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THE ISOENZYMES OF SERUM ALKALINE
PHOSPHATASE IN CATS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

John W. Kramer

1968



This is to certify that the
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of the requirements for

Ph D _____ degree in _____ Education

A handwritten signature in black ink, appearing to read "Samuel S. Corl, III". The signature is fluid and cursive, with a large initial "S" and "C".

Major professor
Samuel S. Corl, III

Date August 4, 1976

ABSTRACT

THE ISOENZYMES OF SERUM ALKALINE
PHOSPHATASE IN CATS

by John W. Kramer

Total serum alkaline phosphatase and serum zymograms of alkaline phosphatase were obtained from normal mature cats and kittens and from kittens with experimentally produced biliary obstruction. Mean total serum alkaline phosphatase was $1.61 \pm 1.9^*$ Sigma units for 12 normal kittens and $0.82 \pm 0.678^*$ Sigma units for 10 normal mature cats. Only a beta-1 globulin alkaline phosphatase was located in serum zymograms of normal mature cats and kittens.

There was no increase in total serum alkaline phosphatase in 2 kittens with ligated common bile ducts. Serum alkaline phosphatase zymograms demonstrated beta-1 globulin and alpha-3 globulin alkaline phosphatases in both kittens within 14 days of ligation.

* One standard deviation

THE ISOENZYMES OF SERUM ALKALINE
PHOSPHATASE IN CATS

By
John W. Kramer

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INTRODUCTION

Enzymes with the same catalytic properties and found within the same species are termed isoenzymes. Introduction of the term isoenzyme is probably a reflection of our advances in enzyme characterization and, as advancement continues, systematic names will probably be applied where numbers are now used.

Isoenzymes have been introduced as a means of monitoring disease conditions in both research and clinical medicine. Frequently changes in permeability of cell membranes are demonstrable by changes in serum enzyme activity before clinical signs are apparent or changes are detectable by means of the light microscope.

Isoenzymes of serum alkaline phosphatase have been identified in many species and are presently used in human medicine as a means of increasing specificity of diagnosis in cases of osteogenic and hepatic diseases.

Cats appear to be the only animals in which biliary obstruction does not result in great elevations of total serum alkaline phosphatase activity. The reason for this is unknown. The purposes of this study were to:

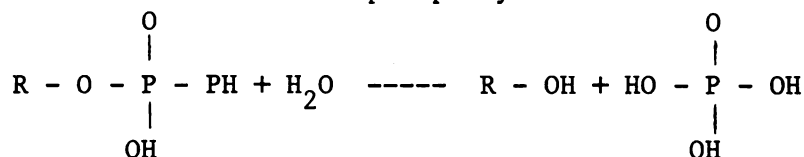
1. Establish normal total serum alkaline phosphatase activity of cats.
2. Characterize isoenzymes of serum alkaline phosphatase of cats by means of electrophoresis.
3. Study the relationship of serum alkaline phosphatase to hepatic biliary obstruction.

LITERATURE REVIEW

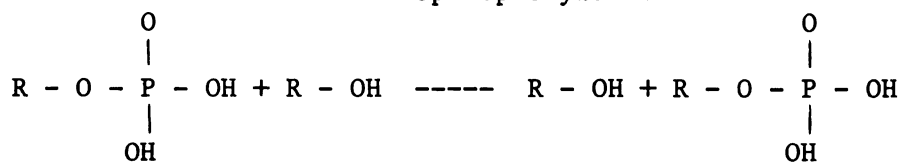
Chemistry

Alkaline phosphatase is a phosphoric monoester hydrolase of animals which demonstrates little or no specificity as to the radical from which it hydrolyzes the phosphate. Its systemic name is orthophosphoric monoester phosphohydrolase and it is capable of catalyzing transphosphorylation as well as dephosphorylation (Enzyme Nomenclature, 1964). Chemical reactions catalyzed are as follows:

Dephosphorylation



Transphosphorylation



As Pearse (1961) has pointed out, the term alkaline in this enzyme's name refers to the optimum pH at which it functions in vitro, not in vivo. Because optimum pH depends on substrate and substrate concentration, it is not possible to define clearly the true optimum pH for this enzyme until the substrates on which it acts in vivo are known.

What was formerly referred to simply as serum alkaline phosphatase is in fact a group of enzymes with similar activity. This will be documented as the review proceeds. The International Union of

Biochemists Committee on Enzymes defines isoenzymes as multiple enzyme forms having the same activity in a single species (Webb, 1964). Thus, it would appear that alkaline phosphatase can be demonstrated in more than one form. However, Wilkinson (1962) stated that because of relative nonspecificity and numerous sources of this enzyme it would be better to regard it as a family of enzymes until a clearer definition can be made.

Early realization that serum alkaline phosphatase is of significance in clinical medicine led to development and use of a number of quantitative tests suitable for use in clinical laboratories (Table 1). These clinical tests differ primarily in the substrate used and, because of this difference, it is difficult to compare values obtained by one method to those obtained by another (Tietz et al., 1967). However, it does become necessary at times to make a comparison between values because of the limited amount of work done in this field (Table 2).

Histochemistry

Gomori (1939) and Takamatsu (1939) independently developed a technique for the demonstration of alkaline phosphatase in tissues. In this technique a calcium salt of the freed phosphate is produced which, and in turn, reacts with silver or cobalt salt at the site of enzyme activity. The resulting silver or cobalt phosphate is then changed to the free metallic form which can be visualized. Since then numerous other substrates have been introduced for this same purpose (Pearse, 1961). Phenyl and naphthyl phosphates are now the more common substrates in use.

Histochemical studies of alkaline phosphatase activity have been carried out with tissues of the cat (Gomori, 1941; Kritzler and Beaubien, 1949; Smith and Freeman, 1954; Wachstein, 1955; Martin, 1951).

