VITAMIN D AND CITRATE METABOLISM: STUDIES ON RACHITIC INFANTS*

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There are many reasons for considering the possibility that the metabolism of citrate is interrelated with the physiological effects of vitamin D and with the equilibrium of calcium and phosphorus in the body. Hamilton and Dewar[10] and Shohl[17] demonstrated that the addition of citrate to rachitogenic diets resulted in prevention or cure of rickets in rats. Subsequently, Shohl and Butler[19] showed that the feeding of mixtures of sodium citrate and citric acid to rachitic infants on a milk diet produced healing of the rickets although no vitamin D was given. Since the administration of dicarboxylic acids which do not form complex ions with calcium does not cause healing of rickets,[17] it is possible that the effect of oral citrate is related to its property of reducing the concentration of ionized calcium by virtue of the formation of an undissociated calcium citrate complex.[11] Dickens[5] added further interest to the possible rôle of citrate in bone formation by his studies of the citrate content of bone. He noted that bone contained appreciable amounts of citrate which was bound to the bone salt so that it was not eluted by water. The citrate content of the bone of a rachitic animal was found to be less than that of normal bone. Nicolaysen[16] extended this particular phase of the study and found that the bone citrate of rachitic rats was reduced to a greater extent than was the calcium so that the molar ratio of citrate to calcium in rachitic bone was less than that found in the bone of normal rats. The presence of citrate in bone could be merely another consequence of its reaction with calcium to form an undissociated complex and may not necessarily indicate any participation of citrate in the primary reactions leading to deposition of bone salt. The possibility that the metabolism of citrate might be altered in the vitamin D deficient subject developed from a study by Freudenberg,[8] which was concerned with the effect of vitamin D on the urinary excretion of organic acids in rachitic infants. Freudenberg suggested that the total organic acid excretion was high in the rachitic infant and was reduced by vitamin D therapy but his results were inconclusive. He had also determined separately the citrate content of the urine, and although there was considerable variation from day to day there did seem to be a real increase of citrate excretion following vitamin

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D therapy. This prompted us to study the concentration of citrate in the serum and the excretion of citrate in the urine of rachitic infants before and after treatment with vitamin D.

METHODS

The subjects were ten infants who had the characteristic clinical, x-ray, and biochemical evidences of active rickets and who had received little or no vitamin D prior to admission to the hospital. These infants were placed on a uniform diet consisting chiefly of whole cow's milk plus ascorbic acid and, for the older infants, supplements of cereal and vegetables. Repeated determinations of serum calcium, phosphorus, alkaline phosphatase, and citrate were made before and after treatment with vitamin D. The chemical methods used were as follows: calcium, Kramer and Tisdall[13];

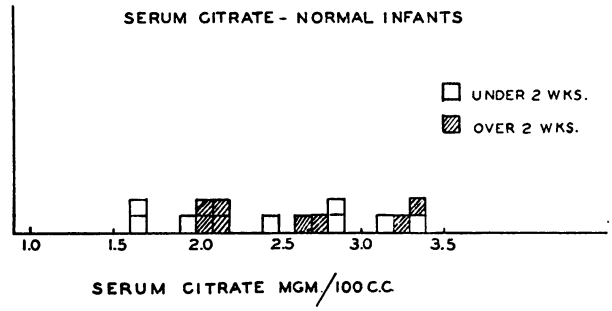

FIG. 1. Distribution of the values for serum citrate in the serum of normal infants. Determinations in infants under two weeks of age designated by open squares, those in infants between two weeks and two years of age by squares with diagonal lines.

phosphorus, Fiske and Subbarow[6]; alkaline phosphatase, Bodansky[3]; and citrate, Natelson, Pincus, and Lugovoy.[14] All blood samples were obtained in the fasting state to avoid changes of serum citrate due to carbohydrate ingestion.[15] In the infants on a four-hour feeding schedule the sample was obtained immediately before a feeding. After a preliminary period of observation, vitamin D_2 was given in a single dose of 600,000 units injected intramuscularly. This route was used in an effort to obtain uniformity of dosage. Serial x-ray examinations of the bones were made to correlate the anatomical evidences of healing with the changes in the serum levels of calcium, phosphorus, and citrate. In three infants the urine was collected quantitatively and the daily excretion of citrate determined before and after vitamin D therapy. The urine was collected in bottles containing 15 to 25 cc. of 10% acetic acid to prevent bacterial destruction of citrate.

