The serum citrate concentration in hereditary vitamin D resistant rickets and in idiopathic hypercalcemia

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Fredric K. Cantor

1962
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IN HEREDITARY VITAMIN D RESISTANT RICKETS
AND IN IDIOPATHIC HYPERCALCEMIA

Fredric K. Cantor
B.A. Yale University, 1958

A thesis presented to the faculty of the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine

Department of Pediatrics
1962
THE SERUM CITRATE CONCENTRATION
IN HEREDITARY VITAMIN D RESISTANT RICKETS
AND IN IDIOPATHIC HYPERCALCEMIA

T. R. Cohen
P. A. Yale University, 1956

A thesis presented to the faculty of the Yale University School
of Medicine in partial fulfillment of the requirements for
the degree of Doctor of Medicine

Department of Pediatrics
1965
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I also wish to thank the gracious patients and family who cooperated so willingly in order to make this study possible.
INTRODUCTION

Many attempts have been made to elucidate the role of citric acid in bone metabolism, both in health and in certain disease states. Documentation of variation in serum citrate concentration in idiopathic vitamin D resistant rickets and in idiopathic hypercalcemia has been uncertain. The studies presented in this paper were undertaken in an attempt to clarify the role of serum citrate in these two disorders of calcium-phosphorus metabolism, to investigate the usefulness of serum citrate measurements in identifying heterozygotes in familial hypophosphatemia and to ascertain the mode of inheritance of familial hypophosphatemia in a previously unstudied kindred.

Parathyroid Hormone and Vitamin D

Attention was first drawn to the existence of a mechanism for the homeostatic regulation of body calcium in 1909 by MacCallum and Voegtlin (1) who observed a lowering of the serum calcium following removal of the parathyroid glands. The occurrence of hypocalcemia with hypoparathyroidism and of hypercalcemia in hyperparathyroidism have long been recognized. The presence of bone lesions in certain cases of hyperparathyroidism has demonstrated the existence of a relationship between parathyroid hormone, calcium metabolism and the mineral content of the skeleton.

That vitamin D, too, has an effect on the mineralization of bone has been borne out by its effective use as a therapeutic agent in deficiency rickets (3). Elevated serum calcium found in instances of overdosage with vitamin D has provided direct evidence of the influence of that vitamin on the concentration of calcium in the blood (3). This relationship forms the basis for the use of vitamin D in treating hypocalcemia in patients with hypoparathyroidism and chronic renal disease.
There have been several attempts to clarify more precisely the relationship between parathyroid hormone activity and the homeostatic regulation of body calcium. The decrease in serum phosphorus found in both vitamin D deficiency rickets and vitamin D resistant rickets with normal or diminished serum calcium implied that skeletal mineralization may be retarded by a deficiency of phosphorus as well as calcium. Reciprocity between serum concentration of calcium and of phosphorus was noted in chronic renal disease. Albright and Reifenstein (2) felt that the prime stimulus for parathyroid gland activity was an elevation of serum phosphorus and that the primary action of parathyroid hormone was to decrease renal tubular reabsorption of phosphorus. According to this schema the changes in serum calcium would be a secondary effect. The development of secondary hyperparathyroidism in cases of chronic renal disease manifesting a high serum phosphorus was cited in support of this concept. Further evidence was provided by an observation of the reduced ability of patients with hypoparathyroidism to excrete phosphates. Rasmussen (6) feels that parathyroid hormone increases the level of serum calcium by increasing renal tubular reabsorption of calcium and, in addition, by decreasing renal tubular reabsorption of phosphorus. In this latter schema a decrease in the concentration of calcium ion in the serum serves as the stimulus for parathyroid gland activity.

Much less is known about the manner in which vitamin D exerts its influence on calcium metabolism. There is evidence that vitamin D increases both intestinal absorption and mobilization of calcium from bone (8). Other observations suggest that vitamin D increases intestinal absorption and tubular reabsorption of phosphorus in vitamin D deficiency rickets (3).

Citric Acid

In 1941 Dickens (9) demonstrated that the concentration of citrate in
bone was greater than that in serum, cartilage or marrow. He also noted that prolonged administration of parathyroid extract to a normal dog tended to increase bone citrate and serum calcium while bone citrate dropped in a cat made rachitic by a high calcium - low phosphorus diet. Many others have observed an apparent relationship between citrate and calcium. Hypercitremia has been found to be associated with hypercalcemia in hyperparathyroidism (2, 10, 11), Paget's disease (2) and in some patients with hypercalcemia following prolonged immobilization (2). Conversely, a decrease in serum citrate has been noted in cases of hypoparathyroidism (2, 10, 12) and in vitamin D deficiency rickets (12).

