



ALKALINE PHOSPHATASE ISOENZYMES
IN NEONATAL JAUNDICE

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ABSTRACT

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By

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Measurements of serum isoenzyme levels of alkaline phosphatase were made on 2 groups of jaundiced infants: (1) those with transient "physiological" jaundice and (2) those with jaundice due to fetal-maternal incompatibilities leading to hemolysis. Assuming that relatively few of those with physiologic jaundice would manifest some degree of immaturity of the hepatic excretory enzyme systems and that most of those with hemolytic jaundice would show varying degrees of the same immaturity, the experiment was designed to reveal any association of immature enzyme systems, glucuronyl transferase and alkaline phosphatase in this instance.

The data revealed no associated decrease in the hepatic fraction of alkaline phosphatase isoenzyme, but an unexpected decrease in the osseous fraction of alkaline phosphatase was encountered in infants with hemolysis.

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INTRODUCTION

This thesis problem was designed to evaluate the relative degrees of maturation of certain critical hepatic functions in the neonate. All of the data were collected on newborns showing varying degrees of hyperbilirubinemia. The principal comparisons in this study are limited to the relationship of serum alkaline phosphatase isoenzymes and hyperbilirubinemia in 2 groups of newborns. The groups were separated on the basis of immunohematological evidence of fetal-maternal incompatibility leading to excessive hemolysis. Those infants showing no incompatibility were designated "normal" infants, displaying physiologic jaundice in varying degrees, while the "abnormal" group displayed a defined fetal-maternal incompatibility leading to hemolysis. The results obtained, in some instances, were unexpected.

Cord bloods were tested electrophoretically for the presence of the isoenzyme fractions of alkaline phosphatase. These data reveal a definite change in total alkaline phosphatase levels in the 2 groups analyzed.

Bilirubin

The degree of hyperbilirubinemia present in the neonate is directly related to the maturity of the newborn liver and the maturity of the enzyme systems involved in the degradation of hemoglobin by-products.

Aged or damaged red blood cells are readily phagocytosed by the reticuloendothelial (RE) cells anywhere in the body, but particularly in the liver, spleen and bone marrow. The heme portion of the released

hemoglobin is converted in the RE cells, by a series of chemical changes, to bilirubin. It was originally believed that bilirubin was carried in the blood in the form of bilirubin-globin. However, it is now recognized to be carried in the blood loosely bound, partly to albumin and partly to α_1 -globulin (Hoffman, 1970).

The bilirubin in normal plasma, although it may circulate in loose combination with proteins, acts as free (indirect) bilirubin. This free bilirubin is insoluble in water. When serum bilirubin passes through the polygonal cells of the liver, it is converted, at least in part, to glucuronide and, as such, is excreted into the bile. About 80% of the bilirubin conjugates with glucuronic acid to form bilirubin glucuronide; an additional 10% conjugates with sulfate to form bilirubin sulfate. The final 10% conjugates with a variety of other solubilizing substances. This converted or conjugated (direct) bilirubin is now highly soluble.

A small portion of the soluble bilirubin formed by the hepatic cells returns to the plasma. This is then either re-excreted by the liver cells directly into the liver sinusoids or it is reabsorbed into the blood from the bile ducts. Regardless of the exact mechanisms by which bilirubin reenters the blood, this results in the presence of a small portion of soluble bilirubin in the body fluids.

Jaundice is a syndrome characterized by hyperbilirubinemia and yellowish pigmentation of the sclerae, skin and mucous membranes. Occasionally, the predominant color is green because of biliverdin, an intermediate product of hemoglobin degradation. Jaundice is predominantly of 2 types: (1) retention jaundice, in which there is overproduction of bilirubin caused by excessive hemolysis, the liver being unable to remove bilirubin as fast as it is produced; and (2) regurgitation jaundice, in which bile mechanically finds its way back into the blood stream because of either

intra-hepatic or post-hepatic obstruction of the bile ducts.

The mechanisms involved in the production of jaundice are not completely clarified. There is probably an overlapping of the 2 types of jaundice in any individual case (Watson, 1937).

Jaundice, caused by a deficiency of the glucuronyl-transferase, is a common occurrence in the neonate. The rate of formation of bilirubin glucuronide in the polygonal cells of the liver determines the rate of hepatic excretion of bilirubin. Although the fetal liver has a poorly developed glucuronyl-transferase system, and the enzyme system gradually improves during fetal development, it is frequently inadequate at the time of birth. During fetal life, the plasma bilirubin is removed by the placenta, apparently by a glucuronide forming mechanism (Claireau, 1960). After birth, the enzyme system in the newborn liver develops rapidly and becomes adequate in a week or two. In the immediate neonatal period, however, all infants have some hyperbilirubinemia, with approximately 30% having a level above 6 mgs./dl., producing visible jaundice in the newborn. In the absence of any hemolytic factor, the jaundice usually does not appear in the first 24 hours, is usually self-limiting and is seldom associated with bilirubin levels above 10 mgs./dl.