Gomori (1941) found alkaline phosphatase activity in cats' pre-capillary and capillary blood vessels; also spleen, thymus, lymph nodes, lingual epithelium, stomach, small intestine, colon, liver, pancreas, bronchi, lung, urinary bladder, testis, epididymis, prostate, ovary, uterus, brain, and adrenal. Gomori (1941), Kritzler and Beaubien (1949), Smith and Freeman (1954) and Wachstein (1955) reported activity in the glomeruli of the normal cat. These same authors did not find activity in the glomeruli of the normal dog, human, monkey, rabbit, guinea pig, rat, gopher, hog, and groundhog.

Smith and Freeman (1954) observed an increase in cytoplasmic lipids of the renal tubules in the mature cat as contrasted to the kitten and half-grown cat. They suggested that alkaline phosphatase activity in these cells was in direct proportion to the lipid material. These same workers and Wachstein (1955) reported activity in association with the brush borders of proximal convoluted tubules. This may be an indication of a part played by the enzyme in the active transfer of molecules across membranes (Pearse, 1961).

Clinical Pathology

Robison (1923) reported alkaline phosphatase in bone which led to his theory concerning the calcification of bony tissue. Gutman (1959) reviewed the work carried out by numerous authors who have associated high serum alkaline phosphatase activity with disease conditions of man. The earliest reports following Robison's (1923) dealt with conditions

which were osteogenic. Later, Roberts (1930) reported raised levels associated with obstructions of the biliary tract and, to a lesser degree, in hepatocellular disease. Elevated levels of serum alkaline phosphatase were of significant use in preclinical diagnosis of osteogenic conditions and early biliary obstruction, as increases in activity occurred prior to a bilirubinemia. However, the need for a means of early differentiation of these two primary diseases remains.

Source of Serum Alkaline Phosphatase

Armstrong and Banting (1935) found detectable alkaline phosphatase activity in many normal canine tissue homogenates. By removing the spleen, kidney and intestine they were unable to demonstrate a decrease in serum activity. They concluded that these organs were not the source of an appreciable amount of normal serum activity. Roberts (1930) established that bile has alkaline phosphatase activity and postulated that this is the route of elimination in man. Armstrong and Banting (1935) ligated the common bile duct of the dog and produced increases in serum alkaline phosphatase activity. This rise could be reversed by eliminating the ligature.

Cats were used for similar studies by Cantarow et al. (1936), Flood et al. (1937), Thannhauser et al. (1937), Dalgaard (1948), and Carlsten et al. (1961). These workers were only able to produce slight or moderate initial rises of serum enzyme activity in cats with ligated common bile ducts. The resulting rise in most cases did not exceed what was considered as normal. Thannhauser et al. (1937) and Flood et al. (1937) ligated the ureters as well as the common bile duct, and this resulted in an increased total serum alkaline phosphatase.

Thannhauser et al. (1937) reported that when ascorbic acid was added to serum from cats with ligated common bile ducts in concentration of 5 mg./ml., total alkaline phosphatase activity of the serum was increased to levels as high as those found in dogs under similar conditions. They suggested that the reason for low levels of serum activity in cats with ligated common bile ducts was not because of the absence of the enzyme but rather absence of an activator, such as ascorbic acid.

Flood et al. (1937) examined urine as well as serum from 12 cats with ligated common bile ducts and found 8 which had transitory increases in urine alkaline phosphatase activity. Only 5 of the 8 exceeded the preoperative normal level.

A number of authors have determined normal levels of total serum alkaline phosphatase of cats (Table 3).

Isoenzymes

Bodansky (1937a) reported results of work dealing with the relationship of bile salts to alkaline phosphatase activity of bone, kidney, and intestine. He concluded that bile salts inhibit the enzyme activity in bone and kidney but not that in intestine. This was probably the first recognition that alkaline phosphatase is not a single enzyme. Thannhauser et al. (1937), Cloetens (1939) and Bodansky (1948) studied the effect of different amino acids and organic and inorganic salts on alkaline phosphatase obtained from different tissues (Table 4). The substrate used was beta-glycerolphosphate.

It has become possible to separate alkaline phosphatase from other tissue components in a number of ways.

On a basis of solubility in nonionic solvents such as ethanol or butanol, the enzyme has been separated from the tissues of dog and man (Norton, 1950; Schlamowitz, 1954a,b; Schlamowitz and Bodansky, 1959; Ahmed and King, 1959; Moss, 1962; and Peacock et al., 1963).

Zone electrophoresis has been carried out on and in such media as paper (Taleisnik et al., 1953; Baker and Pellegrino, 1954), agar gel (Haije and de Jong, 1963; Stevenson, 1961) and starch gel (Hunter and Markert, 1957; Kowlessar et al., 1959; Rosenberg, 1959; Estborn, 1959; Lawrence et al., 1960; Dubbs et al., 1960; Boyer et al., 1961; Kowlessar et al., 1961; Paul and Fottrell, 1961; Moss et al., 1961; Moss and King, 1962; Moss, 1962; Robinson and Pierce, 1964). Specimens most commonly used were serums of man with activity reportedly located in association with alpha-2 and beta-1 globulins. Boyer (1961) reported the location of 16 bands of activity in serums of man, all of which were not present in any one specimen. Stevenson (1961) worked with normal canine serums, as did Lawrence et al. (1960), who, in addition, examined the serums of man, monkey, rabbit, guinea pig, rat, and mouse. Paul and Fottrell (1961) noted a similarity of zymogram patterns in serums from mice, perch, guinea pig, rat, frog, and pigeon.

Separation has also been carried out with a chromatographic technique (Fahey et al., 1958) and by ultracentrifugation (Ahmed and King, 1959).

Once the enzyme was isolated it was characterized in a number of ways. Schlamowitz (1954a,b) and Schlamowitz and Bodansky (1959) produced antiserums in rabbits against alkaline phosphatase of canine intestine, human bone, and human intestine. By the use of these antiserums they demonstrated that bone was the source of about 40 to 59%

of the normal human serum alkaline phosphatase activity.