Neuman and Neuman (8) feel that although the solubility product of calcium ion and phosphate is a constant, it is supersaturated with respect to hydroxy apatite, the mineral phase of bone, in serum and in areas of bone formation and is undersaturated in areas of bone resorption. This implies the presence of a barrier between the interstitial fluid in the bone and the remainder of the extracellular fluid. The authors believe that this barrier may be maintained by a substance which promotes local calcium solubility, possibly a chelating agent or an organic acid. Such an agent would have to be metabolized rapidly by extra-skeletal tissues to be consistent with the relatively meager amount of chelated calcium in the circulation and the existence of a stable body pH. Since "citrate ion is the most potent calcium-complexing agent of all the organic acids yet studied" is plentiful in bone and is rapidly oxidized by extra-skeletal tissues (especially kidney) (8), it is well-suited for maintaining the necessary dynamic relationships between bone and body fluids. Neuman and Neuman (8) postulate that citric acid is manufactured by the cellular elements of bone under the influence of vitamin D and parathormone.

Administration of vitamin D to animals made rachitic by feeding a
high phosphorus - low calcium, vitamin D - free diet as well as to patients with vitamin D deficiency rickets causes an increase in serum calcium, serum phosphorus and serum citrate (3, 13, 17). Bone citrate, which is also depressed in experimental and clinical cases of deficiency rickets, also rises following vitamin D administration (8, 13-17). Harrison has observed that serum citrate is decreased in vitamin D deficiency rickets regardless of the level of serum calcium (12) and that a rise in citrate can be detected before a demonstrable rise in calcium (3). This implies a primary action of vitamin D on citrate metabolism, while citrate, in turn, brings about calcification. Indeed, it has been shown that oral administration of citrate can "cure" radiologically demonstrable lesions of deficiency rickets (12, 18, 19). In addition Joshi and others found a decrease in citrate content and citrogenase activity in rachitic cartilage (20). Following administration of vitamin D, there was a significant increase in both (13).

The fact that blood collected from the spongiosa of bone shows a greater increase in citrate content following parathyroid extract administration than does simultaneously collected arterial blood (21) suggests that parathormone, too, influences the production of citrate by bone.

Familial Hypophosphatemia

Idiopathic vitamin D resistant rickets appears to be caused by an hereditary metabolic error (4). In general the skeletal lesions and biochemical characteristics of this disease resemble those of deficiency rickets. Common metabolic features of both are hypophosphatemia, elevated serum alkaline phosphatase and diminished renal tubular reabsorption of phosphorus. Massive doses of vitamin D, however, are necessary to obtain any healing of the rachitic bone changes in resistant rickets, and, in
some cases, the response is poor and erratic even with administratation of toxic amounts of vitamin D. In contradistinction to deficiency rickets neither serum phosphorus nor renal tubular reabsorption of phosphorus increases following administration of vitamin D even though skeletal calcification takes place (3, 4). "Asymptomatic carriers" of this disease have been identified by the presence of hypophosphatemia (4). Serum citrate has been reported as normal (3, 4) in the few cases of resistant rickets studied. In addition oral administration of citrate does not promote bone calcification in this disease. This suggests a difference between deficiency and resistant rickets. In one case treated with vitamin D, however, serum citrate was observed to rise as healing took place, while both serum calcium and serum phosphorus remained at a constant level (3).

In the majority of cases studied by Winters and his colleagues, resistant rickets seems to be inherited as a sex-linked dominant, the gene (or genes) involved being carried on the X chromosome (4, 22). In a few families the mode of transmission appears to be by means of an autosomal dominant (22), and in one case there was no evidence of resistant rickets in any other members of the family (23). Winters postulates that there may be several genes capable of influencing phosphorus excretion. Accordingly, a change at any one of several loci might be responsible for the appearance of resistant rickets (22).

