Appendix (cont.). Date of birth, date of measurement, height, weight, bone age and body density of individual subjects.

Subject Number	Date of Birth	Date of measurement	1955					1956					1957					1958				
			Height, cm	Weight, kg	Bone age, years	Density, g/ml	Date of measurement	Height, cm	Weight, kg	Bone age, years	Density, g/ml	Date of measurement	Height, cm	Weight, kg	Bone age, years	Density, g/ml	Date of measurement	Height, cm	Weight, kg	Bone age, years	Density, g/ml	Date of measurement
1.	2/13/51	4/06	165.0	45.50	13.5	1.086	4/21	175.0	57.50	14.0	1.063	5/06	182.8	63.10	15.0	1.082	4/29	185.0	68.00	15.75	1.082	
2.	8/18/50	4/28	178.1	65.15	15.5	1.082	5/25	180.5	69.40	17.25	1.065	4/18	181.5	71.80	18.0	1.070	4/29	181.6	74.20	19.0	1.095	
3.	1/12/51	4/07	175.0	59.40	15.25	1.080	5/17	180.0	61.90	16.75	1.090	5/15	182.0	68.40	17.75	1.079	4/29	184.5	69.00	18.5	1.084	
4.	12/25/50	3/15	161.0	46.00	13.0	1.073	4/25	172.0	55.70	13.5	1.073	6/03	181.0	65.90	15.25	1.063	5/02	183.2	73.90	16.75	1.056	
5.	2/18/50	3/30	173.0	60.80	16.0	1.086	5/17	175.0	62.00	17.5	1.076	5/04	175.0	63.40	18.5	1.081	5/07	175.5	66.30	19.0	1.090	
6.	6/18/50	4/05	170.2	57.80	14.0	1.074	6/22	179.5	66.20	14.75	1.075	6/21	182.5	75.50	16.25	1.071	11/18	183.0	79.30	18.42	1.083	
7.	2/13/51	4/26	174.0	65.75	15.0	1.062	6/08	180.0	72.90	15.5	1.052	6/08	183.5	74.60	16.75	1.063	11/22	184.5	74.50	18.75	1.078	
8.	9/14/50	4/08	183.0	78.16	16.5	1.081	6/09	186.5	82.40	18.0	1.091	6/21	187.0	87.70	18.5	1.080	6/20	187.0	88.20	18.75	1.078	
9.	7/10/50	4/01	160.5	47.00	14.0	1.058	5/26	164.5	51.90	16.5	1.063	10/25	166.0	56.40	18.0	1.075	4/26	167.0	56.69	19.0	1.076	
10.	5/09/50	5/14	170.0	66.30	15.15	1.059	6/13	171.5	70.50	17.5	1.068	5/30	172.8	69.80	18.25	1.071	6/13	172.5	73.10	19.0	1.087	
11.	5/11/50	4/22	171.0	66.10	14.75	1.045	5/31	174.5	71.30	16.0	1.037	5/24	176.8	70.50	17.0	1.058	4/30	176.5	71.10	18.25	1.056	
12.	10/16/50	4/13	156.0	44.50	14.5	1.054	5/31	164.0	50.40	13.75	1.070	4/20	169.3	57.60	15.0	1.068	5/02	174.5	63.75	16.0	1.090	
13.	1/13/50	5/20	183.0	60.20	16.0	1.080	5/27	184.5	63.40	17.0	1.064	5/04	185.0	66.50	18.5	1.073	5/24	185.0	66.60	19.0	1.090	
14.	11/06/50	6/04	163.0	43.30	13.75	1.055	5/05	170.5	49.30	12.75	1.037	5/11	179.3	59.60	13.75	1.078	5/08	182.5	64.20	16.5	1.081	
15.	3/25/51	4/08	170.1	56.00	14.5	1.060	5/27	178.0	64.20	16.0	1.056	5/15	180.0	67.50	17.0	1.067	4/23	182.0	69.40	17.75	1.078	
16.	6/21/50	3/31	174.5	61.80	16.0	1.076	5/10	177.5	69.50	17.5	1.061	5/06	178.5	69.80	18.5	1.068	5/26	179.0	73.40	19.0	1.078	
17.	8/20/50	3/24	163.1	47.50	13.5	1.071	5/16	172.0	54.00	14.75	1.059	5/04	175.0	58.50	16.5	1.071	4/25	176.8	60.50	17.5	1.085	
18.	10/25/50	5/27	158.0	44.30	13.0	1.055	4/21	165.5	51.10	13.75	1.092	5/15	173.0	57.90	14.5	1.079	5/08	177.0	61.60	15.5	1.074	
19.	11/18/50	3/31	166.2	49.00	14.0	1.073	5/03	173.0	54.50	15.5	1.080	5/17	175.3	59.40	17.0	1.074	5/13	176.5	61.80	18.5	1.093	
20.	6/21/50	4/05	168.3	53.40	14.5	1.046	5/11	176.0	62.60	15.0	1.073	5/17	180.0	67.80	16.5	1.049	7/04	181.5	72.90	18.0	1.056	
21.	10/28/50	5/18	171.6	59.70	14.5	1.058	6/15	177.0	61.50	16.25	1.065	5/19	179.0	68.80	17.25	1.057	4/26	179.5	70.40	18.25	1.066	
22.	2/06/51	4/21	175.0	60.60	14.5	1.070	5/19	180.0	68.90	16.75	1.049	4/26	181.2	72.10	18.0	1.065	5/12	183.0	75.30	18.75	1.071	
23.	4/22/50	3/15	173.5	67.40	15.5	1.076	6/13	177.5	72.50	17.0	1.074	4/26	178.5	76.50	18.5	1.080	5/08	179.0	78.77	19.0	1.083	
24.	8/26/50	5/31	176.0	60.35	15.25	1.058	5/05	178.0	65.00	16.75	1.067	5/22	179.4	65.00	18.0	1.088	5/29	179.0	66.00	18.75	1.084	
25.	9/11/50	5/03	171.0	55.50	14.25	1.064	5/06	175.0	61.30	15.75	1.051	5/29	177.0	65.30	17.25	1.070	7/02	178.5	68.90	18.5	1.068	
26.	10/23/50	3/17	155.5	45.70	14.25	1.072	5/31	164.0	55.20	15.0	1.064	6/29	168.5	58.40	17.0	1.077	6/06	169.5	62.50	18.0	1.076	
27.	6/02/50	3/30	170.2	53.50	15.0	1.084	5/11	175.0	56.10	17.0	1.055	4/21	176.5	59.10	18.0	1.076	5/13	177.0	62.30	18.75	1.052	
28.	8/09/50	5/12	173.2	57.40	15.0	1.074	5/06	175.5	61.30	17.0	1.073	5/23	176.5	73.80	18.0	1.058	5/03	178.3	66.50	19.0	1.074	
29.	10/25/50	5/13	172.5	57.00	15.25	1.074	5/19	174.5	61.00	17.0	1.058	5/05	175.0	64.10	17.75	1.085	6/03	175.5	64.32	19.0	1.091	
30.	3/20/50	5/13	165.0	47.90	13.5	1.050	5/24	172.5	55.60	15.0	1.051	6/05	178.0	58.00	15.75	1.062	5/16	179.1	61.30	16.5	1.087	
31.	1/21/50	4/21	168.0	68.70	15.25	1.035	5/11	171.0	72.50	17.0	1.039	7/03	171.0	68.60	18.25	1.054	4/26	172.5	76.63	19.0	1.053	
32.	5/23/50	4/22	175.0	54.40	14.0	1.058	5/06	183.0	60.20	14.75	1.062	6/01	186.3	66.30	17.0	1.092	11/20	189.5	70.40	18.0	1.091	
33.	6/13/50	4/14	170.0	56.00	14.75	1.056	5/30	179.5	64.00	15.5	1.054	6/08	183.0	67.60	16.5	1.073	4/26	184.5	70.00	18.0	1.074	
34.	12/11/50	4/12	165.2	47.40	13.0	1.063	6/22	176.0	56.30	13.75	1.075	6/20	180.0	62.20	15.5	1.083	10/21	182.5	64.40	18.0	1.091	
35.	7/20/50	5/06	160.5	46.50	16.0	1.065	6/22	162.0	67.00	18.25	1.077	5/04	162.0	70.60	18.5	1.053	5/28	163.0	76.40	19.0	1.042	
36.	11/11/50	4/14	155.0	47.60	13.5	1.062	5/17	163.5	54.90	14.75	1.052	5/18	166.0	58.70	16.0	1.073	4/30	168.0	61.50	16.75	1.064	
37.	3/17/51	3/30	171.0	62.00	14.5	1.057	6/08	173.5	69.70	16.5	1.043	6/21	178.0	73.30	18.0	1.040	6/12	178.0	79.70	18.5	1.044	
38.	1/19/51	3/31	169.4	66.90	14.0	1.051	6/14	177.0	71.90	15.25	1.048	8/08	178.5	66.90	16.75	1.070	5/17	179.3	67.50	17.5	1.072	
39.	4/21/50	5/04	181.5	59.75	14.5	1.087	5/10	185.0	65.30	15.75	1.082	4/27	187.0	68.30	17.0	1.075	5/14	187.5	67.32	18.25	1.085	
40.	2/03/50	5/17	170.5	62.20	14.75	1.055	6/08	176.0	71.90	15.75	1.048	5/30	179.2	71.10	17.75	1.072	7/08	179.0	74.60	19.0	1.083	

