HISTOCHEMICAL, FLUOROMETRIC AND ELECTROPHORETIC ANALYSIS OF HUMAN LEUKOCYTIC ALKALINE PHOSPHATASE

by

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INTRODUCTION

General Consideration

The fact that phosphoric acid can be liberated from certain compounds by enzymatic action has been known since the beginning of this century. Subsequently, it was shown that a family of enzymes existed with this ability and because of their hydrolytic action on phosphoric acid esters, they have become known as phosphatases. At the present time, the phosphatases are classified into mono-, di-, and triphosphatases according to the substrates upon which they act (92, p. 384). The triphosphatases split the end phosphate from a variety of triphosphate esters; the diphosphatases, acting in a similar manner, split the end phosphate from diphosphate esters and monophosphatases, similarly, hydrolyze monoesters into their respective alcohol and free phosphoric acid. Phosphomonoesterases (monophosphatases) have been further subdivided according to their pH optima and their activation or inhibition by magnesium, into an alkaline phosphatase and three acid phosphatases (105, p. 481). All four phosphomonoesterases are characteristic in that they are non-specific phosphatases and readily hydrolyze many substrates such as α- and β-glycerophosphate, dihydroxyacetone phosphate, phenylphosphate, α- and β-naphthyl phosphates, etc. (120, p. 89, 122, p. 59). Alkaline phosphatase, as its name implies, is most active at the higher pH (8.5-11.0) and is activated by magnesium. As will be later discussed in detail, this enzyme has been found in nearly every tissue of animal origin.

The study reported in this thesis is the characterization of human leukocytic alkaline phosphatase by starch gel electrophoresis. When this project was undertaken, no reports had yet appeared on the

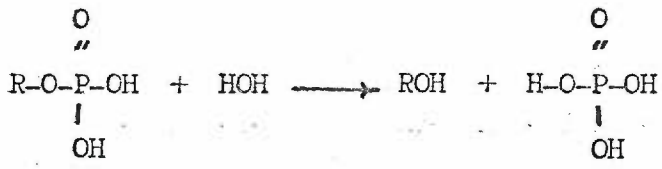
electromobility of leukocytic alkaline phosphatase. The possibility of elucidating the genetics of alkaline phosphatase in leukocytes was an additional stimulus for choosing this problem.

Function of Alkaline Phosphatase

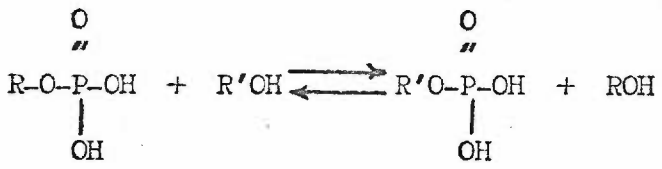
There is estimated to be several thousand articles on alkaline phosphatase, but to date there is relatively little knowledge about its biological function. Robinson and Soames (103) first suggested that bone alkaline phosphatase liberates phosphate ions at ossification centers, thus aiding in the formation of bone. Others have suggested that it plays a role in the formation of fibrous proteins and in the passage of metabolites across cell membranes (16, 27). This latter supposition is supported by its localization to the basement membranes of intestine, kidney, placenta and liver, all of which are involved in active transport (20, p. 160). Placental tissue is especially rich in alkaline phosphatase and is claimed to be the greatest single source of alkaline phosphatase in the body by several investigators (20, 100). Its function in the placenta is thought to be absorption of glucose from maternal blood (4). In the mammary glands, alkaline phosphatase activity increases markedly during pregnancy and is later maintained at high levels during lactation. The function of alkaline phosphatase in this organ is thought to be similar to that in the placenta (33), i.e. transport of glucose from maternal blood to the milk. Finally, Moog (81) has implied a relationship of this phosphatase to the "sodium pump" and the active uptake of sugar molecules in the intestine. Articles by Gutman (41) and Burstone (20, p. 160) contain reviews on the current thinking of the function of alkaline phosphatase.

As previously mentioned, alkaline phosphatase hydrolyzes ortho-

phosphates from phosphmonoesters of various types. The general reaction may be stated:



where R represents an alcoholic or phenolic radical (92, p. 384). In this hydrolysis the cleavage of the phosphate occurs at the oxygen-phosphorous bond (25). Another reaction that probably occurs when alcohol (phenol) is in higher concentration is a transphosphorylation reaction (120, p. 60):



This reaction may be the most important <u>in vivo</u> function of alkaline phosphatase (92, p. 385). Extensive studies on alkaline phosphatase's transphosphorylation ability has been reported by Meyerhof and Green (75). These authors also have reported direct phosphorylation action of alkaline phosphatase when this enzyme was incubated with high concentrations of inorganic phosphate and various alcohols (74).

Kinetics

Much has been written about activators, inhibitors, optimum pH and reaction velocities of alkaline phosphatase, and only a fragment of this material will be cited here. One is referred to review articles for further details (105, p. 484, 120, pp. 60-63, 83, 20, p. 182).

Activators

Divalent cations are, in general, activators of alkaline phosphatase, the two most important ones being Mg^{++} and Zn^{++}. These two metals optimally activate at concentrations of 5×10^{-3} M and 10^{-6} M re-

spectively (66). Others include Mn^{++} Co^{++} and of lesser effect, Fe^{++} Ni^{++} and Ca^{++} (105, p. 486).

Other compounds are also known to increase the activity of alkaline phosphatase; notably, most amino acids (alanine in particular), amino alcohols, peptides, amines, imidazols (120, p. 61) and cyanides in low concentration (10^{-4} - 10^{-5} M) (105). All of these substances are metal-complex-forming compounds, and their mode of action is most likely the chelation of inhibitory divalent cations (66).

Inhibitors

Inhibitors of alkaline phosphatase have been classified by Stadtmen (120) into three types: 1) metal-chelating agents such as ethylene-diaminetetraactate, α,α'- dipyridyl, o-phenanthroline, and KCN in high concentration; 2) some divalent cations, i.e. beryllium (10^{-4} - 10^{-6} M), copper (10^{-5} M) and zinc (10^{-4} M); and 3) orthophosphate and related polyvalent anions including pyrophosphate, borate (140), carbonate, polyestradiol and polyphloretic phosphates.

Optimum pH and Maximum Velocity Constants (Km)

Stadtman (120, p. 66) has stated the following about optimum pH: The "...optimum pH [or Km] for a particular reaction is a function of the nature of the phosphate ester substrate, the concentration of the substrate, the type of enzyme preparation employed, the concentation of enzyme added, the nature and concentration of metal activator and the type of buffer employed." From this it is obvious that any statement about the pH optimum or the Km is meaningless unless the exact conditions are stated. However, the pH optimum for alkaline phosphatase, under most conditions, ranges from pH 8.5 to 10 (20, p. 182, 83, 87).

Folley and Kay (32) have reported an interesting relationship between substrate concentration and pH optimum. They found that decreasing substrate concentrations are associated with a decreased pH optimum of alkaline phosphatase. This work implies that alkaline phosphatase may function optimally at ordinary cytoplasmic pHs, where the substrate concentration is low.

Several maximum velocity constants have been reported. Moss and associates (83) gave the Km (mM) values of human bone, liver, intestine and kidney alkaline phosphatases as 0.110, 0.067, 0.090 and 0.103 respectively utilizing disodium β-naphthyl phosphate as a substrate. In a later article, Moss and King (85) reported differing Km values for isozymes found in individual tissues (bone, liver, intestine and kidney).

Clinical Importance of Alkaline Phosphatase

The voluminous clinical studies of alkaline phosphatase have been summarized by several authors (1, 38, 41, 45, 65). Even though alkaline phosphatase has been found in almost all body tissues and fluids (1, p. 55), only serum and leukocytic alkaline phosphatase are studied clinically. Leukocytic alkaline phosphatase will be discussed in a subsequent section.

Serum Alkaline Phosphatase

Alkaline phosphatase present in serum is detected by measuring the end products of its hydrolytic action (phosphate, phenol, p-nitrophenol) on their respective phosphate esters. The various methods for determining alkaline phosphatase activity have been recently summarized by Deren et al. (28).

The origin and fate of serum alkaline phosphatase has been a sub-

ject of controversy for many years. At the present time serum alkaline phosphatase is believed to originate from osteoblasts (40) which release alkaline phosphatase into their surrounding medium, presumably for participation in bone formation. In an unknown fashion, the enzyme finds its way from bone into the blood stream, only to be removed by liver parenchymal cells. These cells then excrete the enzyme into the bile with eventual destruction in the intestine (41). With this pathway in mind, it is understandable that an elevation of serum alkaline phosphatase activity occurs when there is an increase in osteogenic activity, when there is damage to liver cells or when there is obstruction to biliary flow. Increased amounts of serum alkaline phosphatase are found in normal children, Paget's disease, rickets, osteomalacia, hyperparathyroidism and osteoblastic carcinoma. An elevation associated with the liver and biliary system occurs in hepatitis, cirrhosis and intra- or extrahepatic cholestasis. A substantial increase in serum alkaline phosphatase is also seen in pregnancy and is thought to reflect the release of this enzyme from the placenta into the blood (69, 94).

Another clinical entity, hypophosphatasia, has recently received considerable attention (12, 30, 34, 67, 109, 118). It is a genetically inherited disease which shows a marked decrease in alkaline phosphatase in all parts of the body (98, 99) . Carriers of this disease are detected by low serum alkaline phosphatase levels (99). Korner (60) has reported normal serum alkaline phosphatase electrophoretic patterns in affected individuals.

Human Leukocytic Alkaline Phosphatase

Leukocytic alkaline phosphatase activity today is playing an

important role in the diagnosis of various diseases. Much of the work done on this enzyme, in leukocytes, has centered on the development of a reliable histochemical technique for the demonstration of alkaline phosphatase in blood smears.

Robinson (102) in 1923 reported the first histochemical method for demonstrating alkaline phosphatase. He found that rachitic bone developed an intense black color when first incubated in calcium hexosemonophosphate and then treated with silver nitrate. Control sections of bone treated identically but incubated without calcium hexosemonophosphate showed no staining at all. Robinson's method was only a macroscopic demonstration of alkaline phosphatase activity and it was not until 1939 that Gomori (35) and Takamutsu (123) applied Robinson's technique to microscopie tissue sections. Gomori incubated his tissue sections in a solution containing the substrate, β-glycerophosphate, and the capturing agent, calcium chloride. Phosphatase liberated inorganic phosphate which then combined with the calcium and precipitated at the site of the enzymatic action. After incubation, the sections were first treated with cobalt nitrate resulting in the substitution of cobalt for the calcium and then with ammonium sulfide which replaced the phosphate with sulfide and resulted in the formation of cobalt sulfide, a highly insoluble, black precipitate. Cobalt sulfide can easily be seen under the microscope at the site of enzymatic action.

Various modifications of Gomori's original method have been developed. Menton et al. (73) demonstrated alkaline phosphatase activity by using β-naphthyl phosphate as a substrate and diazotized α-naphthylamine as a capturing agent. This diazotized compound combined with β-naphthol produced in the hydrolysis of β-naphthyl phosphate

forms a chromogenic complex which is highly colored and quite insoluble. Another modification of Menton's method employed a stabilized diazotate of α-naphthylamine. Kaplow (50) first applied Menton's technique to leukocytes. He also developed a "scoring" procedure for a semi-quantitative method of determining leukocytic phosphatase activity (see Materials and Methods for further details of this procedure). In 1958, Burstone (19) introduced a series of substituted naphthol phosphates and recommended naphthol AS-MX phosphate for the demonstration of alkaline phosphatase activity in the neutrophil. More recently, Kaplow (51) found naphthol AS-BI in combination with red violet LB salt (diazotized 5-benzamido-4-chloro-2-toluidine) to give the greatest contrast and the most precise localization of the substituted naphthol phosphates and recommended its use for routine leukocytic alkaline phosphatase determinations. A generalized discussion of alkaline phosphatase histochemistry can be found in books by Pearse (92), Burstone (20) and Danielli (27).

