the fraction containing the particles when prepared from BCG cells was not always immunogenically active. The sediment containing the particles appeared grey in color, and the immunogenic activity was reduced by sonic vibration and by washing with sucrose buffer. 4. A chemical analysis of the particles from both H37Ra and BCG showed that they contain large amounts of lipid and no DNA. The significance of these findings is discussed.

1. Youmans, G. P., Millman, I., and Youmans, A. S., *J. Bact.*, 1955, v70, 557.

2. Millman, I., and Darter, R., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 271.

3. Darter, R. W., and Millman, I., *ibid.*, 1957, v95, 440.

4. Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, v55, 629.

5. Rosenthal, S. R., *Acta Tuberc. Scandinav.*, 1952, v26, 106.

6. Youmans, G. P., and Youmans, A. S., *Am. Rev. Tuberc.*, 1951, v64, 541.

7. Litchfield, J. T., Jr., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 399.

8. Youmans, G. P., and Youmans, A. S., *J. Immunol.*, 1957, v78, 318.

9. Ma, T. S., and Zuazaga, G., *Ind. Eng. Chem.*, Anal. Ed., 1942, v14, 280.

10. Youmans, A. S., and Youmans, G. P., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 566.

11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

12. Marr, A. G., and Cota-Robles, E. H., *Bact. Proc.*, 1956, p113.

13. Robrish, S. A., and Marr, A. G., *ibid.*, 1957, p130.

14. Mudd, S., Takeya, K., and Anderson, H. J., *J. Bact.*, 1957, v72, 767.

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Vit. D and Citrate Metabolism. Inhibition of Vit. D Effect by Cortisol.* (23603)

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The search for metabolic effects of Vit. D which might throw light on its mechanism of action both in promoting bone salt deposition and in regulating serum calcium levels has led to studies of changes in concentrations of citrate in plasma, urine, and tissue associated with deficiency of Vit. D and its correction. Serum citrate levels are reduced in Vit. D deficient infants and are increased by Vit. D administration as is also urinary excretion of citrate(1). If large doses of Vit. D are given to rachitic children serum citrate levels are increased above normal range and hypercitricemia is seen as a manifestation of excessive Vit. D dosage both in patients(2) and experimental animals(3). Administration of Vit. D to Vit. D deficient rats increases the concentration of citrate in serum and in certain tissues such as kidney, bone and intestine although not liver(4). According to Carlsson and Hollunger(5) there is a slight initial decrease of citrate in serum of the Vit. D deficient rat during the first few hours post Vit. D and then a progressive increase which can be detected by 24 hours following Vit. D. The rise of serum citrate after Vit. D administration to some extent parallels the evidences of the antirachitic effect of Vit. D such as increase in serum calcium and phosphorus concentration and bone salt deposition. On the other hand feeding of calcium salts to Vit. D deficient rats given low calcium diets rapidly increases concentration of calcium but concentration of citrate in serum does not show a parallel rise(5). More recently DeLuca, Gran and Steenbock(6) have found that citrate is utilized more rapidly by rat kidney homogenates prepared from Vit. D deficient rats than from animals given Vit. D and they suggest that Vit. D retards conversion of citrate to α keto glutarate, thus leading to intracellular citrate accumulation. The asso-

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TABLE I. Effect of Cortisol on Concentrations of Phosphorus, Calcium and Citrate in Serum, and Calcium and Citrate in Bone of Rachitic Rats.

	Serum			Bone		
Group	Phosphorus	Calcium	Citrate	Calcium	Citrate	Citrate/Calcium
	mg/100 cc			mg/g		
Cortisol group	.8 ± .10 (10)	9.7 ± .17 (8)	1.7 ± .14 (12)	126 ± 3.16 (11)	3.53 ± .16 (11)	28.0
Rachitic controls	2.6 ± .18 (9)	10.1 ± .16 (11)	2.8 ± .18 (14)	109 ± 4.68 (10)	3.38 ± .20 (10)	30.8

Values are means and stand. error of the mean. Concentrations of citrate and calcium in bone are expressed as mg/g of dry fat-free bone. In final column the conc. of citrate in bone is also expressed as mg/g of calcium. Figures in parentheses indicate No. of animals on which the particular determination was made.

ciation of the effect of Vit. D on citrate levels in body fluids with its antirachitic properties has induced speculation that the citrate effect may be the primary effect of Vit. D and that complexing of calcium by citrate is of importance in the calcifying mechanism and in regulation of calcium concentrations of extracellular fluid.

