nutrition, New York, the Century Company, [illegible] p. 130. (b) Sherman, H. C., and Hawley, E.: Calcium and Phosphorus Metabolism in Children, J. Biol. Chem. 32:275 (Aug.) 1932. [illegible] Leitch, I.: The Determination of the Calcium Requirements of Man, Nutrition Abstr. & Rev. 6:153 (Jan.) 1937.

6. (a) Todd, T. Wig: Personal communication to the author. (b) Sherman, H. C., and Chapin, H. L.: Growth and Reproduction on Simplified Food Supply: IV. Improvement in Nutrition Brought about by an Increase in Proportion of Milk in the Diet, J. Biol. Chem. 64:[illegible] (Mar.) 1925.

7. (a) Jeans, P. C.: Vitamin D Milk, J. A. M. A. 104:2016 (June 13), 2159 (June 20) 1935. (b) Cooley, T. B., and [illegible] Lawrence: The Interpretation of X-Ray Films in the Diagnosis of Rickets, J. Pediat. 10:743 (June) 1937.

tional essential is somewhat greater than the amount which barely prevents clinical pathologic changes.

The determination of calcium and inorganic phosphorus of blood serum has a definite but limited usefulness in estimating the vitamin D requirement. In general, rickets cannot develop concurrently with a continuously normal level. The amounts of vitamin D that barely prevent rickets produce serum values within the range considered normal. However, values of this magnitude give little indication as to whether the calcium balance is weakly or strongly positive; it may even be negative. Perhaps one should distinguish between low and high normal levels. When the amount of vitamin D is considered fully adequate, the serum calcium and phosphorus levels then would be in the upper part of the normal range, whereas with amounts of vitamin D considered inadequate the serum values would be at the lower limits of normal.

The rate of growth is an important factor in determining the vitamin D requirement. Since rickets is a condition the severity of which tends to increase with increasing rates of growth, it is more important to prove that rickets has been prevented in a rapidly growing than in a slowly growing infant. Evidence is available also that the rate of growth is increased above the average when the intake of vitamin D is fully adequate.[8]

The most useful criterion of the vitamin D requirements is provided by studies of the utilization of calcium and phosphorus. Observations on the retention of these minerals permit a direct evaluation of the efficiency of utilization at different levels of vitamin D intake instead of the indirect estimates which have been discussed. This direct evaluation is the one procedure available which permits the estimation of an appropriate rather than a minimal requirement for vitamin D. Rickets prevention can be a criterion only in infancy, whereas the retained quantity of calcium and phosphorus offers a criterion for all age periods. Ample rather than minimal retention of calcium and phosphorus is required throughout the period of growth to supply material for new bone and for the replacement of water in bone already formed. Ample retention of these elements is desirable also to aid in compensating for the inevitable mineral losses in association with illness.

The consensus of various studies seems to be that the quantity of vitamin D received by a baby fed customary quantities of milk containing 135 units[9] to the quart is sufficient to prevent the development of rickets in the majority of full term infants but insufficient to prevent moderate or severe rickets in prematurely born infants; that from 300 to 400 units daily as cod liver oil or as milk containing 400 units to the quart allows sufficient vitamin D to prevent rickets of clinical significance in both full term and prematurely born infants.[10]

Early studies reported different normal standards for the serum calcium and phosphorus levels of breast-fed and artificially fed babies. Later studies showed that artificially fed infants given adequate vitamin D had serum values equaling those of infants given human milk.[11] When infants are fed milk containing 135 units of vitamin D to the quart, the serum calcium and phosphorus values are normal, but higher dosages of vitamin D (from 300 to 400 units) permit levels nearer the top of the normal range.[12]

It has been observed that infants given milk containing 135 units to the quart tend to grow at average rates, whereas infants given from 300 to 400 units of vitamin D tend to grow somewhat faster than the average.[8a] This observation has been corroborated recently.[8b]

Studies of calcium and phosphorus retention[13] have shown that infants given no additional vitamin D show marked variability in calcium retention with a low average. When from 60 to 135 units of vitamin D is given daily, the number of low retentions decreases, raising the average retention of the group. A further and significantly greater increase in average retention is obtained (by raising the lower limit of the retention range) when from 300 to 400 units of vitamin D is fed daily. Thus, increasing the vitamin D content of the diet from 0 to 300 or 400 units decreases variability of the infant in regard to ability to retain calcium and tends to raise all infants to the level of those most efficient in calcium retention. These studies show also that the calcium retentions observed when from 300 to 400 units of vitamin D is fed daily are accompanied by excellent dentition and the maintenance of serum calcium and phosphorus at high normal levels as well as by increased growth and development. The average retention observed when from 300 to 400 units of vitamin D is given apparently is ample to provide abundantly the nutritional needs for these minerals even at the somewhat increased rate of skeletal growth observed.

Data are not available to show whether an intake of vitamin D between 135 and 300 units daily would be equally effective in permitting ample retention of calcium and phosphorus. Whatever the minimal intake of vitamin D for good retention of these minerals that level seems to be greater than 135 units.

A few observations indicate that amounts of vitamin D which are considerably in excess of 400 units daily may be detrimental when growth and retentions are used as criteria. In an inpatient study, five infants given 1,500 or more units daily have shown decreased appetite accompanied by retardation of growth after beyond 6 months of age. Decreasing the vitamin D intake of two of these infants has resulted in a greater food intake and an increased rate of growth.[14] Further study will be necessary before the significance of these observations can be evaluated.

Present knowledge of the relationship of vitamin D to growth helps to explain the occasional dis-

8. (a) Stearns, Genevieve, Jeans, P. C., and Vandecar, Nevin: The Effect of Vitamin D on Linear Growth in Infancy, J. Pediat. 9:1 (July) 1936. (b) Shyse, J. J.; Hand, B. M.; Fisk, M. W.; Cooley, T. B., and Mary, Jee G.: Relationship Between Vitamin D Intake and Linear Growth in Infants, Proc. Soc. Exper. Biol. & Med. 37:499 (Dec.) 1937.

9. All units mentioned in this review refer to U. S. P. XI units of vitamin D.

10. (a) Rapoport, Milton, and Steiger, Joseph, Jr.: II. The Antirachitic Value of Irradiated Evaporated Milk and Irradiated Whole Dried Milk in Infants, J. Pediat. 8:153 (Feb.) 1936. (b) Eliot, Martha M.; Nelson, E. M.; Barnes, D. J.; Browne, Florence A., and Jones, Rachel M.: A Study of the Comparative Value of Cod Liver Oil, Viosterol and Vitamin D Milks in the Prevention of Rickets and of Certain Basic Factors Influencing Their Efficacy, ibid. 8:355 (Sept.) 1936. (c) Dodds, T. G. H.; Tisdall, F. F., and Brown, Alan: Irradiated Evaporated Milk in the Prevention of Rickets, ibid. 8:163 (Feb.) 1936; A Comparison of the Antirachitic Effect of Irradiated Cholesterol and Cod Liver Oil, ibid. 10:421 (Oct.) 1936. (d) Davidson, L. T.; Merrill, Katharine K., and Chipman, S. S.: Prophylaxis of Rickets in Infants with Irradiated Evaporated Milk, Am. J. Dis. Child. 53:1 (Jan., pt. 1) 1937; (e) Prophylaxis of Rickets in Infants with Vitamin D Milk, ibid. 51:1 (Jan.) 1936. Jeans[8a]

11. Stearns, Genevieve, and Waters, Edna: Studies of the Blood: I. The Partition of Phosphorus in Whole Blood and Serum, Calcium and Plasma Phosphatase from Birth, J. Biol. Chem. 102:249 (Oct.) 1933.

12. (a) Jeans, P. C., and Stearns, Genevieve: The Retention of Calcium by Infants Fed Evaporated Milk Containing Cod Liver Concentrate, Proc. Soc. Exper. Biol. & Med. 34:1 (Sept.) 1936; (b) McQuarrie, Irene, Thompson, W. H.; Stearns, G., and Jeans, P. C.: The Antirachitic Potency of Evaporated Milk Irradiated by Electrons, J. Pediat. 10:295 (March) 1937.

13. Stearns, Genevieve, and Jeans, P. C.: The Effect of Intake of Vitamin D Intake on Calcium Retention in Infancy, J. Nutr. 11:1 (May) 1936.

14. Stearns, Genevieve, and Jeans, P. C.: Unpublished

rickets by roentgenographic means when rickets probably is not present. The small changes used as criteria in the roentgenographic diagnosis of slight rickets occur when no vitamin D is given and tend ultimately to increase, producing the picture of clinical rickets. When from 60 to 135 units of vitamin D is given daily, a minimum number of these changes is reported in inpatient studies.[7a] When from 300 to 400 units of vitamin D is given and growth is more rapid, a larger number of small changes in growth at the epiphysial line is reported, but these changes (in inpatient studies) never become any more definitely rachitic and accompany excellent retention of calcium with maintenance of serum calcium and phosphorus at high normal levels. From outpatient studies fewer bone changes are reported when large amounts of vitamin D are given. As the rate of growth seems to be nearly maximal with from 300 to 500 units of vitamin D,[8] it seems that the small changes reported as evidence of early or very mild rickets may represent equally well changes due to rapidity of growth except when no vitamin D is given.

Factors other than those which have been discussed must be considered along with the vitamin D requirement. One of these is the relative ease of administration. This has been brought out in several published studies of outpatient groups in which it was found that the amount of rickets occurring was in direct proportion to the amount of cod liver oil prescribed.[15] The apparent supremacy of vitamin D milks in outpatient studies may be ascribed to this cause.

Another factor requiring consideration is the relative efficacy of the different varieties of vitamin D. This question has been discussed at length in a previous review.[7b] It is possible that vitamin D from vegetable sources (related to ergosterol) is less potent for man on a unit for unit basis than that from animal sources (related to cholesterol). The difference in potency between these two varieties, if any exists, is small, almost certainly no greater than 1 : 1.5, and probably less than this. Criteria capable of determining such a narrow difference must be most exacting, and a satisfactory answer to the problem does not seem possible with available information.

A factor of apparently greater importance than the source of vitamin D is its state of concentration or dispersion. It has long been believed on the basis of clinical experience that a greater unitage of vitamin D is required as viosterol in oil than as cod liver oil. It seems probable that a large part of this difference, if not all of it, may be explained on the basis of relative dispersion. Lewis,[16] Erben[17] and Shelling[18] have shown in clinical experiments that irradiated ergosterol in a dispersed state is much more effective than the same material in a high concentration. The best concentration for efficient utilization is not known. Data[7a] from observations of infants receiving cod liver oil and various vitamin D milks) indicate that infants can utilize vitamin D in concentrations up to at least 100 units to the gram as efficiently as they utilize this vitamin in a highly dispersed state. The difference in utilization depending on concentration makes difficult the evaluation of data on the effect of higher dosages, for these high dosages have been given almost exclusively as concentrates or patent pharmaceutic preparations. The effect of the very high dosages in decreasing appetite suggests that a larger absolute amount is absorbed than with the smaller dosages of the dispersed forms; the disproportionate antirachitic effect between concentrated and dispersed forms indicates that a much smaller proportion is absorbed from the concentrates. In view of the strong probability that concentrated forms of vitamin D are not fully absorbed, it seems wise to proceed cautiously in administering to infants high unitages of the more dispersible forms of vitamin D. The actual limit of tolerance may be lower than the amounts which hitherto have been administered without toxic effect.

Tentatively the vitamin D requirement of the infant fed cow's milk may be stated as between 300 and 400 units daily, these amounts having been shown to be efficient in prophylaxis of rickets, in producing ample retention of calcium and phosphorus and in permitting excellent skeletal growth and dentition. This requirement is stated in terms of vitamin D of no greater concentration than is found in average high grade cod liver oil.

Infants Fed Human Milk.—The requirement of breast-fed infants for vitamin D is in general less than that of babies fed cow's milk. Infants fed human milk are less liable to the development of rickets when no vitamin D is given; however, rickets is by no means rare. A study of the literature shows that the calcium retention of infants receiving human milk and no additional vitamin D is somewhat less variable than that of infants receiving cow's milk without vitamin D; but the number of low retention values observed is sufficient to account for the occasional development of rickets. In our laboratory four infants fed human milk were given from 300 to 400 units of vitamin D daily; the calcium retention was less variable than is reported for infants given no additional vitamin D and the average retention was approximately 30 percent higher. It seems that for infants fed human milk as for artificially fed babies, the ingestion of vitamin D decreases the range of retention by increasing the efficiency of those with poor retentions to equal that of infants most efficient without vitamin D. It appears that for many breast-fed babies vitamin D is essential and for most of them it is useful. Though the requirement cannot be stated with accuracy on the basis of available data, it would seem wise to prescribe the same amount as is required by the artificially fed baby.

Prematurely Born Infants.—The vitamin D needs of prematurely born infants are not easy to determine because of the difficulty of supplying ample amounts of calcium and phosphorus during the first few weeks of extra-uterine life. Normally during the last month of full term pregnancy the fetal need of calcium is approximately 0.3 Gm. daily. Obviously it is not possible for a prematurely born infant fed human milk to ingest this quantity of calcium, much less to retain it. Even when the feedings are fortified with dried skimmed milk or calcium caseinate, the total calcium intake is usually under 0.6 Gm., an amount which may be considered scarcely sufficient to permit retention of 0.3 Gm. The prematurely born infant tends to grow

[15] Drake, F. G. H.; Stiehl, F. P., and Brown, Alan: A Study of Antirachitic Values of Cod Liver Oil, Viosterol and Irradiated Milk, Canad. M. A. J. 34:395 (Oct.) 1934. Eliot, Nelson, Barnes, Page and Jeans.

[16] Lewis, J. M.: Clinical Experience with Crystalline Vitamin D: Influence of the Medium on the Effectiveness of the Antirachitic Principle, J. Pediat. 6:102 (March) 1935; Further Observations on the Relative Antirachitic Value of Crystalline Vitamin D Administered in Olive Oil or in Propylene Glycol, ibid. 8:409 (March) 1936.

[17] Erben, Friedrich: Über den Einfluss des Lösungsmittels auf die Therapeutische Wirkung von Fest und Flüssigkeiten Ergosterin, München. med. Wschr. 83:1761 (Oct. 4) 1933.

[18] Shelling, D. H.: Calcium and Phosphorus Studies: XIII. The Effect of Administration on the Potency of Viosterol in Treatment of Rickets in Children, J. Pediat. 10:745 (June) 1937.

more rapidly than the infant born at term. The combination of increased growth and deficient intake is such a handicap that it is doubtful whether the ingestion of vitamin D can compensate entirely, though it may readily compensate to the extent of preventing rickets.

Davidson, Merritt and Chipman[19] reported that irradiated evaporated milk is inadequate to protect prematurely born infants from moderate or severe rickets. The amount of vitamin D received by these babies varied from 24 to 59 units daily at the age of 1 month. These same authors[20] found that cow's milk containing 430 units of vitamin D to the quart protected against moderate and severe rickets but not against the roentgen signs of slight rickets. Reinterpretation of the data in the light of the preceding discussion of roentgenographic evidence of rickets in this review permits the exclusion of these cases of slight rickets and allows the conclusion that all the infants were protected. The amount of vitamin D received by these babies varied from 116 to 245 units with an average of 170 units at the age of 1 month. The average birth weight of the babies was 2,000 Gm., and the average weight at 1 month was 2,500 Gm. Horesh and Russell[21] found complete protection against rickets in prematurely born infants from the use of a protein milk containing 3.8 cc. of cod liver oil to the quart. McQuarrie[22] obtained rickets prevention with 540 units daily as activated ergosterol. Davidson, Merritt and Chipman[19] reported that viosterol fed at a level of 3,150 units daily did not protect against roentgenographic evidence of slight rickets in prematurely born infants receiving human milk, cow's milk or mixed feeding, but again reinterpretation permits the conclusion that rickets was prevented. Stadling[23] observed osteoporosis but not rickets in premature infants given from 3,500 to 4,500 units of vitamin D as viosterol. These reports indicate that rickets is prevented in prematurely born infants by an amount of vitamin D approximately twice that known to protect the majority of full term infants and that rickets is not prevented by an amount less than that required for full term infants. No means are at hand for comparing the relative effects of the same amount of vitamin D in the two groups. The possibility remains that the prematurely born baby requires no more vitamin D for the prevention of rickets than the baby born at term. For the full term infant the amount that permits ample retention of calcium is considerably greater than that which barely prevents rickets. No doubt this holds for the prematurely born baby also. Until further information is obtained it may be tentatively considered that prematurely born babies require twice as much vitamin D as babies born at term, or from 600 to 800 units fed in a diversified form. After the period of most rapid growth in early infancy the requirement should be the same as for the full term infant.

REQUIREMENT DURING CHILDHOOD

The requirement for ingested vitamin D has been assumed to decline after infancy, though little experimental evidence for this supposition exists. Belief in this idea is reflected in the widespread lack of use of vitamin D preparations in childhood compared to the almost universal use in infancy. The assumption of a lesser need may be based on the absence of rickets in childhood and on the presumption that older children get more sunshine than infants. Whether or not the fault is with vitamin D, there is abundant evidence that present customs of diet in this country are not fully adequate for optimal skeletal and dental development. This is attested by the prevalence of dental caries and, in late childhood, the finding of osteoporosis. As a cause of these abnormalities a deficiency of intake of calcium, vitamin D or both substances is probably more common with the American type of diet than an intake deficient in phosphorus. At least some of these defects may be caused by inadequacy of vitamin D.

The criteria of adequacy of intake of vitamin D are fewer for children than for infants. Prevention of rickets is of no value and studies of levels of calcium and phosphorus in the blood are of little assistance. The rate of growth might be useful, but no studies have come to our attention in which this criterion was used when vitamin D was the only factor varied. A criterion which has a considerable degree of usefulness is the prevention and arrest of dental caries. The most satisfactory criterion subject to quantitative measurement is the retention of calcium and phosphorus with and without vitamin D, compared with the calculated requirement for these minerals.

Dental Caries.—Numerous observations have shown that vitamin D is a factor in prevention and arrest of dental caries. In some instances vitamin D has been added to the diet in an orphanage or other group without other change. The effect on the teeth has been variable, as is to be expected. It is worth reiterating that vitamin D does not reduce the minimum requirement for calcium and that vitamin D may not be expected to improve a diet containing an amount of calcium below the minimum requirement. In order to determine the vitamin D requirement by using the criterion of dental caries, the diet must be adequate in all respects other than vitamin D, including adequate calcium and phosphorus.

Mellanby[24] administered cod liver oil in an amount containing from 700 to 1,500 units of vitamin D daily, as a supplement in orphanage diets which happened to be deficient in fruits and animal protein and which contained about 500 cc. of milk daily. Mellanby reported that the children to whom cod liver oil had been given developed fewer new cavities and showed arrest of progress of old caries, although carious activity was not entirely prevented. A somewhat similar type of observation by Day and Sedwick[25] gave results. They found no change in the carious process in a group of school children for whom was prescribed 4,000 units of vitamin D daily as viosterol, as compared to a control group not receiving vitamin D. These children lived at home and the diet was uncontrolled. Anderson and his co-workers[26] found a "marked decrease" in dental caries when vitamin D (4,700 units) was administered to children with presumably good diets. McBeath[27] reported

19. Hess, A. F., and Rosoff, G.: Relationships of the Growth and Nutrition of Premature Infants Fed Irradiated Evaporated Milk, Ohio State M. J. 31:1,359 (May) 1935.

20. Davidson, L. J., Merritt, Katharine K., and Chipman, S. S.: Further Studies of Viosterol in the Prophylaxis of Rickets in Premature Infants, Am. J. Dis. Child. 51:394 (March) 1936.

21. Horesh, D. H., and Hooper, Katherine R.: Calcium and Phosphorus Studies. XII. Six Years' General Experience with Viosterol in Prevention and Treatment of Rickets, Infant and Allied Diseases, Bull. Johns Hopkins Hosp. 58:107 (March) 1936.

22. The Committee for Investigation of Dental Disease: The Effect of Diet on Caries in Children's Teeth (Final Report), Med. Res. Council, Special Report Series, No. 211, London, His Majesty's Stationery Office, 1936.

23. Day, C. D., Mc and Sedwick, H. J.: The Effect of Diet and Dental Caries in Children, J. Nutrition 8:[illegible] (Sept.) 1934.

24. Anderson, P. G.; Williams, C. H. M.; Hadden, W. [illegible] feldt, C., and Agnew, R. G.: The Influence of Vitamin D [illegible] vention of Dental Caries, J. Am. Dent. A. [illegible] 1935.

25. McBeath, E. C.: Nutritional Control of Dental Caries, [illegible] State J. Med. [illegible] (Sept. 15) 1935.

evidence of dental caries in an orphanage group whose diets are recorded as "good," when 10,000 units of vitamin D as viosterol was given daily without other dietary change. It is evident from the observations cited and others in the literature that vitamin D is a factor in the arrest and control of dental caries, but most of these reports give no clue to the vitamin D requirement.

McBeath[26] has reported the prevention of dental caries with the administration of an excellent experimental diet including 3 teaspoonfuls of cod liver oil. He[27] has reported also in a winter study of orphanage children a decrease in the number of new carious processes when the unrecorded diet was altered by the addition of 1 pint of milk and by 1 pint of milk containing 270, 400 and 800 units of vitamin D respectively, the improvement in the dental condition being in the order stated. Decrease in caries produced by the addition of milk alone indicates that the basal diet may not have been adequate. The apparent superiority of 800 over 400 units of vitamin D may not be actual, because these unitages were employed in different orphanages and were not added to the same basal orphanage diet. However, definite superiority of 800 over 270 units was shown.

Boyd and Drain and their co-workers[27] observed rapid arrest of dental caries in children given a well rounded diet which included a quart of milk and about 350 units of vitamin D daily, the latter in the form of cod liver oil. In a recent intensive study of four children with dental decay these authors[28] noted the effect of diets otherwise adequate but with the vitamin D intakes graduated; 0, 155 and 600 units daily were given successively to the same children. The ingestion of a good diet without added vitamin D brought about appreciable lessening of the activity of the caries during the first few weeks of the study in the fall months with the children spending much time outdoors. Subsequently for a period of five months, during which the children received a diet of high protective value aside from its vitamin D content and through both the periods of no added vitamin D and of the 155 unit addition, the caries was stationary with minimal but definite activity. It was not until the higher amount of 600 units of vitamin D had been given for nine weeks that the caries became definitely arrested. The results with 600 units daily showed no advantage of this amount over the 350 units daily intake of previous experiments.

Incidentally it is worthy of mention that the studies of Boyd and Drain have pertained exclusively to dental caries, while most if not all other observers have included both caries of the dentin and of the enamel in their data. Included in the latter designation, lesions due to dystrophy, fracture and disintegration have been considered; any surface irregularities deep enough to catch the exploring tine have been designated as sites of decay. Obviously not all these bear relation to nutrition. This difference of interpretation must be evaluated in comparing studies of tooth decay. The effect of nutrition on enamel disintegration remains to be determined. It is possible that complete prevention of enamel "caries" by a nutritional regimen is not to be expected.

One may conclude from the dental studies that the greatest freedom from dental caries is observed in groups of children receiving an adequate diet containing ample quantities of milk and 350 or more units of vitamin D daily. If the diet is suboptimal, the addition of vitamin D lessens the severity but does not prevent dental caries. Similarly, a diet otherwise adequate but lacking in vitamin D may decrease the incidence but not prevent entirely the development of tooth decay, nor provide for complete arrest of caries already present.

Calcium and Phosphorus Retention.—Many studies of calcium and phosphorus retention of children are available, though in but few have comparative studies been made with and without vitamin D. Unlike studies throughout infancy, wherein the dietary regimen is known for the entire period after birth, observations in childhood are more likely to be handicapped by incomplete knowledge of the previous dietary regimen. Wang[29] has shown that if the child's previous diet has been deficient in calcium, the calcium retention during the period of observation may be unusually high. In our studies there have been observed also a few children whose previous diets had been deficient in calcium but who reacted very slowly to the change in diet; only after several weeks of ingestion of an adequate diet with added vitamin D did the calcium and phosphorus retention reach the average of the group. Because of the influence of previous dietary conditions on the retention observed from a given diet, it has become customary to allow a longer period of adjustment to the experimental diet than was formerly the custom and to make a series of retention studies rather than a single observation with each given dietary. When the experiment is conducted in this manner the results are more reliable.

The adequacy of a diet in calcium, phosphorus and vitamin D may be judged by comparing the retention observed with the quantities considered ample for osseous and dental development. Because the quantity of phosphorus retained varies with the nitrogen as well as the calcium retention, the quantity of calcium retention may be used as the most convenient guide. When the standards set up by Leitch[30] are recalculated into the more customary terms of retention per kilogram, the calcium retention considered desirable decreases from 25 mg. per kilogram for a year old child to from 10 to 12 mg. for a child over 6 years of age. These values are somewhat higher for young children than those postulated as desirable by Sherman and Hawley.[31]

Reports of long term studies of children not given vitamin D stress the variability of calcium retention observed in a group of children[32] or in the same child from period to period of study.[33] The retention observed with an intake of 1 Gm. of calcium daily varied from 6 to 24 mg. per kilogram, and the average retention of some children was far below the amounts postulated as safe by Leitch.

Weld and Sykes[34] determined the calcium and phosphorus retention of six children over a five week

26. McBeath, E. C.: Vitamin D Studies, 1933-1935, Am. J. Pub. Health 26: 1129 (Dec.) 1936.

27. Boyd, J. D.; Dixon, C. L., and Nelson, Martha V.: Dietary Control of Dental Caries, Am. J. Dis. Child. 38: 721 (Oct.) 1929.

28. Boyd, J. D.; Drain, C. L., and Stearns, Genevieve: Nature of Diet in Relationship to Control of Dental Caries, Proc. Soc. Exper. Biol. & Med. 36: 445 (June) 1937.

29. Wang, Chi Cao; Kercher, Millie J., and Frank, Margaret: Metabolism of Undernourished Children. IV. Calcium Metabolism, Am. J. Dis. Child. 36: 8 (July) 1928.

30. Heinecke, Helen A.; Hummel, Frances C., and Macy, Ida G.: Variability of Metabolic Response of Different Children to a Given Intake of Calcium, Proc. Soc. Exper. Biol. & Med. 35: 166 (Oct.) 1936.

31. Porter-Levin, Lindsay: Calcium and Phosphorus Metabolism of Normal Preschool Children. II. Successive Balance Studies. Some Range of Variation in Calcium and Phosphorus Studies, J. Am. Diet. Assn. 9: 2 (May) 1933.

32. Weld, C. B., and Sykes, J. F.: The Effect of Impaired Liver Function on Calcium and Phosphorus Retention in Children, Proc. Soc. Exper. Biol. & Med. 41: 81, 1932.

period with an intake of 0.82 Gm. of calcium and no added vitamin D. During a second five week period 135 units of vitamin D as irradiated ergosterol was added to the diet of four of the children with no significant change observed in the retention.

We[34] studied fifty children aged from 1 to 12 years with varying intakes of milk and with and without added vitamin D. When given, the amount of vitamin D was from 300 to 400 units as cod liver oil daily. For the majority of these children each dietary regimen was maintained for from five to eight weeks. A few were studied for periods up to three months with one dietary regimen. All children were permitted outdoor exercise when weather allowed. The variability of retention noted by others when children were not given vitamin D was also observed in this series. The retention of the younger children was more variable than that of the older but less variable than had been observed in infants not given vitamin D. As with the infants, the addition of vitamin D decreased the variability of retention by decreasing the number of poor retentions. Thus, if a child utilized calcium efficiently without added vitamin D, the addition of the vitamin to the diet had little effect. If, however, the utilization was poor, the ingestion of vitamin D increased the retention of calcium. The increased retention was by no means always observed promptly; two of the children studied did not achieve retention equal to the average of their age group until the diet with vitamin D had been given for a period of from two to three months. One of these children had active dental caries at the beginning of the experiment; the caries remained active much longer than is customarily observed in this clinic. The retention observed in this group of fifty children indicates that the ingestion of vitamin D in no way decreases the quantity of calcium need but that it assures better utilization. A retention equal to that postulated by Leitch as desirable was consistently obtained when the milk intake was 750 cc. daily or higher and when vitamin D was given.

Daniels and her collaborators[35] fed vitamin D in daily amounts varying from approximately 1,200 units (15 cc. of cod liver oil) to 3,000 units or more (15 cc. of cod liver oil plus 8 drops of viosterol). Though these studies were of short duration, the study group was large. An average retention of about 10 mg. per kilogram was observed whether the intake of milk was 1 pint or 1 quart daily. These retention values are lower than those noted by Hunscher and her co-workers in long term studies of children of this age group not receiving additional vitamin D, and considerably lower than the average values observed by us in children of the same age range given from 300 to 400 units of vitamin D daily.

It seems from a summary of the studies which have been cited that the variability of response to the calcium of the diet is marked in young children not given vitamin D. Efficiency of utilization tends to increase with increasing age of the child, but even by 9 or 10 years of age many children seem unable to utilize efficiently the calcium and phosphorus of the diet unless vitamin D is also ingested. The daily allowance of at least 750 cc. of milk together with from 300 to 400 units of vitamin D seems to permit consistently ample retention of calcium and phosphorus for proper development of bone and teeth. The data at hand are insufficient to allow an accurate estimate of the optimal intake of vitamin D for children, though it appears probable that the total quantity needed daily is not greater than the amount required for infants, nor does it seem to be less.

ADOLESCENCE

The literature contains very few reports of studies of either the calcium or the vitamin D requirements of adolescents, notwithstanding the fact that this age period is of special importance because of the rapid skeletal growth. Leitch has estimated that a retention of from 500 to 700 mg. of calcium daily is necessary to maintain normal calcification during this period. The condition of the teeth of the average adolescent and the possible occurrence of osteoporosis[36] during this period of growth indicate inadequacy of previous dietaries. Whether in this state of suboptimal calcification the need is primarily for calcium or for vitamin D is difficult to determine with the data at hand.

Herbst[36] in 1913 reported calcium retention on two boys aged 13 and 14 years, with a calcium intake of 0.62 and 0.64 Gm. daily. The younger, who was growing rapidly, retained 0.3 Gm. of calcium daily, the older 0.15 Gm. Wang and her associates[37] have summarized the scanty data in the older literature and have added twenty-three six day studies of girls from 12 to 15 years of age. Vitamin D was not given. In Wang's study the daily intake of calcium varied from 1.18 Gm. to 1.80 Gm. and the retention from 0.079 Gm. to 0.823 Gm., or from 1 to 19 mg. with an average of 11 mg. per kilogram; the average retention was 417 mg. daily, two thirds of the girls retaining less than the Leitch standard of 500 mg. daily.

Henderson and Kelly[37] studied for twenty-four weeks the calcium retention of five African boys aged from 15 to 17 years. With the control diet containing 0.3 Gm. of calcium the boys lost calcium at a rate of approximately 0.1 Gm. a day. The addition of cod liver oil to the diet of one boy did not decrease the calcium loss. The diets of three of the subjects were then supplemented respectively with 1 pint of milk daily, a mineral mixture equivalent to the minerals of 1 pint of milk daily, and the mineral mixture with added cod liver oil. The calcium retention of each of the three boys was consistently positive, varying from 0.096 Gm. to 0.5 Gm. daily, the higher value being obtained with the addition of milk without cod liver oil. The data are insufficient to permit evaluation of the effect of vitamin D added to the increased diet but do demonstrate that the ingestion of vitamin D did not decrease the minimal requirement for calcium.

The data obtainable from studies of adolescents indicate that individuals are by no means equally efficient in retaining calcium without the ingestion of vitamin D. Apparently the variability in ability to utilize the calcium and phosphorus of the diet is a major factor in determining the quantity of these elements retained by adolescents as well as by young

33. Jeans, P. C., and Stearns, Genevieve: The Vitamin D Requirement of the Child. Proc. Am. Pediat. Soc., Am. J. Dis. Child. 54:185 (July) 1937.

34. Daniels, Amy L., Hixson, Mary K., Kraft, Elizabeth, Everson, Gladys, and Wright, Olive L.: Relation of Ingestion of Milk to Calcium Metabolism in Children. Am. J. Dis. Child. 47:482 (March) 1934. Daniels, Amy L., Hixson, Mary K., Kraft, Elizabeth, Wright, Olive, and Fromm, Mary: Calcium and Phosphorus Needs of Pre-School Children. J. Nutrition 20:153 (Feb.) 1935.

35. Herbst, O.: Calcium and Phosphor beim Wachsenden Menschen. Kindhed. Ztschr. f. Kinderh. 79:161, 1912.

36. Wang, Chi Chu; Kancher, Mildred, and Wing, [illegible]: Metabolism of Adolescent Girls: IV. Mineral Metabolism. Am. J. Dis. Child. 49:[illegible] (July) 1935.

37. Henderson, J. M., and Kelly, S. G.: Influence of Calcium Supplements in Relation to the Calcium Requirements of Growing Natives. J. Hyg. 29:[illegible] (Feb.) 1933.

children. It is indicated also that vitamin D does not lower the minimum requirement for ingested calcium, though no estimate can be made as to the optimal intake of either calcium or vitamin D.

REQUIREMENTS OF ADULTS

Men and Nonpregnant Women.—During adult life the quantity of calcium and phosphorus considered necessary is only that amount sufficient for maintenance. It may be found that some retention of calcium and phosphorus is desirable, at least during the postadolescent period. Whether the addition of vitamin D may be necessary to insure such retention or whether the ingestion of vitamin D would insure better utilization of an intake only slightly above minimal requirements are questions dificult to answer with the few data at hand.

Much of the calcium intake of adults may be from sources other than milk; therefore the acid-base ratio of the diet assumes importance. The ingestion of an acid-ash diet or of acid salts increases the urinary excretion of calcium. If the intake is low, the added excretion may be sufficient to cause a negative calcium balance. Wide differences in intake ratios of calcium and phosphorus apparently have less effect on the retention of these elements by adults[38] than by children.[39]

Leitch[8] has summarized the data for the calcium requirement of women not ingesting vitamin D. She concluded that, at or above a daily intake of 0.55 Gm. of calcium, losses of one period were equaled by gains of another, whereas below this intake output usually exceeded intake. The minimum calcium requirement as set by Sherman[40] is somewhat lower; namely, 0.45 Gm. a day. Few data are available concerning the requirement for men, but such as there are[36] indicate that the daily requirement is about the same as for women.

Most of the published studies of the effect of vitamin D on calcium and phosphorus retention of adults have centered on an attempt to determine whether an increased ingestion of vitamin D decreases the minimal requirement for calcium. The answer is unanimously in the negative.[41] When vitamin D has been given as an addition to a diet containing more than the minimal requirement of calcium, the results have been conflicting. All the subjects studied by Kelly and Henderson[41] and by Bauer and his co-workers[41] retained calcium when the intake of this element was ample and no vitamin D was ingested. A further increase in retention was noted by Kelly and Henderson when cod liver oil was given in addition to the increased mineral intake. Bauer and his collaborators observed no increase in retention when from 30 to 90 mg. of irradiated ergosterol was given in addition to the high calcium diet but did obtain evidence of increased absorption, for the urinary excretion of calcium was decreased and the fecal calcium correspondingly increased when vitamin D was given. Henscher and her co-workers[42] studied three young women whose calcium intake varied from 0.7 to 1.0 Gm. a day, amounts ordinarily considered ample. When no vitamin D was given two of the three women consistently lost 0.1 Gm. of calcium a day, the third was approximately in calcium equilibrium. When 15 Gm. of cod liver oil was given daily for twenty-three days the average calcium losses were not decreased, nor do the data show any significant alteration in the mode of calcium excretion.

Data are not available as to the effect of vitamin D on retention of calcium and phosphorus by middle-aged or elderly persons. The prevalence of senile osteoporosis may be due wholly to the habit common to most American adults of ingesting diets low in calcium. It has been shown[43] that mineralization of bone can be increased in adults past 60 by increasing the intake of calcium and vitamin D. Since elderly persons are less likely to be exposed to sunlight than more vigorous younger adults, the ingestion of moderate amounts of vitamin D, together with a diet ample in calcium and phosphorus, might serve to prevent the development of osteoporosis in the older adult.

Animal studies bearing on these questions include those of Sherman and Campbell,[30] who showed that when young rats are given a diet somewhat subnormal in calcium content, normal growth occurs, but normal calcification of bone is not achieved until nearly middle age. A recent report of these authors[44] lists an ample intake of calcium among the factors resulting in longer life and extension of the period of adult vitality of rats. Even if these observations are assumed to hold for human beings, there still remains to be determined whether and how much vitamin D would contribute to better utilization of calcium by adults.

The observations on adults indicate, as for children, that the ingestion of vitamin D in no way lessens the requirement for calcium. Whether vitamin D, when given in addition to an ample mineral intake, increases the utilization of calcium undoubtedly varies with the person. Although the average adult seems more efficient in the absorption of calcium than the average child, vitamin D may aid the inefficient adult in the same manner as the inefficient child. If this is true, the amount of vitamin D optimal for this purpose remains to be determined.

Reed and others[45] have given vitamin D in enormous dosage to adults, with apparently few untoward effects. The limit of tolerance varies with the individual but seems to be 150,000 units or more. The vitamin in these studies was given always as a very concentrated product. Whether adults utilize the concentrated sources of vitamin D efficiently or not, the enormous intake given can be interpreted as indicating that the tolerance of the adult to vitamin D can be relatively high.

Pregnancy and Lactation.—The calcium and phosphorus requirement during pregnancy and lactation, is

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38. Fairbanks, B. F.; Salter, W. T., and Aub, J. C.: The Effect of the Ingestion of Phosphates on the Excretion of Calcium, J. Clin. Investigation 10: 651 (June) 1931.

39. Sherman, Genevieve, and Jeans, P. C.: Utilization of Calcium Salts by Children, Proc. Exper. Biol. & Med. 28: 428 (Dec.) 1931.

40. Sherman, H. C.: Calcium Requirement of Maintenance in Man, J. Biol. Chem. 42: 21 (Oct.) 1920.

41. Hess, M. C.; Lourie, Inez, and Heyl, F. W.: Effect of Irradiation and Cod Liver Oil on Calcium Balance in the Adult Human, J. Biol. Chem. 76: 143 (Jan.) 1928. Kelly, E. C., and Henderson, L. M.: The Influence of Certain Dietary Supplements on the Retention of the Mineral Salts, J. Nutrition 2: 413 (Feb.) 1930. Bauer, Walter; Marble, Alexander, and Claflin, Dorothy: Studies on the Mode of Action of Irradiated Ergosterol: I. Its Effect on the Calcium, Phosphorus and Nitrogen Metabolism of Normal Individuals, J. Clin. Investigation 11: 1 (Jan.) 1932.

42. Henscher, Helen A.; Duncan, Eva, and Erickson, Betty: Results of the Ingestion of Cod Liver Oil and Yeast on Calcium and Phosphorus Metabolism, J. Nutrition 8: 442 (Sept.) 1934.

43. Mecleuchot, E., and Meyer, A. R.: [illegible] Jeger [illegible] 1936, quoted by Leitch.[8]

44. Sherman, H. C., and Campbell, H. L.: Nutritional Well-Being and Length of Life as Influenced by Different Enrichments of an Adequate Diet, J. Nutrition 11: 139 (Dec. 12) 1936.

45. Reed, C. I.: Symptoms of Vitamin Overdosage in Humans, J. A. M. A. 102: 1745 (May 26) 1934. Wyatt, D. A.; Ferguson, R. A., and Thompson, H. E.: Massive Doses of Vitamin D in the Treatment of Rheumatoid Arthritis, Ann. Int. Med. 10: [illegible] 1936. Slack, L. E.: Clinical Experience in the Treatment of [illegible] with Massive Doses of Vitamin D, Illinois M. J. 71: 93 (March) [illegible] Coter, E. T., and Zan, [illegible]: Treatment of Psoriasis with Massive [illegible] of Crystalline Vitamin D and Irradiated Ergosterol, Pub. Health Rep. 52: 1580 (Nov. 27) 1937.

received much intensive study. The report of Bauer and Aub[46] indicates that with a subminimal intake the pregnant woman is unable to conserve calcium for the needs of the fetus. The Toverud[47] observed that when the mother's diet was deficient, the skull of the infant was softer than normal, the epiphysial lines, particularly of the metacarpals, were fringed and poorly calcified, and the teeth when erupted were often grossly hypoplastic. They observed further that when the mother's diet contained ample calcium and vitamin D, the bones and teeth of the infant were normal. Finola[48] and his co-workers noted a significant increase in the density of bone and a decrease in the size of the fontanel of babies whose mothers received a good diet plus approximately 1.5 Gm. of calcium as dicalcium phosphate and 7,000 units of vitamin D as viosterol, in comparison with infants whose mothers received a good diet without the addition of calcium and vitamin D.

The Toverud[47] and Mellanby[49] both consider a poor diet of the mother during pregnancy as one of the chief predisposing factors in the development of rickets and dental caries in the children. Calcium and vitamin D are two of the factors most often deficient in the mother's diet. Corroborative evidence of the need of increased mineral retention in pregnancy is obtained in the study of Drain and Oberst[50] wherein the dental condition of pregnant women was shown to be improved by a diet rich in calcium and vitamin D.

The work of many investigators[51] demonstrates that a calcium intake of at least 1.4 to 1.6 Gm. daily is necessary in order to permit retention of the amount of calcium needed by the fetus. Below this intake the addition of vitamin D does not insure retention. With a calcium intake of from 1.6 to 2.5 Gm. some persons can retain calcium in ample amounts even without added vitamin D. Studies of multiparas[52] show that the drain of rapidly succeeding pregnancies and periods of lactation may result in poor retention of calcium and phosphorus regardless of intake unless vitamin D is also given. Very few show poor retention with a high intake of calcium even when the vitamin D intake is equivalent to 20 Gm. of cod liver oil. In general the addition of vitamin D in amounts supplied by from 10 to 20 Gm. of cod liver oil daily tends to increase the retention of calcium and phosphorus. Insufficient studies have been made with ample intake of calcium and varying amounts of vitamin D to permit a conclusion or general statement as to the vitamin D requirement during pregnancy.

The studies of lactation[53] indicate that the drain on the mother is more severe than in pregnancy. The need for ample calcium intake is imperative. There seems necessary an intake of at least 1½ quarts of milk, containing 1.8 Gm. of calcium and representing a total day's intake of more than 2 Gm. Even then, relatively few women are efficient enough to prevent loss of calcium from the body unless additional vitamin D is given. Desirable amounts of vitamin D seem to vary with the individual, as shown by the following unpublished study from our laboratory: Two primiparas, aged 21 and 26, ingested good diets, each containing 1,500 cc. of milk and one teaspoonful of cod liver oil (from 350 to 400 units) daily. The 21 year old woman secreted from 1,100 to 1,500 cc. of milk daily and retained calcium continuously. The other woman secreted never more than 600 cc. of milk a day yet continuously lost calcium from the body; increasing the calcium intake decreased the loss but did not prevent it entirely. Again, two other primiparas, aged 17 and 19, were given from 1,800 to 2,000 cc. of milk daily. One retained calcium consistently throughout lactation; the other was unable to retain calcium until from 750 to 800 units of vitamin D daily as cod liver oil was given. The milk output of the latter was approximately 1,500 cc. and that of the former approximately 1,000 cc. a day.

Hunscher's studies[53] tend to show that in rapidly succeeding pregnancies and lactation periods vitamin D is especially necessary and that ample calcium intake does not suffice to prevent loss of calcium from the body unless vitamin D is given.

One factor often overlooked is the effect of the maternal diet on the quality of the milk secreted. Telfer[54] commented on the higher mineral content of milk of country women compared to that of women from cities. The differences were ascribed to diet and to the relative amount of sunlight. The Toverud[47] found a low calcium content in the milk of fourteen mothers whose diets were poor; when the calcium intake was increased the calcium content of the milk of one woman was increased after one week, the maximum increase being about 25 per cent. The effect of added vitamin D was not studied, but the possibility of its ingestion increasing the calcium content of the milk should not be ignored. The content of calcium in human milk varies considerably according to supply.

While available data point strongly to the advisability of giving vitamin D during pregnancy and lactation, it is impossible with our present information to arrive at any conclusion regarding optimal dosage. Individual variability has been demonstrated. The amount of vitamin D to be recommended is that which is desirable for the least efficient in utilizing the calcium of the diet. This amount may be greater, especially during lactation, than at any other period of life.

46. Bauer, Walter; Albright, Fuller, and Aub, J. C.: Studies on Calcium and Phosphorus Metabolism. II. The Calcium Excretion of Normal Individuals on a Low Calcium Diet. Also Data on a Case of Pregnancy, J. Clin. Investigation, 7: 75 (April) 1929.

47. Toverud, K. U., and Toverud, G.: Studies on Mineral Metabolism During Pregnancy and Its Bearing on Disposition to Rickets and Dental Caries, Acta pædiat. 22: (Supp. 2) 111, 1937.

48. Finola, G. C.; Temple, Ruth A., and Gibson, Mozelle: Bone Changes in the Infant Following the Administration of Dicalcium Phosphate and Viosterol to the Pregnant Mother, Am. J. Obst. & Gynec. 18: 955 (Dec.) 1929.

49. Mellanby, May: Diet and the Teeth: III. The Effect of Diet on Dental Structure and Decay in Man, Medical Research Council, Special Report Series, no. 191, London, His Majesty's Stationery Office, 1934.

50. Drain, C. L.; Oberst, F. D., and Oberst, W. E.: Prevention and Control of Dental Caries in Pregnant Mothers, J. Dent. Research 13: 253 (April) 1933.

51. Gamble, R. G., and Stearns, G.: A Review of Recent Work on Dietary Requirements in Pregnancy and Lactation, with an Account of Access Human Requirements, Nutrition Abst. & Rev. 5: 845 (April) 1936. Macy, Ide G., and Hunscher, Helen A.: Evaluation of Maternal Nitrogen and Mineral Needs During Pregnancy and Infant Development, Am. J. Obst. & Gynec. 27: 2[illegible] (Feb.) 1934. Hunscher, Helen A.; Hunscher, Helen V.; Bates, Mary E.; Hunter, Dorothy; Macy, Ide G., and Johnston, J. A.: A Confirmation of the Nutritive Study on the Metabolism of Women During Pregnancy, J. Nutrition 13: 261 (March) 1937. Cooke, Hilda Mae; Schaffer, A. I.; Marshall, G. B., and Cooke, R. E.: Studies in Metabolism During Pregnancy, Oklahoma Agricultural and Mechanical College, Agricultural Experiment Station, Bulletin 221, May 15, 1937. Cooke, Hilda Mae, and Cooke, R. E.: Some Effects of Cod Liver Oil and Wheat Germ on the Retention of Iron, Nitrogen, Phosphorus, Calcium and Magnesium During Human Pregnancy, J. Nutrition 14: 595 (Dec.) 1937. Toverud and Toverud.[47]

52. Macy, Ide G.; Hunscher, Helen A.; Nelson, Ruth, and McCosh, Sylvia S.: Metabolism of Women During the Reproductive Cycle: I. Calcium and Phosphorus Utilization in Pregnancy, J. Biol. Chem. 80: 19 (March) 1929. Hunscher, Helen A.; Hunscher, Helen V.; Bates, Mary E.; Hunter, Dorothy; Macy, Ide G., and Johnston, J. A.: Metabolism of Women During the Reproductive Cycle: VII. Calcium and Phosphorus Utilization in Pregnancy, A Continuous Cycle Study of a Multipara, J. Nutrition 14: 253 (March) 1937.

53. Macy, Ide G.; Hunscher, Helen A.; McCosh, Sylvia S., and Betty: Metabolism of Women During the Reproductive Cycle: IV. Calcium, Phosphorus and Nitrogen Utilization in Lactation Before Supplementing the Usual Home Diet with Cod Liver Oil, J. Biol. Chem. 80: 59 (March) 1929. Hunscher, Helen A.: Metabolism of Women During the Reproductive Cycle: II. Calcium and Phosphorus Utilization in Two Successive Lactation Periods, ibid. 86: 17 (Feb.) 1930. Toverud and Toverud.[47] Footnote 52.

54. Telfer, S. V.: Mineral Metabolism in Infancy: I. The Constituents of Human Milk and Cow's Milk, Glasgow M. J. 130: 210 (May) 1938.

Animal experiments wherein large amounts of vitamin D were given to pregnant rats show that, if the maternal diet is good, the calcium and phosphorus content of the bones of the young rats is increased above that of the control group;[55] large doses of viosterol together with a deficient diet result in decreased content of mineral in the bones of young.[55] Subtoxic doses of vitamin D given to rats receiving a diet deficient in minerals during gestation increase the calcium and phosphorus content of the offspring over that of young born of mothers given the deficient diet without added vitamin D.[56] Hypervitaminosis D has not been reported in women during pregnancy and lactation; consequently the effect is unknown.

SUMMARY

Individual variation in ability to utilize the calcium and phosphorus of the diet without added vitamin D exists at all age periods. A high proportion of infants have poor retention and only a very few retain an ample amount without vitamin D. As the age increases persons in increased proportion are able to retain adequate amounts of these minerals without vitamin D, but at all age periods some persons are found who are not efficient. In defining a standard for the vitamin D requirement it seems desirable to state an amount which will be satisfactory for those who are less efficient. Vitamin D tends to decrease the range of retention of calcium and phosphorus in study groups by increasing the retention of those least efficient to approximate that of those most efficient. Vitamin D ordinarily does not increase the retention of those who have high retention without it.

Vitamin D does not decrease the minimum requirement of calcium and phosphorus and this vitamin cannot produce a good retention in a person who is ingesting less than the requirement for these minerals. Determination of the need for vitamin D must be based on studies of persons who are receiving an ample intake of calcium and phosphorus.

It appears from available evidence that vitamin D is not as well utilized on a unit for unit basis from the more concentrated preparations as from those preparations in which it is more widely dispersed. The most desirable concentration has not been determined, but apparently the concentration found in cod liver oil is as effective as any lesser concentration studied.

On the basis of the preceding premises and of an evaluation of data in the literature the requirement for vitamin D for different age periods and conditions has been tentatively postulated. These requirements are considered in terms of a concentration of vitamin D no greater than is found in average high grade cod liver oil.

The vitamin D requirement of the full term artificially fed baby is probably between 300 and 400 units a day.

Normal babies receiving human milk require less vitamin D than do babies receiving cow's milk, but how much less is not known. However, vitamin D is necessary for many and useful for most breast-fed babies. It would seem wise to prescribe for them the same amount as is required by artificially fed babies.

It is tentatively considered that prematurely born babies may require twice as much vitamin D as full term babies during the early period of most rapid growth, after which time the requirement should be the same as for babies born at term.

For children between infancy and adolescence a daily allowance of at least 750 cc. of milk together with from 300 to 400 units of vitamin D permits consistently ample retention of calcium and phosphorus. The optimal quantity of vitamin D cannot be stated accurately, though it appears probable that the total quantity needed is neither greater nor less than the amount required for the infant.

For adolescents a need for vitamin D exists, but insufficient data are available to permit an estimate of the quantity required. It seems probable that from 300 to 400 units a day would be satisfactory.

For adults the optimal amount of vitamin D, if a need exists, remains to be determined.

It appears strongly advisable to give vitamin D during pregnancy and lactation. The optimal amount is not known. During lactation the requirement may be greater than at any other period of life and a daily dosage of 800 units or more is suggested, together with an abundant intake of calcium and phosphorus.

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55. Nicholas, H. D., and Kuhn, Evelyn M.: Role of Calcium, Phosphorus and Vitamin D in Pregnancy, J. Clin. Investigation 11: 1313 (Nov.) 1932.

56. Sontag, L. W.; Manson, Paul, and Huff, Ellen: Effects on Fetus of Hypervitaminosis D and Calcium and Phosphorus Deficiency During Pregnancy, Am. J. Dis. Child. 51: 302 (Feb.) 1936.

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization or of the Food and Agriculture Organization of the United Nations.

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REQUIREMENTS OF ASCORBIC ACID, VITAMIN D, VITAMIN B_{12}, FOLATE, AND IRON

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(1) Vegetables contribute some 60–85% of the total intake in developing areas, but only 20–50% in Europe and the USA;

(2) Fruits contribute 1–20% in developing countries and 15–45% in the developed areas. Mention has already been made of the high ascorbic acid intakes of population groups whose staple food consists of starchy roots and tubers. Where these foods are not used as a staple they may only contribute about 30% of ascorbic acid intake, as in Europe and the USA.

Vitamin D

This nutrient is obtained in two distinct ways: (*a*) from food, (*b*) through its formation in the human skin by the action of sunlight. Foods that supply vitamin D are, with very few exceptions, animal products. Since the vitamin D that can be derived from exposure to sunlight varies with latitude and environmental conditions, it is difficult to determine exactly the amount that is available to an individual, let alone a population.

Vitamin D intakes in tropical areas are considered to be low because of the nature of the diet; the predominance of cereals and the limited availability of protective foods, especially such good sources of vitamin D as butter, egg yolk, and livers of mammals. However, the sunlight acting on the skin to cause the formation of vitamin D in the body reduces the need for vitamin D from foods.

In developed regions of the temperate zone, the amount obtainable from the diet is also limited, and needs to be supplemented by fortified foods (e.g., margarine, milk preparations), cod-liver oil, or other forms of supplement.

Data on vitamin D intake in populations are virtually non-existent. According to a recent estimate, the range of vitamin D intake among the rural and urban population of the United Kingdom is between 2.90 and 3.33 µg (116 and 133 IU) per person per day (*28*).

Vitamin B_{12}

The effect of income on levels of vitamin B_{12} intake is clearly illustrated by the data available from the USA, where levels of 31, 16, and 2.7 µg per day have been found for persons eating adequate high-cost, adequate low-cost, and poor diets respectively (*47*).

As is to be expected in view of the low consumption of foods of animal origin, vitamin B_{12} intakes in developing countries are low. Table 2 shows levels ranging from 0.39 µg to 3.90 µg per person per day. Intake levels for the majority range from 0.64 to less than 3 µg per person per day. Except in milk, vitamin B_{12} is stable during normal cooking; dietary vitamin B_{12} is as readily absorbed as crystalline vitamin B_{12}.

2962

Minimum requirements

The minimum requirement of a nutrient is usually considered to be the smallest exogenous supply needed to prevent symptoms of deficiency, as judged by clinical signs and symptoms, and/or by parameters of biochemical or physiological function. It is difficult to decide upon the minimum requirement, since the amount is subject to individual variations and will be valid only for a stated set of circumstances. The usual approach is to use a combination of criteria: (*a*) the intake of a particular nutrient in a population that has no apparent deficiency symptoms; (*b*) levels of intake of population groups where deficiency symptoms occur; and (*c*) results of controlled experiments on human subjects to produce, prevent, or cure deficiency.

The first two criteria are often difficult to evaluate, since the information on intakes has to be supplemented by information on the incidence of deficiency at the same time and location. Criterion (*c*) is extremely valuable, but is not always available and suffers from the drawback that only a small number of subjects can normally be employed. It has the advantage, however, that the minimum dosage needed to prevent or cure deficiency symptoms can be assessed fairly accurately. The value of the results will depend on the reliability of the biochemical and clinical criteria that have been developed to diagnose deficiency.

When these investigations are supplemented by turnover studies using radioisotope-labelled nutrients, a clearer picture is obtained and more precise conclusions can be drawn. The results of animal experiments also have to be evaluated, in view of possible differences between species.

When the results of epidemiological surveys, controlled studies on human subjects, and animal experiments all agree, they provide a sound basis for estimation of the requirement of the nutrient concerned.

Recommended intakes

The recommended intakes given in this report for ascorbic acid, vitamin D, vitamin B_{12}, folate, and iron are the amounts considered sufficient for the maintenance of health in nearly all people. It is emphasized that these recommended intakes are the amounts of the nutrients that are actually consumed. In the case of vitamin D, however, the recommended intake may be replaced partially or completely by exposure to sunlight.

The recommended intakes are intended to be used mainly for evaluating the adequacy of the intake of population groups, for planning diets and food supplies, and for planning and evaluating food programmes and public health nutrition programmes. They are not intended to be used as the sole basis for the evaluation of nutritional status, particularly of individuals.

Sources of vitamin D

Vitamin D can be obtained from two sources: exposure of the skin to sunlight and ingestion of the vitamin. The amount of cholecalciferol produced in the skin by exposure to sunlight is not known, but varies with such factors as the duration of exposure, the skin area exposed, the geographical location, and the season of the year. In some countries, exposure to sunlight is prevented or is reduced to a low level, particularly in the case of women and infants, by housing conditions in large cities and by social customs. Foods not fortified with vitamin D generally contain little of the vitamin; exceptions are some animal foods such as fat-rich fish, eggs, liver, and butter. It should be emphasized that milk, human or otherwise, is a poor source of the vitamin.

Requirements of vitamin D

The minimum amount of vitamin D required to maintain normal homeostasis of calcium and phosphate and normal mineralization of bone in man is not known, mainly because information is not available on the amount of vitamin D resulting from exposure to sunlight. Even if the contribution of exposure to sunlight is ignored and only the amount taken orally is considered, there are few pertinent data on which to base the requirements of vitamin D. Those data that are available mainly concern infants who may require more vitamin D because of their high growth rate. In full-term infants, it has been found that a daily intake of 2.5 µg (100 IU)[1] of vitamin D prevents rickets and is commensurate with adequate absorption of calcium in the gut, adequate growth rate, and normal mineralization of bone (*6, 7*). In the same studies, intakes of 7.5–10 µg (300–400 IU) per day were associated with still greater intestinal absorption of calcium and with some increase in the rate of growth. Higher intakes up to 20 µg (800 IU) per day had no greater effect. The finding that 10 µg per day has a more favourable effect than 2.5 µg per day needs confirmation. Nevertheless, it would at present be inadvisable to recommend the lower amount as daily intake since it is not known whether this would meet the requirements of infants insufficiently exposed to sunlight.

No studies are available on the requirements of vitamin D in older children and adults. The only relevant observation is that daily administration of 2.5 µg (100 IU) of vitamin D produced a satisfactory response in patients with osteomalacia (*8, 11, 12, 13*).

The extent of exposure to sunlight of the subjects in the studies considered above is unknown.

[1] See the notes on terminology given in Annex 1.

Recommended daily intakes

The daily intakes of vitamin D recommended by the Group are shown in Table 5. It should be remembered, however, that part or all of the vitamin D required may be supplied through exposure to sunlight.

TABLE 5. RECOMMENDED DAILY INTAKES OF VITAMIN D

Age or status	Recommended daily intake in µg [a]
Birth–6 years	10
7 years and over	2.5
Pregnancy (second and third trimesters)	10
Lactation	10

[a] 2.5 µg of cholecalciferol are equivalent to 100 IU of vitamin D.

Infants

The Group recommends a daily intake of 10 µg (400 IU) of vitamin D. This will cover the requirement even if the child is exposed to no sunlight at all.

Children aged 1–6 years

There are no experimental data on which a recommendation for daily intake for these children can be based. However, there are several parts of the world where rickets is most prevalent in children in this age group, presumably in spite of exposure to sunlight, and the Group therefore recommends that the daily intake should be 10 µg (400 IU).

Older children and adolescents

Active rickets is virtually non-existent in this age group in spite of the fact that the dietary intake of vitamin D is low and supplements of the vitamin are not given regularly. It should be noted that exposure to sunlight is likely to be greater in this age group than in any other. The Group therefore recommends a daily intake of 2.5 µg (100 IU), which will meet the need even during the pubertal growth spurt.

Adults

Little is known of the vitamin D requirement of adults and the elderly. It is known that osteomalacia sometimes occurs in women of all ages and that it responds satisfactorily to the administration of 2.5 µg (100 IU) of vitamin D per day. The Group considers that this level of intake is more than sufficient to prevent osteomalacia and is adequate to meet the health requirements of most people. It therefore recommends a daily intake of 2.5 µg (100 IU) of vitamin D.

Pregnancy and lactation

It is not known whether the requirements of women are higher during pregnancy and lactation than at other times. During these periods calcium metabolism is under physiological stress. Calcium is supplied to the fetus in increasing amounts during the second and third trimesters of pregnancy. During lactation even more calcium is lost by the mother. Taking into account the influence of vitamin D on calcium metabolism, and in order to meet this stress situation adequately, the Group recommends a daily intake of 10 µg (400 IU) during the second and third trimesters of pregnancy and during lactation.

Other factors influencing the requirements

There is no evidence that sex, body weight, or physical activity affect the requirements of vitamin D in any age group.

Apart from sunlight, environmental factors such as temperature, climate, and altitude have no apparent influence on the requirements of vitamin D.

Toxicity

Attention is drawn to the fact that intakes greatly in excess of those recommended here could result in hypervitaminosis D, a harmful condition. Hypercalcaemia, possibly caused by hypervitaminosis D, has been reported in some infants receiving 75–100 µg (3000–4000 IU) per day in their diet for prolonged periods of time (*9*, *10*). The intakes recommended here carry no such risk and will prevent the occurrence of rickets or osteomalacia.

Factors influencing the content and utilization of vitamin D in foods

Vitamin D, whether it occurs naturally in foods or is added as a supplement, is stable with regard to storage, processing, and cooking.

There is very little information on the availability of vitamin D from foods. In the absence of any evidence to the contrary, the Group assumes that the whole of vitamin D in food, whatever its origin, is absorbed from the gastrointestinal tract.

6962

REFERENCES

1. Norman, A. W. (1968) *Biol. Rev.*, 43, 97
2. Steendijk, R. (1968) *Folia med. neerl.*, 11, 178
3. Bordier, P. et al. (1968) *Lancet*, 1, 437
4. Blunt, J. W., Deluca, H. F. & Schnoes, H. K. (1968) *Chem. Comm.*, 14, 801
5. Lawson, D. E. M., Wilson, P. W. & Kodicek, E. (1969) *Nature (Lond.)*, 222, 171
6. Jeans, P. C. & Stearns, G. (1938) *J. Amer. med. Ass.*, 111, 703
7. Jeans, P. C. (1950) *J. Amer. med. Ass.*, 143, 177
8. Morgan, D. B. et al. (1965) *Lancet*, 2, 1089
9. Lightwood, R. & Stapleton, T. (1957) *Ann. paed.*, 188, 270
10. British Paediatric Association (1956) *Brit. med. J.*, 2, 149
11. Fourman, P. & Morgan, D. B. (1969) *Symposium of the Group of European Nutritionists, Cambridge 1968* (in press)
12. Dent, C. E. & Smith, R. (1969) *Quart. J. Med.*, 38, 195
13. Gough, R. K., Lloyd, O. C. & Wills, M. R. (1964) *Lancet*, 2, 1261

TABLE 1. SUMMARY OF RECOMMENDED DAILY INTAKES OF ASCORBIC ACID, VITAMIN D, VITAMIN B_{12} AND FOLATE

		Ascorbic acid (mg)	Vitamin D [b] (µg) [c]	Vitamin B_{12} (µg)	Folate (µg)
Infants,	0–6 months [a]	20	10	0.3	40
	7–12 months	20	10	0.3	50
Children,	1–3 years	20	10	0.9	100
	4–6 years	20	10	1.5	
	7–9 years	20	2.5	1.5	
	10–12 years	20	2.5	2.0	
Boys / Girls	13–19 years	30	2.5	2.0	200
Adults,	men / women	30	2.5	2.0	200
	pregnancy	50 [d]	10 [d]	3.0	400
	lactation	50	10	2.5	300

[a] It is accepted that for infants aged 0–6 months breast-feeding by a well-nourished mother is the best way to satisfy the requirements of ascorbic acid, vitamin B_{12} and folate, but not of vitamin D.

[b] Adequate exposure to sunlight may partially or totally replace dietary vitamin D.

[c] 2.5 µg of cholecalciferol are equivalent to 100 IU of vitamin D.

[d] For 2nd and 3rd trimesters.

TABLE 2. RECOMMENDED DAILY INTAKES OF IRON

		Recommended intake according to type of diet		
		less than 10% of calories from animal foods (mg)	10–25% of calories from animal foods (mg)	more than 25% of calories from animal foods (mg)
Infants,	0–4 months	[a]	[a]	[a]
	5–12 months	10	7	5
Children,	1–12 years	10	7	5
Boys,	13–16 years	18	12	9
Girls,	13–16 years	24	18	12
Adults,	men / non-menstruating women	9	6	5
	menstruating women	28	19	14
	pregnancy / lactation	See text, p. 52		

[a] Breast-feeding is assumed to be adequate.

More data are also required on the "free" and "total" folate content of foods.[1] Furthermore, since many of the naturally occurring folates are

[1] The preferred method for these assays in foods and mixed diets is described in György, P. & Pearson, W. N. (1967) *The vitamins*, London, Academic Press.

EFFECT OF HYPOCALCEMIA AND VITAMIN D-INDUCED HYPERCALCEMIA ON THE INCIDENCE OF METASTASES IN RATS

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To evaluate the effect of serum calcium on tumor metastases, studies were done with Walker sarcoma on hypo-, normo-, and hypercalcemic rats. Hypocalcemia was produced by thyroparathyroidectomy, and hypercalcemia was produced by vitamin D_3 administration. No significant difference was found on the incidence of tumor takes in any group, or at any specific serum calcium level.

THE RECENT DEMONSTRATION OF A STEROL IN the plasma of breast cancer patients capable of producing hypercalciuria in the rat[8] has excited interest in the possible role of sterols in cancer growth. The experiments to be reported in this paper were designed to investigate the effects of vitamin D_3, hypercalcemia, and hypocalcemia on the incidence of tumor takes and metastases in the rat. In previous experiments, we have demonstrated that the suspension stability of tumor cells in solutions could be influenced by alteration in calcium levels in the environment.[5] That report demonstrated a plateau above a calcium level equivalent to 8.5 mg/100 ml where no further loss of suspension stability of the cells could be shown. We hoped to obtain additional information with respect to this point in this study but were unable to lower the serum calcium sufficiently to obtain the critical levels necessary.

MATERIALS AND METHODS

This study presents the results obtained from a total of 110 male, 150 g. Sprague Dawley rats 61 of which were thyroparathyroidectomized (TPT rats) prior to the start of this experiment. The completeness of thyroparathyroidectomy was ascertained by presence of low serum calcium levels in these animals. The animals were divided into 4 groups and were treated as follows: Group I—26 normal rats receiving 0.5 ml saline; Group II—51 normal rats receiving 20,000 u water soluble vitamin D_3† in each injection; Group III—23 TPT rats receiving 0.5 ml saline; and Group IV—40 TPT rats receiving 20,000 u vitamin D_3 in each injection.

Three injections were given subcutaneously on alternate days. At the end of one week each animal was explored under ether anesthesia, and 500,000 Walker sarcoma cells in 0.5 ml saline were injected into a peripheral mesenteric vein. The Walker sarcoma was obtained from an ascitic form maintained in our laboratory by repeated passage for the past 3 years. The animals were sacrificed on the 17th day after tumor inoculation, and the presence of gross tumor was noted in the liver and mesentery. Serum calcium determinations were done prior to the start of the experiment in TPT animals and at the time of tumor injection and sacrifice in all animals.

RESULTS

Effect of Vitamin D_3 Administration on Serum Calcium Levels: Table 1 shows the serum calcium levels before and after treatment in each group. It can be seen that in TPT animals treated with vitamin D_3 (Group IV), mean serum calcium rose from 5.71 ± 0.18 to 10.87 ± 0.34, while in TPT animals treated with saline (Group III), the values

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were 5.26 ± 0.14 initially and 6.76 ± 0.27 after treatment. A marked difference in serum calcium is also noted between normal animals treated with vitamin D_3 (11.3 ± 0.26) and those receiving saline (8.68 ± 0.18).

Effect of serum calcium on tumor takes: As can be seen in Table 2, the incidence of liver takes was 38.4% in Group I, 29.4% in Group II, 34.7% in Group III, and 32.5% in Group IV. Mesenteric takes were 53.8%, 66.6%, 60.8%, and 70.0% in the same groups. In our experience, we find mesenteric growth at the site of injection when a peripheral mesenteric vein is used (as in this experiment) as opposed to a large central mesenteric vein or direct intraportal injection. The differences were not significant. Fig. 1 shows the incidence according to serum calcium level rather than by group. Serum calciums in the range 5 mg/100 ml to 16 mg/100 ml could not be correlated with the incidence of tumor takes.

TABLE 2. Incidence of Tumor Takes in Rats After Intramesenteric Venous Injection of Walker Sarcoma. Number of Takes/Total Animals Inoculated.

Group	I	II	III	IV
Liver	10/26 (38.4%)	15/51 (29.4%)	8/23 (34.7%)	13/40 (32.5%)
Mesentery	14/26 (53.8%)	34/51 (66.6%)	14/23 (60.8%)	28/40 (70.0%)

DISCUSSION

The frequent association of hypercalcemia with malignancies has led some investigators to hypothesize the production of a calcium mobilizing hormone by these tumors. This hypothesis was supported by investigations of Gardner and his associates[4] who found evidence of osteolysis in analysis of the metabolism of calcium and phosphate of breast cancer patients. The demonstration of direct osteolysis by human cancer implanted to the rat calvarium[6] and the subsequent isolation of a sterol from breast cancer patients by Gordan and his associates[7] which was capable of producing hypercalcuria in the rat added further support.

Mimmoto and Honjo[10] demonstrated the enhancement of metastases in rats treated with parathyroid hormone. These investigators felt that the effect of the hormone was independent of the alteration in serum calcium produced. We, therefore, considered it worthwhile to evaluate the effects of sterol-induced hypercalcemia on the incidence of tumor takes and metastases using intraportal inoculations of Walker sarcoma. Recent similar experiments[8,9] failed to show significant effects. We used groups of thyroparathroidectomized animals to avoid the effects of calcitonin on the hypercalcemia[9] and to magnify the sterol effect. Our results clearly indicate that sterol-induced hypercalcemia does not influence tumor takes or metastases in this system.

Cancer cells have been shown to have decreased calcium content when compared to normal cells.[1] The significance of this observation is not clear. It has been demonstrated that the calcium level can influence the total net negative surface charge on the Walker sarcoma cell.[5] However, it was clear from these experiments that the effect was unimportant above a serum calcium level of 9.5 mg/100 ml. In none of our TPT animals could we lower the calcium below this value.

TABLE 1. Serum Calcium Levels in Normal (Groups I and II) and Thyroparathyroidectomized (Groups III and IV) Rats Treated with Saline (Groups I and III) or Vitamin D_3 (Groups II and IV).

Group	I	II	III	IV
Initial serum calcium	—	—	5.26 (±0.14)	5.71 (±0.18)
Serum calcium after treatment	8.68 (±0.18)	11.3 (±0.26)	6.76 (±0.27)	10.87 (±0.41)

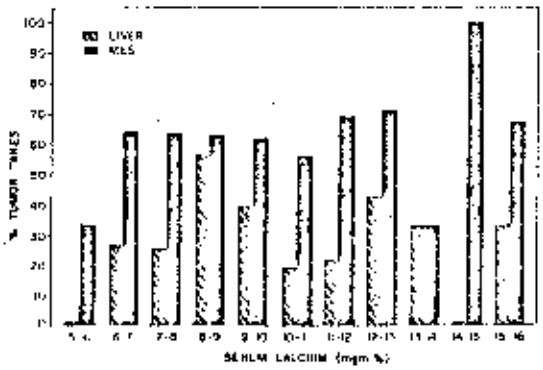

FIG. 1. Note the lack of correlation between the per cent of tumor takes and the serum calcium levels.

We are, therefore, unable to evaluate these experiments with respect to the effect of calcium levels on the net surface charge of circulating tumor cells.

At present we find no evidence to indicate that the sterol secreted by breast cancer patients may have an effect on subsequent spread of the cancer.

REFERENCES

1. DeLong, R. P., Comar, D. R., and Zeidman, I.: The significance of low calcium and high potassium in neoplastic tissue. *Cancer* 3:718, 1950.

2. Fisher, B., and Fisher, E. R.: Role of host and tumor calcium on metastases. *Cancer Res.* 28:1753, 1968.

3. Gardner, B., Eisenberg, E., and Schmidt, H.: Failure of osteolytic agents to induce skeletal metastases in rats with sarcoma. *Cancer* 18:1087, 1965.

4. Gardner, B., Graham, W. P. III, Gordan, G. S., Loken, H. F., Thomas, A. N., and Teal, J. S.: Calcium and phosphate metabolism in patients with disseminated breast cancer: effect of androgens and of prednisone. *J. Clin. Endocr.* 23:1115, 1963.

5. ———, and Stark, A. J.: Effect of calcium on the net surface charge of Walker sarcoma cells. *Surg. Forum* 18:86, 1967.

6. Gordan, G. S., Eisenberg, E., Loken, H. F., Gardner, B., and Hayashida, T.: Clinical endocrinology of parathyroid hormone excess. *Recent Progr. Hormone Res.* 18:297, 1962.

7. ———, Fitzpatrick, M. E., and Lubich, W. P.: Identification of osteolytic steroids in human breast cancer. *Trans. Amer. Ass. Physicians* 80:183, 1967.

8. ———, Cantino, T. J., Erhardt, W., Hause, J., and Lubich, W.: Osteolytic sterol in human breast cancer. *Science* 151:1226, 1968.

9. Hirsch, P. F., and Munson, P. L.: Importance of the thyroid gland in the prevention of hypercalcemia in rats. *Endocrinology* 79:635, 1966.

10. Mizumoto, R., and Honjo, T.: Parathyroid hormone and metastasis of experimental tumor. I. Effects of parathyroid hormone on the metastasis formation of experimental tumor. *Gann* 56:421, 1965.

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HYPERVITAMINOSIS D IN MONKEYS: A CLINICAL AND PATHOLOGIC STUDY *

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The Radiobiological Laboratory of the University of Texas and the United States Air Force, Austin, Texas, and the School of Aviation Medicine, Randolph Air Force Base, Texas, maintain a monkey colony in connection with an experimental program in radiobiology. Because of an error by the manufacturer of the monkey food, the entire colony received excessive amounts of calcium, phosphorus, and vitamin D for a period of nearly three months. Acute vitamin D intoxication developed and resulted in a number of deaths before the diagnosis was established. This opportunity of observing vitamin D intoxication in a large number of higher mammals was not a planned experiment, but developed accidentally. The clinical and pathologic observations made while the colony received the toxic diet and for one year thereafter are the subject of this report.

CLINICAL INFORMATION

At the onset of the high vitamin D diet, there were 558 monkeys (*Macaca mulatta*) in the colony: 283 were in the Austin colony and 275 were in a holding colony at Randolph Air Force Base. Of the 283 animals in the Austin colony, 103 had received whole-body ionizing radiation; 70 had received focal ionizing radiation to the right eye; 21 had received intravenous nitrogen mustard; 89 were control animals which had received neither ionizing radiation nor nitrogen mustard. Thirty-two of the irradiated animals had had positive skin tests for tuberculosis and had received 250 mg. of streptomycin and 59 mg. of isoniazid intramuscularly twice a week (November 2, 1953 to January 15, 1956). All of these animals were being studied to observe the latent effects of ionizing radiation or nitrogen mustard. None of the 275 animals in the Randolph colony had been utilized in the experimental program of the Laboratory. Because the data on the Austin colony are more complete, only they are reported unless otherwise specified.

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The animals varied in age from 3 to 9 years, and in weight from 2.5 to 10.0 kg. Seven of the 283 animals were female; only males are used in the experimental program of the laboratory. The animals were maintained on a daily diet consisting of approximately 114 gm. of monkey meal (whole wheat flour, 47 per cent; soy bean oil, 17.5 per cent; powdered milk, 5 per cent; bone meal, 5 per cent; molasses, 4 per cent; wheat germ meal, 4.5 per cent; cornmeal, 10 per cent; salad oil, 5 per cent; salt, 1 per cent; irradiated yeast, 1 per cent), supplemented with fresh fruit (oranges, grapefruit, apples, or bananas) and vegetables (carrots, lettuce, potatoes, or cabbage) three days per week. Because of the aforementioned error in food manufacture, the diet of the colony was altered so as to include 162,000 U.S.P. units of vitamin D per animal per day (Table I). Each also received approximately 3.5 gm. of calcium and 2.9 gm. of phosphorus per day. The animals in the Austin colony received the high vitamin D diet for 81 days (February 15 to May 7, 1956), and the animals in the Randolph colony for 86 days (January 23 to April 20, 1956) before the error was discovered and the diet was changed. On April 20, 1956, the animals in the Randolph colony, and on May 7, 1956, the animals in the Austin colony, were placed on a diet low in vitamin D. The amount of calcium and phosphorus in the diet was also reduced. In addition, each animal in the Austin colony received 50 mg. of Dicumarol (sodi-

TABLE I

*Analysis of Monkey Meal**

		A†	B†	C†
Total solids	Per cent	91.43	90.88	89.34
Moisture	Per cent	8.57	9.12	10.66
Protein	Per cent	22.19	19.47	20.17
Fat	Per cent	7.38	7.97	4.59
Ash	Per cent	6.92	7.83	5.16
Fiber	Per cent	2.81	2.85	2.40
Carbohydrate	Per cent	52.13	52.81	56.98
Calcium	Grams (per animal, per day)‡	1.54	3.50	0.87
Phosphorus	Grams (per animal, per day)	0.72	2.90	0.62
Vitamin D	U.S.P. units (per animal, per day)	4,252	165,000	40.00

* Analyses were performed by the Food Research Laboratory, Long Island City, N.Y., and by the Fourth U.S. Army Medical Laboratory, Fort Sam Houston, Texas.

† Diet A—Standard monkey diet fed prior to high vitamin D diet.

Diet B—High vitamin D diet.

Diet C—Diet fed subsequently; beginning on August 11, 1956, each animal received a vitamin supplement containing approximately 400 units of vitamin D daily.

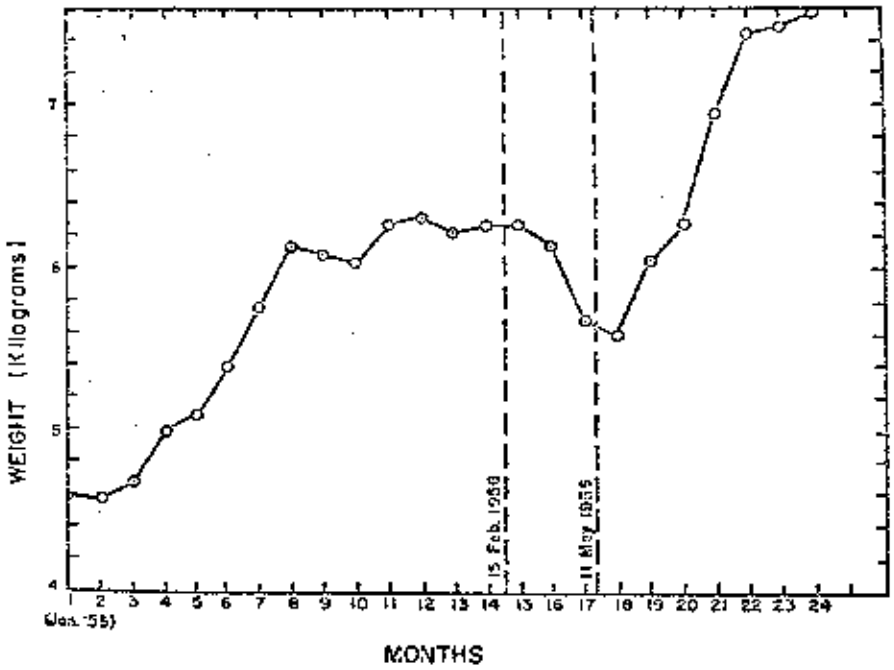
‡ Each animal received approximately 114 grams of the monkey meal per day.

8107

zoleamid... 4 cc. of aluminum hydroxide gel per day for 60 days in an attempt to maintain a slight acidosis. It was thought that this might tend to lessen the deposition of calcium in the tissues and promote its excretion. Antibiotic agents (penicillin, streptomycin, or chloromycetin) were used in the treatment of respiratory and gastrointestinal infections. A vitamin supplement was added to the diet on August 11, 1956, so that thereafter each animal would receive a total of 400 U.S.P. units of vitamin D daily.

The first clinical evidence of hypervitaminosis D was an increased incidence of upper respiratory infection and diarrhea occurring during late April and early May 1956. This was greater than was usually noted during this period of the year.

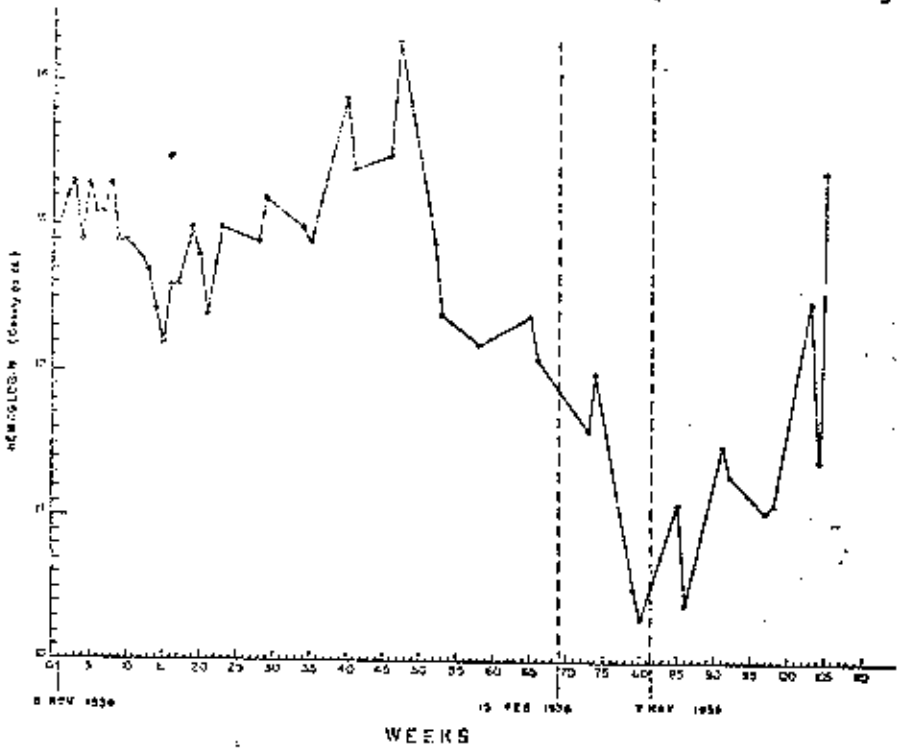
Each animal in the colony is weighed monthly. A graph of the average weight of 20 nonirradiated control animals over a period of 2 years, indicates the onset of weight loss shortly after the inception of the high vitamin D diet (diet B, Text-fig. 1). The weight loss con-



Text-figure 1. Weight of 20 nonirradiated control animals over a period of two years. The vertical lines represent the period the colony was on the high vitamin D diet. Note the weight loss during and shortly after that period.

tinued for approximately one month after diet B was terminated. Immediately following this, the entire group of animals gained weight progressively. A similar weight loss was also noted in the colony as a whole during the high vitamin D diet.

A peripheral blood count and smear are obtained on selected ...als in the colony every 2 weeks to 1 month. Most of these animals had been irradiated. However, a group of nonirradiated control animals was available for study. An appreciable decrease in erythrocytes and hemoglobin during and after the period of excess vitamin D intake was evident (Text-fig. 2). When compared to the period immediately



Text-figure 2. Average hemoglobin values of 11 nonirradiated animals over a period of two years. The vertical lines represent the period the colony was on the high vitamin D diet. Note the drop in hemoglobin during and shortly after this period.

before the high vitamin D diet, the drop in hemoglobin noted over a 28-week period starting shortly after the beginning of the toxic diet is significant to the .001 level using the *t* test of the difference for paired observations. A decrease in red blood cell counts significant to the .01 level was noted during the same period. Similar hematologic findings have been described in human patients who have received high doses of vitamin D over long periods of time.[2]

Blood urea nitrogen (BUN), calcium, and phosphorus determinations on the serum of 40 monkeys were done within two weeks after the high vitamin D diet was terminated and repeated approximately 9 months after termination of the episode. The average BUN for the groups on the first examination was 26.3 (15 to 37) mg. per hundred milliliters and 9 months later was 19.4 (11 to 24) mg. When compared

8104

to the aver JN of 10 "normal" animals, 19.8 (16 to 24) mg.,* this information suggests that a minor degree of renal failure was present in many of the animals near the end of the high vitamin D diet and that the renal function improved during the succeeding period of several months. The average serum calcium level initially (May 18, 1956) was 13.4 (11 to 17) mg. per hundred milliliters and the average serum phosphorus was 4.7 (3 to 7) mg. Nine months later, the average serum calcium value was 12.2 (10 to 14) mg., and the serum phosphorus, 6.8 (4 to 8) mg. This may be compared to values for calcium of 11.36 mg. and for phosphorus of 3.7 mg. given by Wats and Das Gupta[8] for "normal" *Macaca mulatta*. Similar elevations of BUN, calcium, and phosphorus have been described both in human subjects and in experimental animals receiving high vitamin D diets.[4-6]

Electrocardiographic tracings were done on 12 animals shortly after diet B was terminated. Two significant alterations were noted. In some, the T waves were rounded, a feature consistent with the effect of hypercalcemia. Large peaked T waves as seen with hyperpotassemia were also noted. Because of the very rapid heart rate natural in monkeys and their excitement from being handled, evaluation of an abnormality of the QT interval was extremely difficult.

Roentgenograms were made of the torso, including the proximal ends of the humeri and femurs in selected animals. These were obtained at the time the toxic diet was stopped, and at monthly intervals for 6 months. In the long bones no changes characteristic of prolonged vitamin D intoxication were seen. Soft tissue calcifications were not noted in the films, although, as will be shown later, they were observed in necropsy specimens obtained from animals dying during this period.

Catheterized urine specimens were procured from several animals immediately after the high vitamin D diet was terminated. These showed a large amount of sediment consisting chiefly of calcium phosphate crystals. Leukocytes, red blood cells, epithelial cells, and granular casts were also found.

Approximately a month after the high vitamin D diet was terminated, the surviving animals in the colony appeared to be in good health, and no symptoms which could be related to the high vitamin D diet were noted in the period that followed.

Pathologic Study

The following data are based on a study of 114 animals from the Austin colony that died or were sacrificed between June 20, 1955 and May 7, 1957. Twenty-six of these monkeys died between June 20, 1955

* Obtained from the Los Alamos Scientific Laboratories, Los Alamos, New Mexico.

and February 14, 1956, while the colony received the nontoxic diet A (Table I). These animals served as controls. Thirty-one died between February 15, 1956 and May 7, 1956, while the colony subsisted on diet B (high vitamin D). Fifty-six animals died while receiving diet C during the year following the discontinuance of the high vitamin D diet. Most of the tissue procured at necropsy from the 110 animals that died in the Randolph colony from the day that group of animals received diet B until one year later (January 23, 1956 to January 23, 1957) was also studied. The pathologic changes in the tissues from the two groups were very similar. Since the material from the Austin colony was more suitable, it only is used for this report unless otherwise noted.

All of the animals reported were necropsied and tissues were taken for microscopic examination from the parotid gland, submaxillary gland, esophagus, stomach, small and large intestine, pancreas, liver, larynx, trachea, lung, heart, aorta, kidney, urinary bladder, prostate, testis, adrenal, thyroid, pituitary, cerebrum, cerebellum, sternum, rib, vertebrae, head and shaft of the femur, lymph nodes, spleen, skin, and skeletal muscle. The eyes were examined in more than half and the parathyroid glands were available in more than three fourths of the animals. Tissues were fixed in buffered neutral formalin, embedded in paraffin, and sectioned at 6 microns thickness. Sections were stained with hematoxylin and eosin. The von Kossa method was used in demonstrating calcium in tissue.[7] Von Kossa stains were prepared on sections of the kidney, heart, and lung of all the animals reported. In 14 animals dying at the height of the vitamin D feeding episode, von Kossa stains were done on all the tissue. The alizarin red method for staining calcium[7] was applied to occasional tissues. Because the distribution of calcium was shown to be similar by the two methods, the alizarin red method was not used on all tissues. Gomori's method[8] for demonstrating iron in tissue was used regularly.

Results

The characteristic lesions found in the animals with hypervitaminosis D consisted of mineral deposits with or without associated inflammation, depending on the tissue involved. The term "mineral deposits" is used since calcium and iron were found in all of these lesions, and phosphorus was probably present in most (Table II). Other minerals may have been present, but no attempt was made to demonstrate them. In addition, an organic component was present in the lesions,

3104

particularly in the kidneys. This consisted of an amorphous, eosinophilic material which was positive with the periodic acid-Schiff (PAS) stain, metachromatic with toluidine blue and stained blue with Alcian

TABLE II

*Chemical Analysis of Kidney, Heart, and Lung for Calcium and Phosphorus**

Organ	Control animals			Hypervitaminosis D animals		
	Animal number	Calcium†	Phosphorus†	Animal number	Calcium†	Phosphorus†
Kidney	112+	2.63	2.90	V-5	10.16	3.03
	K-8	1.46	2.90	370+	8.83	4.53
	309+	3.10	2.30	Z-5	10.67	7.00
	127	0.95	6.00	116	6.00	10.80
	489	2.40	7.08	133	12.00	9.60
	686	0.48	8.76	61+	6.72	7.80
Average		1.84	4.99		10.56	7.13
Heart	112+	0.24	4.92	V-5	1.97	2.63
	K-8	1.80	0.97	370+	3.30	4.67
	309+	5.28	4.80	Z-5	1.20	3.00
	127	3.60	4.44	116	5.28	8.64
	489	3.84	4.20	133	4.44	4.56
	686	2.88	5.76	61+	2.16	4.92
Average		2.94	4.18		3.06	4.74
Lungs	112+	2.07	2.23	V-5	4.57	2.43
	K-8	1.43	2.20	370+	3.60	2.30
	309+	3.60	2.60	Z-5	2.40	6.96
	127	5.78	7.92	116	5.28	8.64
	489	2.46	6.48	133	3.84	6.00
	686	3.60	6.00	61+	6.96	2.40
Average		3.31	4.57		4.44	4.79

* All determinations were done at the Fourth U.S. Army Medical Laboratory, Fort Sam Houston, Texas, under the supervision of Capt. Arthur C. Dixon.
† All values given as mg. of calcium or phosphorus per 100 mg. of dried tissue.

blue. These characteristics suggest that this material contained an acid mucopolysaccharide. A similar material has recently been described in the soft tissue lesions of rats receiving high doses of vitamin D.[9]

The livers of two animals dying within one month after diet B was terminated, were assayed for vitamin D and compared to similar assays on two control animals and two animals which died 9 months after the termination of diet B (Table III). The vitamin D content in the first two animals was high as compared to the controls. The concentration of vitamin D in the last two animals was near the control level.

Urinary Tract

Characteristic lesions were found more consistently in kidneys than in any other tissue. An animal which died 28 days after the beginning of diet B was the first to show focal mineral deposits. The kidneys of

TABLE III

*Analysis of Liver for Vitamin D**

Animal No.	Group 1†		Group 2‡		Group 3§	
	a	b	1	2	267	639
U.S.P. units of vitamin D per gm. of fresh liver	3.5	1.4	11	10	2.1	4.2
Average	2.45		10.5		3.15	

* Bio-assays performed by Food Research Laboratories, Inc., Long Island City, N.Y.
† Two "normal" animals obtained from Okatie Farms, Hardieville, S.C.
‡ Two animals dying within one month after diet B was terminated.
§ Two animals dying nine months after the termination of diet B.

every animal that died thereafter manifested some degree of mineralization.

The earliest lesions (28 to 54 days) consisted of occasional small deposits in the tubules, often in the basement membranes. Many were not associated with an inflammatory reaction. The number of deposits increased with time so that by the 76th day, severe lesions were noted (Fig. 3). In these cases the deposits could be seen on gross examination as yellowish-white flecks in the cortex and medulla (Fig. 1). They could also be seen in postmortem roentgenograms of the kidneys (Fig. 2).

Microscopically, the deposits appeared in the tubules for the most part or in the surrounding interstitial tissue. The distal convoluted, proximal collecting, and thin loop tubules were involved most frequently. The proximal convoluted tubules and medullary collecting tubules were relatively spared. In the sections stained with hematoxylin and eosin, the deposits varied from a dark or light blue to an almost colorless appearance. Calcium and iron were demonstrated consistently in them. Foreign body type giant cells and mononuclear cells were often found in and about the deposits, and polymorphonuclear cells were noted occasionally (Fig. 4). Many of the lesions were metachromatic when stained with toluidine blue. They were also PAS positive and stained blue with Alcian blue before and after decalcification. In several cases, deposits were noted in the glomeruli and also in the walls of small arteries and veins. Hyaline casts were often present in the tubules. In some instances, the tubules above the

8104

TABLE IV

Distribution of Calcium Deposits in Heart, Kidneys, and Lungs

Organ	Animal number															
Heart	213+	55+	54+	C-8	55+	X-7	135	61+	86+	165+
Left ventricle										
Apex	+		+	+	+	+	+	+	+	
Base	+	○	○	+	+	+	○	+	+	○
Middle	+	+	+	+	+	+	+	+	+	
Right ventricle										
Apex	○	○	○	+	○	○	○	○	○	○
Base	+	○	○	+	○	○	○	○	○	○
Left atrium	+	○	○	+	+	○	○	○	○	○
Right atrium	○	○	○	○	○	○	○	○	○	○
Kidney										
Superior pole										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+
Inferior pole										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+
Middle										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+
Lung										
Upper lobe										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+
Middle lobe										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+
Lower lobe										
Right	+	+	+	+	+	+	+	+	+	+
Left	+	+	+	+	+	+	+	+	+	+

* The quantity of calcium was estimated on the basis of the amount of black precipitate in the von Kossa stained sections: ++++ = most severe calcification noted in a given organ, +++, ++, + = 75, 50, 25 per cent of the maximum.

† Tissue not available for examination.

mineral deposits were dilated. Multiple sections were ta[illegible] from both kidneys of 10 animals dying at the height of the vitamin D feeding episode. Although there was some variation in the number of calcium deposits from slide to slide, no one portion of the kidney was more consistently involved than another (Table IV).

The degree of calcification in the kidneys seemed to decrease about a month after diet B was terminated and continued to do so throughout the remainder of the year. In addition to a decrease in the number of deposits, there was a decrease in the amount of inflammatory reaction as well. For the last half of the year, no inflammation was noted in relation to the renal deposits. One animal dying in the Randolph colony approximately 6 months after the end of the high vitamin D diet showed renal lesions very closely resembling those found in human patients with malignant hypertension. As this was the only animal in which such lesions appeared, it is difficult to relate them to the high vitamin D diet.

The renal pelvis and bladder and the testes were not abnormal nor were stones found in the urinary tract. In one animal, fine granular deposits of calcium were noted in the stroma of the prostate in relation to the elastic fibers, and in two cases small deposits were found in the epithelium lining the seminal vesicles.

Respiratory System

The lungs were often affected but not as regularly as the kidneys: mineral deposits were found in the lungs of 23 of the 39 animals dying between the 55th to 208th day. The early lesions appeared in the mucosa of the small bronchi and alveolar ducts where mineral deposits frequently lay within the basement membranes. The lesions contained both calcium and iron and were often surrounded by mononuclear and foreign body giant cells. The granulomas caused deformity of the overlying epithelium in some cases and occasionally resulted in ulceration. In the animals dying near the 81st day, the deposits were more extensive. In these, the alveolar walls were incrusted with calcium and iron (Fig. 5). Also multiple small granulomas containing calcium and iron were found in the mucous membranes of the bronchi, trachea, and larynx, on and around the basement membranes. Mineral deposits were not found in animals dying in the Austin colony after the 281st day. However, 3 animals from the Randolph group that died almost one year after diet B was terminated showed extensive calcifications. Some of the deposits occurred in the lumens of the alveoli.

In the older control animals, the laryngeal cartilages were often calcified, but this was not the case in the bronchi. In the animals that

8104

received high vitamin D diet, the bronchial cartilages were frequently calcified. The first animal to show this, died on the 68th day. Twenty of the 29 animals dying between the 68th and 230th days had some calcification of the bronchial cartilages. Presumably this was related to the high vitamin D diet.

Cardiovascular System

Cardiac lesions were noted only in the animals dying between the 55th and 86th days: 9 of the 24 animals dying during this period showed lesions. Abnormality was recognized grossly in all 9 animals. This consisted of a scattered grayish-yellow mottling of the myocardium (Fig. 6). Microscopically, the lesions were focal and exhibited calcium and iron deposits. The deposits consisted of fine granules or irregular plaques along the muscle fibers. Evidence of myofibril degeneration and necrosis was found in these areas (Fig. 8). Mononuclear cells also were often found, although the inflammatory reaction was not marked. Foreign body giant cells were seen only occasionally. In the animals with minimal myocardial damage, the lesions were predominantly subendocardial in location. In the more severe cases, lesions were found throughout the myocardium of the left ventricle (Fig. 7). In these cases, intramyocardial vessels were often mineralized, sometimes partially necrotic and occasionally the seat of thrombosis. Multiple sections of the heart in the 9 cases showed that the lesions were always in the left ventricle, occasionally in the left atrium and right ventricle, but never in the right atrium (Table IV).

Focal areas of scarring and chronic myocarditis were found in the left ventricle of 2 animals dying almost a year after the termination of the high vitamin D diet. Although no calcium was seen in these lesions, their distribution suggested that they might represent the residuals of myocardial damage similar to that seen in animals dying between the 55th and 86th days.

Mineral deposits were also found in the aortas of 12 of 34 animals dying between the 55th and 140th days. The deposits were predominantly in the media along the elastic fibers. The lesions were not seen on gross examination and there was no loss of aortic elasticity. In animals from Randolph Field, gross aortic calcifications were found twice, and in the aorta from a third case, a calcified area was surrounded by a dense infiltration of neutrophils. This represented the sole instance of aortic mineralization associated with inflammation.

If mineralization of small vessels in the presence of massive parenchymal calcification be excluded, disseminated lesions of small arteries in soft tissues were encountered in only 3 animals: one animal from the Randolph Field colony and one from the Austin colony died near the end of the high vitamin D diet. The third animal was from the Randolph group and died 11 months after the termination of diet B. The deposits lay along the internal elastic laminas in all cases (Fig. 9).

Digestive Tract

Next to renal involvement, the earliest and most common evidence of calcification was found in the salivary glands: 27 of 43 animals dying between the 47th and 226th days had these lesions. The submaxillary glands were involved almost twice as commonly as the parotid glands. The deposits appeared in the walls or lumens of small ducts (Fig. 10). Duct dilatation was found occasionally, in both the presence and the absence of demonstrable ductal obstruction.

Mineral deposits were noted in the mucosa of the stomach of 10 animals. In most cases they were scanty, but in one they were extensive (Fig. 12). In the latter, the muscularis mucosae was involved but not the other muscle layers. The mineral deposits were often associated with foreign body granulomas. Similar mineral deposits were noted in the jejunum of one animal and the colons of 2 animals. In all 3 cases, the deposits were slight and not associated with an inflammatory reaction.

Lesions attributable to hypervitaminosis D were not found in any of the sections taken from the tongue, esophagus, liver, and pancreas.

Central Nervous System

Several months after the termination of diet B, 6 animals which had received high doses of x-ray irradiation to the head died. Necrotic areas were found in the path of irradiation through the brain. Calcium and iron deposits were found in these. Similar deposits have been noted in monkeys receiving focal ionizing irradiation to the head in the absence of a high vitamin D diet.[10] Other evidences of calcification were not noted in the central nervous system.

Endocrine Glands

Although sections were examined from the pituitary, thyroid, and pancreas in all cases, no mineral deposits were noted in these areas. Laminated deposits were frequently found in the inner portion of the adrenal cortex (Fig. 11), but these were present equally frequently in the control animals.

Although the parathyroid glands were not weighed, they did not appear to be enlarged. They were composed characteristically of uniform cells with dark-staining nuclei and scant cytoplasm. The average

8104

diameter 50 cells from 4 control animals and 4 animals dying at the height of the vitamin D episode were compared. There was no significant difference between the 2 groups as has been described in the dog.[11]

Musculo-skeletal System

There was little evidence of destruction in the sections of bone examined. In one animal the lacunas in the vertebrae were larger than those seen in other cases: however, there was no evidence of an increase in the number of osteoblasts, nor was there any gross evidence of demineralization. The paucity of bone lesions in these animals may be due to the high calcium and phosphorus content of the diet. The bone marrow in a number of cases was atrophic, although this could be related either to high doses of whole-body ionizing radiation or to the administration of nitrogen mustard. Mineral deposits were not noted in the sections of skeletal muscle which were usually taken from the sartorius muscle.

Other Tissues

Although sections were examined from the skin, testes, spleen, lymph nodes, and occasionally from the eyes, mineral deposits as perceived in the other soft tissues were not found.

Discussion

Since hypervitaminosis D was first noted in 1928, it has been described in a number of species, including man.[12-14] Only two reports were found in the literature dealing with this condition in monkeys. Hess and Lewis[15] produced it in monkeys but they were interested primarily in the blood calcium and did not describe the pathologic lesions. The 19 monkeys reported by Cowdry and Scott[16] apparently received more vitamin D than our animals. Despite this, the mineral deposits in the soft tissue were minimal. The high calcium and phosphorus content of the diet of our animals may explain the extensive deposits found in the soft tissue of many.

The quantity of vitamin D received by the animals included in this report was well above the toxic dose. Despite this, 28 days elapsed before any pathologic lesions attributable to hypervitaminosis D appeared in the tissues. It was 55 days before really severe lesions were noted. The alterations were most severe in animals dying about the time the high vitamin D diet was terminated and for one month thereafter. The amount of calcification and the number of organs affected decreased with time, so that animals dying one year after the termination of diet B showed slight mineralization. The animals dying in the Randolph Field colony generally revealed more severe lesions. This

was particularly evident in the necropsy examination of animals during the last half of the year following the termination of diet B. Three animals from the Randolph group during that period had extensive mineral deposits in the lungs, as well as in the kidneys. Two factors probably contributed to the difference in the two groups: first, the Randolph group was on diet B for 5 days longer; second, the Randolph group was housed outdoors and was thus exposed to the sun, whereas most of the Austin colony was maintained indoors.

The distribution of the lesions in these monkeys was similar to that described in other species. However, the degree of vascular involvement seemed to be less than that described in rodents.

The reaction of the tissues to the mineral deposits varied considerably. The deposits in the kidneys, mucosa of the stomach, and the mucosa of the respiratory tree usually were surrounded by mononuclear cells and foreign body giant cells forming small granulomas which distorted the tissues. On the other hand, there was usually no inflammatory reaction to the mineral deposits in the aorta and salivary gland. The muscle fibers in the heart containing mineral deposits showed evidence of degeneration or were frankly necrotic. A mononuclear cell reaction was frequently seen in the myocardium but was usually mild, and foreign body giant cells were observed only occasionally.

The reports in the literature dealing with the pathologic alterations of hypervitaminosis do not mention the presence of inorganic iron in the lesions as observed in our animals. The presence of iron in the mineral deposits raises the possibility that there was systemic disturbance of iron metabolism in addition to that of calcium and phosphorus. This is not likely, for as Bunting[17] has pointed out, iron is usually found in soft tissue calcifications regardless of the cause of the lesions.

Mulligan[18] has suggested that the tissues where acids are formed, are rendered alkaline when these acids are secreted so that calcium deposition is favored. The high incidence of calcification of the acid-secreting kidneys, mucosa of the stomach and lungs, and the absence of calcification of the alkaline-secreting pancreas and liver in our animals adds support to this idea. The tendency for the calcifications to be greater in the left ventricle and atrium than in the right ventricle and atrium, as noted in our animals and as has been reported in other species, may be explained on the same basis; that is, the pH of the arterial blood is higher than the venous blood.

Most of the animals reported on here which died spontaneously, succumbed as a result of acute infection of the lungs or colon. Though the toxic amounts of vitamin D may have lowered the resistance of the colony to infection, it is not unusual to have monkeys in the colony

3104

die of pneumonia or acute colitis, particularly in the spring. Some of the animals apparently died of hypervitaminosis D. In these, the calcification and degeneration in the myocardium were extensive, and there was evidence of congestive heart failure: dilatation of the ventricles, congestion and edema of the lungs, and congestion of the liver. The renal lesions in a number of the animals were extensive, and renal failure was probably a lethal factor. It is difficult to evaluate this, as studies of renal function were not done on any of the animals that died. Blood urea nitrogen determinations were done on 40 animals which did not die, and the levels were elevated in many.

Because many of the animals had received some type of ionizing radiation or nitrogen mustard, the relationship to hypervitaminosis of some of the pathologic lesions found at necropsy was doubtful. Hypoplasia of the bone marrow, atrophy of the spleen, lymph nodes, and thymus are features attributable to radiation or nitrogen mustard administration and have not been described in hypervitaminosis D in other species. When these lesions were found in our animals, they therefore could be related in every case to either ionizing radiation or the administration of nitrogen mustard. On the other hand, widespread soft tissue mineralization accompanied by inflammation or degeneration is not an anticipated reaction to irradiation or nitrogen mustard. When appearing in animals receiving a diet high in vitamin D and not in the control animals, this obviously reflects the effect of the high vitamin D diet.

Summary

A colony of 558 monkeys (*Macaca mulatta*) was inadvertently given a diet high in vitamin D for a period of approximately 3 months. The clinical and pathologic findings noted in these animals during the period of the high vitamin D diet and for a year thereafter have been described, and compared to those reported in other species. There were weight loss, anemia, elevation of blood urea nitrogen and serum calcium, and an increased incidence of diarrhea and upper respiratory infections during the period of acute exposure. Lesions were most common in the kidney, salivary gland and lung, and consisted of calcium and iron deposits which were often associated with a foreign body type reaction. The animals examined a year after the high vitamin D diet was terminated showed few lesions.

References

1. Kleiner, I. S. Human Biochemistry. C. V. Mosby Co., St. Louis, 1945. 573 pp.
2. Scharfman, W. B., and Prego, S. Anemia associated with vitamin D intoxication. *New England J. Med.*, 1956, 255, 1207–1212.

3. Wats, R. C., and Das Gupta, B. M. Comparative biochemical f[illegible]s in blood of normal and malaria-infected monkeys. *Indian J. M. Research*, 1934, 21, 475–481.
4. Adams, F. D. Reversible uremia with hypercalcemia due to vitamin-D intoxication. *New England J. Med.*, 1951, 244, 590–592.
5. Albright, F., and Sulkowitch, H. W. The effect of vitamin D on calcium and phosphorus metabolism: studies on four patients. *J. Clin. Invest.*, 1938, 17, 305–315.
6. Steck, I. E., Deutsch, H., Reed, C. I., and Struck, H. C. Further studies on intoxication with vitamin D. *Ann. Int. Med.*, 1937, 10, 951–964.
7. Pearse, A. G. E. Histochemistry, Theoretical and Applied. J. & A. Churchill, Ltd., London, 1953, 530 pp.
8. Gomori, G. Microscopic Histochemistry: Principles and Practice. University of Chicago Press, Chicago, 1952, 273 pp.
9. Eisenstein, R., and Groff, W. A. Experimental hypervitaminosis D: hypercalcemia, hypermucoproteinemia, and metastatic calcification. *Proc. Soc. Exper. Biol. & Med.*, 1957, 94, 441–444.
10. Vogel, F. S., and Pickering, J. E. Demyelinization induced in the brains of monkeys by means of fast neutrons. *J. Exper. Med.*, 1956, 104, 435–442.
11. Mulligan, R. M., and Stricker, F. L. Metastatic calcification produced in dogs by hypervitaminosis D and haliphagia. *Am. J. Path.*, 1948, 24, 451–473.
12. Bellman, S., and Engfeldt, B. Kidney lesions in experimental hypervitaminosis D. *Am. J. Roentgenol.*, 1955, 74, 288–294.
13. Pfannenstiel, W. Weitere Beobachtungen über Wirkungen bestrahlten Ergosterins im Tierversuch. *München med. Wchnschr.*, 1928, 75, 1113–1114.
14. Morgan, A. F., Axelrod, H. R., and Groody, M. The effect of a single massive dose of vitamin D_2 on young dogs. *Am. J. Physiol.*, 1947, 149, 333–339.
15. Hess, A. F., and Lewis, J. M. Clinical experience with irradiated ergosterol. *J. A. M. A.*, 1928, 91, 783–788.
16. Cowdry, E. V., and Scott, G. H. Effect on monkeys of small doses of a concentrated preparation of viosterol. *Arch. Path.*, 1936, 22, 1–23.
17. Bunting, H. Histochemical analysis of pathological mineral deposits at various sites. *A. M. A. Arch. Path.*, 1951, 52, 458–469.
18. Mulligan, R. M. Metastatic calcification. *Arch. Path.*, 1947, 43, 177–230.

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Legends for Figures

Fig. 1. Kidney. White flecks of calcium are evident, particularly in the cortex. × 2.5.

Fig. 2. Postmortem roentgenogram of the kidney. Note the small opacities throughout the kidney. × 2.5.

Fig. 3. Kidney. Mineral deposits are widely distributed. Von Kossa stain. × 6.

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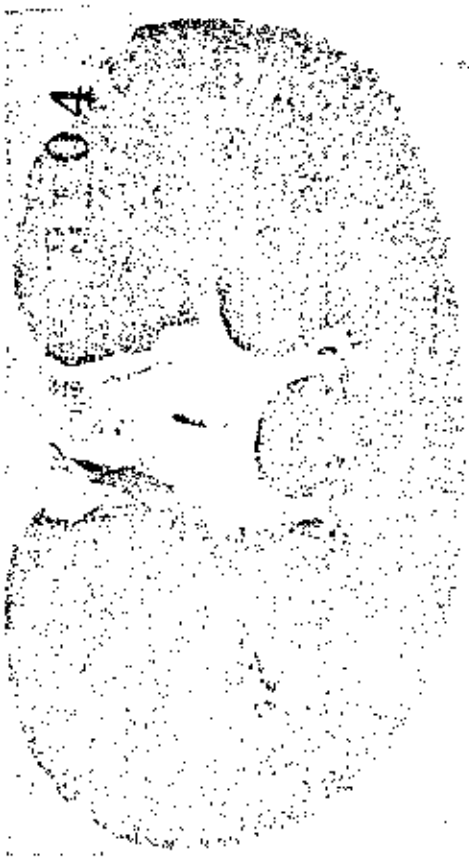

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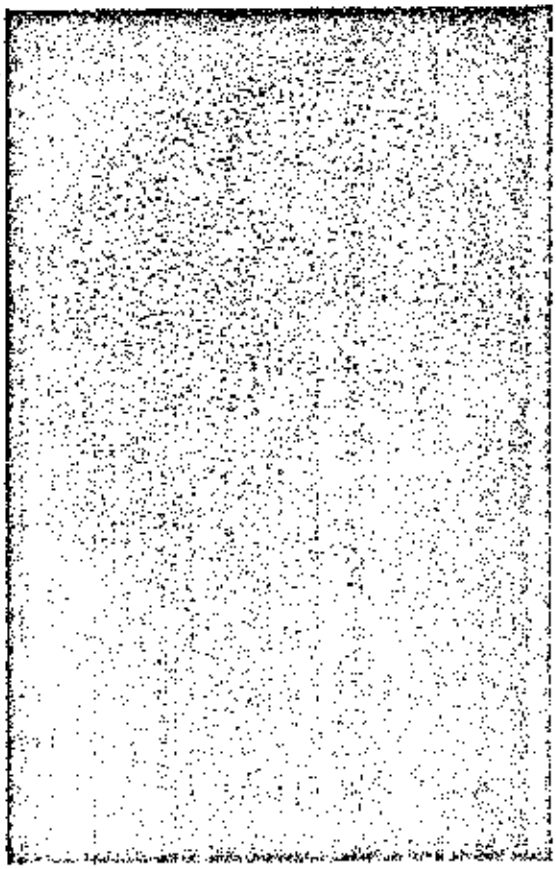

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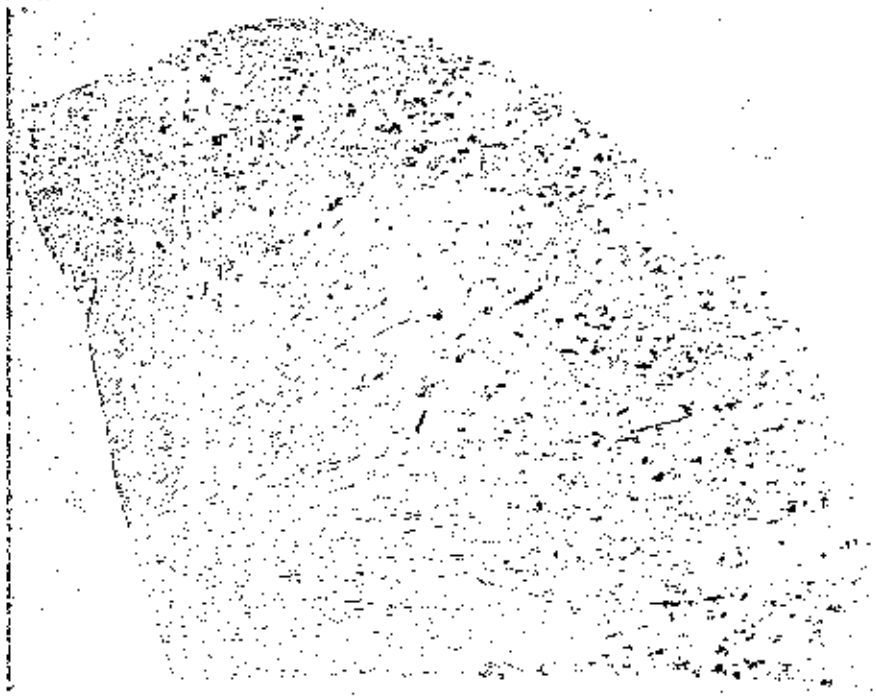

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FIG. 4. Kidney showing granulomatous reaction to the mineral deposits. Hematoxylin and eosin stain. × 250.

FIG. 5. Lung showing extensive calcium deposits. Von Kossa stain. × 6.

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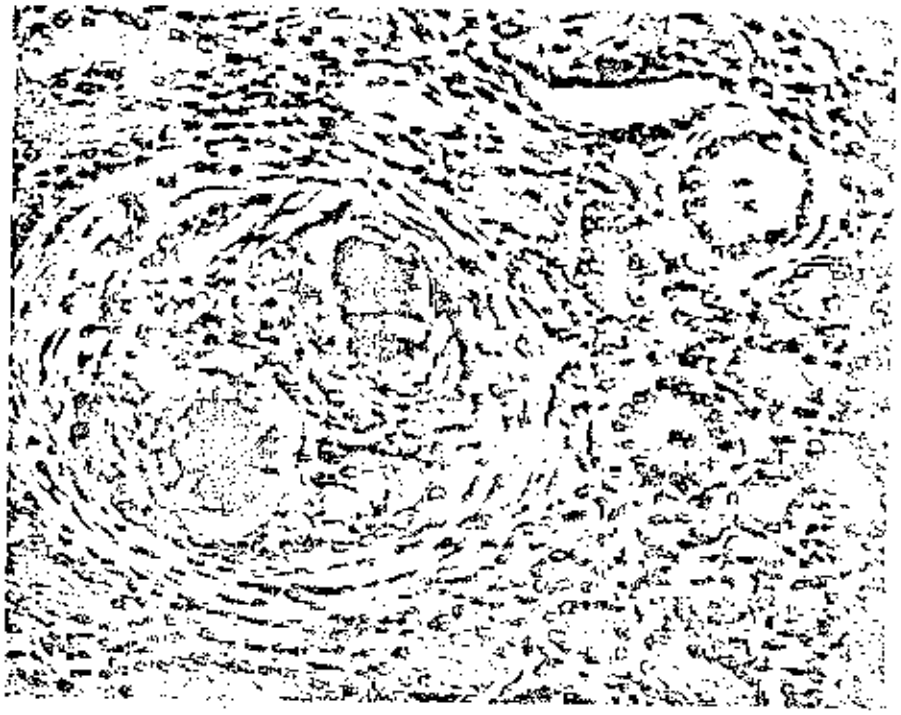

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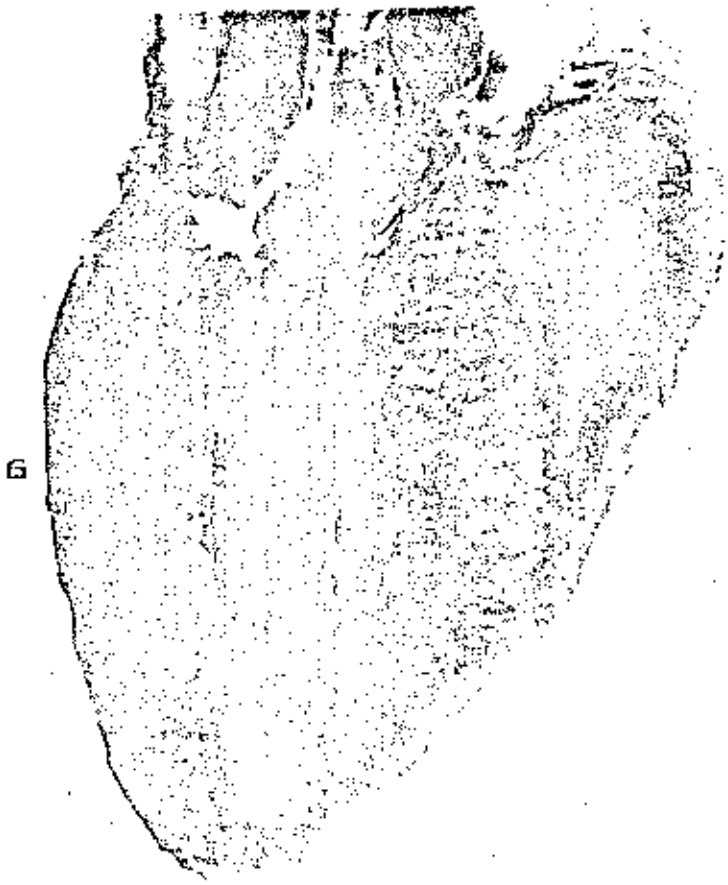

FIG. 6. Heart. Note the mottled appearance of the myocardium. × 2.5.

FIG. 7. Heart. Extensive calcium deposits are found throughout the myocardium. Von Kossa stain. × 12.

FIG. 8. Heart showing calcification and atrophy of muscle fibers. Hematoxylin and eosin stain. × 93.

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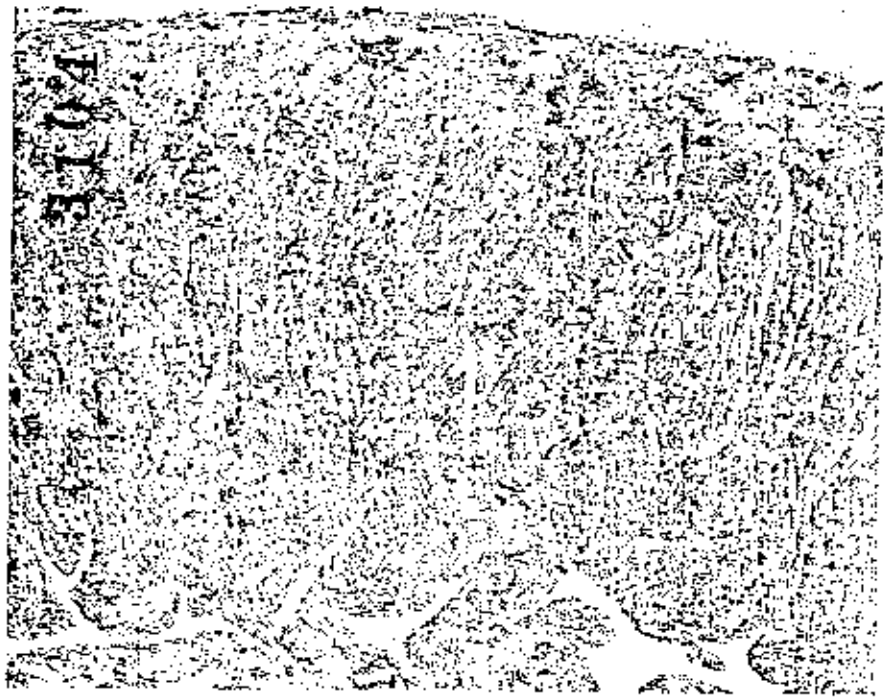

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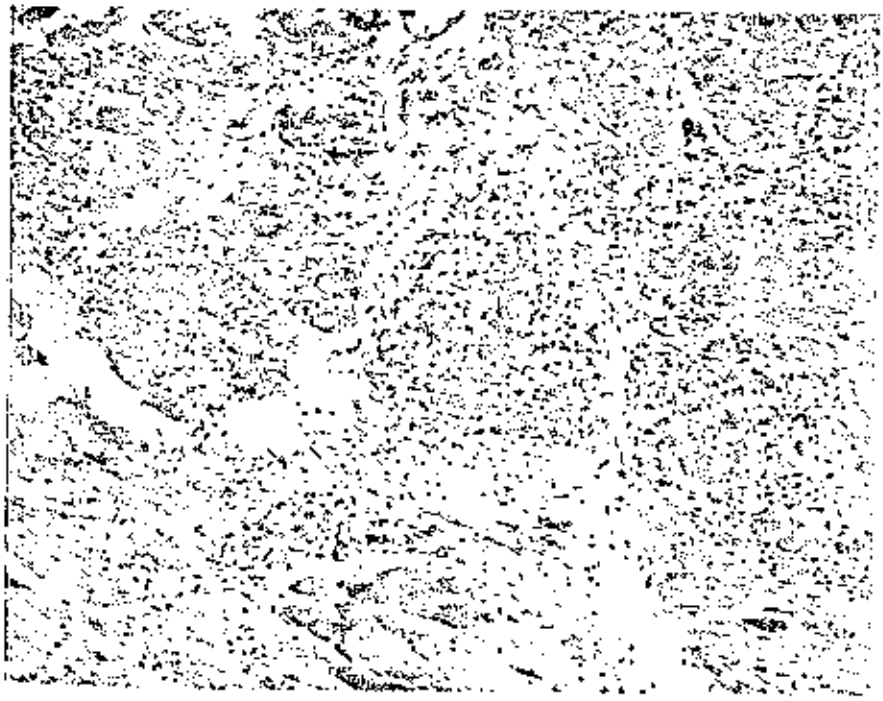

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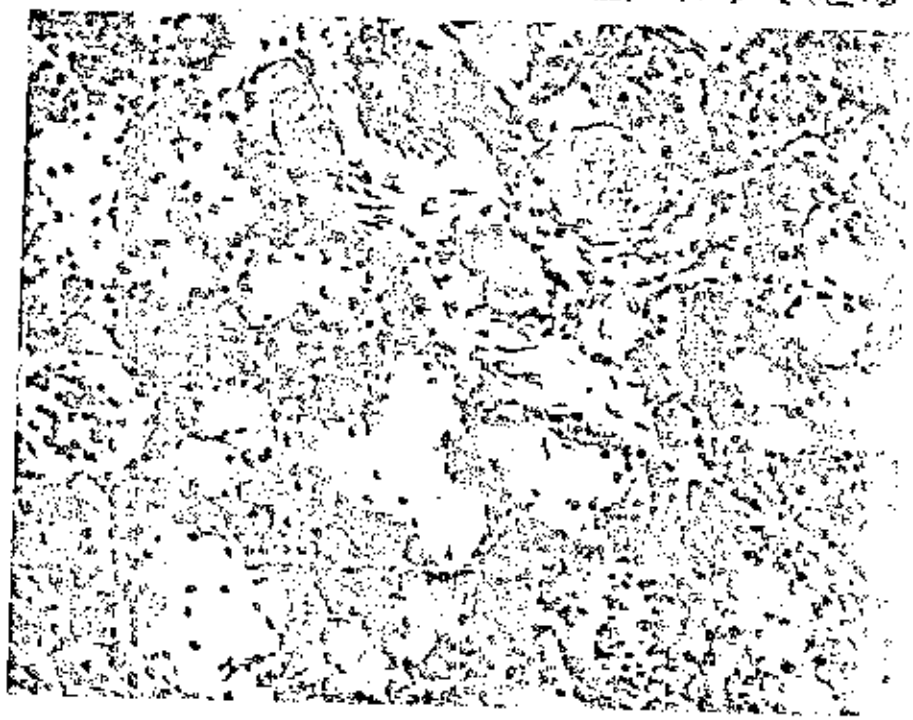

FIG. 9. Small artery from tissue adjacent to an axillary lymph node. The internal elastic lamina is calcified. Von Kossa stain. × 220.

FIG. 10. Submaxillary gland. A mineral deposit is found in a small duct. Hematoxylin and eosin stain. × 150.

11

12

FIG. 11. Adrenal. A calcified mass in the inner portion of the cortex. Hematoxylin and eosin stain. × 60.

FIG. 12. Stomach. Note the extensive calcification in the mucosa. Von Kossa stain. × 85.

Russian Translation

Hypervitaminosis D and Idiopathic Hypercalcemia in Young Children (Infants)

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It is known that vitamin D is stored in the organism and that its excessive and prolonged use may cause toxic phenomena. Most pharmacology textbooks (manuals) state that the toxic dose of vitamin D is several hundred or thousand times greater than the therapeutic dose, and therefore hypervitaminosis D is practically not observed in children. On the basis of observations made at the Tartu City Children's Clinical Hospital during the past years, we cannot agree with this statement. The following example is given.

Kay R., 14 months old. In view of the early onset of rickets, the mother gave to the child for 4 months an alcoholic solution of vitamin D in a total amount of 7.5 million units, i.e. an average of 62,500 units daily. Clinical symptoms of hypervitaminosis were noted during the 3rd month, when the child had received a total of 6 million units of vitamin D. The child showed a loss of appetite, constipation and apathy, and had a subfebrile temperature. Vomiting was rarely observed. The child showed a weight loss of 2100 g during the last 2 months.

Upon being admitted to the hospital, the little girl was apathetic; sometimes capricious, did not want to play, and did not stand up. A complete loss of appetite, strong constipation, exsiccation (desiccation),

an extraordinary dryness of the skin, and a subfebrile temperature in the evening were noted. The Sul'kovich reaction in the urine was strongly positive; the amount of inorganic calcium in the blood plasma was 20 mg %, and the ESR (erythrocyte sedimentation rate) varied from 10 to 42 mm/hr according to Panchenko. Traces of protein were found in the urine and usually individual leucocytes, but sometimes large numbers of leucocytes were also found, and 0 to 15 erythrocytes were observed in the field of vision under large magnification. The arterial pressure was elevated up to 110/90 mm mercury column. After administration of vitamin D was stopped, pathological elements were still observed in the urine for another month, the amount of blood calcium remained high for 1 1/2 month, the subfebrile temperature stayed for 2 months, and the accelerated ESR-for 2 1/2 months.

Thus, only a 10-fold excess of the daily therapeutic dose of vitamin D for 4 months caused a severe hypervitaminosis D in the child. According to the data of V.A. Vlasov and V.K. Stolyarova, no parallel relationship was observed between the amount of introduced vitamin D and intoxication phenomena.

In recent years, data have been published on a special nosological unit in young children (infants), the so-called idiopathic hypercalcemia (R. Lightwood, J. M. P. Dawson et al., K. G. Lowe et al., D. G. Greery and D. W. Neill et al., I. O. Forfar et al.). In this disease, a hypercalcemia (12-20 mg %) is noted in the children, while the content of inorganic phosphates and alkaline phosphatase in the blood serum is normal. The blood alkali reserve is usually normal, and the content of $gamma_2$-globulins is high. A hypercholesterolemia was noted by many authors. Such a condition was observed in children during normal or slightly increased dosage of

vitamin D. The hypercalcemia syndrome is clinically expressed, and for this reason the pattern of the disease is similar to hypervitaminosis D. In severe forms of this disease, an inhibition of the growth and mental development of the child can be noted, as well as phenomena of a general visceral calcinosis. Severe forms of this disease may have a fatal outcome. (Th. Stapleton).

In the so-called idiopathic hypercalcemia, a high intestinal resorption of calcium is noted. Under normal conditions, 35% calcium from cow's milk is absorbed, up to 50% is absorbed when vitamin D is used, and up to 60% calcium is absorbed in idiopathic hypercalcemia (H. G. Morgan et al.). The reasons for the appearance of this disease have not yet been fully clarified. The opinion has been expressed that one of the most frequent causes is the individual greater sensitivity to vitamin D, since a worsening of the disease is noted when this vitamin is taken and an improvement when the intake of vitamin is stopped. The consumption of food with a high calcium content contributes to the development of idiopathic hypercalcemia, and for this reason the disease occurs primarily in artificially fed children, since the calcium content in cow's milk is 4 1/2 times greater than in maternal milk. The consumption of food with an alkaline residue and the use of alkalizing drugs also contribute to the development of hypercalcemia.

The following observations show how different is the reaction of the organism to the introduction of vitamin D.

Khel'gi K., 6 months old. The mother, on her own initiative, gave the child with flourishing rickets an oil solution of vitamin D for 4 months, in a total dosage of 2.5 million units, i.e. on the average 20,833 units daily. After 2 1/2 months, the child became restless and lost its apetite,

followed later by vomiting and constipation. Starting at the age of 4 months, the child was kept on mixed feeding, and at 5 1/2 months - on artificial feeding.

Upon admittance to the hospital, the following symptoms were noted: apathetic child, smiling very rarely, with a dry skin, lowered tissue turgor, first-degree hypotrophy (subnormal growth), lack of appetite, furred tongue, vomiting almost after each feeding, constipation, subfebrile temperature. The Sul'kovich reaction was positive, the amount of calcium in the blood serum was 15.5 mg %, the ESR was 35 mm/hr. No pathological changes in the urine. After the intake of vitamin D was stopped, the mood of the child gradually improved, vomiting stopped, and a weight gain was noted. The blood calcium content returned to normal after 1 month, and the ESR became normal 2 months after intake of vitamin D was stopped.

Thus, only a two-fold excess of the vitamin D dose for 4 months caused a state of hypercalcemia in a child kept on mixed feeding.

Val'deko K., 10 months old. During the past 4 months, the mother regularly gave the child, in view of a light case of rickets, twice a day 12 drops of an oil solution of vitamin D (50,000 units in 1 ml), thus 40,000 units daily. During 4 months, the child was given 2 1/4 flasks of an oil solution of vitamin D, amounting to 3,375,000 units. No clinical symptoms of hypervitaminosis D were noted in this child. The calcium content in blood serum was 11.4 mg %. Only in a X-ray photograph could a more intensive decalcification be observed in the epiphyses of tubular bones.

In this case, even a 10-fold increase in the vitamin D dose for 4 months did not cause any clinical symptoms of hypervitaminosis D in the 10-month-old child.

The above examples show how different can be the reaction of the organism to the introduction of vitamin D. This depends, in all probability, on the individual characteristics of the organism, and on the state of increased sensitivity or resistance of the organism.

The above literature and clinical data show that hypercalcemia phenomena can arise also at a lower dosage of vitamin D. If, during the prevention or treatment of rickets with vitamin D, a loss of appetite without any visible reasons is noted in the child, as well as apathy and a weight loss, the supply of vitamin D should be stopped immediately. In addition, the calcium content of the blood should be determined, and X-ray photographs should be taken of the epiphyses of tubular bones.

The treatment of hypervitaminosis D and the so-called idiopathic hypercalcemia should be directed at reducing the hypercalcemia. The following measures are used by us. The administration of vitamin D and calcium preparations is stopped immediately, and also ultraviolet irradiation. The food should have a low calcium content (less than 300 mg daily), but should have a sufficient amount of calories and a sufficient content of animal protein to restore the weight of the patient. Suckling infants should preferably be given maternal milk, having a calcium content of 25 mg %, while the calcium content of cow's milk is 115 mg %, i.e. 4 1/2 times greater. In order to reduce the amount of calcium in cow's milk, the use of ion-exchange resins is suggested (E.N. Khokhol et al., A.V. Ferguson, C.K. McGowen, G. Anderson et al., H.G. Morgan, Th. Stapleton, et al.). At present, a method has been developed in our faculty (Kh. Yu. Kuus) for obtaining cow's milk, in which the amount of calcium has been reduced by a factor of 3-4 with the aid of the KU-1 exchange resin.

The use of food products with a high calcium content should be avoided, such as cheese, egg yolk, canned fish, caviar, beans, cauliflower, spinach, almonds. For drinking and the preparation of food, distilled water is recommended, since the usual tap water contains about 5-20 mg % calcium. The patient should drink large amounts of water to prevent the development of nephrocalcinosis.

To reduce calcium absorption, the use of cortisone is recommended, which in contrast to the action of vitamin D, inhibits the intestinal absorption of calcium. To improve the metabolism, it is suggested that the patient be given sufficient amounts of vitamins (A, B_1, B_2, PP), which have a regulating affect on the metabolism.

When the doctor prescribes vitamin D preparations for children, he should indicate the exact dose for the course of treatment and warn the parents about its harmful effect when used in excess. In order to achieve an exact dosage, a prescription should be issued both for the pipet and for vitamin D.

Large doses of vitamin A inhibit the development of hypervitaminosis D (S.N. Matsko); for this reason, in the treatment of rickets, together with large doses of vitamin D, it is advisable to administer simultaneously vitamin A in daily doses of 5000 to 10,000 units (E.M. Lepskii).

Conclusions

1. Symptoms of hypervitaminosis D can develop even with insignificant overdosages of vitamin D.

2. The sensitivity of children to vitamin D can be very different individually.

3. During treatment with vitamin D, a careful check (observation) of the child is necessary, and the supply of vitamin D should be stopped with the appearance of the first symptoms of hypercalcemia.

4. When vitamin D preparations are prescribed for children, the parents should be warned about the danger of overdosage.

Bibliography

1. Vlasov V.A., Stolyarova V.K. Voprosy okhr. mat. i det. 1957, No. 2, p. 11.
2. Lepskii E.M. Hypovitaminoses in childhood, Moscow, 1953, p. 71-72 (in Russian).
3. Matsko S.N. In book: Contemporary problems of Soviet vitaminology, Moscow, 1955, p. 182 (in Russian).
4. Khokhol E.N., Ott V.D., Stal'nenko E.S. et al. Abstracts of reports given at the 8th All-Union Congress of Pediatricians, Leningrad, 1957, p. 13 (in Russian).

Summary

The analysis of clinical cases shows that even a two-fold increase of vitamin D given for a long period of time may cause a severe D hypervitaminosis. The individual sensitivity of vitamin D in children is quit different. Pertinent literature data presents a special nosological entity in children of early age -- the so-called idiopathic hypercalcination. One of the most frequent causes of this disease is the individual elevated sensitivity to vitamin D. Hence, when instituting vitamin D treatment, special attention should be paid to the child's condition, and upon appearance of the first symptoms of hypercalcination the administration of vitamin D must be stopped.

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Андреев А. Ф. Труды
ю, 1926, № 1, стб. 24.—
ина при сердечно-сосуди-
Сатаки Е. В. Труды
Чопелева Н. Н. Те-
, 1954, стр. 154.—

Марголяна М. И. Врач. дело, 1939, № 5, стб. 353. — Мартынюк А. Г. Клин. мед., 1937, № 6, стр. 724. — Меерзон Т. И. Роханская Е. Т. Там же, 1938, № 3, стр. 355. — Назиров М. Р. Там же, 1936, № 5, стр. 653. — Николаева М. М. Труды Московск. обл. научн.-исслед. клинического ин-та, 1951, в. 5, стр. 30. — Самарин Г. А., Мясников А. Л. Клин. мед., 1931, № 17, стр. 746. — Слуцкая Ц. С., Ильяшенко М. К. Тер. архив, 1940, № 1, стр. 106. — Тареев Е. М. Труды Моск. обл. Научн.-исслед. клинического ин-та, 1951, в. 5, стр. 114. — Цвиликовская Э. Е., Шувалова Е. М., Халабузарь А. Н. Клин. мед., 1946, № 8, стр. 52. — Tacats G., Trump R. A., Gilbert N. C., JAMA, 1944, v. 125, p. 840. — Macht D. I. Ibid, 1952, v. 148, p. 265.

THE INFLUENCE OF DIGITALIS, STROPHANTHIN AND MERCUSAL ON THE THROMBOGENIC PROPERTIES OF THE BLOOD IN PATIENTS WITH CARDIAC INSUFFICIENCY

I. N. Yakovleva

Summary

The paper presents investigations on the blood coagulation, prothrombin index, bleeding time and thrombocyte count carried out in 96 patients suffering from rheumatic heart defect and atherosclerotic cardiosclerosis with circulatory insufficiency of different stages, who were subjected to digitalis or strophanthin treatment. When the effect of treatment was good there was observed in almost 50% of patients an increase of the prothrombin index and shortening of the blood coagulation time which, however, were within normal values. These changes are associated, apparently, with the improved function of the liver.

Mercusal did not produce any noticable changes in the blood coagulation indices.

О ГИПЕРВИТАМИНОЗЕ D И ИДИОПАТИЧЕСКОЙ ГИПЕРКАЛЬЦЕМИИ У ДЕТЕЙ РАННЕГО ВОЗРАСТА

Кандидат медицинских наук *Л. М. КЕРЕС*

Из кафедры педиатрии (зав. — кандидат медицинских наук З. П. Саар) Тартуского государственного университета на базе Тартуской городской детской больницы (главный врач Х. О. Кяпра)

Как известно, витамин D накапливается в организме, и чрезмерное и длительное его применение может вызвать токсические явления. В большинстве руководств по фармакологии указывается, что токсическая доза витамина D превышает в несколько сот или тысяч раз терапевтическую дозу и поэтому практически D-гипервитаминоза у детей не наблюдается. На основании наблюдений, проведенных в Тартуской городской детской клинической больнице за последние годы, мы с этим не можем согласиться. Приведем пример.

Кай Р., 1 года 2 месяцев. Мать давала ребенку по совету окружающихся рахита в течение 4 месяцев спиртовой раствор витамина D, всего 7,5 млн. единиц, т. е. в среднем по 62 500 единиц в день. Клинические явления гипервитаминоза появились на 3-м месяце, когда ребенок получил в общей сложности 6 млн. единиц витамина D. У ребенка пропал аппетит, появились запоры, апатия, субфебрилитет. Рвота наблюдалась редко. Падение веса за последние 2 месяца 210 г.

При приеме в больницу состояние было апатичным, иногда капризничала, играть не хотело, на ноги не вставала. Наблюдались полное отсутствие аппетита, сильный запор, эксикоз, необычная сухость кожи и по вечерам субфебрильная температура. Реакция Сулковича в моче была резко положительной, в сыворотке крови количество неорганического кальция составляло 20 мг%. РОЭ колебалась в пределах от 10 до 42 мм в час по Панченко. В моче обнаруживались следы белка, обычно единичные лейкоциты, но иногда лейкоциты встречались и в большом количестве, эритроциты от 0 до 15 в поле зрения под большим увеличением. Артериальное давление повышено до 110/90 мм ртутного столба. После прекращения применения витамина D патологические элементы наблюдались еще в течение месяца, количество кальция в крови было повышенным в течение 1½ месяцев, субфебрильная температура держалась в течение 2 месяцев и ускоренная РОЭ — 2½ месяца

Таким образом, только десятикратное превышение суточной терапевтической дозы витамина D в течение 4 месяцев вызвало у ребенка тяжелый D-гипервитаминоз.

По данным В. А. Власова и В. К. Столяровой, параллелизма между количеством введенного витамина D и явлениями интоксикации не наблюдалось.

За последние годы появились данные об особой нозологической единице у детей раннего возраста, так называемой идиопатической гиперкальцемии (R. Lightwood, J. M. P. Dawson и др., K. G. Lowe и др., D. G. Greery и D. W. Neill и др., I. O. Forfar и др.). При этом заболевании у детей наблюдается гиперкальцемия (12—20 мг%), в то время как содержание неорганических фосфатов и щелочной фосфатазы в сыворотке крови нормальное. Щелочный резерв крови бывает обычно нормальным, содержание гамма$_2$-глобулинов повышено. Многие авторы наблюдали гиперхолестеринемию. Такое состояние наблюдалось у детей при обычной или несколько повышенной дозировке витамина D. Клинически выражен синдром гиперкальцемии, и поэтому картина болезни сходна с D-гипервитаминозом. При тяжелых формах болезни можно констатировать задержку роста и умственного развития ребенка, а также явления общего висцерального кальциноза. Тяжелые формы этого заболевания могут оканчиваться как летально, так и выздоровлением (Th. Stapleton).

При так называемой идиопатической гиперкальцемии резорбция кальция в кишечнике повышена. Обычно всасывается 35% кальция коровьего молока, при применении витамина D — до 50%, а при идиопатической гиперкальцемии — 60% (H. G. Morgan и др.). Причины возникновения этого заболевания еще полностью не выяснены. Существует мнение, что одной из наиболее частых причин является индивидуальная повышенная чувствительность к витамину D, так как при приеме этого витамина болезнь ухудшается, при прекращении приема улучшается. Развитию идиопатической гиперкальцемии способствует применение пищи с большим содержанием кальция, поэтому она встречается преимущественно у искусственно вскармливаемых детей, так как содержание кальция в коровьем молоке в 4½ раза больше, чем в грудном. Развитию гиперкальцемии способствует также применение пищи со щелочным остатком и употребление лекарственных препаратов, вызывающих ощелачивание.

Нижеприводимые наблюдения показывают, насколько различной может быть реакция организма на введение витамина D.

Хельги К., 6 месяцев. Мать по собственной инициативе давала ребенку с цветущим рахитом масляный раствор витамина D в течение 4 месяцев, всего 2,5 млн. единиц, т. е. в среднем 20 833 единицы в день. Через 2½ месяца ребенок стал беспокойным, аппетит пропал. Позднее появилась рвота, запор. За последний месяц потеря в весе 700 г. Девочка стала вялой, появилась субфебрильная температура. Начиная с четырехмесячного возраста ребенок на смешанном кормлении, а с 5½ месяцев — на искусственном.

При приеме в больницу имелись следующие симптомы: ребенок вялый, очень редко смеется, кожа сухая, тургор тканей понижен, гипотрофия первой степени, аппетит отсутствует, язык обложен, рвота почти после каждого приема пищи, запор, субфебрилитет. Реакция Сулькозича положительная, количество кальция в сыворотке крови 15,5 мг%; РОЭ 35 мм в час. Моча без патологических изменений. После прекращения приема витамина D настроение ребенка постепенно стало улучшаться, прекратилась рвота, появилось нарастание в весе. Содержание кальция в крови нормализовалось через месяц, а РОЭ — через 2 месяца после прекращения приема витамина D.

Таким образом, только двукратное превышение дозы витамина D в течение 4 месяцев вызвало у ребенка, находившегося на смешанном вскармливании, состояние гиперкальцемии.

Вальдеко К., 10 месяцев. В течение последних 4 месяцев мать давала ребенку по поводу легкого рахита регулярно 2 раза в день по 12 капель масляного раствора

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витамина D (в 1 мл 50 000 еди
4 месяца ребенку было дано 2½
ставляет 3 375 000 единиц. Никак
ка не наблюдалось. Содержание
рентгенограмме можно было отм
ное обызвествление.

В данном случае даже
D в течение 4 месяцев не
ских явлений D-гипервитам

Приведенные примеры
быть реакция организма на
вероятности, от индивидуал
повышенной чувствительно

Вышеизложенные литё
вают, что явления гиперка
кой дозировке витамина D.
та витамином D у ребенка
видимых причин, появляет
ленно прекратить дачу вит
содержание кальция в кро
трубчатых костей.

Лечение D-гипервитам
гиперкальцемии должно бы
мин. Нами применяются с
кращается прием витамина
фиолетовое облучение. Ни
300 мг в день), но в то же
животные белки и достато
больного. Грудным детям
жание кальция в которо
ния содержится 115 мг%.
ства кальция в коровьем
ные смолы (Е. Н. Хе
Г. Андерсон и др., H. G. M
время на нашей кафедре
чению коровьего молока,
смолы КУ-1 уменьшено

Не следует примен
желток, рыбные консер
миндаль. Для питья
дистиллированную воду,
5—20 мг% кальция. Е
отвращения развития и

Для уменьшения ре
кортизон (Th. Stapleton,
действию витамина D за
Для улучшения обмена в
статочном количестве рег
B_2, PP).

Прописывая детям п
точную дозу на курс лечен
действии при излишнем
при выписке рецепта на

Большие дозы витамин
ноза (С. Н. Мацко), поэт
дозами витамина D целесо
в дозах от 5000 до 10 000

витамина D (в 1 мл 50 000 единиц), следовательно 40 000 единиц ежедневно. За 4 месяца ребенку было дано 2¼ флакона масляного раствора витамина D, что составляет 3 375 000 единиц. Никаких клинических явлений D-гиперитаминоза у ребенка не наблюдалось. Содержание кальция в сыворотке крови 11,4 мг%. Только на рентгенограмме можно было отметить в эпифизах трубчатых костей более интенсивное обызвествление.

В данном случае даже десятикратное повышение дозы витамина D в течение 4 месяцев не вызывало у 10-месячного ребенка клинических явлений D-гиперитаминоза.

Приведенные примеры показывают, насколько различной может быть реакция организма на введение витамина D. Это зависит, по всей вероятности, от индивидуальных особенностей организма, от состояния повышенной чувствительности или резистентности организма.

Вышеизложенные литературные и клинические данные показывают, что явления гиперкальцемии могут возникнуть и при более низкой дозировке витамина D. Если при профилактике или лечении рахита витамином D у ребенка наблюдается потеря аппетита без всяких видимых причин, появляется апатия, падение веса, то следует немедленно прекратить дачу витамина D. Кроме того, необходимо выяснить содержание кальция в крови и сделать рентгенограммы с эпифизов трубчатых костей.

Лечение D-гиперитаминоза и так называемой идиопатической гиперкальцемии должно быть направлено на уменьшение гиперкальцемии. Цели применяются следующие мероприятия. Немедленно прекращается прием витамина D и препаратов кальция, а также ультрафиолетовое облучение. Пища должна быть бедной кальцием (менее 300 мг в день), но в то же время достаточно калорийной и содержать животные белки в достаточном количестве для восстановления веса больного. Грудным детям лучше всего давать женское молоко, содержание кальция в котором равно 25 мг%; в коровьем же молоке кальция содержится 115 мг%, в 4½ раза больше. Для уменьшения количества кальция в коровьем молоке рекомендуется применять ионообменные смолы (Е. И. Хохол и др., A. V. Ferguson, C. K. McGowen, Г. Андерсон и др., H. G. Morgan, Th. Stapleton и др.). В настоящее время на нашей кафедре (Х. Ю. Куус) разработана методика по получению коровьего молока, в котором количество кальция при помощи смолы КУ-1 уменьшено в 3—4 раза.