Idiopathic Hypercalcemia

Idiopathic hypercalcemia is a syndrome which usually appears between 1 and 15 months of age. There apparently two types: a benign form, usually lasting no longer than a few months, and a chronic, severe form. The symptoms of the former are those of hypercalcemia - that is, failure to thrive, muscular weakness, polyuria, polydipsia, constipation, anorexia,
nausea, vomiting and weight loss (24). In the severe type elfin facies (low-set ears, a large upper lip, a short upturned nose with nostrils pointing anteriorly and prominent epicanthal folds), osteosclerosis, physical and mental retardation and, sometimes, a heart murmur are found in addition to the symptoms of hypercalcemia. Many patients with the severe form exhibit hypertension (24), azotemia, and renal damage which is probably secondary to the hypercalcemia (26). Elevated free serum cholesterol has been consistently found in both forms of this disease (27), and, in some cases, there have been abnormally high levels of vitamin D activity in the serum (28, 29). In both types of idiopathic hypercalcemia the serum phosphorus is normal or slightly elevated and the alkaline phosphatase is normal. These findings help to differentiate this disease from primary hyperparathyroidism, which may present with very similar symptoms. Smith and others (29) feel that serum citrate is initially elevated in severe idiopathic hypercalcemia and gradually drops to normal during therapy; in their case this paralleled the drop in vitamin D activity in the serum. Forfar and others (27) found that serum and urinary citrate were both low in a group of untreated cases of both types. Following treatment of the latter cases, serum citrate rose to levels greater than normal as the patients' condition improved and finally approached the normal level when the children had almost fully recovered. Fellers and Schwartz (28) also found an elevation of serum citrate in both forms of the disease.

Many authors (28, 29) are of the opinion that the hypercalcemia is caused by defective vitamin D metabolism, although Forfar and others (25) have pointed out the possibility of the existence of a disorder in cholesterol metabolism leading to the production of sterols with 'vitamin D-like' activity. Therapy has generally consisted of a low calcium, vitamin
D-deficient diet and the administration of steroids (29).

Present Studies

The present study showed no relationship between serum citrate and familial hypophosphatemia. Serum citrate concentration in normals was found to vary by age group. Moderate but statistically insignificant elevations of serum citrate were found in a case of severe idiopathic hypercalcemia.

The mode of transmission of familial hypophosphatemia in the kindred studied was consistent with that of a dominant linked to the X chromosome.
CASE MATERIAL AND METHODS

Subjects

Serum samples were collected from 3 brothers, ages 4½, 6 and 8, with the clinical and biochemical characteristics of vitamin D resistant rickets. All were under treatment with large doses of vitamin D. Samples were also obtained from 48 other members of the kindred (Fig. 1) including the mother, aged 31, who had marked skeletal deformities of the lower extremities and the grandmother, aged 57, who had mild bowing of the legs. Neither of these women had evidence of active bone disease. None of the other members of the kindred were known to have bone disease. More than one specimen was taken from several members of the kindred. Samples were also obtained from a 1½ year-old girl from another family who had bow-legs, low serum phosphorus, elevated serum alkaline phosphatase and radiologic signs compatible with rickets despite an intake of vitamin D adequate for a normal child.

Serum was taken at several intervals between 1½ and 2 years of age from a child with the severe type of idiopathic hypercalcemia. She was being treated with prednisone during almost all of this period.

Methods

Processing of samples. Serum for phosphorus and citrate determinations was frozen before or immediately after precipitation with TCA if the chemical determinations were not to be done immediately. Control studies undertaken indicated that these slightly different means of processing the samples introduced no variables into the results with the methods utilized. Samples for calcium determinations were sent directly to another laboratory.

Chemical determinations. The Clark–Collip method (32) was used for
the measurement of serum calcium and the Fiske–SubbaRow procedure (31) for inorganic phosphorus. Serum citrate was determined by the method of Natelson and others (30) modified as follows. The protein in a sample consisting of 0.5cc instead of 0.2cc of serum was precipitated with 15% TCA. Readings were taken in a spectrophotometer with a 1 cm instead of a 5 cm light path. Several samples of a standard solution of citric acid as well as samples of pooled sera were run to verify the accuracy of the method and to show that Beer's Law was obeyed for a range of 1 ug to 20 ug of citrate. Further verification of accuracy was obtained by recovery experiments and by determining that the standard deviation of 7 samples of a standard solution was less than .001 absorbancy units when read in a Beckman DU spectrophotometer. Finally, the color of the pentabromoacetone-thiourea complex was read at 430mu rather than at 445mu in accordance with Elliott's (38) observations.

**Statistical methods.** The curve for normal citrate was derived as follows:

1) The values were divided into consecutive groups of 4. (Since there were 35 normal values in all, the 'extra' value was arbitrarily included in the grouping with the 8 - 10 year-old age group.)