RESULTS

The distribution of the values for serum citrate in a group of normal infants under two years of age is shown in Figure 1. The average value for the entire group of 16 infants is 2.5 mg. per 100 cc. with a standard deviation of $\pm$ 0.59. The variability seems to be somewhat greater in the newborn infants than in the older group. The average value found in the entire group is in good agreement with the average concentration of 2.8 mg. per 100 cc. found in a group of normal children by Natelson, *et al.*,[15] and the

average value of 2.4 mg. per 100 cc. reported by Hagelstam[9] in normal subjects.

Table 1 summarizes the observations in a four-month-old rachitic infant. Prior to therapy the concentrations of serum citrate were found to be 1.5 and 1.6 mg. per 100 cc. Following injection of 600,000 units of vitamin D there was a prompt rise of the serum citrate level which increased progressively to a maximum of 3.6 mg. per 100 cc. approximately two weeks after

TABLE 1

EFFECT OF VITAMIN D UPON THE CONCENTRATIONS OF SERUM CITRATE AND URINARY EXCRETION OF CITRATE IN AN INFANT WITH RICKETS

A.C. AGE 4 MOS.

Day	P	*Serum* Ca *Mg./100 cc.*	*Cit.*	*Urine Cit. Mg./day*
1	3.8	9.1	1.5	
6				67
7	3.8	10.3	1.6	83
8				86
9		*Vit. D, 600,000 units*		
10				100
11				92
12				109
13				103
14	5.2	10.9	2.9	
17				133
18				143
19				126
20	6.4	10.3	3.2	146
21				160
22				162
25	7.3	12.0	3.6	
40	6.7	11.4	3.0	
376	6.2	10.5	2.4	

therapy. The increase in concentration of serum citrate paralleled the rise of the values for serum phosphorus and coincided with healing of the bone lesions as shown by x-ray examinations. A definite elevation of the concentration of serum calcium also occurred, but this appeared to lag behind the increase of the levels of serum citrate. The urinary excretion of citrate rose immediately following the injection of vitamin D, increasing from an average pre-treatment level of 70 mg. per day to a maximum value of 162 mg. per day following vitamin D.

A similar course of events in another infant with rickets (A. S., age 18 months) is shown in Table 2. The serum citrate levels which were 1.9 and

1.8 mg. per 100 cc. before therapy increased to a high value of 3.8 mg. per 100 cc. following the injection of 600,000 units of vitamin D. The urinary excretion of citrate increased from an average pre-treatment level of 83 mg. per day to a maximum value of 155 mg. per day. The rise of the values for serum and urine citrate coincided again with other evidences of vitamin D effect, viz., increase of the concentration of serum phosphorus and, to a lesser extent, of the concentration of serum calcium, in addition to evidences of healing by x-ray.

TABLE 2

EFFECT OF VITAMIN D UPON THE CONCENTRATIONS OF SERUM CITRATE AND URINARY EXCRETION OF CITRATE IN AN INFANT WITH RICKETS

A.S. AGE 18 MOS.

Day	P	*Serum* Ca *Mg./100 cc.*	*Cit.*	*Urine Cit. Mg./day*
1	3.0	8.9	1.9	
3				83
4				79
5				88
6	3.0	9.9	1.8	
7		*Vit. D, 600,000 units*		
8				108
9				115
10				155
11				151
14	4.6	10.6	3.3	
18	6.9	11.2	3.8	