Some of the work carried out previously on serum and tissue homogenates was repeated on individual fractions. Ahmed et al. (1959) demonstrated that ions of Mg and Co were activators and Zn was an inhibitor of alkaline phosphatase activity of alpha-2 globulin of human placenta, bovine kidney, and dog intestine. They reported that amino acids gave variable results as effectors of alkaline phosphatase in these same tissues.

Robinson and Pierce (1964) established that neuraminidase slowed 3 out of 4 bands of alkaline phosphatase activity detected in serums of man by starch gel electrophoresis. The band unaffected by neuraminidase was inhibited by L-phenylalanine, which is known to inhibit intestinal alkaline phosphatase. They speculated that the differences in enzymes may be in the prosthetic portion.

Moss (1960a,b), Moss et al. (1961), and Moss (1964) introduced characterization of alkaline phosphatase isoenzymes by the spectrofluorometric technique. This technique monitors the reaction of the enzyme with the substrate. By introducing different effectors in the presence of certain substrates, the Michaelis constant is determined under different conditions for each of the isoenzymes.

A genetic association of certain isoenzymes with blood groups has also been demonstrated. Arfors et al. (1963), Beckman (1964) and Bamford et al. (1965) have established that number and location of zones of activity produced by zone electrophoresis of human serums were associated with blood groups. Gahne (1963) demonstrated similar patterns of activity between monozygous human twins. Randel et al. (1964) and Randel and Stormont (1964) reported an association between the blood groups of sheep and serum isoenzymes of alkaline phosphatase.

Isoenzymes in Disease

As had been previously recognized, serum alkaline phosphatase activity increased in diseases associated with bone and extra- and intra-hepatic biliary obstruction in man (Gutman, 1959). Keiding (1959) used starch block electrophoresis to study serums of persons with bone and hepatic disorders. He reported an association between hepatic disorders and an increase in serum enzyme activity in the alpha-2 and alpha-1 globulin positions. In instances of bone disease increases of activity were in beta-1 globulin position. Cooke and Zilva (1961) studied a patient with an unnamed neoplasm with no bone metastasis. Serum alkaline phosphatase activity was increased and the rise was associated with the alpha-1 and alpha-2 globulin. In man, Chiandussi et al. (1962) and Hodson et al. (1962) related hepatic disorders with an increase in serum alkaline phosphatase activity associated with faster-moving beta lipoprotein. In human bone diseases such as Paget's osteomalacia and parathyroid osteitis, increases in serum enzyme activity were associated with slower-moving beta globulin (Hodson et al., 1962).

Using agar gel electrophoresis, Haije and de Jong (1963) recorded three zones of alkaline phosphatase activity in serums of man. They located the increase in activity on the cathode side of alpha-2 globulin in serums from patients with Paget's disease and osteoplastic secondary bone tumors. In cases of secondary hepatic tumors, reversible hepatic cellular damage, and malignant reticulosis, the increases in serum enzyme activity were in alpha-2 and alpha-1 globulin.

Other workers have reported results similar to those listed above for serums of man (Latner and Hodson, 1962; Nordeloft Jensen, 1964;

and Newton, 1967). In summary they were:

- a. alpha-2 and alpha-1 globulin -- diseases with hepato-biliary obstruction
- b. beta-1 globulin -- bone diseases

Pulvertaft and Luffman (1967) examined serums of persons who had had partial gastrectomy. They found that the increase in serum alkaline phosphatase level in these patients was probably not intestinal in origin.

Review of Studies Concerned with Alkaline Phosphatase of Cats

Since the work of Cantarow et al. (1935), Flood et al. (1937), and Thannhauser et al. (1937), little has been done to examine the cause of the lack of a sharp increase of serum alkaline phosphatase activity in cats with experimentally produced biliary stasis.

Thannhauser et al. (1937) put forward the concept that apparent increases in serum alkaline phosphatase activity following biliary stasis may be due to the increase of an activator and not to an increase in enzyme concentration. They found that the addition of ascorbic acid to serums of cats with complete biliary stasis increased activity to a level comparable to that of dogs with similar conditions.

Dalgaard (1948) ligated the common bile duct of 10 cats and examined their serums for activity. He described results similar to the earlier works but suggested that it is a mistake to regard the increase in activity as negligible or slight in view of low normal activity. Both he and Flood et al. (1937) had also examined the enzyme's activity in urine and found some increases, although not in all cases of biliary stasis. Flood et al. (1937) suggested urine as a pathway of elimination; however, Dalgaard (1948) nephrectomized cats which had ligated common

bile ducts and no appreciable increases in serum enzyme activity occurred. Dalgaard (1948) reported urea as an inhibitor of alkaline phosphatase activity. The resulting accumulation of urea following the nephrectomy may have suppressed enzyme activity. Dalgaard (1948) reported a difference in total serum alkaline phosphatase levels between young and adult normal cats. Higher levels were reported in cats less than 11 months of age than in adults. Pregnant cats at term also had higher levels.

Carlsten et al. (1961) found a moderate increase in enzyme activity in lymph of the biliary lymphatics of cats with biliary obstruction. They recorded a high level of activity in serum from the hepatic vein in cats with biliary obstruction.

Gibson (1952) reported the death of a 6-year-old cat as the result of cholelithiasis. The calculi consisted of calcium salts of bilirubin.

MATERIALS AND METHODS

Specimens

Serum and whole blood samples were collected from 4 male and 6 female mature cats and from 7 male and 5 female kittens. Male cats with undescended testicles were regarded as kittens and their female litter mates were also regarded as kittens. At the time of sampling, kittens were estimated to be less than 4 months of age. One sample was obtained from each animal. All animals had at least one month's acclimatization prior to sampling.

Hematology

Blood samples were obtained by cardiac puncture from the kittens and jugular puncture from cats. Serums were collected by centrifugation from clotted samples. Three milliliters of whole blood was collected in vials containing 0.05 ml. of a 7.5% aqueous solution of the potassium salt of ethylenediaminetetraacetic acid. Complete blood counts (CBC) were done consisting of hemoglobin determination by the cyanmethemoglobin method, total leukocyte count with hemocytometer, packed cell volume percentage by the microhematocrit method, and differential leukocyte counts. Each determination was done in duplicate.