Gomori (36) was first to describe the histochemical localization of leukocytic alkaline phosphatase. He found it to occur in relatively low concentration in neutrophils. The first detailed study of alkaline phosphatase in leukocytes was published by Wachstein (134) in 1946. Using Gomori's histochemical method, he found that neutrophilic phosphatase activity varied greatly among the neutrophils of any single individual and that some were completely devoid of enzyme activity. He also reported a marked increase in enzymatic activity in individuals with infections and decreased activity in patients with chronic granulocytic leukemia. Subsequent to these two reports, numerous papers have appeared describing leukocytic alkaline phosphatase activity in normal individuals and in various disease states.

In a healthy individual, about 20% of the circulating neutrophils contain demonstrable phosphatase activity (50, 55, 79). In 1963, Kaplow (51) reported a mean value of 61 for normal individuals using his more sensitive azo-dye method. Valentine and associates (124, 129) have reported a quantitative method for determining phosphatase activity. They incubated whole white cells in β-glycerophosphate and then determined the amount of inorganic phosphate liberated. Normal mean values, using this method, are 25.8 mg phosphate per hour per 10^{10} leukocytes or 40 mg phosphate per hour per 10^{10} neutrophils.

Decreased alkaline phosphatase activity in neutrophils is seen consistently in chronic granulocytic leukemia (10, 51, 55, 58, 129, 133, 134, 136), paroxysmal noctural hemoglobinuria (43, 124), and hypophosphatasia (12, 60). Phosphatase studies in chronic granulocytic leukemia will be more extensively discussed in a following section. In hypophosphatasia, Beisel et al. (12) reported no detectable or inducible leukocytic alkaline phosphatase but Korner (60) noticed detectable activity in an affected patient who was suffering from a wound infection.

Neutrophilic alkaline phosphatase activity is elevated in many conditions including most acute infections, "stress", pregnancy and certain hematological disorders. Wachstein (134) first reported increased activity of neutrophilic alkaline phosphatase in infections (pneumonia, osteomyelitis, meningitis, etc.) and observed the degree of activity to correspond with the severity of the disease. Valentine and Beck (129) reported a patient with an infection to have an activity of 275 mg of phosphate, thirteen times the normal activity. Others have reported similar findings (50, 55, 58, 130, 131). Normal values have been reported for chronic infections such as tuberculosis and

syphilis (137). Stressful situations, resulting in increased neutrophilic alkaline phosphatase levels, include surgery, cerebral vascular accidents, trauma, uremia, diabetic acidosis, etc. (50, 52, 130, 132, 137). Peak leukocytic activity occurs 48 to 72 hours after an operation and in uncomplicated cases returns to normal levels in ten to eleven days (52, 130, 137). Marked elevation of alkaline phosphatase activity in neutrophils occurs following the administration of corticosteroids, or ACTH in patients with intact adrenals (130, 133). Thus, the elevated alkaline phosphatase activity in stressful situations may be related to an increased steroid elaboration (130).

Pregnancy is also associated with an elevation in neutrophilic alkaline phosphatase levels. Activity increases throughout pregnancy and the activity just prior to delivery is more than three times above that found in the non-pregnant state. At parturition and in the immediate postpartum period (two to three days), enzyme levels rapidly rise to five or six times that of normal levels and then slowly return to normal over the next six weeks (24, 44, 96, 97).

Several hematological disorders are associated with increased leukocytic activity including leukemoid reactions, polycythemia vera, multiple myeloma and myelofibrosis (51, 58, 80, 136). Alkaline phosphatase scores in these diseases are usually two to three times normal values and not as high as those seen in infections.

Chronic Granulocytic Leukemia

The neutrophils in patients with chronic granulocytic leukemia, a myeloproliferative disease, were first noticed by Wachstein (134) to possess a low alkaline phosphatase activity. In contrast, Wachstein found that acute granulocytic leukemics have a normal alkaline phosphatase activity in their neutrophils. Valentine and Beck (129),

using their quantitative method, found the alkaline phosphatase activity in chronic granulocytic leukemics to range from 0 to 14.4 (mg of phosphate liberated per hour per 10^{10} leukocytes) with a mean of 4.0 mg, a mean significantly lower than the normal of 25.8 mg. These findings (129, 134) have been confirmed by others (70, 136).

Neutrophilic alkaline phosphatase activity appears to increase in chronic granulocytic leukemia when the patient has a concomitant infection or a remission in his disease. Valentine and Beck (129) cited a patient with an extensive skin infection who had chronic granulocytic leukemia and an elevated leukocytic alkaline phosphatase activity. Subsequently, with control of the infection, his phosphatase activity decreased to low levels. Rosen and associates (106) reported a high alkaline phosphatase activity in a patient suffering with ulcerative colitis and chronic granulocytic leukemia. With control of the colitis, the phosphatase activity reverted to low levels. Xefteris and co-workers (138) cited several patients whose alkaline phosphatase returned to normal levels following treatment and remission of their chronic granulocytic leukemia.

The etiology of chronic granulocytic leukemia was first suggested by Nowell and Hungerford (89) to be an abnormally small acrocentric chromosome. This abnormal chromosome has been since shown to represent a deletion of the long arms of a G-group chromosome (2, 8, 125) and appears to be limited to blood and bone marrow cells (8).

Mongolism

Mongolism was described as a clinical entity exactly 100 years ago by J. L. Down (29) and now bears his name. Mongolism occurs once in every 500 or 600 births, making it the most frequent single entity causing severe mental retardation (68, p. 23), and accounting for

approximately 10% of institutionalized retardates.

In 1959, Lejune and associates (61A) reported finding 47 chromosomes, one more than normal, in mongolism. The extra chromosome was a small acocentric chromosome belonging to the G-group and presumably to the 21 pair. Since their article appeared, numerous investigators have confirmed and extended these findings (7, 108, 112, 139), though recent papers have argued that the additional chromosome belongs to the 22 pair.

It is now known that mongolism occurs in two major forms. The first type is that reported by Lejune, 21-trisomic condition, resulting from a nondisjunction, and occurs most frequently in children born to older mothers. Since nondisjunction occurs in the gametes during meiosis or in the early cleavage stages of the zygote, it is generally a non-hereditary form of mongolism (68, p. 18). The second type of mongolism, first reported by Polani et al. (93), is a translocation of the 21st chromosome onto either a D-group or another G-group chromosome. This type of chromosomal derangement may arise in the patient, de novo, or may be found in one of the carrier parents and passed on to daughter cells during meiosis, thus making it a hereditary type of mongolism. In contrast to 21-trisomic mongolism, which is associated with older mothers, translocation mongolism is typically associated with younger mothers.

From the studies of alkaline phosphatase in chronic granulocytic leukemia, where a low enzyme activity is associated with a loss of chromosomal material, Alter et al. (2) reasoned that mongols with extra chromosomal material should have an increase in leukocytic alkaline phosphatase. In their study of 35 healthy mongols and 35 healthy control children, they found a mean leukocytic activity of 139 (mg phosphate per hour per 10^{10} leukocytes) for the mongols and 83 for the

controls (differences significant at the 0.01 level of confidence). Others quickly confirmed their findings (57, 62, 90, 127).

Turbowitz et al. (127) showed a significant difference between mongols and controls when he used the histochemical method of Kaplow (50). O'Sullivan and Pryles (90), reporting on the histochemical phosphatase activity in leukocytes of 200 mongols and 200 controls, found a mean activity of 78 $\pm$ 25.8 for the mongols and 32 $\pm$ 16.6 for the controls with no associated differences in sex. Twenty of their mongols, who had alkaline phosphatase scores in the range of normals, were karyotyped as 21-trisomy. Three translocation mongols had values that fell within the normal range, a finding also reported by others (108, 127).

King et al. (57) have suggested the elevation of phosphatase in leukocytes of mongols is due to an additional gene. Other explanations for this elevation have been cited. Hook and Engel (47) have suggested that the elevation in the alkaline phosphatase activity encountered in mongolism is due to a younger mean age of neutrophils. Supporting this notion, Turpin and Bernyer (128) have reported the Arneth index (the average neutrophilic lobe count which is lower in immature neutrophils) of 42 mongols to be lower than their normal relatives and the general population. This observation has been confirmed by others (13, 77, 78, 111). Lennox and associates (62), however, have correlated the lobe count with the phosphatase score in leukocytes of mongols, and found that the cells with low lobe counts had low alkaline phosphatase activity. Valentine and associates (129) have reported an increase of leukocytic alkaline phosphatase with infection. Mongols are known to have a higher incidence to infection (88, p. 1238), and Turbowitz et al. (127) suggested that infections in mongols may contribute to the elevated

enzyme activity.

With the activity of one enzyme in mongols being abnormal, other enzymes were quickly examined. Brandt (17, 18) reported an elevation in galactose-1-phosphate uridyl transferase in hemolyzed whole blood of mongols. Hsia and his associates (48) found a similar elevation in white cells of mongols but not in their red cells. They found aldolase, pyruvate kinase, lactic acid dehydrogenase and acid phosphatase of erythrocytes and leukocytes, hexose isomerase, phosphoglucomutase and glutathione reductase in whole blood, and glucose-6-phosphate dehydrogenase in 6-phosphate-glucose dehydrogenase in leukocytes to show no significant increase in activity in mongols over normal. Mellman et al. (71) also found an increased galactose-1-phosphate uridyl transferase activity in white cells of 14 mongols they studied and contrary to the finding of Hsia and associates (48), Mellman et al. reported an elevation of glucose-6-phosphate dehydrogenase and acid phosphatase in the leukocytes of their mongols. Rosner and co-workers (108), in a comprehensive study, found leukocytic galactose-1-phosphate uridyl transferase, acid and alkaline phosphatase, and 5-nucleotidase, and erythrocytic glucose-6-phosphate dehydrogenase to be elevated in trisomic mongolism but normal in both translocation mongols and normal individuals. Platelet alkaline phosphatase and glucose-6-phosphate dehydrogenase studied by Shih and Hsia (113) showed no significant variation between controls and mongoloid patients.

Starch Gel Electrophoresis

In 1955, Smithies (115) first described starch gel electrophoresis and showed that it had superior resolving powers over those of other electrophoretic methods. With paper electrophoresis, serum can be

resolved into albumin, alpha-one ($\alpha 1$), alpha-two ($\alpha 2$), beta (β) and gamma-globulin fractions. With the improved resolution of starch gel, Smithies was able to identify the following fractions in serum: pre-albumins, albumin, post-albumins, fast alpha-two globulin ($F\alpha 2$), beta-globulin, haptoglobins (HTgn), slow alpha-two globulin ($S\alpha 2$), β-lipo-protein, and gamma-globulin. The increased resolving power obtained with starch gel is thought to be due to impedence of the migration of larger molecules through the gel matrix even though the larger molecules may have an identical electrical charge to that of smaller molecules (115, 116, 117). By increasing the concentration of starch in a gel which produces smaller pores in the gel, the migration of proteins can be retarded (116). From these two facts, Smithies (116) has concluded that the migration rate of proteins is inversely proportional to the gel concentration and to the molecular size of the protein. Poulik (95) modified Smithies starch gel method by using a discontinuous buffer system and obtained superior resolution. Hunter and Markert (49) applied starch gel electrophoresis to esterase and found the enzyme to separate into multiple bands. Markert and Møller (64) applied the term "isozymes" to these multiple bands. Many enzymes have since been found to exist in multiple molecular forms (isozymes) including alkaline phosphatase.

Although starch gel electrophoresis is the method of choice for demonstrating alkaline phosphatase isozymes, other methods have been tried. By paper electrophoresis, serum alkaline phosphatase migrates with the α_2-globulin fraction of serum (9). With starch block electrophoresis, alkaline phosphatase appears as one or two bands migrating with either α_1-, α_2-, or β-globulin fractions (53, 54, 59, 60, 107). By agar gel electrophoresis, Haije (42) found liver extracts and bile to contain two bands of alkaline phosphatase activity appearing between

α_1- and α_2- globulins while bone was shown to contain one band migrating near the α_2- globulin.

