Hypophosphatemia (Adult Form): Quantitation of Serum Alkaline Phosphatase Isoenzyme Activity in a Large Kindred

José Luis Millán,¹ Michael P. Whyte,² Louis V. Avloll,² and William H. Fishman¹

We used heat inactivation, L-phenylalanine inhibition, and electrophoresis on polyacrylamide gel and cellulose acetate membranes—with and without use of specific antisera against the liver—bone, intestinal, and placental iso-enzymes—to distinguish and quantify the different alkaline phosphatase isoenzymes in sera from 23 adult members of a kindred affected by the adult form of hypophosphatemia. Nine subjects had values for total activity more than two standard deviations below the mean values for age- and sex-matched normal persons. Bone isoenzyme was diminished in all nine, whereas liver isoenzyme was subnormal in only four. Phosphoethanolamine and phosphoserine in the urine of eight hypophosphatasemic individuals correlated inversely with both total and liver alkaline phosphatase activity in their serum, but not with the activity of the bone isoenzyme. Total activity in the serum of adult kindred members correlated best with the circulating liver isoenzyme activity. The findings suggest that altered hepatic metabolism is responsible for the increased urinary excretion of phosphoethanolamine, and perhaps phosphoserine, in hypophosphatemia.

Additional Keyphrases: heritable disorders — genetics — liver disease — phosphoethanolamine and phosphoserine in urine — enzyme activity — bone disease — hypophosphatasemia

Hypophosphatemia, a metabolic bone disorder, was arbitrarily classified by Fraser in 1957 as occurring in three forms: "infantile," "childhood," and "adult" (1). The adult form is rarest, only 15 cases having been described in the world literature by 1978 (2). Patients often have a history of rachitic skeletal deformity and early loss of deciduous teeth in childhood, and they develop osteopenia with recurrent fractures, persistent pseudofractures, and early loss or extraction of permanent teeth in adult life (2, 3). Laboratory findings include: (a) subnormal circulating alkaline phosphatase (ALP; EC 3.1.3.1) activity, (b) defective bone mineralization that results in excess unmineralized osteoid (osteomalacia), (c) increased urinary excretion of phosphoethanolamine and pyrophosphate, and perhaps (d) increased urinary excretion of phosphoserine (2, 3). Although phosphoethanolamine, pyrophosphate, and phosphoserine appear to be natural substrates for ALP (2, 3), precisely how the biochemical abnormalities are interrelated in adult hypophosphatasia remains unknown. Furthermore, the genetic aspects of this disorder require further investigation. Some reports have favored recessive inheritance (1), others have claimed a dominant mode of transmission (2, 4, 5). Studies are needed to define both qualitatively and quantitatively the circulating forms of ALP in adult hypophosphatasia, and to clarify the pathophysiology, pattern(s) of inheritance, and clinical variability of this disorder (6).

In a large kindred where the proposita had the adult form of hypophosphatasia, we determined the isoenzyme distribution of circulating ALP activity and related the findings to previously reported metabolic studies (2).

Methods

Sample Collection

After diagnosis of hypophosphatasia in the proposita (II-3, Figure 1), we screened family members at home for the disorder. Blood was collected on ice, centrifuged in the cold, and frozen within 4 h. Total ALP activity was initially determined by the procedure of Bowers and McComb (7) at the Clinical Chemistry Laboratory, Barnes Hospital, St. Louis, MO, with use of a Centrifichem System-400 centrifugal analyzer (Union Carbide, Rye, NY) where the coefficient of between-assay variation was <10% for ALP activities that were within the normal range. Hypophosphatasemia was deemed to be present if ALP activity was below two standard deviations from the normal mean value for the patient's age and sex. Nine of 12 family members with hypophosphatasemia were subsequently studied as in-patients at the Clinical Research Center at either Barnes or St. Louis Children's Hospital. Urinary phosphoethanolamine and phosphoserine were quantitated in single 24-h collections with an automated amino acid analyzer (at Bio-Science Laboratories, St. Louis Branch, St. Louis, MO). Essentially the same serum samples from 23 adult family members were then analyzed at the La Jolla Cancer Research Foundation Clinical Laboratory, La Jolla, CA, for detailed study of the ALP isoenzymic composition.

Alkaline Phosphatase Fractionation

We fractionated ALP activity into the individual isoenzymes by a combination of methods, including (a) heat inactivation and inhibition by L-phenylalanine and (b) electrophoresis on polyacrylamide gel and cellulose acetate membranes, with and without use of specific antisera against the individual isoenzymes, to retard the migration of the different electrophoretic bands.