Не следует применять продукты, богатые кальцием — сыр, яичный желток, рыбные консервы, икру, фасоль, листную капусту, шпинат, миндаль. Для питья и приготовления пищи можно рекомендовать дистиллированную воду, так как в обычной воде содержится около 5—20 мг% кальция. Больному назначается обильное питье для предотвращения развития нефрокальциноза.

Для уменьшения всасывания кальция рекомендуется применять кортизон (Th. Stapleton, H. G. Morgan), который в противоположность действию витамина D задерживает всасывание кальция в кишечнике. Для улучшения обмена веществ рекомендуется давать больному в достаточном количестве регулирующие обмен веществ витамины (A, B_1, B_2, PP).

Прописывая детям препараты витамина D, врач обязан назначить точную дозу на курс лечения и предупредить родителей о вредном его действии при излишнем применении. Для точной дозировки следует при выписке рецепта на витамин D выписывать и пипетку.

Большие дозы витамина A задерживают развитие D-гиперитаминоза (С. Н. Мацко), поэтому при лечении рахита наряду с большими дозами витамина D целесообразно применять одновременно витамин A в дозах от 5000 до 10 000 единиц в день (Е. М. Ленский).

Выводы

1. Явления D-гиповитаминоза могут развиться даже при незначительных передозировках витамина D.

2. Чувствительность детей к витамину D может быть индивидуально весьма различной.

3. Во время лечения витамином D следует внимательно следить за ребенком и при появлении первых симптомов гиперкальцемии прекращать дачу витамина D.

4. При назначении детям препаратов витамина D необходимо предупреждать родителей об опасности передозирования.

ЛИТЕРАТУРА

Власов В. А., Столярова В. К. Вопр. охр. мат. и дет., 1957, № 2, стр. 11. — Лепский Е. М. Гиповитаминозы в детском возрасте. М., 1953, стр. 71—72. — Мацко С. Н. В кн.: Современные вопросы советской витаминологии. М., 1955, стр. 182. — Хохол Е. Н., Отт В. Д., Стальченко Е. С. и др. Тезисы докл. VII Всесоюзного съезда детских врачей. Л., 1957, стр. 13.

O HYPERVITAMINOSIS AND IDIOPATHIC HYPERCALCINATION IN CHILDREN OF EARLY CHILDHOOD

L. M. Keres

Summary

The analysis of clinical cases shows that even a two-fold increase of vitamin D given for a long period of time may cause a severe D hypervitaminosis. The individual sensitivity of vitamin D in children is quite different. Pertinent literature data presents a special nosological entity in children of early age — the so-called idiopathic hypercalcination. One of the most frequent causes of this disease is the individual elevated sensitivity to vitamin D. Hence, when instituting vitamin D treatment, special attention should be paid to the child's condition, and upon appearance of the first symptoms of hypercalcination the administration of vitamin D must be stopped.

К ВОПРОСУ ОБ ОСЛОЖНЕНИЯХ ПРИ ПЕРВИЧНОМ РАКЕ ЛЕГКОГО

Л. В. ГРИГОРЯН, В. С. ЖДАНОВ

Из кафедры общей хирургии (зав. — проф. В. И. Стручков) лечебного факультета I Московского ордена Ленина медицинского института имени И. М. Сеченова и патологоанатомического отделения (зав. — действительный член АМН СССР проф. И. В. Давыдовский) больницы имени Медсинтруда (главный врач А. П. Тимофеева)

Диагностика рака легких до сих пор еще остается трудной. Это заболевание нередко не распознается даже в специальных учреждениях, иногда же имеет место гипердиагностика рака легкого.

Трудности диагностики связаны прежде всего с тем, что рак легких у огромного большинства больных сопровождается хроническим нагноительным процессом. Он-то нередко и определяет клиническую картину. Вместе с тем хронические воспалительные процессы в легких не всегда являются осложнениями рака, нередко они предшествуют развитию его (С. С. Вайль, И. К. Есипова, В. И. Стручков, Д. Ф. Скрипниченко, А. Т. Хазанова и др.). Важно, однако, подчеркнуть, что атипические изменения эпителия, которые можно условно назвать предраковыми, встречаются нередко у лиц сравнительно молодого возраста — 30 лет (А. В. Григорян, В. С. Жданов, Е. В. Рыжкова). Это свидетельствует о том, что для развития рака на почве хро-

3105

нических воспалительных
лет. У этих больных при
тельный легочный анамне
ских воспалительных проц
без предшествующих хрон
цессы осложняют течение
кмеют, как правило, более
вено, что хорошо собранн
помогает уточнить характе
вии, является ли он перви
витию опухоли.

Приводим два наблюде

Больной Г., 56 лет, поступ
лобами, на кашель с мокротой, б
бость, высокую температуру.

Считает себя больным с 19
боли в левой половине грудной
по поводу односторонней пнеумо
прежнюю работу старшего инспе
усиливалось боли в грудной кле
38,5°. Указанные явления врачеб
ского неспецифического воспале
больной был многократно госпи
Состояние его прогрессивно ухуд
в грудной клетке, увеличивались
с кровью, температура держалась
редной рентгеноскопии грудной
легкого.

При поступлении состояни
ных покровов и видимых слизис
аускультации сухие и влажные
нижнем отделе правой половины
наполнения, ритмичный. Тоны с
ного столба. Язык влажный, обл
реченных пальца из-под реберной
больницы.

При рентгеноскопии и рен
левого легкого. Множественны

24/IV 1948 г. больной уме

Патологоанатомическое иссле
ции 3—4 см от места разветвле
бронха и прорастающая его сте
мущественно у ворот. Опухоль
резекции. В нижней доле
Солидные белые опухолевые
ского неспецифического
скопическое исследование обн
ленного легкого. Опухоль сосуд
ткань и окружающую легочную
но выраженного пневмосклероза
отдельные альвеолы как в лег

У другого больного
была обнаружен одноарны

Больной К., 59 лет, поступ
лами на боль в правой плечевой
кости в виде отдельных узлов
мена кровохаркание в виде отд
Грудной клетки.

При клиническом и рентге
рак правого легкого с метастазами
цо — пульмонэктомия справа
течение.

Патологоанатомическое иссле
верхнедолевого бронха, располагай
нижнего и среднего долевого бро
ны, просвет их заполнен густо
варгина плоскоклеточного бронхог

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NEUROPSYCHIATRIC PRESENTATION OF HYPERCALCEMIA

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Much has been written about the skeletal, renal and gastrointestinal manifestations of hypercalcemia, but relatively little emphasis has been focussed upon the neuropsychiatric symptomatology of this biochemical derangement. Regardless of the etiology of the hypercalcemia, confusion, lethargy and a bizarre organic mental syndrome may be the earliest manifestation. Recently, three patients were referred for neurological study because of severe mental changes. The presenting symptoms were those relating to the involvement of the nervous system. No definite diagnosis was made until a high serum calcium level was discovered. The case reports of these subjects follow.

CASE REPORTS

A 67 year old woman was admitted to The Mount Sinai Hospital for the first time with the chief complaint of confusion and mental deterioration. The patient was apparently well until December 23, 1956, when she was injured in an automobile accident. She sustained a fracture of the left wrist and had bruises and cuts on the left side of her head. There was no loss of consciousness. She was hospitalized in another hospital and x rays of the skull were reported to be negative. She was discharged and remained well until January 27, 1957 when she began to show symptoms of nervous system dysfunction. Her gait became somewhat unsteady; she listered from side to side when she walked. She became progressively more helpless and soon required a nurse to assist her with her personal hygiene. By March 1957 these symptoms were exaggerated and accompanied by severe loss of memory, disorientation and confusion. She became extremely dull and apathetic. There was marked bradyphrenia and bradykinesia. The patient just sat and had to be fed. The gait became extremely unsteady and she required support to get about. The family had noted an increase of water intake, urinary frequency and subsequent incontinence. Various consultants suggested that the patient had cerebral arteriosclerosis and that she be institutionalized in a psychiatric hospital. Since cerebral arteriosclerosis rarely causes such severe mental deterioration within a three month period, the patient was finally advised to have a complete examination prior to such commitment.

The physical examination on admission was essentially negative, except for slight enlargement of the heart. On neurological examination the chief findings were mental changes characterized by disorientation, confusion and marked lapse of memory. There were no gross motor deficits, abnormal tendon or pathological reflexes. Coordination was generally poor. The gait was unsteady.

Laboratory findings on admission revealed the hemogram to be within normal limits. The urine showed a specific gravity of 1.015 with a faint trace of albumin. 15 mg/100 ml and 15 to 20 wbc/hpf. Blood urea nitrogen was 40 mg % and the fasting blood sugar level was 121 mg %. The lumbar puncture revealed a normal pressure and the cerebrospinal fluid

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contained four lymphocytes per cubic mm and a protein of 47 mg %. The sedimentation rate was 8 mm/hr. Skull x rays revealed some thinning of the dorsum sellae and posterior clinoids. There was an irregular, rounded calcification in the left posterior frontal region slightly to the left of the midline, and two small calcifications posteriorly which were interpreted as being the choroid plexuses. A pneumoencephalogram revealed slightly dilated, lateral and third ventricles without displacement.

Following the pneumoencephalogram, the patient had a mild headache but otherwise felt well. She continued to be extremely apathetic, markedly confused and disoriented. She was incontinent of urine. Over the succeeding week the patient's state of consciousness continued to fluctuate and her obtundation continued to increase. There were gross twitchings of the hands and fingers. Of interest was the frequent, sudden, temporary drop of the outstretched hands appearing not unlike myoclonic jerks. There was also a tendency to suddenly relax her grip, on a spoon for example, but this was immediately compensated for. Because of the marked fluctuation in the patient's state of consciousness, it was thought that a subdural hematoma might be present. On March 9, 1957 a left carotid arteriogram was performed which showed good visualization of the carotid siphon and anterior and middle cerebral arteries without displacement or abnormality.

Since the patient's depressed mental status continued, complete work up was undertaken. Her basal metabolic rate was minus 9%. Electroencephalograms on several occasions were abnormal with diffuse slowing and shifting bursts without evidence of a cortical focus. Radioactive iodine uptake by the thyroid gland was 30% in 24 hours. The serum calcium concentration was found to be 15.8 mg %, the phosphorus 1.9 mg %, the sodium 138 meq/L, potassium 3.6 meq/L, chloride 102 meq/L, alkaline phosphatase 9.3 K units, and uric acid 8.0 mg %. Repeat blood chemistries revealed the uric acid concentration to be 7.2 mg %, calcium 15.2 mg % and phosphorus 1.7 mg %. The endogenous creatinine clearance was 10 ml/minute.

Subsequently, x ray examination of the long bones, pelvis and hands showed considerable demineralization. There was a healing fracture in the upper end of the right fibula and in the distal end of the left radius. The appearance was thought to be consistent with, though not pathognomonic of, hyperparathyroidism. An intravenous pyelogram showed prompt excretion bilaterally without any evidence of opaque concretions in the kidney contours. There were some rounded calcifications in the pelvis classified as "unmodified phleboliths".

Upon careful cross examination of the family, it was determined that for the past five to six years the patient had been taking a medication containing 100,000 units of vitamin D as well as other vitamins daily without prescription. In addition, the patient had been eating large quantities of sour cream. From this it was concluded that the clinical picture and the laboratory findings could be explained by the toxic metabolic disorder of serum hypercalcemia secondary to hypervitaminosis D and an increased calcium intake.

The patient was consequently placed on a low calcium diet. During the succeeding two weeks the patient became much brighter and more responsive. The blood calcium level dropped to 14.0 mg %. Her mental status improved and she became more interested in her environment. She began to feel benefit and was able to carry on a good conversation with her family and physicians. Following her discharge from the hospital, she has continued to improve, and has remained well.

On three month follow up examination, her calcium level had fallen to 12.0 mg % and she was almost asymptomatic with virtually a complete subsidence of the organic mental syndrome.

A 62 year old woman was admitted to The Mount Sinai Hospital for the first time on October 1, 1957 with the chief complaint of personality change and epigastric discomfort. At that time she stated that she had enjoyed perfect health until three weeks before admission when she experienced a brief bout of sharp epigastric pain. Subsequently she had repeated episodes of vomiting, frequent loose bowel movements, and loss of appetite. She also com-

3453

plained that "her whole personality had changed". There was no previous history of serious illness and system review revealed no significant symptomatology.

The physical examination was entirely normal. Routine examination of the urine showed a faint trace of albumin, a specific gravity of 1.019, 3 to 5 gm/HPF and 15 to 20 wbc/HPF. The hemoglobin was 12.0 gm % and the white blood count was normal. The sedimentation rate was 33 mm/hour, the blood urea nitrogen 17 mg %, and the fasting blood sugar level 91 mg %. Alkaline phosphatase was 14.6 KA units, the serum amylase 32 units, sodium 139 meq/L, potassium 4.0 meq/L, and chloride 101 meq/L. Radiological studies of the chest and abdomen revealed no abnormalities. Barium enema and intravenous pyelogram were normal.

After her discharge from the hospital on October 9, 1957, the patient continued to vomit frequently, usually right after her meals. These episodes were followed by severe occipital headaches which diminished in intensity over the subsequent hours and were unaccompanied by any other symptoms. Occasionally these occurred without prior vomiting. She was therefore re-admitted on November 8, 1957. Physical examination was again within normal limits. A routine blood count and urine examination on admission were normal. The blood calcium level was 13.4 mg % and the alkaline phosphatase 14.2 KA units. On her second hospital day, immediately following an episode of vomiting, the patient was found to have an irregular tachycardia of 120/min. An electrocardiogram showed the presence of atrial fibrillation. This remitted spontaneously and a subsequent ECG was normal. Repeat radiologic studies of the gastrointestinal tract were normal as was a sternal marrow examination.

On the third hospital day, the patient became confused and disoriented. On neurological examination she confabulated having been to Japan during the previous night. She tended to perseverate and had difficulty in calculation. Occasionally she confused right and left. The face hand test (bilateral simultaneous stimulation of face and hand) showed displacement from either hand to the face. Instead of reporting the sensation in one hand and in the face, she indicated that the stimuli were applied to either side of the face. There was generalized weakness. The outstretched left upper extremity fell in a jerky fashion which the patient claimed she could not control. There were no ataxia or tremors. The reflexes were equal and active throughout and there were no pathological reflexes. The cranial nerve and sensory examinations revealed no defects. Slight resistance to flexion of the neck was noted.

A lumbar puncture showed faintly turbid, colorless fluid under an initial pressure of 15 cm/mg which contained 6 lymphocytes and 550 fresh RBC/cu mm and a protein of 2.0 mg %. An electroencephalogram showed diffuse bursts of 2 to 4 cps activity, most marked anteriorly, which increased on hyperventilation.

Over the course of the next ten days, the patient showed marked fluctuation in her mental status. The remainder of the neurological picture was unchanged. She continued to suffer from severe nausea and vomiting which frequently necessitated intravenous fluid therapy to maintain hydration. She was placed on a Bauer-Aub low calcium diet. The serum calcium fluctuated between 10.9 and 11.5 mg % and the phosphorus between 2.8 and 4.1 mg %. A 24-hour urine specimen of 2600 ml contained 132 mg of calcium and 403 mg of phosphorus. From the 12th hospital day on, the patient became progressively more lethargic and disoriented and on the 16th day became somatose, responding only to painful stimuli. There was resistance to flexion of the neck. The reflexes were equal and the fundi were normal. Occasional myoclonic movements of the right side of the face and right shoulder were described. The blood calcium on that day was 11.5 mg %, the phosphorus 3.9 mg % and the plasma creatinine 1.2 mg %. Over the course of the subsequent two days there was gradual deepening of the coma and the patient appeared pre-terminal.

On the 18th hospital day, an exploration of the neck was performed on the chance that a parathyroid adenoma might be found. However, the pathologist could report only normal thymus, thyroid and malaria road tissue in the specimens which were removed. The patient ceased on the day following operation.

Post-mortem examination revealed an area of focal adenomatous hyperplasia, 2 mm in diameter, in one remaining parathyroid gland. No other evidence of parathyroid tissue was found in the neck or mediastinum. In addition, multiple pulmonary emboli with a right lower lobe pulmonary infarct, and left lower lobe atelectasis secondary to a nonequivalent plug in the left lower lobe bronchus were found. A healed duodenal ulcer and a sessile adenocarcinoma of the rectum without evidence of regional or distant metastases were also present. In the bones examined there was no evidence of osteoclastic activity [illegible]. Examination of the brain revealed no abnormalities.

This case has already been reported at length elsewhere (13). This 18 year old widow was in excellent health until July 1954, at which time she became progressively listless and withdrawn. She noted pain and swelling of her left knee which was treated by her physician with "vitamins" and injections. Over the course of the succeeding two months she became increasingly drowsy and, by the second week in October, spent most of her time asleep.

On October 13, 1954, she was admitted to The Mount Sinai Hospital, where general examination revealed only a small node in the left axilla and a vague abdominal mass.

On neurological examination, she was disoriented in all spheres, she perseverated, and was grossly out of contact. The reflexes were hypoactive but the remainder of the examination revealed no abnormalities.

Roentgenological examination showed no significant abnormalities in the skull, chest, long bones or urinary system. Lumbar puncture was normal. The EEG revealed diffuse, symmetrical 4 to 6 cps activity with high voltage 1.5 to 2 cps bursts. The blood urea nitrogen was 54 mg %, creatinine 2.9 mg %, and phosphorus 4.4 mg %.

It was only after the finding of the elevated blood calcium, that the history was elicited from the patient's physician that she had been taking 100,000 units of Vitamin D daily for six weeks during July and August of 1954.

On a low calcium diet, the blood urea nitrogen decreased to 24 mg % and calcium to 12.5 mg %. Concurrently, the patient became less drowsy and less obtunded. She was discharged on October 30, 1954, remarkably improved.

The patient continued to do well after discharge until three weeks before the second admission on December 29th, when the lethargy had returned and was accompanied by anorexia, constipation, weight loss and pain in the left shoulder. On physical examination, the woman appeared chronically ill and lethargic. An irregular, freely movable mass, 3 by 5 cm, was noted in the left axilla and a rubbery lymph node, 2 by 3 cm, was felt in the right supraclavicular area. The liver edge was palpable one hogerbreadth below the right costal margin and a previously described mass in the left upper quadrant had increased in size. There was tenderness over the lower cervical and upper thoracic vertebrae. Neurologic examination revealed marked lethargy and obtundation. When roused, the patient was disoriented for time and place.

The blood urea nitrogen was 25 mg % and the ESR 91 mm/hr. The hemoglobin was 10.5 gm %, the wbc was 9,700 per cu mm, with 51% neutrophiles, 24% lymphocytes, 20% monocytes, clear cells and 5% eosinophiles. Platelet and reticulocyte counts were normal. The serum calcium was 17.4 mg %, serum phosphorus 3.1 mg %, and the alkaline phosphatase was 9.8 [illegible] units. X-ray studies of the cervical and dorsal spine and of the left shoulder revealed no abnormality. A plain film of the abdomen showed the outline of the left kidney to be obscured. On the second hospital day, a biopsy of the mass in the left axilla was performed and the diagnosis of Hodgkin's Disease in lymph node tissue was reported by the pathologist.

The patient remained afebrile but the obtundation and lethargy continued for the first three hospital weeks. On the sixth hospital day, A.C.T. 60 units per day, was begun, and on the tenth day, cortisone, 100 mg per day was substituted. At the same time, radiotherapy was given to the left axilla, left upper quadrant of the abdomen, and the right cervical region. The patient's lethargy and confusion gradually cleared. By the 26th hospital day, the serum calcium level had fallen to 10.1 mg %, the serum phosphorus to 1.9 mg %, and the blood urea nitrogen to 19 mg %. The patient was discharged on February 4, 1955. At

that time, the masses in the abdomen and left axilla had decreased in size, but new lymph nodes were noted in the left cervical region.

The patient was well for a few weeks after discharge, but then became febrile. Nitrogen mustard therapy was administered at home, after which thrombocytopenia and leucopenia developed. She died suddenly two months after discharge with evidence of central nervous system bleeding. No post mortem examination was performed.

DISCUSSION

The majority of the currently popular texts of medicine, endocrinology and neurology do not emphasize the changes in mental status or consciousness in patients with hypercalcemia nor do they stress that such changes may provide the predominant presenting symptoms. However, sporadic reference has been made to involvement of the nervous system in such patients in the literature of the past two decades. Fitz and Hallman, as recently as 1952, were the first to call attention to the fact, in the American literature, that hyperparathyroid patients may present with mental symptoms which clear after surgical removal of the parathyroid adenoma (1). Barter, in his excellent review on the parathyroid gland and its relation to the nervous system, lists among other symptoms of hypercalcemia: psychosis, depression, listlessness, lethargy, apathy, drowsiness, fatigability and mental retardation as well as weakness, hyporreflexia and hypersensitivity to sensory stimuli (2). Eitinger calls attention to the fact that seven out of the fifty patients he studied with hyperparathyroidism, showed mental changes (3). Other authors mention neuropsychiatric symptoms in hyperparathyroidism, in vitamin D intoxication, and in hypercalcemia associated with androgenic and estrogenic therapy (4-7). Finally, Wenger *et al.*, in reviewing the symptoms of hypercalcemia in the milk-alkali syndrome states: ". . . mental confusion, ataxia, stupor and toxic psychosis may dominate the picture" (8).

The three patients presented here demonstrate the difficulties in diagnosis when the primary and most conspicuous manifestation of hypercalcemia relates to the central nervous system. As is summarized in Table 1, mental changes of varying duration and progressive nature were the prominent presenting complaints in all three cases. In each instance, primary brain disease was suspected, because no obvious cause for the symptomatology was in evidence and neurological consultation was therefore requested. In the first case, the picture was further confused by the history of recent cerebral trauma and therefore the possibility of subdural hematoma was seriously considered. After all studies had failed to demonstrate any evidence of a space-occupying intracranial mass, a complete spectrum of blood chemistries revealed the presence of hypercalcemia. The history of high vitamin D and calcium intake was obtained only after meticulous questioning of the patient's family. In the second patient, the course was extremely rapid and progressive. Despite a rather careful search of the mediastinum at the time of post-mortem examination, no satisfactory cause for the hypercalcemia could be found, except for the small area of adenomatous change in the remaining parathyroid. However, primary hyperparathyroidism remains as the most probable diagnosis.

It is interesting to note that both the first and second patients showed sudden

TABLE I

Age	Sex	Neurological Presenting Symptoms	Duration	Admission Water Chemistry: Ca mg%	P mg%	Alk Phase K.A.U.	Urea N mg%	EEG	Etiology
67	♀	Mental retardation, lassitude, dyscalculia, disorientation, unsteadiness, muscular weakness, polydipsia, anorexia	5 weeks	15.8	1.5	9.3	12	Diffuse sweeps, sporting bursts 1.5–3 cps	Hypervitaminosis D
62	♀	Personality change, nausea, anorexia	5 weeks						
		Confusion, disorientation, dyscalculia, weakness	3 weeks	16.3	2.3	14.2	12	Diffuse bursts 2–3 cps	Fossilized hyperparathyroidism
		Progressively deepening coma	1 week	14.5	3.9				
48	♀	Listlessness, fatigue, drowsiness, confusion, perseveration, disorientation, blurring of vision	3 months	13.8	4.1	10.2	60 43	Diffuse sweeps Diffuse bursts 1.5–2 cps	
		Lassitude and drowsiness	2 months later	17.1	3.1	9.8	26		Hypervitaminosis D Hodgkin's disease

fleeting loss of tone in outstretched extremities which gave the appearance of jerky, almost myoclonic dropping of the limb which was immediately restored to its original position. This symptom was quite prominent. Patients who manifest such symptoms should be suspected of some form of intoxication.

All three patients showed moderate, diffuse slowing in their electroencephalograms; this was accompanied by diffuse bursts of 1.5 to 2.5 cps activity. This type of disturbance is also non-specific and can be seen in cerebral dysfunction due to a wide variety of etiologies (9).

The organic mental syndrome, as described in the literature and displayed by the patients presented, cannot be characterized in any specific manner, except that it is usually accompanied by marked lassitude and drowsiness, occasionally stupor and coma. The patient is often disoriented and confused, has a poor memory, has difficulty in calculating and has a short attention span. The face-hand test is usually positive (10). Incontinence of urine and feces and difficulty in personal hygiene are frequently noted. Such mental changes are characteristic of any type of diffuse cerebral dysfunction. Consequently, diagnoses of brain damage due to pressure of intracranial tumor, subdural hematoma, or hemorrhage are entertained. When these symptoms are accompanied by anorexia, nausea, vomiting, epigastric pain, muscular weakness, constipation or polydipsia and polyuria, hypercalcemia as an etiologic factor should be strongly suspected. However, the possibility of a hypercalcemic syndrome should be borne in mind in the diagnostic evaluation of all patients with organic mental syndromes of obscure etiology.

Prolonged hypercalcemia may cause parenchymatous and tubular deposition

of calcium in the kidneys. Thus pyelonephritis with consequent reduction in renal function may ensue. Furthermore, abrupt elevation of the serum calcium level causes immediate alterations of tubular function such as reduced tubular reabsorption of salt and water (11). The salt and water losses due to the consequent polyuria may cause dehydration and further reduction, albeit reversible, in renal function (12). In prolonged hypercalcemia, irreversible renal damage may be produced. Generally, as renal function falls consequent to hypercalcemia, the level of the serum calcium rises precipitously. However, a fall in renal function may cause an elevation of the serum phosphorus with a consequent reduction in the serum calcium, sometimes obscuring to some degree, the original hypercalcemia. Since, in many cases, the pathologic state causing the hypercalcemia may be correctible, the importance of prompt recognition of this condition by determination of the serum calcium level is self evident.

The following pathologic entities may produce hypercalcemia:

(a) Hypervitaminosis D; usually after intakes in excess of 100,000 units per day for prolonged periods (5, 6).

(b) Hyperparathyroidism (1, 3, 4).

(c) The milk-alkali syndrome; high dietary calcium content, especially when accompanied by insoluble alkali. This entity is most frequently encountered in patients treated for duodenal or gastric ulcer (8).

(d) Boeck's sarcoid.

(e) Hodgkin's disease (11).

(f) Estrogen or testosterone administration in large doses for extended periods (7).

(g) Metastatic malignancy involving bone.

(h) Multiple myeloma.

(i) Prolonged immobilization.

The precise mechanisms by which alterations in the serum calcium level produce functional changes in the intact central nervous system are not clear. There is a considerable body of experimental data which indicates that elevation of calcium above physiologic levels can produce profound alterations in nerve function, both peripheral and central (13, 15). These include: increased threshold to electrical stimulation of myelinated nerve fibers (16, 18); decreased rate of discharge of the post-synaptic neuron in the sympathetic ganglia where the discharge is produced either by administration of acetyl choline or tetanic stimulation by way of the pre-synaptic fiber (18); acceleration of the decrease in successive end plate potentials during repetitive stimulation of the neuro-muscular junction via the motor nerve (19). It has been found that increasing the calcium concentration of the perfusing medium causes a decrease in permeability of the nerve membrane to ions and this has been offered as a possible explanation of the alterations in the electrical properties (20). Also in brain slices, calcium tends to depress respiration (21).

SUMMARY

Not infrequently a patient may present with confusion, lethargy and a bizarre organic mental syndrome as the primary expression of hypercalcemia, regardless

of etiology. Three cases are reported which were referred for neurological consultation because of a peculiar organic mental syndrome, a clinical picture which caused much diagnostic confusion until a high serum calcium level was established as the etiological factor. In two of the patients, fluctuations in the serum calcium levels were observed to be correlated with changes in mental status: confusion increasing with the rise in the serum calcium level. The underlying causes for the hypercalcemia were Hypervitaminosis D, Hodgkin's Disease and parathyroid adenoma in the three patients respectively. It is emphasized that neuropsychiatric symptomatology may be the presenting picture of a hypercalcemic syndrome. Since prolonged hypercalcemia may cause severe renal tubular damage and even death, and since, in many cases therapeutic measures are available for eliminating either the primary cause of the hypercalcemia or lowering the serum calcium level (e.g. by ethylene diamine tetraacetic acid), early diagnosis is extremely important. The possibility of hypercalcemia should therefore be borne in mind in the differential diagnosis of organic mental syndrome of obscure etiology.

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REFERENCES

1. Fitz, T. E., and Hallman, B. L.: Mental Changes Associated with Hyperparathyroidism. A.M.A. Arch. Int. Med., 89: 547, 1952.
2. Barter, F. C.: The Parathyroid Gland and its Relation to Diseases of the Nervous System. Res. Publ. Ass. Res. Nerv. Ment. Dis., 32: 1, 1953.
3. Ettinger, L.: Hyperparathyroidism With Mental Changes. Nord. med., 14: 1581, 1942.
4. Oliver, W. A.: Acute Hyperparathyroidism. Lancet, 2: 240, 1939.
5. Howard, J. E., and Meyer, R. J.: Intoxication with Vitamin D. J. Clin. Endocrinol., 8: 895, 1948.
6. Chaplin, H. Jr., Clark, C. D., and Rogers, N. W.: Vitamin D Intoxication. Am. J. M. Sc., 221: 369, 1957.
7. Herman, J. B., Lipstein, E., and Kramer, J. S.: Hypercalcemic Syndrome Associated with Androgenic and Estrogenic Therapy. J. Clin. Endocrinol., 9: 1, 1949.
8. Wenner, J., Kinsell, L. B., and Palmer, W. L.: Editorial: The Milk Alkali Syndrome. Am. J. Med., 24: 161, 1958.
9. Strauss, H., Ostrow, M., and Greenstein, L.: Diagnostic Electroencephalography. Grune and Stratton, New York, 1952.
10. Benton, M. B.: Disorders in Perception. Charles C. Thomas, Springfield, Ill., 1951.
11. Levitt, M. F., Hamburg, M. H., Poulmenos, D. P., Swartz, A. V., and Gutierrez, D.: The Effect of Abrupt Changes in Plasma Calcium Concentrations on Renal Function and Electrolyte Excretion in Man and Monkey. J. Clin. Invest., 37: 294, 1958.
12. Levitt, M. F.: The Function of the Nephron in Normal and Uremic Salpers. J. Mt. Sinai Hosp., 25: 5, 415, 1958.
13. Karakow, B., Minski, M. F., and King, H. F.: Hypercalcemia in Hodgkin's Disease. New Eng. J. Med., 256: 50, 1957.
14. Kennedy, B. D., and Lewis, P. R.: Electrolytes and Nerve Function: Elliott, K. A. C. et al. (eds.), Neurochemistry, Charles C. Thomas, Springfield, Ill., 1955: 451-455.

15. BRINK, F.: The Role of Calcium Ions in Neural Processes. Pharmacol. Rev., 6: 243, 1954.
16. FRANKENHAEUSER, B., AND HODGKIN, A. L.: The Action of Calcium on the Electrical Properties of Squid Axons. J. Physiol., 137: 218, 1957.
17. FRANKENHAEUSER, B.: The Effect of Calcium on Myelinated Nerve Fibers. J. Physiol., 137: 245, 1957.
18. BRINK, F., BRONK, D. W., AND LARRABEE, M. G.: Chemical Excitation of Nerve. Ann. N. Y. Acad. Sci., 47: 157, 1946.
19. LUNDBERG, A., AND QUILISCH, H.: On the Effect of Calcium on Presynaptic Potentiation and Depression at the Neuromuscular Junction. Acta Physiol. Scand. Supp. 111, 30: 121, 1953.
20. GORDON, H. T., AND WELSH, J. H.: The Role of Ions in Axon Surface Reactions to Toxic Organic Compounds. J. Cell. & Comp. Physiol., 31: 395, 1948.
21. ELLIOT, K. A. C.: Brain Tissue Respiration and Glycolysis in Elliot, K. A. C. et al. (ed's) Neurochemistry, Charles C. Thomas, Springfield, Ill., 1955, 71.

3607

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The Apparent Vitamin D Resistance of Chronic Renal Failure

A Study of the Physiology of Vitamin D in Man

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Biologic assay of the antirachitic activity in serum and tissues has been used, with concomitant measurements of intestinal net absorption of calcium, to study aspects of vitamin D metabolism in man. Patients with chronic renal failure appear to require a greater intake of the vitamin and a higher serum antirachitic activity, to promote calcium absorption, than does the healthy subject. This increased requirement is not due to intestinal malabsorption of the vitamin nor, apparently, to its metabolic inactivation nor loss in the urine. It could be caused by relative unresponsiveness of the target organ; or, if due to an acquired abnormality of vitamin D metabolism, this is likely to involve some metabolic transformation subsequent to the initial hydroxylation.

When the intake of vitamin D is high, the antirachitic activity of serum appears to be determined principally by the amount absorbed from the gut, and its concentration is a power function of the daily oral dose. Under such conditions of high dosage, the vitamin is stored in various tissues, of which voluntary muscle may be quantitatively the most important. The implications of these relationships are discussed.

In healthy British adults, the antirachitic activity of serum is low compared with the concentrations measured in adults in the United States. This is probably due to a habitually low dietary intake of the vitamin in Britain and limited solar exposure. Although adequate to prevent development of vitamin D deficiency, this nutritional state may provide insufficient reserve to meet an increased requirement caused by disease. It is postulated that when chronic renal failure increases the requirement for the vitamin, the British patient is likely to have an effective vitamin D deficiency. The American, with a habitual luxus consumption of vitamin D, may have acquired sufficient body stores to meet the needs imposed by renal impairment. Differences in the nutritional state of the different populations may thus explain the frequency of azotemic rickets and osteomalacia in Europe and Asia, as compared with their reported infrequency in the United States.

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Despite the recent explosive progress in understanding of the metabolism and mode of action of vitamin D [1–11] there is still no simple means of proving that a state of vitamin D deficiency exists in a particular experimental or clinical situation. Deficiency can be defined only in terms of its effects in causing defective osseous mineralization; its possible existence may be inferred from the detection of rickets or osteomalacia and from the demonstration of an associated impairment of calcium absorption by the intestine. Confirmation of the presumptive diagnosis depends on the demonstrable reversal of the intestinal and bony abnormalities by physiologic amounts of the vitamin.

The clinical recognition of rickets [12,13] and later of osteomalacia [14–16,18,23] in patients with advanced renal failure raised the possibility that the metabolism of vitamin D was disturbed in this situation [14–18]. The defective osseous mineralization was histologically indistinguishable from that produced by simple vitamin D deficiency [19–21], and renal failure was shown to be associated with impaired intestinal absorption of calcium [15–17,22]. The bone lesions were proved to be curable, and the intestinal malabsorption of calcium correctable by vitamin D; but the amount of the vitamin required to produce these effects was invariably greater than is needed to correct a simple privational deficiency [15,16,18,23]. This led to the suggestion that chronic renal failure causes a relative insensitivity to the biologic effects of the vitamin, an acquired "vitamin D resistance" or "an increased requirement for the vitamin" [14–18,24].

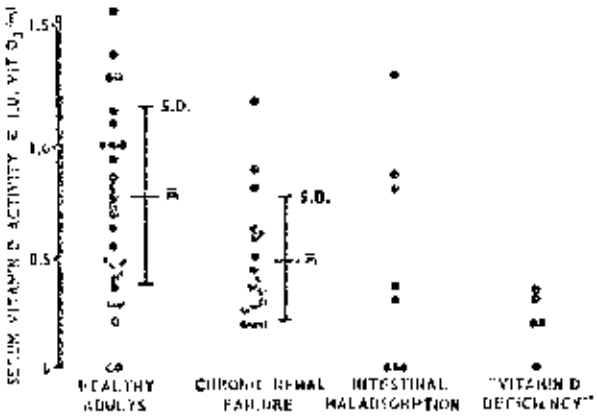

Figure 1. The antirickets activity of human serum as assayed biologically in the rat. The two open circles (○) included with "healthy adults" refer to two patients with structural neurological disease who were randomly selected as metabolically normal controls for radioisotope studies [25]. The cases labeled "vitamin D deficiency" were patients with osteomalacia considered to be caused by dietary deficiency of vitamin D.

In 1963 we commenced studies of vitamin D metabolism in renal failure, using the biologic assay as the only investigative technic then available. This report records the results obtained to date. As the investigation evolved it became necessary to explore aspects of the physiology of vitamin D which, although studied to a variable extent in animals, had received little attention in man. It was also necessary to assess the state of vitamin D nutrition in the indigenous population from which we received patients in whom rickets or osteomalacia had developed as a complication of their chronic renal disease. Consequently, although prompted originally by the problems of renal failure, this investigation is as much concerned with the general biology of vitamin D in man. Only casual reference is made to our continuing studies of the metabolism of radioactive vitamin D in renal failure [24–26].

MATERIAL AND METHODS

Most of the patients investigated had advanced renal failure secondary to primary renal disease or, in Cases 5 and 6, as a complication of primary hyperparathyroidism. All the patients with primary renal disease had azotemic osteodystrophy; abbreviated relevant details are included in Table I. Assays of vitamin D activity in serum were also made in five patients with rickets or osteomalacia attributed to primary vitamin D deficiency, in eight patients with intestinal malabsorption and in a group of healthy adults (Figure 1). None of these subjects had received vitamin D supplementation or therapy.

The clinical methods and the general procedure of the metabolic balance were essentially as described previously [15,27]. Patients consumed a diet of constant composition with which they equilibrated for seven to ten days before metabolic collections were started; urine and feces were collected in four day periods. When a statement is made in the text concerning intestinal malabsorption of calcium, it is based on the analytic results of at least two and usually up to four such collection periods [15,22,27].

The bioassay of vitamin D was based on a standard ten day prophylactic method [28]. A Glaxo strain of Wistar rat, bred by brother-sister mating since 1928, was established as a colony in this laboratory and maintained under conditions appropriate for the production of rickets [29]. Weanlings from the appropriate number of litters and weighing 30 to 40 gm were

distributed into groups of eight. So far as was possible, representatives from all the litters were included in each group. All animals received the ricketogenic diet (diet II) for ten days, and the test substance was administered orally in ten equal daily doses to each rat. Each assay included three groups of eight rats receiving standard doses of vitamin D_2 dissolved in arachis oil (between 0.05 and 1.35 IU/rat/day). The degree of healing rickets was assessed, after silver nitrate staining of the split tibias, by the conventional line test procedure [29]. Potency was measured graphically [29]. The validity of the method was assessed by appropriate statistical methods [30].

In the assay of the total vitamin D activity in serum, the serum was administered in doses between 0.1 and 0.3 ml/day; or, when the level of activity was high, in an appropriate dilution. In some instances a preliminary methanol-chloroform extract of serum was prepared [31] and the extracted lipid administered dissolved in arachis oil. Tissues were either extracted directly by methanol-chloroform or after preliminary saponification with alcoholic potassium hydroxide. Material not assayed on the day of collection was stored in the frozen state at −25°C; no loss of assayable biologic activity was detectable after periods up to three years.

Because of the inevitably limited amount of material, serum could usually be assayed at only one level of dosage. This necessitated the assumption that the slope of the dose response curve of the test material was the same as the standard. This was not usually a problem with the tissue obtained at autopsy. When the vitamin D activity of serum is recorded as apparently zero (see Figure 1), this implies that the degree of ricketic healing produced by the dose of serum used (0.2 to 0.3 ml/rat/day) was less than produced by the lowest standard (0.05 IU/rat/day) or no better than resulted from administration of arachis oil. This meant at most an activity of 0.17 to 0.15 IU/ml in the serum assayed. In some such cases assay of the lipid extract from large volumes of serum indicated that only trivial amounts of activity were present [26].

As has been a general experience in the bioassay of serum activity [32,33], a significant proportion of the trials undertaken were failures that could not be repeated for lack of material. Consequently, in experiments planned to ascertain the daily "requirement" of the vitamin in patients with renal failure, it was usually necessary to compromise with a crudely approximate estimate (see "Results").

Management of Assay Animals. Breeding female animals are maintained on stock diet ("Chardex," No. GR/R/3) until one week prepartum and subsequently fed diet I. Pups are weaned at three to four weeks and maintained on diet I until required for assay (diet II).

Diet I: wholemeal flour 4.2 kg; skimmed milk 1.14 kg; dried yeast powder 0.3 kg; soy flour 0.084 kg; arachis oil 0.36 kg; carotene 13 mg.

Diet II: (ricketogenic: 0.5 per cent calcium; 0.15 per cent phosphorus); white flour 4.25 kg; egg albumin powder 0.5 kg; sodium chloride 0.1 kg; calcium carbonate 0.0475 kg; ferric citrate 0.01 kg; hydrated disodium hydrogen phosphate 0.35 kg.

RESULTS

Serum Vitamin D Activity in the Healthy Adult (Figure 1). The mean value of twenty-six assays, made between 1963 and 1969 on serum specimens from members of the departmental staff, was 0.77 IU/ml (S.D. 0.40 IU/ml) with a range from apparently zero to 1.6 IU/ml. Similar results were obtained from year to year; the mean value obtained in summer months (0.87 IU/ml) was higher than in the winter months (0.66 IU/ml), but the difference was not statistically significant.

Vitamin D Activity in Serum from Patients with Chronic Renal Failure. Satisfactory assays were obtained in seventeen patients with chronic renal failure who had received no previous treatment with vitamin D. The assayed level of serum vitamin D activity varied from 0.2 to 1.2 IU/ml with a mean value of 0.49 IU/ml (S.D. 0.28 IU/ml). Although this mean value was appreciably lower than in the group of healthy subjects, the difference was not statistically significant (Figure 1).

Ten of these patients with chronic renal failure had clear evidence of defective skeletal mineralization in the form of azotemic rickets or osteomalacia [14,15,23]. Metabolic balance studies, carried out in fifteen of the seventeen patients, demonstrated in each an intestinal net absorption of calcium close to zero [15,22].

Serum Vitamin D Activity During Oral Treatment with Ergocalciferol. Figure 2 shows the relationship between the assayed vitamin D activity of serum and the daily oral intake of the vitamin in a group of azotemic patients receiving treatment with ergocalciferol. In six of these patients serial assays were made, either after an increment in oral dose or at intervals of time when the patient was receiving a constant daily dose.

In the individual patient, an increase of daily oral dose invariably produced a corresponding increment in the serum level of vitamin D activity (Figure 2). When the daily intake of ergocalciferol was held constant, the level of vitamin D activity in the serum increased with the duration of treatment. At lower levels of intake (3,000 to 9,000

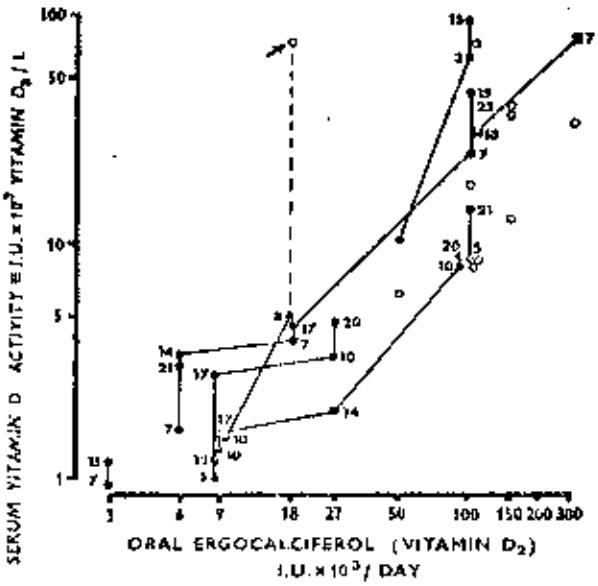

Figure 2. Relationship of the vitamin D activity in the serum of patients with renal failure to the daily oral dose of ergocalciferol. Successive measurements in individual patients are joined by lines. The numerals alongside individual points (closed circles, ●) indicate the number of days the patient had been receiving a particular oral dose of ergocalciferol. The closed squares (■) represent analogous data from a patient with idiopathic hyperparathyroidism similarly treated. The open circles (○) refer to random measurements made in nine different patients who had been receiving oral therapy with ergocalciferol for months. The arrowed point is discussed in the text.

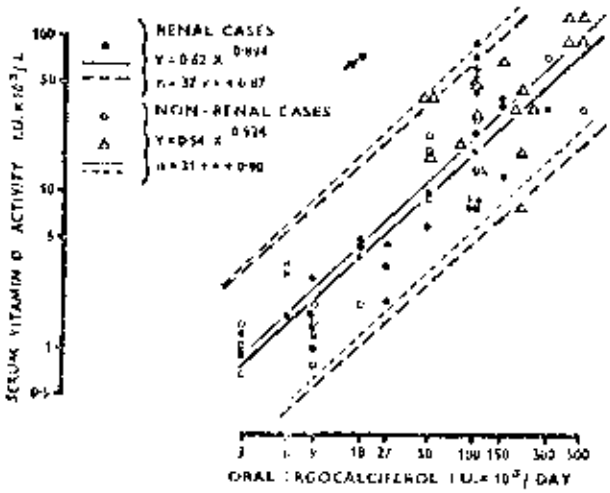

Figure 3. Relationship between serum vitamin D activity and the daily oral intake of ergocalciferol. The data from Figure 2 (●) have been augmented by thirty-one similar measurements in patients who had received oral ergocalciferol for months but who had neither renal nor alimentary disease (○, personal cases; △, data obtained from the literature [33,36]).

IU/day) there might be a two- to threefold increase of serum activity during the course of two to three weeks treatment (Figures 4, 6–8); with greater dosage, the fractional increase was smaller (Figure 2). Thus, in five patients receiving 100,000 IU/day the maximum increase in assayed serum activity between the first and third week of continued treatment with this dose was 12, 35, 42, 47 and 80 per cent. A similar increase of serum activity, on continued treatment with a constant daily oral dose of ergocalciferol, was seen in patients with nutritional osteomalacia (Figures 7 and 8).

Figure 2 also includes the results of serum assays in nine azotemic patients who had been treated for several months with 50,000 to 300,000 IU/day of ergocalciferol. These data (open circles) show a distribution similar to those obtained from patients during the first three weeks of therapy (closed circles). Thus the cumulative effect of a particular daily dose on the level of serum activity appears to be limited and may be at its maximum during the first few weeks of administration.

All thirty seven measurements obtained from the patients with renal failure in Figure 2 have been plotted in Figure 3 (as closed circles), together with thirty-one analogous measurements in patients who had been receiving ergocalciferol orally for weeks or months but who had neither renal nor alimentary disease (open circles and triangles). The separately calculated regressions and confidence limits for the two groups of data are essentially identical (Figure 3). Thus, when the intake is high (3,000 to 500,000 IU/day) the antirachitic activity of serum appears to be a power function of the daily oral dose. This relationship, which presumably reflects mainly the intestinal absorption of the vitamin, is apparently the same in the presence as in the absence of renal failure. It is to be emphasized that the confidence limits are very wide. The daily oral intake of ergocalciferol appears to be the principal determinant of serum vitamin D activity; other factors, such as the cumulative phenomenon described, probably contribute to the scatter of the data; and no allowance has been made for the considerable differences in body weight of the subjects studied. It has also been shown that a single large oral dose of vitamin D produces a brisk increase in serum activity during the subsequent twenty-four hours [34], and it is not possible to be certain that precisely the same time elapsed between the last oral dose and the blood sampling in all subjects.

The arrows in Figures 2 and 3 indicate an apparent exception to the general relationship described. This point relates to a fourteen year old girl with chronic pyelonephritis and azotemic rickets (Case 3, creatinine clearance 13 ml/minute, Table 1). She was treated in the hospital with 18,000 IU/day of ergocalciferol; after eight days treatment the serum vitamin D activity was 4.8 IU/ml, which is a value compatible with the relationship shown in Figures 2 and 3. Four months later, in the outpatient clinic, the serum calcium was found to be 15.2 mg/100 ml, and the serum vitamin D activity was 72 IU/ml. The development of hypercalcemic intoxication within this period and at this level of dosage is outside our therapeutic experience of this disease. Especially in view of the grossly aberrant position of this assay in Figure 3, it is suspected that the patient was provided as an outpatient with 6 tablets daily, each containing 50,000 IU (Tab. Calciferol B.P.) instead of those prescribed to contain 3,000 IU. This is a well recognized hazard in British practice [35]. If the patient's actual intake were 300,000 IU/day, the serum activity of 72 IU/ml would accord with the relationship shown in Figure 3.

The Relationship of the Levels of "Vitamin D Activity" in Serum to the Biologic Effectiveness of Administered Vitamin D. In a group of patients, the production of a definite increase of intestinal net absorption of calcium was used as an index of the biologic effect of administered vitamin D. Serial assays of serum were coupled with measurements of external mineral balance in an attempt to determine the level of serum vitamin D activity associated with detectable biologic action in the individual patient.

TABLE I The Relation of the Dose of Vitamin D and the Prevailing Level of Serum Vitamin D Activity to the Production of Detectable Biologic Effect

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)		(9)		(10)	
							Serum Levels Before and During Effective Vitamin D Therapy					
							Calcium (mg/100 ml)		Phosphorus (mg/100 ml)		Ca × P	
Case No. (Bone Disease)	Age (yr) and Sex	Creatinine Clearance (ml/min)	Highest Ineffective Dose Vitamin D (IU × 10^3/day)	Corresponding Serum Vitamin D Activity (IU/ml)	Lowest Effective Dose Vitamin D (IU × 10^3/day)	Corresponding Serum Vitamin D Activity (IU/ml)	Before	During	Before	During	Before	During
1 (A.O.)	55, F	6.4	18	3.8–4.5	100	24–44	8.5	10.4	3.3	2.4	29	25
2 (A.O.)	44, M	5.0	27	1.6–2.2	100	8.1–9.2	6.0	6.9	4.7	5.7	28	38
3 (A.R.)	14, F	13	9	1.5	18	4.8	9.2	9.3	5.0	4.7	46	44
4 (A.R.)	15, M	12	9	1.0–1.2	27	2.9–4.6	10.3	10.0	4.6	4.6	47	46
5 (Hy: PTX)	74, F	15	3	1.2	...	...	7.1	...	4.2	...	30	...
6 (Hy: PTX)	68, F	9.2	100	6	...	...	10.3	...	4.3	...	45	...
7 (A.R.)	16, M	6.9	50	10.5	100	70	9.3	9.7	6.3	5.0	58	49
8 (A.H.: PTX)	39, F	9.1	150	39	...	...	6.8	...	9.4	...	64	...
9 (A.O.)	30, F	7.4	100	No assay	300	37	7.3	7.0	4.9	6.5	36	45
10 (A.O.)	35, M	8.5	150	13	250	No assay	6.3	8.0	2.5	3.5	21	30
11 (A.O.)	62, M	5.9	...	...	100	8.1	8.7	8.8	3.7	4.1	32	37
12 (A.R.)	10, M	14.6	...	...	100	8.3	9.5	9.8	6.2	5.9	59	58
13 (A.O.)	37, F	3.4	NIL* (D_2)	4.1	...	...	9.4	...	4.6	...	43	...
14 (A.R.)	14, M	26	...	...	4 (D_2)	5.2	8.3	9.2	6.1	5.2	50	48
15 (A.O.)	39, F	5.9	40 (D_2)	9.9	80 (D_2)	13.9	9.0	8.9	6.5	6.4	58	57
16 (A.O.)	43, F	2.5	NIL* (D_2)	0.7	...	...	6.0	...	5.9	...	35	...
			20 (D_2)	7.8	40 (D_2)	5.4	6.2	8.0	5.8	4.0	35	32
17 (A.O.)	56, F	5.0	5 (D_3)	1.2	...	...	6.6	...	4.3	...	28	...
22 (P.O.)	75, F	...	...	...	9	0.8–1.4	8.9	9.7	1.5	2.7	14	26
24 (P.O.)	73, M	...	...	...	3	0.6–1.4	9.0	9.5	2.6	3.4	23	32

NOTE: The initials qualifying the type of bone disease (column 1) signify the following: A.O. = azotemic osteomalacia; A.R. = azotemic rickets; A.H. = azotemic hyperparathyroidism [14,15,18]; A.H.: PTX, = the same condition but after subtotal parathyroidectomy. Hy: PTX = primary hyperparathyroidism following parathyroidectomy. D-def. O. = osteomalacia due to primary deficiency of vitamin D. All patients in this table were studied by metabolic balance. Data in columns 4 and 5 were obtained when the intestinal net absorption of calcium was near zero; in columns 6 and 7, when the vitamin D therapy was producing an unequivocal intestinal net absorption and bodily retention of calcium and phosphorus (see text). Except where otherwise noted (D_1), the form of vitamin D used was ergocalciferol. In Cases 13 and 16, described in column 4 as NIL* (D_2), the patient had received ergocalciferol previously and the assayed serum vitamin D activity (column 5) was attributable to the persistent effect of this therapy. The figures for serum calcium, phosphorus and Ca × P (columns 8–10) represent the mean of several determinations made during the appropriate period of metabolic study in hospital.

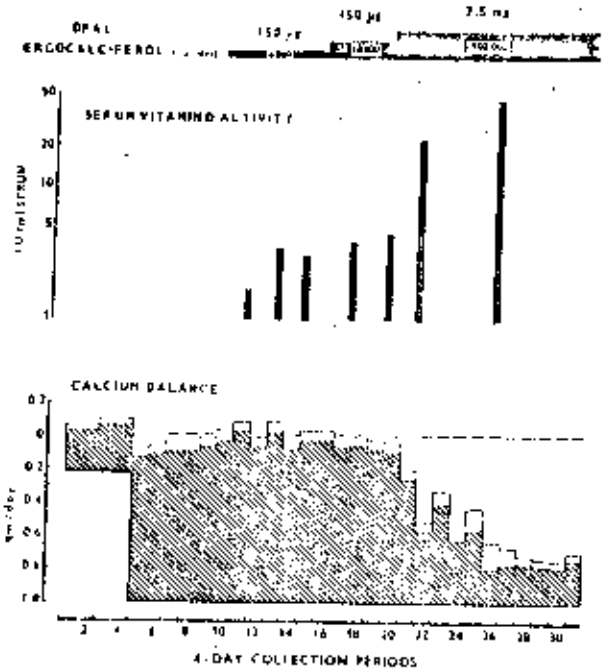

Figure 4. Relation of the level of assayable vitamin D activity in serum to the intestinal absorption of calcium in chronic renal failure. (Case 1; female, aged fifty-five years. Azotemic osteomalacia; creatinine clearance, 6.4 ml/minute.) In this diagram and in Figures 5–8, calcium balance data are charted in the conventional manner [15]; fecal calcium is shown cross hatched and urinary calcium white. (In all these studies simultaneous measurements were also made of phosphorus, nitrogen and, usually, magnesium balance; these data are omitted as largely irrelevant to the present purposes.)

Figure 4 shows a representative study of a patient with azotemic osteomalacia in whom the oral dose of ergocalciferol was increased successively from 6,000 IU (150 μg) to 18,000 IU (450 μg) and 100,000 IU (2.5 mg)/day. The two smaller daily doses increased the serum vitamin D activity to between 3 and 4.5 IU/ml (for four to six times the average level in our healthy subjects, Figure 1) without influencing the intestinal net absorption of calcium (periods 10 to 20, Figure 4). The large increment in dose, to 100,000 IU/day, produced a typical metabolic response, with increased absorption of calcium and bodily retention of both calcium and phosphorus (periods 21 to 31), which ultimately led to clinical cure of the patient's osteomalacia. This metabolic response was associated with assayed serum activity of 24 and 44 IU/ml (Figure 4). Thus a serum level of up to 5 IU/ml was biologically ineffective, and the effective level appeared to lie somewhere between this and 24 IU/ml of serum. Figure 5 shows a similar study in which a serum activity of up to 2.2 IU/ml was ineffective and metabolic response occurred in association with values of 8 to 9 IU/ml.

Figure 6 demonstrates a different order of requirement for vitamin D in a boy of fifteen years with azotemic rickets. A daily dose of 9,000 IU (225 μg) of ergocalciferol increased serum vitamin D activity to 1.2 and 2.9 IU/ml. This appeared to initiate a metabolic response (period 3), but the dose of ergocalciferol was unfortunately increased

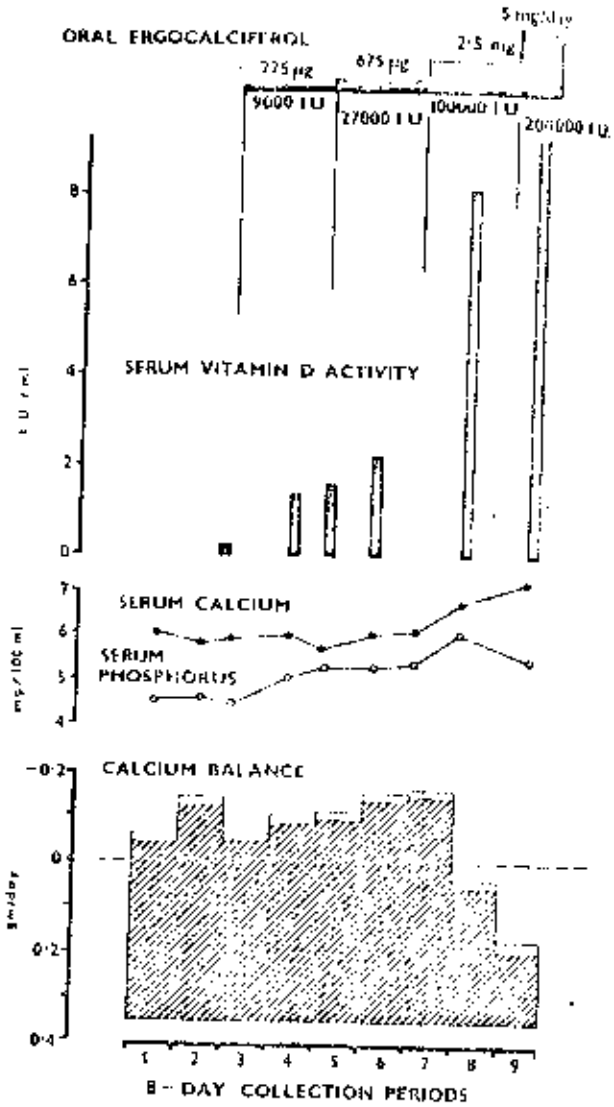

Figure 5. Serum vitamin D activity and the intestinal absorption of calcium in chronic renal failure. (Case 2; male, aged forty-three years. Azotemic osteomalacia; urea clearance, 5 ml/minute.)

before this was appreciated. If the fall in fecal calcium in period 3 (Figure 6) is accepted as significant, this implies a metabolic response in association with serum vitamin D activity some two to three times the average level of healthy subjects and no more than twice the highest of the control values in Figure 1. In any event, the undoubted metabolic response evident in periods 4 and 5 was associated with serum activity between 2.9 and 3.4 IU/ml.

Because of the tedium and time involved for the patient in obtaining valid measurements of intestinal absorption of calcium, and because of failure of several serum assays, it was rarely possible to determine the actual minimum requirement for vitamin D in the individual case. Table I includes all the available relevant data from seventeen patients with azotemic renal failure and osteodystrophy treated with either ergocalciferol (thirteen cases) or cholecalciferol (four cases).

From these data (columns 4 and 5, Table I) it can be seen that, in different patients with renal failure, oral dosage with vitamin D between 3,000 and 150,000 IU/day proved to be biologically ineffective in respect to calcium absorption. This therapy produced levels of total vitamin D activity in serum that were either within the upper half of the range of the healthy control subjects (1.0 to 2.2 IU/ml of serum in Cases 2–5, Table I) or greatly in excess of normal (e.g., Cases 1, 6–9 and 10). Columns 6 and 7 (Table I) show the daily oral dosage of vitamin D and the corresponding levels of serum activity that were observed to be associated with increased intestinal absorption of calcium. Figures 4–6 constitute prototypes of the investigations providing this information. The daily dosage shown in column 6 represents the upper limit of the estimated daily requirement for vitamin D in the individual patient; it may be a several-fold overestimate of the actual need. When it was possible to make the appropriate observations with the patient receiving first an ineffective and then an effective dose of vitamin D (Cases 1, 4, 7, 9, 10, 15 and 16), this involved a 1.6 to 6 times increase of the administered daily dose (see Table I). Since observations were not available at intermediate levels of dosage, the daily requirement of the individual patient might be overestimated by a corresponding factor. Similarly, although an increased intestinal absorption of calcium occurred in association with serum levels of vitamin D activity between 3 and 13 IU/ml (column 7), it is possible that a similar response would have occurred in some patients (especially perhaps Cases 1 and 9) at a lower level of activity. Notwithstanding the inevitable imprecision in studies of this kind, the data of Table I appear to establish that the patient with chronic renal failure requires a higher intake of vitamin D and a higher level of total vitamin D activity in his serum than does a normal subject to produce the biologic effects of the vitamin.

These inferences are reinforced by comparison with similar observations in two patients with nutritional osteomalacia due to dietary deficiency of vitamin D (Figures 7 and 8). In Case 24, the serum vitamin D activity before treatment was 0.2 IU/ml. Treatment with ergocalciferol (9,000 IU or 225 μg/day) progressively increased the serum activity to 1.9 IU/ml; but a definite metabolic re-

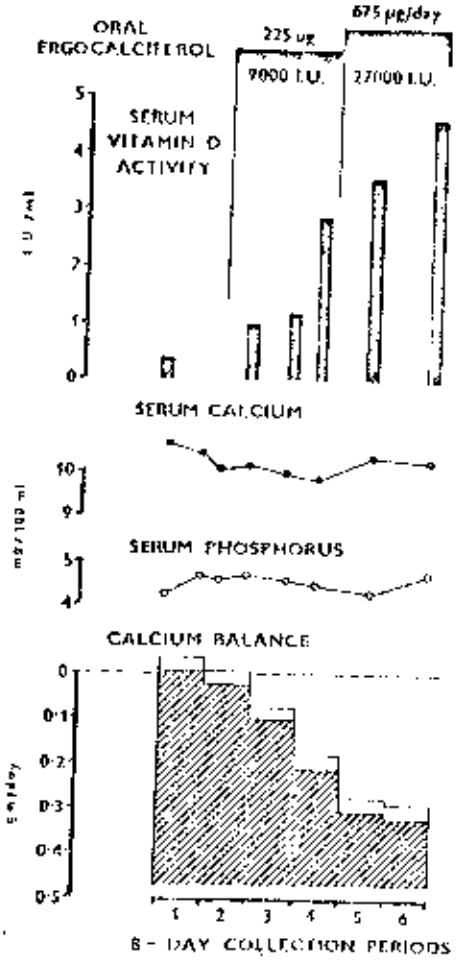

Figure 6. Serum vitamin D activity and the intestinal absorption of calcium in chronic renal failure (Case 4; male, aged fifteen years. Azotemic rickets; creatinine clearance, 13 ml/minute.)

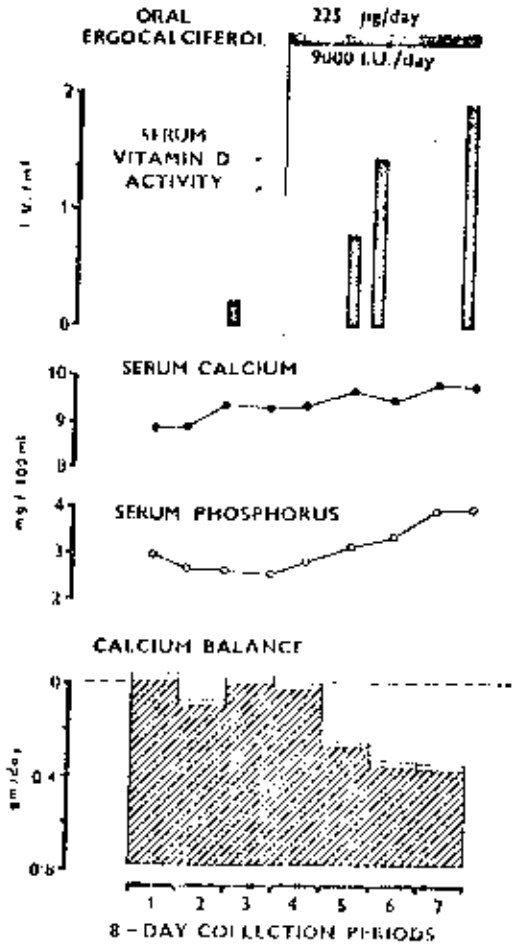

Figure 7. Primary nutritional osteomalacia: the relation of serum vitamin D activity to the intestinal absorption of calcium. (Case 24; male, aged thirty-three years. Epilepsy and mental deficiency.)

sponse occurred (period 5, Figure 7) when the assayed serum activity was between 0.8 and 1.4 IU/ml. In the second case of nutritional osteomalacia (Figure 8) a metabolic response (periods 6 and 7) was associated with a serum vitamin D activity of 0.6 IU/ml and a daily oral intake of 3,000 IU (75 μg) of vitamin D. Thus, in contrast with the behavior in renal failure (Table I), in these otherwise normal subjects it was only necessary to establish a "normal" serum level of vitamin D activity to promote an appropriate intestinal absorption of calcium.

Vitamin D Effect and the Serum Ca × P Product in Chronic Renal Failure. Twelve of the seventeen azotemic patients listed in Table I had clinical rickets or osteomalacia. Before the institution of effective therapy with vitamin D this defective mineralization was associated in different patients with values of the serum Ca × P product between 28 and 59 (Table I). In twelve patients, mean values of the serum calcium, phosphate and Ca × P product were available when the patient was receiving (1) an ineffective dose of vitamin D; (2) an effective dose, i.e., sufficient vitamin D to produce an unequivocal, measured intestinal absorption and bodily retention of calcium and phosphorus. For the twelve cases as a group, the mean values of these three serum biochemical variables were identical before and during effective therapy with vitamin D. Although the latter produced con-

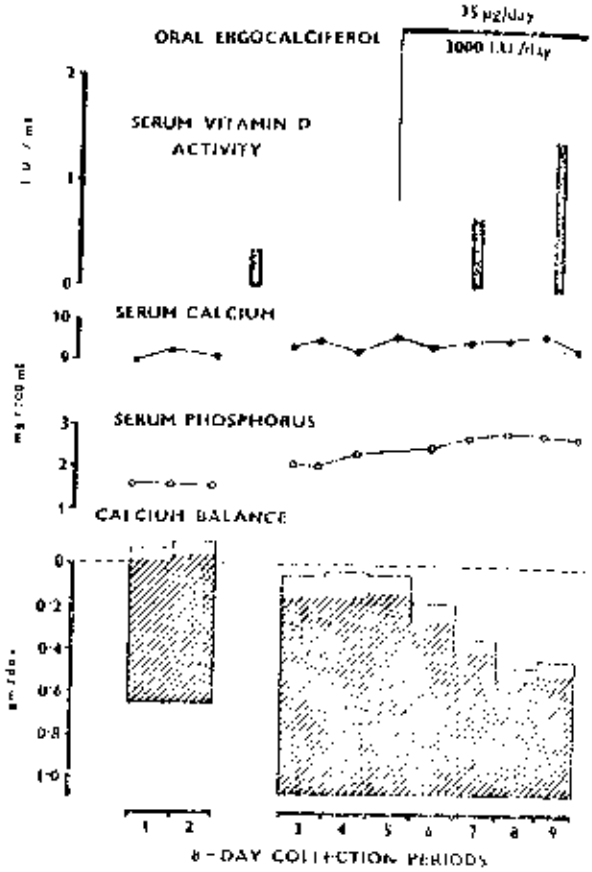

Figure 8. Primary nutritional osteomalacia: relation between the assayed level of serum vitamin D activity and the intestinal absorption of calcium. (Case 23, female, aged seventy-five years.) Metabolic studies were interrupted for a period of three weeks when the patient consumed an ample general ward diet. Assay of serum during this period gave a value of 0.35 IU/ml; an assay on an earlier blood specimen was a technical failure. Although net calcium absorption was higher in periods 3–5 than in periods 1 and 2, suggesting partial correction of her vitamin D deficiency, treatment with 75 μg/day of ergocalciferol produced a typical metabolic response.

oral retention in all twelve patients, only three (Cases 2, 9 and 10) showed an appreciable rise in Ca × P product, and three (Cases 1, 14 and 16) a significant rise in serum calcium, during the few weeks of metabolic study in hospital (Table I). These data support our previous contention [15,18,24] that neither the presence of defective mineralization in renal failure nor the initiation of its healing is predictably related to the plasma Ca × P product. This behavior of patients with renal failure is to be contrasted with the expected rise of the low Ca × P product when the patients with nutritional osteomalacia were treated with vitamin D (Cases 23 and 24, Table I).

The Persistence of Vitamin D Activity in the Blood. After terminating a course of treatment with ergocalciferol in high dosage the vitamin D activity of serum remained elevated above normal

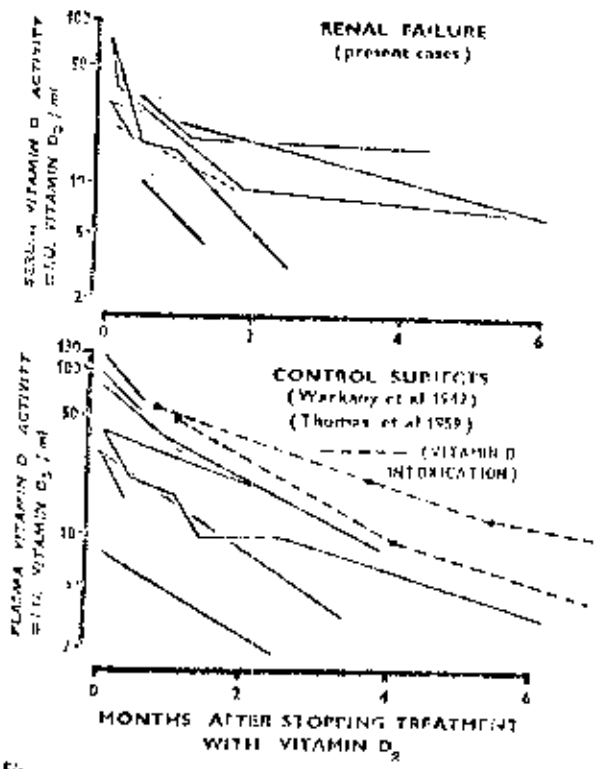

Figure 9. The decay in serum vitamin D activity after stopping oral therapy with large amounts of calciferol. Among the curves for "control cases," the lowest is the normal subject studied by Thomas et al. [39]; the others were patients with rheumatoid arthritis [36]. The two interrupted lines refer to two healthy subjects with accidental vitamin D intoxication studied by ourselves. The interrupted line with the renal cases represents personal observations in a patient with idiopathic acquired hypophosphatemic osteomalacia [101].

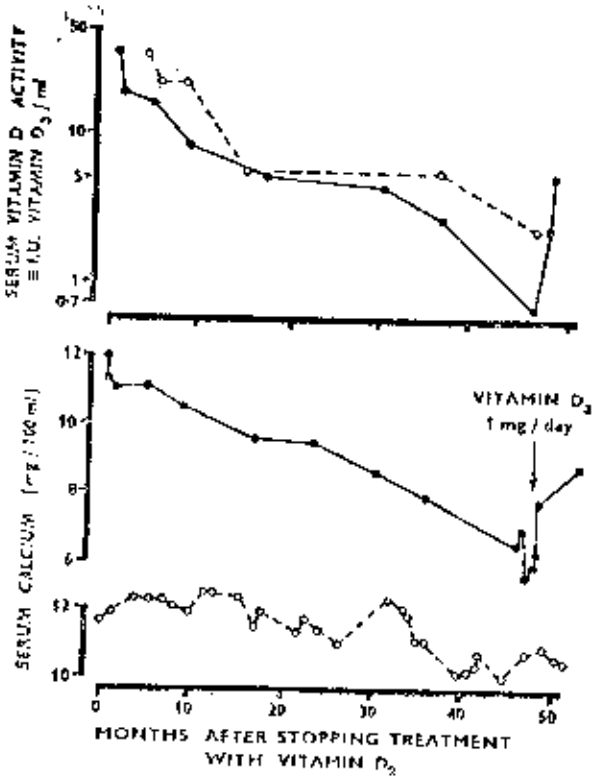

Figure 10. The long persistence of vitamin D activity in serum and its relation to the serum calcium. Case 16. This patient (●) was intoxicated when treated with vitamin D_2 for azotemic osteomalacia. Four years after stopping treatment she required 1 mg/day of vitamin D_2 to control hypocalcemia and recurring osteomalacia (see Table I). Case 18. This patient (○) had chronic renal failure with probable autonomous secondary hyperparathyroidism. Hypercalcemia persisted through four and a half years without vitamin D therapy.

levels for many months (Figures 9 and 10). The upper half of Figure 9 shows the results of assays on serum obtained from six patients with renal failure during the first six months after stopping treatment. The comparable data of Warkany, Guest and Grahill [36], from patients with rheumatoid arthritis treated similarly with ergocalciferol, are plotted below on the same semilogarithmic scale. Measurements extending throughout a period of four years in two of our patients are recorded separately in Figure 10. The patients with renal failure had received from 1.25 to 3.75 mg daily for periods between eight and nineteen months, involving a total dosage of 220 mg to 3.12 gm of ergocalciferol. The patients with rheumatoid arthritis had received comparable dosage [36].

On stopping treatment there was an initial rapid decrease in serum vitamin D activity, followed by a

progressively slower decay (Figure 9). In the present study it was not possible to obtain sufficient measurements to construct complete curves of decay for the individual patient, and the data must be viewed as a whole. In one patient assays on the first and third days after stopping treatment gave an estimated half-time of decay of 3.2 days; in three other patients assays on the first and tenth or twelfth days gave values, for this longer period, of 4.5 to 13.5 days. For the two patients shown in Figure 10, the calculated mean half time of decay, between the fourth and forty-seventh month after stopping treatment, was sixteen and seventeen months, respectively.

For purposes of comparison, Figure 11 includes the only other substantial body of data that we know relating to the prolonged survival of vitamin D in human blood. Figure 11 (above) includes four unpublished curves, from the work of Mawer, Lumb and Stanbury [75], showing the decay in serum levels of radioactive cholecalciferol after the intravenous injection of a test dose. The half-time of decay was one to two days during the first five days after the injection; eighteen to thirty-six days between the sixth and fortieth day; and fifty to sixty days between the fortieth and one-hundredth day of study [25]. The data of Hilp, Antener and Prader [37] have been plotted on a homologous semilogarithmic scale in the lower part of Figure 11. Seven infants received 15 mg of cholecalciferol by intramuscular injection, and

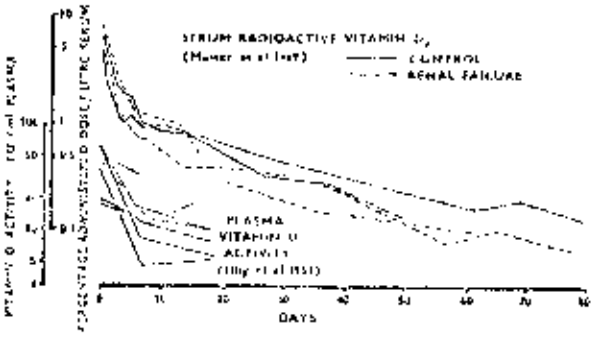

Figure 11. The persistence of vitamin D in serum after administration of a single dose of cholecalciferol. Above, the level of radioactive cholecalciferol in serum after the intravenous injection of [1,2 H_3]- or [4-^{14}C]-cholecalciferol at time 0 [25]. Below, the level of biologically assayed total vitamin D activity in serum after the intramuscular injection of 600,000 IU of cholecalciferol on day 1 [37]. The two differently measured variables are plotted on homologous logarithmic scales to demonstrate that decay in each follows a series of exponential functions (compare with Figures 8 and 9).

assays of serum for vitamin D activity were made on the next day and after approximately one and three weeks. During the first week, the serum activity decayed with a half-time between 2.2 and 13.5 days; during the next two weeks the half-time in four patients was between seventeen and twenty-eight days.

Thus when the serum vitamin D activity has been increased, either by continued administration of ergocalciferol or by acute loading with ergocalciferol, the subsequent decay in levels of biologic activity appears to follow a series of exponential functions. The radioactive data indicate that chromatographically separated cholecalciferol in the serum exhibits a similar pattern of decay. The behavior of patients with and without renal failure was essentially similar (Figures 9 and 11).

Vitamin D Activity in Muscle, Liver and Bile. Assays were made of the vitamin D activity present in tissues obtained at autopsy from three patients with chronic renal failure (Table II). One (Case 20) was treated for azotemic osteomalacia in another hospital in 1963; he then received a total of 3.12 gm or 125 million IU of oral ergocalciferol over a period of ten months. Subsequently, in our care, he received no vitamin therapy for two years. Between July 1965 and May 1966 he received a further 350 mg of ergocalciferol, after which therapy was withdrawn for seven months. Finally, he was treated with 50,000 IU (1.25 mg) daily of ergocalciferol up to the day of his death in January 1968. During this last period of twenty-five months he received approximately 875 mg, and the total dosage received in about four and a half years was thus approximately 4.35 gm or 174 million IU. The second patient (Case 21) had earlier been treated with 100,000 IU (2.5 mg)/day of ergocalciferol for four months (approximate total dose 300 mg or 12 million IU), but she had received no vitamin therapy during the fifteen months prior to her death. The precise intake of the third patient (Case 22), who was not in our personal care, is uncertain; he is, however, known to have received 2.5 mg of ergocalciferol daily for eight days one month before his death (total dose, 800,000 IU).

The level of assayed vitamin D activity in the serum and the liver of each individual patient was essentially the same (Table II). In Case 20, the serum activity was raised to a degree appropriate to the high intake at the time of the patient's death (see Figure 3), and the liver activity was correspondingly high. The assayed activity in the

muscle in Case 22, the patient who had received a relatively small therapeutic dose, was the same as in serum and liver; but in the two patients who had received massive doses of ergocalciferol the level of vitamin D activity in voluntary muscle (Case 21) or cardiac muscle (Case 20) was more than twice that in the serum (Table II).

From the known weight of the liver, and assuming plasma volume to be equivalent to 4 per cent and voluntary muscle to 40 per cent of body weight, the total body content of vitamin D has been crudely computed (see Table II). As has been demonstrated previously in the pig [38], the circulating blood appears to contain more vitamin D than the liver. Our present observations suggest, however, that muscle may be quantitatively the most important site of vitamin D storage in man. This suggestion is compatible with our observations in man using radioactive cholecalciferol [39] and with the autoradiographic demonstration of the radioactive vitamin in the cardiac and voluntary muscle of animals [40].

It is estimated that the daily biliary secretion in man is 0.5 to 1 L and that the hepatic bile is concentrated five to ten times in the gallbladder [41]. Accepting these estimates, the data in Table II suggest that the secreted bile of one patient (Case 20) may have had a vitamin D activity close to that of his serum (6 IU/ml); this would imply a biliary excretion in this patient of perhaps 3,000 to 6,000 IU/day. If the gallbladder bile in the two other patients (Cases 21 and 22) had been similarly concentrated, the secreted bile could have had an activity as low as 0.1 to 0.4 IU/ml with a daily excretion by it is route of perhaps less than 100 or up to 400 IU/day. This difference between behavior in Case 20 and in Cases 21 and 22 raises the speculative question as to whether the content of vitamin D in the liver (or the plasma) determines the concentration of vitamin D in the secreted bile. In patients receiving physiologic amounts of the vitamin little or no free vitamin has been detected in the bile [31]. In a single patient receiving 100,000 IU/day of ergocalciferol, who was subsequently given an injection of radioactive vitamin D_3, we have recovered free cholecalciferol in bile aspirated from the duodenum. After pancreozymin stimulation the total recovery of radioactivity in the aspirated bile was 3×10^{-4} of the administered dose; 61 per cent of this activity was extractable by chloroform and was all present as free cholecalciferol (Mawer and Stanbury, unpublished).

TABLE II Vitamin D Activity in Tissues and Body Fluids

Material	Concentrations of Biologically Assayed Vitamin D Activity (IU/ml of serum or bile; IU/gm of whole wet tissue)		
	Case 20 (34 kg)	Case 21 (40 kg)	Case 22 (60 kg)
Serum	6.1	1.5	(1.9)*
Cardiac muscle	15.5	...	...
Voluntary muscle	...	4.0	1.6
Liver	5.0 (0.98 kg)	1.2 (1.33 kg)	1.6 (1.85 kg)
Gall bladder bile	42.6	1.8	0.9
Compact bone (femur)	...	...	2.0
Material	**Estimated Total Vitamin D in Tissues Assayed ($IU \times 10^3$)**		
Plasma	8.3	2.9	5.25
Liver	4.9	1.6	2.95
Voluntary muscle†	211	78.5	50
"Total in body"	>225	>83	>56
Total known therapeutic dose ($IU \times 10^3$)	174	12	0.8

* No postmortem blood was obtained in Case 22; the "serum" assayed was actually a pericardial effusion containing 3.7 gm/100 ml of albumin and 3.0 gm/100 ml of globulin. The protein content of this fluid was almost identical with that in the last sample of plasma obtained before the patient's death.

† In Case 20, it is assumed that the vitamin D content of voluntary muscle was the same as cardiac muscle.

COMMENTS

Vitamin D Nutrition and the Assayable Vitamin D in Blood. It is not known what should be regarded as a "normal," or minimally adequate, level of biologically assayable vitamin D activity in human serum. The conventionally accepted optimum of approximately 1 IU or 0.025 μg/ml [42] is arbitrary and probably based on the classic assays made by Werkman in the 1930's [32,43,44]. It is evident, however, that the levels actually measured vary considerably in different population groups (Figure 12) and are probably more related to the state of vitamin D nutrition or overnutrition than to biologic requirement. The low levels measured in the healthy control subjects of our present study cannot be dismissed as due to methodologic error; an identical mean value of 0.8 IU/ml of serum was obtained by Thompson [45] in London, and Autenrieth [34,37], using a sensitive bioassay depending on the incorporation of radioactive calcium into bone [46], encountered levels as low as 0.22 IU/

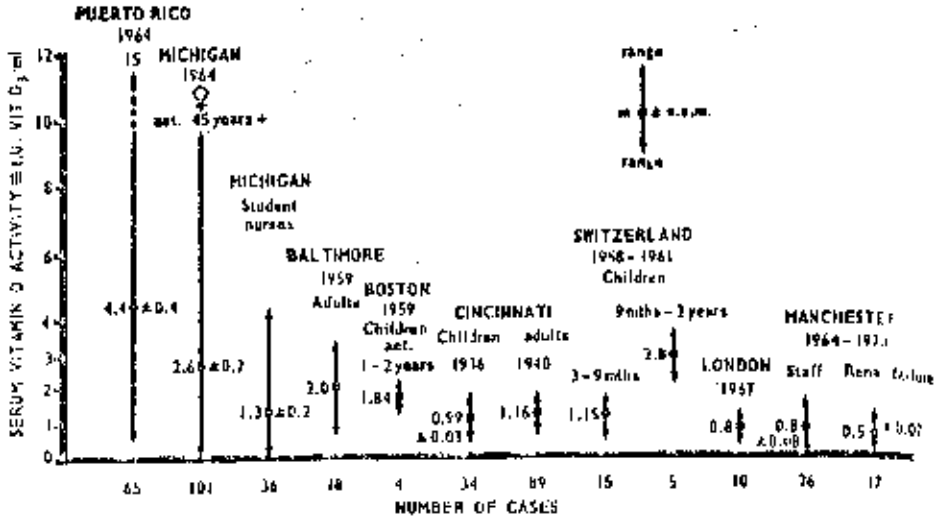

Figure 12. The biologically assayed vitamin D activity of human serum. The data from Figure 1 (Manchester) are compared with similar data available from the literature [33,69,70,73]. The low levels in the student nurses in Michigan were probably attributable to their indoor occupation and institutional feeding. Puerto Rico has the double advantage of food enrichment laws [117] and abundant effective sunshine [69]; London and Manchester have neither.

ml in healthy Swiss babies. It has been demonstrated in our investigation that a curative metabolic response may occur in patients with nutritional osteomalacia when therapy increases serum vitamin D activity to the range 0.6 to 0.8 IU/ml (Figures 7 and 8), but the oral dose of 3,000 to 9,000 IU/day used in these studies was several hundredfold greater than the estimated previous daily intake of the patients, and it is grossly in excess of the actual therapeutic requirement. Fourman et al. [47] produced therapeutic responses, in gastrectomized patients with vitamin D deficiency and osteomalacia, by injecting as little as 100 IU/day. In some Asian adolescents with nutritional late rickets we have found that a mere seven to ten day period of equilibration with a constant diet in the metabolic ward may apparently be sufficient to initiate a metabolic response, similar to that shown in Figures 7 and 8, without the deliberate administration of supplementary vitamin D (Stanbury, unpublished). A similar experience has been reported in nutritional osteomalacia [48]. Our personal experience with the biologic assay suggests that it would not detect the effect of such small doses on the serum vitamin D activity of the individual patient, unless very large volumes of serum were extracted for assay. In several of our healthy control subjects assayable activity was as low as in patients with bone disease attributed to nutritional vitamin D deficiency; in a few, activity was virtually undetectable (Figure 1) although the presence of vitamin D could be demonstrated in lipid extracts of large volumes of serum (Mawer, Lumb, Schaefer and Stanbury, unpublished). These results suggest that the minimum level of vitamin D activity in serum that is compatible with biological action in the organism could be so low as to be effectively unmeasurable. This suggestion is made more feasible with the claim that only nanogram or picogram quantities of "active vitamin" may be required within the nuclei of cells in the target organ [7,8,10].

Recent estimates have placed the requirement for vitamin D in the human adult at about 75 IU/day [49]. Preliminary dietary surveys undertaken by our laboratory in the North West of England indicate that many healthy people ingest as little as 60 to 30 IU/day, and daily intakes above 150 IU are unusual (M. Jones, personal communication). These findings are compatible with the low levels of vitamin D activity assayed in the serum of our healthy control subjects (Figure 1), as compared with the higher levels measured in populations known to have a higher vitamin D intake (Figure 12). Since exposure to effective sunlight is minimal in our climate, this is probably an even less important source of vitamin D than our vitamin D poor diet, or, conceivably, its trivial exposure to solar radiation just suffices to protect the urban British population from developing clinical deficiency. Contemporary experience in Switzerland [50], in Greece [51] and in Egypt, Israel and India [52–57] indicates that casual solar exposure, even in sunny or tropical climates, may fail to counter the effects of inadequate dietary intake of the vitamin in infants. Although cases of nutritional rickets and osteomalacia do occur in Britain [58–59], they are uncommon except in Asian immigrants [60]. The

general state of vitamin D nutrition in Britain is thus probably not optimal but marginally adequate to protect against overt clinical deficiency. Such a nutritional status is peculiarly fitted to display the effects of any increase in requirement for the vitamin that might be produced by disease. A seasonal variation in the whole blood phosphate of adults was recorded in Britain forty-five years ago [61] and, more recently, Europeans have been shown to exhibit seasonal variations in the intestinal net absorption of calcium [62,63]. Both these phenomena may reflect the supplementary contribution made by sunlight to a barely adequate body content of vitamin D; they are probably analogous to the conspicuous fall in blood levels of vitamin D that occurs during winter in sheep reared in the north of Britain [64,65].

In animal husbandry, it is assumed that the blood level of vitamin D activity is a measure of the total amount of the vitamin in the body [38], and a degree of general support for this contention has been obtained in the pig, sheep and cattle [38,64–67]. Earlier observations in man, based on relatively few assays, have been interpreted as showing no predictable relationship between the daily oral intake and assayable serum levels of the vitamin [3,68]. The relationship shown in Figure 3 suggests the converse. The extremely wide confidence limits of the regression indicate how this apparently real relationship could be missed if data were few, or if serum assays were made at only one or two levels of oral intake. It is only necessary to note the fifteen to twenty times variation in the serum level of activity in different subjects ingesting 100,000 IU/day of ergocalciferol (Figure 3). For many reasons, it is not permissible to extrapolate the regression of Figure 3 to levels of intake below 3,000 IU/day, but it seems reasonable to expect that a generally similar relationship would obtain at lower levels of intake. A measure of support for this suggestion is provided by Figure 12 which, we believe, includes all the available series of serum or plasma assays made in man. Only the levels assayed by Warkany in Cincinnati, before vitaminization of foodstuffs became widespread in the United States, approach the nadir of those measured in Manchester and London, England. As already indicated, our own serum assays probably reflect an intake statistically unlikely to exceed 100 to 200 IU/day. We are unaware of any systematic surveys of vitamin D intake in the United States.

The fortification of foodstuffs—milk, milk products, cereals, margarine and others—provide a variable and virtually unavoidable intake of vitamin D which, although difficult to estimate, may often be considerable" [71]. The Committee on Nutrition of the American Academy of Pediatrics estimates that some well cared for children in the United States and Canada could easily ingest as much as 2,000 to 3,000 IU/day [71]. We suggest that this generally higher intake accounts for the higher levels of serum activity in population samples in the United States (Figure 12). If, as appears to be the case, the low British intake is adequate to maintain health in ordinary circumstances it must be near to actual physiologic requirement. In this event, there is a luxus consumption in the United States which, as discussed subsequently, may involve storage of the vitamin in tissues other than plasma [26,72]. This is particularly relevant to the interpretation of studies using radioactive vitamin D to trace the distribution and metabolism of the vitamin in man; it can be shown that particular patterns of distribution kinetics, purported to be caused by disease, may simply reflect the state of vitamin D nutrition (or overnutrition) in the individual [26]. It does, however, seem possible that the different vitamin D nutrition in the two countries could affect the mode of clinical presentation of azotemic osteodystrophy in Britain and the United States [24].

The Apparent Vitamin Resistance of Chronic Renal Failure. Because of the dietary restrictions imposed by chronic renal failure, it is not unexpected to find a lower mean value of serum vitamin D activity in a group of azotemic patients than in healthy members of the same population (see Figure 1; [73]). The difference between the assays in our healthy and azotemic subjects was not significant, indicating that all the data could have been derived from a single group. This in itself suggests that the rickets or osteomalacia present in most of these azotemic patients could not be attributed to simple privational deficiency of vitamin D. This point is not established unequivocably by these data alone, because the results of serum assays obtained in patients with bone disease ascribed to nutritional deficiency overlap with those in renal failure (Figure 1). A more categoric answer is provided by the studies involving simultaneous measurements of external mineral balance and assayable vitamin D activity in serum, during treatment with graded oral doses of ergocalciferol (Figures 4–6 and Table I). These appear to establish that, to produce the biologic effect of vitamin D, the patient with renal failure requires a higher oral intake

of the vitamin and a higher associated serum activity than does the healthy subject. Daily oral doses of ergocalciferol that increased assayable serum activity to the upper extreme of the "normal" range (3,000 to 27,000 IU/day in Cases 1–5 and 16; Figures 4 and 5 and Table I) produced no detectable metabolic effect in patients with renal failure. Comparable dosage and serum activity in simple nutritional deficiency was demonstrably effective (Figures 7 and 8), and the serum levels of activity found in the completely healthy control subjects were presumably biologically adequate. Other cases of renal failure (Cases 6–8, 10 and 15; Table I) were similarly unresponsive to higher levels of serum activity produced by a greater intake of either ergocalciferol or cholecalciferol. Only one index of the vitamin's effect, the production of an unequivocal increase of the intestinal net absorption of calcium, has been used in these studies as a criterion of response. But it has been demonstrated repeatedly that a dose of vitamin D sufficient to produce this effect will always heal azotemic rickets or osteomalacia [15,16,23], and the accompanying changes in calcium, phosphorus and citrate metabolism are indistinguishable from those resulting from correction of simple vitamin D deficiency [15,16,18,23,24]. Thus, defective mineralization in chronic renal failure appears to be due to lack of vitamin D action; and this particular component of azotemic osteodystrophy is caused by an acquired increased requirement for the vitamin or resistance to its biologic action.

The actual apparent requirement varies unpredictably among patients (Table I), and the available data are insufficient to relate requirement to the degree of renal failure present. It appears, however, that some patients with less severe renal failure (Cases 3, 4, 14 and 16; Table II) will show the biologic effect of vitamin D when the level of serum activity is no more than three to six times the average (or two to three times the highest) level found in our indigenous healthy population (Figure 1), and, as discussed with the presentation of results, the requirement may be variably overestimated in these studies. Such therapeutically induced levels of vitamin D activity fall within the range commonly found in the serum of healthy Americans (Figure 12; [33,69,70,71]). It is thus possible that an increased requirement for vitamin D, developing early in renal failure, would be met by the luxurious consumption of the American. With his poorer state of vitamin D nutrition, in the comparable British patient effective vitamin D deficiency would develop, and, if his renal failure were of slow evolution, his bones could be exposed to the effects of such deficiency for some years. This, we suggest, may account for the frequency of the rickettic component of azotemic osteodystrophy in Britain [12–18,21,23,24], China [74] and India (personal communication), and for its reputed infrequency in the United States. By the same token, there could be a greater risk of developing vitamin D deficiency, after gastrectomy or as a complication of intestinal malabsorption, in Britain than in the United States. Vitamin D activity was detectable in the serum of several of our patients considered to have late rickets or osteomalacia due to simple vitamin D deficiency (Figure 1), and low normal levels of serum activity have been recorded previously in nutritional rickets of infants [37]. It is possible that genetic factors [75], sex [76] and other unrecognized influences can cause a major increase in the requirement for the vitamin. The operation of such factors may determine the occurrence of clinical deficiency as much as the absolute dietary intake and solar exposure of the individual, but their influence, like that of early renal failure, may only come to light in a population with a marginally adequate dietary intake.

It is uncertain at what stage, in the progressive evolution of renal failure, the lesion of apparent vitamin D resistance develops. In observations extending over almost twenty years we have not encountered clinical rickets or osteomalacia, nor demonstrated impaired intestinal net absorption of calcium [15,18,22,23], until glomerular filtration is reduced to the region of 30 to 35 ml/minute [77,78], no systematic exploration has been made in less advanced renal failure. Using orally administered radioactive calcium and whole body counting to measure absorption in patients with renal disease, Schaefer et al. [79] found significantly impaired calcium absorption only when the creatinine clearance was less than 25 ml/minute. If this malabsorption of calcium can be accepted as an index of effective vitamin D deficiency, these observations suggest that vitamin D resistance develops relatively late in the course of renal failure. Conversely, recent observations [80,81] suggest that secondary hyperparathyroidism may develop at the earliest stages of chronic renal disease.

Vitamin D Resistance and Vitamin D Metabolism in Chronic Renal Failure. The present data provide no direct evidence concerning the nature of the increased requirement for vitamin D in renal

ture. It is appreciated that what is assayed biologically as the total "vitamin D activity" of human serum may be attributable to several distinct molecular species [1–10]. Of these, two are quantitatively significant: (1) the unchanged native vitamin, and (2) a more polar metabolic "peak IV" which co-chromatographs with authentic 25-hydroxycholecalciferol [82] and which, if man behaves identically with the pig [2–4], may indeed be this chemical species.* Vitamin D ester, which is also antirickettic [83], is present in both normal and uremic human serum in only insignificant amounts [25,26,31,84]. The claim has been made by Avioli and his collaborators [84] that the vitamin D resistance of uremia is attributable to an acquired abnormality of the intermediary metabolism of the vitamin. They postulate that ingested vitamin D is rapidly converted into biologically inert polar metabolites by the uremic patient, and also that both cholecalciferol and "peak IV" are lost in the protein-containing urine [84]. We agree with the latter observation but regard such urinary losses as insignificant: in our studies they have been equivalent to the content of less than 10 ml of plasma/day (Mawer and Stanbury, unpublished). Certain observations made in the present study are difficult to reconcile with Avioli's general hypothesis. When we assay supranormal levels of "vitamin D activity" in the serum of azotemic patients receiving biologically ineffective amounts of the vitamin (Table I, column 5), we are demonstrating an increase above normal in its content of unchanged vitamin, or "peak IV," or both. Thus, the lack of demonstrable biologic effect in the treated patient in this circumstance cannot be attributed to metabolic inactivation or urinary loss of the therapeutically administered vitamin. Hypothetically, it is possible that all the assayed serum activity was attributable to unchanged vitamin. This would be converted to the putative active form, "peak IV" [1–4], by the healthy assay rat [85,86] and be measured as "vitamin D activity," although it could be biologically inert in the donor patient [2–4,88,89]. However, in studies still proceeding that utilize a column chromatographic system capable of separating authentic 25-hydroxycholecalciferol [82], we repeatedly find that the uremic patient rapidly converts administered radioactive cholecalciferol to radioactive "peak IV," which subsequently persists in the circulating plasma [26]. This material is indistinguishable chromatographically from the "peak IV" produced by normal subjects. Moreover, in a few azotemic patients with therapeutically elevated levels of serum total vitamin D activity we have demonstrated that the biologic activity in the serum is fairly evenly distributed between the separated peak III and peak IV fractions [26]. Collectively, our own observations and interpretations conflict with those of Avioli and collaborators [84], which imply that the apparent vitamin D resistance of uremia is effectively due to a deficiency of available 25-hydroxycholecalciferol ("peak IV"). Our observations are more compatible with a state of end organ unresponsiveness that can be overcome by mass action, but they would be equally compatible with failure of some metabolic transformation subsequent to the formation of peak IV. According to Haussler [7,8] and Lawson [10] et al., the active molecular species may be an even more polar metabolite (peak "P" [10], "peak 4b" [7,8], "peak V" [87]), which is apparently formed from peak IV [8,10] and accumulates selectively in target organ nuclei [7,8,10]. Stohs and DeLuca [90] failed to confirm these recent observations, but, if subsequently established, they could imply that what is detectable or measurable in plasma has little relevance to the expression of vitamin D action in the organism. The fallacies that may be incurred when observations on the kinetics of disappearance of injected radioactive vitamin are extrapolated to inferences regarding its metabolism are discussed elsewhere [26].

Vitamin D Absorption, Excretion and Storage Under Conditions of High Therapeutic Intake. Irrespective of the ultimate cause and intimate nature of the vitamin D resistance in uremia, the need to treat patients with large daily doses of the vitamin (Table I; Figures 4, 5, 9 and 10) provided unique opportunities for study. In these circumstances one encounters a different order of biologic phenomena than are involved in the correction of simple deficiency by an appropriate physiological dose. The data of Figure 3, and the more direct observations of Avioli et al. [84], suggest that the patient with renal failure probably

* In contemporary literature it is commonly assumed that this active metabolite in man is actually 25-hydroxycholecalciferol or 25-hydroxyergocalciferol [87]. So far as we are aware, this has not yet been proved by chemical identification of the active metabolite isolated from human material. For this reason we prefer the noncommitted description "peak IV"; chromatographically, this corresponds with "peak IV" of Corchan and DeLuca [85,86], "peak 4a" of Haussler et al. [7] and "peak IVb" of Mawer and Lawson [82].

absorbs administered vitamin D as well as a normal subject. The data of Table II indicate that absorbed vitamin is retained in other tissues besides plasma. In a group of healthy adults the intestinal net absorption of a single oral dose of 1 mg of cholecalciferol was approximately 80 per cent [91]; with a smaller oral dose (~0.1 mg) net absorption was 90 per cent in both healthy control subjects and patients with renal failure [84]. Assuming an intake of 100 to 200 IU/day in our own healthy subjects (Figure 1), it appears from Figures 2 and 3 that a daily intake up to 5,000 times greater produces no more than one hundredfold increase in assayable serum activity, even when such dosage is continued for weeks or months. In members of a family intoxicated from using as cooking oil an animal concentrate containing 5,000,000 IU/gm, we assayed serum activity no higher than 66 IU/ml (to be published). There may thus be a limit to the capacity of serum to accommodate or bind vitamin D active materials. The log/log relationship shown in Figures 2 and 3 carries no direct physiologic implications; it simply expresses mathematically a relationship between independent (oral dose) and dependent variable (serum activity) that is of uncertain significance. It might be explained if the fractional absorption of the administered dose were to diminish with increase in dose or body content of the vitamin; if loss from the plasma occurred, by excretion, or by transfer into storage pools outside the circulating blood; or if metabolic degradation increased with body accumulation of the vitamin. We are unaware of any evidence, from studies in man or in animals, to support the last of these options. In the rat, more of an oral dose of vitamin D is absorbed and retained by vitamin D deficient animals than by those pretreated with the vitamin [92,93]; but it is not known if this is a quantitative relationship or whether it subserves a regulatory function. There are no comparable observations in man. As regards excretion of vitamin D and its metabolites from the blood, it is universally agreed that both in animals [94,95] and in man [31] the principal excretory pathway is through the bile. Vitamin D activity was present in each of the samples of gallbladder bile assayed in the present study (Table II), but the chemical nature of this biologically active material is unknown. Although an isolated observation, it may be significant that appreciably large amounts of vitamin D activity were recovered from the bile only in the patient (Case 20; Table II) who was receiving large amounts of ergocalciferol at the time of death and who had high levels of activity in the serum and liver (Table II). This suggests the possibility that the biliary excretion of vitamin D active materials increases with increase of their level in the serum or in the liver. Biliary excretion may contribute to the initially rapid fall in serum vitamin D activity that occurs when therapy with large doses of vitamin D is stopped (Figures 9–11). This alone is unlikely to constitute an efficient mechanism for eliminating a bodily excess of the vitamin, since vitamin D entering the intestine in bile should be absorbed as effectively as when it is administered orally [84,91]; it is known that most of the cholesterol and bile acids secreted in bile are subsequently reabsorbed.

The demonstration of significant amounts of vitamin D activity in various tissues (Table II) indicates that, at least under conditions of high therapeutic dosage, extravascular pools must share in the accommodation of absorbed vitamin. After the administration of a single, massive dose of vitamin D, assayable vitamin D activity has similarly been demonstrated in many tissues of the rat [92,96], rabbit [97], dog [98–100], pig [38], calf [66,67] and rhesus monkey [92]. The induced rise in levels of assayable activity persisted longer in the blood than in other tissues [38,97,98]. Few of these animal experiments were concerned with the tissue deposition resulting from prolonged high dosage with the vitamin [38], and, with the occasional exception [92,98], little attention was given to vitamin D activity in voluntary muscle. In man, whether measured by direct biologic assay as in the present observations (Table II), or in terms of the bodily distribution of administered radioactive cholecalciferol [39], we always find quantitatively more vitamin D in the whole body musculature than in the circulating blood. It is notable that in Case 2 (Table II), some fifteen months after stopping vitamin D therapy, the concentration of assayable activity in muscle was more than twice that in serum. In another patient, two years after stopping massive dosage with ergocalciferol, the serum vitamin D activity was normal (~ 1 IU/ml) but the concentration of assayed activity in skin, voluntary muscle, subcutaneous fat, compact bone and bone marrow was eight to ten times as high [101]. Assays undertaken earlier in these two patients, when each was receiving treatment with vitamin D, had measured levels of serum activity of 30 IU (Case 21) and 23 IU/ml, respectively. The evidence suggests that

on therapy raises the serum levels of vitamin D, vitamin is transferred from plasma into tissues; this would tend to limit the rise in serum activity during treatment (Figure 3). Continued transfer back from tissues to blood would similarly explain the prolonged persistence of vitamin D activity in serum after stopping therapy (Figures 9–11). If the α_2-globulin of plasma that binds native vitamin D [33,102–105] and peak IV [105] functions as a specific carrier for physiologic amounts of the vitamin, the binding to plasma albumin [33,102–105] might be nonspecific, and this fraction of plasma vitamin D could be regarded as a storage form. Thus considered, the vitamin D bound to plasma albumin and to nonspecific protein acceptors in various tissues would, together with that dispersed in fat [106], constitute the body store of the vitamin.

The Therapeutic Implications For Azotemic Osteodystrophy. The available evidence suggests that the acquired vitamin D resistance persists through to the terminal stages of renal failure [15,26,107]. Even in chronically dialyzed patients, with effectively no residual renal function, the characteristic intestinal malabsorption of calcium can be demonstrated [108]. It is, however, to be emphasized that the mere presence of the metabolic lesion of vitamin D resistance is not a valid indication for treating patients with renal failure or azotemic osteodystrophy with large amounts of vitamin D or 25 hydroxycholecalciferol. As already discussed (vide supra), doses of vitamin D that are metabolically effective in overcoming this resistance will invariably increase the intestinal absorption of both calcium and phosphorus [15]. This is likely to increase the serum phosphate [95,24] and, since the renal capacity to excrete calcium is strictly limited by the renal failure, calcium that is absorbed will also be retained in the body. If the treated patient has rickets or osteomalacia, effectively all the absorbed calcium will be deposited in the skeleton [15,16,23]; the serum calcium level may not rise initially, even though 0.5 gm or more of calcium is being retained daily [15]. There may not even be a significant rise in plasma Ca × P product (Table I), and such patients can be treated with vitamin D in comparative safety [78,109]. The azotemic patient with purely hyperparathyroid bone disease usually has a serum calcium level near to normal, associated with a high plasma phosphate and Ca × P product [110]. Any further increase of Ca × P product produced by vitamin D therapy is likely to cause or increase physical supersaturation of the extracellular fluid; and, although it may be possible to increase skeletal mineralization by such therapy [16,77], it may fail to produce involution of the secondary hyperparathyroidism [81], and it carries a grave risk of inducing metastatic calcification in the soft tissues [78]. The persistence of vitamin D in the body after the administration of large therapeutic doses (Figures 9, 10 and 11, and Table II) intensifies this hazard.

Thus, the only safe indication for the use of vitamin D therapy in azotemic renal failure is the specific treatment of symptomatic defective mineralization of the bone and the growth cartilage. Survey of bone obtained by biopsy or at autopsy in European patients with renal failure has revealed a high incidence of florid rickets and osteomalacia [14,111]. In a recent Australian survey [112] defective mineralization was an infrequent finding; although there appear to have been no systematic studies in the United States since those of Follis [19,20], the same may be true also of American patients with renal failure. The present investigation probably explains the geographic differences in the incidence of the rickets and osteomalacic components of azotemic osteodystrophy. The beneficial effects and relative safety of vitamin D therapy in European patients with azotemic rickets or osteomalacia have been fully documented [15,16,19,23,113], but these results cannot be extrapolated directly to patients with renal failure in the United States. Protected by his luxus consumption of vitamin D (vide supra) the American patient may largely avoid the development of defective mineralization, and his bone lesions may be predominantly those of hyperparathyroidism. The disenchantment with the results of vitamin D therapy in the United States [114] could thus stem partly from its use in inappropriate situations. It could be that only the exceptional American patient develops the defective mineralization which is the specific indication for treatment with vitamin D; and, before implementing a potentially hazardous therapy, it is incumbent on the physician to prove the presence of such bony lesions [109]. When bone disease develops in chronically dialyzed patients, it has usually appeared to be an osteoporotic osteitis fibrosa [115,116]; unless the individual patient is proved also to have osteomalacic bone disease, therapy with large amounts of vitamin D is absolutely contraindicated. It could only increase the

liability to metastatic calcification which is already a major problem in these patients.

The valid uses and the hazards of vitamin D therapy in the treatment of azotemic rickets and osteomalacia and for the remineralization of the skeleton after parathyroidectomy have been discussed elsewhere [18,24,78,107,109,113]. It should finally be emphasized that once defective mineralization has been healed by vitamin D therapy, the need for such therapy has been eliminated. Its continuation in such circumstances is as hazardous as in the treatment of osteitis fibrosa with vitamin D [78].

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REFERENCES

1. DeLuca HF: Mechanism of action and metabolic fate of vitamin D. Vitamins Hormones (NY) 25: 315, 1967.
2. DeLuca HF: Recent advances in the metabolism and function of vitamin D. Fed Proc 28: 1678, 1969.
3. Blunt JW, Tanaka Y, DeLuca HF: The biological activity of 25-hydroxycholecalciferol, a metabolite of vitamin D. Proc Nat Acad Sci USA 61: 717, 1968.
4. Blunt JW, DeLuca HF: The synthesis of 25-hydroxycholecalciferol, a biologically active metabolite of vitamin D. Biochemistry 8: 671, 1969.
5. Norman AW, Lund J, DeLuca HF: Biologically active forms of vitamin D, in kidney and intestine. Arch Biochem 108: 12, 1964.
6. Norman AW: The mode of action of vitamin D. Biol Rev 43: 97, 1968.
7. Haussler MR, Myrtle JF, Norman AW: The association of a metabolite of vitamin D with intestinal mucosa chromatin in vivo. J Biol Chem 243: 4055, 1968.
8. Myrtle JF, Haussler MR, Norman AW: Evidence for the biologically active form of cholecalciferol in the intestine. J Biol Chem 245: 1190, 1970.
9. Carlow RK, Kodicek E, Thompson GA: Metabolism of tritiated vitamin D. Proc Roy Soc (Biol) 164: 1, 1966.
10. Lawson DEM, Wilson PW, Kodicek E: A new cholecalciferol metabolite, involving loss of hydrogen at C-1, in chick intestinal nuclei. Biochem J 115: 269, 1969.
11. Wasserman RH, Corradino RA, Taylor AN: Vitamin D-dependent calcium-binding protein. Purification and some properties. J Biol Chem 242: 1978, 1967.
12. Barker HW: Renal dwarfism. Quart J Med 14: 205, 1921.
13. Parsons LG: Bone changes occurring in renal and coeliac infantilism, and their relation to rickets, renal rickets. Arch Dis Child 2: 1, 1927.
14. Stanbury SW: Azotaemic renal osteodystrophy. Brit Med Bull 13: 57, 1957.
15. Stanbury SW, Lumb GA: Metabolic studies of renal osteodystrophy. I. Calcium, phosphorus and nitrogen metabolism in rickets, osteomalacia and hyperparathyroidism complicating chronic uraemia and in the osteomalacia of the adult Fanconi syndrome. Medicine (Balt) 41: 1, 1962.
16. Dent CE, Harper CM, Phepoe GR: Treatment of renal glomerular osteodystrophy. Quart J Med 30: 1, 1961.
17. Liu SH, Chu HI: Studies of calcium and phosphorus metabolism with special reference to pathogenesis and effect of dihydrotachysterol (A.T.10) and iron. Medicine (Balt) 22: 103, 1943.
18. Stanbury SW, Bone complications of renal disease. Renal Disease (Black DAK, ed), Oxford, Blackwell Scientific Publications, 1962.
19. Follis RH Jr: Renal rickets and osteitis fibrosa in children and adolescents. Bull Johns Hopkins Hosp 87: 593, 1950.
20. Follis RH Jr, Jackson RA: Renal osteomalacia and osteitis fibrosa in adults. Bull Johns Hopkins Hosp 72: 232, 1943.
21. Garner A, Ball J: Quantitative observations on mineralised and unmineralised bone in chronic renal azotaemia and intestinal malabsorption syndromes. J Path Bact 91: 543, 1966.
22. Stanbury SW: The intestinal absorption of calcium in normal adults, primary hyperparathyroidism and renal failure. Nutrition in Renal Disease (Berlyne GM, ed), Edinburgh, E. & S. Livingstone Ltd, 1968.
23. Stanbury SW, Lumb GA: The osteomalacia of vitamin D deficiency. L'Osteomalacie (Hioco D, ed), Paris, Masson et Cie, 1967.
24. Stanbury SW, Lumb GA, Mawer EB: Osteodystrophy

developing spontaneously in the course of chronic renal failure. Arch Intern Med (Chicago) 124: 274, 1969.

25. Mawer EB, Lumb GA, Stanbury SW: Long biological half-life of vitamin D_2 and its metabolites in human serum. Nature (London) 222: 482, 1969.

26. Mawer EB, Lumb GA, Schaefer K, Stanbury SW: The metabolism of isotopically labelled vitamin D_2 in man. I. The influence of the state of vitamin D nutrition. Clin Sci 40: 301, 1971.

27. King RG, Stanbury SW: Magnesium metabolism in primary hyperparathyroidism. Clin Sci 39: 281, 1970.

28. Glaxo Laboratories Ltd: Standard methods.

29. Bills CE: Vitamin D assay—fine tests and chemical methods. Biol Symp 12: 409, 1947.

30. Finney DJ: Statistical Method in Biological Assay, 2nd ed. London, Charles Griffin & Co Ltd, 1964.

31. Avioli LV, Lee SW, McDonald JE, Lund J, DeLuca HF: Metabolism of vitamin D-^{3}H in human subjects. Distribution in blood, bile, feces and urine. J Clin Invest 46: 983, 1967.

32. Warkany J: Estimation of vitamin D in blood serum. Amer J Dis Child 52: 831, 1936.

33. Thomas WC Jr, Morgan HG, Connor TB, Haddock L, Bills CE, Howard JE: Studies of antirickettic activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D in human serum. J Clin Invest 38: 1078, 1959.

34. Antenor I, Gentil C, Paupe J: Dosage de la vitamine D dans le sérum humain. Technique et résultats chez l'enfant normal. Path Biol (Paris) 16: 373, 1969.

35. Dent CE: Dangers of vitamin-D intoxication (letter). Brit Med J 1: 834, 1964.

36. Warkany J, Guest GM, Grabill FJ: Vitamin D in human serum during and after periods of ingestion of large doses of vitamin D. J Lab Clin Med 27: 557, 1942.

37. Illig R, Antenor I, Prader A: Die Wirkung von Dihydrotachysterin (DHT) bei Mangelrachitis und die antirachitische Activität des Serums nach Verabreichung von DHT und Vitamin D. Helv Paediat Acta 16: 469, 1961.

38. Quarterman J, Dalgarno AC, Adam A, Fell BF, Boyne R: The distribution of vitamin D between the blood and the liver in the pig, and observations on the pathology of vitamin D toxicity. Brit J Nutr 18: 65, 1964.

39. Mawer EB, Schaefer K: The distribution of vitamin D metabolites in human serum and tissues. Biochem J 114: 74P, 1969.

40. Kodicek E: The turnover and distribution of vitamin D and its mode of action. The Transfer of Calcium and Strontium Across Biological Membranes New York, Academic Press, 1963.

41. Sherlock S: Diseases of the Liver and Biliary System, 2nd ed. Oxford, Blackwell Scientific Publications, 1968.

42. Fourman P, Royer P: Calcium Metabolism and the Bone, 2nd ed. Oxford, Blackwell Scientific Publications, 1968.

43. Warkany J: Über die Bestimmung des Vitamin-D-Gehaltes des Blutserums. Biochem Z 293: 415, 1937.

44. Warkany J, Mabon HE: Estimation of vitamin D in blood serum. II. Level of vitamin D in human blood serums. Amer J Dis Child 60: 606, 1940.

45. Thompson GR, Neale G, Lewis B, Watts M, Booth CC: Plasma vitamin D-like activity and vitamin D absorption in man. L'Osteomalacie (Hioco DJ, ed). Paris, Masson et Cie, 1967.

46. Antenor I: Dosage biologique de la vitamin D à l'aide du Ca^{45}. Bull Soc Suisse Chim Clin 1: 79, 1960.

47. Morgan DB, Pulvertaft CN, Spiers FW, Fourman P: Osteomalacia after gastrectomy: a response to very small doses of vitamin D. Lancet 2: 1089, 1965.

48. Gough KR, Lloyd OC, Wills MR: Nutritional osteomalacia. Lancet 2: 1251, 1964.

49. Dent CE, Smith R: Nutritional osteomalacia. Quart J Med 38: 195, 1969.

50. Prader A, Fanconi A: Mineral Metabolism in Pediatrics (Barltrop D, Burland WL, eds). Oxford, Blackwell Scientific Publications, 1969.

51. Latapsanis P, Delyannt V, Doxiadis SP: Vitamin D deficiency rickets in Greece. J Pediat 73: 195, 1968.

52. Abou Dahab YW: Clinical studies on rickets in Cairo. J Egypt Public Health Ass 38: 203, 1963.

53. Costeff H, Breslaw Z: Rickets in southern Israel. Some epidemiologic observations. J Pediat 61: 919, 1962.

54. Grosh S, Sarin S, Shepal SK: Study of rickets. J Indian Pediat Soc 1: 253, 1962.

55. Arneil GC, Crosbie JC: Infantile rickets returns to Glasgow. Lancet 2: 423, 1963.

56. Exton Smith AN, Hodkinson MH, Stanton BR: Nutrition and metabolic bone disease in old age. Lancet 2: 939, 1966.

57. Anderson I, Campbell ACR, Dunn A, Runciman JBM: Osteomalacia in elderly women. Scot Med J 11: 429, 1966.

58. Chalmers J, Conacher WDH, Gardner DL, Scott PJ: Osteomalacia, a common disease in elderly women. J Bone Joint Surg 49B: 403, 1967.

59. Richards IDG, Sweet EM, Arneil GC: Infantile rickets persists in Glasgow. Lancet 1: 803, 1963.

60. Dunnigan MG, Paton JPJ, Haase S, McHardy GW, Garner MD, Smith CM: Late rickets and osteomalacia in the Pakistani population of Glasgow. Scot Med J 7: 159, 1962.

61. Havard HF, Reay GA: Normal variations of the inorganic phosphate of the blood. Biochem J 19: 882, 1925.

62. McCance RA, Widdowson EM: Seasonal and annual changes in the calcium metabolism of man. J Physiol 102: 42, 1943.

63. Malm OJ: Calcium requirement and adaptation in adult man. Scand J Clin Lab Invest 10 (supp 36): 1, 1958.

64. Quarterman J, Dalgarno AC, Adam A: Some factors affecting the level of vitamin D in the sheep. Brit J Nutr 18: 79, 1964.

65. Dalgarno AC, Hill R, McDonald I: Vitamin D in the blood of sheep. Brit J Nutr 16: 91, 1962.

66. Guerrant NB, Morck RA, Bechdel SI, Hilston NW: Storage of vitamin D in the tissues of growing calves. Proc Soc Exp Biol Med 38: 827, 1938.

67. Eaton HD, Spielman AA, Lepsli JK, Thomas JW, Newton CL, Turk KL: The effect of a massive dose at birth of vitamins A and D upon blood plasma levels and liver storage in Holstein calves. J Dairy Sci 30: 795, 1947.

68. Cuthbertson WFJ: Vitamin D activity in plasma of idiopathic hypercalcaemia. Proc Nutr Soc 22: 146, 1963.

69. Smith RW Jr, Rizek J, Frame B: Determinants of serum antirachitic activity. Amer J Clin Nutr 14: 93, 1964.

70. Fellers FX, Schwartz R: Etiology of the severe form of idiopathic hypercalcemia in infancy. New Eng J Med 259: 1050, 1958.

71. American Academy of Pediatrics. Committee on Nutrition: The prophylactic requirement and safety of vitamin D. Pediatrics 31: 512, 1963.

72. Mawer EB, Lumb GA, Schaefer K, Stanbury SW: The metabolism of isotopically labelled vitamin D_3 in man. II. The distribution of vitamin D and its metabolites in human tissues. In preparation.

73. Bell NH, Gill JR, Bartter FC: On the abnormal calcium absorption in sarcoidosis. Evidence for increased sensitivity to vitamin D. Amer J Med 36: 580, 1964.

74. Snapper I. Medical Clinics on Bone Diseases, New York, Interscience Publishers Inc, 1944.

75. Sonino JR, Fichman A, Stark H, Edelman CM. Deficiency type rickets due to decreased sensitivity to vitamin D. J Pediat 68: 227, 1966.

76. Chiras B, Cantoni S, Dyke MK. Observations on sex differences in human biology. Bull Johns Hopkins Hosp 110: 134, 1962.

77. Stanbury SW: Bone disease in uremia. Amer J Med 44: 714, 1968.

78. Stanbury SW: Renal Disease, 2nd ed (Black DAK, ed), Oxford, Blackwell Scientific Publications, 1967.

79. Schaefer K, Schaefer P, Poeppe P, Opitz A, Hoffer R: Uraemic osteopathy. The relationship between disturbances in intestinal calcium absorption and renal function. German Med Monthly 13: 575, 1968.

80. Reiss E, Canterbury JM, Bartter FC: Experience with radioimmunoassay of parathyroid hormone in human sera. Trans Ass Amer Physicians 81: 104, 1968.

81. Reiss E, Canterbury JM, Kanter A: Circulating parathyroid hormone in chronic renal insufficiency. Arch Intern Med (Chicago) 124: 417, 1969.

82. Mawer EB, Backhouse J: An improved system for the separation of metabolites of isotopically labelled vitamin D_3 on silicic acid columns. Biochem J 112: 293, 1969.

83. Bell NH, Bryan P: Absorption of vitamin D oleate in the rat. Amer J Clin Nutr 22: 425, 1969.

84. Avioli LV, Birge S, Lee SW, Slatopolsky E: The metabolic fate of vitamin D_3 in chronic renal failure. J Clin Invest 47: 2239, 1968.

85. Ponchon G, DeLuca HF: The role of the liver in the metabolism of vitamin D. J Clin Invest 48: 1273, 1969.

86. Ponchon G, Kennan AL, DeLuca HF: "Activation" of vitamin D by the liver. J Clin Invest 48: 2032, 1969.

87. Suda TH, DeLuca HF, Schnoes K, Blunt JW: The isolation and identification of 25-hydroxyergocalciferol. Biochemistry 8: 3515, 1969.

88. Olson EB, DeLuca HF: 25-Hydroxycholecalciferol: direct effect on calcium transport. Science 165: 405, 1969.

89. Trummel C, Raisz LG, Blunt JW, DeLuca HF: 25-hydroxycholecalciferol: stimulation of intestinal calcium resorption in tissue culture. Science 163: 1450, 1969.

90. Stolis SJ, DeLuca HF: Subcellular location of vitamin D and its metabolites in intestinal mucosa after a 10 I.U. dose. Biochemistry 6: 3738, 1967.

91. Thompson GR, Lewis B, Booth CC: Absorption of vitamin D_3-^{14}C in control subjects and patients with intestinal malabsorption. J Clin Invest 45: 94, 1966.

92. Blumberg A, Aebi H, Hunni H, Schonholzer G: Das Verteilungsmuster von C^{14}-Ergocalciferol (Vitamin D_2) bei der Ratte und beim Meerschweinchen. Helv Physiol Acta 18: 55, 1960.

93. Norman AW, DeLuca HF: The preparation of H^3 vitamins D_2 and D_3 and their localization in the rat. Biochemistry 2: 1160, 1963.

94. Heyman W: Metabolism and mode of action of vitamin D. IV. Importance of bile in the absorption and excretion of vitamin D. J Biol Chem 122: 249, 1937.

95. Bell NH, Bryan P: On the metabolism of ^{14}C vitamin D_3 in the rat. J Lab Clin Med 66: 852, 1966.

96. Cruikshank LM, Kodicek E: Vitamin D balance and distribution in rats given a massive dose of ergocalciferol. Biochem J 54: 337, 1953.

97. Heyman W. Metabolism and mode of action of vitamin D. II. Storage of vitamin D in different tissues in vivo. J Biol Chem 118: 371, 1937.

98. Morgan AF, Shimston N: The absorption and retention by dogs of single massive doses of various forms of vitamin D. J Biol Chem 147: 189, 1943.

99. Coppens PA, Metz GA: Untersuchungen über die Verteilung des bestrahlten Ergosterins (D Vitamins) im Tierorganismus nach Verabreichung per os. Arch Neerl Physiol 18: 407, 1933.

100. Metz GA, Coppens PA: Long en Vitamine D. Nederl Geneesk 78: 769, 1934.

101. Stanbury SW, Ball J, Lumb GA, Mawer EB: Metabolic studies of acquired hypophosphatemic glycosuric osteomalacia in the adult; its association with sarcoma and observations on vitamin D metabolism. In preparation.

102. Chalk KJI, Kodicek E: The association of ^{14}C-labelled vitamin D_3 with rat serum proteins. Biochem J 79: 1, 1961.

103. Chen PS Jr, Lane K: Serum protein binding of vitamin D_3. Arch Biochem 112: 70, 1965.

104. De Crousaz PH, Blanc B, Antener I: Vitamin D and

tivity in normal human serum and serum proteins. Helv Odont Acta 9: 151, 1965.

105. Rikkers H, DeLuca HF: An in vivo study of the carrier proteins of ^{3}H-vitamins D_2 and D_3 in rat serum. Amer J Physiol 213: 380, 1967.

106. Rosenstreich SJ, Volwiler W, Rich C: Evidence for vitamin D storage in adipose tissue. Clin Res 17: 162, 1969.

107. Stanbury SW, Lumb GA, Nicholson WT: Elective subtotal parathyroidectomy for renal hyperparathyroidism. Lancet 1:703, 1969.

108. Curtis FK, Davidson RC, Fendrias JP: Metabolic bone disease and chronic dialysis. Abstracts, 3rd International Congress of Nephrology, vol 2, Washington, 1966. p 176.

109. Stanbury SW: The treatment of renal osteodystrophy. Ann Intern Med 65: 1133, 1966.

110. Stanbury SW, Lumb GA: Parathyroid function in chronic renal failure. Quart J Med 35: 1, 1966.

111. Berner A: Les osteodystrophies d'origine rénal. Etude systematique du squelette dans 138 cas de maladies rénales. Helv Med Acta 11: 741, 1944.

112. Ireland AW, Cameron DA, Stewart JH, Posen S: Quantitative histology of bone in advanced renal failure. Calcif Tissue Res 4: 282, 1969.

113. Stanbury SW: Diseases of the Kidney 2nd ed (Strauss WB, Welt LG, eds), Boston, Little, Brown & Co, 1971.

114. Rubini ML, Coburn JW, Massry SG, Shinaberger JH: Renal osteodystrophy. Some therapeutic considerations relative to long-term dialysis and transplantation. Arch Intern Med (Chicago) 124: 663, 1969.

115. Jowsey J, Massry SG, Coburn JW, Kleeman CR: Microradiographic studies of bone in renal osteodystrophy. Arch Intern Med (Chicago) 124: 639, 1969.

116. Schorr C: Disturbance of calcium metabolism in transplanted patients and patients on maintenance haemodialysis. EDTA Proc 5: 408, 1969.

117. Wilson CD, Fisher KH, Fugua ME: Principles of Nutrition 2nd ed, New York, John Wiley and Sons, 1967.

Gunma J. Med. Sci. 12(2) 109-18, (1963)

EFFECTS OF VITAMINES D_2 AND D_3 UPON THE CALCIFICATION OF NORMAL RABBIT DENTINE

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It was reported by several workers (Klein, 1931; Mizuno, 1932; Blackberg, 1932; Weinman, 1933; Ito, 1948 etc.) that an adequate dose of vitamin D (hereafter abbreviated simply to D) improved the development and calcification of the bone and the tooth, while an overdose as well as deficiency inhibited them. As to the comparison of their antirachitic action between D_2 and D_3, many experiments have been carried out. It was reported that the difference between them was at least in part due to the mode of the administration, species and individual differences of used animals. The concensus for the moment is that in oral administration D_3 is more active than D_2. According to Pees (1939), Türk (1940), Jesserer (1950), Kenn (1950), et al., also in parenteral administration, that is, intramuscular injection of oleovitamin, D_3 is stronger than D_2 in antirachitic action. Especially with regard to the calcifying effect on bone, Harnapp (1939) reported that D_3 was superior to D_2, while some workers found that the action of D_3 was nearly equal to that of D_2. Those results were obtained in most cases on rachitic animals.

Recently, various pure D preparations with different structures, such as D_2, D_3 etc., have been synthesized, thus making it possible to compare the action of both vitamins. The present investigation was instituted in the hope that comparative studies using pure D_2 and D_3 of Difco might cast some further light on the action of the vitamins upon the calcification of dentine in normal animals.

Experimental Methods

a) The degree of dentine calcification was determined by means of stripe figure on dentine sections. To know exactly the time of the change of the figure, vital staining with lead acetate (Okada-Mimura, 1938) was performed.

b) D_2 and D_3 of Difco in pure powdered form (D_2, 1 mg=800 I.U., D_3, 1 mg=4,000 I.U.) were dissolved in sterilized olive-oil in adequate concentration and injected subcutaneously.

c) The teeth (an incisor and a mandibular molar) were decalcified in a 0.2 N HCl solution saturated with H_2S gas, and after freezing sliced into sections.

d) Qualitative change in dentine calcification was observed in a hematoxylin stained section

of a incisor, which shows stripes regularly stained in light and medium blue in normal cases, the former being formed during the daytime, and the latter during the night. Faint staining indicates poor calcification, while deep staining sufficient one.

e) Quantitative change in dentine development, that is, the degree of lateral formation and longitudinal growth were determined by measuring the intervals between vital staining-lines on a sagittal section of a mandibular molar.

f) Collagenic fiber arrangement of dentine was photographed with a Leitz's polarization microscope.

g) The amount of serum calcium was measured by the Clark-Collip's method, and urinary calcium by the Shohl-Pedley's method.

h) Inorganic phosphate in serum and urine was measured by the Fisk-Subbarow's method.

i) The activity of alkaline phosphatase in serum was measured by the Bodansky's method.

j) Blood pH was measured with a glass electrode pH-meter, serum protein level by a refractometer.

Results

1) *Effects of vitamin D_2*

a) Administration of overdoses (400,000 u/kg., u=I.U., 100,000 u/kg. and 10,000 u/kg., once daily for 3 consecutive days, subcutaneously, each 4 to 5 cases) caused anorexia and loss of body weight, leading to death 5-7 days after injection. In the figure of stripes, deeply stained layer was observed, and no inhibitory picture was seen in the case of 100,000 u/kg. or 10,000 u/kg. (Fig. 1). Syste-

Fig. 1. Acceleration of calcification by the administration of an overdose of vitamin D_2. A longitudinal section of a rabbit incisor hematoxylin stained. Three small arrows with a letter D_2 indicate the times of subcutaneous injection of 100,000 u/kg. of D_2 once daily for 3 consecutive days. The arrows with figure 1, 2 and 3 indicate the time of vital staining with lead acetate, which performed every 72 hours. The right hand part of the vital staing arrow NO. 2 (← d →) is stained deeply blue with hematoxylin owing to D_2 administration. D: dentine; P: pulp

8770

nic symptoms were usually accompanied by inhibition of calcification, but in this experiment an accelerative picture was strongly manifested, while an inhibitory figure was observed only limitedly in the case of 400,000 u/kg. administration. There were also cases in which the development of the tooth was quantitatively inhibited. No change of optical rotation was observed in the polarization microscope. In all these cases serum calcium decreased, while serum inorganic phosphate increased conversely. Serum protein level was appreciably diminished.

b) 5,000 u/kg. was administrated subcutaneously once daily for 3 consecutive days to 8 cases. By this dose calcification was accelerated without any side effects, and only deep-blue layer was formed continuously for 8 days following the injection (Fig. 2). There was no change in the degree of dentine formation.

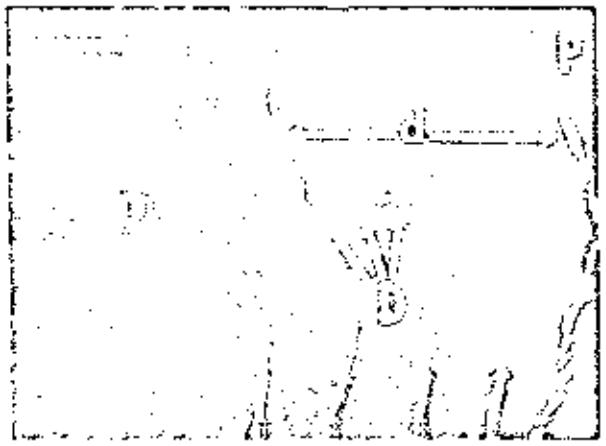

Fig. 2. Accelerated calcification by the administration of an effective dose of vitamin D_2. The arrows with figures 1- 4 indicate lead lines. The right-hand part of the lead line NO. 2 (---d→) is stained more deeply blue with hematoxylin than the control part.

As to the changes in blood components, serum calcium increased in some cases, and decreased in others, without showing any consistent tendency (Fig. 3); serum inorganic phosphate increased gradually, keeping high level as late 126 hours after the injection, and attaining a level twice as high as the normal (Fig. 4). Daily urinary output of calcium increased remarkably, becoming 2 to 3 times as much as the normal. The activity of blood alkaline phosphatase diminished gradually, falling to about a half of the normal controls 54 hours after the injection, and thereafter still continuing to decline (Fig. 5). Serum protein level also decreased.

As to the relationship between the formation of deep-blue stained layer and

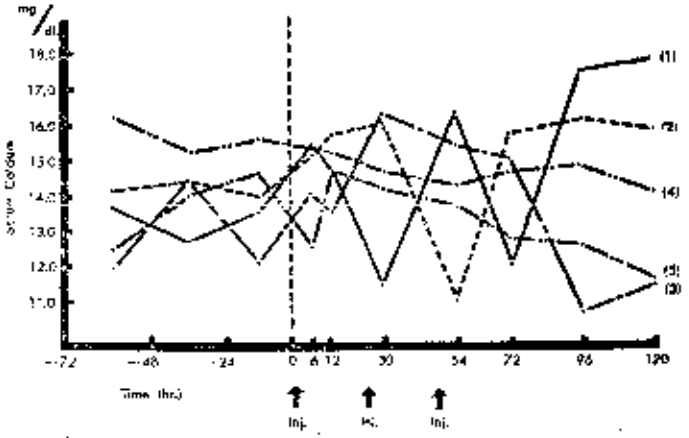

Fig. 3. Effect of vitamin D_2 on serum calcium level (5,000 u/kg. of D_2 was administered subcutaneously three times once daily).

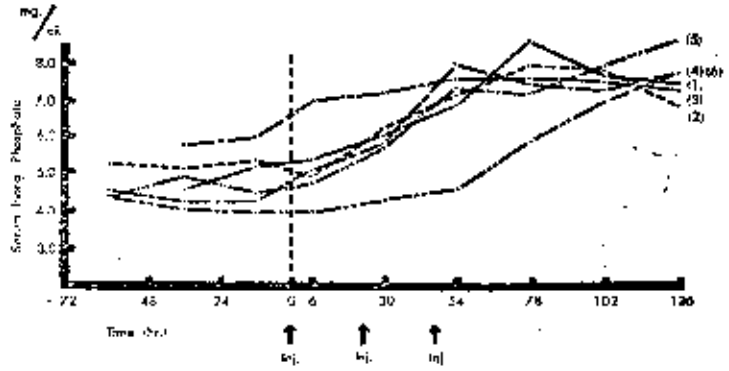

Fig. 4. Effect of vitamin D_2 on serum phosphate level (The same experiments as in Fig. 3).

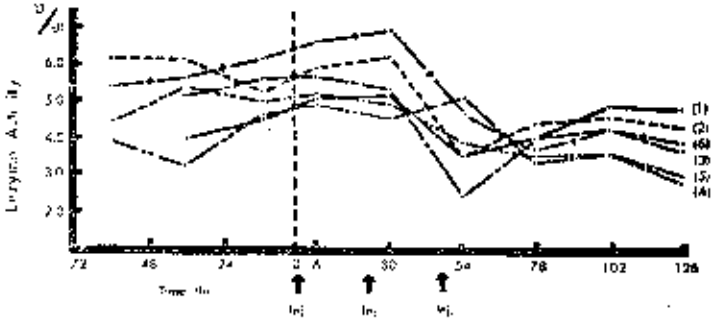

Fig. 5. Effect of vitamin D_2 on the activity of serum alkaline phosphatase (The experiments as in Fig. 3).

9720

the changes in blood components, it was observed that the ratio of calcium to phosphate became smaller than the normal, alkaline phosphatase activity diminished and the deep-blue layer was formed concomitantly. There was little change in blood pH.

c) 3,000 u/kg., once daily for 3 consecutive days subcutaneously, 5 cases. The deep-blue stained layer was observed in 2 of the 5 cases (40%)-effective dose).

On the basis of these data, the range of effective dose without side actions was regarded to be 3,000 to 10,000 u/kg.

2) *Effects of vitamin D_3*

a) D_3 was injected subcutaneously in a dose of 800,000 u/kg. once daily for 3 consecutive days to 10 cases. In the strip figure deep-blue layer were observed covering about a 8 day's space (Fig. 6). There were scarcely any side actions, and very slight effect on collagen arrangement of dentine matrix was observed in one or two cases.

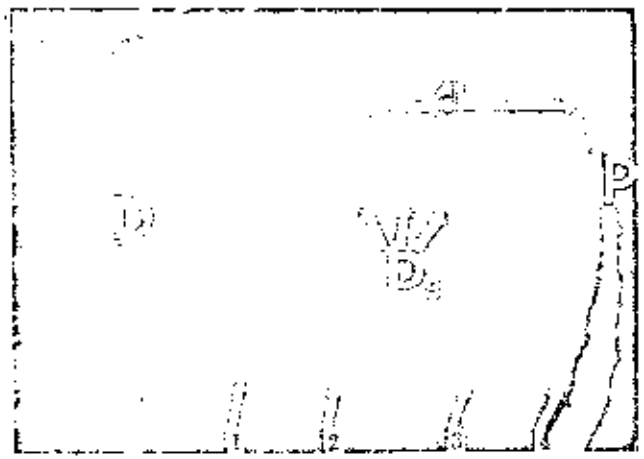

Fig. 6. Accelerated calcification by an effective dose of vitamin D_3. Small arrows indicate the times of subcutaneous injection of 800,000 u/kg. of D_3. The right-hand part of lead line NO. 2 (—d—) is stained with hematoxylin more intensively than the control part owing to the injection.

As to the changes in the blood components, serum calcium gradually increased in most cases after second or third injection (Fig. 7), while serum phosphate increased more or less after first and second injection and slowly returned to the normal level, in most cases after 96-120 hours (Fig. 8). Urinary output of calcium was remarkably augmented, being 3-5 times the normal 1-5 days after the injec-

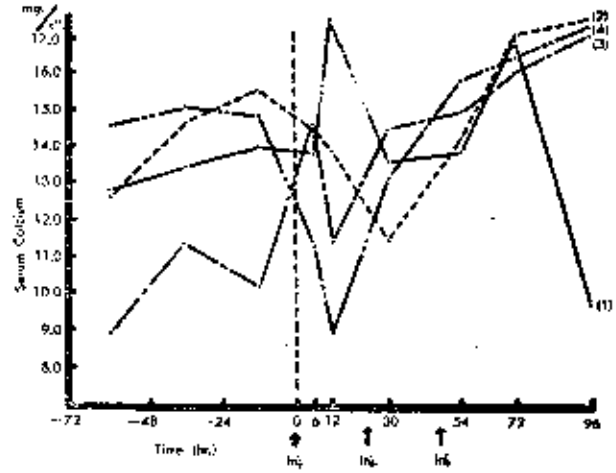

Fig. 7. Effect of vitamin D_3 on serum calcium level (800,000 u/kg. of D_3 was administered subcutaneously three times once daily).

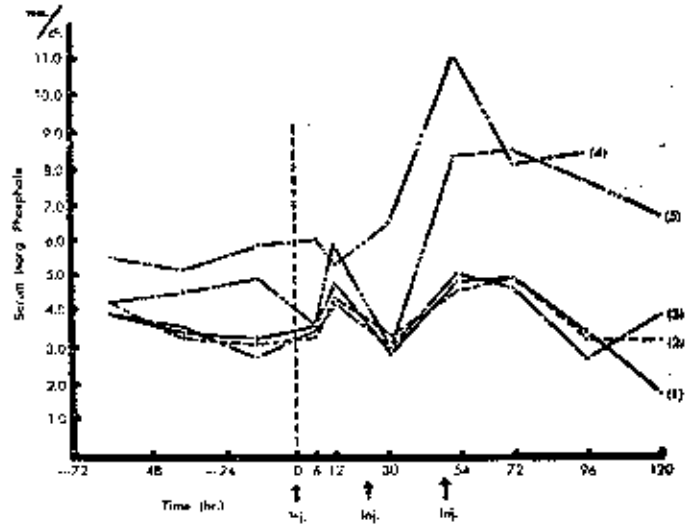

Fig. 8. Effect of vitamin D_3 on serum phosphate level (The same experiments as in Fig. 7).

tion. Urinary output of inorganic phosphate was temporarily decreased. Serum protein level was elevated, while blood pH changed little. The change of serum alkaline phosphatase activity was shown in Fig.9. The same relationship as in the case of D_2 was found between the formation of the deep-blue layer and the

changes in blood constituents.

b) 400,000 u/kg., once daily for 3 consecutive days subcutaneously. 5 cases. The deep-blue stained layer was observed in 2 of the 5 cases (40%-effective dose).

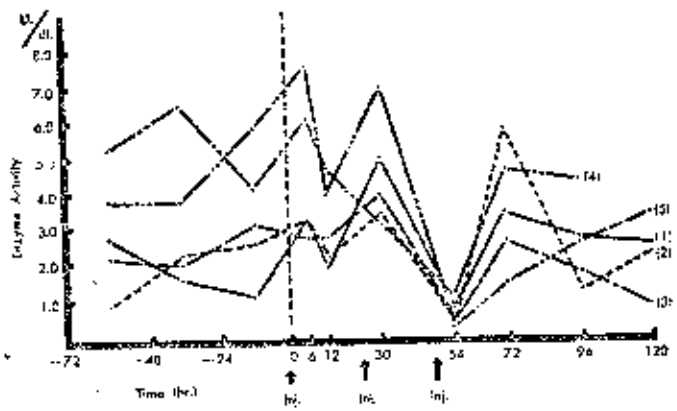

Fig. 9. Effect of vitamin D on the activity of serum alkaline phosphatase (The experiments as in Fig. 7).

Discussion

There are many reports on the remarkable effects of vitamin D preparations on calcification, and it was demonstrated that their effects are observed mainly on the bone and the enamel, and only slightly on the dentine (Mizuno, 1932; Klein, 1931; Blackberg, 1932; etc.). Reports concerning the dentine are far less than those concerning the bone and enamel (Weinman, 1933; Ito, 1948). Previous experiments of Mimura were performed on the effect of Vigantol, a D preparation, by means of the stripe figure of calcification, using the Okada-Mimura's vital staining method, but evident effects could not be observed, assumedly because of the impurity of the D preparation.

In contrast to hitherto reported works indicating that D had little effect on dentine calcification and that its administration in an overdose inhibited calcification, in our present experiments subcutaneous injection of overdose of D_2 and D_3 into normal rabbits was found to have accelerating effect on qualitative calcification. When, however, the dose was larger (400,000 u/kg.), the dentine development was quantitatively inhibited in several cases, though qualitatively improved, in agreement with previous works.

Since D_2 and D_3 have scarcely any growth promoting action, they can be considered, from their action on calcification, as a qualitative stimulant similarly to calcium preparation (Matsumoto, 1950), acethylcholine (Fuse, 1948) and Chondron (Matsuda, 1951).

In previous experiments doses of D_2 and D_3 were varying, and in some cases they were compared by weight., and in others by the international or U.S.P. units. In our experiments D_2 and D_3 preparations of Difco were used, and doses were expressed by the international unit. As described above, D_2 accelerated the calcification in a far smaller dose than that of D_3, when given subcutaneously. In contrast to the results obtained by many workers that D_3 was stronger than D_2 in antirachitic action. Also viewed from hematoxylin stainability of sections, D_2 was found to affect more remarkably than D_3. We may say in conclusion that D_2 is superior to D_3 so far as calcifying action in the normal rabbit dentine concerns, and that since our method has high reliability in determining the degree of calcification, errors in our data must be very small. As to the range of effective doses, D_2 was far broader than D_3 (D_2, 3,000 to below 10,000 u; D_3, 400,000 to above 800,000 u), and assumedly the former may accelerate calcification even in a dose of some 1,000,000 units without side actions.

Concerning their effects on the changes in blood and urinary components, which have an intimate correlation to the calcification of dentine, there were some differences in details between D_2 and D_3 (Table 1), but as a whole D_2 was more

Table 1. Comparative studies on the effects of vitamins D_2 and D_3 upon calcification of dentine, and urinary and blood components of normal rabbits.

	Calcification accelerating dose	Serum calcium	Serum phosphate	Serum alkaline phosphatase	Serum protein level	Blood pH	Daily output of urinary calcium	Daily output of urinary phosphate
D_2	5,000 u/kg. subcut. 3 time	No definite tendency	Increase	Decrease	Decrease	Little change	Increase	Increase
D_3	400,000 u/kg. subcut. 3 time	Gradual increase	Increase	Decrease	Increase	Little change	Increase	Transient decrease

effective than D_3 namely as in calcification. The effects of D_2 and D_3 on serum components were found to be consistent with previous reports (Harris and Innes, 1931; Munro, 1928; Cantarow, 1940; etc.). Namely, greater change was observed in inorganic phosphate rather than in calcium, thus the ratio between them as well as between their urinary daily outputs being greatly altered, and the deep-

3720

blue stained layer seemed to be formed concomitant with a decrease in alkaline phosphatase activity. There has been no report on the relationship between serum alkaline or acid phosphatase and calcification. As above mentioned, when the D preparations were administered, hypercalcification was accompanied by a decrease of alkaline phosphatase activity. It remains to be investigated what an implication serum phosphatase has for calcification.

Now we are attempting to induce experimental rachitis in rabbits to examine pathological changes in the stripe figure, and further to compare the effects of D_2 and D_3 on dentine pattern with each other. The results will be reported in a subsequent paper.

Conclusion

To compare the effects of vitamin D_2 and D_3 on the dentine calcification with each other, pure preparations (Difco) were administered subcutaneously to normal rabbits in varying doses. The results were as follows.

1) Vitamins D_2 and D_3 qualitatively accelerated the dentine calcification in the normal rabbits when subcutaneously injected in adequate doses (D_2 5,000 u/kg. and D_3 800,000 u/kg. once daily for 3 consecutive days).

2) D_2 is superior to D_3 in accelerating the dentine calcification.

3) Their effects on certain blood constituents were found to have nearly the same tendency, though D_2 was stronger than D_3 in the action.

4) The range of effective doses of D_3 was wider than that of D_2.

5) As to the relationship between the calcification and the changes in blood constituents, it was found that the deep-blue stained layer was formed when the ratio between calcium and inorganic phosphate levels was upset and the activity of alkaline phosphatase was lowered.

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Acknowledgment

The authors wish to express their deep gratitude to prof. Yamazoe in the Department of Biochemistry of School of Medicine, who gave us advices and various conveniences.