Serum from most individuals, healthy or otherwise, invariably contains one band of alkaline phosphatase which migrates with β-globulin (14, 22, 26, 37, 46, 135) and occasionally a second, more slowly migrating band which is genetically determined (26, 46, 114) and which is discussed in detail in the following section. Bone alkaline phosphatase from bone extracts or from sera of patients with bone diseases migrates as a single band of activity corresponding to β-globulin (22, 37, 46). Sera from patients with hepatocellular and obstructive biliary diseases, liver extracts and bile give one alkaline phosphatase band corresponding to β-globulin, and two or three bands migrating between the β-lipoprotein and the origin (22, 26, 37, 46, 85). Small intestine yields bands migrating behind β-globulin in the haptoglobin region and one or two bands near the origin (22, 46, 86, 135). Kidney has been found to contain one isozyme moving behind β-globulin in the haptoglobin region (22). Placental isozymes will be discussed in the following section.

Immunological studies supplemented by starch gel electrophoresis have delineated three fairly distinct antigenic classes of alkaline phosphatase. Liver, bone, spleen and kidney comprise one class, intestine another, and placenta a third (15, 110). The intestine and placenta show some cross reactions with each other and with kidney (15).

Alkaline Phosphatase Genetics

The existence of alkaline phosphatase polymorphisms was first reported by Boyer (14). Studying the electrophoretic mobility of alkaline phosphatase in sera and placentae, he found two additional bands of

phosphatase activity associated with pregnancy. These bands migrated faster than serum alkaline phosphatase and were designated as "A" and "B" zones of activity. During the third term of pregnancy, either one or both of these zones were present. Sera from umbilical cord blood of more than thirty infants contained neither A or B components, even though the mothers of these infants possessed one or both in their own sera.

When placental alkaline phosphatase was studied, he found that certain placentae gave only a single band corresponding to the zone of the serum; others contained a band identical to the B zone, and a third type of placenta possessed three bands - one migrating with the A zone, one with the B zone, and a third between the other two zones. He also established the frequency of occurrence of these bands and showed that there was a difference between ethnic groups.

Robson and Harris (104) in examining over 300 placentae, found six alkaline phosphatase phenotypes which they denoted as F, FI, I, SI, FS, and S (letters representing their relative migration rates - fast, intermediate and slow). The F, I and S types of phosphatase were present only as single bands, while types FI, SI, and FS were composed of three bands each. When studying placentae from Negro mothers, they found an excess of phenotype S, showing, as had Boyer, an ethnic variation. The sex of the fetus appeared to play no factor in determination of the resulting phenotype. After studying 380 placantae from 190 dizygotic twins, they observed a close correlation between the expected and observed phenotypes, and a higher than expected proportion of differing phenotypes in the paired placentae.

Alkaline phosphatase in serum has been found to be under genetic control. Hodson et al. (46) noted that sera often contained two bands

of alkaline phosphatase activity on starch gel electrophoresis. The slower migrating phosphatase, they suggested, originated from the intestine. Cunningham and Rimer (26) also noted the occurrence of two bands in sera and in a series of 50 sera found the slower migrating band to be present in 60% of the cases. Arfors and associates (5) classified individuals into types 1 and 2, where serum from type 1 contained only the fast migrating alkaline phosphatase band (A) and from type 2 contained both the fast (A) and the slow (B) enzyme bands. They concluded from twin studies that the B band was under genetic control and was associated with the ABO blood group, and in a later paper (6), with the Lewis blood group and the secretor-phenomenon (ABH). Recently, Bechman (11), Shreffler (114) and Evans (31) have concluded from larger samplings that the appearance of serum B phosphatase is inhibited in individuals of blood group A and in nonsecretors. They also reported no correlation with the Lewis blood group.

Fluorometric Measurement of Alkaline Phosphatase

Relatively little has been reported about the fluorometric detection of alkaline phosphatase activity. Moss (83) described the photofluorometric spectra for α-naphthol and disodium α-naphthyl acid phosphate. Greenberg (39) extended Moss' work by citing the spectra of β-naphthol and disodium β-naphthyl acid phosphate. Activation maxima of both α-and β-naphthyl acid phosphate, he reported, were 290 mμ (α), and 275 and 315 mμ (β), while fluorescence maxima were 360 and 345 mμ, respectively. The corresponding phenols had activation maxima at 335 and 350 mμ, and fluorescence maxima at 455 and 410 mμ, respectively. Measurements were performed in 0.1 M Tris, pH 10.4. Maximum fluorescence of β-naphthol occurred at pH 10.4 which is near the pH optimum

for alkaline phosphatase. Alkaline phosphatase was found to hydrolyze the sodium salt of β-naphthyl phosphate three times as rapidly as the corresponding α-salt.

By using selected wavelengths, an excitation of 350 mμ and fluorescence of 410 mμ, β-naphthol can be distinguished from its corresponding ester. Thus alkaline phosphatase hydrolysis can be continuously recorded. Applying this system, Greenberg was capable of detecting as little as 10^{-14} moles of alkaline phosphatase. Using a system similar to the above, Tierney (126) was able to measure the alkaline phosphatase fluorescence activity in single cells.

MATERIAL AND METHODS

Immediately after usage, all glassware and washable instruments were stored in soapy water until they could be washed. After they had been washed, they were rinsed twenty times in tap water, ten times in distilled water and air dried. Then they were stored inverted on a shelf or sealed with aluminum foil. Pipette washing differed from that of other glassware in that they were rinsed once each in distilled water, 5 M NaOH, 5 M HNO_3, twenty more times in distilled water and finally three times in acetone. Distilled water was produced by a Bellco Glass Still.

Blood Specimens

Whole blood was obtained by venipuncture from normal healthy volunteers, healthy mongols 18 years of age or older, healthy women in the immediate post partum period, and Multnomah County Hospital patients with pyogenic infections. In most instances, forty ml of blood were drawn from each subject. Of this specimen, thirty ml were heparinized (3 units of Heparin, Upjohn/ml of blood), eight ml were allowed to clot for serum and two ml were heparinized with one or two drops of heparin for leukocyte counts and blood smears.

A convenient coding system was devised for this study. Each subject was given the code letter "H" and a number, i.e. H61; all specimens subsequently collected from this person were then labeled with the code. Peripheral blood smear slides were designated by adding a number "1" to the code (H61-1), serum by "2" (H61-2), and white cell extracts by "3" (H61-3). Multiple ampules or slides from one type of specimen were labeled with additional numbers by numbering them consecutively 1

to 6 (H61-1-1 to H61-1-6). Blood slides number 1 and 2 were stained with Wright's stain while numbers 3 through 6 were used for determination of leukocytic alkaline phosphatase activity. Six ampules of serum and usually six ampules of white cell extract were prepared from all subjects. Each ampule was denoted by an additional number as were the blood slides.

Serum

Serum from each subject was collected by allowing 8 ml of whole blood to clot. The clot was allowed to contract at room temperature for one hour, and then either centrifuged at 1600 g for ten minutes or allowed to stand at 4° C. overnight. Serum was removed with a Pasteur pipette, placed in ampules, labeled and stored at -20° C.

White Cell Counts

White cell counts were performed on a portion of the two ml of heparinized blood. A volume of 0.5 mm^3 was drawn into an 11 mm^3 Hemex white cell pipette, and diluted to 11 mm^3 with 5% (v/v) acetic acid. The pipette was immediately agitated for three minutes on a pipette shaker and the leukocytes were then counted with a Spencer bright-line Neubauer counting chamber.

Leukocytic Alkaline Phosphatase Histochemistry

From the two ml of heparinized blood, six blood smears were made. Two of these were stained with Wright's stain for a differential white cell count. The other four slides were fixed and stained according to the method of Kaplow[51]. The slides were fixed by dipping them for thirty seconds in cold (-4° C.), 10% neutral formalin-methanol solution. This fixative was made by mixing ten ml of neutral stock formaldehyde (38%) with 90 ml of absolute methanol. Neutral formalin was prepared

by adding several chips of calcium carbonate to stock formaldehyde a few days in advance of use. After fixation the slides were rinsed for 15 seconds in distilled water, air dried and stored below 0° C. until used.

Histochemical activity of the leukocytic alkaline phosphatase was demonstrated by incubating the slides for ten minutes at room temperature in a staining mixture prepared as follows: five mg of Sigma naphthol AS-BI phosphate substrate were mixed with 0.3 ml of dimethylformamide and added to 60 ml of 0.05 M Tris (Matheson Coleman and Bell: 2-amino-2 (hydroxymethyl)-1, 3-propanediol) buffer adjusted to pH 9.7 with citric acid. Forty mg of Sigma red violet LB salt (diazotized 5-benzamido-4-chloro-2-toluidine) was then added to the buffer. The resulting solution was mixed vigorously, filtered through one thickness of Whatman No. 1 filter paper into a Coplin staining jar and used immediately. After incubation, the slides were washed for ten to fifteen seconds in tap water and counterstained with Mayer's aqueous hematoxylin for three to eight minutes. The exact staining time was found to depend on ripeness of the dye solution.

Karyotypes

Karyotypes of the mongoloid patients were done by Ronald Marcum. Standard procedures currently in use by the karyotype laboratory in the Division of Experimental Medicine were employed. The mongoloid patients were easily sorted into pure trisomy-21 and mosaic-trisomy/normal-categories.

Leukocyte Isolation and Enzyme Extraction

Leukocytes were isolated from the thirty ml specimen of blood by a modification of the method devised by Peabody *et al.*[91] utilizing

dextran. Six ml of a 5% solution of dextran (Sigma Chemical Company: type 100C, average molecular weight of 124,000) in 0.7% sodium chloride were added to 30 ml of blood, followed by gentle mixing. The erythrocytes were then allowed to settle for 45 minutes, at which time the plasma was transferred to a second tube. Cells in the plasma were settled by centrifugation for ten minutes at 800 g in a model CL International Clinical centrifuge. The plasma was then decanted, leaving a pellet of leukocytes which was invariably contaminated with red cells, but the latter contain no alkaline phosphatase.

Alkaline phosphatase was extracted by two different methods: one described by Robinson et al.[101], and another developed in our laboratory. In the Robinson method, leukocytes were washed three times in 0.9% saline, then suspended in five ml of ice-cold Tris-alanine-citrate buffer which was prepared by diluting 100 ml of a stock solution to a total volume of 2000 ml. The stock solution was 1.5 M Tris-0.2 M DL-alanine titrated with citric acid to a pH of 9.5. Leukocytes were sheared at 0° C. in a glass grinder (Kontes No. 44) at approximately 900 r.p.m. using 20 up and down strokes of the tube. Then two ml of n-butanol (0° C.) were added slowly, with an additional ten strokes of homogenization. With continuous stirring, the homogenate was heated to 37° C. for five minutes, then placed in an ice bath for another five minutes. The homogenate was then centrifuged at 20,000 g at 0° C. for 20 minutes in a Servall RC-2 refrigerated centrifuge. The lower aqueous layer containing the solubilized alkaline phosphatase was aspirated and dialyzed in Visking tubing overnight at 4° C. against two liters of the Tris-alanine-citric acid buffer. After dialysis, the sample was centrifuged at 20,000 g at 0° C. for 20 minutes to remove any insoluble parti-

cles. The enzyme was then concentrated by vacuum dialysis in Visking tubing [119] at 4° C. for 12 hours. The final sample was then stored frozen in ampules labeled as previously described.

The second method of extraction developed in our laboratory was identical to that of Robinson et al. except as follows: after the third washing in saline, the leukocytes were suspended in five ml of 0.9% saline and in two ml of n-butanol at 4° C. The mixture was then added to a 10 ml Vir-Tis glass homogenizing flask and homogenized in an ice bath with a "45" Vir-Tis homogenizer for 60 seconds at full rheostat setting but using only 70 volts line current provided by a Power Stat. After centrifugation and aspiration, as previously described, the aqueous phase was dialyzed overnight at 4° C. against two liters of a 1:20 dilution of stock buffer (0.1 M Tris buffer, pH 8.6). The remainder of the procedure was identical.