Serum Chemistries

The biuret reaction (Kolmer, 1959) was used to determine total serum protein.

Total serum alkaline phosphatase activity was determined by use of a commercially prepared kit.* The test incorporated p-nitrophenyl phosphate as a substrate. The substrate is colorless, but the product is yellow in an alkaline solution and has an optimum absorption of 400 m μ . The unknown serum sample was incubated for 30 minutes in a buffered solution of substrate. Following incubation the solution was diluted with 0.02N NaOH and the optical density was determined in a spectrophotometer at 410 m μ . The optical density was compared with a standard curve prepared by use of a known concentration of p-nitrophenol. The reason for using the wave length of 410 m μ and not the optimum of 400 m μ was to compensate for presence of hemoglobin. Hemoglobin has less absorption at 410 m μ than at 400 m μ and p-nitrophenol absorption is not appreciably different over this range (Sigma, 1963). A serum of known alkaline phosphatase activity was run as a control with each series.

Serum alkaline phosphatase activity was expressed in terms of Sigma units per ml. of serum. One Sigma unit will liberate 1 μ M of p-nitrophenol per 60 min. at 37 C. and pH 10.5.

Serum was stored at -20 C. after total serum protein and total serum alkaline phosphatase determinations were carried out.

Electrophoresis. The technique of Crawly and Eberhardt (1962) was used with some modification. The medium was changed from ionagar to agarose.** A 1% agarose gel was prepared in pH 8.6 Veronal buffer*** of 0.037 ionic

* Phosphatase, acid, alkaline, prostatic. Sigma Chemical Co., St. Louis, Mo.

** Agarose, Bausch and Lomb, Rochester, N.Y.

*** Half strength Buffer B-2, Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

strength. Five milliliters of the agarose gel was pipetted onto a 16-cm.-long strip of 35-mm. unperforated plain photographic film leader^{*} and allowed to set. A trench 3 cm. x 0.5 cm. was cut into the gel midway along and across the strip.

Electrophoresis was carried out in a Spinco-Durum cell^{**} from which the paper support stand and wick supports were removed. A pH 8.6 Veronal buffer^{***} of 0.075 ionic strength was the electrolyte. For purposes of maintaining uniformity, the maximum number of strips the cell would hold (8) was always placed in it. With strips in place 0.1 ml. of serum was pipetted into the trench.

Electrophoresis was carried out for 60 minutes at 150 volts. Albumin migrates approximately 5 cm. from the point of application under these conditions.

Each sample was fractionated at least twice, but never in the same cell at the same time.

Once electrophoresis was completed the strips were removed and cut in half lengthwise. One-half of the electrophoregram was used for protein staining and the other for a zymogram.

Staining procedure. Procion Brilliant Blue, M-RS[#] was used as a protein stain because of its high specificity and reduced background staining (Fazekas de St. Groth, 1962).

* DuPont P-40B Cronar, E.I., DuPont de Nemours Co., Cleveland, Ohio.

** Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

*** Buffer B-2, Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

Colab Laboratories, Chicago Heights, Ill.

Solution:

Conc. HCl	20 ml.
Absolute methanol	980 ml.
Procion Brilliant Blue M-RS	5 gm.

Strips were immersed in the staining solution for 5 to 15 minutes. Four 15-minute rinses in absolute methanol followed. One final 5-minute rinse in distilled water was carried out to insure complete removal of buffer salts in the agarose. Following rinses, the strips were air-dried.

Zymogram. A simple modification of the standard histochemical staining techniques (Pearse, 1961) was used for locating alkaline phosphatase activity on the electropherogram. One-half of the electropherogram not stained for protein was immersed in the substrate solution for 2 hours at 37 C. The period may be reduced when serum containing more than 3 Sigma units of activity is used. Following incubation the strips were rinsed in distilled water and immersed in a freshly prepared aqueous solution of the diazonium salt Fast Red Violet LB Salt* (5-benzamido-4-chloro-o-toluidine).

Substrate solution:

Na naphthyl AS-MX phosphate	100 mg.
Magnesium sulfate	602 mg.
Tris (Gomori) buffer pH 8.3	360 ml.
Dionized water	640 ml.

After staining, the strips were rinsed in distilled water, fixed in 70% methanol for 15 minutes and air-dried.

* Sigma Chemical Company, St. Louis, Mo.

Positioning Enzyme Activity

Once dry, electropherograms stained for protein were scanned by the use of a Spinco Analytrol* with the Scan-A-Tron* attachment. A slit width of 0.5 mm. and a B5 balancing cam were used. The absorption wave length for Procion Brilliant Blue is 602 m μ (Fazekas de St. Groth, 1962); however, because of the large amount of serum used and, in turn, the relatively great amount of stain present a 500 m μ interference filter was used to reduce sensitivity. The zymograms were also scanned under these same conditions; however, in some instances staining was too faint to record on the scanning device but was apparent to the eye. In such instances calipers were used to measure the distance from the point of application to the stained band. This distance was used to accurately position bands of enzyme activity on the zymogram in relation to areas of protein separation.

Standardization of the technique was carried out with human and dog serums.

Biliary Stasis

Two kittens between 3 and 4 months of age were anesthetized with sodium thiamylal.** By aseptic surgery a midline abdominal incision was made and the common bile duct located at the head of the pancreas. The duct was doubly ligated approximately 2 cm. above its entrance into the pancreas. The incision was closed and the cats made uneventful recoveries. Two preoperative serum and blood samples were taken.

* Spinco Division, Beckman Instrument Co., Belmont, Calif.