References

Blackberg, S. and J. Berke: J.D. Res. **12**: 349, 1932.
Gantarow, A. and M. Trumper: Clinical Biochemistry. 4th ed. 1949. W.B. Sunders Co., Philadelphia. P. 174.
Fuse, S.: Kokubyō Z. **16**: 53 and 193, 1948. (In Japanese with German abstract)
Harnap, G.: D. Med. Wschr. **65**: 1114, 1939.
Harris, L. and Jones: Biochem. J. **25**: 567, 1931.
Harris, L. and T. Moore: Biochem. J. **22**: 1461, 1928.
Ito, M.: Kokubyō Z. **16**: 329, 1948. (In Japanese with German abstract)
Jesserer, H.: Transactions of the sixth international congress of pediatrics, Zürich, 1950. Wien Klin. Wschrschr. **63**: 855, 1951.
Kern, M.: Mschr. f. Kinderh. **98**: 479. 1950.
Klein, H.: J.A.M.A. **16**: 666, 1931.
Matsuda, N.: J.J.S.S. **10**: 1, 1961. (In Japanese with English abstract)
Matsumoto, M.: Kokubyō Z. **26**: 1723. 1959. (In Japanese)
Mellanby, M.: Brit. D.J. **45**: 515, 1930.
Mitani, M.: The Sapporo Med. J. **8**, 130, 1955. (In Japanese)
Mizuno, S.: Nihon no Shikai. **151**: 723, 1932. (In Japanese)
Nadrai, Pers: Arch. Kinderhk. **116**: 235, 1939.
Okada, M. and T. Minura: J.J. Med. Sci. IV, pharmacology **6**: 116, 1938.
Sano, N.: Niigata Igaku **67**: 891, 1953. (In Japanese)
Weisman, J.: D. Mschr. f. Zhk. **51**: 577, 1933.
Türk, E.: Arch. Kinderh. **121**: 46, 1910.

3770

Proc. Soc. Exp. Biol. and Med. 57(1): 29-31, 1944.

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Chronic Toxicity and Hypercalcemic Effects of Various Activated Sterols in the Albino Rat.

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Toxicity studies on the various activated sterols have generally been carried out on white mice according to the technic of Holtz, Laqueur, Kreitmair, and Moll:[1] a determination of the so-called "toxic borderline dose" or "T.B.D." Data available in this laboratory indicate that the T.B.D.'s of vitamins D_2, D_3, and dihydrotachysterol are, respectively, 50 γ, 80 γ, and 6 γ, based on the weights of the crystalline substances.* The values reported in the literature have been tabulated by McLean,[2] and are of the same general magnitude.

Recently Mrazek, Novak, and Reed[3] reported a study of the chronic toxicity of vitamins D_2 and D_3 for the albino rat. Dosages of 75 I.U. per g per day were given to selected groups of 9 rats until all had died. They found that the average survival time of rats treated with vitamin D_2 was 43 days, and with vitamin D_3, 22 days. Thus it appears that vitamin D_3 is more toxic for the rat than vitamin D_2, but as pointed out above, less toxic than D_2 for the mouse. Morgan, Shimotori, and Hendricks[4] also concluded that vitamin D_3 is more toxic for rats than D_2, but Jung[5] concluded that the toxicities were identical.

The purpose of this study has been to compare, in the albino rat, the toxic and hypercalcemic effects of crystalline vitamins D_2 and D_3 and of commercial dihydrotachysterol with the object of determining whether there is any causal relationship between the two effects. This point cannot be regarded as settled at the present. It is the belief of Reed,[6] for example, that the toxic and hypercalcemic effects of vitamin D are not causally related. The hypercalcemic effects of single doses of the preparations named have been studied by McChesney and Kocher,[7] but data indicating their relative effects on continued administration to the rat are not available.

Experimental Methods. Albino rats of both sexes from our regular breeding colony served as experimental subjects. They ranged in age from 225 to 275 days at the start of the experiments, and weighed from 300 to 600 g. They were given our regular breeding diet[8] (Analysis: Ca, 0.49%; P, 0.53%) and water, *ad libitum*. The medications were given by stomach tube in corn oil, and each rat received 0.125 cc per 100 g body weight per day. Medications were not given on Sundays, therefore 0.1875 cc per 100 g was given on Saturdays and Mondays. (The concentrations of the solutions used ranged from 0.8-2.4 mg/cc.) The rats were weighed twice weekly and the doses were adjusted to these weights. Medications were continued until the animals died. The results of the experiments on chronic toxicity are given in Table 1.

In order to determine the hypercalcemic effects of the activated sterols when administered daily, the same procedures were used. The animals were sacrificed by decapitation after 14 days of treatment since it was at

[1] Holtz, F., Laqueur, F., Kreitmair, H., and Moll, T., *Biochem. Z.*, 1931, **237**, 247.

* The commercial solution of dihydrotachysterol (Hytakerol) contains about 2250 γ of the active principle per cc. Hytakerol is a registered trademark of the Winthrop Chemical Co.

[2] McLean, F. C., *J. A. M. A.*, 1941, **117**, 609.

[3] Mrazek, R., Novak, C. R., and Reed, C. I., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 49.

[4] Morgan, A. F., Shimotori, N., and Hendricks, J. E., *J. Biol. Chem.*, 1940, **134**, 761.

[5] Jung, A., *Schweiz. med. Wochschr.*, 1943, **73**, 17.

[6] Reed, C. I., Struck, H. C., and Steck, I. E., *Vitamin D: Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations*, Univ. of Chicago Press, 1940, p. 152.

[7] McChesney, E. W., and Kocher, H., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 156.

[8] Chimenko, D. R., and McChesney, E. W., *ibid.*, 1942, **51**, 157.

TABLE I.
Survival of Albino Rats Receiving Various Activated Sterols Daily Until Death.

Preparation	Experiment	No. of rats	Dose, mg/kg/day	Survival time, days: Range	Survival time, days: Avg (median)	Equivalent toxic dose*
Vitamin D_2	A§	12	3.0	13-67	33 ± 5‡ (22)	3.00
" "	C	12	2.4	10-72	43 ± 3.3 (42)	
" "	D	10	3.0	11-82	33 ± 5.6 (25)	
" D_3	A	12	2.0	9-32	18 ± 1.5 (17)	2.30
" "	B	8	1.5	19-124	59 ± 10 (44)	
" "	B	8	2.0	10-43	29 ± 2.5 (28)	
" "	C	11	1.75	12-70	30 ± 4.3 (34)	
" "	D	10	2.25	7-74	27 ± 4.7 (20)	
Dihydrotachysterol	A	12	1.0	8-181†	34 ± 10 (18)	1.00
"	C	12	1.12	9-67	24 ± 2.6 (20)	
"	D	10	1.25	9-35	15 ± 5.4 (12)	

* Mg/kg/day—permitting a median 20-day survival time, as estimated from the data of Experiments A, C, and D.

† Animal sacrificed on the 181st day and serum Ca found to be 14.3 mg%. Judging from its condition the animal might still have survived for a considerable period of time.

‡ Probable error of the mean.

§ The experiment numbers are given in order to indicate which animals were run at the same time.

TABLE II.
Final Serum Calcium Values of Albino Rats Medicated Daily with Various Activated Sterols for 14 Days.

Preparation	No. of rats	Dose, mg/kg/day	Serum calcium, mg%: Range	Serum calcium, mg%: Avg	Dose required to produce serum Ca 15.3 mg%‡
Vitamin D_2	8	2.0	13.1-17.3	15.74 ± 0.38*	1.85
" D_3	8	1.0	13.6-15.4	14.61 ± 0.23	1.1
" "	8	1.5	15.3-17.1	16.30 ± 0.16	
Dihydrotachysterol	8	1.0	14.2-17.0	15.29 ± 0.24	1.0

* Probable error of the mean.

† Estimated from the data, as mg/kg/day for 14 days, assuming that the rise above the normal level of 11.3 mg% is proportional to the log of the dose.[12]

about this time that deaths generally began to occur in the chronic toxicity experiments. Blood was collected from each animal in a separate centrifuge tube, and calcium was determined on the serum by the method of Clark and Collip.[9] The results are given in Table II.

Discussion. The results as to the toxicity of vitamins D_2 and D_3 for the rat are in good agreement with the work of Mrazek, Novak and Reed, considering that slightly higher doses were used in this work. It is further established that dihydrotachysterol, on a weight basis, is more toxic than either vitamin

[9] Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, 63, 461.

[12] McChesney, E. W., and Kocher, H., *Endocrinology*, 1942, 30, 787.

3821

D_2 or D_3.

The toxicities of the 3 preparations do not completely correlate with their ability to elevate serum calcium. In the case of vitamins D_2 and D_3 the correlation is as satisfactory as can be expected from the experimental procedures: an equivalent toxic dose of vitamin D_2 is 57% greater, and an equivalent hypercalcemic dose 54% greater than that of vitamin D_3. However, if the toxicity of dihydrotachysterol depended on its hypercalcemic effect alone, one would expect the equivalent toxic dose (as determined experimentally) to be about 1.6 mg/kg/day instead of 1 mg/kg/day. This might have been anticipated since dihydrotachysterol as marketed is a mixture and its physiological action may be the summation of the effects of several substances.[10] It is difficult to conceive of crystalline materials such as vitamins D_2 and D_3 containing a hypercalcemic and an antirachitic principle distinct from each other. Correll and Wise,[11] using vitamin D from cod liver oil, were able to produce toxic effects in chicks without raising the blood calcium. On the other hand, toxic doses of irradiated ergosterol and of dihydrotachysterol did elevate the serum calcium.

The survival times reported must be considered as approximate since individual animals or groups of animals are evidently rather variable in their responses from time to time, due to factors beyond control. For example, in 2 experiments with vitamin D_2 the average survival times were 33 and 42 days although in the latter case the daily dose was 20% larger. Attention is also called to the fact that a small decrease in dose level may result in a disproportionate lengthening of the survival time. Thus, in Experiment B a decrease of 25% in the daily dose more than doubled the survival time. If the dose is not sufficiently large to kill the animals within the first three weeks, they appear to develop a tolerance which may permit them to survive for a considerable period of time. For this reason it is felt that the median survival times may be more significant than the averages.

Summary. For the albino rat, medicated daily until death, crystalline vitamin D_3 is about 55%, and commercial dihydrotachysterol about 260% more toxic than crystalline vitamin D_2 (based on the actual weights of the active principles). So far as vitamins D_2 and D_3 are concerned, the toxicities correlate very well with the hypercalcemic effects of the preparations. However, in the case of dihydrotachysterol the toxicity and ability to elevate serum calcium are not in that same proportion. In view of the difference in response of different species, these considerations do not necessarily apply to any species other than the albino rat.

[10] Werder, F. von, *Z. physiol. Chem.*, 1939, **260**, 119.

[11] Correll, J. T., and Wise, E. C., *J. Nutrition*, 1943, **26**, 641.

National Academy of Sciences. 1972

Annual Poundage Reported per Substance
by NAS and FEMA User Firms.

National Academy of Sciences, Washington, D.C.

104; 478-480, (1962) 4269

14. Results of Calcium Infusion and Renal Biopsy Studies in Refractory Rickets. G.

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Eleven cases of refractory hypophosphatemic rickets have been seen and investigated at the Children's Hospital, Halifax. These included 1 case of Lowe's syndrome (oculo-cerebral-renal syndrome), 1 case of cystinosis, 5 cases of the "simple" type of hypophosphatemic rickets including identical twins, 2 cases with glycosuria, acidosis, and amino-aciduria, and 2 cases with hypophosphatemia, glycosuria, and acidosis.

A standardized calcium infusion procedure was carried out in 7 patients with measurement of creatinine and phosphate clearances, and the results were expressed as amount of phosphate reabsorbed per minute and as per cent tubular reabsorption phosphorus (TRP). In 4 cases of the "simple type" of refractory rickets there was an increase in the per cent TRP with a reduction of the excessive urinary output of phosphorus throughout the period of calcium infusion. observations support those of Lainy, , et al., Fraser et al., and Field and Reiss suggesting extrarenal factors are in part responsible for the pattern of increased phosphate excretion. In 3 patients with acidosis, hypophosphatemia, with and without amino-aciduria, no similar decrease in phosphorus excretion or increase in per cent TRP occurred after calcium infusion.

Percutaneous renal biopsy was done with a modified Vim Silverman needle in 8 cases. Histological and histochemical examinations were done including special stains for diphosphopyridine nucleotide diaphorase (DPND) and alkaline phosphatase. The 2 chief histological findings were the patchy distribution of intratubular calcium deposits either in the form of obstructing casts or as small particles and dilatation of tubules. Calcification was found in all cases except one. All cases had been treated with large doses of vitamin D, and in spite of frequent clinical examination and periodic serum calcium determination, it

* By invitation.

is felt the pathological findings are the result of hypervitaminosis D. Histochemical studies revealed a decrease in DPND and alkaline phosphatase in cells with structural change. There was also evidence of alkaline phosphatase casts associated with calcium deposits.

The pathological findings should be of concern to clinicians treating refractory rickets with large doses of vitamin D. It is conceivable that, although healing of the bony abnormalities may occur with large doses of vitamin D, irreversible renal changes may result in spite of careful clinical and laboratory evaluation during therapy.

It is felt that possibly infrequent periods of hypercalcemia may occur while on large doses of vitamin D resulting in brief, but significant, hypercalciuria producing immediate calcium precipitation in the distal or collecting tubules with retrograde tubular damage.

Discussion

DR. GUNNAR B. STICKLER: I wonder whether you have other normal values of alkaline phosphatase in children older than 5 days?

The second question I have is whether these children you treated with vitamin D had normal calcium levels throughout the period of observation, or did they have periods of hypercalcemia?

One comment that could be made is that perhaps not many patients who have received vitamin D for periods up to 20 years have shown any clinical evidence of renal failure.

DR. G. NIGRIN: Regarding your first question, I would like to say that besides the 5-day-old control, we had 2 other 3-year-old controls with histochemical studies including alkaline phosphatase.

Regarding your second question, the calcium levels in our routine clinical evaluation did not reach values higher than 12.5 mg. %, and yet these patients presented on renal biopsy extensive intratubular calcification.

In support of the damage vitamin D therapy does to the kidneys, I can mention the study that Robinson and Nelson did in 1945 (AMER. J. DIS. CHILD. 69:323, 1945) in which they described 2 out of 6 vitamin-D-resistant rickets patients receiving large doses of vitamin D for 1½ years who presented evidence of a decreased rate of glomerular filtration. It is possible that if we look more carefully we will be able to note that the concentrating ability of the kidneys may be affected before failure of glomerular filtration.

DR. LAWSON WILKINS: It was not clear to me whether or not you have had histochemical studies on any cases before vitamin D therapy.

DR. NIGRIN: No. Unfortunately, we have not been lucky enough. We have not done kidney biopsies in children with vitamin-D-refractory rickets before the administration of vitamin D. We are considering doing so in the near future.

DR. WILKINS: I would say also that Dr. John Eager Howard, who is very much interested in this condition in adults, would certainly agree with your thesis that every case that is corrected as far as the bone lesion is concerned has undergone some intratubular calcification and that one cannot cure this disease with vitamin D alone without doing some damage to the kidney.

For that reason, in his department and now in ours we are using high loads of oral phosphate routinely and are able to reduce the amount of vitamin D required to maintain healing.

DR. WILLIAM H. BERGSTROM: Could you contrast the renal phosphate clearance response in normal patients to that which was shown on the slide?

I gather that the inference is drawn that the depression of phosphate clearance with calcium infusion suggests that there may have been hyperparathyroidism, but I have the impression that normal patients also diminish their phosphate clearance with calcium infusion.

Will you clarify this point, please?

DR. NIGRIN: First of all, I would like to say that all the patients who received calcium infusions were controlled by having the same type of infusion done the preceding day without the addition of calcium. Infusion without calcium did not increase the tubular absorption of phosphate in these cases. Studies by others of calcium infusion in normal individuals have shown that administration of calcium increases the tubular reabsorption of phosphate. Calcium infusion can be effective in increasing tubular reabsorption in 2 ways: (1) by depression of the parathyroid effect, or (2) directly on the tubular transport of phosphate.

DR. LAURENCE FIRKIN: We have confined your fear of high doses of vitamin D in refractory rickets on purely clinical grounds, where we have found it difficult to steer between clinical effectiveness and toxicity. We have had renal impairment occur where it was clinically obvious.

The serum calcium level, followed very closely, has not really been very helpful in picking up these patients. We have had probably more clinical success in looking at calcium excretion in urine on a 24-hour basis and by observing serum phosphorus levels. In our experience, when these get to the normal range, the patients are almost always intoxicated. . .

Because of these problems Dr. Harrison suggested a few years back that we try to have dihydrotachysterol as a therapeutic agent instead

of vitamin D. This sterol turns out to be very effective in handling these patients. We have thought that it is a little more sensitive, enabling us to judge more quickly when toxicity occurs. It has the advantage that when the early signs of toxicity are manifest, withdrawal results in a very prompt return to a normal state.

There does not seem to be very much storage of this steroid. Have you had any experience with this?

Dr. Nichin: No. We have not had any experience with dihydrotachysterol.

Dr. Charles Reiner: I would like to comment on the histochemical procedures that were done.

It is very tempting to try to quantitate histochemical procedures. However, as designed, most of these demonstrate only localization and that the enzyme activity at a given locus is above or below a certain threshold level.

It is not safe to try to draw quantitative conclusions from a qualitative histochemical method.

Have you done any biochemical procedures to support this quantitation?

Dr. Nichin: We have not done extensive enzyme determinations in these patients. We agree with Dr. Reiner that this is an excellent suggestion. Dr. E. V. Pollack and collaborator in their article (*Arch. Intern. Med.* 109:473-481 [April] 1962) bring out the same criticism, emphasizing the danger of drawing conclusions on a quantitation based on the histochemical staining method only. In the future, in these patients ultramicrochemical assays of enzyme activity of kidney biopsies is indicated.

Dr. Benjamin Kramer: I wonder what the urinary pH was in these patients.

The reason I raise this question is that the literature has several references to treatment of these refractory cases along with either alkaline ash diet or alkalinizing. I think that Dr. Harrison reported refractory cases that recovered on ordinary therapeutic doses of vitamin D along with sodium carbonate.

We had a case very recently with very severe multiple fractures that responded very well with ordinary doses of vitamin D.

Dr. Nichin: In the case that had acidosis, the urine pH was alkaline, but in all the other cases it was within normal limits.

Dr. Daniel C. Darrow: Have you determined the concentration of acid soluble phosphate in ultrafiltrates of serum? The presence of high Ca may produce aggregates of phosphate that are not filtrated. Consequently, the calculated reabsorption of phosphate may be seriously in error when Ca is injected.

Dr. Nichin: No, we have not.

Dr. W. A. Cochrane: The point was made that no cases of refractory rickets treated with large doses of vitamin D over a 20-year period have been reported to develop severe renal complications. It is of interest that as one reviews the literature some of these cases do develop later acidosis and changes in renal concentration which may well be due to chronic hypervitaminosis D.

The question of growth in refractory rickets is also of interest. Improvement in the bony abnormalities can be observed after vitamin D therapy and initially some improvement in linear growth. It has been our observation that many of these children seem to grow initially perhaps for the first 1 to 2 years, but from then on growth is extremely slow. The interesting relationship of growth failure and renal disease remains unexplained, but we wondered if perhaps the slow growth in refractory rickets after treatment could be related to renal damage as a result of the high doses of vitamin D therapy.

Dr. Wilkins: I have been under the impression that the growth curve increases as the rickets heals and slows when a relapse occurs.

Dr. Cochrane: This has not been our experience, sir. We have found that the rickets radiologically improves, but growth is very slow.

Dr. Nichin: Dr. Steendijk, of Amsterdam, in a recent paper (*J. Pediat.* 60:340:345, 1962) has done studies on growth in refractory rickets which seem to confirm the preceding comments.

Dr. Wilkins: Before calling on the next speaker, I would like to go back and make another remark about the paper on refractory rickets, because someone asked me what I considered a very pertinent question during the intermission. I was not able to give him a definite answer myself, but I asked Dr. Harriet Guild. The question was: In a woman who has refractory rickets and is being maintained on a relatively high level of vitamin D, is there any danger of vitamin D intoxication of the fetus if she becomes pregnant?

Dr. Guild tells me she has had 2 women with refractory rickets who became pregnant and carried on with the same level of vitamin D, around 100,000 units a day, on which they had been maintained before pregnancy. There was no sign whatsoever of vitamin D intoxication. The children during the first 3 or 4 months showed no signs, either radiologically or chemically, of refractory rickets, but after 3 or 4 months I think each of these children had refractory rickets.

* By invitation.

4376

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Regulation of Vitamin D Metabolism and Function

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I. Introduction 327
II. Physiological and Biochemical Basis of Vitamin D Action 328
A. Intestinal calcium absorption 328
B. Role of vitamin D in mineralization and resorption of bone 340
C. Role of vitamin D in renal tubular transport mechanisms 346
D. Summary of physiologic action of vitamin D 347
III. Vitamin D Metabolites and Their Functional Role 348
A. Historical 348
B. 25-Hydroxycholecalciferol (25-OHD$_3$) 348
C. 1,25-Dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) 349
D. Other vitamin D metabolites 352
E. Absorption, storage, transport, and excretion of vitamin D 354
F. Potential use of vitamin D metabolites in treatment of bone disease 355
IV. Biologically Active Analogs of Vitamin D 356
A. Dihydrotachysterol$_3$ 356
B. 1α-Hydroxyvitamin D$_3$, 5,6-*trans*-25-hydroxyvitamin D$_3$, and 5,6-*trans*-vitamin D$_3$ 358
V. Regulation of Vitamin D Metabolism 358
A. Control of 25-hydroxylation 358
B. Regulation of 25-OHD$_3$-1-hydroxylase and 25-OHD$_3$-25-hydroxylase 359
C. Role of DNA transcription and protein synthesis 363
D. Vitamin D as a prohormone 365

I. INTRODUCTION

Vitamin D has long been delegated a catalytic role in calcium metabolism; however, its metabolism to active forms and the feedback regulation of this metabolism have not been appreciated or even considered until recently. This is not surprising since vitamins are by definition catalytic in their action and usually do not have feedback-regulated functions. It has been assumed since 1919 that the

#4376

antirachitic substance in cod liver oil is a vitamin because of the demonstration by Sir Edward Mellanby (127, 128) that rickets can be produced by dietary manipulation and that this disease can be prevented by the administration of cod liver oil. Consequently concepts regarding the functional metabolism and mechanism of action of vitamin D have been governed by the general principles applicable to the B vitamins.

Huldshinsky (96) made the important observation that rickets can be prevented or cured by exposure of the patient to either sunlight or artificially produced ultraviolet light. Certainly if man exposed a sufficient area of his body to sunlight in an unpolluted environment, vitamin D would not be required in the diet. However, the civilization of man has converted a substance that is normally manufactured in the skin to a dietary requirement. In this respect vitamin D should not be considered a vitamin in the true sense of the word. It also is possible that other compounds classified as fat-soluble vitamins may not function in a manner common to the water-soluble B vitamins (42).

In this review unequivocal evidence is cited to demonstrate that: *1)* vitamin D must first be metabolically altered before it functions; *2)* functional metabolites of vitamin D are generated in organs other than their sites of action; *3)* the rates of synthesis and secretion of these functional forms are feedback regulated; *4)* this regulation is effected through humoral agents that probably involve the parathyroid hormone and possibly calcitonin; and *5)* the final functional form of vitamin D that controls calcium movement from both intestine and bone is a major humoral substance responsible for regulating plasma calcium concentration. Vitamin D therefore must be considered not simply a catalytic material but one whose metabolism is dynamically interrelated with its functions in a complex system that plays a major role in the strict regulation of plasma calcium and phosphate concentrations.

This review summarizes the areas of vitamin D investigation in which marked advances have been registered in recent years. Many important aspects not covered here may be found in previous reviews (37, 38, 53, 142, 184, 185, 213, 214).

II. PHYSIOLOGICAL AND BIOCHEMICAL BASIS OF VITAMIN D ACTION

A. Intestinal Calcium Absorption

1) Historical. In 1923 Orr et al. (155) reported high fecal calcium loss in rachitic children and suggested that ultraviolet therapy cured the disease by increasing intestinal calcium absorption. This idea was not initially accepted because of the belief that vitamin D inhibits the retrograde plasma to intestine movement of calcium (74). However, in 1937 Nicolaysen (137–142) conclusively demonstrated by a variety of physiologic methods that vitamin D acts to increase intestinal calcium absorption in rachitic rats, thereby establishing the hypothesis first put forth by Orr et al. (155).

For approximately 30 years after Nicolaysen's work it was assumed that vitamin D per se evoked an action in the intestine. Consequently during this period most investigative efforts were confined to defining the mechanism whereby calcium is translocated across the intestinal epithelial cell barrier. However, success in synthesizing tritium-labeled vitamin D with a high specific activity in 1966 (134) made feasible investigations into the metabolism of vitamin D at physiological doses, an area that had received little attention from the time of the vitamin's identification in the early 1930's (5, 227). Since 1966 progress has been rapid in the area of active vitamin D metabolites, and at present four metabolites of the vitamin have been isolated and identified. At least two of these are synthesized in organs other than their end-organ targets and are feedback regulated; this suggests that it would be more accurate to classify the metabolites as hormones rather than vitamins. New insights into the mechanism of vitamin D-stimulated calcium transport, obtained from studies using these metabolites, are discussed in detail. It seems advisable, however, to briefly review the current concepts regarding intestinal calcium translocation per se before discussing the action of vitamin D and its metabolites in stimulating the calcium transport system.

2) Physiological and biochemical properties. Both calcium balance and in situ isolated-loop techniques were used by Nicolaysen (137–142) to demonstrate an impaired intestinal calcium absorption in vitamin D-deficient rats. Since this discovery numerous in vitro and in situ methods have been used in studying the mechanism of calcium translocation. Although in vitro preparations of excised intestinal tissue have the inherent disadvantage of not being maintained in a physiological state, they do provide an excellent means of studying the transport system under defined conditions. Schachter and Rosen (181) successfully applied the in vitro everted-gut-sac technique of Wilson and Wiseman (226) to the study of calcium transport. They demonstrated that the intestinal transport system for calcium was finite in capacity, required metabolic energy, and moved the cation from the mucosal to serosal surface against a concentration gradient. Both Schachter (177) and Wasserman et al. (220) provided evidence that calcium is transported against an electrochemical potential gradient, thereby establishing that it moves by an active transport process. These results have been confirmed by both in vitro and in vivo techniques (76, 142, 165, 219). More recently a modification of the Ussing apparatus has been used with both short-circuit and non-short-circuit techniques. Walling and Rothman (211) and Martin and DeLuca (123) demonstrated saturation kinetics for calcium uptake at the mucosal border of intestinal epithelial cells and calculated an apparent K_m of between 1.25 and 2 mM. It was shown further that the transport process requires cellular energy, is carrier mediated, and translocates calcium independent of phosphorus against an electrochemical gradient. Adams and Norman (1) confirmed and extended the above in vitro work by Walling and Rothman (211) and Martin and DeLuca (123); with short-circuit methods they provided additional convincing evidence that calcium is actively translocated according to Ussing's definitions (209).

Martin and DeLuca (123) found that the initial velocity of calcium uptake by +D tissue was approximately 2 times that observed for −D tissue. Such

calcium movement into the mucosal cell appeared to depend on oxidative metabolism; however, this matter remains somewhat equivocal because other investigators (180, 213) have been unable to demonstrate a metabolic energy requirement. However, in studies of calcium uptake only the measurement of initial uptake rates with in vitro techniques is meaningful, because the basal-lateral membrane presents an apparent transport barrier. Nevertheless it now appears that the transfer of calcium across the brush-border surface is the rate-limiting process in vitamin D deficiency; this suggests that a specific brush-border component is instrumental in the transfer of calcium into the epithelial cell.

Addition of filipin to isolated −D chick intestine prompted the active translocation of calcium, a stimulation not observed in similarly treated tissue from chicks given vitamin D (2). Adams et al. (2) propose that filipin effects a structural reorganization of the −D microvillar membrane such that calcium gains ready entrance to the mucosal cell, thereby overcoming the apparent major barrier to calcium transport in −D animals. That vitamin D may alter microvillar membrane organization, and/or composition, prompted an investigation into possible changes in lipid composition of microvillar membranes from vitamin D_3-replete and -deficient chick intestine (63). Vitamin D_3 treatment increased the proportion and changed the composition of phospholipid and cholesterol esters. How an apparent change in lipid composition can enhance the movement of calcium across the microvillar border is not clear, however.

3) Possible functional calcium transport components. How calcium penetrates the microvillar region, moves across the epithelial cell, and then enters the bloodstream is a puzzle that only recently has assumed a recognizable form. Isolation of an intestinal calcium binding protein, identification of a microvillar vitamin D-stimulated calcium-dependent ATPase, the demonstration of a dependency on sodium at the basement membrane, and the appearance of possible calcium granules in mitochondria have all contributed to a clearer insight into the possible mechanism of calcium translocation in intestinal mucosal cells.

Discovery of a vitamin D-dependent intestinal calcium-binding protein by Wasserman and Taylor (222) has increased interest and optimism regarding isolation of an intestinal "carrier molecule" for calcium. The calcium-binding protein has been detected in several species (100, 101, 220, 223, 224), and estimates of its molecular weight have ranged from 25,000 in chicks (99, 217) to 8000 in rats (46). An isoelectric point of 4–4.4 is observed for the chick protein, and maximal binding of calcium does not involve sulfhydryl groups. However, calcium binding depends on conformational integrity of the molecule (99).

The experiments on de novo synthesis of the calcium-binding protein are somewhat equivocal. MacGregor et al. (121) administered the vitamin in various ways and studied [^{3}H]leucine incorporation into the chick calcium-binding protein. An increased relative incorporation of [^{3}H]leucine into the calcium-binding protein led the investigators to suggest de novo synthesis for the calcium-binding protein. However, alternative interpretations of their data could be made, e.g., the concept of a precursor calcium-binding protein molecule. In fact experiments using double-label techniques suggest the existence of a calcium-binding protein

4376

precursor in rats (47). Thus de novo synthesis may not be required for calcium-binding protein production in response to vitamin D.

Measurement of calcium-binding protein activity under physiological conditions known to alter calcium absorption has revealed a high correlation between the binding protein and calcium absorption (152, 153, 181, 203, 218). Although the extent to which the calcium-binding protein participates in the translocation of intestinal calcium is not known, its apparent location in the surface coat microvillar region (204) puts it in a position to function by enhancing the movement of calcium across the luminal border of the epithelium. The appearance of calcium-binding protein and the increased uptake and translocation of calcium by mucosal cells in vitamin D-repleted animals should occur nearly simultaneously. To date, time course studies involving calcium-binding protein appearance and calcium transport have shown that increased calcium transport precedes calcium-binding protein synthesis (50, 73, 153). Furthermore, calcium-binding protein production continues to increase even after the rate of calcium absorption has plateaued (50, 73), and cortisol-treated rats maintain high levels of calcium-binding protein while showing depressed rates of intestinal calcium transport (51, 104). Such findings raise questions concerning the role for calcium-binding protein in calcium transport. However, the calcium-binding protein may function in the initial uptake of calcium at the luminal surface.

Additional studies by Martin et al. (124) on the initial uptake of calcium by the mucosal cell attempted to link the energy requirement for calcium uptake at the luminal surface to a component in the brush-border fraction. The studies revealed that intestinal brush-border preparations possess calcium-dependent ATPase activity that can be increased by vitamin D (124). An increase in calcium ATPase activity followed closely the characteristic rise in calcium absorption after vitamin D repletion (126) and thus suggested a possible role for the calcium ATPase in the uptake of calcium at the luminal brush border. Later studies in the chick (83) revealed a correspondence between the elevation in both alkaline phosphatase and ATPase activity and intestinal calcium absorption. L-Phenylalanine, beryllium, and zinc—all known inhibitors of alkaline phosphatase—also inhibited calcium ATPase activity, suggesting that one enzyme complex in brush-border preparations contained both enzyme activities (83). The increase in alkaline phosphatase activity after vitamin D_3 repletion closely paralleled the increase in intestinal calcium absorption where both enzyme and absorption responses were blocked in vivo by actinomycin D and cycloheximide. Whether this enzyme complex acts as a nonspecific phosphatase or a calcium-requiring ATPase is not clear. Until definitive information is obtained regarding this point, the issue would be clarified by employing the term calcium ATPase-alkaline phosphatase to denote this type of enzyme activity in brush-border preparations. In any case the location of the calcium ATPase-alkaline phosphatase enzyme complex in the brush-border regions of intestinal mucosa suggests that the enzyme complex could function in the movement of calcium into the mucosal cell.

Once calcium has passed the luminal border of the epithelial cells, it proceeds to the serosal border. Since intracellular calcium concentration may reach

as high as 5 mM during the absorption process (214) it appears that most of this calcium would have to be bound to prevent deleterious effects to the cell. Therefore movement of calcium across the cell would most likely occur as a soluble protein-bound complex and/or associated with cellular vesicles (e.g. mitochondria). It has been observed recently, using autoradiographic and microincineration techniques, that intestinal mitochondria from vitamin D-treated rats contain numerous electron-dense granules (176). These granules appear limited to the microvillus portion in rachitic animals, being sparse in the mitochondria. After repletion, however, there is an increase in the density of granules in mitochondria, and an inverse relationship exists in the amount of granules appearing in the mitochondria and in the microvillus (176). Although these techniques are incapable of measuring soluble-bound calcium, their results suggest that the transcellular movement of calcium is reduced in vitamin D deficiency. Hamilton and Holdsworth (72) recently have conducted studies using mitochondria from intestinal mucosal cells loaded in vivo with ^{45}Ca. The degree of ^{45}Ca release from mitochondria was related to the amount of calcium-binding protein present in the incubation medium. The authors suggest that the release of calcium from mitochondria stimulated by the calcium-binding protein aids in the release of calcium from mitochondria at the serosal cell membrane into the extracellular fluid. Although the hypothesis is inviting, there still remains an equivocal point regarding the cellular location of the calcium-binding protein. To effect the release of calcium from mitochondria, the calcium-binding protein must function in the cytosol of the cell; however, the only results to date regarding the cellular location for the calcium-binding protein suggest that the protein is not present in the cytosol but rather is confined to the microvillar portion of the epithelial cell.

The final step in calcium translocation across the epithelial cell involves the transfer of calcium out of the cell and into the extracellular fluid. Schachter et al. (178, 180) previously had suggested that this process had a finite capacity for calcium, required energy, and moved calcium against an apparent thermodynamic gradient. In contrast Wasserman and coworkers (219, 221) have demonstrated that vitamin D increases the flux of calcium from blood to lumen as well as lumen to blood and thus discounted the possibility of a basal membrane-located calcium pump. More recent studies by Martin and DeLuca (122) have demonstrated the stimulatory effect of sodium on the in vitro transport of calcium and have suggested that sodium acts at the basal-lateral membrane of the epithelial cell. Although the translocation of calcium was related to the concentration of sodium, the uptake of calcium at the microvillar border appeared to be independent of sodium.

Ouabain, a known inhibitor of the sodium-potassium-dependent ATPase, has little effect in vitro on the calcium translocation across rat intestinal preparations (122). This result was expected in studies with rat tissue, due to the reported insensitivity of the rat sodium-potassium-dependent ATPase to digitalis (12). Ouabain in the buffer bathing the luminal side of a chick preparation also showed no inhibition in the calcium flux (87); this is not surprising since the sodium-potassium-dependent ATPase is located in the serosal membrane (35). More re-

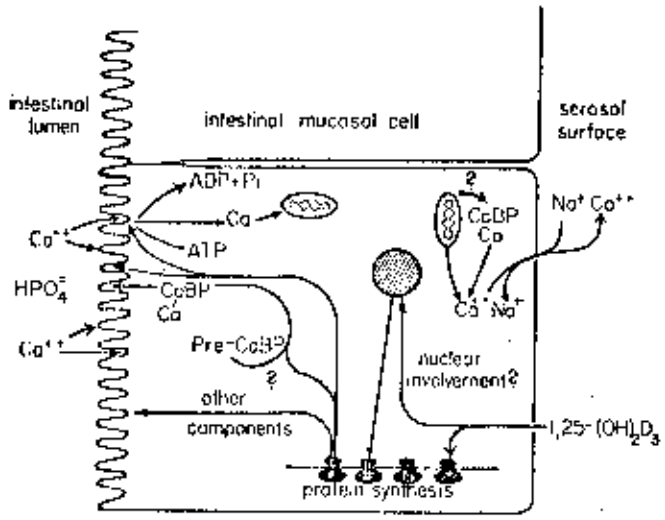


FIG. 1. Schematic summary of possible function(s) of 1,25-$(OH)_2D_3$ in calcium translocation in the intestinal mucosal cell.

cently it has been observed that inhibition of calcium flux does occur when ouabain is present in the medium bathing the serosal surface of chick intestinal preparations. Therefore it now appears that the inward movement of sodium across the serosal boundary into the epithelial cell is capable of being coupled to the exit of calcium, thus completing the translocation of calcium across the epithelial cell. Furthermore this action of sodium appears restricted to the basal-lateral membrane, which suggests a coupling of the calcium flux to the well-known sodium-potassium ATPase.

Figure 1 summarizes the current concepts regarding intestinal calcium translocation. The major obstacle to calcium transport in vitamin D-deficient animals appears to be the cation's ability to traverse the microvillar border. Two known components of the luminal border (the calcium-binding protein and calcium-dependent ATPase-alkaline phosphatase) conceivably act together to move calcium into the cell. The calcium-binding protein has been referred to as a microvillar concentrator (215), whereas the calcium ATPase-alkaline phosphatase most likely functions as a pump for calcium's movement into the cytosol. The necessity for having a "calcium pump" located in the luminal membrane is questionable, although the movement of calcium from the lumen across the brush border appears to be an "uphill" transfer (180) that would require a pump. The activity of the calcium-binding protein and possibly the calcium-dependent ATPase-alkaline phosphatase in the luminal membrane may be dependent on an interaction with other membrane components. For example lysolecithin has been shown to form a complex with chick calcium-binding protein that markedly alters the charge properties of the protein (216). Such phospholipid-protein interactions have been well documented, but the importance in vivo of such interactions remains to be evaluated.

There is no definitive study indicating how calcium moves across the mucosal epithelial cell. The best available evidence suggests that the movement of calcium across the cell involves mitochondria. However, major questions must be answered before this phenomenon can be assigned such a major physiological role. For example: 1) why does calcium precipitate in the mitochondria, and how is precipitated calcium solubilized for movement into the cytosol and across the basal membrane; 2) can such a mechanism occur fast enough to satisfy the observed in vivo absorption rates for calcium?

The movement of calcium across the basal-lateral membranes requires sodium. The transport process likely involves a coupling of the sodium-potassium-dependent ATPase to the efflux of calcium across the serosal boundary; again more direct studies are required to establish such a mechanism.

The influence of vitamin D on intestinal phosphorus absorption obviously has been neglected in this brief survey of calcium absorption. There is a paucity of information on this subject. Nicolaysen (137, 138, 140) initially demonstrated that phosphate absorption in rats is independent of vitamin D treatment. Any change in phosphate absorption appeared to be secondary to a change in calcium absorption, where phosphate appeared to be absorbed in association with calcium. Later in vitro work by Harrison and Harrison (77) showed that calcium was required for the concentrative transport of phosphate and the stimulatory effect of vitamin D. These authors suggested that an increased intracellular calcium concentration activated the concentrative transport of phosphate across the intestine. From a more recent in situ study (205), it appears that the absorption of phosphate is sensitive to changes in dietary calcium. Chicks fed a diet low in calcium show an increase in phosphate absorption. The absorption process appears to be independent of luminal calcium content; however, the possibility that calcium is still required intracellularly cannot be disallowed since calcium in the extracellular fluid has ready access to the mucosal cell.

Whether phosphate enters the cell via a transferase type of action or requires a carrier molecule and how vitamin D and its metabolites function in this transport process are currently uncertain. However, the general area of phosphate transport has recently received new impetus from the discovery that calcium and possibly phosphate play an important role in the control of vitamin D metabolism (see sect. V).

4) Adaptation to varying calcium diets. It has been known for several years that animals fed a low-calcium diet adapt by increasing their efficiency of intestinal calcium absorption (52, 174). Nicolaysen (141) found that adaptation to a low-calcium diet depends on the presence of vitamin D, as deficient animals show no change in intestinal calcium absorption regardless of dietary calcium treatment. The adaptation phenomenon is most readily demonstrated in young animals and appears not to be mediated via the hypophysis, adrenal, gonad, or thymus (105, 141, 181). Animals adapted to a low-calcium intake show an apparent increase in carrier affinity for calcium in intestinal calcium transport (212). This decrease in the apparent Michaelis constant is associated with an increase in the active transport of calcium from mucosal to serosal surface, whereas the retrograde move-

ment of calcium is not affected (211). Calcium-binding protein increases in concentration when animals adapt to a low-calcium diet (23, 154, 181, 223), again suggesting that the calcium-binding protein functions in some manner at the mucosal boundary in uptake and/or translocation of calcium across the epithelial cell. The adaptation to the low-calcium diet and the resultant enhancement in calcium transport depend on protein synthesis, inasmuch as puromycin blocks the increase in calcium-binding protein activity and calcium absorption (23).

How animals detect their calcium needs and thereby adapt to low-calcium intakes is not understood. However, the possibility that the response is mediated via the parathyroid glands must be considered since parathyroid hormone plays such a vital role in calcium homeostasis. Unfortunately experimental results on mediation of the adaptation response by the parathyroid gland are not clear. Results range from the marked inhibition of calcium absorption in parathyroidectomized animals (187) to experiments in which parathyroidectomy seemingly had no effect on the adaptation phenomenon or calcium transport and absorption in general (105, 175). Additional experiments regarding the role of the parathyroid glands in adaptation to dietary calcium are required before this important issue can be settled.

Nicolaysen (144) originally proposed that a humoral "endogenous factor" from bone acts in conjunction with vitamin D to control calcium absorption from the intestine. Unfortunately work for the past 20 years has afforded little insight into this possibility, but we are left with the term "endogenous factor" when possible mechanisms are discussed. However, a recent reevaluation in our laboratory of the vitamin D requirement for the adaptation response revealed that animals fed a low-calcium diet metabolize 25-hydroxyvitamin D_3(25-OHD_3) mainly to 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), whereas the major metabolite in normal or high-calcium fed animals is 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$), with little 1,25-$(OH)_2D_3$ detectable. The recent discovery that 1,25-$(OH)_2D_3$ is responsible for the initiation of intestinal calcium transport suggests that intestinal calcium absorption may be controlled through the modulation of 1,25-$(OH)_2D_3$ synthesis. In such a theory the Nicolaysen endogenous factor could act by controlling the synthesis of the 1,25-$(OH)_2D_3$. To provide evidence for such a theory it is necessary to satisfy at least two criteria: *1)* that the correlation between intestinal calcium absorption and 1,25-$(OH)_2D_3$ is positive and highly significant; and *2)* that animals given 1,25-$(OH)_2D_3$ should demonstrate no change in intestinal calcium absorption with variation of dietary calcium.

Boyle et al. (19, 20) initially followed the in vivo metabolism of 25-OHD_3 in animals fed diets varying in calcium content. Their results revealed that animals fed a low-calcium diet accumulate mainly 1,25-$(OH)_2D_3$ in the intestine whereas animals fed a high-calcium diet accumulate little 1,25-$(OH)_2D_3$. Similar studies were conducted in chicks in which changes in the rate of intestinal calcium absorption were correlated with the rate of in vitro 1,25-$(OH)_2D_3$ synthesis (J. L. Omdahl and H. F. DeLuca, unpublished observations). Although these studies are not complete it now is evident that animals fed a low-calcium diet exhibit an increased rate of intestinal calcium absorption and a high rate of 1,25-$(OH)_2D_3$

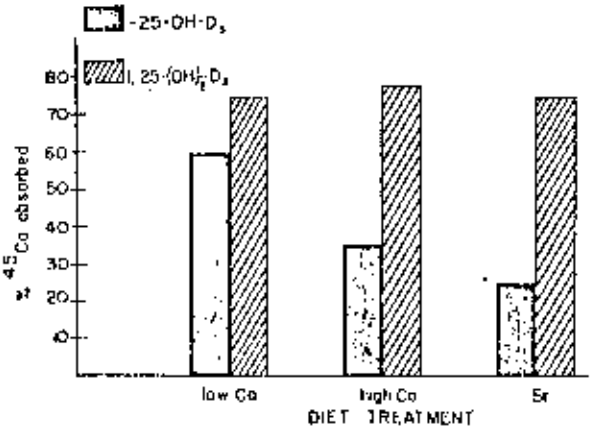


FIG. 2. Influence of 25-OHD$_3$ and 1,25-(OH)$_2$D$_3$ on intestinal calcium absorption in chicks fed a low- (0.08%) or high- (2%) calcium or strontium (2.35%) diet. Chicks were fed a vitamin D-deficient diet until they were 2 weeks old; they were then divided into 3 groups and fed indicated diets. At the same time half of each group received 25-OHD$_3$ (130 pmoles) and other half was given 1,25-(OH)$_2$D$_3$ (130 pmoles). Vitamins were given daily as an oral dose. Calcium absorption was measured at 4 days (strontium group) or 11 days (low- and high-calcium groups) after initiation of dietary and vitamin treatment. Each value is mean of 5 observations.

synthesis. In contrast animals fed a high-calcium diet show depressed intestinal calcium absorption and 1,25-(OH)$_2$D$_3$ synthesis. If indeed the animal is capable of regulating the rate of 1,25-(OH)$_2$D$_3$ synthesis in response to dietary calcium, then providing the animal with 1,25-(OH$_2$)D$_3$ should bypass the regulatory point and thus eliminate the animals' ability to adapt to varying calcium diets. To test this hypothesis chicks were fed a low- or high-calcium diet and were given either 25-OHD$_3$ or 1,25-(OH)$_2$D$_3$ (Fig. 2). As previously shown for vitamin D, the 25-OHD$_3$-treated animals showed an adaptation to the low-calcium intake as evidenced by the enhanced efficiency for intestinal calcium absorption. In contrast animals that received 1,25-(OH)$_2$D$_3$ showed no adaptation to the low-calcium diet. In fact both dietary calcium treatments resulted in the same rate of intestinal calcium absorption (Fig. 2). From these results it is possible to suggest that 1,25-(OH)$_2$D$_3$ plays a central role in the endogenous control of intestinal calcium absorption and most likely the endogenous factor functions by modulating the metabolism of 25-OHD$_3$. Factor(s) instrumental in the regulation of 1,25-(OH)$_2$D$_3$ synthesis are the topic of current investigation and most likely represent the remainder of Nicolaysen's "endogenous factor."

5) *Inhibitors of vitamin D action in the intestine.* A) ACTINOMYCIN D AND CYCLOHEXIMIDE. As previously discussed, deficient animals show a marked enhancement in their intestinal calcium transport after vitamin D repletion. There is an observed lag period, however, between administration of the vitamin and detection of an increase in intestinal calcium transport. It was apparent from early studies that the vitamin effected assembly of the calcium transport system during this period, a process that required protein synthesis (37, 38, 145). What other events occurred during this lag period did not become clear until it was observed that a prerequisite step for vitamin D action was its hydroxylation in the liver at carbon 25 (15, 158, 160). The end product is not 25-OHD$_3$; rather 25-OHD$_3$ is metabolized

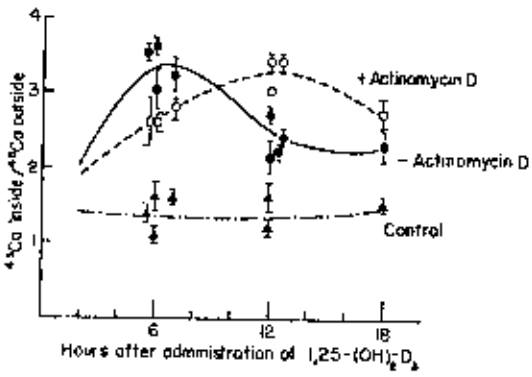


FIG. 3. Intestinal calcium transport response to 1,25-$(OH)_2D_3$ in presence of actinomycin D. Rats were fed a vitamin D-deficient diet (0.47% calcium, 0.3% phosphate) for 4–5 weeks prior to experimentation. Actinomycin D was given intravenously (1 μg/g body wt) 2–5 hr prior to intravenous dose of 1,25-$(OH)_2D_3$ (65 pmoles). Control animals received vehicles only (i.e. 0.9% NaCl and 95% ethanol for actinomycin D and 1,25-$(OH)_2D_3$, respectively). Animals were killed at indicated times and intestinal calcium transport was measured by in vitro gut sac technique. Individual values represent mean ± SD for 5 observations.

in the kidney to 1,25-$(OH)_2D_3$. It is now apparent that 1,25-$(OH)_2D_3$ is responsible for stimulation of intestinal calcium transport as observed both in metabolism studies of 1,25-$(OH)_2D_3$ and in studies in which nephrectomized rats were used (details of these studies are given in sect. III). Basic to this discussion involving inhibitors of intestinal calcium transport, however, is the fact that 1,25-$(OH)_2D_3$ is responsible for stimulating intestinal calcium transport.

Early work showed that vitamin D's action is blocked by the prior administration of actinomycin D, an inhibitor of DNA transcription (229, 230). It was suggested that protein synthesis occurred in the intestine after the vitamin's administration and was responsible for intestinal calcium transport. There may be alternative interpretations of those results, however; it has recently been shown that under experimental conditions in which vitamin D's action is blocked by actinomycin D there is little or no synthesis of 1,25-$(OH)_2D_3$ (see sect. III), and this explains why no increase in intestinal calcium transport was observed. Moreover rats poisoned with actinomycin D give a nearly normal intestinal calcium transport response when dosed with 1,25-$(OH)_2D_3$ (Fig. 3) (Y. Tanaka and H. F. DeLuca, unpublished observations). Use of cycloheximide as a protein synthesis inhibitor in the same type of experiment unfortunately has not been successful. The results to date suggest that 1,25-$(OH)_2D_3$ acts by a process not involving transcription of DNA, but it is unknown whether its action involves translation at the ribosomal level or the conversion of precursors to transport proteins.

In chicks both actinomycin D and cycloheximide appear capable of blocking 1,25-$(OH)_2D_3$-stimulated intestinal calcium transport (J. L. Omdahl and H. F. DeLuca, unpublished observations). However, such results must be evaluated in the light of the fact that actinomycin D and cycloheximide are extremely toxic agents in the chick. Therefore results that show depressed rates of calcium absorption in chicks treated with inhibitors of protein synthesis may actually reflect a general toxic effect that influences respiration and cardiac output; respiration and cardiac output must be maintained at a consistent level if meaningful results are

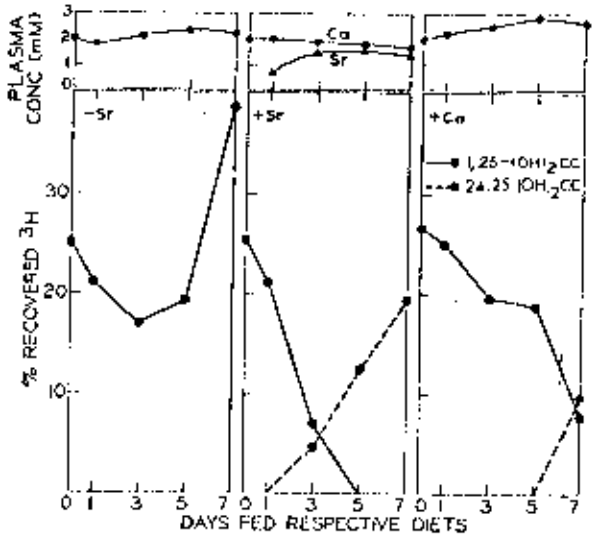


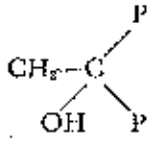
FIG. 4. Influence of dietary calcium and strontium on in vitro production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ by isolated kidney mitochondria. Vitamin D-deficient chicks (2.5 weeks old) started receiving vitamin D supplement (200 pmoles daily) and were fed either a (−) strontium (0.03% calcium, no strontium), (+) strontium (0.03% calcium, 2.35% strontium), or (+) calcium (1.5% calcium, no strontium) diet at zero time. Chicks were killed at indicated times and in vitro production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ was assayed using isolated kidney mitochondria; values are mean of 2 observations. Plasma calcium concentration was also determined and is average from 4 chicks.

to be obtained with in vivo techniques. In vitro gut-sac techniques exclude such toxic factors and are presently being developed for use with chick tissue. More thorough studies in the chick are required before a difference in mode of action for 1,25-$(OH)_2D_3$ in the chick and rat can be stated with certainty.

B) DIETARY STRONTIUM. Animals fed a strontium diet have low plasma calcium and develop rachitic bone lesions (30). Also feeding of stable strontium markedly reduces intestinal calcium transport and formation of the calcium-binding protein (30, 150). In considering strontium's mechanism of action in the intestine it appeared to us that a block in 1,25-$(OH)_2D_3$ synthesis would be reflected as a lowering of intestinal calcium absorption and could explain the inhibitory action of strontium. An initial metabolism study indicated that low amounts of ^{3}H-1,25-$(OH)_2D_3$ accumulated in tissues from strontium-fed chicks injected with ^{3}H-vitamin D_3 or ^{3}H-25-$(OH)D_3$ (150). Apparently the block in 1,25-$(OH)_2D_3$ production was not occurring in the liver but rather in the kidney, where 25-$(OH)D_3$ is normally hydroxylated to 1,25-$(OH)_2D_3$. More direct in vitro studies showed that isolated kidney mitochondria from strontium-fed chicks fail to metabolize 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ but rather produce a different dihydroxy compound, namely 24,25-$(OH)_2D_3$ (Fig. 4). Direct proof that the decrease in calcium transport was due to a block of 1,25-$(OH)_2D_3$ synthesis was obtained when 1,25-$(OH)_2D_3$ but not 25-$(OH)D_3$ was found to restore intestinal calcium absorption to control levels in strontium-fed chicks (Fig. 2). Whether 1,25-$(OH)_2D_3$ also will correct the bone lesions is not known and may prove difficult to determine. Regardless, it is now apparent that strontium is a potentially useful tool in the study of the regulation of 25-$(OH)D_3$ metabolism.

C) DIPHOSPHONATES. The group of diphosphate compounds is characterized

by a P—C—P bond, e.g.



In vivo these compounds prevent soft-tissue calcification (54). When ethane-1-hydroxy-1,1-diphosphate (EHDP) is administered at high doses (10 mg phosphorus/kg body wt) an inhibition of normal calcification is observed (106) that corresponds to a depression in intestinal calcium absorption (60). Such imbalances lead to the development of rachitic bone lesions, a malady refractory to vitamin D supplements. With the prior knowledge regarding the actinomycin D and strontium block of 1,25-$(OH)_2D_3$ synthesis, it occurred to us that one possibility for EHDP's suppressive action on intestinal calcium absorption might involve a block in 1,25-$(OH)_2D_3$ synthesis. Subsequent studies in chicks revealed an inhibition in 1,25-$(OH)_2D_3$ synthesis due to EHDP (L. M. Baxter and H. F. DeLuca, unpublished observations). Concurrently it was observed that inhibition of intestinal calcium absorption in EHDP-treated rats could be corrected by giving 1,25-$(OH)_2D_3$ (J.-P. Bonjour, unpublished observations). Apparently EHDP blocks 1,25-$(OH)_2D_3$ synthesis, which results in low calcium absorption and thus contributes to the development of rickets. The mechanism by which such a response is evoked is not known and is a topic of current investigation.

b) CORTICAL STEROIDS (CORTISOL). Administration of adrenal cortical steroids reduces the elevated serum calcium concentration that occurs in vitamin D intoxication, sarcoidosis, and idiopathic hypercalcemia of infancy (13, 103, 104). It is particularly interesting that cortisol or other glucocorticoid administration decreases intestinal calcium absorption (76, 104). This action of cortisol has been referred to as antagonistic with respect to vitamin D, since large doses of the vitamin fail to reverse the action of cortisol on calcium transport. Attempts to demonstrate an alteration in vitamin D metabolism due to glucocorticoid treatment have yielded somewhat equivocal results. Metabolism studies in man (7) showed a decrease in plasma half-life of ^{3}H-vitamin D_3 and a decrease in accumulation of biologically active metabolites due to prednisone treatment. In contrast Kimberg et al. (104) found no appreciable difference in the tissue accumulation of ^{3}H-vitamin D_3 in cortisone-treated rats. It also was interesting that cortisone stimulated the activity of intestinal calcium-binding protein (104), a finding difficult to explain since cortisone decreases intestinal calcium transport. It should be noted that in the original work only *total* calcium binding protein activity was measured in heat-treated supernatant solutions from rats. No attempt was made to separate the vitamin D-dependent calcium-binding protein from the vitamin D-independent calcium-binding protein. However, more recent studies with rats have shown an increase in the vitamin D-dependent calcium-binding protein after hydrocortisone treatment (51). This discrepancy between high activity of calcium-binding protein and depressed calcium absorption has not been resolved.

4376

Clearly the action of cortisol on intestinal calcium absorption could involve depressed 1,25-$(OH)_2D_3$ production, as previously discussed for strontium and EHTP. However, additional metabolism studies with current chromatographic techniques must be conducted before a definite statement can be made regarding a possible effect of glucocorticoids on vitamin D or 25-OHD_3 metabolism.

E) ANTICONVULSANT DRUGS. Kruse (113) recently observed an increased incidence of rickets in children chronically dosed with multiple anticonvulsant drugs for over 5 years, and Richens and Rowe (168) have shown a positive correlation between total dose of anticonvulsant drugs and degree of hypocalcemia in an adult epileptic population. Interestingly vitamin D supplementation was effective in the treatment of both rachitic (113) and osteomalacic (44) changes in bone. It was subsequently suggested that abnormal vitamin D metabolism occurring in such patients could manifest itself in appearance of the various bone lesions. Accordingly recent studies have explored a possible effect of Dilantin (5,5'-diphenylhydantoin) on the metabolism of vitamin D_3 and 25-OHD_3 (29; M. Garabedian and H. F. DeLuca, unpublished observations). Acute dosing of Dilantin in rats failed to produce rickets (29) and had no effect on vitamin D_3-, 25-OHD_3-, or 1,25-$(OH)_2D_3$-induced intestinal calcium transport and bone mineral mobilization (M. Garabedian and H. F. DeLuca, unpublished observations). The only detectable difference in the Dilantin group involved a more rapid disappearance of ^{3}H-vitamin D_3 and ^{3}H-25-OHD_3 from the serum. However, the accumulation of metabolites from either ^{3}H-vitamin D_3 or ^{3}H-25-OHD_3 showed no detectable change due to Dilantin treatment, suggesting no effect on metabolism per se. However, these experiments represent short-term treatment with anticonvulsant drugs, whereas patients showing changes in calcium metabolism have been treated for years (113). Clearly long-term chronic administration to animals will be required before conclusions can be reached regarding vitamin D metabolism.

Recently Hahn et al. (69) suggested that patients on chronic phenobarbital therapy convert ^{3}H-vitamin D_3 to polar metabolites more rapidly than the normal controls. This increased rate of ^{3}H-vitamin D_3 metabolism could be induced in patients and rats by administration of phenobarbital. Increased in vitro conversion of vitamin D_3 and 25-OHD_3 to polar metabolites occurred in liver homogenates, with the major activity concentrated in the microsomal fraction. This study suggests that anticonvulsant therapy may increase the rate of vitamin D_3 and 25-OHD_3 conversion to presumably inactive polar compounds, which in turn would increase occurrence of osteomalacia. These results are suggestive, but clear identification of metabolites affected by the anticonvulsant drugs must be provided, especially for the in vitro experiments, which may produce artifactual "metabolites."

B. Role of Vitamin D in Mineralization and Resorption of Bone

1) Mineralization. The role of vitamin D in the accretion of mineral in bone has been recognized almost since the beginning of experimental work on the antira-

4376

FIG. 5. Rachitic child with obvious epiphyseal swelling of wrist and bowed legs.

chitic factor. Clinical rickets were studied long before the first demonstration by Sir Edward McHardy in 1919 that rickets could be produced in experimental animals (37). In addition the disease osteomalacia was also recognized and later shown to be a result of vitamin D deficiency in adults. Certainly the major end effect of the vitamin is to bring about normal calcification of bone.

The mineral component of bone imparts rigidity to the collagen fibril matrix, whereas the organic portion of bone by itself is unable to carry out the structural role assumed by bone. This becomes especially evident in rickets, in which a lesion occurring in the mineralization process results in the characteristic deformities especially evident in long bones, ribs, and skull (Fig. 5). A discussion of the pathology and histology associated with vitamin D deficiency is beyond the scope of this review and interested readers are directed elsewhere for this information (184, 185). From a biochemical point of view it can be stated that in vitamin D deficiency the calcification process fails to take place at a rate equal to the synthesis of new organic matrix of bone. The extent of this failure depends on the degree of vitamin D deficiency as well as on the deficiency of either calcium or phosphorus.

The fact that vitamin D brings about normal calcification of bone has led many investigators to consider that the vitamin must function directly on the mineralization process. Although this concept is intellectually satisfying, direct evidence is lacking despite attempts by clinical investigators and basic scientists to gather such evidence over the past 50 years. Certainly clinical investigators have

presented evidence of bone calcification under conditions where the plasma calcium and phosphorus concentrations do not change in response to vitamin D (17, 193). The major problem with these experiments is that the calcium and phosphate concentrations have not been monitored during the entire calcification period. Dikshit and Patwardhan (45) have attempted, using rat bone slices, to demonstrate a direct action of the vitamin on the mineralization process. In their experiments animals were pretreated with vitamin D and bone slices were taken before measurable calcification could be detected by the silver nitrate staining method. Slices thus obtained were found to calcify more rapidly in vitro than slices taken from completely deficient animals. The primary problem here is that silver nitrate staining methods may not detect small nucleation centers initiated by the rise in calcium and phosphorus of the extracellular fluid in response to vitamin D. When incubated in an adequate medium of calcium and phosphorus the slices would calcify more rapidly than slices that had no nucleation centers started. Clearly there is no evidence as yet to support the idea that vitamin D must participate in the calcification process per se. Until a positive experiment can be performed this question must remain unresolved.

The major defect, if not the only defect, in the calcification process resulting from vitamin D deficiency is a failure of the supply of calcium and phosphate to the bone (37, 38). This concept is not new, inasmuch as Howland and Kramer (95) provided strong evidence that in vitamin D deficiency the product of calcium times phosphorus of the blood drops to levels at which calcification would not be expected to occur. In fact the low calcium-times-phosphorus product was used as a diagnostic test of rickets in early clinical work. This idea, although of great clinical importance, was ignored until 1958, when Neuman and Neuman (133) provided the concept that blood is normally supersaturated with calcium and phosphate. It therefore followed that the calcification of bone is essentially a catalyzed crystallization from a supersaturated solution, assuming of course that the extracellular fluid bathing bone approximates the extracellular fluid of the blood compartment. In follow-up studies Neuman (132) showed that rachitic blood is undersaturated with calcium and phosphorus relative to the hydroxyapatite of bone mineral. Incubation experiments of Shipley et al. (188, 189) also support the idea that in rickets there is a defective supply of calcium and phosphorus to the calcification sites. When the experimental data available from the animal kingdom are examined closely it is evident in all cases where calcification fails in D deficiency that there is an accompanying depression of blood calcium and/or phosphorus concentration. In any case there is little doubt that a deficiency of vitamin D reduces plasma calcium and/or phosphorus concentration, which in turn is responsible for the observed failure of bone calcification (10, 191).

2) Calcium mobilization from bone. A) VITAMIN D-INDUCED MOBILIZATION. Because of the great interest in the calcification process in response to vitamin D, little attention was paid to an important aspect of vitamin D function, namely the mobilization of calcium from bone. Prior to 1950 it had been recognized that large amounts of vitamin D bring about a decalcification of bone rather than its mineralization (142). It was primarily the work of Carlsson and coworkers (9, 24, 25)

that conclusively demonstrated that an important function of vitamin D is to bring about the mobilization of calcium from previously formed bone. These investigators not only provided conclusive physiologic experiments but by means of radioisotopic methods demonstrated that the mobilization process occurs even at physiologic doses of the vitamin. Lindquist (116) also showed that the mobilization of calcium from bone increases with the log of dose even to massive amounts of vitamin D, whereas the intestinal calcium transport response becomes saturated at very low doses of the vitamin. Nicolaysen and Eeg-Larsen (143) confirmed the work of Carlsson's group (9, 24, 25) by showing that vitamin D repletion of rats fed a low-calcium diet prompts an elevation in serum calcium concentration. Since the only source of calcium under such circumstances is bone, vitamin D at physiologic doses must in some manner cause the mobilization of calcium from bone. This important process of calcium mobilization from bone has now been studied in vivo using radioisotopic techniques and in vitro using bone organ culture systems. It is evident from such studies that bone mineral mobilization must be viewed in the context of contributing calcium and phosphorus to the blood pool in a manner analogous to the intestine. It therefore contributes to the (calcium) × (phosphorus) product of the plasma, the maintenance of which is necessary for normal calcification of bone.

B) PARATHYROID HORMONE-VITAMIN D INTERACTION. The parathyroid hormone also functions in the mobilization of calcium from bone, and it has been believed that this represents the major way in which the parathyroid hormone brings about the rise in plasma calcium concentration (166). The parathyroid glands sense reduced plasma calcium concentration and in response secrete parathyroid hormone into the systemic circulation. The hormone is rapidly sequestered in bone, where it functions to bring about the mobilization of calcium. Harrison et al. (80) in 1958 made the important observation that vitamin D is required for bone calcium mobilization. The full significance of this finding was not appreciated until Rasmussen et al. (167) confirmed the work of Harrison et al. (80) and focused on the fact that very small amounts of vitamin D could function in a permissive way to allow the expression of parathyroid hormone action on the mobilization of calcium from bone. Later Au and Raisz (6) carried out experiments suggesting that the failure of parathyroid hormone to produce a response in vitamin D deficiency is due to the lack of calcium. Thus the parathyroid responsiveness could be restored by injecting calcium or by providing calcium through the stimulation of intestinal calcium absorption by lactose. However, the source of calcium that appears in the plasma in response to the parathyroid hormone was not shown conclusively to come from bone mineral. In any case the parathyroid hormone cannot mobilize calcium from bone in vitamin D-deficient animals. Vitamin D and the parathyroid hormone therefore must operate in concert on this important calcium homeostatic mechanism.

The discovery of calcitonin raised the question whether this hormone could function under conditions of vitamin D deficiency. Work from this laboratory (41, 129) provided evidence that calcitonin can exert its effects in inhibiting bone calcium mobilization in the presence or absence of vitamin D. The complex

interaction of calcitonin, vitamin D, and parathyroid hormone is not well understood, especially in regard to the molecular mechanisms involved in their actions on bone.

The use of bone organ culture (62, 163) has provided much information, but unfortunately even these techniques have failed to supply data on reactions at the subcellular level. Several models to explain the interaction between vitamin D, parathyroid hormone, and calcitonin on the sites of bone mineral mobilization have been advanced (41, 125, 136, 199). They represent useful models for stimulating experimentation and for aiding the student to recall the many known effects of the agents. Whether any one of the models is valid can only be decided when investigations at the molecular level provide the missing information.

c) VITAMIN D METABOLITES. The mobilization of calcium from bone is a response to metabolites of vitamin D rather than to the parent vitamin. When 25-OH-D_3 was isolated, identified (15), and synthesized (14) it was shown to be more effective and to act more rapidly in the mobilization of calcium from bone than vitamin D_3 (16). In organ cultures as little as 65 pmoles of 25-OHD_3 per milliliter of culture fluid induced measurable bone resorption, whereas 500 times that amount of vitamin D_3 produced no effect (206). With the isolation and identification of 1,25-$(OH)_2D_3$ (92, 93), a further metabolite of 25-OHD_3, it could be shown that the 1,25-$(OH)_2D_3$ works much more rapidly than even the 25-OHD_3 (81, 152). Of importance, physiological doses of 25-OHD_3 do not induce bone calcium mobilization in nephrectomized rats, whereas 1,25-$(OH)_2D_3$ does (90). In addition in the organ culture system 1,25-$(OH)_2D_3$ is approximately 100 times more effective on a weight basis than 25-OHD_3 (164) (Fig. 6). At physiologic doses of the vitamin, the 1,25-$(OH)_2D_3$ (or a further metabolite thereof) appears to be the metabolically active form in the mobilization of calcium from bone. However, at higher doses of vitamin D, 25-OHD_3 may well be the primary bone calcium mobilizer. Certainly it is effective directly in bone without further metabolism, but the concentrations required are higher than for 1,25-$(OH)_2D_3$.

In 1965 Zull et al. (231) demonstrated that the response of the bone calcium mobilization system to vitamin D is blocked by the prior administration of ac-

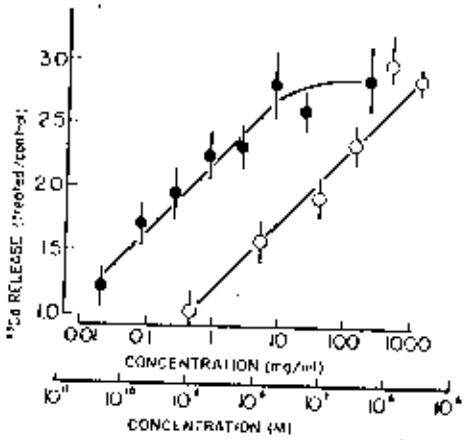


FIG. 6. Fetal bone calcium mobilization in response to 1,25-$(OH)_2D_3$ (●) or 25-OHD_3 (○). Bones labeled with ^{45}Ca were first cultured for 24 hr and then paired cultures were treated with or without 1,25-$(OH)_2D_3$ or 25-OHD_3 at indicated doses. Points indicate mean ± SE for the 48 hr ^{45}Ca release in 4–16 pairs of cultures. [From Rasiz et al. (164).]

4376

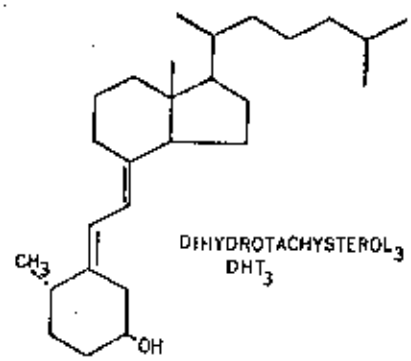


FIG. 7. Structural formula for dihydrotachysterol$_3$ (DHT$_3$).

tinomycin D, the antibiotic that prevents transcription of DNA. Actinomycin D is unable to block the response if administered after vitamin D. This observation has now been extended to include responses to 25-OHD$_3$ and 1,25-(OH)$_2$D$_3$ (200). These results suggest that the expression of vitamin D activity on the calcium mobilization system from bone involves the transcription of DNA and probably protein synthesis. Beyond this observation little can be said about the molecular mechanisms involved in the mobilization of calcium from bone.

b) DIHYDROTACHYSTEROL$_3$ AND 25-HYDROXYDIHYDROTACHYSTEROL$_3$. A discussion of vitamin D and bone calcium mobilization is incomplete without a discussion of dihydrotachysterol. This vitamin D analog is a product of tachysterol reduction and can be regarded as a reduction product of vitamin D. The compound, whose structure is shown in Figure 7, has two important structural differences from vitamin D. First, the C_{10-19} methylene group has been replaced by a methyl of unknown configuration. In addition, ring A is rotated 180°; this places the 3-hydroxyl in quite a different geometric position from that occupied by C_3-OH of vitamin D$_3$. Dihydrotachysterol has been used for many years to raise the plasma calcium concentration, especially in patients suffering from hypoparathyroidism (18, 79, 171, 198). It is only slightly effective in the calcification tests and other assays of vitamin D activity; e.g., it is approximately 1/450 as effective in the calcification of bone and in the promotion of intestinal calcium transport as vitamin D$_3$ (198). At high doses, however, it is much more effective than either vitamin D$_2$ or vitamin D$_3$ in the elevation of plasma calcium concentration. Recently it has been shown that the active agent in the mobilization of calcium from bone is 25-hydroxydihydrotachysterol rather than dihydrotachysterol$_3$ itself. The rationale behind its effectiveness is discussed in an ensuing section.

C. *Role of Vitamin D in Renal Tubular Transport*

In 1941 Harrison and Harrison (75) first focused attention on the possible role of vitamin D in renal tubular reabsorption of phosphate, with the demonstration that the administration of vitamin D increases the tubular reabsorption of phosphate. Unfortunately these early experiments suffered from the fact that

they were performed on dogs with intact parathyroid glands. It is possible that administration of vitamin D elevated plasma calcium concentration, which in turn repressed parathyroid secretion. The parathyroid hormone is known to induce a phosphate diuresis. Conceivably the increased tubular phosphate reabsorption observed by Harrison and Harrison might have been due to a suppression of parathyroid secretion. A possible role of vitamin D in tubular reabsorption of calcium has also been reported by Gran (64), but similar criticisms can also be made of that work.

The kidney could exert an important control over plasma calcium concentration. It filters gram quantities of calcium per day but reabsorption is about 99.8% efficient because in normal man as little as 100 mg calcium/day is excreted. Although the tubular reabsorption of phosphate is not as efficient as that of calcium, the kidney nevertheless handles a large amount of phosphate. Existing work in rats indicates that urinary calcium and phosphate are not markedly decreased by the administration of vitamin D to vitamin D-deficient animals. It is difficult to assess the quantitative contribution of such a renal effect by vitamin D on overall calcium metabolism, but it seems likely that the renal mechanisms are quantitatively much less important than the intestine and bone effects.

Availability of metabolites of vitamin D has focused attention on the possible role of vitamin D in renal tubular reabsorption. Puschett (162) used a vitamin D-sufficient, mildly volume-expanded dog to demonstrate that physiologic doses of vitamin D and 25-OHD_3 will increase tubular reabsorption of phosphate, calcium, and sodium ions. He extended his observations to the new metabolite 1,25-$(OH)_2D_3$ and found that 1,25-$(OH)_2D_3$ and the 25-OHD_3 have about the same effect, but the 1,25-$(OH)_2D_3$ increases tubular reabsorption of phosphate within 30 min after its administration whereas a longer time is required for the 25-OHD_3 to function. Puschett's experiments were carried out on thyroparathyroidectomized dogs to eliminate the possibility that the observed effects resulted from parathyroid suppression. Although he has carefully controlled other factors in these experiments, volume expansion and large amounts of vasopressin were used for a demonstration of vitamin D action. Unfortunately the experiments were not performed on vitamin D-deficient dogs. An increased tubular reabsorption of phosphate in response to vitamin D in vitamin D deficient rats has been shown by micropuncture techniques (22). Therefore it seems likely that an effect of vitamin D on calcium and phosphate reabsorption will be directly demonstrable.

The parathyroid hormone induces a phosphate diuresis primarily through the cyclic AMP mechanism (26, 27), and this phenomenon separates the physiologic actions of the parathyroid hormone and vitamin D. Certainly only large doses of vitamin D induce a phosphate diuresis (135). Because of the dependence of parathyroid hormone on vitamin D for its action in the mobilization of calcium from bone, it is of interest to consider whether phosphate diuresis caused by the parathyroid hormone requires the presence of vitamin D. Measurements of plasma phosphate concentration led Arnaud et al. (4) to conclude that the phosphate diuretic effect of the parathyroid hormone is not dependent on vitamin D. On the other hand Harrison and Harrison (78, 79), using intact rats, concluded that

4376

the parathyroid hormone could not function in phosphate diuresis in the absence of vitamin D. Arnaud et al. (4) used whole perfused animals to provide evidence that phosphate diuresis in vitamin D-deficient rats could be induced by parathyroid hormone provided plasma calcium levels were equalized. The degree of response, however, in the vitamin D-deficient animal was less than that seen in animals given vitamin D. It is not clear now which viewpoint is valid, but the correct conclusion may lie somewhere between the two positions. There seems to be some vitamin D dependence of the parathyroid hormone-phosphate diuretic system, but it is not as vitamin D dependent as the bone calcium mobilization system.

More work is needed on renal effects of vitamin D and its metabolites and on the interaction between vitamin D, parathyroid hormone, and calcitonin in renal functions. The availability of new metabolites of vitamin D has renewed interest in the possible renal function of vitamin D, and research soon will clarify the quantitative physiologic role of the vitamin.

D. *Summary of Physiologic Action of Vitamin D*

There are two important physiologic functions of vitamin D (Fig. 8). First, it is necessary for normal calcification of bone; second, it is an essential homeostatic agent for the control of plasma calcium concentration. Vitamin D is first metabolized to functional forms (described in a later section) and it is these forms that carry out the two important functions. First, they initiate intestinal calcium absorption and possibly intestinal phosphate absorption. The calcium transport process of the intestine is an active cation-oriented process whose molecular mechanism may involve calcium-binding protein and/or a calcium ATPase or some unknown factor. Phosphate is the normal accompanying anion in the calcium transport system but a phosphate transport system also has been described that shows vitamin D dependence (77). In addition to these systems vitamin D brings about the mobilization of calcium and phosphate from bone to contribute to the calcium and phosphate pool of the plasma. This process not only is important for calcification but represents an important calcium homeostatic mechanism. It is not clear whether the kidney is a site of action of vitamin D, but preliminary re-

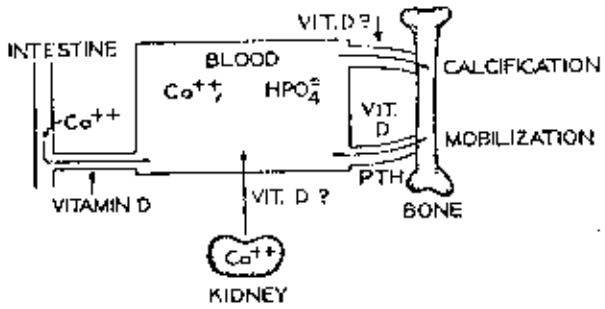


FIG. 8. Schematic summary of physiological actions of vitamin D.

4376

sults suggest that vitamin D increases tubular reabsorption of phosphate and calcium. These three mechanisms then provide adequate amounts of calcium and phosphate for normal calcification. Blood calcium concentration is thus maintained, assuring normal neuromuscular activity.

III. VITAMIN D METABOLITES AND THEIR FUNCTIONAL ROLE

A. Historical

It had been assumed after the definitive work of Windaus et al. (228) and Schenck (183) that vitamin D must function in the body without alteration. This assumption was reinforced by the work of Kodicek and his collaborators during the 1950's. Kodicek first considered the possibility that vitamin D must be metabolized before it can function (33, 34, 109, 110). After massive doses of vitamin D he could account for, by laborious biological assay, only 20% of the original dose. Apparently the remainder was biologically inactive and unable to induce calcification of bone. He concluded correctly that vitamin D must be metabolized to inactive products. In 1955 Kodicek and his collaborators biosynthesized radioactive vitamin D_2 (108). The low specific activity of the material precluded experiments with physiologic doses of the vitamin, and thus he (111) failed to detect biologically active metabolites of vitamin D.

B. 25-Hydroxycholecalciferol (25-OHD_3)

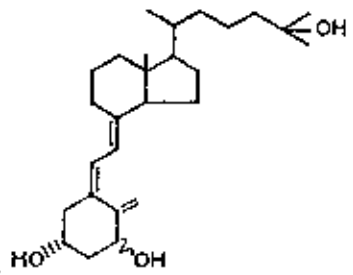
Chemical synthesis of radioactive vitamin D of high specific activity was achieved by three different routes and provided an important breakthrough (43, 98, 134). These labeled materials at physiologic dosages revealed conclusively that vitamin D is in fact metabolized to biologically active metabolites (119). At least three metabolites could be demonstrated in blood, liver, bone, intestine, and kidney (119, 147). The first was clearly identified by Lund et al. (120) and by Fraser and Kodicek (55, 56) as an ester of vitamin D and long-chain fatty acids. The second, which is somewhat less polar than vitamin D_3, is biologically active but remains unidentified. The most important fraction from silicic acid columns was that designated as peak IV. This material elicited a biological activity at least equal to the parent vitamin, and it acted much more rapidly than the parent vitamin in initiating intestinal calcium transport. This suggested that the peak IV fraction contained a metabolically active form or a precursor of an active form of vitamin D (130). Modification of the original chromatographic method facilitated isolation of the major biologically active metabolite of the blood, and it was identified unequivocally as 25-OHD_3 (15). This metabolite subsequently was synthesized chemically (14) and shown to be formed primarily in the liver (158, 160). Horsting and DeLuca (91), using both perfused liver and liver homogenates, demonstrated that an enzyme system of liver carries out the 25-hydroxylation. This hydroxylation system appears unique as it is not blocked by carbon monoxide-oxygen mixtures or diphenylparaphenylene diamine.

The 25-OHD$_3$ is about 4 times more effective than vitamin D$_3$ in the treatment of pseudo-vitamin D-deficiency disease (8; D. R. Fraser, unpublished observations) and is about 1.5 times more effective than vitamin D$_3$ in supporting calcification of rachitic bones of rats and chickens (16). Significantly, 25-OHD$_3$ acts much more rapidly to initiate intestinal calcium absorption than vitamin D$_3$ and it also acts much more rapidly to induce bone calcium mobilization (16). In perfused intestine (149) and in bone organ cultures (207), representing end organs of vitamin D action, the 25-OHD$_3$ elicits a response, whereas massive amounts of vitamin D$_3$ fail to produce a response. The doses of 25-OHD$_3$ used in these systems, however, are superphysiological, at least for the calcium absorption response of perfused intestine. In any case, the results suggest that 25-hydroxylation is essential for vitamin D action.

C. *1,25-Dihydroxycholecalciferol* (*1,25-(OH)$_2$D$_3$*)

The synthesis of 25-OHD$_3$ (14) permitted preparation of 25-OHD$_3$ labeled in the 26 and 27 positions (194). With this material it was demonstrated that the 25-OHD$_3$ is rapidly metabolized both to less polar and more polar materials (31, 32). The less polar compounds primarily represent esters of 25-OHD$_3$, and again the polar metabolites are of major importance. Polar metabolites of 25-OHD$_3$ accumulate rapidly in intestine, bone, and other tissues. From the silicic acid column profiles they were designated as peaks V and VI. A number of questions were raised by the unexpected finding that polar metabolites could be found in both target and nontarget tissues very rapidly after 25-OHD$_3$ administration.

Norman and his colleagues first demonstrated the existence in intestine of a metabolite of [^{3}H]vitamin D$_3$ more polar than 25-OHD$_3$ that possessed intestinal calcium transport activity (82, 115, 131, 159). In Kodicek's laboratory the availability of vitamin D$_3$ labeled with tritium in the 1α position made possible a unique series of experiments. To their surprise it could be shown that the tritium from the 1 position was lost completely from a metabolite that appeared in the intestine (115). This loss of tritium led Kodicek and his collaborators to suggest that an important metabolite of vitamin D in the intestine involves a modification at the 1 position. In our laboratory (159) the polar metabolite of vitamin D was detected in intestine and bone but the biological activity was not impressive. The failure of the silicic acid column to separate the polar metabolites of 25-OHD$_3$ led to the development of a new method of gel-liquid partition chromatography on Sephadex LH-20, an isopropyl ether derivative of Sephadex G-25 (88). This method not only resolves virtually all the peak V metabolites of blood but also is rapid, convenient, and provides close to 100% recoveries of applied metabolites. This method permitted isolation and identification of the polar metabolite of vitamin D in intestine (92, 93). From 1500 chickens given tritiated vitamin D$_3$ 2 μg of pure metabolite were obtained. Mass spectrometry, ultraviolet absorption spectra, and specific chemical reactions established the structure of the metabolite unequivocally as 1,25-(OH)$_2$D$_3$ (Fig. 9). Lawson et al. (114) also independently provided mass spectra of the "tritium"-deficient metabolite generated by chick kidney homogenate but the 1,25-(OH)$_2$D$_3$ structure was not completely identified chemically. A



1, 25 DIHYDROXYCHOLECALCIFEROL

FIG. 9. Structural formula for 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$).

still later report (148) provided additional confirmatory mass spectral data for the metabolite generated by chicken kidney in vitro but also failed to provide chemical identification of the structure.

It is important to recognize that the suggested α configuration of the 1-hydroxyl of 1,25-$(OH)_2D_3$ was not established by the identification work. However, chemical synthesis of the 1,25-$(OH)_2D_3$ has recently been completed that establishes the configuration as α, i.e. 1α,25-$(OH)_2D_3$ (186).

The 1,25-$(OH)_2D_3$ acts even more rapidly than 25-OHD_3 in the initiation of intestinal calcium transport (81, 131, 152) and in the initiation of bone calcium mobilization (200) (Fig. 10). Note that for intestinal calcium transport the effect of the 1,25-$(OH)_2D_3$ is short-lived relative to that produced by 25-OHD_3. In isolated perfused rat intestine 1,25-$(OH)_2D_3$ is effective at a level of 65-325 pmoles/intestine as compared to 60,000 pmoles of 25-OHD_3 (E. B. Olson and H. F. DeLuca, unpublished observations). As previously discussed, the 1,25-$(OH)_2D_3$ is approximately 100 times more effective than 25-OHD_3 in the mobilization of calcium from cultures of bone (164) (Figure 6). These results suggest that at physiologic doses it is the 1,25-$(OH)_2D_3$ and not 25-OHD_3 that is the metabolically active form in both the intestine and in the mobilization of calcium from bone. Direct proof for this was obtained by showing that actinomycin D given 2-5 hr before 25-OHD_3 completely prevented the intestinal calcium transport response (202), whereas when it was given 2-5 hr before 1,25-$(OH)_2D_3$ it was unable to block the response. This coupled with the earlier demonstration that actinomycin D prevents the metabolism of 25-OHD_3 to 1,25-$(OH)_2D_3$ (201) provides strong evidence that 1,25-$(OH)_2D_3$, or a further metabolite thereof, and not 25-OHD_3 is the form of vitamin D_3 metabolically active in the stimulation of intestinal calcium transport. Experiments with radioactive 1,25-$(OH)_2D_3$ provide firm evidence that the 1,25-$(OH)_2D_3$ is not metabolized further before it increases intestinal calcium transport (58; C. A. Frolik and H. F. DeLuca, unpublished observations). Therefore 1,25-$(OH)_2D_3$ seems to be the final form of the vitamin metabolically active in the initiation of intestinal calcium transport.

Fraser and Kodicek (57) made the important discovery that the tritium-deficient metabolite (1,25-$(OH)_2D_3$) is synthesized exclusively in the kidney. This result was quickly confirmed in our laboratory and extended to demonstrate that it was not the uremia resulting from nephrectomy that prevented the conversion

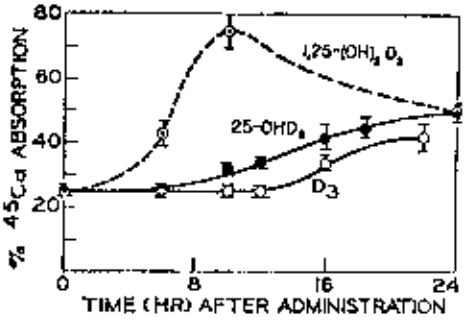


FIG. 10. Stimulation of intestinal calcium absorption in vitamin D-deficient chicks by vitamin D and its metabolites. Intestinal ^{45}Ca absorption was measured in situ at various times after a single 325-pmole oral (1,25-(OH)$_2$D$_3$) or intravenous (25-OHD$_3$ and vitamin D$_3$) dose of respective compounds. Values represent mean ± SE from 5 observations. (From Omdahl et al. (152).)

but rather the lack of kidney tissue per se (66). Furthermore kidney homogenates from vitamin D-deficient chicks are able to carry out this conversion. This enzyme system is found exclusively in the mitochondria of kidney tissue. Its activity is supported by molecular oxygen and reduced pyridine nucleotide and is inhibited by carbon monoxide-oxygen mixtures and to some extent by diphenylparaphenylene diamine (R. W. Gray, J. L. Omdahl, and H. F. DeLuca, unpublished observations). The Michaelis constant for the 25-OHD$_3$ is 2.7×10^{-6} M for intact mitochondria.

Because the kidney is the site of synthesis of 1,25-(OH)$_2$D$_3$ it was readily demonstrated that nephrectomized, vitamin D-deficient rats do not respond to 25-OHD$_3$ at physiologic doses in terms of intestinal calcium absorption, but they do respond to physiologic doses of 1,25-(OH)$_2$D$_3$. Again this provides convincing evidence that 1,25-(OH)$_2$D$_3$ and not 25-OHD$_3$ is the final metabolically active form in the intestine (21). Similar experiments could be carried out with regard to bone calcium mobilization providing firm evidence that 1,25-(OH)$_2$D$_3$ or a further metabolite thereof is the metabolically active form in that system as well (90).

It is clinically significant that the 1,25-(OH)$_2$D$_3$ is of kidney origin, because it can be considered a hormonal form of vitamin D that arises from the kidney and performs its function in organs that do not carry out the synthesis (39, 40, 57). Osteodystrophy related to chronic renal disease is well known (102, 107, 118, 190) and is associated with poor intestinal calcium absorption, hypocalcemia, and a resultant secondary hyperparathyroidism. Bone disease ultimately makes its appearance both in the form of osteomalacia and as osteitis fibrosa cystica. It appears logical that the defect in calcium metabolism associated with this disease results from a failure of the kidney to synthesize 1,25-(OH)$_2$D$_3$ and/or other metabolites of kidney origin. Without these metabolites the intestine cannot absorb calcium, resulting in a hypocalcemia and secondary hyperparathyroidism. Likely either the 1,25-(OH)$_2$D$_3$ or a close analog would be extremely useful in the treatment of the defect in calcium metabolism associated with chronic renal disease.

1,25-(OH)$_2$D$_3$ showed little activity when given as a single dose 7 days prior to examination of calcification by the antirachitic or calcification line test assay (151; Y. Tanaka, H. Frank, and H. F. DeLuca, unpublished observations). Furthermore, when it is given in oral doses of 1 unit/day in oil for up to 7 days, little calcification occurs. It was even more suprising that daily oral doses up to

TABLE 1. *Comparative effectiveness of chronically administered 1,25-(OH)$_2$D$_3$ by intrajugular injection or stomach tube in rats fed low-calcium diets*

Compound Given	Method of Administration	Serum Calcium, mg/100 ml
Oil vehicle	Stomach tube	4.3 ± 0.1
Ethanol vehicle	Intravenous	4.5 ± 0.2
25-OHD$_3$ in oil	Stomach tube	6.4 ± 0.3
1,25-(OH)$_2$D$_3$ in oil	Stomach tube	4.8 ± 0.1
1,25-(OH)$_2$D$_3$ in ethanol	Intravenous	8.1 ± 0.2

Male weanling rats were fed a low-calcium (0.02%) diet for 2 weeks. One group of rats was then given 325 pmoles of 1,25-(OH)$_2$D$_3$ intravenously while another group received .325 pmoles of either 25-OHD$_3$ or 1,25-(OH)$_2$D$_3$ in 0.1 ml Wesson oil by stomach tube daily for 5 days. Individual values represent the average from 5 rats ± SD 24 hr after administration.

325 pmoles of 1,25-(OH)$_2$D$_3$ failed to maintain plasma calcium concentrations of rats on a low-calcium diet, while the same dose of 25-OHD$_3$ given in the same way was extremely effective. The results were confusing since a single pulse dose of 1,25-(OH)$_2$D$_3$ given intravenously or orally was effective in the elevation of plasma calcium concentration. The results shown in Table 1 illustrate that if the 1,25-(OH)$_2$D$_3$ is given daily as an intravenous dose instead of an oral dose it is at least as effective as 25-OHD$_3$ in the maintenance of serum calcium concentration of rats on a low-calcium diet. Furthermore daily intravenous doses of 1,25-(OH)$_2$D$_3$ are approximately 2–5 times more effective than even 25-OHD$_3$ in supporting calcification of bone and in the elevation of plasma phosphate of rats on a low-phosphorus diet (Y. Tanaka, H. Frank, and H. F. DeLuca, unpublished observations). At least in rats it is evident that 1,25-(OH)$_2$D$_3$ must be given parenterally and chronically to be continuously effective.

When given orally in oil to vitamin D-deficient rats, the 1,25-(OH)$_2$D$_3$ appears to be effective as an initial dose but progressively loses its effectiveness until it becomes essentially ineffective. When animals deficient in vitamin D are given 1,25-(OH)$_2$D$_3$ orally, they have an elevated rate of metabolism of 1,25-(OH)$_2$D$_3$ relative to animals maintained in a deficient state. Such results suggest that 1,25-(OH)$_2$D$_3$ in the intestine induces enzymes that carry out its destruction. This represents an important new aspect of the control of vitamin D action in the intestine and possibly bone. As shown in later sections, the synthesis of 1,25-(OH)$_2$D$_3$ is strongly controlled at least in part by the parathyroid hormone. This control would be ineffective if 1,25-(OH)$_2$D$_3$ were not turned over rapidly. The exact nature of the reactions effecting the degradation of 1,25-(OH)$_2$D$_3$ in intestine is currently under investigation. These findings nevertheless reveal that in rats and probably in man 1,25-(OH)$_2$D$_3$ will be more effective when administered parenterally rather than orally.

D. Other Vitamin D Metabolites

Two other metabolites of vitamin D$_3$ have been isolated and identified. They are the 24,25-dihydroxyvitamin D$_3$ (24,25-(OH)$_2$D$_3$) and 25,26-dihydroxy-

vitamin D_3 (25,26-$(OH)_2D_3$) derivatives. What was originally believed to be the 21,25-dihydroxyvitamin D_3 (21,25-$(OH)_2D_3$) (196) has, on more complete characterization, proved to be the 24,25-$(OH)_2D_3$. This metabolite is synthesized by the kidney, especially in animals on high-calcium or -strontium diets and with normal amounts of vitamin D. It also represents the metabolite formed under circumstances where the 1,25-$(OH)_2D_3$ is not synthesized (151). 24,25-$(OH)_2D_3$ is synthesized in kidney mitochondria by a reduced pyridine nucleotide-dependent hydroxylase that has not been thoroughly studied (J. Knutson and H. F. DeLuca, unpublished observations). Originally it was shown to have preferential activity in increasing bone calcium mobilization. It has slight activity in increasing active intestinal calcium transport. Both of these measurements were performed in completely vitamin D-deficient animals that were hence devoid of any residual 1,25-$(OH)_2D_3$ or 25-OHD_3. Interestingly the 24,25-$(OH)_2D_3$ is the second most abundant vitamin D metabolite found in the extracellular fluid and tissues of animals under conditions of normal dietary intakes and vitamin D supplementation (J. T. Boyle, R. W. Gray, and H. F. DeLuca, unpublished observations).

The 25,26-$(OH)_2D_3$ was isolated from the plasma of pigs given high doses of vitamin D_3 (197). Its presence, however, can be demonstrated in the plasma of chicks and rats given physiologic doses of the vitamin, although its biological role and site of formation are presently unknown. It has little biological activity either in the calcification of bone or in the mobilization of calcium from bone. However, it does have significant biological activity in intestinal calcium absorption. Because other sterols are known to be hydroxylated in this fashion prior to cleavage and elimination, it is possible that this reaction is simply one that brings about destruction and elimination of vitamin D_3.

After the isolation and identification of 25-OHD_3, the 25-hydroxyergocalciferol (25-OHD_2) was isolated from the plasma of pigs given large doses of vitamin D_2 and its structure was clearly identified (193). Apparently vitamin D_2 is metabolized, at least initially, very much as vitamin D_3 is metabolized. It is interesting that chicks and other birds (192, 210) as well as New World monkeys (97) respond poorly to vitamin D_2 in contrast to their response to vitamin D_3. On the other hand, man, rats, and other animals appear to utilize vitamin D_2 and vitamin D_3 equally well (184, 185). The phenomenon of differential response of chicks to vitamin D_2 and D_3 was first noted by Steenbock and his colleagues (192); it was Windell who used this as a basis for suggesting that there must be a vitamin D derived from the cholesterol series, a prediction later proved correct by the chemical work of Windaus and his collaborators (228). The efficacy ratio of 10:1 (D_3:D_2) in chicks appears to be correct for almost all parameters of antirachitic activity (28).

The reason for the poor response of chicks to vitamin D_2 is not yet known. With ^{14}C-labeled D_2 and tritium-labeled D_3, it was demonstrated that the vitamin D_2 is metabolized and excreted more rapidly than is the vitamin D_3 (98). The point of discrimination appears to be beyond the 25-hydroxylation stage because chicks form both 25-OHD_3 and 25-OHD_2; yet they show little response to 25-OHD_2, whereas 25-OHD_3 gives a very good response (48). The present hypothesis suggests that the 25-OHD_2 is rapidly metabolized and excreted in chicks, thus

#4376

reducing its effectiveness in initiating intestinal calcium absorption and bone calcium mobilization. However, a clear elucidation of the mechanism of discrimination must await the synthesis of additional radioactive vitamin D_2 and 25-OHD_2 for continued investigation.

E. Absorption, Storage, Transport, and Excretion of Vitamin D

Little is known concerning intestinal absorption of vitamin D although early work of Heymann (85, 86) and Greaves and Schmidt (67), which was later confirmed by Schachter et al. (179), demonstrated the need for bile salts in the absorption of vitamin D. It also is not clear where the bulk of vitamin D is absorbed. Early work by Kodicek (110) suggested that the distal ileum is the site of vitamin D absorption, an observation supported by work at Wisconsin (146). Schachter and his colleagues, however, provided evidence that vitamin D is more rapidly absorbed in the upper duodenum. More recently another investigation indicated that vitamin D is absorbed throughout the small intestinal tract (173). Vitamin D may be esterified during its absorption; however, supporting evidence is meager.

Once vitamin D is absorbed, presumably through the lacteal system in association with chylomicrons, it becomes associated with a vitamin D-binding protein that migrates with the α-globulins (36, 169, 170, 206). Although some vitamin D is associated with the lipoproteins, most is bound to the transport protein that migrates with a α-globulins (169, 170). Recently this vitamin D carrier protein has been purified and found to have a molecular weight of 53,000. It has been partially characterized and shown to be present in blood at a concentration of 5 μg/ml (157). It seems likely that vitamin D or its metabolites in the blood are normally associated with a vitamin D transport protein that has a higher affinity for 25-OHD_2 than for the parent vitamin D_3. These relative affinities have been recognized in the development of a competitive binding assay for vitamin D and 25-OHD_3 (11). In addition a cytoplasmic binding protein from kidney tissue has been utilized by Haddad and his collaborators in the development of a competitive binding assay for 25-OHD$_3$ as well as vitamin D (68). These assay systems provide fruitful information for clinicians interested in measuring the levels of vitamin D and its metabolites in various disease states.

The primary excretory route for vitamin D appears to be the bile (85, 86, 134, 159). As much as 30% of a dose appears in the bile within 24–48 hr, whereas only 2% of the dose normally appears in the urine. No metabolites of vitamin D have been identified either from the bile or from the urine, so much remains to be learned concerning the intermediary metabolism of the D vitamins and their terminal excretion products.

A discussion of vitamin D would be incomplete without some mention of storage. It was shown by Heymann in 1937 (84) that as long as 6 months after a single dose of vitamin D is given to rats, detectable amounts of the vitamin still are present in the liver. It has been assumed primarily from the work of Heymann and later from the work of Kodicek (109, 110) that the liver is the primary site for this

prolonged storage of vitamin D within the body. Although this idea has been questioned it still remains popular. However, a recent study suggests that the primary storage site for vitamin D is not the liver but largely the fat depots throughout the body (172). Certainly vitamin D is distributed in many tissues, with skeleton and muscle accumulating much of the radioactive vitamin D (98, 134, 159). Rather than being stored in a specific site vitamin D appears in virtually every region of the body; it concentrates in the lipid-rich tissues and cell components.

F. Potential Use of Vitamin D Metabolites in Treatment of Bone Disease

Classical forms of vitamin D-resistant rickets must be considered when discussing vitamin D metabolism and function. Particularly interesting is the x-linked dominant familial hypophosphatemia (225, 229). This disease is characterized by low plasma phosphorus, high plasma alkaline phosphatase, normal plasma calcium, often severe bone disease, and a lack of growth. It has been believed for many years that the primary defect in this disease is a failure in the tubular reabsorption mechanisms for phosphate. Recent advances in our understanding of vitamin D metabolism have stimulated the thought that this disease may be due to a block in the functional metabolism of vitamin D. It is clear that familial hypophosphatemia does not result from a block in 25-hydroxylation, because 25-OHD$_3$, although better than vitamin D, is not always effective in the treatment of this disease and when it is, large amounts are required (49). The polar metabolites of 25-OHD$_3$, namely 1,25-(OH)$_2$D$_3$, 24,25-(OH)$_2$D$_3$, and 25,26-(OH)$_2$D$_3$, have not yet been tested in this disease. However, recent work with parathyroid hormone assays suggests that the circulating parathyroid hormone levels in this disease are either normal or low rather than elevated as might be expected if the disease resulted from a defect in vitamin D metabolism (3). Scriver and his collaborators (61) have suggested that there are two mechanisms for renal tubular reabsorption of phosphate and that one of these does not operate in this disease. Although new information has been obtained recently, it will not be clear whether this disease involves a defect in vitamin D metabolism until it can be successfully treated by physiological doses of a missing metabolite.

Another class of rickets known as vitamin D-dependency rickets or pseudovitamin D-deficiency disease was discovered by Prader and his colleagues in Switzerland (161). This disease is completely curable by the administration of large doses of vitamin D, approximately 100,000 units/day. This contrasts with familial hypophosphatemia, which is never completely cured regardless of the dose of vitamin D given. The vitamin D-dependency disease also does not respond to physiologic doses of 25-OHD$_3$ although 25-OHD$_3$ is 4 times more effective than vitamin D in its treatment. Nevertheless the true defect in this disease appears to be beyond the 25-hydroxylation stage of functional vitamin D metabolism.

It must be emphasized that the treatment of bone disease by the vitamin D metabolites has just begun. It appears that the use of 25-OHD$_3$ represents a marked advance over the use of vitamin D in the treatment of bone disease. The

4376

advantage of the 25-OHD$_3$ over vitamin D is that it is more effective on a weight basis, e.g. in the treatment of pseudo-vitamin D deficiency. Another advantage is that 25-OHD$_3$ can bypass the regulatory step in the liver that may represent an important point of variation among individuals and may account for a difference in sensitivity to the vitamin. Because 25-OHD$_3$ is turned over more rapidly than the parent vitamin, toxicity that may be encountered in its clinical use should be easily reversed by withdrawal of the metabolite. In contrast hypercalcemia due to vitamin D toxicity often requires corticosteroid treatment for the return to normal plasma calcium values, even after vitamin D has been withdrawn for weeks.

It is likely that the 1,25-(OH)$_2$D$_3$ or a close analog of it will be even more effective than 25-OHD$_3$ in specific diseases. Although the 1,25-(OH)$_2$D$_3$ and its analogs will be more costly than 25-OHD$_3$, it seems almost certain that a hydroxyl in the 1 position or in the geometric position normally occupied by the 1-hydroxyl of 1,25-(OH)$_2$D$_3$ should be therapeutically effective in chronic renal disease and hypoparathyroidism. They may also effect cures of familial hypophosphatemia and other vitamin D-resistant disease, but additional investigation is needed to answer these practical questions.

IV. BIOLOGICALLY ACTIVE ANALOGS OF VITAMIN D

A. *Dihydrotachysterol*$_3$

The dihydrotachysterols (Fig. 7) are well-known reduction products of the vitamin D series. An isomer of vitamin D, tachysterol, is reduced to dihydrotachysterol. During the course of reduction the C_{10-19} methylene function is reduced to a methyl group and ring A is rotated 180°. The hydroxyl on the 3 position now is in the general geometric area normally occupied by the 1-hydroxyl of 1,25-(OH)$_2$D$_3$ (70, 71). The dihydrotachysterols have very poor antirachitic activity, with about 1/450 the activity of vitamin D$_3$ (198). This poor activity may be related to the short duration of its action or because structural modification makes it a poor substitute for vitamin D. It is interesting that the dihydrotachysterols at high doses are much more effective in the elevation of plasma calcium concentration of either hypoparathyroid patients or thyroparathyroidectomized rats than the corresponding D vitamin (79, 168). In fact this action of dihydrotachysterol has prompted its wide use when large doses of vitamin D are required. This is exemplified by its use as a hypercalcemic agent in hypoparathyroidism (79), for treatment of the intestinal defect in renal osteodystrophy (102, 117), and as a therapeutic agent in vitamin D-resistant rickets.

The reason for the marked effectiveness of the dihydrotachysterols under conditions of high dosage has remained a mystery until recently. The 25-hydroxydihydrotachysterol$_3$ (25-OHDHT$_3$) has been chemically synthesized and tested for biological activity (198). It proved to be more effective than dihydrotachysterol$_3$ in all respects, including calcification of bone, mobilization of calcium from bone in vivo, and mobilization of calcium from bone in organ cultures (208). In the last system the 25-OHDHT$_3$ was approximately as effective as 25-OHD$_3$, whereas

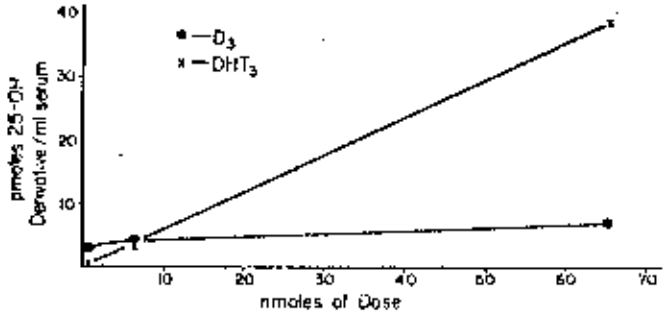


FIG. 11. Appearance of 25-OHD$_3$ and 25-OHDHT$_3$ in plasma of rats given either 1,2-[^{3}H]vitamin D$_3$ or 1,2[^{3}H]dihydrotachysterol$_3$. Rats were dosed intravenously with 0.65, 6.5, or 65 nmoles of either [^{3}H]vitamin D$_3$ (D$_3$) or [^{3}H]dihydrotachysterol$_3$ (DHT$_3$) and killed 1 hr later. Serum from each rat was analyzed for 25-hydroxy derivative with Sephadex LH-20 chromatography (88) (1:1 $CHCl_3$:Skellysolve B).

dihydrotachysterol$_3$ itself was essentially without effect. More recently it has been possible to synthesize radioactive dihydrotachysterols chemically with tritium in positions 1 and 2 (70); furthermore it has been possible to synthesize 25-OHDHT$_3$ labeled in positions 26 and 27 (R. B. Hallick and H. F. DeLuca, unpublished observations). With [^{3}H]dihydrotachysterols it has been shown that hydroxylation of dihydrotachysterol occurs at the 25 position by a mechanism that apparently is not feedback controlled (M. Bhattacharyya and H. F. DeLuca, unpublished observations). This contrasts markedly with the regulated 25-hydroxylation of vitamin D. The data shown in Figure 11 demonstrate that the 25-OHDHT$_3$ appears in the blood as a direct function of the administered dose of dihydrotachysterol$_3$. In contrast the rate of appearance of 25-OHD$_3$ levels off at fairly low doses of vitamin D$_3$ and does not build up as a function of dose of the original vitamin (M. Bhattacharyya and H. F. DeLuca, unpublished observations). It therefore appears that the dihydrotachysterol is hydroxylated completely to the 25-OHDHT$_3$ regardless of dose size and is not subject to feedback control. In this way the dihydrotachysterol can bypass a metabolic control step to which vitamin D$_3$ is subjected. In continued work it has been demonstrated that 25-OHDHT$_3$ is probably the metabolically active form of dihydrotachysterol$_3$ in both intestine and bone (71).

Both dihydrotachysterol$_3$ and 25-OHDHT$_3$ are effective in stimulating intestinal calcium absorption and bone calcium mobilization in nephrectomized animals (71). These interesting experiments demonstrated that if the 25-OHDHT$_3$ must be metabolized further before it is effective it does not require function of the kidney. Thus it seems unlikely that 1-hydroxylation of the dihydrotachysterols occurs. This is understandable on a chemical basis if it is recognized that the 3-hydroxyl of dihydrotachysterol is close to the geometric position normally occupied by the 1-hydroxyl of 1,25-$(OH)_2D_3$ (70). Thus the dihydrotachysterol, by virtue of the position of its 3 hydroxyl, bypasses still another important metabolic control step of vitamin D, namely 1-hydroxylation in the kidney. It is therefore understandable that high doses of dihydrotachysterol are much more effective than vitamin D$_3$. Its poor antirachitic activity and its poor activity at low doses

4376

probably relates to the fact that the diene system of the dihydrotachysterol is not a very good substitute for the *cis*-triene structure of the D vitamins.

B. *1α-Hydroxyvitamin D_3 (1α-OHD_3), 5,6-Trans-25-Hydroxyvitamin D_3 (5,6-Trans-25-OHD_3), and 5,6-Trans-Vitamin D_3 (5,6-Trans-D_3)*

The work on dihydrotachysterol described above prompted us to examine analogs of vitamin D that would approach the 25-OHDHT$_3$ in chemical structure and in biological activity. The 5,6-*trans*-25-OHD_3 (91), the 5,6-*trans*-D_3 (89), and the 1α-OHD_3 (E. J. Semmler, M. F. Holick, H. K. Schnoes, and H. F. DeLuca, unpublished observations) have now been synthesized and their biological activity tested. It is not surprising that the 5,6-*trans*-25-OHD_3 is about as effective as the 25-OHDHT$_3$ in stimulating intestinal calcium transport of nephrectomized rats (91). The fact that the 5,6-*trans*-D_3 also functions in this capacity in nephrectomized rats would first suggest that the 5,6-*trans*-D_3 is hydroxylated in the 25 position. Although this possibility has not yet been excluded, present evidence suggests that the 5,6-*trans*-D_3 is effective without 25-hydroxylation. Note that 5,6-*trans*-25-OHD_3 has little or no effect in the mobilization of calcium from bone, whereas the 5,6 *trans*-D_3 is quite effective (89). This result has two important implications; first, it suggests that the 5,6-*trans*-25-OHD_3 may be the compound of choice in circumstances where intestinal calcium transport is to be stimulated without stimulation of bone calcium mobilization. Such a case would be renal osteodystrophy, and hence it seems possible that the 5,6-*trans*-25-OHD_3 may be the compound of choice in the treatment of the calcium defect associated with chronic renal disease. Second, it also demonstrates that the bone calcium mobilization system is different biochemically from the intestinal calcium transport system. This is also suggested from the fact that the 1,25-$(OH)_2D_3$-activated system for bone calcium mobilization can be completely inhibited by actinomycin D, whereas the intestinal calcium transport response to 1,25-$(OH)_2D_3$ is not actinomycin D sensitive, at least in rats (202). Finally, the 1α-OHD_3 is extremely effective in all systems, including bone calcium mobilization, intestinal calcium transport, and the calcification of bone. When given orally the 1α-OHD_3 is more active than 1,25-$(OH)_2D_3$ in curing rickets. Possibly the 1α-OHD_3 must undergo 25-hydroxylation before it acts, which would suggest that it may be subject to feedback regulation at that stage. The area of analogs of 1,25-$(OH)_2D_3$ is one of great potential and is currently the center of much activity. The promise of compounds with special biological properties in calcium and phosphorus metabolism appears great.

V. REGULATION OF VITAMIN D METABOLISM

A. *Control of 25-Hydroxylation*

Once it was known that the hydroxylation of vitamin D in the 25 position occurs in the liver (158, 160), it was possible to use homogenates of liver to study

the hydroxylation of vitamin D in vitro. These studies revealed that liver homogenates prepared from animals given a prior dose of vitamin D hydroxylate vitamin D in the 25 position very slowly at best (94; M. Bhattacharyya and H. F. DeLuca, unpublished observations). This "turning-off" mechanism is effective within 3 hr after the administration of vitamin D to animals low in vitamin D. Although the exact nature of the turning off is not presently understood, it can be demonstrated readily that 25-OHD_3 added in vitro to homogenates can inhibit hydroxylation of labeled vitamin D_3 (94). In addition it has been observed from in vivo studies that the elaboration of 25-OHD_3 in the blood of animals is not directly related to the dose of vitamin D_3 given (Fig. 11). At low vitamin D doses a high proportion of the vitamin appears in the blood as 25-OHD_3, whereas at high doses the proportion is very small. This contrasts with the lack of control discussed for the 25-hydroxylation of dihydrotachysterol. Whether the product inhibition by 25-OHD_3 turns off hydroxylation or whether there is some more elaborate regulation of the 25-hydroxylation system remains unknown. This bottleneck in vitamin D metabolism is undoubtedly important in: *1)* protecting against vitamin D toxicity, and *2)* conserving vitamin D supplies for periods when both the intake and formation of vitamin D via ultraviolet irradiation of skin are low.

B. Regulation of 25-OHD_3-1-Hydroxylase and 25-OHD_3-24-Hydroxylase

The renal mitochondrial hydroxylation systems may be the most elaborate part of the regulatory system involved with vitamin D metabolism. To date 25-OHD_3 metabolism appears to occur predominantly in the kidney, with the metabolite of major importance being 1,25-$(OH)_2D_3$. Since 1,25-$(OH)_2D_3$ is the metabolically active form of vitamin D in intestine, it was reasoned that animals could possibly control their rate of calcium absorption via modulation of 1,25-$(OH)_2D_3$ synthesis. It therefore was decided to see if the metabolism of ^{3}H-1,25-$(OH)_2D_3$ is altered in animals fed a low-calcium diet, because animals adapt to a low calcium intake by increasing their rate of intestinal calcium absorption (105, 144, 174). Boyle et al. (19, 20) were able to demonstrate that as dietary calcium is decreased the ability of animals to produce 1,25-$(OH)_2D_3$ increases. In contrast little 1,25-$(OH)_2D_3$ was produced in animals fed a high-calcium diet. This suppression of 1,25-$(OH)_2D_3$ synthesis appeared to require vitamin D, although the turn-off mechanism could be demonstrated in vitamin D-deficient animals fed a high-calcium diet (3%) that contained lactose. It is interesting that as the 1-hydroxylation system is suppressed there is a compensatory increase in the appearance of 24,25-$(OH)_2D_3$ (Fig. 12). The exact role of the 24,25-$(OH)_2D_3$ is not known, but its appearance in response to a normal or hypercalcemic situation suggests that it may have an important regulatory role (19, 20, 151). More important, the synthesis of 1,25-$(OH)_2D_3$ can be markedly regulated by dietary calcium (Fig. 12). Nicolaysen and coworkers (144) first suggested that there is an endogenous factor that directs the intestine to absorb calcium in response to body needs, and 1,25-$(OH)_2D_3$ may well represent all or part of Nicolaysen's endogenous factor. To evaluate this hypothesis it was determined whether animals maintained on 1,25-$(OH)_2D_3$ as their source of vitamin D would be able to adapt to dietary

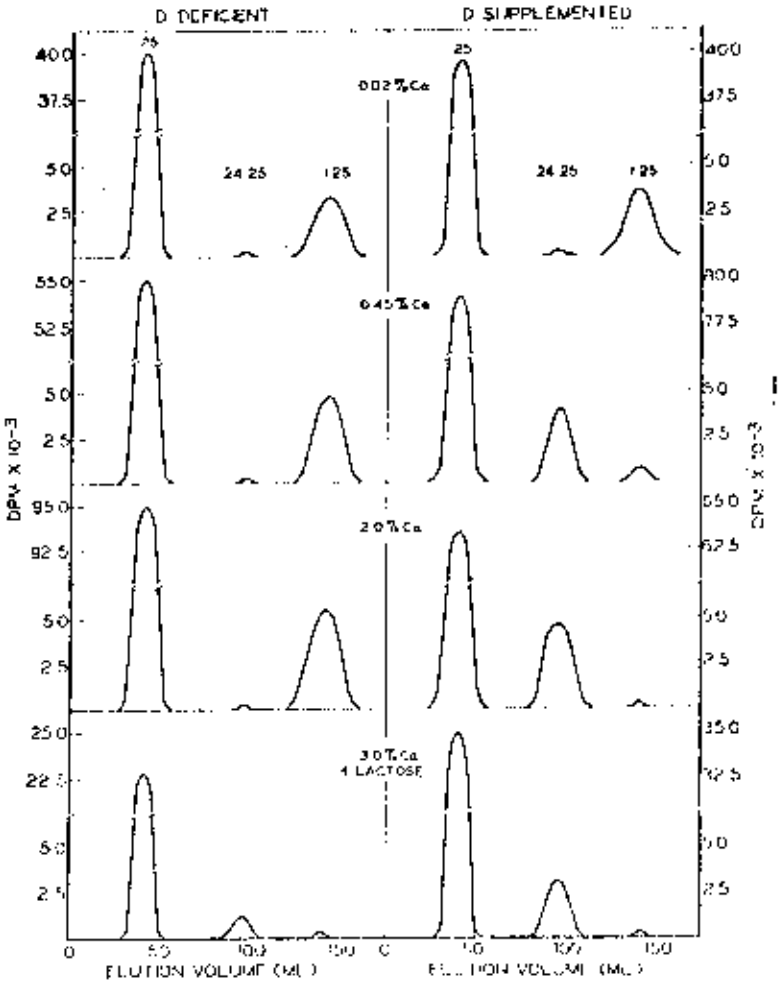


FIG. 12. Sephadex LH-20 chromatographic profiles of serum extracts from rats on diets varying in calcium content and given [^{3}H]25-(OH)D$_3$. Weanling rats received indicated diets for a total of 3 weeks, and half of each group was given a daily oral supplement of vitamin D (1 IU) for last 2 weeks of treatment. At 12 hr prior to sacrifice all rats received 325 pmoles [^{3}H]25-(OH)D$_3$. Harvested plasma was extracted and chromatographed on Sephadex LH-20 in 65:35 chloroform:petroleum ether (88). Pooled samples were used for all groups except 3% calcium + lactose, which accounts for lower recovered radioactivity in this group. [From Boyle et al. (19).]

calcium in terms of intestinal calcium absorption. The results shown in Figure 2 illustrate that chicks maintained on 1,25-(OH)$_2$D$_3$ have a high rate of intestinal calcium absorption unaffected by dietary calcium levels. It therefore seems likely that 1,25-(OH)$_2$D$_3$ represents at least a portion of Nicolaysen's endogenous factor.

With the knowledge that 25-(OH)D$_3$ metabolism is altered by serum calcium (Fig. 13) the next question was, what is the mechanism of the modulation? Two possibilities were initially considered: *1)* direct action of plasma calcium on the kidney, and *2)* release of a humoral factor(s) in response to the plasma calcium levels, which in turn acted on the kidney.

Addition of calcium to mitochondrial suspensions from chick kidneys does not reduce appreciably the rate of 1-hydroxylation of 25-OHD$_3$ unless unreasonably large (200 mM) amounts of calcium are added; these excessive concentrations uncouple oxidative phosphorylation and inhibit respiration. Production of 1,25-(OH)$_2$D$_3$ in chick kidney slices is likewise refractory to added calcium. Furthermore when animals are shifted from a low-calcium to a high-calcium diet there is a considerable time lag (days) before the 1-hydroxylation system is turned off and 24-hydroxylation is turned on (I. T. Boyle, R. W. Gray, and H. F. DeLuca, unpublished observations), despite the fact that plasma calcium levels respond rapidly (within 24 hr) to the dietary shift. These results suggest that the regulation cannot be due to serum calcium concentration per se, and therefore some humoral factor sensitive to plasma calcium levels probably is the effector.

Because the parathyroid glands are sensitive to plasma calcium levels, it appeared possible that the parathyroid hormone might be the active humoral agent. Experiments therefore were designed to test this possibility (59). Removal of the thyroparathyroid glands is accompanied by virtual disappearance of the 1-hydroxylase system and the appearance of the 24-hydroxylase system (Fig. 14). Administration of parathyroid hormone every 6 hr to the parathyroidectomized animals restores the 1-hydroxylation to preoperative levels within 24 hr. It therefore is clear that the parathyroid glands sense the serum calcium concentration and respond by secreting parathyroid hormone, which in some unknown way regulates or stimulates the production of the 1-hydroxylase enzyme and decreases production of the 24-hydroxylase system (Fig. 14). Apparently the parathyroid hormone acts as a tropic hormone for the 1,25-(OH)$_2$D$_3$ hormone, which in turn is responsible for intestinal calcium absorption and bone calcium mobilization.

In addition to parathyroid hormone regulation, another factor is involved in control of calcium metabolism, because rats on a high-calcium, low-phosphorus diet will make 1,25-(OH)$_2$D$_3$. Since rats under these circumstances have a high plasma calcium concentration, the plasma parathyroid hormone levels should be low. Exactly how these results can be reconciled with the parathyroid hormone data is unclear, and the problem requires additional investigation.

The feedback-regulation loop is illustrated in Figure 15, which shows that the 25-hydroxylation is regulated by an incompletely understood mechanism and

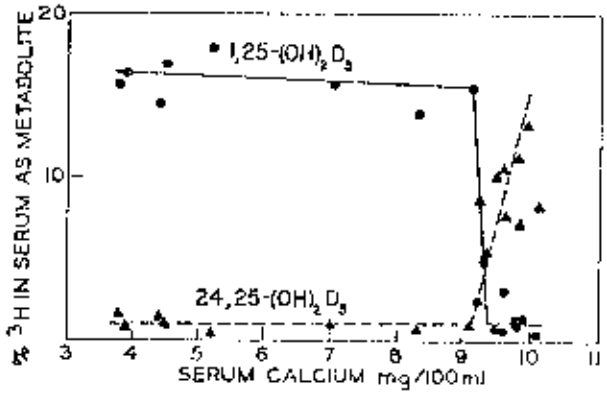


FIG. 13. Relationship between serum calcium at time of injection of 325 pmoles [^{3}H]25-OHD$_3$ and relative amounts of 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ present in serum 12 hr later. Both vitamin D-supplemented and deficient animals were used in order to obtain various serum calcium concentrations.

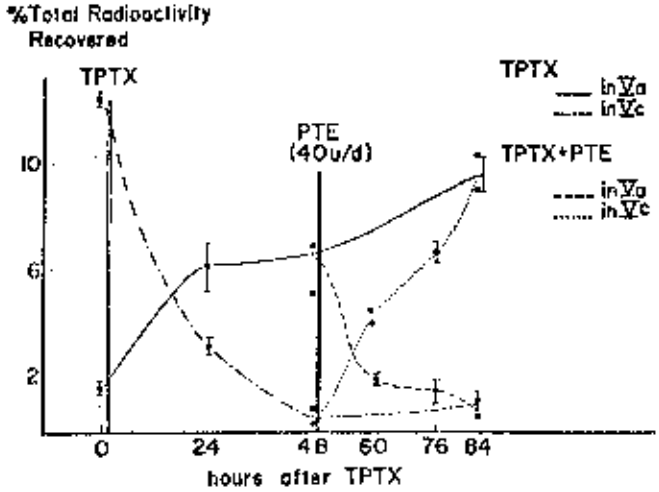


FIG. 14. Time course of change in production of two polar metabolites of 25-OHD_3 as a result of TPTX and PTE treatment. Rats were fed a vitamin D-deficient low-calcium (0.02%) diet and received 1,25-$(OH)_2D_3$ supplements (325 pmoles each) at 48, 24, and 4 hr before surgical removal of thyroid and parathyroid glands (TPTX). Both sham controls and TPTX rats received 325 pmoles of 1,25-$(OH)_2D_3$ at 24-hr intervals after surgery. Parathyroid extract (PTE 10 units) was given every 6 hr beginning 48 hr postsurgery. [From Garabedian et al. (59).]

that the 1-hydroxylation regulation is effected by the parathyroid hormone and perhaps an additional factor such as cellular phosphate concentration.

The fact that the parathyroid hormone is involved in the regulation of 1,25-$(OH)_2D_3$ synthesis raises a number of interesting clinical possibilities regarding hypoparathyroidism. It has long been known that hypoparathyroid patients require large doses of vitamin D for the maintenance of plasma calcium concentration. In addition vitamin D-resistant hypoparathyroidism has been reported as an unusual abnormality (156). It is indeed possible that without parathyroid hormone the normal synthesis of 1,25-$(OH)_2D_3$ cannot occur. It is not surprising then that hypoparathyroidism is characterized by a resistance to vitamin D. Obviously these diseases would best be treated with 1,25-$(OH)_2D_3$ or a close analog.

Perhaps the division of roles between parathyroid hormone and 1,25-$(OH)_2D_3$ in calcium homeostasis poses a question of even greater significance. It has been supposed that parathyroid hormone by itself is the major agent in the regulation of plasma calcium concentration. The question now can be legitimately asked whether the systemic maintenance of plasma calcium concentration could be controlled largely by 1,25-$(OH)_2D_3$ and whether a major role of the parathyroid is in the stimulation of synthesis of the 1,25-$(OH)_2D_3$. The exact role of 1,25-$(OH)_2D_3$ in the maintenance of serum calcium in the parathyroidectomized animal is a question of considerable importance currently under examination. In any case it is newly recognized that the kidney has a regulatory function in calcium homeostasis, a function that involves it as an endocrine organ secreting

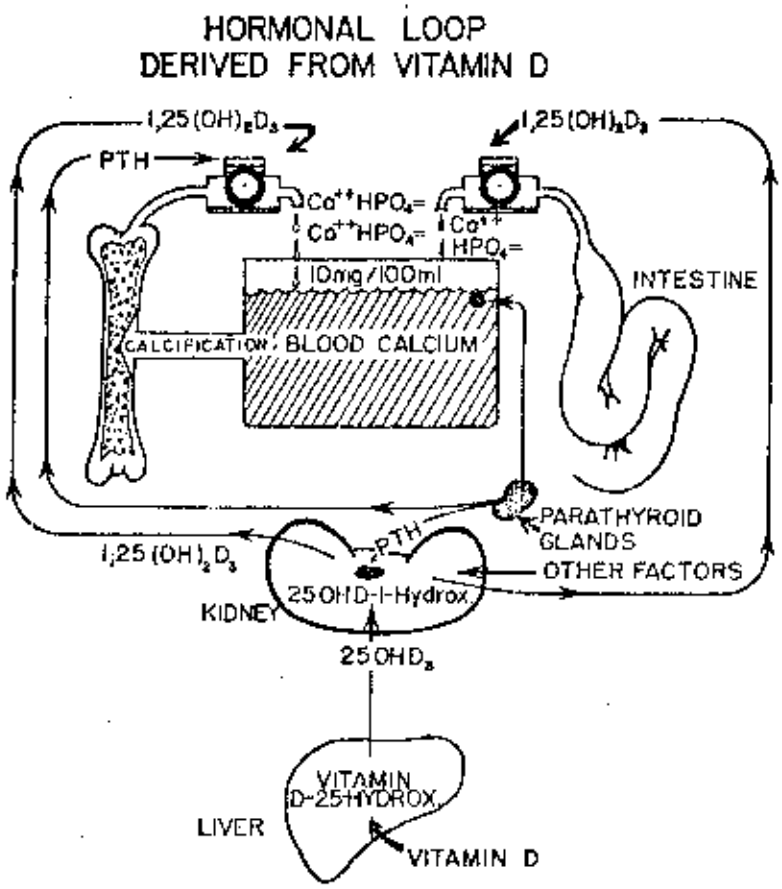


FIG. 15. Schematic designation of hormonal control loop for vitamin D metabolism and function. A drop below set point for serum calcium of 10 mg/100 ml prompts a proportional secretion of parathyroid hormone that acts to increase bone resorption and thus elevates serum calcium. Parathyroid hormone also directs metabolism of 25-OHD$_3$ to 1,25-(OH)$_2$D$_3$ in kidney, where "hormone" 1,25-(OH)$_2$D$_3$ acts both on bone and intestine to mobilize calcium from bone and intestinal contents. As serum calcium increases toward its set point, parathyroid hormone secretion is proportionately decreased.

the metabolically active or hormonal form of vitamin D in response to parathyroid secretion.

C. Role of DNA Transcription and Protein Synthesis

In 1965 it was demonstrated that actinomycin D and other RNA and protein synthesis inhibitors could prevent the well-known functions of vitamin D in the mobilization of calcium from bone and in the stimulation of intestinal calcium transport (230, 231). At the same time Norman (145) reported that actinomycin inhibits vitamin D stimulation of calcium absorption in chicks. At that time the results were considered to indicate that the expression of vitamin D action in the

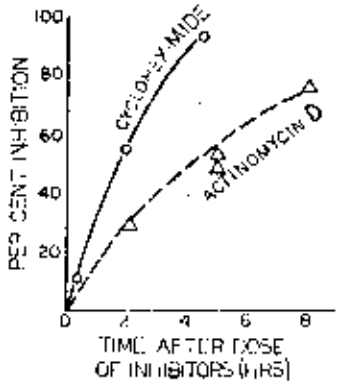


FIG. 16. Diminution of 25-OHD_3-1-hydroxylase of chick kidney after either actinomycin D or cycloheximide administration. Chicks were fed a vitamin D-deficient diet for 3–4 weeks prior to receiving either cycloheximide (5 μg/g body wt) or actinomycin D (1 μg/g body wt) by intravenous injection (0.05 ml). Animals were killed at specified time intervals, kidney homogenates were prepared, and in vitro production of 1,25-$(OH)_2D_3$ was assayed. Results are expressed as percent inhibition of 1,25-$(OH)_2D_3$ production for control tissue.

intestine and in the bone must involve transcription of DNA and protein synthesis. Recently it has been demonstrated that actinomycin D given 2–5 hr before tritiated 25-OHD_3 will prevent the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ (65, 201). Furthermore it was possible to demonstrate in the presence of actinomycin D that 1,25-$(OH)_2D_3$ and not 25-OHD_3 could stimulate intestinal calcium transport in the rat (202). These results suggested that the intestinal calcium transport response to 1,25-$(OH)_2D_3$ does not involve transcription of DNA and may not involve protein synthesis. It also provided additional evidence that it is 1,25-$(OH)_2D_3$ and not 25-OHD_3 that must be considered the metabolically active form of vitamin D in the intestine. It was surprising that actinomycin D could prevent the conversion of 25-OHD_3 to the 1,25-$(OH)_2D_3$ derivative, because in extremely vitamin D-deficient chickens it is possible to demonstrate in vitro a high degree of 1-hydroxylation of 25-OHD_3 (56, 66). Clearly the enzyme is present in deficient animals and does not require induction. These results demanded a more thorough examination of the question of actinomycin D inhibition of 25-OHD_3 metabolism in the kidney. When actinomycin D is given to chicks 5–8 hr before the kidneys are removed, there is a marked diminution of 25-OHD_3-1-hydroxylase activity as measured in vitro (Fig. 16). If cycloheximide is given 5 hr before enzymatic activity is measured in vitro, again a diminution of enzymatic activity to almost negligible levels can be demonstrated (Y. Tanaka and H. F. DeLuca, unpublished observations). These results suggest that the 1-hydroxylase system is turned over very rapidly, with the enzyme having a half-life of about 2.5 hr and its messenger having a half-life of approximately 6 hr. The action of actinomycin D in blocking the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ does not indicate an induction, but rather indicates that the maintenance of the 1-hydroxylase activity requires frequent transcription and protein synthesis. Such a mechanism seems reasonable for a highly regulated enzymatic system. This rapidly turning-over system would then be more sensitive to changes in hormone levels or cellular phosphate levels and could thus be considered in all respects a system under tight hormonal regulation.

D. Vitamin D as a Prohormone

The mode of action of vitamin D is unique among the vitamins. Under normal circumstances it is reasonable to expect that vitamin D is not required in the diet since adequate ultraviolet irradiation of skin will produce sufficient vitamin D. In that respect vitamin D should not be considered a normal dietary requirement but instead a prohormone, one that is metabolized first in the liver to the 25-OHD_3 derivative and proceeds to the kidney, where it is converted to either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, depending on the systems in control. Unfortunately little is presently known about 24,25-$(OH)_2D_3$ except that its production is controlled, which suggests a functional role for the metabolite. In contrast a great deal more is known about the 1,25-$(OH)_2D_3$ metabolite and its mode of action. The conversion of 25-OHD_3 to the 1,25-$(OH)_2D_3$ derivative is stimulated by the parathyroid hormone and low cellular phosphate and thus the parathyroid hormone can be considered as a tropic hormone for the "secretion" of 1,25-$(OH)_2D_3$. The 1,25-$(OH)_2D_3$ is then responsible for the initiation of intestinal calcium transport by a mechanism that apparently does not involve transcription of DNA. It also is involved in the initiation of calcium mobilization from bone by a process sensitive to actinomycin D. The 1,25-$(OH)_2D_3$ may induce the formation of enzymes that bring about its rapid further metabolism and hence both its action and its lifetime are short. The enzyme responsible for its synthesis is rapidly turned over, as is perhaps the messenger for the enzyme. For this reason the 1-hydroxylase system may be sensitive to actinomycin D. The 1,25-$(OH)_2D_3$ brings about an elevation of plasma calcium concentration from both the bone and intestine that is immediately sensed by the parathyroid glands; the parathyroid in turn regulates its secretion of parathyroid hormone. An elevation in parathyroid hormone secretion enhances the 1-hydroxylase enzyme activity in the kidney. When the secretion of the parathyroid hormone is shut off, the 1-hydroxylase activity will decrease to negligible levels.

Although much remains to be learned regarding metabolism and the mechanism of action of vitamin D, progress in recent years has been rapid. The identification of active metabolites of vitamin D and the demonstration of the renal endocrine system, which uses as its prohormone 25-OHD_3 and the parathyroid hormone as its tropin, have placed before us many new possibilities in our understanding of calcium homeostasis and the treatment of metabolic bone diseases.

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REFERENCES

1. ADAMS, T. H., AND A. W. NORMAN. Studies on the mechanism of action of calciferol. I. Basic parameters of vitamin D-mediated calcium transport. *J. Biol. Chem.* 245: 4421–4431, 1970.
2. ADAMS, T. H., R. G. WONG, AND A. W. NORMAN. Studies on the mechanism of action of calciferol. II. Effects of the polyene antibiotic, filipin, on vitamin D-mediated calcium transport. *J. Biol. Chem.* 245: 4432–4442, 1970.
3. ARNAUD, C., T. GLORIEUX, AND G. SCRIVER.

4376

Serum parathyroid hormone in x-linked hypophosphatemia. *Science* 173: 844–847, 1971.

4. ARNAUD, C., H. RASMUSSEN, AND C. ANAST. Further studies on the interrelationship between parathyroid hormone and vitamin D. *J. Clin. Invest.* 45: 1955–1964, 1966.

5. ASKEW, F. A., R. B. BOURDILLON, H. M. BRUCE, R. G. C. JENKINS, AND T. A. WEBSTER. The distillation of vitamin D. *Proc. Roy. Soc. (London), Ser. B* 107: 76–90, 1931.

6. AU, W. Y. W., AND D. G. RAISZ. Restoration of parathyroid responsiveness in vitamin D-deficient rats by parenteral calcium or dietary lactose. *J. Clin. Invest.* 46: 1572–1576, 1967.

7. AVIOLI, L. V., S. J. BURGE, AND S. W. TEE. Effects of prednisone on vitamin D metabolism in man. *J. Clin. Endocrinol.* 29: 1541–1546, 1969.

8. BALSAN, S., AND M. GARABEDIAN. 25-Hydroxycholecalciferol. A comparative study in deficiency rickets and different types of resistant rickets. *J. Clin. Invest.* 51: 749–759, 1972.

9. BAUER, G. C. H., A. CARLSSON, AND B. LINDQUIST. Evaluation of accretion, resorption, and exchange reactions in the skeleton. *Kungl. Fysiograf. Sällskapets I Lund Förhandl.* 25: 3–18, 1955.

10. BAYLINK, D., M. STAUFFER, J. WERGEDAL, AND G. RICH. Formation, mineralization, and resorption of bone in vitamin D-deficient rats. *J. Clin. Invest.* 49: 1122–1134, 1970.

11. BELSEY, R., H. F. DeLUCA, AND J. T. POTTS, Jr. Competitive binding assay for vitamin D and 25-OH vitamin D. *J. Clin. Endocrinol. Metab.* 33: 554–557, 1971.

12. BERG, G. G., AND J. SZIRKOCZIS. The sodium and potassium activated ATPase. II. Comparative study of intestinal epithelium and red cells. *J. Cell Physiol.* 67: 487–500, 1966.

13. BIRD, P. C., G. M. A. PALMIERI, AND I. R. ELLIS. Modification by cortisone of the plasma Ca and P responses to ionophore and theophylline. *Endocrinology* 88: 1267–1273, 1971.

14. BLUNT, J. W., AND H. F. DeLUCA. The synthesis of 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. *Biochemistry* 8: 671–675, 1969.

15. BLUNT, J. W., H. F. DeLUCA, AND H. K. SCHNOES. 25-Hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. *Biochemistry* 7: 3317–3322, 1968.

16. BLUNT, J. W., Y. TANAKA, AND H. F. DeLUCA. The biological activity of 25-hydroxycholecalciferol, a metabolite of vitamin D_3. *Proc. Natl. Acad. Sci. US* 61: 1503–1509, 1968.

17. BORDIER, P., J. MIRAVET, S. TUN CHOT, AND D. HIOCO. Evidences of a direct effect of vitamin D_3 or 25-hydroxycholecalciferol upon human adult bone mineralization. In: *Calcium Metabolism for Calcium Transfer and Homeostasis*, edited by G. Nichols, Jr. and R. H. Wasserman. New York: Academic, 1971, p. 409–410.

18. BOSMANN, H. B., AND P. S. CHEN, Jr. Comparative effects of dihydrotachysterols and dihydrotachysterols in chicks. *J. Nutr.* 90: 141–147, 1966.

19. BOYLE, I. T., R. W. GRAY, AND H. F. DeLUCA. Regulation by calcium of *in vivo* synthesis of 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol. *Proc. Natl. Acad. Sci. US* 68: 2131–2134, 1971.

20. BOYLE, I. T., R. W. GRAY, J. L. OMDAHL, AND H. F. DeLUCA. Calcium control of the *in vivo* biosynthesis of 1,25-dihydroxyvitamin D_3: Nicolaysen's endogenous factor. In: *Proc. Intern. Symp. Endocrinol., 3d, 1971*, edited by S. Taylor. London: Heinemann, 1972, p. 469–476.

21. BOYLE, I. T., J. MIRAVET, R. W. GRAY, M. F. HOLICK, AND H. F. DeLUCA. The response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxy vitamin D in nephrectomized rats. *Endocrinology* 90: 605–608, 1972.

22. BRODEHL, J., W. P. KAAS, AND H. P. WEBER. Vitamin D deficiency rickets: renal handling of phosphate and free amino acids. *Pediat. Res.* 5: 591–604, 1971.

23. BRONNER, F., AND V. T. MADDAIAH. Comment. In: *Symposium on Membrane Proteins*. New York: Little, Brown, 1969, p. 133–136.

24. CARLSSON, A. Tracer experiments on the effect of vitamin D on skeletal metabolism of calcium and phosphorus. *Acta Physiol. Scand.* 26: 212–222, 1952.

25. CARLSSON, A., AND B. LINDQUIST. A comparative study of the mode of action of dihydrotachysterol and vitamin D on the calcium metabolism. *Acta Endocr. Stockholm* 44: 548–558, 1963.

26. CHASE, L. R., AND G. D. AURBACH. Parathyroid function and the renal excretion of 3′,5′-adenylic acid. *Proc. Natl. Acad. Sci. US* 58: 568–575, 1967.

27. CHASE, L. R., AND G. D. AURBACH. Renal adenyl cyclase: anatomically separate sites for parathyroid hormone and vasopressin. *Science* 159: 545–547, 1968.

28. CHEN, P. S., Jr., AND H. B. BOSMANN. Effect of vitamin D_3 and D_4 in serum calcium and phosphorus in rachitic chicks. *J. Nutr.* 83: 135–139, 1964.

29. CLARK, R. L., J. P. KUHN, AND C. A. DUJOVNE. Absence of rickets after chronic dilantin administration: experimental radiological observations in rats. *Invest. Radiol.* 6: 152–154, 1971.

30. CORRADINO, R. A., J. G. EBLE, P. H. CRAIG, A. N. TAYLOR, AND R. H. WASSERMAN. Calcium absorption and the vitamin D_3-dependent calcium-binding protein. I. Inhibition by dietary stimulation. *Calcified Tissue Res.* 7: 81–92, 1971.

31. COUSINS, R. J., H. F. DeLUCA, AND R. W. GRAY. Metabolism of 25-hydroxycholecalciferol in intact and nephrectomized rats. *Biochemistry* 9: 3649–3652, 1970.

32. COUSINS, R. J., H. F. DeLUCA, T. SUDA, T. UHEN, AND Y. TANAKA. Metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa. *Biochemistry* 9: 1449–1451, 1970.

33. CRUICKSHANK, E. M., AND E. KODICEK. Vitamin D balance and distribution in rats given a massive dose of ergocalciferol. *Biochem. J.* 54: 337–343, 1953.

34. CRUICKSHANK, E. M., E. KODICEK, AND P. ARMITAGE. The vitamin D content of tissues of rats given ergocalciferol. *Biochem. J.* 58: 177–175, 1954.

35. CZAKY, T. Z., AND Y. HARA. Inhibition of active intestinal sugar transport by digitalis. *Am. J. Physiol.* 209: 467–472, 1965.

36. DEGROUSAZ, P., B. BLANC, AND J. ANTENER. Vitamin D activity in normal human serum and serum proteins. *Helv. Odontol. Acta* 9: 151–156, 1965.

37. DeLUCA, H. F. Mechanism of action and metabolic

fate of vitamin D. *Vitamins Hormones* 25: 315–367, 1967.

38. DeLUCA, H. F. Recent advances in the metabolism and function of vitamin D. *Federation Proc.* 28: 1678–1689, 1969.

39. DeLUCA, H. F. 1,25-Dihydroxycholecalciferol: isolation, identification, regulation and mechanism of action. In: *Proc. Intern. Symp. Endocrinol., 3rd, 1971*, edited by S. Taylor. London: Heinemann, 1972, p. 452–467.

40. DeLUCA, H. F. Regulation of vitamin D metabolism: a new dimension in calcium homeostasis. *Biochem. J.* In press.

41. DeLUCA, H. F., H. MORII, AND M. J. MELLANCON, JR. The interaction of vitamin D, parathyroid hormone and thyrocalcitonin. In: *Parathyroid Hormone and Thyrocalcitonin (Calcitonin)*, edited by R. V. Talmage and L. F. Belanger. Amsterdam: Excerpta Med. Found., 1968, p. 448–451.

42. DeLUCA, H. F., AND J. W. SUTTIE (editors). *The Fat-Soluble Vitamins*. Madison: Univ. of Wisconsin Press, 1970.

43. DeLUCA, H. F., M. WILLER, J. W. BLUNT, AND P. P. GEVILL. Synthesis, biological activity, and metabolism of 22,23-H-vitamin D₃. *Arch. Biochem. Biophys.* 121: 122–128, 1968.

44. DENT, C. E., A. RICHENS, D. J. F. ROWE, AND T. C. B. STAMP. Osteomalacia with long-term anticonvulsant therapy in epilepsy. *Brit. Med. J.* No. 4: 69–72, 1970.

45. DIKSHIT, P. K., AND V. N. PATWARDHAN. Mode of action of vitamin D: its effect on the calcifying capacity of rachitic epiphyses. *Indian J. Med. Sci.* 6: 107–110, 1952.

46. DRESCHER, D., AND H. F. DeLUCA. Vitamin D stimulates calcium binding protein from rat intestinal mucosa. Purification and some properties. *Biochemistry* 10: 2302–2307, 1971.

47. DRESCHER, D., AND H. F. DeLUCA. Possible precursor of vitamin D stimulated calcium binding protein in vitro. *Biochemistry* 10: 2308–2312, 1971.

48. DRESCHER, D., H. F. DeLUCA, AND M. H. IMRIE. On the use of discrimination of chicks against vitamin D₃. *Arch. Biochem. Biophys.* 130: 657–661, 1969.

49. FARP, H. S., R. L. NEY, H. J. GITELMAN, R. RICHMAN, AND H. F. DeLUCA. Effects of 25-hydroxycholecalciferol in patients with familial hypophosphatemia and vitamin D-resistant rickets. *New Engl. J. Med.* 283: 627–630, 1970.

50. EBEL, J. G., A. N. TAYLOR, AND R. H. WASSERMAN. Vitamin D induced calcium binding protein of intestinal mucosa in relation to vitamin D dose level and lag period. *Am. J. Clin. Nutr.* 22: 431–436, 1969.

51. EILON, G., E. MOR, H. KARAMAN, AND J. MENGEL. Effect of cortisol on calcium-binding protein in rat duodenum. In: *Cellular Mechanisms for Calcium Transfer and Homeostasis*, edited by G. Nichols, Jr. and R. H. Wasserman. New York: Academic, 1971, p. 501–502.

52. FAIRBANKS, B. W., AND H. H. MITCHELL. The relation between calcium retention and the store of calcium in the body with particular reference to the determination of calcium requirements. *J. Nutr.* 11: 551–572, 1936.

53. FIESER, L. F., AND M. FIESER. Vitamin D. In: *Steroids*. New York: Reinhold, 1959, p. 160–168.

54. FLEISCH, H., R. G. G. RUSSELL, S. BISAZ, P. A. CASEY, AND R. G. MÜHLBAUER. The influence of pyrophosphate analogues (diphosphonates) on the precipitation and dissolution of calcium phosphate *in vitro* and *in vivo*. *Calcified Tissue Res.* 2, Suppl. 10: 1968.

55. FRASER, D. R., AND E. KODICEK. Vitamin D esters: their isolation and identification in rat tissues. *Biochem. J.* 96: 590–600, 1965.

56. FRASER, D. R., AND E. KODICEK. The synthesis of vitamin D esters in the rat. *Biochem. J.* 100: 670, 1966.

57. FRASER, D. R., AND E. KODICEK. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature* 228: 764–766, 1970.

58. FROLIK, C. A., AND H. F. DeLUCA. 1,25-Dihydroxycholecalciferol: the metabolite of vitamin D responsible for increased intestinal calcium transport. *Arch. Biochem. Biophys.* 147: 143–147, 1971.