However, physiologic neonatal hyperbilirubinemia may not be entirely due to inadequate glucuronyl-transferase. Some of it may be due to:

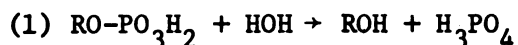
- (1) inhibiting effect of maternal pregnadiol present in the breast milk;
- (2) increased enterohepatic recirculation of bilirubin because of the absence of bacteria in the gastrointestinal tract which convert bilirubin to urobilinogen; and (3) formation of bilirubin from sources other than hemoglobin (Brown, 1962). When, because of extraneous factors causing excessive hemolysis, the amount of bilirubin formed is much greater than normal, the serum-insoluble bilirubin may rise above 20 mgs./dl. The

brain cells of the newborn infant may be sensitive to such levels of unconjugated bilirubin, resulting in the condition known as "kernicterus." This condition is characterized by cerebral manifestations with convulsions, and is frequently fatal.

Phosphatases

The blood plasma of normal man contains representatives of 2 classes of "nonspecific" phosphomonoesterases, one exhibiting optimal activity at pH 9 with substrate in high concentration, and the other at pH 5, hence alkaline and acid phosphatase. The proteins endowed with these enzymatic properties are separated with the alpha-globulins by alcohol fractionation of the plasma proteins (Edsal, 1951).

The "non-specific" phosphatases, unlike the "specific" phosphatases (such as glucose-6-phosphatase) which are more selective in their substrates, act upon the orthophosphoric monoesters of a wide variety of phenolic, alcoholic, carbohydrate and other compounds. Two types of reactions are catalyzed. In one, the enzyme acts as the hydrolase, with cleavage of the P-O bond and removal of the phosphoryl group to liberate inorganic orthophosphate (Axelrod, 1956):



The second type of reaction, a transphosphorylation in which the enzyme acts as a phosphotransferase, does not involve the intermediate formation of inorganic phosphate in transmission of the phosphoryl group to the acceptor:



This reaction, which results in phosphoric ester synthesis, does not require the presence of adenosine triphosphate (ATP) or other high energy phosphates (Morton, 1958). Note that reaction (1) is really a special case of reaction (2).

It has been generally assumed that reaction (1) characterizes the action of alkaline phosphatase in its natural environment, and all methods of estimating the enzyme are based on this premise. Whether the enzyme predominantly catalyzes reaction (1) or (2) appears to depend upon competition between water and other hydroxyl containing compounds for sites at the surface of the enzyme-donor-complex. Nothing definite is known as yet of the substrate binding groups at the active center of the enzyme.

Phosphatase, in splitting off the phosphate group from p-nitrophenyl phosphate, liberates p-nitrophenol which is yellow in alkaline solution and therefore serves as an indicator. Thus, alkaline phosphatase activity is measured by the intensity of yellow color in alkaline media in the presence of a specific substrate. Activity units are arbitrarily defined on the basis of method and conversion charts are available for conversion of either Bodansky or King-Armstrong units to International units (see Appendix).

The normal values for plasma alkaline phosphatase are 2.0 to 4.5 Bodansky units/dl. in adults, and 3.5 to 11.0 Bodansky units/dl. for children. Since alkaline phosphatase is normally excreted by the liver, the values are increased in obstructive jaundice. In a purely hemolytic jaundice there is usually no rise. Unfortunately, various other factors may affect phosphatase activity so that the results of this test must be correlated with clinical findings and with other tests (Harper, 1965).

HISTORICAL REVIEW

Robinson's discovery of alkaline phosphatase in bone in 1923, and his theory of the role of this enzyme in bone formation, stimulated investigation of the relationship of skeletal disease to the serum alkaline phosphatase. Markedly increased alkaline phosphatase activity in the serum has been demonstrated in Paget's disease, hyperparathyroidism (osteitis fibrosa), rickets, osteosarcoma and carcinoma with widespread metastases, just to mention a few (Gutman, 1936; Bodansky and Jaffe, 1934).

A second category of diseases, involving the hepatobiliary system, was found to be associated with increased phosphatase activity in the plasma by Roberts (1933). He noted augmented values in patients with jaundice due to obstruction of the extrahepatic biliary tract and normal or slightly elevated levels in patients with catarrhal, infective, toxic and hemolytic jaundice. Roberts therefore proposed use of the determination of alkaline phosphatase to distinguish obstructive from other forms of jaundice. However, Bodansky and Jaffe (1933) found extensive overlapping of total alkaline phosphatase results in cases of hepatogenous jaundice and suggested discontinuing the use of this determination to differentiate obstructive from hepatogenous jaundice. The level of total serum alkaline phosphatase activity was later found to be a more useful index of certain forms of hepatobiliary disease than was first appreciated, since it reflects obstruction not only of the extrahepatic biliary tract, with or without overt jaundice, but also obstruction of the intrahepatic biliary tract, with or without jaundice. Some metastatic carcinomas of the liver have

been seen wherein the alkaline phosphatase activity is increased in advance of hyperbilirubinemia (Meranze *et al.*, 1938). It was concluded from these early studies that the determination of serum alkaline phosphatase affords, in man, a sensitive criterion of the patency of the excretory biliary channels, extrahepatic and intrahepatic (Gutman, 1940).

By 1940, investigation of the serum alkaline phosphatase had thus revealed markedly increased levels of activity in 2 general categories of human disease: disorders of the skeleton characterized by overactivity of substantial numbers of osteoblasts, and disorders of the hepatobiliary system, notably those characterized by obstruction of the extrahepatic or intrahepatic biliary tract.

