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Prenatal Diagnosis of Hypophosphatasia: Genetic, Biochemical, and Clinical Studies

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INTRODUCTION

Hypophosphatasia is a rare inborn error of metabolism characterized by abnormal bone mineralization associated with a deficiency of alkaline phosphatase (E.C.3.1.3.1). Since first described as a separate disease entity by Rathbun in 1948 [1], a great deal has been added to our knowledge of the clinical and biochemical features of this syndrome [2–7].

The clinical findings include: (1) skeletal abnormalities consistent with impaired ossification; (2) a pattern of familial occurrence indicating autosomal recessive inheritance; and (3) variable severity and age of onset. Fraser noted that when sibs were affected, the age of onset, severity, and clinical course were usually almost identical [5]. The severest form of the disease occurs in utero; infants are often stillborn or die shortly after birth due to inadequate bony support for the cranial and thoracic cavities. The incidence is about one per 100,000 live births. The biochemical findings include: (1) low levels of alkaline phosphatase (ALP) in the serum and tissues (liver, bone, and kidney); (2) increased excretion of phosphoethanolamine in the urine; and (3) frequently hypercalcemia.

This report details investigations of three approaches to the prenatal diagnosis in two pregnancies of a family with a previous history of the severe, early onset form of hypophosphatasia. (1) Ultrasound evaluations of the fetuses were performed for evidence of abnormal bone mineralization. (2) Amniotic fluid supernatant was analyzed to quantitate the total ALP activity as well as the contributions made by the various isozymes (liver, bone, intestine, and placenta) to determine the effect of an affected fetus on the total activity and on that portion accounted for by the liver and bone isozymes. (3) Cultured amniotic fluid cells were assayed for ALP activity to determine the enzyme activity levels of cell populations from affected and control pregnancies.

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METHODS

Cell Samples

Amniotic fluid cells collected by amniocentesis between the 14th and 20th week of gestation were cultured in McCoy's 5A medium containing 30% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.0 mM glutamine. Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air. Confluent cell cultures (75cm^2 flask) at either the second or third passage were harvested with 0.25% trypsin, washed several times with 0.9% NaCl at 4°C, centrifuged, and then stored at −20°C. Skin fibroblasts obtained by biopsy were cultured and harvested as described for amniotic fluid cells except that the medium contained 10% fetal calf serum.

Cell lysates for ALP determinations were prepared just before assay by suspending the cell pellets in 0.5 ml of cold 0.01 M Tris-HCl, 1.0 mM $MgCl_2$, pH 7.5, and sonicating for 10–15 seconds at 50 W on ice with a Branson Sonifier Cell Disruptor. Protein determinations were performed according to the method of Lowry et al. [8].

Tissue Samples and Electrophoresis

Extracts of liver, bone, jejunum, kidney, and placenta prepared by butanol extraction of the homogenized tissues were stored at −20°C until assayed [9]. Starch gel electrophoresis of the tissue extracts was carried out at 5°C using Poulik's Tris-borate discontinuous buffer system, pH 8.0–8.6. The samples were characterized both with and without neuraminidase (Sigma type VI St. Louis, Mo.) treatment [9].

Alkaline Phosphatase Assays

Adult sera, cord sera, amniotic fluids, and tissue extracts were assayed spectrophotometrically at 30°C in diethanolamine buffer, pH 9.8, employing *p*-nitrophenyl phosphate (Sigma 104) as the substrate. Cultured amniotic fluid cell lysates were assayed fluorometrically in the same buffer using 4-methylumbelliferyl phosphate as the substrate.

The procedure for estimating the contributions of the liver/bone, intestinal, and placental isozymes to the total ALP enzyme activity in a mixture is described elsewhere [10]. The general protocol is described in the Appendix.