Fluorometric Assay

Alkaline phosphatase activity in each serum sample and leukocyte extract was assayed by a modification of Greenberg's[39] fluorometric technique. To a three ml quartz cuvette were added two ml of 0.1 M Tris pH 10.2 containing 1.5×10^{-3} M $MgCl_2$; 0.7 ml of 0.005 M Tris pH 7.0; 0.2 ml of 1.5×10^{-3} M Sigma β-naphthyl phosphate; and 0.1 ml of sample or blank. With dilution, final concentrations were calculated to be 0.066 M Tris, 10^{-3} M MgCl, and 10^{-4} M β-naphthyl phosphate. The substrate, β-naphthyl phosphate, was made on the day of use, by mixing 3.37 mg with ten ml of distilled water.

Enzyme activity was detected with a Model 111 Turner Fluorometer. A Corning 7-60 filter was used to provide the excitation wave length (ca. 330mμ) and a Wratten 2-A filter was employed to select the fluo-

rescence wave length (ca. 415mμ) of the hydrolysis product (β-naphthol). Even with the fluorometer set at its lowest possible sensitivity, there was excessive fluorescence, requiring that a Wratten 10% transmission neutral density filter be inserted into the fluorescence pathway. The activity was continuously recorded on the linear scale of a Model 43 Varicord recorder. The reaction was allowed to proceed until the full scale of the recorder had been utilized or until there was a line long enough to make accurate measurements. Units of activity were arbitrarily defined as the number of cm vertical increase of fluorescence per minute of time measured on the chart paper.

Starch Gel Electrophoresis

Horizontal starch gel electrophoresis of each leukocyte extract and serum sample was performed by a modification of Smithies' method[115] using the electrolyte solution recommended by Poulik[95]. The procedure differed from that described by Smithies in the preparation of the gel, in utilization of a circulating pump between electrode vessels, and in the power supply.

The gels were made with hydrolyzed potato starch in amounts, usually 10 to 11 gm for each 100 ml of buffer, recommended by the manufacturer (Connaught Medical Research Laboratory of Toronto). The gel buffer used was 0.076 M Tris (9.1 gm/l) adjusted to pH 8.6 with citric acid (ca. 1.05 gm/l). To 300 ml of this buffer was added the measured amount of starch which was mixed thoroughly and poured into a liter vacuum flask. The flask was then placed in a boiling water bath and the starch mixture heated to 88° C. with constant stirring. The hot starch was degassed by vacuum aspiration, immediately poured onto a 24x13.5x0.9 cm glass tray, allowed to gel and stored in a refrigerator until used. Samples were introduced into the gel by saturating four 10x8 mm pieces of

No. 1 Whatman filter paper and applying these pieces to the cut surface (8 cm from and parallel to one end) of the gel. After loading, the cut surfaces were again approximated and the gel was placed in the electrophoretic cell with the anode at the greatest distance from the filter papers. A potential difference of 5 v/cm was applied with a servo-controlled power supply specially designed and built by the Research Instrument Service to our specifications. Thus, a constant voltage could be maintained between two points continuously monitored in the gel. Gels were run for approximately twelve hours. During electrophoresis the gels were cooled by pumping water (2° C.) from a Model 209 Forma-Temp Jr. unit through the cooling plate of the cell. A Corman No. 11877 rotary pump was used to circulate the electrode buffer. The electrophoretic cells were built to our own specifications by Mr. Hage of the Department of Anatomy.

After electrophoresis was completed, the gel was sliced horizontally with a 34 gauge stainless steel wire. The upper half of the gel was stained for protein using 1% amido black[115]. Alkaline phosphatase was detected by incubating the lower half of the gel in 100 ml of 0.076 M Tris-citric buffer pH 8.6 containing 130 mg $MgCl_2$, 100 mg Sigma β-naphthyl phosphate, and 100 mg Sigma fast blue RR diazonium salt. After mixing, this solution was immediately filtered through one thickness of No. 1 Whatman filter paper directly onto the gel. The gel was incubated at 37° C. for six hours and afterward washed in tap water for another six hours. The gels were then photographed with Kodak Process Ortho cut film using transmitted light with an "f" stop of 20 and a shutter speed of one second. The film was developed in Kodak D-11 for five minutes at 20° C. Prints were made on F-3 Kodabromide paper.

RESULTS

White blood cell counts and differential counts, neutrophilic alkaline phosphatase scores, serum and leukocytic alkaline phosphatase activities, isozyme patterns and clinical conditions of each individual included in this study are shown in Table I. Sample H36, H65, and H69 are from the same individual at eight and two month intervals respectively. Samples H61, H66, and H71 were also from the same individual. The first sample was taken at the height of his infection, the second two months later when he was almost free from infection and the third when he was completely recovered.

Yields of White Blood Cells

With the method of Peacock (91) for separating leukocytes from erythrocytes, as much as 87% recovery of the white cells was achieved. Table II gives detailed figures for five separations.

TABLE II

Yields of White Blood Cells After Separation

Sample Number	WBC per cm^3 Before Separation (In Thousands)	Total Blood Volume-cc	Total WBC (In Thousands)	WBC per cm^3 After Separation (In Thousands)	Total WBC Separated (In Thousands)	Yield %
H68-3	10,650	30	319,500	45,000	225,000	70.5
H69-3	6,000	30	720,000	46,750	420,750	58.5
H70-3	6,700	30	201,000	32,200	161,000	80.0
H71-3	5,750	120	690,000	16,750	150,750	22.8
H72-3	14,750	30	442,000	38,850	388,500	87.5

Leukocytic Alkaline Phosphatase Histochemistry

Figures 1 to 4 are representative of the histochemical alkaline

TABLE I

Tabulation of Results

(1) Neutrophilic alkaline phosphatase scores were determined by the method of Kaplow (50) by summing the individual scores of 100 consecutive neutrophils.

(2) Fluorometric assay of sera and leukocytic extracts for alkaline phosphatase is reported as the number of cm vertical increase of fluorescence for one minute measured on the chart paper. Hb indicates the extract was contaminated with hemoglobin.

(3) The letters C, F, I, S, and FI refer to the migration rate of the neutrophilic alkaline phosphatase: complete, fast, intermediate, slow and fast-intermediate. If the letter occurs in parenthesis, it indicates that the enzyme migrated differently on another occasion.

(4) Alkaline phosphatase from leukocytes was extraced from samples H67-3T, H69-3 and H72-3 by homogenizing the cells in 0.9% saline with a Vir-Tis homogenizer, samples H69-4 and H72-5 were first frozen and then homogenized as for the previous samples, and samples H64-3, H65-3, H66-3, H67-3A and H72-4 were homogenized in 1:20 Tris-alanine-citrate buffer with a glass homogenizer.

Patient's Number	Sex	WBC per mm³	White Cell Differential						Neutrophile Alkaline Phosphatase Score (1)	Fluorometric Assay (2)		Isozyme Type (3)	Comments
			Neutrophil		Lympho-cytes	Eosino-phils	Mono-cytes	Baso-phils		Sera	Leuko-cytic Extracts (4)		
			PMN %	Bands %	%	%	%	%					
H35	F	-	65	0	19	6	7	0	42	5.1	27.7	I	Healthy, normal individual
H36	M	-	64	0	24	1	10	0	9	1.8	3.6	F	Healthy, normal individual
H60	F	7,150	74	0	24	0	1	0	39	4.7	7.1	S (C)	Healthy, normal individual
H63	M	6,750	58	1	29	7	5	0	110	4.6	22 Hb	F	Healthy, normal individual
H65	M	7,400	59	0	37	3	1	0	7	4.7	-	F	Healthy, normal individual
H71	M	5,750	52	2	38	1	7	0	24	15.3	7.2 Hb	F	Same patient as H61 & H66 - No infection
H69	M	6,000	46	1	48	1	4	0	21	4.5	H69-3, 6.3	F (FI)	Healthy, normal individual
								Average	45		H69-4, 1.6		
H38	M	-	35	14	47	4	1	0	111	14.8	9.4	I	21-Trisomy
H39	M	-	45	17	37	1	0	0	31	6.8	19.1	I	21-Trisomy
H40	M	-	49	19	29	2	1	0	20	2.8	15.4	I (FI)	21-Trisomy
H41	M	-	47	12	36	3	0	0	41	8.9	4.5	I	21-Trisomy/Normal Mosaic
H42	M	-	48	8	38	4	0	4	106	6.9	6.3	I (FI)	21-Trisomy/Normal Mosaic
H43	F	4,300	43	4	46	1	2	4	103	5.8	24.8	FI (F)	21-Trisomy
H44	F	7,500	39	6	48	6	0	1	65	5.3	5.6	I	21-Trisomy
H45	F	8,000	43	19	33	2	3	0	115	3.0	36.8	I	21-Trisomy
H46	F	3,300	37	15	46	0	1	1	118	3.5	9.0	I	21-Trisomy/Normal Mosaic
H47	F	6,600	65	6	27	1	0	1	142	3.3	7.4	FI (I)	21-Trisomy
								Average	85.2				
H59	M	19,500	72	5	19	1	1	2	106	9.5	168	S	Leg and arm infection, osteomyelitis, temp. 101.8° F.
H61	M	15,750	69	21	10	0	0	0	198	11.6	6.8 Hb	F	Infected leg ulcer, diabetes mellitus
H62	M	10,100	65	19	16	0	0	0	160	4.8	177.6	C (S)	Cellulitis of left leg, diabetes mellitus, temp. 101° F.
H64	M	6,400	61	14	22	0	2	1	122	6.8	35.8	F & S	Infected right hand, temp. 99.6° F.
H66	M	8,000	53	16	24	5	1	1	124	8.2	69.4	I	Same pt. as H61 but 2 months later, slight infection.
H70	M	6,700	66	18	16	0	0	0	217	11.1	128 Hb	F & S	Cellulitis of entire left leg, treated with steroids
H67	M	15,600	62	1	24	2	4	1	88	4.5	H67-3A, 49	I	Pneumococcal and micrococcal furnucle on chest
								Average	145		H67-3T, 40	F & S	
H68	F	10,650	68	13	16	0	3	0	116	22.3	217.6	I	6 hours postpartum - full term infant
H72	F	14,750	70	5	18	5	2	0	219	17.7	H72-3, 78.9	I	20 hours postpartum, afebrile, treated with stilbesterol
								Average	167		H72-4, 46.0	FI	
											H72-5, 37.6	FI	

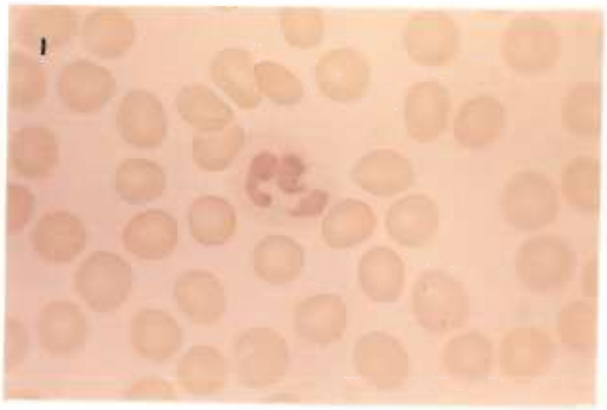
Figures 1 through 4 show the results of Kaplow's histochemical technique (51) for leukocytic alkaline phosphatase.

Figure 1. Neutrophil from a normal individual (H65) with no alkaline phosphatase activity: a score of zero. Enlarged 800 times.

Figure 2. Neutrophil from a lady (H68) who had just delivered a child showing fine cytoplasmic granules of alkaline phosphatase activity: a score of one. Enlarged 1,000 times.

Figure 3. Two neutrophils from an infected patient (H70). In this photograph the cells are touching each other and show different degrees of activity. The granulocyte on the left has a score of two, while the one on the right has a score of three. The cytoplasmic granules in these two cells are also larger than those seen in the cell of Figure 2. Enlarged 1,000 times.