** Surital, Parke, Davis & Co., Ann Arbor, Michigan.

Serum samples were taken every second or third day postoperatively. Total serum alkaline phosphatase levels were determined on each sample. After 7 postoperative days total serum bilirubin was also determined (Kolmer et al., 1959). The kittens were necropsied 14 and 16 days postoperatively. Upon examination it was observed that the common bile duct of both kittens had been completely ligated.

RESULTS

Total mean serum alkaline phosphatase for 12 kittens was $1.61 \pm 1.9^*$ Sigma units and $0.82 \pm 0.678^*$ Sigma units for 10 cats. Higher levels of activity occurred in kittens than in mature cats ($P < .05$).^{**}

Alkaline phosphatase activity was detected on 18 of 22 zymograms. Samples in which no activity was detectable were from mature cats with low total enzyme activity. Only one band of activity was located in each sample. It was located near the peak of beta-1 globulin (Figure 1).

Electropherograms of 2 samples with well demarcated bands of enzyme activity were stained with Oil Red O for lipoprotein. The band of enzyme activity was located within the band of beta lipoprotein.

In some samples the band of enzyme activity was narrow and intensely stained. It appeared as a dense band in the beta globulin (Figure 1). This narrow band of activity was found in 8 out of 12 kittens and 1 out of 7 mature cats. The denser band was more common ($P < .05$)^{***} in serums of kittens than in serums of mature cats.

* One standard deviation.

** $T = 2.71$.

*** As determined by the Chi-square Test, Chi-square value of 4.91.

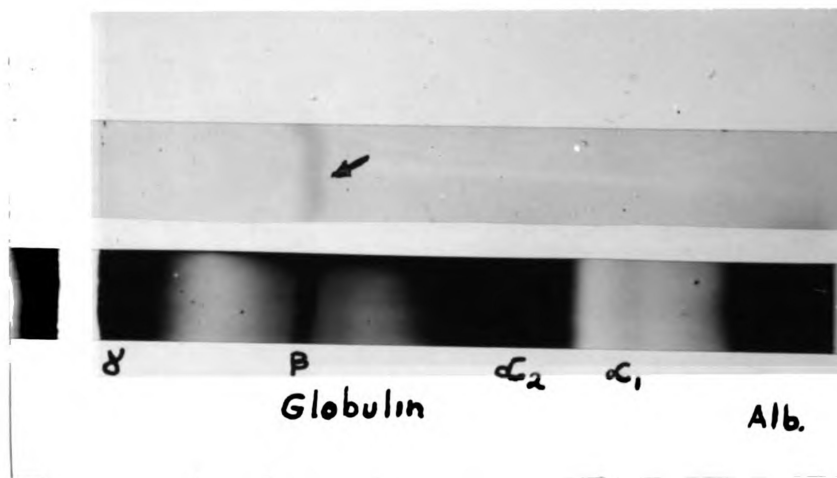


Figure 1. Electropherogram of normal cat serum. Upper strip is a zymogram of serum alkaline phosphatase (arrow: site of enzyme activity) and lower portion is stained for protein.

Zymograms from 2 normal and 1 abnormal human serums were also produced. In 2 of these, more than one band of enzyme activity was detected in the same positions as Haije and de Jong (1963) had reported when ion-agar was used.

Total leukocyte counts were relatively high in most cats. This was considered a physiological leukocytosis on the basis of the increase being due primarily to a lymphocytosis.

The total serum alkaline phosphatase levels of 2 kittens with ligated common bile ducts were unchanged. The range of activity was 1.0 to 2.0 units. Serum bilirubin values were 27.9 mg./100 ml. serum for kitten 101 and 18.9 mg./100 ml. serum for kitten 102, 14 days post-operatively. Zymograms of preoperative serums demonstrated a single band of activity in beta-1 globulin. Fourteen days postoperatively 2 bands were demonstrated in both cats' serums. One band was beta-1 globulin and the other in alpha-3 globulin.

DISCUSSION

Normal Cats

The single band of serum alkaline phosphatase in normal mature cats and kittens in beta-1-globulin suggests that one of the two isoenzymes located in normal human and dog serums is absent or at too low a level to detect by the method used here.

Proteins are known to exist in states of equilibrium in which a single protein may be capable of deconjugating or conjugating with another protein or nonprotein substance. The existing state is dependent on many factors, one of which is protein concentration.

Electrophoresis separates and concentrates proteins. This change in concentration provides an opportunity for protein to shift its state of equilibrium from one form to another. This change in form may result in alteration of mobility so that at low concentrations it may be found in one position and in higher concentrations another. Regardless of change, enzyme activity may or may not be altered.

Because cat serum has a low level of alkaline phosphatase, it was found necessary to use a larger sample size than that used by workers with other species' serums (Haije and de Jong, 1963; Stevenson, 1961; Yong, 1967). To compare the effect of the size of the serum sample, zymograms of human and dog serums were prepared with both large and small samples. No appreciable difference was detected. Greater sample size, then, is probably not the reason for a single band of activity in zymograms of normal cat serum.

The source of beta-1 globulin serum alkaline phosphatase of normal cats is speculative. Other workers using similar methods have also found activity in this same region in serums of normal children and puppies (Haije and de Jong, 1963; Stevenson, 1961; and Yong, 1967). They reported alkaline phosphatase from normal bone in beta-1 globulin and because of normally high bone growth in children and puppies suggested that beta-1 serum alkaline phosphatase is of bone origin. Beta-1 globulin serum alkaline phosphatase is seldom seen in adults except in cases of an active osteoblastic disease (note literature review).

Higher total serum alkaline phosphatase in kittens than in mature cats and no change in mobility as a result of maturity leads me to conclude that at least half of the enzyme activity found in beta-1 globulin of kittens has its origin in bone. While it is difficult to draw conclusions across species lines the work of Haije and de Jong (1963), Stevenson (1961), Yong (1967), and Paul and Tottrell (1961) demonstrated many similarities of alkaline phosphatase mobility between species.