59. GARABEDIAN, M., M. F. HOLICK, H. F. DeLUCA, AND I. T. BOYLE. Control of 25-hydroxycholecalciferol metabolism by the parathyroid glands. *Proc. Natl. Acad. Sci. USA* 69: 1673–1676, 1972.

60. GASSER, A. B., D. B. MORGAN, H. A. FLEISCH, AND L. J. RICHELLE. The influence of two diphosphonates on calcium metabolism in the rat. *Clin. Sci.* 43: 31–45, 1972.

61. GLORIEUX, F., AND C. R. SCRIVER. Loss of a parathyroid-sensitive component of phosphate transport in a clinical hypophosphatemia. *Science* 175: 997–1000, 1972.

62. GOLDHABER, P. Oxygen-dependent bone resorption in tissue culture. In: *The Parathyroids*, edited by R. O. Greep and R. V. Talmage. Springfield: Thomas, 1961, p. 243–254.

63. GOODMAN, D. B. P., M. R. HAUSSLER, AND H. RASMUSSEN. Vitamin D₃ induced alteration of mitochondrial membrane lipid composition. *Biochem. Biophys. Res. Commun.* 46: 40–46, 1972.

64. GRAN, F. C. The retention of parenterally injected calcium in rachitic dogs. *Acta Physiol. Scand.* 50: 152–170, 1960.

65. GRAY, R. W., AND H. F. DeLUCA. Metabolism of 25-hydroxycholecalciferol and its inhibition by actinomycin D and cycloheximide. *Arch. Biochem. Biophys.* 145: 276–282, 1971.

66. GRAY, R. J. BOYLE, AND H. F. DeLUCA. Vitamin D metabolism: the role of kidney tissue. *Science* 172: 1232–1234, 1971.

67. GREAVES, J. D., AND C. L. A. SCHMIDT. The role played by bile in the absorption of vitamin D in the rat. *J. Biol. Chem.* 192: 101–112, 1951.

68. HADDAD, J. G., JR., AND K. J. CHYU. 25-Hydroxycholecalciferol-binding globulin in human plasma. *Biochem. Biophys. Acta* 240: 471–481, 1971.

69. HAHN, J. J., S. J. BURGE, C. R. SCHARP, AND L. V. AVIOLI. Phenobarbital-induced alterations in vitamin D metabolism. *J. Clin. Invest.* 51: 741–748, 1972.

70. HALLICK, R. B., AND H. F. DeLUCA. 25-Hydroxydihydrotachysterol₃ biosynthesis *in vivo* and *in vitro*. *J. Biol. Chem.* 246: 5735–5738, 1971.

71. HALLICK, R. B., AND H. F. DeLUCA. Metabolites of dihydrotachysterol₃ in target tissues. *J. Biol. Chem.* 247: 91–97, 1972.

72. HAMILTON, J. W., AND E. S. HOLDSWORTH. The release of ^{45}Ca from mitochondria of chicken

intestinal mucosa by calcium binding protein. *Biochem. Biophys. Res. Commun.* 40: 1329–1330, 1970.

73. HARMEYER, J., AND H. F. DELUCA. Calcium-binding protein and calcium absorption after vitamin D administration. *Arch. Biochem. Biophys.* 133: 247–254, 1969.

74. HARRIS, L. J. Vitamins. *Ann. Rev. Biochem.* 3: 247–294, 1934.

75. HARRISON, H. E., AND H. C. HARRISON. The renal excretion of inorganic phosphate in relation to the action of vitamin D and parathyroid hormone. *J. Clin. Invest.* 20: 27–35, 1941.

76. HARRISON, H. E., AND H. C. HARRISON. Transfer of Ca^{45} across intestinal wall *in vivo* in relation to action of vitamin D and cortisol. *Am. J. Physiol.* 199: 265–271, 1960.

77. HARRISON, H. E., AND H. C. HARRISON. Intestinal transport of phosphate: action of vitamin D, calcium, and potassium. *Am. J. Physiol.* 201: 1007–1012, 1961.

78. HARRISON, H. E., AND H. C. HARRISON. The interaction of vitamin D and parathyroid hormone on calcium, phosphorus and magnesium homeostasis in the rat. *Metabolism* 13: 952–958, 1964.

79. HARRISON, H. E., H. C. HARRISON, AND F. LIPSHITZ. Interrelation of vitamin D and parathyroid hormone: the response of vitamin D-depleted and of thyroparathyroidectomized rats to ergocalciferol and dihydrotachysterol. In: *Parathyroid Hormone and Vitamin D Assay*, edited by R. V. Talmage and L. F. Belanger. Amsterdam: Excerpta Med. Found., 1968, p. 395–400.

80. HARRISON, H. E., H. C. HARRISON, AND E. A. PARK. Vitamin D and citrate metabolism: effect of vitamin D in rats fed diets adequate in both calcium and phosphorus. *Am. J. Physiol.* 192: 432–440, 1958.

81. HAUSSLER, M. R., D. W. BOYCE, E. T. LITELDIKE, AND H. RASMUSSEN. A rapidly acting metabolite of vitamin D. *Proc. Natl. Acad. Sci. USA* 68: 177–181, 1971.

82. HAUSSLER, M. R., J. F. MYRTLE, AND A. W. NORMAN. The association of a metabolite of vitamin D with intestinal nuclear chromatin in vivo. *J. Biol. Chem.* 243: 4055–4064, 1968.

83. HAUSSLER, M. R., L. A. NAGODE, AND H. RASMUSSEN. Induction of intestinal brush border alkaline phosphatase by vitamin D and interaction with Ca^{45}ATPase. *Nature* 226: 1199–1201, 1970.

84. HEYMANN, W. Metabolism and mode of action of vitamin D. II. Storage of vitamin D in different tissues in vivo. *J. Biol. Chem.* 148: 371–376, 1943.

85. HEYMANN, W. Metabolism and mode of action of vitamin D. IV. Importance of bile in the absorption and excretion of vitamin D. *J. Biol. Chem.* 122: 249–256, 1937.

86. HEYMANN, W. Metabolism and mode of action of vitamin D. V. Intestinal excretion of vitamin D. *J. Biol. Chem.* 122: 257–262, 1937.

87. HOLDSWORTH, E. S. Vitamin D and calcium absorption in the chick. *Biochem. J.* 475: 483, 1965.

88. HOLICK, M. F., AND H. F. DELUCA. A new chromatographic system for vitamin D_3 and its metabolites: resolution of a new vitamin D_3 metabolite. *J. Lipid Res.* 12: 460–465, 1971.

89. HOLICK, M. F., M. GARABEDIAN, AND H. F. DELUCA. The 5-trans isomers of cholecalciferol and 25-hydroxycholecalciferol: substitutes for 1,25-dihydroxycholecalciferol in anephric animals. *Biochemistry* 11: 2715–2719, 1972.

90. HOLICK, M. F., M. GARABEDIAN, AND H. F. DELUCA. 1,25-Dihydroxycholecalciferol: metabolite of vitamin D_3 active on bone in anephric rats. *Science* 176: 1146–1147, 1972.

91. HOLICK, M. F., M. GARABEDIAN, AND H. F. DELUCA. 5,6-Trans-25-hydroxycholecalciferol, vitamin D analog effective on intestine of anephric rats. *Science* 176: 1247–1248, 1972.

92. HOLICK, M. F., H. K. SCHNOES, AND H. F. DELUCA. Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D_3 metabolically active in the intestine. *Proc. Natl. Acad. Sci. USA* 68: 803–804, 1971.

93. HOLICK, M. F., H. K. SCHNOES, H. F. DELUCA, T. SUDA, AND R. J. COUSINS. Isolation and identification of 1,25-dihydroxycholecalciferol. A metabolite of vitamin D active in intestine. *Biochemistry* 10: 2799–2804, 1971.

94. HORSTING, M., AND H. F. DELUCA. *In vitro* production of 25-hydroxycholecalciferol. *Biochem. Biophys. Res. Commun.* 36: 254–261, 1969.

95. HOWLAND, J., AND B. KRAMER. Calcium and phosphorus in the serum in relation to rickets. *Am. J. Dis. Children* 22: 105–149, 1921.

96. HULDSCHINSKY, K. Heilung von rachitis durch ultraviolettes Bestrahlung. *Deut. Med. Wochschr.* 45: 712–713, 1919.

97. HUNT, R. D., U. AY GARCIA, AND D. M. HUGSTED. A comparison of vitamin D_2 and D_3 in new world primates. I. Production and regression of osteodystrophy fibrosa. *Lab. Animal Care* 17: 222–234, 1967.

98. IMRIE, M. H., P. F. NEVILLE, A. W. SNELL-GROVE, AND H. F. DELUCA. Metabolism of vitamin D_2 and vitamin D_3 in the rachitic chick. *Arch. Biochem. Biophys.* 20: 535–542, 1967.

99. INGERSOLL, R. J., AND R. H. WASSERMAN. Vitamin D-induced calcium-binding protein. *J. Biol. Chem.* 246: 2808–2814, 1971.

100. KALEJTA, F. A., A. N. TAYLOR, AND R. H. WASSERMAN. Vitamin D-induced calcium-binding factor in rat intestinal mucosa. *Proc. Soc. Exptl. Biol. Med.* 125: 54–58, 1967.

101. KALEJTA, F. A., AND R. H. WASSERMAN. Correlation between ^{45}Ca absorption and intestinal calcium-binding activity in the golden hamster. *Proc. Soc. Exptl. Biol. Med.* 139: 77–79, 1972.

102. KAYE, M., G. CHATTERJEE, G. F. COHEN, AND S. SAGAR. Arrest of hyperparathyroid bone disease with dihydrotachysterol in patients undergoing chronic hemodialysis. *Ann. Internal Med.* 73: 225–233, 1970.

103. KEMBERG, D. V. Effects of vitamin D and steroid hormones on the active transport of calcium by the intestine. *New Engl. J. Med.* 280: 1361–1365, 1969.

104. KIMBERG, D. V., R. D. BAIRD, E. GERSHON, AND R. T. GRANDUSIUS. Effect of cortisone treatment on the active transport of calcium by the rat small intestine. *J. Clin. Invest.* 50: 1309–1321, 1971.

105. KIMBERG, D. V., D. SCHAGGELEN, AND H. SCHENKER. Active transport of calcium by intestine: effects of dietary calcium. *Am. J. Physiol.* 200: 1256–1262, 1961.

106. KING, W. R., M. D. FRANCIS, AND W. R. MICHAEL. Effect of disodium ethane-1-hydroxy-

1,1-diphosphonate on bone formation. *Clin. Orthopaed. Related Res.* 78: 251–270, 1971.

107. KLEEMAN, C. R., S. G. MASSRY, J. W. COBURN, AND M. M. POPOVTZER. Calcium and phosphorus metabolism and bone disease in uremia. *Clin. Orthopaed. Related Res.* 68: 210–237, 1970.

108. KODICEK, E. The biosynthesis of ^{14}C-labelled ergocalciferol. *Biochem. J.* 60: xxv, 1955.

109. KODICEK, E. Metabolic studies on vitamin D. In: *Ciba Found. Symp. Bone Structure and Metabolism*, edited by G. W. E. Wolstenholme and C. M. O'Connor. Boston: Little, Brown, 1956, p. 161–174.

110. KODICEK, E. The metabolism of vitamin D. In: *Fourth Intern. Congr. Biochem. 4th, Vitamin Metabolism*. London: Pergamon, 1960, vol. xi, p. 198–208.

111. KODICEK, E. Turnover and distribution of vitamin D and its mode of action. In: *The Transfer of Calcium and Strontium Across Biological Membranes*, edited by R. H. Wasserman. New York: Academic, 1963, p. 135–156.

112. KRAWETT, F. L., AND H. F. SCHMIDT. In vivo calcium transport by rat small intestine. *Am. J. Physiol.* 214: 232–236, 1968.

113. KRUSE, R. Osteopathien bei antiepileptischer langzeittherapie. *Monatsschr. Kinderheilk.* 116, 378–381, 1968.

114. LAWSON, D. E. M., D. R. FRASER, E. KODICEK, H. R. MORRIS, AND D. H. WILLIAMS. Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. *Nature* 230: 228–230, 1971.

115. LAWSON, D. E. M., P. W. WILSON, AND E. KODICEK. Metabolism of vitamin D. A new cholecalciferol metabolite, involving loss of hydrogen at C-1 in chick intestinal nuclei. *Biochem. J.* 115: 269–277, 1969.

116. LINDQUIST, B. Effect of vitamin D on the metabolism of radiocalcium in uremic rats. *Acta Paediat.* 41, Suppl. 86: 1952.

117. LIU, S. H., AND H. F. CHU. Studies of calcium and phosphorus metabolism with special reference to parathyroidism and effects of dihydrotachysterol (A.T. 10) and iron. *Medicine* 22: 103–143, 1943.

118. LUMB, G. A., E. R. MAWER, AND S. W. STANBURY. The apparent vitamin D resistance of chronic renal failure. A study of the physiology of vitamin D in man. *Am. J. Med.* 50: 421–441, 1971.

119. LUND, J., AND H. F. DELUCA. Biologically active metabolite of vitamin D₃ from bone, liver, and blood serum. *J. Lipid Res.* 7: 739–744, 1966.

120. LUND, J., H. F. DELUCA, AND M. HURSTING. Formation of vitamin D₃ esters in vivo. *Arch. Biochem. Biophys.* 120: 513–517, 1967.

121. MACGREGOR, R. R., J. W. HAMILTON, AND D. V. COHN. The induction of calcium binding protein biosynthesis in intestine by vitamin D₃. *Biochim. Biophys. Acta* 222: 487–495, 1970.

122. MARTIN, D. L., AND H. F. DELUCA. Influence of sodium on calcium transport by the rat small intestine. *Am. J. Physiol.* 216: 1351–1359, 1969.

123. MARTIN, D. L., AND H. F. DELUCA. Calcium transport and the role of vitamin D. *Arch. Biochem. Biophys.* 134: 139–148, 1969.

124. MARTIN, D. L., M. J. MELANCON, JR., AND H. F. DELUCA. Vitamin D stimulated, calcium-dependent adenosine triphosphatase from brush borders of rat small intestine. *Biochem. Biophys. Res. Commun.* 35: 819–823, 1969.

125. MATTHEWS, J. L., J. H. MARTIN, C. ARSENIS, R. EISENSTEIN, AND K. KUETTNER. The role of mitochondria in intracellular calcium regulation. In: *Cellular Mechanisms for Calcium Transfer and Homeostasis*, edited by G. Nichols, Jr. and R. H. Wasserman. New York: Academic, 1971, p. 239–255.

126. MELANCON, M. J., JR., AND H. F. DELUCA. Vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. *Biochemistry* 9: 1658–1664, 1970.

127. MELLANBY, E. An experimental investigation on rickets. *Lancet* 1: 407–412, 1919.

128. MELLANBY, E. The part played by accessory food factors in the etiology of rickets. *J. Physiol. (London)* 52: 1–10, 1919.

129. MORII, H., AND H. F. DELUCA. Relationship between vitamin D deficiency, hypocalcemia, and parathyroid hormone. *Am. J. Physiol.* 213: 358–362, 1967.

130. MORII, H., J. LUND, P. F. NEVILLE, AND H. F. DELUCA. Biologic activity of a vitamin D metabolite. *Arch. Biochem. Biophys.* 120: 508–512, 1967.

131. MYRTLE, J. F., AND A. W. NORMAN. Vitamin D: a cholecalciferol metabolite highly active in promoting intestinal calcium transport. *Science* 174: 79–82, 1971.

132. NEUMAN, W. F. On the role of vitamin D in calcification. *Am. Med. Assoc. Arch. Pathol.* 66: 204–209, 1958.

133. NEUMAN, W. F., AND M. W. NEUMAN. *The Chemical Dynamics of Bone Mineral*. Chicago: Univ. of Chicago Press, 1958.

134. NEVILLE, P. F., AND H. F. DELUCA. The synthesis of [1,2-^{3}H]-vitamin D₃ and the tissue localization of a 0.25 μg (10 IU) dose per rat. *Biochemistry* 5: 2291–2297, 1966.

135. NEY, R. L., G. KELLY, AND F. C. BARTTER. Actions of vitamin D independent of the parathyroid glands. *Endocrinology* 82: 760–766, 1968.

136. NICHOLS, G., JR., P. HIRSCHMANN, AND F. ROGERS. Bone cells, calcification, and calcium homeostasis. In: *Cellular Mechanisms for Calcium Transfer and Homeostasis*, edited by G. Nichols, Jr. and R. H. Wasserman. New York: Academic, 1971, p. 211–237.

137. NICOLAYSEN, R. Studies upon the mode of action of vitamin D. II. The influence of vitamin D on the fecal output of endogenous calcium and phosphorus in the rat. *Biochem. J.* 31: 127–131, 1937.

138. NICOLAYSEN, R. Studies upon the mode of action of vitamin D. III. The influence of vitamin D on the absorption of calcium and phosphorus in the rat. *Biochem. J.* 31: 122–129, 1937.

139. NICOLAYSEN, R. Studies upon the mode of action of vitamin D. IV. The absorption of calcium chloride, xylose and sodium sulphate from isolated loops of the small intestine and of calcium chloride from the abdominal cavity in the rat. *Biochem. J.* 31: 323–328, 1938.

140. NICOLAYSEN, R. Studies upon the mode of action of vitamin D. V. The absorption of phosphates from isolated loops of the small intestine in the rat. *Biochem. J.* 31: 1686–1688, 1937.

141. NICOLAYSEN, R. The absorption of calcium. *Acta Physiol. Scand.* 6: 201–209, 1943.

142. NICOLAYSEN, R., AND N. EGGE-LARSEN. The

4876

biochemistry and physiology of vitamin D. *Vitamins Hormones* 11: 29–60, 1953.

143. NICOLAYSEN, R., AND N. EGG-LARSEN. The mode of action of vitamin D. In: *Ciba Found. Symp. Bone Structure and Metabolism*, edited by G. W. E. Wolstenholme and C. M. O'Connor. Boston: Little, Brown, 1956, p. 175–186.

144. NICOLAYSEN, R., N. EGG-LARSEN, AND O. J. MALM. Physiology of calcium metabolism. *Physiol. Rev.* 33: 424–444, 1953.

145. NORMAN, A. W. Actinomycin D and the response to vitamin D. *Science* 149: 184–186, 1965.

146. NORMAN, A. W., AND H. F. DeLUCA. The preparations of H³-vitamins D₂ and D₃ and their localization in the rat. *Biochemistry* 2: 1160–1168, 1963.

147. NORMAN, A. W., J. LUND, AND H. F. DeLUCA. Biologically active forms of vitamin D₃ in kidney and intestine. *Arch. Biochem. Biophys.* 103: 12–21, 1964.

148. NORMAN, A. W., J. F. MYRTLE, R. J. MIDGETT, H. G. NOWICKI, V. WILLIAMS, AND G. POJAK. 1,25-Dihydroxycholecalciferol: identification of the proposed active form of vitamin D₃ in the intestine. *Science* 173: 51–54, 1971.

149. OLSON, E. B., AND H. F. DeLUCA. 25-Hydroxycholecalciferol: direct effect on calcium transport. *Science* 165: 465–467, 1969.

150. OMDAHL, J. L., AND H. F. DeLUCA. Avitamin induced rickets: metabolic basis. *Science* 174: 949–951, 1971.

151. OMDAHL, J. L., R. W. GRAY, E. T. BOYLE, J. KNUTSON, AND H. F. DeLUCA. Regulation of metabolism of 25-hydroxycholecalciferol by kidney tissue *in vitro* by dietary calcium. *Nature (New Biol.)* 237: 63–64, 1972.

152. OMDAHL, J. L., M. HOLICK, T. SUDA, Y. TANAKA, AND H. F. DeLUCA. Biological activity of 1,25-dihydroxycholecalciferol. *Biochemistry* 10: 2049–2050, 1971.

153. OMDAHL, J. L., AND P. A. THORNTON. Dihydrotachysterol induction of intestinal calcium-binding activity in the chick. *Biochem. Pharmacol.* 21: 231–238, 1972.

154. OMDAHL, J. L., AND P. A. THORNTON. Intestinal calcium absorption and calcium binding protein. Influence of dietary calcium. *Proc. Soc. Exptl. Biol. Med.* 140: 979–985, 1972.

155. ORR, W. J., L. E. HÜLÍT, JR., L. WILKINS, AND F. H. HOONE. The calcium and phosphorus metabolism in rickets with special reference to ultraviolet ray therapy. *Am. J. Diseases Children* 26: 302–332, 1923.

156. PAK, C. Y. C., H. F. DeLUCA, J. M. CHAVEZ DE LOS RIOS, T. SUDA, B. RUSKIN, AND C. S. DELEA. Treatment of vitamin D-resistant hypoparathyroidism with 25-hydroxycholecalciferol. *Arch. Internal Med.* 126: 239–247, 1970.

157. PETERSON, P. A. Isolation and partial characterization of a human vitamin D-binding plasma protein. *J. Biol. Chem.* 246: 7243–7254, 1971.

158. PONCHON, G., AND H. F. DeLUCA. The role of the liver in the metabolism of vitamin D. *J. Clin. Invest.* 48: 1273–1279, 1969.

159. PONCHON, G., AND H. F. DeLUCA. Metabolites of vitamin D₂ and their biologic activity. *J. Nutr.* 99: 157–167, 1969.

160. PONCHON, G., A. L. KENNAN, AND H. F. DeLUCA. "Activation" of vitamin D by the liver. *J. Clin. Invest.* 48: 2023–2037, 1969.

161. PRADER, A., R. ILLIG, AND E. HEIERLI. Eine besondere form der primären vitamin D-resistenten rachitis mit hypocalcämie und autosomal-dominantem erbgang: die hereditäre pseudo-mangelrachitis. *Helv. Paediat. Acta* 16: 452–468, 1961.

162. PUSCHETT, J. B., J. MORANZ, AND W. S. KURNICK. Evidence for a direct action of cholecalciferol and 25-hydroxycholecalciferol on the renal transport of phosphate, sulfate, and calcium. *J. Clin. Invest.* 51: 373–385, 1972.

163. RAISZ, L. G., AND C. L. TRUMMEL. Induction of bone resorption in tissue culture: interaction of humoral agents and ions. In: *Cellular Mechanisms for Calcium Transfer and Homeostasis*, edited by G. Nichols Jr. and R. H. Wasserman. New York: Academic, 1971, p. 441–451.

164. RAISZ, L. G., C. L. TRUMMEL, M. F. HOLICK, AND H. F. DeLUCA. 1,25-Dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science* 175: 768–769, 1972.

165. RASMUSSEN, H. The influence of parathyroid function upon the transport of calcium in isolated sacs of rat small intestine. *Endocrinology* 65: 517–519, 1959.

166. RASMUSSEN, H. (editor). *International Encyclopedia of Pharmacology and Therapeutics. Parathyroid Hormone, Thyrocalcitonin and Related Drugs.* Oxford: Pergamon, 1970, sect. 51, vol. 1.

167. RASMUSSEN, H., H. DeLUCA, C. ARNAUD, C. HAWKER, AND M. VON STEDINGK. The relationship between vitamin D and parathyroid hormone. *J. Clin. Invest.* 42: 1940–1946, 1963.

168. RICHENS, A., AND D. J. F. ROWE. Disturbance of calcium metabolism by anticonvulsant drugs. *Brit. Med. J.* 4: 73–76, 1970.

169. RIKKERS, H., AND H. F. DeLUCA. An *in vivo* study of the carrier proteins of 25-hydroxyvitamin D₂ and D₃ in rat serum. *Am. J. Physiol.* 212: 380–386, 1967.

170. RIKKERS, H., R. KLEITZIEN, AND H. F. DeLUCA. Vitamin D binding globulin in the rat: specificity for the vitamins D. *Proc. Soc. Exptl. Biol. Med.* 130: 1521–1524, 1969.

171. ROBORGH, J. R., AND T. J. DEMAN. The hypercalcemic activity of dihydrotachysterols and dihydrotachysterols and of the vitamins D₂ and D₃ after intravenous injection of the aqueous preparations. *Biochem. Pharmacol.* 3: 277–282, 1960.

172. ROSENSTIRICH, S. J., C. RICH, AND W. VOLAWILER. Deposition in and release of vitamin D₃ from body fat; evidence for a storage site in the rat. *J. Clin. Invest.* 50: 679–687, 1971.

173. ROSENSTIRICH, S. J., W. VOLAWILER, AND C. RICH. Metabolism and plasma protein transport of vitamin D₃ in the baboon. *Am. J. Clin. Nutr.* 24: 897–905, 1971.

174. ROTTENSTEIN, K. V. GENNI. The effect of body stores on the efficiency of calcium utilization. *Biochem. J.* 32: 1205–1209, 1938.

175. SAMMON, P. J., R. E. STACLY, AND F. BRONNER. Role of parathyroid hormone in calcium homeostasis and metabolism. *Am. J. Physiol.* 218: 479–483, 1970.

176. SAMPSON, H. W., J. L. MATTHEWS, J. H. MARTIN, AND A. S. KUNIN. An electron microscopic localization of calcium in the small in-

testine of normal, rachitic, and vitamin D-treated rats. *Calcified Tissue Res.* 5: 305–316, 1970.

177. SCHACHTER, D. Vitamin D and the active transport of calcium by the small intestine. In: *The Transfer of Calcium and Strontium Across Biological Membranes*, edited by R. H. Wasserman. New York: Academic, 1963, p. 197–210.

178. SCHACHTER, D., E. B. DOWDLE, AND H. SCHENKER. Accumulation of Ca^{45} by slices of small intestine. *Am. J. Physiol.* 198: 275–279, 1960.

179. SCHACHTER, D., J. D. FINKELSTEIN, AND S. KOWARSKI. Metabolism of vitamin D. I. Preparation of radioactive vitamin D and its intestinal absorption in the rat. *J. Clin. Invest.* 43: 787–797, 1964.

180. SCHACHTER, D., S. KOWARSKI, J. D. FINKELSTEIN, AND R. WANG MA. Tissue concentration differences during active transport of calcium by intestine. *Am. J. Physiol.* 211: 1131–1136, 1966.

181. SCHACHTER, D., S. KOWARSKI, AND P. REID. Molecular basis for vitamin D action in the small intestine. *J. Clin. Invest.* 46: 1113–1114, 1967.

182. SCHACHTER, D., AND S. M. ROSEN. Active transport of Ca^{45} by the small intestine and its dependence on vitamin D. *Am. J. Physiol.* 196: 357–362, 1959.

183. SCHENCK, F. Über das kristallisierte vitamin D_3. *Naturwissenschaften* 25: 159, 1937.

184. SEBRELL, W. H., JR., AND R. S. HARRIS (editors). Vitamin D group. In: *The Vitamins*. New York: Academic, 1954, chapt. 6, p. 131–265.

185. SEBRELL, W. H., JR., AND R. S. HARRIS (editors). Vitamin D group. In: *The Vitamins*. New York: Academic Press, 1971, chapt. 7, p. 155–301.

186. SEMMLER, E. J., M. F. HOLICK, H. K. SCHNOES, AND H. F. DELUCA. The synthesis of 25-dihydroxycholecalciferol—a metabolically active form of vitamin D_3. *Tetrahedron Letters* 40: 4147–4150, 1972.

187. SHAH, B. G., AND H. H. DRAPER. Depression of calcium absorption in parathyroidectomized rats. *Am. J. Physiol.* 211: 1063–1066, 1966.

188. SHIPLEY, P. G., B. KRAMER, AND J. HOWLAND. Calcification of rachitic bones in vitro. *Am. J. Diseases Children* 30: 37–51, 1925.

189. SHIPLEY, P. G., B. KRAMER, AND J. HOWLAND. Studies upon calcification in vitro. *Biochem. J.* 20: 379–387, 1926.

190. STANBURY, S. W. Bone disease in uremia. *Am. J. Med.* 44: 714–724, 1968.

191. STEENBOCK, H., AND D. C. HERTING. Vitamin D and growth. *J. Nutr.* 32: 449–468, 1946.

192. STEENBOCK, H., S. W. F. KLETZIEN, AND J. G. HALPIN. The resources of the chicken for irradiated ergosterol and irradiated yeast as compared with natural vitamin D in the hen and *J. Biol. Chem.* 97: 289–294, 1923.

193. STEENDIJK, R. Metabolic bone disease in children. *Clin. Orthopaed. Related Res.* 77: 247–275, 1971.

194. SUDA, T., H. F. DELUCA, AND R. B. HALLICK. Synthesis of [20-27-H^3]-25-hydroxycholecalciferol. *Anal. Biochem.* 43: 139–140, 1971.

195. SUDA, T., H. F. DELUCA, H. SCHNOES, AND J. W. BLUNT. 25-Hydroxyergocalciferol: a biologically active metabolite of vitamin D_2. *Biochem. Biophys. Res. Commun.* 35: 182–185, 1969.

196. SUDA, T., H. F. DELUCA, H. K. SCHNOES, G. PONCHON, Y. TANAKA, AND M. F. HOLICK. 21,25-Dihydroxycholecalciferol. A metabolite of vitamin D_3 preferentially active on bone. *Biochemistry* 9: 2917–2922, 1970.

197. SUDA, T., H. F. DELUCA, H. K. SCHNOES, Y. TANAKA, AND M. F. HOLICK. 25,26-Dihydroxycholecalciferol, a metabolite of vitamin D_3 with intestinal calcium transport activity. *Biochemistry* 9: 4776–4780, 1970.

198. SUDA, T., R. B. HALLICK, H. F. DELUCA, AND H. K. SCHNOES. 25-Hydroxydihydrotachysterol. Synthesis and biological activity. *Biochemistry* 9: 1651–1657, 1970.

199. TALMAGE, R. V. Calcium homeostasis-calcium transport-parathyroid action. *Clin. Orthopaed. Related Res.* 67: 219–224, 1969.

200. TANAKA, Y., AND H. F. DELUCA. Bone mineral mobilization activity of 1,25-dihydroxycholecalciferol, a metabolite of vitamin D. *Arch. Biochem. Biophys.* 146: 574–580, 1971.

201. TANAKA, Y., AND H. F. DELUCA. Inhibition of the metabolism of 25-hydroxycholecalciferol by actinomycin D and cycloheximide. *Proc. Natl. Acad. Sci. US* 68: 603–608, 1971.

202. TANAKA, Y., H. F. DELUCA, J. OMDAHL, AND M. F. HOLICK. Mechanism of action of 1,25-dihydroxycholecalciferol on intestinal calcium transport. *Proc. Natl. Acad. Sci. US* 68: 1286–1288, 1971.

203. TAYLOR, A. N., AND R. H. WASSERMAN. Correlations between the vitamin D-induced calcium binding protein and intestinal absorption of calcium. *Federation Proc.* 28: 1844–1848, 1969.

204. TAYLOR, A. N., AND R. H. WASSERMAN. Immunofluorescent localization of vitamin D-dependent calcium-binding protein. *J. Histochem. Cytochem.* 18: 107–115, 1970.

205. TAYLOR, A. N., AND R. H. WASSERMAN. Intestinal phosphate (^{32}P) absorption: effect of vitamin D and other variables (abstr.). *Federation Proc.* 31: 698, 1972.

206. THOMAS, W. C., JR., H. G. MORGAN, T. B. CONNOR, L. HADDOCK, C. E. BILLS, AND T. E. HOWARD. Studies of antirachitic activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D in human sera. *J. Clin. Invest.* 38: 1076–1086, 1959.

207. TRUMMEL, C. L., L. G. RAISZ, J. W. BLUNT, AND H. F. DELUCA. 25-Hydroxycholecalciferol: stimulation of bone resorption in tissue culture. *Science* 163: 1450–1451, 1969.

208. TRUMMEL, C. L., L. G. RAISZ, R. B. HALLICK, AND H. F. DELUCA. 25-Hydroxydihydrotachysterol-stimulation of bone resorption in tissue culture. *Biochem. Biophys. Res. Commun.* 44: 1096–1101, 1971.

209. USSING, H. H. Some aspects of the application of tracers in permeability studies. *Adv. Enzymol.* 13: 21–65, 1952.

210. WADDELL, J. The provitamin D of cholesterol. I. The antirachitic efficacy of irradiated cholesterol. *J. Biol. Chem.* 105: 711–720, 1934.

211. WALLING, M. W., AND S. S. ROTHMAN. Phosphate-independent, carrier-mediated active transport of calcium by rat intestine. *Am. J. Physiol.* 217: 1114–1118, 1969.

212. WALLING, M. W., AND S. S. ROTHMAN. Apparent increase in carrier affinity for intestinal cal

4376

cium transport following dietary calcium restriction. *Am. J. Physiol.* 243: 5007-5011, 1970.

213. WASSERMAN, R. H. Vitamin D and the absorption of calcium and strontium *in vivo*. In: *The Transfer of Calcium and Strontium Across Biological Membranes*, edited by R. H. Wasserman. New York: Academic, 1963, p. 211-228.

214. WASSERMAN, R. H. Calcium transport by the intestine: a model and comment on vitamin D action. *Calcified Tissue Res.* 2: 301-313, 1968.

215. WASSERMAN, R. H. The vitamin D-dependent calcium-binding protein. In: *The Fat-Soluble Vitamins*, edited by H. F. DeLuca and J. W. Suttie. Madison: Univ. of Wisconsin Press, 1970, p. 21-37.

216. WASSERMAN, R. H. Interaction of vitamin D-dependent calcium-binding protein with lysolecithin: possible relevance to calcium transport. *Biochim. Biophys. Acta* 203: 176-179, 1970.

217. WASSERMAN, R. H., R. A. CORRADINO, AND A. N. TAYLOR. Vitamin D-dependent calcium-binding protein: purification and some properties. *J. Biol. Chem.* 243: 3978-3986, 1968.

218. WASSERMAN, R. H., R. A. CORRADINO, AND A. N. TAYLOR. Binding proteins from animals with a possible transport function. *J. Gen. Physiol.* 54: 1145-1175, 1969.

219. WASSERMAN, R. H., AND F. A. KALLFELZ. Vitamin D and unidirectional fluxes across rachitic chick duodenum. *Am. J. Physiol.* 203: 221-224, 1962.

220. WASSERMAN, R. H., F. A. KALLFELZ, AND C. L. COMAR. Active transport of calcium by rat duodenum *in vivo*. *Science* 133: 883-884, 1961.

221. WASSERMAN, R. H., AND A. N. TAYLOR. The nonessentiality of sodium ions for intestinal calcium transport. *Proc. Soc. Exptl. Biol. Med.* 114: 479-482, 1963.

222. WASSERMAN, R. H., AND A. N. TAYLOR. Vitamin D-induced calcium-binding protein in chick intestinal mucosa. *Science* 152: 791-793, 1966.

223. WASSERMAN, R. H., AND A. N. TAYLOR. Vitamin D-dependent calcium-binding protein: response to some physiological variables. *J. Biol. Chem.* 243: 3987-3993, 1968.

224. WASSERMAN, R. H., AND A. N. TAYLOR. Evidence for a vitamin D-induced calcium-binding protein in new world primates. *Proc. Soc. Exptl. Biol. Med.* 136: 25-28, 1971.

225. WILLIAMS, T. L., R. W. WINTER, AND C. H. BURNETT. Familial (hereditary) vitamin D-resistant rickets with hypophosphatemia. In: *The Metabolic Basis of Inherited Disease*, edited by J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson. New York: McGraw-Hill, 1966, p. 1179-1201.

226. WILSON, T. H., AND G. WISEMAN. The use of sacs of the everted small intestine for the study of the transference of substances from the mucosal to serosal surface. *J. Physiol. (Lond.)* 124: 116-125, 1954.

227. WINDAUS, A., O. LINSERT, A. LÜTTRINGHAUS, AND G. WEIDLICH. Crystalline-vitamin D₂. *Ann.* 492: 226-241, 1932.

228. WINDAUS, A., F. SCHENCK, AND F. VON WERDER. Über das antirachitisch wirksame Bestrahlungsprodukt aus 7-dehydro-cholesterin. *Z. Physiol. Chem.* 241: 100-103, 1936.

229. WINTERS, R. W., J. B. GRAHAM, T. F. WILLIAMS, V. W. FALLS, AND C. W. BURNETT. A genetic study of familial hypophosphatemia and vitamin D-resistant rickets with a review of the literature. *Medicine* 37: 97-142, 1950.

230. ZULL, J. E., E. CZARNOWSKA-MISZTAL, AND H. F. DeLUCA. Actinomycin D inhibition of vitamin D action. *Science* 149: 182-184, 1966.

231. ZULL, J. E., E. CZARNOWSKA-MISZTAL, AND H. F. DeLUCA. On the relationship between vitamin D action and actinomycin-sensitive processes. *Proc. Natl. Acad. Sci. US* 55: 177-184, 1966.

Oniwa, T.: STUDIES ON THE INFLUENCE OF VITAMIN D ON THE FEMALE SEXUAL FUNCTION OF RATS. III. EFFECT OF ESTROGEN AND VITAMIN D ON THE FEMALE SEXUAL FUNCTION OF RATS. Bitamin, Vol. 22 (1), pp. 68-84, 1961. Department of Gynecology and Obstetrics, Iwate Medical College (Director: Prof. Y. Hata)

Study on vitamin D has been conducted primarily in relation to rickets, and papers providing a systematic report of a clinical or experimental study on vitamin D in relation to reproductive functions are confined to those published by Okamoto (1925), Guggisberg (14) (1929), Alder Boltink (9) (1929), Watanabe (2,4)(1932), Tochiku (3) (1933), Dodds (10) (1934), Freedmann (11)(1951), and Kayane (17,18) of this faculty (1958,60). Kayane demonstrated that vitamin D deficiency in animals disturbed their ability to conceive while an appropriate dosage of it stimulated the ovarian activity and the ability to conceive, and a prolonged, large dose administration caused the atrophy of sexual organ. The author carried out the following experiments as a part of the study on sexual function and vitamin D.

EXPERIMENTAL PROCEDURE

White rats grown successively at this faculty were used. A group of young rats were castrated on their 40th day after birth and a group of mature rats, on their 140th day after birth to be used for the study of vitamin D in relation to castration. The experiments in which the animals were given vitamin D-deficient or -enriched feed were begun from the 3rd day after castration The animals were raised in a dark room. The vitamin D-deficient feed comprised 76 parts of corn starch (ether extracted), 20 parts of soybean powder (ether extracted), 3.0 parts of official calcium carbonate, and 1.0 part of official common salt. The feed given to the animals for the experiment in which the effect of excessive vitamin D was studied was the normal diet from which only liver oil had been eliminated, i.e., with a composition of 60 parts of unpolished rice powder, 20 parts of wheat powder, 13 parts of skim milk powder, 5.0 parts of soybean oil, 1.0 part of official calcium carbonate, and 1.0 part of common salt. As the source of vitamin D, an oily Chokora D_2 injection solution and an aqueous Chokora D_2 solution were employed.

The observation of sexual cycle was based on Zondek-Aschheim's classification (1925-1926) consisting of 4 periods (diestrus, preestrus, estrus, and metestrus). Each group consisted of 3 to 5 rats.

For histologic examination, specimens were stained by the hematoxylin-eosin double staining method, and, if necessary, with a Van-Gieson's solution.

The calculation of ovarian follicles and corpora lutea followed the basic procedure employed by Matsumoto, Tarui, and Koga (5) (1936), i.e., serial sections of an entire ovary were prepared, and the numbers of ovarian follicles and corpora lutea for the entire sections and 20 central sections were compared. Since no appreciable difference in calculated value between the two results was noted, 30 sections were prepared from 3 places in the central part, and the ovarian follicles and corpora lutea were counted. Averages were obtained for the right and left sides. The sizes of ovarian follicles and corpora lutea were classified according to Koga's method, in which follicles larger than 500 u are considered as large, those between 500 and 200 u, medium size, and those smaller than 200 u, small, and corpora lutea larger than 1000 u, large, those between 1000 and 500 u, medium size, and those smaller than 500 u, small.

I. RELATIONSHIP BETWEEN CASTRATION, VITAMIN D DEFICIENCY, AND ESTROGEN

(1) CASTRATION AND VITAMIN D DEFICIENCY

1. YOUNG CASTRATED WHITE RATS

The vitamin D deficient diet was given to young castrated rats weighing approximately 45 g for 90 days, and the same diet and daily injection of D 50 i.u. were given to the control group.

EXPERIMENTAL RESULTS

i) General Condition. The rats given the D-deficient feed showed less movement with time but no other significant change. The control group remained normal.

ii) Rate of Weight Gain. There was no appreciable difference between the two groups, the rates for the D-deficient group and the control group being 164.7% and 194.8%, respectively (Figure 1).

iii) Vaginal Smear. The vitamin D-defient group were in the diestrus at all times, whereas the control group showed the sign of cornification in an average of 65.5 days (56-78 days), with subsequent indication of sexual cycle.

iv) Histologic Impression. The average weight of the uterus of the 1st group was 99.7 mg as compared to the 214 mg of the control, obviously smaller (Table 1). The 1st group indicated the atrophy of the uterus, whereas the control group showed thick mucous epithelial cells with deep-set nuclear arrangement, normal uterine glands, interstice, and muscular layer, and the infiltration of eosinophilic cells (photograph 1).

The vaginal mucous membrane of the control comprised basal cells, 6 - 8 layers of rectangular cells, and 2 - 3 layers of flat cells, whereas that of the vitamin D deficiency group indicated atrophy of the stratified flat epithelium.

2. MATURE CASTRATED WHITE RATS

The vitamin D deficient diet was given to mature castrated white rats for 90 days, and the control group received the same diet and daily injection of D 50 i.u.

EXPERIMENTAL RESULTS

i) General Condition. One of the vitamin D deficiency group (No. 173) died on the 73rd day. The surviving rats became less and less active with time.

ii) Rate of Weight Gain. The rates for the deficiency group and the control group were 16.8 and 25.1%, respectively (Table 2, Figure 2).

iii) Vaginal Smear. Both groups remained in the diestrus at all times.

iv) Histologic Impression. The average weight of the uterus was 204.8 mg for the control and 115.8 mg for the D deficiency group (Table 2). The tissue of each layer appeared atrophied, but the control showed atrophy of lesser severity, with the infiltration of eosinophilic cells (photograph 2).

(2) CASTRATION, VITAMIN D DEFICIENCY, AND ESTROGEN

1. YOUNG CASTRATED WHITE RATS

After castrated, young rats weighing approximately 45 g were given the vitamin D deficient feed and daily injection of OvH*1 20 i.u. for 90 days, while the control animals were given the same amount of OvH and D 20 i.u.

(*1 OvH = ovahormone)

EXPERIMENTAL RESULTS

i) General Condition. Both groups became inactive and indicated lack of appetite.

ii) Rate of Weight Gain. The rates were 222.7% and 226.8% for the OvH group and the OvH + D group, respectively, with no appreciable difference (Table 3, Figure 3).

iii) Time up to the Estrus. The signs of estrus were observed in an average of 1.9 days for the OvH group and 1.8 days for the OvH + D group.

iv) Histologic Impression. Both groups indicated the thickening of the mucous membrane epithelium of the uterus, favorable growth of uterine glands, and enlargement of the muscular layer, with notable infiltration of eosinophilic cells. The control group showed orderly arrangement of mucous epithelial cells and uterine cells, with well developed muscular layer (photographs 3,4).

2. MATURE CASTRATED WHITE RATS

Two groups of rats were used, one given the basal diet, and the other, the vitamin D deficient diet. From the 30th day, OvH 10 i.u. was given twice daily for 5 days, and the vaginal smear was examined.

EXPERIMENTAL RESULTS

There was no difference between the two groups (Figures 3,5).

(3) SUMMARY

The vitamin D deficiency groups, both young and mature, indicated pronounced atrophy of the uterus, but it was interesting to note that some of the young control group, castrated but given vitamin D injections, showed enlarged uterus and complete cornification of vaginal smear.

With regard to the relationship between castration, vitamin D deficiency, and estrogen, there was no appreciable difference between the vitamin D deprived young andmature castrated white rats given OvH injections, and those given OvH + vitamin D, except for the fact that the latter indicated somewhat more favorable growth of uterine layers.

II. ADMINISTRATION OF VITAMIN D TO YOUNG WHITE RATS

(1) NON-CASTRATED YOUNG WHITE RATS

1. LARGE DOSE ADMINISTRATION OF VITAMIN D

Forty-day old white rats weighing approximately 50 g were divided into 4 groups,

and an injection of special sesame oil was given at 1-day intervals at 1000, 2000, 10000 and 0 i.u., and changes were observed for 60 days.

EXPERIMENTAL RESULTS

i) General Condition. The D 1000 i.u. group showed reduced appetite and the roughness of hair, whereas the 3 other groups indicated no abnormality, with favorable appetite and active movement.

ii) Rate of Weight Gain. The rates were 274.5, 297.7, 279.6, and 153.3% for the control, 1000, 2000, and 10000 i.u. groups, respectively (Figure 6).

iii) Sexual Cycle. The administration of vitamin D did not result in early production of sexual cycle. The control group showed irregular sexual cycle in an average of 25.4 days from the onset of experiment, and the cycle became increasingly regular with time. The D 1000 i.u. group exhibited signs of estrus in an average of 27 days, but after a temporary increase in the frequency of estrus, the phase became less frequent, with some rats indicating prolonged diestrus. The D 2000 i.u. group began to show sexual cycle in an average of 22.8 days, with the estrus lasting for 4 - 9 days each time and shifting to prolonged diestrus in 14 - 18 days (Table 4, Figure 7). The D 10000 i.u. group failed to show sexual cycle.

iv) Histologic Impression

(1) D 1000 i.u. group. The ovary of the rats with irregular sexual cycle weighed an average of 19.5 mg and that with prolonged diestrus, 14.5 mg. The former indicated a degenerative image of small and medium ovarian follicles but no abnormality in the primitive ovarian follicles. The corpora lutea were characterized by severe invasion of connective tissue and ill-defined cellular boundary but without vacuole or congestion. The latter showed hardly any healthy large or medium size follicles, but numerous degenerative small follicles. There were minimal corpora lutea with pronounced invasion by connective tissue and distention. The cellular boundary was ill-defined, and the interstitial tissue revealed nuclear concentration, infiltration of leukocytes, and congestion. Primitive follicles remained normal.

The uterus weighed an average of 316.8 mg for the former and 245.2 mg for the latter. The mucous membrane of the former was characterized by obscure boundary of enlarged cells, lightly stained nuclei, and low-set, disorderly or overlapping nuclear arrangement.

The growth of uterine glands was relatively favorable, but no convolution and expansion of glandular lumen was observed. The interstice was notably thickened, containing slightly stainable, irregularly round or oval giant nuclei. The muscular layer remained normal, with pronounced infiltration of eosinophilic cells just as in the interstice. For the latter, the epithelium of the mucous membrane was thickened, the cell membrane destroyed, and the nuclei overlapped (photograph 5). The interstice revealed no atrophy, and the growth of uterine glands was normal, but the muscular layer was slightly thin.

(2) D 2000 i.u. group. The ovary, the average weight of which was 16 mg, contained no large follicles, and a minimal amount of medium follicles, most of which had underwent cystic degeneration. Most of small follicles were degenerated. Corporal lutea were large (photograph 6), with multi-angled cells of various

sizes and invasion of connective tissue. Medium and small corpora lutea indicated severe invasion of connectivetissue, characterized by ill-defined cellular boundary and vacuoles in the cells, forming alveoli. The interstitial tissue revealed concentrated nuclei, infiltration of leukocytes, vacuolar formation, vascular distention, and congestion.

The uterus weighed an average of 447.2 mg, and contained 4 - 6 phymata (photographs 7,8). Histologic comparison of a portion without a phyma and a portion with a phyma revealed that, in the former, the mucous epithelium became thicker and projected into the uterine cavity in a wave-like fashion, with poorly defined cellular boundary and notably irregular nuclear arrangement (photograph 9). The uterine glands were poorly developed, the interstice remained without appreciable change, the muscular layer,appeared thickened but coarse, and the blood vessels, distented. The latter showed the destruction of cell membrane of the mucous epithelium with small nuclei arranged in an disorderly manner, most of them having lost their original form. A large proportion of the interstice was occupied by phyma tissue, with no sign of uterine glands, and the phymata were surrounded by the covering of the connective tissue (photograph 10). Themuscular layer was thin, and coarse, with muscular fibers roughly arranged, and with pronounced vascular distention and dilatation.

(3) D 10000 i.u. group. The average weight of ovary was 8.6 mg. The ovary generally contained no large follicles but degenerative medium and small follicles, and intact primitive follicles. There was no corpus luteum. The ovary was filled with interstitial tissue over a wide area (photograph 11). The uterus weighed an average of 72.6 mg, with marked atrophic image of all layers.

2. SMALL DOSE ADMINISTRATION OF VITAMIN D

Young noncastrated white rats weighing approximately 45 g and raised on the basal diet were divided into 2 groups. One group was given D 200 i.u., and the other, the control, daily injection of purified sesame oil for 25 days.

EXPERIMENTAL RESULTS

i) General Condition. Both groups maintained normal activity and good appetite, with no significant change.

ii) Rate of Weight Gain. The rate was 160.9% for the D 200 i.u. group, and 132.4% for the control group (Table 5, Figure 8).

iii) Vaginal Smear. All experimental cases indicated cornification on the 9th day, and prolonged cornification images were observed from the 16th day, but the control group showed no cornification during this period (Table 5).

iv) Histologic Impression. The uterus weighed an average of 191.6 mg for the vitamin D group, and 45.3 mg for the control. Vitamin D stimulated the growth of the uterus (photograph 12-A,B), and slightly enlarged the uterine cavity, with somewhat thickened mucous epithelium and epithelial cells. The growth of uterine glands was relatively favorable, but the internal cavity was small, and the interstitial and muscular layers yet to be developed, with coarse arrangement of muscular fibers (photograph 14). The control group exhibited less favorable growth of uterine layers, with narrow internal cavity and uterine glands yet to be formed (photograph 13).

(2) CASTRATED YOUNG WHITE RATS

Young castrated white rats 3 days after castration, weighing approximately 45 g, and raised on the basal diet were divided into 3 groups, and given D 200 i.u., 1000 i.u., and purified oil (control) daily for 25 days.

EXPERIMENTAL RESULTS

i) General Condition. All 3 groups remained active with favorable appetite, but the D 1000 i.u. group appeared subdued compared to other groups.

ii) Rate of Weight Gain. The rate was 168.1% for the D 200 i.u. group, 163.4% for the 1000 i.u. group, and 122.9% for the control group, the first 2 groups showing slightly more favorable rates (Table 6, Figure 8).

iii) Vaginal Smea[illegible] The D 200 i.u. group exhibited cornified cells on the 9 - 10th day, and the ima[illegible] f complete cornification remained from the 15th day. The 1000 i.u. group [illegible] to show cornified cells from the 5 - 7th day, and an incomplete image of cornifi[illegible] on lasted for 2 - 4 days, shifting to the diestrus thereafter. The control grou[illegible] owed no vaginal dilatation at all (Table 6).

vi) Histologic Impression. The average weights of the uterus were 122, 43.3, and 44 mg for the D 200 i.u. group, and 1000 i.u. group, and the control group, respectively. The D 200 i.u. group showed notable uterine hypertrophy (photograph 12 C) with slightly enlarged inner cavity, thickened mucous epithelium, and swollen cell nuclei arranged in an orderly manner without vacuolar formation. The interstitial and glandular growth was favorable, the glandular lumen, round, andthe growth of muscular layer, favorable, with well preserved arrangement of muscular fibers (photograph 15). The D 1000 i.u. group showed pronounced uterine atrophy (photograph 12-i), with poorly defined boundary between the layers of uterine wall, shrinking of mucous epithelium, poorly developed uterine glands, and degeneration of uterine glands. The degeneration of the interstice and poor development of the muscular layer were prominent, and the submucous tissue exhibited hyaline degeneration. The control group showed somewhat poor develoment of layers of uterine wall as compared to the D 200 i.u. group.

(3) SUMMARY

In the experiment in which large doses of vitamin D were given to non-castrated young rats, the D 1000 i.u. group and D 2000 i.u. group underwent vaginal dilatation at the same time as the control. At 10000 i.u., no sexual cycle was produced. The castrated young white rats showed sexual cycle earlier, but indicated an early shift to prolonged diestrus without complete cornification of vaginal smear.

A small dose of D given to young white rat produced no difference in the time of vaginal dilatation between noncastrated and castrated groups, all animals indicating prolonged estrus as observed in the vaginal smear, Histologically, both groups exhibited favorable uterine growth.

III. EFFECT OF VITAMIN D ON THE ACTION OF ESTROGEN

(1) NONCASTRATED MATURE WHITE RATS

1. SMALL-DOSE ADMINISTRATION OF ESTROGEN

Three groups of rats were used. One group received OvH 2 i.u. daily for 20 days, and the other two groups, OvH + D 1000 i.u., and OvH + 10000 i.u.

EXPERIMENTAL RESULTS

i) General Condition. No significant change was observed.

ii) Sexual Cycle. No appreciable change was shown by the OvH group. The OvH + D 1000 i.u. group exhibited increased frequency and prolonged duration of estrus. The OvH + D 10000 i.u. group showed irregularity of estrus (Figure 9).

iii) Histological Impression

a) OvH Group. The average weight of ovary was 28.9 mg. There was no significant change. The number of corpora lutea remained normal, and the interstitial tissue, without change. The uterus weighed an average of 368.6 mg. The mucous epithelium became thickened, with nuclei showing a tendency of being located lower yet occasionally overlapping. Leukocytal infiltration and vacuolar formation were minimal. The growth of uterine glands, interstice, and muscular layer was favorable, with strong infiltration of eosinophilic cells.

b) OvH + D 1000 i.u. Group. The ovary weighed an average of 30 mg, with numerous, healthy, well-developed ovarian follicles. There were a large number of corpora lutea with relatively clear cell boundary. Some of them contained vacuoles. The interstitial tissue was well packed (photograph 16). The uterus weighed an average of 617.8 mg, and was characterized by thick mucous epithelium and interstice, and well developed uterine glands and muscular layer (photograph 17).

c) OvH + D 10000 i.u. Group. The ovary weighed an average of 25.8 mg. All of large, medium size, and small follicles became degenerated. Large follicles in particular showed notable changes (photograph 18). Corpora lutea were mostly small or medium size, and the interstitial tissue was coarse and congested. The uterus weighed an average of 385.6 mg. All layers of the uterine wall were generally thick but the growth of mucous epithelium and interstice was less favorable than that of the previous two groups (photograph 19).

2. MEDIUM DOSE ADMINISTRATION OF ESTROGEN

The rats were divided into 3 groups. One group received OvH 20 i.u. daily for 20 days, and the other 2 groups, OvH + D 1000, and OvH + D 10000 i.u.

EXPERIMENTAL RESULTS

i) General Condition. All groups remained without notable change.

ii) Sexual Cycle. All indicated prolonged estrus.

iii) Histologic Impression

a) OvH Group. The ovary weighed an average of 36.4 mg. There were few healthy large follicles, but medium size follicles were generally healthy. Corpora lutea were large, with some newly produced (photograph 20). The uterus weighed an average of 721.6 mg. The uterine cavity was large, with thick mucous

epithelium. Many of uterine glands contained large lumen. The growth of uterine glands and interstice was favorable (photograph 21).

b) OvH + D 1000 i.u. Group. The ovary weighed an average of 41.2 mg. There were more corpora lutea than those of the first group, and a greater proportion of them exhibited vacuolar formation and degeneration (photograph 22). The uterus weighed an average of 863 mg. The uterine wall was well developed, and the mucous epithelial cells enlarged. Uterine glands were slightly less in number than those of the OvH group, but the convolution and dilatation of glandular lumen was more notable and the interstice and muscular layer, thicker (photograph 23).

c) OvH + D 10000 i.u. Group. The ovary weighed an average of 33 mg. There was no healthy large or medium size ovarian follicles, and most of smaller follicles were degenerated. Corpora lutea were mostly small or medium size, with advanced vacuolar formation (photograph 24). The uterus weighed an average of 543 mg. The uterine cavity was large. Epithelial cell nuclei displayed an irregular arrangement. Uterine glands were poorly developed. The infiltration of eosinophilic cells in the interstice and muscular layer was most minimal among the 3 groups (photograph 25).

3. LARGE DOSE ADMINISTRATION OF ESTROGEN

Of 2 groups, one group received daily injection of OvH 10000 i.u. for 50 days, and the other group, OvH + D 1000 i.u.

EXPERIMENTAL RESULTS

i) General Condition. Both groups showed poor appetite and severe reduction of body weight from the 20th day, with reduced bodily movement, diarrhea, and hyposthenia. However, no death occurred (Table 7, Figure 10).

ii) Sexual Cycle. Both groups exhibited signs of prolonged estrus.

iii) Histologic Impression. There was no clear difference between the 2 groups. The ovary was pale and smooth, weighing an average of 12.4 mg for the OvH group and 10.6 mg for the OvH + D group. Neither group contained large follicles, and most of ovarian follicles, small or medium size, displayed a degenerative image. Primitive cells were also minimal. The interstice occupied the greater proportion due to the degeneration of ovarian follicles. There were only small corpora lutea, but the severe invasion of connective tissue obscured the boundary from the interstitial tissue (photograph 26). The uterus, edematous in all cases, weighed an average of 1.5 mg for the OvH group and 1.7 mg for the OvH + D group. The uterine cavity was large, and the mucous epithelium, thick, and the interstice and muscular layer became compressed by the enlarged uterine cavity (photograph 27).

(2) MATURE CASTRATED WHITE RATS

1. ADMINISTRATION OF ESTROGEN AND VITAMIN D

The animals were divided into 4 groups. One group received OvH 20 i.u. twice daily for 5 days, and 3 other groups, D 1000 i.u., 10000 i.u., and 5000 i.u. once daily in addition. The duration of estrus was observed.

EXPERIMENTAL RESULTS

As shown in Figure 11, there was no significant difference between the 4 groups.

2. ADMINISTRATION OF VITAMIN D FOLLOWED BY THE INJECTION OF ESTROGEN

The animals were divided into 2 groups. One of them received D 1000 i.u. for 20 days, while the other group received purified sesame oil. Both groups received OvH 20 i.u. twice daily for 5 days from the 21st day. The duration of estrus was observed.

EXPERIMENTAL RESULTS

As shown in Figure 12, no appreciable difference was noted between the two groups.

3. ADMINISTRATION OF ESTROGEN AND A SMALL DOSE OF VITAMIN D TO NONCASTRATED YOUNG WHITE RATS

White rats weighing approximately 45 g were divided into 2 groups. One group received OvH 100 i.u. daily, and the other, D 200 i.u. as well as OvH for 20 days.

EXPERIMENTAL RESULTS

i) General Condition. Both groups remained active with favorable appetite.

ii) Rate of Weight Gain. The first group showed a rate of 143.4% and the second group, 153.9% (Table 8).

iii) Vaginal Smear. In the 1st group, cornification occurred on the 2nd day after injection. The second group showed cornified cells in 1 - 3 days, an average of 1.3 day.

iv) Histologic Impression. The uterus weighed an average of 105 mg for the 1st group and 356.6 mg for the 2nd group, the latter obviously showing a more favorable growth. Uterine growth was favorable in both cases. The uterine glands were numerous and well developed. The interstice and muscular layer were thickened, and muscular fibers arranged regularly with round cell nuclei. The average weight of the ovary was 34.6 mg for group 1 and 35.6 mg for group 2, with no appreciable difference between the two. The 2nd group showed well developed follicles and numerous corpora lutea, whereas the 1st group showed many mature follicles but relatively fewer corpora lutea.

4. ADMINISTRATION OF ESTROGEN AND A SMALL DOSE OF VITAMIN D TO CASTRATED YOUNG WHITE RATS

White rats weighing approximately 48 g and 3 days after castration were divided into 3 groups. Group 1 received OvH 100 iu, group 2, OvH 10000 i.u. + D 200 i.u., and group 3, OvH 100 i.u. + D 200 i.u. daily for 25 days.

EXPERIMENTAL RESULTS

i) General Condition. Group 2 exhibited reduced movement, with diarrhea starting from the 10th day, but other groups showed no abnormality.

ii) Vaginal Smear. Cornified cells began to appear slightly earlier in groups 2 and 3, but all groups exhibited prolonged complete cornification from the 6 - 7th day (Table 9).

iii) Histologic Impression. The uterus was enlarged, with an average weight of 45.6 mg for group 3, 550.6 mg for group 2, and 348.6 mg for group 1. The uterine cavity was enlarged in an edematous fashion in all 3 groups, with pronounced hypertrophy of mucous epithelium- The thickening of epithelium was particularly notable in group 3, with a strong proliferative tendency. On the other hand, some exhibited degenerative image indicating nuclear destruction and vacuolar formation with only the membrane left in sight. The interstice contained a large proportion of connective tissue fibers, with extremely favorable growth of uterine glands and muscular layer with distented cell nucleus. In group 2, the mucous membrane of the uterus became thickened but no proliferative tendency was observed. In some instances, the mucous epithelium had dropped off and epithelial cell nuclei contained little chromatin and displayed a disorderly arrangement. The interstice was coarse with no sign of additional glandular growth, but muscular fibers became enlarged with the nuclei forming large spheres.

5. SUMMARY

The combined use of a small dose of estrogen and D 1000 i.u. in mature white rats resulted in more frequent occurrence of estrus, than in the single use of estrogen, but an increase in the dosage of D to 10000 i.u. produced an inhibitory action. When OvH 10000 i.u. was given to castrated young rats, and D 200 i.u. subsequently, cornified cells occurred earlier than in the group given OvH only.

Histologically, the mature OvH + D 1000 9.u. group indicated normal growth, mature follicles, increased number of copora lutea, enlargement and thickening of various uterine tissues, but the OvH + D 10000 i.u. group exhibited degenerative changes in the ovaries. The uterus of castrated young animals particularly those given estrogen and D 200 i.u., showed the potentiation of the action of estrogen.

DISCUSSION

(1) CASTRATION, VITAMIN D DEFICIENCY, AND ESTROGEN

Guggisberg (14) denied the possibility of reduced female sexual activity in osteomalacia (1929), but neuweiler (16) maintained in 1940 that vitamin D deficiency resulted in the atrophy of sexual organ, cystic dgeneration of ovarian follicles, and changes in the anterior pituitary. Kato (1932), Tochiku (2) (1933), Watanabe (4)(1932), and Hashimoto (7)(1941) stated that, in white rats, the sexual cycle shifted to prolonged diestrus due to vitamin D deficiency. Richter (13)(1943) observed a similar tendency, but reported that ultraviolet irradiation brought back normal sexual cycle. Kayano (18)(1960) stated that vitamin D deficiency distrubed the sexual activity of female animals and atrophy of their sexual organ, particularly when the animals were young.

The relationship between castration and vitamin D has been discussed by many. Shimamura and Matsuba castrated 60-day old white rats and observed poor development of long bones. According to Aoyanagi (6)(1930), animals given rickets diet developed the disease earlier as a result of castration. Landauer (15)(1949) experimentally demonstrated that estrogen played a preventive role against rickets.

The results of the present experiment show that the white rats, both young and mature, raised on the D-deficient diet showed poor growth when castrated, as compared to the noncastrated animals. Even when vitamin D was supplemented, the growth of the castrated animals was less favorable than the noncastrated animals.

Histologically, both young and mature castrated rats raised on the vitamin D deficient diet indicated pronounced atrophy of the uterus, and the control group given vitamin D injections showed less severe uterine atrophy. In young rats in particular, the administration of vitamin D thickened the uterine wall and produced the image of complete cornification in vaginal smear. Such findings are in agreement with Kayane's observation that vitamin D exerted proliferative and stimulatory action on female sexual organ.

Studies on the effect of vitamin D deficiency and estrogen are few. Richter (13) (1943) observed that the external appearance and symptoms of vitamin D deprived rats resembled those of pituitectomized rats, and held that vitamin D manifested its effect by stimulating the anterior pituitary. Agduhr (12)(1935) repotted that irradiated ergosterol stimulated the anterior pituitary of mice and increased their body weight, which suggests a correlation between the anterior pituitary and vitamin D.

It is still difficult to draw a conclusion affirmative of these observations based on the results of the present experiment, but the small dose administration of D seems to have intensified the effect of estrogen, which is confirmed by experiment III.

(2) ADMINISTRATION OF VITAMIN D TO YOUNG WHITE RATS

Adler-Boltink (9)(1929) administered vitamin D to white rats and observed increased frequency of estrus. Dodds (10)(1934) administered a large dosage of vitamin D to castrated white rats, and noted increased frequency of estrus and enlargement of the uterus. He concluded that vitamin D possessed an estrogen-like action. Tochiku (3)(1932) reported that a small amount of vitamin D given to white rats normalized their sexual cycle. According to Watanabe (2,4)(1932), a small amount of Bigantol increased the frequency of estrus and stimulated female sexual organ, but a medium or large dose of the compound produced a degenerativeeffect. Kayane (18)(1960) observed an estrual image in vaginal smear following the large dose administration of vitamin D (10000 i.u. daily) to castrated white rats, and also obtained similar histologic findings following the administration of estrogen.

With regard to the effect of vitamin D on animals, Tochiku (3)(1933) denied any specific action of vitamin D on the reproductive glands, based on the histologic impression at the time of vaginal dilatation.

In the present experiment, a large dose of vitamin D was administered to noncastrated young white rats, but no change in sexual cycle was observed. The prolongation of the treatment caused increased frequency and duration of estrus, and further continuation of treatment subsequently reduced the incidence of estrus, then suppressed the phase completely. Vitamin D at 10000 i.u. failed to produce sexual cycle, as Kayane (17) had previously reported (1958) with regard to mature castrated animals. Furthermore, the animals did not show reduced body weight or symptoms characteristic to excessive intake of vitamin D even when the disturbance in sexual cycle took place.

Histologically, some degree of degenerative change in the ovary was noted in coincidence with the irregularity and termination of sexual cycle. However, the uterus revealed no tendency of atrophy at 1000 or 2000 i.u. of D, but it formed phymata. This can be related to the proliferative, stimulatory action of vitamin D. At 10000 i.u., the sexual activity was inhibited, and the atrophic image of uterine layers was considerable.

The karge dose administration of D to castrated young rats produced vaginal dilatation earlier than in intact rats, but their vaginal smear exhibited no complete cornification image, the image quickly shifting to prolonged diestrus.

Histologically, the uterus displayed a pronounced atrophy, specific characteristics including the narrowing of vaginal cavity, poor development of uterine glands, and atrophied interstice. Occasionally, however, there were relatively well developed uterine glands. These findings can be interpreted as that the large dose administration of D to castrated young rats temporarily exerts a stimulatory effect, but consequently fails to eliminate the effect of castration.

At a small dose of D, there was no significant difference between the castrated group and the intact group of young rats with regard to the time of vaginal dilatation, all indicating an image of prolonged estrus in vaginal smear and the enlargement of the uterus. This sbustantiates the findings from the vitamin D deficiency experiment, that a small amount of vitamin D exerts a stimulatory and proliferative action on female sexual function particularly in young rats, and is in agreement with the statement by Dodds (10)(1934), Freedman (11)(1951), and Kayane (18)(1960) to the effect that vitamin D has an estrogen-like action.

(3) THE ACTION OF ESTROGEN AND THE ADMINISTRATION OF VITAMIN D

There is no paper concerning the relationship between vitamin D and estrogen. The reports which contain any reference to this relationship are confined to those by Adler Boltink (9)(1939), Dodds (10)(1934), Freedman(11)(1951), and Kayane (18) (1960) who merely held an assumption that vitamin D possesses an estrogen-like action.

When a small dosage of OvH and D 100 i.u. were given to mature white rats, the estrus occurred more frequently than in the group receiving OvH alone, and when the dosage of D was increased to 10000 i.u., the effect was reverse, i.e., inhibitory. The combined administration of OvH 100 i.u. and D 200 i.u. to castrated young rats produced cornified cells in vaginal smear earlier than the administration of OvH alone, and the histologic findings showed a similar pattern. More specifically, at 1000 i.u., mature animals showed increased production of ovarian follicles and corpora lutea, and the combined administration with D 200 i.u. to castrated young rats produced notable, proliferative changes in all layers of the uterus. At 10000 i.u., however, the number of follicles dropped, and pronounced degenerative changes occurred to corpora lutea.

These findings are contradictory to the findings introduced in the previous section on the administration of vitamin D alone, which demonstrated that a small amount of D exerted a stimulatory action on female sexual function. In other words, when a small amount of vitamin D was combined with a large dosage of OvH, the stimulatory effect on the female sexual activity was less than that exhibited by OvH alone. Thus, it was found that vitamin D exerted a stimulatory action on

the effect of estrogen within a specific quantitative relationship, and a large quantity of estrogen and a large dosage of vitamin D exhibited a mutually suppressive action.

CONCLUSION

1. Vitamin D deficiency in castrated white rats causes pronounced uterine atrophy, but the administration of vitamin D prevents the symptom. In young rats, the administration of vitamin D enlarges the uterus.

2. An adequate dosage of vitamin D stimulates the growth of ovarian follicles and the uterus, but an excessive dosage and prolonged duration of treatment result in a negative effect , and inhibits sexual function.
3. A large dosage of vitamin D temporarily stimulates the sexual activity of young animals, but prolonged treatment even after vaginal dilatation disturbs the sexual function of the animals before it affects their general condition.

4. The effect of vitamin D is stronger in young rats than in mature rats.

5. Vitamin D potentiates the action of estrogen only within a specific quantitative relationship, but a large dosage of vitamin D exerts an inhibitory action on estrogen.

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REFERENCES

1. Sekiguchi, S.: Jikken Igaku Zasshi, Vol. 14, p. 1059, 1930.
2. Watanabe, F.: Hokuetsu Igaku Zasshi, Vol. 47, p. 569, 1932.
3. Tochiku, C.: Aichi Igaku Zasshi, Vol. 40, No. 3, p. 355, 1933.
4. Watanabe, F.: Hokuetsu Igaku Zasshi, Vol. 47, p. 823, 1932.
5. Koga, K.: Nippon Fujinka Gakkai Zasshi, Vol. 31, p. 2177, 1936.
6. Aoyanagi, Y.: Jikken Igaku Zasshi, Vol. 14, p. 996, 1930.
7. Hashimoto, K.: Okayama Igaku Zasshi, Vol. 51, p. 2342, 1941.
8. Imura, T.: Tokyo Igaku Zasshi, Vol. 45, p. 9, 1942.

17. Kayane, R.: Bitamin, Vol. 14, p. 3, 1958
18. Kayane, R.: Bitamin, Vol. 19, p. 3, 1960
19. Sato, T., and R. Kayane: Sanfujinka no Sekai, Vol. 9, p. 11, 1957.

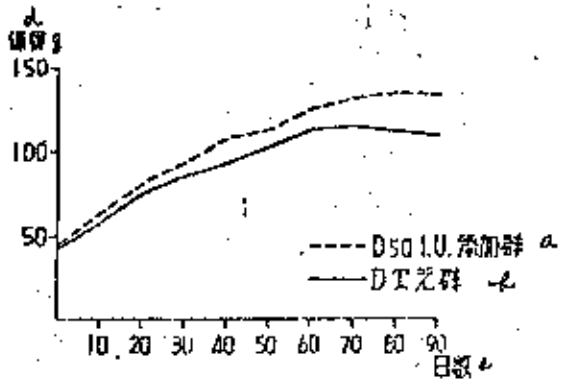

Figure 1. Growth Curves of Young Castrated White Rats Given Vitamin D Deficient Diet
KEY: a, D 50 i.u. group; b, D-deprived group; c, no. of days; d, body weight.

TABLE 1. CASTRATED YOUNG WHITE RATS RAISED ON VITAMIN D DEFICIENT DIET

a 動物番号	b 体重 (g) f 初体重	g 最高体重	h 終体重	c 日数 i 飼育期間	j 最高体重時	d 屠殺	e 子宮重量 (mg)
160	50.5	124	124	90	90	k 殺	114
161	40	120.5	102.5	90	73	k 殺	97
162	49	119	109	90	68	k 殺	102.5
163	41.5	112.5	112	90	77	k 殺	96
164	33.5	113	110.5	90	88	k 殺	89
l D 50 i.u. 添加群の子宮平均重量							214

KEY: a, animal no.; b, body weight; c, no. of days; d, reversion; e, weight of uterus; f, initial body weight; g, maximum body weight; h, final body weight; i, period of observation; j, time of maximum body weight; k, killed; l, average weight of uterus of the D 50 i.u. group.

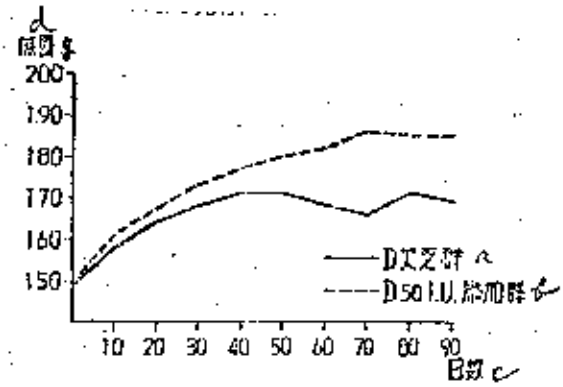

Figure 2. Growth Curve of Castrated Mature White Rats Raised on Vitamin D Deficient Diet
KEY: a, D deficiency group; b, D 50 i.u. group; c, no. of days; d, body weight

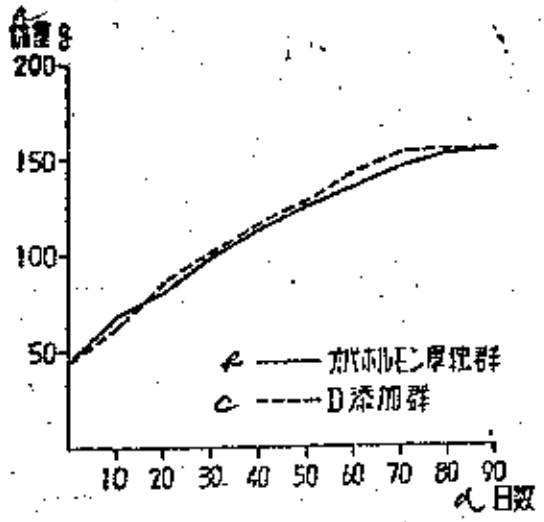

Figure 3. Growth Curve of Young Castrated White Rats Raised on the Vitamin D Deficient Feed and Given OvH.
KEY: a, body weight; b, OvH group; c, OvH + D group; d, No. of days

TABLE 2. MATURE CASTRATED WHITE RATS ON A VITAMIN D-DEFICIENT DIET

a 動物番号	b 体重 (g)			c 日数		d 転帰	e 子宮重量 (mg)
	f 初体重	g 最高体重	h 終体重	i 飼育期間	j 最高体重時期		
170	148.5	176	172.5	90	49	k 殺	119
171	140	167	166	90	53	殺	116
172	146.5	173.5	165	90	56	殺	107
173	154	179	152	73	38	k' 死亡	
174	156.5	174.5	174	90	66	殺	120
l D 50 i.u. 添加群の子宮平均重量							204.8

KEY: a, animal number; b, body weight; c, no. of days; d, reversion; e, weight of uterus; f, initial body weight; g, maximum body weight; h, final body weight; i, period of observation; j, time of maximum body weight; k, killed; k', died; l, average weight of uterus of the D 50 i.u. group.

TABLE 3. YOUNG CASTRATED WHITE RATS ON A VITAMIN D DEFICIENT DIET AND TREATED WITH OvH

a 動物番号	b 体重 (g)			c 日数		d 転帰	e 子宮重量 (mg)
	f 初体重	g 最高体重	h 終体重	i 飼育期間	j 最高体重時期		
180	46	163.5	157.5	90	82	k 殺	278
181	52.5	167	167	90	90	殺	261
182	46.5	149.5	146.5	90	73	殺	266
183	57	159	158	90	76	殺	263
184	42	152	150	90	85	殺	272
l D 20 i.u. 添加群の子宮平均重量							288

KEY: a, animal number; b, body weight; c, no. of days; d, reversion; e, weight of uterus; f, initial body weight; g, maximum body weight; h, final body weight; i, period of observation; j, maximum body weight (time of) k, killed; l, average weight of uterus of the D 20 i.u. group.

TABLE 4. CHANGES IN SEXUAL CYCLE IN YOUNG NONCASTRATED WHITE RATS DUE TO D 2000 i.u. INJECTIONS AT 1-DAY INTERVALS

a 動物番号	b 初体重 (g)	c 発情期徴出現時体重 (g)	d 発情期徴出現時期 (日)	e 発情期徴持続期間 (日)	f 持続的発情期出現時期 (日)	g 持続的発情期出現時体重 (g)	h 持続的発情期出現時子宮重量 (mg)
246	45	96.5	21	8	17	169	453
247	50	97	25	9	14	171	453
248	48	90.5	22	6	18	162.5	457
249	44	93	21	5	15	155	398.5
250	48	89	20	4	18	175	477.5

KEY: a, animal number; b, initial body weight; c, body weight at the time of first estrus (g); d, time of estrual signs (days); e, duration of estrus (days); f, time of prolonged diestrus (days); g, body weight at the first prolonged diestrus (g); h, weight of uterus at the first prolonged diestrus.

TABLE 5. SMALL DOSE ADMINISTRATION OF D TO YOUNG NONCASTRATED WHITE RATS

a 群	b 動物番号	c 体重(g) d 初体重	e 終体重	f 観察期間 (日)	g 発情徴出現時期 (日)	h 子宮重量 (mg)
D 200 i.u.	321	50	70	25	8	160
	322	44	70	25	9	215
	323	40	60	25	9	200
i 平均		43.6	70.6		8.6	191.6
j ゴマ油	324	40	60	25	k 持続	40
	325	42	58	25	k 持続	50
	326	51	62	25	k 持続	60
i 平均		45.3	60			45.3

KEY: a, group; b, animal number; c, body weight; d, initial body weight; e, final body weight; f, period of observation (days); g, time of cornification (days); h, weight of uterus (mg); i, average; j, sesame oil; k, diestrus

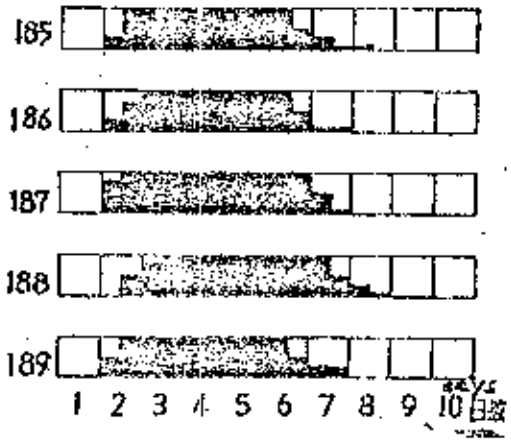

Figure 4. Durations of estrus of mature castrated rats on basal diet, given OvH 10 i.u. twice daily for 2 days.

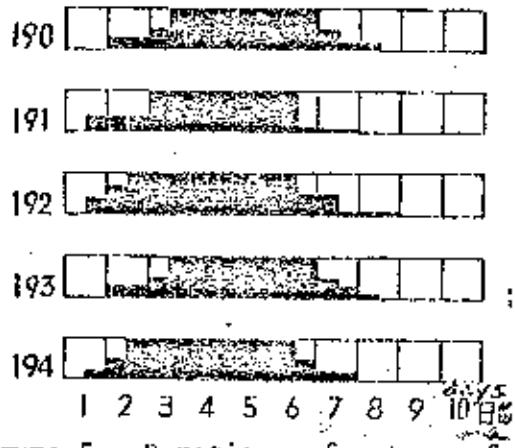

Figure 5. Durations of estrus of mature castrated rats on D-deficient diet, given OvH 10 i.u. twice daily for 5 days.

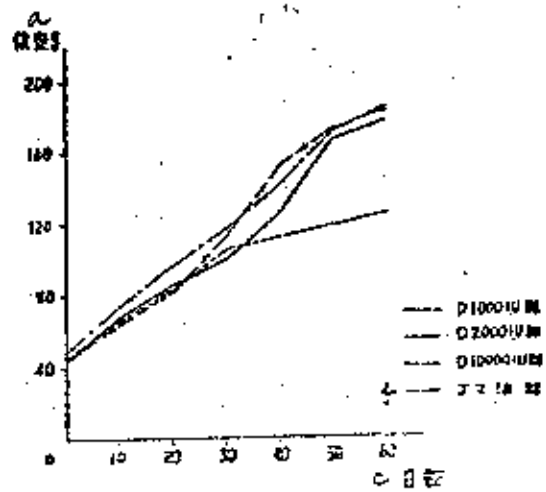

Figure 6. Growth Curves of Young Noncastrated rats Given Excessive Doses of Vitamin D
KEY: a, body weight; b, sesame oil; c, number of days

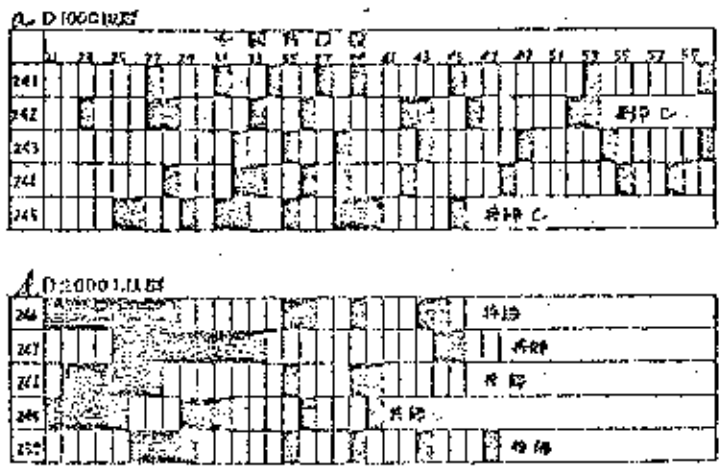

Figure 7. Changes in the Occurrence of Estrus in Young Noncastrated Rats Given D 1000 i.u. and D 2000 i.u. at 1-day Intervals
KEY : a, D 1000 i.u. group; b, number of days; c, prolonged; d, D 2000 i.u. group.

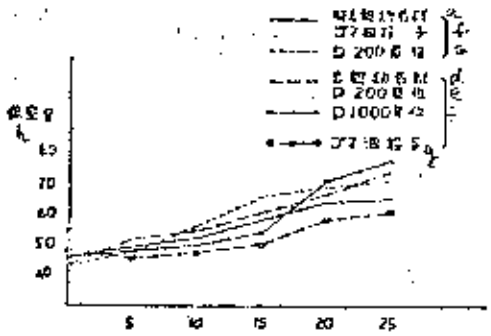

Figure 8. Growth Curves of Young Rats given Small Doses of Vitamin D

KEY: a, young noncastrated group; b, sesame oil; c, D 200; d, young castrated group; e, D 200; f, D 1000; g, sesame oil; h, body weight

TABLE 6. SMALL DOSE ADMINISTRATION OF VITAMIN D TO YOUNG CASTRATED WHITE RATS

群 a 別	動物番号 b	体重(g) c 初体重 d	体重(g) c 終体重 e	飼育期間 f (日)	角化細胞出現時 g 期 (日)	子宮重量 h (mg)
D200 I.U.	327	44	76	25	10	120
	328	48	78	25	9	130
	329	40	68	25	9	110
平均 i		44	74		9.3	122
D 1000 I.U.	330	44	82	25	5	41
	331	51	71	25	6	44
	332	40	73	25	7	45
平均 i		46	75.3		6	43.3
ゴマ油 j	333	44	58	25	k 静止	46
	334	42	60	25	静止	37
	335	52	64	25	静止	39
平均 i		49.3	60.6			44

KEY: a, group; b, animal number; c, body weight; d, initial body weight; e, final body weight; f, period of growth (days); g, time of cornification (days); h, weight of uterus; i, average; j, sesame oil; k, diestrus

TABLE 7. ADMINISTRATION OF DAILY OvH 10000 i.u. TO MATURE NONCASTRATED WHITE RATS FOR 50 DAYS

a 群	b 動物番号	c 初体重 (g)	d 最高体重 (g)	e 最高体重出現時期 (日)	f 最高時体重 (g)	g 結果	h 子宮
i OvH 単独	291	145	157.5	19	138	j 殺	k 子宮溜水腫
	292	150.5	169	8	124	殺	子宮溜水腫
	293	150	156	27	123	殺	子宮溜水腫
	294	148.5	152	35	143	殺	子宮溜水腫
	295	157	177.5	38	169	殺	子宮溜水腫
OvH + D	296	143.5	168	8	129.5	殺	l 子宮溜水腫
	297	154.5	157.5	13	130	殺	子宮溜水腫
	298	147	152.5	7	131.5	殺	子宮溜水腫
	299	151.5	165	21	130	殺	子宮溜水腫
	300	153.5	173	34	149	殺	子宮溜水腫

KEY: a, group; b, animal No.; c, initial body weight; d, maximum body weight; e, time of maximum body weight (days); f, body weight at the time of reversion; g, reversion; h, uterus; i, OvH group; j, killed; k, edema

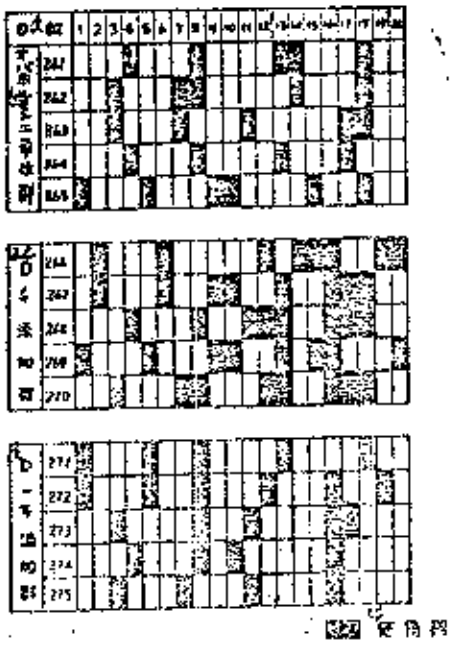

Figure 9. Occurrence of Estrus in mature noncastrated rats given daily OvH 2 i.u., daily OvH 2 i.u. + D 1000 i.u., and daily OvH 2 i.u. + D 10000 i.u.
KEY: a, No. of days; b, OvH i.u. group; c, OvH + D 1000 i.u. group; d, OvH + D 10000 i.u. group; e, Estrus

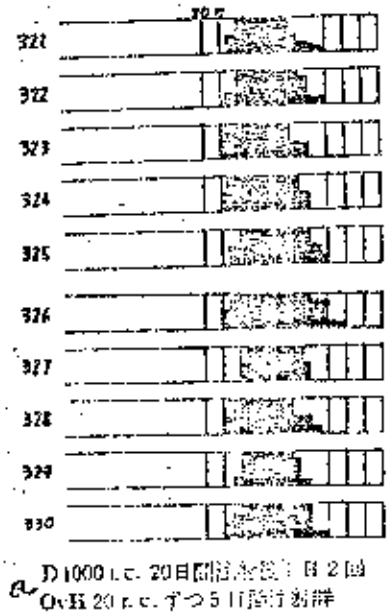

Figure 12. Administration of Purified Sesame Oil for 20 Days, Followed by OvH 20 i.u. injections twice daily for 5 days.
(a) D 1000 i.u. for 20 days, then OvH 20 i.u. twice daily for 5 days.

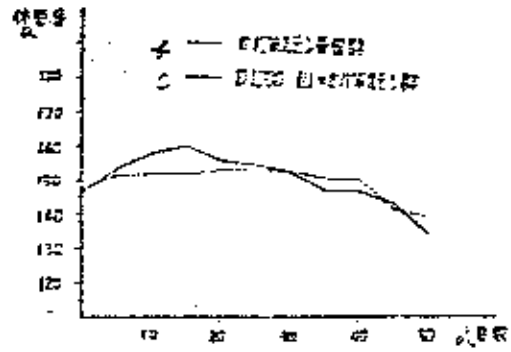

Figure 10. Growth Curves of Mature noncastrated white rats given daily OvH injections and OvH + D 10000 i.u.

KEY: a, body weight; b, OvH group; c, D 1000 i.u. + OvH group; d, No. of days

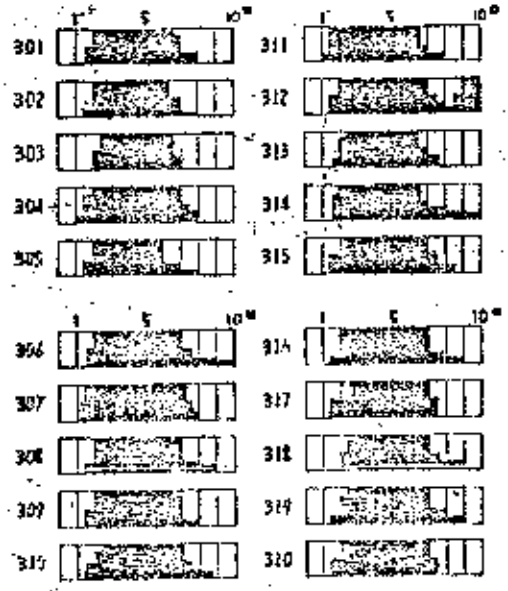

Figure 11. a, OvH 20 i.u. twice daily for 5 days
b, OvH + D 1000 i.u. once daily for 5 days
c, OvH + D 10000 i.u. once daily for 5 days
d, OvH + D 5000 i.u. once daily for 5 days.

TABLE 8. ADMINISTRATION OF SMALL DOSES OF VITAMIN D AND OvH TO YOUNG NONCASTRATED WHITE RATS

a 群別	b 動物番号	c 体重 (g) d 初体重	e 終体重	f 角化細胞出現時期 (日)	g 角化細胞	h 子宮重量 (mg)	i 卵巣重量 (mg)
D 200 I.U. OvH 100 I.U.	336	56	76	1	持続	260	36
	337	42	68	1	〃	380	30
	338	46	68	2	〃	430	38
平均 k		46	67.3	1.3		356.6	34.6
OvH 200 I.U.	339	44	66	2	〃	165	36
	340	44	62	2	〃	220	30
	341	45	67	2	〃	220	38
平均 k		47.3	68.3	2		195	34.6

KEY: a, group; b, animal number; c, body weight; d, initial body weight; e, final body weight; f, time of initial cornification (days); h, cornified cells; h, weight of uterus; i, weight of ovary; j, prolonged; k, average

TABLE 9. ADMINISTRATION OF SMALL DOSES OF VITAMIN D AND OvH TO YOUNG CASTRATED WHITE RATS

a 群別	b 動物番号	c 体重(g) d 初体重	e 終体重	f 角化細胞出現時期 (日)	g 角化細胞	h 子宮重量 (mg)
D 200 I.U. OvH 100 I.U.	342	54	81	3	持続	665
	343	39	76	3	〃	472
	344	50	77	3	〃	500
平均 i		48	78	3		545.6
D 200 I.U. OvH 1000 I.U.	345	52	74	3	持続	572
	346	54	72	3	〃	520
	347	50	70	3	〃	560
平均 i		52	72	3		550.6
OvH 100 I.U.	348	44	66	3	持続	266
	349	40	62	4	〃	380
	350	50	77	4	〃	400
平均 j		44.6	68.3	3.6		348.6

KEY: a, group; b, animal No.; c, body weight; d, initial body weight; e, final body weight; f, time of initial cornification (days); g, cornification signs; h, weight of uterus; i, average; j, prolonged

EXPLANATIONS OF PHOTOGRAPHS NOS. 1 - 27

No. 1. Young Castrated Group : D-deficient diet + D 50 i.u., daily.
Uterus: mucous epithelium, thickened; uterine glands, interstice, and muscular layer well developed.

No. 2. Mature Castrated Group : D deficient diet + D 50 i.u. daily.
Uterus: pronounced infiltration of eosinophilic cells in all layers.

No. 3. Young Castrated Group : D deficient diet + OvH 20 i.u. + D 20 i.u.
Uterus: mucous epithelium thickened, uterine glands well developed, muscular layer, enlarged.

No. 4. Young Castrated Group : D deficient diet + OvH 20 i.u. + D 20 i.u.
Uterus: mucous epithelium, thickened; uterine glands, well developed; muscular layer, enlarged.

No. 5. Young Noncastrated Group : D 1000 i.u. daily
Uterus: mucous epithelium, thickened; cell membranes, destroyed; nuclei, overlapping.

No. 6. Young Noncastrated Group : D 2000 i.u. daily.
Ovary: ovarian follicles, degenerated; numerous corpora lutea.

No. 7. Young Noncastrated Group : D 2000 i.u. daily
Uterus: a few phymata formed.

No. 8. Sections of Phymata.

No. 9. Young Noncastrated Group : D 2000 i.u. daily
Uterus: mucous epithelium, thickened, projecting into the uterine cavity in a wave-like fashion; cellular boundary, ill-defined; nuclei, irregularly arranged.

No. 10. Young noncastrated Group : D 2000 i.u. daily.
Uterus: The phyma is surrounded by the covering of the connective tissue.

No. 11. Young Noncastrated Group : D 10000 i.u. daily.
Ovary: no large ovarian follicle; small and medium size follicles, degenerated; numerous primitive follicles; increase and congestion of interstitial connective tissue noted.

No. 12. Macroscopic impression of the uterus of young white rats following the administration of a small dosage of vitamin D and OvH.
A, noncastrated rat given purified sesame oil daily; B, noncastrated rat given D 200 i.u. daily; C, castrated rat given D 200 i.u. daily; F, castrated animal given D 200 i.u. + OvH 100 i.u.; H, castrated animal given OvH 100 i.u.; I, castrated rat given D 1000 i.u. daily.

No. 13. Young Noncastrated Group : sesame oil.
Uterus: uterine layers, poorly developed; growth of uterine glands, not visible.

No. 14. Young Noncastrated Group : D 200 i.u.
Uterus: uterine cavity, slightly enlarged; mucous epithelium, thickened; (illegible) , relatively well developed.

No. 15. Young Castrated Group : D 200 i.u.
Uterus : uterine cavity, enlarged; mucous epithelium, thickened; interstice, muscular layer, well developed; glandular lumen, round.

No. 16. Mature Noncastrated Group : OvH 2 i.u. + D 10000 i.u.
Ovary : healthy, mature follicles; clearly defined cellular boundary; (illegible) tissue, well packed.

No. 17. Mature Noncastrated Group : OvH 2 i.u. + D 1000 i.u.
Uterus : Mucous epithelium and interstice, thickened; uterine glands and muscular layer, well developed.

No. 18. Mature Noncastrated Group : OvH 2 i.u. + D 10000 i.u.
Ovary : all of large, small, and medium size ovarian follicles, degenerated; (the rest, illegible).

No. 19. Mature Noncastrated Group : OvH 2 i.u. + D 1000 i.u.
Uterus : all layers, slightly thickened.

No. 20. Mature Noncastrated Group : OvH 20 i.u. daily.
Ovary : numerous corpora lutea.

No. 21. Mature Noncastrated Group : OvH 20 i.u. daily.
Uterus : uterine cavity, enlarged; mucous epithelium, thickened; glandular lumen, dilated.

No. 22. Mature Noncastrated Group : OvH 20 i.u. + D 100 i.u.
Ovary : corpora lutea, degenerated.

No. 23. Mature noncastrated Group : OvH 20 i.u. + D 1000 i.u.
Uterus : uterine wall, winding; glandular lumen, convoluted; interstice and muscular layer, thickened.

No. 24. Mature Noncastrated Group : OvH 20 i.u. + D 10000 i.u.
Ovary : a large number of small and medium size corpora lutea; cells indicate clear vacuolar formation.

No. 25. Mature Noncastrated Group : OvH 20 i.u. + D 10000 i.u.
Uterus : uterine cavity, spacious, epithelial cells display irregular nuclear arrangement; uterine glands, poorly developed.

No. 26. Mature Noncastrated Group : OvH 1000 i.u. + D 1000 i.u.
Ovary : small and medium size closed ovarian follicles are numerous, but primitive follicles are minimal in number.

No. 27. Mature Noncastrated Group : OvH 10000 i.u. _ D 1000 i.u.
The uterine interstice and muscular layer are compressed due to the enlarged uterine cavity.

4382

B. Tamin (Kyoto) 22 (1), 68, 1961

ビタミンDの雌性性機能におよぼす影響にかんする実験的研究

(Ⅲ) シロネズミの雌性性機能にたいするエストロゲンおよびビタミンD投与の影響

岩手医科大学産婦人科学教室（主任　桑　良隆教授）

大　庭　ト　シ

STUDIES ON THE INFLUENCE OF VITAMIN D ON THE FEMALE SEXUAL FUNCTION OF RATS

(Ⅲ) EFFECT OF ESTROGEN AND VITAMIN D ON THE FEMALE SEXUAL FUNCTION OF RATS

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The author studied the influence of vitamin D on the sexual function of albino rats. An administration of vitamin D to the castrated animal prevents the atrophy of the uterus to a considerable degree. Administration of an optimal dose of vitamin D accelerates the growth of ovarian follicles and uterus of noncastrated young and adult animals, on the contrary, an excess of vitamin D, especially when administered for a long period, restrains and suspends the sexual function of the animal. Young animals administered with a large dose of vitamin D show the temporary acceleration of sexual function, but the successive administration of an excess-dose causes the disorder of sexual function prior to that of general conditions. In general the effect of administration of vitamin D to the young animals is more prominent than that to the adult ones. The action of estrogen is enhanced by the simultaneous administration of an adequate dose of vitamin D, but it is reduced by a large dose.

Dの研究は従来主としてクル病との関係に向けられ生殖機能におよぼす影響についてはまだ臨床的にも実験的にも詳細な系統的報告は少なく屬本(1925), Gugisberg[10](1929), Adler-Bölkink[9](1929), 渡辺[29](1932), 本間[3](1933), Dodds[8](1941), Freedman[11](1957)および馬場他[27][28](1958, 1960)の報告をみるくらいである。それによりDの欠乏は動物の妊娠能力を減退し甚だしきのときは卵巣機能を衰弱してついに不妊性を惹起しやすいが大量長期投与では性腺の萎縮をおこすことを観察している。わたくしは本邦にひきつづき性機能とDの関係について以下の実験を行なつたのでその成績を報告する。

実　験　方　法

実験動物は当教室にて繁殖飼育しているシロネズミを使用し実験開始時では生後40日目に去勢した群では生後140日目に去勢し欠乏および過剰群はいずれも去勢後3日目より行なつた。なお実験動物の管理は常法で行なつた。飼料にはD欠乏食としてトコフェロコシ粉76(エーテル抽出)，大豆粉200(エーテル抽出)，局方炭酸カルシウム3.0，局方食塩1.0の配合のものを使用しD過剰群には普通食より粉末のみ除去し米粉80，小麦粉30，脱脂粉乳10，大豆粉5.0，局方炭酸カルシウム1.0，局方食塩1.0の配合を使用しD剤として

油性チョコラ D_2注射液および水性 チョコラ D_2液を使用した。

性周期の観察はZondek・Aschheim(1925～1926)の方法にしたがい4期(静止期，発情前期，発情期，発情後期)に区別して観察した。なお動物は3～5匹を1群として実験を行なつた。

組織学的検索はヘマトキシリン・エオジン重染色を主とし必要に応じてVan Gieson染色をおこなつた。

卵胞および黄体の計算は松本，庄野，吉野[5](1936)の報告を参考にして，まず一応卵巣全体の連続切片を作成して計算し，ついで中央部の連続20切片について計算したものとを比較した。その結果両者間の計算値に大差ないことを知つたので中央部0.4g所より30切片を作つて計算し左右両側の平均をとつた。卵胞および黄体の大小の区別は古賀の分類にしたがつて卵胞は1000μ以上を大，500～200μを中，200μ以下を小とし黄体は2000μ以上を大，1000～500μを中，500μ以下を小とした。

I. 去勢とD欠乏およびエストロゲンとの関係

1) 去勢とD欠乏

1. 去勢幼若シロネズミのばあい

体重15g前後の去勢幼若シロネズミにD欠乏食を90日間授与し対照群にはD欠乏食のほか同時にD50 I.U.を1日1回ずつ注射補給した。

実験成績

i) 一般状態　D欠乏動物の運動は時日の経過とともに不活潑となつたがその他には異変がなかつた。また対照群ではまつたく異常がみられなかつた。

ii) 体重増加率　D欠乏群154.7%，対照群191.6%で大差がなかつた(図1)。

iii) 性周期　D欠乏群では終始発情期を示したが対照群では平均65.5日(56～78日)で角化細胞の出現を認め性周期が発来した。

iv) 組織学的所見　子宮の平均重量は対照群の214mgにたいしD欠乏群では99.7mgであきらかに小さかつた(表1)。そして後者では組織学的にも子宮の萎縮が著明であつたが，前者では粘膜上皮線腺厚し核は偽重性に配列し子宮腺，間質，筋層の萎縮がなくエオジン好性細胞の増殖を認めた(写真1)。

膣粘膜は対照群では表層細胞，6～8層の方形細胞，2～3層の扁平細胞よりなりD欠乏群では粘膜の重層扁平上皮は萎縮像を呈していた。

2) 去勢成熟シロネズミのばあい

去勢成熟シロネズミにD欠乏食を90日間授与し対照群には同一食餌のほかにD50 I.U.を1日1回ずつ注射補給した。

実験成績

i) 一般状態　D欠乏群中1例(No.173)がついに73日目に死亡し，その他は時日の経過とともに運動がいちじるしくにぶくなつたが死を免れた。

ii) 体重増加率　D欠乏群16.8%，対照群25.1%であつた(表2,図2)。

図1　去勢幼若シロネズミのD欠乏食授与による発育曲線

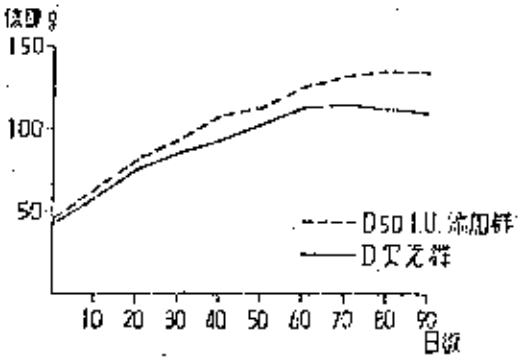


表1　去勢幼若シロネズミのD欠乏飼育

番号	体重(g) 初体重	最高体重	終体重	日数 飼育期間	最高体重時期	性周期	子宮重量(mg)
[illegible]	50.5	121	124	90	90	発	114
[illegible]	46	120.5	122.5	90	73	発	97
[illegible]	49	119	109	90	68	発	102.5
[illegible]	41.5	112.5	112	90	77	発	96
[illegible]	38.5	113	110.5	90	88	発	89
D 50 I.U. 注射群の子宮平均重量							214

図2 去勢成熟シロネズミのD欠乏食投与による発育曲線

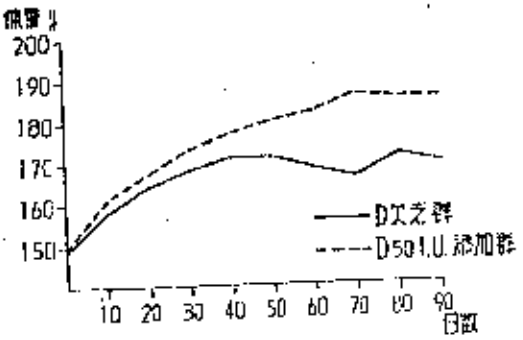
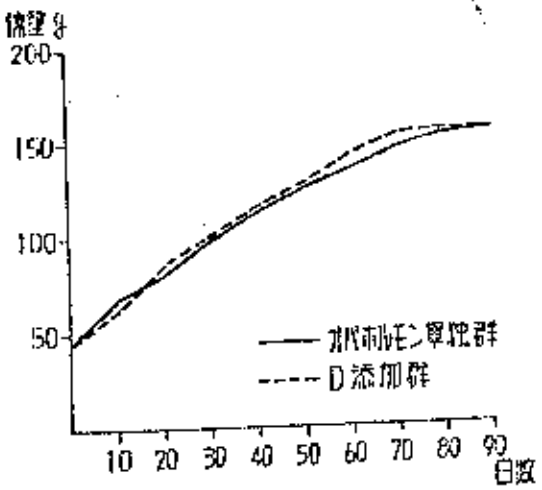


図3 D欠乏食飼育の去勢幼若シロネズミにOvHを投与したさいの発育曲線



iii) ちつ脂帯 両群ともつねに静止期像を示した。

iv) 組織学的所見 子宮の平均重量は対照群の204.8mgにたいしD欠乏群では115.8mgで(表2)，子宮各層組織の萎縮像を認めたが前者では萎縮の傾向が少なく各層にエオジン好性細胞の浸潤がみられた(写真2)。

(2) 去勢とD欠乏およびエストロゲン

1) 去勢幼若シロネズミのばあい

体重45g前後の幼若成熟を去勢したのちD欠乏飼育と同時に1日1回ずつOvH[*1]20 I.U.を注射し対照群には同量のOvHと同時にD20 I.U.ずつを投与し，いずれも90日間飼育観察した。

実験成績

i) 一般状態 両群ともしだいに運動が不活発となり食欲不振があらわれた。

ii) 体重増加率 OvH単独群222.7%，D添加群226.8%でまったく差がなかった(表3，図3)。

iii) 発情期像出現時期 OvH単独群平均1.9日，D添加群1.8日で，これにも差がなかった。

iv) 組織学的所見 両群とも子宮粘膜上皮が肥厚し子宮腺の発育良好で筋層の肥大を認めエオジン好性細胞の浸潤が著明であった。ことに対照群では粘膜上皮細胞，子宮細胞の配列が整然としており筋層の発育がよかった(写真3，4)。

2) 去勢成熟シロネズミのばあい

動物を2群に分けそれぞれ基本食とD欠乏食を投与し30日目から1日2回ずつOvH10 I.U.を5日間注射して膣脂膏の状態を観察した。

実験成績

図3，5にみるように両者間に差がみられなかった。

(3) 小 括

D欠乏群では幼若群，成熟群ともに子宮の萎縮がつよいがDを補給した幼若対照群中には去勢動物でありながら子宮の肥厚，膣脂膏の完全角化を示したものがあることは興味ぶかい。

また去勢と欠乏およびエストロゲンの関係についてはD欠乏去勢幼若ならびに成熟シロネズミにOvHを投与したものとOvHと同時にDを投与したものとの間に顕著な差がなく，わずかに後者で子宮各層の発育がまさっているかにみえたにすぎなかった。

II. 幼若シロネズミにたいするD投与実験

(1) 非去勢幼若シロネズミのばあい

1) D大量投与

生後40日目体重50g前後のシロネズミをそれぞれ1000，2000，10000 I.U.投与群および対照群(特製ゴマ油を隔日に注射)の4群にわけ，ともに60日間観察した。

実験成績

i) 一般状態 D10000 I.U.群では投与30日目ごろから食欲不振，毛のあれをきたしたが他の3群ではいずれも食欲良好，運動活発で異常を認めなかった。

*1) OvH=Oxahormone

表2　去勢成熟シロネズミのD欠乏飼育

動物番号	体重(g)			日数		転帰	子宮重量(mg)
	初体重	最高体重	終体重	飼育期間	最高体重時期		
170	149.5	176	172.5	90	49	殺	119
171	140	167	166	90	53	殺	116
172	146.5	173.5	165	90	56	殺	107
173	154	179	152	73	38	死亡	
174	156.5	174.5	174	90	66	殺	120
D 50 i.u. 添加群の子宮平均重量							204.8

表3　去勢幼若シロネズミにOvHを投与したさいのD欠乏食飼育

動物番号	体重(g)			日数		転帰	子宮重量(mg)
	初体重	最高体重	終体重	飼育期間	最高体重時期		
180	46	163.5	157.5	90	82	殺	278
181	52.5	167	167	90	90	殺	261
182	46.5	149.5	146.5	90	73	殺	266
183	57	159	158	90	76	殺	263
184	42	152	150	90	85	殺	272
D 20 i.u. 添加群の子宮平均重量							288

表4　非去勢幼若シロネズミに隔日D 2000 i.u. 投与による性周期の変動

動物番号	初体重(g)	発情期像出現時体重(g)	発情期像出現時期(日)	発情期像持続期間(日)	持続的静止期出現時期(日)	持続的静止期出現時体重(g)	持続的静止期出現時子宮重量(mg)
246	45	96.5	21	8	17	169	453
247	50	97	25	9	14	171	453
248	43	90.5	22	6	18	162.5	457
249	44	93	21	5	15	155	398.5
250	48	89	26	4	18	175	477.5

ii) 体重増加率　対照群274.5%，D 1000 i.u.群279.7%，2000 i.u.群279.6%，10000 i.u.群153.3%(図6)で10000 i.u.群がやゝおとつていた。

iii) 性周期　D投与によつて性周期発現の促進はみられなかつた。対照群では実験開始後平均25.4日で不稔な性周期があらわれ，しだいに延長となつた。D 1000 i.u.群では実験開始後平均27日で発情期像が出現し一時頻発したが，のちにはしだいに希発傾向となり一部ではついに持続的静止期に移行した。2000 i.u.群では実験開始後平均22.5日で4～9日間持続する発情期像が出現し，その後11～18日で持続的静止期となつた(表4，図7)。10000 i.u.群では性周期の発現をみなかつた。

iv) 組織学的所見

表5　非去勢幼若シロネズミにたいする少量D投与

群別	動物番号	体重(g)		飼育期間(日)	角化開始時期(日)	子宮重量(mg)
		初体重	終体重			
D 200 i.u.	321	50	76	25	8	160
	322	44	70	25	9	215
	323	40	60	25	9	200
平均		43.6	70.6		8.6	191.6
ゴマ油	324	40	60	25	静止	40
	325	42	56	25	静止	50
	326	54	62	25	静止	60
平均		45.3	60			45.3

図4 基本食飼育去勢成熟シロネズミにOvH 10 i.u., 1日2回 2日間投与群の発情持続期間

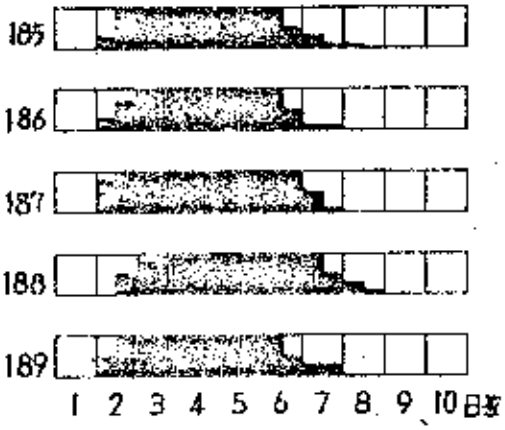
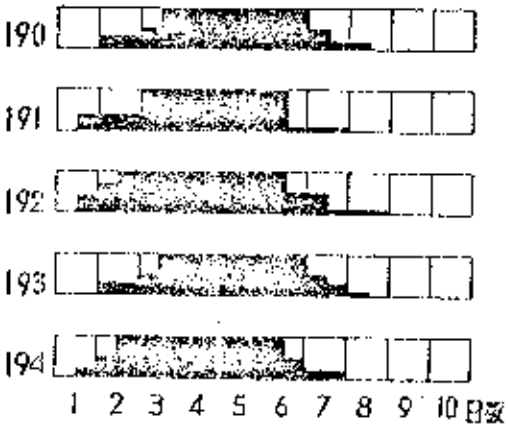


図5 D欠乏食飼育去勢成熟シロネズミにOvH 10 i.u., 1日2回5日間投与群の発情持続期間



(1) D 1000 i.u.群　不整性周期群の卵巣は平均重量19.5mg，持続的静止期をきたしたものでは14.5mg，前者は中小卵胞の退行性多く原始卵胞には異常をみなかつた。黄体は結合織の進入が強く細胞境界不鮮明で空胞形成，充血をみなかつた。後者では大・中卵胞にはほとんど健全なものがなく小卵胞の退行像多数を認めた。黄体は少なく結合織の進入が著明で充血があり細胞境界は不鮮明となり間質組織は核が濃縮し白血球の浸潤，充血をみた。原子卵胞には異常がなかつた。

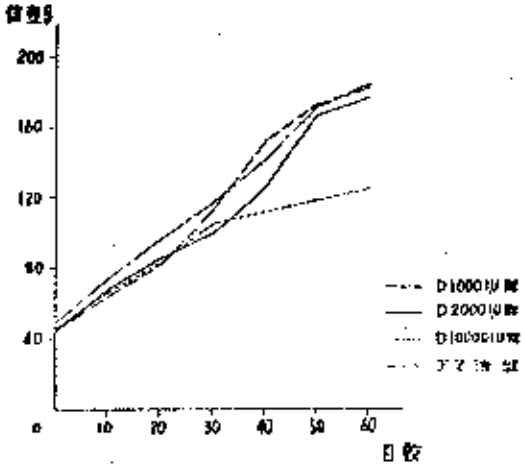
子宮は前者では平均重量316.8mg，後者では245.2mg，前者の粘膜は肥厚細胞の境界が不鮮明で核は淡染し偏在性かつ混雑にあるいは重積して配列していた。

子宮腺の発育は比較的良好であつたが腺腔の逆曲拡大はなかつた。間質は著しく肥厚し淡染する不正円形ないし楕円形の大なる核を有していた。筋層に異常がなく間質とともにエオジン好性細胞の浸潤が著明であつた。後者では粘膜上皮は前者と同様肥厚し細胞膜の破壊，核の重積をみた(写真5)。間質には萎縮の傾向がなく子宮腺の発育も正常であつたが，筋層はやゝうすかつた。

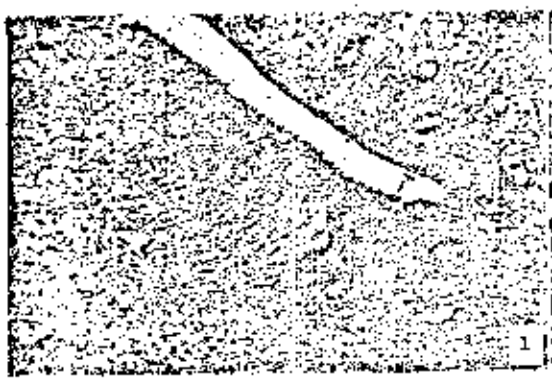
(2) D 2000 i.u.群　卵巣の平均重量は16mgで大卵胞をみず中卵胞も少数で多くは発泡状に変性し小卵胞にも退行したものが多かつた。黄体には大黄体を多数みとめ(写真6)，細胞は大小多角形で結合織の進入をみた。中小黄体では結合織の進入が強く細胞境界が不鮮明となり細胞は空胞を形成し腫瘍状をなすものがおおかつた。間質組織では核が濃縮し白血球の浸潤，腫瘍形成，血管拡張，充血をみた。

子宮は平均重量447.2mgで4～6層の腫瘍形成を認めた(写真7，8)。腫瘍のない部分と腫瘍部とに分けて組織学的に検索すると，前者では粘膜上皮が肥厚して波状に子宮腺内に突出し細胞境界の不鮮明，核の配列不整がはなはだしかつた(写真9)。子宮腺は発育不良，間質はおおむね著変がなく筋層は肥厚してみえるが粗で，血管の拡張を認めた。後者では粘膜上皮は細胞膜が破壊され核は小さく混雑に配列し，ほとんどその形態をとゞめていなかつた。間質の大部分は腫瘍組織によつて占められ子宮腺を認めず腫瘍の周囲は結合織の被覆に包まれていた(写真10)。筋層はうすく筋線

図6 非去勢幼若シロネズミのD過剰投与による発育曲線



去勢幼若群：D欠乏食＋D50 i.u. 連日投与



子宮：粘膜上皮肥厚，子宮腺，間質，筋層発育良好

去勢成熟群：D欠乏食＋D50 i.u. 連日投与

子宮：子宮各層のエオジン好性細胞浸潤著明

去勢幼若群：D欠乏食＋OvH 20 i.u. ＋D20 i.u. 添加

子宮：粘膜上皮肥厚，子宮腺発育良好，筋層肥大

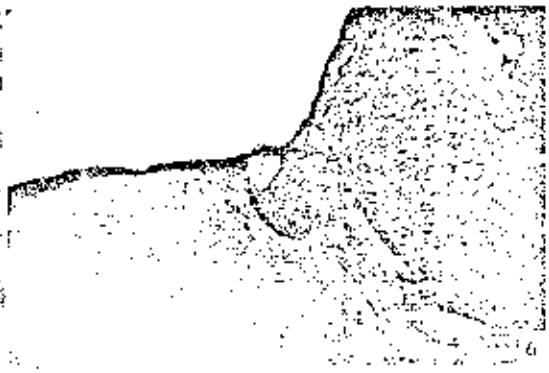
去勢幼若群：D欠乏食＋OvH 20 i.u. ＋D20 i.u. 添加

子宮：粘膜上皮肥厚，子宮腺発育良好，筋層肥大

非去勢幼若群：D 1000 i.u. 連日投与

子宮：粘膜上皮肥厚，子宮腺の発育良好，筋層の肥厚

非去勢幼若群：D 2000 i.u. 連日投与



卵巣：卵胞退行，黄体形成

4382

非去勢幼若群：D 2000 i. u. 連日投与

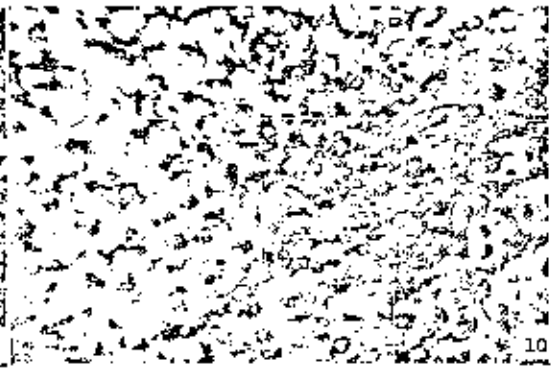
子宮：2～3 ヵ の腺腫形成

腫瘤割面

非去勢幼若群：D 2000 i. u. 連日投与

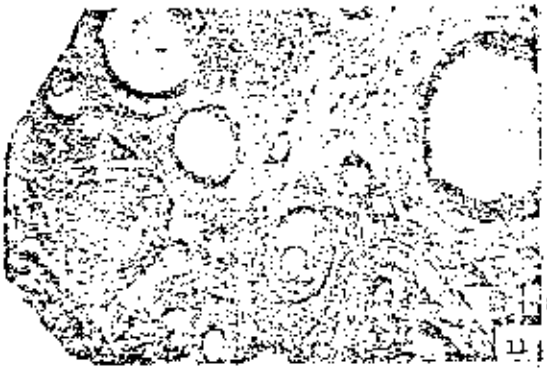
子宮：粘膜上皮肥厚，波状に腔内に突出，細胞境界不鮮明，核の配列不整

非去勢幼若群：D 2000 i. u. 連日投与

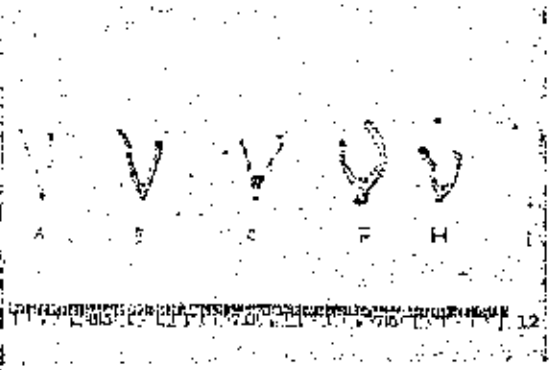


子宮：腫瘤の周囲は結合織の鞘膜に包まれる

非去勢幼若群：D 10000 i. u. 連日投与



卵巣：大卵胞気，中小卵胞の退行，原始卵胞多数，間質結合織増加充血をみる



幼若シロネズミにD少量，OvH 投与による子宮の肉眼所見
A：非去勢動物に新製ゴマ油日投与
B：非去勢動物にD200 i. u. 連日投与
C：去勢動物にD 200 i. u. 連日投与
F：去勢動物にD 200 i. u. + OvH 100 i. u. 投与
H：去勢動物にOvH 100 i. u. 投与
I：去勢動物にD 1000 i. u. 連日投与

図7　非去勢幼若シロネズミに隔日D1000r.u., 2000r.u.投与による発情期像出現図

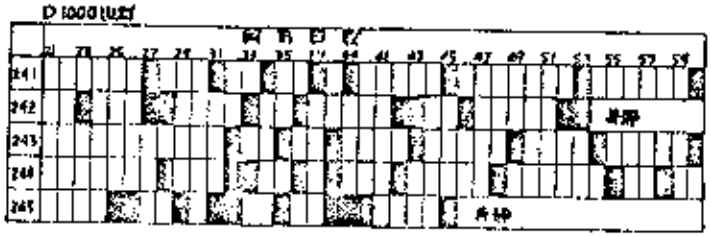


図8　幼若シロネズミD少量投与による発育曲線

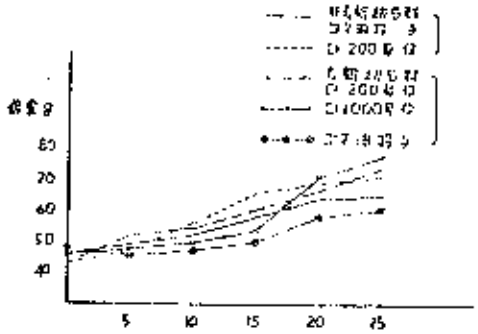


表6　去勢幼若シロネズミにたいするD投与

群	動物番号	体重(g) 初体重	終体重	観察期間(日)	発情期(日)	子宮重量(mg)
D200 r.u.	327	44	76	25	10	120
	328	46	78	25	9	130
	329	40	68	25	9	110
平均		44	74		9.3	122
D 1000 r.u.	330	44	82	25	5	41
	331	54	71	25	6	44
	332	40	73	25	7	45
平均		46	75.3		6	43.3
ゴマ油	333	44	58	25	静止	46
	334	42	60	25	静止	37
	335	52	61	25	静止	39
平均		49.3	60.0			44

線の伸展が緩で相となっており血管の充血および拡張が明瞭であった。

(3) D 10000 r.u. 群　卵巣の平均重量は8.6mg, 大小混在し中・小卵胞はほとんど退行像を示したが原始卵胞には異常がなかった。黄体は欠如し間質組織が広い部分を占めていた(写真11)。子宮は平均重量72.6mgで内腔の萎縮が著明であった。

2) D少量投与

米本式で飼育した45g前後の非去勢幼若シロネズミを2群に分け1群にはD 200 r.u.を，他群には対照としてゴマ油を連日注射して25日間観察した。

実験成績

i) 一般状態　両群とも運動活発，元気良好で著変を認めなかった。

ii) 体重増加率　D 200 r.u.群 160.9%，対照群132.4%であった(表5, 図8)。

iii) ちつ脂膜　D投与開始9日目に全例角化細胞の出現をみ，16日目よりいずれも持続的角化所見を示したが対照群ではこの期間内には角化細胞の出現をみなかった(表5)。

iv) 組織学的所見　子宮の平均重量はD投与群191.6mg, 対照群45.3mgでD投与により子宮の発育が著しく促進され(写真12-A, B)子宮内腔は拡大し粘膜上皮がやゝ肥厚し上皮細胞形成をみた。子宮腺の発育は比較的良好であったが，その内腔がせまく間質，筋層はまだうすく結合織の増殖は軽であった(写真14)。対照では子宮各層の発育がおとり内腔はせまくて管腔の発育はまだほとんどみられなかった(写真

表7　非去勢成熟シロネズミにOvH 1日1回ずつ10000 r.u.投与50日間飼育

群	動物番号	初体重 (g)	最高体重 (g)	最高体重出現時期 (日)	振歸時体重 (g)	振歸	子宮
OvH 単独	291	145	157.5	19	138	殺	子宮溜水腫
	292	150.5	169	8	124	殺	子宮溜水腫
	293	150	156	27	123	殺	子宮溜水腫
	294	148.5	152	35	143	殺	子宮溜水腫
	295	157	177.5	38	169	殺	子宮溜水腫
OvH + D	296	143.5	168	8	129.5	殺	子宮溜水腫
	297	154.5	157.5	13	130	殺	子宮溜水腫
	298	147	152.5	7	131.5	殺	子宮溜水腫
	299	151.5	165	21	130	殺	子宮溜水腫
	300	153.5	173	34	149	殺	子宮溜水腫

図9　非去勢成熟シロネズミに1日1回OvH 2 r.u.注射した群とD 1000 r.u., 10000 r.u.添加した群の発情期像出現状況

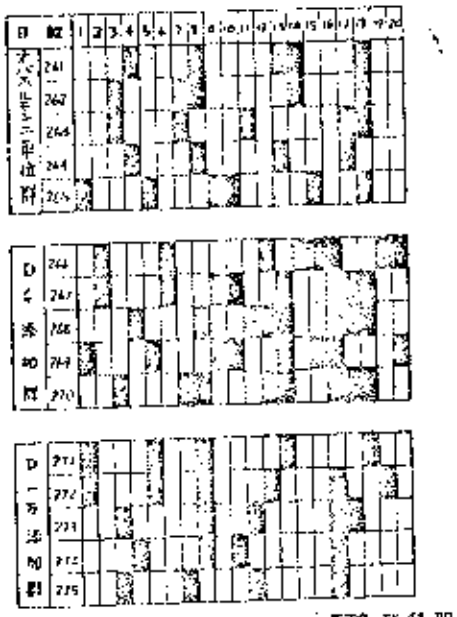
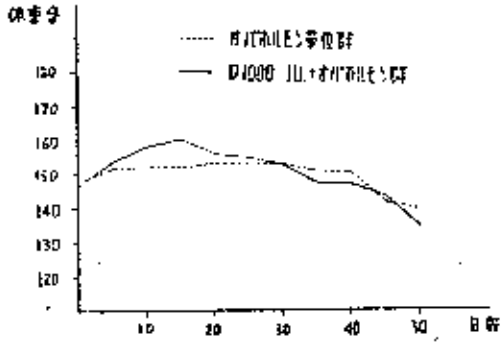


図10　非去勢成熟シロネズミにOvH 1日1回ずつ10000 r.u.投与群の発育曲線



13).

（2）　去勢幼若シロネズミのばあい

基本食で飼育した45g前後，去勢後3日目の去勢幼若シロネズミを3群に分け，それぞれD200 r.u., 1000 r.u.および精製ゴマ油（対照）を連日注射し25日間観察した．

実験成績

i)　一般状態　　3群とも運動活発，食欲良好で悪変をみなかつたがD 1000 r.u.群は他群に比し運動がやゝ不活発となつた．

ii)　体重増加率　　D 200 r.u.群168.1%，1000 r.u.群153.4%，対照群122.9%で前2者がやゝまさつていた（表6，図8）．

iii)　ちつ腟膏　　D 200 r.u.群では投与開始後9～10日目に角化細胞が出現し15日目には完全角化像を呈しそのまゝ持続した．1000 r.u.群では5～7日目で角化細胞が出現し不完全角化像が2～4日間持続し，その後静止期像となり対照群ではまつたく腟開口をみなかつた（表6）．

iv)　組織学的所見　　子宮の平均重量はD200 r.u.群122mg，1000 r.u.群43.3mg，対照群44mgで，D200 r.u.群では子宮の肥大が著るしく（写真1-C），内腔は極度に拡大し粘膜上皮の肥厚，細胞核の腫大がみられ，配列は雑然で腺胞形成はほとんどみられなかつた．間質および線の発育も良好で腺腔は円形，筋層の発育も

良好で筋線維の走行はみだれていなかつた(写真15). D 1000 i.u.群では子宮の萎縮が高度(写真12-i)で，子宮壁各層の境界不明瞭，粘膜上皮もとく萎縮し子宮腺の発育不良，かつ退行変性を認めた．また間質の退行変性と筋層の発育不良がめだち結膜下組織にガラス様変性をみた．対照群ではD 200 i.u.群より子宮壁各層の発育がおとつていた．

（3） 小　括

非去勢幼若シロネズミにたいするD大量投与のうちD 1000 i.u.群，2000 i.u.群では膣開口発現時期は対照群と差がなかつた．さらに大量の10000 i.u.投与では性周期の出現をみなかつた．去勢幼若シロネズミでは性周期が早くあらわれたが，卵巣内に完全角化像を認めずまもなく持続的発情期に移つた．

幼若シロネズミにたいするDの少量投与では膣開口発現時期に非去勢群と去勢群と差がなく全例早期に膣脂内に持続的発情期像があらわれた．組織学的にも両群とも子宮の発育の良好な所見をみた．

Ⅲ. エストロゲンの作用にたいするD投与の影響

（1） 非去勢成熟シロネズミのばあい

1） エストロゲン少量投与群

動物を3群に分け1群には1日1回OvH2 i.u.を20日間注射し他の2群はOvHと同時にそれぞれD 1000 i.u.および10000 i.u.を注射した．

実験成績

i） 一般状態　各群とも著変を認めなかつた．

ii） 性周期　OvH単独群ではとくべつの変化はなかつた．D 1000 i.u.群ではしばしば発情期の延長，短縮を認めD 10000 i.u.群ではいくぶん不整な発情期の出現をみた(図9)．

iii） 組織学的所見

a） OvH単独群　卵巣平均重量28.9mgでとくべつの変化はなかつた．黄体の増加はみられず間質組織に著変はなかつた．子宮は平均重量368.6mg，粘膜上皮は肥厚し核は腺腔側の傾向を示したが乳頭に富むあい間細胞の増殖，腺腔形成は少なかつた．子宮腺，間質，筋層の発育は良好でエオジン好性細胞の増加がつよかつた．

b） D 1000 i.u.添加群　卵巣は平均重量20mgで多数の健全な黄体形成を認めた．白色多く細胞境界は明瞭であつた．一部の黄体細胞には空胞がみられた．間質細胞はふつうであつた(写真16)．子宮は平均重量617.8mg，粘膜上皮および間質の肥厚，子宮腺，筋層の発育は良好であつた(写真17)．

c） D 10000 i.u.添加群　卵巣は平均重量25.8mgで大・中・小各卵胞とも退行し，とくに大卵胞に顕著であつた(写真18)．黄体はふるい中・小黄体が多く間質組織は粗で充血が強かつた．子宮は平均重量385.6mg，子宮壁各層ともやゝ肥厚を認めたが粘膜上皮，間質の発育は前記2群より劣つていた(写真19)．

2） エストロゲン中等量投与群

動物を3群に分け1群には1日1回OvH20 i.u.を20日間注射し他の2群はOvHと同時にそれぞれD1000 i.u.，10000 i.u.を注射した．

実験成績

i） 一般状態　各群とも著変を認めなかつた．

ii） 性周期　すべて持続的発情期像を示した．

iii） 組織学的所見

a） OvH単独群　卵巣は平均重量36.4mg，健全な大卵胞が少なく中卵胞には健全なものが多かつた．黄体は大きいものが多く新鮮黄体も認められた(写真20)．子宮は平均重量721.6mg，子宮壁はひろく粘膜上皮肥厚し子宮腺の多くは腺腔拡大を示した．子宮腺，間質の発育は良好であつた(写真21)．

b） D 1000 i.u.添加群　卵巣は平均重量41.7mg，OvH単独群に比してさらに黄体の数が多く，その大部分に腺腔形成を認め退行変性を示した(写真22)．子宮は平均重量863mg，子宮壁は肥厚，粘膜上皮細胞の肥厚をみた．子宮腺の数はOvH単独群よりやゝ少なかつたが，腺腔式大拡張がつよく間質，とくに筋層の肥厚が著明であつた(写真23)．

c） D 10000 i.u.添加群　卵巣は平均重量53mg，健全な大・中卵胞が少く小卵胞の増加をみたが多くは退行変性を示していた．黄体は中・小黄体が多く黄体の変性壊死はもつとも著明であつた(写真24)．子宮は平均重量513mg，子宮壁は広く粘膜上皮細胞の増例は不良，子宮腺の発育は不良，間質，筋層のエオジン好性細胞の出現は3群中もつとも少なかつた(写真25)．

3） エストロゲン大量投与群

動物を2群に分け1群は1日1回OvH 10000 i.u.を50日間注射し他群はOvHと同時にD 1000 i.u.を注射した．

実験成績

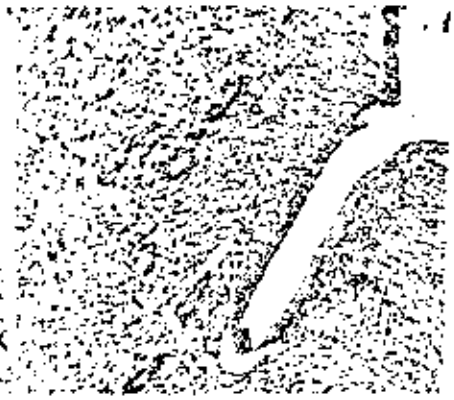
i） 一般状態　両群とも投与30日目頃になると食欲不振がひどく体重減少し運動が不活発となり下痢をおこし衰弱が著しくなつたが死亡例はなかつた(表

4382

非去勢幼若群：ゴマ油投与

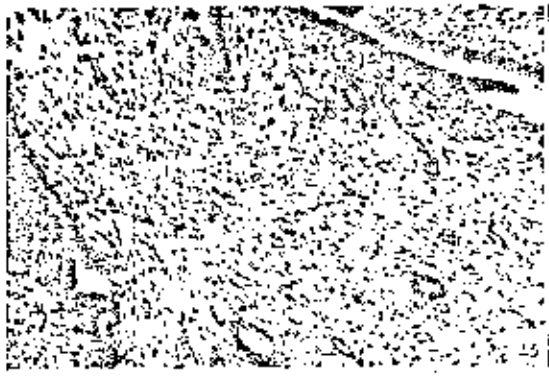
子宮：子宮各層の発育不良，子宮腺の発育全くみられず

非去勢幼若群：D 200 r. u.



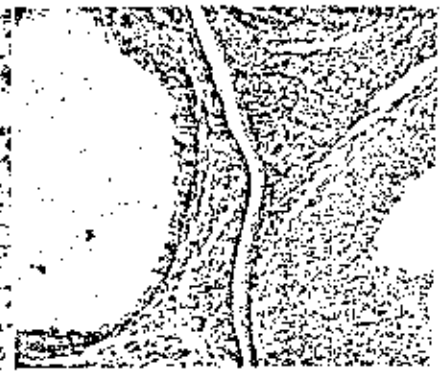
子宮：子宮壁軽度拡大，粘膜上皮肥厚，発育やゝ良好

去勢幼若群：D 200 r. u.



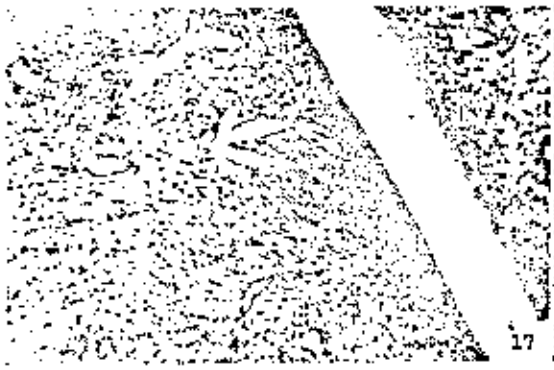
子宮：子宮腺拡大，粘膜上皮肥厚，間質，筋層発育良好，腺管円形

非去勢成熟群：OvH 2 r. u. + D 10000 r



卵巣：健全な発育卵胞，結合織界鮮明組織密である

非去勢成熟群：OvH 2 r. u. + D 1000 r. u. 添加



子宮：粘膜上皮間質の肥厚，子宮腺，筋層発育良好

非去勢成熟群：OvH 2 r. u. + D 10000

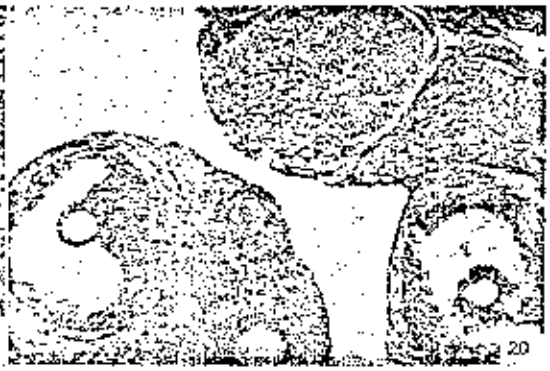
卵巣：大中小各卵胞の退行

4382

非去勢成熟群：OvH 2 i.u. + D 10000 i.u.添加

子宮：各層やゝ肥厚

非去勢成熟群：OvH 20 i.u. 連日投与



卵巣：大黄体多数

非去勢成熟群：OvH 20 i.u. 連日投与

子宮：子宮腔拡大，粘膜上皮肥厚，腺腔の拡大

非去勢成熟群：OvH 20 i.u. + D 1000 i.u.添加

卵巣：黄体の退行変性

非去勢成熟群：OvH 20 i.u. + D 1000 i.u.添加

子宮：子宮粘膜の肥厚，腺腔の拡大，間質，筋層の肥厚

非去勢成熟群：OvH 20 i.u. + D 10000 i.u.添加

卵巣：小中黄体多数，細胞の腔胞形成高度

図 11

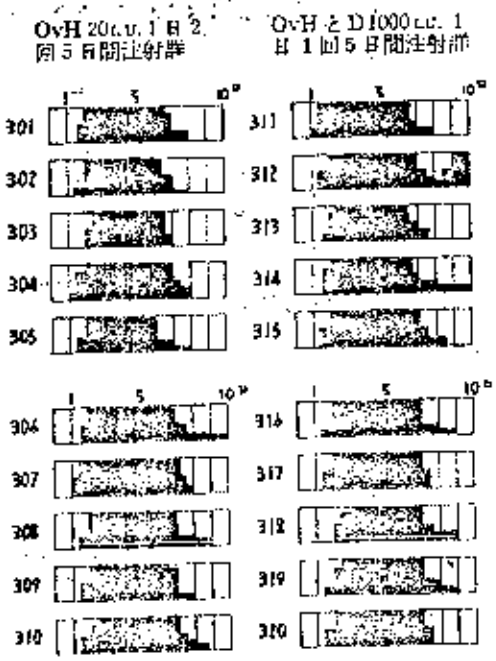

OvH 20 r.u. 1日2回5日間注射群
OvHとD 1000 r.u. 1日1回5日間注射群
OvHとD 10000 r.u. 1日1回5日間注射群
OvHとD 50000 r.u. 1日1回5日間注射群

図12 精製ゴマ油20日間注射後同時に1日2回OvH 20 r.u. ずつ5日間注射群

D 1000 r.u. 20日間注射後1日2回OvH 20 r.u. ずつ5日間注射群

7. 図10).

ii) 性周期　両群とも持続的発情関係を示した。

iii) 組織学的所見　両群間に差を認めがたく卵巣は蒼白，平滑で平均重量はOvH単独群12.4mg，D添加群は10.6mgであつた。両群とも大卵胞が欠如し中・小卵胞も大部分退行変性を示し原始細胞も少なかつた。間質は卵胞の退行により広い部分を占めていた。黄体にはわずかに小黄体を認めたが結合織の混入が著明なため間質組織との境界が判然としなかつた（写真26）。子宮はすべて淵水腫を形成し平均重量はOvH単独群1.5mg，D添加群1.7mgであつた。両群とも子宮腔が広く粘膜上皮の肥厚があつたが間質，筋層は子宮腔の拡大により圧迫されていた（写真27）。

（2） 去勢成熟シロネズミのばあい

1) エストロゲンとD同時投与群

動物を4群に分け1群には1日2回OvH 20 r.u. を5日間注射し他の3群にはOvHと同時に1日1回D 1000 r.u.，10000 r.u.，50000 r.u. ずつをそれぞれ注射して発情持続期間を観察した。

実験成績

図11に示すようにOvH単独群とD添加群との間に有意差を認めなかつた。

2) D投与後エストロゲン投与群

動物を2群に分け1群にはD 1000 r.u. を20日間注射し他群には精製ゴマ油を20間注射した。そして21日目にいたり両群とも1日2回OvH 20 r.u. を5日間注射し発情持続期間を観察した。

実験成績

図12のごとくで両群間に有意差を認めなかつた。

（3） 非去勢幼若シロネズミにエストロゲンとD少量を投与したばあい

体重45g前後のシロネズミを2群に分け第1群には1日1回OvH 100 r.u.，第2群にはOvHと同時にD 200 r.u. をそれぞれ20日間注射した。

実験成績

i) 一般状態　両群とも運動活発，食欲良好であつた。

ii) 体重増加率　第1群143.4%，第2群153.9%であつた（表8）。

iii) ちつ胞膜　角化細胞の出現は第1群ではいずれも注射開始後2日目，第2群では1～3日，平均1.3日であつた。

iv) 組織学的所見　子宮の平均重量は第1群105，第2群356.6mgで後者が明らかにまきつていたが，ともに発育がよく胞大が著明であつた。子宮腔も多数存在し発育が良好であつた。間質，筋層も肥厚し筋線維

4382

表8　非去勢幼若シロネズミに少量のDとOvH投与

群別	動物番号	体重(g) 初体重	体重(g) 終体重	角化細胞出現時期(日)	角化細胞	子宮重量(mg)	卵巣重量(mg)
D 200 i.u. OvH 100 i.u.	336	56	76	1	持続	260	36
	337	42	68	1	〃	380	30
	338	46	68	2	〃	430	38
平均		46	67.3	1.3		356.6	34.6
OvH 200 i.u.	339	44	66	2	〃	165	36
	340	44	62	2	〃	220	30
	341	45	67	2	〃	220	38
平均		47.3	68.3	2		195	34.6

表9　去勢幼若シロネズミに少量のDおよびOvH投与

群別	動物番号	体重(g) 初体重	体重(g) 終体重	角化細胞出現時期(日)	角化像	子宮重量(mg)
D 200 i.u. OvH 100 i.u.	342	51	81	3	持続	665
	343	39	76	3	〃	472
	344	50	77	3	〃	500
平均		48	78	3		545.6
D 200 i.u. OvH 10000 i.u.	345	52	74	3	持続	572
	346	54	72	3	〃	520
	347	50	70	3	〃	566
平均		52	72	3		550.6
OvH 100 i.u.	348	44	66	3	持続	206
	349	40	62	4	〃	380
	350	50	77	4	〃	400
平均		44.6	68.3	3.6		348.6

の走行は明らかで細胞核は丸味をおびていた。卵巣の平均重量は第1群34.6mg，第2群35.6mgで差がなく，とくに第2群では健全な発育卵胞，黄体が多く第1群では発育および成熟卵胞は多かつたが黄体が比較的少いようであつた。

(4)　去勢幼若シロネズミにエストロゲンとD少量を投与したばあい

去勢後3日目の体重48g前後のシロネズミを3群には第1群には1日1回OvH 100 i.u.，第2群にはOvH 10000 i.u.と同時にD 200 i.u.を，第3群にはOvH 100 i.u.と同時にD 200 i.u.をそれぞれ5日間注した。

実験成績

i)　一般状態　第2群ではしだいに運動が不活発となり10日目以降より下痢を認めたが他群には異常がなかつた。

ii)　膣脂膜　角化細胞の出現はD併用群にいくぶん早いようにみえたが，いずれも6～7日目には持続的完全角化像となつた(表9)。

iii)　組織学的所見　子宮は3群とも肥大し平均重量は第3群545.6mg，第2群550.6mgで第1群の348.6mgがもつとも小であつた。子宮内膜は3群とも水腫様に肥大し粘膜上皮はともに肥厚を明，とくに第3群では肥厚が高度で増殖傾向がつよかつたが反面一部には棒酸壊し膜のみ残存し，あるいは病胞形成をきたし退行像に近い所見もみられた。間質には結合織組織が多く子宮腺の発育はきわめて良好，前層の発育もよく細胞核は膨大していた。第2群では子宮粘膜の肥厚はあつたが増殖傾向はほとんどなく粘膜上皮の脱落があり上皮細胞核は肥大しクロマチンに乏しく核は大小乱雑に配列していた。間質は粗となり腺の増加はみられなかつたが，筋組織だけは肥大し核が大きく透明を呈していた。

(5)　小　括

成熟シロネズミでは少量のエストロゲンとD 1000 i.u.の併用はエストロゲン単独投与よりも発育期間の頻発をまねくが，さらにDをふやして10000 i.u.にすると，むしろ抑制的傾向があらわれた。また去勢幼若シロネズミにOvH 10000 i.u.を投与し，同時にD 200 i.u.を追加するとOvH単独投与群よりも早期に膣脂膜に角化細胞の出現を認めた。

組織学的には成熟群のD 1000 i.u.添加例では健全な

発育ならびに成熟卵胞の存在，黄体の増加，子宮各組織の肥大，肥厚をみたが，D 10000 i.u. 併用例では卵巣に退行変化の強まる傾向がみられた．去勢幼若動物の子宮ではとくにD 200 i.u. 併用群でエストロゲン作用の増強がみられた．

考　　察

(1) 去勢とD欠乏およびエストロゲン

Guggisberg[14](1929)は女性骨軟化症では性機能の低下をみずとしたがNeuweiler[15](1940)はD欠乏は性器の萎縮，卵巣の濾胞変性をまねき下垂体前葉にも変化をきたすとのべている．加藤(1932)，都筑[2](1933)，渡辺[3](1932)，橋本[4](1951)などはシロネズミではD欠乏により性周期が持続的静止期に移行するとのべ，Richter[16](1943)も同様静止期像を認めたが紫外線照射により性周期が回復したとのべている．茅根[17](1960)はD欠乏が雄性動物の性機能を障害し性器の萎縮をまねき，とくに幼若動物にその影響が大きいことを報告した．

去勢とDとの関係については島村，松葉は生後60日以内のシロネズミを去勢すると長骨の発育が不良になるといい，青柳[9](1930)はクル病食餌を与えた動物では去勢により発病が促進されるとのべ，またLandauer[10](1919)はエストロゲンがクル病発生にたいして抑制的役割を演ずることを実験的に証明した．

わたくしの実験成績からみるとD欠乏幼若シロネズミでは幼若群，成熟群ともに去勢例の発育に非去勢例に劣り，このさいべつにDを補給したものでも去勢例は非去勢より発育が劣っていた．

組織学的には去勢D欠乏例に幼若，成熟群とも子宮に顕著な萎縮像を認め対照のD添加群では萎縮の傾向が軽度で，とくに幼若群ではD投与により子宮壁の肥厚を認め腺腔内に充血像をみたものもあった．かゝる所見は学者がDは雄性性器にたいして増強促進的に作用するとのべた見解に一致するものである．

D欠乏とエストロゲンにかんする研究は少なく Richter[16](1943)はD欠乏シロネズミの症状，外観が下垂体切除ネズミとの間に共通性を有するのに気づきDは下垂体前葉の刺激により間接にその効果を発揮するものと考えられるとのべ，Aghdhr[18](1935)はマウスに照射コルゴステロールを投与すると下垂体前葉を刺激し卵巣の増加をきたすと報告しDと下垂体前葉との関連性を認めている．

上記わたくしの実験成績からただちにそれらの点を決定づけることはむりであるが少量のD投与はエストロゲンの効果をより有効ならしめるものと思われ，このことは量の実験により実証されたわけである．

(2) 幼若シロネズミにたいするD投与実験

Adler・Fröhlink[9](1929)はシロネズミにDを投与して発情期の頻発をみ，Dodds[19](1934)は去勢シロネズミに大量のDを投与して発情期像の出現と子宮の肥大を認め，DにはエストロゲンにたいするDの作用があるとのべ，また都筑[3](1933)はシロネズミに少量のDを与えると性周期が正調となるものが多いと報告し，渡辺[3](1932)は少量のビタミン-D投与は発情期の頻発をきたし雄性性器に増強的に作用するが中等量たいし大量では退行変像を認めたといい，茅根[17](1960)は去勢シロネズミにD大量投与実験（1日10000 i.u.）を行ない発情期に所に発情期像を認め子宮，膣にも組織学的にエストロゲン投与時所見と同様の所見をえている．

動物にたいするDの影響にかんしては都筑[3](1933)が胚胎期の組織学的所見よりみて生殖腺にたいするDの特異作用をみとめずと報告している．

わたくしは非去勢幼若シロネズミにDの大量を投与したが性周期変現はD投与例と対照群との間に差をみなかった．そしてD投与続行により発情期像の頻発ないし持続をきたし，なおも投与を続けると，まもなくかえって発情期像の消失，さらには停止をきたすことを認めた．またD 10000 i.u. を投与したものではついに性周期の出現をみとめず，これはちょうど茅根[17](1958)が去勢成熟動物について報告したところとことなっている．しかもこれら性周期の障害された時期においてもなお動物は体重減少やD過剰症の徴候をしめさなかった．

組織学的には不整あるいは停止した性周期に応じて卵巣に大なり小なりの退行変化を認めたが子宮ではD 1000 i.u. および 2000 i.u. 群では萎縮の傾向がなく腺腔の形成をみた．これはDの増強促進的作用にもとづくものと考えられる．さらにD 10000 i.u. では逆に性機能は抑制され子宮各層の萎縮像が著明であった．

去勢幼若シロネズミに大量のDを投与すると非去勢群より早く腟開口を示したが，腟塗抹には完全角化像をみることがなくまもなく持続的静止期となった．

組織学的に子宮は明瞭な萎縮像を示し子宮腺の減少，子宮筋は一様に発育不良，間質の増加をみた．これら退行変像を認めた反面，一部分には充血増強傾向と思われる子宮腺もみられた．つまり去勢幼若動物にDの大量を投与するとさいしょ一時的には刺激作用が

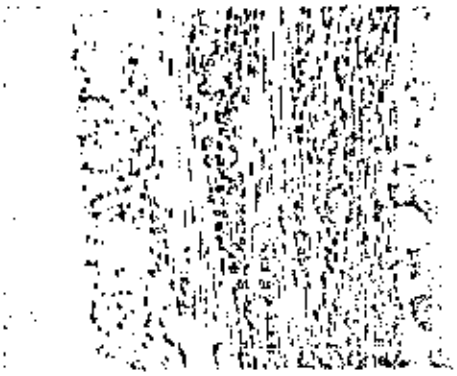
非去勢成熟群：OvH 30 r. u. ＋D10000 r. u. 添加

子宮：子宮壁広く上皮性増殖型不正，腺の発育不良

非去勢成熟群：OvH 10000 r. u. ＋D 1000 r. u.