Alkaline phosphatase has been extensively used in diagnosis during the past 3 decades, and there has been much discussion concerning the origin of the serum enzyme in hepatobiliary disease. Bone appears to be the principal source of alkaline phosphatase, and there is strong evidence indicating that this enzyme is mainly excreted in the bile (Gutman, 1959). Many other tissues, including the intestine, kidney and placenta, are known to contain this enzyme (Bodansky, 1948; Ross *et al.*, 1956). The possibility remains that some other tissue might contribute to an increased serum alkaline phosphatase activity.

Among the procedures which have been applied in attempts to identify the tissue(s) of origin of the serum-enzyme activity are chromatographic and electrophoretic techniques. These methods have led eventually to recognition of its heterogeneity. The existence of serum alkaline phosphatase in more than one form was first indicated by paper electrophoresis by Baker and Pellegrino in 1954. After starch-gel electrophoresis, activity was extracted from 2 zones, one of which moves more slowly than the slow α_2 -globulin and the other slightly more slowly than the β -globulin.

(Kowlessar, 1958). Both zones show increased activity in liver disorders, but there is an increase in the β -globulin region only in bone disease. The test-paper method for visualizing the zones of activity was introduced by Estborn in 1959. This has demonstrated a major band which travels slightly more slowly than β -globulin and a second faint band corresponding to what has been described as the prealbumin (acid α_1 -glycoprotein) zone.

The application of ion-exchange chromatographic techniques has also shown that the human serum enzyme consists of at least 2 distinct components. Fahey, McCoy and Goulian (1958) detected 2 peaks of activity, both of which appear long before the single peak of acid phosphatase activity, using chromatography on DEAE cellulose columns.

Quantitative studies with the aid of starch block electrophoresis also indicate that the major zone of the serum alkaline phosphatase activity migrates with the α_2 -globulins (Rosenberg, 1959), but Keiding (1959) succeeded in resolving this into an " α_2 -component" migrating near the α_2 -globulin and the " β -component" located between the α_2 -globulin and the β -globulin. Both workers found a minor band in the α_1 -globulin region in the sera of patients with liver disease. Since the β -globulin-fraction is considerably increased during childhood, and is particularly prominent in the sera of patients with Paget's disease or bone metastases, it would appear to be largely derived from bone. The α_1 -component appears to originate in the liver, and the finding that an appreciable fraction of the alkaline phosphatase of the bile migrates as an α_1 -globulin is consistent with this conclusion. Further support for this view is provided by Cooke and Zilva (1961), who reported a case in which extensive metastases to the liver, but no bony metastases, were found at autopsy.

After starch-block electrophoresis, about 50% of the serum alkaline phosphatase activity was found in the α_1 -peak.

Haiji and deJong (1963) applied agar-gel electrophoresis to the study of the isoenzyme patterns of the alkaline phosphatases of human sera and tissues. The results resembled those obtained by starch-block electrophoresis in showing 3 main bands of activity: Band I was slightly slower and Band II slightly faster than the α_2 -globulins, while Band III had a mobility between that of the α_1 - and α_2 -globulins. A single band also appeared between I and II, and was attributed to superimposing of these 2 bands as seen in patients with diseases involving both liver and bone. This may also be detected by mixtures of serum containing individual bands. There is general agreement in the interpretation of the results of Haiji and deJong (1963) obtained by starch-block electrophoresis: Band I appears to be associated with bone diseases, while the faster fractions, Bands II and II, predominate in liver disease.

The improved resolution of protein fractions obtainable by starch-gel electrophoresis confirmed the existence of these phosphatase components (Markert and Moller, 1959). Estborn (1959) showed that the principal alkaline phosphatase in the serum of a patient with obstructive jaundice due to cholelithiasis migrated as a distinct band between the α -globulin and haptoglobin fractions. In certain other sera, this band was accompanied by a weak band of activity occurring in the prealbumin (acid α_1) fraction. This component was also found in bile, but the principal phosphatase of this fluid migrated somewhat more slowly than the main serum component. Estborn concluded that the serum alkaline phosphatase migrates separately from the main serum protein fractions and that the association with the α_1 - and α_2 -globulins reported by investigators using starch grains as support media is coincidental. Hodson, Latner and Raine (1962) came to

the same conclusion as a result of their observation that the positions of the alkaline phosphatase bands relative to the major serum proteins may be varied by using a discontinuous buffer system.

Numerous investigators have separated the alkaline phosphatase components of human and animal serum and tissue by starch-gel electrophoresis and, though the patterns obtained differ in detail, certain general conclusions may be drawn. The principal alkaline phosphatase band of liver extract migrates close to the transferrin (β -globulin) fraction of serum; minor bands in the β -lipoprotein and slow α_2 -globulin zones are usually detected. There may also be activity near the point of application. The main band of kidney extracts occurs in the haptoglobin zone, while minor bands may be seen in the β -lipoprotein and transferrin (β -globulin) regions. Most of the alkaline phosphatase of intestinal origin moves more slowly than the main band of the liver enzyme; the slower fractions in the β -lipoprotein region and near the origin may be detected. Bone alkaline phosphatase activity is usually found close to the β -globulin region. Slower bands have been reported by some investigators (Moss, 1962).

Evidence that the slow-moving bands of alkaline phosphatase isoenzymes originate in the liver has been provided by elution of the enzymes after starch-gel electrophoresis. The slow band of alkaline phosphatase also exhibits considerable 5'nucleotidase activity, whereas the fraction associated with the β -globulin is devoid of such activity (Kowlessar *et al.*, 1961). Since elevation of the serum 5'nucleotidase is highly specific for liver diseases, these observations are consistent with the view that the liver is the source of the slow components of the serum alkaline phosphatase.