Figure 4. Neutrophil from patient H68. The activity is so great and the cytoplasmic granules so numerous that they are almost indistinguishable. This cell would have a score of four. Enlarged 800 times.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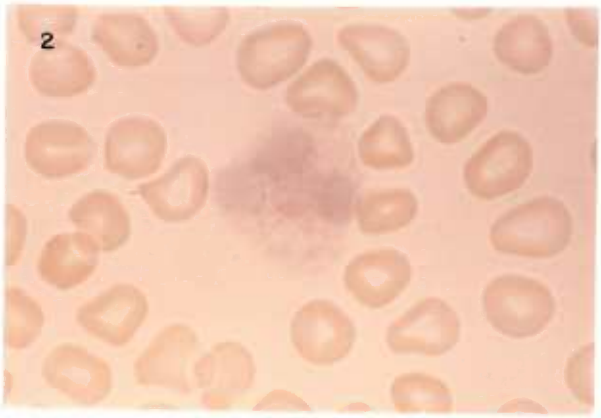
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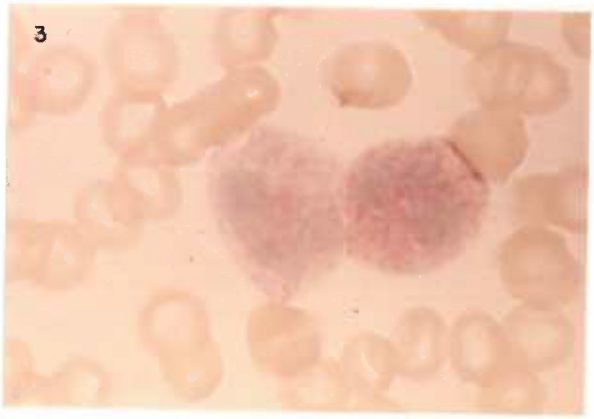
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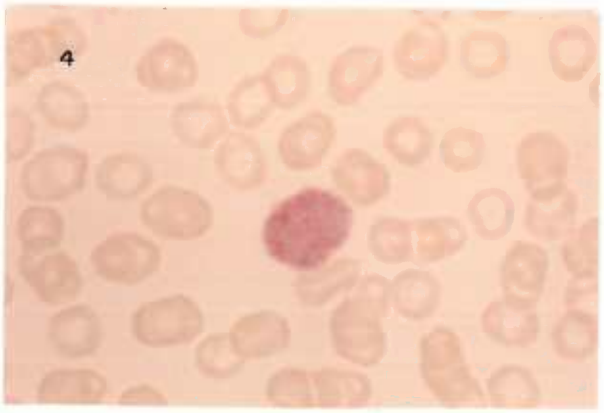
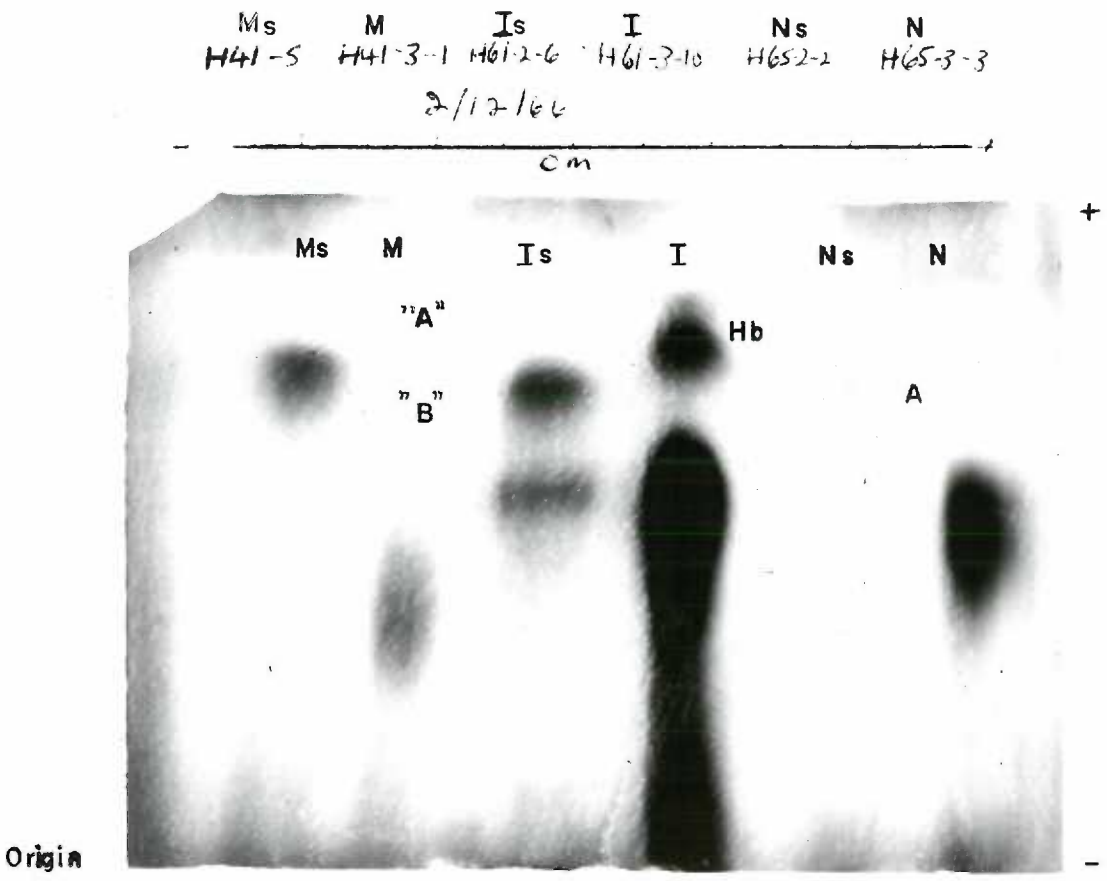

4

Figure 5. Comparison of serum and neutrophilic alkaline phosphatase from mongoloid (H41), infected (H61) and normal (H65) individuals. In all three instances, the "A" band of serum migrates more rapidly than the neutrophilic alkaline phosphatase. The "B" band, seen in H61-2, migrates with the main concentration of neutrophilic alkaline phosphatase from that patient (H61-3). The effect of over-loading the gel with too much enzyme is demonstrated in this Figure by comparing the phosphatase pattern from H61-3 to that of H65-3. Over-loading, with much trailing, is present in H61-3, while H65-3 was of sufficient concentration to show good localization of phosphatase activity. The mongoloid leukocytic phosphatase migrates in an intermediate position. In this and subsequent Figures, the origin and cathode of the gel are at the bottom of the picture and the anode at the top. Electrophoresis was at pH 8.6, 5 v/cm for 16 hours. Abbreviations: I-Infected individual, M-Mongoloid person, N-Normal subject, and P-Postpartum (pregnancy): Subscript "s" indicates serum.

Figure 6. Phosphatase activity from the leukocytes of one mongol (H47-3), two normal individuals (H60-4, H63-3) and two infected patients (H64-3, H70-3) which both show two bands of activity. In a previous gel the majority of enzyme of sample H70-3 was present in the faster migrating band but, for unexplained reasons, the majority of enzyme seen here migrates with the slower band. The normal serum "A" band is present in H60-2. Electrophoresis was at pH 8.6, 10 v/cm for three hours.


Ms M Is I Ns N
H41-5 H41-3-1 H61-2-6 H61-3-10 H65-2-2 H65-3-3
2/12/66
cm
+
Ms M Is I Ns N
"A"
Hb
"B"
A
Origin
-

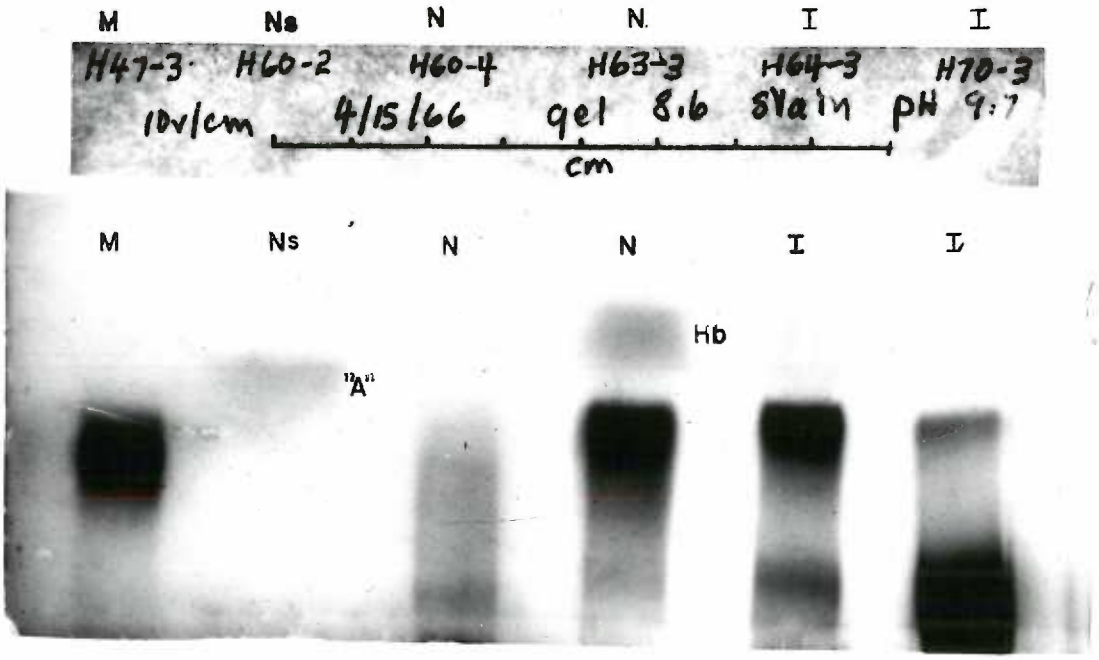
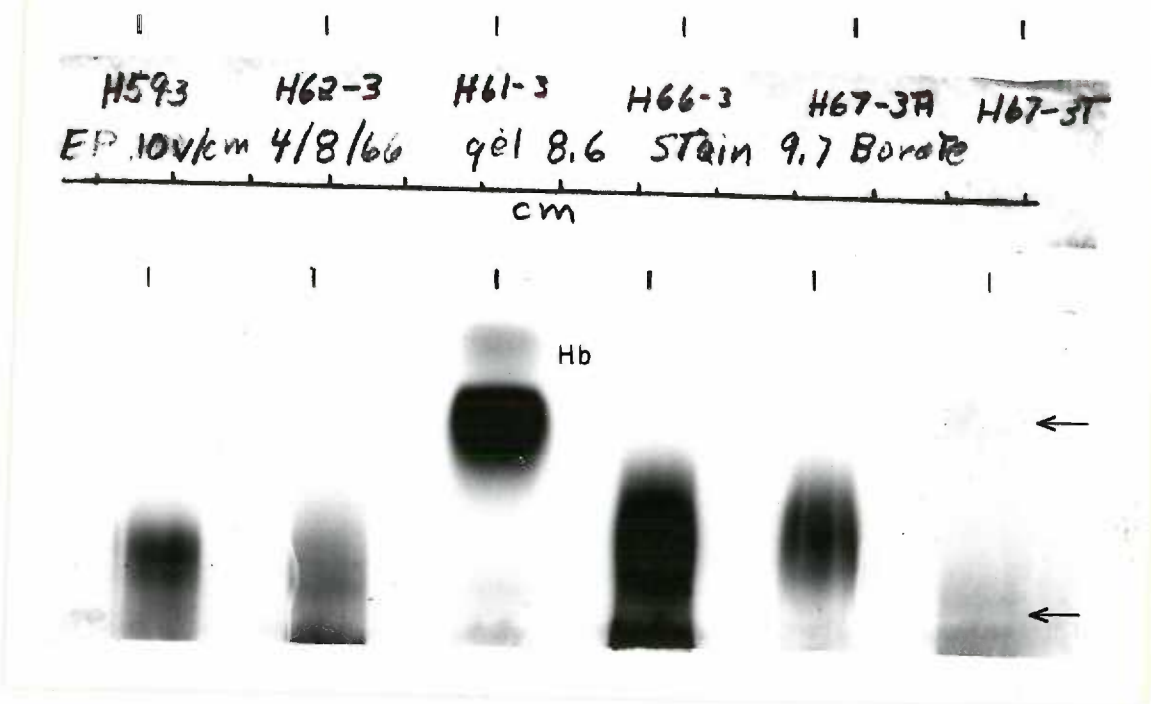

M Ns N N I I
H47-3 H60-2 H60-4 H63-3 H64-3 H70-3
10v/cm 4/15/66 gel 8.6 Stain pH 9.7
cm
M Ns N N I I
Hb
"A"

Figure 7. Neutrophilic phosphatase patterns from infected individuals. Samples H61-3 and H66-3 are from the same individual (H61-3 was obtained at the height of the infection and H66-3 was drawn two months later when the infection was almost gone). Samples H67-3A and H67-3T are also from the same individual. Samples H61-3 and H67-3T were homogenized in 0.9% saline using a Vir-Tis homogenizer, while H66-3 and H67-3A were homogenized with 1:20 Tris-alanine-citrate buffer in a glass homogenizer. H67-3T activity does not appear distinctly in this photograph but was composed of two bands, one migrating near the origin and the other migrating near the borate line (arrows). Sample H59-3 and H62-3 are both slow migrating alkaline phosphatases and were homogenized in 0.9% saline in a Vir-Tis homogenizer. Electrophoresis was at pH 8.6, 10 v/cm for three hours.