卵巣：中小卵胞卵胞多数，原始卵胞は少ない

非去勢成熟群：OvH 10000 r. u. ＋D 1000 r. u.



子宮：間質，腺腔，子宮壁の肥大により圧迫

みられる卵巣は去勢本来の目的を損しまないことがわかる。

幼若シロネズミにたいするDの少量投与では睡眠に発現時期に差異が認められ，成熟群ではなく幼若群に腟開口に持続時の発情徴候がみられ，子宮の肥大がみとめられる。このことは前の実験実験の項でDの少量添加が雌性生殖器にたいしてとくに幼若期に増殖作用亢進効に働くという所見を与えるものであり，さらにDodds[9](1931), Freedman[10](1951), 宇田[11](1960)らのDにはエストロゲン様作用があると述べているところと一致する所見であるといえよう。

(3) エストロゲンの作用とD投与

Dとエストロゲンとの関係についての報告はなくAdler-Bellink[12](1939), Dodds[9](1931), Freedman[10](1951), 宇田[11](1960)らはDにはエストロゲン様作用があるとのべているにすぎない。

わたしは成熟シロネズミに低単位のOvHとD 1000 r. u. を併用するとOvH単独群より発情期が頻発し，さらに高単位のD(10000 r. u.)ではむしろ反対に抑制的効果をみた。また去勢幼若シロネズミでもOvH100 r. u. とD 200 r. u. の併用はOvH単独群より早期に腟開口の亢進刺激作用をきたし組織所見もこれと一致した。すなわちD 1000 r. u. 併用成熟動物では卵胞，黄体の発育増強傾向を認め去勢幼若D 200 r. u. 併用群では子宮各層に著明な増殖所見を認めた。しかしD 10000 r. u. 併用群では発育程度に減少を，黄体に著明な退行性変化を認めた。去勢幼若群OvH増量(10000 r. u.)併用例でも同様の所見をみし子宮は著明に萎縮し，卵巣に退行性変化を認めた。

これらの所見は前のD単独投与実験の項でDの少量添加が雌性生殖機能にたいして促進的に働くという実験成績とは異なりDの少量にOvH増量併用例ではOvH単独群より雌性性腺刺激増強効果は弱る傾向を認めDはエストロゲンの作用にたいし一定の量的関係において促進的作用を有し大量のエストロゲン，大量のD投与は反対にたがいに抑制的に作用することがわかる。

結　　論

1) D欠乏去勢シロネズミでは著明な子宮の萎縮をきたすがD投与によりこの萎縮傾向は防止される。またとくに幼若動物ではそのさい子宮の肥大を認める。

2) Dの適量投与は卵巣の発育ならびに子宮の発育をうながす。しかし投与量，投与期間の過大にかえって反対の結果をまねく危険性が推測される。

3) 幼若動物に大量のDを投与すると一時生殖器の亢

進がみられるが離開日後も投与を続けると，また全身状態のおかされないまえにすでに性機能障害があらわれる。

4)　D投与の影響は幼若動物において成熟動物よりもつよくあらわれる。

5)　Dはエストロゲンの作用にたいし一定の量的関係において促進的に作用するがきわめて大量のD投与は抑制的に作用する。

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(緑資源研究員)

文　　献

1)　関口三郎：実験医学 14, 1059 (1930)
2)　渡辺福明：北越医誌 47, 569 (1932)
3)　都筑千秋：愛知医誌 40, 3, 355 (1933)
4)　渡辺福明：北越医誌 47, 823 (1932)
5)　古澤陵八郎：日産会誌 31, 2177 (1935)
6)　吉柳　豊：実験医誌 14, 996 (1930)
7)　橋本　清：岡山医誌 54, 2212 (1941)
8)　井村俊郎：東京医誌 45, 9 (1943)
9)　Adler, K., Hüttink, E.: Geburtsh. Gynäk. 132, 19 (1929)
10)　Dodds, E. C.: Lancet 987 (1934)
11)　Freedman, Hathan: Am. J. Obst. Gynec. 62, 1273 (1951)
12)　Agduhr: Z. Vitaminforsch. 4, 74 (1935)
13)　Richter, Rice: Am. J. Physiol. 139, 693 (1943)
14)　Guggisberg: Ges. Gynäk. Geburtsh. 37, 401 (1936)
15)　Landauer, W.: Endocrinology 55, 636 (1954)
16)　Neuweiler W: Geburtsh. Gynäk. 109, 322 (1940)
17)　茅野義平：ビタミン 14, 3 (1958)
18)　茅野義平：ビタミン 19, 3 (1960)
19)　佐藤友義・茅野義平：産婦の世界 9, 11 (1957)

コリン生合成におけるメチル供与体

コリン生合成にかんする Stekol, J. A. (Am. J. Clin. Nutr. 6, 200, 1958) の仮説を検討するためその中間体と考えられる化合物についてヒナノンチョウのヒナのコリン欠乏による骨変形および体重減少にたいする効果をメチオニン存在下で試験した。その結果 Aminoethanol, HCHO を反応させた Aminoethanol, Hydroxymethylserine はいずれも無効で Monomethylaminoethanol は骨変形にコリンと同等の効力を示したが発育では劣り Dimethylaminoethanol は骨変形，発育ともにコリンと同等の効果を示した。この結果メチル供与体としてベタイン，メチオニンを与えても Aminoethanol は無効であつたことからみてメチル受容体はむしろ Cephalin に近い化合物ではなかろうかと思われる。(Vohra, P., Lanyer, B. W., Kratzer, F. H.: Poultry Sci. 39, 626, 1960)

(鈴田)

ニワトリの *Coccidium* 病とビタミンA栄養

Eimeria tenella および *E. acervulina* を人工感染させたニワトリに種々量のAまたはβ-カロチンを投与し発育，飼料量および肝中A量に及ぼす影響を観察した。飼料中8000i.u./lbのAを与えると800i.u./lbのものより食欲が回復し発育も良好であつた。感染1週後の肝中A量は正常対照動物よりも著しく低かつた。β-カロチン投与は飼料量，発育，肝中A量においてA投与のばあいよりも劣つていた。Coccidiosisの程度はA投与量にかゝわらず大差がなかつたがA量を増すと生残つた動物の発育が良好となつた。肝中のAが0.8～0.9 i.u./g に低下すると，その原因がA摂取不足であつても *Coccidium* 感染であつても運動失調を来すものである。(Erasmus, J., Scott, M. L., Levine, P. P.: Poultry Sci. 39, 564, 1960)

(鈴田)

To Dr. Sassoon
Just to see Aerosthes
See Rotty & Reidy

Israel J. Med. Sci
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ALTERATIONS IN THE MINERAL COMPOSITION AND METABOLISM OF RAT FETUSES AND THEIR PLACENTAS INDUCED BY MATERNAL HYPERVITAMINOSIS D_2

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ABSTRACT

Four thousand, 20,000 and 40,000 IU vitamin D_2 were given daily by intragastric intubation to nonpregnant and pregnant rats. The fetuses were removed on the 22nd day of pregnancy.

Hypercalcemia was seen only in the nonpregnant rats which received 20,000 and 40,000 IU vitamin D_2/day. In the group receiving 40,000 units a decrease in wet weight, ash weight and in calcium and phosphorus content of the whole fetus and of pooled fetal bones were observed. The magnesium content of fetal bone was increased. Twenty thousand and 40,000 units of vitamin D_2 reduced the size, wet weight and ash content of the placenta.

The findings suggest that vitamin D_2 may pass through the placental barrier and directly alter the mineral composition of fetal bones.

Hypervitaminosis D_2 is known to induce changes in the metabolism of calcium, phosphorus and magnesium (1-4). Pathological changes in different organs have been observed in the fetuses of mothers with hyperparathyroidism or hypervitaminosis D (5, 6). Alterations in organogenesis, litter size and placental structure, a decrease in fetal weight and reduction in the size of maternal and fetal parathyroids have been reported in rats as a consequence of maternal hypervitaminosis D (5, 7, 8).

Changes in the estrus cycle, fertilization and interference with the course of pregnancy have been observed. High doses of the vitamin given from the day of mating or a few days before or after fecundation modified the structure of decidua, altered the process of implantation, and produced degeneration and resorption of the implanted blastocysts. Administration after the fifth day of pregnancy does not alter gestation, and apparently normal birth occurs (8).

The purpose of the present investigation was to study the effect of hypervitaminosis D_2 on the mineral composition of rat fetuses, fetal bones and placentas and on the maternal serum levels of calcium and phosphorus.

MATERIAL AND METHODS

Four thousand, 20,000 and 40,000 IU of vitamin D_2 (Teva, Israel) in 1 ml of olive oil solution

the fetus. It might be presumed that vitamin D passes through the yolk sac placenta and later is transferred by the internal vitelline membrane. It has been reported that lipids pass to the rat fetus through the yolk sac placenta (17). It has also been shown that damage to the internal vitelline membrane facilitates the transfer of teratogens in mice, although the chorionic placenta does not show structural damage (18). A much larger amount of vitamin D was found in fetuses of rabbits treated with high doses of vitamin D than in control fetuses (19). In our experiment the effect of 40,000 units vitamin D_2 was more pronounced on the fetuses than on the mothers. The maternal serum calcium and phosphorus levels remained within the normal range. Retardation of fetal development and changes in placental structure were present as indicated by reduced fetal and placental weight. No evidence was found that large amounts of minerals were deposited in the affected fetus, and this suggests that the fetal maldevelopment was not caused by large amounts of calcium but by a direct effect of vitamin D_2, probably at a cellular level (20).

Twenty thousand units of vitamin D_2/day administered prior to mating produce changes in the estrus cycle. The same dose administered shortly after fecundation will prevent pregnancy (8). These effects of the vitamin are presumably mediated by an imbalance in hormonal status. Four thousand and 20,000 units administered from the ninth day of pregnancy had no apparent deleterious influence on fetal development. Forty thousand units did, however, cause a significant disturbance in development. Placentas, fetuses and bones were all small, and the progeny were nonviable. These results may indicate an interference with maternal hormonal balance or a direct effect of vitamin D on the fetus or both.

The processes of mineralization seem to be slowed down even more than the general development of the bone, as reflected in the reduction in ash content. Osteoporosis and micromelia in rat fetuses due to hypervitaminosis D have been described by Yukioka (6). However, in adult rats the ash percentage of osteoporotic bone is not significantly lower than that of normal bone (21). Hypermagnesemia and increased absorption of magnesium due to vitamin D in nonpregnant rats have been reported (2, 3, 22). The increased content of magnesium in the fetal bone in our experiments is consistent with this finding. An elevated magnesium content has also been reported in rachitic bone (23).

Although the mineral composition of the fetal bones in our experimental animal might suggest rickets, the histological picture of the osteogenesis did not show any evidence of rachitic bone development. Details of histological observations on the osteogenesis, fetal differentiation and placental structure will be reported elsewhere.

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REFERENCES

1. Harrison, H. E. Physiology of vitamin D. *Helv. paediat. Acta* 14: 434, 1959.
2. Harrison, H. E. and Harrison, H. C. The interaction of vitamin D and parathyroid hormone on calcium, phosphorus and magnesium homeostasis in the rat. *Metabolism* 13: 952, 1964.
3. Hanna, S. Influence of large doses of vitamin D on magnesium metabolism in rats. *Metabolism* 10: 735, 1961.
4. Frederick, A. Vitamin D metabolism physiological and pharmacological action. *Path. et. Biol.* 13: 434, 1965.
5. Potvliege, P. R. Hypervitaminosis D_2 in gravid rats. *Arch. Path.* 73: 371, 1962.
6. Yukioka, K., Yamamoto, Y., Tondike, H., Inaba, T., Miyoshi, T. and Akama, G. Experimental studies on the effect of D hypervitaminosis on rat fetuses. *J. Osaka Cy. med. Cent.* 8: 34, 1959.
7. Neuweiler, W. Vitamin D_2 Hypervitaminosis

und Schwangerschaft. *Int. Z. Vitamin-forsch.* 25: 203, 1953.

8. NEBEL, L. and ORNSTEIN, A. Effect of hypervitaminosis D_2 on fertility and pregnancy in rats. *Israel J. med. Sci.* 2: 14, 1966.
9. FISKE, C. H. and SUBBAROW, Y. The colorimetric determination of phosphorus. *J. biol. Chem.* 66: 375, 1925.
10. GRETTE, K. Micromethod for the determination of calcium and magnesium in serum. Titration with ethylene-diamine tetracetate. *Scand. J. clin. Lab. Invest.* 5: 151, 1953.
11. SCHEFFÉ, H. "Analysis of variance?" New York, John Wiley, 1959.
12. MANTEL, N. "Rapid estimation of standard errors of means for small samples." Bethesda, Md., National Cancer Institute, 1963.
13. FEASTER, J. P., HANSARD, S. L., OGTHER, J. C. and DAVIS, G. K. Placental transfer of calcium in the rat. *J. of Nutr.* 58: 399, 1958.
14. DE ALVAREZ, R. Renal glomerulotubular mechanism during normal pregnancy. I. Glomerular filtration rate, plasma flow and creatine clearance. *Amer. J. Obstet. Gynec.* 25: 931, 1958.
15. BERLINER, D. L., JONES, J. E. and SHALANIC, H. A. The isolation of adrenal like steroids from the human placenta. *J. biol. Chem.* 223: 1043, 1956.
16. PEARLMAN, W. H. and CERNEO, E. The isolation of progesterone from human placenta. *J. biol. Chem.* 198: 79, 1952.
17. EVERETT, J. W. Morphological and physiological studies of placenta in the albino rat. *J. exp. Zool.* 20: 243, 1935.
18. NEBEL, L. and HAMBURGH, M. Observations on the penetration and uptake of trypan blue in embryonic membranes of the mouse. *Z. Zellforsch.* 75: 129, 1966.
19. FRIEDMAN, W. F. and ROBERTS, W. C. Vitamin D and the supravalvar aortic stenosis syndrome. The transplacental effects of vitamin D on the aorta of the rabbit. *Circulation* 34: 77, 1966.
20. SCARPELLI, D. G., TREMBLAY, G. and PEARSE, A. G. E. A comparative cytochemical and cytologic study of vitamin D induced nephrocalcinosis. *Amer. J. Path.* 36: 331, 1960.
21. MENCZEL, J., SCHRAER, R., PARIS, G., POSNER, A. S. and LIKINS, R. C. Effect of low calcium diet on bone crystalinity and skeletal uptake of Ca^{45} in rats. *Proc. Soc. exp. Biol. (N.Y.)* 112: 128, 1963.
22. MEINTZER, R. B. and STEENBOCK, H. Vitamin D and magnesium absorption. *J. Nutr.* 55: 285, 1955.
23. VON EULER, H. and RYDBOM, M. The influence of magnesium salts on bone formation and rickets. *Biochemistry* 14: 241, 1931.

Urine Proteins in Nutritionally Induced Ovine Urolithiasis

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SUMMARY

Urine proteins were not different in yearling sheep fed alfalfa hay or a calculogenic, high-grain diet. The concentrate diet and additions of citric acid, calcium citrate, vitamin D, or chlortetracycline were self-fed 113 wether lambs. Stones developed in 55 lambs. Lambs developing calculi had significantly higher urine protein values than noncalculi lambs. Most of the urine protein was mucoprotein. Concentrate diets per se were not responsible for the increase. Differences in mineral requirements must have been involved. Vitamin D enhanced urolithiasis. Addition of calcium citrate to the calculogenic diet prevented urolithiasis and reduced urine volume, total protein, mucoprotein, and phosphotungstic acid precipitable Tyrosine excretion. Serum protein appeared in the urine only in severe, terminal urolithiasis. Protein bound hexosamine excretion and total nondialyzable solids (TNDS) were not altered by urolithiasis, but variation occurred between diets. Increased protein excretion might facilitate calculogenesis but the minerals and matrix appear as noncrystalline coprecipitates. The urolith matrix was a mucoprotein that made up 25% of the stone, had no laminar form, stained periodic acid-Schiff (PAS) positive, and had an infrared pattern similar to the TNDS. An x-ray diffraction pattern of the stones showed an amorphous rather than crystalline form.

Urinary proteins may play an important role in initiating calculogenesis in human beings. Attempts to correlate changes in urinary protein excretion with urolithiasis in ruminants have been inconclusive. Ruminant calculi contain a mucoproteineous material.[7,8,16] Increases in the concentration or total excretion or both of various fractions of urinary protein upon changing sheep and cattle from alfalfa hay to concentrate rations have been reported.[6,7,27,28,30] In these studies it was assumed that alfalfa hay is a noncalculogenic control and that the concentrate rations are calculogenic even when calculi were not produced during the experiment.[6,27] The increased protein excretion observed on concentrate rations is not necessarily a primary factor in calculogenesis. Increases have been observed even in the absence of calculi.[6,27] Increasing the concentrate to roughage ratio significantly ($P < 0.05$) increased urinary mucoprotein excretion without significantly increasing urolithiasis.[28] Dietary sodium chloride reduced urolithiasis without causing any significant change in total urinary mucoprotein excretion although the concen-

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tration was reduced.[31] The protein bound hexosamine excretion decreased slightly in one experiment[29] and increased significantly in the 2nd experiment[31] upon increasing the salt content of the diet. Finally, in the experiments where calculi were produced and mineral data are available, significant alterations in mineral metabolism were observed simultaneously with the increases in urine proteins.[7,28] The following experiments were designed to gather more information on the role of urinary proteins in ovine urolithiasis. The experimental diet of Packett *et al.* has consistantly produced high incidence of urolithiasis under Texas[24] and Indiana feedlot conditions.[14,23] Modifications of this diet afforded the opportunity to measure urine protein changes with nutrition, determine composition of calculus matrix, and measure urine protein of animals with clinical and subclinical urolithiasis as well as those free from calculi.

Experimental Procedures

Texas-bred Rambouillet wethers were used in both experiments. In experiment 1 (during fall), 2 groups of 10 yearlings previously on pasture were fed either alfalfa hay or a pelleted, concentrate diet 1 (Table 1), ad libitum. After 4 weeks, the sheep were placed in metabolism cages and fed 2.2 lb. of diet 1 or 2.8 lb. of hay. After 3 days, a 24-hour urine collection was made and immediately analyzed. It was found that small fluctuations in feed intake did not affect urinary protein excretion, so the long equilibration periods commonly used in digestion trials were unnecessary.

TABLE 1—Composition (%) of 6 Diets

Ingredient (%)	Diet 1	2	3	4	5	6
Ground grain sorghum	45	42	42	...	45	45
Ground corn	...	...	...	45	...	...
Cottonseed hulls	27	27	27	27	27	27
Cottonseed meal	10	10	10	10	10	10
Alfalfa	8	8	8	8	8	8
Molasses	10	10	10	10	10	10
Additives	...	3*	2.05**	...	...†	OTC‡

* Sodium citrate 0.2 H_2O, supplied by Chas. Pfizer, Terre Haute, Ind. ** Calcium citrate (2.725%) and 4 H_2O, 0.225% anhydrous citric acid, supplied by Chas. Pfizer, Terre Haute, Ind.

† Vitamin D_2, 200 I.U./lb. of feed, supplied by Napco Chemical Co., Harrison, N.J. ‡ Crystalline chlortetracycline hydrochloride, 10 mg./lb. of feed.

In experiment 2 (winter experiment), 112 (Table 3) fattening lambs were allotted to 6 groups and each group fed one of the 6 diets given (Table 1). The diets were mechanically mixed, pelleted, and made available in a self-feeder along with salt and warm water. The diets meet National Research Council nutrient requirements for fattening lambs. Urine samples were taken from as many sheep as possible during the 12-week trial. Collections were made as described, and lambs from at least 5 of the groups were included in each collection period. During a given collection, all lambs were given the same amount of feed. In order to further increase the reliability of the results, 2 consecutive 24-hour collections were made.

Concentration of urine proteins by ultrafiltration of dialysis against polyvinyl pyrrolidione followed by paper electrophoresis was used initially; however, ultrafiltration was time consuming, polyvinyl pyrrolidione was expensive, and most samples had only a single broad band in the α globulin region upon electrophoresis. The following procedure was developed for this study. Urine was collected in bottles containing a chelating agent (10.0 Gm. of citric acid in experiment 1 and 10.0 Gm. of a 2:3 mixture of disodium:tetrasodium ethylenediaminetetraacetate in experiment 2) to prevent sedimentation and 0.2 Gm. of sodium azide to reduce microbial growth. Duplicate 5-ml. aliquots of urine were placed in dialysis tubing (8/32-inch diameter) and dialyzed for 3 days at 4 C. against several changes of distilled water. The dialysate was transferred to a 10-ml. volumetric flask and made to volume with rinsings from the tubing. This solution, 1 ml., was used for total protein determination,[9] using crystalline bovine serum albumin as a standard.

Mucoprotein was isolated, using a modification of the seromucoid procedure of Wiazler *et al.*[4] A 5.0-ml. aliquot of diluted dialysate was treated with 20.0 ml. of 0.75 M perchloric acid, allowed to stand exactly 10 minutes, and filtered through Whatman No. 43 filter paper. Filtrate, 10.0 ml., was placed in a centrifuge tube, treated with 2.0 ml. of 5% phosphotungstic acid (PTA) in 2 N HCl, allowed to stand 15 minutes, and centrifuged. The precipitate was washed with 2.0 ml. of PTA reagent and centrifuged. After adding 1.0 ml. of water, mucoprotein was determined.[5] Total PTA precipitable protein was determined by treating 1.0 ml. of diluted dialysate with 0.2 ml. of PTA reagent, centrifuging, washing, and determining as for mucoprotein.

For determination of total nondialyzable hexosamine, 1 ml. of diluted dialysate was hydrolyzed with 1 ml. of 2 N HCl in a sealed, 12-ml., graduated centrifuge tube for 12 hours at 100 C. The hydrolysates were neutralized to a phenolphthalein end point with 4 N sodium hydroxide. Then 0.5 N HCl was added dropwise until the solution became colorless. After adjusting the volume to 4 ml., the solution was filtered through Whatman

No. 42 filter paper, and 3 ml. was placed in a 10-ml. volumetric flask, acetylated, and assayed for hexosamine.[1]

Total urinary nondialyzable solids were isolated by lyophilization and analyzed for nitrogen (micro-Kjeldahl), hexosamine,[1] tyrosine,[25] and reducing sugars.[12] All determinations except total nitrogen were run on neutralized hydrolysates. Infrared patterns were obtained using potassium bromide pellets.

The hydrolysates were chromatographed for amino acids using n-butanol:pyridine:water (1:1:1) followed by 80.0% phenol with 0.5% ammonium hydroxide. Sugar chromatograms were prepared using ethyl acetate:pyridine:water (120:56:40). The lyophilized material was subjected to cellulose acetate electrophoresis * in cells using a tris buffer ** at 3 ma. for 2 hours. The strips were stained with amido black 10 B according to Gelman Manual 17.

Fresh calculi were decalcified.[4] Some were examined histologically. Others were pooled, lyophilized, and analyzed by the methods described for total nondialyzable solids.

Oven-dried calculi were analyzed for phosphorus,[11] calcium,[23] magnesium,[27] carbonate,[5] and citrate.[24] Spot tests were made for sulfate, cysteine, and uric acid.[5] Fresh stones were dissolved in 2 N sulfuric acid for ammonia determination by the standard micro-Kjeldahl procedure. The ammonia:phosphorus ratio in the solution was used to calculate the theoretical percentage of ammonia in the oven-dry stone. X-ray diffraction patterns were made using copper radiation with nickel foil.

Results

The procedure described for determining mucoprotein consistently yielded mucoprotein values equal to about one-half of the total protein. Since, in most cases, visible precipitate was not formed upon treatment of the dialysate with perchloric acid and only one band was detected upon electrophoresis, an effort was made to determine the cause of the discrepancy between total and mucoprotein. About 15% of the total nondialyzable tyrosine was ultrafilterable and not precipitable with PTA. Sufficient protein was adsorbed on the filter paper to account for the remaining protein. The filter paper error has been studied in detail.[19] It was concluded that normal ovine urinary protein is predominantly a single mucoprotein or group of very similar mucoproteins plus a small amount of lower weight material and occasional traces of serum proteins.

Several observations supported the hypothesis that urine mucoprotein originated in the uriniferous tubules. First, albumin and other serum proteins were rarely detected in the concentrated urine either by perchloric acid precipitation or by electrophoresis. In urine samples with positive reactions to benzidine tests (blood in urine), electrophoretic patterns similar to those in serum were obtained. This indicated that the procedure used would detect serum proteins when they comprised a significant portion of the urinary protein. Since it is unlikely that one protein would enter the urine by glomerular filtration in such large quantities without others accompanying it, the bulk of normal ovine urinary protein does not appear to originate from the serum.

A second clue of ovine urinary protein origin was that fresh urine from the distended bladders of animals with urethral blockage did not have the increased protein concentration which would be expected if the proteins were being secreted by the bladder transitional epithelium. This is in agreement with Edwards *et al.*,[9-11] who

* In Spinco Model R Durrum cells, Beckman Instruments, Inc., Palo Alto, Calif.

** Arnesson buffer, pH 8.9, Gelman Technical Manual 17, Gelman Instrument Co., Chelsea, Mich.

TABLE 2—Urine Protein Excretion on Alfalfa Hay and Basal Grain Sorghum Ration

Treatment*	Urine vol. (ml./day)	Mucoprotein mg./100 ml.	Mucoprotein mg./day	Total proteins mg./100 ml.	Total proteins mg./day
Grain sorghum (10)	1,331±646	12.1	122±41	25.8	278
Alfalfa hay (10)	946±201	12.8	121±27	29.7	240

* Numbers in parentheses are numbers of sheep included in the averages.

concluded that the bladder epithelium of the dog had no role in mucoprotein secretion. The constancy of protein concentration in the distended bladders also eliminates the possibility that the accessory sex glands make a significant contribution to protein excretion. Since the protein does not appear to originate from the serum or lower urinary tract, it most likely originates in the renal tubules. An increase in PAS positive material in the renal tubules simultaneously with urolithiasis has been observed.[8]

Under our conditions there were no statistically significant differences in urine volume, protein concentration, or total protein excretion between sheep given alfalfa hay and the basal concentrate ration (Table 2). In fact, contrary to the results of Udall,[30] Cornelius *et al.*,[6,7] and Swingle and Cornelius,[27] the sheep fed the pelleted diet tended to excrete more, not less, urine than those fed hay. However, the sheep fed the pelleted diet did have much greater variation between individuals than the others. Packett and Butcher[23] found dietary sodium citrate increased urine volume almost threefold without reducing urolithiasis.

Considerable incidence of urolithiasis was produced in all groups of lambs in experiment 2 with the exception of lambs consuming calcium citrate (Table 3); they did not have any urolithiasis. Vitamin D supplementation produced the highest incidence. Similar effects from calcium citrate and vitamin D had been observed in a previous summer experiment. The calculi occurred almost exclusively in the bladder. They were usually fine, chalky particles. Occasionally, stones weighing several milligrams were found. They were primarily $Mg_3(PO_4)_2$ with some calcium phosphate and about 25% organic matter (Table 4). Stones of higher calcium content have resulted from this diet, but the dietary ingredients were from a different source. X-ray diffraction indicated that the minerals were deposited in an amorphous rather than crystalline form. This substantiates previous findings.[7]

TABLE 3—Calculi Incidence

Group	Treatment	No. of lambs	Urolithiasis: Clinical cases	Urolithiasis: Cases at slaughter
1	Grain sorghum	26	10	5
2	Sodium citrate	26	0	4
3	Calcium citrate	15	0	0
4	Corn	15	1	5
5	Vitamin D	15	6	6
6	Chlortetracycline	16	6	3

TABLE 4—Calculi Composition

Component	Percentage*
Phosphorus	15.7±0.9 (6)
Magnesium	17.9±1.0 (6)
Calcium	1.9±0.7 (6)
Organic nitrogen	1.7±0.8 (4)
Ammonia nitrogen	0.6±0.2 (6)
Volatile at 600 C.	26.3±2.3 (2)

* Numbers in parentheses are numbers of calculi analyzed; ± values are standard deviations.

Histologically, laminar forms were not found in the matrix. Instead, the matrix appeared to have enclosed and possibly cemented together the mineral matter. Erythrocytes, leukocytes, and epithelial cells were observed in almost every instance, but the number varied considerably. Histochemically, the matrix was PAS positive. Quantitative analysis detected 3% reducing sugar, 2% hexosamine, and 4% tyrosine. Chromatography indicated the amino acid and sugar composition was similar to that reported by other workers.[16-18] The amino acids detected were aspartic acid, glutamic acid, serine, glycine, threonine, alanine, valine, methionine, leucine, isoleucine, phenylalanine, lysine, arginine, histidine, and tyrosine. It is interesting that hydroxylysine, an amino acid frequently associated with calcified proteins,[26] has never been detected in stone matrix. Sugars detected were glucosamine, galactose, glucose, mannose, and a possible trace of fucose.

The matrix material contained 8.0% ni-

TABLE 5—Urinary Total Protein, Mucoprotein, and Phosphotungstic Acid Precipitable (PTA) Tyrosine Excretion in Experiment 2

Diet treatment	No. of 24-hr. collections†	Urine volume (ml./day)	Total protein mg./100 ml.	Total protein mg./day	Mucoprotein mg./100 ml.	Mucoprotein mg./day	PTA tyrosine mg./100 ml.	PTA tyrosine mg./day
A—No Clinical Urolithiasis or Bloody Urine								
Grain sorghum	22 (7)	1,347	28.0	305±56	14.5	149	21.1	228
Sodium citrate	16 (6)	1,265	30.5	329±59	15.1	159	17.6	237
Calcium citrate	24 (10)	617	54.1	259±26**	28.7	139*	40.7	198**
Corn	12 (6)	743	50.0	309±56	28.7	175	31.0	247
Vitamin D	16 (7)	911	44.7	350±43	24.5	178	32.6	259
CTC	8 (3)	766	66.0	383±91	31.0	201	43.3	263
B—Developed Clinical Urolithiasis or Bloody Urine								
Grain sorghum	6 (3)	533	142	516	33.8	150	119	537
Sodium citrate	6 (3)	2,117	30.0	442	9.6	205	15.5	328
Calcium citrate	0 (0)	.....	....	....	.....	.....	.....	.....
Corn	4 (2)	1,456	56.7	797	19.0	272	50.3	679
Vitamin D	6 (3)	976	50.3	427	23.4	220	36.3	358
CTC	12 (4)	654	70.7	473	33.9	310	60.3	406

* Significantly different from groups 1, 3, 4, 5, and 6 ($P < 0.05$). ** Significantly different from groups 1, 2, 4, 5, and 6 ($P < 0.01$). † Numbers in parenthesis are numbers of lambs from which measurements were taken. CTC = Crystalline chlortetracycline hydrochloride.

trogen, and the stones contained 1.0% organic nitrogen. Some of the latter was undoubtedly occluded nitrogenous compounds. However, assuming that the entire 1.0% came from the matrix, the stones contained an average of 13.0% matrix. This is high compared to the 2.5% reported for human calculi.[8] However, it is not unreasonable considering that the stones were only 75.0% ash and gave an amorphous x-ray diffraction pattern. Also, calculi from steers have been reported to contain 3.0 to 5.0% protein.[38]

In addition to preventing urolithiasis, calcium citrate supplementation significantly reduced total urinary protein, mucoprotein, and PTA precipitable tyrosine excretion (Table 5). To prevent secondary complications induced by acute urolithiasis from influencing the group results, data from sheep which developed bloody urine or clinical urolithiasis at any time during the 12-week experiment were tabulated separately. The data (Table 5, part A) should reflect the influence of diet treatment. There is no doubt that clinical urolithiasis causes increases in urine protein excretion over that of sheep on the same diet but not developing urolithiasis (Table 5, part B). While occasional bloody urine was encountered, some lambs with negative benzidine tests still had protein excretions of 600 mg. per day.

In 4 instances where, by coincidence, urine collections were obtained just prior to urethral blockage, electrophoresis of the

TABLE 6—Analysis of Total Urinary Nondialyzable Solids and Calculus Matrix

Group treatment	No. of samples	N (%)	Hexosamine (%)	Tyrosine (%)	Reducing sugars (%)
Total Urinary Nondialyzable Solids					
Grain sorghum	5	8.8	4.5	5.0	10.6
Sodium citrate	3	8.3	4.2	4.4	9.9
Calcium citrate	6	9.9	6.1	4.5	13.9
Corn	2	9.8	4.6	4.8	10.4
Vitamin D	4	9.0	5.1	4.7	10.0
Chlortetracycline	3	9.5	5.0	4.7	12.8
Pooled Matrix					
	1	7.6	2.1	4.3	3.0

lyophilized total nondialyzable solids showed a new band in addition to the usual one. However, even where the new band predominated, the infrared pattern and chemical analysis were unchanged from the normal results and did not approach the matrix values (Table 6; Fig. 1). The ma-

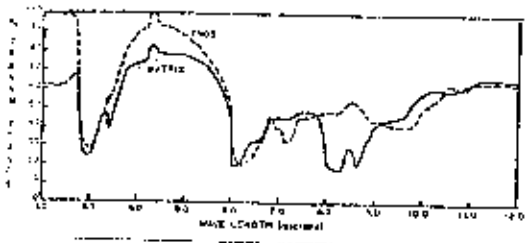

Fig. 1—Infrared patterns of pooled calculi matrix and urinary total nondialyzable solids (TNDS).

trix had a much lower carbohydrate content than the total nondialyzable solids. The infrared patterns, while similar, contain some significant differences possibly due to the reduced carbohydrate content of the matrix. The matrix may have resulted from the breakdown of normal urinary protein. It is also possible that calcification and decalcification procedure altered the matrix. Chromatography of the total nondialyzable solids showed the amino acid and sugar composition to be similar to that reported for the matrix.

TABLE 8—Urinary Total Nondialyzable Solids (TNDS) Calculated on Bovine Serum Albumin Basis vs. Lyophilized and Gravimetrically Determined

Group	Dietary treatment	No. of lambs	Total protein excretion (mg./day) Albumin basis	Total protein excretion (mg./day) TNDS basis
1	Grain sorghum	6	418	989
2	Sodium citrate	5	380	1,057
3	Calcium citrate	6	269	638
4	Corn	2	484	973
5	Vitamin D	6	370	910
6	CTC	5	355	882

CTC = Crystalline chlortetracycline hydrochloride.

Urinary protein-bound hexosamine excretion showed less variation than the nondialyzable tyrosine excretion (Table 7). This had been observed previously, so the main effort of this experiment was placed on the tyrosine measurements.

The values for protein excretion which appear in the tables were calculated relative to bovine serum albumin. When the total nondialyzable solids were lyophilized and weighed, the actual excretions were about 2.5 times as great (Table 8). Group 3 (calcium citrate) still had the lowest excretion.

Discussion

The data clearly indicate that changes in urinary proteins are associated with

TABLE 7—Urinary Protein-Bound Hexosamine Excretion

Group	Dietary treatment	No. of lambs	Total* (mg./day)	PTA** (mg./day)	Mucoprotein (mg./day)
	A—No Clinical Urolithiasis or Bloody Urine				
1	Grain sorghum	3	39.1	16.3	11.5
2	Sodium citrate	2	57.7	32.8	14.5
3	Calcium citrate	4	39.3	15.0	11.9
4	Corn	2	46.8	23.1	15.0
5	Vitamin D	3	51.2	19.4	15.4
6	CTC	2	38.9	14.6	11.6
	B—Developed Clinical Urolithiasis or Bloody Urine				
1	Grain sorghum	1	38.7	26.3	6.7
2	Sodium citrate	1	64.4	29.2	19.4
3	Calcium citrate	0	.....	.....	.....
4	Corn	1	62.4	37.1	15.8
5	Vitamin D	1	57.1	31.2	17.4
6	Chlortetracycline	1	42.3	18.2	12.1

* Total nondialyzable hexosamine. ** Total phosphotungstic acid precipitable hexosamine.
CTC = Crystalline chlortetracycline hydrochloride.

ovine urolithiasis occurring under feedlot conditions. However, the question remains, are the changes in urinary proteins the primary stimulus for gross calculi formation, or are they secondary factors? The strongest evidence that urinary proteins have a specific role in calculogenesis in man comes from the immunochemical studies[4] where a protein substance, common to all human calculi matrices tested, was found only in the urine of persons with current or recent histories of urolithiasis. However, there are some sharp differences between human and ruminant urolithiasis which presently make it impossible to automatically interchange data between the two. Human calculi are formed in acid urine and are predominantly calcium oxalate or phosphate stones. The deposits are usually quite well crystallized and the matrix well organized.[5] Also, most cases are not associated with excessive mineral excretion. Upon standing, human urine usually produces a slight sediment. In ruminants, however, stones are formed in an alkaline urine. In sheep fed concentrate diets, magnesium and calcium phosphates seem to predominate. The deposits are frequently amorphous[7] and the matrices poorly organized,[8] although Keeler[10] did find laminar structure in bovine siliceous calculi. Gross alterations in mineral metabolism have been associated with calculogenic diets.[7,14,23,25] In our preliminary experiments we tested methods of avoiding the large amount of sediment formed within a few minutes after excretion. Combinations of sodium azide, sodium glycocholate, and sodium fluoride were used to inhibit bacterial growth. The urine bottles were placed in a 37-C. water bath and purged with nitrogen. However, due to the high mineral concentration in the urine, only the use of powerful chelating agents such as sodium citrate, citric acid, and EDTA averted formation of sediment. It would seem difficult to attribute such massive sedimentation to urinary proteins.

Abnormalities in phosphorus metabolism were detected in mineral metabolism studies of the sheep used in this experiment which were much more closely correlated with a given sheep's susceptibility to calculi than was the urinary protein excretion.[14,25] It is known that high urinary phosphorus levels can cause renal tubular damage.[18,21,22] Possibly the high phosphorus excretion resulting in all sheep except those on the calcium citrate diet caused alterations in the renal tubules which resulted in increased protein excretion. Significantly higher serum and urine phosphorus levels were observed after 3 weeks on the calculogenic diets in lambs that subsequently developed gross calculi.[25] Serum magnesium also increased in all sheep on calculogenic diets.[25] Calcium citrate prevented such changes and calculi development.

Therefore, we conclude that under the conditions of our experiments, urinary proteins play only a secondary role in calculi formation. The primary role of the matrix seems to be one of cementing microcalculi together rather than one of initiating crystallization.

References

1. Boas, N. F.: Method for the Determination of Hexosamines in Tissues. J. Biol. Chem., 204. (1953): 553–563.

2. Boutwell, J. H., Elam, J. F., and Skidmore, W. D.: Biochemistry. U. S. Naval Medical School, Bethesda, Md., 1957.

3. Boyce, W. H., and Sulkin, N. M.: Biocolloids of Urine in Health and in Calculous Disease. III. The Mucoprotein Matrix of Urinary Calculi. J. Clin. Invest., 35, (1956): 1067–1079.

4. Boyce, W. H., Kinz, J. S., and Fielden, M. L.: Total Nondialyzable Solids (TNDS) in Human Urine. XIII. Immunological Detection of a Component Peculiar to Renal Calculous Matrix and in Urine of Calculous Patients. J. Clin. Invest., 41. (1962): 1180–1189.

5. Conway, E. J.: Microdiffusion Analysis and Volumetric Error. Crosby Lockwood and Sons, London, 1957.

6. Cornelius, C. E., Bishop, J. A., Berger, L. H., and Pangborn, J.: Ruminant Urolithiasis. IV. Ultrafiltration, Electrophoretic and Chromatographic Studies on Urinary Biocolloids of Sheep. Am. J. Vet. Res., 22. (Nov., 1961): 1000–1011.

7. Cornelius, C. E., Moulton, J. E., and McGowan, B.: Ruminant Urolithiasis. I. Preliminary Observations in Experimental Ovine Calculosis. Am. J. Vet. Res., 20, (Sept., 1959): 863–871.

8. Cornelius, C. E., and Moulton, J. E.: Ruminant Urolithiasis. II. Histochemical Studies in Experimental Ovine Calculosis. J. Urol., 84. (1960): 223–237.

9. Edwards, C. N., Boyce, W. H., and King, J. S., Jr.: Studies on Urothelium. I. Characteristics of Canine Transitional Epithelium Following Isolation from the Urinary Stream. J. Urol., 85. (1961): 802–809.

10. Edwards, C. N., Boyce, W. H., and Drummond, C. S.: Studies on Urothelium. II. Effect of Parathyroid Extract, Vitamin D and Dihydrotachysterol on Some Histochemical Characteristics of Canine Transitional Epithelium. J. Urol., 86. (1961): 364–366.

11. Edwards, C. N., Garver, F. K., and Boyce, W. H.: Studies of Urothelium. III. Experimental Vesical Stone Formation in the Dog. J. Urol., 89. (1963): 207–213.

12. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus. J. Biol. Chem., 66, (1925): [illegible]-400.

13. Fourman, J.: Two Distinct Forms of Experimental Nephrocalcinosis in the Rat. Brit. J. Exptl. Path., 40, (1959): 464-473.

14. Hauschild, J. P.: Calcium, Phosphorus and Magnesium Interactions in Ovine Urolithiasis. M.S. Thesis, Purdue University, West Lafayette, Ind., 1963.

15. Horvath, S. M., and Knehr, C. A.: Adaptation of the Folin-Malmros Micro Blood Sugar Method to the Photoelectric Colorimeter. J. Biol. Chem., 140, (1941): 569-577.

16. Kesler, R. F.: The Internal Structure and Composition of Siliceous Urinary Calculi of Bovine Origin. Am. J. Vet. Res., 21, (May, 1960): 428-436.

17. King, J. S., and Boyce, W. H.: Amino Acid and Carbohydrate Composition of the Mucoprotein Matrix in Various Calculi. Proc. Soc. Exptl. Biol. & Med., 95, (1957): 163-167.

18. King, J. S., and Boyce, W. H.: Analysis of Renal Calculous Matrix Compared with Some Other Matrix Materials and with Uromucoid. Arch. Biochem., 82, (1959): 178-181.

19. Ledvina, M., and Cechalova, S.: Die Adsorption der Mucoproteide des Blutserums an Filterpapier. Clin. Chim. Acta., 6, (1961): 16

20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193, (1951): 265-275.

21. MacFarlane, D.: Experimental Phosphate Nephritis in the Rat. J. Path. & Bact., 52, (1941): 17-24.

22. MacKay, E. M., and Oliver, J.: Renal Damage Following the Ingestion of a Diet Containing an Excess of Inorganic Phosphate. J. Exptl. Med., 61, (1935): 315-324.

23. Packett, L. V., and Butcher, G. A.: Effects of Dietary Sodium Citrate and Oxytetracycline Upon Fattening Lambs. J. Anim. Sci., 22, (1963): 1100-1103.

24. Packett, L. V., Watkins, T. D., and Kunkel, H. O.: Influence of Dietary Chlortetracycline on Incidence of Urinary Calculi in Sheep. Proc. Soc. Exptl. Biol. & Med., 97, (1959): 860-863.

25. Parkett, L. V., and Hauschild, J. P.: Phosphorus, Calcium, and Magnesium Relationships in Ovine Urolithiasis. J. Nutr., 84, (1964): 185-190.

26. Pies, K. A.: Amino Acid Composition of Some Calcified Proteins. Science, 134, (1961): 841-842.

27. Swingle, K. F., and Cornelius, C. E.: Ruminant Urolithiasis. V. Excretion of Certain Urinary Biocolloid Fractions by Sheep on Alfalfa and Grain Rations. Am. J. Vet. Res., 23, (Sept., 1962): 972-976.

28. Udall, R. H., Deem, A. W., and Maas, D. D.: Studies on Urolithiasis. I. Experimental Production Associated with Feeding in Steers. Am. J. Vet. Res., 19, (Oct., 1958): 825-839

29. Udall, R. H.: Studies on Urolithiasis. III. The Control by Force Feeding Sodium Chloride. Am. J. Vet. Res., 20, (May, 1959): 423-428.

30. Udall, R. H.: Studies on Urolithiasis. IV. The Effects of the Ration on the Predisposition as Measured by the Urinary Mucoproteins. Am. J. Vet. Res., 20, (May, 1959): 426-429.

31. Udall, R. H.: Studies on Urolithiasis. V. The Effects of Urinary pH and Dietary Sodium Chloride on the Urinary Excretion of Proteins and the Incidence of Calculosis. Am. J. Vet. Res., 23, (Nov., 1962): 1241-1248.

32. Willis, J. B.: The Determination of Metals in Blood Serum by Atomic Absorption Spectroscopy. II. Magnesium. Spectrochim. Acta., 15, (1963): 273-278.

33. Willis, J. B.: Determination of Calcium and Magnesium in Urine by Atomic Absorption Spectroscopy. Analyt. Chem., 33, (1961): 556-559.

34. Winzler, R. J., Devor, A. W., Mell, J. W., and Smyth, J. M.: Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation. J. Clin. Invest., 27, (1948): 604-616.

Nutrition

1st in a series of 5

Vitamin D: A Reawakening

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Clear descriptions of the clinical picture of rickets were published in the 17th century although mention of various aspects of the syndrome goes back even further. That a chemical factor could reverse the rachitic process was well settled by the mid-1920s. It is now clear that a number of sterols can be rendered antirachitic under the influence of ultraviolet irradiation. The significant sterols, in this regard, are ergosterol (derived from plant sources) and 7-dehydrocholesterol (from animals). When appropriately irradiated, these compounds form ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), respectively. Historically, "vitamin D_1" was really found to be a mixture of substances and not a separate entity.

Vitamin D in Man

Of course, irradiating many types of plant or animal sterols will produce therapeutic amounts of D_2 or D_3 that can prevent or cure rickets when ingested. But it has been known for generations that irradiating the patient himself will produce similar effects. Areas of skin in most adults contain 3% to 4% 7-dehydrocholesterol (7-DHC) and all of it is beneath the stratum corneum. Because of this unique anatomical relationship it can be seen that production of D_3 from 7-DHC can be partially regulated by the amount of natural or induced pigment in the corneum. Birds and some fur-covered mammals secrete 7-DHC onto their skin appendages. After irradiation, the activated sterol (D_3) is eaten during preening or grooming. It is noteworthy that human infants have more than twice the adult concentration of 7-DHC in their skin and that, regardless of race, their corneum is always thinner and less pigmented than that of their parents.[1]

Accepted Actions of Vitamin D

Vitamin D_3 or its metabolites, influences gut mucosal cells directly and brings about the active absorption and transport of calcium.[2] In this regard, parathyroid hormone plays a synergistic role, but vitamin D can produce the same effect in parathyroidectomized animals. If an animal is placed on a totally calcium-free diet, an injection of vitamin D will still cause an elevation of serum calcium. This response is due to the mobilization of calcium from hydroxyapatite crystals located in older bone, an osteolytic effect. Vitamin D probably has a direct effect on osteoblasts and will influence calcification of cartilage, especially in the epiphyses or other growing areas of bone. The vitamin exhibits a variety of complex direct effects on the kidney. Most of these renal effects are obscure, but vitamin D does depress renal citrate oxidation.[3] A highly active metabolite of vitamin D is also *formed* in the normal kidney.

Excess Vitamin D

Most commercial milks, baby foods, margarines, and breakfast cereals are fortified with vitamin D. When this is added to endogenous production (from exposure to sunlight) plus that *naturally* present in foods, the average American may receive several times the daily amount of vitamin D he requires. Not all persons are alike in their responsiveness to an "excessive" daily dose of vitamin D. A small number shows profound toxicity to amounts only slightly above the daily recommended dose. The syndromes of idiopathic hypercalcemia of infancy and congenital supravalvular aortic stenosis may be examples of this toxicity.[4] Such persons may metabolize the vitamin in an excessively efficient manner and thus are susceptible to vitamin D intoxication. However, in the normal patient, continued dosage exceeding 100,000 international units (IU) per day is needed to produce obvious hypercalcemia, resulting in nausea, vomiting, constipation, polyuria, polydipsia, dehydration, and generalized weakness. Metastatic calcification occurs in the kidneys of such patients and their plasma cholesterol levels may rise.

Prophylactic Requirements

Nutritional rickets or osteomalacia, in the absence of intestinal malabsorption, is now rare in the United States. Infants, women in the latter half of pregnancy, and lactating mothers have the highest vitamin D requirements. These individuals may require up to 400 IU/day as oral vitamin D supplements. Most other persons certainly need no more than 400 IU of vitamin D per day *from all sources*.[5] Intermittent exposure to sunlight plus ingestion of the ordinary American diet will easily provide this amount to all except the recluse who is also a strict vegetarian. It has been estimated that the total minimum requirement for the normal adult is only about 70 IU of vitamin D per day.[6] It must be mentioned that, because of melanin pigment interference with irradiation, members of dark-skinned races convert 7-DHC to cholecalciferol (D_3) less readily than light-skinned persons under equal sunlight exposure.

Newer Concepts

Until relatively recently it was universally accepted that ergocalciferol (D_2) or cholecalciferol (D_3) were directly active in the whole animal, producing their various antirachitic effects without further metabolism. During the past five years, Deluca[7] and several of his colleagues have described and synthesized a highly active metabolite of D_3, 25-hydroxycholecalciferol (25-HCC). That substance (Fig 1), is formed in the liver, is less lipid-soluble and more polar than D_3, and is more potent in antirachitic activity *in vivo*. 25-HCC will stimulate calcium resorption in bone tissue culture where D_3 produces no effect.[8] There is also evidence that 25-HCC promotes the active transport of

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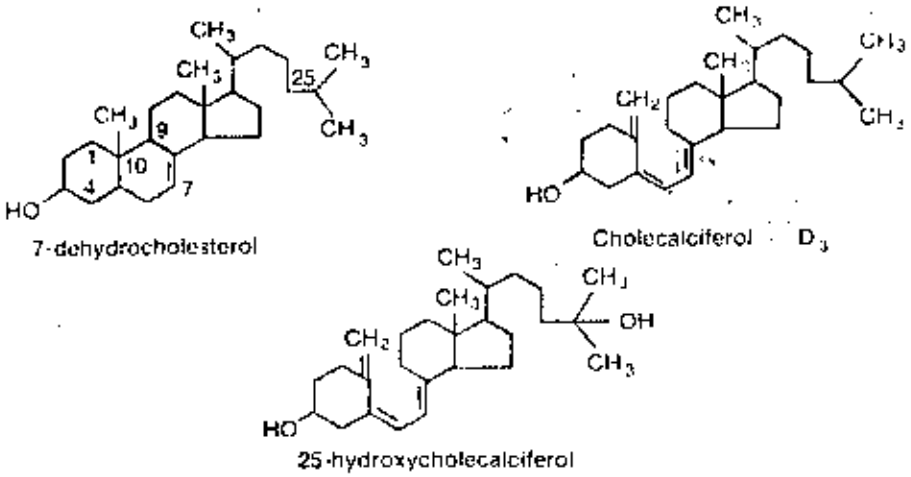
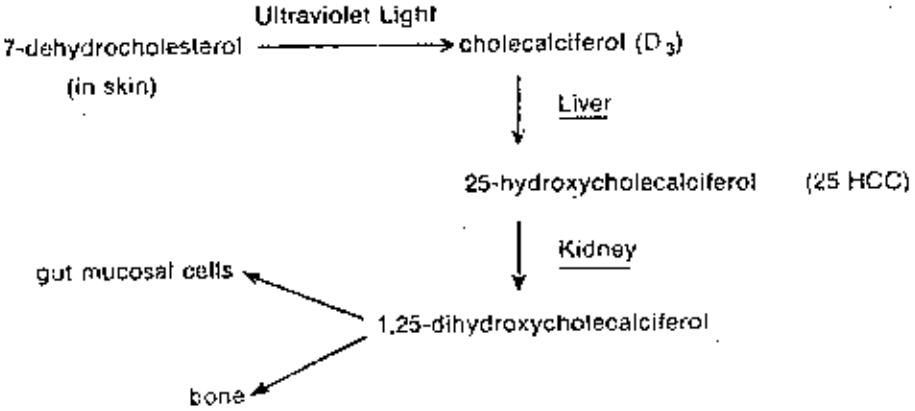


Fig 1.—Structural formulae of vitamin D and associated compounds.

Fig 2.—Flow diagram of probable events in metabolism of vitamin D.



calcium from gut lumen to serum.[7]

For some time it was held that 25-HCC was the active circulating form of vitamin D.[7] But now, additional evidence has been provided by Fraser and Kodicek[8] and Gray and associates[9] that an even more polar metabolite of 25-HCC is produced in kidney tissue and that this latter substance is very likely the most active form of vitamin D in influencing intestinal absorption of calcium and bone metabolism (Fig 2). This highly active polar metabolite has now been shown to be 1,25-dihydroxycholecalciferol.[10] These findings may well explain the mechanism of osteodystrophy or rickets in some patients with chronic renal disease.[11] Concurrently with these research breakthroughs in vitamin D metabolism, considerable light is emerging in regard to the complex series of events that "active" vitamin D initiates in the intestinal mucosal cells.[12,13]

As these research endeavors become further extended and characterized, physicians will be better able to understand a number of clinical situations manifested by disturbances in calcium economy. An acquired kidney disorder, renal osteodystrophy, was mentioned above. Other forms of vitamin D "resistant" rickets can now be partially explained by considering the various steps in the metabolism or binding of vitamin D. In the syndrome of vitamin D dependency, the cellular mechanisms for calcium absorption in the gut are operating, but the patients require large doses of vitamin D to prevent rickets.[14] Presumably these people have an autosomal recessive trait characterized by inability to produce adequate amounts of 25-HCC unless given extra D. This subject has been recently commented on in more detail by Scriver.[1]

At the opposite end of the spectrum are several hereditary or congenitally acquired clinical abnormalities characterized by marked hyper-reactivity to doses of vitamin D only very slightly in excess of recommended daily doses: hypercalcemia of infancy, supravalvular aortic stenosis, renal acidosis, and mineralized arteriosclerosis of infancy). Seelig[4] published a provocative review of this aspect of vitamin D dysfunction in 1969. It may eventually be shown that some of these children have an enhanced capacity to convert D to more active metabolites, eg, 25-HCC, on a genetic basis. Even more interesting is a report that barbiturates are capable of inducing liver microsomal enzymes that accelerate this conversion in the liver.[17]

In any event, the recent breakthroughs in the understanding of vitamin D metabolism will certainly encourage additional clinical research.[18] If and when the various active metabolites become available in adequate amounts, new therapeutic agents will be ready for clinical use.

References

1. Loomis WF: Skin-pigment regulation of vitamin D biosynthesis in man. *Science* 157:501-506, 1967.
2. Scriver CR: Vitamin D dependency. *Pediatrics* 45:361-363, 1970.
3. DeLuca HF, Steenbock H: An *in vitro* effect of vitamin D on citrate oxidation by kidney mitochondria. *Science* 126:258, 1957.
4. Seelig MS: Vitamin D and cardiovascular, renal, and brain damage in infancy and childhood. *Ann NY Acad Sci* 147:527-552, 1969.
5. Committee on Nutrition, American Academy of Pediatrics: The prophylactic requirement and the toxicity of vitamin D. *Pediatrics* 41:512-521, 1964.
6. Dent CE, Smith R: Nutritional osteomalacia. *Q J Med* 38:195-209, 1969.
7. DeLuca HF: 25-Hydroxycholecalciferol. *Arch Intern Med* 124:442-450, 1969.
8. Trummel CL, et al: 25-Hydroxycholecalciferol: Stimulation of bone resorption in tissue culture. *Science* 163:1453-1454, 1969.
9. Cousins RJ, DeLuca HF, Gray RW: Metabolism of 25-hydroxycholecalciferol in target and nontarget tissues. *Biochemistry* 9:3649-3652, 1970.
10. Fraser DR, Kodicek E: Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature* 228:764-766, 1970.
11. Gray R, Boyle I, DeLuca HF: Vitamin D metabolism: The role of kidney tissue. *Science* 172:1232-1234, 1971.
12. Holick MF, Schnoes HF, DeLuca HF: Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D, metabolically active in the intestine. *Proc Nat Acad Sci USA* 68:803-804, 1971.
13. Raisz LG: A confusion of vitamin D's. *N Engl J Med* 287:926-927, 1972.
14. Kimberg DV: Effects of vitamin D and steroid hormones on the active transport of calcium by the intestine. *N Engl J Med* 280:1396-1405, 1969.
15. Norman AW: The mode of action of vitamin D. *Biol Rev* 43:97-137, 1968.
16. Arnaud C, et al: Vitamin D dependency: An inherited postnatal syndrome with secondary hyperparathyroidism. *Pediatrics* 46:871-880, 1970.
17. Hahn TJ, et al: Phenobarbital-induced acceleration of vitamin D metabolism. *J Clin Invest* 49:39 (abstract), 1970.
18. DeLuca HF: Vitamin D: A new look at an old vitamin. *Nutr Rev* 29:179-181, 1971.

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Renal function and histology after long-term vitamin D therapy of vitamin D refractory rickets

Since large amounts of vitamin D are potentially nephrotoxic, renal function and histology were studied in 14 patients with vitamin D refractory rickets treated with high dosages of vitamin D for long periods. One had previously had severe and protracted hypercalcemia; this patient showed altered renal function and histology. Renal function was normal in the other patients, but one had small amounts of microscopic medullary calcification. It is concluded that vitamin D therapy is relatively safe if strictly controlled to avoid hypercalcemia. Significant permanent lesions causing impaired renal function occurred only after severe and prolonged hypercalcemia.

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THE VALUE of vitamin D in treating various types of vitamin D refractory rickets is established, but the amount required to produce significant benefit is very large. Often, the therapeutic dose for a given patient is close to the amount that produces a toxic response, the individual tolerance varying from case to case. An excessive intake of vitamin D produces hypercalcemia with which is associated impairment of renal function ranging from temporary loss of concentrating capacity[2] to irreversible renal insufficiency and nephrocalcinosis.[3-5] The degree

4473

and permanence of the renal impairment are related to the magnitude and duration of the hypercalcemia.

For several years, a number of patients with vitamin D refractory rickets of various types have been regularly attending the outpatient service of this hospital for treatment with large doses of vitamin D, and during this time they have received close clinical and laboratory supervision. In view of the potential hazards of vitamin D, it was decided to assess the renal function and structure in the entire group and to correlate the findings with the duration and intensity of treatment and with episodes of hypercalcemia. In particular, it was important to establish whether, over a period of time the large amount of vitamin D used in these patients had produced insidious renal injury in spite of overt normocalcemia and absence of vitamin D intoxication.

MATERIAL AND METHODS

Patients and methods of treating vitamin D refractory rickets. The series consisted of 14 patients, 8 boys and 6 girls (Table I); 11 had hypophosphatemic vitamin D refractory rickets of the simple type, and 3 had vitamin D dependent rickets with aminoaciduria. The classification and diagnostic criteria used at this hospital have been defined in previous publications.[6, 7] The ages of the patients ranged between 7 and 16 years at the time of the renal studies.

Table I. Clinical status of the patients

Case	*Sex*	*History No.*	*Type of refractory* rickets*	*Chronological age (years)*	*Height age† (years)*	*Dose of vitamin D_2 (I.U./day)*
1	M	183471	Aminoaciduria	16	12½	50,000-70,000
2	F	247040	Aminoaciduria	15	11½	75,000-100,000
3	F	244412	Simple	16	12½	50,000-175,000 (stopped at age 14 yr.)
4	F	253823	Simple	15	11½	100,000-125,000
5	M	198426	Simple	14½	11	75,000-100,000
6	M	271139	Simple	11½	9½	75,000-175,000
7	F	436138	Simple	13½	10½	100,000
8	M	397715	Simple	11	8	100,000-125,000
9	M	302011	Simple	11½	6½	100,000-125,000
10	F	266997	Simple	12½	11½	75,000-100,000
11	F	317400	Simple	12½	7½	150,000
12	M	395252	Aminoaciduria	7½	6	25,000-50,000
13	F	398432	Simple	7	5	25,000-50,000
14	F	523188	Simple	14	5	250,000

Normal values

*Abbreviations for types of refractory rickets: simple = vitamin D refractory rickets of the simple type[6, 7]; aminoaciduria = vitamin

†From data of Stuart, H. C., et al., Department of Maternal and Child Health, Harvard School of Public Health, Boston, Mass.

‡Mean values on treatment.

4473

All patients attended the Metabolic Outpatient Clinic of this hospital, and their management was supervised continuously by one of us (D.F.). With one exception (Case 13), treatment was initiated at the clinic, and uniform records of physical, radiological, and biochemical progress were available from the time of diagnosis.

All patients were treated with large doses of vitamin D_2 by therapeutic procedures described elsewhere.[6, 8] At the time of study, 11 patients had received therapy for 8 years or more, 2 for 6 years, and one for one year. Treatment was begun with an oral dose of 25,000 to 50,000 I.U. vitamin D per day. At the initiation of medication, each patient was seen in clinic every month, subsequently less frequently. On each occasion, the effect of therapy was assessed by history, physical examination, radiological study of the skeleton, and determination of the serum calcium and inorganic phosphorus levels and the alkaline phosphatase activity. The dosage of vitamin D was increased stepwise as indicated by these studies. When radiological and biochemical evidence of satisfactory improvement in the rickets had become apparent, the dosage of vitamin D was kept constant. In the early stages of treatment and whenever the dose of vitamin D was changed, the serum calcium level was measured at intervals of 4 weeks, and later was measured every 2 to 3 months, never less frequently than every 6 months.

Duration of therapy (years)	*Serum Ca‡ (mg./100 ml.)*	*Serum P‡ (mg./100 ml.)*	*Serum alkaline phosphatase‡ (K.A. units)*	*Hypercalcemic episodes*
14	9.3	2.2	35	0
12½	9.0	3.9	12	Age 6 yr.: Ca 16.6 mg./100 ml. had returned to normal in 4 mo.
12	8.9	1.2	11	Age 5 yr.: a single Ca of 12.8 mg./100 ml.
11½	9.6	1.2	15	Age 6 yr.: Ca 15.7 mg./100 ml. had returned to normal in 6 mo.
10½	9.4	2.5	22	Age 12 yr.: Ca 14.1 mg./100 ml. had returned to normal in 4 mo.
9½	9.0	2.9	25	0
9½	9.4	2.3	18	Age 11 yr.: a single Ca of 11.2 mg./100 ml.
9	9.8	2.7	26	0
9	10.0	3.0	20	Age 6 yr.: a single Ca of 11.1 mg./100 ml.
8½	9.9	2.5	17	Age 6 yr.: Ca 14.3 mg./100 ml. had returned to normal in 1 mo.
8	9.6	2.1	16	0
6½	9.0	5.2	19	Age 14 mo.: Ca 15.8 mg./100 ml. had returned to normal in 10 days
6	9.7	2.6	14	Age 1 yr.: severe, protracted hypercalcemia (see text)
1½	9.3	1.9	19	0
	9.0-10.5	3.0-5.5	10-20	

D dependent rickets with aminoaciduria.[5, 29]

Since, according to the regimen described, the serum calcium level was the most important index of excessive vitamin D dosage, the accuracy of the measurement of serum calcium was of prime importance. Serum calcium levels were determined using a micro-EDTA titration with calcein indicator.[9, 10] The normal range in this laboratory was 9.0 to 10.5 mg. per 100 ml. The determinations were carried out under research conditions and the reproducibility of the method was maintained at ± 1 per cent (1 standard deviation) by appropriate quality control techniques. As an important measure in regulating vitamin D dosage, patients and their parents were taught to recognize the early clinical symptoms of hypercalcemia, such as polyuria, polydypsia, anorexia, nausea, vomiting, and headache.

The dose of vitamin D required to produce optimal healing in individual patients was between 50,000 and 250,000 I.U. per day (Table I). The incidence of hypercalcemic episodes and the degree of hypercalcemia are indicated in the table. Each of 9 patients had a single episode of vitamin D intoxication. As soon as these were discovered, vitamin D therapy was reduced immediately or temporarily stopped. In Case 12, serial determinations indicated that serum calcium had returned to normal in 10 days. It was not feasible to monitor the rate of recovery from hypercalcemia in other patients. However, except for Case 13, the symptoms were of short duration, and the episodes were judged to have been of mild severity. In all instances, cooperation of the patients and parents was good, hence occurrence of unrecorded serious episodes of hypercalcemia during follow-up at this hospital is considered unlikely.

As part of an experimental study,[11] 4 patients (Cases 7, 9, 10, 11) received oral supplements of isotonic sodium phosphate (approximately 60 mmoles per day) for periods ranging from 4 to 18 months. In Case 7, this therapy was terminated 1 month prior to the renal studies; in the other 4 patients, periods ranging from 1 to 6 years had elapsed since termination of oral phosphate administration.

Assessment of renal function. Several parameters of renal function were assessed in each patient. Routine urinalysis and measurement of blood urea nitrogen were carried out on each visit. At the time of detailed

Table II. Renal findings in the patients

Case	BUN (mg./100 ml.)	G_{IN} (ml./min./m.²)	G_{PAH} (ml./min./m.²)	Tm_{PAH} (mg./min./m.²)	Max urine osmolality (mOsm./Kg. H_2O)	G_{Ca}+/m.²
1	14.0	94	485	46	1,100	1.23
2	9.0	68	410	37	1,260	1.0
3	8.0	58	425	35	1,250	1.13
4	14.5	74	480	37	1,300	0.8
5	15.0	59	405	48	1,080	1.60
6	15.0	62	360	33	970	1.78
7	11.4	77	300	38	1,350	1.01
8	15.8	73	396	39	1,300	1.37
9	7.8	62	363	55	1,100	1.85
10	18.0	75	400	38	1,300	1.0
11	11.2	66	312	42	1,390	1.25
12	14.0	75	440	54	1,180	1.62
13	28.0	55	272	16	690	1.32
14	12.0	63	358	30	1,280	1.85
Normal values	8-20	59-80[13]	298-392[13]	37-51[13]	> 900	1.96-0.81[14]

*Plastic-embedded tissue.

investigation of renal function, the following procedures were performed.

1. Routine urinalysis and bacteriological culture of a catheterized specimen of urine.
2. Intravenous pyelography.
3. Inulin clearance (C_{IN}), para-aminohippurate clearance (C_{PAH}), and maximal rate of tubular secretion of para-aminohippurate (Tm_{PAH}) were measured during 3 consecutive periods according to the techniques of Smith,[12] and the results were expressed as the average. Typical normal values for children have been recorded by Stalder.[13] Inulin in serum and urine was determined by the method of Roe and associates,[14] and PAH by the method of Smith and associates.[15]
4. Urine concentration test. Commencing after the evening meal, the children were deprived of liquids until the afternoon of the following day, during which interval only a dry breakfast was given. Urine was voided spontaneously and each specimen was collected separately. Urine osmolality was measured with an Advanced Osmometer.
5. Acid-load test. A 3 day acid-load test was carried out as described by Elkinton[16] and the renal "clearance indices" of hydrogen ion (C_{H^+}) ammonium ion ($C_{NH_4^+}$) and titratable acidity (C_{TA}) were compared with normal values recorded in the literature.[16, 17] The patients received NH_4Cl, 0.1 Gm. per kilogram per day, and a 24 hour urine specimen was collected commencing at the beginning of the third day. The titratable acidity was measured by the method of Henderson and Palmer as described by Peters and Van Slyke[18] and urinary ammonia by the method of O'Brien and Ibbott.[19]
6. Percutaneous renal biopsies were obtained with a 14 gauge Vim-Silverman needle. Light microscopic examination was made of paraffin-embedded sections stained with hematoxylin and eosin, PAS, von Kossa, and Alcian blue, and of half-micron-thick sections of Epon-embedded tissue stained with toluidine blue. Small portions of cortex and medulla were processed for electron microscopy by a standard osmium-Epon schedule.[20, 21]

Since the adequacy of sampling is important in the interpretation of biopsy findings, the amounts of cortex and medulla were estimated in the largest histological section

$C_{NH_4^+}$/m.^{2}	C_{TA}/m.^{2}	Urinalysis and culture	Renal biopsy findings: Cortex (mm.)	Renal biopsy findings: Medulla (mm.)	Renal biopsy findings: Histopathology
0.86	0.37	Normal	3.0*	5.8	Normal
0.70	0.44	Normal	—	—	—
0.72	0.41	Normal	—	—	—
0.55	0.25	Normal	5.9	2.0*	Normal
1.30	0.50	Normal	—	—	—
1.15	0.64	Normal	1.9	2.9	Normal
0.66	0.38	Normal	5.6	2.0	Normal
0.50	0.87	Normal	0	3.8	Normal
1.00	0.85	Normal	6.4	0	Normal
0.61	0.39	Normal	4.0	0	Normal
0.87	0.38	Normal	2.5	3.8	Normal
1.07	0.55	Normal	3.25 2.25	4.0 2.8	Calcification in medulla
0.50	0.87	Pyuria, bacteriuria	4.9	0	Chronic pyelonephritis and calcium deposits
0.95	0.9	Normal	—	—	—
1.38-0.46[16]	0.58-0.35[16]				

from each biopsy with the use of a calibrated eyepiece. The biopsy diameters were between 0.9 to 1.0 mm. and the lengths of cortex and medulla are shown in Table II. Plastic-embedded tissues provided additional material for study, but the lengths were listed only when suitable paraffin-embedded tissue was not available.

RESULTS

Clinical aspects. All patients were in good general health. From the standpoints of skeletal development and mineral metabolism, all patients were considered to have benefitted from the vitamin D therapy. Radiographic evidence of active rickets at the metaphyseal plates was no longer present, and deformities of the lower extremities had usually improved. Some residual "coarseness" of the trabecular pattern of the long bones was frequently present. Serum alkaline phosphatase activity had decreased in all patients, in most cases to normal or nearly normal values (Table I). None of the patients had hypercalcemia at the time of study. Despite therapy, hypophosphatemia was still present in most of the children, and, taking the group as a whole, the stature was diminished, though in some individuals the height was within the low range of normal.

None of the patients had overt clinical evidence of renal dysfunction, edema, or anemia. Blood pressures, serum electrolytes, and the blood pH and CO_2 content were normal in every case.

Renal studies.

Urinalysis. Except for 1 patient (Case 13), urinalyses including microscopic examination were consistently normal, and catheter specimens were sterile (Table II).

Renal function. The results of blood urea nitrogen (BUN), glomerular filtration rate (C_{IN}), renal plasma flow (C_{PAH}), maximal tubular secretion of PAH (Tm_{PAH}), hydrogen ion clearance indices (C_{H^+}, $C_{NH_4^+}$, C_{TA}),[16] and maximum urine osmolality are shown in Table II. The intravenous pyelogram was normal in all patients and nephrocalcinosis was not detected radiographically.

Only 1 patient (Case 13) had impaired renal function. This child was of particular interest, because she was the only patient in the series who had a history of previous severe and protracted vitamin D intoxication. The diagnosis of vitamin D refractory rickets was made at another hospital when the child was 9 months of age on the basis of rachitic lesions of the bones and the fact that 2 other members of the family were affected with the disease. The infant was immediately started on large doses of vitamin D and soon developed vitamin D intoxication. After 4 months of therapy, vitamin D was stopped, but the hypercalcemia persisted for approximately 15 months and was still present when the child was first seen at this hospital.

At the time of the renal study, the patient was known to have chronic pyelonephritis and previously had had a few episodes of acute urinary tract infection with fever and positive urine culture. The intravenous pyelogram, cinecystogram, and a urological examination failed to demonstrate an obstructive lesion of the urinary tract. Despite long-term treatment with various antibiotics, chronic pyuria and bacteriuria persisted. The renal function tests demonstrated markedly reduced Tm_{PAH}, slightly reduced renal plasma flow and concentrating power, and a glomerular filtration rate at the lower limit of normal. The capacity to excrete hydrogen ion in response to an acid load was normal.

Renal biopsy findings. Biopsies were obtained from 10 patients. In 6 patients the specimens included both cortex and medulla, in 3 patients cortex only, and in 1 patient medulla only (Table II). In 8 patients, no abnormality was found by either light or electron microscopy. In particular, there was no evidence of calcification on examination of step-sections from each biopsy. The 4 children who had received isotonic sodium phosphate supplements were included in this group.

Histological abnormalities were present in the biopsies of 2 patients. In 1 patient (Case 12), renal function tests were completely normal but the biopsy specimen showed some small deposits of calcium in the outer zone

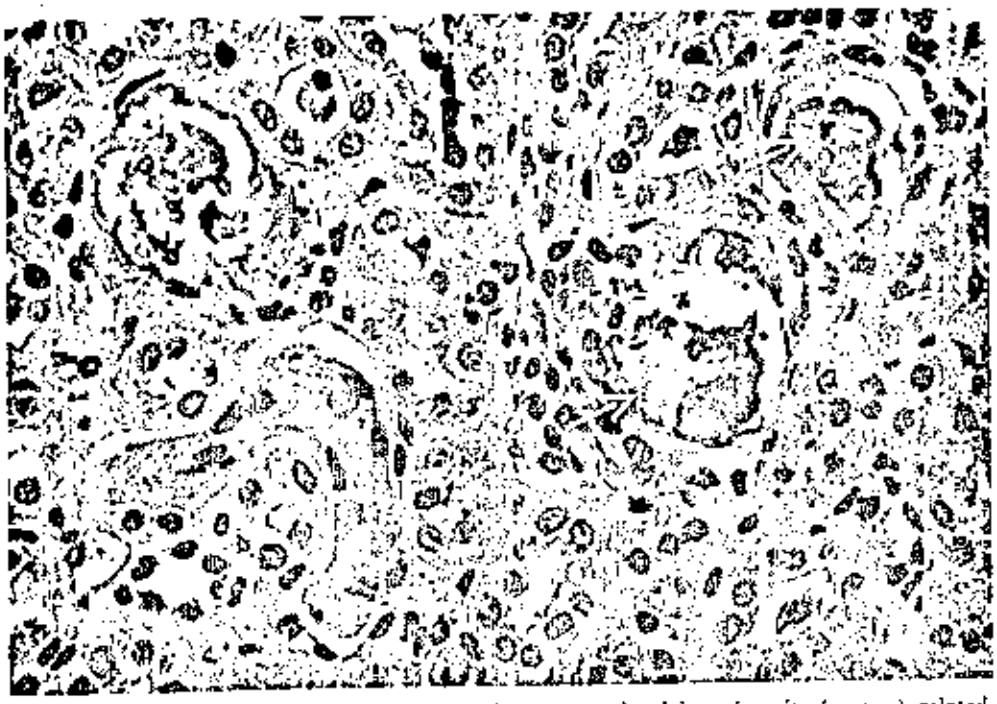

Fig. 1. Case 12, Outer medulla showing cellular casts and calcium deposits (arrows) related to loops of Henle and collecting tubules. (Hematoxylin and eosin. ×650.)

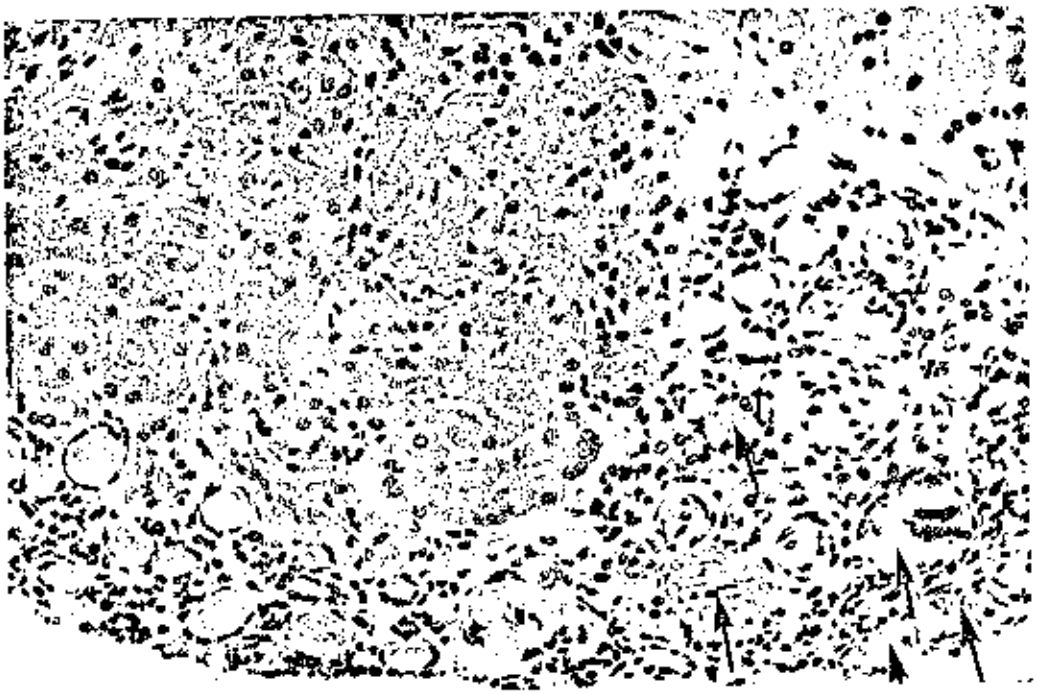

Fig. 2A. Case 13, Chronic pyelonephritis. Cortex. There is an area of scarring with calcium deposits (arrows), inflammatory cell infiltration, hyalinized glomeruli, thick-walled arterioles, and reduction in number of tubules, many of which contain colloid casts. (Hematoxylin and eosin. ×250.)

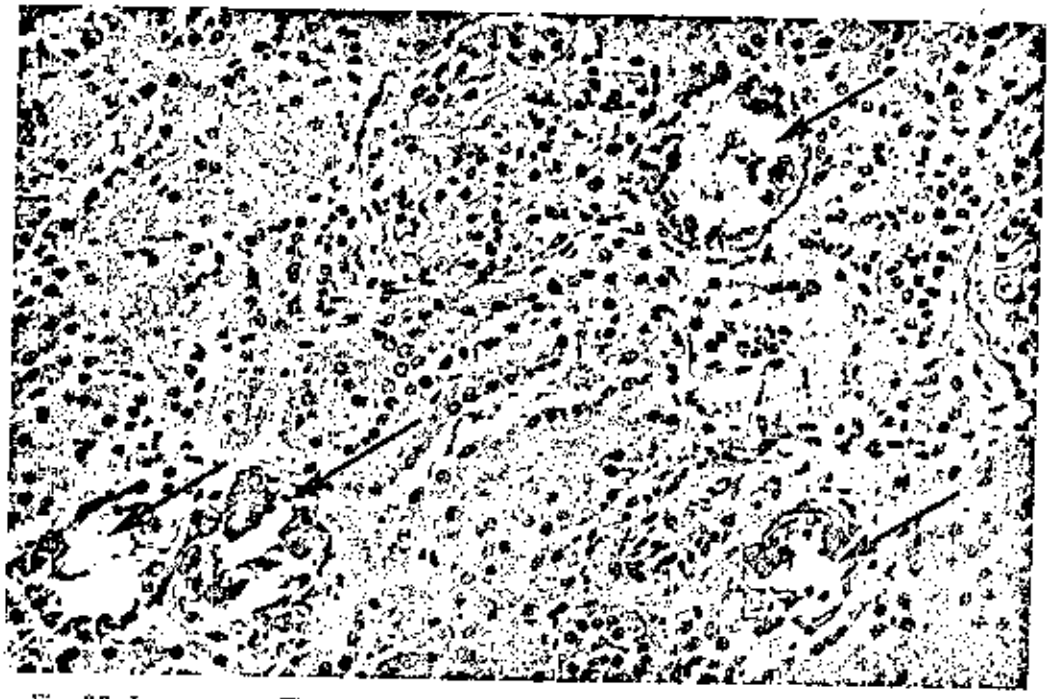

Fig. 2B. Inner cortex. There are calcium deposits (arrows) around tubules, without inflammation or scarring. (Hematoxylin and eosin. ×250.)

Fig. 2C. Inner cortex. Note annular arrangement of calcium deposits in one-half micron thick, plastic-embedded tissue. Area indicated by arrow is shown in Fig. 2D. (Phase contrast—toluidine blue. ×650.)

Fig. 2D. Inner cortex. Electron micrograph of the area designated in Fig. 2C showing edge of a normal proximal convoluted tubule and needle-shaped crystalline calcium deposits (arrows). These deposits can easily be distinguished from collagen (C). (Lead hydroxide. ×8,000.)

Fig. 2E. Electron-micrograph of calcium deposits in an area of scarring from Case 13. (Lead hydroxide. ×11,000.)

4473

of the medulla in and around loops of Henle and collecting tubules (Fig. 1). In some areas there was destruction of the basement membrane and tubular degeneration with production of cellular casts. Cellular infiltration was not seen. No abnormality was found in the glomeruli, tubules, or interstitium of the cortex. Two separate specimens were obtained at the time of biopsy, and they were similar. The one-half micron thick, plastic-embedded specimens prepared for electron microscopy from Case 12 consisted of cortex, and this appeared normal. Unfortunately, medullary tissue was not present for electron microscopic study.

Much more marked histological changes were observed in Case 13, who was known to have had severe and protracted vitamin D intoxication, and who had clinically evident chronic pyelonephritis. On examination by light microscopy, there were calcium deposits, some associated with the typical histological changes of pyelonephritis (Fig. 2A), some occuring around tubules in the inner cortex in areas where there was no cellular infiltration or scarring (Fig. 2B). In the plastic-embedded sections also, annular deposits of calcium were occasionally present (Fig. 2C). Electron microscopy of the biopsy of Case 13 revealed 2 types of calcium deposition. One type consisted of electrondense needleshaped particles (Fig. 2D) and was found in areas where surrounding tubules appeared normal and no scarring was present. It was difficult to relate this calcification precisely to basement membrane or intracellular regions because the deposits were large and sections frequently tore during preparation and were distorted in the electron beam. A second type of calcification consisted of rounded masses of electrondense material (Fig. 2E). This calcification was found in areas of scarring and frequently appeared to be in the wall of blood vessels.

DISCUSSION

Although it is well known that the large doses of vitamin D required for treating vitamin D refractory rickets are not without hazard, no systematic studies of renal function have previously been carried out in treated patients, and renal morphology has been investigated in only one group of 7 patients.[22] In that group, investigated by Nigrin and associates,[22] nephrocalcinosis was described in all 7. No information was given regarding control of vitamin D therapy or incidence of hypercalcemic episodes, and the status of renal function was not mentioned.

In the present series, Case 13 was the only patient whose lesions resembled those described by Nigrin and associates.[22] This child, who was known to have been severely intoxicated with vitamin D, had histological evidence of nephrocalcinosis and chronic pyelonephritis, and there was reduced renal function compatible with this diagnosis. Pyelonephritis can lead to secondary calcium deposition, but it is also known that nephrocalcinosis can predispose to pyelonephritis.[23] Since the child in question had had severe and protracted hypercalcemia in infancy, and since the biopsy revealed that some of the calcium deposits were not associated with the lesions of pyelonephritis (Figs. 2B, 2C, and 2D), it is assumed that vitamin D intoxication led to nephrocalcinosis, with pyelonephritis as the final result. The results from this patient emphasize that vitamin D therapy must be closely supervised to avoid the constant danger of hypercalcemia.

Each of the 13 other patients in our series had normal renal function. Renal tissue was examined in 9 of the latter. In one (Case 12), small deposits of calcium were found in the renal biopsy. Six years previously this child had had a hypercalcemic episode which lasted for 10 days; the maximum serum calcium level was 15.8 mg. 100 ml. Although other patients had similar hypercalcemic episodes, no evidence of nephrocalcinosis was detected by light or electron microscopy in these individuals.

It is known that calcifications following hypervitaminosis D are diffusely distributed in the renal tubules.[5] The fact that we did not find calcification in the biopsies of 8 patients does not prove that there might not have been a focus of calcification elsewhere in the kidney. However, taken in conjunction with

the function tests, histological studies provide encouraging evidence for the relative safety of long-term vitamin D therapy if carefully controlled. They exclude extensive renal damage in this series of patients. At the same time, Case 12 illustrates that normal renal function tests cannot alone be accepted as an indication that a minor degree of renal calcification may not have occurred in any given case.

The ideal therapeutic dose of vitamin D is difficult to establish. Obviously, it must be such that vitamin D toxicity is avoided, but at the same time the dose must be sufficient to produce the best possible healing of the rachitic lesions. It is our opinion,[6] shared by others,[24-26] that it is usually not possible to completely correct the skeletal and biochemical abnormalities in most types of vitamin D refractory rickets (exception: vitamin D dependent rickets with aminoaciduria[6, 8, 27]) by administration of large doses of the vitamin, although there is no question that such therapy is beneficial. Because of this problem and because of the inherent variability in the severity of the rachitic lesions in individuals, it is impossible to compare the results of therapy from patient to patient or from center to center.

Although other methods have been advocated to prevent over-dosage of vitamin D in refractory rickets,[28] renal function and morphology have not yet been assessed in patients treated by such methods. It is our opinion that an increase of the serum calcium level above the upper limit of normal provides a reliable signal of vitamin D toxicity, provided that the determination is performed accurately and at suitably frequent intervals. In this series of patients, permanent manifestations of vitamin D toxicity have been almost entirely avoided by applying this criterion.

SUMMARY

Renal function (clinical assessment, routine urinalysis, intravenous pyelography, blood urea nitrogen, C_{IN}, C_{PAH}, Tm_{PAH}, acid-ion clearance, and concentrating power) and renal histology were studied in 14 patients with vitamin D refractory rickets. All had been treated with large doses of vitamin D, 13 for more than 6 years, and all were considered to benefited. Twelve patients (including 4 who had also received isotonic sodium phosphate supplements as a therapeutic trial) had normal renal function; the 8 kidney biopsies obtained from this group did not show any histological abnormalities by light and electron microscopy. An additional patient had normal renal function, but moderate calcification of the medulla was present at biopsy. Another patient, who had had a long period of severe vitamin D intoxication, was found to have impaired renal function and there were histological lesions of nephrocalcinosis and chronic pyelonephritis.

Our results indicate that therapy of refractory rickets with high doses of vitamin D is relatively safe if the serum calcium level is carefully controlled. Evidence of impaired renal function and prominent histological lesions occurred only as a result of severe prolonged hypercalcemia.

REFERENCES

1. Paunier, L., Fraser, D., Conen, P. E., and Gibson, A. A. M.: Appraisal of renal function and morphology after long-term therapy of vitamin D refractory rickets, J. Pediat. 67: 1029, 1965. (Abst.)
2. Manitius, A., Levitin, H., Beck, D., and Epstein, F. H.: On the mechanism of impairment of renal concentrating ability in hypercalcemia. J. Clin. Invest. 39: 693, 1960.
3. Debré, R.: Toxic effects of overdosage of vitamin D_2 in children, Am. J. Dis. Child. 75: 787, 1948.
4. David, N. J., Verner, J. V., and Engel, F. L.: The diagnostic spectrum of hypercalcemia. Case reports and discussion, Am. J. Med. 33: 88, 1962.
5. Parfitt, A. M.: Hypercalcaemic nephropathy, M. J. Australia 2: 127, 1964.
6. Fraser, D., and Salter, R. B.: The diagnosis and management of the various types of rickets, Pediat. Clin. North America 5: 417, 1958.
7. Fraser, D.: Rickets, *in* Conn, H. F., Clohecy, R. J., and Conn, R. B., editors: Current diagnosis, Philadelphia, 1966, W. B. Saunders Company, p. 391.
8. Fraser, D.: Rickets, *in* Gellis, S. S., and Kagan, B. M., editors: Current pediatric therapy, 1966-67, Philadelphia, 1966, W. B. Saunders Company, p. 409.

9. Coleman Instruments: Calcium Ultramicro Analytical Program, No. D 298, 1960.
10. Bachra, B. N., Dauer, A., and Sobel, A. E.: The complexometric titration of micro and ultramicro quantities of calcium in blood, serum, urine and inorganic salt solutions, Clin. Chem. 4: 107, 1958.
11. Fraser, D., Geiger, D. W., Munn, J. D., Slater, P. E., Jahn, R., and Liu, E.: Calcification studies in clinical vitamin D deficiency and in hypophosphatemic vitamin D refractory rickets: The induction of calcium deposition in rachitic cartilage without the administration of vitamin D, Am. J. Dis. Child. 96: 460, 1958. (Abst.)
12. Smith, H. W.: Principles of renal physiology, New York, 1956, Oxford University Press, p. 196.
13. Stalder, G.: Clearance-Untersuchungen in Kindesalter. Methodik und Befunde, Bibl. Paediat. 74: 195, 1960.
14. Roe, J. H., Epstein, J. H., and Goldstein, N. P.: A photometric method for the determination of inulin in plasma and urine, J. Biol. Chem. 178: 839, 1949.
15. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M.: Renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man J. Clin. Invest. 24: 388, 1945.
16. Elkinton, J. R.: Renal acidosis: Diagnosis and treatment, M. Clin. North America 47: 935, 1963.
17. Peonides, A., Levin, B., and Young, W. F.: The renal excretion of hydrogen ions in infants and children, Arch. Dis. Childhood 40: 33, 1965.
18. Peters, J. P., and Van Slyke, D. D.: Quantitative clinical chemistry. II. Methods, Baltimore, 1932, The Williams and Wilkins Company, p. 825.
19. O'Brien, D., and Ibbott, F. A.: Laboratory manual of pediatric micro- and ultramicrobiochemical techniques, ed. 3, New York, 1962, Harper & Row Publishers, p. 35.
20. Luft, J. H.: Improvements in epoxy resin embedding methods, J. Biophys. & Biochem. Cytol. 9: 409, 1961.
21. Caulfield, J. B.: Effects of varying the vehicle for O_sO_4 in tissue fixation, J. Biophys. & Biochem. Cytol. 3: 827, 1957.
22. Nigrin, G., Cochrane, W. A., Jannigan, D., and Ernst, A.: Results of calcium infusion and renal biopsy studies in refractory rickets, Am. J. Dis. Child. 104: 478, 1962. (Abst.)
23. Harrison, A. R., and Ghose, R. R.: Nephrocalcinosis, Proc. Roy. Soc. Med. 56: 925, 1963.
24. Pierce, D. S., Wallace, W. M., and Herndon, C. H.: Long-term treatment of vitamin-D resistant rickets, J. Bone & Joint Surg. 46: 978, 1964.
25. Stamp, W. G., Whitesides, T. E., Field, M. H., and Scheer, G. E.: Treatment of vitamin-D resistant rickets: A long-term evaluation of its effectiveness, J. Bone & Joint Surg. 46: 965, 1964.
26. Tapia, J., Stearns, G., and Ponseti, I. V.: Vitamin-D resistant rickets: A long-term clinical study of eleven patients, J. Bone & Joint Surg. 46: 935, 1964.
27. Prader, A., Illig, R., and Heierli, E.: Eine besondere Form der primären vitamin-D-resistenten Rachitis mit Hypocalcämie und autosomal-dominantem Erbang: die hereditäre Pseudo-Mangelrachitis, Helvet paediat acta 16: 452, 1961.
28. Sterns, G.: A guide to the adequacy of therapy in resistant rickets due to familial or essential hypophosphatemia, J. Bone & Joint Surg. 46: 959, 1964.

Acta Pædiat. 43: 541, 1954

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Vitamin D and Phosphorylation of Thiamin

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The thiamin resorbed from the food is mainly found in unbound, free form. The thiamin develops its physiologically active phosphorylated form inside the living cell, where it is also protected from the influence of splitting phosphatases. If a person's diet is rich in thiamin, the content of cocarboxylase increases slowly in all the cells. The easiest way of investigating this phenomenon is by analyzing the content of cocarboxylase in blood. All the cocarboxylase in the blood is found inside the blood cells.

In the spring of 1951 we investigated the cocarboxylase content in blood in a great number of children at the Children's Clinic in Helsinki (RÄIHÄ and FORSANDER 1953). If the children got thiamin intramuscularly the cocarboxylase in the blood began to increase slowly, but, if they simultaneously got an injection of vitamin D, the cocarboxylase content increased in a high degree in many cases (RÄIHÄ and FORSANDER 1951). This influence of the vitamin D was more obvious in children than in adults. This may have been due to the possibility of the subjects having been "saturated" with vitamin D before the experiment, and an additional supply of vitamin D could not then be observed.

Because an extensive material would have been necessary for proving statistically an activation of the phosphorylation by vitamin D in humans and, further, because it is difficult to determine the "degree of saturation" of vitamin D in humans, we have performed experiments with white rats, by which it is easier to get identical conditions.

Fifteen white rats, 3 weeks old, from two different litters of 7 and 8 youngs, were mixed and divided into 3 groups of 5 youngs in each group and placed in cages in a dark cellar. For 21 days they were all given plenty of water and a special diet, which resulted in a deficiency of vitamin D. Group I (the normal group) was given every 7 days 1000 IU of vitamin D mixed in the food. Nineteen days after the beginning of the experiment the animals in groups I and II were given 1000 IU of vitamin D and 10 mg of thiamin mixed in the food. Group III were given only 10 mg of thiamin. Two days later blood samples were drawn from the animals directly from the heart under ether anæsthesia. The 2 ml of blood needed for the analysis were not obtained from all the animals. The cocarboxylase in blood was determined according to Westen-

TABLE I

	Cocarboxylase per 100 ml blood
Group I. Normal group	
Rat 1	12.9
" 2	13.4
	M = 13.2
Group II. Vitamin D and thiamin	
Rat 1	9.9
" 2	13.1
" 3	12.3
" 4	9.4
" 5	10.3
	M = 11.0
Group III. Thiamin	
Rat 1	9.2
" 2	8.9
" 3	10.0
	M = 9.4

brink's manometric method (WESTENBRINK *et al.*). Crystalline cocarboxylase from La Roche was used as the standard. Alkaline-washed yeast was prepared from dried yeast (obtained from Oranjeboom, Rotterdam). An X-ray examination of the skeleton of the rats showed that the animals from groups II and III were distinctly rickety, while the animals from group I were completely healthy. The results of the analyses are shown in Table 1.

The rats from group I (the normal group) had the highest amount of cocarboxylase in the blood. This group was also the most lively and had the best appetite and, therefore, may perhaps have succeeded in getting more thiamin than the other groups. The animals from groups II and III developed apparently equally, but still the cocarboxylase content was considerably higher in group II than in group III. The vitamin D had here considerably intensified the phosphorylation of the thiamin.

In order to make sure that all the animals got the same amount of thiamin and also of vitamin D, a new experiment was performed. Instead of mixing the vitamins in the food they were injected directly into the animals. Otherwise the experiment was identical with the previous one, and the number of animals was the same. Groups I and II were given 50,000 IU of vitamin D_2 (devitol forte, Medical Factory Orion) and 5 mg of thiamin (Neuramin, Medical Factory Orion), while group III were only given 5 mg of thiamin. The results are shown in Table 2.

The blood of the rats in groups I and II contained this time almost the same amount of cocarboxylase, while the blood of the animals in group III con-

TABLE 2

	Cocarboxylase per 100 ml blood
Group I. Normal group	
Rat 1	15.9
» 2	12.8
» 3	11.1
» 4	13.1
	M – 13.2
Group II. Vitamin D and thiamin	
Rat 1	14.8
» 2	13.1
» 3	11.0
	M – 13.0
Group III. Thiamin	
Rat 1	9.8
» 2	10.0
» 3	8.5
» 4	8.8
	M – 9.3

tained much less cocarboxylase. The X-ray examination showed that the animals in group I were quite healthy, the animals in group II were only slightly rickety and the animals in group III were very rickety. The means of groups I and III were about the same as in the first experiment while the mean of group II was much higher. The variations in the animals of the different groups were considerable.

In deficiency of vitamin D the phosphorylation of thiamin was intensified if vitamin D was added at the same time as thiamin. This experiment with rats gave the same result as the previous investigations in children. The experiment does not, however, explain the question: which process is stimulated when vitamin D is added? ZETTERSTRÖM has, however, shown that vitamin D, which has been made water-soluble by esterifying with phosphoric acid, strongly accelerates the aerobic oxidation in kidney. In such an oxidation the amount of cocarboxylase ought also to increase in order to keep step with the increased formation of pyruvic acid. In the intensified oxidation the phosphorus metabolism is also increased. As the thiamin gets its pyrophosphate group from ATP, a higher supply of ATP ought to be found in this case for the synthesis, hence the possibility of phosphorylation is increased. Another explanation is that vitamin D activates the enzyme, the thiaminokinase, which catalyzes the transfer of phosphate from ATP to thiamin in the same way as vitamin D phosphate accelerates the activity of alkaline phosphatase (ZETTERSTRÖM and LJUNGGREN).

Summary

The observation that thiamin is phosphorylated faster in children, if vitamin D is injected at the same time, is repeated with young white laboratory rats under standard conditions. Rickety rats phosphorylated thiamin faster if they were given thiamin and vitamin D as injection or by mouth than if they were given thiamin only.

References

RÄIHÄ, C.-E. and FORSANDER, O.: The cocarboxylase content of the blood in children. Acta pædiat. 42: 514, 1953.

RÄIHÄ, C.-E. and FORSANDER, O.: The activation by vitamin D of the phosphorylation of thiamin. Science 115: 242, 1952.

WESTENBRINK, H. G. K., STEY-PARVÉ, E. P., VAN DER LINDEN, A. C. and VAN DEN BROEK, W. A.: The determination of aneurin-pyrophosphate in blood. Ztschr. Vitaminforsch. 13: 218, 1943.

ZETTERSTRÖM, R.: Activation of aerobic oxidation in kidney by phosphorylated vitamin D_2. Acta chem. scandinav. 5: 343, 1951.

ZETTERSTRÖM, R. and LJUNGGREN, M.: The Activation of alkaline phosphatase from different organs by phosphorylated vitamin D_2. Acta chem. scandinav. 5: 283, 1951.

4691

Australian J. Exp. Biol. Med. Sci. 11:49-52, 1933

THE EFFECTS OF MODERATE OVERDOSAGE OF VITAMIN D AND OF VITAMINS A+D ON THE GROWTH RATE AND LONGEVITY OF THE WHITE MOUSE

Part II

by

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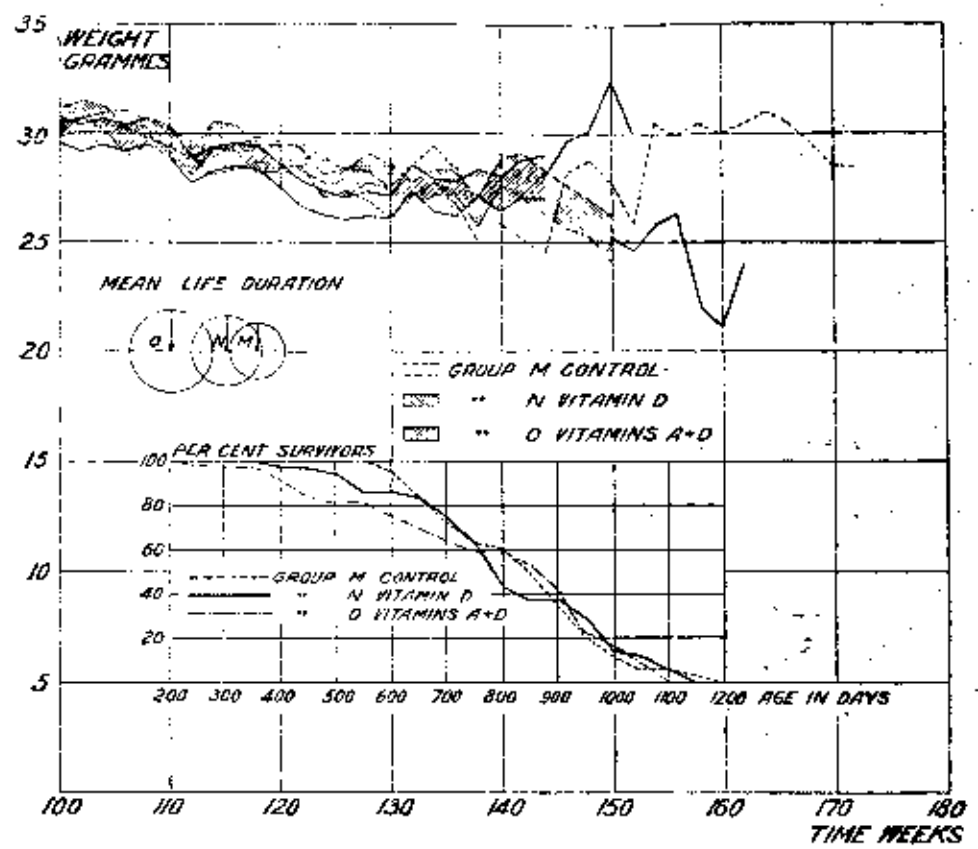
In a previous paper (Robertson, *et al.*, 1932) the conduct of this experiment was described in some detail, and the results obtained during the first 100 weeks of the lives of the animals were discussed.

The growth curves from 100 weeks of age to the death of the last animal are given here, together with the mortality statistics.

A few words must suffice to recapitulate the data of the earlier paper. Three groups, each of 36 male animals, were fed on a mixed diet. The individuals of one group (N) received daily a supplement of 50 rat-units of vitamin D dissolved in 0·05 c.c. of olive oil, while those of the second group (O) received 50 rat-units of vitamin D and 460 rat-units of vitamin A dissolved in the same quantity of oil. The individuals of the third group (M), which acted as a normal control, received merely the daily dose of 0·05 c.c. of the solvent, olive oil.

The growth of the mice in group N was favourably influenced over the first 30 weeks of the experiment. The beneficial effect of excess of vitamin D was entirely counteracted by administering simultaneously an excess of vitamin A. After 35 weeks of age the mean weight of the individuals comprising the three groups was not significantly different. Group N, however, maintained an apparent though not statistically significant superiority in weight over the others until they were about 90 weeks of age.



From this time until death the weight of the animals was not affected by the difference in treatment.

If we consider the mortality statistics it becomes apparent from the survival curves that, while early deaths were less frequent in control group M than in either of the groups O or N, after the lapse of 750 days the total number of deaths in each group was approximately equal. A calculation revealed the fact that the mean duration of life of animals still existing at 750 days did not differ significantly in the three groups.

The life duration of mice dying before 750 days of age differed appreciably in groups M and O, with group N occupying an intermediate position.

While the response to moderate overdosage of vitamin D in an earlier experiment was an insignificant increase in longevity (*loc. cit.*, 1932), the life duration of the animals receiving similar treatment in the present experiment was slightly

less than that of the controls, and one may conclude that an excess of vitamin D of this order, administered throughout life, has no appreciable effect on longevity. When, however, a large excess of vitamin A was simultaneously administered with the overdosage of D the expectation of life of the young animals was reduced. The expectation of life of those animals which survived to 750 days was not significantly altered.

TABLE 1.

Group M. Controls.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
25	100	30·14	·55
24	102	30·38	·31
23	104	30·59	·46
22	106	30·66	·42
22	108	30·23	·39
22	110	29·86	·42
22	112	29·30	·36
22	114	28·57	·42
19	116	28·07	·53
18	118	29·28	·56
17	120	29·12	·32
17	122	28·94	·42
15	124	27·63	·57
13	126	27·88	·55
11	128	27·64	·68
11	130	26·86	·73
9	132	27·89	·70
8	134	28·62	·83
7	136	27·64	·84
6	138	25·83	·80
4	140	25·97	—
3	142	25·17	—
3	144	24·50	—
3	146	28·25	—
2	148	28·75	—
2	150	27·55	—
2	152	25·75	—
1	154	30·50	—
1	156	30·00	—
1	158	30·50	—
1	160	30·00	—
1	162	30·50	—
1	164	31·00	—
1	166	30·00	—
1	168	29·50	—
1	170	28·50	—
1	172	28·50	—

TABLE 2.

Group N. Vitamin D.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
26	100	30·50	·54
24	102	31·02	·47
24	104	30·73	·55
23	106	29·61	·64
21	108	30·07	·56
21	110	29·65	·66
18	112	28·69	·62
15	114	29·97	·65
15	116	29·87	·55
14	118	28·93	·70
14	120	28·80	·63
13	122	28·02	·59
13	124	28·08	·55
13	126	27·77	·59
13	128	28·46	·67
13	130	28·08	·62
13	132	27·23	·56
10	134	27·70	·59
9	136	27·72	·52
8	138	26·87	·66
6	140	28·25	·66
5	142	28·10	1·08
5	144	27·30	1·01
4	146	26·62	·
4	148	26·12	—
4	150	25·42	—
2	152	24·50	—
2	154	27·75	—
2	156	26·25	—
2	158	22·00	—
2	160	21·00	—
1	162	24·00	—

TABLE 3.

Group O. Vitamins A + D.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
22	100	30·07	·63
21	102	29·74	·61
21	104	30·19	·57
21	106	29·79	·59
21	108	30·14	·61
21	110	29·52	·63
21	112	28·36	·61
20	114	28·85	·55
20	116	29·05	·56
20	118	28·87	·53
20	120	28·10	·54
19	122	27·26	·60
19	124	26·82	·52
16	126	26·59	·54
15	128	26·77	·53
14	130	26·64	·49
10	132	27·90	·61
8	134	27·12	·77
8	136	27·00	·80
7	138	27·71	·65
6	140	27·25	·86
6	142	27·91	·88
6	144	27·99	1·09
4	146	29·75	—
4	148	30·12	—
2	150	32·50	—
1	152	30·00	—

MORTALITY STATISTICS.

(Accidental deaths excluded.)

At Age in Days.	Percentage of Survivors. Group M. Control.	Group N. Vitamin D.	Group O. Vitamins A + D.
200	100	100	100
250	100	100	97·2
300	100	100	97·2
350	100	100	97·2
400	100	97·1	91·7
450	100	97·1	83·3
500	100	94·3	80·6
550	100	85·7	80·6
600	94·3	85·7	75·0
650	82·9	82·9	69·4
700	71·4	74·3	63·9
750	62·9	62·9	58·3
800	60·0	42·9	58·3
850	48·6	37·1	52·8
900	34·3	37·1	41·7
950	20·0	28·6	22·2
1,000	11·4	14·3	16·7
1,050	5·7	11·4	8·3
1,100	5·7	5·7	0·0
1,150	2·9	0·0	
1,200	0·0		

Mean Duration of Life.

Group M 825±17 days.
Group N 806±22 „
Group O 771±26 „

Difference between M and O 54±31 days.

Mean Duration of Life of Mice still alive at 750 days.

Group M 924±14 days.
Group N 920±17 „
Group O 944±11 „

Difference between M and O 20±18 days.

Mean Duration of Life of Mice dying before 750 days.

Group M 658± 9 days.
Group N 612±21 „
Group O 529±24 „

Difference between M and N 46±23 days.
N and O 83±32 „
M and O 128±25 „

REFERENCES.

Robertson, Thorburn Brailsford, Marston, Hedley R., Dawbarn, Mary C., Walters, J. W., and Wilson, J. D. O. (1932): Aust. Journ. Exper. Biol. and Med. Sci., 9, p. 203.

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METABOLIC ACTIVITIES OF VITAMIN D IN ANIMALS
VI. PHYSIOLOGICAL ACTIVITIES OF VITAMIN D SULFATE

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In the previous paper, the present authors reported the occurrence and isolation of vitamin D sulfate in fresh mammalian milk (1). Further researches were made on the physiological activities of pure synthetic vitamin D_2 sulfate.

First, the toxicity of synthetic vitamin D_2 sulfate in saline solution for mice was tested after intraperitoneal injection. The lethal dose (LD_{50}) was estimated to be about 2,500,000 I.U./kg. Then, the antirachitic effect of the compound was tested on the vitamin D-deficient rats fed on the Steenbock diet, and found that the prophylactic dose of the compound was the just same as that of vitamin D_2. Finally, hypervitaminosis by administering the compound was tested. No toxic symptom was observed even when given 5,000 I.U. of the compound per head orally daily.

EXPERIMENTAL AND RESULTS

1. *Materials*

Vitamin D_2 sulfate preparation used was synthesized in our laboratory according to the method described in the preveous paper as follows. The compound was synthesized by the reaction of vitamin D_2 in pyridine with pulverized sulfamic acid, and the product was recrystallized from ethanol and ether, yielding pure ammonium vitamin D_2-sulfate melting at 113—115° (uncor.). Analysis was performed by $SbCl_3$ coloration and spectrophotometry. The maximum absorbance lay at 500 mμ ($E^{1\%}_{1cm}$ = 1740). The sodium salt was prepared by treating the ammonium salt with sodium bicarbonate.

2. *Toxicity Test of Vitamin D_2 Sulfate in Saline Solution upon Mice*

This experiment was carried out in order to find the minimal lethal dose and also to observe symptoms caused by the compound. Saline solution of vitamin D_2 sulfate (as ammonium or sodium salt) was intraperitoneally injected to mice, weighing about 20 g. The death rate of the compound seemed to be extremely lower than free vitamin D_2. LD_{50} was estimated to be about 2,500,000 I.U./kg. (Table I).

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TABLE 1
Toxicity of Vitamin D_2 Sulfate in Mice

Material	Injected dose[b,c]	Death rate after 24 hr[a]	Death rate after 48 hr[a]
	10^3 I.U. (as vitamin D_2)		
Ammonium vitamin D_2 sulfate	2	0/10	0/10
	4	0/10	0/10
	6	0/10	0/10
	12	0/10	0/10
	30	0/10	0/10
	40	0/ 0	0/10
	45	4/10	6/10
	50	8/10	10/10
Sodium vitamin D_2 sulfate	2	0/10	0/10
	4	0/10	0/10
	6	0/10	0/10
	12	0/10	0/10
	30	0/10	0/10
	40	0/10	0/10
	45	4/10	6/10
	50	6/10	10/10

[a] The number of the dead mice/the number of mice used.
[b] The dose per 20 g of the body weight of the mice.
[c] Each volume injected per head was 0.5 ml of saline solution.

3. *Tests for Antirachitic Ability of Vitamin D Sulfate in Rats*

White rats, weighing about 40 g, were previously fed on Steebock's diet consisting of, in per cent, yellow corn meal 76, glutein (supplemented with 0.5% of L-lysine) 20, $CaCO_3$ 3, and NaCl 1. The animals were divided at random into three groups, to one of which was given daily 10 I.U. vitamin D_2 in 0.01 ml of olive oil per head *per os*. The second group was orally given daily 10 I.U. of ammonium vitamin D_2 sulfate in 0.01 ml olive oil per head. The third group was served as a

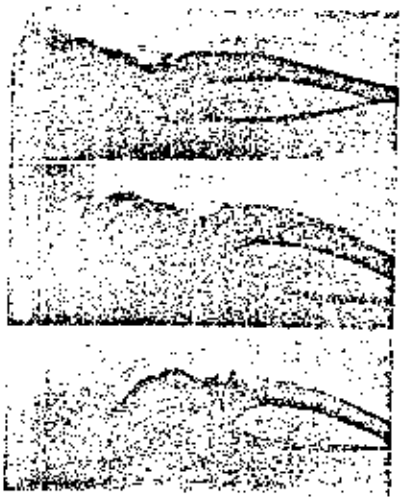

FIG. 1 *X-Ray Tests for Antirachitic Potency of Rats Receiving Vitamin D_2 Sulfate*

negative control. During 8-week feeding, the growth curves were recorded. Then, the animals were killed, and the calcium content of left tibia was determined according to the AOAC method. Further, the antirachitic potency of vitamin D_2 sulfate in rats was also tested by the ordinary X-ray test. As shown in Fig. 1, both vitamin D_2 and its sulfate were similarly antirachitic, whereas the control was not.

4. Tests for Hypervitaminosis in Rats by Administering Orally Vitamin D_2 Sulfate in a Large Dosage.

The rats, weighing about 120 g, were previously fed on the ration consisting of, in per cent, α-starch 65, casein 20, soy bean oil 7, McCollums salt (No. 185) 4, Vitamin A oil 3, vitamin mixture 1. The animals were divided at random into three groups, to one of which was given 5,000 I.U. of vitamin D_2 sulfate per head *per os* daily. The second group was orally given 5,000 I.U. of free vitamin D_2 per head daily. The third group was served as a positive control, receiving 10 I.U. of vitamin D_2 per head *per os* daily. As shown in Fig. 2, both groups receiving 5,000 I.U. of vitamin D_2 sulfate and 10 I.U. of vitamin D_2 gained body weight till 15 days, whereas that receiving 5,000 I.U. of vitamin D_2 failed to gain weight after 10 days following the appearance of severe symptoms of hypervitaminosis.

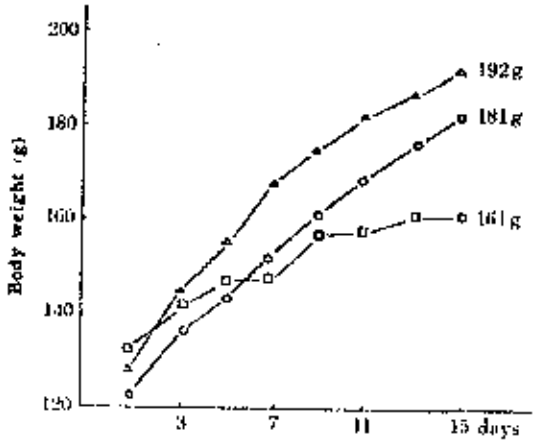

FIG. 2 *Growth Curves of Rats Receiving Ammonium Vitamin D_2 Sulfate in Large Doses*

△, group receiving 5,000 I.U. of vitamin D_2 sulfate/head/day; ○, group receiving 10 I.U. of free vitamin D_2/head/day; □, group receiving 5,000 I.U. of free vitamin D_2 head/day.

SUMMARY

1. Toxicity of vitamin D_2 sulfate in saline solution in mice was tested by intraperitoneal injection. The lethal dose, LD_{50}, in mice, weighing about 20 g, was about 2,5000,000 I.U./kg.

2. Antirachitic potency of vitamin D_2 sulfate was tested by prophylactic method and it was found that the compound had about the same effect as vitamin D_2.

4990

3. By administering 5,000 I.U. of the compound or 10 I.U. of vitamin D_2 per head *per os* daily, gained the body weight till 15 days, whereas those receiving 5,000 I.U. of vitamin D_2 per head *per os* daily resulted in the failure to gain weight after 10 days.

ACKNOWLEDGEMENTS

This work was supported in part by Research Grant from the Lipid-Soluble vitamin Research Committee to which the authors' thanks are due. The authors wish to thank the laboratory of Nippon Formula Feed Manufacturing Company Ltd. for the kindness throughout this experiment.

REFERENCES

1. Sahashi, Y., Suzuki, T., Higaki, M., and Asano, T., *J. Vitaminol.* 13, 33 (1967).

SCHAEFMAN, W. B. and TROPP, S. **Anemia associated with vitamin D intoxication.** *New Engl. J. Med.*, 1956, **255**, 1207–1212. [Dept. Med., Albany Med. Coll., N.Y.]

Four patients with normocytic, normochromic anaemia of unknown origin and resistant to treatment were all found to have taken from 50,000 to 150,000 "units" vitamin D daily for years. They all had signs of illness compatible with vitamin D intoxication, and a varying degree of renal impairment. None of them was hypertensive; all had high blood N. Treatment was with a diet high in fluid and low in calcium, on which the patients soon felt much better, but renal improvement was slow, and the anaemia responded slowly. It is believed that anaemia is almost always present with vitamin D excess, and results from the damage to the kidney, or, less probably, from direct toxic action on the bone marrow.—E. M. Hume.

German Translation

Vitamins and Tissue Growth

H.O. Schmid (From the Heidelberg Surgical University Clinic - Director: Prof. Dr. M. Kirschner).

Source: Klinische Wochenschrift 16(13): 405, 1937

The question as to whether the growth of tissues can be influenced by vitamin supply has been checked many times. Tests on the effect of vitamins on the liver have already been carried out earlier by several researchers (Windaus, Genkin, Dessauer, A.W. Schmidt, etc.). In 1932, Dietrich and Sander reported epithelium proliferations on the rabbit ear induced by vitamin D used in the form of irradiated ergosterol.

The well-known observations reported by Askanazy that malignant tumors can be induced by tomato juice were also initially often attributed to the action of vitamins. A check-up of these findings, performed as many institutes, yielded mostly negative results; however, Bellow, among others, also obtained tumors in some of his tests, which were identified histologically as sarcomas, but their formation was attributed to saprophytes. In my own work at the Pathological Institute of Tubingen University (Director: Prof. Dr. Dietrich), sterile juice from tomatoes partly irradiated with a quartz lamp and partly exposed to strong sun light was injected intraperitoneally into a series of 100 rats. We have never observed anything more than occasional typical resorption granulomas. Feeding experiments were carried out on a large number of animals, which gave negative results.

In 1932, Nordmann and Horger reported that the growth of tissue cultures was greatly enhanced under the effect of irradiated ergosterol, whereas no effect on the growth could be noted in control tests with nonirradiated

ergosterol or pure linseed oil. Lohr has also referred to these studies. In the course of tests, it was found further that highly concentrated vitamin D has a toxic effect.

In 1933, Matsudaira reported the mode of action (effect) of vitamins A to D. He introduced small gallstones into the gall bladder of guinea pigs by means of an operation reported by Kazama and described below in this article. At the same time, the animals were fed a low vitamin A diet. After a long observation time, tumors were found, which were illustrated and designated (characterized) by Matsudaira as adenocarcinomas. The induction and growth of these tumros was influenced by a variable supply (administration) of vitamins, particularly vitamin A. After administration of vitamins C and D, weaker and in part precancerous changes with cellular atypia and mitoses were noted. The tumor illustrations (figures) reported by Matsudaira were not characterized (designated) by Dietrich, however, as clearcut adenocarcinomas.

In 1934, Kemmler reported that vitamin D solutions applied to wounds accelerate the healing of such wounds. He ascribed this fact, on the basis of tests, to proliferation processes in the epithelium.

Stimulated by Matsudaira's experiments, in my work at Tubingen, I have exposed the gall bladder of a large number of animals (guinea pigs) by means of a costal arch pararectal incision, and, after ligature of the ductus cysticus, emptied (evacuated) the gall bladder by puncture and filled it up again. As a filling, a 0.2% solution of irradiated ergosterol in linseed oil was used. An amount of 0.5 cc (irradiated ergosterol in linseed oil (?) - not clear) was injected, and the injection site was ligated. X-ray control tests performed after Thorotrast filling showed that Thorotrast

could be detected over 1 year later. At the same time, the same tests, using nonirradiated ergosterol and pure linseed oil, were carried out on other animals. In this last series, autopsy revealed in part a complete resorption of the bladder content, without any particular changes in the gall bladder wall, and in part hydropic alterations. A weight loss was soon observed in the animals treated with irradiated ergosterol; changes were mostly noted in the gall bladder and its vicinity. Small tumors had formed in the gall bladder, and these were identified histologically in part as atypical epithelium formations with giant cells and very numerous Luschka's crypts, or merely as adenoid proliferations or brown tumors. A destructive growth or metastase formation was not observed. Even before these tests were completed, the calcinose factor was isolated, along with other products, from irradiated ergosterol, and described by Holtz as AT. 10.

I have continued these tests at the Heidelberg Surgical University Clinic, employing the same technique and using AT. 10. After an observation period of 4 months, several of the animals treated with AT. 10 died, while refusing to eat and exhibiting a weight loss. Tumors similar to those noted in animals exposed to irradiated ergosterol were found. In part, the gall bladder exhibited changes comprising cysts as large as a chicken egg, and in part small, bean-sized, firm and whitish structures were noted. Histologically, a large number of Luschka's crypts with chronic wall thickening, mitoses and atypical epithelial cell formations were observed. These epithelial cell formations exhibited a good demarcation against the surrounding area. No metastases could be found.

The above findings were noted already 4 months after the operation. Further tests, also with other animals, are in progress. Therefore, we are

not yet able to draw any conclusions from these tests or to evaluate them in a clearcut manner. It is also impossible to draw any kind of conclusions (or inferences) from these tests with regard to therapeutic measures.

Bibliography

1. Askanazy, Z. Krebsforsch. 34, 362, (1932).
2. Bellow, Z. Krebsforsch. 34, 348 (1932).
3. Blumenthal, Erg. exper. Krebsforsch. u. Krebsther. 1934.
4. Caspari, Strahlenther. 37, 719 (1930).
5. Dietrich u. Sander, Naturwiss. med. Ver. Tubingen, Sitzung vom 6, VI. 1932.
6. Genkin II u. J.D. Dmitruk, Z. Krebsforsch. 36, 104 (1932).
7. Haga, Z. Krebsforsch. 12, 525 (1913).
8. Holtz, Ther. Gegenw. 1936, 6-7.
9. Y. Kazama, Z. jap. Chir. Ges. 25, H. 5.
10. Kemmler, Mitt. Grenzgeb. Med. u. Chir. 43, 453 (1934).
11. M. Matsudaira, Mitt. path. Inst. d. Med. Fak. Nugata. Juni 1933.
12. Nordmann-Horger, Arch. exper. Zellforsch. 13, 430 (1932).
13. Plonskier, Z. Path. 55, 56 (1932).
14. Z. Krebsforsch. 37, 499 (1932).
15. R. Seyderhelm, Klin. Wschr. 1932, 628.
16. Schmidt, Virchows Arch. 291, 432 (1933).
17. Weitere Literatur bei Blumenthal.

German Translation

Relation Between Vitamin D Administration to Gravidae and Children and a Deformation Syndrome Accompanied by Supravalvular Aortic Stenosis

Comment by a Commission of the German Pediatric Society (compare this journal, Vol. 114, No. 9, p. 457, 1966)

Source: Monatsschr. Kinderheilkunde 115: 531, Oct. 1967

During the meeting of the Northwest German Pediatric Society in Bremen on 6-8 May 1966, Beuren (Gottingen) called attention to a syndrome consisting in a combination of supravalvular aortic stenosis, a special facies with low-set ears, turned up lips, broad nose saddle, and a more or less pronounced intelligence lowering. In addition, in some cases, aplasia of dental germs, other vascular deformities, multiple peripheral pulmonary stenoses and peripheral arterial stenoses are noted, as well as a generalized pulmonary and aortic hypoplasia, and finally an alteration of vascular walls, in particular a media hypertrophy and intima proliferation of the aorta. Of particular importance was the report that the extent of vascular changes can increase as a result of the retarded growth of the injured points, and that the syndrome can be connected with a severe idiopathic hypercalcemia. Vitamin D, and especially the massive (stoss) vitamin D doses used in Germany, was considered to be the cause responsible for the appearance of this syndrome. Excessive (1) iron medication was also considered as a possible cause. The impression made by this report, together with the recent thalidomide catastrophy, created an understandable

feeling of alarm among the public.

The German Pediatric Society soon set up a commission, which was to check the data reported by Beuren. It was made possible for this commission to perform postexaminations of sick children in Gottingen, to talk to their mothers and fathers, and to review photocopy sets of the papers prepared by Beuren prior to their publication in this journal. During two meetings held on 29 Oct. 1966 and 21 Jan. 1967, the Commission arrived at the following results:

1. There can be no doubt about the existence of the supravalvular aortic stenosis syndrome, described by Beuren and by European and foreign authors both before and after him, with all its local phenomena of external aspect and vascular changes.

2. A connection between this syndrome and the severe form of so-called idiopathic hypercalcemia is highly probable in view of the common occurrence of both diseases in some patients and the presence of histological changes in large vessels in individual cases of severe idiopathic hypercalcemia, which are similar to those described by Beuren.

3. A constitutional hypersensitivity towards vitamin D, apparently due to genetic causes, was detected in individual children with this particular syndrome, as well as in their mothers (Antener, Beuren, Bonham-Carter).

4. In all probability, the syndrome of supravalvular aortic stenosis has an intrauterine origin. A heart murmur, which can be traced back to the aortic stenosis, was detected in many patients already during the first months or weeks of life. Administration of toxic

doses of vitamin D to pregnant animals also induces a supravalvular aortic stenosis, along with other vascular injuries, in part of their offspring.

5. A connection between the appearance of the supravalvular aortic stenosis syndrome and the prevention of rickets by massive vitamin D doses (stoss therapy) has not been confirmed sofar. The syndrome can also appear in extremely rare cases during administration of moderate and low doses of vitamin D, and possibly even without an external supply of vitamin D. The patients with a so-called severe idiopathic hypercalcemia with and without supravalvular aortic stenosis, as well as their mothers, described in the foreign literature, certainly did not receive any massive (stoss) vitamin D doses.

6. This observation must be understood at first in such a way that, when vitamin D hypersensitivity is present (and this happens in an extremely small number of people), every administration of vitamin D can contribute to the appearance of the syndrome during pregnancy, and perhaps even the vitamin D production of the body itself. After birth, the syndrome can develop under the same conditions, since vitamin D hypersensitivity remains.

7. According to present knowledge, the complete suppression of the syndrome seems to be impossible, even when every kind of medicinal vitamin D prophylaxis is excluded. A test, which so far does not exist, for detecting individual hypersensitivity to vitamin D would be very desirable.

8. In order to prevent as far as possible the appearance of this syndrome, the following suggestions can be made for the prevention of rickets:

a) The use of massive (stoss) vitamin D doses should be completely avoided during pregnancy.

b) During the postnatal life of full-term infants, in the medicinal prevention of rickets, the continuous administration of 500-1000 IU (international units) vitamin D should be given preference over the use of massive doses (stoss prevention), since vitamin D incorporated in massive (stoss) form is liberated from storage organs of the skin, liver, muscles, intestinal walls, etc. in amounts unknown so far, and thus a process which has already started can go out control. On the other hand, such a process can be stopped more easily, during long-term prevention, in the case of appearance of overdosage symptoms such as lack of appetite, constipation, weight maintenance, hypercalcemia, and positive urine Sulkowitch test. For the majority of infants and small children, the avoidance (rejection) of every kind of medicinal rickets prevention is undisputable under any circumstances.

9. Financial considerations on the part of the public should play no role whatsoever in the substitution of stoss prevention by continuous prevention, since this substitution appears to be urgently needed also for other reasons.

10. Since the margin between therapeutic and toxic dose of vitamin D is too narrow (a 5-fold dose in rats), the administration of vitamin D should be done by a physician.

11. Members of the commission did not have a uniform opinion concerning the question of the so-called mute prevention of rickets by addition of vitamin D to the infant's milk. This question requires further consultation and discussion.

5153

Über den Zusammenhang zwischen Vitamin D-Verabreichung an Schwangere und Kinder und einem mit supravalvulärer Aortenstenose einhergehenden Mißbildungssyndrom

Stellungnahme einer Kommission der Deutschen Gesellschaft für Kinderheilkunde (vgl. diese Z., Bd. 114, 1966, Nr. 9, S. 457)

Während der Tagung der Nordwestdeutschen Gesellschaft für Kinderheilkunde in Bremen (6. 8. 5. 1966) wurde von Beuren, Göttingen, auf ein Syndrom aufmerksam gemacht, das aus einer Kombination von supravalvulärer Aortenstenose, einer besonderen Facies mit tiefstehenden Ohren, aufgeworfenen Lippen sowie einem breiten Nasensattel und einer mehr oder weniger starken Intelligenzminderung besteht. Hierzu kommen in einzelnen Fällen eine Aplasie von Zahnanlagen, andere Gefäßmißbildungen wie multiple periphere Pulmonalstenosen, periphere arterielle Stenosen und eine generalisierte Pulmonalis- bzw. Aortenhypoplasie, schließlich noch eine Veränderung der Gefäßwand, im besonderen eine Medinhypertrophie und Intimaproliferation der Aorta. Besonders wichtig war die Mitteilung, daß das Ausmaß der Gefäßveränderungen durch Zurückbleiben des Wachstums der geschädigten Stellen zunehmen kann und daß das Syndrom mit einer schweren idiopathischen Hypercalcämie verbunden sein kann. Ursächlich wurde für die Entstehung des Syndroms das Vitamin D, besonders aber der in Deutschland verbreitete Vitamin D-Stoß verantwortlich gemacht. Eine (überreichliche?) Eisenmedikation wurde ebenfalls ursächlich in Betracht gezogen. Unter dem Eindruck dieser Mitteilung und der erst kürzlich hereingebrochenen Thalidomid-Katastrophe entstand eine verständliche Beunruhigung der Öffentlichkeit.

Die Deutsche Gesellschaft für Kinderheilkunde stellte alsbald eine Kommission zusammen, die die Bremensche Angaben nachprüfen sollte. Sie hatte zum Teil die Möglichkeit, die erkrankten Kinder in Göttingen nachzuuntersuchen, mit den Müttern und Vätern zu sprechen und noch vor Erscheinen der von BEUREN für die Monatsschrift für Kinderheilkunde zusammengestellten Publikationen diese in Fotokopie einzusehen. In 2 am 29. 10. 1966 und 21. 1. 1967 abgehaltenen Sitzungen kam die Kommission zu folgenden Ergebnissen:

1. Die Existenz des von BEUREN und von europäischen und außereuropäischen Autoren vor sowie nach ihm beschriebenen Syndroms der supravalvulären Aortenstenose mit allen Teilerscheinungen des äußeren Aspektes und der Gefäßveränderungen kann nicht bezweifelt werden.

2. Mit einem hohen Grad von Wahrscheinlichkeit sprechen für Zusammenhänge zwischen diesem Syndrom und der schweren Form der sog. idiopathischen Hypercalcämie das gemeinsame Vorkommen beider Krankheiten bei einzelnen Patienten und das Vorkommen histologischer Veränderungen an den großen Gefäßen bei einzelnen Fällen von schwerer idiopathischer Hypercalcämie, die den von BEUREN beschriebenen ähnlich sind.

3. Bei einzelnen Kindern mit dem einschlägigen Syndrom sowie ihren Müttern wurde eine offenbar genetisch bedingte, konstitutionelle Überempfindlichkeit gegenüber Vitamin D nachgewiesen (ANTENER, BEUREN, BONHAM CARTER).

4. Das Syndrom der supravalvulären Aortenstenose entsteht aller Wahrscheinlichkeit nach intrauterin. Das auf die Aortenstenose zurückzuführende Herzgeräusch wurde bei vielen Patienten schon in den ersten Lebensmonaten oder -wochen festgestellt. Auch läßt sich durch toxische Gaben von Vitamin D an trächtige Tiere bei einem Teil ihrer Jungen eine supravalvuläre Aortenstenose neben anderen Gefäßschäden erzeugen.

5. Zusammenhänge zwischen dem Auftreten des Syndroms der supravalvulären Aortenstenose und der Rachitisprophylaxe durch Vitamin D-Stöße sind bislang nicht bewiesen. Das Syndrom kann auch bei mäßigen und niedrigen Dosen von Vitamin D in äußerst seltenen Fällen, möglicherweise sogar ohne äußere Zufuhr von Vitamin D auftreten. Die im ausländischen Schrifttum beschriebenen Patienten mit sogenannter schwerer idiopathischer Hypercalcämie mit und ohne supravalvulärer Aortenstenose erhielten ebenso wie ihre Mütter mit Sicherheit keine Vitamin D-Stöße.

6. Diese Feststellung muß zurückhaltend verstanden werden, daß bei vorhandener Vitamin D-Überempfindlichkeit, deren nur bei einer außerordentlich geringen Zahl von Menschen zu rechnen ist, jede Gabe von Vitamin D an der Entstehung des Syndroms während der Gravidität beteiligt sein kann, vielleicht sogar auch die körpereigene Vitamin D-Produktion. Postnatal kann sich das Syndrom unter den gleichen Bedingungen ausbilden, da die Vitamin D-Überempfindlichkeit bestehen bleibt.

7. Eine völlige Verhinderung der Entstehung des Syndroms scheint nach den heutigen Erkenntnissen selbst bei Aussetzung jeglicher medikamentöser Vitamin D-Prophylaxe unmöglich zu sein. Wünschenswert ist ein bis heute nicht existierender Test zur Erkennung der individuellen Vitamin D-Überempfindlichkeit.

8. Um die Entstehung des Syndroms möglichst weitgehend zu verhindern, können für die Rachitisprophylaxe folgende Vorschläge gemacht werden:

a) Während der Gravidität sollte eine Vitamin D-Stoßprophylaxe ganz unterbleiben.

b) Während des postnatalen Lebens ist bei medikamentöser Rachitisprophylaxe der kontinuierlichen Verabreichung von 500—1000 IE Vitamin D bei ausgetragenen Säuglingen der Vorzug vor der Stoßprophylaxe zu geben, da einmal in Stoßform inkorporiertes Vitamin D in bisher nicht bekannten Mengen aus den Speicherungsorganen der Haut, Leber, Muskulatur, Darmwand usw. abgegeben wird und damit ein einmal angelaufener Prozeß außer Kontrolle geraten kann. Die protrahierte Prophylaxe kann dagegen bei Auftreten von Überdosierungserscheinungen (Appetitlosigkeit, Obstipation, Gewichtsstillstand, Hypercalcämie, positive Sulfosalicylsäure-Probe im Urin) ein solcher Prozeß aber gestoppt werden. Im Vergleich auf jegliche medikamentöse Rachitisprophylaxe ist für das Gros der Säuglinge und Kleinkinder unter keinen Umständen diskutabel.

9. Finanzielle Überlegungen seitens der öffentlichen Hand sollten bei dem Ersatz der Stoßprophylaxe durch eine kontinuierliche Prophylaxe, der auch aus anderen Gründen vorzugsweise geboten erscheint, keine Rolle spielen.

10. Da die Spanne zwischen therapeutischer und toxischer Dosis beim Vitamin D zwar groß ist (Pfäffenhofer Brauch), gehört die Verabfolgung von Vitamin D in die Hand des Arztes.

11. Zur Frage der sogenannten stramen Prophylaxe durch D-Vitaminierung der Stillenden [illegible] Kommissionsmitglieder keine einheitliche Meinung, die Frage bedarf weiterer Beratung und Besprechung.

5217

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PERSONAL VIEW

Are American Children Still Getting an Excess of Vitamin D?

Hyperreactive Children at Risk

MILDRED S. SEELIG, M.D., M.P.H.*

THE safety of vitamin-D supplementation has been the subject of editorials and statements by pediatric associations, both in Great Britain[1-7] and in the United States[8-11] for more than 12 years, ever since infantile hypercalcemia was correlated with hypervitaminosis D.** Frank overdose with vitamin D, and hyperreactivity to amounts of vitamin D far lower than those usually deemed toxic, have been implicated in the acute phase of infantile hypercalcemia, and also early in life by the normocalcemic disturbance characterized by supravalvular aortic stenosis, involvement of other arteries, renal calcinosis and severe mental retardation, associated with an elfin facies. This combination of stigmata is often referred to as the Williams syndrome, or more frequently in the United States as the congenital supravalvular aortic stenosis syndrome.**

Vitamin D-fortified milk has proven effective in eradicating rickets. The lower incidence of infantile hypercalcemia in America than in Great Britain may well be attributed to the addition of no more than 400 units of vitamin D per quart of milk since 1941,[15] and to the repeated official *** recommendation that no more than 400 units of vitamin D, from all sources, be consumed daily.[8-11] This paper calls attention to evidence that the usual intake of vitamin D by American infants may not be universally safe, not only because of individual variation in response, but because of the addition of vitamin D to milk.

Early Studies on Daily Vitamin D Requirement and Effect of Vehicle

Several early findings on the safety and efficacy of vitamin D are startling in their implications. Vitamin D_2 (irradiated ergosterol or calciferol) was significantly more toxic in experimental animals than is natural (fish oil) vitamin D (vitamin D_3), particularly as regards cardiovascular and renal calcification.[16, 17]

Even more significant are the data on the influence of milk on the dose of vitamin D required for prophylaxis and for cure of rickets. It was shown more than 30 years ago that the activity of vitamin D increased manifold when it was added to milk. Lewis[18] observed, in 1935, that 90 units of crystalline vitamin D in milk were superior to those obtained with 900 units in oil in treating rachitic children. He commented that 10 to 15 drops of vitamin D in oil were necessary to cure rickets, whereas only one drop was necessary if it was incorporated in milk.

Stearns *et al.*[19] reported, in 1936, that infants given 300 to 400 units of vitamin D in cod liver oil grew at faster than average rates, whereas infants getting all of their vitamin D from cod liver oil emulsified in milk grew at about the same rate on only 135 units per quart.

Drake[20] reported from Canada, the same year, that children of British and northern European stock were cured of moderate or advanced rickets by 300 units of vitamin D; they were fully protected by 95 units daily in irradiated milk, or 150 units as cod liver oil or viosterol.

Lewis[21] found a considerable range in the amount of vitamin D necessary to prevent rickets in his population of New York City children of Negro, Puerto Rican and Italian derivation, the white children requiring less vitamin D than the black. Of 58 infants given 145 or 290 units in oil or in propylene glycol, 14 per cent and 10 per cent, respectively, developed moderate to severe rickets. When the same amounts of vitamin D were added to milk, only 5 per cent and 2 per cent exhibited early roentgenographic evidence of rickets. Among 42 infants who received 1,450 units of vitamin D in oil, only one (a Negro child) developed a mild form of the disease. To protect even such hyperreactive children from rickets, Lewis[21] recommended that 332 units be added to

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** For reviews, see references 10, 12-14.

*** Council of Nutrition, American Academy of Pediatrics.

milk to achieve the potency of at least 1,450 units in oil.

A later paper by Glaser *et al.*[32] (1949) presented evidence that 100 units daily of all preparations of vitamin D in an oily vehicle were prophylactic against clinical rickets, even in premature and in Negro infants. However, because nine infants on that dose developed roentgenographic evidence or suspicion of rickets, among the 166 who were followed for eight months, the authors commented that 400 to 800 units (in oil) daily would allow a greater margin of safety in cases of illness or temporary neglect. The enhancing effect of milk, or of lactalbumin, on the potency of vitamin D was also shown in rats by Supplee *et al.*[23]

A 1963 statement on vitamin D requirements by the Committee on Nutrition of the American Academy of Pediatrics pointed out that daily intakes of 250 units are at least as effective as larger intakes, and that 300 to 500 units daily are therapeutic.[11] The committee also recalled that prior to the era of supplementation and fortification of food with vitamin D, a balanced diet and outdoor play "practically always provided sufficient vitamin D to prevent detectible evidence of vitamin D deficiency . . ." (in the preschool and school child). In 1961 Bakwin[9] observed that during spring and summer months when sunlight is ample and babies are exposed freely, no supplementation is necessary, and the use of vitamin D-reinforced milk is superfluous. That exposure to the sun of only the cheeks of fair-skinned infants and children, for as little as three hours daily, is probably sufficient to generate 400 units vitamin D, has recently been stated by Loomis.[24] On the other hand, Bakwin[9] suggested that Negro infants might require vitamin D supplementation additional to that in fortified milk.

By 1953, irradiation of milk (which gave rise to vitamin D_3) was almost entirely superseded by addition of 400 units vitamin D_2 to each quart of fresh milk in most states.[25, 26] Crystalline vitamin D_2 in equivalent amounts is added to evaporated milk by the major milk companies. Since the removal of the governmental restriction against such fortification of dried milk (recommended in 1966[27]) an equivalent amount of vitamin D (usually D_2) is beginning to be added to many dried skim milks.

Potentialities of Risk of Vitamin D in Higher Than Prophylactic Doses

The amount of vitamin D that currently is added to almost all marketed fluid and dried milks delivers considerably more than prophylactic requirements for most infants; this dose even exceeds the amount needed to cure rickets in many cases.

Since the hazard of overuse of vitamin D was emphasized in 1961[9] and in 1963,[11] the amount of vitamin D provided in vitamin concentrates and added to other foods has been curtailed. Vitamin supplements, for example, now deliver up to 400 units daily. A recent analysis of consumption of vitamin D delivered by food and vitamin preparations[28] revealed that among infants, the daily vitamin D intake was from 130 to 914 units. In the preschool group, the range of intake was 21 to 1,137 units. In most instances, the major source of vitamin D was fortified milk.

A possibly significant corollary to the greater susceptibility of deeply pigmented infants to rickets is the seemingly greater frequency in fair-skinned children of infantile hypercalcemia, Williams syndrome, and possibly other sequelae such as renal calcinosis (see review[14]). For infants who are constitutionally hyperreactive, the amount of vitamin-D activity provided by 400 units added to a quart of milk may well be within the toxic range.

Reference should be made to Black's 1964 discussion[12] of theories to explain the metabolic abnormalities or variations that may be responsible for hyperreactivity to vitamin D. He also cautioned that the rat-bone line test (used for decades in the bioassay of vitamin D in milk) does not detect every agent in the serum which is capable of raising the serum calcium level of the infant. For example, although dihydrotachysterol has the same activity in man weight for weight as has vitamin D, the rat-line test indicated only 1/400 of the activity of vitamin D.

More recent studies suggest that among the individual metabolic differences may be variations in ability to convert vitamin D or related cholesterol derivatives to active or to inert forms. Blunt *et al.*[29] have shown a metabolic product of vitamin D (25-hydroxycholecalciferol or 25-HCC) to be 40 per cent more active than its precursor. Possibly hyperreactive infants produce 25-HCC more effectively from vitamin D; hyporeactive infants may provide less, or may more effectively degrade vitamin D to inert metabolic products, as described by Avioli *et al.*[30]

Taussig[31, 32] has discussed the possibility that an inborn variation in man's ability to metabolize vitamin D may be responsible for the injury to the cardiovascular system seen, not only in infantile hypercalcemia and in supravalvular aortic stenosis, but also in other cardiovascular diseases.

Norman's[33] current view and evaluation of new laboratory work has led him to postulate that vitamin D does not act as a vitamin or as a cofactor for enzymes, but rather as a steroid hormone. Minimal alterations in its chemical structure may either increase or abolish its biologic activity.