Copyright © 1976 International Pediatric Research Foundation, Inc.

Printed in U.S.A.

Pediat. Res. 10: 650-656 (1976)

Alkaline phosphatase
hypophosphatasia
leukocyte

Hypophosphatasia: A Developmental Anomaly of Alkaline Phosphatase?

RAFAEL GORODISCHER,⁽²⁸⁾ RONALD G. DAVIDSON, LUIS L. MOSOVICH, AND SUMNER J. YAFFE

Department of Pediatrics, State University of New York at Buffalo, Buffalo, New York, USA

Extract

This report deals with quantitative and qualitative investigations of alkaline phosphatase in two unrelated infants with the severe infantile form of hypophosphatasia. Both affected infants had no detectable leukocyte alkaline phosphatase activities and both sets of parents and one sibling tended to have low but variable leukocyte enzyme activities. Normal duodenal juice alkaline phosphatase activity was present in the one patient in whom it was measured and a wide range of variation in enzymic activity was observed in the stools. There was no significant difference in the stool enzyme activity between both patients with hypophosphatasia (42.01 ± 9.77 U) and control infants (40.55 ± 6.29 U). However, the heterozygous parents had values significantly lower than the control adults (2.10 ± 0.47 as compared with 19.10 ± 4.44 U). Intestinal bacteria did not contribute significantly to the stool alkaline phosphatase activity. Enzyme activity was present in the bile of one of the patients and nearly absent in that of the other.

Three "inducers" of alkaline phosphatase were given to both patients (phenobarbital, vitamin A, and corticosteroid). No clinical improvement or rise in serum alkaline phosphatase activity was observed during the trial of therapy with these agents. However, a significant increase in the activity of serum acid phosphatase was demonstrated during the course of vitamin A administration, suggesting an *in vivo* action of vitamin A on the lysosomes through decreasing the stability of the membrane and releasing acid phosphatase to the serum.

Quantitative determination of tissue alkaline phosphatases from autopsy tissues was highly variable: no activity was found in bone, lungs, or spleen of either infant; there was a discrepancy in liver and kidney alkaline phosphatase values (zero in one patient and present in the other) and activity was present in the intestinal mucosa of both.

Qualitative analysis of kidney, liver, and intestinal alkaline phosphatase revealed some differences between the patients and control subjects in heat inactivation and phenylalanine inhibition

(Table 3). Starch gel electrophoresis of the liver preparation of one patient disclosed a single band which had greater mobility than that of six control subjects matched for age. Liver extracts from a premature and from full term newborns showed two bands. The single band of the patient's liver enzyme corresponded to the newborn's fast moving component. In addition, the intestinal enzyme prepared from the same patient had an extra band when compared with age-matched control subjects.