Heat inactivation and inhibition by L-phenylalanine: Depending on the volume of serum available for study, two aliquots of either 500 or 100 μL were taken from each serum sample and processed as follows:

1. One 500-μL aliquot was placed in a 10 × 75 mm disposable Pyrex tube, stoppered, and incubated in a 55 (range 0.01) °C constant-temperature water bath for exactly 16 min, then immediately chilled in crushed ice. If the amount of serum was a limiting factor, 100 μL of serum was placed in a 100-μL disposable DADE Accupette pipet, which was then stoppered with Plastiline, and heat-inactivated as above. Both procedures had previously been performed on 22 samples of different ALP activities and composition. When the percentages of ALP heat-inactivated in tube (x) and pipets (y) were con-

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trasted, regression analysis resulted in the equation \( y = 1.02x - 1.2 \), with a correlation coefficient of 0.991 (\( p < 0.001 \)), indicating excellent agreement between the two procedures.

2. The other aliquot was treated as in step 1, except that it was heat-inactivated for exactly 5 min at 65 (range 0.01) °C.

After the two heat-inactivation procedures were completed, we did two ALP activity determinations on the original serum and both heat-inactivated aliquots by the kinetic procedure of Hausamen et al. (8), with a slight modification, as follows. In one determination we used a buffered substrate composed of, per liter, 15 mmol of \( p \)-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO), 0.5 mmol of \( MgCl_2 \), 5 mmol of D-phenylalanine (Calbiochem, La Jolla, CA) in 1 mol/L diethanolamine buffer (pH = 9.8); this gave us the "D activity." The other determination, with use of the same constituents but with L-phenylalanine (Calbiochem) replacing D-phenylalanine, gave us the "L activity." The reaction rates were determined at a controlled temperature of 30 °C (range 0.1 °C) with a Gilford 222-A spectrophotometer set at 405 nm. The coefficients of variation for the D activity were 1.6% at 40 U/L and 0.9% at 180 U/L, and for L activity 1.5% at 34 U/L and 2.3% at 163 U/L. The molar absorptivity (molar absorption coefficient) for \( p \)-nitrophenol was determined to be 18,396 L mol\(^{-1}\) cm\(^{-1}\) at 405 nm. After ALP activity was assayed as above, the following calculations, mathematical expressions of nomograms published previously (9), were performed:

\[
\text{% L-phenylalanine inhibition} = \left[ (\text{total D} - \text{total L})/\text{total D} \right] \times 100
\]

% heat inactivation
\[
= \left[ (\text{total L} - 16 \text{ min} 55 ^\circ \text{C L})/\text{total L} \right] \times 100
\]

where

\[
\text{% intestinal + placental ALP} = 1.46 \times (\% \text{L-phenylalanine inhibition}) - 15
\]

% liver ALP = 308.8 - 3.318 \times (\% \text{heat inactivation})

Total activity = total D

Intestinal + placental activity
\[
= \text{total D} \times \% (\text{int. + plac. acty.})
\]

Liver activity = \[ \text{total D} - (\text{int. + plac. acty.}) \] \times \% \text{liver acty.}

Bone activity = \[ \text{total D} - (\text{int. + plac. acty.}) \] - liver acty.

Placental activity = D activity of 5 min 65 °C aliquot.

**Electrophoresis:** Electrophoresis on 75 g/L polyacrylamide gel (10) or cellulose acetate membranes (11) was performed on both heated and unheated serum aliquots in the presence and absence of specific antisera against liver–bone, intestinal, and placental ALP. Each time, 45 μL of serum was mixed with 5 μL of a 10-fold dilution of the different antisera. Antisera in the final dilutions retarded the specific electrophoretic bands. Typically, the following electrophoreses were used for each patient’s serum: (a) total, (b) 16 min 55 °C, (c) 16 min 55 °C + anti-liver–bone, (d) 16 min 55 °C + anti-intestinal, (e) 5 min 65 °C, and (f) 5 min 65 °C + anti-placental ALP.

After the electrophoresis, ALP isoenzyme bands were made visible by an appropriate staining procedure. For polyacrylamide gel electrophoresis 50 mg of \( \alpha \)-naphthyl phosphate (Sigma) and 50 mg of Variamine Blue (Dajac) were dissolved

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**Table 1. Quantitation of Serum ALP Isoenzyme Activity and Urinary Phosphoethanolamine and Phosphoserine in Adult Members of the H. H. Kindred**

<table>
<thead>
<tr>
<th>Patient no. (Fig. 1)</th>
<th>Total ALP (52-148)</th>
<th>Liver iso. (11-78)</th>
<th>Bone iso. (18-96)</th>
<th>Intest. iso.</th>
<th>Phospho-ethanolamine ( \mu \text{mol/g of creatinine per day} )</th>
<th>Phosphoserine ( \mu \text{mol/g of creatinine per day} )</th>
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*a* Numbers in parentheses represent the normal reference ranges (±2 SD of the log normal distribution) for the La Jolla Cancer Research Foundation, determined with 72 normal adults.