Of 120 patients with a variety of diseases and having elevated serum alkaline phosphatase activities, only 2 exhibited bands in other than the β -globulin and haptoglobin I zones, and Boyer (1961) therefore concludes that simple starch-gel electrophoresis is not likely to be useful as a diagnostic tool. Hodson *et al.* (1962) point out that serum isoenzyme separation might aid in the differential diagnosis of jaundice; for cases of obstructive jaundice they noted a doublet of phosphatase activity near the origin which is less prominent in sera from patients with hepatocellular disease.

Disagreement remains concerning the origin of the alkaline phosphatase in normal human serum. Most of the activity occurs close to the β -globulin region, a fraction attributed by Kowlessar (1959) to bone phosphatase and by Hodson (1962) to the liver enzyme. Chianducci *et al.* (1962) conclude that it would be of either hepatic or osseous origin.

A fourth fraction in cord blood, migrating with the β -globulins, has been reported by Chianducci *et al.* (1962), who also found a similar band in placental extracts. Boyer (1961), while concurring with this finding in cord blood, also found a fourth fraction in serum from women in labor. The main band is similar to that of bone extract, but different from that in the placenta and liver.

Quantitation of the isoenzyme fractions remains a difficult task because of overlapping of fraction patterns. Nevertheless, utilizing an established electrophoretic technique for isoenzyme fractionation, with only minor changes, the results indicate that normal levels previously established for infants may not be valid when hyperbilirubinemia is present.

MATERIALS AND METHODS

Source of Specimens

Blood from the fetal-maternal cord was obtained at the time of delivery by one of the staff obstetricians. The cord was cut and a 10-ml. sample collected from the fetal side. All specimens were centrifuged at 2000 rpm for 10 minutes when received and the resultant serum removed and separated into aliquots of 0.5 to 1.0 ml. each. The labelled samples were placed in the refrigerator (4 to 6 C.) until electrophoresis was carried out.

Total Alkaline Phosphatase

Method. Dilute 1 drop (0.04 ml.) of substrate concentrate with 1.0 ml. of water and warm to 37 C. Add 0.1 ml. of serum and mix. After exactly 20 minutes add 5.0 ml. of color stabilizer and read the absorbance at 550 nm against a reagent blank without serum. Determine the alkaline phosphatase activity from the standard curve.

Standardization. Dilute 1.0 ml. of the stock phenolphthalein standard to 50.0 ml. with water in a volumetric flask. To 1, 2, 3, and 4 ml. of working standard (0.05 μ mole/ml.) and 5.14, 4.14, 3.14, and 2.14 ml. of color stabilizer, respectively, mix and read the absorbance at 550 nm. Results are expressed in International units. The unit is defined as the amount of enzyme that will liberate 1 μ mole of product in 1 minute. The above standards are equivalent to 25, 50, 75, and 100 U., respectively.

The precision of the total alkaline phosphatase method as reported by Babson *et al.* (1966) was 40.3 to 41.4 U., with a mean of 40.9 U. The coefficient of variation was 0.8%.

Alkaline Phosphatase Isoenzymes - Electrophoresis

Reagents and apparatus

1. Electrophoresis cell*
2. Power Supply (Beckman Duo-stat)**
3. Cellulose acetate strips, 1 x 6-1/2 in.*
4. Sample applicator (Beckman 1 in.)**
5. Barbitol buffer, pH 8.6, ionic strength 0.025

Method. Cold barbitol buffer (700 ml.) is poured into the cell chambers and the chambers equilibrated. Cellulose acetate strips are saturated with buffer in a small tray, removed, laid on blotter paper and blotted to near dryness.

Sample application. A 0.01-ml. pipet*** calibrated at 0.01, 0.008, and 0.006 is used to deliver 0.006 ml. of serum to the edge of the applicator, which is then brought into contact with the moist cellulose acetate strip resting on the blotter paper in a horizontal straight line about 1/2 in. cathodic to the center of the strip. Instant absorption occurs by capillary action in a narrow band of serum across the strip. The strips are

* Gelman Instrument Co., Ann Arbor, Michigan.

** Beckman Instruments, Palo Alto, California.

*** Spinco Division, Beckman Instruments, Palo Alto, California.

placed into the cell, and the magnetic rubber gripping devices are then attached, which act as a tension device holding the strips in place. The cell cover is replaced and a voltage of 17.5 v/cm. (of cellulose acetate strip length) is applied for 40 min. at 4 to 6 C.--conveniently in a refrigerator. [Note: This experiment was conducted by placing the Gelman cell on a crushed ice bath.]

Detection

Reagents

1. Buffered phenolphthalein monophosphate substrate
(see Appendix)
2. Color stabilizer

Method. Dilute 0.2 ml. of the phenolphthalein monophosphate substrate concentration with 2.0 ml. of water. This is sufficient quantity to saturate 3 cellulose acetate strips. Pour the diluted substrate into a shallow tray and immerse 3 clean strips into the solution. Using forceps, remove the strips from the tray, allowing excess solution to drip off, and lay them on a clean, flat, glass plate approximately 10 x 5 in. For conservation of cellulose acetate strips, the strips may be cut in half and placed on a 1 x 3 in. glass hematology slide. Disconnect the current to the cell and remove the strips containing the separated specimens. With care to avoid any air pockets from forming, superimpose them onto the strips containing the substrate. Place the glass plate or slides containing the specimens into a moist atmosphere, such as a large plastic box containing a few milliliters of water. Incubate the specimens in a 37 C. bacteriologic incubator for 2 hours.