Speculation

Among the many quantitative and qualitative alterations in alkaline phosphatases found in these two infants with the severe form of hypophosphatasia, the most striking finding was the presence in the liver of one of the cases of what appeared to be a form of the enzyme normally present only during very early postnatal life. The defective regulation of the various alkaline phosphatase isozymic forms during development may thus represent the basic defect in this disease.

Progress has been made in the understanding of hypophosphatasia since Rathbun (18) described it as a separate disease entity in 1948. The clinical features have been well defined and a number of biochemical abnormalities (low alkaline phosphatase in serum and various tissues, increased phosphoethanolamine and pyrophosphate in the urine (3), hypercalcemia) have been recognized (1, 5, 9). However, fundamental questions remain unanswered. It is not known, for example, how these biochemical abnormalities are interrelated. The name of the disease suggests that alkaline phosphatase plays a central pathophysiologic role, but comprehensive characterization of this enzyme has not been carried out. An unsettled controversy exists regarding the presence or absence of alkaline phosphatase in extraskeletal tissues. Although inhibitors have been investigated, it is unclear as to whether the alkaline phosphatase deficiency is the primary defect or merely a secondary phenomenon.

Other unexplained phenomena are the tendency towards failure to thrive, the development of pulmonary infections and increased intracranial pressure, and the extreme variability in the clinical picture.

This report deals with quantitative and qualitative investigations of alkaline phosphatase in two unrelated infants with a severe form of the disease.

CASE REPORTS

CASE 1

TB, the first child of a healthy 29-year-old gravida 6 para 1 woman and a 32-year-old man, was admitted to the Buffalo Children's Hospital at 5 months of age. The pregnancy was complicated by excessive weight gain and hypertension for which the mother received treatment with hydrochlorothiazide, restricted caloric intake, and dextroamphetamine. The infant was born after approximately 36 weeks of gestation and weighed 3,050 g. He appeared healthy at birth, but poor weight gain was noted from the first month in spite of adequate caloric intake. The usual vitamin supplement of A, C, and D was given from the second month. The infant smiled at 5 weeks, was able to grasp objects at 4 months, but had not developed head control by 5 months. "Protuberances" in the ribs were noted by the parents, and the infant was referred for evaluation.

The mother had five spontaneous abortions, each occurring between 2 and 4 months of pregnancy. The family history was otherwise negative with no known consanguinity. Both parents are of Northern European descent.

Physical examination revealed a 5-month-old, small, poorly developed, and undernourished male infant with mottled skin, who weighed 4,500 g and measured 58 cm. The anterior fontanel was soft and large (7 × 5 cm), the sutures were wide, and the posterior

fontanel was open. Cartilages of the external ears and trachea were soft. Marked costochondral beading was visible as well as palpable and the anteroposterior diameter of the chest was decreased. Other skeletal anomalies included widening of the wrists, double malleolus, bilateral talus valgus, and lumbar kyphosis. The sclerae were blue, there was a grade II/VI ejection systolic heart murmur along the left sternal border; blood pressure was 105/68; the musculature of the abdominal wall was thin and weak; the liver edge was palpable 6.5 cm below the right costal margin; and the spleen tip was also palpable. A right-sided hydrocele and inguinal hernia were present and the skeletal muscles were hypotonic. Deep tendon reflexes were hyperactive, and the infant had no head control. However, he was able to hold a bottle and transfer objects from hand to hand. Allowing for the muscular disability, his fine motor ability, adaptive language, and personal-social behavior were age-appropriate, and he was not felt to be mentally retarded.

X-rays disclosed large central lucent defects and vertical striations in the metaphyseal ends of all long bones, decreased ossification of the skull with large sutures and large fontanels, and delayed ossification in all centers of the spine and pelvis.

Laboratory studies revealed a calcium level of 9.8 mg/100 ml, phosphorus level of 6.4 mg/100 ml, and serum alkaline phosphatase level of 0.1 Bodansky units. Increased urinary excretion of phosphoethanolamine (588 $\mu\text{M}/\text{m}^2/24 \text{ hr}$) was found and the diagnosis of hypophosphatasia was thus confirmed.

Additional laboratory studies included hematocrits ranging from 25 to 31% with reticulocyte counts of from 0.9 to 3.9%. White blood count and serum electrolytes were within normal limits. Urinalysis revealed failure to concentrate the urine past a specific gravity of 1.010 after over 10 hr of water deprivation. White blood cells in the urine ranged from zero to many per high power field and urinary calcium excretion was 2.0 mEq/24 hr. Blood urea nitrogen was normal and although endogenous creatinine clearances were low (35.9–40 ml/min/1.73 m^2), inulin clearance was normal (121 ml/min/1.73 m^2); aldosterone was 3.0 μg in a 24-hr urine specimen. Serum T_4 , lactate dehydrogenase, SGOT, SGPT, and protein electrophoresis were all within normal limits. There was no impairment in the absorption of glucose and fats from the gastrointestinal tract, as determined by oral glucose and vitamin A-absorption tests. Excretion of mucopolysaccharides in the urine was normal in amount and distribution, and the serum parathormone level was 0.25 ng/ml (normal 0.3–0.4 ng/ml), as measured by radioimmunoassay. There were no detectable anomalies of the cardiovascular system by EKG, vectocardiogram, and chest roentgenograms. EEG, electromyogram, and nerve conduction time were normal. At 7 months of age serum and tracheal γ -globulins were within normal limits. Six months after Salk vaccination, the appropriate antibody response was found both in the serum and in the tracheal secretions. The infant's karyotype was normal, as were those of both his parents.

Therapeutic trials included oral administration of hydrocortisone (15 mg/day for 6 weeks), sodium phenobarbital (30 mg/day for 3 weeks and 60 mg/day for 3 weeks) and vitamin A (25,000 units/day for 3 weeks and 50,000 units/day for 6 weeks) successively. However, his clinical condition did not improve and serum alkaline phosphatase activity and bony x-ray changes were not modified by the administration of these drugs. During the trial therapy period with vitamin A, acid phosphatase activity in the serum increased gradually from 0.06 Bessey-Lowry units before therapy to 0.55–0.75 Bessey-Lowry units between the 28th and 61st days of vitamin A administration.