CASE HISTORIES

Case II-2

A 22-year old black female (I-3) came for counseling following the delivery of a macerated, stillborn male infant (II-2) after a term pregnancy (fig. 1). Although the infant at delivery was reported to have an enlarged head, autopsy examination detected no fetal abnormalities including hydrocephalus. She had a healthy 5-year-old daughter (II-1) whose father was different from that of the stillborn. This patient's subsequent pregnancies (II-3 through II-6), fathered by a third consort, are the subjects of this report.

Case II-3

This pregnancy was uncomplicated until 34 weeks gestation when polyhydramnios was noted. B-mode ultrasound examination at that time failed to define a fetal vertex. A 2,500 g female infant (Apgar score: 1 at 1 min and 5 at 5 min) was delivered at 37 weeks gestation following

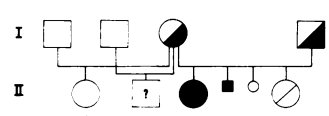

FIG. 1.—Family pedigree. Shading indicates heterozygote parents and two homozygous children. 1 = normal; 2 = stillborn; 3 = hypophosphatasia: died 1 day of age; 4 = hypophosphatasia: elective abortion; 5 = elective abortion; 6 = normal: possible heterozygote.

spontaneous labor. On physical examination, the head circumference was 30 cm, chest circumference 32 cm, and length 40 cm. The calvarium was remarkably soft to palpation. There was shortening of all the extremities more marked proximally, with bowing and internal rotation of the extremities (fig. 2*A*). Serum alkaline phosphatase was 3 IU (normal: 50–200 IU). The radiographic findings were consistent with hypophosphatasia (fig. 2*B*). The infant expired at 2 hr of age of progressive respiration insufficiency. The G-banded karyotype was normal (46,XX).

Case II-4

B-mode ultrasound examination was performed at 16, 18, and 19 weeks gestation as estimated by menstrual history and clinical examination. On each of these occasions, sonography failed to define the fetal head. With this information and the 25% recurrence risk for hypophosphatasia, the patient requested termination of the pregnancy. Maternal serum and amniotic fluid alpha-fetoprotein were normal for the gestational age. The karyotype of the cultured amniotic fluid cells was 46,XY. ALP assays of the amniotic fluid supernatant and cultured amniotic fluid cells were performed.

Abortion was induced by the intra-amniotic instillation of prostaglandin $F_2\alpha$. Radiographic examination of the maternal abdomen prior to abortion revealed an enlarged uterus without detectable fetal skeletal structures. Six hours following prostaglandin instillation, the patient aborted a male fetus weighing 151 g with marked shortening, internal rotation, and bowing of all extremities (fig. 3*A*). Radiographic examination of the fetus demonstrated marked undermineralization of the skeleton (fig. 3*B*). Histopathologic examination of the long bones showed normal cartilage with no evidence of calcification of the metaphyseal cartilage. ALP assays were performed on tissue samples obtained at autopsy.