Elution of activity. The cellulose acetate strips are removed from the incubator and the isoenzyme bands of activity, which may or may not be completely visible, are cut at 1 cm. intervals from the origin to the marker band of albumin-bound bilirubin. The segments are placed into 13 x 100 mm. tubes to which 3.0 ml. of the color stabilizer has been added. Mix the tubes either by inversion for several minutes or by using a glass rod, until all the visible color has been eluted from the strip. A blank is prepared by cutting a segment of the strip free of enzyme activity and placing it into 3.0 ml. color stabilizer. The alkaline phosphatase activity is read in 12 x 75 mm. cuvettes on a spectrophotometer at 550 nm against the reagent blank. Conversion of the %T (transmission) readings to O.D. (optical density) was made and the results were read from a curve prepared under similar conditions. A semiquantitative estimate of the percentage of enzyme activity attributed to each isoenzyme can be calculated by the following equation:

$$\frac{\text{absorbance of isoenzyme-reagent blank} \times 100}{\text{total absorbance-reagent blank}} = \% \text{ activity}$$

Standard curve. A standard curve was prepared for use in this experiment. Dilutions and combinations of Versatol E and EN^{*} were prepared in the following manner:

* Commercial control sera from General Diagnostics Division, Warner-Chilcotte, Morris Plains, New Jersey.

<u>Combination</u>	<u>Unit Value</u>	<u>O.D.</u>
1. Ver. EN	5.8	0.029
2. E + EN + 1 vol. Saline	8.5	0.041
3. (3E + 1 EN) + 1 vol. Saline	11.3	0.061
4. 1 E + 1 saline	14.1	0.066
5. E + EN	17.1	0.071
6. 3E + 1 EN	22.7	0.076
7. Ver. E	28.3	0.089

EN = 5.8 International units (normal)

E = 28.3 International units (abnormal)

The curve was plotted to a straight line and utilized in the calculation of the alkaline phosphatase units described in this thesis. The dilutions prepared were subjected to electrophoresis in the same manner as the serums and then eluted to obtain the optical density readings listed.

Experiment

Prior to the electrophoretic analysis of the cord bloods, several parameters were tested. Initially, storage time and temperature were determined. Sera from 5 patients were analyzed by a manual method for determining alkaline phosphatase activity. Aliquots were prepared, one set frozen at -20 C. and the second set stored at 4 C. Daily for 5 days, 1 aliquot from each thermal store was analyzed for total alkaline phosphatase activity. No appreciable difference was detected in the total values over the 5-day period. However, when electrophoresis was carried out on each daily sample, results obtained after the third day showed decreasing values in the isoenzyme fractions of the bone and liver. The use of fresh sera for isoenzyme fraction was considered an important factor for the data collection to be relevant.

Electrophoresis was then carried out on sera from patients with elevated levels of alkaline phosphatase, predetermined from manual testing to be either osseous or hepatic in origin. Following electrophoretic analysis, elution of the isoenzyme activity was compared to the values ascertained from the manual thermolabile technique. Mixtures of sera from patients with metastatic bone tumors and obstructive jaundice were analyzed electrophoretically, the 2 isoenzyme bands were eluted and again compared to manual techniques. The results were comparable once the operation of the electrophoretic equipment was mastered.

The length of time for optimal migration of the isoenzyme activity was determined by the staining of several different cellulose acetate strips for protein. Protein migration was well defined at 40 minutes of electrophoresis time and this time was used throughout the experiment. The hepatic and osseous alkaline phosphatase isoenzyme bands were well defined with little or no visible overlapping at 40 minutes when the 2 fractions were present in mixed sera.

The temperature of the barbital buffer during electrophoresis was a critical factor, as was the number of times the buffer was charged. It was found that a 2 C. rise in the buffer during electrophoresis destroyed a considerable amount of the activity of the osseous fraction as well as causing burning effects on the cellulose acetate strip itself.

Romel *et al.* (1968) suggest refrigeration of the cell but as this was not feasible in our situation, an alternative was constructed. A styrofoam box, with 1-in. walls and 3/4 in. cover was cut down so that the Gelman cell would fit snugly inside. Space beneath the cell, about 4 in., was filled with crushed ice. This device kept the precooled buffer at 4 C. to 1 C. throughout the period of electrophoretic analysis.

The temperature was checked before and after each analysis until known to be stable, thereafter checked periodically.

The number of times the buffer could be charged was determined by repeated use of the same serum. At each new application of the current, the electrical terminals were reversed so migration was - to +, + to -, etc. After 2 charges, it was noted that the buffer failed to conduct adequately, resulting in a poor migration pattern. This experiment was conducted by using the same buffer twice only. Fresh buffer was used at the beginning of each day analysis was carried out.

Refrigeration of the Gelman cell for a period of one-half hour before use made it possible to begin electrophoresis 5 minutes after the cell had been transferred to the "cooling box" and the cold buffer had equilibrated.