Although there were brief periods of improvement in the hospital, the general course was gradually downhill. The first episode of pneumonia developed shortly after admission and repeated respiratory infections ensued in spite of vigorous use of antibiotics and supportive treatment. The infant's weight remained essentially unchanged from 5 to 10 months of age, except for periods during which edema of undetermined etiology developed. At the age of 10 months, he had a cardiorespiratory arrest and expired.

CASE 2

HP, the second child of a healthy gravida 2, para 2, 26-year-old woman and a 27-year-old man was hospitalized seven times between the ages of 3 and 14 months. She was the product of a full term, uncomplicated pregnancy. Birth weight was 2,900 g and the infant gained weight slowly, appeared pale, had episodes of screaming, and the parents noted a very large "soft spot." She was admitted to hospital at age 3 months for investigation of failure to thrive. The family history was entirely negative with no known consanguinity. The mother is of Scottish descent and the father is an American Indian.

Developmental evaluations at 13, 28, and 54 weeks indicated severe neuromotor retardation with hypotonia and muscular weakness. However, the child appeared to function normally in adaptive behavior and she obtained a developmental quotient of 96 at 54 weeks of age.

Physical examination on the first admission revealed a 3-month-old, pale, undernourished, and underdeveloped infant with a prominent forehead. She weighed 3,700 g and measured 54.5 cm in length. Head circumference was 39 cm. Positive findings included prominent veins in the head, a large bulging anterior fontanel (6.5×6.5 cm), an open posterior fontanel, and wide sutures. The sclerae were blue; there was a rachitic rosary and mild hypotonia.

Skeletal x-rays disclosed radiolucent defects in the metaphyses of long bones and laboratory studies revealed a calcium level of 13.5 mg/100 ml, phosphorus level of 5.8 mg/100 ml, and alkaline phosphatase of 0.7 Bodansky units. Increased urinary excretion of phosphoethanolamine ($1,324 \mu\text{M}/\text{m}^2/24$ hr) confirmed the diagnosis of hypophosphatasia.

Additional laboratory values revealed a hematocrit of 23% with reticulocyte counts varying from 1.7 to 3.4%. The white cell count was within normal limits and the peripheral smear revealed normocytic and normochromic red cells with moderate polychromasia and some basophilic stippling. Erythrocyte enzymes were unremarkable although there was a slightly increased concentration of ATP. Serum electrolytes were normal as was the blood urea nitrogen and serum protein electrophoresis.

Urinalysis revealed a fixed specific gravity at a maximum of 1.008 even after up to 12 hr of withholding fluids. There was a trace of protein, 0–2 red cells, and 0–10 white blood cells per high powered field. Creatinine clearance was $38.4 \text{ ml}/\text{min}/1.73 \text{ m}^2$ and 24-hr urinary calcium excretions ranged between 0.5 and 0.7 mEq. An intravenous pyelogram was normal. Serum immunoglobulins were within normal limits and the serum contained normal levels of parathormone as measured by radioimmunoassay (0.22 ng/ml). Electroencephalograms were normal and a ventriculogram at age 5 months showed normal ventricles. A repeat skeletal survey at 13 months of age disclosed thick, abnormally dense zones of provisional calcification, abnormal radiolucency of the submetaphyseal zones, cortical thinning, generalized osteoporosis, and pathologic fractures of the humerus and radius.

The clinical course was characterized by poor nutrition, episodes of hyperpyrexia of obscure origin, hypercalcemia, increased intracranial pressure, and repeated episodes of pneumonia. Gradually the bulging fontanel became more tense and spinal fluid pressure rose from 196 mm of water at 3 months to over 400 at 12 months. Head circumference increased by only 3.2 cm during this period and skull x-rays remained unchanged. Neurosurgical risk was considered high because of the infant's general condition and craniectomy was deferred because of this, along with the fact that the cranial bones failed to ossify. The infant grew only 4.5 cm in length from 3 to 10 months of age and her weight, before she died of pneumonia at age 13 months, was 3,800 g.

SPECIAL ALKALINE PHOSPHATASE STUDIES: MATERIALS AND METHODS

QUANTITATIVE DETERMINATIONS OF ENZYME ACTIVITY

In *Serum*, *Duodenal Juice*, *Urine*, *Stool*, and *Intestinal Flora*. The colorimetric method described by Bessey, *et al.* (2), using

p-nitrophenylphosphate as substrate and glycine buffer, pH 10.5, was employed.

In *Leukocytes*. Semiquantitative histochemical determination was performed by the method of Kaplow (13). Positive controls obtained from pregnant women were stained simultaneously in each determination.

Other Tissues. For this study, 1 M 2-amino-2-methyl-1-propanol buffer, pH 10.0 (14), was used. Samples were incubated at various times (usually 0, 10, 20, and 30 min); then enzymic reaction was stopped by the addition of 5 ml 0.02 N NaOH; absorbance at 410-nm was read in a Gilford model 300 microsample spectrophotometer against tissue and reagent blanks; the changing absorbance per minute was determined from the straight line plot of the early time-source of the reaction. Because we found that 0.005 M L-phenylalanine (8) did not produce inhibition of the enzyme activity in the presence of 2-amino-2-methyl-1-propanol buffer, glycine buffer, pH 10.5, was employed for the phenylalanine inhibition experiments. Thermal stability was determined by incubating the enzyme preparation for 30 min at 56° and assaying enzymic activity thereafter.

All quantitative determinations were made at least in duplicate. Protein concentrations were measured according to the method of Lowry *et al.* (15).

PREPARATION OF MATERIAL FOR QUANTITATIVE ANALYSIS AND ELECTROPHORESIS

Stools. Fecal materials from healthy subjects (eight adults and seven infants) and from *HP*, *TB* and their immediate families were homogenized in a Waring Blendor in distilled water. For quantitative analysis 1:400 to 1:4,000 suspensions in distilled water were prepared while 10 times more concentrated samples were employed in the starch gels. Specimens so prepared were kept frozen at -19° until assayed.