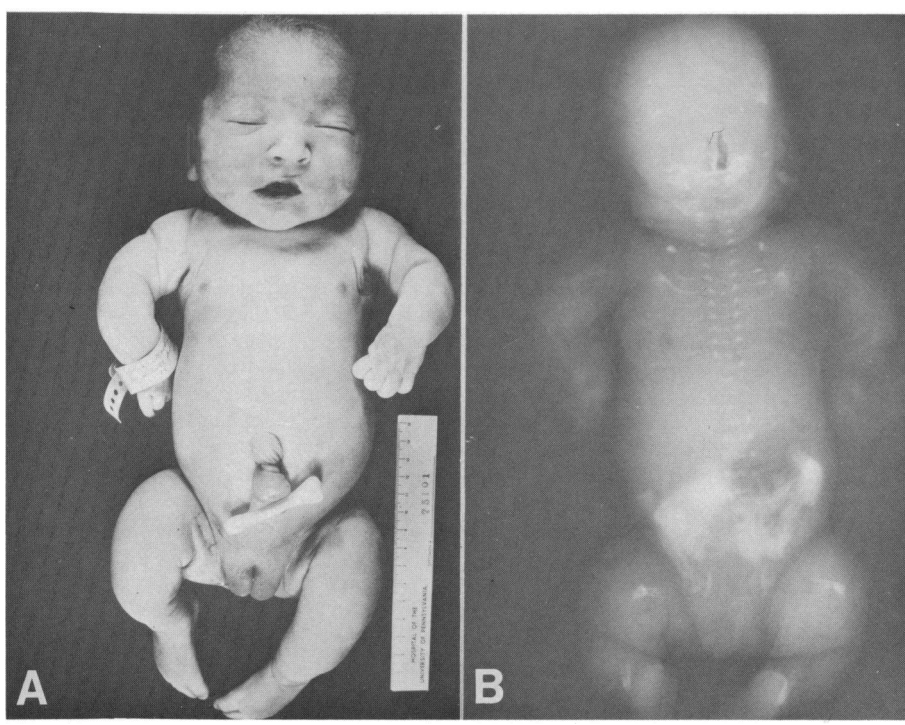

FIG. 2.—Case II-3. *A*, Postmortem appearance displaying shortening and bowing of extremities; *B*, radiologic examination showing marked undermineralization of bone.

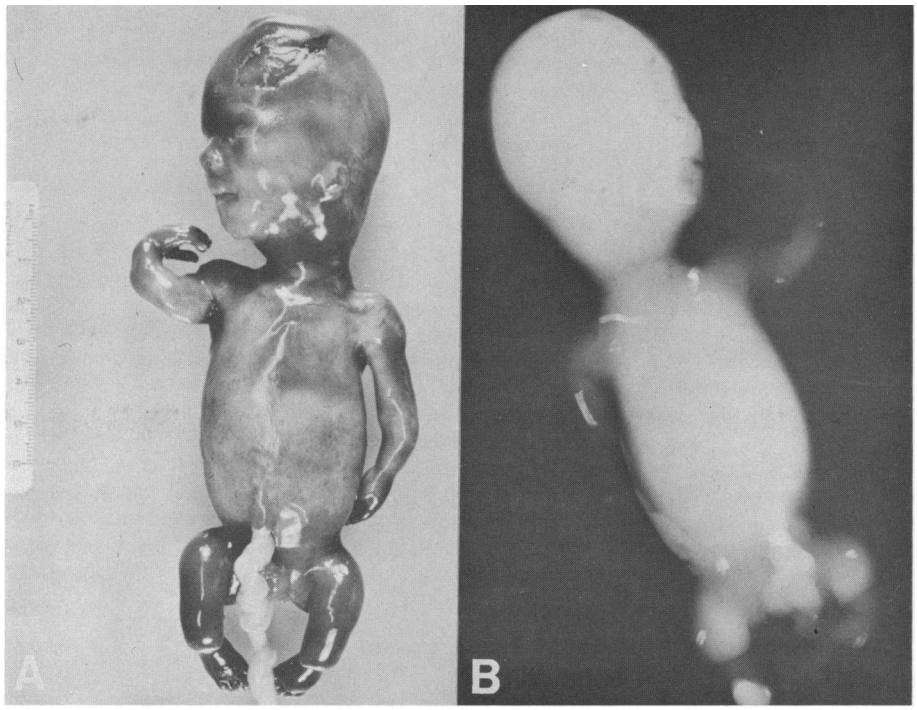

FIG. 3.—Case II-4. *A*, Postmortem appearance of fetus displaying shortening and bowing of extremities; *B*, radiologic examination showing an almost complete absence of ossification.

Case II-5

An elective abortion was performed at 8 weeks gestation.