It should be noted that bilirubin bound to albumin serves as a good migration marker, and, as such, was a check on electrophoresis time. It was found that this marker band had migrated 3.5 to 4.0 cm. from the point of origin after 40 minutes. Extremely high bilirubin levels did cause drag of the marker band but generally created no problems in the elution method. However, if the marker drag is greater than 0.5 cm., complete elution of the hepatic fraction is difficult.

Serum obtained from submitted cord samples were separated into 3 aliquots of 0.5 to 1.0 ml. (when blood was obtained in microcapillary tubes, 0.1 ml. of serum was aliquoted). Fresh serum was subjected to electrophoresis initially. A 0.006 ml. sample was placed on the cellulose acetate strip as previously described and electrophoresis was carried out for 40 min. in the Gelman cell. A standard electrophoresis dye was used with each set of sera processed to check the electrode function and direction of migration.

It was found that the substrate could be adequately applied to a 1 x 3 in. cellulose acetate strip for the incubation phase (cut a regular strip in half). The substrate-soaked strips were then placed on a 1 x 3 in. laboratory glass slide. The processed strip was then cut 1 cm. cathodic to the origin placement and 2 cm. anodic to the marker band and this was superimposed on the 1 x 3 in. substrate strip. These strips were then placed in a 37 C. bacteriological incubator for 2 hours.

During incubation of the first samples, one of the aliquots of the original specimen was inactivated for 15 minutes at 56 C., followed immediately by immersion in an ice bath. After cooling, this sample was subjected to electrophoresis by the established procedure. The electrical terminals were reversed and electrophoretic analysis was carried out for 40 min.

During incubation of the second sample, a third aliquot was inactivated for 30 min. at 56 C. and processed in the manner just described.

Following the 2-hour incubation phases, each set of strips was cut at 1-cm. intervals from the origin to the marker band and elution was carried out in a 16 x 100 mm. tube containing 3.0 ml. color stabilizer. The blank was prepared as previously described. The results of elution were read at 550 nm. on a Coleman Junior II spectrophotometer using 12 x 75 mm. cuvettes. The elution of the total color generally took about 15 minutes. The %T readings were converted to O.D. values and the alkaline phosphatase units read directly from the standard curve.

The second and third aliquots were cut at 1-cm. intervals only for convenience of elution as only a total alkaline phosphatase value was recorded.

The cut segments of the original, fresh sample represent the 3 isoenzyme fractions in this manner:

0-1 cm.	placental fraction
1-2 cm.	osseous fraction
2-3 cm.	hepatic fraction

RESULTS

The results obtained are listed on the following tables.

Table 1. Electrophoretic analysis of alkaline phosphatase isoenzymes in "normal" infants (results in International units)

Specimen	Total Alkaline Phosphatase	Liver (unstable) 30' 56°	Bone (unstable) 15' 56°	Placental (stable)
1	35.5	2.3	31.4	1.7
2	28.8	2.1	26.1	0.8
3	29.1	1.5	25.2	1.7
4	24.3	0.8	22.2	0.8
5	42.2	2.2	38.5	1.9
6	37.5	1.9	35.2	0.2
7	29.3	1.2	27.2	2.0
8	43.6	1.6	38.0	2.5
9	34.7	2.3	29.9	1.9
10	41.0	2.0	37.0	2.0
11	39.3	1.3	35.2	2.9
12	34.0	1.2	30.0	1.2
13	42.6	2.2	38.0	2.0
14	35.7	2.3	32.4	1.9
15	36.2	1.0	33.6	1.8
16	28.4	1.3	25.3	1.9
17	33.8	1.1	30.6	1.9
18	37.7	1.3	33.8	2.3

Table 1 (cont'd.)

Specimen	Total Alkaline Phosphatase	Liver (unstable)		Bone (unstable)		Placental (stable)
		30'	56°	15'	56°	
19	31.2	0.9		28.4		1.7
20	42.1	2.5		38.6		1.2
21	39.3	1.5		34.3		4.1
22	36.7	1.7		33.5		1.5
23	29.6	1.2		27.2		1.4
24	41.9	2.2		36.9		2.6
25	36.3	0.9		33.6		1.8
26	33.5	1.5		29.8		2.1

Table 2. Electrophoretic analysis of alkaline phosphatase isoenzymes in infants with an ABO incompatibility (results in International units)

Specimen	Total Alkaline Phosphatase	Liver	Bone	Placental
1	16.8	2.3	13.1	1.4
2	26.2	4.4	21.0	0.8
3	33.8	2.7	28.2	1.9
4	26.1	1.9	21.5	2.8
5	25.6	1.8	22.0	1.9
6	29.1	2.4	24.9	1.8
7	27.5	1.9	23.8	1.8
8	21.6	1.2	17.6	2.8
9	18.9	2.5	16.0	0.4
10	24.3	0.5	22.2	1.6
11	26.1	1.8	21.9	2.4
12	25.6	1.8	22.0	1.8
13	28.8	1.6	25.4	1.8
14	24.4	1.3	21.6	1.5
15	24.6	1.8	22.0	0.8
16	24.3	0.5	22.2	1.6
17	19.6	0.9	19.6	4.0

Table 3. Electrophoretic analysis of alkaline phosphatase isoenzymes in infants with an Rh incompatibility (results in International units)

Specimen	Total Alkaline Phosphatase	Liver	Bone	Placental
1	28.6	3.9	22.8	1.9
2	41.9	2.2	35.9	3.8
3	19.1	0.2	13.3	5.6
4	18.8	0.6	16.8	1.4
5	15.6	0.8	10.9	3.9