In Figure 2 are summarized the results of similar studies of M. N., an eight-month-old rachitic infant. In this boy the concentrations of serum citrate before therapy were quite low, 1.2 and 1.3 mg. per 100 cc. Following the injection of 600,000 units of vitamin D the levels of serum citrate and of serum phosphorus increased slightly and the concentration of serum calcium rose. The concentrations of serum citrate and phosphorus did not, however, show the progressive increase seen in the previous patients and on x-ray there was little evidence of healing. Accordingly 25 days later a second injection of 600,000 units of vitamin D was given. The serum citrate level increased rapidly following this second dose to a maximum of 4.6 mg. per 100 cc. and the concentration of serum phosphorus likewise rose to levels compatible with the rapid healing of the rickets which was now evident by x-ray. The urinary excretion of citrate increased progressively after the first dose of vitamin D, but there was little further change following the second course despite the continued rise of serum citrate.

In Figure 3 are charted the concentrations of serum citrate of all ten infants with rickets arranged with respect to the time before or after the administration of vitamin D. One other infant besides patient M. N. (Fig. 2) showed little therapeutic effect following the first dose of 600,000 units of vitamin D, and a second dose of 600,000 units of vitamin D was given to produce complete healing. For these two patients the data are plotted with respect to the time of administration of the second course of vitamin

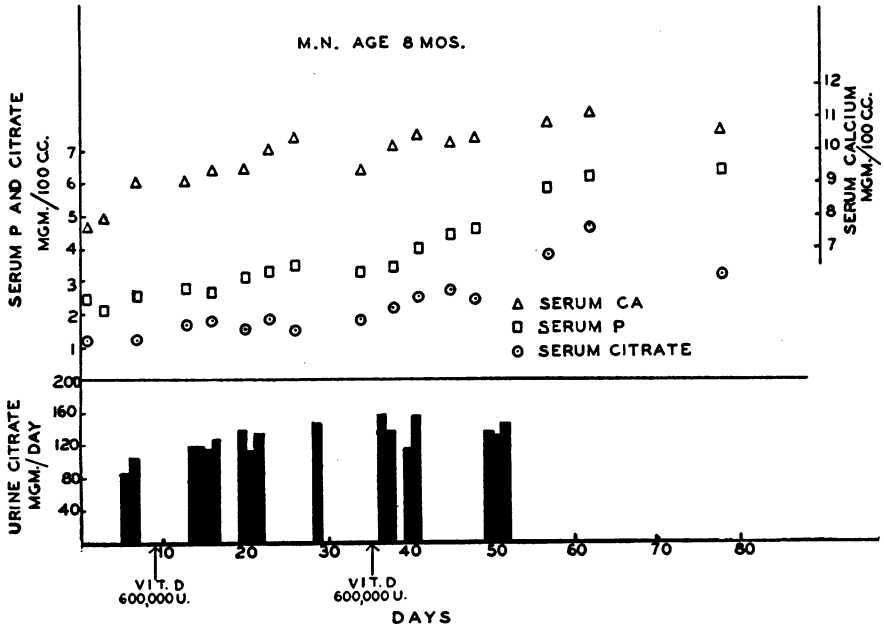

FIG. 2. Effect of vitamin D upon the urinary excretion of citrate, and upon the concentrations of citrate, phosphorus, and calcium in the serum of a rachitic infant, M. N., age 8 months. The urinary citrate is shown by the solid columns, serum citrate by ⊙, serum phosphorus by □, and serum calcium by △. The days on which vitamin D was injected are designated by the arrows.

D. The average value of 21 determinations of serum citrate in the ten rachitic patients before administration of any vitamin D was 1.5 ± 0.38 mg. per 100 cc. in comparison with the average value of 2.5 mg. per 100 cc. in the group of normal infants. This difference is statistically significant, $p < .01$. Following treatment with vitamin D the concentrations of serum citrate rose in all patients to values of 2.5 mg. or more. The rate of increase was variable so that in some instances a definite rise was found within five days, whereas in other cases ten or more days elapsed before the serum citrate rose to values above 2.0 mg. per 100 cc. In no instance was the level of serum citrate found to be low in a patient who showed evidences of healing of the rickets by x-ray. In several patients the concentration of serum citrate rose to levels above 3.5 mg. per 100 cc. and as high as 4.6 mg.

per 100 cc., which are above the normal range. These excessively high values of serum citrate are presumably evidences of temporary hypervitaminosis D, as they gradually dropped again to normal levels over a period of several months.