Case II-6

B-mode ultrasonography was performed at 16 and 18 weeks gestation by menstrual history and clinical examination. A normal fetal vertex with a biparietal diameter consistent with the gestational age was found at each of these examinations. Amniocentesis was performed at 19 weeks gestation. Amniotic fluid (25 ml) was withdrawn for study, and 12 ml of contrast medium (Hypaque) was instilled for amniography. The amniogram defined a normal fetal humerus, femur, and tibia with ossification of the calvarium and vertebral column. The upper and lower extremities were proportional in length to the fetal head and trunk. Maternal serum and amniotic fluid alpha-fetoprotein were normal. The karyotype of the cultured amniotic fluid cells was 46,XX. ALP assays were performed on amniotic fluid supernatant and cultured amniotic fluid cells.

A normal female infant weighing 2,780 g was delivered following elective induction of labor at 38 weeks gestation. Apgar scores were 9 at 1 min and 9 at 5 min. Cord blood was obtained for ALP assays. Skeletal survey on the second day of life was normal. Growth and development at 6 months were normal.

RESULTS

Carrier Detection

Serum levels of ALP are a reflection of the bone and liver ALP activity present in

those tissues. Occasionally, the intestinal isozyme is also found in serum depending upon the blood group type, secretor status, and diet of the individual [11]. Carriers of hypophosphatasia have been shown to have reduced levels of bone/liver ALP activity in their serum [2–7]. Table 1 compares the bone/liver isozyme contributions to the total ALP activities for a healthy control group of 12 men and 13 nongravid women and the parents of cases II-3 and II-4 with documented hypophosphatasia. Both parents, apparently heterozygous for hypophosphatasia, have enzyme activity levels substantially lower than the control group.

ALP Activity of Fetal Tissues

Tissue samples obtained postmortem from the affected fetus (II-4) and three age-matched control fetuses were assayed for ALP activity (table 2). Extracts of liver, kidney, and bone from the affected fetus show drastically reduced activities, while the activity in placenta and jejunum was similar to the controls. Starch gel electrophoresis revealed no detectable activity for the bone, liver, and kidney of the affected fetus, while the placental and intestinal extracts had isozyme patterns identical to those from corresponding tissues from controls (fig. 4).

Amniotic Fluid ALP

A series of amniotic fluids collected between the 14th and 20th week of gestation for other purposes and those from fetus II-4 (affected) and II-6 (unaffected) were assayed to determine total, bone/liver, placental, and intestinal ALP activity (table 3). Two bloody amniotic fluids were omitted from the control group. None of the amniotic fluids studied were meconium stained.

It can be seen that most of the ALP activity is accounted for by an intestinal isozyme. There is a small contribution by the bone/liver isozymes and a smaller contribution by the placental isozyme. The normal sibling had values very close to the mean of the controls for all four alkaline phosphatase determinations. The affected fetus had values within the normal range for all determinations except that there was no detectable activity attributable to the bone/liver isozymes.

Although no amniotic fluids were found with no ALP activity, some had activities just at the level of detection. Similarly, it can be seen that the contributions to the total ALP activity made by the various isozymes varied widely.

TABLE 1

BONE/LIVER ALP ACTIVITY IN SERUM IN HYPOPHOSPHATASIA CARRIERS

Subjects	No.	Mean ± SD	Range
Controls:			
Males	12	136.8 ± 35.1	75–190
Females	13	129.3 ± 45.5	68–211
Parents:			
Father	1	53.2	...
Mother	1	26.8	...

NOTE.—Activities are expressed as nmol *p*-nitrophenol/min/ml. SD = standard deviation.

TABLE 2

ALP ACTIVITY OF FETAL TISSUE

Subjects	Jejunum	Liver	Kidney*	Bone	Placenta
Control Fetuses:					
I	1,990.9	146.1	242.3	...	...
II	706.4	215.5	241.9	204.4	268.8
III	380.0	...	...	...	...
Affected Fetus†	377.4	1.6	1.6	1.7	152.8

NOTE. — Activities are expressed as nmol *p*-nitrophenol/min/OD_{280}.

* The ALP found in kidney extracts is indistinguishable by inhibition and thermostability characteristics from liver ALP.

† Skin from the affected fetus had an activity of 1.1. No control material was examined.