Citrate levels in serum are reduced by cortisone and other steroids with similar biological properties such as cortisol and Δ_1 cortisol. The mechanism of this effect is unknown but it was thought possible to utilize this reaction to determine first whether the Vit. D effect on citrate was suppressed by adrenocortical steroids and second whether the antirachitic potency of Vit. D was influenced by interference with the citrate effect.

Procedure. Three-week-old male rats of the Sprague-Dawley strain were placed upon a synthetic high calcium, low phosphorus rachitogenic diet previously described(7). Following 2 to 3 weeks on this ration when skeletal changes are pronounced, one group of rats was continued without change, while another group was given this same diet to which cortisol was added in concentration of 30 mg/100 g diet. The steroid treated rats continued to eat amounts of diet comparable to the rachitic controls but failed to gain weight. After 10 days animals of both groups were given 100 units of Vit. D and continued on the respective diets while other animals were maintained on the two diets without Vit. D, as controls. Six to 8 days after Vit. D administration, the rats were anesthetized with sodium pentobarbital by intraperitoneal injection and blood obtained by puncture of the abdominal aorta. One tibia was taken for chemical analysis and the other for histological section. Determinations of calcium, phosphorus and citric acid were made on serum and on bone. Bone was dried to constant weight at 105° and defatted by extraction with ethyl ether. It was then dissolved in 8% trichloracetic acid and all analyses were made on trichloracetic acid filtrate. Calcium was determined in serum by a micro method(8) and in bone by the Clark and Collip method(9). Phosphorus was determined by the method of Fiske and Subbarow(10) and citrate by the method of Natelson, Pincus and Lugovoy(11). Tibia for histological section was fixed in 10% formalin, then embedded in celloidin and sections of the proximal third cut without decalcification. Sections were stained with silver nitrate followed by hematoxylin and eosin to reveal the pattern of calcification.

Results. Concentrations of calcium, phosphorus and citrate in serum and bones of Vit. D deficient rats on the rachitogenic diet with and without cortisol are shown in Table I. The low serum citrate levels characteristic of Vit. D deficient rats are further reduced by steroid feeding. Serum calcium concentrations are not altered by steroid feeding but serum phosphorus values are markedly reduced—so low in some animals as to be hardly measurable. Analyses of bone show an actual increase in calcium content of bone of the cortisol treated rats in comparison with control rachitic animals. This is of interest in light of the histological findings in bone discussed below. Citrate concentrations in bone of treated rats are somewhat higher than in controls when values are expressed in terms

TABLE II. Effect of Cortisol on Concentrations of Phosphorus, Calcium and Citrate in Serum, and Calcium and Citrate in Bone of Rachitic Rats Given Vit. D.

	Serum			Bone		
	Phosphorus	Calcium	Citrate	Calcium	Citrate	Citrate/Calcium
	mg/100 cc			mg/g		
Cortisol group		11.6	1.3	141	3.83	27.2
	2.6	10.6	1.5	105	2.46	28.7
		11.5	1.3	123	2.85	24.7
	3.5	12.1		143	3.53	25.0
	4.5	11.1	1.2	137	3.76	27.5
	4.5		1.3	132	3.54	26.8
Control Group 1	5.6 ± .15 (6)	11.5 ± .13 (7)	4.3 ± .13 (7)	130 ± 4.13 (7)	6.09 ± .35 (8)	46.8
Control Group 2	6.1 ± .82 (4)	11.6 ± .24 (4)	5.1 ± .31 (4)	177 ± 7.9 (4)	8.19 ± .44 (4)	46.2

Values in individual rats of cortisol fed, vit. D treated group are compared with mean values of control vit. D treated group (control group 1) and also with mean values of a group of rats which was given vit. D prophylactically in dosage of 100 units/wk while on the rachitogenic diet (control group 2). The data are expressed in the same units as in Table I.

of dried fat-free bone but are reduced below controls when expressed as mg/g of bone calcium. This latter concentration is probably the more significant since it indicates concentration of citrate in the crystals of bone salt. Table II compares similar determinations in Vit. D treated rachitic rats with and without cortisol feedings. For comparison findings in rats given Vit. D prophylactically are also shown (Control Group 2). The obvious finding is the marked reduction of serum citrate concentration in the cortisol group. The Vit. D treated controls show the expected increase of citrate values both in serum and bone. In cortisol fed animals serum citrate levels are actually lower than in D deficient rats despite Vit. D treatment. An increase in concentration of calcium and phosphorus in serum due to Vit. D occurs, however, despite suppression of the citrate effect although serum phosphorus values of cortisol fed rats remain somewhat lower than in controls. Concentrations of calcium in bone are the same in cortisol fed and control Vit. D treated rats. Concentrations of citrate in tibias of control animals are, however, much higher than in those of the cortisol treated group. Administration of Vit. D to control rats resulted in an increase of bone citrate parallel to the increase of serum citrate levels whereas cortisol administration suppressed this Vit. D effect both on serum citrate and bone citrate concentrations. Suppression of the citrate effect did not inhibit healing of the rickets, however, as indicated by histological changes in bone.