In order to see whether the levels of serum citrate were correlated with either the levels of serum calcium or of serum phosphorus, the concentra-

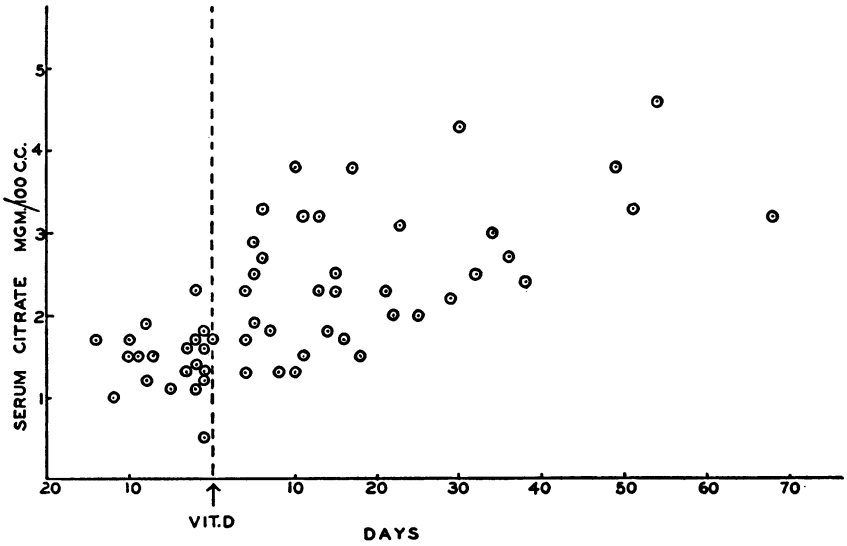

FIG. 3. Concentrations of serum citrate in group of ten rachitic infants plotted with respect to time before or following administration of vitamin D.

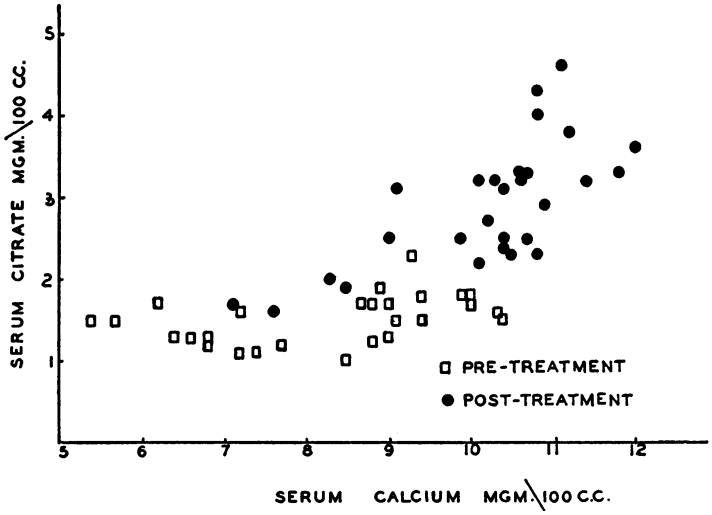

FIG. 4. Relationship of concentration of serum citrate to that of serum calcium in rachitic infants. Values before treatment with vitamin D shown by open squares, values following treatment with vitamin D by filled circles.

tions of serum citrate on the ordinate were plotted against the concentrations of serum calcium on the abscissa in Figure 4 and against the concentrations of serum phosphorus in Figure 5. The pre-treatment data are indicated by the open squares and the post-treatment values by the solid circles. Although in Figure 4 there is an apparent correlation between the concentration of serum citrate and that of serum calcium, closer analysis reveals that the data fall into two separate groups. There is no correlation between the levels of serum citrate and serum calcium before treatment, but