Biliary Stasis

The reason is unclear for the appearance of a second band of enzyme activity without an increase in total enzyme activity in serums of cats with biliary stasis. However, as was noted at the beginning of this discussion, protein mobility may be altered by conjugation or deconjugation with other substances. In this case the other substances may be related to biliary obstruction. There is also the possibility that there is an absolute increase in alpha-3 globulin enzyme and a loss of some beta-1 globulin enzyme.

Alpha-3 globulin of cats corresponds in mobility to alpha-2 globulin of humans and dogs. It is alpha-2 globulin serum alkaline phosphatase

which is reported by Haije and de Jong (1963) and Yong (1967) as being increased in biliary obstruction in man. It is my personal observation that this is also true in dogs.

If again conclusions may be drawn across species lines, there is reason to believe that the alpha-3 globulin serum alkaline phosphatase may arise from liver. However, why there is not an increase in total serum enzyme activity is not clear.

SUMMARY

Total serum alkaline phosphatase and serum zymograms of alkaline phosphatase were obtained from normal mature cats and kittens and from kittens with experimentally produced biliary obstruction. Mean total serum alkaline phosphatase was $1.61 \pm 1.9^*$ Sigma units for 12 normal kittens and $0.82 \pm 0.678^*$ Sigma units for 10 normal mature cats. Only a beta-1 globulin alkaline phosphatase was located in serum zymograms of normal mature cats and kittens.

There was no increase in total serum alkaline phosphatase in 2 kittens with ligated common bile ducts. Serum alkaline phosphatase zymograms demonstrated beta-1 globulin and alpha-3 globulin alkaline phosphatases in both kittens within 14 days of ligation.

* One standard deviation.

Table 1. Clinical tests for total serum alkaline phosphatase

Originator	Substrate	Unit
Bodansky, O. (1937)	beta glycerolphosphate	1 mg.P/100 ml. serum/ 60'
King-Armstrong (1934)	phenylphosphate	1 mg. phenol/100 ml. serum/30'
Bessey-Lowry-Brock (1946)	p-nitrophenyl phosphate	1 μ M p-nitrophenol/ 100 ml. serum/30'
Sigma (1952)	p-nitrophenyl phosphate	1 μ M p-nitrophenol/ 1 ml. serum/60'

Table 2. Factors for the conversion of total serum alkaline phosphatase units

- a. Bessey-Lowry-Brock units to King-Armstrong: multiply
the former by 2.5 (Cornelius and Kaneko, 1963).
- b. Bodansky units to Bessey-Lowry-Brock units: multiply
the former by 1.8 (Gutman, 1959).

Table 3. Normal serum alkaline phosphatase activity in the cat

Author	No. of Specimens	Units
Flood <u>et al.</u> (1937)	21	0.6 - 5.0 Bodansky units/ 100 ml.
Cantarow <u>et al.</u> (1936)	45	0.95 - 3.84 Bodansky units/100 ml.
Dalgaard (1948)	24 (adult)	4.1 $\pm$ 1.7 King-Armstrong units/100 ml.
Bloom (1957)	10	0.0 - 7.1 (mean 3.35) Bodansky units/100 ml.
Bekemeier (1962)	7	3.4 (1.9 - 7.9) King- Armstrong units/100 ml.

Table 4. Effectors of alkaline phosphatase

Tissue	Inhibitor	Activators
Intestine, brain, kidney	high levels - L-alanine, L-glutamate, L-lysin, L-histidine and glycine KCN	The same as those which are inhibitors at high levels are activators at low levels. Mg
Serum	Zn, Cu	Fe, Mn, Co, Ni, Mg

Thannhauser et al. (1937)
 Cloetens (1937)
 Bodansky (1948)

BIBLIOGRAPHY

- Ahmed, Z., Abdul-Fadl, M. A. M., and King, E. J. 1959. The co-enzyme factor of alkaline phosphatase. *Biochem. Biophys., Acta*, 36: 228-240.
- Ahmed, Z., and King, E. J. 1959. Placental phosphatases. *Biochem. Biophys., Acta*, 34: 313.
- Arfors, K. E., Beckman, L., and Lundin, L. G. 1963. Further studies on the association between human serum phosphatases and blood groups. *Acta Genet.*, 13: 366-368.
- Armstrong, A. R., and Banting. 1935. The site of formation of the phosphatase of serum. *Canad. Med. Assoc. J.*, 33: 243-246.
- Baker, R. W. R., and Pellegrino, C. 1954. The separation and detection of serum enzymes by paper electrophoresis. *Scand. J. Clin. Lab. Invest.*, 6: 94-101.
- Bamford, K. F., Hanis, H., Luffman, J. E., Robson, E. B., Cleghorn, T. E. 1965. Serum- alkaline-phosphatase and the ABO blood groups. *Lancet I*, (January-June, 1965): 530-531.
- Beckman, L. 1964. Associations between human serum alkaline phosphatases and blood groups. *Acta Genet.*, 14: 286-297.
- Bekemeier, H. 1962. Einige normale Laboratoriumswerte der Hauskatze. *Deutsche Tierärztliche Wochenschrift*, 69: 250-252.
- Bessey, O. A., Lowry, O. H., and Brock, M. J. 1946. A method for the rapid determination of alkaline phosphatase with five millimeters of serum. *J. Biol. Chem.*, 164: 321-329.
- Bloom, F. 1957. Some blood chemical constituents of normal cats. *N. Am. Vet.*, 38: 114-117.
- Bodansky, O. 1937a. Notes on the determination of serum inorganic phosphate and serum phosphatase activity. *J. Biol. Chem.*, 120: 167-175.
- Bodansky, O. 1937b. Are the phosphatases of bone, kidney, intestine and serum identical? The use of bile salts in their differentiation. *J. Biol. Chem.*, 118: 341-362.
- Bodansky, O. 1948. The inhibitory effects of alanine, L glutamic acid, L lysine and L histidine on the activity of intestinal, bone and kidney phosphatase. *J. Biol. Chem.*, 174: 465-476.