Typical sections of proximal tibias of rats of various groups are shown in Fig. 1-3. Tibia of cortisol fed, Vit. D deficient rat shows a retardation of cartilage growth which is in accord with suppression of weight gain. The overall architecture, however, is that of severe advanced rickets, *viz*: accumulation of proliferative cartilage, degenerative changes in this cartilage, and on the shaft side irregular in-

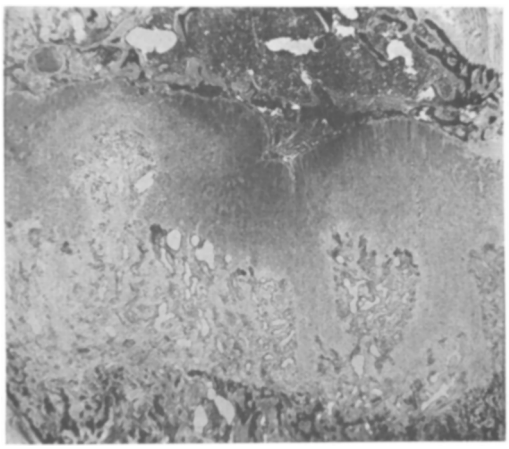

FIG. 1. Section of tibia of control rat fed rachitogenic diet. In this and subsequent Figures the epiphysis is at the top and the metaphyseal bone at bottom of photomicrograph. Magnifications are the same in all Figures (× 17). This section shows the extremely broad zone of uncalcified proliferative cartilage and accompanying degenerative changes.

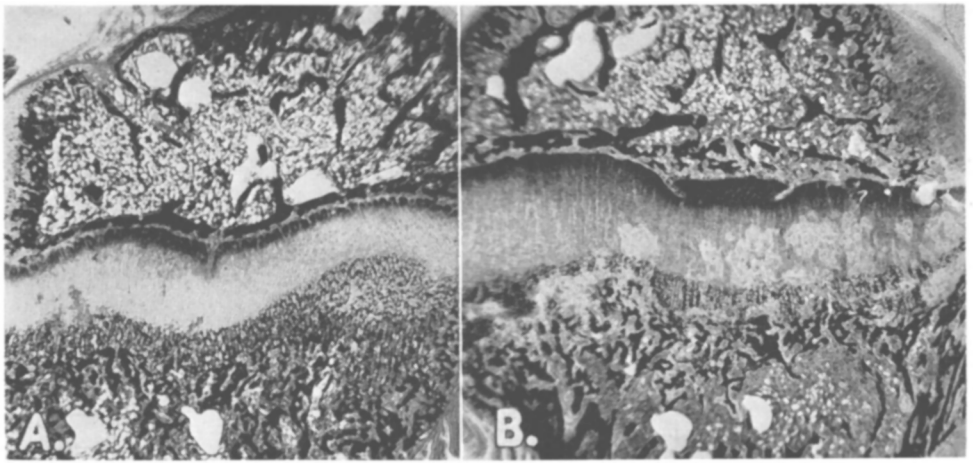

FIG. 2. Sections of tibias of rats given cortisol supplement without vit D. Inhibition of cartilage growth is shown by narrowing of proliferative cartilage. There is also a zone of calcification in the cartilage which borders on the shaft.

vasion of cartilage by vascular elements which sprout into it from the shaft at scattered points with tongues of cartilage in between. The trabeculae also bear witness to severity of the rickets by heavy encasements of osteoid. The most conspicuous difference from the severe rickets of control rats is that in tibia of cortisol fed rats the cartilage mass, which borders on the shaft, is heavily calcified. Calcification extends into the horizontal partitions of matrix separating cells from each other so that many individual cells are surrounded by calcium phosphate precipitate. It is of interest that cartilage cells in the calcified zone are intact with well preserved nuclei since in normal sequence the cartilage cell dies as the surrounding matrix becomes calcified under influence of invading capillary osteoblast complex. There is, further, little evidence of invasion by the capillary osteoblast complex into the calcified zone preliminary to its transformation into bone. In the shaft osteoid encasements of trabeculae are thick. Slight depositions of calcium phosphate may have occurred here and there in the osteoid. It is difficult to estimate osteoblastic activity