Cultured Amniotic Fluid Cell and Fibroblast ALP

Cells cultured from 68 control amniotic fluids and those from fetus II-4 (affected) and II-6 (unaffected) were assayed for ALP activity. Based upon thermostability and L-phenylalanine and L-homoarginine inhibition studies, the ALP produced by these cultures was found to be indistinguishable from the liver/bone isozymes (table 4).

Cells from the affected fetus had an average specific activity which was 40% of the lowest control value and 4% of the mean of the control cultures. Cells from the normal sibling had an average specific activity well within the normal range.

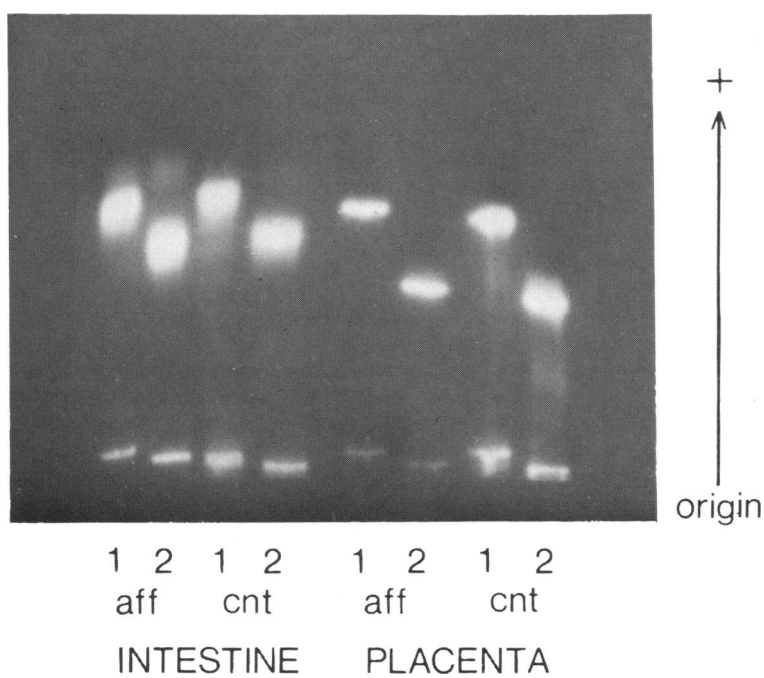

FIG. 4. — Starch gel electrophoresis of fetal intestine and placenta from affected (aff) fetus, II-4; and an age matched control fetus (cnt); without (1) and with (2) neuraminidase treatment.

TABLE 3

ALP ACTIVITY IN AMNIOTIC FLUIDS

Subjects	No.	Mean ± SD	Range
Total:			
Controls	74	38.76 ± 23.34	4.0–118.0
Affected fetus	1	78	...
Normal sibling	1	44	...
Bone/Liver:			
Controls	44	6.23 ± 3.76	0.61–18.59
Affected fetus	1	0	...
Normal sibling	1	2.16	...
Placenta:			
Controls	48	1.48 ± 1.17	0.08–6.04
Affected fetus	1	0.59	...
Normal sibling	1	1.45	...
Intestinal:			
Controls	45	34.86 ± 20.76	0.11–95.98
Affected fetus	1	77.41	...
Normal sibling	1	40.39	...

NOTE. — Fluids were centrifuged to remove cells. Activities are expressed as nmol *p*-nitrophenol/min/ml. SD = standard deviation.

Figure 5 shows a histogram of the values obtained for the 68 control amniotic fluid cell cultures. The distribution of activities is skewed to the right and departs significantly from normal (coefficient of skewness $g_1 = 2.847, P < .001$; coefficient of kurtosis $g_2 = 11.54$, $P < .001$). However, log transformation normalizes the distribution ($g_1 = 0.356$, $.3 < P > .2$; $g_2 = 2.47$, $.4 < P > .3$). Taking $\log_{10}$ of the activity values, the mean for the control series is -0.0025, and the standard deviation is 0.457. The transformed value for the affected fetus is -1.161 which is 2.54 standard deviations below the mean. The transformed value for the normal sib, 0.535, is 1.18 standard deviations above the mean of the controls.