A recent postulate to receive favorable consideration by a number of authorities[8, 10, 18, 34] is that the initial metabolic and anatomic lesions of infantile hypercalcemia and supravalvular aortic stenosis syndromes are received *in utero*. These

proposals, initially made by clinicians who had recognized characteristics of infantile hypercalcemia in infants five to nine months old,[35, 36] led to laboratory studies of the effect of excess vitamin D given to rabbits during pregnancy.[37] Pregnant animals who were given exceedingly high doses of vitamin D, and some of their offspring, developed aortic lesions resembling those seen in the human disease. Since the mothers of hypercalcemic infants, from whom histories of prenatal supplementation were available, had either received no vitamin D,[38] or vitamin D in amounts not over 1,000 units daily,[12, 30, 40] this postulate is not proven.[14, 41] However, whether or not hyperreactivity to vitamin D in the mother provokes the lesion in the fetus, exposure of hyperreactive infants to even moderately elevated vitamin D intake has been reported to cause lesions.[13, 14,† 35, 38, 39]

In Great Britain, where hundreds of infants were reported to develop hypercalcemia during a period when they were given excessive amounts of vitamin D (calculated to be as high as 4,000 units daily in some instances),[6, 7] halving the amount of vitamin D in dried milk formulas, and in other supplements, resulted in halving the incidence of hypercalcemia.[1] The disease, however, has not been eliminated.[1, 2, 12, 14, 42, 43]

On the other hand, the incidence of rickets did not increase from the level reported before reducing the vitamin supplementation, during the same period of time, in an analysis that considered the colored immigrants separately.[1] The feeding histories of the immigrant children, however, revealed that they had not been fed the fortified infant foods to the same extent as had the native-born British children.[1, 44]

Editorial approval was given to the compromise reached in providing vitamin supplementation at a level that protects against rickets in the majority of the population, and causes disease only in the minority of infants with lower than normal tolerance for this vitamin.[2]

The official position, in 1965, of the American Academy of Pediatrics, referring to their earlier acceptance[11] of the likelihood that vitamin D intake is related to infantile hypercalcemia and supravalvular aortic stenosis, was to recommend public health measures which may reduce the incidence of one form of mental retardation and one form of congenital heart disease.[8] Such recommendations were considered justified only if a much larger normal population were not placed simultaneously in jeopardy from vitamin-D deficiency. Coleman,[45] who reported ECG changes in 12 of the 13 infants with hypercalcemia admitted to a Glasgow hospital in 1962–1963, has commented that in selecting the prophylactic dose of vitamin D it is necessary to make responsible inquiry into the nature of the residual effects of vitamin D deficiency and toxicity, and to consider their relative importance. By the time infantile hypercalcemia is diagnosed, irreversible brain and cardiovascular damage may have developed. Rickets can be diagnosed early in the course of the disease; the early bone lesions can be reversed by appropriate therapy.

It is infinitely preferable to prevent both diseases, rather than to weigh the sociologic value of preventing one or the other. For the child whose brain and heart are permanently damaged, the frequency with which this happens is irrelevant. A concerted effort should be undertaken to develop simplified techniques to ascertain the individual response to vitamin D very early in infancy. Supplementation with vitamin D, appropriate to individual requirements, should then be prescribed. The amount of vitamin D required for the prophylaxis of rickets in one child may be toxic for another.

There should be re-investigation of the delivered potency of the 400 units of vitamin D, added to milk. I have been able to find no disproof of the early work that showed markedly increased antirachitic activity of vitamin D in milk. We should exert every effort to develop an improved program which will prevent rickets without causing toxic reactions, even in a minority of our infant population.

† For details of cases of infantile hypercalcemia developing on daily intakes up to 2,000 units of vitamin D, see also references 43–45, 56, 61, 66, 67, 77, 91, 92, 95–98 in Seelig.[14]

References

1. Oppé, T. E.: Infantile hypercalcemia, nutritional rickets, and infantile scurvy in Great Britain; a British Paediatric Association report. Brit. Med. J. 1: 1659, 1964.
2. Editorial: Vitamin D as a public health problem. *Ibid.* 1: 1654, 1964.
3. Editorial: Aetiology of idiopathic hypercalcemia. *Ibid.* 1: 535, 1960.
4. Editorial: Idiopathic hypercalcaemia of infants. Lancet 2: 138, 1960.
5. Editorial: Infantile hypercalcaemia. Brit. Med. J. 1: 159, 1956.
6. Lightwood, R., Sheldon, W., Harris, C. and Stapleton, T.: Hypercalcaemia in infants; subcommittee report, British Paediatric Association report. Lancet 271: 138, 1956.
7. ——, ——, —— and ——: Hypercalcaemia in infants and vitamin D; subcommittee report, British Paediatric Association report. Brit. Med. J. 2: 149, 1956.
8. Committee on Nutrition, American Academy of Pediatrics: Vitamin D intake and the hypercalcemic syndrome. Pediatrics 35: 1022, 1965.
9. Bakwin, H.: The overuse of vitamins in children. J. Pediat. 59: 154, 1961.
10. Committee on Nutrition, American Academy of Pediatrics: The relation between infantile hypercalcemia and vitamin D; public health implications in North America. (Report written by Fraser, D.) Pediatrics 40: 1050, 1967.

11. ——: The prophylactic requirement and the toxicity of vitamin D. *Ibid.* 31: 512, 1963.
12. Black, J. A.: Idiopathic hypercalcaemia and vitamin D. German Med. Monthly 9: 290, 1964.
13. Fraser, D., Kidd, B. S. L., Kooh, S. W. and Paunier, L.: A new look at infantile hypercalcemia. Pediat. Clin. N. Amer. 13: 503, 1966.
14. Seelig, M. S.: Vitamin D and cardiovascular, renal, and brain damage in infancy and childhood. Ann. N. Y. Acad. Sci. 147: 537, 1969.
15. Bing, F. C.: Vitamin D milk containing 600 USP units of vitamin D to the quart is not eligible for list of accepted foods. JAMA 116: 413, 1941.
16. Harris, R. S., Ross, B. D. and Bunker, J. W. M.: Histological study of hypervitaminosis D. Amer. J. Dig. Dis. 6: 81, 1939.
17. Morgan, A. F., Kimmel, I., and Hawkins, N. C.: A comparison of the hypervitaminoses induced by irradiated ergosterol and fish liver oil concentrates. J. Biol. Chem. 120: 85, 1937.
18. Lewis, J. M.: Clinical experience with crystalline vitamin D; the influence of the menstruum on the effectiveness of the antirachitic factor. J. Pediat. 6: 362, 1935.
19. Stearns, G., Jeans, P. C. and Vandecar, V.: The effect of vitamin D on linear growth in infancy. *Ibid.* 9: 1, 1936.
20. Drake, T. G. H.: Comparison of the antirachitic effects on human beings of vitamin D from different sources. Amer. J. Dis. Child. 53: 754, 1937.
21. Lewis, J. M.: Further observations on the comparative antirachitic value of crystalline vitamin D administered in milk, cod oil, or in propylene glycol. J. Pediat. 8: 308, 1936.
22. Glaser, K., Parmelee, A. H. and Hoffman, W. S.: Comparative efficacy of vitamin D preparations in prophylactic treatment of premature infants. Amer. J. Dis. Child. 77: 1, 1949.
23. Supplee, G. C., Ansbacher, S., Bender, R. C. and Flanigan, G. L.: The influence of milk constituents on the effectiveness of vitamin D. J. Biol. Chem. 114: 97, 1936.
24. Loomis, W. F.: Skin pigment regulation of vitamin D biosynthesis in man. Science 157: 501, 1967.
25. Wickel, K. G.: Theory and practice of vitamin D fortification of milk. Quart. Rev. Pediat. 8: 224, 1953.
26. Spur, B.: Laboratory evaluation and control of homogenized and vitamin D milk. *Ibid.* p. 240.
27. Council on Foods and Nutrition: Fortification of nonfat milk solids with vitamins A and D. JAMA 197: 1107, 1966.
28. Dale, A. E. and Lowenberg, M. E.: Consumption of vitamin D in fortified and natural foods and in vitamin preparations. J. Pediat. 70: 952, 1967.
29. Blunt, J. W., De Luca, H. F. and Schnoes, H. K.: 25-hydroxycholecalciferol; a biologically active metabolite of vitamin D. Biochem. 7: 3317, 1968.
30. Avioli, L. V., Williams, T. F., Lund, J. and De Luca, H. F.: Metabolism of vitamin D_3-^3H in vitamin D-resistant rickets and familial hypophosphatemia. J. Clin. Invest. 46: 1907, 1967.
31. Taussig, H. B.: Possible injury to the cardiovascular system from vitamin D. Ann. Intern. Med. 65: 1195, 1966.
32. ——: On the evolution of our knowledge of congenital malformations of the heart. Circulation 31: 768, 1965.
33. Norman, A. W.: The mode of action of vitamin D. Biol. Rev. 43: 97, 1968.
34. Rowe, R. D. and Cooke, R. E.: Vitamin D and craniofacial and dental anomalies of supravalvular stenosis. Pediatrics 43: 1, 1969.
35. Garcia, R. E., Friedman, W. F., Kaback, M. M. and Rowe, R. D.: Idiopathic hypercalcemia and supravalvular aortic stenosis. New Eng. J. Med. 271: 117, 1964.
36. Jue, K. L., Noren, G. R. and Anderson, R. C.: The syndrome of idiopathic hypercalcemia of infancy with associated congenital heart disease. J. Pediat. 67: 1130, 1965.
37. Friedman, W. F. and Roberts, W. C.: Vitamin D and the supravalvular aortic stenosis syndrome; the transplacental effects of vitamin D on the aorta of the rabbit. Circulation 34: 77, 1966.
38. Stapleton, T., Macdonald, W. B. and Lightwood, R.: Management of "idiopathic" hypercalcaemia in infancy. Lancet 1: 932, 1956.
39. Schlesinger, B. E., Butler, N. R. and Black, J. A.: Severe type of infantile hypercalcaemia. Brit. Med. J. 1: 127, 1956.
40. Wilkse, H. E., Goldbloom, R. B., Antia, A. H., Ottesen, O. E., Rowe, R. D. and Cooke, R. E.: Infantile hypercalcemia syndrome in twins. New Eng. J. Med. 275: 1157, 1966.
41. Editorial: Supravalvular aortic stenosis. JAMA 198: 1302, 1966.
42. Barr, D. G. D. and Forfar, J. O.: Oral calcium-loading test in infancy, with particular reference to idiopathic hypercalcaemia. Brit. Med. J. 1: 477, 1969.
43. Coleman, E. N.: Infantile hypercalcaemia and cardiovascular lesions; evidence, hypothesis, and speculation. Arch. Dis. Child. 40: 535, 1965.
44. Bransby, E. R., Berry, W. T. C. and Taylor, D. M.: Study of the vitamin-D intakes of infants in 1960. Brit. Med. J. 1: 1661, 1964.

hyper-reactivity to vitamin d*

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"It is significant that the high incidence of 'idiopathic' hypercalcemia of infancy in England in the years 1953-57 was correlated with the excessive fortification with Vitamin D, of National Dried Milk for infant formulas (and other foods) during that period."

PRECIS

The author claims that selection of the approved prophylactic dosage of vitamin D, and of the vehicle in which it should be administered, was based on findings in infants with relatively high requirements of vitamin D. More recent recognition of the similarity of several pediatric diseases to syndromes caused by hypervitaminosis D, calls for a re-evaluation of the safety of a uniform high prophylactic dosage of this nutrient.

Whether toxicity will develop when vitamin D is administered, constitutes a peculiarly medical problem. The physical status of the patient, and the degree of his responsiveness to vitamin D, establishes a set of conditions which determines whether he will react adversely or respond adequately to prophylactic doses of vitamin D. Patients at one extreme react adversely to doses below recommended requirements. At the other extreme, administration of doses more than 50 times the advised therapeutic dose may be necessary for amelioration of some of the physical signs of vitamin deficiency, or even to prevent rickets. Fanconi[1] has presented an analysis of pathological conditions in childhood, which can be differentiated by their completely different reactions to vitamin D: vitamin D-resistant rickets at one extreme, and idiopathic hypercalcemia at the other (Figure 1, see next page). Infants with rickets, characterized not only by deficient intestinal absorption of calcium and phosphorus, but also by diminished renal tubular reabsorption of phosphorus,[2] have long been known to require and to tolerate much larger doses of vitamin D than normal infants.[3,4] Avioli et al.[5] have reported that familial vitamin D-resistant rickets is caused by a genetic abnormality in the metabolism of vitamin D, with increased formation and excretion of its water-soluble, inert degradation products.

A disease associated with elevated serum calcium and/or phosphorus may increase susceptibility to vitamin D toxicity. Hypercalcemia and/or hyperphosphatemia can result from primary or secondary failure of renal excretion. Increased mobilization of calcium from body stores may be caused by hyperparathyroidism, immobilization with atrophy of disuse, or primary or metabolic neoplasms of bone. Disturbances of calcium metabolism in sarcoidosis, and in idiopathic hypercalciuria has been attributed to hyper-reactivity to vitamin D,[6] as has

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*Vitamin D_2 = irradiated ergosterol or calciferol
Vitamin D_3 = natural fish oil vitamin D

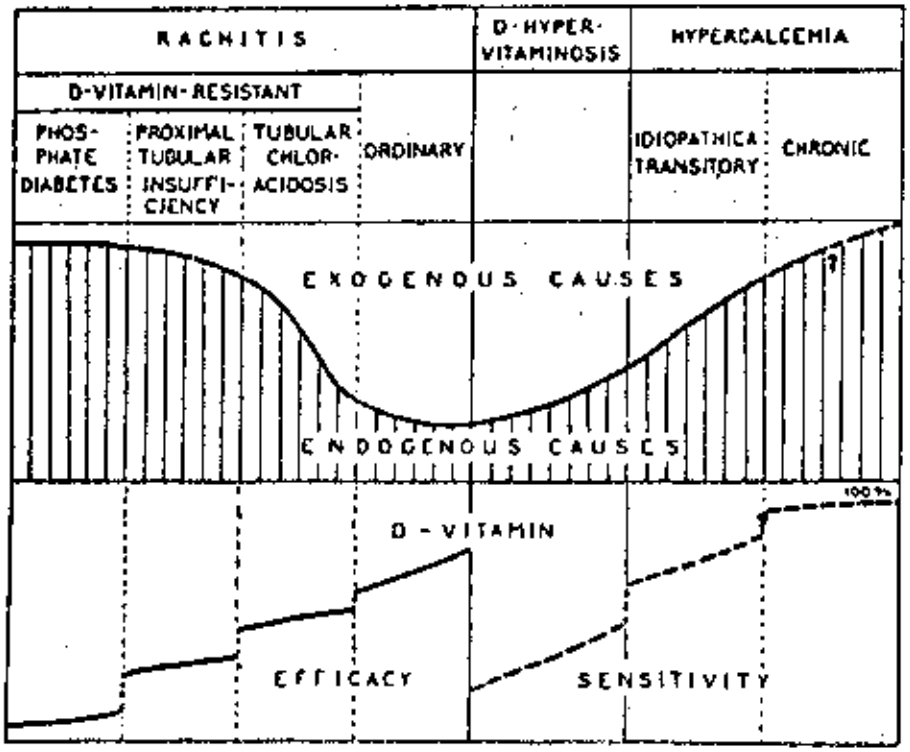


Figure 1. Spectrum of varying reactions to vitamin D. (G. Fanconi) Bone Structure and Metabolism. Ed. by G. E. W. Walstenholme and C. M. O'Connor (Little Brown & Company, Boston, Massachusetts, 1956)

infantile hypercalcemia and its sequellae[7] *(infra vide)*. Taussig[8] has proposed that there may be a genetic trait that, because it allows for better than average utilization of vitamin D, results in greater resistance to rickets and greater susceptibility to vitamin D toxicity.

Norman[5] has presented a thought-provoking postulate that vitamin D does not act as a vitamin or as a co-factor for enzymes, but as a steroid hormone. As such, vitamin D or one of its metabolites may interact on either gene activation or regression, or on one of the other steps in the regulation of calcium metabolism. Norman has concluded that vitamin D probably plays a crucial role, with parathyroid hormone and calcium, in a DNA, gene-dependent, homeostatic control mechanism for calcium metabolism. Since only slight alterations in the chemical structure of vitamin D may increase or abolish its activity, it is possible that differences in its metabolic handling in the body may explain the differences in reactivity to administered vitamin D in the clinic. For example, De Luca et al.[10,11] have recently identified a metabolic product of vitamin D (25-hydroxycholecalciferol, or 25-HCC), formed in the liver from vitamin D, that has 1.5 times the antirachitic potency of vitamin D, and that is probably the physiologically active form of vitamin D. Possibly hyper-reactive infants more effectively produce 25-HCC from vitamin D or the precursors. Hypo-reactive infants may produce less 25-HCC or may more effectively degrade vitamin D to inert metabolic products.[5]

Relative Hypo-reactivity to Vitamin D; Rickets Prophylaxis

The early investigators into the susceptibility to rickets of children, who had not received exogenous vitamin D, found differences in amounts of vitamin D required, depending on the population groups studied. Drake[12] reported from Toronto that only 13% of 244 infants (of British or Northern European stock) had detectable rickets, although none had received vitamin D supplementation. After five months observation, during which no vitamin D was added to their diets, only an additional 8% had active advancing rickets. As little as 300 units of vitamin D_2, administered as irradiated ergosterol, was therapeutic in rickets of moderate or marked degree.[12] The lowest amount tested: 95 units vitamin D_2 daily in irradiated milk, or 150 units of vitamin D_3, as cod liver oil, was fully prophylactic, as determined by roentgenographic examination.

In a New York City study of darker skinned children (Puerto Rican and Italian: 45%, and Negro 55%), Lewis[13] found that among 22 infants who had received no vitamin D during a five month observation period, eight, all of whom were black, developed rickets. This constituted 62% of the 13 Negro infants in this group. Eight percent, or 27 of 355 children given vitamin D_2 for five winter months, in doses ranging from 145 units in corn oil, propylene glycol or milk to 1,450 units in corn oil, developed rickets. Among the 155 Negro infants in this study, 21 or 14% developed rickets; among the 200 Puerto Rican and Italian infants, six or 3% developed rickets. None of the infants given vitamin D_2 in milk, even in doses as low as 145 units/28 ounces, developed moderate or advanced rickets; 3 developed mild rickets. However, one black child developed a mild

form of the disease, even on as much as 1,450 units of crystalline vitamin D_2 in corn oil.

Autopsy studies by Follis et al.,[14,15] of a series of 1,303 infants under two years of age, revealed that half of those who had received no vitamin D had rickets. The rachitic lesions were more prevalent and severe in Negro children than in white.

The failure to prevent rickets in one infant given 1,450 units vitamin D in oil suggested to Lewis[13] that 2,000-2,500 units might have been more fully protective. Three (5%) of the 58 infants given 145 units vitamin D_2 in milk having developed mild rickets, Lewis[13] recommended adding 332 units of vitamin D_2 to each quart of milk. A later paper by Glaser, et al.[16] presented evidence that all preparations of vitamin D in an oily vehicle in daily doses of 100 units were prophylactic against clinical rickets, even in premature and in Negro infants. However, because nine (5.6%) developed roetgenographic evidence or suspicion of rickets, among the 166 who were followed for the full observation period of eight months, the authors recommended the general use of 400-800 units (in oil) as a prophylactic dose. (For further data on effect of vehicle on potency, *infra vide.*)

Infantile Hypercalcemia: an Expression of Hyper-reactivity to Vitamin D

Infantile hypercalcemia has developed in children on doses of vitamin D considered prophylactic. Anorexia, vomiting, fretfulness, constipation, and weight loss or failure to gain are frequent during the first weeks or months. Renal and cardiac damage may be detectable next, and severe mental retardation, often associated with a peculiar "elfin" facies may become manifest later in the first year of life. Excessive absorption of calcium from the gut, in response to given doses of vitamin D, and/or abnormal metabolism of this vitamin or of related cholesterol derivatives have been demonstrated in this condition.[17-25] The similarity of the renal and cardiovascular findings in infantile hypercalcemia to those of experimental hyper-vitaminosis D, in the face of intakes that were not excessive has led many to suggest that the afflicted children are hyper-reactive to vitamin D.[1,7,8,17-54]

Lightwood[55] reported the first case of infantile hypercalcemia, that was probably caused by hyperreactivity to vitamin D. The infant had received only "physiological" amounts of vitamin D at a time (1932) when there was no vitamin D-supplementation of milk. She developed hypercalcemia, albuminuria, and mental retardation, was dwarfed, and died at 27 months of age. Her mother had taken no vitamin D during pregnancy.

In 1936, Thatcher[26] attributed the progressive failure in health of an infant who had thrived until six months of age, to idiosyncratic response to prophylactic administration of vitamin D. He had been given 100-400 units/day, and exposed to sunlight or ultraviolet irradiation from six months until he died of renal damage at almost a year of age. An infant who died at 15 weeks of age with hypercalcemia, and who had pathological signs of generalized arteriosclerosis of infancy at autopsy, had never received more than 500 units daily.[45] Infantile hypercalcemia, diagnosed in the first half year of life, has been reported to develop on vitamin D intakes of 130-1,000 units daily.[41,53] The condition of these children improved when the vitamin D and calcium intakes were restricted. Among the infants diagnosed as hypercalcemic at 6-20 months of age, the early vitamin D intakes were estimated at not over 2,000 units daily in over 50 cases.[17-21,23,26,29,33-36,44,45,51] Among five children diagnosed in late childhood, as presenting late stigmata of infantile hypercalcemia, reported by Black and Bonham-Carter,[54] two had had "usual" supplementation, and three had been breast-fed for one and a half to six months before receiving "usual" supplements. Seven such patients had histories of irregular vitamin supplementation,[56] no more than "usual" prophylactic doses,[36,57] 1,000-1,500 units daily for 5 years,[38] and less than 1,000 units daily.[50] In no instance, in which the vitamin intake of the mother had been recorded, had pre-natal vitamin D intake been over 1,000 units daily.[23-50,52,53]

The responses to the administration of vitamin D to infants with hypercalcemia have been found to differ from case to case. In one of the early studies, Bonham-Carter et al.,[18] in an attempt to identify a pathogenic factor, found that loading a hypercalcemic baby in remission with high doses of vitamin D_2 caused an increase in plasma calcium and a deterioration of his condition. His intake of vitamin D and calcium was then reduced with subsequent improvement. However, the hypercalcemic response to vitamin D and to its removal, seems to differ from case to case. Spontaneous fluctuations in serum calcium, as great as 2-3 mg/100 ml, have been reported by Fraser et al.[51] during the acute phase of the disease. Forfar[22] administered large doses of vitamin D_2 (50,000-100,000 units) for about a week

to three infants with hypercalcemia. In two instances, after a few days, the serum calcium rose significantly. It took about three weeks for the serum calcium to fall to dosage load levels. In one case, the serum calcium level fell following the administration of calciferol, and rose again to the pre-calciferol load level within a day of stopping treatment. Girardet[58] has stated that in certain individuals, even large doses of vitamin D may produce signs of renal toxicity without significant hypercalcemia.

Either hyper-reactivity to administered vitamin D,[18,31] or its defective inactivation,[40] is suggested by metabolic balance studies that show the high percentage of dietary calcium that is absorbed (60%) after administration of vitamin D to hypercalcemic infants. Measurements of vitamin D activity in the plasma of hypercalcemic infants have shown both insignificant differences from normal values in one series,[59] and markedly higher than normal values in others.[10,31,44] Garcia et al.[44] reported a serum value of 1,540 units in an infant who had hypercalcemia and supravalvular aortic stenosis, and whose daily vitamin D intake was 400 units from milk and 1,000 units from a supplement. (The normal range of serum vitamin D in this institution was 60-400 units.) Fellers and Schwartz,[15] reported that three patients with hypercalcemia had serum vitamin D activity that was 20 to 30 times that seen in normal infants, although all exogenous vitamin D had been removed from their diets. Two additional hypercalcemic infants were reported by Manios and Antener[40] to respond to vitamin D (700 units in oil for four days) by a twofold rise in serum vitamin D levels from pretreatment values of 520 and 580 (as compared with the normal mean in this laboratory of 133 units: range = 50 to 200 units).

These observations suggest that this condition is associated with abnormal metabolism of vitamin D. Forfar and his co-workers[20-22] found that hypercalcemic infants also have a marked increase in free serum cholesterol, a finding reported also by Lefebvre.[25] In view of the vitamin D-like effect of some cholesterol derivatives, Forfar and his co-workers have suggested that this disease may be related primarily to a disorder of cholesterol metabolism. They postulated that even a small amount of calciferol may act as a chemical mediator in this disease, by activating endogenous vitamin D precursors that may be present in excess in susceptible infants.[21,22]

Williams' Syndrome or Supravalvular Aortic Stenosis — (SAS) Syndrome: Late Expression of Infantile Hypercalcemia

It is possible that the SAS or Williams' Syndrome, an abnormality characterized by supravalvular aortic stenosis, a peculiar facies, and severe mental retardation[60] (Figure 2) is a late expression of hyper-reactivity to vitamin D during infancy.[7] Black and Bonham-Carter[54] first observed the similarity in the stigmata of children who survived infantile hypercalcemia, to those seen in children with supravalvular aortic stenosis (Figure 3). Both conditions have been associated with cardiac damage, often with renal impairment, "hypercalcemic facies" and mental retardation.[7,8,48,51,52,60-74] One of the children, reported by Black and Bonham-Carter,[54] later died at 16 years of age with renal failure, hypertension, and left ventricular failure[61] (Figure 4). Garcia et al.[44] reported the Williams' syndrome in an infant with biochemically diagnosed hypercalcemia and a markedly elevated vitamin D blood level (Figure 5). Bauman and Bauman[62] reported two children with hypercalcemia and cardiac damage, in one of whom all symptoms, signs and abnormal laboratory findings disappeared five years later (Figure 6). The only significant treatment had been curtailment of vitamin D intake. Beuren et al.[63,64] reported supravalvular aortic stenosis with mental retardation and "hypercalcemic facies" in German children with definite histories of hypercalcemia. Many of these children had received excessive vitamin D parenterally during infancy. In some instances, the mothers had also been given "usual" prophylactic doses of vitamin D pre-natally (in multivitamin preparations.)

The possibility that pre-natal insult (caused by excess vitamin D during pregnancy) is the most important contributing factor to this disease has been proposed by several authorities.[65,75-77] They suggest that the excessive response of the infant to vitamin D may be caused by metabolic and anatomic damage to the fetus. Support for this concept was obtained largely from the experimental work of Friedman et al.,[78,79] who had earlier shown aortic lesions in some of the young rabbits, and in all of their mothers, given toxic doses of vitamin D during pregnancy.[80] However, there is no evidence that excessive vitamin D had been taken during pregnancy by any of the mothers of children with hypercalcemia or the SAS syndrome.[7,67A] The mother of the child with infantile hypercalcemia, who had been reported by Smith et al.[37] was later found to

Figure 2 (Top): Four patients with supravalvular aortic stenosis (courtesy of J. C. P. Williams. **(Bottom):** Profiles of children in front row (reproduced by permission of J. C. P. Williams, B. G. Barratt-Boyes, and J. B. Lowe, and Circulation, 24:1311-1318, 1961).

respond to test with a large dose of vitamin D by development of hypercalcemia, and by symptoms of mental agitation.[63] It is of interest that her second daughter, who had been reported earlier by Kenny et al.[41] to have had symptomless hypercalcemia that was diagnosed at three and a half months because of the history of the older sister with the infantile hypercalcemia syndrome, improved on a low vitamin D regimen, and developed normally. Another mother of a child with infantile hypercalcemia became pregnant shortly after being given large dosage vitamin D to see if she was hyper-reactive. She was immediately taken off all vitamin D, and after a normal gestation, gave birth to a normal infant.[61]

Friedman[13] has stated that the SAS syndrome is not uncommon, and that its association with infantile hypercalcemia may be missed. He has commented on the fact that not all of the infants who developed hypercalcemia had received large doses of vitamin D, and reviewed the evidence that an inborn error of metabolism of vitamin D — possibly with production of a cholesterol derivative with anti-rachitic activity (Forfar[20]) — might be responsible.[18] Taussig[48] has suggested that the two types of supravalvular stenosis syndrome — that associated with, and that without the characteristic facies and severe mental retardation — may represent different responses to the same underlying condition. She has suggested that the inborn variation in man's ability to metabolize vitamin D may be responsible for the injury to the cardiovascular system seen both in infantile hypercalcemia and in supravalvular aortic stenosis.[8] The increase in blood cholesterol, often seen in hyper-vitaminosis D, further sug-

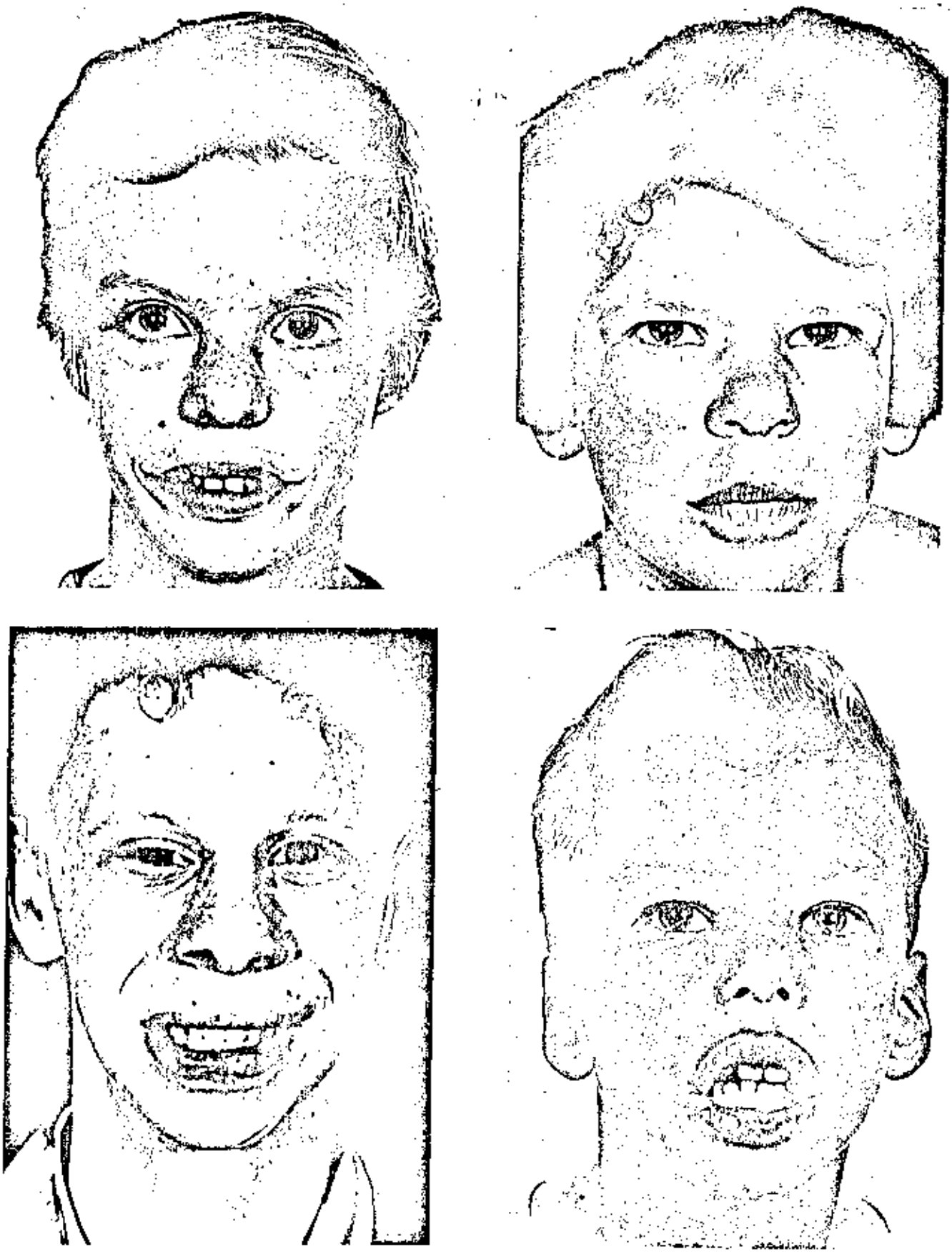


Figure 3. Survivors of infantile hypercalcemia. (Reproduced by permission of J. A. Black and P. E. B. Bonham-Carter and Lancet, 2:745-746, 1953.)

A

B

C

Figure 4 (A, B). Patient with hypercalcemia at 21 months of age. (Reproduced by permission of B. E. Schlesinger, N. R. Butler, and J. A. Black and Brit. Med. J. 1:127-134, 1958). **(C).** Same child at 11 years of age (reproduced by permission of J. A. Black and R. E. B. Bonham-Carter and Lancet 2:745-748, 1963).

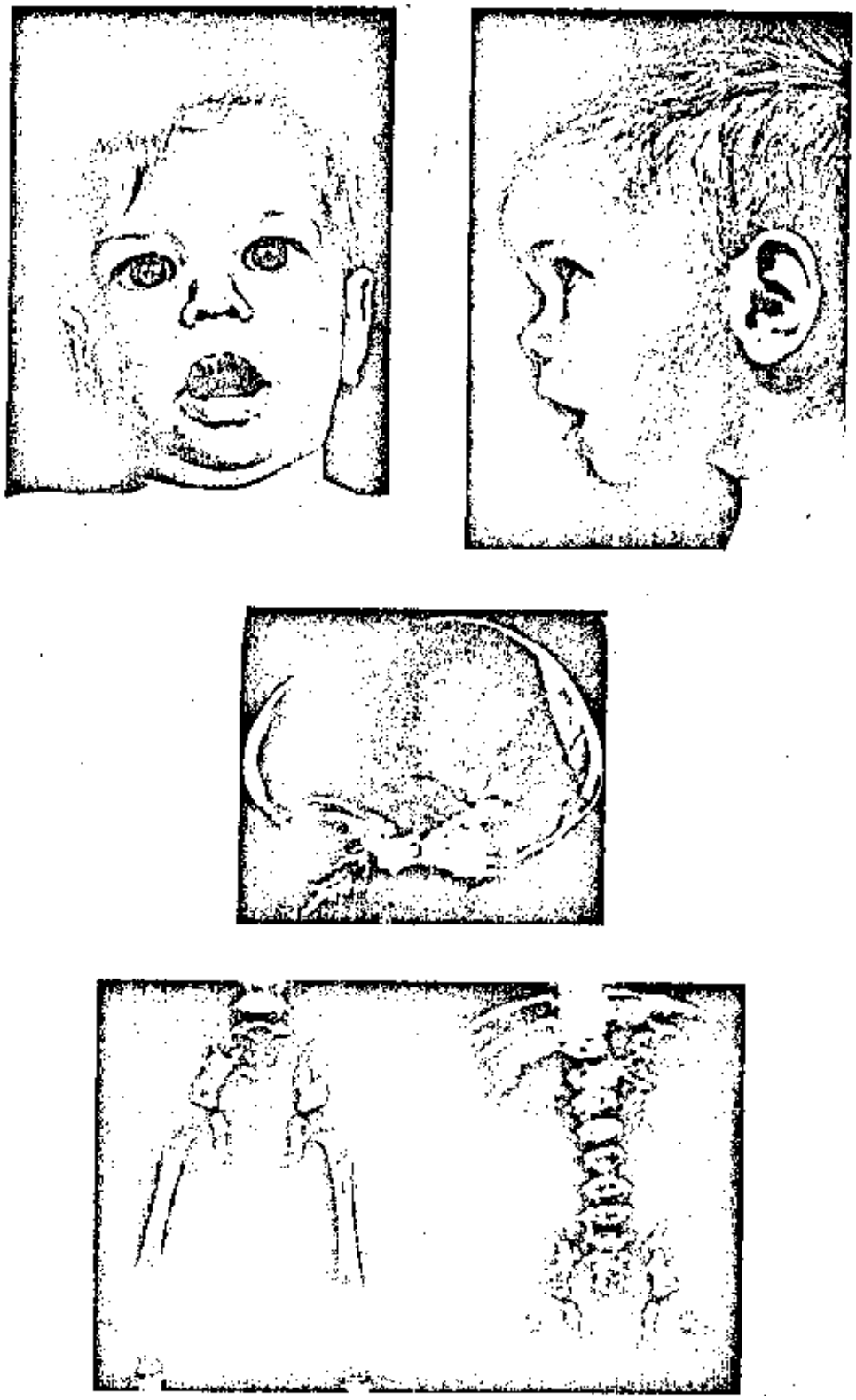
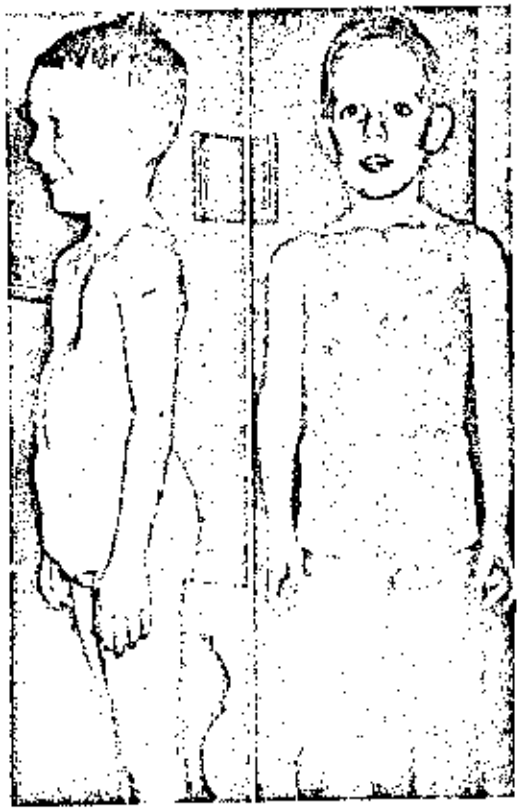


Figure 5. Hypercalcemic infant with supravalvular aortic stenosis. **(Top)** Note the high, prominent forehead, epicanthal folds, underdeveloped bridge of the nose and mandible and overhanging upper lip. **(Center and Bottom)** Roentgenograms of the skull, pelvis and long bones and spine, showing appreciable sclerosis of the calvaria and base of the skull, as well as diffuse osteosclerosis, with bands of increased density at the metaphysical ends of the long bones and the epiphyseal margins of the vertebras. (Reproduced by the permission of R. E. Garcia, W. F. Friedman, and R. D. Rowe and New Eng. J. Med., 271:117-120, 1964.)

A

B



C

Figure 6. Hypercalcemic patient, who recovered on curtailment of vitamin D. **A.** Hypercalcemic infant: Note malnutrition, poor development, low position of pinnas. **B.** Roentgenogram, showing cardiac enlargement; "coeur en sabot" configuration. **C.** Patient, five years later: Note alert appearance, good muscular development. [Reproduced by permission of A. Bauman and C. G. Bauman and N. Y. State J. Med., 65:1910-1917, 1965.]

gests that such variation in susceptibility to vitamin D may be related to the far commoner vascular lesions such as arteriosclerosis, which is associated with abnormal cholesterol metabolism. Black[24] has reviewed this and additional theories that have been proposed to explain the metabolic abnormalities that may be responsible for hyper-reactivity to vitamin D.

Generalized Arteriosclerosis of Infancy and Vitamin D

The possibility that hypervitaminosis D may be a causal factor in generalized arterial calcification of infancy, a disease diagnosed only at autopsy, was considered in 1947 by Stryker.[82] He recorded the available information as to vitamin D intake in his series of five cases, as well as in 15 cases tabulated from literature. One stillborn infant had coronary and aortic calcification as well as myocardial necrosis and calcification. Four who died between two and seven months of age also had coronary calcification; two had myocardial infarctions. In the case of an infant who died at three months, the family physician had suspected hypervitaminosis D, but could elicit no history of unusual vitamin intake by either the mother or the infant. Menten and Fetterman[83] reported an infant who died of generalized arteriosclerosis and myocardial infarction, born to a mother who had taken no vitamin D during pregnancy, but who had taken two capsules of dicalcium phosphate daily. He had received five drops of Oleum Percomorph daily from two weeks of age. In his second month of life he began to show symptoms (i.e. vomiting and fretfulness) that are commonly seen early in infants with infantile hypercalcemia. At autopsy, all of the superficial coronary branches were found to be thickened, sclerotic and calcified, and the aorta was diffusely thickened. Wilkerson's[43] hypercalcemic infant, who had never received more than 500 units vitamin D/day, had severe calcinosis of arteries, kidneys, and soft tissues on autopsy at 17 weeks.

Infants who died early in the course of hypercalcemia, as well as those with generalized arteriosclerosis of infancy, had histologic aortic, myocardial, and renal changes that resemble markedly the lesions of experimental hypervitaminosis D in rats reported by Gillman and Gilbert[64] and by others reviewed elsewhere.[7] Intimal and sub-endothelial thickening with fragmentation, degeneration and calcification of the elastica, as well as medial and myocardial degenerative changes have been found on autopsy of infants and children reported here[7,23,27,31,43,45,55,72,74] as well as in the laboratory models of vitamin D toxicity.[7,64]

The renal damage seen in the picture of generalized arteriosclerosis of infancy has been emphasized by Stryker.[82] Moran and Becker,[85] who reviewed the published cases to date in 1959, suggested that renal damage might have predisposed to the metastatic arterial calcification seen in this condition.

Renal Acidosis and Vitamin D

Another disease of infancy with which vitamin D has been associated, and that may be related to hypercalcemia of infancy, is renal tubular acidosis. Lightwood[86] observed that there was a steady increase in the incidence of this disease in Great Britain from 1950 (the first date in his tabulation) through 1953, after which there was a sharp drop in incidence (See Figure 7). Since infants with this syndrome often exhibit elevated serum calcium levels, he proposed that there was clearly a biochemical overlap between infantile hypercalcemia and infantile renal tubular aci-

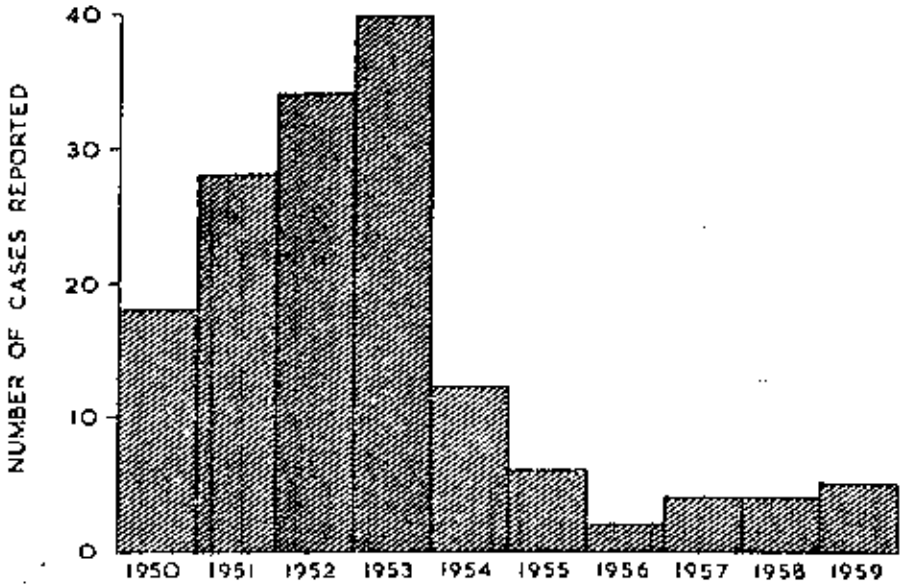


Figure 7. Infantile renal tubular acidosis: 153 cases by years of occurrence (14 centres). Information is lacking for 1950 for Liverpool. (Reproduced by permission of R. Lightwood and N. Butler and Brit. Med. J. 1:855-857, 1963.)

doses. However, the drop in incidence occurred in 1954, three years before the excessive fortification with vitamin D_2 of Dried Milk Formula in England was corrected.[7,87] The reduced incidence of infantile renal acidosis coincided with the sharp rise in incidence of hypercalcemia of infancy that occurred the year after the Ministry of Food recommended increasing the vitamin D content of dried milk from 280 to 500 I.U. per ounce.[7,88] It is possible that infants who developed the full blown syndrome of hypercalcemia of infancy, which includes renal impairment and calcinosis, might have developed only renal tubular acidosis on half the amount of vitamin D.

Graham[13] reported the association of renal acidosis with infantile hypercalcemia in his series of 47 infants with hypercalcemia. Except for the low plasma CO_2 in those with renal acidosis, the findings of all the affected infants were essentially the same. Renal calcinosis is common to both diseases. The author commented that he rarely saw renal acidosis uncomplicated by hypercalcemia.

In a study of 60 cases of nephrocalcinosis infantum, detected only at autopsy, Shanks and MacDonald[89] observed that only 12 had been diagnosed ante-mortem as having had "renal acidosis." Prolonged administration of fortified milk and/or vitamin supplements had been reported in the histories of 52. The authors postulated that symptomless hypercalcemia is a more widespread condition than has been appreciated and that the renal lesions may be detected only in children that died of some other condition.

Risk of Mass Medication with High Dose Vitamin D in Milk

Mass uniform prophylactic therapy, intended to prevent one disease, is justified only when the practice does not, at the same time, cause another. Evidence has accrued that the amount of vitamin D added to milk (which is enough to prevent, or even to cure, rickets in children who require very large amounts) is sufficient to cause renal, cardiovascular, and brain damage in those who are hyperreactive. Such children are now subject to diseases caused by hypervitaminosis D. Vitamin D is not only added to milk, but is commonly added to many other foods, as well as taken as specific supplements, so that apparent vitamin D intakes of as much as 2000 units/day may be reached.[90]

Since vitamin D is much more active in milk than it is in oil, and since there is evidence that vitamin D_2 is more toxic than vitamin D_3, the addition of 400 units of vitamin D_2 to a quart of milk may provide far more than a safe intake for susceptible infants. For children with a high intake of milk, the daily intake of vitamin D is undoubtedly higher.

Effect of Vehicle on Potency of Milk

Increases in potency, 3-10 fold, have been reported when vitamin D is added to milk, as compared with its potency in oil.[12,13,91-93] Stearns et al.[91] calibrated the effect of different doses of vitamin D_3 against the growth of infants. Their work suggested that infants given 300-400 units of vitamin D_3 in cod liver oil grew at faster than average rates, whereas infants getting all of their vitamin D_3 from cod liver oil emulsified in milk required only 135 units per quart for the same rate of growth. Lewis[92] has demonstrated that 90 units of vitamin D_2 in milk healed all nine rachitic infants in his study, whereas of the nine infants who received 90 units in oil, three showed no healing at six weeks and the rachitic process became worse at the end of the eight weeks in two. On the basis of his studies with vitamin D_2 in both vehicles in the treatment of rickets (see Table 1, following page) he observed that the therapeutic results obtained from 90 units in milk were superior to those obtained with 900 units in oil.

Supplee et al.[93] explored the factors in milk that might have accounted for this enhancement effect. Using the rachitic rat "line test," they found marked differences in the anti-rachitic response, depending on the vehicle in which the vitamin D had been delivered. Milk, or even an isolated milk constituent, lactalbumin, was shown to enhance the potency of vitamin D.

It should be noted here that Black[24] and Thomas et al.[94] have cautioned that this assay does not detect every substance in the serum that is capable of raising the serum calcium (i.e., to pathogenic levels). For example, dihydrotachysterol has 10 times the activity of vitamin D in causing calcifying cardiomyopathy (in rats),[95] but has only 1/400 the anti-rachitic activity of vitamin D by the rat "line test," and has hypercalcemic activity approximately equal to that of vitamin D_2 in man.[94]

The decision as to the amount of vitamin D_2 to be added to milk was based largely on the early work done by Lewis,[13] Drake,[12] and Glaser.[16] These investigators found that 100 to 290 units of vitamin D_2 in oil were sufficient to prevent rickets in over 90% of the children on test. To assure adequate prophylaxis in all children, Glaser[16] recommended 400-

Table I

COMPARISON OF HEALING, AMONG 36 RACHITIC INFANTS, BROUGHT ABOUT BY CRYSTALLINE VITAMIN D INCORPORATED IN MILK OR IN CORN OIL

CASE	AGE (MO.)	DATE BEGUN	MENSTRUUM IN WHICH CRYSTALLINE VITAMIN D WAS INCORPORATED	NO. OF RAT UNITS (STEENBOCK) GIVEN	ROENTGENOLOGIC RICKETS: AT ONSET	HEALING AFTER 4 WK.	HEALING AFTER 6 WK.	HEALING AFTER 8 WK.	HEALING AFTER 10 WK.	COMMENT
C. M.	8	1/23	Milk†	45	Moderate	+	++	+++	++++	Porto Rican
B. G.	6	1/25			Moderate	+	+±	++	++±	Porto Rican
F. R.	13	1/26			Moderate	0	0			Negro
E. D.	6	2/ 7			Slight	+	++	+++±		Italian
C. N.	8	2/18			Moderate	+	+±	++	+++	Porto Rican
C. W.	22	2/17			Marked	+±	++	++±		Negro
G. B.	8	2/21			Moderate	+±	++±	+++±		Negro
J. N.	6	2/24			Mild	0	0	0 (worse)		Negro
V. L.	6	1/13	Milk‡	90	Slight	++	+++	Normal		Italian
S. Q.	4	1/13			Moderate	+±	++±	+++±	++++	Porto Rican
J. A.	5	1/13			Slight	+++	++++	Normal		Negro
R. L.	5	1/19			Moderate	+++	Normal	Normal	Normal*	Negro
C. H.	20	1/25			Marked	+++	+++±	++++	Normal	Negro—1/25: calcium, 9.8 mg.; phosphorus, 3 mg. 2/23: calcium, 10.6 mg.; phosphorus, 5 mg.
K. S.	4	2/22			Slight	++	+++	Normal		Negro
B. C.	7	3/ 2			Moderate	+				Porto Rican
F. S.	15	3/17			Moderate	+	+±			Negro
B. R.	6	3/17			Moderate	+±	+++			This infant had received 90 units of crystalline vitamin D in oil for one month, and no healing resulted
T. R.	5	2/ 4	Oil§	90	Moderate	+±	++±	+++	++++	Porto Rican
S. L.	7	2/12			Slight	†	+	+±		
B. M.	4	2/14			Slight	+	++		++	Negro
B. R.	5	2/16			Slight	0 (worse)				Negro
V. B.	14	2/16			Marked	+	+±	++	+++	Negro
D. R.	7	2/21			Moderate	0	0	0 (worse)	0	Negro
C. Q.	5	2/23			Slight	†	+	+±	++±	Porto Rican
C. B.	8	2/28			Moderate	0	0	0 (worse)		Negro
T. B.	8	3/19			Moderate	0	0			Negro
J. H.	3	2/16	Oil‖	900	Slight	++		Normal	Normal	Negro
P. G.	6	2/ 9			Slight	++	++++			Italian
J. B.	6	2/16			Slight	++	++±	+++	+++±	Negro
T. B.	6	2/16			Slight	++	++++	Normal		Negro
C. M.	6	2/16			Moderate	++				Negro
R. P.	17	2/17			Marked	+				Negro
H. B.	4	2/17			Slight	++	+++	+++±	Normal	Negro
J. D.	7	2/17			Moderate	++	++±	++±	+++±	Negro
N. E.	3	2/17			Slight	+	++	++±		2/15: calcium, 7.2 mg.; phosphorus, 5.3 mg. 3/13: calcium, 10.3 mg.; phosphorus, 6.1 mg.
J. D.	24	1/30			Marked	+	+	+	+±	Negro; at 12 weeks healing ++

§One drop contained 15 units.
‖One drop contained 150 units.

+Denotes slight healing; ++, moderate healing; +++, marked healing; ++++, healed rickets.
* X-ray picture taken at 13 weeks.
†One liter contained 60 units.
‡One liter contained 120 units.

From LEWIS, J.M.: Clinical Experience With Crystalline Vitamin D: The Influence of the Menstruum on the Effectiveness of the Antirachitic Factor. J. of Pediatrics 6: 362-373 (1935)

5218

800 units in oil; Lewis,[13] who had found vitamin D_2 in milk to be far more potent than vitamin D_2 in oil, suggested adding 332 units to milk, in order to protect even infants with very high vitamin D requirements.

Quite apart from the effect of the vehicle on the potency of vitamin D, is the type of vitamin D provided. Forfar and his co-workers[21,22] have suggested that the vitamin D_2 provided in milk, to a greater extent than natural fish oil vitamin D_3, might activate chemical precursors of anti-rachitic sterols in sensitive infants. Furthermore, direct evidence was provided over thirty years ago that vitamin D_2 is significantly more toxic than is vitamin D_3, particularly as regards cardiovascular and renal toxicity.[96,97]

It is significant that the high incidence of "idiopathic" hypercalcemia of infancy in England in the years 1953-1957 was correlated with the excessive fortification with vitamin D_2 of National Dried Milk for infant formulas, and of other Welfare and proprietary foods during that period[7,87] (see Table II). In 1956, the British Pediatric Association recommended reconsidering the vitamin D needs of normal infants before advising on the amount of vitamin D to be added to milk; they suggested its omission from infant cereals, as well as from milk until more data were obtained.[87] The Government recommendation, in 1957,[99] was that (1) the vitamin D content of dried milk be reduced to a minimum of 90-100 units/dry ounce, (2) the vitamin D content of infant cereals be reduced to 300 units/dry ounce, and (3) the vitamin D content of cod liver oil be reduced to 400 units/teaspoonful. Consumption of only one of these sources provides the maximum daily intake or more. Intake of either fortified cereal or oil may be marginally safe in hyper-reactive children; intake of fortified milk with or without the other sources may be toxic.

The compromise of providing vitamin D at a level that protects against rickets in the majority of the population, and that causes disease only in the minority of infants with lower than normal tolerance for this vitamin was approved editorially in 1964.[100] Halving the amount of vitamin D in fortified foods and supplements, was reported to halve the incidence of hypercalcemia;[101] it did not increase the incidence of rickets (in an analysis that considered the colored immigrants separately, since they had not been fed the fortified foods to the same extent as had the native British children[101,102]). Coleman,[47] who reported ECG changes in 12 of 13 Scottish infants with hypercalcemia (admitted to a hospital in the year 1962-1963), has cautioned that it is necessary to make responsible inquiry into the nature of the residual effects of vitamin D deficiency and toxicity, in selecting prophylactic dosage.

In North America, where the Council on Foods and Nutrition of the American Medical Association has long considered acceptable only milk containing no more than 400 units of vitamin D[103], there have been many published reports on infants with hypercalcemia[7,17,30,33-41,43-45,50,56,57,104-106] and other reports on the Williams' Syndrome.[8,44,48,62,63,67,67A,69,70,75,78,106] The American Academy of Pediatrics accepted, in 1963, the likelihood that vitamin D intake is related to infantile hypercalcemia and supervalvular aortic stenosis.[60] In 1965, they recommended public health measures which may reduce one form of congenital mental retardation

TABLE II

FORTIFICATION OF NATIONAL DRIED MILK
(Great Britain)

Date	I.U. Vitamin D per Dry Ounce	Recommendation by
1945	280	Ministry of Health[99]
1953	500	Ministry of Food[87]
1957	90-100	Ministry of Health[99]

CALCULATED DAILY INTAKE OF VITAMIN D (1956)[87]

1 1/2 pints dried milk (460 U/dry oz.):	1,725	I.U.
1 ounce cereal (1,000-1,500/dry oz.):	1,000 - 1,500	I.U.
1 tsp. cod liver oil	700 - 800	I.U.
Total	3,525 - 4,025	I.U.

(Top): Changes in amounts of vitamin D added to dried milk in Great Britain. (Bottom): Possible intake of vitamin D at time of peak fortification, if all three sources were consumed.

and heart disease, provided a much larger population was not simultaneously placed in jeopardy of rickets.[107]

Readily detectable, early bone changes of rickets are reversible; the cardiovascular, renal and brain lesions of hypercalcemia are not. It is infinitely preferable to prevent both diseases, rather than to weigh the sociologic value of preventing one or the other. The minimum toxic dose of vitamin D for infants has been given by Stewart et al.[108] as only five to ten times that recommended for optimum growth. If the potency of vitamin D_2 in milk is increased only five fold, and the work cited indicates that it may be increased even more, and may be more toxic than "natural" vitamin D_3, a toxic dose may be reached for the more susceptible children in one quart of milk/day without any other source — as supplement, food, or sunlight. Our objective should be to identify hyper- and hypo-reactive infants, and to see to it that they ingest the amount of vitamin D that they can tolerate and/or that they require.

Identification of Hyper-reactive Infants

It is noteworthy that with only one exception, all of the children with infantile hypercalcemia reported in the literature reviewed in this paper were fair-skinned. That hyper-reactivity is not limited by race, however, is indicated by the fact that generalized infantile arteriosclerosis (which most resembles acute vitamin D toxicity) has been reported in two Negro infants[45] although most affected infants have been white.[86] Daeschner, et al.[109] reported that in a clinic with a significant proportion of Negro or Latin American children, there was a much higher incidence of urinary tract calculi in the white children. As for Williams' syndrome, it was initially described as a disease seen in fair-skinned children.[90] Several dark-skinned children with supravalvular aortic stenosis, however, have been reported.[67,69]

Warkany and Mabon[110] have tested blood samples from 34 white children and 26 Negro children (See Table III) and found that the average value of 122.6 anti-rachitic units for the white children was about 15% higher than that for the Negro children (106.5 units). Perhaps more significant in considering factors that may play a role in diseases caused by either hypersusceptibility to vitamin D or hyposusceptibility, is the distribution of values. The highest blood levels reported (165 units) were seen in 18% of the white and 4% of the black children; levels half that high were found in only 9% of the white, and in 36% of the black children.

The possibility that the generally higher anti-rachitic blood levels and greater response to vitamin D in white than in heavily pigmented children may be a consequence of the screening of ultra-violet rays by the skin pigment must be considered. On the basis that one square centimeter of white human skin synthesizes 18 units vitamin D in three hours, Loomis[111] has calculated that 400 units/day can be synthesized by the cheeks of Northern European infants exposed to the sun. On the other hand, Negro stratum corneum filters out 50-95% of the solar ultra-violet irradiation. That this explanation can account solely for the extremes in vitamin D-reactivity, however, seems unlikely. Generalized infantile arteriosclerosis and infantile hypercalcemia at one end of the distribution curve, and familial vitamin D-resistant rickets at the other, probably reflect mutations which cause changes in the metabolism of vitamin D, and which can occur irrespective of skin pigment.

Routine diagnostic tests to detect reactivity to vitamin D, preferably in the first week of life, should be developed. Since vitamin D toxicity is known to cause hypercalcemia and hypercholesterolemia (particularly of the free fraction),[22] perhaps a simple screen for the serum calcium and free cholesterol response to a standard dose of vitamin D in

Table III

U. S. P. Units per 100 Cc. of Blood Serum	Total No. Tested	White Adults	White Children	Negro Children
165	9	2	6	1
142	27	10	11	6
110	31	14	10	7
94	6	2	4	2
83	13	1	3	9
66	1	1	0	0
Total	89	30	34	25
Average vitamin D level, units per 100 cc.	110.4	117.6	122.6	106.5

Frequency Distribution of Persons According to Levels of Vitamin D in Blood Serum

(Reproduced by permission of J. Warkany and H. E. Mabon and Amer. J. Dis. Children, 60:606-614, 1940.)

milk, may suffice. The range of responses should be established—with special attention directed to new-born siblings of children with supravalvular aortic stenosis or of children with familial vitamin D-resistant rickets, as well as of the patients themselves, and of their parents (fathers, as well as mothers). More complicated tests, as of serum levels of anti-rachitic factors, including the recently discovered 25-HCC,[10,11] may provide important additional data on the nature of individual differences in response to vitamin D.

With such data, individualized vitamin D requirements should be able to be determined early in life. Until suitable tests are available and widely employed, milk should be made available in two forms; one that is free of added vitamin D, and one that is fortified, preferably at the same price so that economics not play a role in the milk bought. The private or public health physician should then recommend the source and dosage of vitamin D to be taken.

SUMMARY

1. Disease states or genetic differences that cause altered intestinal absorption or renal excretion of calcium and phosphorus, or altered synthesis or metabolism of Vitamin D, affect individual requirements of Vitamin D or susceptibility to its toxicity.

2. Vitamin D requirements are not uniform, white children generally requiring significantly less Vitamin D than do black children for prevention or cure of the skeletal lesions of hypovitaminosis D.

3. Hyper-reactivity to Vitamin D has been widely implicated as a pathogenic factor in infantile hypercalcemia, a disease that results in irreversible damage to kidneys, heart, arteries and brain.

4. There is evidence that the Williams' Syndrome (supravalvular aortic stenosis, usually associated with renal damage, severe mental retardation, and peculiar facies) is a late expression of infantile hypercalcemia.

5. The cardiovascular and renal lesions of generalized arteriosclerosis of infancy resemble those of infants dying early in the course of infantile hypercalcemia, and of animals with experimental hypervitaminosis D.

6. Renal tubular acidosis and nephrocalcinosis infantum, diagnosed only at autopsy, have also been associated with either Vitamin D overdosage or hyper-reactivity to Vitamin D.

7. The three- to ten-fold enhanced activity of Vitamin D in milk, as compared to the same unitage in oil, and the greater toxicity of Vitamin D_2 as compared with Vitamin D_3, results in consumption of potentially harmful amounts of Vitamin D by those drinking milk freely.

8. Four hundred units in oil has been shown to be prophylactic even in the group most susceptible to rickets; 90 units in milk has been reported to be prophylactic in the population group most reactive to Vitamin D.

9. There is urgent need for development of tests for reactivity to a standard dose of Vitamin D, to be applied in infancy.

10. Milk should be provided in 2 forms; (1) free of added Vitamin D, and (2) fortified as at present, the physician to recommend the source and amount of Vitamin D to be taken.

11. The question of how best to protect against rickets should be reinvestigated, with greater individualization of dosage considered a vital objective.

BIBLIOGRAPHY

1. Fanconi, G.: Variations in sensitivity to vitamin D: From vitamin D resistant rickets, vitamin D avitaminotic rickets and hypervitaminosis D to idiopathic hypercalcaemia. Bone Structure and Metabolism, ed. G. E. W. Wolstenholme and C. M. O'Connor. Little, Brown, and Co., Boston, 1956.
2. Harrison, H. E.: Vitamin D and calcium and phosphate transport. Pediatrics 28:531, 1961.
3. Harris, L. J.: The mode of action of vitamin D. The "parathyroid" theory. Clinical hypervitaminosis. Lancet 1:1031, 1932.
4. Harrison, H. E.: The Fanconi syndrome. J. Chronic Diseases. 7:346, 1958.
5. Avioli, L. V., Williams, T. F., Lund, J., and Deluca, H. F.: Metabolism of vitamin D_3 ^3H in vitamin D-resistant rickets and familial hypophosphatemia. J. Clin. Invest. 46:1907, 1967.
6. Jackson, W. P. U., and Dancaster, C.: A consideration of the hypercalciuria in sarcoidosis, idiopathic hypercalciuria, and that produced by vitamin D. A new suggestion regarding calcium metabolism. J. Clin. Endocrinol. and Metabolism. 19:558, 1959.
7. Seelig, M. S.: Vitamin D and cardiovascular, renal, and brain damage in infancy and childhood. Ann. N. Y. Acad. Sci. 147: [Art. 15]:537, 1969.
8. Taussig, H. B.: Possible injury to the cardiovascular system from vitamin D. Ann. Int. Med. 63:1195, 1966.
9. Norman, A. W.: The mode of action of vitamin D. Biol. Rev. 43:97, 1968.
10. Blunt, J. W., Deluca, H. F., and Schnoes, H. K.: 25-Hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. Biochem. 7:3317, 1968.
11. DeLuca, H. G.: Recent advances in the metabolism and function of vitamin D. Fed. Proc. 28:1678, 1969.
12. Drake, T. G. H.: Comparison of the antirachitic effects on human beings of vitamin D from different sources. Am. J. Dis. Child. 53:754, 1937.
13. Lewis, J. M.: Further observations on the comparative antirachitic value of crystalline vitamin D administered in milk, corn oil, in propylene glycol. J. Pediat. 8:300, 1936.
14. Follis, R. H., Jr., Park, E. A., and Jackson, D.: The relationship of vitamin D administration to the prevalence of rickets observed at autopsy during the first two years of life. Bull. Johns Hopkins Hosp. 92:426, 1953.
15. Follis, R. H., Park, E. A., and Jackson, D.: The prevalence of rickets at autopsy during the first two years of age. Bull. Johns Hopkins Hosp. 91:480, 1952.
16. Glaser, K., Parmelee, A. H., and Hoffman, W. S.: Comparative Efficacy of vitamin D preparations in prophylactic treatment of premature infants. Am. J. Dis. Children. 77:1, 1949.
17. David, N. J., Verner, J. V., and Engel, F. L.: The diagnostic spectrum of hypercalcemia. Am. J. Med. 33:98, 1962.
18. Benham-Carter, R. E., Dent, C. E., Fowler, D. J., and Harper, C. M.: Calcium metabolism in idiopathic hypercalcaemia of infancy with failure to thrive. Arch. Dis. Child. 30:309, 1955.
19. Fellers, F. S. and Schwartz, R.: Etiology of the severe form of idiopathic hypercalcemia of infancy. A defect in vitamin D metabolism. New Eng. J. Med. 259:1050, 1958.
20. Forfar, J., Bell, C. L., Maxwell, G. M., and Tompsett, S. L.: Idiopathic hypercalcemia of infancy. Lancet 1:981, 1956.
21. Forfar, J. O., Tompsett, S. L., and Forshall, W.: Biochemical studies in idiopathic hypercalcemia of infancy. Arch. Dis. Child. 34:525, 1959.
22. Forfar, J. O.: Clinical and metabolic studies of idiopathic hypercalcemia of infancy. M.D. Thesis, University of St. Andrews, 1958.
23. Schlesinger, B. E., Butler, N. R., and Black, J. A.: Severe type of infantile hypercalcaemia. Brit. Med. J. 1:127, 1956.
24. Black, J. A.: Idiopathic hypercalcaemia and vitamin D. German Medical Monthly (English Language Ed.) 9:290, 1966.
25. Lefebvre, G.: En cherchant l'hypervitaminose D_2 et l'hypercalcemie idiopathique: Un syndrome tres voisin. Pediatrie. 14:576, 1959.
26. Thatcher, L.: Hypervitaminosis D. Lancet. 1:20, 1936.
27. Lowe, K. O., Henderson, J. L., Park, W. W., and McGreal, D. A.: The idiopathic hypercalcemic syndromes of infancy. Lancet. 2:101, 1954.
28. Stapleton, T. and Evans, I. W. J.: Idiopathic hypercalcemia in infancy. Helvet. Paediat. Acta. 10:149, 1955.
29. MacDonald, W. B. and Stapleton, T.: Idiopathic hypercalcaemia of infancy. Studies of the mineral balance. Acta Paediat. 44:569, 1955.
30. Rhaney, D. and Mitchell, R. G.: Idiopathic hypercalcemia of infants. Lancet 1:1028, 1956.
31. Morgan, H. G., Mitchell, R. G., Stowers, J. M., and Thomson, J.: Metabolic studies on two infants with idiopathic hypercalcemia. Lancet. 1:925, 1956.
32. Unsigned Editorial: Infantile Hypercalcemia. Brit. Med. J. 1:150, 1956.
33. Bongiovanni, A. M., Eberlein, W. R., and Jones, J. T.: Idiopathic hypercalcemia of infancy, with failure to thrive. New Eng. J. Med. 257:951, 1957.
34. Daeschner, G. L. and Daeschner, C. W.: Severe idiopathic hypercalcemia of infancy. Pediatrics 19:362, 1957.
35. Snyder, C. H.: Idiopathic hypercalcemia of infancy. Am. J. Dis. Child. 96: 370, 1958.
36. O'Brien, D.: Idiopathic hypercalcemia of infancy. Pediatrics. 23:640, 1959.
37. Smith, D. W., Blizzard, R. M., and Harrison, H. E.: Idiopathic hypercalcemia. A case report with assays of vitamin D in the serum. Pediatrics 24:258, 1959.
38. Gribetz, D. and Wolf, B. S.: Idiopathic hypercalcemia. Am. J. Med. 126:936, 1959.
39. Mitchell, R. G.: The prognosis of idiopathic hypercalcemia of infants. Arch. Dis. Childhood. 35:393, 1960.
40. Michael, A. F., Hong, R., and West, C. D.: Hypercalcemia of infancy. Am. J. Dis. Child. 104:235, 1962.
41. Kenny, F. M., Aceto, Jr., T., Purisch, M., Harrison, H. E., Harrison, H. C., and Blizzard, R. M.: Metabolic studies in a patient with idiopathic hypercalcemia of infancy. J. Pediat. 62:531, 1963.
42. Cuthbertson, W. F. J.: Vitamin D activity of plasma in idiopathic hypercalcemia. Proc. Nutr. Soc. 22:146, 1963.
43. Rushkind, W. J., Golinko, R., and Arcasoy, M.: Cardiac findings in idiopathic hypercalcemia of infancy. J. Pediat. 58:300, 1964.
44. Garcia, R. E., Friedman, W. F., Kaback, M. M., and Rowe, R. D.: Idiopathic hypercalcemia and supravalvular aortic stenosis. New Eng. J. Med. 271:117, 1964.
45. Wilkerson, J. A.: Idiopathic infantile hypercalcemia, with subcutaneous fat necrosis. Amer. J. Clin. Path. 41:390, 1964.
46. Unsigned Editorial: Idiopathic hypercalcemia of infancy. A review. The National Vitamin Foundation Inc., 11, Sect. 2:1, 1965
47. Coleman, E. N.: Infantile hypercalcemia and cardiovascular lesions. Evidence, hypothesis, and speculation. Arch. Dis. Child. 40:515, 1965.
48. Taussig, H. B.: On the evolution of our knowledge of congenital malformations of the heart. Circulation. 31: 768, 1965.
49. Manios, S. G. and Antoner, L.: A study of vitamin D metabolism in idiopathic hypercalcemia of infancy. Acta Paediat. Scand. 55:600, 1966.
50. Wiltse, H. E., Goldbloom, R. B., Antia, A. U., Ottesin, O. E., Rowe, R. D., and Cooke, R.E.: Infantile hypercalcemia in twins. New Eng. J. Med. 275:1157, 1966.
51. Fraser, D., Kidd, B. S. L., Kook, S. W., and Paunier, L. A.: A new look at infantile hypercalcemia. Pediat. Clin. N.A. 13:503, 1966.
52. Stapleton, T., MacDonald, W. B., and Lightwood, R.: Management of "idiopathic" hypercalcemia in infancy. Lancet 1:24:932, 1956.
53. Graham, S.: Idiopathic hypercalcemia, Postgrad. Med. 25:67, 1959.
54. Black, J. A. and Bonham-Carter, R. E. B.: Association between aortic stenosis and facies of severe infantile hypercalcaemia. Lancet 2:746, 1963
55. Lightwood, R.: A case of dwarfism and calcinosis with widespread arterial degeneration. Arch. Dis. Childhood. 7:193, 1932.
56. Hayles, A. B. and Nolan, R. H.: Idiopathic hypercalcemia. Proc. Staff Meeting of the Mayo Clinic. 33:307, 1958.
57. Shiers, J. A., Nauhauser, E. B. D., and Bowman, J. R.: Idiopathic Hypercalcemia. Am. J. Roentgen. Rad. Ther. and Nuc. Med. 78:19, 1957.
58. Girardet, P.: Hypersensibilité à la vitamin D? Helvet. Paediat. 11:118-124, 1956.
59. Cuthbertson, W. F. J.: The vitamin D activity of plasma of children with idiopathic hypercalcaemia. Brit. J. Nutr. 17:627, 1963.
60. Williams, J. C. P., Barratt-Boyes, B. G., and Lowe, J. B.: Supravalvular aortic stenosis. Circulation 24:1311, 1961.
61. Black, J. A., Butler, N. R., and Schlesinger, B. E.: Aortic stenosis and hypercalcaemia. Lancet 2:546, 1965.
62. Bauman, A., and Bauman, C. C.: Idiopathic hypercalcemia of infancy simulating congenital heart disease. N.Y. State J. Med. 65:1910, 1965.
63. Ehrhardt, A. A. and Money, J.: Hypercalcemia—a family study of psychological functioning. Johns Hopkins Med. J. 121:14, 1967.
64. Beuren, A. J., Schulze, C., Eberle, P., Harmjanz, D., and Apitz, J.: The syndrome of supravalvular aortic stenosis, peripheral pulmonary stenosis,

mental retardation and similar facial appearance. Am. J. Cardiol. 13:471, 1964.

65. Beuren, A. J., Apitz, J., and Harmjanz, D.: Supravalvular aortic stenosis in association with mental retardation and certain facial appearance. Circulation 26:1235, 1962.

66. Beuren, A. J., Apitz, J., Stoermer, J., Kaiser, B., Schlange, H., Berg, W., and Jaergensen, G.: Vitamin D-hypercalcemic cardiovascular disease. Mschr. Kinder. 114:457, 1966 [Ger.].

67. Antia, A. U., Rowe, R. D., Otteson, O. E., Levin, S., and Cooke, R. E.: Pathogenesis of the supravalvular aortic stenosis syndrome in children. Circulation 32:44, 1965.

67A. Antia, A. U., Rowe, R. D., Levin, S.: Pathogenesis of the supravalvular aortic stenosis syndrome. J. Pediatrics 71:431, 1967.

68. Williamson, D. A. J.: Supravalvular aortic stenosis associated with mental and physical retardation and a characteristic facies. Proc. Soc. Med. 57:118, 1964.

69. Myers, A. R. and Willis, P. W.: Clinical spectrum of supravalvular aortic stenosis. Arch. Int. Med. 118:553, 1966.

70. Otteson, O. E., Antia, A. U., and Rowe, R. D.: Peripheral vascular anomalies associated with the supravalvular aortic stenosis syndrome. Radiology 86:430, 1966.

71. Priest, J. H. and Lang, D. M.: Aortic stenosis and hypercalcaemia. Lancet 1:1335, 1967.

72. Dunham-Carter, R. E. B. and Sutcliffe, J.: A syndrome of multiple arterial stenosis, in association with the severe form of idiopathic hypercalcaemia. Arch. Dis. Child. 39:478, 1964.

73. Friedman, W. F.: Supravalvular aortic stenosis. Amer. J. Dis. Child. 109:201, 1965.

74. Schlesinger, B., Butler, N., and Black, J.: Londoner Fall. Helv. Paediat. Acta. 7:335, 1952.

75. Rowe, R. D. and Cooke, R. E.: Commentaries. Vitamin D and craniofacial and dental anomalies of supravalvular stenosis. Pediatrics 43:1, 1969.

76. Committee on Nutrition, American Academy of Pediatrics: Vitamin D intake and the hypercalcemic syndrome. Pediatrics 35:1022, 1965.

77. Committee on Nutrition, American Academy of Pediatrics: The relation between infantile hypercalcemia vitamin D—public health implications in North America. (Report written by Fraser, D.) Pediatrics 40:1050, 1967.

78. Friedman, W. F.: Vitamin D and the supravalvular aortic stenosis syndrome. Adv. Teratol. 3:85, 1968.

79. Friedman, W. F. and Mills, L. F.: The relationship between vitamin D and the craniofacial and dental anomalies of the supravalvular aortic stenosis syndrome. Pediatrics 43:12, 1969.

80. Friedman, W. F. and Roberts, W. C.: Vitamin D and infantile hypercalcemia. Circulation 34:77, 1966.

81. Forbes, G. B., Caferelli, C., and Manning, J.: Vitamin D and infantile hypercalcemia. Pediatrics 42:293, 1968.

82. Stryker, W. A.: Arterial calcification in infancy with special reference to the coronary arteries. Am. J. Path. 23:1007, 1947.

83. Menten, M. L. and Fetterman, G. H.: Coronary sclerosis in infancy. Am. J. Clin. Path. 18:803, 1948.

84. Gillman, J. and Gilbert, C.: Calcium, phosphorus and vitamin D as factors regulating the integrity of the cardiovascular system. Exp. Med. Surg. 14:130, 1956.

85. Moran, J. J. and Becker, S. M.: Idiopathic arterial calcification of infancy. Am. J. Clin. Path. 31:517, 1959.

86. Lightwood, R. and Butler, N.: Decline in primary infantile renal acidosis: aetiological implications. Brit. Med. J. 1:855, 1963.

87. Lightwood, R., Sheldon, W., Harris, C., and Stapleton, T.: Hypercalcemia in infants and vitamin D. Brit. Med. J. 2:149, 1956.

88. Woodcock, R. H.: Letter to editor, Lancet 267:244, 1954.

89. Shanks, R. A. and MacDonald, A. M.: Nephrocalcinosis infantum. Arch. Dis. Child. 34:215, 1959.

90. Committee on Nutrition, American Academy of Pediatrics. The prophylactic requirement and the toxicity of vitamin D. Pediatrics 31:512, 1963.

91. Stearns, G., Jeans, P. C., and Vandecar, V.: The effect of vitamin D on linear growth in infancy. J. Pediat. 9:1, 1936.

92. Lewis, J. M.: Clinical experience with crystalline vitamin D: The influence of the menstrum on the effectiveness of the antirachitic factor. J. Pediat. 6:326, 1935.

93. Supplee, G. C., Ansbacher, S., Bender, R. C., and Flanigan, C. E.: The influence of milk constituents on the effectiveness of vitamin D. J. Biol. Chem. 114:97, 1936.

94. Thomas, W. C., Morgan, H. G., Connor, R. B., Haddock, L., Bills, C. E., and Howard, J. E.: Studies of Antirachitic activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D in human serum. J. Clin. Invest. 38:1078, 1959.

95. Selye, H.: The pluricausal cardiopathies. p. 160. Publ. Charles C. Thomas, Springfield, Illinois, 1961.

96. Moyan, A. F., Kimmel, L., and Hawkins, N. C.: A comparison of the hypervitaminosis induced by irradiated ergosterol and fish liver oil concentrates. J. Biol. Chem. 120:85, 1937.

97. Harris, R. S., Ross, B. D., and Benker, J. W. M.: Histological study of hypervitaminosis D. The relative toxicity of the vitamin D of irradiated ergosterol and tuna liver oil. Am. J. Digest. Dis. 6:81, 1939.

98. Seelig, M. S.: Are American children still getting an excess of vitamin D? Hyper-reactive children at risk. Clin. Ped. 9: in press.

99. Report of the Joint Sub-Committee on Welfare Foods. Ministry of Health, Dept. of Health for Scotland. London, Her Majesty's Stationery Office, 1957.

100. Editorial: Vitamin D as a public health problem. Brit. Med. J. 1:1654, 1964.

101. Oppé, T. E.: Infantile hypercalcaemia, nutritional rickets, and infantile scurvy in Great Britain. A British Paediatric Association Report. Brit. Med. J. 1:1659, 1964.

102. Bransby, E. R., Berry, W. T. C., and Taylor, D. M.: Study of the vitamin D intakes of infants in 1960. Brit. Med. J. 1:1061, 1964.

103. Bing, F. C.: Vitamin D milk containing 600 U.S.P. units of vitamin D to the quart is not eligible for list of accepted foods. J. Am. Med. Assoc. 116:413, 1941.

104. O'Brien, D., Peppers, T. D., and Silver, H. K.: Idiopathic hypercalcemia of infancy. J. Am. Med. Assoc. 174:1100, 1960.

105. Joseph, M. C. and Perrott, D.: Severe infantile hypercalcaemia with special reference to the facies. Arch. Dis. Child. 33:385, 1958.

106. Jue, K. J., Nuren, G. R., and Anderson, R. C.: The syndrome of idiopathic hypercalcemia of infancy with associated congenital heart disease. J. Pediat. 67:1130, 1965.

107. Committee on Nutrition, American Academy of Pediatrics. Vitamin D intake and the hypercalcemic syndrome. Pediatrics 35:1022, 1965.

108. Stewart, W. K., Mitchell, R. G., Morgan, H. G., Lowe, K. G., and Thomson, J.: The changing incidence of rickets and infantile hypercalcaemia as seen in Dundee. Lancet 1:659, 1964.

109. Daeschner, C. W., Singleton, E. B., Curtis, J. C.: Urinary tract calculi and nephrocalcinosis in infants and children. J. Pediat. 57:721, 1960.

110. Warkany, J., Mabon, H. E.: Estimation of vitamin D in blood serum. II. Level of vitamin D in human blood serums. Am. J. Dis. Child. 60:606, 1940.

111. Loomis, W. F.: Skin Pigment Regulation of vitamin D biosynthesis in Man. Science 157:501, 1967.

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5230

HUMAN REQUIREMENTS OF MAGNESIUM FACTORS THAT INCREASE NEEDS

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Introduction

Determination of Magnesium Requirements

Serum or Plasma Magnesium

Tissue Magnesium

Magnesium Exchange

Urinary Excretion of Magnesium Test Dose

Metabolic Balance

Dietary Factors that Increase Magnesium Requirements

Hard Water.

Calcium/Magnesium interrelationships

Increased Calcium Intake → Magnesium Loss (Clinical)

Increased Calcium Intake → Magnesium Loss (Animal)

Calcium Loss Secondary to Magnesium Deficit (Adults)

Neonatal Hypocalcemia Secondary to Hypomagnesemia

Phosphorus/Magnesium Interrelationships

Increased Phosphate Intake → Magnesium Deficit (Clinical)

Increased Phosphate Intake → Magnesium Deficit (Animal)

Vitamin D and Magnesium Requirements

Neonatal Hypocalcemia, Hypomagnesemia, and Vitamin D

Hypoparathyroidism in Hypocalcemic Response to Magnesium Deficit or Vitamin D Administration

Animal Magnesium Deficit: Effect on Response to Vitamin D; Role of Parathormone

Infantile Hypercalcemia, Vitamin D and Magnesium

Vitamin D and Magnesium in the Adult

Protein/Magnesium Interrelationships

High Protein → Decreased Magnesium Retention (Clinical)

High Protein → Magnesium Deficiency (Clinical)

High Protein → Increased Magnesium Requirements (Animal)

Requirement of Magnesium for Protein Synthesis (Animal)

Carbohydrates and Lipids: Interrelationships with Magnesium

Human Magnesium Requirements

During Formation of New Tissue

Magnesium and Pregnancy

Magnesium Balance During Normal Pregnancy

Magnesium and Lactation

Magnesium in Infancy and Childhood

Magnesium Deficits in Infancy

Normal Magnesium Requirements During Infancy and Childhood

Concluding Remarks

INTRODUCTION

THE place of magnesium deficit in medicine is still not universally accepted. The difficulties in measuring magnesium have, until relatively recently, deterred the accumulation of data. It is predominantly an intracellular ion, with plasma concentration normally maintained within very narrow limits. The fact that plasma magnesium often differs only slightly from normal, even in the presence of clinically demonstrable deficiency, makes laboratory detection of clinical magnesium deficiency difficult. And the fact that early changes are subtle, first involving functional, and later structural aberrations of the cardiovascular, renal and neuromuscular systems, further complicates diagnosis of early or chronic magnesium deficits.

The program of this symposium is evidence of the growing concern for the manifestations of clinical magnesium deficit that are less dramatic than those of acute hypomagnesemia. To extend the recognition of the medical significance of magnesium deficit we must improve our methodology for detection of chronic, as well as acute deficiencies. We must establish what the magnesium requirements are, not only in health under different physiologic conditions such as pregnancy and infancy, but also in disease.

Complicating study of the place of this ubiquitous element in medicine is the fact that its requirement is influenced by so many factors. During periods of rapid formation of new tissues, the magnesium requirement goes up. Conditions that interfere with magnesium absorption, or enhance its urinary excretion, increase magnesium requirements. This paper presents evidence that high intakes of calcium, phosphates, protein, and vitamin D each increases the magnesium requirements.

With such a multiplicity of factors that influence the absorption and excretion of magnesium, as well as the amount needed for metabolic processes, no wonder there is dispute as to what the magnesium requirement is. There is probably no uniform optimal need. The best we can hope to do is establish recommended ranges that available evidence suggests should protect against the occult manifestations of magnesium deficit. Our knowledge of what these manifestations are must be based largely on findings from animal studies of chronic, low-grade insufficiencies. Investigation of the magnesium requirements of patients with disorders similar to those seen in magnesium-deficient animals, may disclose both unsuspected clinical deficits, and conditions in which the magnesium requirements are increased. Certainly, the requirements of patients who have been shown to have hypomagnesemia, or to respond favorably to magnesium therapy should be further studied. Recommendation for magnesium intake should be in accord with demonstrated requirements which should be determined by acceptable procedures.

DETERMINATION OF MAGNESIUM REQUIREMENTS

How are we to ascertain whether the magnesium in a given subject's diet meets his specific needs? If they do not, is the patient magnesium deficient, and if so, what diagnostic criteria can be employed to identify the condition? * To determine the magnesium status, several types of investigations are needed. Most urgent, is the need for establishment of normal values, so that the clinician can more readily diagnose even subacute or long-term magnesium deficits, and determine the adequacy of response to supplements.

SERUM OR PLASMA MAGNESIUM

Since the serum levels of magnesium are maintained within very narrow limits, we must establish what the normal range actually is. Table I summarizes the major techniques that have been used to measure serum magnesium values of magnesium as well as the ranges and means that have been reported for normal adults by the different procedures. Recent reviews reflect the published confusion as to what is normal. WATSON'S (355) most complete review cites values, given as normal, that range from 1.25 to 2.14 mEq/L. Other reviews have similarly reported wide ranges of values accepted by investigators as normal (109, 148, 236, 305, 340). Most of those publications indicated normal mean values of 1.60 to 2.9 mEq/L, with a few above and a few below. Serum or plasma Mg levels in infants and children, except for the neonatal period, are about the same as in adults (Table II) (17, 171, 394, 322), or slightly lower (323). Despite these wide ranges, accepted as normal in the literature, most experts agree that limits of the normal range of serum Mg are very narrow (109, 200, 201, 204, 340, 360).

There is evidence that the physiochemical state of the magnesium in the plasma may deviate from normal in several conditions associated with magnesium deficit (18, 31, 43, 52, 66, 78, 115, 228, 254, 255, 304, 313, 347-349, 353-356, 358).

Factors that alter magnesium binding, ultrafiltrability, and complexing must also be studied.

(*) DURMAN and ROUSSEAU (84 A, 268 A) have presented practical approaches to the problem of determining the clinical magnesium status.

TABLE I

MAGNESIUM SERUM VALUES (MEq/L) REPORTED AS NORMAL

PROCEDURE	INVESTIGATORS AND YEAR	NO. SUBJECTS IN STUDY	RANGE	MEAN
Molybdivanate precipitation	Simonson et al (1947)[306]	21	1.58 - 2.07	1.72
	Stutzman and Amatuzio (1953)[317]	17		1.82 ± 0.2
	Smith and Hannarsten (1958)[312]	40	1.14 - 1.82	1.48 ± 0.17
	Thoren (1963)[325]	90	1.43 - 1.93	1.68 ± 0.125
Titan Yellow (Refs. 106, 137, 246 (Accuracy stated unreliable because of interfering substances in biological material 48,136,344	Heagy and Burton (1948)[137]	10		1.89 ± 0.015
	Orange and Rhein (1951)[246]	45	1.58 - 2.07	1.89
	Baldwin et al (1952)[24]	?	1.7 - 2.2	
	Albert et al (1953)[5]	43	1.36 - 1.94	1.61 ± 0.14
	Graham et al (1960)[121]	7	1.83 - 2.44	2.15 ± 0.20
	Barker (1960)[26]	64		1.82 ± 0.37
	Prasad et al (1961)[254]	?		1.81 ± 0.13
	Durlach (1969)[86]	64		1.98 ± 0.13
EDTA, Eriochrome Black-T (Refs. 48, 50, 309, 367)	Smith (1955)[309]	24	1.75 - 2.50	2.1 ± 0.2
	Carr and Frank (1956)[48]	20	1.60 - 1.96	1.72 ± 0.08
	Kim et al (1961)[168]	?		1.60 ± 0.20
	Walser (1961)[354]	20	1.68 - 2.08	1.92 ± 0.12
	Wallach et al (1962)[350]	77	1.75 - 2.34	2.0 ± 0.15
	Opie et al (1964)[245]	?	1.5 - 1.75	
With Semi-Automatic or Automatic equipment (Refs. 111, 165, 204, 205)	Sones et al (1960)[314]	?	1.7 - 2.0	1.8 ± 0.07
	Jones and McGuckin (1964)[165]	40		1.98 ± 0.11
	McPherson (1965)[204]	100		1.90 ± 0.2
		227		1.80 ± 0.2
	Gitelman et al (1966)[111]	25	1.45 - 1.87	1.66 ± 0.11
Fluorometric (Refs. 273, 346)	Schachter (1961)[273]	14		1.80 ± 0.26
	Bateakis et al (1964)[24]	113	0.80 - 2.80	1.88 ± 0.40
	Tein (1968)[323]	21		1.90 ± 0.036
Flame Spectrophotometry (Refs. 6, 7, 93, 335, 341, 342)	Wacker & Vallee et al (1957)[343] (1960)[335, 345]	14	1.85 - 2.56	2.05 ± 0.18
		30	1.60 - 2.40	2.10 ± 0.16
	Telsh (1958)[324]	25		2.04 ± 0.14
	MacIntyre et al (1960)[7] (1967)[200]	76	1.50 - 1.80	1.66 ± 0.01
	Hanna (1961)[126]	12	1.50 - 1.90	
	Montgomery (1961)[224]	46	1.35 - 2.05	1.70
	Fawcett and Wynn (1961)[93]	10		1.74 ± 0.07
	Hughes and Tonks (1965)[159]	36	1.92 - 2.41	2.3
Atomic Absorption Spectrometry (Refs. 74, 108)	Stewart et al (1963)[316]	60 ♂		1.74 ± 0.11
		40 ♀		1.75 ± 0.12
	Heaton et al (1963)[142] (1967)[139]	?	1.43 - 1.90	
	Lim et al (1964)[191]	87	1.30 - 1.90	1.67 ± 0.14

TABLE II

SERUM MAGNESIUM: INFANCY AND CHILDHOOD

PROCEDURE	INVESTIGATOR	AGE	NUMBER OF DETERMINATIONS	TOTAL Mg	% UF Mg
TITAN YELLOW	Silverman & Gardner (1954)[304]	3 Mos. - 12 Yrs.	43	1.77 ± 0.02	1.16 ± 0.02
	Anast (1964)[10]	1-5 Days	238	1.92 ± 0.27	
FLAME SPECTRO-PHOTOMETRY	Hüther (1964)[162A]	1-14 Years.	98	1.80 ± 0.26	
	Kobayashi (1967)[171]	0-5 Days	13	1.64 ± 0.19	
		6-10 Days	9	1.53 ± 0.15	
		11-30 Dasy	16	1.68 ± 0.16	
		31-60 Days	6	1.78 ± 0.17	
		2 Mos - 11 mos.	23	2.26 ± 0.15	
		1 - 5 11/12 YRS.	40	2.25 ± 0.16	
		6 - 15 11/12 YRS	15	2.16 ± 0.17	
FLUOROMETRIC	Teh (1968)[323]	At Birth	18	1.53 ± 0.05	1.05 ± 0.04
		First Week	20	1.35 ± 0.05	0.88 ± 0.04
		2-4 Weeks	13	1.54 ± 0.09	1.03 ± 0.08
		2 Mos.- 1 Yr.	12	1.64 ± 0.07	1.08 ± 0.06
		2 Yrs. - 10 yrs.	13	1.68 ± 0.66	1.17 ± 0.05
		(Young Adults)	21	(1.90 ± 0.04)	1.26 ± 0.02
ULTRA-MICROSCOPIC METHOD (MANN DYE)	Bajpai et al (1966)[17]	First Week	56	1.51 ± 0.12 (1.20 ± 1.80)	

TISSUE MAGNESIUM

Since most of the body's magnesium is intracellular, establishment of ranges of normal values of readily accessible cellular tissue is desirable. The reported ranges of soft tissue magnesium values, and some of the difficulties in interpreting them, have been presented by WALSER (355). How reliable an index of magnesium deficit the erythrocyte levels are, is not settled. TUFTS and GREENBERG (330) have suggested that the erythrocyte magnesium reflects the status of magnesium at the time the red cells are produced. GINSBERG *et al.* (110) have shown that the younger the erythrocytes, the higher their magnesium concentration, an observation made also by ROWLEY and MORRIS (269). Therefore, the magnesium level of a given sample of erythrocytes will be influenced, not only by the magnesium available at the time of erythropoiesis, but by the age-mix of the erythrocytes in the sample. Perhaps the erythrocyte magnesium level is a better index of a long-term deficit, than of a more or less acute deficit. For example, in chronic experimental animal deficiency, TUFTS and GREENBERG (330, 331) found the erythrocyte magnesium level to drop more slowly, and to persist longer at a low level than did serum magnesium. DURLACH *et al.* (84-86) have found that erythrocyte magnesium levels are significantly lower than normal in their spasmophilic patients. On the other hand, clinical magnesium depletion studies have shown the depression of erythrocyte Mg levels to be less marked than the drop in plasma Mg (83, 300-302). The erythrocyte Mg is not grossly abnormal in kwashiorkor (MONTGOMERY (226), PRETORIUS *et al.* (256), a condition associated with acute magnesium deficit.

Even skeletal muscle magnesium is an uncertain index of magnesium depletion. Adult subjects of acute magnesium-deficiency studies have not manifested lowered muscle magnesium (83). On the other hand, patients with chronic magnesium deficits with manifestations of hypomagnesemia have had lowered Mg levels in their skeletal muscle (34, 91, 203, 250). Infants with kwashiorkor consistently have had depressed muscle levels of magnesium (47, 213, 225, 226, 333). We are confronted with the paradox that hypomagnesemia may be associated with normal or abnormal magnesium muscle levels. Review of the experimental evidence shows that functional, subcellular, and histologic damage can exist in magnesium-deficient animals, even in the absence of measurable reduction of soft tissue magnesium (355).

The question of intracellular magnesium deficiency is the most difficult to resolve, but it is the status of the intracellular magnesium that lies at the root of the syndromes associated with long-term magnesium deficit. The meaning of tissue levels of magnesium may be difficult to interpret. Ultramicroscopic studies have indicated that magnesium may be found in damaged mitochondria (i.e., of the heart) as precipitates of phosphate salts, rather than as functional magnesium in association with the intracellular

enzymes (35, 36, 164, 286). High magnesium levels in arterio-sclerotic arteries (69) and in kidneys of magnesium-deficient animals (41) have been attributed to precipitation of inorganic salts of magnesium, as well as of calcium, in the damaged tissue. Therefore, analyses of body or organ content of magnesium, not accompanied by histologic examination of the tissues, may obfuscate the true magnesium picture.

MAGNESIUM EXCHANGE

Investigators have attempted to calculate the status of the magnesium stores and the magnesium requirements by determining the distribution and excretion of tagged magnesium (2-4, 16, 25, 27, 77, 91, 119, 121, 181, 265-267, 303, 352, 372, 375). Whether determination of magnesium exchange can be relied upon as an index of magnesium deficit, however, has been questioned (77, 355). DIMICH and WALLACH (77) have shown that most patients with magnesium deficits of alcoholism or malabsorption had normal kinetics of magnesium exchange, or even enlarged cellular exchange pools (91). Their data also indicated that calculated exchangeable body pools of magnesium are not reliable parameters of magnesium turnover. They concluded that moderate magnesium deficiency, and/or moderate hypomagnesemia, need not interfere with the ability of the cell membranes to exert control over the transport of magnesium into the cell. WALSER (355), who has reviewed the significance of isotope exchangeability, has concluded that our present knowledge permits us to say little more than that the absence of tagged magnesium in specific tissues or fluids means merely that it was not transferred to those sites.

URINARY EXCRETION OF MAGNESIUM TEST DOSE

The test that may prove most feasible in clinical medicine is the measurement of the 24-48 hour urinary magnesium after a load dose. Retention of a significant amount (i.e., as indicated by urinary excretion of less than 70 % of the administered dose) has been considered indicative of magnesium deficiency (96, 102, 103, 1 5, 125, 148, 166, 180, 225, 245, 256, 318, 325, 326, 366, 373). The first indication that this might be so was the observation by FITZGERALD and FOTHERGILL (96) of renal retention of magnesium after a period of experimental magnesium deprivation, by two healthy young men. They excreted only a small percentage of an intravenous dose of magnesium, that had been calculated to restore the magnesium lost during the period of dietary magnesium deprivation. Despite their magnesium losses, that had been shown by metabolic balance studies, their plasma magnesium had remained essentially unchanged. A prerequisite for this test, as FREEMAN and PEARSON (103) have pointed out, is normal renal function in conserving magnesium. In aldosteronism, where there is increased renal clearance of magnesium (131, 156, 173, 219, 340) and in patients with reduced glomerular filtration rates (52, 340) high and low percentage clearance of magnesium respectively, in response to magnesium load would not be indicative of the adequacy of the magnesium stores.

METABOLIC BALANCE

Perhaps, despite its difficulties, the most reliable means of determining magnesium requirements is the metabolic balance technique. Metabolic ward conditions, in which the total dietary intake of magnesium is analyzed, as is the total magnesium content of the excreta, give the most reliable information. The findings from metabolic balance studies, done with volunteers on controlled diets on an outpatient basis are not as reliable, but provide supportive data with larger populations of healthy subjects than is feasible for metabolic ward studies.

In an earlier paper (287), analyses of such studies on healthy young adults on different dietary intakes of magnesium suggested that the daily requirement of magnesium is at least 6 mg/kg. Taking into account the published animal and clinical evidence that demonstrated that high dietary intakes of calcium, protein, vitamin D, phosphate (as phytates), and alcohol each increased the magnesium requirements, it was proposed that 7-10 mg/kg/d might more adequately meet the needs of the normal adult. Metabolic studies done since then have verified the need for at least 6 mg/kg as a minimal daily intake (8, 84, 162 B). Additional work, that has further elucidated the effect of these and other nutrients on magnesium requirements, is discussed in this paper.

In addition to metabolic balance studies done in normal subjects, we should ascertain how much magnesium is needed for equilibrium of patients with "idiopathic" cardiovascular and renal disorders. Similar studies during normal and pre-eclamptic pregnancies and in infancy, comparing the requirements of smaller than normal neonates with that of normal-weight infants, may provide important baseline data. Long-term supplement studies in high-risk subjects, like those undertaken to evaluate the effect of unsaturated fats, should provide important epidemiologic data, in terms of incidence of fatal cardiovascular disease, or of incidence of kidney stones in the non-supplemented, as compared with the supplemented groups.

5219

DIETARY FACTORS THAT INCREASE MAGNESIUM REQUIREMENTS

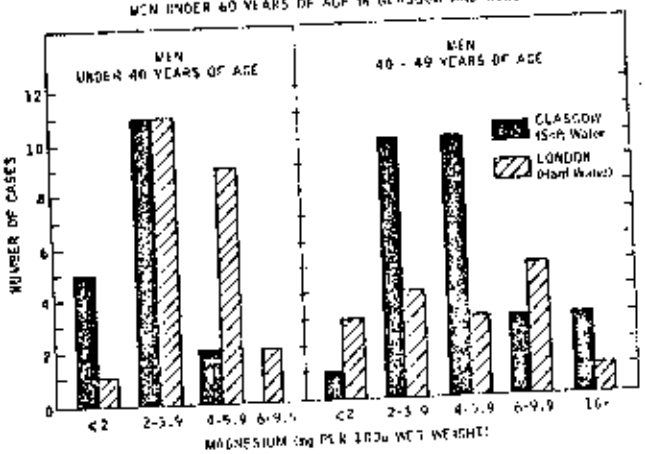
HARD WATER

Before considering the specific factors that influence magnesium requirements, we should take into account the magnesium supplied in the drinking water. Recent epidemiological studies have demonstrated that there is a significantly higher death rate from cardiovascular disease in soft water areas than where the water is hard (11, 32, 68, 69, 172, 229, 232, 277-280, 332, 334). The nature of the « water factor » is still controversial, but a number of investigators consider the magnesium in hard water as a likely protective agent (20, 30, 38, 69, 235, 248). NEAL and NEAL (235) studied the effect of naturally-hard water and of distilled water with and without either calcium or magnesium, on the development of atherosclerosis in rabbits on an atherogenic diet. They found that the rabbits given hard water to drink had less arterial damage than did those given distilled water. The calcium added to distilled water exerted no protection; the magnesium-supplemented distilled water completely protected against the arteriosclerosis. The study by CRAWFORD and CRAWFORD (69) provides interesting insight into the clinical significance of these findings. They showed that young men under 40 from a soft water area, who had died in accidents, had lower coronary magnesium levels and greater evidence of prior myocardial disease, than did comparable men from a hard water area (Figure 1). The authors

Figure 1

CORONARY MAGNESIUM IN ACCIDENT CASES

MEN UNDER 40 YEARS OF AGE IN GLASGOW AND LONDON



From Crawford, T. and Crawford, M.D., Lancet I 229-232, 1967

commented that the increased magnesium levels in the older age groups may have been caused by deposition of mineral deposits in established arterial lesions. It may be such abnormal deposition of magnesium that is responsible for the lack of significant difference found by SCHRODER *et al.* (280) in tissues from people from geographic areas where the dietary intakes of magnesium differ substantially.

In evaluating such epidemiological studies, it must be kept in mind that water intake is highly variable, and the amount of magnesium in each water supply is different (280). Therefore, future studies considering magnesium metabolism and requirements should take into account the magnesium supplied to each subject by the water he imbibes.

CALCIUM/MAGNESIUM INTERRELATIONSHIPS*

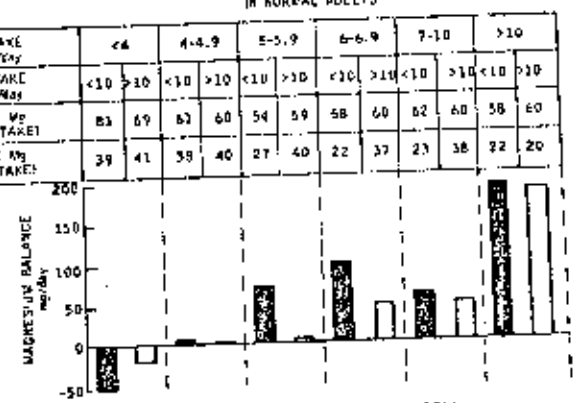
INCREASED CALCIUM INTAKE → MAGNESIUM LOSS-CLINICAL

Analysis of metabolic figures from normal young adults (Figure 2) reveals that on low magnesium intakes, there is magnesium loss regardless of the calcium intake. On marginal magnesium intake of 5 to 5.9 mg/kg and above, the greater the calcium intake, the less magnesium is retained (287) (Figure 2).

Figure 2

INFLUENCE OF CALCIUM INTAKE ON MAGNESIUM BALANCE IN NORMAL ADULTS

Mg INTAKE mg/kg/day	<4		4-4.9		5-5.9		6-6.9		7-10		>10	
Ca INTAKE mg/kg/day	<10	>10	<10	>10	<10	>10	<10	>10	<10	>10	<10	>10
FECAL Mg (% OF INTAKE)	83	69	62	60	54	59	58	60	62	60	58	60
URINE Mg (% OF INTAKE)	39	41	39	40	27	40	22	37	23	38	22	20



Adapted from M.S. Seelig, Am. J. Clin. Nutr., 14: 342-390 (Fig. 31), 1964

Three additional published metabolic studies indicate the effect on magnesium of increasing the calcium intake in health and disease. PARLIER *et al.* (247) reported magnesium balance determinations with 12 normal subjects on dietary intakes of 4.0 to 4.5 mg/kg/d, and with patients suffering from idiopathic and corticoid-osteoporosis, osteomalacia, hyperparathyroid osteolytic disease, and/or with renal disease. With three exceptions who were in negative balance, and one who was on the highest magnesium intake and who retained 100 mg/kg, the normal subjects remained in equilibrium or in slightly positive magnesium balances on intakes of 204 to 391 mg/d. On the average, they excreted 70 % of ingested magnesium in their feces, and 28 % in their urine, figures that are similar to those of the composite values (287). Most of the patients with osteopathies were in substantially negative magnesium balance, as were the patients with hypercalciuria

(*) For further data, see LARYOR and DERSACH (178 A, 178 B).

or renal insufficiency. The patients with osteopathies, who were given calcium supplements, tended to develop additional magnesium losses while on that therapy, as did patients with hypercalciuria (Table III). The negative balances of the patients with

Table III

EFFECT OF CALCIUM-THERAPY ON MAGNESIUM BALANCE IN OSTEOPOROSIS AND IN HYPERCALCIURIA

	NUMBER	MEAN INCREASE IN DAILY LOSS OF Mg
OSTEOPOROSIS (COMMON: IMMOBILIZATION)	6	9 mg
OSTEOPOROSIS (IDIOPATHIC)	6	15 mg
OSTEOPOROSIS (CORTICOSTEROID)	6	57 mg (↑ Fecal Mg)
HYPERCALCIURIA	6	148 mg (↑ Urinary Mg)

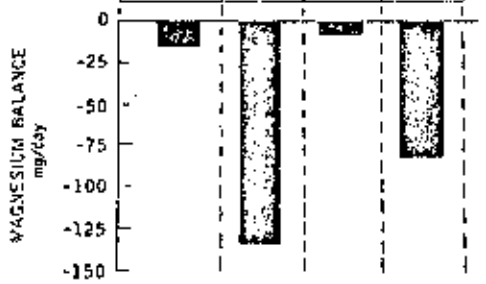
Adapted from R. Parlier, D. Hioco, and R. Leblanc [247] Rev. Franc. Endocr. Clin. 4: 93-135, 1963

osteomalacia were caused primarily by defective intestinal absorption of magnesium, which was intensified by the oral administration of supplemental calcium. In the osteolytic disorders of hypercalciuria or hyperparathyroidism, there was excessive urinary excretion of magnesium. These investigators also demonstated that intravenous perfusion of calcium (400-800 mg) caused a 30 to 40 % diminution of serum Mg in all subjects. The normal subjects excreted 88 % more magnesium in their urine during the calcium perfusion; patients with osteoporosis or osteomalacia excreted 38 % and 20 % more, respectively. This lesser magnesiuric response was attributed to the lesser bone stores of magnesium in the patients with bone-wasting disorders. AMIOT *et al.* (8) found that the magnesium supplied by the French hospital diet, or by the diet at home, was about 4 mg/kg/d and was not sufficient to prevent negative Mg balance, either in the patients with osteopathies or in normal members of their families. Increasing the calcium intake approximately twofold (to two and a half grams daily in 114 patients), interfered with the intestinal absorption of magnesium, particularly in men, with a resultant increased loss of magnesium during the period of calcium supplementation (Figure 3). CLARKSON *et al.* (58) studied the effect of calcium loads on magnesium in eight patients with chronic renal failure and in three normal subjects under classic metabolic ward conditions. On about the same daily intake of magnesium as they normally ingested (about 200-250 mg Mg/d), increasing their normal calcium intake by 2 grams daily caused a diminution of intestinal absorption of magnesium and a decrease in its urinary output, with no net change in magnesium balance. The plasma levels of magnesium tended to be somewhat low (1.66 to 1.84 mEq/L in the normal subjects; 1.36 to 1.77 in

Figure 3

EFFECT ON MAGNESIUM BALANCE OF CALCIUM SUPPLEMENTATION IN PATIENTS WITH OSTEOPATHIES

	MEN		WOMEN	
DAILY MAGNESIUM INTAKE (mg)	234	273	202	179
DAILY CALCIUM INTAKE (mg)	<1200	>2600	<1200	>2600
FECAL MAGNESIUM (% OF INTAKE)	74%	120%	73%	115%
URINARY MAGNESIUM (% OF INTAKE)	37%	35%	37%	30%



Adapted from D. Amiot, D.J. Hioco, and J. Durlach [8] (Int'l Congr. on Mg, Argentina, April 1968)

four of the patients and 1.82 to 1.99 in three) before the calcium loading period and to drop further in seven of the entire group of eleven. In one case it dropped from 1.48 to 0.98 mEq/L on calcium administration. Another patient with osteoporosis, reported by HEATON *et al.* (141), whose magnesium intake was higher (344 mg/d), responded to a 2 + gram daily load of calcium by increased fecal excretion of magnesium and decreased urinary magnesium, with a net reduction in positive Mg balance (from + 33 to + 6 mg/d).

INCREASED CALCIUM INTAKE → MAGNESIUM LOSS (ANIMAL)

There has been much laboratory evidence that the magnesium-deficiency syndrome, including renal and cardiovascular necrosis and calcinosis, is intensified by increasing the dietary calcium (40, 59, 97, 124, 153, 208, 221, 239, 240, 242, 243, 275, 331, 336). Acute intravenous loading of dogs with calcium has also been shown to increase the renal excretion of magnesium (51, 351). WALLACH *et al.* (348) have further studied such dogs and found that inducing hypercalcemia by intravenous infusion caused a significant increase in the fraction of serum magnesium that was ultrafiltrable, indicating that the additional binding of calcium to plasma proteins reduced the fraction of magnesium that was protein-bound. (The increase in ultrafiltrability of the magnesium may contribute to the increase in its renal excretion.)

Animal studies have demonstrated, also, that magnesium administration to animals on sub-optimal intakes, improves their calcium absorption and retention (54-57, 88, 97, 179, 207). Laboratory studies have also shown that magnesium is important in the metabolism of bone (3, 57, 79, 88), and that magnesium deficiency causes defects in bones (80, 357) and

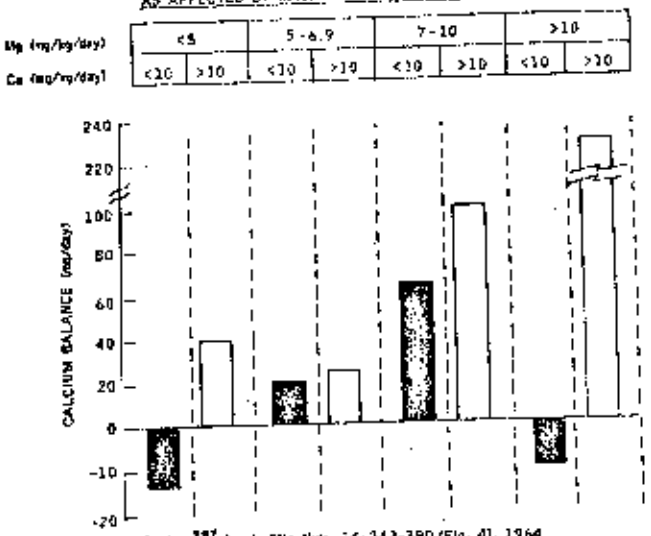
causes mobilization of bone calcium (79, 80). These observations bear on the clinical evidence of magnesium deficits in patients with osteopathies (8, 141, 247).

Calcium Loss Secondary to Magnesium Deficit * (Adults)

Analysis of the calcium balances of normal adults on low and high calcium and magnesium intakes (287) (Figure 4) reveals that when both minerals were in

Figure 4

CALCIUM BALANCE IN NORMAL ADULTS AS AFFECTED BY MAGNESIUM AND CALCIUM INTAKES

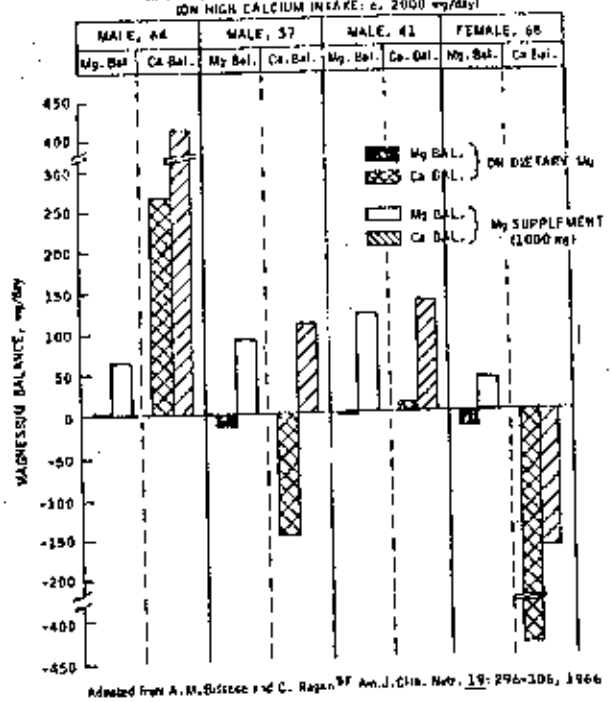


Adapted from M. S. Seelig,[287] Am. J. Clin. Nutr. 14: 342-390 (Fig. 41, 1964

short supply, there was negative calcium balance. Positive calcium balance developed when the magnesium intake improved. In view of the ample evidence that soft tissue calcium deposition occurs in animals on low magnesium and high calcium intakes, the demonstration of strongly positive calcium balances in subjects on diets with a high Ca/Mg ratio, is probably not indicative of a salutory balance. Batson and Ragan (37) have more recently shown that increasing the magnesium intakes of three men with histories of alcoholism, and of a 68-year-old woman with rheumatoid arthritis, by one gram daily for three weeks, improved their absorption and retention of calcium (Figure 5). Two had been in substantially negative calcium balance prior to the magnesium supplementation. Three of these patients had had serum magnesium levels of 1.4, 1.6, and 1.5 mEq/L prior to the study, that rose to 1.5, 1.8, and 2.0 mEq/L during the period of supplementation. This observation, in patients not magnesium deficient by the usual clinical parameters is in line with the enhancement by magnesium administration, of calcium retention

Figure 5

EFFECT OF INCREASING MAGNESIUM INTAKE ON CALCIUM AND MAGNESIUM BALANCE IN PATIENTS WITH POSSIBLE MAGNESIUM DEFICITS (ON HIGH CALCIUM INTAKE: c. 2000 mg/day)



Adapted from A. M. Batson and C. Ragan,[37] Am. J. Clin. Nutr. 19: 296-306, 1966

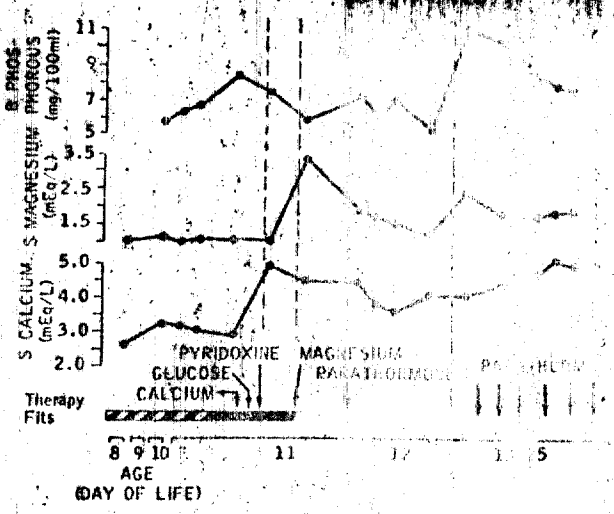
and/or elevation of serum calcium levels in patients with malabsorption, who have clinically manifest magnesium deficits (34, 120, 130, 139, 140, 203, 234, 245, 251, 373), or in whom magnesium deficit had been experimentally induced (300).

Neonatal Hypocalcemia Secondary to Hypomagnesemia

In the past few years there has been a spate of papers on symptomatic hypomagnesemia of infancy, usually associated with hypocalcemia that responds to treatment with magnesium (73, 78, 104, 249, 250, 270, 272, 307, 370). In two instances (73, 78), the condition seemed to be consequent to maternal hypomagnesemia and hyperparathyroidism. The infant reported by Davis *et al.* (73), who developed generalized convulsions at eight days, was found to have both hypomagnesemia (0.9 mEq/L) and hypocalcemia (2.6 mEq/L). As is seen in Figure 6, not until magnesium treatment was started, did the fits cease. Dooling and Stern (78) reported another such infant born to a woman who had hyperparathyroidism and hypomagnesemia. This infant's hypocalcemia was detected when he developed convulsions on his sixth day of life; his hypomagnesemia was not recognized until six days later, following failure of anticonvulsant therapy and calcium gluconate to control his convulsions. Treatment with magnesium promptly corrected both the convulsions and the hypocalcemia. Electrocardiographic changes (low T waves) that are consistent with the hypomagnesemic state [Caddell

(*) See also *Hypoparathyroidism in Hypocalcemic Response to Magnesium Deficit* (*Infra vide*), and Larvor and Dorbach (178 B).

Figure 6
RESPONSE OF NEONATAL HYPOCALCEMIA [illegible] CALCIUM, AND [illegible]



From: J.A.Davis, D.R.Harvey, & J.S.Yu, Arch. Dis. Childh. 40: 286-2[illegible] 1965

(44, 45), SEELIG (284)] were corrected on continuing the magnesium therapy. It should be noted that the severity of this infant's hypocalcemia may have been complicated by his having been given an exchange transfusion of citrated blood. The use of citrated blood has been shown to cause a decrease in ionized plasma magnesium (18, 43) as well as calcium (9[illegible]).

The infant reported by PAUNIER *et al.* (249, 2[illegible]) developed neuromuscular irritability, repeated convulsions, and protracted periods of depressed consciousness that led to diagnosis of hypomagnesemia only when his hypocalcemia failed to respond to treatment with intravenous calcium gluconate. It soon became manifest that the hypocalcemia was secondary to hypomagnesemia (0.3-0.4 mEq/L). Institution of parental $MgSO_4$ therapy resulted in increases of both serum magnesium and calcium levels, as well as neuromuscular improvement. This child required 30-36 mg/kg/d of Mg to maintain his serum Mg at levels that were considered normal by these investigators (1.1-1.3 mEq/L), but that are considerably below the usually accepted norm. Muscle biopsy analysis revealed low levels of both magnesium and potassium*, that dropped further on temporary discontinuation of the daily magnesium supplement. Although renal function studies were normal, a renal biopsy showed some glomerular damage that the authors considered due to the Mg deficit. Administration of parathormone or high dosage vitamin D elevated the serum calcium but had either no effect or produced a gradual fall in serum magnesium despite a high Mg intake. On the basis of their extensive studies, PAUNIER *et al.* (2[illegible]) suggested that the abnormality in this boy might be a defect in intestinal [illegible] of magnesium. There was no evi[illegible] a defect in renal conservation [illegible]. They suggested that possibly there [illegible] unresponsiveness to parathormone, rather than [illegible] hypoparathyroidism. The sluggish [illegible] to parathormone depicted in figure 6 may also reflect a target unresponsiveness in the infant reported by [illegible] *et al.* (73). A similar infant was reported by [illegible] *et al.* (270). Failure of perfusion of calcium or of high dosage vitamin D therapy to correct the convulsions and hypocalcemia led to diagnosis of hypomagnesemia (0.8 mEq/L). Subsequent studies revealed that continued magnesium supplementation was necessary for prevention of hypocalcemia. The authors concluded that this child suffered from a specific defect in intestinal absorption of magnesium requirements. Two additional infants, whose hypocalcemia was considered secondary to magnesium deficits caused by specific malabsorption of magnesium, have been reported by SKYBERG *et al.* (30[illegible]) and by FRIEDMAN *et al.* (104). The latter group (101) found that the infant could be kept in positive magnesium balance only when he was given unusually large amounts of magnesium (358-[illegible]00 mg/d). On three occasions, withdrawing or decreasing the magnesium supplements led to a fall both in plasma Mg and Ca and to recurrence of convulsions. Since so many nearly identical cases of hypomagnesemia have been identified in a short period of time, in widely separated medical centers, FRIEDMAN *et al.* (104) suggest that this cause of infantile convulsions may be more common than has been realized.

ATWELL (15) has presented case histories of three infants who developed symptomatic hypomagnesemia subsequent to intestinal surgery that resulted in interference with their absorption of magnesium. As demonstrated in Figure 7, oral supplementation with 60 mg/d corrected not only the hypomagnesemia and terminated the convulsions, but also more gradually corrected the hypocalcemia.

PHOSPHORUS/MAGNESIUM INTERRELATIONSHIPS

INCREASED PHOSPHATE INTAKES → MAGNESIUM DEFICIT (CLINICAL)

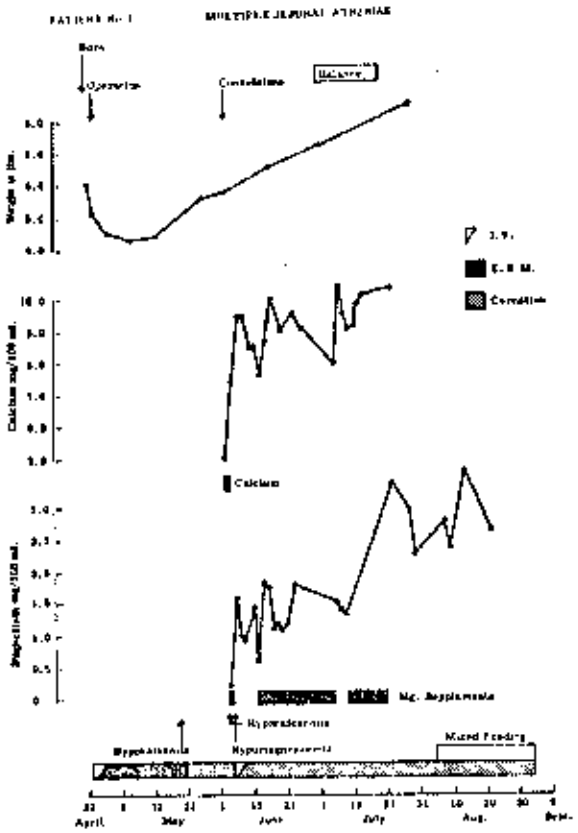
The magnesium balance values, during phosphate loading of patients with renal stones or metabolic bone disease reported by HEATON *et al.* (141), showed that phosphate loading reduced intestinal absorption of magnesium. Although there was a decrease in urinary excretion of magnesium, as well as a decrease in urinary calcium, the increased fecal excretion of magnesium usually resulted in a net increased loss. Unlike the perfusion of calcium, which lowered the serum magnesium, intravenous perfusion of neutral

(*) See WELT (359), SEELIG (286), WHANG (362, 363), WACKER *et al.* (340, 368); for discussions of decreased cellular K, with loss of cellular Mg.

5219

Figure 7

POSTOPERATIVE SERUM Ca and Mg IN INFANT: NOTE RISE AND FALL OF Ca CORRESPONDING TO ORAL ADMINISTRATION OF Mg



From J.D. Atwell, J. Ped. Surg. 1, 427-440, 1966

sodium phosphate did not significantly affect the serum or urine magnesium levels. EDWARDS *et al.* (89) investigated the effect of 12 grams daily of disodium hydrogen phosphate on the urine mineral content and on the metabolic balance of men with calcium-containing renal stones. Of the three whose magnesium balances were reported, increasing the phosphorus intake by two grams daily caused a slightly negative magnesium balance in one, and slightly increased retention in two. Like the previous investigators (141), this group (89) also found that the phosphate load resulted in decreased magnesium levels in the urine.

Another patient, who had kidney stones and suffered increasing hypertension, had been under long-term treatment with calcium supplements plus cellulose phosphate. He was reported by SUTTON (319) to have and markedly low urinary magnesium levels, and hypomagnesemia (0.86 mEq/L). Since it was not felt that he was suffering any untoward effects from his hypomagnesemia, the calcium and phosphate therapy was continued for another year. During that time, his serum magnesium remained between 0.6 and 1.2 mEq/L, and his urinary magnesium between 2 and 15 mg/24 hours. Discontinuation of the phosphate resulted in a drop in both fecal calcium and magnesium excretion, and an increase in urinary calcium and magnesium. After a brief period of positive magnesium balance, there was again magnesium loss, but the serum magnesium had risen to 1.6 mEq/L, which was considered normal. During the transient period of positive magnesium balance, the patient had retained 166 mg of magnesium. On reinstituting the phosphate cellulose, the fecal magnesium again exceeded the intake. Within 10 days the patient lost 350 mg of magnesium, and his serum magnesium again fell to 0.8 mEq/L.

The effect of phosphate loads on serum levels of magnesium during infancy has been considered in studies of neonatal tetany, associated with feeding cow's milk. COUSSONS (67), recently referring to the work published in 1950 by GARDNER *et al.* (105), pointed out that the decrease in serum magnesium, as well as calcium, that is seen in newborns fed cow's milk but not in those fed breast milk (10, 117, is a phenomenon related to high serum levels of phosphate. The greater amount of phosphate in cow's milk than in human milk has been shown to exceed the capacity of the immature renal tubules to prevent hyperphosphatemia (60, 105, 253). It should be observed that the infants recognized as having hypocalcemia secondary to hypomagnesemia had also been on cow's milk.

INCREASED PHOSPHATE INTAKES → MAGNESIUM DEFICIT (ANIMAL)

It has been known for almost 20 years, that increased phosphate dietary intakes intensify the soft tissue damage and calcification produced in experimental animals that are kept on inadequate magnesium intakes (61, 158, 208). MORRIS and O'DELL (231, 243) found that increasing the phosphate intake of guinea pigs increases their magnesium requirements by decreasing their intestinal absorption of magnesium, thereby intensifying their symptoms of deficiency, and increasing the soft tissue deposit of inorganic salts of calcium phosphate (230). Increase of the damage caused by magnesium deficits has been produced in dogs on low magnesium and high phosphorus intakes by BUNGE *et al.* (40). They have more recently demonstrated that the effect of dietary phosphorus on the magnesium balance of weanling rats depends upon the level of magnesium intake (42). High intakes of phosphorus lowered the apparent absorption of magnesium when the magnesium intake was ample, but improved its absorption when the dietary content of magnesium was low. Despite the improved magnesium balance on the higher phosphorus intake, increasing the phosphorus intake of each level of dietary magnesium resulted in increased renal calcium, and intratubular calcium casts, an early sign of magnesium deficit.

Phosphate loads have been demonstrated to intensify pharmacologically-induced renal and cardiovascular damage and calcification by SELYE, BAJUSZ, and coworkers (19, 21-23, 289-291, 295, 297). LEHR *et al.* (183-186) and other investigators (87, 222, 223, 238, 257, 258, 329). The protective effect exerted by prior and concurrent magnesium administration (21, 22, 222, 289-294, 296, 297) suggests phosphate-induced magnesium deficit as a pathogenic factor.

VITAMIN D AND MAGNESIUM REQUIREMENTS

Whether vitamin D will cause increased or decreased retention of magnesium depends upon the relationship of the supply of vitamin D to the need for it. When given in physiologic doses, vitamin D plays a role in the absorption both of calcium and magnesium. Little clinical work is available on the physiologic importance of vitamin D in the absorption of magnesium. Some animal work has indicated only limited effects on magnesium of dietary vitamin D in rats (210) and chicks (371). SCHACHTER and ROSEN (274) have shown that small intestines from vitamin D deficient rats exhibit impaired active transport of both calcium and magnesium. More recent intensive studies with baby pigs by MILLER *et al.* (214-217) have shown that vitamin D deficiency impairs the absorption of magnesium, and that supplementary magnesium exerts a vitamin D-sparing effect. HUETHER (162 A) has reported that five of fifteen children with florid rickets had definite hypomagnesemia, in addition to hypocalcemia.

On the other hand, it has long been recognized, also predominantly in animal studies, that high doses of vitamin D cause magnesium loss. Although vitamin D has been shown to increase the intestinal absorption of magnesium, in large doses it caused a greater increase in its urinary excretion and gave rise to hypomagnesemia, as well as to hypercalcemia (13, 127, 129, 133, 259, 260, 310, 347, 351, 359).

The histopathologic renal and cardiovascular damage induced by excess vitamin D has been reviewed elsewhere (SELYE, 288). That some of these lesions may be due to magnesium loss is suggested by studies of animals with subacute and chronic magnesium deficits that reveal remarkably similar histopathologic changes: myocardial degenerative changes (14, 61, 123, 143, 145, 170, 195, 220, 227, 294, 298, 336, 337, 361), early elastica degeneration of the arteries (14, 94, 227, 233, 337-339), renal tubular degenerative changes (14, 28, 94, 122, 123, 151, 167, 170, 195, 202, 220, 233, 241, 244, 298, 311, 336, 339, 357, 359, 364), as well as calcinosis of these tissues. Very interesting is RICO's (261-264) evidence that markedly increasing the magnesium intake of rats protects against the cardiovascular damage induced by diets rich in all of these dietary constituents: calcium, phosphate, protein and vitamin D, as well as in fat.

NEONATAL HYPOCALCEMIA, HYPOMAGNESEMIA, AND VITAMIN D

It is, perhaps, the effect of vitamin D in cow's milk, as well as the increased concentration of phosphate, that is responsible for the greater decrease in serum magnesium that is seen in new-born infants on formula, as compared with that seen in breast-fed newborns (10, 15, 67, 73, 105, 117). The many cases of infantile convulsions, tremors, and tetany in infants fed cow's milk, who have been found to have both hypomagnesemia and hypocalcemia (73, 78, 104, 249, 250, 270, 272, 307, 370), militate against the role of vitamin D-induced hypercalcemia as a mediator in this form of hypomagnesemia. FINCUS *et al.* (253) demonstrated that it was the vitamin D in cow's milk that was responsible for neonatal hypocalcemia, more than the high phosphate content. ATWELL (15) has proposed that the vitamin D in the evaporated milk formulas might have directly contributed to hypomagnesemia in three infants who had undergone intestinal surgery during the neonatal period. The better retention of magnesium while one of the infant received magnesium supplementation of cow's milk, as compared with the retention of magnesium when it was not added to the vitamin D-enriched milk, or was given with sodium lactate, is shown in Figure 8.

HYPOPARATHYROIDISM* IN HYPOCALCEMIC RESPONSE TO MAGNESIUM DEFICIT OR VITAMIN D ADMINISTRATION

As has been noted, the syndromes of neonatal hypocalcemia associated with hyperphosphatemia (78, 105) and that associated with primary hypomagnesemia, which may be associated with normal or low serum phosphate (73, 250), have each been attributed to hypoparathyroidism either as a transient neonatal condition, or as a functional lack of target organ response. A comparable blunting of response to parathormone and/or to vitamin D has been attributed to prolonged magnesium deficit in the adult. HANNA *et al.* (130) first showed that patients with prolonged magnesium deficits, who had hypocalcemia, did not have a satisfactory response to even enormous doses of vitamin D until magnesium was given. ZIMMET (373, 374) has proposed that the hypocalcemia of adults with long-term magnesium deficits caused by malabsorption may be caused by failure of parathormone to mobilize ionized calcium from bone and soft tissue stores. One of his patients became refractory to calcium and vitamin D until the magnesium deficit was corrected. MULLOWNEY *et al.* (234) have reported several similar cases. Five of their nine patients with intestinal malabsorption had hypomagnesemia, hypocalcemia, and tetany, that responded to magnesium but not to parathormone. The hormone injection caused increased phosphaturia, but did not elevate the serum calcium until magnesium had been given. Refractoriness to administered para-

(*) See LARVOR and DUBIAU (178 B) for further data on magnesium and the parathyroids.

Phosphate loads have been demonstrated to intensify pharmacologically-induced renal and cardiovascular damage and calcification by SELYE, BAJUSZ, and coworkers (19, 21-23, 289-291, 295, 297). LEHR *et al.* (183-186) and other investigators (87, 222, 223, 238, 257, 258, 329). The protective effect exerted by prior and concurrent magnesium administration (21, 22, 222, 289-294, 296, 297) suggests phosphate-induced magnesium deficit as a pathogenic factor.

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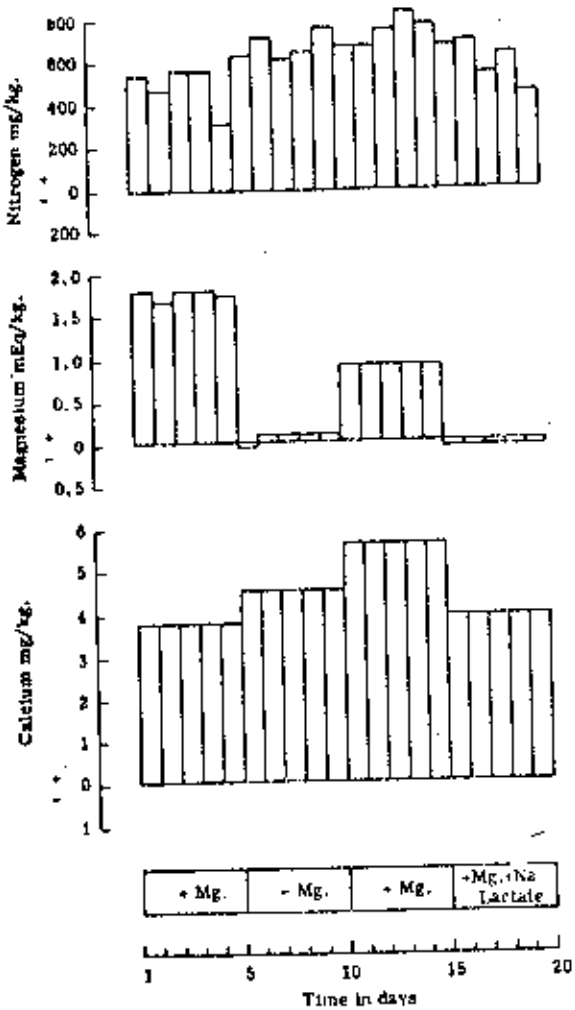
(*) See LARYOR and DURRAGH (178 B) for further data on magnesium and the parathyroids.

Figure 8

BALANCE STUDY IN INFANT DURING POST-OPERATIVE COURSE; EVAPORATED MILK FEEDING



From J. D. ATWELL, *J. Ped. Surg.*, *1*: 427-440, 1966.

thormone and to vitamin D until magnesium was administered also has been reported by ESTIER *et al.* (90) in hypomagnesemic alcoholic patients. These observations suggest that DURLACH's (86) magnesium-deficient spasmophilic patients, whose calcemic response to vitamin D is restored on correction of their magnesium deficit, may similarly have had a blunting of target organ response.

The possibility that the magnesium requirements of hypoparathyroid adults are not usually met is suggested by the report by JONES and FOURMAN (166). They found that all 22 hypoparathyroid adults in their series had significantly lower than normal plasma magnesium levels. Twelve of the patients who were studied in greater detail had low urinary levels of magnesium and were in apparent deficit, as indicated by their retention of high percentages of parenterally-administered magnesium. It is noteworthy that administration of neither calcium nor vitamin D caused a calcemic response until their magnesium deficit was corrected.

The refractoriness of hypomagnesemic patients to vitamin D may lead to administration of very high doses of vitamin D to correct the hypocalcemia. SALTER *et al.* (270) have pointed out the risk of trying to correct the hypocalcemia that is associated with hypomagnesemia, by increasing vitamin D dosage. They found that administration of very large doses did raise the plasma calcium somewhat, but precipitated acute hypomagnesemia and convulsions. If parathyroid deficit or target unresponsiveness persisted in this child, this toxic response to vitamin D is directly analogous to the vitamin D toxicity that has been reported in several parathyroidectomized adults (128, 157, 182, 196), HANKA *et al.* (128), and more recently, LEESON and FOURMAN (182) reported hypomagnesemia in such patients. LYNCH *et al.* (196), who did not find lowered serum magnesium in his patient, nonetheless treated her symptomatic hypercalcemia effectively with intravenous $MgSO_4$.

Perhaps re-evaluation of children diagnosed as having vitamin D resistant rickets, specifically for their magnesium requirements and metabolism, may be rewarding. ANAST (9), for example, has reported such a child, who responded to high dosage vitamin D with a drop in serum magnesium from 1.7-1.9 mEq/L to 1.2-1.5 mEq/L.

ANIMAL MAGNESIUM DEFICIT: EFFECT ON RESPONSE TO VITAMIN D; ROLE OF PARATHORMONE

Recent animal work may explain the contrast in response to Vitamin D in clinical magnesium deficiency, depending on the status of parathyroid function. Magnesium deficiency has been shown by several groups of investigators to cause increased parathyroid secretion in experimental animals (39, 98, 112-115, 179, 201, 299, 360, 365). LARVOR *et al.* (178) have demonstrated parathyroid hyperplasia in magnesium-deficient calves. This explains the hypercalcemia and hypophosphatemia seen with hypomagnesemia in the presence of normally functioning parathyroids (39, 56, 113, 114, 360).

Magnesium-deficient parathyroidectomized animals, in contrast, have been shown by GITTIMAN *et al.* (113, 114) to develop a decreased concentration of plasma ionic calcium, without a decrease in plasma phosphate (114). CLARK's (54) metabolic studies of parathyroidectomized rats, on different dietary intakes of magnesium, showed that there was impairment of intestinal absorption of calcium and increased urinary phosphate when the magnesium intake was inadequate. There was a resultant negative calcium balance and low plasma Ca, as well as low plas-

ma Mg. P balance and plasma levels of P were little affected. These findings are very much like those seen in neonatal hypomagnesemia, associated with hypoparathyroidism (73, 250). The paradoxical hypocalcemic, hyperphosphatemic response to vitamin D, demonstrated by PINCUS *et al.* (253) in newborn infants, was attributed by this group to transient hypoparathyroidism. Support for this postulate has recently been provided by ARNAUD *et al.* (13) who showed that normal parathyroid function is essential for the phosphaturic response of rats to vitamin D.

The recent animal work by LIFSHITZ *et al.* (189, 190) may contribute to our understanding of vitamin D-refractoriness seen in clinical magnesium deficiency. Weanling rats that were magnesium deficient did not respond to low or moderate dosage of vitamin D by increased intestinal absorption of calcium, but exhibited increased phosphaturia (189). High dosage vitamin D restored the plasma calcium to normal (190). Their earlier paper (188) showed that high doses of vitamin D given to magnesium-deficient rats caused decreased bone calcium; possibly the source of the increased serum calcium seen in response to the high dosage vitamin D.

INFANTILE HYPERCALCEMIA, VITAMIN D AND MAGNESIUM

When parathyroid response is normal, either magnesium deficit or hypervitaminosis D can cause hypercalcemia. Whether magnesium deficit contributes to the syndrome of infantile hypercalcemia, and to its sequellae, the WILLIAMS Syndrome or the supravalvular aortic stenosis syndrome, has not been studied. Both low and normal levels of plasma magnesium have been reported in hypercalcemic infants (70, 194, 199). MACDONALD and STAPLETON (199) studied the metabolic balances of three such infants on vitamin D-fortified and on vitamin D-free milk. The infants were either in slightly negative magnesium balance or in equilibrium while on vitamin D; their magnesium balances became slightly positive when put on vitamin D-free milk. DALDERUP (71) has suggested that the damage caused by hypervitaminosis D in infancy might be mediated by loss of magnesium.

We know that this condition has been reported most often in countries where vitamin D is administered generously, and particularly where it is administered in milk (283, 285, 288). Whether in addition to acute hypercalcemia and its serious sequellae in children who are hyperreactive to vitamin D, its excessive administration induces magnesium loss in the average individual, thereby contributing to cardiovascular and renal disease, can neither be averred nor denied with certainty at present. DELANGEN and DONATH (76) and HASS (134) have discussed the possibility that hypervitaminosis D (76, 134) and magnesium deficit (134) may each be implicated in human arteriosclerosis. As observed earlier, each causes elastica degeneration and calcinosis.

VITAMIN D AND MAGNESIUM IN THE ADULT

Indirect evidence that clinical hyper-reactivity to vitamin D may be a cause, not only of hypercalcemia but also of magnesium loss, is provided by a few studies of sarcoid patients. Sarcoidosis is known to be associated with hyper-reactivity to vitamin D (12, 147, 163, 175, 187, 321). HENDRIX (147) has reported that withholding vitamin D entirely from such patients results in improved retention of magnesium. LYNCH *et al.* (196) have reported that the hypercalcemia of sarcoidosis, as well as that of metastatic carcinoma and of vitamin D toxicity responds to $MgSO_4$ therapy. That infantile hypercalcemia and adult sarcoidosis may be caused by similar metabolic disturbances is suggested, at least inferentially, by HUNT *et al.* (155) report of a father with sarcoidosis, who had a child with the infantile hypercalcemia syndrome. The similarity between the hypercalcemias seen in the infantile form, and that in sarcoid has been commented upon by KOHUT and NEUMANN (175).

There is little direct evidence that vitamin D excess in man causes magnesium loss. The metabolic studies by CHU *et al.* (53) with normal adults on ample dietary magnesium (8 mg/kg/d) showed that high doses (12,000 units/day) of vitamin D reduced the positive magnesium balance to 3 % of the daily intake, from the 10 % they had retained prior to the vitamin D loads. HANNA *et al.* (130) reported hypomagnesemia (0.20 and 0.9 mEq/L) and negative magnesium balance in two patients (one with malabsorption and one with osteomalacia due to renal failure) whose hypocalcemia had been treated with enormous doses of vitamin D. After parenteral repletion of the magnesium deficit, and on discontinuation of the vitamin D, the magnesium balances became positive. HEATON *et al.* (141) showed that administration of vitamin D to patients with osteopathy or with idiopathic hypercalciuria caused increased urinary loss of magnesium in 4 of the 7 patients, and decreases in plasma magnesium from 1.9 to 1.5 mEq/L in one and from 1.6 to 1.4 mEq/L in another.

PROTEIN/MAGNESIUM INTERRELATIONSHIPS

HIGH PROTEIN → DECREASED MAGNESIUM RETENTION (CLINICAL)

Very few studies of the influence of proteins on magnesium balance in the normal human subject have been found since the work of MCCANCE *et al.* (198) and SCHOFIELD and MORRELL (135, 276) was reviewed in 1964 (287). Young men on a magnesium intake of 7-8.6 mg/kg were in more positive magnesium balance on a high protein intake than on a low (198). Preadolescent children on low to marginal magnesium intakes (3.9-9.5 mg/kg/d) retained magnesium better on the low than on the high protein intakes (135, 276). In the recent paper on 18 young women by HUNT and SCHOFIELD (162), those

given insufficient protein to maintain nitrogen balance, went from negative to slightly positive magnesium balance when their low magnesium and protein intakes were slightly increased (i.e., 166 to 178 mg Mg/d). When their diets were supplemented with 48 mg/d animal protein (casein and egg white), there was better retention of magnesium, even though the daily intake of magnesium was still low (180-196 mg). Preliminary findings recently reported by STEPHENSON *et al.* (315) indicate that on a more adequate magnesium intake (5.73 mg Mg/kg/d), the source of the protein influences the retention of magnesium. The best absorption and retention of magnesium was seen in those who received their protein as casein-lactalbumin. Beef protein resulted in satisfactory retention, and peanut flour protein seemed to allow for least effective absorption of magnesium. Their findings also indicated that the magnesium requirement for humans is lower at low protein intakes than at high. The authors commented that their findings suggest that the present generally recommended magnesium allowance should be considerably higher than it is to cover the needs of all persons on mixed diets.

That a high intake of protein can interfere with the retention of both magnesium and calcium has been reported (82, 193). Dull (82) found that administration of 50 gm/d of gelatin caused a substantial increase in urinary output of the divalent cations, which was not a consequence of their increased intestinal absorption. LINDEMAN *et al.* (193) found that ingestion of single doses of 50 and 100 gm of casein produced striking increases in rates of urinary excretion of calcium and magnesium.

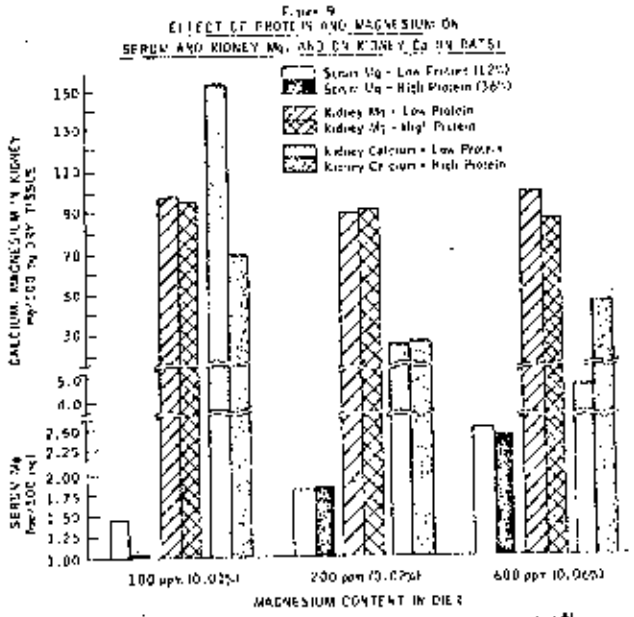
HIGH PROTEIN → MAGNESIUM DEFICIENCY (CLINICAL)

Although it is difficult to relate the protein intake of normal subjects to their magnesium requirements, a dramatic demonstration of intensification of acute magnesium deficiency has been reported in children with kwashiorkor (protein-calorie malnutrition) when their protein deficits are restored. MONTGOMERY (225, 226) and MERCHAN *et al.* (213) demonstrated that this syndrome is associated with markedly low muscle magnesium levels, and with prolonged low urinary outputs of magnesium during magnesium repletion (225). Balance studies showed this low urinary output of magnesium to be due to retention of absorbed supplements, and not to fecal loss. The magnitude of the magnesium deficit was demonstrated by MONTGOMERY (225), who showed that as much as 480 mg Mg was retained by a 20-month-old baby during a three-week repletion period. LINDER *et al.* (192) verified these findings with longer term balance studies. They commented that the very high magnesium requirements of these children is probably due to their extremely rapid rate of growth during the recovery period, when their protein deficit is also being repaired.

The risk of repleting these children with the standard protein-calorie-vitamin-mineral supplements, without special magnesium enrichment, has been well documented by CAMPBELL (44-46). She has described the rapid response to magnesium of the neuromuscular and cardiac manifestations of the acute magnesium deficiency syndrome seen in infants with kwashiorkor on high protein diets, that did not meet their enhanced magnesium requirements. She noted that the high milk intake also increased the magnesium requirement because of its high calcium and phosphorus content (45).