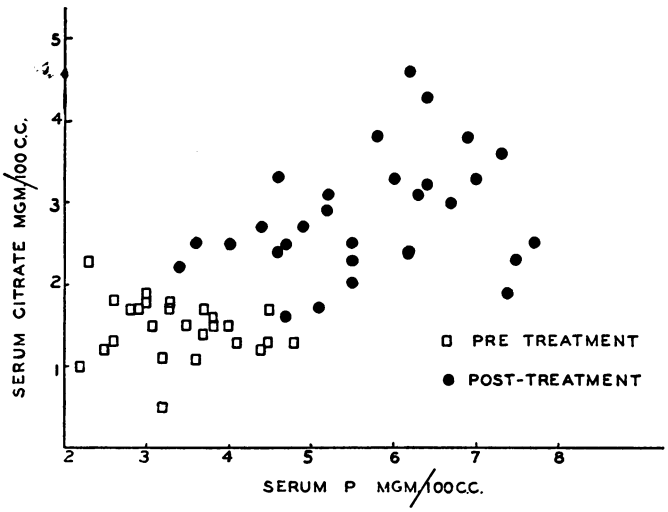

FIG. 5. Relationship of concentration of serum citrate to that of serum phosphorus in rachitic infants. Values before treatment with vitamin D shown by open squares, values following treatment with vitamin D by filled circles.

there is a rise of the concentration of serum calcium as well as of serum citrate after vitamin D. The data for the levels of serum citrate and serum phosphorus in Figure 5 show the same separation into two groups with no correlation between the concentrations of serum phosphorus and serum citrate in the pre-treatment group, whereas in the post-treatment group the increase of serum citrate is seen to be roughly parallel to the increase of serum phosphorus.

DISCUSSION

The data show unequivocally that the concentration of citrate in the blood serum and the urinary excretion of citrate are influenced by vitamin D. There are variations in the extent and the rate of rise in the levels of serum citrate following administration of a standard dose of vitamin D to infants and children with rickets. The intramuscular administration of total doses of 1,200,000 units of vitamin D to two children with rickets resulted in temporary rises of serum citrate to supernormal levels despite slow

response to an initial dose of 600,000 units. The essential problem is whether the effect of vitamin D on the level of citrate in the serum and the urinary excretion of citrate affords us a clue to the mode of action of vitamin D on tissue cells, or whether it is simply a phenomenon secondary to the effect of vitamin D on the levels of calcium and phosphorus in the body fluids.

In a recent review of the Krebs citrate cycle,[18] citrate is considered to be an integral link in the series of transformations of organic acids which are required for the oxidation of the "activated acetate" which is formed in the metabolism of protein, fat, and carbohydrates. Citrate is therefore continuously being formed and broken down in tissue cells, and the levels of citrate in the blood serum should reflect the balance of these processes in tissue cells. Temporary reductions of serum citrate follow the administration of glucose or the injection of insulin with subsequent return to the basal level.[15] Under *in vitro* conditions the synthesis of citrate is inhibited by addition of calcium,[13] but in the intact animal conditions which cause hypocalcemia are associated with reduction of serum citrate, and increase of the serum calcium is accompanied by increase of serum citrate. For example, it has been reported that in two patients with hypoparathyroid tetany the serum citrate was reduced to 1.3 and 1.6 mg. per 100 cc.,[1] whereas in two patients with hyperparathyroidism the serum citrate was elevated to 4.0 mg. per 100 cc. Shorr, *et al.*,[20] also reported a rough parallelism between urinary excretion of calcium and of citrate in a patient with hyperparathyroidism and in a subject with hypoparathyroidism given parathyroid extract. Since the metabolism of citrate has been studied in but few patients with disturbances of parathyroid function the results can only be considered as suggestive. In intact dogs an increase of serum citrate coincident with increase of serum calcium has been reported following administration of parathyroid extract.[1] Freeman and Chang[7] determined the concentrations of serum calcium and citrate of thyroparathyroidectomized dogs and found that the level of serum citrate was reduced as hypocalcemia developed. When large doses of vitamin D were given to these animals, the concentrations of serum citrate and of calcium both increased. All of these effects of parathyroid hormone and of vitamin D might be interpreted as indicating that the citrate levels of the body fluids are influenced by the concentrations of serum calcium, or, conversely, that the changes in the level of calcium in the serum produced by both parathyroid hormone and vitamin D are in some way related to effects of these agents upon the metabolism of citrate.