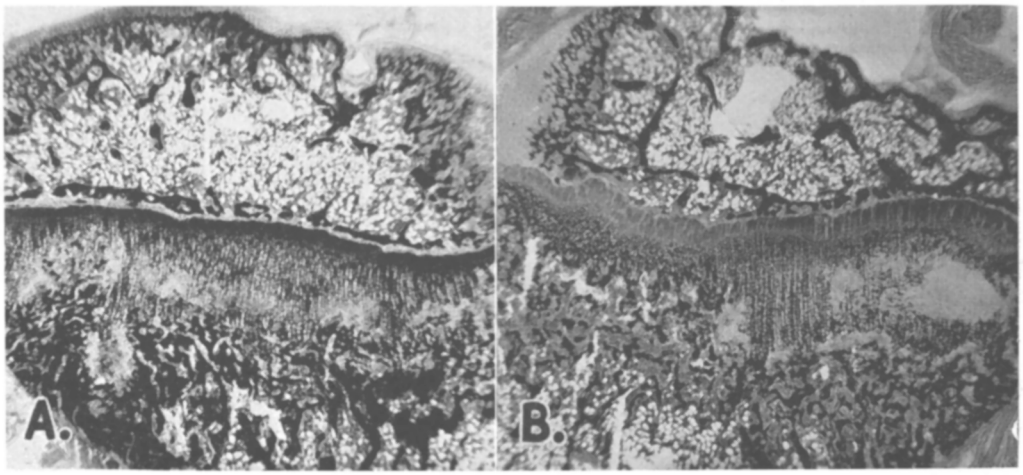

FIG. 3. Sections of tibias of rats given cortisol supplement and treated with vit D. Calcification of cartilage occurred in 2 zones, one just above the metaphyseal bone and another more distal at usual site of calcification in healing rickets. At some points the 2 zones have coalesced. These sections show both the growth inhibitory effects of cortisol and the antirachitic effect of vit D.

in these preparations but it appears that the osteoblastic coverings of the trabeculae are scantily developed and few osteoclasts are seen so that both formative and destructive aspects of cellular activity have been inhibited.

Tibias of cortisol fed rats treated with Vit. D show a characteristic zone of calcification high up in the proliferative cartilage, in the zone where healing normally occurs whether induced by Vit. D, citrate feeding or starvation. In all but one animal a fragmentary zone of calcification at the level seen in the cortisol fed Vit. D deficient rats can also be distinguished. The separate existence of this zone is obliterated in places where the zone of calcification resulting from Vit. D treatment has become broad enough to coalesce with cortisol induced calcification. In the one rat failing to show two separate zones of calcification the probable reason is that coalescence is complete. In the shaft the osteoid is calcifying as shown by diminished width of osteoid borders and deposits of streaks and sheets of precipitate in its substance. Capillary and cellular activity also appear to have been somewhat stimulated and there are signs of capillary invasion in the metaphysis, rows of osteoblasts are more prominent, and osteoclasts are also easily found. Some osteoblasts and osteoclasts are strikingly large. Administration of Vit. D to cortisol fed rachitic rats appears to have visibly increased cellular activity both of formative elements and destructive elements of bone.

Discussion. Feeding of cortisol in doses which inhibit growth reduces the concentration of citrate in serum and bones of Vit. D deficient rats and suppresses the enhancing effect of Vit. D on serum and bone citrate. Despite prevention of the citrate effect of Vit. D, cortisol does not block the antirachitic action of physiologic doses of Vit. D as measured both by rise of serum calcium and phosphorus concentrations and by calcification of rachitic cartilage. An antivitamin D action of cortisone has been suggested because of its effect in lowering the elevated serum calcium levels of patients with sarcoidosis and hypervitaminosis D(12). The present studies indicate a dissociation between antirachitic property of Vit. D and its effect upon citrate concentrations in body fluids and demonstrate that changes in serum and bone citrate levels are not a necessary concomitant of the healing of rickets. Citrate accumulation in bone may simply represent citrate uptake by bone crystals in contact with extracellular citrate and in cortisol Vit. D treated rats bone crystal deposition occurs in the presence of low extracellular citrate concentrations. The source of the extracellular citrate increment which usually follows Vit. D administration remains unanswered. Increased content of citrate in kidney and intestine of Vit. D treated rats(4) and decreased utilization of citrate by kidney homogenates of these rats(6) suggest that increased extracellular citrate reflects intracellular accumulation of citrate. It is clear, however, that increased citrate concentrations in serum and bone are not prerequisites for acceleration of the calcification mechanism by Vit. D.