Figure 8. Comparison of the migration of neutrophilic phosphatase from mongols (H39, H43, H46 and H47) to that of an infected individual (H61), a normal person (H69) and of a lady who just delivered an infant (H72). Samples H39, H46, and H72 have intermediate bands, while H43 and H47 are fast, but not as fast as H61 and H69. Electrophoresis was at pH 8.6, 10 v/cm for three hours.


H593 H62-3 H61-3 H66-3 H67-3A H67-3T
EP 10v/cm 4/8/66 gel 8.6 Stain 9.7 Borate
cm
Hb

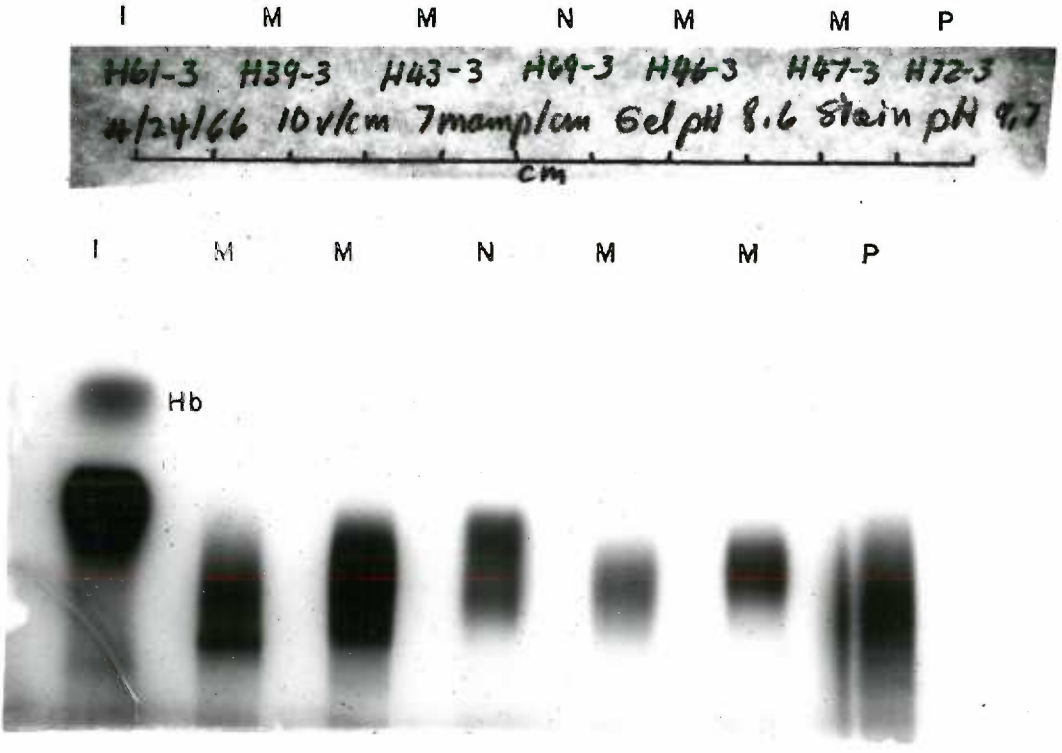
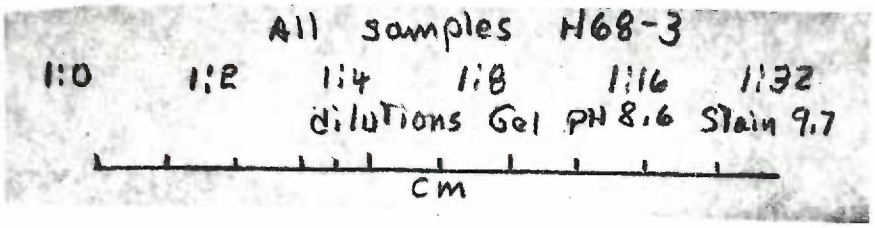
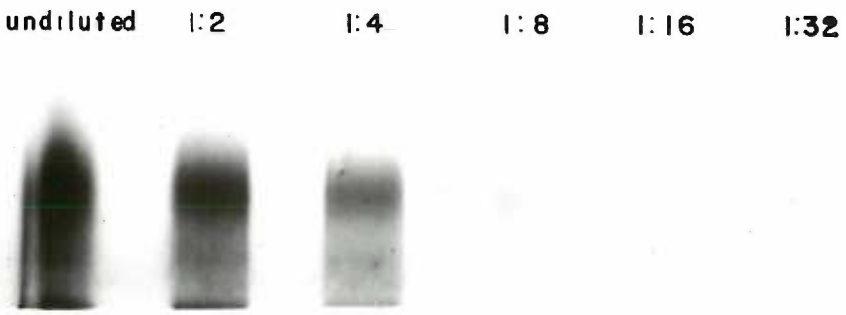

I M M N M M P
H61-3 H39-3 H43-3 H69-3 H46-3 H47-3 H72-3
4/24/66 10v/cm 7mamp/cm Gel pH 8.6 Stain pH 9.7
cm
Hb

Figure 9. Electrophoresis of leukocyte phosphatase from a postpartum patient showing how enzyme concentration effects the migration pattern. The undiluted sample (1:0) which is an over-load, gives much trailing and poor localization of the enzyme, while the 1:4 dilution gives a distinct band with little trailing. The undiluted sample has an activity of 220 units, while the 1:4 dilution has an activity of about 60 units. Electrophoresis was at pH 9.0, 5 v/cm for six hours.

Figure 10. Protein patterns of leukocyte extracts from normal (H69), infected (H61) and mongoloid (H39, H43, H46 and H47) individuals after starch gel electrophoresis (three hours at pH 9.0 and 10 v/cm). All samples except H72-3, which has only a faint staining 5 band, contain bands 1 and 5, while band 2 appears only in the normal and infected individuals, and bands 3 and 4 only in mongols. Other than bands 1 and 5, H43 contains only band 3, H39 has only band 4, and H46 and H47 contain both bands 3 and 4. H61 also contains a hemoglobin band (Hb). Figure 10 is a protein stain of the other half of gel shown in Figure 8 for phosphatase activity.


All samples H68-3
1:0 1:2 1:4 1:8 1:16 1:32
dilutions Gel pH 8.6 Stain 9.7
cm


undiluted 1:2 1:4 1:8 1:16 1:32

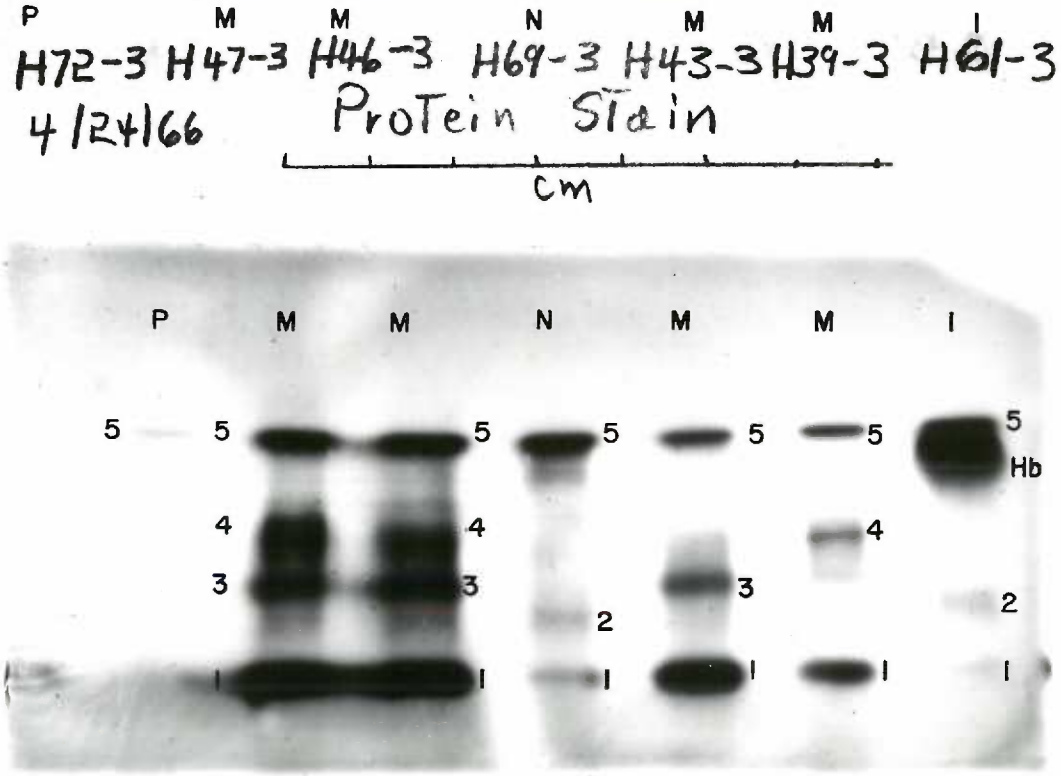
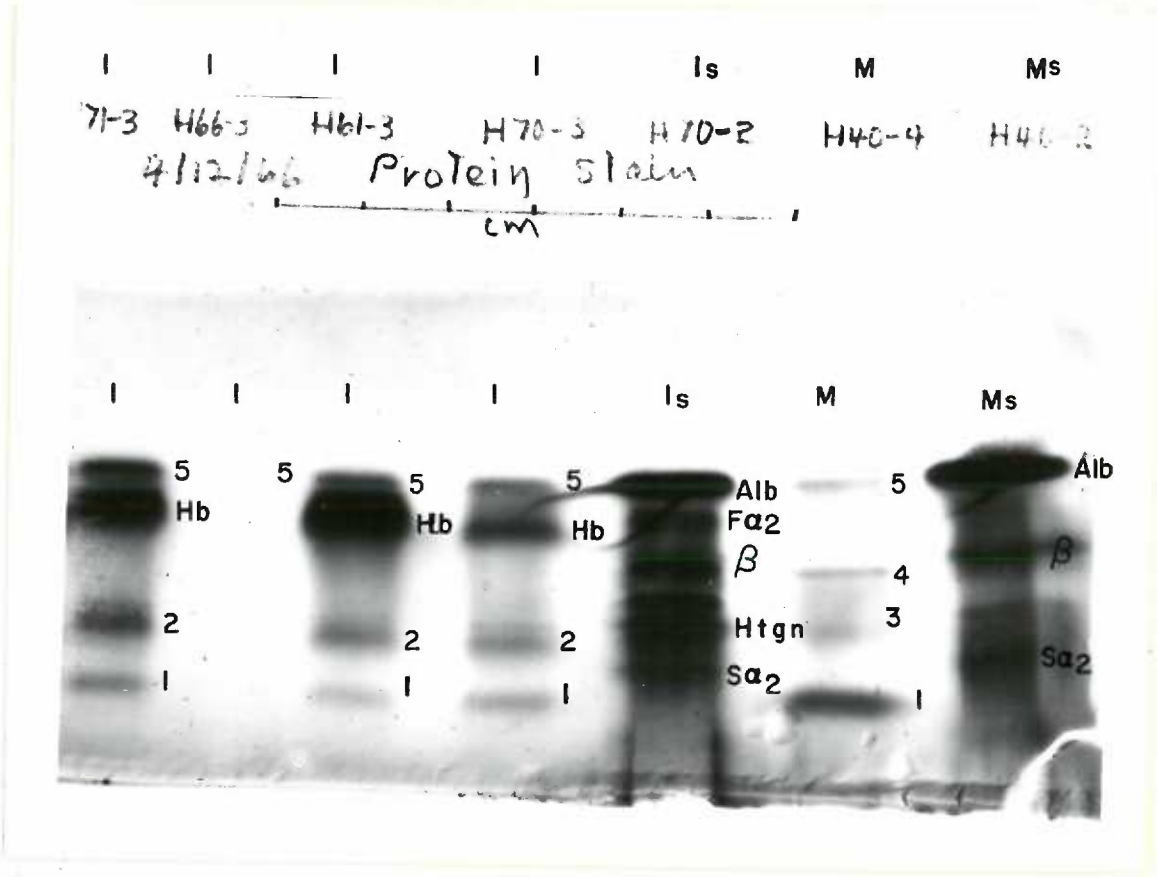