Table 4. Tabulation of the mean and standard deviation (S.D.) of alkaline phosphatase fractions in "normal" and "abnormal" newborns

	Total Alkaline Phosphatase	Liver	Bone	Placental
<u>Normal cases: N = 26</u>				
Mean	35.5	1.7	32.0	1.8
S.D.	5.2	0.6	4.6	0.6
Coef. Var.	14.6	40.0	14.6	37.4
<u>ABO Incompatibilities: N = 17</u>				
Mean	24.9	1.8	21.4	1.8
S.D.	4.0	0.9	3.5	0.8
Coef. Var.	16.4	49.9	16.4	46.3
<u>Rh Incompatibilities: N = 5</u>				
Mean	24.8	1.5	19.9	3.3
S.D.	10.7	1.5	9.9	1.6
Coef. Var.	43.2	98.6	50.0	51.0

Note: Mean and S.D. expressed in International units.

Table 5. Mean values obtained for "normal" and "abnormal" newborns and the results of the application of the t-test for unpaired data

Fraction	Normal	Abnormal
Liver	1.7	1.7
Bone	32.0	21.1
Placenta	1.8	2.1
Total	35.5	25.0
<u>t-test</u>		
Liver	t = 0.41	P = <50 >25%
Bone	t = 7.9	P = 0.1%
Placenta	t = 0.41	P = <50 >25%
Total	t = 7.13	P = 0.1%

Note: Fractional values expressed in International units.

DISCUSSION

The alkaline phosphatase levels in jaundiced infants are consistently either at the low end of the normal range or below. This decrease is due almost entirely to a low concentration of the isoenzyme fraction derived from bone. Since there is no reason to believe that there is stimulation of hepatic excretory processes by jaundice, it would appear that bilirubin in some way inhibits the activity of osteoblasts in the formation of alkaline phosphatase, or that hyperbilirubinemia and decreased phosphatase levels have a common cause. Consider, then, the possibility that osteoblasts possess surface antigens similar or identical to those present on erythrocytes and that, when there is sufficient antibody derived from the maternal side to destroy many erythrocytes, a significant number of osteoblasts are also damaged. Undoubtedly the quantitative relationships between the concentrations of various specific protein types are important in this concept. Thus, we must picture the osteoblasts aligned on spicules of osteoid but separated from the plasma compartment by interstitial space, extracellular fluid, and the endothelial lining of the capillary wall (Figure 1). If detectable amounts of phosphatase arising from osteoblasts are present in plasma, the concentration of this protein immediately adjacent to the osteoblasts must be relatively high. However, the fact that detectable levels of enzyme do appear in the plasma establishes the ability of proteins to pass from osteoblasts to the plasma compartment. With a steady influx of antibody protein into the fetal plasma from the maternal circulation, inevitably some of this protein, as well as complement,

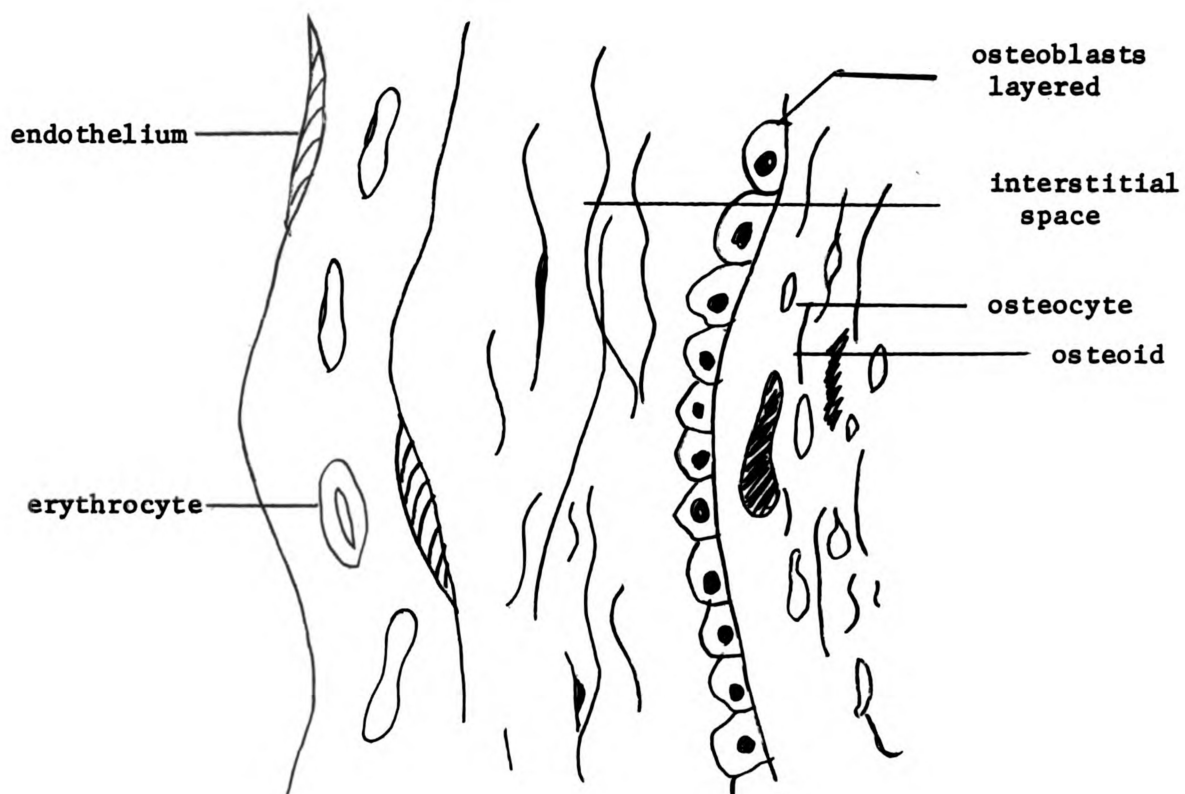


Figure 1. A diagram of the proposed site of action of the maternal antibodies on the neonatal osteoblasts.

must reach the osteoblasts in sufficient concentration to interfere with the production of alkaline phosphatase.