The marked hypophosphatemia of the cortisol fed Vit. D deficient rat cannot be ascribed to an effect of cortisol upon renal excretion of phosphate since on low phosphate diet the urine is free of phosphate both in controls and cortisol fed animals. The depletion of extracellular phosphate must result from diminished intestinal absorption of phosphate or increased tissue uptake. It is unlikely that soft tissue uptake of phosphate is increased in view of inhibition of growth resulting from cortisol. There is evidence, however, that bone uptake of phosphorus is greater in cortisol treated rats than in controls. Calcification in rachitic cartilage in these rats, despite extremely low levels of serum phosphorus suggests that inhibition of cartilage growth by cortisol is associated with some change in cartilage matrix which results in local precipitation of bone salt despite depletion of extracellular phosphate. Cellular processes of bone destruction and resorption of bone salt may also be inhibited by cortisol. Tibias of steroid treated rats are heavier in proportion to body weight and have a higher concentration of calcium and phosphorus than those of control rats. If total phosphorus in a single tibia is adjusted to body weight, the average tibial phosphorus/100 g of body

weight is 9.3 mg in Vit. D deficient cortisol fed rat in comparison with 5.9 mg in comparable control rats. Following Vit. D administration this value is 12.9 mg in cortisol fed rats and 8.1 mg in controls. Suppression of body weight increment by cortisol is thus not accompanied by proportionate decrease of bone salt mass. Histological sections indicate that cartilage cell proliferation is inhibited by cortisol, and also that osteoblasts and osteoclasts are less prominent. The cellular activity of bone tissue seems to have been checked so that resorption as well as formation of bone is depressed. In bones of Vit. D treated rats not only has calcification of proliferative cartilage and osteoid been initiated but also the inhibitory effect of cortisol on bone cell activity appears to have been overcome.

Summary. Correlation of the effect of Vit. D in augmenting concentrations of citrate in plasma and bone with its antirachitic action has been studied. Rats were made rachitic by feeding a Vit. D deficient diet which was also low in available phosphorus. Addition of cortisol to the diet of such rats reduced concentration of citrate in serum and also blocked the effect of Vit. D in increasing serum and bone citrate levels. Antirachitic action of Vit. D as measured by rise of serum phosphorus concentrations and by histological evidences of calcification of rachitic cartilage and osteoid was not suppressed by cortisol. The antirachitic action of Vit. D and its effect upon citrate metabolism can, therefore, be separated. The tibias of Vit. D deficient cortisol fed rats show evidences of increased calcification in comparison with rachitic controls which might in part be due to inhibition of bone resorption as well as retardation of cartilage growth by cortisol. This increased calcification in cortisol fed rats is associated with extreme depletion of extracellular phosphate.

1. Harrison, H. E., and Harrison, H. C., *Yale J. Biol. Med.*, 1952, v24, 273.

2. Harrison, H. E., *Pediatrics*, 1954, v14, 285.

3. Freeman, S., and Chang, T. S., *Am. J. Physiol.*, 1950, v160, 341.

4. Steenbock, H., and Bellin, S. A., *J. Biol. Chem.*, 1953, v205, 985.

5. Carlsson, A., and Hollunger, G., *Acta Physiol. Scand.*, 1954, v31, 317.

6. DeLuca, H. F., Gran, F. C., and Steenbock, H., *J. Biol. Chem.*, 1957, v224, 201.

7. Harrison, H. E., and Harrison, H. C., *ibid.*, 1950, v185, 857.

8. ———, *J. Lab. Clin. Med.*, 1955, v46, 662.

9. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, v63, 461.

10. Fiske, C. H., and Subbarow, Y., *ibid.*, 1925, v66, 375.

11. Natelson, S., Pincus, J. F., and Lugovoy, J., *ibid.*, 1948, v175, 745.

12. Anderson, J., Dent, C. E., Harper, C., and Philpot, G. R., *Lancet*, 1954, v2, 720.

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Rapidly Sedimenting Properties of Specifically Precipitating Component of a Hashimoto's Disease Serum. (23604)

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Evidence has been presented that chronic thyroiditis in humans may be the result of an autoimmunization process in which an individual produces antibodies against tissue components of his own thyroid. Thus, Rose and Witebsky have shown that thyroiditis similar to that in humans can be produced in dogs and rabbits by injecting the animal with a thyroid extract, either of other thyroids of the same species or even a portion of the individual's own thyroid(1,2). Roitt, Doniach, Campbell and Hudson have reported, moreover, that the serum of patients with Hashimoto's disease contains materials capable of