- Boyer, S. H. 1961. Alkaline phosphatase in human sera and placentae. *Science*, 134: 1002.
- Cantarow, A., and Stewart, H. L. 1935. Alteration in serum bilirubin and bromsulphalein retention to morphological changes in the liver and bile passages in cats with total biliary stasis. *Am. J. Path.*, 11: 561-581.
- Cantarow, A., Stewart, H. L., and McCool, A. G. 1936. Serum phosphatase of cats with total bile stasis. *Proc. Soc. Exper. Biol. Med.*, 35: 87-89.
- Carlsten, A., Edland, Y., and Thulesius, O. 1961. Bilirubin, alkaline phosphatase and transaminases in blood and lymph during biliary obstruction in the cat. *Acta Physiol. Scand.*, 53: 58-67.
- Cawley, L. P., and Eberhardt, L. 1962. Simplified gel electrophoresis. I. Rapid technic applicable to the clinical laboratory. *Am. J. Clin. Path.*, 358: 539-547.
- Chiandussi, L., Greene, S. F., and Sherlock, A. 1962. Serum alkaline phosphatase fractions in hepato-biliary and bone diseases. *Clin. Sci.*, 22: 425-434.
- Cloetens, R. 1939. Identification de deux phosphatases alcalines dans les organes animaux. *Enzymol.*, 6: 46-56.
- Cooke, K. B., and Zilva, J. F. 1961. Serum alkaline phosphatase fractionation as an aid to diagnosis. *J. Clin. Path.*, 14: 500-501.
- Cornelius, C. E., and Kaneko, J. J. 1963. *Clinical Biochemistry of Domestic Animals*. Academic Press, New York.
- Dalgaard, J. B. 1948. Phosphatase in cats with obstructive jaundice. *Acta Physiol. Scand.*, 15: 290-303.
- Dubbs, C. A., Vivonia, C., and Hilburn, J. M. 1960. Subfractionation of human serum enzymes. *Science*, 131: 1529-1531.
- Enzyme Nomenclature Recommendations (1964) of the International Union of Biochemistry. 1965. Elsevier Pub. Co., New York.
- Estborn, B. 1959. Visualization of acid and alkaline phosphatase after starch-gel electrophoresis of seminal plasma serum and bile. *Nature*, London, 184: 1636.
- Fahey, J. L., McCoy, P. F., and Goulian, M. 1958. Chromatography of serum proteins in normal and pathological sera: The distribution of protein bound carbohydrate and cholesterol, siderophilin, thyroxine-binding protein, alkaline and acid phosphatase, radioiodinated albumin and myeloma proteins. *J. Clin. Invest.*, 37: 272-284.

- Fazekas de St. Groth, A., Webster, R. F., and Datyner, A. 1962. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochima et Biophysica Acta*, 71: 377-391.
- Flood, C. A., Gutman, E. B., and Gutman, A. B. 1937. Serum and urine phosphatase activity in the cat after ligation of the common bile duct. *Am. J. Physiol.*, 120: 696-702.
- Gahne, B. 1963. Genetic variation of phosphatase in cattle serum. *Nature*, 199: 305-306.
- Gibson, K. S. 1952. Cholelithiasis and choledocholithiasis in a cat. *J.A.V.M.A.*, 121: 288-289.
- Gomori, G. 1939. Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. Exp. Biol. N.Y.*, 42: 23-26.
- Gomori, G. 1941. The distribution of phosphatase in normal organs and tissues. *J. Cell. Comp. Physiol.*, 17: 71-83.
- Gutman, A. B. 1959. Serum alkaline phosphatase activity in disease of the skeletal and hepatobiliary systems. A consideration of the current status. *Am. J. Med.*, 27: 875-901.
- Haije, W. G., and de Jong, M. 1963. Iso-enzyme patterns of serum alkaline phosphatase in agar gel electrophoresis and their clinical significance. *Clin. Chem. Acta*, 8: 614-620.
- Hodson, A. W., Latner, A. L., and Raine, L. 1962. Isozymes of alkaline phosphatase. *Clin. Chem. Acta*, 7: 255-261.
- Hunter, R. L., and Markert, C. L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science*, 125: 1294.
- Keiding, N. R. 1959. Differentiation into three fractions of the serum alkaline phosphatases and the behavior of the fractions in diseases of bone and liver. *Scand. J. Clin. Lab. Invest.*, 11: 106-112.
- King, E. J., and Armstrong, A. R. A. 1934. A convenient method for determining serum and bile phosphatase activity. *Canad. M. A. J.*, 31: 376-381.
- Kritzler, R. A., and Beaubien, J. 1949. Microchemical variation of alkaline phosphatase activity of liver in obstructive and hepatocellular jaundice. *Am. J. Path.*, 25: 1079-1097.
- Kolmer, J. A., Spaulding, E. H., and Robinson, H. W. 1959. *Approved Laboratory Technic*, 5th ed. Appleton-Century-Crofts, Inc., N.Y.