Seven control skin fibroblast cultures from adults, children, and infants produced ALP of the bone/liver type (mean ± SD: 7.36 ± 12.15; range: 0.37–34.15). Six of the seven control fibroblast cultures had activities within the range observed for the cultured amniotic fluid cells (fig. 5). The one culture not within this range had a higher activity. The specific activities for duplicate skin (0.081, 0.079) and rib (0.058, 0.055) fibroblast cultures from the affected fetus were clearly lower than the controls and almost identical to those observed for the cultured amniotic fluid cells from the same fetus.

TABLE 4

ALP ACTIVITY IN CULTURED AMNIOTIC FLUID CELLS

Subjects	Mean ± SD	Range	Individual Cultures
Controls	1.77 ± 2.37	0.179–12.53	...
Affected fetus	0.069	...	0.059, 0.082, 0.066
Normal sibling	3.43	...	2.38, 3.45, 4.47

NOTE. — Activities are expressed as nmol 4-methylumbelliferone/min/mg protein. SD = standard deviation.

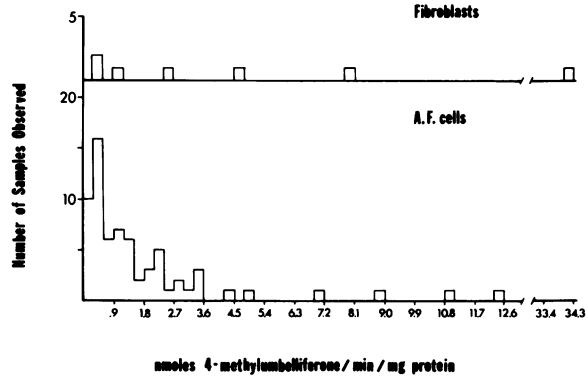

FIG. 5.—Histogram of ALP activities observed for 68 amniotic fluid cell cultures *(bottom)* and seven fibroblasts cultures *(top)*. Only the results obtained with the control cultures are shown.

Cord Blood ALP of Normal Sibling

Serum ALP of cord bloods from 13 control newborns and the normal sibling (II-6) were assayed. Starch gel electrophoresis, thermostability, and L-phenylalanine and L-homoarginine inhibition studies revealed that all of the activity present in cord blood was accounted for by the bone/liver isozymes of ALP. The sib's cord blood activity (84.1) was significantly lower than those of the controls (mean ± SD: 306.2 ± 92.4 nmol/*p*-nitrophenol/min/ml; range: 190.5–463.5) which suggests that she is a carrier of hypophosphatasia.

DISCUSSION

Since 1976, three reports on the prenatal diagnosis of hypophosphatasia have appeared in the literature. One report considered only cultured amniotic fluid cell ALP [12]; the second investigated ultrasonography, supernatant amniotic fluid ALP, and cord blood ALP [13], and the third considered ultrasonography, supernatant amniotic fluid ALP, and ALP levels in cells cultured from amniotic fluid, amnion, and skin [14]. The results of these investigations were not always in agreement. The observations presented in this paper verify some of the previous results and help to explain some of the discrepancies.

The alkaline phosphatases are a group of similar enzymes having an alkaline pH optimum which hydrolyze monophosphate esters. The most common and best characterized forms of these enzymes, named for the tissue in which they are found, are the isozymes of bone, liver, intestine, and placenta. In hypophosphatasia, only the bone and liver isozyme activities are reduced or absent. Any biochemical diagnosis of hypophosphatasia based upon ALP levels in tissues or fluids therefore requires that the specific bone and liver isozyme contributions to the total activity be determined.