Duodenal Juice. This was obtained by intubation in *HP* and in a control infant of the same age; the site of the tip of the catheter was confirmed radiologically.

Bacteria. Rectal swabs from *TB* and a normal adult were cultured in Difco "brain heart infusion" for approximately 24 hr. Suspensions were then centrifuged at $9,000 \times g$ for 10 min, and pellets washed twice and resuspended in 0.25 M sucrose.

Tissues. Tissue specimens were obtained from autopsy performed within 4 hr after death. The source of most control tissues was a 10-month-old female infant with congenital heart disease; control liver, mucosa of the small intestine, and kidney specimens were also obtained from infants whose ages varied between 2 days (including one premature 32-week gestation infant) and 12 months, who expired because of various unrelated conditions. All tissues so obtained were frozen immediately at -19° for a maximum of 15 days before enzymatic assays were performed. Bone and cartilage enzymes were prepared according to the method of Boyer (4). The mucosa of the small intestine was washed thoroughly and scraped. For quantitative assays tissues were homogenized with an equal volume of cold distilled water in a glass homogenizer with a Teflon pestle. All tissues, except brain and intestinal mucosa, were then centrifuged for 10 min at $2,000 \times g$. Tissue preparations were partially purified for electrophoretic studies: intestinal mucosa, by autolysis in ethanol and a mixture of ethylacetate and toluene pH 7.0 for 72 hr (20); liver, by extracting the enzyme with butanol (4).

STARCH GEL ELECTROPHORESIS OF ALKALINE PHOSPHATASE

Horizontal starch gel electrophoresis was performed at 4° using a discontinuous buffer system, pH 8.7. The gels were made from a buffer containing 0.1 M tris-(hydroxymethyl)-aminomethane and HCl; 0.3 M sodium borate buffer was placed in the electrode vessels. Enzyme preparations were subjected to electrophoresis for 5 hr. The total enzymic activity placed in the slots was approximately the same in each gel. Gels were sliced longitudinally and zones of enzyme activity were developed directly using a solution containing 0.002 M sodium naphthyl acid phosphate, 0.005 M MgSO_4 , 0.05% fast blue RR salt in 0.06 M sodium borate buffer,

pH 9.7, at 37° for 1 hr. For the intestinal alkaline phosphatase inhibition studies, phenylalanine was added to the developing solution (5×10^{-3} M).

SPECIAL ALKALINE PHOSPHATASE STUDIES: RESULTS

SERUM AND LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITIES

Both affected infants had no detectable enzyme activity in either serum or leukocyte extracts. Using quantitative and qualitative techniques, both sets of parents and one sibling of *HP* tended to have low but variable alkaline phosphatase activities, all within the wide ranges found among apparently healthy individuals.

DUODENAL JUICE ALKALINE PHOSPHATASE

Activity was near normal in *HP* but not assayed in *TB*. The enzyme activity was markedly inhibited by 0.005 M phenylalanine in both the patient (91%) and a control (75%). Electrophoresis revealed two anodic bands with the same mobility from the patient and the control.

URINE

Urine from nine normal children and from one of the affected infants (*HP*) had no enzymic activity; *TB* had some detectable enzyme activity (0.39 Bessey-Lowry units).

STOOLS

A wide range of variation in enzymic activity was observed in the four groups studied (Table 1). Infants had significantly higher

values than adults ($p < 0.005$); there was no significant difference in the stool enzyme activity between both hypophosphatasia patients and control infants. However, the heterozygous parents had values significantly lower than the control adults ($p < 0.005$). The enzyme obtained from the stools of the two patients and from normal subjects was inhibited to a similar extent (66–81%) by 0.005 M phenylalanine as the intestinal enzyme. Intestinal bacteria did not contribute significantly to the stool alkaline phosphatase activity since the cultured fecal flora had little or no enzyme activity. Moreover, sterile meconium from a newborn infant had high values and "sterilization" of the intestine (oral Kanamycin 40 mg every 6 hr for 3 days) had little effect on the stool enzyme activity. Additionally, in starch gel, stool and intestinal preparations (obtained from the same subject) had different electrophoretic characteristics, and butanol extractions of the stool enzyme followed by incubation of the intestinal enzyme with trypsin had no effect on the electrophoretic mobility.

BILE

Enzymic activity was present in *TB*'s bile (28.3 Bessey-Lowry units) and practically absent in *HP*'s bile (0.2 Bessey-Lowry units; mean of four controls: 61.1 ± 17.3 (SE)).

INVESTIGATION OF ACTIVATORS AND INHIBITORS

When normal serum was incubated with *HP*'s or with *TB*'s sera, the experimental result was almost identical with the average of both of them measured independently. Similarly, no inhibition of the serum enzyme nor activation of the stool enzyme was detected by incubation of a mixture of serum and stool preparations.

QUANTITATIVE DETERMINATION OF TISSUE ALKALINE PHOSPHATASE (TABLE 2)

Of particular note are the following observations: no activity was found in bone, lungs, or spleen of both infants; there was a discrepancy in liver and kidney alkaline phosphatase values in both infants; and activity was present in the intestinal mucosa.