In the present studies on rickets there is a partial correlation between the levels of serum citrate and of serum calcium, but the data plotted in Figure 4 do not indicate a direct relation between the concentrations of total serum calcium and of serum citrate. In this diagram the points fall into two distinct groups. In the rachitic pre-treatment group the concentrations of serum citrate are low, ranging between 1 and 2 mg. per 100 cc., and there

is no correlation with the concentrations of serum calcium which vary between 5 and 10.5 mg. per 100 cc. Following treatment, the levels of both serum citrate and calcium rise so that there is a correlation between these two variables in the post-treatment group. These results suggest that the increased concentration of serum citrate following vitamin D is not the result of increase in the level of serum calcium but that both of these changes are manifestations of vitamin D effect. Similar comparison of the levels of serum inorganic phosphorus and of serum citrate reveals a correlation between these two variables, but separation of the data into pre-treatment and post-treatment groups again suggests that there is no direct relation between the concentrations of serum phosphorus and of serum citrate, but that the simultaneous increase of the levels of serum phosphorus and serum citrate following vitamin D therapy are both manifestations of vitamin D effect. It is possible, of course, that there may be changes in the concentrations of intracellular calcium or of organic phosphoric acid esters which may influence the metabolism of citrate, and that such variations in the intracellular state of calcium and phosphorus are not directly related to the concentrations of these ions in extracellular fluid.

There is a large body of data which show that the urinary excretion of citrate is influenced by the acid-base equilibrium of the body. Administration of alkali results in an increased excretion of citrate, whereas acidifying salts produce a decreased excretion of citrate. The acid-base ratio of the diet is of significance in the experimental rickets of rats. Certain neutral or alkaline ash diets which are not rachitogenic produce rickets when the acid intake is increased.[18] In the human variety of rickets due to vitamin D deficiency without abnormal ratios of calcium to phosphorus in the diet, there is no evidence of any disturbance of acid-base equilibrium which could account for the reduction of the concentration of citrate in serum and urine or for the increase in these values following vitamin D therapy.

The diet of the infants was composed chiefly of milk which contains about 0.16% citrate.[4] There is no direct evidence concerning the efficiency of absorption of dietary citrate, but it seems highly unlikely that increased absorption of citrate could explain the increase in serum citrate levels resulting from vitamin D therapy. When citrate is administered orally in large amounts it is rapidly utilized so that there is little increase in urinary excretion of citrate. Following oral or intravenous administration of citrate to normal subjects it is removed rapidly from the blood and the concentration of serum citrate returns to basal levels.[3] Persistent changes in the concentration of citrate in the serum probably indicate changes in the metabolic processes involved in the formation and transformation of citrate in the tissues.

The biochemical mechanisms involved in the interrelation of vitamin D and the metabolism of citrate and of calcium are unexplained. Further studies are in progress which may throw light on this problem.

Summary

Studies of citrate metabolism were made in ten rachitic infants. In this group the concentration of citrate in the serum was determined before and following the administration of vitamin D. In three infants measurements of the urinary excretion of citrate were also made and the effect of vitamin D studied.

The average level of serum citrate in the rachitic infants was found to be reduced to 1.5 mg. per 100 cc. in comparison with the average value of 2.5 mg. per 100 cc. in normal infants. Following intramuscular injection of either a single dose of 600,000 units of vitamin D or a total of 1,200,000 units in two injections the level of serum citrate rose progressively. In several infants the concentration of citrate in the serum increased following treatment to values above the normal range and then returned to normal levels. The urinary excretion of citrate increased following vitamin D treatment in each of the three infants studied.

The increase in concentration of citrate in the serum was found to coincide with the other manifestations of vitamin D effect which were studied, i.e., rise of the level of serum phosphorus, increase in the concentration of serum calcium, and healing of the bone lesions as demonstrated by x-ray examination.

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