2) The means were found for each group.

3) A curve of best fit (spline-fitted) was fitted to the means. It was felt that the curve obtained by the procedure outlined above resulted in as accurate a fit as might be obtained by following the more traditional procedure of deriving a mathematical function to fit the data (37).

The standard deviation for citrate was obtained from the following formula:

\[ S.D. = \sqrt{\frac{(y-y')^2}{n}} \]
where \( y \) = the obtained value for serum citrate
\( y' \) = the predicted " " " for that age
\( n \) = the number of normal cases.

Since the citrate curve represents predicted rather than mean values for serum citrate, it was felt that the above formula would be the most accurate expression of the S.D. (37).

The S.D. obtained was 0.78mg%.
RESULTS

Phosphorus

The range of serum phosphorus found in the 51 members of the kindred studied is shown in Fig. 2 where the values are plotted against the age of each subject. The normal range for age and sex for serum phosphorus established by Greenberg and others (33) in an examination of the sera of 908 normal people was used to classify the results obtained in this study. The distribution of values in unaffected individuals shown in Fig. 2 suggests a variation by age group.

Eight individuals were found to have concentrations of serum phosphorus more than $2\frac{1}{2}$ S.D. (99% confidence limit) below the mean for their respective ages using the standards set by Greenberg and others (33). Four of these were the 3 boys (IV-6, IV-4, IV-5 in Fig. 1) and the 1\frac{1}{2} year-old girl with active bone disease described above, and one was the mother of these boys (III-3), also described previously, who had skeletal deformities but no active bone disease. The 7\frac{1}{4} year-old male (I-1) with hypophosphatemia was the maternal great-grandfather of the 3 boys mentioned above, and the two remaining abnormal subjects were sisters - in - law (I-3, I-4) to the great-grandfather. None of these 3 people had clinical evidence of bone disease.

Two females (II-1, II-9) were found to have values for serum phosphorus between 2 S.D. (95% confidence limit) and $2\frac{1}{2}$ S.D. below the mean for their respective ages. One of these women, the grandmother of the 3 boys described above, had mild bowing of the legs, and the other was her sister. These results are summarized in Table 1. The individuals with hypophosphatemia and skeletal deformities are indicated in Fig.1.
Citrate

The values of serum citrate for 39 members of the kindred under study are plotted against age in Fig. 3. For purposes of analysis those individuals without clinical bone disease whose serum phosphorus was within 2 S.D. of the mean were considered "normals". The curve shown in Fig. 3 was fitted to the values obtained from citrate determinations on 33 members of the kindred with normal serum phosphorus and no bone disease. The method used for deriving the curve is discussed in the "Methods" section above. The S.D. found was 0.78 mg%. The distribution suggests an age-related variation of serum citrate.

Concentrations of serum citrate for those subjects whose serum phosphorus was more than 2 S.D. below the mean for age and sex (33) are shown in Table 1. Although 5 out of 7 had serum citrate concentrations below the expected value for that age, all had serum citrates within 2 S.D. of the predicted value.

No statistically significant relationship was found between citrate and phosphorus when serum citrate was plotted against serum phosphorus.  
(r = 0.29, .10 p .05)

Idiopathic Hypercalcemia

Results of serum citrate determinations on the child with idiopathic hypercalcemia are shown in Table 2 with the corresponding values for serum calcium. Although all of the citrate determinations are higher than that predicted for her age, none are more than 2 S.D. above predicted values.
DISCUSSION

Normal Phosphorus

Greenberg and others found that serum phosphorus varied with age in a study of 908 normals (33). In general his values declined with age except in females where they tended to rise somewhat after reaching a minimum mean value of approximately 3.4 mg% at 47 - 48 years of age. The results obtained in the present study (Fig. 2) corroborated the study cited above.

Familial Hypophosphatemia

Winters and his colleagues feel that a concentration of serum phosphorus more than $2\frac{1}{2}$ S.D. below the mean for age and sex is diagnostic of familial hypophosphatemia (1, 33). Based on this criterion, 7 members of the kindred under study may be classified as having familial hypophosphatemia or, in the case of those with active bone disease, vitamin D resistant rickets. In addition the 1$\frac{1}{2}$ year-old female from another family fits this criterion.

If one considers the 2 females (II-4, II-9) with serum phosphorus more than 2 S.D. but less than $2\frac{1}{2}$ S.D. below the mean as affected members of the kindred, then the mode of transmission of this disease in this kindred is consistent with a sex-linked dominant gene on the X chromosome. This would agree with the conclusions of Winters and his colleagues who feel that this is the most common pattern of inheritance found in this disease (1, 22).