HIGH PROTEIN → INCREASED MAGNESIUM REQUIREMENTS (ANIMAL)

It has long been recognized that increased protein intakes intensify the signs of acute magnesium deficiency in rats (59, 211, 221). BUNCE *et al.* (41) have demonstrated that the magnesium requirements of rats and chicks are directly related to their protein intake. They found that on an intake of magnesium that is generally considered adequate (200 ppm in the diet), a high (36 %) protein intake resulted in development of symptoms of acute magnesium deficiency. They referred to the findings of GERSHOFF and ANDRES (107) who found that on twice that intake (400 ppm), minimal calcium deposits developed in the kidneys of 1/4 of the rats on a 15 % casein diet. Their own studies (Figure 9) revealed low



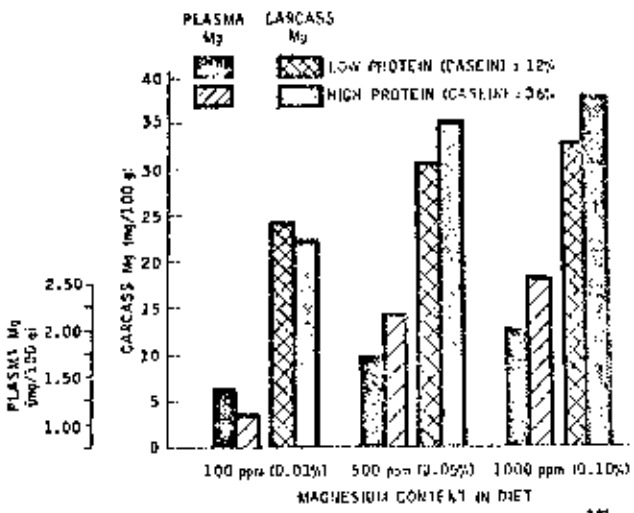
Adapted from G. Bunce, P. Beyers, T. Oka, R. Sauberlich, J. Nutr., 79: 270-276, 1963[41]

serum Mg and high renal levels of both calcium and magnesium in kidneys of rats on low magnesium intakes. They speculated that Mg found in high levels in the kidneys of deficient animals might be as non-functional Mg, in association with crystalline deposits of apatite. The serum Mg rose as the dietary Mg was increased, and the kidney Ca level fell. The metabolic balance studies revealed a rise in urinary Mg with the higher protein intake, that

resulted in negative balance only on the lowest Mg intake. FEATHERSTON *et al.* (94, 95, 233) confirmed the observations that higher levels of dietary Mg were necessary to prevent the Mg deficiency syndrome on a high protein intake than in those on a low protein intake in chicks and dogs. SCHWARTZ *et al.* (281) confirmed the increase in magnesium requirements for maximum growth and development of rats on a high protein intake, as compared with that on a low protein diet (Figure 10). HEINICKE *et al.* (144) demonstrated that for maximal growth response of guinea pigs to specific amino acid supplementation of their diets, it is necessary to provide additional magnesium supplements.

Figure 10

EFFECT OF PROTEIN AND MAGNESIUM ON PLASMA AND CARCASS MAGNESIUM IN RATS



Adapted from R. Schwartz, F. Wang, & R. Woodcock, J. Nutr., 97: 385-393, 1968 [281]

As in the cited preliminary clinical study (315), the source of the dietary protein also affects magnesium retention in animals. FORBES (99, 100) has shown that the magnesium requirement is higher for weanling rats fed a soy protein-lactose diet than for those fed a casein-glucose diet. In a subsequent study, FORBES (101) showed that rats on soy protein retained less magnesium than did those on egg protein, regardless of the sugar used. Similarly, MILLER *et al.* (216) and HENDRICKS *et al.* (146) have shown that the magnesium retention of baby pigs is greater on a casein diet than on a soy protein diet.

REQUIREMENT OF MAGNESIUM FOR PROTEIN SYNTHESIS (ANIMAL)

Since KRUSE *et al.* (176) demonstrated in 1934 that magnesium depleted dogs develop negative nitrogen balance, and KLEIBER *et al.* (169) in 1941, showed decreased efficiency of protein utilization in magnesium deficient rats, magnesium has been known to be important in protein metabolism. SALBERLICH and BAUMANN (271) demonstrated in 1949, that mice kept on a magnesium-deficient diet ceased growing after several weeks, and began to show increased urinary excretion of amino acids. A few years later, MENAKER and KLEINER (211, 212) commented on the importance of magnesium in protein synthesis in growing rats. CARRILLO *et al.* (49) then showed that only when the dietary intake of weanling rats was adequate did doubling their protein intake from 10 to 20 % of the diet produce significantly greater rates of growth. The early reported increased urinary amino acid excretion seen in magnesium deficiency (271) has been verified more recently (41). At low protein intakes, the mean amino acid excretion was much higher in rats on low Mg intakes than in those on high Mg intakes. FEATHERSTON *et al.* (95) made a similar observation with chicks, and suggested that the increased urinary nitrogen in the chicks on low Mg/high protein intakes was due to excretion of amino acids and/or protein as a result of kidney damage. MAZZACCO *et al.* (209) demonstrated significantly increased urinary excretion of amino acids by rats as their magnesium deficiency progressed. They attributed the aminoaciduria of their Mg-deficient rats to renal tubular dysfunction. Explanation of renal tubular dysfunction (i.e., such as might account for aminoaciduria) has been provided by KO *et al.* (170) who reported loss of phosphatase activity in the renal tubules of magnesium-deficient rats. It should be noted that HESS *et al.* (151) have related the loss of oxidative enzyme activity in the kidneys of magnesium-deficient rats to mitochondrial swelling [*].

Depressed synthesis of liver proteins and plasma albumin and decreased levels of alkaline phosphatase have also been seen in magnesium deficiency. SCHWARTZ *et al.* (282) have demonstrated diminished amino acid uptake into liver proteins of magnesium-deficient rats, and decrease in serum albumin synthesis. LARVOR *et al.* (178) have reported significant diminution in serum alkaline phosphatase levels in magnesium-deficient calves, an observation that has been made, also in magnesium-deficient children with protein-caloric malnutrition (45).

CARBOHYDRATE AND LIPIDS: INTERRELATIONSHIPS WITH MAGNESIUM

The influence of carbohydrates and fats on magnesium metabolism has been discussed at length by DURLACH and LARVOR (84 B, 178 A). For discussion of interrelationships between fats and magnesium, see LARVOR and DURLACH (178 A) and SEELIG and VITALE (285 A). The evidence cited, that high sugar intakes tend to increase the urinary excretion of magnesium, and the evidence that carbohydrate metabolism is magnesium-dependent (84 B, 178 A), suggests that subjects ingesting diets high in sugar may have increased requirements for magnesium. The evidence that high dietary fat interferes with magnesium intestinal

(*) See SEELIG (286) for a discussion of the role of magnesium in maintaining mitochondrial integrity.

absorption (178 A, 285 A) points to an increased need for magnesium when diets are rich in fats. Similarly, the evidence of changes in blood and tissue levels of lipids in magnesium deficiency (285 A) supports the premise that adequate magnesium intakes are necessary for normal fat utilization. Therefore the amount of magnesium required by patients on diets rich in fats may well be higher than the amount required by those on a more spartan intake.

HUMAN MAGNESIUM REQUIREMENTS DURING FORMATION OF NEW TISSUE

The laboratory evidence that optimal amounts of magnesium are necessary for normal growth and for protein synthesis, is in accord with the demonstration, both in the laboratory and clinic, of increased magnesium requirements during protein synthesis (i.e., during rapid growth). However, since DUCKWORTH and WARNOCK'S (81) calculations in 1942, of the probable magnesium requirements during gestation, lactation, and growth, few additional studies have been published that pertain directly to this aspect of magnesium metabolism in the normal human subject.

MAGNESIUM AND PREGNANCY

Magnesium deficiency in pregnancy is rarely considered, despite the fact that eclampsia is characterized by hypomagnesemia, and that the acute symptoms respond rapidly to parenteral magnesium therapy. There is evidence that the dietary requirement of magnesium may not be routinely met during pregnancy, and that not only the mother but the fetus may be adversely affected by the combination of conditions that lead to the acute hypomagnesemia of toxemia.

MAGNESIUM BALANCE DURING NORMAL PREGNANCY

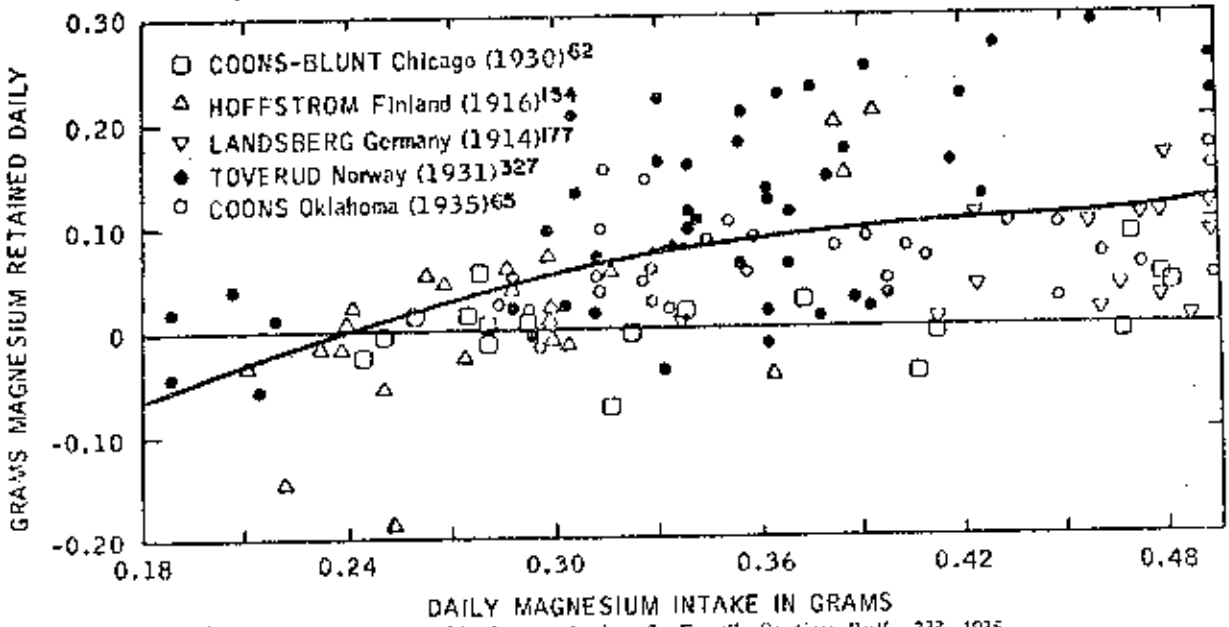
Magnesium balance determinations, done in the 1930's have provided the data that have led occasional reviewers (26, 344, 345) since DUCKWORTH and WARNOCK (81) calculated human Mg requirements, to recommend a dietary intake of substantially more magnesium (400 mg daily) during pregnancy than they considered necessary for other adults. Further scrutiny of the data analyzed by DUCKWORTH and WARNOCK (81) and consideration of the evidence of reduced magnesium plasma levels during normal pregnancies (1, 33, 75, 126, 174, 191, 237, 350, 358, 369), which is intensified in toxemic pregnancies (1, 126, 136, 152) suggest that this daily intake may not be optimal.

COONS *et al.* (62-65) published metabolic balance studies performed on pregnant women. In their 1930 study, COONS and BLUNT (62) were interested in magnesium, primarily to see whether an excess of magnesium intake (i.e., of milk of magnesia for laxative effect) would have an adverse effect on the retention of calcium. No interference with calcium retention was seen, even on magnesium intakes of as much as 810 mg/d. They reported a tendency towards negative magnesium balance, even on intakes of over 400 mg/d towards the end of pregnancy.

In a subsequent study, COONS *et al.* (65) compared their findings with those reported by other investigators (Figure 11). The composite curve, as well as

Figure 11

COMPILATION OF PUBLISHED DATA ON THE RETENTION OF MAGNESIUM DURING HUMAN PREGNANCY SHOWING RELATION OF STORAGE TO INTAKE



From COONS C. M., *Oklahoma Agric. & Expt'l Station Bull.*, 223, 1935.

the scatter diagram, shows low or negative balances on intakes below 300 mg of magnesium daily. The balances rose with the intakes. An average of 16 % of the intake was retained, although on occasion, as much as 30 % of the intake was retained. COONS (64) took note of the preponderance of positive balances in their study, carried out in Oklahoma, which contrasted with an earlier Chicago study (62). In the Chicago study, half of the metabolic periods showed negative magnesium balances. The possibility that exposure to the sun in Oklahoma might have explained the difference was considered, and the effect of cod liver oil administration on magnesium retention, investigated in a young primipara (64). This study covered 101 days of continuously regulated diet during the last half of pregnancy, and included 18 metabolic periods of 14 days each. Despite an apparently adequate intake of magnesium (369 - 561 mg/d), three negative balances occurred during three of the metabolic periods, and there was an average daily retention of only 18 mg/d, which was considered poor. The women receiving Oklahoma sunlight had stored an average of 63 mg daily from an average daily intake of 394 mg Magnesium. Cod liver oil supplementation improved the retention only slightly.

The ranges of magnesium intakes and of balances from the published metabolic studies during pregnancy are indicated on Table IV. From these studies, it appears that magnesium intakes of not less that 400 mg/d may be sufficient to meet both fetal and maternal needs. However, it is worth looking at the findings reported by HUMMEL *et al.* (161). They reported 28 balance periods with a pregnant woman cited as unusually healthy, with a successful record of gestation, lactation, and the birth of healthy infants. Her mean intake of magnesium was 600 mg/d, and her retention of magnesium (15.5 grams) during the second half of her pregnancy, was in excess of that reported in detail by the other investigators. This case is in particular contrast to the young mother, reported by COONS and COONS (64) who apparently did not retain more than an average of 18 mg/d, despite a magnesium daily intake of 455 mg. It is of interest that COONS's patient developed moderate hypertension during the final two weeks of gestation.

The large retention of magnesium during the last third to half of gestation is explicable, both on the basis of fetal and maternal needs. GIVENS and MACY (118) reported that fetal demands for magnesium are greatest during the final trimester. MCCANCE and WIDDOWSON (197) have verified the increasing fetal requirements for magnesium as gestation progresses. As new cells are formed, and as the skeleton is mineralized, the fetal uptake of magnesium increases. COONS (65) considered that the retention by the mothers, of an amount of magnesium greater than that would be indicated as necessary for fetal needs by analysis of fetal composition, to be due to maternal storage in preparation for lactation.

MAGNESIUM AND LACTATION

It is of interest that the first clear demonstration of damage caused by magnesium loss, outside of laboratory conditions, was that seen in lactating cows in the early spring (14, 268). The comparable syndrome in ewes, called grass tetany, has been specifically associated with the early period of heavy lactation, and has responded to, and been prevented by supplementing the forage with magnesium oxide (149, 150). In a paper describing the efficacy of MgO dietary supplementation in preventing this syndrome

TABLE IV
MAGNESIUM INTAKES AND RETENTIONS DURING PREGNANCY

	NO. OF OBSERVATIONS		Mg INTAKE	Mg RETENTION
	WOMEN	METABOLIC PERIODS	RANGE (MEAN) (mg/day)	RANGE (MEAN) (mg/day)
HOFFSTROM[154] (FINLAND)	1	23	177-395 (282)	-176 to +188 (+13)
LANDSBERG[177] (GERMANY)	14	14	338-512 (457)	+2 to +159 (+56)
TOVERUD [327] (NORWAY)	16	27	186-688 (341)	-47 to +255 (+99)
TOVERUD [327] (NORWAY)	23	30	186-688 (368)	0 to +322 (+132)
COONS-BLUNT[62] (CHICAGO, ILLINOIS, U.S.A.)	9	23	236-810 (375)	-311 to +81 (-85)
COONS[64] (OKLAHOMA, U.S.A.)	6	23	285-471 (394)	-3 to +154 (+63)
COONS & COONS[64] (OKLAHOMA, U.S.A.)	1	18	369-561 (455)	-7 to +63 (+18)
HUMMEL et al [161] (DETROIT, MICHIGAN, U.S.A.)	1	28	480-760 (600)	+30 to +230 (+110)
HUMMEL et al [160] (DETROIT, MICHIGAN, U.S.A.)	1	13	350-430 (390)	+10 to +160 (+60)

in sheep, HERD (149) has observed that flocks of sheep subject to grass tetany, also have an unusually high incidence of toxemia before lambing.

Hypomagnesemic tetany has also been reported in a young mother who was lactating heavily (125). It is noteworthy that the investigators commented that her serum Mg concentration rose steadily from its markedly depressed initial level without replacement therapy while on a diet that delivered 500 mg magnesium daily. Nonetheless, her retention of magnesium from a loading test given on the sixth hospital day, was about 78 %, suggesting an underlying magnesium deficit.

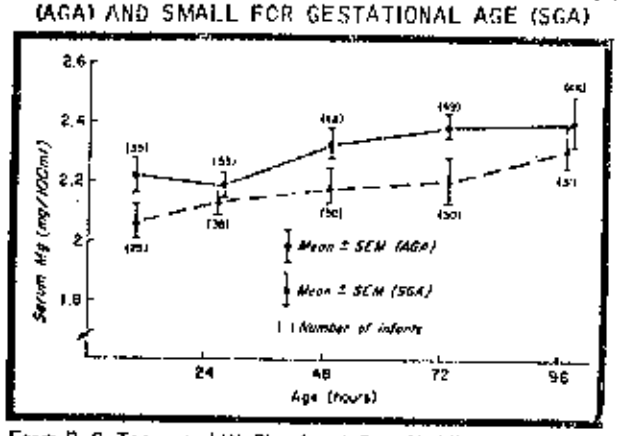
MAGNESIUM IN INFANCY AND CHILDHOOD

MAGNESIUM DEFICITS IN INFANCY.

Evidence is beginning to accumulate that indicates that requirements for magnesium during infancy are influenced by the status of the maternal dietary supply during gestation, as well as by her health. DURLACH (86) has speculated on the possibility that a low plasma Mg during gestation may contribute to development of neuromuscular irritability in the child. Infants born of toxemic mothers have been shown to have lower magnesium levels than do those born of normal mothers. They probably thus have higher than usual magnesium needs. The recent study by TSANG and OH (328), demonstrates low serum magnesium levels in infants of low birth weights, in infants born of primiparas, and in particular of infants born of toxemic mothers (Fig. 12). They

Figure 12

SERUM Mg LEVELS IN PRETERM INFANTS WITH BIRTH WEIGHTS APPROPRIATE FOR GESTATIONAL AGE (AGA) AND SMALL FOR GESTATIONAL AGE (SGA)



From R.C. Tsang and W. Oh, Am.J.Dis.Childh. 120: 44-48, 1970

found that all infants of toxemic mothers were small for gestational age. They postulate that the lower Mg levels in infants who were small for gestation suggest that altered fetal Mg metabolism may be part of the intrauterine malnutrition syndrome. That the low Mg blood levels of toxemic women (1, 126, 136, 152) reflect inadequate stores of magnesium, and that the supply to the fetus may be inadequate for optimal growth should also be considered. Whether the lower birth size and lower Mg levels of infants born to primiparas than to multiparas reflects the lesser magnesium stores of young girls who might still be in or had recently completed an active phase of growth themselves, is another point requiring elucidation.

Some of the infants reported (*supra vide*), with hypomagnesemia associated with transient or functional hypoparathyroidism, were born to women with recognized hypomagnesemic hyperparathyroidism during gestation (73, 78). Other infants who developed symptomatic hypomagnesemia, were born to women after complicated births, or as multiple births and who underwent surgery. ATWELL (15) has presented three such infants, who had to undergo abdominal surgery in the neonatal period for intestinal obstruction. Two had been born after pregnancies complicated by hydramnios; one had been one of twins. Resections or short circuiting of segments of the small intestine were necessary in each instance. It was observed that this would be expected to interfere with the absorption of magnesium, which is largely absorbed in the small intestines (121). Complicating the postoperative course, and causing further losses of magnesium was the increased loss of magnesium from the intestinal contents, caused by diarrhea in two of the infants, and by ileostomy dysfunction and fecal fistulae in the third. (Such discharges have been shown by THORÉN (325) to contain 6 - 14 mEq of magnesium per liter.)

Still another infant who developed convulsions with hypomagnesemia, after surgery and development of diarrhea, has been reported by Savage and McADAM (272). This infant had undergone surgery at 5 weeks for suspected intestinal obstruction; at 3 months he developed severe diarrhea, subsequent to which he developed hypomagnesemic convulsions, despite somewhat elevated calcium levels. He responded well to intramuscular magnesium. These investigators attributed the intensification of the magnesium deficit to the ingestion of cow's milk formula, on the basis of the high Ca/Mg ratio in cow's milk.

Cognizant of the risk of magnesium depletion in the neonate or young child, particularly after surgery or severe diarrhea, WILKINSON and HARRIS (366) have recently recommended examination of magnesium deficit in such infants who fail to respond to the usual therapy. They gave an intravenous load dose of magnesium to nine such infants, and found that five retained 63 - 96 % of the dose (were severely depleted), one retained 49 % (slight depletion), and three excreted 72 % in their urine, retaining only 28 % (not depleted). Recovery followed rapid replacement of magnesium in each of the five who were severely depleted.

That hypomagnesemia, without notable hypocalcemia, can cause tremor and convulsions in infants and children has been reported by WONG and TEAI (370). They measured serum magnesium levels in forty children with convulsions, tremors or muscular twitching selected at random from three pediatric wards and found hypomagnesemia in thirteen. In six of the cases, magnesium therapy in doses insufficient to cause drowsiness, controlled the tremors. The remainder of the cases showed clinical improvement in several days, with elevation of the serum Mg without magnesium therapy.

Now that magnesium analytic techniques are more readily available, the syndrome of magnesium depletion in infancy, with and without hypocalcemia, is being recognized more frequently. Search through the older literature would probably reveal other cases of magnesium deficit that had gone undetected. GITLEMAN *et al.* (116) for example, reported a high incidence of hypocalcemia on the first day of life, particularly in prematures, or in infants born after complicated pregnancies (i.e., 20 % of infants born by Caesarian section). Magnesium levels were not measured. The boy reported by MILLER (218) in 1944, who had tremors and convulsions during infancy, and tremors throughout his childhood, was diagnosed as hypomagnesemic as the age of six. He responded to oral magnesium with striking relief of his tremors and dizziness. It seems very likely that earlier examination for magnesium levels might have detected the deficit in magnesium when his early symptoms developed.

NORMAL MAGNESIUM REQUIREMENTS DURING INFANCY AND CHILDHOOD

We are dependent, for our recommendations of magnesium requirements during infancy, on DUCKWORTH and WARNOCK'S (81) analysis of the published data, up to 1942. They calculated the magnesium requirements of infants and children by using the available data for magnesium content of soft and hard tissues and correlating these values with published growth curves. Their calculations suggested that the annual increment of magnesium increases from about 1 - 3 grams, or 2 to 8.5 mg/d from 1 to 13 years of age. However, study of the sparse metabolic balance data indicated that much higher daily retentions of magnesium actually take place. The mg/kg retentions, in general, tended to be greater for the smaller than for the larger children and ranged from 0.6 to 4.9 mg/kg d. The total daily retentions of magnesium ranged from 10 to 78 mg. On the basis of these calculations, they estimated the magnesium requirements for infants as 10 - 20 mg/kg/d, and for children up to 8 years of age as 13 mg kg/d.

Subsequent metabolic work with children suggests that the optimal magnesium intake may even be somewhat higher. DANIELS (72) reported that three boys 3 - 5 years of age were in magnesium equilibrium or negative balance on intakes of less than 11 mg/kg. They retained 16 to 25 mg daily on intakes of 14 - 15.7 mg/kg/d. MACY (206) also found, in their study of 15 boys and girls, 4 - 7 years of age, that retentions of magnesium were better on 15 - 17 mg/kg/d than they were on 10 - 14 mg/kg/d. PETRUSKINA (252) found that children 6 - 8 years of age were in negative magnesium balance on daily intakes of less than 10 mg/kg/d, and were in equilibrium or slightly positive balance (+ 7 mg) on 10 - 30 mg/kg/d. On intakes of 37 - 50 mg/kg/d, they were in strong positive balance (13 to + 21 mg/d). Does this mean that children do best on such high intakes of magnesium? Additional studies are necessary.

The optimal requirement of magnesium by infants is even more uncertain. The status of maternal magnesium stores and of parathyroid function has been shown to influence the neonatal magnesium requirements. The nature of the infant's feeding also affects his magnesium needs. Cow's milk, by providing higher calcium and phosphorus intakes, as compared with that of magnesium and the perhaps excessive supplementation with the vitamin D in milk (283, 288) all increase the magnesium requirements of the infant on cow's milk formula, as compared with that of infants on mother's milk. SWANSON (320) and SLATER (308) found that the mineral retentions, particularly of calcium and to a lesser degree of magnesium were substantially greater by infants on cow's milk than on breast milk. Is this « supermineralization » a reflection only of increased mineral deposition only in the skeleton?

CONCLUDING REMARKS

It is difficult to provide precise mg/kg/d recommendations for optimal magnesium intakes, because the amount needed to prevent pathology in experimental models is influenced by so many factors. Increasing the intakes of calcium, phosphate, vitamin D, and protein, all plentiful in the Occidental dietary, each increases the magnesium requirements. Not included in this paper is the evidence that fats and sugars may also increase the magnesium requirements. No longer in question is the effect of alcohol ingestion on magnesium.

The risk of not providing optimal magnesium intakes may be deduced from examination of magnesium-deficient animals. That the symptoms of acute hypomagnesemia in man resemble those produced in experimental animals is now recognized. Now that analytic facilities for measuring magnesium are more readily available, hypomagnesemia is more frequently looked for — perhaps not at first, but at least as the need for anticonvulsant therapy persists.

The similarity of some of the histopathologic findings in human cardiovascular and renal disease, to those seen in animals on long-term magnesium

deficits, is rarely noted. The laboratory evidence that magnesium administration can prevent damage to the cardiovascular and renal systems that is caused by a number of pharmacologic agents, as well as by prior magnesium deficiency, is rarely applied to the clinical situation. There is far more interest in magnesium, as a useful cardioprotective agent, in Europe, than there is in the United States. In the United States, there is more interest in the use of magnesium to prevent calcareous kidney stones.

Emphasis has been placed on the blunting, by magnesium deficiency, of the calcemic response to parathormone and to vitamin D, because of the common administration of very high doses of these agents to patients with hypocalcemia, with osteopathies, or possibly even with vitamin D-resistant rickets, to increase their absorption of calcium. Early attention to their magnesium levels, and to possibly increased magnesium requirements by such patients may allow for more physiologic enhancement of calcium retention. Similarly, correction of magnesium deficit has been shown to improve the calcemic response to administered calcium. This is an important point, since treatment with each causes intensification of the magnesium deficit.

Since no animal or clinical evidence has been uncovered that indicates that large oral doses of magnesium are harmful to subjects with normal renal function, it is suggested that we revise upwards the recommendations for daily intakes. Normal adults may require 7-10 mg kg/d; pregnant and lactating women probably require more, perhaps 15 mg/kg/d. Infants and rapidly growing children may require as much as 15-30 mg/kg/d. Perhaps members of families with histories of frequent and early cardiovascular and calcifying renal disease, or with neuromuscular lability, have relative intestinal malabsorption of magnesium and require further supplements. Long term epidemiologic studies of such subjects are necessary to prove or disprove the preventive role of higher magnesium intakes in man.

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ABSTRACT

Magnesium Requirements

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The magnesium requirements must be established for conditions of health in periods of new tissue formation and when growth is complete, and in abnormal conditions. To extend recognition of the medical significance of magnesium deficit, it is necessary to simplify methodology and establish reliable normal values. Although metabolic balance studies provide the most complete data, a more practical clinical means of detecting magnesium deficiency may be determination of percentage urinary excretion of parenterally administered load of magnesium. Many factors influence magnesium requirements. Conditions or dietary constituents that interfere with absorption or retention of magnesium make a higher intake necessary to maintain equilibrium. The calcium/magnesium relationship is complex. A high calcium intake increases the magnesium requirement by interfering with its intestinal absorption and increasing its renal excretion. In disease associated with calcium loss, correction of magnesium deficit enhances calcium retention. Whether magnesium deficit will interfere with calcium retention or cause hypercalcemia (i.e., in response to vitamin D) seems to depend on the state of the parathyroids. Normally, magnesium deficit causes increased parathyroid secretion. However, chronic magnesium deficit may blunt the target organ responses in terms of calcium absorption in response to parathormone or to vitamin D. Phosphate excess and vitamin D excess also increase magnesium requirements, as does a high protein intake. Since protein synthesis is magnesium-dependent, during periods of new tissue formation, there are substantial increased magnesium requirements. The hypomagnesemias of pregnancy and infancy are correlated with a postulated magnesium deficit at those times. It is suggested that toxemia of pregnancy and neonatal convulsions of hypocalcemia/hypomagnesemia are associated with failure to meet magnesium requirements. Since no animal or clinical evidence has been found that indicates that large oral doses of magnesium are harmful when renal function is normal, it is suggested that the recommendations for daily intake of magnesium be revised upwards. On the basis of metabolic studies, and taking into account the effect of other dietary components on magnesium requirements, it is suggested that the normal adult may require 7-10 mg/kg/d. Pregnant and lactating women may need as much as 15 mg/kg/d. Infants and rapidly growing children may need as much as 15-30 mg/kg/d. More work is needed to determine the requirements of patients subject to diseases that have characteristics seen in chronic experimental magnesium deficiency.

RÉSUMÉ

Les besoins magnésiques

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Pour maintenir la bonne santé au cours de la formation de tissu nouveau, quand la croissance est complète, et dans des conditions anormales, on doit déterminer le besoin magnésique en chaque état. Pour étendre la connaissance de l'importance médicale d'un manque de magnésium, il est nécessaire de simplifier les méthodes et d'établir des valeurs normales et sûres. Bien que les recherches au sujet de l'équilibre métabolique fournissent les données les plus complètes, un moyen plus pratique de découvrir d'une manière clinique un déficit de magnésium peut être de déterminer le pourcentage d'un dosage parentéral de magnésium éliminé dans l'urine. La quantité exigée de magnésium subit beaucoup d'influences. A cause des conditions ou des éléments du régime alimentaire qui interviennent dans l'absorption ou dans la conservation de magnésium, une ingestion augmentée est obligatoire afin de maintenir l'équilibre. Les rapports de calcium/magnésium sont complexes. Quand l'apport

calcique est élevé, l'absorption intestinale est diminuée et l'élimination urinaire est augmentée. Par conséquent il faut augmenter le magnésium alimentaire. Dans une maladie caractérisée par la perte de calcium, quand on améliore le déficit magnésique il y a une meilleure rétention de calcium. Soit que le déficit magnésique intervienne dans la conservation du calcium ou produise un état d'hypercalcémie (i.e., en réponse à vitamine D) il apparaît dépendre de l'état des parathyroïdes. Normalement, le déficit magnésique produit une sécrétion parathyroïdique qui est élevée. Cependant le déficit magnésique chronique peut diminuer la réponse des organes qui sont les buts de l'action de la vitamine D ou de la parathormone, quant à l'absorption de calcium. Un excès de phosphore ou de vitamine D ou de protéine, produit aussi un besoin augmenté de magnésium. Puisque la synthèse protéinique dépend du magnésium, beaucoup plus de magnésium est nécessaire pour la formation de tissu nouveau. L'hypomagnésémie de la gestation et de l'enfance se trouvent en corrélation avec un déficit magnésique hypothétique en pareilles époques. On propose que l'éclampsie et les convulsions d'enfance s'associent au défaut de fournir assez de magnésium. Puisqu'aucune evidence ni normale ni clinique n'a été trouvée pour indiquer que les grandes doses magnésiques font du mal si la fonction rénale est normale, on propose que l'on augmente les recommandations pour la ration quotidienne de magnésium. Fondé sur les études métaboliques et considérant les effets des autres constituants du régime alimentaire sur les besoins magnésiques, on propose que l'adulte normal a besoin de 7-10 mg/kg/j. Les femmes enceintes et celles qui allaitent leurs nourrissons peuvent avoir besoin de 15 mg/kg/j. Les nourrissons et les enfants qui se développent rapidement peuvent avoir besoin de 15-30 mg/kg/j. Plus de travail est nécessaire pour déterminer les besoins des patients enclins aux maladies semblables à celles des animaux qui souffrent d'un déficit magnésique chronique.

AUSZUG

Der Magnesiumbedarf muss einerseits unter gesunden

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Bedingungen zu Zeiten neuer Gewebsbildung und nach abgeschlossenem Wachstum, andererseits unter abnormen Bedingungen bestimmt werden. Um das Verständnis der medizinischen Bedeutung des Magnesiummangels zu erweitern, ist es notwendig, die Methodik zu vereinfachen und verlässliche Normalwerte festzulegen. Obwohl Untersuchungen der Stoffwechselbilanz die vollständigsten Daten liefern, ist möglicherweise die Bestimmung des mit dem Urin ausgeschiedenen Anteils einer parenteral verabreichten Magnesiumgabe ein praktischerer klinischer Weg, Magnesiummangel zu entdecken. Viele Faktoren beeinflussen den Magnesiumbedarf. Wenn gewisse Bedingungen oder Nahrungsbestandteile die Absorption oder Retention von Magnesium eingreifen, ist eine vermehrte Zufuhr erforderlich, um das Gleichgewicht einzuhalten. Das Calcium/Magnesium Verhältnis ist verwickelt. Eine hohe Calciumaufnahme führt zu erhöhtem Magnesiumbedarf dadurch, dass die intestinale Absorption beeinträchtigt und die renale Ausscheidung vermehrt ist. In Erkrankungen, die mit einem Calciumverlust einhergehen, fördert die Ausgleichung des Magnesiumdefizits die Calciumretention. Ob ein Magnesiumdefizit die Calciumretention beeinträchtigt oder eine Hypercalcämie verursacht (z.B. nach Vitamin D-Zufuhr) scheint vom Zustand der Nebenschilddrüsen abzuhängen. Normalerweise verursacht Magnesiummangel eine vermehrte Nebenschilddrüsensekretion. Ein chronisches Magnesiumdefizit stumpft möglicherweise die Zielorgane in Bezug auf ihre Fähigkeit zur Calciumabsorption nach Parathormon- oder Vitamin D-Zufuhr ab. Phosphatüberschuss und Vitamin D-Überschuss ebenso wie hohe Eiweisszufuhr vermehren den Magnesiumbedarf ebenfalls. Da Eiweisssynthese magnesiumabhängig ist, ist der Magnesiumbedarf zu Zeiten neuer Gewebsbildung beträchtlich vermehrt. Hypomagnesämien während Schwangerschaft und Säuglingsalter stehen in Beziehung zu dem für diese Zeitabschnitte angenommenen Magnesiumdefizit. Es besteht Grund zur Annahme, dass Schwangerschaftstoxämie und Neugeborenenkrämpfe auf der Basis von Hypocalcämie/Hypomagnesämie mit einem Versagen in der Deckung des Magnesiumbedarfs einhergehen. Da keine tierexperimentellen oder klinischen Beweise dafür vorliegen, dass grosse orale Magnesiumgaben bei normaler Nierenfunktion schädlich sind, wird empfohlen, die tägliche Magnesiumaufnahme höher anzusetzen. Auf der Grundlage von Stoffwechselversuchen und unter Berücksichtigung der Wirkung anderer Nahrungsbestandteile auf den Magnesiumbedarf wird vorgeschlagen, beim normalen Erwachsenen einen täglichen Bedarf von 7-10 mg/kg Körpergewicht anzunehmen. Schwangere und stillende Frauen benötigen möglicherweise bis zu 15 mg/kg täglich. Säuglinge und Kinder zu Zeiten beschleunigten Wachstums brauchen möglicherweise bis zu 15-30 mg/kg täglich. Weitere Studien sind erforderlich, um den Bedarf bei Patienten zu bestimmen, die an Erkrankungen leiden, welche charakteristische Besonderheiten mit chronischem experimentellem Magnesiummangel gemeinsam haben.

BIBLIOGRAPHY

1. Achari G., Mishra K., Achari K., Ramkissen R., Upadhyay S. — Serum magnesium in women in the normal state and in certain conditions. *J. Indian Med. Assoc.*, 1961, *36* : 93-95.
2. Aikawa J. — Magnesium turnover in man. *Dynamic Clinical Studies with Radioisotopes*. Editor R. M. Kniselew, 1964, 565-580.
3. Aikawa J. — The role of magnesium in biologic processes. *Electrolytes and Cardiovascular Diseases*. Editor E. Bajusz, 1965, 9-27.
4. Aikawa J., Gordon G., Thomas B. — Magnesium metabolism in human beings: Studies with Mg^{28}. *J. Appl. Physiol.*, 1960, *15* : 803-807.
5. Albert D., Morita Y., Isert L. — Serum magnesium and plasma sodium levels in essential vascular hypertension. *Circ.*, 1958, *17* : 761-764.
6. Alduce N. — Concentration of magnesium in human plasma or serum. *Nature*, 1965, *206* : 89-90.
7. Alfrey N., MacIntyre I., Rabe I. — The determination of magnesium in biological fluids and tissues by flame spectrophotometry. *J. Clin. Path.*, 1960, *13* : 506-510.
8. Amiot D., Hugo D., Durlach J. — Fréquence du déficit magnésique chez le sujet normal et dans diverses osteopathies. *J. de Médecine de Besançon*, 1969, *5* : 371-378.
9. Anast C. — Magnesium studies in relation to vitamin D-resistant rickets. *Pediatrics*, 1967, *40* : 425-435.
10. Anast C. — Serum magnesium levels in the newborn. *Pediatrics*, 1964, *33* : 969-974.
11. Anderson T., Richie W., Mackay J. — Sudden death and ischemic heart disease. *New Eng. J. Med.*, 1969, *280* : 805-807.
12. Anning S., Dawson J., Dolby D., Ingram J. — The toxic effects of calciferol. *Quart. J. Med.*, 1948, *17* : 203-228.
13. Arnaud C., Rasmussen H., Anast C. — Further studies on the interrelationship between parathyroid hormone and vitamin D. *J. Clin. Invest.*, 1966, *45* : 1955-1964.
14. Arneil R., Fischam I. — Cardiovascular and pulmonary calcification apparently associated with dietary imbalance in Jamaica. *J. Comp. Path.*, 1950, *60* : 51-66.

15. ATWELL J. — Magnesium deficiency following neonatal surgical procedures. *J. Pediat. Surg.*, 1966, *1* : 427-440.
16. AVIOLI L., LYNCH T., BERMAN M. — Digital computer compartmental analysis of Mg^{28} kinetics in normal subjects, Paget's disease, and thyroid disease. *J. Clin. Invest.*, 1963, *42* : 915.
17. BAJPAI P., SEGDEN D., KAMOS A., STERN L. — Serum Mg levels in the newborn and older child. *Arch. Dis. Childh.*, 1966, *41* : 424-427.
18. BAJPAI P., SEGDEN D., STERN L., DENTON R. — Serum ionic magnesium in exchange transfusion. *J. Pediat.*, 1967, *70* : 193-199.
19. BALÁSZ E. — Experimental pathology and histochemistry of heart muscle. *Meth. Achiev. Exp. Path.*, 1967, *2* : 172-223.
20. BALÁSZ E. — Heart disease and soft and hard water. *Lancet*, 1967, *1* : 726-727.
21. BALÁSZ E. — Nutritional aspects of cardiovascular diseases. J. B. Lippincott Co., Philadelphia and Montreal, 1965.
22. BALÁSZ E., SELYE H. — Conditioning factors for cardiac necroses. *Trans. N.Y. Acad. Sci.*, 1959, *21* : 659-667.
23. BALÁSZ E., SELYE H. — Influence of variations in dietary intake of chlorides upon diverse necrotizing cardiopathies. *Am. Heart J.*, 1960, *60* : 266-280.
24. BALDWIN D., ROBINSON P., ZIEGLER K., EILENBERG J. — Interrelations of magnesium, potassium, phosphorus, and creatine in skeletal muscle of man. *J. Clin. Invest.*, 1952, *31* : 850-858.
25. BASF H., GLICKSMAN A., SCHWARTZ M., NICELSON J. — Distribution and physiologic exchange of Mg^{28} in animals and man. *Clin. Res. Proc.*, 1956, *4* : 127.
26. BARKES E. — Physiologic and clinical aspects of magnesium metabolism. *J. Chron. Dis.*, 1960, *11* : 278-291.
27. BARNES B., BROWNELL G. — A comparison of the distribution of magnesium-28 with that of potassium-42 and calcium-45. *Proc. Second International Conference*, Geneva, September, 1958.
28. BARON G., BROWN S., PEARSON P. — Histological manifestations of a magnesium deficiency in the rat and rabbit. *Proc. Soc. Exper. Biol. & Med.*, 1949, *70* : 220-223.
29. BAYSOKES J., MADERA-ORSINI F., SELLES D., BRIERE R. — Fluorometric estimation of magnesium in serum and urine. *Am. J. Clin. Path.*, 1964, *42* : 541-546.
30. BERSHEIAN D. — Cardiovascular Deaths. *J.A.M.A.*, 1962, *179* : 825.
31. BETTER O., KLEIMAN C., GONICK H., VARRAIN P., MAXWELL M. — Renal handling of calcium, magnesium and inorganic phosphate in chronic renal failure. *Israel J. Med. Sci.*, 1967, *3* : 60-79.
32. BJORCK G., BOSTROM H., WIDSTROM A. — On the relationship between water hardness and death rate in cardiovascular diseases. *Acta Medica Scandinavica*, 1965, *178* : 239-251.
33. BOGERT L., PLASS E. — Placental transmission. I. The calcium and magnesium content of fetal and maternal blood serum. *J. Biol. Chem.*, 1923, *56* : 299-307.
34. BOOTH C., BARBOURIS M., HANNA S., MACINTYRE I. — Incidence of hypermagnesium in intestinal malabsorption. *Brit. Med. J.*, 1963, *2* : 141-143.
35. BRIERLEY G. — Ion transport by heart mitochondria. *J. Biol. Chem.*, 1967, *242* : 1115-1122.
36. BRIERLEY G., MURER E., BACHMANN E., GREEN D. — The accumulation of inorganic phosphate and mg ions by heart mitochondria. *J. Biol. Chem.*, 1963, *238* : 3482-3489.
37. BRINSON A., RAGAN C. — Effect of magnesium on calcium metabolism in man. *Amer. J. Clin. Nutr.*, 1956, *10* : 296-306.
38. BROWSE S. — Magnesium and cardiovascular disease. *Brit. Med. J.*, 1963, *2* : 118.
39. BUCELE R., CARE A., COBLER C., GUEHMAN H. — The influence of plasma magnesium concentrations on parathyroid hormone secretion. *J. Endocr.*, 1968, *42* : 529-534.
40. BUNCE G., CHEMCHARSKI Y., PHILLIPS P. — The mineral requirements of the dog. IV. Effect of certain dietary and physiologic factors upon the magnesium deficiency syndrome. *J. Nutr.*, 1962, *76* : 23-29.
41. BUNCE G., REYES P., OTA T., SAUBERLICH H. — Influence of the dietary protein level on the magnesium requirement. *J. Nutr.*, 1963, *79* : 220-226.
42. BUNCE G., SAUBERLICH H., REYES P. — Dietary phosphorus and magnesium deficiency in the rat. *J. Nutr.*, 1965, *86* : 406-414.
43. BUNKER J., BENDIXEN H., MURPHY A. — Hemodynamic effects of intravenously administered sodium citrate. *The New Eng. J. Med.*, 1962, *266* : 372-377.
44. CABRILL J. — The effect of mg therapy on cardiovascular and electrocardiograph changes in severe protein-calorie malnutrition. *Trop. Geogr. Med.*, 1969, *21* : 33-38.
45. CALSOLA J. — Magnesium in the therapy of protein-calorie malnutrition of childhood. *Pediatr.*, 1955, *66* : 392-413.
46. CAMPBELL J. — Studies in protein-calorie malnutrition. II. A double-blind clinical trial to assess magnesium therapy. *New Eng. J. Med.*, 1967, *276* : 535-546.
47. CAMPBELL J., GOMEZ D. — Studies in protein-calorie malnutrition. I. Chemical evidence for magnesium deficiency. *New Eng. J. Med.*, 1967, *276* : 533-535.
48. CARR M., FRANK H. — Improved method for determination of calcium and magnesium in biologic fluids by EDTA titration. *Am. J. Clin. Path.*, 1956, *26* : 1157-1165.
49. CARRILLO B., POND W., KIRKK L., LOVEMORE F., LOPEZ J. — Response of growing rats to diets varying in magnesium, potassium and protein content. *Proc. Soc. Exp. Biol. & Med.*, 1961, *107* : 793-796.
50. CARUBELLI R., SARTH W., HAMMARSTEN J. — Determination of magnesium and calcium in urine. *Clin. Chem.*, 1959, *5* : 45-49.
51. CHARBON G., HOEKSTRA M. — On the competition between calcium and magnesium. *Acta Physiol. Pharmacol. Neerlandica*, 1962, *11* : 141-150.
52. CHESLEY L., TEPPER I. — Some effects of magnesium loading upon renal excretion of magnesium and certain other electrolytes. *J. Clin. Invest.*, 1958, *37* : 1362-1372.
53. CHU H., LU S., HSU H., CHIA H., CHIU S. — Calcium, phosphorus, nitrogen and magnesium metabolism in normal young Chinese adults. *Chinese Med. J.*, 1941, *59* : 1-33.
54. CLARK I. — Effects of magnesium on calcium and phosphate metabolism in parathyroidectomized rats. *Endocrinol.*, 1969, *85* : 1103-1113.
55. CLARK I. — Effects of magnesium ions on calcium and phosphorus metabolism. *Am. J. Physiol.*, 1968, *214* : 348-356.
56. CLARK I. — Metabolic interrelations of calcium, magnesium and phosphate. *Am. J. Physiol.*, 1969, *217* : 871-878.
57. CLARK I., BELANGER L. — The effects of alterations in dietary magnesium on calcium, phosphate and skeletal metabolism. *Calc. Tiss. Res.*, 1967, *1* : 204-218.
58. CLARKSON E., WARREN R., MCDERMOTT S., m. WARDENER H. — The effect of a high intake of calcium on magnesium metabolism in normal subjects and patients with chronic renal failure. *Clin. Sci.*, 1967, *32* : 11-18.
59. COBB R., FRYE C. — Effect of feeding high levels of protein and calcium in rat rations on magnesium deficiency syndrome. *Am. J. Physiol.*, 1951, *166* : 409-412.
60. CONNELLY J., CRAWFORD J., WATSON J. — Studies on neonatal hyperphosphatemia. *Pediat.*, 1962, *30* : 425-432.
61. CONSTANT M., PHILLIPS P. — The occurrence of a calcinosis syndrome in the cotton rat. I. The effect of diet on the ash content of the heart. *J. Nutr.*, 1952, *47* : 317-326.
62. COONS C., BUNSE K. — Retention of nitrogen, calcium, phosphorus and magnesium by pregnant women. *J. Biol. Chem.*, 1930, *86* : 1-16.
63. COONS C., COONS R., SCHRIVERSON A. — The acid-base balance of the minerals retained during human pregnancy. *J. Biol. Chem.*, 1934, *104* : 757-768.
64. COONS C., COONS R. — Some effects of cod liver oil and wheat germ on the retention of iron, nitrogen, phosphorus, calcium, and magnesium during human pregnancy. *J. Nutr.*, 1935, *10* : 289-310.
65. COONS C., SCHRIVERSON A., MARSHAL G., COONS R. — Studies in metabolism during pregnancy. *Experiment. Station Bull.*, 1935, 223.
66. CUP C., WILLY B. — The ultrafiltrable serum magnesium in hyperthyroidism. *Biochem. J.*, 1942, *36* : 413-415.
67. COUSSINS H. — Magnesium metabolism in infants and children. *Postgrad. Med.*, 1969, *46* : 135-139.
68. CRAWFORD M., GARDNER J., MORRIS J. — Mortality and hardness of local water-supplies. *Lancet*, 1968, *1* : 827-831.

69. Crawford T., Crawford M. — Prevalence and pathological changes of ischaemic heart-disease in hard-water and in a soft-water area. *Lancet*, 1967, *I*: 229-232.

70. Cripery R., Nitti D. — Idiopathic hypercalcemia in infants with failure to thrive. *Lancet*, 1954, *267*: 110-114.

71. Dalderup L. — The role of magnesium in osteoporosis and idiopathic hypercalcemia. *Voeding*, 1960, *21*: 424-435.

72. Daniels A. — Magnesium needs of preschool children. *Am. J. Dis. Childh.*, 1941, *62*: 568-576.

73. Davis J., Harvey D., Yu J. — Neonatal fits associated with hypomagnesemia. *Arch. Dis. Childh.*, 1965, *40*: 286-290.

74. Dawson J., Hughes E. — The determination of magnesium in biological materials by atomic absorption spectrophotometry. *Biochem. J.*, 1964, *89*: 99-106.

75. De Jorge B., Delascio D., Barros de Ulhoa Cintra A., Antunes M. — Magnesium concentration in the blood serum of normal pregnant women. *Obst. & Gyn.*, 1965, *25*: 253-254.

76. De Langen C., Dienth W. — Vitamin D sclerosis of the arteries and the danger of feeding extra vitamin D to older people, with a view on the development of different forms of arteriosclerosis. *Acta Med. Scand.*, 1956, *156*: 317-323.

77. Dimich A., Wallach S. — Magnesium transport in humans with hypomagnesemia. *J. Clin. Endocrin. and Metab.*, 1969, *29*: 496-505.

78. Dooling E., Stern L. — Hypomagnesemia with convulsions in a newborn infant. *Canad. Med. Ass. J.*, 1967, *97*: 827-831.

79. Duckworth J., Godden W. — The replenishment of depleted skeletal reserves of magnesium. *Biochem. J.*, 1943, *37*: 597-598.

80. Duckworth J., Godden W., Warnock G. — The effect of acute magnesium deficiency on blood formation in rats. *Biochem. J.*, 1940, *34*: 97-108.

81. Duckworth J., Warnock G. — The magnesium requirements of man in relation to calcium requirements, with observations on the adequacy of diets in common use. *Nutr. Abstr. and Rev.*, 1942, *12*: 167-183.

82. Dull T. — Effect of dietary acidin on Ca, Mg, and P metabolism. *Clin. Res.*, 1963, *11*: 404-405.

83. Dunn M., Walser M. — Magnesium depletion in normal man. *Metabolism*, 1966, *15*: 884-895.

84. Durlach J. — Le magnésium métabolisme, besoins. Incidences diététiques et thérapeutiques. *Nouvelles Archives Hospitalières*, 1970, *42*: 31-41.

84 A. Durlach J. — Le magnésium en pathologie humaine. Problèmes pratiques et incidences diététiques. *Gazette Méd. de France*, 1967, *74*: 3303-3320.

84 B. Durlach J. — Les relations entre magnésium et glucides. *Le Diabète*, 1971, *19*: (in press).

85. Durlach J., Dirry C., Lebrun R., Turbrat F., Baratier L. — Étude statistique du magnésium érythrocytaire dans la spasmophilie constitutionnelle idiopathique. *Ann. d'Endocrinologie*, 1962, *23*: 83-88.

86. Durlach J. — Spasmophilie and magnesium deficit. Masson & Co. (ed.). Paris, 1969.

87. Dutcheshead J., Mair R. — Biochemical Studies on experimental cardiopathy: Electrolytes in rat tissues. *Brit. J. Exper. Path.*, 1959, *40*: 250-254.

88. Ebel J., Chaff C. — Effect of dietary magnesium on strontium calcium discrimination and incorporation into bone of rats. *J. Nutr.*, 1968, *96*: 403-408.

89. Edwards N., Russell R., Hodgkinson A. — The effect of oral phosphate in patients with recurrent renal calculus. *Brit. J. Urol.*, 1965, *37*: 390-398.

90. Eaton H., Silva W., Walthouse C., Hoff R., Hechanu W., Tucker S. — Hypocalcemia due to hypomagnesemia and reversible parathyroid hormone unresponsiveness. *J. Clin. Endocrinol. and Metab.*, 1969, *29*: 842-848.

91. Eankobien D., Rasaie D., Dimich A., Wallach S. — The significance of hypomagnesemia in alcoholic patients. *Ann. J. Med.*, 1964, *37*: 802-812.

92. Farquhar J., Smith H. — Clinical and biochemical changes during exchange transfusion. *Arch. Dis. Childh.*, 1958, *33*: 142-149.

93. Fawcett H., Wynn V. — The determination of magnesium in biological materials by flame photometry. *J. Clin. Path.*, 1961, *14*: 437-439.

94. Fetherston W., Morris E., Phillips P. — Influence of lactose and dried skim milk upon the magnesium deficiency syndrome in the dog. I. Growth and biochemical data. *J. Nutr.*, 1963, *79*: 431-436.

95. Fetherston W., Roeder J., Parker H. — Metabolic interrelations of magnesium and protein in the chick. *Fed. Proc.*, 1964, *23*: 290.

96. Fitzgerald M., Forman P. — An experimental study of magnesium deficiency in man. *Clin. Sci.*, 1956, *15*: 635.

97. Forbes R. — Effects of dietary CA:MG:P ratios on mineral utilization in the rat. *Fed. Proc.*, 1961, *20*: 293.

98. Forbes R. — Magnesium deficiency and the parathyroid. *Nutr. Rev.*, 1961, *8*: 167-168.

99. Forbes R. — Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium and phosphorus. *J. Nutr.*, 1963, *86*: 321.

100. Forbes R. — Mineral utilization in the rat. II. Restoration of normal tissue levels of magnesium and calcium following magnesium deficiency. *J. Nutr.*, 1964, *83*: 44-46.

101. Forbes R. — Mineral utilization in the rat. III. Effects of calcium, phosphorus, lactose, and source of protein in zinc-deficient and in zinc-adequate diet. *J. Nutr.*, 1964, *84*: 225-233.

102. Forman P., Morgan D. — Chronic magnesium deficiency. *Proc. Nutr. Soc.*, 1962, *21*: 34-41.

103. Freeman R., Pearson E. — Hypomagnesemia of unknown etiology. *Ann. J. Med.*, 1966, *41*: 645-656.

104. Freeman M., Hatcher G., Watson L. — Primary hypomagnesemia with secondary hypocalcemia in an infant. *Lancet*, 1967, *I*: 703-705.

105. Gardner L., McLachlan E., Pick W., Terry M., Butler A. — Etiologic factors in tetany of newly born infants. *Pediat.*, 1950, *5*: 228-240.

106. Gauner R. — Colorimetric determination of magnesium in plasma or serum by means of titan yellow. *Biochem. J.*, 1946, *40*: 828-831.

107. Gershoff S., Andrus S. — Dietary magnesium, calcium, and vitamin B6 and experimental urolithiasis in rats: calcium oxalate calculi, urate nephrocalcinosis. *J. Nutr.*, 1961, *73*: 308-316.

108. Gibeily E., Massey A., Bonsnes R. — Determination of calcium and magnesium in serum, urine, diet, and stool by atomic absorption spectrophotometry. *Clin. Chem.*, 1967, *13*: 204-214.

109. Gikn H. — Treatment of potassium and magnesium abnormalities. *Modern Treatment*, 1968, *5*: 663-675.

110. Ginsburg S., Smith J., Gassoumis E., Bowman J., Arkawa H. — Magnesium metabolism of human and rabbit erythrocytes. *Blood*, 1962, *20*: 722-729.

111. Gitelman H., Hoff C., Letwak L. — An automated spectrophotometric method for magnesium analysis. *Anal. Biochem.*, 1966, *14*: 106-120.

112. Gitelman H., Kukolj S., Welt L. — Inhibition of parathyroid gland activity by hypermagnesemia. *Am. J. Physiol.*, 1968, *215*: 483-485.

113. Gitelman H., Kukolj S., Welt L. — The partition of plasma calcium in magnesium depletion. *Clin. Res.*, 1965, *13*: 79.

114. Gitelman H., Kukolj S., Welt L. — The influence of the parathyroid glands on the hypercalcemia of experimental magnesium depletion in the rat. *J. Clin. Invest.*, 1968, *47*: 118-126.

115. Gitelman H., Welt L. — Magnesium deficiency. *Ann. Rev. Med.*, 1960, *20*: 233-242.

116. Goldman L., Pincus J., Schwarzacher E., Sayo M. — The incidence of hypocalcemia on the first day of life in mature and premature infants. *Am. J. Dis. Children*, 1955, *90*: 573-574.

117. Goldman L., Pincus J., Schwarzacher E. — Interrelationship of calcium and magnesium in the mature neonate. *Am. J. Dis. Children*, 1964, *107*: 119-124.

118. Gross M. — The chemical composition of the human fetus. *J. Biol. Chem.*, 1933, *102*: 7-17.

119. Gruebsman A., Schwartz M., Bier H., Roberts K., Rasoul H. — Physiologic distribution and excretion of Mg^{28} in dogs and man. Endocrines and metabolism. *Clin. Res. Proc.*, 1956, *4*: 14.

120. Gourley A., van Fossan D., Barry E. — Magnesium deficiency in celiac disease. *Pediat.*, 1962, *29*: 948-952.

121. Graham J., Calsar J., Burgess A. — Gastrointestinal absorption and excretion of Mg^{28} in man. *Metabolism*, 1960, *9*: 646-650.

122. Greenberg D. — Mineral metabolism calcium, magnesium and phosphorus. *Ann. Rev. Biochem.*, 1939, *8*: 269-304.

5219

123. GREENBERG D., ANDERSON C., TUFTS E. — Pathological changes in the tissues of rats reared on diets low in magnesium. *J. Biol. Chem.*, 1936, *114*: 43.

124. GREENBERG D., TUFTS E. — The nature of magnesium tetany. *Am. J. Physiol.*, 1938, *121*: 416-423.

125. GREENWALD J., DEBIN A., CARSON L. — Hypomagnesemic tetany due to excessive lactation. *Am. J. Med.*, 1963, *35*: 854-860.

126. HALL D. — Serum magnesium in pregnancy. *Obstet. & Gynec.*, 1957, *9*: 158-162.

127. HANNA S. — Influence of large doses of vitamin D on magnesium metabolism in rats. *Metabolism*, 1951, *10*: 735-743.

128. HANNA S. — Plasma magnesium in health and disease. *J. Clin. Path.*, 1961, *14*: 410-414.

129. HANNA S., ALGOOD N., LAZARUS B., MILLAN B. — Changes in gut and urinary citrates following a low calcium and magnesium diet and administration of vitamin D. *J. Lab. & Clin. Med.*, 1963, *61*: 220-229.

130. HANNA S., HARRISON M., MACINTYRE I., FRASER R. — The syndrome of magnesium deficiency in man. *Lancet*, 1960, *2*: 172-175.

131. HANNA S., MACINTYRE I. — The influence of aldosterone on magnesium metabolism. *Lancet*, 1960, *2*: 348-350.

132. HANNA S., NORTH K., MACINTYRE I., FRASER R. — Magnesium metabolism in parathyroid disease. *Brit. Med. J.*, 1961, *2*: 1253-1256.

133. HARRISON H., HARRISON H. — The interaction of vitamin D and parathyroid hormone on calcium phosphorus and magnesium homeostasis in the rat. *Metabolism*, 1964, *13*: 952-958.

134. HASS G. — Experimental inquiry into pathogenesis of human atheroarteriosclerosis. *Geriatrics*, 1961, *16*: 581-597.

135. HATHAWAY M. — Magnesium in human nutrition. Home Economic Research Report No. 19. Agricultural Research Service. United States Dept. of Agriculture, Washington, D.C., 1961.

135. HAURY V., CASTROW A. — Variations of serum magnesium in 52 normal and 340 pathologic patients. *J. Lab. Clin. Med.*, 1942, *27*: 616-622.

137. HEAGY F., BURTON A. — Effect of intravenous injection of magnesium chloride on the body temperature of the unanesthetized dog, with some observations on magnesium levels and body temperature in man. *Am. J. Physiol.*, 1948, *152*: 407-416.

138. HEATON F. — Determination of magnesium by the titan yellow and ammonium phosphate methods. *J. Clin. Path.*, 1960, *13*: 355-360.

139. HEATON F., CLARK C., GRIFFITHS J. — Magnesium deficiency complicating intestinal surgery. *Brit. J. Surg.*, 1967, *54*: 41-45.

140. HEATON F., FISHMAN P. — Magnesium deficiency and hypocalcemia in intestinal malabsorption. *Lancet*, 1965, *2*: 50-52.

141. HEATON F., HIRSCHSON A., ROSE G. — Observations on the relation between calcium and Mg metabolism in man. *Clin. Sci.*, 1964, *27*: 31-40.

142. HEATON F., PARSONS L. — Magnesium metabolism in patients with parathyroid disorders. *Clin. Sci.*, 1963, *25*: 475-485.

143. HEGGAVIT H., HERVAS L., MISHRA R. — Cardiac necrosis and calcification in experimental magnesium deficiency. *Am. J. Path.*, 1964, *45*: 757-782.

144. HEINTZE H., HARPER A., ELVEHJEM C. — Protein and amino acid requirements on the guinea pig. *J. Nutr.*, 1966, *88*: 269-280.

145. HEITERSTEIN A., NALAMURU M., HRISTIU D., VITALE J. — Studies on the interrelationships between dietary magnesium, quality and quantity of fat, hypercholesterolemia and lipidosis. *J. Nutr.*, 1960, *71*: 339-345.

146. HENDRICKS D., MILLER E., ULLREY D., HOEFER J., LUECKE R. — Effect of source and level of protein on mineral utilization by the baby pig. *J. Nutr.*, 1969, *100*: 235-240.

147. HERBRICH J. — Abnormal skeletal mineral metabolism in sarcoidosis. *Ann. Int. Med.*, 1966, *64*: 797-805.

148. HERZER J., DE WITT M., RIEDSTRA G. — Significance of magnesium and zinc metabolism in the surgical patient. I. Magnesium. *Arch. Surg.*, 1957, *95*: 974-990.

149. HIRD R. — Grass tetany in sheep. *Australian Veterinary J.*, 1966, *42*: 160-164.

150. HIRD R. — Supplementary magnesium for the prevention of hypocalcaemia, hypomagnesemia and grass tetany in sheep. *Australian Veterinary J.*, 1966, *42*: 369-373.

151. HISS R., MACINTYRE I., ALLCOCK N., PEARSE A. — Histochemical changes in rat kidney in magnesium deprivation. *Brit. J. Exper. Path.*, 1959, *40*: 80-86.

152. HIRSCHFELDER A. — Clinical manifestations of high and low plasma magnesium. *J.A.M.A.*, 1934, *1002*: 1138-1141.

153. HOLESHA W. — Recent observations on mineral interrelationships. *Fed. Proc.*, 1964, *23*: 1068-1076.

154. HOLMSTROM K. — Eine stoffwechseluntersuchung während der schwangerschaft. *Skandinavisches Archiv Fur Physiologie*, 1916, *23*: 326-420.

155. HOOFT C., VERMASSEN A., EECKELS R., VANHAVE R. — Familial incidence of hypercalcemia. Extreme hypersensitivity to vitamin D in an infant whose father suffered from sarcoidosis. *Helv. Paediat. Acta*, 1961, *3*: 199-210.

156. HORTON R., BIGLIERI E. — Effect of aldosterone on the metabolism of magnesium. *J. Clin. Endocrin. & Metab.*, 1962, *22*: 1187-1192.

157. HOSSAIN M. — Vitamin D intoxication during treatment of hypoparathyroidism. *Lancet*, 1970, *1*: 1149-1151.

158. HOUSE W., HOGAN A. — Injury to guinea pigs that follows a high intake of phosphates. *J. Nutr.*, 1955, *55*: 507-517.

159. HUGHES A., TINKER R. — Platelets, magnesium, and myocardial infarction. *Lancet*, 1965, *1*: 1044-1046.

160. HUMMEL F., HUNSCHER H., BUTTS M., BOSSER P., MACY I. — A consideration of the nutritive state in the metabolism of women during pregnancy. *J. Nutr.*, 1937, *13*: 263-278.

161. HUMMEL F., SHERSHEROW H., HUNSCHER H., MACY I. — Metabolism of women during the reproductive cycle. *J. Nutr.*, 1936, *11*: 235-255.

162. HUNT S., SCHOFIELD F. — Magnesium balance and protein intake level in adult human female. *Am. J. Clin. Nutr.*, 1959, *22*: 367-373.

162 A. HUTHER W. — Magnesiumuntersuchungen bei gesunden und kranken kindern. *Mnchr. Kinderheilk.*, 1964, *112*: 16-21.

162 B. IRWIN M. A., FERRY R. M. — Frequency and size of meals and serum lipids, nitrogen and mineral retention, fat digestibility and urinary thiamine and riboflavin in young women. *Amer. J. Clin. Nutr.*, 1967, *20*: 816-824.

163. JACKSON W., DONCASTER C. — A consideration of the hypercalciuria in sarcoidosis, idiopathic hypercalciuria, and that induced by vitamin D. A new suggestion regarding calcium metabolism. *J. Clin. Endocrinol. & Metab.*, 1959, *19*: 658-680.

164. JENNINGS R. — Symposium on the prehospital phase of acute myocardial infarction. Part II. Early phase of myocardial ischemic injury and infarction. *Am. J. Card.*, 1969, *24*: 753-765.

165. JONES J., MCCUTCHIN W. — Complexometric titration of calcium and magnesium by a semiautomated procedure. *Clin. Chem.*, 1964, *10*: 267-280.

166. JONES K., FISHMAN P. — Effects of infusions of Mg and of calcium in parathyroid insufficiency. *Clin. Sci.*, 1966, *30*: 139-150.

167. KASHIWA H. — Magnesium deficiency in intact, in adrenalectomized and in hypophysectomized rats. *Endocrinol.*, 1961, *68*: 80-91.

168. KIM Y., ANDREWS C., RUTH W. — Serum magnesium and cardiac arrhythmias with special reference to digitalis intoxication. *Am. J. Med. Sci.*, 1961, *242*: 87-92.

169. KELLER M., BOESTER M., GOLDBERG D. — Fasting catabolism and food utilization of magnesium deficient rats. *J. Nutr.*, 1941, *21*: 363-372.

170. KO K., FIELDS C., CRAIG J. — Observations on magnesium deficiency in the rat. *Lab. Invest.*, 1962, *11*: 294-305.

171. KOBAYASHI A. — Neonatal hypomagnesemia. *Lancet*, 1967, *2*: 100.

172. KOBAYASHI J. — On geographical relationship between the chemical nature of river water and death-rate from apoplexy. *Berichte Ohara Inst.*, 1957, *11*: 12-21.

173. KOBAYASHI A., ICHASHI Y. — Serum magnesium level in the salt-losing type of congenital adrenal hyperplasia. *Arch. Dis. Childh.*, 1968, *43*: 223-228.

174. Koberlin W., Mischel W. — Der magnesiumgehalt des blutserums bei der frau während der schwangerschaft und bei spätgestosen. *Zentralblatt Gynäk.*, 1958, *80*: 226-232.

175. Kozel M., Neumann L. — Renal involvement in Boeck's sarcoidosis. *Pediat.*, 1963, *28*: 410-424.

176. Kruse H., Schmidt M., McCollum E. — Studies on magnesium deficiency in animals. *J. Biol. Chem.*, 1934, *106*: 553-572.

177. Langsdorf L. — Eiweiss und mineralstoffwechseluntersuchungen bei der schwangeren frau nebst fütterversuchen mit besonderer berücksichtigung der funktion endokriner drüsen. *Zeitschrift für Geburtshilfe und Gynäkologie*, 1914, *75*: 53-98.

178. Larvor P., Girard A., Bouchard M., Parrot A., Sebastre J. — Etude de la carence expérimentale en magnésium chez le veau. I. Observations cliniques, biochimiques et anatomopathologiques. *Ann. Biol. Anim. Biochim. & Biophys.*, 1964, *4*: 343-369.

178 A. Larvor P., Delbach J. — Relations entre magnésium et nutrition. First International Symposium on Magnesium Deficit, Vittel, 1971.

178 B. Larvor P., Delbach J. — Relations entre magnésium et glandes endocrines. First International Symposium on Magnesium Deficit, Vittel, 1971.

179. Larvor P., Girard A., Bouchard M. — Etude de la carence expérimentale en magnésium chez le veau. II. Interférence entre la carence en magnésium et le métabolisme du calcium. *Ann. Biol. Anim. Biochim. Biophys.*, 1964, *4*: 371-382.

180. Lazzare R., Yu S., Black W., Walsh J., Burch G. — Magnesium and potassium metabolism in patients with idiopathic cardiomyopathy and chronic congestive heart failure. *Proc. Soc. Exp. Biol. Med.*, 1965, *120*: 110-115.

181. Lazzara R., Hyatt K., Low W., Crosnoe J., Burch G. — Tissue distribution, kinetics, and biologic half-life of Mg in the dog. *Am. J. Physiol.*, 1963, *204*: 1086-1094.

182. Lefson P., Fourman P. — Increased sensitivity to vitamin D after vitamin D poisoning. *Lancet*, 1956, *1*: 1182-1185.

183. Lehr D. — The role of certain electrolytes and hormones in disseminated myocardial necrosis. Electrolytes and Cardiovascular Diseases. Ed. E. Bajusz, Basel/New York, 1965, pp. 248-274.

184. Lehr D., Kretkowski M. — About the mechanism of myocardial necrosis induced by sodium phosphate and adrenal corticoid overdosage. *Ann. N.Y. Acad. Sci.*, 1963, *105*: 135-182.

185. Lehr D., Kretkowski M., Cotos R. — A simple method for the production of acute cardiorenal necrosis. *Arch. Int. Pharmacodyn.*, 1967, *168*: 251-271.

186. Lehr D., Kretkowski M., Ricciuti G. — Role of the parathyroid in cardiorenal injury of phosphate-mineralocorticoid poisoning. *Fed. Proc.*, 1959, *18*: 414.

187. Lerman H. — Hypercalcemia and impairment of renal function in sarcoidosis. *Nordisk Med.*, 1954, *51*: 670-671.

188. Lieberitz F., Harrison H., Harrison H. — Effects of vitamin D on magnesium metabolism in rats. *Endocrinol.*, 1967, *81*: 849-853.

189. Lieberitz F., Harrison H., Harrison H. — Intestinal transport of calcium and phosphate in experimental magnesium deficiency. *Proc. Soc. Exp. Biol. and Med.*, 1967, *125*: 19-25.

190. Lieberitz F., Harrison H., Harrison H. — Response to vitamin D of magnesium deficient rats. *Proc. Soc. Exp. Biol. and Med.*, 1967, *125*: 472-476.

191. Lim P., Jacob E., Dunn S., Kroo O. — Values for tissue magnesium as a guide in detecting magnesium deficiency. *J. Clin. Path.*, 1969, *22*: 417-421.

192. Linder G., Hanna J., Kasanen C. — The metabolism of magnesium and other inorganic cations and of nitrogen in acute kwashiorkor. *Pediat.*, 1963, *31*: 552-568.

193. Landman R., Alder S., Yunoist M., Beard E. — Influence of various nutrients on urinary divalent cation excretion. *J. Lab. Clin. Med.*, 1967, *70*: 236-245.

194. Lowe K., Hendersson J., Park W., McGreal D. — The idiopathic hypercalcemic syndromes of infancy. *Lancet*, 1954, *2*: 101-110.

195. Lowenthal E., Schulman M., Gittenberg D. — Basic histologic lesions of magnesium deficiency in the rat. *Arch. Path.*, 1950, *49*: 427-433.

196. Lynch H., Limas H., Henk M., Ericsson R., Gierstad R. — Vitamin D intoxicated patient with hypoparathyroidism. Hypercalcemia, acute cerebellar ataxia and EEG changes: magnesium sulfate therapy. *Arch. Intern. Med.*, 1961, *114*: 375-380.

197. McCance R., Widdowson E. — Mineral metabolism of the fetus and newborn. *Brit. Med. Bull.*, 1961, *17*: 132-136.

198. McCance R., Widdowson E., Lehmann H. — The effect of protein intake on the absorption of calcium and magnesium. *Biochem. J.*, 1942, *36*: 686-691.

199. Mawdsley W., Stapleton T. — Idiopathic hypercalcemia of infancy. Studies of the mineral balance. *Acta Pediat.*, 1955, *44*: 509-518.

200. McIntyre I. — Magnesium metabolism. Advances in Internal Medicine, ed. W. Dock and I. Snapper, Year Book Medical Publishers, Inc., 1961.

201. McIntyre I., Boss S., Hendrikes V. — Parathyroid hormone and magnesium homeostasis. *Nature*, 1963, *196*: 1068-1069.

202. McIntyre I., Davidson D. — The production of secondary potassium depletion, sodium retention, nephrocalcinosis and hypercalcemia by magnesium deficiency. *Biochem. J.*, 1956, *62*: 400-402.

203. McIntyre I., Hanna S., Boyle C., Read A. — Intracellular Mg deficiency in man. *Clin. Sci.*, 1961, *20*: 291-305.

204. Mortenson G. — Stable calcium isotopes as tracers in studies of mineral metabolism. *Acta Orthopaed. Scand.*, Suppl. 18, Munksgaard Copenhagen, 1960.

205. Magnusson B., Hallgrensson T. — Automatic titration of calcium or magnesium in blood serum. *Clin. Chem.*, 1959, *5*: 50-56.

206. Macy I. — Nutrition and Chemical growth in childhood. Published Charles C. Thomas, Springfield, Illinois, Vol. 2, 1946.

207. Martin H., Wilson M. — Effect of magnesium deficiency on serum and carcass electrolyte levels in the rat. *Metabolism*, 1960, *9*: 484-491.

208. Maynard L., Bruce D., Fisk G., Shelin D. — Dietary mineral interrelations as a cause of soft tissue calcification in guinea pigs. *J. Nutr.*, 1958, *64*: 85-97.

209. Mazurkiewicz V. — Generalized aminoaciduria in the magnesium deficient rat. *Proc. Soc. Exp. Biol. Med.*, 1960, *125*: 405-408.

210. Meinzer R., Steinbrook H. — Vitamin D and magnesium absorption. *J. Nutr.*, 1955, *56*: 285-294.

211. Menaker W. — Influence of protein intake on magnesium requirement during protein synthesis. *Proc. Soc. Exp. Biol. Med.*, 1954, *85*: 149-151.

212. Menaker W., Kellner I. — Effect of deficiency of magnesium and other minerals on protein synthesis. *Proc. Soc. Exp. Biol. Med.*, 1952, *81*: 317-318.

213. Metcoff J., Frank S., Antonowicz I., Gornillo C., Lopez E. — Relations of intracellular ions to metabolic sequences in muscle in kwashiorkor. *Pediat.*, 1960, *26*: 960-972.

214. Miller E., Ullrey D., Zutaut C., Baltzer B., Schmidt D., Hoefer J., Luecke R. — Magnesium requirement of the baby pig. *J. Nutr.*, 1965, *85*: 13-20.

215. Miller E., Ullrey D., Zutaut C., Hoefer J., Luecke R. — Mineral balance studies with the baby pig: Effects of dietary vitamin D₂ level upon calcium, phosphorus and magnesium balance. *J. Nutr.*, 1965, *85*: 255-259.

216. Miller E., Ullrey D., Zutaut C., Hoefer J., Luecke R. — Comparisons of Caseins and Soy proteins upon mineral balance and vitamin D₂ requirement of the baby pig. *J. Nutr.*, 1965, *85*: 347-354.

217. Miller E., Ullrey D., Zutaut C., Baltzer B., Schmidt D., Vincent B., Hoefer J., Luecke R. — Vitamin D requirement of the baby pig. *J. Nutr.*, 1964, *83*: 140-198.

218. Miller M. — Tetany due to deficiency in magnesium. *Am. J. Dis. Children*, 1944, *67*: 117-119.

219. Milne M., Mitchell R., Aird I. — Primary aldosteronism. *Quart. J. Med.*, 1957, *26*: 317-334.

220. Mishra R. — Studies on experimental Mg deficiency in the albino rat. I. Functional and morphologic changes associated with low intake of Mg. *Rev. Canadienne de Biologie*, 1960, *19*: 122-135.

221. Mishra R. — Studies on experimental magnesium deficiency in the albino rat. 3. Influence of certain salts and dietary constituents on the electroseizure threshold of rats on Mg-deficient diet. *Rev. Canadienne de Biol.*, 1960, *19*: 143-153.

5219

222. MISHRA R. — Studies on experimental Mg deficiency in the albino rat. 5. Influence of Mg deficiency regime on steroid-PO_4-cardiopathy. *Rev. Canadienne de Biologie*, 1960, *19*: 156-162.

223. MISHRA R. — Studies on experimental magnesium deficiency in the albino rat. 6. Effect of phosphate-iontophoresis into the hearts of animals on Mg deficient diet. *Rev. Canadienne de Biol.*, 1960, *19*: 163-167.

224. MONTGOMERY R. — The estimation of magnesium in small biological samples by flame spectrophotometry. *J. Clin. Path.*, 1961, *14*: 460-462.

225. MONTGOMERY R. — Magnesium balance studies in nutrosmic kwashiorkor. *J. Pediat.*, 1961, *59*: 119-123.

226. MONTGOMERY R. — Magnesium metabolism in infantile protein malnutrition. *Lancet*, 1960, *2*: 74-76.

227. MOORE L., HAMMOND E., SCOTT L. — Cardiovascular and other lesions in cattle fed diets low in magnesium. *Arch. Path.*, 1956, *1*: 828-838.

228. MORRIS E., O'DELL B. — Effect of magnesium deficiency in guinea pigs on kidney function and plasma extracellular ions. *Exp. Biol. & Med.*, 1961, *152*: 105-110.

229. MORRIS J., CRAWFORD M., HEADY J. — Hardness of local water-supplies and mortality from cardiovascular disease. *Lancet*, 1962, *2*: 860-861.

230. MORRIS E., O'DELL B. — Magnesium deficiency in the guinea pig: mineral composition of tissues and distribution of acid-soluble phosphorus. *J. Nutr.*, 1961, *75*: 77-83.

231. MORRIS E., O'DELL B. — Relationship of excess calcium and phosphorus to magnesium requirement and toxicity in guinea pigs. *J. Nutr.*, 1963, *81*: 175-181.

232. MORRIS J., CRAWFORD M., HEADY J. — Hardness of local water-supplies and mortality from cardiovascular disease in the county boroughs of England and Wales. *Lancet*, 1961, *1*: 860-862.

233. MORRIS M., PLATHIRISIOR W., PHILLIPS P., MCNULT S. — Influence of lactose and dried skim milk upon the magnesium deficiency syndrome in the dog. II. Pathological changes. *J. Nutr.*, 1963, *79*: 437-441.

234. MOSHOWITZ F., MCKENNA T., KUSE L., FRANKEL R., SWAN M. — Parathyroid-like effect of magnesium replenishment in steatorrhea. *New Eng. J. Med.*, 1966, *2*: 61-68.

235. NEAL J., NEAL M. — Effect of hard water and Mg SO_4 on rabbit atherosclerosis. *Arch. Path.*, 1962, *73*: 400-403.

236. NELSON N. — Magnesium metabolism in clinical medicine. *G.P.*, 1966, *34*: 97-102.

237. NEWTON R. — Serum electrolytes in pregnancy, parturition, and puerperium. *Obstet. & Gynec.*, 1957, *10*: 51-55.

238. NICKERSON M., KARR G., DRESEL P. — Pathogenesis of « electrolyte-steroid cardiopathy ». *Fed. Proc.*, 1959, *18*: 427.

239. NIGHAN D., EDWARDS H. — Effect of calcium and phosphorus on magnesium metabolism in chicks. *Fed. Proc.*, 1961, *20*: 294.

240. NIGHAN D., EDWARDS H. — Influence of dietary Ca and P levels on the Mg requirement of the chick. *J. Nutr.*, 1963, *80*: 184-188.

241. O'DELL B. — Magnesium requirement of non-ruminant animals. Magnesium and Agriculture. A Symposium, 1959 (Sept.), 116-123.

242. O'DELL B. — Magnesium requirement and its relation to other dietary constituents. *Fed. Proc.*, 1960, *19*: 648-654.

243. O'DELL B., MORRIS E., REGAN W. — Magnesium requirement of guinea pigs and rats. Effects of calcium and phosphorus and symptoms of Mg deficiency. *J. Nutr.*, 1960, *70*: 103-110.

244. OLIVER J., MACDOWELL M., WHANG R., WEST L. — The renal lesions of electrolyte imbalance. *J. Exp. Med.*, 1966, *124*: 263.

245. ORR J., HUSE B., FINLAY J. — Massive small bowel resection with malabsorption and negative magnesium balance. *Gastroenterology*, 1964, *47*: 415-420.

246. ORNAGE M., RIEGE H. — Microestimation of magnesium in body fluids. *J. Biol. Chem.*, 1951, *189*: 379-386.

247. PARLIER R., HERIO D., LEBLANC R. — Le métabolisme du magnésium et ses rapports avec celui du calcium. I. A propos d'une étude des bilans magnésiens chez l'homme normal, dans les ostéoarthies et les néphropathies. *Rev. Franç. Endocr. Clin.*, 1963, *4*: 93-135.

248. PARSONS R., BUTLER T., SELLARS E. — Hardness of local water-supplies and mortality from cardiovascular disease. *Lancet*, 1961, *2*: 215.

249. PAUNICK L., RABBE I., KOHN S. — Primary hypomagnesemia with secondary hypocalcemia. *J. Pediat.*, 1963, *63*: 945.

250. PAUNICK L., RABBE I., KOHN S. — Primary hypomagnesemia with secondary hypocalcemia in an infant. *Pediat.*, 1968, *41*: 865-882.

251. PETERSEN V. — Metabolic studies in clinical magnesium deficiency. *Acta Med. Scand.*, 1965, *113*: 285-298.

252. PETROSKINY A. — Über die Bilanzen von Natrium, Kali, Magnesium, Phosphor und Eisen bei Kindern von 1-8 Jahren. *Zeitschrift f. Kinderheilk.*, 1964, *56*: 219-226.

253. PIKUS J., GITELMAN L., SOBEL A., SCHMERZLER E. — Effects of vitamin *D* on the serum calcium and phosphorus levels in infants during the first week of life. *Pediat.*, 1954, *13*: 158-165.

254. PRASAD A., FLINK E., MCCOLLISTER R. — Ultrafiltration studies on serum magnesium in normal and disease states. *J. Lab. & Clin. Med.*, 1961, *58*: 531-541.

255. PRASAD A., FLINK E., ZINKHAN H., MCCOLLISTER R. — Magnesium-protein relationship and status of ultrafiltrable magnesium in normal and abnormal human sera. *Clin. Res.*, 1958, *6*: 206-204.

256. PRIDEKEUS P., WEIMEYER A., THERON J. — Magnesium balance studies in South African Bantu children with kwashiorkor. *Am. J. Clin. Nutr.*, 1963, *13*: 331-339.

257. PRIDEKEUS P. — Role of potassium in the pathogenesis of the « electrolyte-steroid-cardiopathy with necrosis ». *Clin. Res.*, 1962, *10*: 182-183.

258. PRIDEKEUS P. — Sodium and potassium in the pathogenesis of the « electrolyte-steroid-cardiopathy with necrosis ». *Canad. J. Biochem. & Physiol.*, 1963, *41*: 965-968.

259. RICHARDSON J., HEFFINES W., WEST L. — The effect of concomitant hypercalcemia and potassium depletion on the rat kidney. *Metabolism*, 1963, *12*: 500-509.

260. RICHARDSON J., WEST L. — The hypomagnesemia of vitamin D administration. *Proc. Soc. Exp. Biol. Med.*, 1965, *118*: 512-514.

261. RIGO J. — Veränderungen des Kollagens der aorta beim altern und durch calciferol und magnesiumbehandlung. *Gerontologia*, 1965, *11*: 25-33.

262. RIGO J., LI B., ZELLES T., SZILENYI L., SOS J. — The effect of a magnesium rich diet on experimental vascular changes. *Acta Physiol. Acad. Sci. Hung.*, Suppl., 1965, p. 40.

263. RIGO J., SIMON G., HEGYVARI C., SOS J. — Effect of magnesium on dietary infarctoid changes in the heart. *Acta Med. Acad. Sci. Hung.*, 1964, *19*: 231-236.

264. RIGO J., SZILENYI L. — Die wirkung von magnesium auf die neurogene und alimentäre hyperkinese bei ratten. *Acta Physiol. Acad. Sci. Hung.*, 1963, *24*: 253-259.

265. ROGERS T. — The exchange of radioactive magnesium in erythrocytes of several species. *J. Cell. and Comparative Physiol.*, *57*: 119-121.

266. ROGERS T., MARAN P. — Exchange of magnesium in soft tissues. *Fed. Proc.*, 1959, *18*: 130.

267. ROGERS T., MARAN P. — Exchange of radioactive magnesium in the rat. *Proc. Soc. Exp. Biol. & Med.*, 1959, *109*: 235-239.

268. ROOK J., STORRY J. — Magnesium in the nutrition of farm animals. *Nutr. Abstracts and Reviews*, 1962, *32*: 1055-1077.

268 A. ROUSSEL F., DURRAN J. — Exploration pratique du métabolisme magnésique en clinique humaine (in press).

269. ROWLEY P., MORRIS J. — Reticulocyte maturation: Magnesium and the decline in protein synthesis. —*Clin. Res.*, 1966, *14*: 325.

270. SAYLE J., POBROWSKI C., DE GRUYON F., PEAN G., MEURGEY B., FOURNIER J. — Tétanie hypocalcémique récidivante par hypomagnésémie congénitale. *Arch. Françaises de Pédiatrie*, 1966, *23*: 749-768.

271. SAUBERLICH H., BAUMANN C. — Excretion of amino acids by mice fed certain deficient diets. *Arch. Biochem.*, 1949, *20*: 305-314.

272. SAVAGE D., MCADAM W. — Convulsions due to hypomagnesemia in an infant recovering from diarrhea. *Lancet*, 1967, *2*: 234-236.

273. SCHWEIGER D. — Fluorometric estimation of magnesium with 8-hydroxy-5-quinolinesulfonate. *J. Lab. & Clin. Med.*, 1961, *58*: 495-498.

274. SCHACHTER D., ROSEN S. — Active transport of Ca^{45} by the small intestine and its dependence on vitamin D. *Am. J. Physiol.*, 1959, *196*: 357-362.

275. SCHMIDT C., GREENBERG D. — Occurrence, transport and regulation of calcium, magnesium and phosphorus in the animal organism. *Physiol. Rev.*, 1935, *15*: 297-434.

276. SCHROEDER F., MORRILL E. — Calcium, phosphorus and magnesium. *Fed. Proc.*, 1950, *19*: 1011-1016.

277. SCHROEDER H. — Municipal drinking water and cardiovascular death rates. *J.A.M.A.*, 1966, *195*: 125-129.

278. SCHROEDER H. — Relations between hardness of water and death rates from certain chronic and degenerative diseases in the United States. *J. Chron. Dis.*, 1960, *12*: 586-591.

279. SCHROEDER H., DERAS J. — Hardness of local water supplies and mortality from cardiovascular disease. *Lancet*, 1961, *1*: 1171.

280. SCHROEDER H., NASON A., TIPTON I. — Essential metals in man-magnesium. *J. Chron. Dis.*, 1969, *21*: 815-841.

281. SCHWARTZ R., WANG F., WOMACK N. — Effect of varying dietary protein-magnesium rations on nitrogen utilization and magnesium retention in growing rats. *J. Nutr.*, 1968, *97*: 165-193.

282. SCHWARTZ R., WOMACK N., BLAKELY J., WANG F., KEMMERER E. — Effect of magnesium deficiency in growing rats on synthesis of liver proteins and serum albumin. *J. Nutr.* 1970, *100*: 123-128.

283. SELIG M. — Are American Children still getting an excess of vitamin D? *Clin. Pediat.*, 1970, *9*: 380-382.

284. SELIG M. — Electrographic patterns of magnesium depletion appearing in alcoholic heart disease. *Ann. N.Y. Acad. Sci.*, 1969, *162*: 908-917.

285. SELIG M. — Hypersensitivity to vitamin D. *Med. Counterpoint.*, 1970, *2*: 28-46.

285 A. SELIG M. VITALE J. J. — Lipids and magnesium deficit. First International Symposium on Magnesium Deficit. Vittel, 1971.

286. SELIG M. — Myocardial loss of functional magnesium. I. Effect on mitochondrial integrity and potassium retention and II. In Cardiomyopathies of Diverse Etiology. *J. Mol. Cell. Cardiol.* (in press).

287. SELIG M. — The requirement of magnesium by the normal adult. *Am. J. Clin. Nutr.*, 1961, *14*: 342-390.

288. SELIG M. — Vitamin D and cardiovascular, renal, and brain damage in infancy and childhood. *Ann. N.Y. Acad. Sci.*, 1969, *147*: 537-582.

289. SELYE H. — The chemical prevention of cardiac necroses. The Ronald Press Co., New York, 1958.

290. SELYE H. — On the site of interaction between NaH_2PO_4 and $MgCl_2$ or KCl in the prevention of phosphate-steroid-cardiopathy. *Acta Endocrinologica*, 1958, *28*: 273-278.

291. SELYE H. — The Pluricausal cardiomyopathies. Publ. Charles C. Thomas, Springfield, Illinois, 1961.

292. SELYE H. — Prevention of experimental hyperparathyroidism by magnesium and potassium salts. *Endocrin.*, 1958, *63*: 216-225.

293. SELYE H. — Prophylactic treatment of an experimental arteriosclerosis with magnesium and potassium salts. *Am. Heart J.*, 1958, *55*: 803-809.

294. SELYE H., BARSZ E. — Provocation and prevention of potassium deficiency by various ions. *Proc. Soc. Exp. Biol. Med.*, 1958, *98*: 580-583.

295. SELYE H., BOIS P. — Effect of corticoids on the resistance of the kidney to an excess of phosphates. *Am. J. Physiol.*, 1956, *187*: 41-44.

296. SELYE H., MISHRA R. — Prevention of the «phosphate-steroid-cardiopathy» by various electrolytes. *Am. Heart J.*, 1958, *55*: 163-173.

297. SELYE H., RENAUD S., NAHOUM V. — Ability of various steroids to produce «infarctoid cardiopathy» in rats conditioned by NaH_2PO_4. *J. Pharm. & Exper. Therap.*, 1958, *122*: 504-511.

298. SETA K., HEITSELLIN E., VITALE J. — Myocardium and plasma electrolytes in dietary magnesium and potassium deficiency in the rat. *J. Nutr.*, 1965, *87*: 179-188.

299. SHERWOOD L., HERRMAN L., BASSETT C. — Parathyroid hormone secretion *in vitro*: regulation by calcium and magnesium ions. *Nature*, 1970, *225*: 1056.

300. SHILS M. — Experimental human magnesium depletion. *Medicine*, 1969, *48*: 61-65.

301. SHILS M. — Experimental human magnesium depletion. I. Clinical observations and blood chemistry alterations. *Am. J. Clin. Nutr.*, 1964, *15*: 133-143.

302. SHILS M. — Experimental production of magnesium deficiency in man. *Ann. N.Y. Acad. Sci.*, 1969, *162*: 847.

303. SILVER L., ROBERTSON J., DAIN L. — Magnesium turnover in the human studied with Mg^{28}. *J. Clin. Invest.*, 1960, *39*: 420-425.

304. SILVERMAN S., GARDNER L. — Ultrafiltration studies on serum magnesium. *New Engl. J. Med.*, 1954, *250*: 938-941.

305. SIMON K. — Magnesium-Physiology, Pharmacology, Clinic. Publ. Wissenschaftliche Verlagsgesellschaft MBH, Stuttgart, 1964.

306. SIMONSEN D., WESTOVER L., WURMAN M. — The determination of serum magnesium by the molybdivanadate method for phosphate. *J. Biol. Chem.*, 1947, *169*: 39-47.

307. SKIBBE D., STRAUSS J., KESSAKEN R., HARRIS K. — Congenital primary hypomagnesemia, an inborn error of metabolism. *Acta Pediat. Scand.*, Suppl. 1967, *177*: 26-27.

308. SLATER J. — Retentions of nitrogen and minerals by babies one week old. *Brit. J. Nutr.*, 1961, *15*: 83-93.

309. SMITH A. — A colorimetric method for the estimation of serum magnesium. *Biochem. J.*, 1955, *60*: 522-527.

310. SMITH P. — Calcium and magnesium metabolism in calves. 2. Effect of dietary vitamin D and ultraviolet irradiation on milk-fed calves. *Biochem. J.*, 1958, *70*: 201-205.

311. SMITH W., BAXTER D., LINDNER A., GINN H. — Effect of magnesium depletion on renal function in the rat. *J. Lab. Clin. Med.*, 1962, *59*: 211-219.

312. SMITH W., HAMMARSTEN J. — Serum magnesium in clinical disorders. *South M. J.*, 1958, *51*: 1116-1119.

313. SOLOMON L., COHN C., GROSSMAN E., JACOBS M., SOMERSON H. — Magnesium partition studies in Graves' disease and in clinical experimental hyperthyroidism. *J. Clin. Invest.*, 1941, *20*: 429-432.

314. SONIS D., MCGUCKIN F., WALSH K. — Determination of intracellular magnesium by an automatic titration procedure. *Clin. Chem.*, 1960, *6*: 564-571.

315. STEINBERG M., BUTLER L., ADAMS Y. — Effect of protein source on magnesium balance of young women. *Fed. Proc.*, 1970, *29*: 606.

316. STEWART W., HUTCHINSON F., FLEMING L. — The estimation of magnesium in serum and urine by atomic absorption spectrophotometry. *J. Lab. & Clin. Med.*, 1963, *61*: 858-872.

317. STUCZAYN F., ANTUZZO D. — Blood serum magnesium in portal cirrhosis and diabetes mellitus. *J. Lab. Clin. Med.*, 1953, *41*: 215-219.

318. SULLIVAN L., LANGFORD H., SWERTZ M., FARRELL C. — Magnesium metabolism in alcoholism. *Am. J. Clin. Nutr.*, 1963, *13*: 297-303.

319. SUTTON R. — Hypomagnesemia and magnesium deficiency. *J. Roy. Coll. Physcns. Lond.*, 1968, *2*: 356-370.

320. SWANSON W. — The composition of growth. II. The full-term infant. *Am. J. Dis. Children*, 1932, *43*: 10-18.

321. TAYLOR R., LYNCH H., WEBER W. — Seasonal influence of sunlight on the hypercalcemia of sarcoidosis. *Am. J. Med.*, 1963, *34*: 721-727.

322. TEH Y. — Serum magnesium levels. *Arch. Dis. Childh.*, 1956, *43*: 502.

323. TEH Y. — Total and ultrafiltrable serum Mg and Ca in normal children and adults in Singapore. *J. Singapore Paediatric Soc.*, 1968, *10*: 115-117.

324. TIDDY H. — Estimation of magnesium in serum by means of flame spectrophotometry. *Amer. J. Clin. Pathol.*, 1958, *30*: 129-132.

325. THORÉN L. — Magnesium deficiency in gastrointestinal fluid loss. *Acta Chirurgica Scandinavica* (Suppl. 306). 1963.

326. THORÉN L. — Magnesium deficiency. Studies in two cases of acute fulminant ulcerative colitis treated by colectomy. *Acta Chir. Scand.*, 1962, *124*: 134-143.

327. TOVERUD K., TOVERUD G. — Studies on the mineral metabolism during pregnancy and lactation and its bearing on the disposition to rickets and dental caries. *Acta Paediatrica*, 1931, *12*: 5-116.

328. TSANG R., OH W. — Serum magnesium levels in low birth weight infants. *Am. J. Dis. Child.*, 1970, *120*: 44-48.

5219

329. TUCKER V., HANNA H., KAISER C., DARROW D. — Cardiac necrosis accompanying potassium deficiency and administration of corticosteroids. *Circ. Res.*, 1963, *13* : 420-431.

330. TUFTS E., GREENBERG E. — The biochemistry of magnesium deficiency. I. Chemical changes resulting from magnesium deprivation. *J. Biol. Chem.*, 1938, *122* : 693-701.

331. TUFTS E., GREENBERG D. — The biochemistry of magnesium deficiency. II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of the dietary calcium level thereon. *J. Biol. Chem.*, 1937, *122* : 715-726.

332. UNSIGNED. — Hardness of water and cardiovascular disease. *Brit. Med. J.*, 1963, *1* : 1429.

333. UNSIGNED. — Magnesium and malnutrition. *Lancet*, 1967, *1* : 712-713.

334. UNSIGNED. — The water story. *Lancet*, 1969, *1* : 1012-1013.

335. VALLEE B. — Simultaneous determination of sodium, potassium, calcium, magnesium and strontium by a new multichanneled flamespectrometer. *Nature*, 1954, *174* : 1050-1051.

335 A. VALLEE B., WACKER W., ULMER D. — The magnesium deficiency tetany syndrome in man. *New Eng. J. Med.*, 1960, *262* : 155-161.

336. VITALE J., HELLERSTEIN E., HIGGINS D., NAKAMURA M., FARBMAN A. — Studies on the interrelationships between dietary magnesium and calcium in atherosclerosis and renal lesions. *Am. J. Clin. Nutr.*, 1959, *7* : 13-22.

337. VITALE J., HELLERSTEIN E., NAKAMURA M., LOWN B. — Effects of magnesium deficient diet upon puppies. *Circ. Res.*, 1961, *9* : 383-394.

338. VITALE J., VELEZ H., GUZMAN C., COSTICA P. — Magnesium deficiency in the cebus monkey. *Circ. Res.*, 1963, *12* : 642-650.

339. VITALE J., WHITE P., NAKAMURA M., HIGGINS D., ZAMCHECK N., HELLERSTEIN E. — Interrelationships between experimental hypercholesterolemia, magnesium requirement, and experimental atherosclerosis. *J. Exp. Med.*, 1957, *106* : 757-766.

340. WACKER W., PARISI A. — Magnesium metabolism. *New Eng. J. Med.*, 1968, *278* : (I) 658-663, (II) 712-717, (III) 772-776.

341. WACKER W., INA C., FUWA K. — Accuracy of determinations of serum magnesium by flame emission and atomic absorption spectrometry. *Nature*, 1964, *202* : 659-662.

342. WACKER W., INA C., FUWA K. — Answer to Alcock *et al. Nature*, 1965, *206* : 90.

343. WACKER W., VALLEE B. — A study of magnesium metabolism in acute renal failure employing a multichanneled flame spectrometer. *New Eng. J. Med.*, 1957, *257* : 1254-1262.

344. WACKER W., VALLEE B. — Magnesium metabolism. *New Eng. J. Med.*, 1958, *259* : 431-438, 475-482.

345. WACKER W., VALLEE B. — Pathological abnormalities of magnesium metabolism. *Med. Clin. N. Am.*, 1960, *44* : 1557-1570.

346. WALLACH D., ESSKID M. — Fluorescence techniques in the micro-determination of metals in biological materials. III. Method for complexometric determination of magnesium in small serum samples. *Analy. Biochem.*, 1964, *7* : 67-73.

347. WALLACH S., BELLAVIA J., SCHORR J., GAMBINO P. — Effect of vitamin D on tissue distribution and transport of electrolytes, Ca and Mg. *Endocrinol.*, 1965, *79* : 773-782.

348. WALLACH S., BELLAVIA J., SCHORR H., REIZENSTEIN D. — Tissue distribution of electrolytes, Ca^{47} and Mg^{28} in acute hypercalcemia. *Am. J. Physiol.*, 1964, *207* : 553-560.

349. WALLACH S., BELLAVIA J., SCHORR J., SCHAFFER A. — Tissue distribution of electrolytes, Ca, and Mg in experimental hyper- and hypoparathyroidism. *Endocrinol.*, 1966, *78* : 16-25.

350. WALLACH S., CARREL L., ROGAN F., JONES H. — Plasma and erythrocyte magnesium in health and disease. *J. Lab. & Clin. Med.*, 1962, *59* : 195-210.

351. WALLACH S., CARTER A. — Metabolic and renal effects of acute hypercalcemia in dogs. *Am. J. Physiol.*, 1961, *200* : 359-366.

352. WALLACH S., RIZEK J., DIATCH A. — Radiomagnesium kinetics in normal and uremic subjects. *J. Clin. Invest.*, 1965, *44* : 1107-1108.

353. WALSER M. — Determination of free magnesium ions in body fluids. Improved methods for free calcium ions, total calcium, and total magnesium. *Anal. Chem.*, 1960, *32* : 711-717.

354. WALSER M. — Ion association. VI. Interactions between calcium, magnesium, inorganic phosphate, citrate and protein in normal human plasma. *J. Clin. Invest.*, 1961, *40* : 723-730.

355. WALSER M. — Magnesium metabolism. *Rev. Physiol., Biochem. and Exp. Pharm.*, 1967, *59* : 185-296.

356. WALSER M. — The separate effects of hyperparathyroidism, hypercalcemia of malignancy, renal failure, and acidosis on the state of calcium, phosphate, and other ions in plasma. *J. Clin. Invest.*, 1962, *41* : 1454-1471.

357. WATCHORN E., MCCANCE R. — Subacute magnesium deficiency in rats. *Biochem. J.*, 1937, *31* : 1379-1390.

358. WATCHORN E., MCCANCE R. — VI. Inorganic constituents of cerebrospinal fluid. II. The ultrafiltration of calcium and magnesium from human sera. *Biochem. J.*, 1932, *26* : 54-64.

359. WEIT L. — Experimental magnesium depletion. *Yale J. Biol. Med.*, 1964, *36* : 325-349.

360. WEIT L., GUTMAN H. — Disorders of magnesium metabolism. Disease-a-Month. Year Book Medical Publishers, Inc., Chicago, 1965, pp. 1-22.

361. WESER J., PINTAR K., SIMON M., MOTOLA R., FRIEDMAN R., MAYMAN A., SCHECHTER R. — The effects of prolonged hypomagnesemia on the cardiovascular system in young dogs. *Am. Heart. J.*, 1964, *67* : 221-231.

362. WHANG R. — Some aspects of the interrelationship of magnesium and potassium. *J. Chron. Dis.*, 1968, *21* : 207-210.

363. WHANG R., MOROSE H., ROGERS D., REYES R. — The influence of sustained magnesium deficiency on muscle potassium repletion. *J. Lab. & Clin. Med.*, 1967, *70* : 895-902.

364. WHANG R., OLIVER J., WEIT L., MACDOWELL M. — Renal lesions and disturbance of renal function in rats with magnesium deficiency. *Ann. N.Y. Acad. Sci.*, 1969, *162* : 706.

365. WHANG R., WEIT L. — Observations in experimental magnesium depletion. *J. Clin. Invest.*, 1963, *42* : 305-313.

366. WILKINSON A., HARRIS J. — Mg depletion in the newly born of young child. *Brit. J. Surg.*, 1969, *56* : 628.

367. WILKINSON R. — A micromethod for serum calcium and serum magnesium. *J. Clin. Path.*, 1957, *10* : 126-135.

368. WILLIAMS R., WACKER W. — Cation balance in biological systems. *J.A.M.A.*, 1967, *201* : 96-100.

369. WOLFF R., JORRAND, BUCHOUARD A. — Le magnésium sérique dans la grossesse. *C. R. Soc. de Biol.*, 1937, *126* : 345-347.

370. WONG H., TIU Y. — An association between serum-magnesium and tremor and convulsions in infants and children. *Lancet*, 1968, *2* : 18-21.

371. WORKER N., MIGNOWSKY B. — Effect of vitamin D on the utilization of beryllium, magnesium, calcium, strontium and barium in the chick. *J. Nutr.*, 1961, *74* : 490-494.

372. YUN T., LAZZARA R., BLACK W., WARSH J., BURCH G. — The turnover of magnesium in control subjects and in patients with idiopathic cardiomyopathy and congestive heart failure studied with magnesium^{28}. *J. Nuclear Med.*, 1966, *7* : 177-187.

373. ZIMMET P. — Interrelationship of Mg and ionized calcium in Human Mg deficiency. *Med. J. Australia*, 1968, *2* : 496-500.

374. ZIMMET P., BRIDGER H., NAYLER W. — Plasma ionized calcium in hypomagnesaemia. *Brit. Med. J.*, 1968, *1* : 622-623.

375. ZUMOFF B., BERNSTEIN E., TAMARISIO H., HELLMAN L. — Radioactive magnesium (Mg^{28}) metabolism in man. *Clin. Res.*, 1958, *6* : 260.

5384

The Turnover and Transport of Vitamin D and of a Polar Metabolite with the Properties of 25-Hydroxycholecalciferol in Human Plasma

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ABSTRACT. Four normal men were injected intravenously with physiological doses (6 μg) of vitamin D_3 12-^{3}H. Serial samples of plasma were collected for 30 days. Total lipid extracts were chromatographed on silicic acid columns or thin-layer plates in order to characterize the radioactive components. Labeled vitamin D_3 disappeared rapidly from plasma (initial half-life approximately 12 hr); after 7 days unchanged vitamin D_3 represented less than 1% of circulating radioactivity. Coincident with vitamin D_3 disappearance a more polar labeled metabolite appeared with chromatographic and other properties identical with those of 25-hydroxycholecalciferol. The disappearance of the more polar metabolite was relatively slow with a half life of 19.6 ± 0.6 days. A similar half-life was seen in a 6th subject, injected with 80 μg of vitamin D_3 ^{3}H. Most (approximately 92%) of the plasma total radioactivity was represented by this component throughout the study. Plasma samples collected at various times were adjusted to density (d) 1.21 and were ultracentrifuged to separate plasma lipoproteins from proteins with d > 1.21. In all samples, almost all (mean 94%) of the radioactivity was found in association with proteins of d > 1.21. This observation was confirmed by bioassay, measuring uptake of ^{45}Ca by intestinal slices. All plasma bioassayable vitamin D was found in association with proteins of d > 1.21; 55% of ^{3}H activity was found in the chromatographic fraction corresponding to 25-hydroxycholecalciferol and 11% in the fractions representing vitamin D_3. Since both vitamin D_3 and its 25-hydroxy metabolite are lipid soluble sterol derivatives, the finding that these components do not circulate in association with the known plasma lipoproteins provides presumptive evidence for the existence of a specific transport protein of d > 1.21. The transport protein for the polar metabolite has been partly characterized by gel filtration on Sephadex G-200 and by electrophoresis on polyacrylamide gel. The protein has an apparent size slightly smaller than plasma albumin (approximate mol wt 50,000-60,000) and an electrophoretic mobility very slightly greater than that of albumin. Studies are in progress to fractionate further and to characterize the transport protein.

INTRODUCTION

Recent studies of vitamin D metabolism have demonstrated that vitamin D is rapidly converted into a more polar metabolite after its intravenous injection into humans (1) or other animals (2-4). This metabolite, isolated from swine plasma, was identified as 25-hydroxycholecalciferol by Blunt, DeLuca, and Schnoes (3), and was shown to be biologically more active than cholecalciferol (vitamin D_3) itself. The conversion of vitamin D_3 to its 25-hydroxy "active" metabolite appears to occur in the liver (2) and may be an essential step in the activation of vitamin D for target tissues such as the intestinal mucosa (5) or bone (6). After its formation, 25-hydroxycholecalciferol is presumably transported from the liver to the target tissues where still more polar metabolites are formed and found (7-9) and where biological effects occur. It has also been suggested recently by Fraser and Kodicek (10) and confirmed by DeLuca (11) that the kidney is the site of conversion of 25-hydroxycholecalciferol to a still more polar active metabolite which is found in intestinal mucosa.[a]

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[a] *Note added in proof.* This metabolite has very recently been identified as 1,25-dihydroxycholecalciferol [Lawson, D. E. M., D. R. Fraser, E. Kodicek, H. R. Morris,

Limited information is available about the turnover and the plasma transport of vitamin D_3 and of its 25-hydroxy metabolite. In 1962, Avioli, Lee, McDonald, Lund, and Delaney reported that cholecalciferol-^{3}H disappeared rapidly from the plasma of normal subjects with a half-life of 20–30 hr (11). Coincident with the disappearance of cholecalciferol, a more polar metabolite with the properties of 25-hydroxycholecalciferol appeared. A slower plasma disappearance rate of vitamin D_3-^{3}H and a reduction in the amount of ^{3}H appearing as the polar metabolite were observed in patients with vitamin D-resistant rickets (12). More recently, Mawer, Lund, and Strachan reported that, after the initial rapid disappearance of labeled vitamin D_3 from human plasma, a prolonged second phase was seen with a half-life of 20–24 days (13). A large proportion of the radioactivity found in plasma during the slow second phase was present as polar metabolites. In this study the amounts of labeled vitamin D injected were 10–20 times the recommended daily allowance for vitamin D (14).

Before the isolation and identification of 25-hydroxycholecalciferol, several studies were conducted to examine the transport of vitamin D in human (15–17) and in rat (18, 19) serum. Human serum proteins were separated by agar gel electrophoresis by de Crousaz, Blum, and Antoine who found vitamin D activity, by bioassay, in the albumin and α-globulin fractions (16). Thomas et al. also found vitamin D activity in association with the α-globulin and albumin fractions of human serum (15). Using labeled vitamin D_3, Jeffries and Delaney found that both the vitamin and its polar metabolite were associated with several protein bands, but particularly with an α-globulin, after disc gel electrophoresis (19). No information is available about the plasma transport of 25-hydroxycholecalciferol in man.

We now report a study of the turnover of vitamin D_3 and of its polar metabolite in humans after the administration of small, physiological doses of the labeled vitamin. The transport of the vitamin and particularly of its polar metabolite has been examined in some detail. Neither the vitamin nor its polar metabolite circulates in association with the known plasma lipoproteins. The transport protein for the polar metabolite has been partly characterized with regard to apparent molecular size and to electrophoretic mobility.

METHODS

Experimental design. Five normal male volunteers, 24–28 yr of age, participated in this study. All subjects ate their usual diets throughout the studies; none of them received, or had been taking, vitamin supplements. Each subject was given an intravenous injection of ^{3}H-labeled cholecalciferol. Serial samples of blood were collected, after 5, 1, 2, 4, 7, 20 and 45 days, and then at weekly intervals for a total of 28 wk, in syringes moistened with a solution of heparin. Relatively large samples, of approximately 100 and 200 ml, were collected on the 2nd and 7th days, respectively, for attempted fractionation of the transport protein. Blood cells were eliminated by centrifugation at 2000 rpm for 30 min at 4°C. Portions of the plasma samples were then dialyzed, extracted with $CHCl_3$-CH_3OH (see below), subjected to ultracentrifugation in order to separate lipoproteins from other plasma proteins (see below), or stored in the frozen state at −20°C for later study.

In the first, preliminary experiment, subject C. H. was given 70 μg (2.80 μCi) of cholecalciferol-1,2-^{3}H, specifically labeled (see below) (Amersham/Searle, Arlington Heights, Ill.), dissolved in 0.2 ml absolute ethanol in a volume sterile syringe (Cut of Ardell and Sons, Chicago, Ill.). The radioactive purity of the injected material was tested 99% as assessed by thin-layer chromatography (TLC).[*]

In the second and major study, each of four subjects received 10 μg (2.50 μCi) of a high specific radioactivity (Amersham/Searle) preparation of cholecalciferol-1,2-^{3}H, dispersed in an isotonic NaCl solution. The cholecalciferol was purified by TLC on silica gel, using benzene–ethyl acetate, 3:1 (v/v) as solvent just before use. The purified cholecalciferol-^{3}H was dissolved in 1 ml of ethanol, and under sterile conditions was dispersed through a 30-G hypodermic needle into 20 ml of sterile 0.9% NaCl solution. Each subject was injected with 4.5 ml of the final dispersion.

Extraction and chromatography. Samples of whole plasma or of plasma fractions were extracted with 20 volumes of $CHCl_3$-CH_3OH, 2:1 (v/v) containing 1 mg of butylhydroxytoluene (BHT) per 100 ml as an antioxidant.

TABLE I

Elution Scheme Employed for Silicic Acid Column Chromatography

Fraction No.	Eluted with ml	Solvent[*]	Total ^{3}H after fractionation[†]
1	300	7% benzene in hexane	
2	25	85% benzene in hexane	
3	15	85% benzene in hexane	
4	25	85% benzene in hexane	
5	15	85% benzene in hexane	
6	20	85% benzene in hexane	
7	40	Chloroform	
8	25	25% methanol in chloroform	
9	50	10% methanol in chloroform	
10	50	60% methanol in chloroform	
11	25	Methanol	

* 25% benzene in hexane (Mallinckrodt, Spectrophotometric Grade, 25:75 (v/v)) was used for the elution of cholesterol from silicic acid (Mallinckrodt, 100 mesh).

† The total sample obtained from subject F.R. 24 hr after the intravenous injection of 10 μg cholecalciferol-1,2-^{3}H.

Extraction of the plasma fractions. Samples of whole plasma or of plasma fractions were extracted with 20 volumes of $CHCl_3$-CH_3OH, 2:1 (v/v) containing 1 mg of butylhydroxytoluene (BHT) per 100 ml as an antioxidant.

[*] New England Nuclear Corp., Boston, Mass.

[†] *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; RBP, retinol binding protein; TLC, thin-layer chromatography; TMS, trimethylsilyl.

and D. H. Williams. 1971. *Nature (London).* 230: 228; Holick, M. F., D. G. Schnoes, H. F. DeLuca, T. Suda, and R. J. Cousins. 1971. *Biochemistry.* 10: 2799; Norman, A. W., J. F. Myrtle, R. J. Midgett, H. G. Nowicki, V. Williams, and G. Popjak. 1971. *Science (Washington).* 173: 51.

g of unlabeled carrier cholecalciferol[5] was added to each fraction. Each extraction mixture was split into two [illegible] by addition of 5 volumes of 0.05% H_2SO_4, and the [illegible] $CHCl_3$ phase was collected and evaporated to dryness under a stream of nitrogen. Portions of the total lipid extracts so obtained were assayed directly for radioactivity; other portions were subjected to column or thin-layer chromatography.

Column chromatography was performed with another glass column of 1 cm diameter containing 5 g of silicic acid (Grill, 100-200 mesh). 12 fractions were eluted from each column in a stepwise procedure as shown in Table I. This elution scheme is a modification of the elution schemes employed in this laboratory for the separation of plasma total lipids and phospholipids (20, 21). The scheme shown in Table I was designed to achieve a clean separation between free sterols (e.g., cholecalciferol) and 25-hydroxy sterols (e.g., 25-hydroxycholecalciferol) from each other and from still more polar lipids. Experiments with the reference compounds demonstrated that, in this scheme, cholecalciferol is eluted in fractions 3, 4, and 5, and 25-hydroxycholecalciferol in fraction 8. During this study the mean (± S.D.) recovery of radioactivity applied to the column was 102 ± 2% for 47 chromatographic runs.

Portions of the total lipid extracts of the sorbents with density (d) greater than 1.21 were chromatographed on Kieselgel G thin-layer plates with benzene-ethyl acetate, 8:1, in an atmosphere of nitrogen (c) in the dark. Carrier 25-hydroxycholecalciferol[6] was added to some but not all samples before TLC. After chromatography the plates were fully sprayed with a solution of Rhodamine 6G, and the isolated cholecalciferol (R_f 0.55-0.6) and 25-hydroxycholecalciferol (R_f 0.20) bands were visualized under ultraviolet light, scraped, and eluted with chloroform. The remainder of the plate was divided into bands of approximately 1 cm each, which were each scraped and eluted. The eluates were concentrated and assayed for radioactivity.

Trimethylsilyl (TMS) ether derivatives. TMS ether derivatives of the total lipid extracts of the products of d > 1.21 were prepared from the plasma samples collected from subjects A. B. and F. B. 36 days after the injection of 1 microcurie of ^{45}Ca. 1 mg of carrier 25-hydroxycholecalciferol was added to the extraction mixture for each of these samples. The total lipid extract was suspended in 200 μl of a solution of pyridine-hexamethyldisilazane-trimethylchlorosilane, 9:3:1 (22) (Sil-prep[7] and allowed to react at 60° 1 min. Fractions of the reaction mixtures were then subjected to T.L.C. on silica gel plates in two different systems, (a) benzene-ethyl acetate, 8:1, and (b) successively with benzene-hexane, 1:1, followed at right angles by methyl ethyl ketone-acetonitrile, 7:3, 80% saturated with silica gel. The area of the carrier was located as described above, and the scraped bands or spots were eluted with benzene-ethyl ether, 1:1.

Isolation of lipoproteins. Plasma lipoproteins were separated from pooled plasma with hydrated density gradient ultracentrifugation, using a simplified modification of the method of Havel, Eder, and Bragdon (23). For each fraction, 10 ml of fresh plasma was placed in a 5 × 3 in. tube (Lab containing 3.3% g of KBr). After the KBr had dissolved, the tubes were filled with a d 1.21 solution of KBr-NaCl and centrifuged in a No. 40 rotor of a Spinco model L ultracentrifuge at 40,000 rpm for 36-48 hr. The tubes were sliced in the clear zone, and the floating (lipoprotein, d < 1.21) and sedimenting (d > 1.21) fractions were collected separately.

Gel filtration and electrophoresis. Gel filtration was carried out on columns of Sephadex G-200[8] in a cold room at 4-5°C. The columns were eluted with 0.02 M phosphate buffer, pH 7.3, containing 0.2 M NaCl. The size of the column varied in different experiments, but the volume of the sample applied was restricted to 3% or less of the column bed volume. The samples subjected to gel filtration included the following: (a) a large sample of pooled plasma obtained from subjects A. B., F. B., B. H., and T. L. on the 5th day after the injection of cholecalciferol-^3H; (b) plasma obtained from subject C. H. on the 13th day after injection; and (c) a pooled sample of plasma obtained from subject C. H. on the 5th, 29th, and 41th days of study.

Preparative polyacrylamide gel electrophoresis was performed with a Buchler[9] "Poly prep 200" apparatus as described previously (24).

Dosage. The vitamin D activity of plasma and plasma fractions was estimated by the in vitro uptake of ^{45}Ca by intestinal slices (25, 26). The samples which were assayed included total lipid extracts of whole plasma and of the lipoprotein (d < 1.21) and d > 1.21 fractions of plasma. The cholecalciferol containing and 25-hydroxycholecalciferol-containing fractions (fractions 3+4+5 and fraction 8, respectively) after silicic acid column chromatography of extracts of whole plasma were also bioassayed. Carrier vitamin D was not added at any time to these samples, and all organic solvents contained 1 mg BHT per 100 ml. The $CHCl_3$ extracts of column fractions derived from 20 ml of plasma were concentrated under nitrogen to approximately 5 ml and transferred to a vial containing 1 g of olive oil with 0.1% BHT (w/w). The samples were flushed with nitrogen until the solvent was fully evaporated. The olive oil solutions and dilutions of USP reference vitamin D were administered with calibrated droppers to vitamin D deficient rats. The rats were prepared by feeding rachitogenic diet No. 2[10] for 5 wk to weanling male rats of the Sherman strain[11] kept in covered cages. Each rat received two drops initially and three drops 12 hr later. The intestinal uptake studies were conducted 44-50 hr after giving the initial dose. The intestines were removed and incubated as described by Gordan and Schachter (26) except that ^{45}Ca was used instead of ^{47}Ca. The incubated intestinal slices (approximately 200 mg) were removed from the incubation medium, blotted dry, and homogenized with 2 ml of 1 M EDTA in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 12,000 g for 30 min, and 0.2 ml of the supernatant solutions each was suspended in 1 ml of 1 N hyamine hydroxide in methanol. After the suspensions became clear, 10 ml of the counting solution described by Bray (27) was added, and the vials were shaken vigorously and cooled overnight. After standing overnight at room temperature, the samples were assayed for ^{45}Ca in a Packard Tri-Carb liquid scintillation counter.

Other procedures. Radioassay for ^3H was carried out by dissolving samples in 15 ml of 0.5% 1-naphthylamide toluene followed by assay with a Packard liquid scintillation

[5] Mallinckrodt Biochemicals Inc., Cleveland, Ohio.
[6] Hoffmann-La Roche Inc., Nutley, N. J.
[7] Applied Science Laboratories Inc., State College, Pa.
[8] Pharmacia Fine Chemicals Inc., Piscataway, N. J.
[9] Buchler Instruments, Inc., Fort Lee, N. J.
[10] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[11] Carworth Research Institute, Inc., Wayne, N. J.

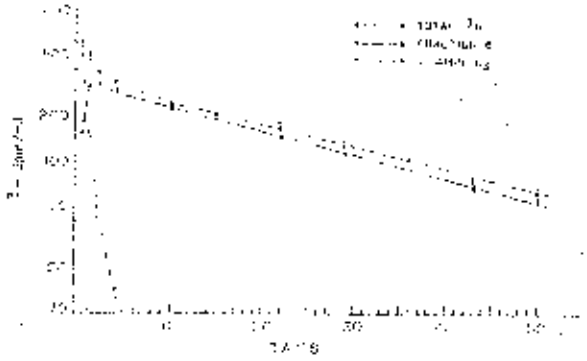

FIGURE 1 The turnover of plasma vitamin D_3 after the injection of 6 μg of cholecalciferol-^{3}H into each of four subjects. The points show the mean ±SEM values (in brackets) for plasma total radioactivity, and for plasma-^{3}H recovered in the cholecalciferol-containing fractions (labeled: vitamin D_3) and in the 25-hydroxycholecalciferol-containing fraction (labeled: fraction 8) after silicic acid column chromatography.

counter. Quenching was corrected for, where appropriate, by means of an automatic external standardization system.

Saponification of the total lipid extract of a d > 1.21 sample was conducted in 2.15 ml of 2.2% KOH in 63% ethanol at 65–70°C under nitrogen for 2 hr. 1 ml of water was added, and the unsaponifiable lipids were collected by hexane extraction, each time with 5 ml of hexane.

The half-life of the slow, semilogarithmic disappearance of radioactivity from plasma (see Results) was determined for each subject on the eight observations made between the 4th and 14th days after the injection of cholecalciferol-^{3}H. For subject C. H. nine observations between the 7th and 21st days were used. Linear regression lines of the logarithm of the concentration of radioactivity vs. time in days were calculated by the method of least squares (29), using a Wang 700 model 700 electronic calculator. The half-lives were calculated from the regression lines.

RESULTS

Turnover of radioactive vitamin D_3. Fig. 1 shows the results obtained with the four subjects who were injected with 6 μg of cholecalciferol-^{3}H. The semilogarithmic plot of the concentration of plasma total radioactivity vs. time described a curve during the first 8 days, whereas beyond this time the plot followed a straight line.

Extracts of each of the plasma samples collected in this study were analyzed by silicic acid column chromatography. In all samples, quantitatively significant amounts of ^{3}H were only recovered in the cholecalciferol-containing fractions (fraction 3 + 4 + 5) and in the 25-hydroxycholecalciferol-containing fraction (fraction 8). A small amount of ^{3}H was also consistently recovered in fraction 10, which contributed a mean (±SEM) of 2.4 ±0.3% of the recovered ^{3}H for the samples analyzed. (See Table I for a representative example.) No information is available about the labeled compounds which were recovered in fraction 10.

During the early, curvilinear part of the plasma total radioactivity plot, labeled cholecalciferol disappeared rapidly from plasma, with an initial half-life, during the first 48 hr, of 12 hr (Fig. 1). Cholecalciferol represented only 13% of the total plasma radioactivity after 48 hr and less than 1% after 7 days in the two subjects receiving the 6 μg dose. In subject C. H., who received the 80 μg dose, cholecalciferol-^{3}H disappearance was rapid with an initial half-life of 34 hr. In this subject cholecalciferol represented 47% of the total plasma radioactivity after 48 hr and 5% after 12 days.

Coincident with cholecalciferol-^{3}H disappearance a more polar labeled component appeared with the chromatographic properties of 25-hydroxycholecalciferol eluted in silicic acid column fraction 8, and reached a peak. This component represented approximately 92% of the plasma total radioactivity throughout the linear portion of the semilogarithmic turnover plot. The disappearance of this polar metabolite was relatively slow with a half-life of 19.6 ±0.6 days (Table II). The semilogarithmic disappearance rate of plasma total radioactivity was identical with that of the polar metabolite (Fig. 1 and Table II). As shown in Table II, the turnover rate of the polar metabolite was similar in the subject (C. H.) who received the 80 μg dose to the rate observed in the subjects given 6 μg.

Characterization of the polar metabolite. The labeled metabolite eluted in fraction 8 from silicic acid column exhibited chromatographic and other properties of 25-hydroxycholecalciferol. The methods used to compare the properties of the labeled polar metabolite with those of authentic 25-hydroxycholecalciferol (in addition to silicic acid column chromatography) included TLC in the formation of the TMS ether derivative and TLC in two different systems, and saponification and chromatography of the nonsaponifiable lipids.

TABLE II

Turnover of Plasma Total Radioactivity and of the Labeled Polar Metabolite of Vitamin D_3

Subject	$t_{\frac{1}{2}}$ of total plasma-^{3}H	$t_{\frac{1}{2}}$ of polar metabolite-^{3}H*
	days	*days*
A. B.	18.1	18.1
F. B.	17.2	18.0
R. H.	20.4	20.1
T. L.	21.4	20.6
C. H.	20.7	21.1
Mean ± SEM	19.6 ±0.8	19.6 ±0.6

* Metabolite with the properties of 25-hydroxycholecalciferol (see text).

On TLC in benzene–ethyl acetate, 3:1, the radioactivity in column fraction 8 was found to comigrate with authentic 25-hydroxycholecalciferol. In a representative experiment, a pooled extract of plasma proteins of d > 1.21 obtained 48 hr after injection was analyzed by TLC. 11% of the ^{3}H rechromatographed with carrier cholecalciferol and 83% with carrier 25-hydroxycholecalciferol. The remaining very small amount (6%) of the radioactivity was mainly smeared throughout the area from the origin to the 25-hydroxycholecalciferol band. On silica gel column chromatography of an extract of this pool of whole plasma, 13% of the ^{3}H was eluted in the cholecalciferol-containing fractions (fractions 3 + 4 + 5) and 81% in fraction 8.

TMS ether derivatives were formed with the extracts of two samples obtained on the 36th day of the study, which contained 82% of their ^{3}H content as the polar metabolite (eluted in column fraction 8) (see Methods). On subsequent TLC, most of the ^{3}H rechromatographed with the simultaneously formed TMS ether derivative of authentic carrier 25-hydroxycholecalciferol. After TLC in benzene–ethyl acetate (3:1) 81 and 85% (for the two samples) of the recovered ^{3}H rechromatographed with the TMS carrier band (R_F 0.85); the remaining radioactivity was spread in a broad trailing zone behind this band. After two dimensional TLC, 58% and 73% of the recovered ^{3}H (for the two samples) rechromatographed with the TMS carrier spot. Since the recovery of the TMS derivative of 25-hydroxycholecalciferol after its formation and TLC was not expected to be quantitative, these data support the conclusion that the labeled metabolite in column fraction 8 had properties virtually identical with those of 25-hydroxycholecalciferol.

By saponification it was confirmed that the polar metabolite was not an ester or other saponifiable derivative of vitamin D_3. A sample of plasma proteins of d > 1.21 was obtained from subject C. H. 56 days after injection of cholecalciferol-^{3}H. After extraction and silicic acid column chromatography, 56% of the ^{3}H in this sample was recovered in the 25-hydroxycholecalciferol-containing fraction. After saponification of a portion of the extract, 58% of the ^{3}H was recovered with the nonsaponifiable lipids. The labeled nonsaponifiable lipid was eluted from a silicic acid column in a pattern identical with that seen for the labeled lipid in the extract before saponification (96% of the ^{3}H recovered in the 25-hydroxycholecalciferol containing fractions).

Ultracentrifugation at density 1.21. Plasma samples were separated into lipoproteins (d < 1.21) and proteins of density greater than 1.21 at several times during the study. The distribution of radioactivity between these two fractions, for the subjects who were given 6 μg of cholecalciferol-^{3}H, is shown in Table III. Almost all (>90%, mean 94%) of the ^{3}H was consistently found to be associated with the proteins of density greater than 1.21. In these four subjects, slightly more radioactivity (mean of 8% of total ^{3}H) was found in the lipoprotein fractions (d < 1.21) at 2 days after isotope injection than at later intervals. On column chromatography of extracts of the 2-day samples, relatively more of the ^{3}H in the lipoprotein fractions than in the d > 1.21 fractions was found as esterified and as unchanged vitamin D_3. Similarly, with the subject who received the 80 μg dose, 90, 97, and 98% of the plasma total ^{3}H was found associated with the proteins of density greater than 1.21 after 5, 19, and 56 days, respectively. In this subject, during the interval 12 hr–3 days (four samples) 84–87% of the ^{3}H was found in the d < 1.21 fraction. The results with the earliest samples collected suggest that a very small portion of the injected labeled vitamin D_3 was circulating in association with the plasma lipoproteins during the early time intervals. Most of the vitamin D_3, however, and virtually all of the polar metabolite with the properties of 25-hydroxycholecalciferol circulated in association with the proteins of density greater than 1.21 and not with the known plasma lipoproteins. This conclusion was confirmed by bioassay (see below).

TABLE III

*Distribution of Radioactive Vitamin D after Ultracentrifugation at Density 1.21**

Fraction	Distribution of radioactivity†
	%
Lipoproteins (d < 1.21)	6.1 ±0.8
Proteins with d > 1.21	93.9 ±0.8

* 14 samples were analyzed, which were collected from subjects A. B., F. B., R. H., and T. L. 2, 4, and 7 days after the injection of label, and from subjects R. H. and T. L. after 36 days.

† Mean ±S.E.M.

Gel filtration. Gel filtration on Sephadex G-200 was performed with three plasma samples, each containing almost all of its radioactivity as the polar metabolite (see Methods for more details). These experiments provide information about the apparent molecular size of the transport protein for the polar metabolite. The results of a representative experiment are shown in Fig. 2. An identical elution pattern was obtained in each of five chromatographic runs. In each instance, almost all of the radioactivity was eluted in a peak with an elution volume very close to that of plasma albumin. The center of the peak of radioactivity was consistently found to be eluted very slightly after the center of the plasma albumin peak (see Fig. 2). These results suggest that the polar metabolite

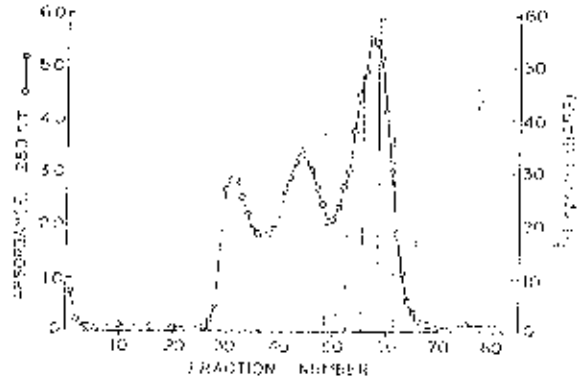

FIGURE 2. Gel filtration of whole plasma containing radioactive vitamin D metabolites. 6 ml of whole plasma from a sample of pooled plasma collected from four subjects on day 7 (see Methods) was dialyzed successively three times, each time against 4 liters of 0.02 M Na phosphate buffer, pH 7.5, containing 0.2 M NaCl. The dialyzed sample was applied to a 2.5 × 63 cm column (bed volume, 300 ml) of Sephadex G-200. The elution was conducted with the same buffer at a flow rate of 15 ml/hr. Fractions of 3 ml each were collected.

of vitamin D_3 circulates in association with a protein of apparent molecular size very close to, but slightly smaller than, that of human plasma albumin.

Preparative polyacrylamide gel electrophoresis. The fractions comprising the ^{3}H-containing peak after gel filtration were combined, and the protein was then subjected to preparative polyacrylamide gel electrophoresis.

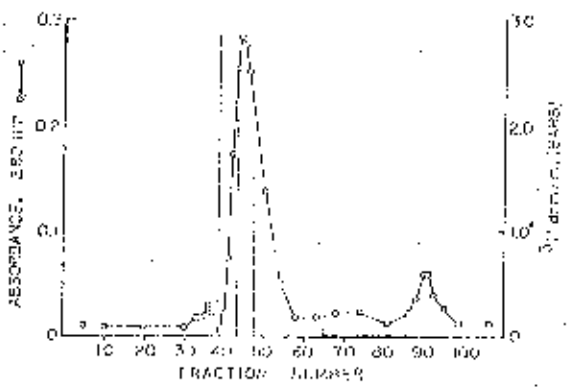

FIGURE 3. Preparative polyacrylamide gel electrophoresis. The fractions comprising the ^{3}H-containing peak after gel filtration (e.g. fractions 50–66 in Fig. 2) of the pooled sample from subject C. H. were concentrated by ultrafiltration (Diaflo UM-2 membrane; Amicon Corp., Lexington, Mass.) and dialyzed against water. 8 ml of the concentrated sample containing 10 mg of protein per ml in 5% sucrose was applied to a 9.5 cm high column of 7% acrylamide gel. The electrophoresis was conducted at a constant voltage of 400 v and approximately 20 ma, with an elution rate of 40 ml/hr. Fractions of 6 ml were collected.

TABLE IV
Distribution of Vitamin D Activity in Plasma by Bioassay

Sample assayed*	Distribution of total bioassay-able activity
	%
Whole plasma	100
Lipoproteins (d < 1.21)	0
Proteins with d > 1.21	100
Fractions 3 + 4 + 5‡	11
Fraction 8‡	55

* The samples assayed were derived from a sample of pooled plasma collected from subjects A. B., F. B., and B. [illegible] the 50th day of the study. The pooled plasma was [illegible] to contain a total vitamin D activity equivalent to [illegible] ng of cholecalciferol per ml.
‡ After silicic acid column chromatography of an extract of whole plasma.

As shown in Fig. 3, the radioactivity migrated in a single sharp peak with a mobility very slightly greater than that of plasma albumin. In each of three experiments, the peak of radioactivity was found to be mainly located along the leading edge of the albumin peak. The mobility of the ^{3}H-containing peak was distinctly less than that of plasma prealbumin, but considerably more than that of proteins with α-mobility. The fractions containing the proteins with α-mobility, identified by the fluorescence of retinol-binding protein (24), contained only 4% of the total radioactivity.

Bioassay. The results of a bioassay study are presented in Table IV. In this experiment, virtually all of the biological activity of whole plasma was recovered after ultracentrifugation at density 1.21, in association with the sedimenting proteins of density greater than 1.21. Slightly more than half of the biological activity of plasma was found in fraction 8 after silicic acid column chromatography of an extract of plasma; the remainder was found in the cholecalciferol-containing fractions (fractions 3 + 4 + 5).

These observations were confirmed in a later experiment using plasma collected from a normal 31 yr old male subject who had not been injected with labeled cholecalciferol. In this subject, the vitamin D activity of an extract of whole plasma was equivalent to 26 ng of cholecalciferol per ml of plasma. After ultracentrifugation, an extract of the proteins with d > 1.21 contained bioactivity equivalent to 21 ng of cholecalciferol per ml of plasma. The compounds recovered in fractions 3 + 4 + 5 and in fraction 8, after silicic acid column chromatography, had bioactivity equivalent to 0 and 11 ng of cholecalciferol per ml of plasma, respectively.

DISCUSSION

The studies reported here demonstrate that the labeled polar metabolite which appears in plasma after the injection of radioactive vitamin D_3 has a relatively slow turnover with a half-life of 19.6 ±0.6 days. In these studies, four of the five subjects were injected with a small, physiological dose (6 μg) of vitamin D_3, which was well within the recommended daily allowance of vitamin D (14). After injection the labeled vitamin D_3 disappeared rapidly from plasma, with an initial half-life of approximately 12 hr. After 7 days, labeled unchanged vitamin D_3 represented less than 1% of the circulating radioactivity. Coincident with the disappearance of labeled vitamin D_3 a non-polar component appeared, with chromatographic and other properties of 25-hydroxycholecalciferol. Most (approximately 92%) of the plasma total radioactivity was represented by this component throughout the later part of the study.

The properties of the labeled polar metabolite were compared directly with those of authentic 25-hydroxycholecalciferol. The labeled metabolite cochromatographed with 25-hydroxycholecalciferol on silicic acid columns and on thin layers of silica gel. The two components also cochromatographed during TLC in two different systems after formation of the TMS ether derivatives. Finally, the labeled polar metabolite was shown to be a non-saponifiable lipid whose chromatographic properties were not altered by saponification. These findings indicate that the labeled polar metabolite in plasma was either 25-hydroxycholecalciferol itself or an extremely similar compound with properties identical with those of 25-hydroxycholecalciferol in the systems examined (or a mixture of such nearly identical compounds). In view of the demonstrated biological activity of the fraction containing the polar metabolite it is reasonable to conclude that the labeled polar metabolite was probably 25-hydroxycholecalciferol.

A slow disappearance of plasma total radioactivity, after the injection of labeled vitamin D_3 into human subjects, has been reported by Mawer et al. (13). In contrast with our findings, however, Mawer et al. reported that after the initial rapid disappearance of labeled vitamin D_3 from plasma and the appearance of labeled polar metabolites, both the unchanged vitamin D_3 and its polar metabolites exhibited a prolonged phase with a slow turnover rate. The half-life of labeled vitamin D_3 during the phase of slow turnover was 19.36 days, while the plasma total radioactivity was 20.41 days (13). The differences between these observations and our own may possibly be explained by the relatively large doses of labeled vitamin D_3 (110 and 216 μg) injected by Mawer et al. Consistent with this explanation is our finding that the disappearance rate of labeled vitamin D_3 in the subject who received an 80 μg dose was considerably slower and more prolonged than that seen in the subjects who were given 6 μg. This explanation is also supported by the results of Schneider, Koch, Opitz, von Herrath, and Knoop (29) who injected small doses of labeled vitamin D_3 (12.5 μg) into normal humans and patients with renal disease and found that unchanged vitamin D_3 represented only approximately 2% of the plasma-PH after 7 days. Moreover, more recently Mawer, Lumb, Schneider, and Stanbury have reported that the disappearance rate of radioactive vitamin D_3 was slower in subjects receiving large doses of vitamin D than in patients relatively deficient in vitamin D (30).

By combining the results of the bioassay experiments with those of the turnover studies, an estimate can be obtained of the steady-state turnover of plasma 25-hydroxycholecalciferol in man. The bioassay studies suggested that the plasma samples contained 8–9 ng of cholecalciferol per ml and an amount of 25-hydroxycholecalciferol with bioactivity equivalent to 10–11 ng of cholecalciferol per ml. The biological activity of 25-hydroxycholecalciferol has been reported to be 1.1 times (31) and 1.78 times (8) that of cholecalciferol, on a per unit mass basis. By using a mean of these values, we can conclude that the samples bioassayed contained approximately 7 ng of 25-hydroxycholecalciferol per ml. If we now estimate the plasma volume as 3 liters and the extravascular (and extracellular) compartment as being similar to that for serum albumin (1.1 times the intravascular compartment [31]), we can estimate the sizes of the intravascular and extravascular pools of 25-hydroxycholecalciferol. These calculations indicate that the plasma compartment contained a total of approximately 21 μg, and the extravascular compartment a total of approximately 29 μg, giving a total extracellular pool of approximately 50 μg of 25-hydroxycholecalciferol. Given a half-life of 19.6 days and assuming a fully miscible extracellular compartment which behaved as a single pool during the later phase of the turnover study, these data suggest that the steady state turnover of 25-hydroxycholecalciferol in the extracellular compartment was approximately 1.3 μg per day. This is equivalent to a steady-state turnover of 80–85 IU of biological activity per day. A comparable estimate cannot be made for vitamin D_3 itself because of the likelihood that the disappearance rate observed during the first days of study represented an unsteady state with regard to the turnover and metabolism of the labeled material.

The experiments reported here conclusively demonstrate that significant amounts of neither vitamin D nor its polar metabolite (presumably 25-hydroxycholecalciferol) circulate in plasma in association with any of the known plasma lipoproteins. In each of the several time intervals studied, almost all (usually 95% or more) of the plasma radioactivity was found associated with the

proteins of density greater than 1.21. A very small portion of the injected labeled vitamin D_3 was found in association with the plasma lipoproteins during the shortest time intervals studied. The bioassay experiments demonstrated that biological activity associated with both vitamin D_3 and with the 25-hydroxycholecalciferol-containing fraction, was found in association with the plasma proteins of density greater than 1.21. In contrast, cholesterol and other lipids (except for FFA [32] and lysolecithin [33]) circulate in plasma mainly as part of plasma lipoprotein complexes with hydrated densities of less than 1.21 (23, 34). Since both cholecalciferol and its 25 hydroxy derivative are steroid derivatives and non-saponifiable lipid alcohols, the fact that these molecules circulate in association with proteins of density greater than 1.21 provides presumptive evidence for the existence of a specific transport protein for provitamin D and for these compounds.

This conclusion is supported by recent studies in our laboratory dealing with the transport of retinol (vitamin A alcohol) in plasma. Beginning with the observation that retinol circulates in plasma in association with proteins of density greater than 1.21, we were able to develop a fractionation sequence for the isolation of retinol-binding protein (RBP), the specific transport protein for plasma vitamin A (24). RBP has now been characterized to a considerable extent (24, 35, 36).

The plasma protein responsible for the transport of 25-hydroxycholecalciferol has been partly characterized with regard to apparent molecular size and to electrophoretic mobility. Since the plasma samples which were examined in these experiments contained almost all their radioactivity as the polar (25 hydroxy) metabolite, comparable information is not available about the transport protein for vitamin D_3 itself. It remains for future studies to determine whether the same or different proteins are responsible for the transport of vitamin D and of 25 hydroxycholecalciferol. Gel filtration on Sephadex G-200 indicated that the polar metabolite (presumed to be 25-hydroxycholecalciferol) circulates in association with a protein of apparent molecular size very close to, but slightly smaller than, that of human plasma albumin. The mol wt of the protein can be estimated as approximately 50,000-60,000. On polyacrylamide gel electrophoresis the transport protein displayed mobility very slightly greater than that of plasma albumin. If we assume that the protein carries one binding site for one molecule of 25-hydroxycholecalciferol and that the protein circulates almost entirely as the holo-protein containing a molecule of bound 25-hydroxycholecalciferol, then the concentration of the transport protein would be of the order of 1 mg/liter of plasma. These assumptions are based upon our experience with plasma RBP (24, 36). Should plasma vitamin D be transported by the same protein, then the level of the protein in plasma would be of the order of 2-3 mg/liter. Studies are in progress in our laboratory in an attempt to further fractionate and characterize the transport protein.

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REFERENCES

1. Avioli, L. V., S. W. Lee, J. E. McDonald, J. Lund, and H. F. DeLuca. 1967. Metabolism of vitamin D_3 in human subjects: distribution in blood, bile, feces, and urine. *J. Clin. Invest.* 46: 983.
2. Boyd, G., A. L. Kumar, and H. F. DeLuca. 1969. "Activation" of vitamin D by the liver. *J. Clin. Invest.* 48: 2032.
3. Blunt, J. W., H. F. DeLuca, and H. K. Schnoes. 1968. 25-Hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. *Biochemistry.* 7: 3317.
4. DeLuca, H. F. 1969. Recent advances in the metabolism and function of vitamin D. *Fed. Proc.* 28: 1618.
5. Olson, E. B., and H. F. DeLuca. 1969. 25-Hydroxycholecalciferol: direct effect on calcium transport. *Science (Washington).* 165: 405.
6. Tanaka, Y., H. G. Raisz, J. W. Blunt, and H. F. DeLuca. 1969. 25-Hydroxycholecalciferol: stimulation of bone resorption in tissue culture. *Science (Washington).* 163: 1443.
7. Gossins, R. J., H. F. DeLuca, and R. W. Gray. 1970. Metabolism of 25 hydroxycholecalciferol in target and nontarget tissues. *Biochemistry.* 9: 4649.
8. Myrtle, J. F., and A. W. Norman. 1971. Vitamin D_3 cholecalciferol metabolite highly active in promoting intestinal calcium transport. *Science (Washington).* 171: 79.
9. Hunsaker, M. R., D. W. Boyce, R. T. Little-Ely, and J. Rasmussen. 1971. A rapidly acting metabolite of vitamin D_3. *Proc. Nat. Acad. Sci. U. S.* 68: 177.
10. Fraser, D. R., and E. Kodicek. 1970. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature (London).* 228: 764.
11. DeLuca, H. F. 1971. Editorial: role of kidney tissue in metabolism of vitamin D. *N. Engl. J. Med.* 284: 53.
12. Avioli, L. V., T. P. Williams, J. Lund, and H. F. DeLuca. 1967. Metabolism of vitamin D_2 in vitamin D resistant rickets and familial hypophosphatemia. *J. Clin. Invest.* 46: 1966.
13. Mawer, E. B., G. A. Lumb, and S. W. Stanbury. 1968. Long biological half-life of vitamin D_3 and its polar metabolites in human serum. *Nature (London).* 217: 482.
14. National Research Council, Report of the Food and Nutrition Board. 1968. Recommended dietary allowances. National Academy of Sciences-National Research Council. Washington. 7th edition. 24.
15. Thomas, W. C., Jr., H. G. Mayer, T. B. Gaze, L. Diedrick, C. E. Ellis, and J. E. Edward. 1971. Sci

ies of antirickettic activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D in human serum. *J. Clin. Invest.* **38**: 1078.

16. de Crousaz, P., B. Blanc, and I. Antenor. 1965. Vitamin D activity in normal human serum and serum proteins. *Helv. Odontol. Acta* **9**: 151.
17. Chen, P. S., Jr., and K. Lam. 1965. Serum protein binding of vitamin D_3. *Arch. Biochem. Biophys.* **112**: 70.
18. Chalk, K. J. L., and F. Kodicek. 1961. The association of ^{14}C-labelled vitamin D_3 with rat serum proteins. *Biochem. J.* **79**: 1.
19. Rikkers, F., and H. F. DeLuca. 1967. An in vivo study of the carrier proteins of ^{3}H-vitamin D_3 and D_4 in rat serum. *Amer. J. Physiol.* **212**: 380.
20. Goodman, DeW. S., and T. Shiratori. 1961. Fatty acid composition of human plasma lipoprotein fractions. *J. Lipid Res.* **5**: 307.
21. Whyte, M., A. Karmen, and DeW. S. Goodman. 1963. Fatty acid esterification and chylomicron formation during fat absorption: 2. Phospholipids. *J. Lipid Res.* **4**: 322.
22. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6**: 397.
23. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345.
24. Kanai, M., A. Raz, and DeW. S. Goodman. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47**: 2025.
25. Schachter, D., D. V. Kimberg, and H. Schenker. 1961. Active transport of calcium by intestine: action and bioassay of vitamin D. *Amer. J. Physiol.* **200**: 1263.
26. Gordan, G. S., and D. Schachter. 1963. Vitamin D activity of normal and neoplastic human breast tissue. *Proc. Soc. Exp. Biol. Med.* **113**: 260.
27. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**: 279.
28. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. 6th edition. Iowa State University Press, Ames, Iowa. 50 and 135.
29. Schaefer, K., H. U. Koch, A. Opitz, D. von Herrath, and H. Kunop. 1970. Vitamin D-Stoffwechsel und Niereninsuffizienz. *Klin. Wochenschr.* **48**: 1129.
30. Mawer, E. B., G. A. Lumb, K. Schaefer, and S. W. Stanbury. 1971. The metabolism of isotopically labelled vitamin D_3 in man: the influence of the state of vitamin D nutrition. *Clin. Sci. (London)*. **40**: 39.
31. Becker, W. L., W. Vulwiler, P. D. Goldsworthy, L. E. Garby, W. E. Reynolds, R. Stogsdill, and R. S. Stemler. 1962. Studies of I^{131}-albumin catabolism and distribution in normal young male adults. *J. Clin. Invest.* **41**: 1312.
32. Fredrickson, D. S., and R. S. Gordon, Jr. 1958. Transport of fatty acids. *Physiol. Rev.* **38**: 585.
33. Switzer, S., and H. A. Eder. 1965. Transport of lysolecithin by albumin in human and rat plasma. *J. Lipid Res.* **6**: 506.
34. Fredrickson, D. S., R. I. Levy, and R. S. Lees. 1967. Fat transport in lipoproteins: an integrated approach to mechanisms and disorders. *N. Engl. J. Med.* **276**: 34.
35. Raz, A., T. Shiratori, and DeW. S. Goodman. 1970. Studies of the protein-protein and protein-ligand interactions involved in retinol transport in plasma. *J. Biol. Chem.* **245**: 1903.
36. Smith, F. R., A. Raz, and DeW. S. Goodman. 1970. Radioimmunoassay of human plasma retinol-binding protein. *J. Clin. Invest.* **49**: 1754.