QUALITATIVE ANALYSIS OF KIDNEY, LIVER, AND INTESTINAL ALKALINE PHOSPHATASE

Results of heat inactivation and inhibition with phenylalanine of intestinal and kidney enzymes are indicated in Table 3. Starch gel electrophoresis (Fig. 1) of the liver preparation of *TB* disclosed a

Table 1. *Stool alkaline phosphatase*

Group	No. of deter- mina- tions	No. of sub- jects	Mean $\pm$ SE (μ M <i>p</i> -nitrophenol/ 30 min/mg protein)
Patients (<i>TB</i> + <i>HP</i>)	4	2	42.01 ± 9.77
Heterozygotes	6	4	2.10 ± 0.47
Control infants	8	7	40.55 ± 6.29
Control adults	12	8	19.10 ± 4.44

Table 2. *Tissue alkaline phosphatase activities (nanomolar concentration of p-nitrophenol per min per mg protein)*

	<i>HP</i>	<i>TB</i>	Control (mean $\pm$ SE)	Control subjects ¹
Liver	0.00	0.36	12.80 ± 1.5	(7)
Kidney	0.00	11.20	32.00	(2)
Heat inactivation (%)		50	88 ± 10	
Phenylalanine inhibition (%)		81	51 ± 4	
Spleen	0.00	0.00	9.60	(1)
Thymus	0.01	0.41	13.00	(1)
Lymph node		2.20	2.80	(1)
Muscle		0.39	0.33	(1)
Pancreas	0.58	0.34	2.40	(1)
Adrenals		2.00	39.00	(1)
Intestine	18.00	358.00	338.00 ± 15	(4)
Heat inactivation (%)		9	33 ± 7	
Phenylalanine inhibition (%)	95	86	74 ± 4	
Bone (skull)	0.00	0.00	2.20	(1)
Costochondral junction	0.10	0.00	1.30	(1)
Cartilage (trachea)	0.00	0.48	6.90	(1)
Lung	0.00	0.00	7.70	(1)
Heart	0.89	0.00	1.50	(1)
Brain, gray matter		0.00	28.00	(1)
Brain, white matter		2.00	18.70	(1)

¹ Numbers in parentheses indicate the number of control subjects tested.

Table 3. Heat inactivation and phenylalanine inhibition

Intestine	Kidney
Heat inactivation	Heat inactivation
Control 29%; 37% (2)	Control 98%; 78% (2)
<i>TB</i> 9%	<i>TB</i> 50%
Phenylalanine inhibition	Phenylalanine inhibition
Control 77%; 72% (2)	Control 48%; 55% (2)
<i>TB</i> 86%	<i>TB</i> 81%
<i>HP</i> 95%	

single band which had greater mobility than that of controls matched for age (4–12 months). Liver extracts from a premature (32 weeks of gestation) and full term newborns (2-day-old infants) showed two bands. The single band of *TB*'s liver enzyme corresponded to the newborns' fast moving component. On starch gel the intestinal enzyme preparation obtained from *TB* had an extra slow moving band when compared to five age-matched controls; a faint band in the same area was observed in *HP*'s intestinal preparation (Fig. 2A). This extra component was also sensitive to 5×10^{-3} M phenylalanine.

Electrophoresis of intestinal alkaline phosphatase was also performed in cellulose acetate gel (26) using 0.05 M Tris-HCl buffer, pH 7.5, and 0.6 mA/cm. The developing solution consisted of 0.25% naphthyl-sodium-acid phosphate, 0.25% fast blue RR salt, and 0.30% magnesium sulfate in 0.3 M sodium borate buffer, pH 10.0. In this system a single band was seen in the control preparation and an additional fast moving band in *TB* (Fig. 2B). No additional band was visible in a preparation from *HP*.

DISCUSSION

Although a voluminous literature has been published concerning the alkaline phosphatases, the biological function of this group of enzymes has remained a mystery (for review, see References 7 and 17). Perhaps the study of this enzyme in pathologic states in which alkaline phosphatase is deficient will help to elucidate its physiologic role.

If a deficient alkaline phosphatase were the basic abnormality in hypophosphatasia, attempts to increase the catalytic activity of this enzyme could result in improvement of the condition. Three "broad spectrum" and specific alkaline phosphatase "inducers" were given to *TB* and *HP*: phenobarbital (24), vitamin A (19), and corticosteroid (10). The suggestion has been made that normal alkaline phosphatase is vitamin A dependent (19) and therefore the possibility that hypophosphatasia was a "vitamin A dependency syndrome" was an attractive one. No clinical improvement or rise in serum alkaline phosphatase was observed during trial therapy with these agents. An interesting phenomenon was observed, however, during vitamin A administration. In order to determine whether the vitamin preparation was having systemic effects, serum acid phosphatase was measured; a significant increase in the activity of this enzyme was demonstrated, which paralleled the rise in the serum vitamin A level. Although this does not prove a cause-effect relationship, this is likely and it could represent an *in vivo* action of vitamin A on the lysosomes through decreasing the stability of the membrane and releasing acid phosphatase to the serum (23).

A number of therapeutic agents have been of no value in hypophosphatasia and at present there is no generally accepted mode of treating this disease. The effect of corticosteroids and phosphate salts has not been uniform (21). Since some patients with hypophosphatasia have spontaneous rapid improvement, evaluation of various forms of therapy should be particularly critical.

The study of the stool alkaline phosphatase suggests an intestinal origin, in spite of the different electrophoretic mobility of these

two preparations, and our results are consistent with those previously reported (12, 17). The wide variation in stool enzymic activity among different individuals correlates well with the reported variability of intestinal alkaline phosphatase values. The variability in the specific activity and electrophoretic mobility of stool alkaline phosphatase probably is due also to other factors, such as intestinal transit, action of various proteolytic enzymes, turnover rate of intestinal mucosa, and diet. The normal stool alkaline phosphatase activity found in both *TB* and *HP* suggested that they had normal intestinal enzymic activity. Quantitation of

Fig. 1. A: starch gel electrophoresis of liver alkaline phosphatase of *TB* and six control infants; 1, premature infant, 22 weeks; 2, premature infant, 32 weeks; 3 and 4, full term newborns, 2 days; 5, *TB*; 6, infant, 8 months. B: a diagram of A. C: starch gel electrophoresis of *TB* and two age-matched controls. HGB: hemoglobin.

Fig. 2. *A*: starch gel electrophoresis of intestinal alkaline phosphatase from both patients (*TB* and *HP*) and age-matched controls (*N*). The extra slow moving band is marked with an arrow. *B*: Cellogel electrophoresis of intestinal alkaline phosphatase.

the enzyme in the intestinal preparations obtained at autopsy proved that this assumption was correct.