Supernatant Amniotic Fluid ALP

Total ALP activities of supernatant amniotic fluid have been reported for two affected fetuses. In one case no ALP was detected [14], while the second case had normal ALP levels [13]. In the present study, the affected fetus had a normal total supernatant amniotic fluid ALP activity. This result is not surprising, however, since

most of the activity is intestinal in origin with contributions from the bone/liver and placental isozymes. The bone/liver and placental isozymes contribute only about 16% and 4%, respectively, to the total activity. On average, therefore, only 16% of the total activity found in amniotic fluid could aid in the diagnosis of hypophosphatasia, and it is not known for certain whether this activity is maternal or fetal in origin. If the bone/liver isozyme activities are contributed by the mother, we would expect them to be lower than normal since she is an obligate heterozygote and has low levels of these isozymes in her serum.

A comparison of the bone/liver isozyme activities in the amniotic fluids of the affected and control fetuses does reveal a difference. No bone/liver ALP activity was detected in the affected fetus. While the undetectable bone/liver isozyme activity may be indicative of disease, we do not feel it is useful diagnostically, as some of the control amniotic fluids have barely detectable levels of these isozymes. This is also true for total supernatant amniotic fluid ALP activity. Amniotic fluid from several normal fetuses had total ALP activities which were just at the level of detection.

Cultured Amniotic Fluid Cell ALP

In the present study, the ALP activities in amniotic fluid cell cultures from an affected fetus were compared with those observed in 68 control cultures. It was found that the ALP activity in the controls had the characteristics of the liver/bone isozymes. A considerable variation in the levels of activity from culture to culture was also observed. A similar wide range of activities has been reported for normal human diploid fibroblasts [15, 16].

The distribution of ALP activities for the cultured amniotic fluid cells was skewed toward the right and was consistent with a log normal distribution. Cells from the affected fetus had an average ALP specific activity 4% of the mean and 40% of the lowest control value. In $\log_{10}$ activity units, the value for the affected fetus was 2.5 standard deviations below the mean. The probability of obtaining this result, or one more extreme, in a random series of normal individuals is about 0.6%. The risk of having hypophosphatasia is therefore extremely high for a fetus whose sibling had hypophosphatasia and whose cultured amniotic fluid cells have an ALP activity 2.5 standard deviations below the mean of the controls.

Consistent with our results are those of Clark et al. [12] who observed an affected female fetus with an ALP activity 3% of the mean of seven control cultures; no range was given for the controls. Benzie et al. [14], however, found that cultured skin fibroblasts, but not amniotic fluid cell cultures from an affected female fetus had an ALP specific activity lower than control cultures. The exact reason for this discrepancy is not clear, but it is possible that the amniotic fluid cell cultures contained cells of maternal origin.

Genotype of Normal Sibling

Cord blood. Serum normally contains the bone and liver isozymes of ALP, and assays of fetal serum ALP activity should be diagnostic of hypophosphatasia. Sutcliffe and Brock [17] studied fetal and maternal sera from 13 to 41 weeks gestation and found greater bone/liver isozyme activity in fetal serum compared to maternal serum at all

gestational ages. In the present study, we examined the cord blood from the normal sibling (II-6) and 13 controls at delivery and showed that term cord bloods ordinarily have more than twice the enzymatic activity of adult sera. The ALP activity of the normal sibling was below that of the controls, consistent with the heterozygous state.

One report which compared the cord blood of a 19 week fetus with hypophosphatasia to an age matched control fetus failed to show any differences in ALP activity [13]. This result is surprising since the values obtained for both fetuses were identical and quantitatively were at least an order of magnitude less than expected.

Amniotic fluid cells. In contrast to the heterozygote levels of ALP activity found in cord blood, the ALP activity observed in cultured amniotic fluid cells from the normal sibling was greater than the mean for the controls. Three cultures were analyzed, and all three had values greater than the mean.

Unfortunately, no one has extensively studied the ALP activity of cultured fibroblasts either heterozygous or homozygous for hypophosphatasia, but we would have expected lower ALP activity levels if the normal sib was a heterozygote. The reason for this discrepancy is not presently understood.