*b* Normal reference range values are in parentheses.
in 25 mL of 2-amino-2-methyl-1,3-propanediol buffer (1 mol/L, pH = 9.68) supplemented with, per liter, 3 mmol of MgCl₂ and 233 µmol of ZnSO₄. A trace of charcoal was added, and the solution was filtered and used immediately. After the gels were incubated with substrate-in-dye mixture for a few minutes at 37 °C, a brownish localized color appeared, indicating the position of the isoenzyme bands. After electrophoresis on cellulose acetate membranes, the membrane was placed upside down in a Petri dish on a layer of agarose (10 g/L) containing 2 mmol of naphthol AS-MX phosphoric acid (Sigma) per liter, buffered with 2-amino-2-methyl-propanol (0.2 mol/L, pH 11.2). The covered Petri dish was incubated at 37 °C until the isoenzyme bands were clearly visible under ultraviolet light.

Thus, in each case, electrophoresis with catalytic staining and immunological retardation confirmed the presence of the ALP isoenzymes quantitated previously with the heat-inactivation/L-phenylalanine-inhibition procedure.

### Statistical Analyses

Linear regression equations were calculated by the least-squares method. Correlation coefficients (r) were assigned p values, to reflect the probability that the observed relationship between the two variables could have occurred by chance alone.

### Results

Figure 1, the complete kindred pedigree of the proposita (H.H.), is based on serum total ALP activity assayed in St. Louis (2). Table 1 summarizes the activities of the serum total and isoenzyme ALP activity in the adult kindred members as assayed in La Jolla, and the previously reported daily urinary excretion of phosphoethanolamine and phosphoserine in eight of the nine who were hypophosphatasemic (2). Subjects III-18 to IV-4 were deliberately excluded from our study because they were children.
Placental ALP activity was not detected in any individual, as none were pregnant. Not all blood samples were from fasted subjects, so intestinal ALP activity was detected in the sera of several. Assay of serum total ALP activity in La Jolla identified the same nine adults who had shown total ALP values below two standard deviations from the mean in St. Louis. Bone ALP isoenzyme activity was subnormal in each, whereas only four (II-3, II-9, II-11, and III-17) showed decreased liver isoenzyme. A high degree of correlation (r = 0.89, p <0.001) was noted between serum total ALP activity and liver isoenzyme activity in the 23 adult family members (Figure 2). When total ALP activity was contrasted to bone isoenzyme activity, however, the degree of correlation was less striking (r = 0.56), but still statistically significant (p <0.01) (Figure 3). Figure 4 shows the regressions, equations, and correlation coefficients obtained when serum total ALP activity was related to liver or bone isoenzyme activity in the 72 normal adults who were the La Jolla reference population. Identical correlation coefficients were obtained for both regressions, and the slopes of the curves were essentially the same.

Assay results for serum total ALP activity in La Jolla correlated inversely with both urinary phosphotanalamine and phosphoserine in the eight hypophosphatasemic individuals (r = -0.60, p <0.01 for phosphotanalamine and r = -0.40, p <0.05 for phosphoserine) (Figure 5), as reported previously with assay of serum total ALP activity in St. Louis (2, 5). The high correlation between total ALP activity and liver isoenzyme activity in these individuals suggested a statistically significant negative correlation between urinary phosphotanalamine and phosphoserine and liver ALP isoenzyme activity. This was indeed the case (Figure 6) (r = -0.80, p <0.01 for phosphotanalamine and r = -0.64, p <0.05 for phosphoserine). There was no correlation, however, between
These activity clinically the function offers physiological nature, as shown in Fig. 5. Relationship between urinary phosphoethanolamine (PEA, ●) and phosphoserine (PS, △) and serum total ALP activity in eight hypophosphatasemic adult kindred members.

![Graph showing relationship between urinary phosphoethanolamine (PEA) and phosphoserine (PS) and serum total ALP activity.](image)

Urinary phosphoethanolamine and phosphoserine and the circulating bone ALP isoenzyme activity ($r = 0.07, p > 0.1$ for phosphoethanolamine and $r = 0.13, p > 0.1$ for phosphoserine).

**Discussion**

Assays of ALP activity have been used in clinical chemistry for several decades. Nevertheless, little is known about the physiological role of this enzyme (12). An “experiment of nature,” where a genetic defect diminishes circulating ALP, offers an opportunity to enhance our understanding of the function of this enzyme.