P M M N M M I
H72-3 H47-3 H46-3 H69-3 H43-3 H39-3 H61-3
4/24/66 Protein Stain
cm
P M M N M M I
5
4
3
2
1
Hb

Figure 11. Protein patterns in infected and mongoloid patients. Sample H61-3 is a white cell extract from an infected patient at the height of the infection and sample H71-3 is from the same patient but when he is well. Protein patterns in both samples (H61-3 and H71-3) are identical. Sample H70 is another infected individual, and like H61-3 and H71-3, shows only bands 1, 2 and 5. H40-4 is a mongol showing bands 1, 3, 4 and 5. Band 2 appears to migrate in the haptoglobin region while band 4 migrates with the β-globulin fraction of serum. Electrophoresis was at pH 8.6, 10 v/cm for about three hours. Abbreviations: Alb-Albumin, Fα2-Fast alpha two-globulin, β-Beta-globulin, HTgn-Haptoglobin, Sα2-Slow alpha two-globulin and β-lipo-Beta-lipoprotein.

Figure 12. Comparison of the protein patterns of a leukocytic extract from a mongol (H43-3) with her serum pattern (H43-2) and the serum pattern from a postpartum patient (H72-2). Band 1 migrates between the β-lipoprotein and the Sα2 fractions of serum, band 3 migrates in the haptoglobin region and band 5 corresponds to the albumin fraction. White cell extracts from H72 are not of sufficient concentration to stain for proteins. Serum protein patterns are labeled according to Chiandussi et al. (22). Electrophoresis was at pH 8.6, 10 v/cm for three hours.


I
I
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Is
M
Ms
H71-3
H66-3
H61-3
H70-3
H70-2
H40-4
4/12/66 Protein Stain
cm
I
I
I
I
Is
M
Ms
5
Hb
2
1
Alb
Fα2
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Sα2
4
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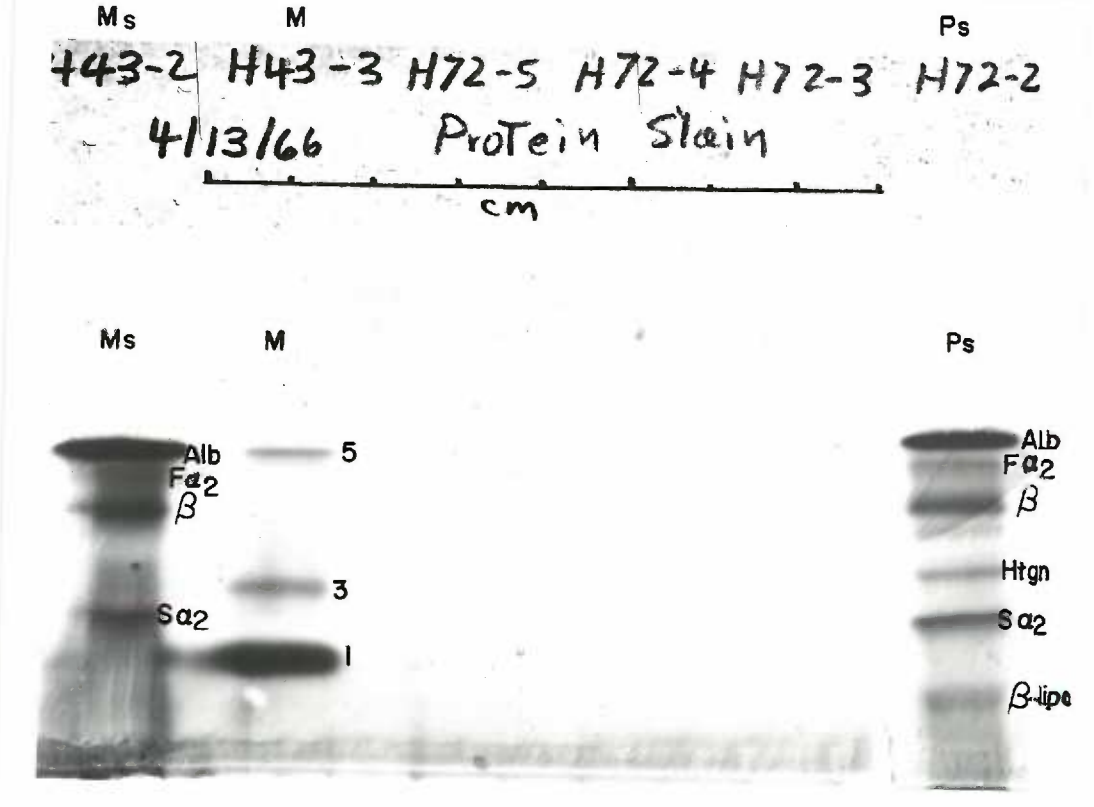
Ms
M
Ps
H43-2
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H72-5
H72-4
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Htgn
β-lipo

phosphatase activity seen in neutrophils when Kaplow's method (51) is employed. Activity was not seen in lymphocytes, monocytes, eosinophils, basophils or erythrocytes.

Gel Electrophoresis of Leukocytic Alkaline Phosphatase

Figures 5 through 9 show typical migration patterns for leukocytic alkaline phosphatase. No characteristic isozyme pattern was observed for any group of patients studied except possibly in those persons with mongolism. Slow, intermediate and fast migrating bands were found for normal and infected individuals (Figure 7), and intermediate and fast were observed for the two postpartum patients (Figures 8 and 9). Several mongols (H38, H39, H40, H41, H45 and H46) possessed slow-intermediate bands while other mongols (H43 and H47 in Figure 8) possessed bands that migrated faster but would still be classed as "intermediate" and which never migrated as rapidly as the "fast" type (H61 and H65 in Figure 8). All leukocyte samples gave just one band of activity with the exception of three individuals who each possessed two bands of activity (H64-3 and H70-3 in Figure 6, H67-3T in Figure 16).

Protein Patterns

Figures 10 and 11 demonstrate the protein patterns found in various leukocytic extracts. As indicated in these Figures, five distinct bands can be observed. Table III lists the frequency of these bands. Serum patterns and a leukocytic extract pattern can be compared in Figure 12.

TABLE III

Occurrence of Leukocytic Protein Bands

Number of Patient	Clinical Condition	Bands Observed
H69	Normal	1,2,5
H71*	Diabetes	1,2,5, Hb
H70	Infection	1,2,5
H61*	Infection	1,2,5, Hb
H39	Trisomy-21	1,4,5
H40	Trisomy-21	1,3,4,5
H43	Trisomy-21	1,3,5
H44	Trisomy-21	1,3,(4?),5
H45	Trisomy-21	1,3,4,5
H46	Mosaic Mongol	1,3,4,5
H47	Trisomy-21	1,3,4,5

*H61 and H71 are samples from the same patient, but taken at an interval of three months.
Hb indicates that a hemoglobin band was present.

Trailing

Trailing is defined as the smearing or spreading of enzyme activity in the gel and was a major obstacle in this study. Figures 5 (H61-3) and 9 (undiluted sample) demonstrate trailing. The most effective means of minimizing trailing were found to be: limiting the enzyme concentration to between 20 and 40 units per load, running the gels at 10 v/cm instead of 5 v/cm, and allowing the front created by the migration of borate ions (borate line) to move only 4 cm past the origin instead of 8 cm. The improved resolution can be seen for sample H61-3 in Figure 7.

Detection of Enzyme Activity

For good visualization of enzyme activity on starch gels, 20 units of alkaline phosphatase was found to be necessary. In the case of sera, where the activity was usually about 5 units, the 20 unit minimum was achieved by using four pieces of No. 1 Whatman filter

paper. Leukocytic extracts with activity greater than 20 units required only one piece of filter paper. Optimum enzyme localization occurred with about 40 units of enzyme per sample. After the leukocytic extract was concentrated, it was diluted to 40 units if it possessed a greater activity.

To be able to convert from units determined by the fluorometric procedure defined in this paper into standard units (King-Armstrong), activities of the same serum sample for three patients were determined by the fluorometric method and by the method of King and Armstrong (56) with the Auto-analyzer in the Clinical Pathology Department. The ratios of fluorometric units to King-Armstrong units for sera H65, H70 and H71 were calculated to be 1.25, 1.56 and 1.52 respectively.

Effect of pH on Alkaline Phosphatase Migration

Various gel and electrode buffer pHs were tested and found to have very little effect on the relative migration rates of white cell alkaline phosphatases or to cause significant loss of resolution.

DISCUSSION

Discussion of Results

Leukocyte Alkaline Phosphatase Isozymes

Leukocyte alkaline phosphatase activity is elevated in infections (50, 51, 129, 134, 137), mongolism (2, 3, 71, 90, 108, 127), myeloproliferative diseases (50, 51, 58, 129), "stress" (52, 130, 133) and pregnancy (24, 44, 96, 97), and depressed in chronic granulocytic leukemia (10, 51, 134). The extra chromosome 21 found in mongolism (associated with an increase in leukocyte phosphatase) and the partially deleted chromosome 21 found in patients with chronic granulocytic leukemia (associated with a decreased leukocyte phosphatase activity), has led several investigators (3, 57, 125, 127) to suspect that a gene is present on chromosome 21 which controls or regulates the production of alkaline phosphatase. This hypothesis and most other information about leukocytic alkaline phosphatase activity has been based on histochemical techniques, such as the one described by Kaplow (51) or on white cell incubation procedures, such as the one developed by Valentine and Beck (129).

Until recently, no one had studied starch gel electrophoretic patterns of alkaline phosphatase of leukocytes in mongolism, chronic granulocytic leukemia or other clinical conditions where the phosphatase activity deviates from that found in normal individuals. Within the last year, however, Robinson et al. (101) have reported isozyme patterns for normal individuals, and patients with acute or chronic granulocytic leukemia. For seven normals, they found two bands of alkaline phosphatase activity at one and three cm from the origin when the borate line

had moved eight cm past the origin (they mentioned no variation among their controls). In four patients with chronic granulocytic leukemia, of whom three had received treatment for their disease, they found one intense band at two cm and three faint bands at 0.4, 1.0 and 5.0 cm. Only two of their cases revealed Philadelphia chromosomes. Leukocyte extracts from four cases of acute granulocytic (myelogenous) leukemia revealed essentially the same pattern as that observed in normals, except for a slight retardation of the mobility of the three cm band. From their data, they could neither substantiate or refute the hypothesis of genetic control of leukocyte alkaline phosphatase by a gene on the 21st chromosome but they suggested that the normal mechanism for controlling alkaline phosphatase is either altered or suppressed in chronic granulocytic leukemia.