Ultrasonography

From 16 weeks gestation, a well-defined fetal skull may be visualized by ultrasonography because of the acoustic differences between the calcified fetal skull and amniotic fluid. Failure to visualize a fetal skull coupled with normal levels of alpha-fetoprotein in amniotic fluid has suggested the prenatal diagnosis of hypophosphatasia for the three cases in which they were studied, since the alternative diagnosis of anencephaly is associated with elevated alpha-fetoprotein [18].

In one case, no fetal head was observed at either 16, 18, or 19 weeks [14]. In the second study, sonograms at 16, 18, and 19 weeks revealed a very thin, faint, circular outline of a fetal head [13]. In the present study, no fetal head was observed at either 16, 18, or 19 weeks gestation. When no fetal head was observed at 16 weeks, serial ultrasonography was performed to eliminate any error due to gestational dating.

It has been suggested that fetuses heterozygous for hypophosphatasia might manifest a delay in ossification which could lead to an error in diagnosis. However, ultrasound examination at 16 and 18 weeks were normal for the normal sibling (II-6) who had heterozygote levels of cord blood alkaline phosphatase. The ultrasound findings were confirmed by the observation of normal skeletal structures employing amniography.

SUMMARY

This report has considered three approaches to the prenatal diagnosis of the severe, early onset form of hypophosphatasia. Two of these approaches, ultrasonography and the determination of the bone/liver isozymes of alkaline phosphatase (ALP) in cultured amniotic fluid cells, have proven useful diagnostically. The third method, assay of the bone/liver isozyme activity or total activity in supernatant amniotic fluid, was not informative for the affected fetus we studied.

Failure to visualize a well-defined fetal skull after 16 weeks of pregnancy when the level of alpha-fetoprotein in the amniotic fluid is normal should arouse the suspicion of hypophosphatasia. Because the disease is known to manifest clinical variability,

studies to detect both the biochemical defect as well as the structural manifestations should be considered. The combined use of ultrasonography, analysis of amniotic fluid alpha-fetoprotein, and the measurement of the bone/liver ALP in cultured amniotic fluid cells would appear to be the best approach to the prenatal diagnosis.

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APPENDIX

ANALYSIS OF MIXTURES OF HUMAN ALKALINE PHOSPHATASES

Assuming that the total alkaline phosphatase activity is the sum of the liver and bone $(L + B)$ plus the intestinal (I) plus the placental (P) activities, then total activity $= (L + B) + (I) + (P)$. It is possible to define a set of three equations employing the data obtained from (1) heat denaturation, (2) L-phenylalanine inhibition, and (3) L-homoarginine inhibition studies whose solution yields a quantitative estimate of the contributions of $(L + B)$, (I), and (P) to the total activity of a mixture.

The total activity remaining after heat denaturation performed to destroy all nonplacental activity is defined as:

$$\mathrm{Act}_{(\mathrm{HEAT})} = (P). \tag{1}$$

The total activity remaining after L-phenylalanine inhibition is defined as:

$$\mathrm{Act}_{(\mathrm{PHE})} = a(\mathrm{L} + B) + b(I) + c(P). \tag{2}$$

The total activity remaining after L-homoarginine inhibition is defined as:

$$\mathrm{Act}_{(\mathrm{HARG})} = d(L + B) + e(I) + f(P). \tag{3}$$

Letters a–f represent percentages of activity remaining which are determined using isozyme standards prepared from appropriate tissues. Solving equations (2) and (3) for $(L + B)$:

$$(L + B) = \frac{e}{ea - db}\,[\mathrm{Act}_{(\mathrm{PHE})}] - \frac{b}{ea - db}\,[\mathrm{Act}_{(\mathrm{HARG})}] + \frac{bf - ec}{ea - db}[\mathrm{Act}_{(\mathrm{HEAT})}]$$

and

$$(I) = \text{total activity} - [(L + B) + (P)].$$

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