Our findings on bile alkaline phosphatase can be added to the evidence against the hypothesis that bile alkaline phosphatase represents the serum enzyme in the process of being excreted by the liver. Since *HP* had low levels of activity in both serum and bile, the low serum values in hypophosphatasia probably are not due to the loss of enzyme in the bile. The presence of readily detectable alkaline phosphatase activity in the bile of *TB* suggests that the enzyme was produced in the liver cells or bile canaliculi.

Quantitative measurements of tissue alkaline phosphatase in hypophosphatasia have been reported in bone, liver, kidney, intestine, lung, and leukocytes. One report (6) stresses the finding of normal values in intestinal mucosa biopsied from patients with the mild form of the disease and other data indicate that under certain circumstances the intestinal alkaline phosphatase is the major component of serum alkaline phosphatase activity (22).

Our results, as well as those from the literature, illustrate the marked tissue variability in alkaline phosphatase activity in hypophosphatasia. One could speculate that symptoms are related to the deficiency of enzyme activity in the involved organs.

It is of interest to note that *HP*, who expired at 14 months of age, had much lower tissue enzymic values than *TB*, who died at the age of 10 months. In addition, probably the four presumed heterozygous parents had low intestinal alkaline phosphatase activities, since the levels in their stools were so much decreased. This is remarkable because, as is the case for other genetically determined enzyme deficiencies, we would have expected to

encounter little activity in the homozygous infants and values somewhere in between those of the homozygous and the normals in the heterozygous parents.

Qualitative abnormalities in alkaline phosphatase from kidney, intestine, and liver from *TB* and *HP* were evident upon further characterization of the enzyme by heat inactivation, phenylalanine inhibition, and electrophoresis in starch gel. When liver alkaline phosphatase preparations obtained from premature and full term newborn infants were compared electrophoretically with the liver enzyme of *TB*, it became apparent that *TB*'s liver alkaline phosphatase could represent a vestige of the fast moving band of early postnatal life.

Thus, our results confirm and expand described changes in electrophoretic mobility of alkaline phosphatase in tissues of human fetuses and infants (11, 16) and, from our unexpected findings in liver and intestinal patterns, one could hypothesize that the early postnatal enzyme failed to be turned off in the tissues involved at the proper time during biologic development, while the normal infantile form failed to be induced. Eventually, at a later stage, the enzyme of the early postnatal period might be gradually turned off, resulting in loss of enzymic activity and failure of some critical function.

The marked tissue variability in alkaline phosphatase, as well as the obvious genetic heterogeneity in hypophosphatasia, may be explained by the subunit hypothesis of the structure of proteins and isozymes. Zinkham *et al.* (25) have proposed that the expression of an abnormality in inborn metabolic errors is restricted to those tissues in which the mutant gene controls the synthesis of the major isozyme component.

SUMMARY

Studies of both qualitative and quantitative activities of alkaline phosphatases are reported in two patients with the severe infantile form of hypophosphatasia. Clinical and autopsy studies revealed quantitative and qualitative heterogeneity between these two patients and among their close relatives and control subjects. The nature of the basic defect and the relationship between the disease and alkaline phosphatase activities in various tissues remain an enigma.

REFERENCES AND NOTES

1. Bartter, F. C.: Hypophosphatasia. In: J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson: *The Metabolic Basis of Inherited Disease*, Ed. 3, pp. 1295-1304 (McGraw-Hill Book Company, New York, 1973).
2. Bessey, D. A., Lowry, O. H., and Brock, M. J.: A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.*, **164**: 321 (1946).
3. Bongiovanni, A. M., Album, M. M., Root, A. W., Hope, J. W., Marino, J., and Spencer, D. M.: Studies in hypophosphatasia and response to high phosphate intake. *Amer. J. Med. Sci.*, **255**: 163 (1968).
4. Boyer, S. H.: Human organ alkaline phosphatases: Discrimination by several means including starch gel electrophoresis of antienzyme-enzyme supernatant fluids. *Ann. N.Y. Acad. Sci.*, **103**: 938 (1963).
5. Currarino, G., Newhauser, E. B. D., Reyersboch, G. C., and Sobel, E. H.: Hypophosphatasia. *Amer. J. Roentgenol.*, **78**: 392 (1957).
6. Danovitch, S. H., Baer, P. N., and Laster, L.: Intestinal alkaline phosphatase activity in familial hypophosphatasia. *New Engl. J. Med.*, **278**: 1253 (1968).
7. Fishman, W. H.: Perspectives on alkaline phosphatase isoenzymes. *Amer. J. Med.*, **56**: 617 (1974).
8. Fishman, W. H., Green, S., and Inglis, N. I.: L-phenylalanine: An organ specific, stereospecific inhibitor of human intestinal alkaline phosphatase. *Nature*, **198**: 685 (1963).
9. Fraser, D.: Hypophosphatasia. *Amer. J. Med.*, **22**: 730 (1957).
10. Griffin, M. J., and Cox, R. P.: Studies on the mechanism of hormonal induction of alkaline phosphatase in human cell culture. *J. Cell. Biol.*, **29**: 1 (1966).
11. Hernandez-Jodra, M., and Gancedo, C.: Isoenzymes of alkaline phosphatase in human meconium and small intestine during development. *Enzyme*, **12**: 682 (1971).
12. Horrigan, F. D., and Danovitch, S. H.: The origin of human fecal alkaline phosphatase. *Amer. J. Digest. Dis.*, **19**: 603 (1974).
13. Kaplow, L. S.: Cytochemistry of leukocyte alkaline phosphatase. *Amer. J. Clin. Path.*, **39**: 439 (1963).
14. Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S., and Crawford, E. J.: The quantitative histochemistry of brain. II. Enzyme measurements. *J. Biol. Chem.*, **207**: 19 (1954).
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**: 265 (1951).