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III. EARLY STUDIES OF VITAMIN D REQUIREMENT DURING GROWTH

Genevieve Stearns, Ph.D.

When Dr. Jeans and I started our studies of the nutritional requirements of infants, very few quantitative studies of infants had been carried out in this country. I had assisted Dr. Amy Daniels in her first studies of infant metabolism, and together we had worked out the techniques of such studies. No one, so far as I know, had assumed complete control of a group of infants for from six to nine months in order to have exact knowledge of the results of a given dietary regimen. Dr. Jeans characteristically reminded me of our legal and ethical responsibilities in such an undertaking by suggesting that we draw up a code of ethics for our own guidance. Its first item was: (1) the study should

benefit [illegible] objects as well as science; (2) no procedure would be used on any infant that we would not willingly use on an infant of our own families. This rule was broken only once, and then only because the need for information was great and the danger considered small.

At the time the studies were started, the literature was getting more and more confused as to the infant requirement for vitamin D. Standardization of fish oils, the first known sources, had been delayed for some years after the discovery of the vitamin. In the meantime, the need for prevention of rickets was so great that some type of fish oil must be given, and oils of many degrees of purity, age, and odor were used; their potencies later proved to vary from zero to several hundred units per gram. Early units varied with the investigators. Finally, an international unit was chosen, and methods were worked out for preservation of potency during storage.

Milk, irradiated directly or obtained by feeding a large unitage of vitamin D as irradiated yeast to the cows, contained only 135 units per quart, but was believed by many to be superior to a higher unitage of cod-liver oil as a rickets preventive. Dr. Daniels, in her studies of infants, had found that babies given irradiated milk had retained more calcium and phosphorous than those given cod-liver oil.[1] Looking back, these findings are not surprising. The oil was bought on the open market before standardization was developed, and was kept in a warm coat closet for the entire two years of the study. Its potency must have disappeared. It is a wonder no baby got rickets. But irradiated milk became the most popular source of vitamin D, for it eliminated the fishy odor of the baby and the indelible yellow stains left by the oil on clothing.

Milk obtained from cows fed large amounts of vitamin D as irradiated yeast was also used, and had the same unitage as milk irradiated directly. Addition of any substance to milk was against most state laws. Concentrated oily sources of vitamin D that used oils without color or odor also came on the market, and the use of single doses of a million or more units was advocated as an annual rickets preventive. It was reported that vitamin D from plant sources (irradiated ergosterol) and that from animal sources (irradiated cholesterol) were equally used by rats, but not by fowl. Were infants like rats or like chickens? Clearly, quantitative studies of vitamin D requirement using known unitages and sources were needed.[2]

Basic Studies

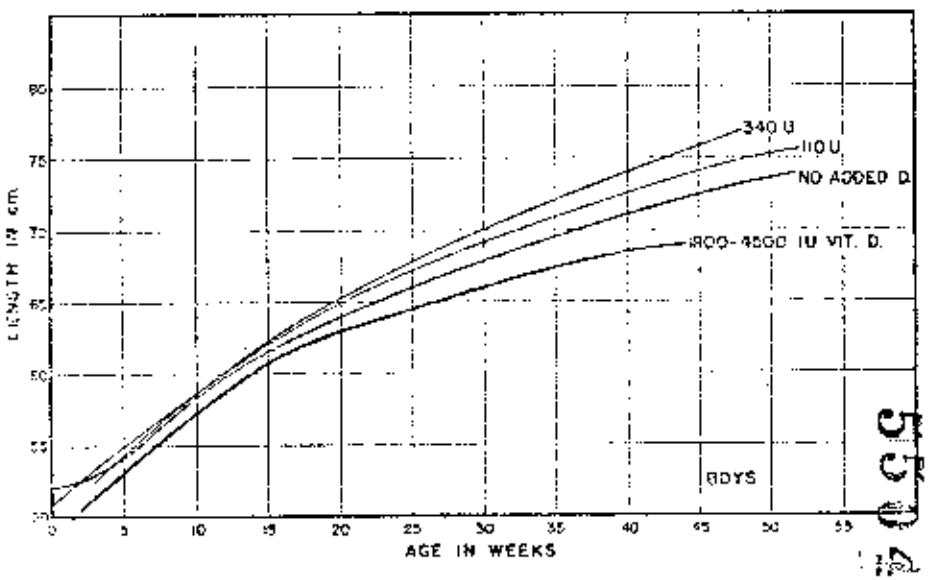
Our first studies necessarily were directed to the establishment of a basic dietary regimen, which we could feel was eminently satisfactory for the growth and development of artificially fed infants. The basic feeding chosen was of soured whole milk, a feeding developed first as a dysentery preventive in an era when refrigeration was nonexistent in poor homes. This feeding permitted growth and development equal to that of breast-fed infants, and was far better than feedings of condensed milk, the only other known safe, nonrefrigerated feeding for infants. Condensed milk is nearly 50 per cent sucrose, and was diluted 1:8 to make it calorically equal to breast milk. It is not surprising that it provided insufficient nutrients for the rapidly growing infant.

One teaspoonful of a standard cod-liver oil, which contained 340 IU of vitamin D, was given each infant daily. All infants were closely housed to prevent additional vitamin D activity from sunlight. Supplemental iron-containing foods were added after the third or fourth month of age. After three years of trial of various minor modifications of the regimen, we had amassed data from a group of 35 infants, each studied for a minimum of six months under carefully controlled conditions.[3-8] We had as complete a file of data on each infant as we could devise; we even photographed each baby at monthly intervals to show his muscular achievement at the time, for Alfred Hess had commented on the flabby musculature of rachitic infants.[9]

Assembling our data for this standard group, we found their retention of mineral and nitrogen greater than those reported from random studies of breast-fed and other infants of various ages.[9] Linear growth was significantly more rapid than that reported for Iowa City infants in 1920[10] before the discovery of vitamin D. Rate of dentition was earlier than reported,[10] and muscular achievements were precocious. On reporting these findings, we were subjected to the criticism of raising "monsters," for the data necessarily had to be compared with those obtained from breast-fed infants not receiving vitamin D. It took several years to show that a selected group of healthy breast-fed infants, given equal amounts of vitamin D, grew and developed at the same rate as our artificially fed infants.[11] It is interesting that, although fashions in infant feeding have changed greatly, "well-born" babies today grow at the same rates as those established for our groups over 30 years ago.[12]

We then proceeded to the study of the different types and dilutions of vitamin D. Figure 1 shows the linear growth of several groups of Iowa infants. Those studied by Baldwin received no added vitamin D, but they probably got more of this vitamin from sunlight on the skin than infants in large cities where sunlight was obscured by soot and smoke. Yet babies getting 135-unit milk, and receiving only from 60 to 160 units of vitamin D daily, grew faster and none had rickets. Yeast milk and irradiated milk were equivalent when given in the same unitage; thus, the baby, as expected, behaved like other mammals and not like birds. Babies

Figure 1—Linear growth of well infants in relation to vitamin D intake. Data for the curve "no added D" were taken from Baldwin's study of Iowa City infants in 1920.[10]



given 340 units of vitamin D daily grew faster than those given 135-unit milk.[16] They also retained more calcium and phosphorus, had earlier dentition, and were more advanced in muscular achievement than those fed irradiated milk. The amount of vitamin D needed to prevent rickets, therefore, is about one-third the amount needed to permit more rapid growth and development.

The use of concentrated oily sources of vitamin D showed that the effect of a given unitage was always much less than the same unitage from more dilute sources. This fact, of course, is not true of the modern water-miscible concentrates of vitamin D which are very well absorbed.

In the mid-30's it was observed clinically that some healthy infants showed decelerated linear growth, beginning at about six months of age. Each of these babies had been getting from 2,000 to 3,000 units of vitamin D daily, a dosage customarily used along the eastern seaboard where rickets had been rampant and where early experiences with cod-liver oil had been unsatisfactory. From our own results with closely housed infants who received little or no stray vitamin from sunlight, we felt that such high dosages were unnecessary and might be deleterious. Few believed us, and the high doses were far more commonly used than our moderate dosage. In view of possible permanent effects on growth, we felt the effect of these higher dosages must be studied. With careful watching, and continuous scrutiny of data obtained, it seemed such studies could be stopped before serious or permanent damage occurred, if such damage did indeed occur.

Altogether, 22 infants were given either milk containing 2,000 units of vitamin D homogenized in it per quart, or 1,500 units given daily as an oily concentrate. The mean rate of linear growth of these infants is shown in the lower curve of Figure 1. The average size of the group was somewhat small at birth. But they grew very well to about four to five months of age, some of them to six months. On the average, a definite slowing became evident by about five months. The first three infants were carried on the high dosage to nine months of age; the slowing was marked. The remaining babies were given the high dosage until significant slowing of growth was noted for three consecutive two-week measurements. They were then returned to the 340-unit customary dosage. Each infant, including the first three, showed slow growth for a month or so after the high dosage was stopped then grew very rapidly until the length expected from their early growth was attained, at which time the infant was again considered normal. Because of the importance of these findings, the results of the first year's study of nine infants was reported immediately[71]; the remaining infants followed the same pattern, so further publication was omitted at the time.

The only other finding attributable to the high vitamin D dosage was consistent though mild falling off of appetite, so that the food intake of these babies was significantly less than usual.[72] No other symptoms of toxicity were noted. Appetite was regained when the D dosage was dropped.

Comparative Studies

Figure 2 shows the rates of linear growth noted by other investigators studying large outpatient groups of infants given varying amounts of vitamin D. All infants given 135-unit milk[15,16] showed the same rate of growth as the smaller group fed similarly. Macy's group[15] also fed from 250 to 600 IU of vitamin D to one group of infants; they grew at the same rate as our 340-unit group, and as a small group whom we fed 800 units daily.[17] Stokes et al.[16] reported that our outpatient group, given 1,500 units of vitamin D daily, grew at the same rate as those given 135-unit milk; i.e., a slower rate of growth than that of infants given from 340 to 800 units. Thus, the critical upper safe level for continuous intake of vitamin D must be somewhere between 800 and 1,500 units daily. Pentman and Higgons[18] reported a large series of infants from private practice who were given from 2,000 to 3,000 units of vitamin D daily. These babies were larger at birth than our group, grew very well to about 20 weeks of age, as ours did, then showed definite slowing of linear growth—not quite to the extent that our quantitatively studied babies did, but a significant slowing.

It is emphasized that the slowing of growth noted with excessive vitamin D dosage does not appear in most infants until about five or even six months of age (from 21 to 26 weeks). We concluded that "in order to determine the effect of the dietary regimen, growth should be studied well past six months of age, preferably during the greater part of infancy."[74]

Fomon and others[19] have reported no decrease in linear growth of infants during the earlier months of life when fed 1,320 to 9,170 units of vitamin D daily. Three groups of infants were studied, beginning at the eighth day of life and ending at the 168th day (5½ months). It would have been of real interest to know the rates of linear growth of each of these infants for another two to three months, with the high dosage of vitamin D continued.

Infants vary as much as older people, and we still need to know the amount of vitamin D that will produce mild toxicity in those infants most susceptible to such toxicity, and to learn more of the range of time in which such toxicity first appears. We have ample proof that excessive ingestion of vitamin D can be

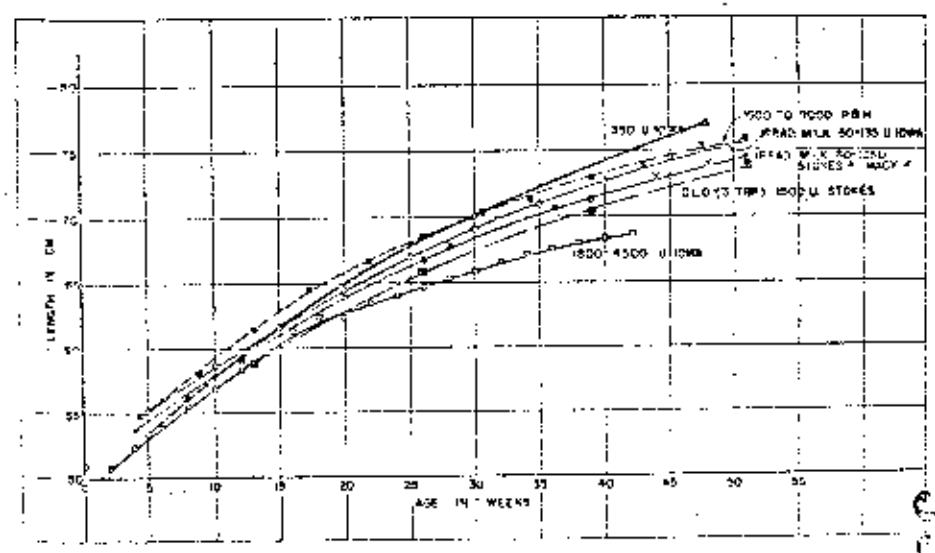
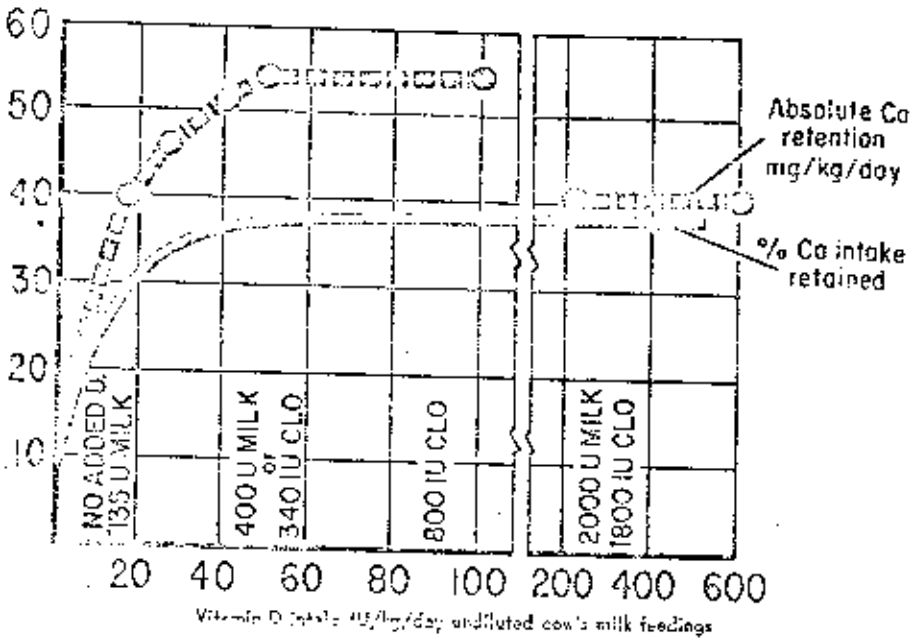


Figure 2—Linear growth of well infants in relation to vitamin D intake. Outpatient studies of others: irradiated milk (65-110 IU) from Macy's group,[17] also from Stokes, et al.[16] cod-liver oil, 1,500 IU daily, also from Stokes[16]; cod-liver oil, 1,000-3,000 IU daily, from Pentman and Higgons.[18] The Iowa data are shown for comparison.

Figure 3—[illegible]tion of calcium by infants in relation to the per kg daily intake of vitamin [illegible]. Data are shown as mg/kg/day retention and retention as percentage of intake. The total daily intakes of vitamin D are also given. Data for the "no added D" group are taken from the older literature.[9]



toxic and should be avoided; we also have ample proof that from 300 to 400 IU of vitamin D daily is sufficient—not merely for rickets prevention—but to provide for excellent growth and development. To give routinely from three to four times this optimal dosage seems scarcely unwise.

It is fashionable now to question whether slow growth is not better than fast. We all know that slow growth, under certain very strict conditions, can mark in greatly increased longevity.[29] Such increase, however, is attained at very great cost to an animal; namely, the marked shortening of the period of maturity. On the other hand, rats fed improved diets throughout life showed a marked lengthening of the period of vigorous maturity.[11] Well-fed animals, given vigorous exercise after growth has ceased, will show significant increase in the length of the period of vigorous maturity and a shortening of the period of senility. Man, in his own stumbling toward a better life, has progressed along these latter lines, we think correctly so. Animal studies have long shown us that any healthy young animal will grow at a rate determined by the nutrient which is in least supply. Human studies corroborate these findings. Neither will grow at full potential unless each nutrient is amply provided.[27]

Figure 3 gives the mean retention of calcium in mg/kg daily in relation to the mean vitamin D intake per kg[22] (dotted line). The lowered retention from the high vitamin intake can be due to lower milk intake, lowered absorption, or both. The solid line shows calcium retention in terms of per cent of intake; the same percentage of intake was retained with high dosage as with moderate dosage; the lowered retention, therefore, was wholly due to the lower intake of this group.

When the American Medical Association Council on Foods considered the addition of vitamin D to market and other milk, our total data were sent in to provide the quantitative information. Although our standard teaspoonful of cod-liver oil contained 340 units of vitamin D, the human error in measuring and administering was such that a range of from 300 to 400 IU might be more nearly true than a closer figure. The decision of the council to promote the addition of vitamin D to milk, and to limit its amount to 400 units per quart, was a great step forward in the prevention of infantile rickets with safety to all infants. The addition of this dosage to evaporated milk practically wiped out infantile rickets overnight in this country and improved the health and growth of all infants. The efficiency of this level of vitamin D in milk has been restudied recently and reaffirmed[23] as providing a safe dosage for growth and for rickets prevention.

Our studies of children over one year of age were planned to determine both the requirement of calcium as milk and the need for added vitamin D. Figure 4 shows the theoretical retention we expected for each age group during childhood in order to maintain well-calcified bone. Children from one to 12 years of age were given good diets including milk in amounts of 500, 750 and 1,000 ml daily, with or without the 300 to 400 IU of vitamin D daily. The figure shows the mean retention values observed with 500 ml of milk without added vitamin D and 1,000 ml of milk with the added vitamin, the adolescent and maximum curves excepted. The other retention curves fell between these limits rather regularly. We concluded that the average child needs added vitamin D during growth, for in each age group some children retained calcium poorly, when no additional vitamin D was given. Up to 12 years of age, 750 ml

Figure 4—Retention of calcium by children from one to 12 years old in relation to age and to the calcium and vitamin D intake. Mean retention values from 500 ml of milk daily without added vitamin D and from 1,000 ml of milk with 300-400 IU of vitamin D daily are shown against the "theoretical retention," as minimum and maximum mean values observed.

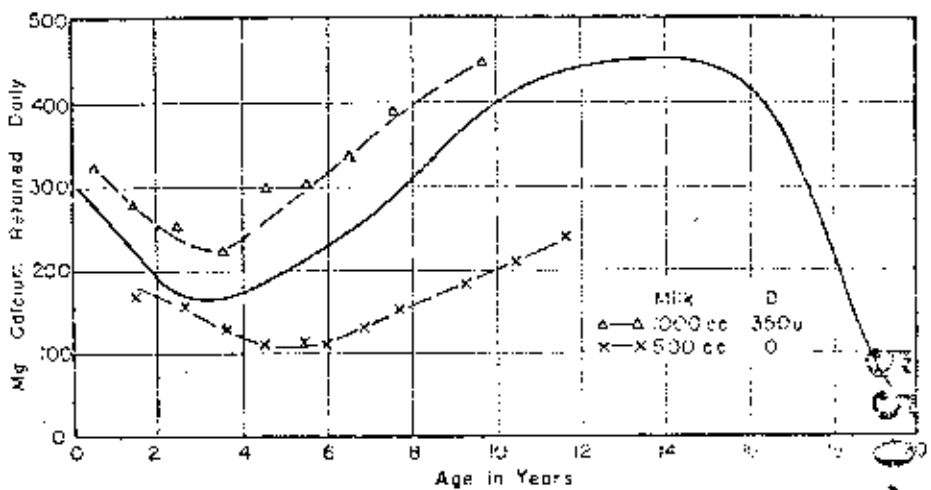
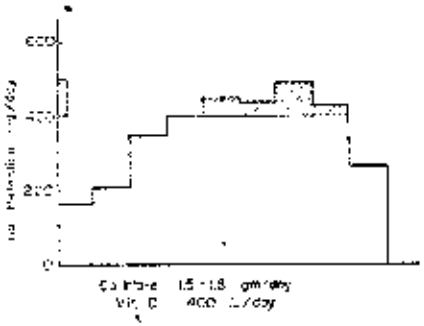


Figure 5—Calcium retention of adolescent girls from a relatively poor environment, given an excellent diet containing one quart of 400 IU vitamin D milk daily. Each block represents one month of study; the cross-hatched areas represent theoretically adequate retention. The mean calcium retention increased steadily for five months, remaining at an adequate level thereafter, until a deliberately imposed emotional stress the last month of the study resulted in sharply lowered retention.



(three glasses) of vitamin D fortified milk or its equivalent suffice for excellent mineral retention. During puberty and adolescence, up to 1,000 ml of vitamin D fortified milk may be necessary for good mineral retention. When other needed foods are eaten in sufficient quantity, at no age did we find evidence of damage from milk intakes to 1,000 ml daily.

Children who have been poorly fed for some time often cannot use a good diet efficiently as soon as it is given. The longer the period of malnutrition, and the older the child, the longer the period of recovery. Figure 5 shows the calcium retention of a group of adolescent children (11 to 16 years), whose former diet had not been good but was far better than the diet that many American children receive today. It was simple in all known nutrients, and its calcium and vitamin D content were kept constant for nine months. On the average, it took five months for the retention to reach a plateau. It was then maintained at that level for four months when an emotional problem, deliberately generated, had a sharply deleterious effect on retention. This delaying effect of previous diet on retention is not observed in pregnancy, but the deleterious effect of emotional distress may be even more devastating.

From our data we concluded that children who are well fed and protected from emotional distress can and do retain sufficient bone minerals to maintain a well-calcified skeleton throughout growth. Therefore, the "physiological osteoporosis," which Wingate Todd observed during the thirties in the adolescents he studied, is not truly physiological but nutritional, and can and should be avoided.

Summary

The end of our 25-year study saw the end of infantile rickets, but not the end of excessive intake of vitamin D. At present, far more babies in America are being overdosed with both vitamins A and D than those receiving inadequate intakes of these vitamins. Breakdown and excretion of both these vitamins are slow, and they can easily increase to toxic levels. Children, on the other hand, often are being refused milk intakes adequate to permit good mineral retention for fear of a damage never shown to occur in a healthy child.

REFERENCES

1. Daniels, A. L.; Stearns, G.; and Hutson, M. K. Calcium and Phosphorus Metabolism in Artificially Fed Infants. I. Influence of Cod Liver Oil and Fat Upon [illegible]. Am. J. Dis. Child. 37:[illegible] (Feb.), 1929.
2. [illegible], P. G. Vitamin D Milk. J.A.M.A. [illegible] (June 13, 29), 1936.
3. [illegible], P. G. and Stearns, G. Growth and Retention of Calcium, Phosphorus and Nitrogen of Infants Fed Evaporated Milk. Am. J. Dis. Child. 46:[illegible] (July), 1933.
4. ———, Effectiveness of Vitamin D in Infancy [illegible] Prog. Soc. Exper. Biol. & Med. 31:[illegible], 1936.
5. ———, Retention of Calcium by Infants Fed Evaporated Milk Containing Cod Liver Oil Concentrate. [illegible] (June), 1938.
6. [illegible], P. G.; Stearns, G.; McKinley, J. B.; Goff, E. A.; and [illegible]. Factors Possibly Influencing the Retention of Calcium, Phosphorus and Nitrogen by Infants Given Whole Milk Feedings. J. Pediat. 8:403 (April), 1936.
7. Pirquet, Baron C. F. Nutrition and Health. Philadelphia, Dan Lea & Febiger, 1939.
8. [illegible], A. Dr. and Keller, A. Der Kinder Ernährung, Ernährungs-Störungen und Ernährungs-Therapie. Leipzig, Johann Ambr. Barth, 1932.
9. [illegible], B. T. Physical Growth of Children from [illegible] Months. University of Iowa Studies in Child Welfare, 1, No. 1 (Jan.), 1931.
10. [illegible], T. Dr. and Stearns, G. Ion Balance of Vitamin D as the Age of the Infant at the Time of Operation of the Ion Deficiency Period. J. Pediat. [illegible] (Feb.), 1939.
11. [illegible], G. Growth of Selected Groups of Breast Fed Infants in Iowa City. Pediatrics 11: 30 (Jan.), 1953.
12. [illegible], G.; Wonderlich, R.; Ilgar, M.; and [illegible], T. Dr. and Lewis, N. P. Growth of "Well Baby" Artificial Infants Fed Human and Cow's Milk. Pediat. 21:[illegible] (Mar.), 1941.
13. Stearns, G.; Jeans, P. C.; and Vandecar, V. The Effect of Vitamin D on Linear Growth in Infancy. J. P. Pat. 9:1 (July), 1936.
14. Jeans, P. C. and Stearns, G. The Effect of Vitamin D on Linear Growth in Infancy: The Effect of Intakes Above 1500 USP Units Daily. J. Pediat. 13: [illegible] (Nov.), 1938.
15. Sieber, F.; Hamil, B. M.; Poole, M. W.; Conley, T. B.; and Mace, J. G. Relationship Between Vitamin D Intake and Linear Growth in Infants. Proc. Soc. Exper. Biol. & Med. 33:[illegible] (Dec.), 1937.
16. Shank, T. Dr. Report, Mrs. Kennedy, Mr. and Jeans, P. C. Studies in the Growth and Development of Milk Children Receiving Evaporated Milk. The Effect of Various Vitamin Supplements on Growth in Length and Incidence of Rickets During the First Two Years of Life. J. Pediat. 19:169 (Aug.), 1941.
17. Jeans, P. C. and Stearns, G. Unpublished [illegible].
18. Drummond, J. C. and Hilmes, T. A. Growth [illegible] Child in the Age of Five Years. A Study of Children Based Upon Pediatric and Clinic Cases. Am. J. Dis. Child. [illegible] (Feb.), 1936.
19. Fierson, [illegible]; Rosenberg, M. Kit and Fierson, [illegible]. Influence of Vitamin D on Linear Growth of Normal Pediatric Infants. J. Nutrition [illegible] (Dec.), 1936.
20. Mellby, C. Mae Maxwell, L. A.; Spellman, [illegible]. Growth, [illegible] Daily Vitamin D and Age Changes in the Affect Diet After Feeding Diets Restricted in Calories. Pediat. 18, [illegible].
21. Chenoweth, H. C. Calcium and Phosphorus in Foods and Nutrition. New York, N. Y.: Columbia University Press, 1947, p. 65,113.
22. Stearns, G. Infant Metabolism. Proceedings of the World Health Organization's Seminar Held at Leyden and Scheveningen, October-November, 1950. New York, N. Y.: Macmillan, 1956, p. 84.
23. Report of Committee on Nutrition, American Academy of Pediatrics. The Prophylactic Requirement and Toxicity of Vitamin D. Pediatrics 31: 532 (March), 1963.

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TABLE I—*Continued*

No.	1000 units/K/day	Days	Kidney Mg Ca/100 gm. Dry Tissue	Max. Blood Ca	Microscopic: Cell. Degen.	Microscopic: Ca Stain	Wt. Loss Per Cent	Other Symptoms of Toxicity	
39	25	70	—	—	—	—	18	?	Good condition when killed
40	25	79	—	—	—	—	8	Moderate	Found dead
41	25	50	—	—	—	—	22	Severe	Died in coma
42	25	56	—	—	—	—	5	Severe	Died in coma
43	25	16	—	—	—	—	20	Severe	Died of distemper
44	20	38	228	13.26	0	0	0	0	Good condition when killed
45	20	41	186	12.90	0	0	3	0	Good condition when killed
46	20	67	174	11.78	0	0	5	Mild	Good condition when killed
47	20	83	203	12.02	0	0	0	0	Good condition when killed
48	20	120	93	11.00	0	0	+7	0	Good condition when killed
49	20	60	—	—	—	—	0	Mild	Died of distemper
50	20	93	—	—	—	—	0	0	Good condition when killed
51	20	80	—	—	—	—	7	?	Allowed to recover
52	20	55	—	—	—	—	0	0	Good condition when killed
53	20	40	—	—	—	—	0	0	Good condition when killed
54	15	62	212	16.85	?	0	5	Slight	Died from distemper
55	15	136	147	11.83	0	0	0	Slight	Good condition when killed
56	15	70	86	12.15	0	0	0	0	Good condition when killed
57	15	153	262	10.80	0	0	+16	0	Good condition when killed
58	15	56	248	11.50	0	0	0	0	Good condition when killed
59	15	61	—	—	—	—	+12	0	Good condition when killed
60	15	61	—	—	—	—	0	0	Good condition when killed
61	15	67	—	—	—	—	0	0	Good condition when killed
62	15	90	—	—	—	—	+10	0	Good condition when killed
63	15	47	—	—	—	—	0	0	Good condition when killed
64	15	30	—	—	—	—	5	0	Good condition when killed

Early experience with impure preparations of vitamin D, particularly abroad, has led to a great deal of misunderstanding and fear of overdosage on the part of those who have had little acquaintance with the fundamental mechanisms involved. This point has been adequately discussed by Bills.[9] Suffice it to say that most of this earlier work must be disregarded when considering the effects produced by the highly purified preparations now available.

EXPERIMENTS ON TOXICITY OF VITAMIN D FOR DOGS

The effects of massive doses of vitamin D must be judged on the basis of the dose per unit body weight and not on the absolute size of the dose. When considered in this light the order of increasing susceptibility appears to run as follows: rat, dog, human, rabbit, with little difference between the dog and human, while the rat is very much more resistant, and the rabbit much less so.

In the experiments on dogs, vitamin D was administered in the form of a solution of activated ergosterol in corn oil * (1,000,000 units per gm.) or of calciferol † dissolved in corn oil. Most of the administration was done orally. A few animals received intravenous injections in which form the material is slightly more effective. The results of experiments on 64 adult healthy dogs are shown in table 1. The daily dose ranged from 15,000 to 500,000 units per kilogram of body weight as shown in the first column. An effort was made to adjust the dose to decreasing body weight so that the ratio between the dose and the weight of metabolizing tissue remained fairly constant.

The figures in the second column represent the actual number of days of administration of the vitamin D and, therefore, the number of days the animals *survived the treatment*. Usually those that did not die were killed on the day following the last dose, others within two to three days. With amounts greater than 50,000 units daily the average survival period was 12 days; with amounts between 20,000 and 50,000, 39 days; and with 20,000 units or less, 68 days.

At death, tissues were taken for chemical analysis and for microscopic examination. In an earlier report[10] on analyses of 13 different tissues in a series of dogs, it was shown that the kidney is the most vulnerable of any tissue to the calcifying action of vitamin D. In order to conserve space, only the figures for the calcium content of the kidney are included. In all other tissues the findings were very inconstant. Analyses of tissues of normal dogs have shown a calcium content in the kidneys ranging from 29 to 301 mg. per 100 gm. of dried tissue with a mean of 85 mg. The calcium content in the kidneys of 33 of the 64 dogs is shown in the third column of table 1.

With a daily dose above 50,000 units per kg. the average content of calcium in the kidney was 564 mg. per 100 gm. of dried tissue; between 20,000 and 50,000, 921 mg.; and with 20,000 units or less, 183 mg. The lower average in the first group may possibly be related to the shorter period of survival. The average in the third group is obviously lower because the dose was not great enough to cause as much deposition.

In the fourth column the figures represent the maximum concentration of

* Supplied mainly by Mead Johnson and Company, also by Abbott Laboratories, Parke Davis and Company, and Winthrop Chemical Company.

† Supplied by Mead Johnson and Company, Winthrop Chemical Company, and Glaxo Laboratories.

plasma calcium found at any time. The level was not constant in any group. Very high levels were seldom constantly maintained. In figure 1 is shown the distribution of the figures observed in dogs in relation to the daily dose of vitamin D. It is obvious that there is little correlation between the two factors. With daily doses greater than 20,000 units per kg. every animal at some time showed very high concentrations, but the level fluctuated greatly in each animal. With lower doses the figures are within the normal range for heparinized plasma.

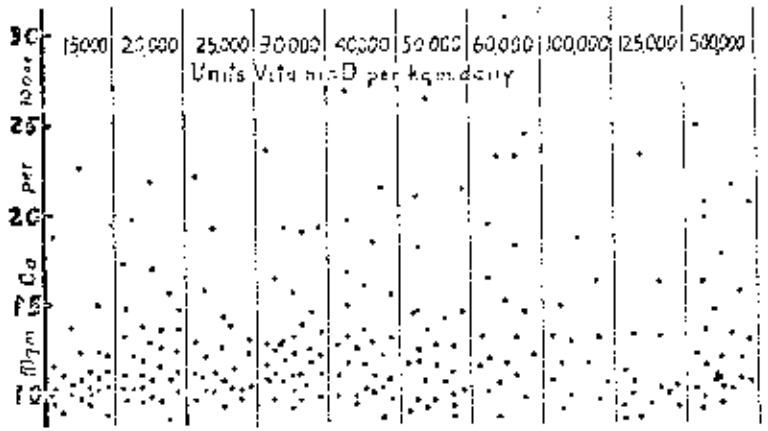

FIG. 1. The blood calcium in 43 normal dogs under treatment with vitamin D. In each column are shown the total number of determinations made on all in each dosage group. Successive determinations on the same dog appear on the same ordinate.

For purposes of simplicity of recording and comparing the microscopic findings, a scale of 1 to 5 arbitrary units was used, although this method is open to some objection. The only striking microscopic findings were degeneration of individual cells and the presence of deeply stained areas of calcium deposition. In recording the degree of cell injury, a unit of 1 indicates the presence of only occasional degenerated cells, two or three in a single field. A unit of 5 indicates a stage in which considerable masses of contiguous cells had undergone degeneration. However, this stage always occurred in conjunction with a comparable stage of calcium deposition. It will be seen that the relationship between the degrees of cell destruction, calcium deposition and calcium content of the tissue varies considerably, but that in general those tissues having the higher calcium content by analysis showed the more extensive injury. These injuries appeared first and were most numerous in the collecting tubules but later were extended quite generally throughout the organ. In only one animal were calculi found in the tubules (No. 21, table 1).

It appears that cellular injury occurs first and that calcium deposition then takes place at the sites of these injuries. In four animals (Nos. 7, 18, 27, 31) showing extensive damage to the kidney, there was no evidence of

cellular injury nor of excessive calcium deposition in any other tissue examined.

From evidence obtained on 12 other animals not included in the table, it appears that considerable loss of weight may occur before there is any evidence of injury to cells, but this was not invariably the case.

Our observations do not confirm those of Appelrot[11] to the effect that hypervitaminosis D produces medial thickening in arteries. In only four of the 64 dogs examined, i.e., numbers 18, 21, 27 and 29, was this observed. In each case there was some medial thickening in the aorta and occasionally in smaller arteries.

Loss of weight was marked in 36 out of the 43 dogs receiving more than 20,000 units per kg. per day. One retained constant weight, one gained 17 per cent. As previously suggested,[12] the early weight loss appears to be due mainly to impoverishment of fat depots. When dog No. 28 was examined at autopsy it was almost impossible to find any macroscopic fat deposits. This dog lost 48 per cent of the original weight, yet showed only mild objective symptoms of toxicity and was lively and active at the termination of the experiment. There was practically no microscopic evidence of cell injury. The plasma calcium was maintained at a higher average level over the period of the experiment than in any other animal in our experience.

On the other hand, dog No. 21 lost only 15 per cent of the initial weight, although the kidney content of calcium was higher than in any other animal. These two animals serve to show clearly that weight loss is not an inevitable accompaniment of fatal intoxication in dogs.

One of the characteristic features of fatal hypervitaminosis D is the premortal coma. This condition is usually, though not always, preceded by partial paralysis, slow, shallow respiration, fine, thready, rapid pulse, salivation, and often by psychic changes of such a nature that a previously tame, friendly dog may become unmanageable and even vicious. Very often the symptoms resemble those following an injection of oil of wormwood. This condition may persist for several days but usually appears from two to three hours before death. It is probable that all of the dogs labelled "found dead" passed through this stage during the night, but the actual train of symptoms could not be observed. None of the animals that were killed were in this stage.

The objective symptoms of toxicity were much the same as those previously described for the human,[1] such as weakness and lassitude, anorexia, polydipsia, polyuria, psychic disturbances, diarrhea. Bloody feces were passed by 11 of the 64 dogs. In addition, petechial hemorrhages were found in the mucosa of the stomach and intestines at autopsy.

With eight exceptions all of the 43 dogs receiving more than 20,000 units per kg. per day died spontaneously. Nine of these died from distemper. Of the nine, three were in such a condition that early death in coma was predictable. Of the eight exceptions, one was allowed to recover. Of

the seven that were killed, two would probably have died. The other five would probably have recovered with cessation of the treatment.

Among the 20 dogs receiving 20,000 units or less per kg. there were no evidences of cell injury, insignificant weight loss, very little evidence of toxic symptoms, and with the exception of two dogs that died from distemper and one that was allowed to recover all were in good condition when killed.

Thus, it may be concluded that vitamin D up to 20,000 units per kg. per day for periods ranging up to 153 days is not seriously injurious to normal dogs. In greater amounts there is a wide range of susceptibility entirely unpredictable from any data at present available.

That the toxic effects may be characterized as true hypervitaminosis D is proved by the fact that in four instances (numbers 7, 17, 29, 34) the vitamin preparation administered was crystalline calciferol (40,000,000 units per gm.) in solution in corn oil. This preparation contained no toxic by-products and yet the results were quite comparable with those from the same dose of activated ergosterol which may contain some inert material but practically no toxisterol.

In another series of experiments the dogs were brought to a stage of extreme toxicity with vitamin D and the administration was then discontinued. The state of toxicity was manifested by loss of weight, anorexia, listlessness and paralysis, and, in six animals, prostration. After varying intervals, when there was objective evidence of complete recovery, the animals were killed and subjected to the same examination as in the other series. The results are shown in table 2. In the first column are shown the periods

TABLE II

No.	Days	Recovery Days	Units/K/Day	Kidney Ca	Max. Blood Ca	Microscopy: Cell. Degen	Microscopy: Ca Stain	Wt. Loss Per Cent	
1	24	126	15,000	257	18.59	0	0	6	Good condition
2	15	9	20,000	48	19.59	0	0	13	Good condition
3	80	102	20,000	212	17.11	0	0	7	Good condition
4	10	48	25,000	323	22.13	0	0	30	Good condition
5	5	113	30,000	240	19.14	0	0	43	Good condition
6	5	107	35,000	242	19.43	0	0	30	Good condition
7	35	20	50,000	151	16.87	0	0	10	Good condition
8	18	115	50,000	272	14.62	0	0	29	Good condition
9	26	38	50,000	300	19.30	0	?	45	Fair condition. Still 15 per cent under weight
10	15	8	105,000	218	18.72	—	—	30	Fair condition. 10 per cent under weight

of administration of the vitamin, in the second the daily dose per kg., and in the third the recovery interval allowed. Actually in the dogs No. 4, 6, 7 and 8 complete recovery of weight was accomplished in 53, 86, 73 and 60 days respectively. Animals No. 9 and 10 were killed before weight

recovery was complete because both were definitely overweight when the experiments were begun.

Eight additional dogs were originally included in this series but all of these were in the terminal stages of toxicity described above when the treatment was discontinued, and all died within two to seven days. Six of these were already prostrate when administration was discontinued.

In all but one (dog No. 4) the kidney content of calcium was within the normal range, although all showed figures in the higher limits of the normal range except Nos. 2 and 7. The average weight loss approximated that in comparable dosage ranges in the first series.

The maximum concentrations of plasma calcium observed were all high. Microscopic examination of the tissues showed no definite evidence of calcium deposition and no cellular injury. In Nos. 2 and 5 there was some distortion of the collecting tubules in the kidney by fibrosis. No vascular lesions were found.

From these experiments it appears that dogs may recover from extreme stages of toxicity and that whatever tissue injury occurs may be reparable. In extremes the result may be fatal before repair can be effected.

In the experiments on animals reported by others, so far as the potency of the dose can be calculated, toxicity has occurred only with doses above 20,000 units per kg. per day. Since the relation of dosage to weight has not been stressed before, many other workers have failed to record data which would make it possible to calculate the dose in terms of units per kilogram.

In both of our series it is apparent that the total amount of vitamin administered is not the most important determinant of the degree of toxicity since large doses have been tolerated well over long periods while smaller doses have produced drastic effects in a few days. Nor is the magnitude of the daily dose the sole factor. In general both dogs and human subjects appeared to be less sensitive during winter months.

TABLE III

Dog No.	1	2
Units/kg./day	60,000	30,000
Initial plasma Ca	11.60	10.89
5th day	12.03	14.62
7th day	12.00	13.90
14th day	14.00	12.44
15th day	11.30	11.79
16th day	12.57	11.12
21st day	12.10	12.15
22nd day	11.34	..
23rd day	12.00	13.25
24th day	dead	—
26th day		12.24
30th day		9.70
31st day		13.00
32nd day		11.93
33rd day		dead

It has been emphasized many times that the diet is a very important factor in conditioning toxicity. All of these animals were kept on the stock kennel diet throughout and no variations were introduced at any time.

It is also apparent that the concentration of plasma calcium is not closely correlated with toxicity. Many investigators have been inclined to use the terms "hypercalcemia" and "toxicity" interchangeably. Many experiments have been done which clearly disprove a causal relation between these conditions but the two experiments reported in table 3 will serve to illustrate the point. Both of the animals showed some hypercalcemia, but it was by no means pronounced. Number 1 received twice the dose of number 2, but except for the earlier death there was no striking difference. When one contrasts these figures with the high figures for some of the animals that survived as in tables 1 and 2, it is a fair assumption that hypercalcemia per se is not the cause of toxicity.

Observations on Human Subjects

The enormous absolute doses of vitamin D that have been administered to human subjects have naturally aroused some question. If, however, one bears in mind that human and canine susceptibility seem closely approximated, and if one gives due consideration to the weight/dosage relationship, it appears that few of the human subjects have ever received amounts comparable to the highest doses tolerated by the dogs of this series.

Table IV

	Male			Female		
	Total No.	No. Toxic	Per Cent	Total No.	No. Toxic	Per Cent
Postoperative tetany	2	0	0	15	4	26.4
Hay fever and asthma	178	13	7.3	322	24	7.4
Arthritis	43	5	11.6	107	11	9.4
Miscellaneous	12	1	8.3	23	3	13
Normal subjects	63	1	1.5	8	1	12.5
	298	20	6.7	475	43	9

Total subjects 773
Number toxic 63
Per cent toxic 8

Table 4 shows the status of 773 human subjects who have received amounts of vitamin D above 100,000 units daily. The doses *routinely* given ranged upward from 200,000 units total daily dose for periods ranging from seven days to five years. With such a dose a 50 kilo subject would receive only 4,000 units per kg. per day. With 300,000 units a 60 kg. subject would receive 5,000 units per kg. per day. This table, of course, does not take into account the *varying* dosages, so that one cannot draw conclusions as to comparative susceptibility in the various groups of subjects. However, it appears, from other statistics too voluminous to include here, that the order of decreasing susceptibility among the different groups of patients is: arthritis, normal subjects, hay fever alone, hay fever with asthma, tetany.

Table V

Units/kg./day	No.	No. Toxic at any Stage	Per Cent Toxic
1500–3000	3	5	100
3000–5000	555	25	4.5
6000–7000	123	18	14.6
8000–15,000	70	11	15.7
15,000–25,000	16	3	18.8
25,000–35,000	4	1	25
	773	63	8+

In table 5 the incidence of toxicity at each range of dosage is shown. This analysis does not take into account the duration of administration since, as was indicated by the experiments on dogs, the total amount does not seem to be the most important factor. Nor does it take into account the effect of simultaneous administration of yeast as a protective measure which was done with many human subjects. This procedure has already been discussed rather fully.[4,6] The first five subjects may be disregarded since they became nauseated from corn oil as readily as from viosterol, so that they do not represent true hypervitaminosis D, but rather some kind of sensitivity not related to the vitamin. It is probable that some of those subjects included in the toxic groups at higher dosage were of this type. The shortest period of administration that produced intoxication in the group on 3,000 to 5,000 units per kg. per day, was 87 days. Since the condition in this instance developed very suddenly and without any weight loss, it is possible that some unrecognized disturbance rendered the subject temporarily more susceptible. On discontinuance of administration of vitamin D prompt recovery occurred, and four days later the treatment was resumed and continued four months without further disturbance.

In the group on 6,000 to 7,000 units per kg. per day the shortest period for development of toxicity was 60 days. In this instance there was a loss of four pounds in two days. The condition was abated sufficiently to resume only after eight days without treatment. A second mild intoxication occurred three months later.

In the last two groups the high doses were not continued beyond 10 days except in one case, regardless of whether intoxication occurred. In this case one of the authors, a normal subject, took 35,000 units per kg. per day (3,000,000 units total daily) for 15 days without any evidence of disturbance of any kind.

Vrtiak and Lang[7] have recently reported 100 per cent incidence of toxicity in a series of 22 human subjects to whom massive doses of vitamin D

were administered. The discrepancy between this high incidence and the relatively low incidence in our series is difficult to explain at present.

The symptoms of hypervitaminosis D in human subjects were described fully in an earlier paper.[9] It remains now to correlate these findings with the experimental results on animals. Tentatively we suggest that the course of events is as follows: First, cellular degeneration occurs, more commonly in the kidney. Concurrently there may be loss of weight and other objective symptoms. If weight loss occurs before other symptoms it is probably due solely or mainly to increased fat catabolism. Second, deposition of calcium occurs at the sites of cell injury; apparently this does not occur except as a secondary result of such injury. Third, up to advanced stages of toxicity these processes may be reparable in dogs if the vitamin D administration is discontinued.

From the results of previously reported work[10,12] it appears that the calcium removed from the tissues during recovery is excreted in the urine. At least, repair is not complete until the urine calcium excretion becomes normal.

The increased excretion of calcium that usually takes place[12,13] under massive administration of vitamin D in both human subjects and dogs is not, however, due solely to removal of the microscopic deposits in the soft tissues, because the increase in the urine begins before there is any microscopic or chemical evidence of excessive deposition in soft tissues. The source of this initial increase in the urine has not yet been determined, but from Shelling's discussion it probably comes from the trabeculae of the bones. Generally the average level of blood calcium is decreased after the excretion is increased.

We have made no examination of blood pressure in dogs, but a large number of the human subjects were examined at frequent intervals over long periods. Since there have been no significant changes the data are omitted. In most instances there seems to be some tendency to a slight decrease in the general level. This, of course, does not eliminate the possibility of medial proliferation in the arterioles.

Our human subjects ranged in age from 17 to 76 years. Older subjects were generally less readily intoxicated but recovered less readily when intoxication did occur, and seemed to be somewhat more sensitive thereafter.

There are on record only two instances of death in human subjects, certainly due to hypervitaminosis D, since the more highly purified preparations became available. One of these, recently reported by Thatcher,[11] was probably a case of idiosyncrasy to vitamin D. It is difficult to determine from the report the unit dosage. However, it *is* clear that the administration was continued after intoxication was markedly developed.

We wish to stress that administration of vitamin D should be discontinued at once when the symptoms of intoxication appear. Neither animal nor human subjects in our experience have ever recovered from the toxic condition while administration continued.

The other case has not yet been reported in detail in the literature, but the reports of the coroner and the attending physician[15] reveal the following facts. A physician, aged 74, weight 290 pounds, undertook self medication with a concentrated solution of activated ergosterol. Owing to an *error* in calculation of the dosage he received 2,300,000 units daily for 18 days or approximately 18,000 units per kg. per day, a dose 10 times that intended. Since he was very obese the dose per kilogram of actively metabolizing tissue was much greater. The symptoms described were quite typical of hypervitaminosis D, with hypercalcemia, so that there is no doubt that in this case the treatment was the immediate cause of death. However, the presence of generalized arteriosclerosis suggests that this was a fundamental handicap to his recovery after discontinuing the treatment.

The administration of similar or larger amounts in our series without serious disturbance should not be interpreted to mean that such treatment can be undertaken without caution. In fact, our experience indicates clearly that administration of massive doses of vitamin D should not be undertaken for any cause except under the careful supervision of a physician who can and will carefully check the patient's condition at frequent intervals and who will see to it that the treatment is discontinued promptly on the appearance of the first signs suggestive of toxicity.

It is probable that any suggestion of kidney dysfunction should constitute an absolute contraindication. Until further information is available arteriosclerosis also should probably be considered a contraindication. Consequently, this form of treatment should be administered to older subjects only with extreme caution.

Nevertheless, if these precautions are observed, massive doses of vitamin D may be utilized therapeutically as safely as many other agents administered daily. That its misuse has resulted in death should not prejudice its controlled use under circumstances of possible value.

In view of the extensive experience in administration of vitamin D to human subjects with a relatively low incidence of toxicity, and the correlation of the results of animal experiments with the observations on human subjects, we believe that the burden of proof now rests on those who maintain the undesirability of the use of this form of therapy. Its actual practical value in particular clinical conditions will, of course, be determined only by more extensive clinical experience.

It must be admitted that the mechanism of toxicity is still unexplained. Our findings do not agree entirely with those of Ham and Lewis.[18] It is possible that the dose of approximately 600,000 per kg. per day or more, which these observers administered to rats, was a factor in producing a different type of lesion. Up to the present time conceptions of the physiology of vitamin D have perhaps been too circumscribed because of its striking effect on calcium metabolism. That the thyroid plays an important part in the action of vitamin D is indicated by the results of another investigation.[7]

Also, it appears that the pathologic effects are greatly accentuated in a hypothyroid state. This may be an important factor in the variability in sensitivity to intoxication.

The results obtained by Gelfan [illegible] on isolated frog muscle suggest that vitamin D exercises a catalytic effect in peripheral tissues. If this should be confirmed, it might serve as a forward step in explaining the injury to isolated cells in peripheral tissues. It is conceivable that the metabolism of individual cells might be accentuated to such a point as to result in the disintegration of the cells themselves. We have not been able to recognize the cellular changes preliminary to disintegration so that we can offer no explanation of the actual nature of this process.

Summary and Conclusions

1. Observations on 64 dogs and 773 human subjects receiving massive doses of vitamin D have been made and data recorded as to dose per unit of body weight, and on the nature of the process of intoxication.

2. Both human subjects and dogs generally survive the administration of 20,000 units per kilogram per day for indefinite periods without intoxication.

3. Hypervitaminosis D first produces cell injury followed by calcium deposition. This process is reversible and reparable if administration is discontinued promptly.

4. Intoxication for short periods does not result in any permanent injury that can be recognized by the methods employed in this investigation.

Addendum. After this manuscript was written there came to our attention a very comprehensive study by Cowdry and Scott [19] on normal monkeys under treatment with vitamin D in which it was suggested that pathological changes may occur in tissues without clinical symptoms. However it was also suggested that there might be a species peculiarity.

Bibliography

1. Reed, C. I., and Steck, I.: The treatment of clinical tetany with irradiated ergosterol. Endocrinology, 1933, xvii, 136–148.
2. Rappaport, B. Z., and Reed, C. I.: Viosterol of high potency in seasonal hay fever and allergy, Jr. Am. Med. Assoc., 1933, ci, 105–109.
3. Rappaport, B. Z., Hathaway, M. L., Reed, C. I., and Struck, H. C.: The treatment of hay fever and asthma with viosterol of high potency. Jr. Allergy, 1934, v, 541–553.
4. Steck, C. I.: The symptoms of viosterol overdosage in human subjects, Jr. Am. Med. Assoc., 1934, ciii, 1745–1748.
5. Dreyer, L., and Reed, C. I.: The treatment of arthritis with massive doses of vitamin D, Arch. Phys. Therapy, 1935, xvi, 537–540.
6. Crimm, P. D., et al.: Vitamin therapy in pulmonary tuberculosis, Am. Rev. Tuberc., 1932, xxvi, 112–123; *Ibid.*, 1933, xxviii, 202–216; Am. Jr. Med. Sci., 1934, clxxxvii, 557–562; Jr. Lab. and Clin. Med., 1934, xix, 966–971.
7. Vernan, H. G., and Lane, R. S.: Observations on the treatment of chronic arthritis with vitamin D, Jr. Am. Med. Assoc., 1936, cvi, 1162–1163.
8. Sherling, D. H.: The parathyroids in health and disease, 1935, C. V. Mosby Co., St. Louis, Mo.
9. Bills, C. E.: Physiology of the steroids, including vitamin C, Physiol. Rev., 1935, xv, 1–97.

10. Reed, C. I., Dillman, L. M., Thacker, E. A., and Klein, R. L.: The calcification of tissues by excessive doses of irradiated ergosterol, Jr. Nutr., 1933, vi, 371–381.
11. Arthurot, S.: Hypervitaminosis D and blood pressure in dogs. Am. Jr. Physiol., 1933, cv, 294.
12. Reed, C. I., Thacker, E. A., Dillman, L. M., and Welch, J. W.: The effects of irradiated ergosterol on the metabolism of normal dogs. Jr. Nutr., 1933, vi, 355–370.
13. Hathaway, M. L., Rappaport, B. Z., Reed, C. I., and Struck, H. C.: A study of blood constituents in pollerosis with and without treatment. Jr. Allergy, 1936, viii, 1–21.
14. Thacher, L.: Hypervitaminosis D, Lancet, 1936, i, 20–22.
15. Kerr, W. J., and Cara, J. L.: Personal communications.
16. Ham, A. W., and Lewis, M. D.: Experimental intimal sclerosis of the coronary arteries of rats, Arch. Path., 1934, xvii, 356–361.
17. Deutsch, H., Reed, C. I., and Struck, H. C.: The rôle of the thyroid in the calorigenic action of vitamin D, Am. Jr. Physiol., 1936, cxii, 1.
18. Gelfan, S.: The effect of viosterol upon oxygen consumption of frog's muscle, Am. Jr. Physiol. 1935, cxiii, 464–476.
19. Cowdry, E. V., and Scott, G. H.: Effect on monkeys of small doses of a concentrated preparation of viosterol, Arch. Path., 1936, xxii, 1–23.

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Intestinal Calcium Transport: Stimulation by Low Phosphorus Diets

Abstract. *Rats maintained on a low phosphorus diet supplemented with 25-hydroxyvitamin D_3 show high intestinal calcium transport activity as compared to rats similarly treated but fed a diet containing adequate phosphorus. This increased transport activity is correlated with an increased biosynthesis of 1,25-dihydroxyvitamin D_3, the probable metabolically active form of the vitamin in the intestine.*

Vitamin D must be hydroxylated in the C-25 position by the liver (*1, 2*) and in the C-1 position by the kidney (*3*) before it can function to stimulate both intestinal absorption of calcium (*4*) and mobilization of bone calcium (*5*). In addition, strong evidence indicates that the resulting 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] does not require further metabolic alteration before it initiates both intestinal calcium absorption and bone calcium mobilization (*6*). Therefore, it appears that 1,25-$(OH)_2D_3$ is at least one of the hormonal forms derived from vitamin D responsible for the mobilization of calcium from these two organs. This concept was strengthened by the find-

Table 1. Stimulation of intestinal calcium transport and accumulation of 1,25-$(OH)_2D_3$ in blood and intestine of rats fed a low phosphorus diet. Weanling rats were fed a vitamin D–deficient diet containing 1.2 percent calcium and either 0.1 or 0.3 percent phosphorus for 2 weeks. At that time the rats receiving 0.1 percent phosphorus weighed 76 ± 6 g (24 rats) and those receiving 0.3 percent phosphorus weighed 88 ± 10 g (20 rats). At this stage a third of the rats in each group received 130 pmole of 25-OHD_3 orally each day in cottonseed-soybean (Wesson) oil; another third received 130 pmole of 25-$OH[^3H]D_3$ orally each day in Wesson oil, and the other third received only Wesson oil each day. All rats were killed by decapitation 2 weeks after this treatment was begun. The tissues from three or four rats receiving 25-$OH[^3H]D_3$ were pooled for each group, extracted, and chromatographed (*13*) to determine the amount of 1,25-$(OH)_2D_3$, while the other rats (at least six per group) were used to measure intestinal calcium transport by the everted sac method (*12*). Serum calcium and phosphorus were measured in all animals. Standard deviations are given.

Treatment	Body weight (g)	Serum calcium (mg/100 ml)	Serum phosphorus (mg/100 ml)	Calcium transport (inside/outside)	1,25-$(OH)_2D_3$ in Intestinal mucosa (pmole/g)	1,25-$(OH)_2D_3$ in Serum (pmole/ml)
		0.1 percent dietary phosphorus				
Oil	104 ± 10	10.7 ± 0.4	1.7 ± 0.3	1.6 ± 0.1		
25-OHD_3	116 ± 11	13.1 ± 0.7	6.5 ± 0.8	7.9 ± 0.6*	0.760	1.07
		0.3 percent dietary phosphorus				
Oil	154 ± 9	11.6 ± 0.4	3.9 ± 0.3	2.0 ± 0.2		
25-OHD_3	165 ± 16	11.7 ± 0.4	9.8 ± 0.7	3.9 ± 0.3†	0.322	0.395

* Significantly different from † ($P < .001$).

ing that the biosynthesis of 1,25-$(OH)_2D_3$ in the kidney is subject to strong feedback regulation in some manner by serum calcium, which is in turn determined to some degree by dietary calcium (*7*). Thus, the adaptation of intestinal calcium absorption to dietary calcium can be accounted for at least in part by regulation of 1,25-$(OH)_2D_3$ synthesis.

The parathyroid glands sense the serum calcium concentration and secrete parathyroid hormone in response to hypocalcemia. The parathyroid hormone plays a determining role in the synthesis of 1,25-$(OH)_2D_3$ (*8*). Hypophosphatemia also triggers 1,25-$(OH)_2D_3$ synthesis, and it appears that a low concentration of inorganic phosphorus in the renal tubule cell may actually be responsible for increased 1,25-$(OH)_2D_3$ synthesis in response to parathyroid hormone (*9*).

Morrissey and Wasserman demonstrated that chicks on low phosphorus diets show increased rates of calcium absorption and increased intestinal production of calcium-binding protein (*10*). It seemed likely that these observations might be accounted for by an increased production of 1,25-$(OH)_2D_3$, the hormonal form of vitamin D_3 responsible for increased intestinal calcium absorption.

Weanling male rats from a low vitamin D colony (Holtzman, Madison, Wisconsin) were fed a vitamin D–deficient diet (*11*) containing 1.2 percent calcium and either 0.1 or 0.3 percent phosphorus for a total of 4 weeks. The animals were maintained in hanging wire cages and had free access to food and water. For each concentration of phosphorus, a third of the rats received 130 pmole daily of nonradioactive 25-hydroxyvitamin D_3 (25-OHD_3) orally in 0.1 ml of cottonseed-soybean (Wesson) oil for the last 2 weeks. Another third of the rats received 130 pmole of tritiated 25-OHD_3 orally each day, while the remaining third received the 0.1 ml of Wesson oil vehicle daily during the last 2 weeks. At this time, all rats were killed by decapitation. Serum was collected from each rat. The rats receiving the oil vehicle or the nonradioactive 25-OHD_3 were used for measurements of intestinal calcium transport by the everted sac method (*12*). Intestine, kidney, and serum were immediately collected from the rats receiving the 25-OH[^{3}H]D_3. Intestinal mucosa, kidney, and blood were homogenized with five volumes of distilled water, extracted with a methanol-chloroform mixture (*1*), and chromatographed on a Sephadex LH-20 column prepared and developed in chloroform, Skellysolve B (65:35, by volume) (*13*). Portions of tissue homogenates or blood were dissolved in Nuclear-Chicago solubilizer to determine total radioactivity. Column effluents were collected and dried under a stream of air. To each sample was added a toluene counting solution (*1*), and ^{3}H was determined in a Packard liquid scintillation counter (model 3375) equipped with automatic external standardization. The amount of metabolite in each tissue was calculated from the specific radioactivity of the 25-OH[^{3}H]D_3 given, since these animals had no other source of vitamin D.

Table 2. Dietary phosphorus and the metabolism of 25-OH[^{3}H]D_3. The rats were fed vitamin D–deficient diets containing 1.2 percent calcium and either 0.1 or 0.3 percent phosphorus for 2 weeks. At this time each rat received 135 pmole of 25-OH[^{3}H]D_3 orally in Wesson oil daily for 14 days. The rats were killed by decapitation for the radioactivity determinations. The samples of serum or tissue homogenate were solubilized in Nuclear-Chicago solubilizer to measure the total radioactivity in the tissues. For the determination of 1,25-$(OH)_2D_3$ the tissues were extracted and chromatographed (*13*). The tissues from four rats in each group were pooled and analyzed; dpm, disintegrations per minute.

Dietary phosphorus content (%)	Radioactivity in: Intestinal mucosa (dpm per gram of tissue)	Radioactivity in: Serum (dpm/ml)	Radioactivity in: Kidney (dpm per gram of tissue)	Radioactivity as 1,25 = $(OH)_2D_3$ in: Intestinal mucosa (dpm per gram of tissue)	Radioactivity as 1,25 = $(OH)_2D_3$ in: Serum (dpm/ml)
0.1	11,650	31,425	16,125	2,867	4,044
0.3	11,125	31,620	16,875	1,216	1,489

Serum calcium concentration was determined with a Perkin-Elmer atomic absorption spectrometer (model 402) in the presence of 0.1 percent lanthanum chloride. Inorganic phosphorus was measured by the method of Chen *et al.* (*14*).

The 25-OHD_3 used was kindly supplied by John Babcock of the Upjohn Company, Kalamazoo, Michigan. The 25-OH[^{3}H]D_3 was prepared in this

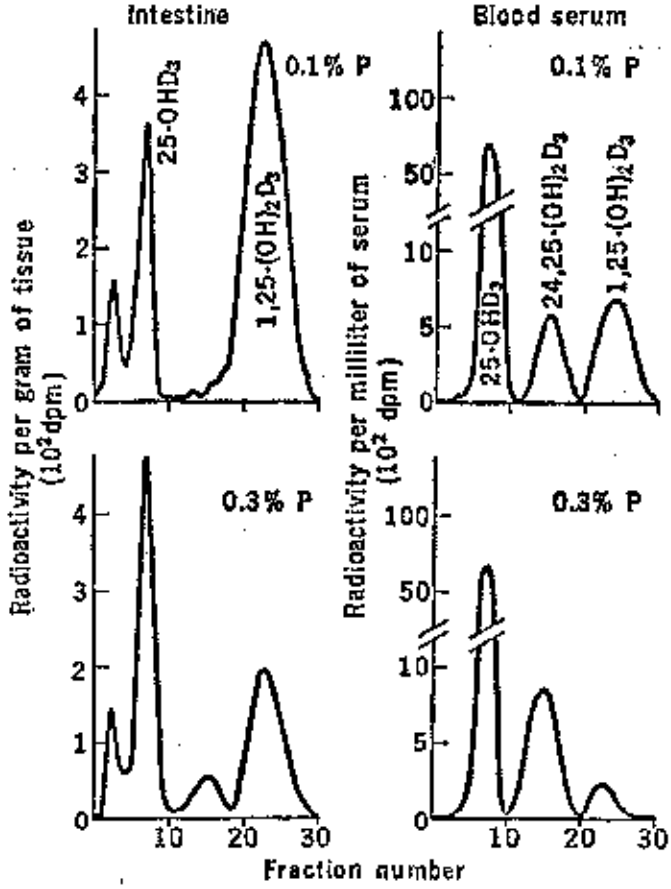

Fig. 1. Chromatographic profiles of methanol-chloroform extracts of intestinal mucosa and serum of rats fed a diet containing low phosphorus (0.1 percent) or normal phosphorus (0.3 percent). The 2 by 12 cm column contained 10 g of Sephadex LH-20 and was eluted with chloroform, Skellysolve B (65 : 35) (Skellysolve B is a light petroleum ether fraction redistilled at 67° to 69°C) as described by Holick and DeLuca (*13*). Fractions of 5.5 ml were collected, and the radioactivity in each was determined; dpm, disintegrations per minute.

laboratory (*15*), and Sephadex LH-20 was purchased from Pharmacia, Piscataway, New Jersey.

Rats on the low phosphorus diet develop severe hypophosphatemia, which is partially corrected by the administration of 25-OHD_3 (Table 1). These animals have marked intestinal calcium transport activity, even more marked than that observed in rats on low calcium diets (*16*). Rats fed a normal amount of phosphorus (0.3 percent) show a hypophosphatemia that is corrected by the 25-OHD_3 treatment. Although calcium transport is high, it is approximately half that of rats on the low phosphorus diet. Thus, in agreement with Morrissey and Wasserman (*10*), low phosphorus diets result in high rates of calcium absorption in rats as well as in chicks.

Because low phosphorus diets that result in hypophosphatemia have been shown to result in increased biosynthesis of 1,25-$(OH)_2D_3$ (*9*), the vitamin D–derived hormone responsible for the initiation of intestinal calcium absorption, it was of interest to determine if the increased transport of calcium could be correlated with the production of 1,25-$(OH)_2D_3$ and its accumulation in the intestine. The low phosphorus diet stimulates the accumulation of 1,25-$(OH)_2D_3$ in blood and intestine (Fig. 1); there is more than twice as much 1,25-$(OH)_2D_3$ in the intestine and blood of rats on the low phosphorus diet than in those of animals on the normal phosphorus diet (Table 1). More 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] is observed in the chromatographic profiles of rats on the 0.3 percent phosphorus diet than in those from rats on the 0.1 percent phosphorus diet.

Dietary phosphorus does not affect the accumulation of radioactivity in blood serum, kidney, and intestine, whereas it markedly affects the amount of that radioactivity appearing as 1,25-$(OH)_2D_3$ (Table 2). Thus, low phosphorus diets and the resulting hypophosphatemia stimulate 1,25-$(OH)_2D_3$ production, which is probably responsible for increased calcium transport and may contribute further to the hypercalcemia. The increased accumulation of 1,25-$(OH)_2D_3$ under conditions of hypercalcemia and hypophosphatemia, in which there should be little or no parathyroid secretion, demonstrates that parathyroid hormone is not necessary for 1,25-$(OH)_2D_3$ production in the hypophosphatemic animal. It also provides support for the idea that the inorganic phosphate concentration in renal cells is an important determinant of 1,25-$(OH)_2D_3$ production.

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References and Notes

1. J. W. Blunt, H. F. DeLuca, H. K. Schnoes, *Biochemistry* 7, 3317 (1968).
2. G. Poncher, A. L. Kenan, H. F. DeLuca, *J. Clin. Invest.* 48, 2032 (1969); M. Horsting and H. F. DeLuca, *Biochem. Biophys. Res. Commun.* 36, 251 (1969).
3. M. F. Holick, H. K. Schnoes, H. F. DeLuca, *Proc. Nat. Acad. Sci. U.S.A.* 68, 803 (1971); ——, T. Suda, J. Cousins, *Biochemistry* 10, 2799 (1971); D. R. Fraser and E. Kodicek, *Nature* 228, 764 (1970).
4. I. T. Boyle, L. Miravet, R. W. Gray, M. F. Holick, H. F. DeLuca, *Endocrinology* 90, 603 (1972).
5. M. F. Holick, M. Garabedian, H. F. DeLuca, *Science* 176, 1146 (1972); R. G. Wong, A. W. Norman, C. R. Reddy, J. W. Coburn, *J. Clin. Invest.* 51, 1287 (1972).
6. C. A. Frolik and H. F. DeLuca, *Arch. Biochem. Biophys.* 147, 143 (1971); *J. Clin. Invest.* 51, 2900 (1972).
7. I. T. Boyle, R. W. Gray, H. F. DeLuca, *Proc. Nat. Acad. Sci. U.S.A.* 68, 2131 (1971); I. T. Boyle, R. W. Gray, J. L. Omdahl, H. F. DeLuca, in *Endocrinology 1971*, S. Taylor, Ed. (Heinemann, London, 1972), pp. 468–476.
8. M. Garabedian, M. F. Holick, H. F. DeLuca, I. T. Boyle, *Proc. Nat. Acad. Sci. U.S.A.* 69, 1673 (1972); H. Rasmussen, M. Wong, D. Bikle, D. B. P. Goodman, *J. Clin. Invest.* 51, 2502 (1972)
9. Y. Tanaka and H. F. DeLuca, *Arch. Biochem. Biophys.* 154, 566 (1973).
10. R. L. Morrissey and R. H. Wasserman, *Amer. J. Physiol.* 220, 1509 (1971).
11. T. Suda, H. F. DeLuca, Y. Tanaka, *J. Nutr.* 100, 1049 (1970).
12. D. L. Martin and H. F. DeLuca, *Amer. J. Physiol.* 216, 1351 (1969).
13. M. F. Holick and H. F. DeLuca, *J. Lipid Res.* 12, 460 (1971).
14. P. S. Chen, Jr., T. Y. Toribara, H. Warner, *Anal. Chem.* 28, 1756 (1956).
15. T. Suda, R. B. Holick, H. F. DeLuca, H. K. Schnoes, *Biochemistry* 9, 1651 (1970).
16. D. V. Kimberg, D. Schachter, H. Schenker, *Amer. J. Physiol.* 200, 1256 (1961).
17. Supported by PHS grant AM-14881, AEC contract AT(11-1)-1663, and by the Food Research Institute of the University of Wisconsin, Madison.

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5703

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Possible Injury to the Cardiovascular System from Vitamin D

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THE INTEREST IN VITAMIN D as a possible source of injury to the cardiovascular system lies in the recent advances in our knowledge of supravalvular aortic stenosis. When, in 1958, Denie and Verhougt (1) reviewed the literature on supravalvular aortic stenosis, they found that 12 cases had been described, and they added 1 case of their own. After this, a number of such cases were recognized. In 1961, Wooley and associates (2) described the occurrence of supravalvular aortic stenosis in several members of one family. The existence of this so-called familial type has been confirmed by others and is now generally accepted as a clinical entity. The same year (1961) Williams, Barratt-Boyes, and Lowe (3) described a second type in which no familial aggregation was found but in which the supravalvular aortic stenosis was associated with mental retardation and a peculiar facies. These authors commented that the children looked "more like one another than like their own sisters and brothers." Moreover the facies were so distinctive that they suggested that the diagnosis might be made at sight. As so frequently occurs when a new clinical syndrome is recognized, a number of cases were immediately reported from various parts of the country and, indeed, from various parts of the world. In 1964, Beuren and coworkers (4) reported from Göttingen, West Germany, that peripheral pulmonary stenosis and an abnormality in the dentine were also part of the clinical syndrome.

In 1963, Black and Bonham-Carter (5) recognized the similarity of the facies of those patients with supravalvular aortic stenosis to the facies of the children who had suffered from severe idiopathic hypercalcemia in infancy. These investigators then reviewed the autopsy material of infants who had died of the severe type of idiopathic hypercalcemia and found that in addition to the renal calcinosis these infants showed a wide variety of arterial stenoses, including renal artery stenoses and coarctation of the aorta; and although none had supravalvular aortic stenosis, abnormalities of the aortic valve were common among these infants. These observations immediately aroused the interest of pediatric cardiologists.

Idiopathic hypercalcemia has been known to pediatricians since Lightwood's (6) first report in 1952. This syndrome, too, is usually divided into two classes, the mild and the severe type of idiopathic hypercalcemia. The mild type is readily corrected by dietary measures. The severe type of idiopathic hypercalcemia is associated with anorexia, vomiting and failure to thrive, marked hypotonia, hypertension, a systolic heart murmur, renal insufficiency, and severe mental retardation. The high blood calcium level characteristic of hypercal-

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cemia suggested that it was related in some way to vitamin D intake or vitamin D metabolism (7).

During the next few years an increasing number of infants were recognized as suffering from this condition. Indeed, England suffered from a veritable epidemic of this disease. The epidemic occurred at a time when 1,000 units of vitamin D were being added to each quart of milk and vitamin D supplement was being added to many foodstuffs, such as cereals, bread, and flour. It was calculated that the majority of infants in Great Britain at that time received between 2,000 and 3,000 units of vitamin D/day. As soon as the vitamin D was taken out of the foodstuffs and the vitamin D content of milk was reduced to 400 units/qt, the outbreak of severe idiopathic hypercalcemia came to an abrupt end. Indeed, the decrease in the prevalence of idiopathic hypercalcemia was nearly as striking as the elimination of phocomelia in Germany after thalidomide was withdrawn from the market. The decrease in the severe form of idiopathic hypercalcemia that occurred with the reduction of the amount of vitamin D given to these infants is an incontrovertible fact, and it is a strong argument for the existence of some relation between the two conditions.

Idiopathic hypercalcemia has been extensively studied. The severe form is believed to be related to an abnormality in the metabolism of vitamin D of such a nature that these infants respond to a given dose of vitamin D with a greater rise and a more prolonged maintenance of a high level of vitamin D in the blood than occur in the average individual. Bongiovanni, Eberlein, and Jones (8) and Fellers and Schwartz (9) have considered this variation in metabolism to fulfil the criteria of "an inborn error" of metabolism; that is, the infants were born with an abnormality in the manner in which they metabolized vitamin D. This "inborn error" of metabolism is of such a nature that the infant is able to get a greater effect from a smaller amount of vitamin D than does the so-called "normal" individual. In other words, before the days of isolation of vitamin D, these infants could use the vitamin D that occurred in nature more effectively than could the so-called "normal" individual, and thus these individuals were less prone to rickets; now, because we can add unlimited amounts of vitamin D to our diet, we say those who can use it more effectively suffer from an "inborn error" of metabolism. The condition is more accurately described as an inborn "variant" in metabolism.

The important consideration in the present discussion is that some infants are born with an ability to metabolize vitamin D of such a nature that they can be seriously and severely injured by a dose of vitamin D that is apparently not injurious to other children. Moreover, this dose is approximately only eight times the usual amount required for normal growth and development. The figure eight times the usual amount is derived because a decrease from approximately 3,000 units/day to 400 units/day virtually eliminated the severe form of idiopathic hypercalcemia.

It is also of interest that Blizzard (10) has seen a family in which two children have suffered from idiopathic hypercalcemia. When the mother of these two children was given a relatively high dose of vitamin D over a period of several weeks, she developed nervousness and a high blood calcium level, while the father showed no such reaction with the same dose of vitamin D over the same period of time. It is also of interest that, although these children have both shown a heart murmur from time to time, neither of the children has supravalvular aortic stenosis or any clinical evidence of a cardiac abnormality at the present time. Both children do show a rise in blood calcium when exposed to excessive sunlight and are therefore kept out of the full sun in the summer, and they are never given food to which

vitamin D has been added. In this instance the basic disturbance appears to be one of an abnormality in calcium metabolism.

To return to the problem of supravalvular aortic stenosis—in the spring of 1964 Garcia, Friedman, Kaback, and Rowe (11) reported an infant who was referred to the Cardiac Clinic of the Children's Medical and Surgical Center because of a cardiac murmur. On careful study this child was found to have a high blood calcium level, a high blood level of vitamin D, and supravalvular aortic stenosis. This, we believe, is the first reported case in which both hypercalcemia and supravalvular aortic stenosis have been reported.

In the summer of 1965 Black, Butler, and Schlesinger (12) reported that one of their original patients with a severe form of idiopathic hypercalcemia died of hypertension and renal failure at the age of 16 years. Autopsy showed renal calcinosis, massive left ventricular hypertrophy, and supravalvular aortic stenosis.

Antia and associates (13) studied 14 children referred to our clinic with supravalvular aortic stenosis with both the familial type and the type associated with mental retardation. They found that these patients, regardless of the type of facies that they had, showed a wide spread in the range of their I.Q.s; many of them were slightly, although not severely, retarded, and those with a normal I.Q. showed curious variations in their response to certain mental tests. One patient with normal facies showed an abnormality of the dentine suggestive of that seen in patients with supravalvular aortic stenosis and mental retardation. These findings suggested that the two types of supravalvular aortic stenosis represent different responses to the same underlying condition.

Recently, Beuren (14) studied two infants who had been incorrectly diagnosed as having infantile tetany and had been given massive doses of vitamin D. Both infants at the age of 8 months had the full-blown picture of supravalvular aortic stenosis, peripheral pulmonary stenosis, and severe mental retardation. Beuren has started vitamin D loading studies and has advised me that not only do the patients with supravalvular aortic stenosis and mental retardation show an abnormal response to vitamin D, but also, those patients with the familial type of supravalvular aortic stenosis appear to be abnormally sensitive to vitamin D. Beuren suspects that patients with valvular aortic stenosis respond abnormally to vitamin D. This suggestion that aortic stenosis of the valvular type may be of similar or related origin does not surprise me because Bonham-Carter and Suffice (15) have reported that many infants dying of idiopathic hypercalcemia showed abnormalities of the aortic valve. Exactly what determines whether an infant with a high blood calcium subsequently develops supravalvular aortic stenosis is certainly not clear. Nevertheless, all present evidence indicates that injury to the cardiovascular system occurs when there is an abnormally high blood calcium or an abnormality of vitamin D metabolism or the combination of these two factors. Under such a combination of circumstances the patient may develop what has generally been considered to be a congenital malformation of the heart.

This observation introduces a new aspect to the etiology of malformations. We are all well aware that nearly all grades of severity of a malformation exist in almost all types of malformations, as for example, a large and a small ductus or a mild and a severe stenosis; and furthermore we recognize that the most obstructive lesions become more severe over a period of years. Also, we recognize that the extreme of one malformation may merge into another; for example, an infant with tetralogy of Fallot and a pulmonary atresia, who survives the closure of the ductus arteriosus, has a functional truncus arteriosus. These observations concerning hypercalcemia and vi-

tamin D indicate that at least in some instances the fetus can be injured and that such injury may result in a congenital malformation. Moreover, this injury that is associated with a disturbance in the metabolism of calcium and vitamin D causes both mental retardation and widespread injury to the vascular tree, which may result in supravalvular aortic stenosis, peripheral pulmonary stenosis, renal artery stenosis, possible aortic valvular stenosis, or perhaps even in some instances coarctation of the aorta.

During this past winter (1965 to 1966) Friedman and Roberts (16) approached the problem experimentally. They gave pregnant rabbits high doses of vitamin D to determine whether vitamin D crossed the placenta and to explore the relation between maternal hypervitaminosis and supravalvular aortic stenosis. Briefly, they found that the blood level of vitamin D in the pregnant rabbits given vitamin D was 18 times higher than that in the control rabbits and that the vitamin D assay of the offspring of the rabbits fed high doses of vitamin D was 9 times higher than that of the control. Among the 84 offspring whose mothers had received high doses of vitamin D, 14 showed abnormalities of the aorta. Moreover, 6 rabbits showed lesions that were anatomically similar to supravalvular aortic stenosis in man. In addition, 1 rabbit had a nonconstricting fibrous band above the aorta such as has been reported in man, and another had a microscopic abnormality in the proximal aorta; all of the controls were normal. In another experiment 6 offspring of mothers receiving high doses of vitamin D were given relatively small doses of vitamin D at 3 months of age. These rabbits developed generalized vitamin D toxicity of the advanced type seen in adults given massive doses of vitamin D, namely, generalized irregularities of the wall with degeneration and calcification. Friedman and Roberts emphasize the necessity for caution in drawing analogies between the experimental animal model and the clinical disease. Nevertheless, their experiments suggest that in certain animals —not necessarily all animals—an in utero derangement in vitamin D metabolism on the part of the mother or of the fetus, or both may be responsible for supravalvular aortic stenosis, especially when the lesion is associated with infantile hypercalcemia. In any event, these experiments showed that vitamin D can pass through the placenta and that abnormalities can occur in the offspring of the mothers who receive massive doses of vitamin D and that smaller doses at a later date can severely injure the aorta.

Obviously, experiments are needed to determine whether animals can be rendered hypersensitive to vitamin D, or only when mothers are fed excessive vitamin D may their offspring be rendered hypersensitive to vitamin D. Furthermore, their experiments also suggest that rabbits that have received an excessive amount of vitamin D in utero are susceptible to further injury if they are again fed vitamin D.

The question arises: how widespread is this phenomenon? At the present time three variations in the metabolism of vitamin D are known: [1] the "hypersensitive" response related to idiopathic hypercalcemia; [2] the normal or usual response; and [3] the children with vitamin D-resistant rickets. Are there just three points or is there a wide spectrum in the manner in which people metabolize vitamin D? Or is it all primarily related to disturbances in calcium metabolism? These observations open a tremendous field for study, and many of the problems lie in the realm of internal medicine.

For example, as one considers the various types of aortic stenosis, one wonders whether the calcific aortic stenosis of advancing years, that condition that has long defied pathologists as to whether it was congenital or rheumatic in origin, may not be of this nature, namely, the result of a

5703

combination of some genetic factor or increased sensitivity to vitamin D and the amount of sunlight and vitamin D that the individual received over a period of years.

In all probability there is some additional unknown factor of vital importance. The phenomenon of calciphylaxis that Selye (17) has studied may be of great significance. He has shown that animals may be given a dose of vitamin D, and subsequently at a critical time the animal is given another substance that acts as a challenger and produces calcification in various places. By varying the amount of vitamin D and the challenger Selye has produced calcification almost at will.

These observations open up many problems—I shall not speculate. Nevertheless, I do seriously wonder how great may be the variation in metabolism of calcium and of vitamin D and whether harm is being done by the widespread use of vitamin D in relatively high doses. Certainly we have virtually eliminated rickets, but some infants tolerate less than others and some infants need more than others; and perhaps some adults with a special genetic background may be injured.

Another interesting problem concerns the relation between calcium metabolism and hypercholesterolemia. In this connection, in 1963, Hooft, Vermassen, and Blanquaert (18) reported two cases. The first patient was a child who was known to have severe idiopathic hypercalcemia. He was treated with thyroxin and a low calcium diet until 3 years of age; at 6 years of age he had a normal renal function, but he had hypercholesterolemia. The second was a patient with characteristic facies and a typical history of idiopathic hypercalcemia, marked mental retardation, and increase of density of the long bones, who at the age of 8 years had hypercholesterolemia and aortic stenosis. In this instance, the type of aortic stenosis is not known since the family of the latter patient refused to permit cardiac catheterization or angiocardiography. Be that as it may, the observation raises the question whether the problem is basically one of calcium metabolism or of vitamin D metabolism or the interaction of one upon the other, and what its relation is to cholesterol metabolism.

Whatever may be the outcome of these many problems, a new horizon has opened in the etiology of pathological conditions, both congenital and acquired, and we have further evidence of the late effects of early insults. My plea to you all is that, if large doses of vitamin D are not necessary and there is any possibility that they may do harm, do follow the old adage, "If you cannot do good, be sure not to do harm."

Summario in Interlingua

Le possibilitate del lesionage del systema cardiovascular per vitamina D es suggestionate per le evidentia que un certe relation existe inter hypercalcemia idiopathic e le typo de stenosis aortic supravalvular que es associate con periphere stenosis pulmonar, retardamento mental, e peculiaritates del facie.

Iste ultime characteristica esseva recognoscite como frappantemente simile al facie de juveniles qui ha supervivite a formas sever de hypercalcemia idiopathic. In plus, necropsias in infantes con hypercalcemia idiopathic ha revelate evidentia de un extense lesionage del systema cardiovascular in association con anomalitates del valvules aortic. Un subjecto con hypercalcemia idiopathic qui moriva al etate de 16 annos habeva stenosis aortic supravalvular. Un infante con stenosis aortic supravalvular habeva un alte nivello sanguinee de calcium e monstrava un responsa a vitamina D simile a illo que occurre in subjectos con hypercalcemia idiopathic.

Hypercalcemia idiopathic es cognoscitemente relationate al quantitate de vitamina D ingerite e/o al metabolismo de vitamina D e es reguardate como un "error innate" in le metabolismo de vitamina D.

Le studios experimental effectuate per Friedman in conilios ha monstrate que vitamina D pote passar cis-trans le placenta, que grande doses de vitamina D alimentate al mater causava in le prole un anormalmente alte nivello de vitamina D a nato, e que septimanas plus tarde un dose medie de vitamina D causa un reac-

5708

tion toxic simile a illo que occurre post le alimentation de quantitates massive de vitamina D.

Iste observationes aperi nove vistas in le etiologia de malformationes e suggestiona fortemente que, in subjectos susceptibile, vitamina D pote esser nocive al systema cardiovascular ante e post nato. Il seque que meticulose attention es recommendabile in le uso de doses massive de vitamina D in patientes de omne etates.

References

1. Denie, J. J., Verhelst, A. P.: Supravalvular aortic stenosis. *Circulation* 18: 902, 1958.
2. Wooley, C. F., Hosier, D. M., Booth, R. W., Molnar, W., Sirak, H. D., Ryan, J. M.: Supravalvular aortic stenosis. Clinical experiences with four patients including familial occurrence. *Amer. J. Med.* 31: 717, 1961.
3. Williams, J. C., Barratt-Boyes, B. G., Lowe, J. B.: Supravalvular aortic stenosis. *Circulation* 24: 1311, 1961.
4. Beuren, A. J., Schulze, C., Eberle, P., Harmjanz, D., Apitz, J.: The syndrome of supravalvular aortic stenosis, peripheral pulmonary stenosis, mental retardation and similar facial appearance. *Amer. J. Cardiol.* 13: 471, 1964.
5. Black, J. A., Bonham-Carter, R. E.: Association between aortic stenosis and facies of severe infantile hypercalcaemia. *Lancet* 2: 745, 1963.
6. Lightwood, R.: Idiopathic hypercalcemia with failure to thrive (abstract). *Arch. Dis. Child.* 27: 362, 1952.
7. Forfar, J. O., Ball, C. L., Maxwell, G. M., Tomlinson, S. L.: Idiopathic hypercalcemia of infancy: clinical and metabolic studies with special reference to aetiological role of vitamin D. *Lancet* 1: 981, 1956.
8. Bongiovanni, A. M., Eberlein, W. R., [illegible] I. T.: Idiopathic hypercalcemia of [illegible] with failure to thrive; report of three [illegible] with a consideration of the possible [illegible]. *New Eng. J. Med.* 257: 951, 1957.
9. Fellers, F. X., Schwartz, R.: Etiology of the severe form of idiopathic hypercalcemia of infancy. A defect in vitamin D metabolism. *New Eng. J. Med.* 259: 1050, 1958.
10. Blizzard, R.: Personal communication.
11. Garcia, R. E., Friedman, W. F., Kalack, [illegible] M., Rowe, R. D.: Idiopathic hypercalcemia and supravalvular aortic stenosis: documentation of a new syndrome. *New Eng. J. Med.* 271: 117, 1964.
12. Black, J. A., Butler, N. R., Schlesinger, B. E.: Aortic stenosis and hypercalcemia (abstract). *Lancet* 2: 547, 1965.
13. Antia, A. U., Rowe, R. D., Ottesen, O. E., Levin, S., Cooke, R. E.: Pathogenesis of the supravalvular aortic stenosis syndrome in children (abstract). *Circulation* 32: 44, 1965.
14. Beuren, A.: Personal communication. Spring 1966.
15. Bonham-Carter, R. E., Suffice, J.: Reported at the British Pediatric Meeting (abstract). Spring 1964.
16. Friedman, W. F., Roberts, W. C.: Vitamin D and the supravalvular aortic stenosis syndrome: the transplacental effects of vitamin D on the aorta of the rabbit (abstract). *Circulation* 34: 77, 1966.
17. Selye, H.: *Calciphylaxis* (abstract). University of Chicago Press, Chicago, Ill., 1962.
18. Hoolt, C., Vermassen, A., Blancquaert, A.: Observations concerning the evolution of the chronic form of idiopathic hypercalcaemia in children. *Helv. Paediat. Acta* 18: 139, 1963.

5713

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THE TOXIC EFFECTS OF EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL UPON YOUNG ANIMALS.

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In the earlier stages of these investigations full-grown dogs were employed practically entirely. It was stated in a previous communication (1931) that the smallest dose which caused death in these animals was, weight for weight, about 20 times the therapeutic dose for the human subject. The minimal lethal dose for adult dogs was based upon the potency of the irradiated ergosterol as originally assayed by the manufacturers. This material was designated 10,000 *D*, which meant that its potency was 10,000 times that of cod-liver oil, or 40 times that of the therapeutic material (250 *D*). Later this material was stated by Bills (1931) to be more than 100 times the potency of the therapeutic material (250 *D*), and was therefore designated 10,000 *X* (25,000–30,000 *D*). Therefore the irradiated ergosterol which we employed in our experiments upon adult animals had a potency 2½–3 times greater than the original estimate. In a desire to determine the effect, if any, of a single maximal dose of irradiated ergosterol upon a young dog, a collie of between 3 and 4 months of age, and weighing 4 kilos was selected and given 1 c.c. of irradiated ergosterol (10,000 *X*) by mouth. Three days later the animal appeared bright and active, and apparently normal in every way. The serum calcium was not determined. Two c.c., an admittedly enormous dose, was then given. Extreme weakness and vomiting followed within a few hours, and death occurred within 48 hours after this second dose had been given. The typical signs of acute ergosterol overdosage described in previous papers were found post-mortem. The mucosa of the stomach and intestines showed as intense a reaction as any we have seen. It seemed to us that this animal, which had received 3 c.c. of the sterol in two doses of 1 c.c. and 2 c.c. over a short period, had succumbed more rapidly than one would expect an older dog to do. In the course of earlier experiments we had also been given the general but definite impression that the younger adult dogs were somewhat less resistant than older ones to overdosage.

TABLE I.—*Effect of Irradiated Ergosterol on Pups above 4 months old.*

No. of animal.	Weight before administration in kilos.	Daily dose of irradiated ergosterol 10,000 *X* in c.c.				Number of days from commencement of administration to death.	Serum calcium mgm. per 100 c.c. and symptoms.	Weight at, or shortly before, death in kilos.	Post-mortem findings.
		Total.	Per kilo.	Equivalent of 250 *D* per kilo.	*X* T* per kilo.				
1	2·71	0·3	0·10	10	83	11	Hypercalcemia (18 mgm.), conjunctivitis, vomiting, diarrhœa, blood in stools	1·7	Flushing of gastric mucosa with small extravasations of blood. Hæmorrhagic areas in lungs.
2	3·77	0·25	0·06	6·6	55	30	Hypercalcemia (16 mgm.) conjunctivitis and inflammation of lids with loss of eyelashes. Profound emaciation. Vomit and diarrhœa in early part of experiment	2·77	Stomach and intestines apparently normal.
3	4·76	0·20	0·04	4·0	33	50	Hypercalcemia (14 mgm.) shortly after commencement followed by recovery to normal	2·31	Stomach and intestines apparently normal.
4	3·69	0·15	0·04	4·0	33	15	Calcium 11 mgm. Loss of weight. No other definite symptoms until 24 hours before death, when vomiting and great weakness, ending in collapse occurred	2·50	Intussusception of lower 12 in. of ileum into colon, invaginated bowel, hæmorrhagic and sections gangrenous.
5	4·11	0·7	0·02	2·0	17	60	Slight rise in serum calcium during first 2 weeks. Fall after this which reached normal 2 weeks later	2·12	Nothing abnormal noted in viscera.

* T = maximal therapeutic dose (0·12 c.c. 250 *D* per kilo).

5713

In order to investigate the apparently greater susceptibility of growing animals to irradiated ergosterol, two groups of pups were selected. The first group comprised 5 animals of about 4 months of age, and weighing from 2·71 to 4·76 kilos (Table I). There were 6 animals in the second group about 2 to 2½ months old, and weighing from ·910 to 1·56 kilos (Table II).

The members of the first group were given ergosterol (10,000 *X*) which for convenience of administration was diluted 1 in 10 with corn oil. The diluted material had a potency, therefore, ten times that of the therapeutic material (250 *D* or 100 *X*). The material was administered by mouth in doses ranging from 3 to 1 c.c. per day. That is, pup No. 1 received 3 c.c., pup No. 2 2·5 c.c., and so on, the dose being reduced by 0·5 c.c. with each successive animal, so that pup No. 5 received 1 c.c. only. Pup No. 1 was the first to die. This animal was, so far as we could judge, 4 to 6 weeks younger than the others. It was also the lightest in weight, and received the largest dose. The first definite symptoms were evident within a few days after the administration was commenced. There was rapid loss in weight, loss of appetite, vomiting, diarrhœa. The serum calcium rose to a high level. There was profound weakness, especially noticeable in the hind quarters. Apathy and lethargy were pronounced features. There was a purulent conjunctivitis, which kept the lids almost completely closed, and death occurred on the 11th day. The changes in the gastric and intestinal mucosa were much less intense than we had been accustomed to find in adult dogs. A general flushing and a few ecchymoses alone were found. No. 4 was the next to die, on the 15th day. This animal received next to the smallest dose. It weighed 3·60 kilos and was no younger than the rest. Its death came as a surprise, and we did not believe that its death was the result of overdosage. The calcium was scarcely raised above the normal, which fact confirmed us in this conclusion. At post-mortem the stomach showed no abnormality, but the lower 12 in. of the ileum was invaginated into the colon. The imprisoned section of bowel was hæmorrhagic and in parts gangrenous. The intussusception which was certainly the cause of death, was looked upon at the time as probably a fortuitous occurrence, independent of overdosage. We believe now, however, that ergosterol predisposes the young dog to the development of intussusception. Since the condition was observed in this animal a large proportion of young animals have died from this cause following ergosterol overdosage. Of 30 puppies which died as a result of ergosterol overdosage, intussusception was the cause of death in 10. In one other there was definite evidence that an intussusception had existed, but had been released, and in others the lower ileum was strongly contracted to a small cord, while the adjoining colon was widely dilated. The two sections of bowel in this instance were each in a state which must have been conducive to the development of intussusception. In three of the instances the intussusception was double. That is, the ileum had passed for a distance of from 6 to 12 in. within the colon and the latter had again become invaginated into itself. In one animal intussusception was the cause of death after a single large dose of irradiated ergosterol had been injected intravenously. These effects of activated ergosterol upon the motor mechanisms of the bowel cannot be dealt with further in the present paper, but it may be said that of the large number of young dogs of approximately the same age

5719

TABLE II.—*Effect of Irradiated Ergosterol on Pups 2–2½ months old.*

No. of animals.	Weight before administration in kilos.	Daily dose of irradiated ergosterol 10,000 *X* in c.c.				Number of days from commencement of administration to death.	Serum calcium mgm. per 100 c.c. and symptoms	Weight at death in kilos.	Post-mortem findings
		Total.	Per kilo.	Equivalent of 250 *D* per kilo.	*X* T* per kilo.				
100	1·18	0·35	0·29	29·0	240	8	Hypercalcæmia (20·8 mgm.), vomiting, great weakness, especially of hind quarters, blood highly concentrated	0·80	Deep flushing of gastric mucosa, haemorrhagic patches in lungs, intussusception of 3 in. of lower ileum into colon, bone-marrow haemorrhagic.
101	1·25	0·30	0·24	24·0	200	11	Hypercalcæmia (22·6 mgm.), emaciation, weakness leading to complete prostration	0·87	Ileum contracted, a thin cord, caecum widely dilated, conditions appear preparatory for development of intussusception. Lungs show haemorrhagic areas 1 cm. square, bone-marrow haemorrhagic.
102	1·25	0·25	0·2	20·0	166	12	Hypercalcæmia probably present, but calcium determinations not made. Symptoms similar to preceding	0·90	Intussusception of 3 in. of lower ileum. Gastric mucosa shows flushing only.
103	1·22	0·20	0·14	14·0	116	14	Hypercalcæmia (24·0 mgm.). Symptoms similar to those of preceding animals	0·90	Gastric mucosa injected, a few ecchymotic areas. Bowel filled with dark brown fluid. Lungs show well-defined haemorrhagic areas. A section of ileum about 3 in. long deeply congested and showing a haemorrhagic area ½ in. long. The mesentery of this section of the ileum injected, vessels dilated. Ileum on either side of this area is quite pale and normal in appearance.
104	0·91	0·15	0·16	16·0	136	6	Diarrhœa, weakness, particularly of hind-quarters, comatose last few hours of life	0·78	Lungs haemorrhagic, stomach mucosa colour normal, bone-marrow haemorrhagic.
105	1·56	0·10	0·06	6·0	50	13	Hypercalcæmia (19·1 mgm.)	1·03	No haemorrhages in lungs. Stomach bright red in pyloric region, duodenum pale, bone-marrow haemorrhagic, ileum firmly contracted, in sections separated by dilated pouches, but there is no intussusception.

* T = maximal therapeutic dose [illegible]

employed as controls, only 2 died. The cause of death in these animals was not determined, but in neither was intussusception found. There is no reason, moreover, to believe that intussusception is a common occurrence in young dogs under laboratory conditions. We must therefore conclude from these results that in young dogs irradiated ergosterol induces a disturbance in the motor mechanisms of the bowel which predisposes to the condition. Intussusception is an effect of the administration which usually supervenes early and causes death before the other signs of overdosage are apparent.

Of the remaining three pups of Group I all were alive one month after the commencement of the ergosterol administration. In all, the serum calcium was elevated and all had lost a large proportion of their body-weight. The rapid loss in weight was especially noticeable in pups Nos. 3 and 5. The latter animal had received only 0·10 c.c. of irradiated ergosterol (10,000 *X*) daily. At the beginning of the experiment its weight was 4·11 kilos. Two weeks later, even upon a casual observation, its extreme emaciation was very noticeable. Its weight shortly before death was 2·12 kilos, which represented a loss of nearly 50 per cent. of the body-weight. The three other animals showed a loss of weight to a somewhat less degree. It is to be remembered that all were young animals, and consequently the figures do not represent the full effect which irradiated ergosterol exerts upon the body-weight. These animals, if untreated, would have been expected to add from 1 to 2 kilos to their weights during periods of similar duration.

The second group of 6 animals (Nos. 100–105), though considerably younger, were given similar doses of irradiated ergosterol. The undiluted material (10,000 *X*) was employed, and the bulk of each dose was correspondingly smaller. Thus pup No. 100 received 0·35 c.c., pup 101 0·3 c.c., and so on. Pup 105 received only 0·1 c.c. of this material. All these animals died within 2 weeks. The results are shown in Table II.

Evidently in these two groups of animals the minimal lethal dose of irradiated ergosterol had not been reached. In order therefore to arrive at some idea of what this might be, it was decided to give two large doses in succession to each animal in a group and note the survival periods. A large dose of irradiated ergosterol is apparently either not quickly absorbed, but remains in the gastro-intestinal tract for some days, or continues to circulate in the blood and exert its effects for a considerable period after its administration. It was therefore considered justifiable, in order to arrive at a tentative estimate of the minimal daily dose of irradiated ergosterol that would produce signs of overdosage, to take the quantity of the total dose in c.c. administered to these animals, and divide it by the survival time in days. Four young pups about 2 months old were given 0·5, 0·4, 0·3 and 0·2 c.c. respectively of viosterol (10,000 *X*) by mouth on two successive days. The results are shown in Table III.

In the foregoing series the animals were fed stock laboratory diets consisting of milk and bread or dog biscuit and a small quantity of meat. No vitamins were added. Although puppies thrive and grow upon this diet, it was thought possible that it was not adequate, and that a more liberal diet including the necessary vitamins might enable the animals to resist better the effects of ergosterol overdosage. In the next series particular attention was therefore

TABLE III.—*Effect of Two Large Doses of Irradiated Ergosterol.*

Pup No.	Weight in kilos	Number of days after first dose till death.	Total dose (10,000 X) in c.c.	Calculated dose per day = total dose ÷ days of survival.	Calculated dose per kilo per day in c.c.	Vitosterol (250 D) equivalent per kilo per day in c.c.	X/T*
110	1·46	7·5	1·0	0·13	0·08	8·0	66
111	1·39	8	0·8	0·10	0·07	7·0	58
112	0·91	5	0·6	0·12	0·13	13·0	108
113	1·6	8	0·4	0·05	0·03	3·0	25

* T = maximal therapeutic dose.

paid to the diet in order to ensure that it did not lack any requisite for health and normal growth. Six police pups of the same litter, about 2 months of age, were chosen for this experiment. They were fed whole milk, dog biscuit and 20 gm. of meat daily. Vitamins B and C were supplied by adding suitable amounts of bemax and tomato-juice. Upon admission to the laboratory they were strong, well-nourished animals, full of vigour and apparently in perfect condition. They were placed upon the above diet, and kept under observation for two weeks after admission. Some weight was lost during the first week, but a gain was shown at the end of the second week. Irradiated ergosterol administration was then commenced. The dosage ranged from 0·05 c.c. to 0·005 c.c. of 10,000 X per kilo, as shown in Table IV. Four of the animals had lost weight at the end of 10 days of treatment, and all weighed less at the end of 3 weeks. The animal receiving the largest dose died on the 18th day. The one receiving the smallest dose survived for 2 months; a slight gain in weight occurred during the first 5 weeks, but from this time on there was a steady decline. The dose received by this animal was only four times the maximal therapeutic dose. Though caution must always be exercised in drawing conclusions applicable to man from experiments upon animals, these results certainly suggest that the administration of irradiated ergosterol to infants in amounts not exceeding so very greatly those at present recommended may be fraught with danger.

In animals receiving comparatively small, though still toxic, doses of irradiated ergosterol over long periods hypercalcæmia does not always occur. For this reason we are forced to fall back upon other less reliable criteria of overdosage, such as loss of body-weight, lowered vitality and survival period. Nevertheless, though snuffles or pneumonia occasionally caused the death of the control animals, the deterioration of the animals which invariably followed the administration of ergosterol leaves little room for doubt, when one considers the number of animals employed, that overdosage was the cause of death. Recently we have been following the calcium excretion of animals so treated, with the view of obtaining a more precise index of overdosage. We have found so far that in doses comparable with those given to the preceding groups a very definite increase in total calcium excretion occurs. The results of these experiments will be reported shortly.

The final series of growing animals treated with irradiated ergosterol were somewhat older than those in the preceding groups. These animals, 8 in number, were about 3 months of age, vigorous and apparently in good health

TABLE IV.—*Effect of Irradiated Ergosterol on Pups about 8 weeks old, fed on a Diet containing Adequate Vitamins B and C.*

Puppy No.	Weight in kilos. Jan. 26, 1931.	Feb. 5.	Feb. 14.	Mar. 2.	Mar. 9.	Mar. 16.	Mar. 23.	Mar. 30.	Daily dose per kilo 10,000 X in c.c. from Jan. 26, 1931.	Daily dose in equivalent of 250 D in c.c.	X T.*
1	2·39	2·20	Died Feb. 13. Wt. 1·60	..	..	..	..	..	0·05	5	42
2	2·53	2·38	2·33	2·5	Died Mar. 9. Wt. 1·75	..	..	..	0·04	4	33
3	2·24	2·19	Died Feb. 14. Wt. 1·62	..	..	..	..	..	0·03	3	25
4	2·32	2·25	2·30	2·61	2·52	Died Mar. 14. Wt. 1·68	..	..	0·02	2	17
5	2·40	2·45	2·21	1·89	Died Mar. 3. Wt. 1·6	..	..	..	0·01	1	8
6	2·40	2·46	2·24	2·49	2·30	2·25	2·04	Died Mar. 27. Wt. 1·32	0·005	0·5	4

* T = maximal therapeutic dose.

at the commencement of the experiment. They were fed upon a diet of whole milk, bread, meat and the necessary vitamins. The animals were divided into two groups of 4 each. One group received irradiated ergosterol in doses ranging from 0·3 to 0·7 c.c. of 10,000 *X* per kilo daily. The other group served as controls. Every precaution was taken to keep the animals in good condition. They were removed from all other animals in the laboratory and placed in a separate room in another wing of the laboratory and were allowed to roam in the open-air for several hours daily.

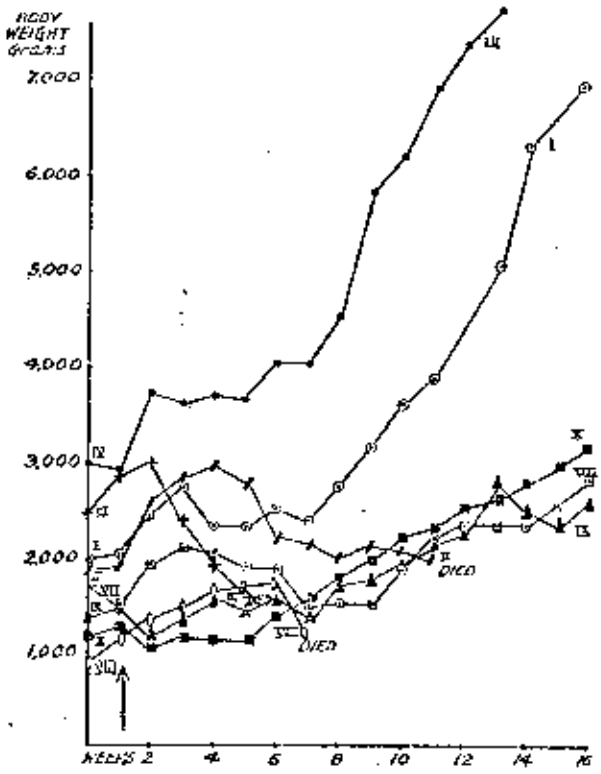

FIG. 1.—Growth-curve of pups of Table V. Animals II, III, VIII and X treated with irradiated ergosterol. Arrow indicates time administration was commenced. Nos. I, IV, VII and IX are the control animals.

The ergosterol administration was not commenced until all animals had become accustomed to their new surroundings and were gaining in weight. The experiment extended over a period of 25 weeks. Of the 4 treated animals, those receiving the three largest doses died in from 4 to 10 weeks. The animal receiving the smallest dose survived and was alive at the end of 6 months. All the control animals survived for the same length of time, and were then used for other experiments. The treated animals received irradiated ergosterol (10,000 *X*) once daily. The dosage and survival times of the four treated animals are given in Table V. The growth-curves of the 8 animals are shown in Fig. 1.

TABLE V.—*Effect of Irradiated Ergosterol on Pups about 3 months old, fed on a Diet containing Adequate Vitamins B and C.*

Pup No.	Weight in kilos.	Survival time, weeks.	Dose 10,000 *X* per kilo in c.c.	Equivalent to 250 *D* per kilo in c.c.	*X* T.*
II	2·29	10	0·07	7·0	58
III	2·27	4	0·06	6·0	50
VIII	1·20	6	0·04	4·0	35
X	1·60	Until conclusion of experiment	0·03	3·0	25

* T = maximal therapeutic dose.

It will be observed that there was not complete correspondence between the size of the doses and the survival periods. Of those which died, pup No. II, though it received the largest dose, lived the longest. This, however, is not surprising, since we have found that animals varied rather widely in their resistance to overdosage. The fourth animal (pup No. X) also tolerated a dose considerably larger than some pups in other series, which may be accounted for by the fact that the animals in this last series were somewhat older than the majority of those in the earlier experiments.

All these animals, including the controls, showed a flattening or decline in the growth-curve some time between the second and ninth weeks (middle of June to end of July) of the experiment. This was probably due in large part at any rate, to the intensely hot weather which occurred during this time.

SUMMARY.

Experiments upon the effects of irradiated ergosterol on young dogs are described. The doses employed were on the whole smaller than those used in previous experiments upon adult animals, and the administration was extended over longer periods.

The resistance of individual animals to the toxic effects of irradiated ergosterol varies considerably. In some animals a daily dose less than ten times the maximal infant dose, which is taken to be 0·12 c.c. of 250 *D* per kilo, proved definitely toxic. On the other hand, one animal remained apparently unaffected by a daily dose which was twenty-five times the maximal therapeutic dose.

REFERENCES.

BILLS, C. E., HONEYWELL, E. M., WINICK, A. M., AND NUSSMEIER, M.—(1931) *J. Biol. Chem.*, **90**, 619.

TAYLOR, N. B., WEED, C. B., BRANTON, H. D., AND KAY, H. D.—(1931) *Can. Med. Ass. J.*, **24**, 763.—(1931) *ibid.*, **25**, 20.

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Is Vitamin D Treatment Carcinogenic?

by A. Touraine and M. Zureick

This question was posed by Desaux and Stauffer in 1947, by Degos in 1948, by Hornberger in 1949 and by Acquasciati in 1950. It is important because it is well known how widespread the use of vitamin D_2 is today in the treatment of numerous pathological conditions, especially rickets and tuberculosis cutis. There is no doubt that if the question is answered in the affirmative, its use will require stringent restrictions, or at least, a revision of its method of application.

Desaux's case is doubtful; it involved a symmetrical adenoma of the face, of Balzer's type, situated near a lupus, and Civatte wondered if this was not a case of the beginning of a transformation into epithelioma.

At the 29th Congress of the Swiss Society of Dermatology and Venerology at Geneva (September 13-14, 1947), Stauffer reported seeing several small cancers which had developed in cases of lupus during the course of vitamin therapy; these cancers were shown to be less sensitive to radiation than usual.

More recently, in 1949, Hornberger (from Tubingen) noted the considerable increase in cancers in cases of lupus in his private practice when they reached the level of 10.5% of all lupus; the malignancy and the tendency to recur increased also. Reviewing these instances of increase, he considered the possible role of the widespread use of radiotherapy, radiation limits, treatments with vitamin D or with thiosemicarbazone.

This carcinogenic effect of vitamin D_2 has found several arguments

supporting it.

There is first of all, the chemical composition of the D vitamins, which as is known are sterols of the phenanthrene group, in other words, substances whose carcinogenic capacity is well known.

There is also the tendency, pointed out by Lapière and Van Runckelen, that scars from lupus treated with vitamin D_2 show dyskeratoses of the skin more often than occurs after older methods of therapy. Dyskeratosis is a histological alteration which characterizes several precancerous skin conditions, such as Bowen's disease and certain erythroplasias.

Finally, a relatively high number of cancers in cases of lupus treated by the Charpy-Fanielle method have been published since the usuage of this method was established. Besides the cases of Dessux and Stauffer already noted, let us mention those of Bondet and Fayart in 1946, of Colin-Jones in 1947, of Degos and Delzant, of Jaeger (3 cases), of Consentino and Musumeci in 1948, of Corrente-Siergo, of Touraine and Miss Lambergeon in 1949.

Are these arguments sufficient to question the value of a treatment, which has previously shown real effectiveness against lupus tuberculosus? Do we have the right at the present time to put reservations on the long-term prognosis for vitamin D_2 therapy by emphasizing the possibility of cancer arising, as a more or less long shot, in patients who have been subjected to this therapy?

Given the actual state of our knowledge, it does not seem possible to answer these questions.

We have tried to estimate from recent literature, the frequency of cancers in cases of lupus treated with vitamin D_2. We have found no utilizable data. If the literature abounds in publications on the immediate or

short-term clinical and histological results, we still lack information on the long-term future of these patients. Only the observation of Degos and Delzant specifies that two years passed between the end of the treatment and the beginning of the cancer. It is very likely that if a cause and effect relationship exists, the necessary delay may be much longer.

In order to allow an exact appreciation of the facts, it would be necessary to know the frequency of cases of cancer after vitamin therapy, in relation to the total of all cases of lupus tuberculosus, appearing either on the cutaneous lesions themselves, or on the skin at another location or in another organ.

Following that, it will still remain to be known whether or not this frequency is greater than that of cancer in cases of lupus not treated with vitamin therapy. It should be remembered that these are not negligeable. We have put together the statistics from Leloir, Sequeira, Reyn, Wichmann, Thieme, Stumpke and Mecking all dating from before the advent of vitamin therapy; they noted 93 cases of cancer in 8,945 cases of lupus vulgaris, in other words, a percentage of 1.04; Darier found the proportion to be 4%. Still, is it known whether the incidence of cancer increases in cases of long-term lupus (generally of more than 12 to 15 years); this "incubation period" must then be taken into account in order to compare the incidence with that of post-vitamin therapy cancer.

Perhaps it will be the same with vitamin D_2, as the cases with radiotherapy, formerly widely used, and accused of favoring the local development of cancer. Several authors (Wichmann Silberstein, Mecking, etc.) have showed that in effect, cancer is not more frequent among lupus patients treated with X-rays than among the others.

If later it appears that vitamin D_2 is carcinogenic, it will be necessary to do research in order to specify the conditions for and the mechanism of carcinogenesis, to reconsider the intensity and the duration of the vitamin therapy, to study the delay which takes place between the therapy and the beginning of the treatment, and, as a consequence, to study whether modifications of the treatment might allow avoidance of the risk.

Finally, experimental research in animals would be necessary in order to study more closely, not only the reality but also the histological or chemical processes of the carcinogenesis. The experiments of Mrs. Chevrel-Bodin and M. Cormier, on the rabbit in 1948, with high and prolonged doses of cod-liver oil showed no evidence of an estrogenic effect or carcinogenic action.

It is necessary, therefore, in order to judge the carcinogenic action of vitamin D_2 to collect the greatest possible number of detailed observations. We ask all those who are using vitamin D_2 therapy to use their form to record their observations on the following data:

1. Frequency of cancer, both in cases of lupus and in other patients treated with vitamin D_2; percentage of cases of cancer in relation to all cases of lupus vulgaris seen and treated with other therapy.
2. Method, duration and intensity of the vitamin treatment prescribed.
3. Delay between the treatment and the beginning of the cancer.
4. Clinical and histological type of cancer.
5. Response of the cancer to accepted methods of treatment.

Only an accumulation of well-documented cases will resolve this question, the importance of which is undeniable.

Summary

The authors recall the arguments that vitamin D_2 so widely used as therapy, especially against lupus tuberculosus, may exert a carcinogenic action.

These arguments are not, however, sufficient to decide this important question. It is necessary to gather precise, detailed data, and only the accumulation of this data will enable us out to find out precisely whether cancers in cases of lupus occur with clearly greater frequency after vitamin therapy than they do spontaneously.

5820

LE TRAITEMENT PAR LA VITAMINE D EST-IL CANCÉRIGÈNE ?

par A. Touraine et M. Zurbick
(Paris, France)

La question a été posée par Desaux et par Stauffer en 1947, par Degos en 1948, par Hornberger en 1949, par Acquasciati en 1950. Elle est d'importance, puisque l'on sait avec quelle libéralité la vitamine D2 est aujourd'hui utilisée dans le traitement de nombreux états pathologiques, en particulier dans celui du rachitisme et de la tuberculose cutanée. Nul doute que, si elle devait se résoudre par l'affirmative, l'emploi de cette médication appellerait de sérieuses restrictions ou, tout au moins, une révision de sa méthode d'application.

Le cas de Desaux est douteux ; il s'agissait d'un adénome symétrique de la face, du type Balzer, situé près du lupus, pour lequel Civatte se demandait si l'on n'assistait pas au début d'une transformation en épithélioma.

Au *29e Congrès de la Société suisse de Dermatologie et de Vénéréologie*, à Genève (13-14 septembre 1947), Stauffer disait avoir vu plusieurs petits cancers se développer sur un lupus au cours de la vitaminothérapie ; ces cancers s'étaient montrés moins radiosensibles que d'habitude.

Plus récemment, en 1949, Hornberger (de Tubingen) signalait l'augmentation considérable des cancers sur lupus, dans sa pratique personnelle, puisqu'elle atteignait 10,5 % de tous les lupus ; la malignité et la tendance à la récidive s'accroissaient aussi. Passant en revue les causes de cette augmentation, il envisageait le rôle possible de la vulgarisation de la radiothérapie, des rayons-limite, des traitements par la vitamine D ou par la thiosemicarbazone.

Cette action cancérigène de la vitamine D2 trouve plusieurs arguments qui plaident en sa faveur.

C'est d'abord la constitution chimique des vitamines D qui, on le sait, sont des stérols du groupe du phénanthrène, c'est-à-dire de corps dont on connaît le pouvoir cancérigène.

C'est aussi la tendance, signalée par Lapière et Van Runckelen, qu'ont les cicatrices de lupus traités par la vitamine D2 à présenter une dyskératose de l'épiderme plus marquée qu'après les anciennes thérapeutiques. Or, la dyskératose est une altération histologique qui caractérise plusieurs états cutanés éminemment précancéreux, tels que la maladie de Bowen, certaines érythroplasies.

Enfin, un nombre relativement élevé de cancers sur lupus traités par la méthode de Charpy-Fauielle ont été publiés depuis la diffusion de cette méthode. Outre ceux de Desaux, de Stauffer déjà signalés, mentionnons ceux de Bocage, de Gaté, Bonder et Fayart en 1946, de Colin-Jones en 1947, de Degos et Delzant, de Jaeger (3 cas), de Cosentino et Musumeci en 1948, de Corrente-Siergo, de Touraine et Mlle Lambergeon en 1949.

⁂

Ces arguments sont-ils suffisants pour diminuer la valeur d'un traitement qui, par ailleurs, a montré sa réelle efficacité contre le lupus tuberculeux ? A-t-on le droit de formuler, dès aujourd'hui, des réserves sur le pronostic lointain de la vitaminothérapie D2, en soulignant la possibilité d'une cancérisation à plus ou moins longue échéance chez les sujets qui lui ont été soumis ?

Dans l'état actuel de nos connaissances, il ne nous paraît pas possible de répondre à ces questions.

Nous avons cherché à estimer, d'après la littérature récente, la fréquence des cancers sur lupus traités par la vitamine D2. Nous n'avons trouvé aucune donnée utilisable. Si les publications abondent sur les résultats cliniques et histologiques immédiats ou à courte échéance, nous manquons encore de renseignements sur l'avenir lointain des malades. Seule l'observation de Degos et Delzant précise qu'il s'est écoulé 2 ans entre la fin du traitement et le début du cancer. Il est très probable que, s'il existe une relation de cause à effet, le délai nécessaire pourra être beaucoup plus long.

Pour permettre une appréciation exacte des faits, il serait nécessaire de connaître la fréquence, par rapport à la totalité des lupus tuberculeux, des cas de cancer après vitaminothérapie, soit sur les lésions cutanées elles-mêmes, soit sur la peau à distance, soit dans un autre organe.

Il resterait ensuite à savoir si cette fréquence est, ou non, supérieure à celle du cancer sur lupus non soigné par la vitaminothérapie. On se rappellera que celle-ci n'est pas négligeable. Nous avons réuni les statistiques de Leloir, Sequeira, Reyn, Wichmann, Thieme, Stümpke, Mecking, toutes datant d'avant la vitaminothérapie ; elles signalent 93 cas de cancer sur 8.945 lupus vulgaires, c'est-à-dire un pourcentage de 1,04 ; Darier portait même cette proportion à 4 %. Encore sait-on que l'incidence du cancer s'élève d'autant plus qu'il s'agit de lupus anciens (généralement de plus de 12 à 15 ans) ; il faudra donc tenir compte de ce « temps d'incubation » pour le comparer avec celui des cancers post-vitaminiques.

Peut-être, alors, en sera-t-il, pour la vitamine D2, comme pour la radiothérapie, jadis très utilisée, qui a été accusée de favoriser le développement local du cancer,

5820

Divers auteurs (Wichmann, Silberstein, Mecking, etc.) ont montré, en effet, que le cancer n'était pas plus fréquent chez les lupiques traités par les rayons X que chez les autres.

Si, ensuite, il apparaît que la vitamine D2 est cancérigène, il faudra chercher à préciser les conditions et le mécanisme de la cancérisation, considérer l'intensité et la durée de la cure vitaminique, le délai qui s'est écoulé entre celle-ci et le délai du cancer et, en conséquence, chercher si des modifications de cette cure ne permettent pas d'éviter ce risque.

Enfin, des recherches expérimentales, chez l'animal, seraient nécessaires pour étudier de plus près non seulement la réalité mais aussi le processus histologique ou clinique de la cancérisation. Les expériences de Mme Chevrel-Bodin et M. Cormier, chez le lapin, en 1948, avec l'huile de foie de morue à hautes doses prolongées, ne sont d'ailleurs en faveur ni d'un effet œstrogène ni d'une action cancérigène.

***

Il importe donc, pour juger de l'action cancérigène de la vitamine D2, de recueillir le plus grand nombre possible d'observations détaillées. Nous demandons à tous ceux qui ont recours à la vitaminothérapie D2 d'apporter leurs documents personnels, en attirant leur attention sur les précisions suivantes :

1° Fréquence du cancer, soit sur le lupus, soit ailleurs, chez les malades traités par la vitamine D2 ; pourcentage par rapport à tous les cas de lupus vulgaire observés et soumis à d'autres thérapeutiques.

2° Mode, durée et intensité du traitement vitaminique suivi.

3° Délai entre ce traitement et le début du cancer.

4° Type clinique et histologique du cancer.

5° Obéissance de ce cancer aux traitements usuels.

Seule, l'accumulation de cas bien observés permettra de résoudre cette question dont l'importance n'échappera à personne.

RÉSUMÉ

Les auteurs rappellent les arguments qui ont quelquefois fait penser que la vitamine D2, si utilisée en thérapeutique, notamment contre le lupus tuberculeux, pouvait avoir une action cancérigène.

Ces arguments ne sont cependant pas suffisants pour trancher cette importante question. Il est nécessaire de réunir des faits précis, détaillés, dont l'accumulation permettra, seule, de préciser si le cancer sur lupus est nettement plus fréquent après vitaminothérapie que spontanément.

SUMMARY

The question of the carcinogenic action of vitamine D2 was raised by Desaux and Stauffer (1947), Degos (1948), and Hornberger (1949). There are several arguments in favour : the chemical composition of the vitamins D, the tendency to the formation of dyskeratoses in the scars of lupus treated with vitamin D2 (and dyskeratoses are a feature of several precancerous skin conditions), and the relatively high incidence of skin cancer in cases of lupus treated by the method of Charpy-Fanielle. No conclusion can be reached on the data available at present and the authors request all doctors prescriving vitamin D2 therapy to use their form on which the following data should be recorded :

1° Incidence of cancer, both in cases of lupus and in other conditions treated with vitamin D2 ; percentage of cases of cancer in all cases of lupus vulgaris seen.

2° Method, duration, and osage of vitamin D2 employed.

3° Time interval between commencement of treatment and appearence of cancer.

4° Clinical and histological type of cancer.

5° Response of cancer to accepted methods of treatment.

If vitamin D2 is shown to be carcinogenic, research of this type will be necessary to discover whether modification of the regime will allow it to be continued without risk, and to discover experimentally the exact means by which the cancer is produced.

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CLINICAL PHARMACOLOGY
Office of
J. W. HINMAN, PH.D.

February 21, 1974

Humphrey F. Sassoon, Ph.D.
Tracor Jitco, Inc.
1300 East Gude Drive
Rockville, Maryland 20851

Dear Dr. Sassoon:

Dr. Babcock discussed with me your phone call of February 7 and forwarded a copy of your letter.

In response, in the hope that it could prove of some value to you, we are enclosing a copy of our clinical brochure but with the request that it be retained in confidence and not be included among the documents published by NTIS. In this spirit, however, the references cited to published material may be of some value to you although we suspect you will have seen most of them.

For the record, the following statement we believe to be a fair summarization for 25-OHD_3 and may be incorporated in whole or in part if you are so inclined:

> "Questions have been raised concerning the current status of the active metabolites of Vitamin D_3, 25-OHD_3 and 1,25-$(OH)_2D_3$, and the closely related analog, 1αOHD_3. In support of basic research by Dr. DeLuca and by clinical scientists working in the field, The Upjohn Company, Kalamazoo, Michigan, prepared a Master File, completed 6-month toxicology in the rat and dog, and provided unit dosage forms to investigators who had effective INDs, with approval of the FDA. More recently, they have filed an IND and are sponsoring clinical studies to establish conditions under which 25-OHD_3 is safe and effective in treating specific indications which have represented problems in the use of available forms of the vitamin.

While it is premature and inappropriate to draw conclusions at this time, it may be noted that the use of Vitamin D has been associated with several therapeutic difficulties, including: individual variability of response from subject to subject; lag time between dosing and response; danger of overdose due to retention of the vitamin in tissues; and absence of a clinically convenient assay to monitor serum levels. Because of the multi-step enzymatic pathway involved in the activation of Vitamin D, it may be hoped that management of these therapeutic problems will be relieved in part through the use of 25-OHD_3. For specific indications, it may be expected that 1α-OHD_3 and 1,25-$(OH)_2D_3$ will be uniquely useful. Clinical availability of the active metabolites should provide alternative approaches to the treatment of resistant patients and eliminate the necessity of using heroic doses of Vitamin D itself with the long-term hazards involved."

Sincerely,

J. W. Hinman, Ph.D.

JWH:sd

Encl.

VASCULAR ENZYME ACTIVITY CHANGES ACCOMPANYING THE INDUCTION OF EXPERIMENTAL ATHEROSCLEROSIS

PART 2. RATS FED EXCESS VITAMIN D

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(Received December 12th, 1964)

INTRODUCTION

Feeding an excess of vitamin D causes a type of mediocalcinosis in the arteries of rats[1]. A combination of vitamin D intoxication with cholesterol feeding and physical exercise[2], or with feeding of egg yolk and cholic acid, together with induced hypothyroidism[3-5] causes atherosclerosis-like lesions, even in the rat. CONSTANTINIDES recently described a further modification of technique for inducing atherosclerotic lesions very quickly (within 6 weeks) in rats, using a "lipaemia-producing" diet and feeding the animals a large excess of vitamin D for only 3 days. The diet used in these experiments was essentially the same as that described by HARTROFT AND THOMAS[6], but without excess butter.

In view of the data from the above-mentioned studies on the successful combination of various procedures with calciferol-induced lesions, it seemed to be important to know what kind of vascular enzyme activity changes develop in rat aortae as a result of calciferol feeding. It may be added that such an investigation might be of additional interest in view of the fact that in old rats there appears a kind of spontaneous arteriosclerosis, possessing the characteristics of mediocalcinosis[7]. This applies particularly to female rats which have been bred repeatedly[8-11]. According to ZÁHOŘ AND CZABANOVÁ[12], there is a connection between lactation and the appearance of such lesions.

MATERIAL AND METHODS

Adult albino male rats were used. Both the control and the experimental rats were [illegible] ordinary laboratory diet (per kg: coarse meal 623 g, casein 163 g, dried whole milk 109 g, lucerne 32.5 g, cod liver oil 7 g, hydrogenated vegetable fat 47 g, $CaCO_3$ 16 g, and NaCl 2.5 g). The experimental rats received, in addition, a dose of 30,000 units of vitamin D in oil (Calciferol Spofa) daily, for a period of 5 or 9 days. The [illegible] was fed to unanaesthetised animals by means of a syringe with a short length of [illegible] attached. The animals were killed by decapitation at

times indicated below, and immediately after killing the aortae were removed, rinsed several times in ice-cold saline, and carefully freed of peri-aortic tissue and almost the entire adventitia.

The preparation of homogenates and extracts was done in the same way as described in the preceding paper[13], and the methods used for biochemical determination of enzyme activities were also identical. In addition, the activities of dehydrogenases in some of the experimental series were determined by a technique in which phenazine methosulphate and neotetrazolium hydrochloride (NT) were used as electron-acceptors. In all determinations, the incubation vessels contained 1.5 ml of Krebs-Ringer phosphate buffer at pH 7.4, 1.5 ml of the appropriate substrate (i.e. 0.2 *M* solution of sodium lactate, malate or succinate), 0.4 ml of 0.5 % NT, 0.2 ml 10^{-3} *M* solution of phenazine methosulphate, and 0.2–0.4 ml of the aortic extract. In succinate dehydrogenase activity determinations, however, 10 mg of aortic powder prepared using liquid nitrogen, was used instead of the aortic extract. In lactate and malate dehydrogenase determinations, 0.2 ml of NAD (2×10^{-3} *M* freshly prepared), and 0.2 ml of 0.1 *M* NaCN were also added. Following incubation (45 min at 38°C) and centrifugation (3000 r.p.m., for 5 min), the diformazan was extracted with 2 × 5 ml redistilled acetone, for 20 hours. The amount of diformazan was estimated at 470 mμ, and calculations were performed on the basis of the protein content of the aortic extract, or in the case of succinate dehydrogenase on the basis

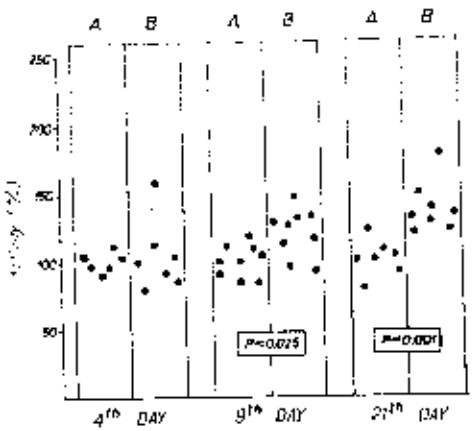

Fig. 1. The activity of phosphomonoesterase I in the aortae of calciferol-intoxicated rats. A: control rats. B: rats fed calciferol and killed on the 4th, 9th or 21st day of the experiment, respectively. Average activity in the aortae of control rats: 10.05 units (μmoles of phenol liberated/killing protein of aortic extract). Results expressed as percentage differences.

of the DNA content of the aortic tissue, determined according to the method of SCHNEIDER[14] and CERIOTTI[15].

RESULTS

As shown by Figs. 1–4, no significant change in the activities of the aortic

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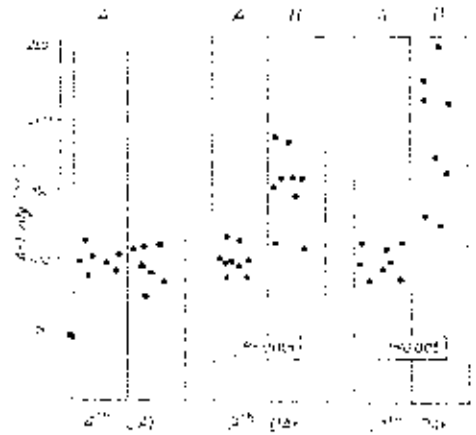

Fig. 2. The activity of phosphomonoesterase II in the same aortae as in Fig. 1. Average activity in the aortae of control rats: 1.05 units (nmoles of phenol liberated/h/1 mg protein of aortic extract). Results expressed as percentage differences.

enzymes studied could be found in calciferol-fed rats on the fourth day of the experiment. However, in rats which had been fed calciferol daily and were killed on the ninth day of the experiment, a significant decrease in the activity of group-specific carboxylic esterase occurred, and this contrasted with increases in the activi-

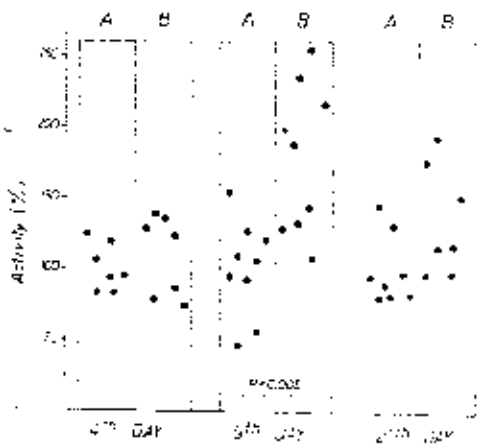

Fig. 3. The activity of 5'-nucleotidase in the same aortae as in Fig. 1. Average activity in the aortae of control rats: 1.61 units (μg of phosphate liberated/30 min/100 μg of protein of aortic extract). Results expressed as percentage differences.

ties of phosphomonoesterase I, phosphomonoesterase II and 5'-nucleotidase. The activities of phosphomonoesterase I and II remained significantly elevated in rats fed calciferol for 9 days and killed on the 21st day, while in the activities of 5'-nucleotidase and carboxylic esterase, merely a trend toward increase or decrease, respectively, could be observed. In the activity of aortic adenylpyrophosphatase, no difference between experimental and control animals could be shown.

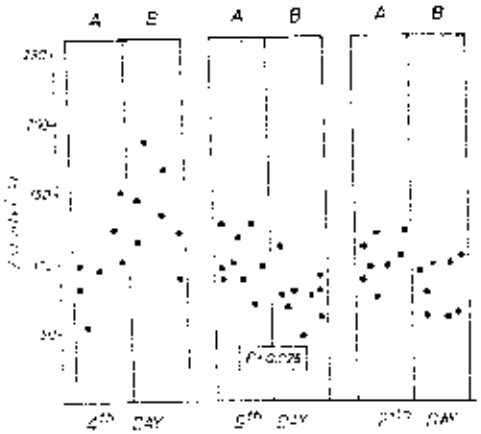

Fig. 4. The activity of group-specific carboxylic esterase in the same aortae as in Fig. 1. Average activity in the aortae of control rats: 22.56 units (μg of β-naphthol/h/100 μg of protein of aortic extract).

In another experimental series (Fig. 5), in which calciferol feeding lasted for only 5 days, an increase in the activity of phosphomonoesterases could again be observed on the ninth day of the experiment, together with a trend towards a decrease in carboxylic esterase activity. In addition, there was also a tendency toward an elevation of phosphomonoesterase I activity as early as the fifth day. This finding is in agreement with the very early increase in phosphomonoesterase activity demonstrated by histochemical means in the muscle cells of the media, surrounding deposits of calcium phosphate (see LOJDA *et al.*, to be published together with the whole histological picture). It is interesting that at this early stage of the experiment there also appears a slight but significant increase in the activity of adenylpyrophosphatase, which is not demonstrable at later stages.

In the activities of lactate and malate dehydrogenase, no change could be observed in these very early stages of the experiment, either by the "optical test" based upon changes in NAD concentration, or by the combined phenazine methosulphate and neotetrazolium method. Much later, however, there appeared a clear tendency towards a decrease in dehydrogenase activity, in particular in the succinate dehydrogenase system[16].

DISCUSSION

From the data gained in studies mentioned in the introduction, it seems to be established that the combination of a lipaemia-producing diet of the cholesterol-thiouracil cholate (HARTROFT) type with calciferol-feeding induces, even in rats, lesions similar to those of spontaneous atherosclerosis in man.[4]

The detailed morphological picture of the arteries in calciferol-fed rats is well known, *e.g.* from the excellent study of GILLMAN *et al.*[17], and no attempt will be made here to repeat their statements. Suffice it to mention, however, that in contrast to

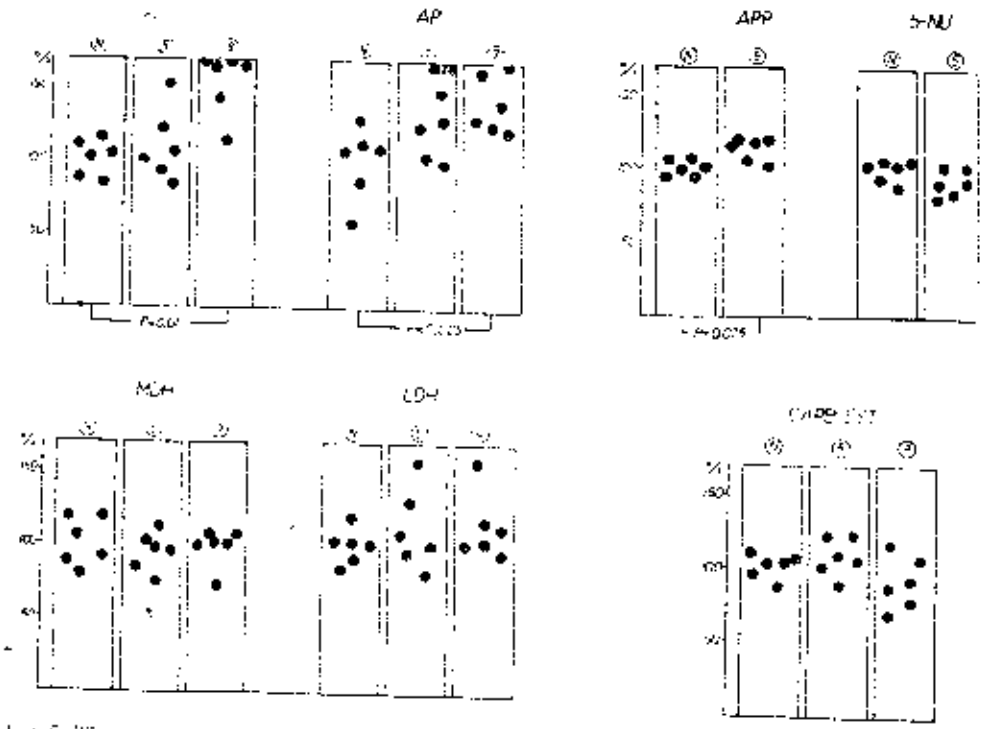

Fig. 5. The activity of some enzymes in a further series of rats, fed on the basis of eighth day of the experiment. AP: phosphomonoesterase (pH 9); ACP: phosphomonoesterase (pH 5); APP: adenosyl-phosphatase; 5'-NU: 5'-nucleotidase; MDH: malate dehydrogenase (Pennington et al., 1961); lactate dehydrogenase (Cappiello et al., 1961); carb. est.: gamma-naphthyl carboxylic esterase. Average activities of the aortae of control rats (in units as indicated in text to Figs. 1–4): AP, 8.78; ACP, 1.24; APP, 8.61; 5-NU, 2.12; carb. est., 41.65. Average activity of MDH, 221.79 (unit of 1.191, 47.90 Wróblewski units/100 mg of protein of aortic extract). Results expressed as percentage differences.

what is seen in the arteries of rats fed HARTROFT's diet[18], a characteristic feature of the aortae of calciferol-fed rats is a very early and intense reaction of the connective tissue. This is characterised, histochemically, by an increase and depolymerisation of mucoproteins, and by a derangement of collagenous structures, leading to the formation of a sort of vascular scar tissue[11]. It may be added that such tissue changes facilitate not only metastatic calcification[18], a well-known feature of the arteries of these rats, but are also, according to GILLMAN *et al.*[17,19], a prerequisite for lipid accumulation.

Both experimental and spontaneous atherosclerosis are characterised by connective tissue changes as well as by an accumulation of lipids. Therefore, as already previously stated[13], study of the above two experimental models separately (e.g. as though they are) may yield valuable information about the enzymic changes associated with the two facets of atherosclerotic lesions.

As seen from our results, a related feature of the aortae of calciferol-fed rats is

an early increase in the activities of phosphomonoesterase (and of 5'-nucleotidase). In view of the facts mentioned above it is reasonable to suppose that the increases in these enzyme activities might reflect an increase in vascular connective tissue metabolism. It is only fair to point out that so far little of a definite nature is known of the exact physiological role of these enzymes. Nevertheless, it has been suggested by several authors (although refuted by others) that phosphomonoesterase (and 5'-nucleotidase) are in some way connected with tissue calcification. Another much disputed function, ascribed to the former enzyme, would be in the formation of fibrous precursors and in vascular collagen fibres[21]. Such views seem to be consistent with our findings and their interpretations.

Concerning phosphomonoesterase II, this enzyme can be found in lysosomes[22], and according to NAVIKOFF[23] lysosomal enzymes play an important role in pinocytosis, phagocytosis and lytic processes.

The decrease in the activity of carboxylic esterase could be expected from our previous experience with this enzyme activity[15] as well as with the lipolytic activity in the aortae of such animals[24]. This phenomenon might be associated with the accumulation of abnormal mucopolysaccharides or calcium salts in the aorta, as both factors under certain circumstances inhibit the lipolytic activity of aortic tissue[25,26].

Our results show that there is no change in the activity of malate dehydrogenase in the early stages of calciferol feeding, and the same was found to apply to succinate dehydrogenase, in another series. Changes in the activities of dehydrogenases of the tricarboxylic acid cycle appeared only late in the experiment (*i.e.*, after 3 or more weeks).

It must be pointed out that the enzymic changes just described are in sharp contradistinction to those found in the experimental model characterised only by lipid accumulation without connective tissue reaction[13]. The decreased activity of tricarboxylic acid cycle enzymes is a feature common to the arteries of rats fed HARTROFT's diet[18], the arteries of cholesterol-fed rabbits[27,28], and human arteries or those segments of arteries in which the extent and severity of atherosclerosis is characteristically of a higher degree[29–31]. On the other hand, with the exception of the rats fed HARTROFT's diet, the arteries in those situations just mentioned show an increase in the activity of some phosphomonoesterases.

Such findings are consistent with the view that changes in phosphomonoesterase activities (including 5'-nucleotidase) might reflect primary or secondary connective tissue changes in experimental and spontaneous human atherosclerosis also. On the other hand, as suggested in our previous paper[13], the decreased activity of respiratory enzymes might be related to the accumulation of lipids in the vessel wall.

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SUMMARY

Male albino rats were fed a large excess of vitamin D in oil (Calciferol Spofa), and changes in certain enzyme activities were studied. It is known that a characteristic feature of the aortae of such rats is a very early and intense reaction of their connective tissue, leading to the formation of a sort of vascular scar tissue.

It was found that the aortae of calciferol-fed rats showed, in addition to a decrease in esterolytic activity, early increases in the activities of phosphomonoesterase I and II, and of 5'-nucleotidase. The activities of the tricarboxylic acid cycle enzymes do not change in these early stages of the experiment.

These results oppose those found in rats fed "HARTROFT's diet", where the aortae display endothelial lipid accumulation without a connective tissue reaction.

Such findings are consistent with the view that changes in vascular phosphomonoesterase activities (including 5'-nucleotidase) might reflect primary or secondary connective tissue alterations, while the decreased activity of respiratory enzymes might in some way be related to other events, in particular to the accumulation of lipids in the vessel wall.

REFERENCES

1 [illegible], *Arch. Path.*, 281 (1932) 151.
2 J. H. DUNCAN, *J. Pathol. Bacteriol.*, 33 (1930) 597.
3 G. F. WILGRAM, *Exhibition Proc.*, 16 (1957) 270.
4 G. F. WILGRAM, *Proc. Soc. Exptl. Biol. Med.*, 99 (1958) 496.
5 P. CONSTANTINIDES, *Circulation*, 28 (1963) 663.
6 W. S. HARTROFT AND W. A. THOMAS, *J. Am. Med. Assoc.*, 164 (1957) 1899.
7 K. B. HEMMES AND L. L. BARNES, *Am. J. Pathol.*, 14 (1938) 121.
8 D. L. SNIDER AND G. F. BAKER, *Record Progr. Harrison Res.*, 8 (1953) 143.
9 H. C. WEXLER AND B. L. MILLER, *Science*, 127 (1958) 590.
10 H. C. WEXLER, *J. Atherosclerosis Res.*, 1 (1961) 57.
11 T. GILMAN AND M. HATHORN, *Nature*, 183 (1959) 1139.
12 Z. ZÁVODA AND V. CYRANOVÁ, *J. Atheroscler. Res.*, 1 (1961) 435.
13 T. ZEMPLÉNYI, J. HLOUSKA AND J. MIKUŠOVÁ, *J. Atherosclerosis Res.*, 5 (1965) 540.
14 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
15 F. CERIOTTI, *J. Biol. Chem.*, 198 (1952) 297.
16 T. ZEMPLÉNYI, J. MIKUŠOVÁ, Z. LOJDA AND D. GRANSCHEIER, In: H. PRUSIK, Z. REPISÍS AND D. RIEDL (Eds.), *Metabolismus Parietis Vasorum*, State Medical Publ. House, Prague, 1962, p. 63.
17 T. GILMAN, R. A. GRANT AND M. HATHORN, *Brit. J. Exptl. Pathol.*, 41 (1960) 1.
18 G. V. KRUSHAN AND A. N. HOWARD, *Brit. J. Exptl. Pathol.*, 41 (1960) 395.
19 A. E. SOBEL, *Ann. N. Y. Acad. Sci.*, 60 (1955) 713.
20 T. GILMAN, *Lancet*, ii (1958) 901.
21 B. S. GOLDS, *Urinary Hormones*, 18 (1960) 89.
22 E. L. KUFF AND G. H. GOLDSMITH, In: H. HAMMER (Ed.), *Hormones, Units of Biological Structure and Function*, Academic Press, New York, N.Y., 1956, p. 235.
23 A. D. NOVIKOFF, In: J. BRACHET AND A. E. MIRSKY (Eds.), *The Cell*, Academic Press, New York, N.Y., 1961, p. 423.

24 D. GRANSCHEIER AND T. ZEMPLÉNYI, *Arteriosclerosis*, 18 (1962) 85.
25 S. GEORGE, J. GEROCZY, F. DEVÉNYI, S. VIRÁG, J. SZÉKELY AND L. JAVOR, In: H. PRUSIK, Z. REPISÍS AND D. RIEDL (Eds.), *Metabolismus Parietis Vasorum*, State Medical Publ. House, Prague, 1962, p. 34.
26 D. GRANSCHEIER AND T. ZEMPLÉNYI, *Gerontologia*, 3 (1961) 63.
27 T. ZEMPLÉNYI, Z. LOJDA AND J. MIKUŠOVÁ, In: M. SANDLER AND G. H. BOURNE (Eds.), *Atherosclerosis and its Origin*, Academic Press, New York, N.Y., 1963, p. 456.
28 J. MIKUŠOVÁ, T. ZEMPLÉNYI AND Z. LOJDA, *J. Atheroscler. Res.*, 3 (1963) 11.
29 T. ZEMPLÉNYI, In: R. PAOLETTI AND D. KRITCHEVSKY (Eds.), *Advances in Lipid Research*, Academic Press, New York, N.Y., 1964, p. 235.
30 T. ZEMPLÉNYI AND J. MIKUŠOVÁ, Paper read at the *International Conference on Lipidology and Internal Medicine*, Bandung, October 23 and 24, 1964, *Acta Medica Indonesiana*, for the press.
31 T. ZEMPLÉNYI, J. MIKUŠOVÁ AND D. GRANSCHEIER, Paper read at the *Symposium on Coronary Heart Disease*, Antwerp, November 10, 1964, published in *Bull. Soc. Int. Chir. Suppl. d'Antwerp*, 37 (1965) 55.

6394

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