If the mode of transmission in this family is a sex-linked dominant, one would expect to find evidence of bone disease in the 71 year-old great-grandfather (I-1) of the 3 affected brothers since his serum phosphorus is more than $2\frac{1}{2}$ S.D. below normal for age and sex. Either this may represent a false positive for hypophosphatemia or there is a variation in the expres-
sivity of this gene in affected males. Fourteen out of 16 males with a serum phosphorus more than \(2.5\) S.D. below the mean had evidence of bone disease in a large kindred studied by Winters and others (4). Greenberg and others (33) point out that their range for normal mean serum phosphorus in the older age groups may be less accurate because of the smaller number of subjects tested, but they feel that the effect would be to widen rather than to narrow the zone of normalcy.

If this man does not have familial hypophosphatemia, the mode of transmission in this kindred would still be consistent with a dominant linked to the X chromosome if his wife demonstrated hypophosphatemia. Although this woman (I-2) was deceased, 2 of her sisters (I-3, I-4) did have concentrations of serum phosphorus more than \(2.5\) S.D. below normal.

**Citrate**

There does not appear to be consistent agreement on a 'normal' value for serum citrate in humans (5, 10, 11, 29, 34, 35, 36). The lowest value considered to be normal in the studies cited above was 1.5 mg% (34) while the highest was 4 mg% (36). Lemon and others (5) obtained a mean of 2.7 mg% with a S.D. of 0.7 for 27 normal males while 41 normal adult females were found to have a mean of 3.8 mg% with a S.D. of 2.4.

The data presented in Fig. 3 suggest that citrate varies with age in normal subjects, being higher in children than in adults. The small number of subjects in the older age groups makes interpretation of this portion of the curve difficult. Natelson and others (35) have previously observed that citrate levels tend to be higher in fasting children than in fasting adults. It is important to note that the majority of those cited above as reporting values for normal citrate use methods identical or very similar to that of Natelson and others (30).
Serum citrate did not differ significantly from normal in any of the hypophosphatemic subjects studied. These findings are in agreement with previously reported studies of serum citrate in idiopathic vitamin D resistant rickets (3,4). It is not clear why serum citrate in resistant rickets should be normal in contradistinction to the low levels observed in vitamin D deficiency rickets.

Idiopathic Hypercalcemia

Serum citrate in the child with idiopathic hypercalcemia was consistently higher than the expected value for the appropriate age but was never as much as 2 S.D. above normal. Other authors (27, 29) have demonstrated elevated serum citrate in patients being treated for this disease and have suggested that the level falls toward normal as treatment with steroids has an effect.

Forfar and others (27) found a high correlation between serum levels of calcium and citrate in this disease. In this patient, also, serum calcium and citrate showed some tendency to fluctuate together.

Normal levels of vitamin D activity* were found in this patient's serum at a time when concentrations of calcium and citrate in her serum appeared to be elevated. This suggests that factors other than vitamin D or a vitamin D-like substance can influence the course of this disease. Alternatively, it is possible that certain metabolic readjustments must take place after vitamin D activity in the serum drops before serum calcium and serum citrate also fall to normal.

*Assay kindly performed by Dr. F. Fellers
SUMMARY

1) The age-related variation in serum phosphorus in normal people was confirmed.

2) The mode of transmission of familial hypophosphatemia in a previously unstudied kindred appears to be a sex-linked dominant on the X chromosome.

3) Serum citrate was also found to vary with age in normal subjects.

4) The concentration of serum citrate was within normal limits in seven subjects with hypophosphatemia. Four of these had active vitamin D resistant rickets and two had skeletal deformities.

5) Serum citrate was consistently higher than the predicted values in a child with idiopathic hypercalcemia but was never more than 2 S.D. higher than the predicted values. The serum citrate tended to fluctuate with the serum calcium in this case.
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37) Sheffield, F. Personal communication

<table>
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<th>Serum Citrate mg%</th>
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<td>h.6</td>
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*The first citrate determination was kindly done by Dr. Harold Harrison.*
FIGURE 1

Diagram of a family tree with symbols indicating different conditions:
- Normal
- Serum Pi between 2 and 2.5 S.D. below mean
- Serum Pi more than 2.5 S.D. below mean
- Status of bones unknown
- Known bone disease
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