- Kowlessar, O. D., Haeffner, S. J., and Riley, E. M. 1961. Localization of serum leucine aminopeptidase, 5-nucleotidase and nonspecific alkaline phosphatase by starch-gel electrophoresis: Clinical and biochemical significance in disease states. *Ann. N. Y. Acad. Sci.*, 94: 836.
- Kowlessar, O. D., Pert, J. H., Haeffner, S. J., and Sleisenger, M. H. 1959. Localization of 5-nucleotidase and nonspecific alkaline phosphatase by starch-gel electrophoresis. *Proc. Soc. Exper. Biol.*, 100: 191-193.
- Latner, A. L., and Hodson, A. W. 1962. Iso-enzyme of alkaline phosphatase. Meeting of the Assoc. of Clin. Pathologists, London, 1961. *J. Clin. Path.*, 15: 93-94.
- Lawrence, S. H., Melnick, P. J., and Weimer, H. E. 1960. A species comparison of serum proteins and enzymes by starch gel electrophoresis. *Proc. Soc. Exp. Biol. Med.*, 105: 572-575.
- Martin, B. F. 1951. Alkaline phosphatase in the large intestine. *J. Anat.*, 85: 140-158.
- Moss, D. W. 1960a. Kinetics of phosphatase action on naphthyl phosphates, determined by a highly sensitive spectrofluorometric technique. *Biochem. J.*, 76: 32p.
- Moss, D. W. 1960b. An improved spectrofluorometer for biochemical analysis. *Clin. Chem. Acta*, 5: 283-288.
- Moss, D. W., Campbell, D. M., Anagnostou-Kakaras, E., and King, E. J. 1961. Characterization of tissue alkaline phosphatases and their partial purification by starch gel electrophoresis. *Biochem. J.*, 81: 441-447.
- Moss, D. W. 1962. Iso-enzymes of alkaline phosphatase in autolysed and butanol-extracted liver preparations. *Nature, Lond.*, 193: 981-982.
- Moss, D. W., and King, E. J. 1962. Properties of alkaline phosphatase fractions separated by starch gel electrophoresis. *Biochem. J.*, 84: 192-195.
- Moss, D. W. 1964. Properties of alkaline phosphatase fractions in human small intestine. *Biochem. J.*, 92: 16p.
- Newton, M. A. 1967. The clinical application of alkaline phosphatase electrophoresis. *Quart. J. Med.*, 36: 17-28.
- Nordentoft Jensen, B. 1964. Behavior of the fractions of serum alkaline phosphatase in the course of diseases of liver or bone. *Acta Med. Scand.*, 176: 705-710.

- Norton, R. K. 1950. Separation and purification of enzymes associated with insoluble particles. *Nature*, 166: 1092-1095.
- Paul, J., and Fottrell, P. F. 1961. Molecular variation in similar enzymes from different species. *Ann. N. Y. Acad. Sci.*, 94: 668-677.
- Peacock, A. C., Reed, R. A., and Highsmith, E. M. 1963. Ethanol fractionation of human serum alkaline phosphatase. *Clin. Chem. Acta*, 8: 914-917.
- Pearse, A. G. E. 1961. *Histochemistry, Theoretical and Applied*, 2nd ed. Little, Brown and Co., Boston.
- Pulvertaft, C. N., and Luffman, J. E. 1967. Isoenzymes of alkaline phosphatase in patients operated upon for peptic ulcer. *Lancet*, 7484: 237-239.
- Randel, J., Aaland, O., Freeland, R. A., Möller, F. 1964. The relationship between the alkaline phosphatase polymorphism and blood group O in sheep. *Genetics*, 50: 973-986.
- Randel, J., and Stormont, C. 1964. Variants of ovine alkaline serum phosphatases and their association with the R-O blood groups. *Proc. Soc. Exp. Biol. Med.*, 115: 853-856.
- Roberts, W. M. 1930. Variations in the phosphatase activity of blood in disease. *Brit. J. Exp. Path.*, 11: 90-95.
- Robison, R. 1923. The possible significance of heosephosphoric esters in ossification. *Biochem. J.*, 17: 286-293.
- Robinson, J. C., and Pierce, J. E. 1964. Differential action of neuramidase on human serum alkaline phosphatase. *Nature, Lond.*, 204: 472-473.
- Rosenberg, I. N. 1959. Zone electrophoretic studies of serum alkaline phosphatase. *J. Clin. Invest.*, 38: 630-644.
- Schlamowitz, M. 1954a. Specificity of dog intestinal phosphatase antiserum. *J. Biol. Chem.*, 206: 369-374.
- Schlamowitz, M. 1954b. Production of antibodies against dog intestinal phosphatase. *J. Biol. Chem.*, 206: 361-367.
- Schlamowitz, M., and Bodansky, O. 1959. Tissue source of human serum alkaline phosphatases as determined by immunochemical procedures. *J. Biol. Chem.*, 234: 1433-1437.
- Sigma Technical Bulletin No. 104. 1963. Sigma Chemical Co., St. Louis, Mo.

- Smith, C., and Freeman, B. L. 1954. Distribution of lipids, lipase and alkaline phosphatase in renal tubules of the cat. *Proc. Soc. Exp. Biol. Med.*, 86: 775-778.
- Stevenson, D. E. 1961. Demonstration of alkaline phosphatase activity following agar gel electrophoresis. *Clin. Chem. Acta*, 6: 142-143.
- Takamatsu, H. 1939. *Trans. Soc. Path., Japan*, 29: 429, cited by Pearse, A. G. E. 1961. *Histochemistry, Theoretical and Applied*, 2nd ed. Little, Brown and Co., Boston.
- Taleisnik, S., Paglini, S., and Zeitune, V. 1953. Localisation de la phosphatase alcaline due serum dans les fractions proteïniques séparées par electrophoresis. *Comt. Rend. Soc. de Biol.*, 149: 1790, cited by Gutman, A. B. 1959. *Am. J. Med.*, 27: 875-901.
- Thannhauser, A. J., Reichel, M., Grattan, J. F., and Maddock, J. J. 1937. Studies on serum phosphatase activity. II. The effect of experimental total biliary obstruction on the serum phosphatase activation in dogs and cats. *J. Biol. Chem.*, 121: 709-714.
- Tietz, N. W., Woodrow, D., and Woodrow, B. 1967. A comparative study of the Bodansky and the Bessey, Lowry and Brock methods for alkaline phosphatase in serum. *Clin. Chem. Acta.*, 15: 365-367.
- Wachstein, M. 1955. Histochemical staining reaction of the normal functioning and abnormal kidney. *J. Histochem., and Cytochem.*, 3: 246-270.
- Webb, E. C. 1964. Nomenclature for multiple enzyme forms. *Nature, Lond.*, 203: 821.
- Wilkinson, J. H. 1962. *Introduction to Diagnostic Enzymology*. Williams and Wilkins, Baltimore, Md.
- Yong, J. M. 1967. Origins of serum alkaline phosphatase. *J. Clin. Path.*, 20: 647-653.

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