We studied a large kindred in which the proposita had all the features of the adult form of hypophosphatasia, and other members were found to be hypophosphatasemic although not clinically affected. Initial studies (2) showed that transmission of hypophosphatasemic trait through three generations was compatible with autosomal dominant inheritance. Incomplete penetrance and sex influence in the expression of the trait were also suggested by our finding (with assay of total circulating ALP activity in St. Louis) that only one out of 11 men at risk in younger generations (III and IV, Figure 1) was hypophosphatasemic.

In the present study, assay of serum total ALP activity in La Jolla identified hypophosphatasemia in the same adults (II-1, II-3, II-7, II-9, II-11, III-1, III-8, III-14, and III-17) found previously in St. Louis to have subnormal circulating total ALP activity. Bone ALP isoenzyme activity was subnormal in all, but the hepatic fraction was decreased in only four.

One kindred member (III-16) who was at risk because her mother (II-9) was hypophosphatasemic, had modestly decreased total ALP activity in her serum as measured in La
Jolla, but normal values as measured in St. Louis. Her bone and liver isoenzyme activity were both normal. We are, therefore, uncertain whether or not to consider her “affected” at this time, but think it unlikely. In considering the discrepancy between her normal and subnormal serum total ALP activity in St. Louis vs La Jolla, it must be recalled that the normal range for serum total ALP activity in adults, as assayed in La Jolla, could not account for age- and sex-related differences.

One subject (II-12, Figure 1), a woman apparently not at risk because she was not a “blood relative,” was also found to be hypophosphatasemic as measured in La Jolla, but not as measured in St. Louis. She had normal liver ALP isoenzyme activity, but decreased bone isoenzyme activity. It may be that she is a heterozygous “carrier” for hypophosphatasia. If this is so, some future offspring could be severely affected with the “infantile” form of this disease because it appears that this form of hypophosphatasia results from a double dose of the gene that is defective in adults (2). However, her subnormal serum total and bone ALP activity could also represent normal values that are below the 4.6 percentile for the reference population.

Subject III-9, whose father and sister were normal (Figure 1), had slightly diminished serum total ALP activity as assayed in La Jolla, but normal bone and liver isoenzyme activity. Three other individuals with normal serum total ALP activity had subnormal bone isoenzyme activity but normal liver isoenzyme activity (I-1, III-11, and III-15, Figure 1). If the isoenzyme values are accurate, identification of these individuals illustrates the utility of isoenzyme quantitation in this disorder and suggests that the pattern of inheritance should also be interpreted as showing “skip generations.” Further detailed study of these individuals will be essential to confirm the implications of the ALP isoenzyme assays.

It is generally accepted that the expression of the intestinal ALP isoenzyme is not impaired in hypophosphatasia. In agreement with previous reports (13, 14), several hypophosphatasemic members of this family showed intestinal isoenzyme contributing to their serum total ALP activity.

Several other conclusions may be drawn from this study. Subnormal circulating total ALP activity in adults with hypophosphatasia is associated with decreased bone isoenzyme activity, although the liver isoenzyme may be subnormal in some hypophosphatasemic individuals. Excretion of phosphoethanolamine and phosphoserine in hypophosphatasemic adults is inversely correlated with circulating liver but not bone ALP activity. These findings suggest that the increased excretion of phosphoethanolamine, and perhaps phosphoserine, in hypophosphatasia results from diminished hepatic metabolism of this substrate and that there is a concomitant metabolic disorder in the liver. The fact that urinary excretion of phosphoethanolamine is increased in patients with this disorder, and that both urinary phosphoethanolamine and phosphoserine correlate inversely with serum total and liver ALP activity, supports the idea that they are physiological substrates for ALP, specifically for the liver isoenzyme. The skeletal expression of hypophosphatasia probably results from a paucity of bone isoenzyme activity.

Findings seemingly at variance to ours have been reported by Bixler et al. (15, 16), who found dominantly inherited hypophosphatasemia in a kindred where subnormal circulating total ALP activity was ascribable to decreased liver isoenzyme activity. Bone isoenzyme activity was normal. Phosphoethanolamine excretion was not increased, and their patients showed dental disease but no skeletal abnormalities. Recent reassessment of their findings, however, revealed that both bone and liver isoenzyme activity were decreased in affected individuals, and phosphoethanolamine excretion was increased (D. Bixler, personal communication).

Studies to demonstrate whether the liver and bone ALP isoenzymes are products of the same gene (but undergo different post-translational modifications) or represent independent gene products would be important to explain our findings. Development of immunological assays for liver and bone ALP isoenzymes should enable us to ascertain whether the defect in hypophosphatasia is quantitative (less enzyme being synthesized) or qualitative (synthesis of immunoreactive ALP isoenzymes that are functionally inactive), or even a combination of these. Studies are in progress in our laboratories to attempt to answer these questions.

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References