The neutrophil isozyme patterns reported in this thesis do not resemble those described by Robinson et al. Most individuals in this study (see Table I) possessed only one alkaline phosphatase band which migrated at either a fast, slow or intermediate rate. Three infected patients (H64-3, H70-3 in Figure 6 and H67-3T in Figure 7), however, possessed two isozymes. In these cases, the slowest migrating band corresponds approximately to the two cm band of Robinson's chronic granulocytic leukemics, but the faster band does not correspond to any of the isozymes reported by them. Slowly migrating isozymes were also seen in two other infected patients (H59-3, H62-3, in Figure 7) and one normal individual (H60-3 in Figure 6). The fact that a second slowly migrating band appeared in some infected cases (H64, H67, H70) and that it occurred more frequently in infected individuals than in other clinical conditions, suggests that infection may stimulate the produc-

tion of a second type of alkaline phosphatase. Sample H61-3 in Figure 7 was collected when the patient was infected and sample H71-3 (not pictured) was obtained after recovery from his infection. Both samples contained alkaline phosphatase isozymes with identical electrophoretic patterns. This data tends to refute the above hypothesis both because there is no slow phosphatase band in this patient and because there was no change in the isozyme pattern from the infected to the non-infected state.

Although infected individuals commonly possessed a slow migrating isozyme, both intermediate (H66-3, H67-3A in Figure 7) and fast types (H61-3 in Figures 5, 7 and 8, H64-3 and H70-3 in Figure 6 and H67-3T in Figure 7) were also occasionally observed. The intermediate migrating alkaline phosphatase in these cases was extracted by Robinson's method (101), while leukocyte alkaline phosphatase from these same cases, extracted by the method developed in our laboratory, possessed a fast band in H61-3 and both a slow and a fast band in H67-3T. This indicates that the method of extraction may influence the migration pattern of the phosphatase.

Alkaline phosphatase patterns in eight mongols differed from those in other clinical conditions, in that an atypical intermediate band appeared (H41-3 in Figure 5, H39-3, H46-3 in Figure 8). The other two mongols (H43-3 and H47-3 in Figure 8) had "fast-intermediate" isozymes which migrated just behind but were distinct from the "fast" isozymes (H61-3 and H69-3 in Figure 8). The preponderance of the atypical intermediate migrating enzymes in mongols suggests that mongolism may, in some way, alter the slow or fast migrating alkaline phosphatase or replace it with another type.

Three of five normal individuals (H63-3 in Figure 6, H65-3 in

Figure 5 and H71-3) possessed fast migrating phosphatase bands which, again, did not correspond to any isozyme described by Robinson et al. The mosaicism found in mongols H41, H42, and H46 did not seem to influence their isozyme patterns, the white cell protein patterns or the neutrophil alkaline phosphatase scores.

Serum Alkaline Phosphatase Isozymes

Considerable alkaline phosphatase activity is normally present in serum in a dynamic steady state which is presumably controlled by the production and release of alkaline phosphatase by bone and its removal from serum by the liver (41, 121). Other enzymes, such as lactic dehydrogenase, glutamic-oxalacetic transaminase and amylase, are not normally found in the serum in large quantities, but become elevated when there is necrosis or trauma of the tissue in which they are found (76). The appearance of these enzymes in the blood stream is generally associated with death of the cell and subsequent release of the intracellular enzymes which then gain entrance into the blood. One might then reason that destruction of neutrophils might, by the same mechanism, contribute to the serum alkaline phosphatase. The isozyme patterns obtained in this study indicate, however, that serum and leukocytic alkaline phosphatase are distinctly different as can be seen in Figure 5. Each serum in Figure 5 (H41-2, H61-2 and H65-2) contains the common "A" band of Arfors et al. (5) which migrates more rapidly than the leukocyte phosphatase band and corresponds to the beta-globulin fraction of serum proteins. All other sera examined also contained the "A" band of leukocyte phosphatase. In sample H61-2 of Figure 5, the "B" band, which migrates more slowly than the "A" band, is also present and, in this case, corresponds to the migration of the

leukocyte enzyme from that patient. This was also true for the "B" band of patient H43, but not for patient H35 whose leukocytic alkaline phosphatase migrated more slowly than the "B" band. The "B" band is thought to be derived from the intestine (46, 86), and was not observed in most normal persons studied for the thesis.

As discussed in the preceding section, homogenization has an effect on the migration pattern of leukocyte phosphatase. To test the effects of homogenization on serum alkaline phosphatase, serum from individual H69 was homogenized and extracted with butanol by the method developed in our laboratory. After such treatment, the alkaline phosphatase migration pattern is unaffected, thus indicating that the homogenization procedure does not account for the observed differences between the isozyme patterns of the serum and leukocytes.

From the above findings, it can be stated with reasonable assurance that serum alkaline phosphatase is not derived from leukocytes and that the serum is not the source of leukocyte alkaline phosphatase.

Leukocytic Protein Patterns

Apparently there is no published information describing the starch gel electrophoretic pattern of human leukocyte proteins. The absence of literature on this subject may reflect the difficulty in visualizing leukocyte proteins due to their very low concentration in white cell extracts. The ability to concentrate leukocyte proteins was greatly facilitated by ultrafiltration, i.e. vacuum dialysis (119), which permits the rapid concentration of proteins without heating or freezing the sample.

Protein patterns shown in Figures 10, 11 and 12 represent proteins found in the leukocyte extracts prepared for alkaline phosphatase

studies and, in each case, the opposite half of the gel was stained for alkaline phosphatase. However, Figures 8 and 12 are the only complementary gels shown in this paper.

As can be seen in Figure 10, there are five distinct protein bands in the white cell extracts which are labeled one through five, and a distinct hemoglobin band. It can be seen that bands 1 and 5 occur in all samples except H72-3 which has only band 5 (H72-3 was likely not sufficiently concentrated), that bands 3 and 4 are found only in mongols (H39, H43, H46 and H47), and that band 2 appears only in non-mongols (H61 and H69). Bands 3 and 4 seem to occur in a distinct pattern in that patient H43 possesses only band 3, patient H39 possesses only band 4, and patients H46 and H47 possess both band 3 and band 4. These data suggest that mongols have a different type of leukocyte protein than normal individuals and that they may represent the enzymes known to occur in excess in mongolism (17, 18, 71, 108). It is also possible that a genetic mechanism, such as a polymorphism, is controlling the occurrence of bands 3 and 4. All of these protein bands were seen best in samples that received insufficient heparin and had some clumping of the white cells, and may represent thrombin, fibrin, or some other artifact.

Leukocyte Alkaline Phosphatase Kinetic Studies

Moss and King (85) have reported Michaelis constants (Kms) of 6.7 x 10^{-5} to 11.8 x 10^{-5} M (β-naphthyl phosphate) for alkaline phosphatase isozymes from different organs (kidney, liver, bone, and intestine). In conjunction with this thesis, Dr. Richard Lyons and Mr. James Beck have determined Km values for serum and leukocytic alkaline phosphatases using the same substrate and buffer. Preliminary results show a serum

Km of 6.24×10^{-5} M (sample H61-2), leukocyte "intermediate" phosphatase Km of 4.82×10^{-5} M (sample H67-3A) and a "fast" phosphatase Km of 3.46×10^{-5} M (sample H72-4). The differences between serum and leukocyte intermediate and fast phosphatase Km values appear to be significant. This is additional support for the idea that the various leukocyte bands demonstrated on starch gel are actually different isozymes and not merely artifacts due to the methods employed in this study.

Discussion of Materials and Methods

Enzyme Resolution

One of the major difficulties encountered in this study was the problem of good enzyme localization (resolution) on the starch gels. Earlier zymograms revealed poor localization and, in an attempt to resolve this difficulty, numerous factors were investigated. These included ultracentrifugation at 100,000 g[(a)] to remove microsomes, different grades of filter paper, different gel and electrode buffer pHs, freezing and thawing the leukocytes, and homogenization of the leukocytes in a glass homogenizer. None of these procedures improved the resolution. It was then found that by restricting enzyme loads on the gels to about 40 units of activity, by using gel voltages of 10 v/cm, and by allowing the borate line to migrate only 4 cm past the origin[(b)], more exact localization of enzyme could be achieved (compare H61-3 in Figure 5 with the same sample in Figure 7). Restricting the enzyme load prevents exceeding the carrying capacity of the starch gel, a higher voltage results in a more uniform migration of proteins and a

[(a)]Suggested by Dr. Elliot Vesell, personal communication
[(b)]Suggested by Dr. J. C. Robinson, personal communication

shorter total electrophoretic migration gives a more narrow, concentrated band of activity. Even with the improved resolution, achieved with the above technique, some trailing persisted (see sample H63-3 in Figure 6). Chilson et al. (23) have reported marked effects on both enzyme activity and electrophoretic mobility after freezing and thawing. This latter effect on alkaline phosphatase mobility has not been reported and, due to a lack of time, it has not been investigated in this thesis.

Staining for alkaline phosphatase in gels at a pH of 8.6 caused significant background staining which interfered with the quality of photographs obtained. With the dye solution recommended by Boyer (14), which has a pH of 9.7, gels did not develop as much background stain and resulted in photographs with much higher contrast (compare Figures 5 and 6: gel in Figure 5 was stained at pH 8.6 and the other gel was stained at pH 9.7).

Heparin

Late in this study, it was discovered that the earlier blood samples (H35, H36, H38 to H47, H69 and H71) had not received enough heparin. They had received one unit per ml of blood, which apparently was enough to prevent gross coagulation of the blood, but not enough to inhibit white cell clumping. Such treatment did cause a decreased yield of white blood cells as can be seen with sample H71-3, Table II, which received an insufficient amount of heparin (1 unit per ml), resulting in a yield of only 22%. Other samples in Table II received adequate heparin (3 units per ml) and had correspondingly higher yields. For ideal anti-coagulation with heparin, Miller (76) recommends 10 units per ml of whole blood.

Alkaline Phosphatase Histochemical Scores

Kaplow's histochemical technique (51) for demonstrating neutrophil alkaline phosphatase activity proved to be a simple and relatively reliable procedure. Scoring of the neutrophils, however, was time consuming and liable to considerable subjective error. Figures 1 through 4 illustrate the various classifications of enzyme activity. The average neutrophil scores were 45 for healthy controls, 85 for mongols, 145 for infected individuals, and 167 for postpartum persons. In the small sample size available, it is difficult to draw any conclusion regarding activity in these conditions, but the differences have been well documented by others (see Introduction).

The histochemical score of neutrophil alkaline phosphatase has been shown to have no correlation with the white cell count or the serum alkaline phosphatase activity (50, 77, 78, 129). These studies are further confirmed by comparing the white blood count, serum phosphatase level and neutrophil score of various patients listed in Table I. In particular, patient H70 had a severely infected leg, a normal white count, and a high neutrophil phosphatase score. The high phosphatase score was probably related to both the infection and the steroid which he was receiving. This case demonstrates that neutrophil alkaline phosphatase may increase independently of the white cell count and serum activity.

Fluorometric Assay

Fluorometry was found to be a reliable and sensitive technique for the quantitative determination of alkaline phosphatase activity. The method is reliable if uniform conditions are maintained, and is so sensitive that the alkaline phosphatase activity in twenty lambda (0.02 ml)

of serum could be accurately measured. Difficulty in assaying activity was encountered when the leukocyte extract was contaminated with hemoglobin. Hemoglobin apparently interfered with the absorption of exciting light by the substrate, caused quenching of fluorescence, or caused excess light scatter, thus reducing the amount of detectable phosphatase activity. No solution to this difficulty was found other than further dilution of the hemoglobin. Fluorometry was performed mainly for dilution of the enzyme to about 40 units of activity in an effort to achieve better gel resolution.

For a more complete study, protein determinations of the leukocyte extracts would be desirable. However, only the relative enzyme activity was necessary to determine the amount of enzyme to load on a gel. It would still be possible to obtain protein concentrations on most of the samples if knowledge of absolute enzyme activity were desired.

SUMMARY

Alkaline phosphatase isozymes have been demonstrated to be present in neutrophils. Fast, intermediate and slow migrating isozymes were possessed by normal and infected individuals. Intermediate migrating isozymes predominated in mongols.

Serum alkaline phosphatase was found to migrate faster than leukocyte alkaline phosphatase in starch gels suggesting that leukocytes do not contribute to alkaline phosphatase activity in serum.

Leukocyte extracts were found to contain five protein bands, two of which occurred only in mongols, and one of which appeared only in non-mongols.

Serum alkaline phosphatase activity, alkaline phosphatase activity of leukocyte extracts and leukocyte phosphatase histochemical activity for most of the individuals studied were presented and their significance was discussed.

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