During the analysis of the data, it was found that the liver fraction of alkaline phosphatase was unchanged when the 2 groups were compared. This finding was not expected, since the project was designed to specifically look at the enzyme maturation of alkaline phosphatase in the liver. As is stated above, the unexpected findings in the osseous fraction changed the direction of the conclusions that were drawn. One can conclude, however, that the effect of hyperbilirubinemia in the neonate is unrelated to maturation of the liver alkaline phosphatase isoenzyme.

The statistics noted for placental alkaline phosphatase were not a primary consideration at this time, but were calculated to complete the data.

SUMMARY AND CONCLUSIONS

Measurements of serum isoenzyme levels of alkaline phosphatase were made on 2 groups of jaundiced infants: (1) those with transient "physiological" jaundice and (2) those with jaundice due to fetal-maternal incompatibility leading to hemolysis. Assuming that relatively few of those with physiologic jaundice would manifest some degree of immaturity of the hepatic excretory enzyme systems and that most of those with hemolytic jaundice would show varying degrees of the same immaturity, the experiment was designed to reveal any association of immature enzyme systems, glucuronyl transferase and phosphatase in this instance.

The data reveal no associated decrease in the hepatic fraction of phosphatase enzymes. However, an unexpected decrease in the osseous fraction of alkaline phosphatase was encountered in the infants with hemolysis. The hypothesis is offered that osteoblasts have surface antigens in common with erythrocytes and that sufficient complement and hemolytic antibodies reach the osteoblasts to produce an appreciable drop in their output of alkaline phosphatase.

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APPENDIX

APPENDIX

Total Alkaline Phosphatase Reagents

1. Buffered substrate concentrate: Dissolve 65 mM phenolphthalein monophosphate in 7.8 M 2-amino-2-methylpropanol buffer at pH 10.15. The concentrate is stable under refrigeration and should be warmed to room temperature before use.
2. Color stabilizer: Disodium hydrogen phosphate-trisodium phosphate, 0.1 M, at pH 11.2.
3. Phenolphthalein stock standard, 2.5 mM: Dissolve 79.6 mg. of phenolphthalein in 50 ml. of alcohol in a 100-ml. volumetric flask and dilute to 100-ml. with water. The solution is stable at room temperature.
4. Barbitol buffer: Dissolve 5.12 gms. sodium barbitol and 0.92 gms. of barbituric acid in a 1-liter volumetric flask. Dilute to 1 liter.

Note: Barbitol buffer, color stabilizer and the substrate can be purchased in pre-pack form from General Diagnostics-Warner Chilcotte, Morris Plains, New Jersey.

Conversion of International Units of Alkaline Phosphatase to:

1. Bodansky units - multiply I.U. by 0.12.
2. King-Armstrong units - multiply I.U. by 0.36.
3. Shinowara-Jones-Reinhart units - multiply I.U. by 0.18.

Heat Stability:

1. Osseous fraction is destroyed by exposure to 56 C. for 15 minutes.
2. Liver fraction is destroyed by exposure to 56 C. for 30 minutes.
3. Placental fraction stable.

VITA

The author was born in Parnell, Michigan, on April 15, 1938. She lived there until graduation from St. Patrick's High School in 1956. In September of that year, she entered Mercy College of Detroit, on a Silverman Honors Scholarship, graduating with a B.S. degree in 1960 after completion of a 12-month internship for Medical Technologists at Mt. Carmel Mercy Hospital, Detroit. Later that same year, she became certified with the Registry of Medical Technologists of the American Society of Clinical Pathologists.

In June 1960 she began working at Ferguson-Droste-Ferguson Hospital and Proctology Clinic in Grand Rapids, Michigan. She remained there for 5 years, 3 years of which she was Chief Technologist of the laboratory.

In July 1965 she moved to Ann Arbor, Michigan, where she worked as supervisor of hematology, later becoming supervisor of the Blood Bank at St. Joseph Mercy Hospital.

In September 1966 she entered the AABB approved school at Michigan Community Blood Center in Detroit, Michigan, for a year of training in Blood Banking. She was certified by AABB and the American Society of Clinical Pathologists in April 1968.

She was employed at Edward W. Sparrow Hospital, Lansing, Michigan, in September 1967, as Supervisor of Blood Bank and Immunoserology, the position she currently holds.

She entered Michigan State University in September 1968 in the Clinical Laboratory Science program, Department of Pathology. She worked

full time at E. W. Sparrow Hospital during the graduate program.

The author is an active member of the Lansing area, Michigan, and American Society of Medical Technologists, and the Michigan and American Association of Blood Banks.

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