16. Pataryas, H. A., and Christodoulou, C.: Alterations in human organ alkaline phosphatases during foetal development. *Human Hered.*, 20: 420 (1970).
17. Posen, S.: Alkaline phosphatase. *Ann. Intern. Med.*, 67: 183 (1967).
18. Rathbun, J. C.: Hypophosphatasia, a new developmental anomaly. *Amer. J. Dis. Child.*, 75: 822 (1948).
19. Riley, P. A., and Sperman, R. I. C.: Vitamin A-induced synthesis of alkaline phosphatase. *Science*, 160: 1006 (1968).
20. Schlamowitz, M., and Bodansky, O.: Tissue sources of human serum alkaline phosphatase, as determined by immunochemical procedures. *J. Biol. Chem.*, 234: 1433 (1959).
21. Teree, T. M., and Klein, L.: Hypophosphatasia: Clinical and metabolic studies. *J. Pediatr.*, 72: 41 (1968).
22. Warshaw, J. G., Littlefield, J. W., Fishman, W. H., Inglis, N. R., and Stolbach, L. L.: Serum alkaline phosphatase in hypophosphatasia. *J. Clin. Invest.*, 50: 2137 (1971).
23. Weissman, G.: Labilization and stabilization of lysosomes. *Fed. Proc.*, 23: 1038 (1964).
24. Yaffe, S. J., Levy, G., Matauzawa, T., and Baliah, T.: Enhancement of glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital. *New Engl. J. Med.*, 275: 1461 (1966).
25. Zinkham, W. H., Blanco, A., and Kupchik, L.: Isozymes: Biological and clinical significance. *Pediatrics*, 37: 120 (1966).
26. Cellogel, Chemetron, Milan, Italy; available through Kalex Scientific Co., Manhasset, N.Y.
27. The authors are indebted to Drs. M. Crea, L. Schiff, and D. Weinstein for the opportunity to study the patients; to Dr. H. B. Robinson, Department of Pathology, Children's Hospital of Buffalo, for providing us with the autopsy specimens for alkaline phosphatase studies; to Dr. I. A. Schafer, Department of Pediatrics, Cleveland Metropolitan General Hospital, for the determination of urinary mucopolysaccharides; to Dr. L. M. Sherwood, Beth Israel Hospital, Boston for serum parathormone determinations and to the following investigators of the Children's Hospital of Buffalo for their help in the clinical evaluation of the patients: L. G. MacDougall, D. Kerr-Grant, P. L. Ogra, J. Rahill, W. Georgi, D. Klein, and R. Munschauer. We wish to express our gratitude to the staff of the Clinical Research Center for the care the patients received during their prolonged hospitalizations.
28. This research was supported in part by Public Health Service Grants FR-05493, FR-77, HD-06321; Department of Health, Education and Welfare Maternal and Child Health Services, Project 417; and a grant from the United Health Foundation of Western New York.
29. Requests for reprints should be addressed to: R. Gorodischer, M.D., Kupat-Holim, P.O.B. 151, The Soroka Medical Center, Beer-Sheba (Israel).
30. Accepted for publication January 28, 1976.

Copyright © 1976 International Pediatric Research Foundation, Inc.

Printed in U.S.A.

Pediat. Res. 10: 656-660 (1976)

Aorta
blood flow
blood pressure
oxygen
umbilical artery catheter

Effect of Umbilical Artery Catheters on Blood Flow and Oxygen Supply to Extremities

ROBERT F. HUXTABLE,⁽¹³⁾ KENNETH G. PROCTOR, AND ANTHONY V. BERAN

Department of Pediatrics, College of Medicine, University of California, Irvine, California, USA

Extract

The effect of catheter placement in the abdominal aorta on the blood flow and oxygenation of lower extremities was evaluated in 30 New Zealand rabbits, weighing 0.55-3.5 kg, by inserting 3.5 and 5.0 French polyvinyl chloride catheters, of the type commonly used for umbilical artery catheterization, through the femoral artery, advancing 15-20 cm, and leaving in place for 10-30 min. Arterial blood pressure (BP), common iliac artery blood flow (BF), gracilis muscle tissue oxygen availability (O_{2a}), and subcutaneous temperature (T) in the foot were continuously monitored before and during catheter placement and after withdrawal. There were no changes in the physiologic variables measured in the contralateral leg when the catheter remained below the aortic bifurcation; however, when the catheter was advanced 15-20 cm into the abdominal aorta, a decrease in lower extremity BF, O_{2a} , and T occurred. Because the length of catheter insertion was maintained constant in each animal, the decreases in BF, O_{2a} , and T are related to the relative dimensions of the vessel and the catheter. The ratio of catheter to vessel diameter, in addition to the site of catheter placement, should be considered during the clinical application of arterial catheters. Reduction in blood flow could be detected by continuous differential monitoring of core and extremity temperature or extremity muscle oxygen availability.

Speculation

Placement of catheters in the aortas of animals of physical sizes comparable to those of newborn infants may produce an appreciable

obstruction to aortic flow. Until a suitable noninvasive method of assessing oxygenation in newborns is available, umbilical artery catheters should be of the smallest possible outside diameter and placed below the bifurcation of the abdominal aorta.

The patency of the umbilical vessels at birth allows long term catheterization. In 1959, James (5) reported the use of an umbilical artery catheter to obtain repeated samples for blood gas and pH analysis. Since that time, catheterization of umbilical vessels has become a routine procedure in the management of diseased neonates. Various authors (7) have suggested catheter placement in: (1) the abdominal aorta, distal to the renal and inferior mesenteric arteries and proximal to the aortic bifurcation (6), (2) the thoracic aorta, distal to the ductus arteriosus (6), or (3) the iliac artery, distal to the aortic bifurcation (1, 11). Although this procedure facilitates the care of sick infants, especially those requiring respiratory support and fluid maintenance, it may lead to serious complications including thrombosis, infection, vessel perforation, extremity blanching, and visceral necrosis (7, 8).

A catheter placed in an artery produces a partial acute mechanical obstruction equivalent to coarctation of the aorta, and measurement of this effect has not been previously reported. Poiseuille's law was used as the theoretical rationale for this study.

$$\dot{Q} = \frac{\pi(P_2 - P_1)}{8\lambda L} \left[r_2^4 - r_1^4 - \frac{(r_2^2 - r_1^2)^2}{\ln \frac{r_2}{r_1}} \right]$$