A BIOCHEMICAL COMPARISON OF ALKALINE PHOSPHATASE ACTIVITY IN THE FETAL THYMUS WITH THAT OF NORMAL AND LYMPHOMATOUS TISSUES

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BY
RACHAEL ALLEN FLOYD

DEPARTMENT OF BIOLOGY

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ABSTRACT

FLOYD, RACHAEL ALLEN

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A Biochemical Comparison of Alkaline Phosphatase Activity
in the Fetal Thymus with that of Normal and
Lymphomatous Tissues

Advisor: Dr. Judith Rae Lumb

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Although the association of alkaline phosphatase
(APase) with murine fetal thymus up to 16 days gestation
and murine thymic lymphoma has been established, very
little work has been done to determine if the murine
lymphoma APase is similar to the fetal thymus APase. In an
effort to determine the nature of the relationship a bio-
chemical characterization was performed. The placenta and
spleen were also included since previous biochemical exper-
iments had shown lymphoma APase similar to these APases.
The parameters included pH optimum, heat inactivation, sub-
strate ratios, Michaelis constant, activation and
inhibition. In addition, the electrophoretic mobility was
determined in the presence and absence of neuraminidase using the substrates alpha-naphthyl phosphate (NP) and p-nitrophenyl phosphate (pNPP). The results indicate that the lymphoma APase is very similar to fetal thymus, placenta, and spleen APases. All APase activities had a pH optimum of 10. Approximately 55% of the activity was inactivated at 55°C in four minutes. The APase activities were all inhibited by ethylenediamine tetra-acetic acid, L-homoarginine, and L-phenylalanine at the level of 100, 80 and 7.71% respectively. Magnesium activated each APase activity by approximately 46%. Furthermore, the nucleophilic buffers diethanolamine, 2-amino-2-methyl-1,3-propanediol, and tris(hydroxymethyl)aminomethane activated the APase activities. The electrophoretic mobility of all APases was similar and neuraminidase slowed the migration of these APase activities. The localization of each APase was identical with the substrates NP and pNPP. These results lend support to the hypothesis that the APase activity which appears in thymic lymphoma might represent a derepressed embryonic function. Thus, the murine thymic lymphoma APase may be termed a cell membrane-bound carcino-fetal enzyme.
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CHAPTER I

INTRODUCTION

The association of alkaline phosphatase (APase) with murine fetal thymus up to 16 days gestation and with murine lymphoma has been established. Murine lymphoma APase activity has been previously compared with that of several normal tissues in addition to the APase activity in the whole fetus and placenta at 16 days gestation. Because the lymphoma APase was found to be similar to that of the spleen and placenta APases, it was suggested that the APase of lymphoma may represent a derepressed embryonic function. However, to establish this, it is also necessary to compare the lymphoma APase activity with the APase activity of the fetal thymus.

The sensitive fluorometric assay for APase has made such a comparison possible. The fluorometer has the advantages of being extremely sensitive and specific when compared with a spectrophotometer. Fluorescence is a direct measure of the absorbed light whereas the spectrophotometer measures the absorbed light indirectly as the difference between the reference or 100% beam and the
transmitted beam. When the spectrophotometer is used to measure low concentrations, the inaccuracies of measurement approach this difference and cannot be measured.

The goal of this research was to support or refute the hypothesis that the APase activity which appears in thymic lymphoma might represent a derepressed embryonic function by biochemical comparisons. The substrate alpha-naphthyl phosphate (NP) was chosen not only because it gives a fluorogenic product but because it is the primary substrate used to correlate the histochemical association of APase in the murine fetal thymus and murine lymphoma.

The biochemical parameters investigated in this work were pH optimum, heat inactivation, substrate ratio, Michaelis constant \((K_m)\), activation and inhibition. In addition, electrophoresis was performed in the presence and absence of neuraminidase with the substrates NP and p-nitrophenyl phosphate (pNPP). The tissues used in this comparison were the fetal thymus, the placenta, the spleen and lymphomatous tissues. The placenta and spleen were used since previous experiments had shown the APase activity in these tissues to be similar to the APase activity in the lymphoma.
CHAPTER II

LITERATURE REVIEW

Alkaline Phosphatase

In vitro experiments show that alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) catalyzes two basic types of reactions. The first type involves the hydrolysis of monoesters of orthophosphoric acid, thiophosphoric acid, pyrophosphate, fluorophosphate and creatine-N-phosphate. There are systems in which APase hydrolyzes monoesters of orthophosphoric acid at a level two times greater than the monoesters of thiophosphoric acid (Neumann et al., 1971, 1974; Fernley, 1971). In contrast, there are reports of APases which have the unique capacity to differentiate between oxygen and sulfur links between the alcohol moiety and the phosphoryl group. This indicates that the phosphorothioate esters are not hydrolyzed in mammalian systems (Eckstein, 1966; Neumann, 1968; Fernley, 1971). In the second type of reaction APase acts as a phosphotransferase in which the phosphoryl group is transferred to an acceptor molecule without the appearance of inorganic phosphate (Axelrod, 1948; Morton,
APase is found in a large number of species. Non-mammalian sources include yeasts (Parish and Pelli, 1974), bacteria (Heppel, 1971), echinoderms (Hsiao and Fjuii, 1963), insect larvae (Beckman and Johnson, 1964), annelids (Bhoomittra, 1964), teleosts and elasmobranches (Bodansky et al., 1931), amphibians (Licht, 1964), reptiles (Richards et al., 1965) and birds (Motzok and Branion, 1959). Most mammalian species have been shown to possess APase in tissues, blood, or secretions. The main mammalian tissues with high APase levels are those concerned with active transport mechanisms such as the intestine and kidney.

The ultrastructural localization of APase in E. coli is in the periplasmic space between the outer and inner cell membrane (Heppel, 1971). In Dictyostelium discoideum APase is in part associated with the cell surface (Parish and Pelli, 1974). The intestinal APase is on the microvillar membranes of the absorbing epithelial cells of the intestinal mucosa. The reaction is also prominent in the Golgi zone, the smooth reticulum cisternae, and in the dense bodies (Hugon and Borgers, 1966). Likewise, the liver APase appears on the microvilli of the bile
canaliculi, but it does not extend into the intracellular spaces beyond the terminal bar (Fishman and Lin, 1973).

Hulstaert et al. (1973) localized placental APase not only along the plasma membrane lining the microvilli and in the vesicles of the syncytiotrophoblast but also along the basal surface of the syncytiotrophoblast. APase lies in the microvilli of the proximal tubular cells and in the whole cell surface membrane bordering intercellular spaces in the kidney (Goldfisher et al., 1964; Molbert et al., 1960; Schmidt and Dubach, 1972).

The subcellular localization of APase activity in HeLa cells is somewhat controversial. There are reports of nuclear localization (Cox et al., 1967; Spencer and Macrae, 1972) and of plasma membrane localization (Bosman et al., 1968). Recently, Singer et al. (1974) showed ultrastructural evidence for APase activity localized on the plasma membrane in HeLa cells.

In their ultrastructural studies on Regan and non-Regan isoenzymes of APase in human ovarian cancer cells, Sasaki and Fishman (1973) correlated the ultrastructural localization of APase with amino acid inhibition pattern. In cases in which the Regan isoenzyme was present, the ovarian cancer cells had enzymatic activity on the inner
and outer membranes of the mitochondria which was sensitive to L-phenylalanine (L-phe). In cases in which the non-Regan isoenzyme was present, the cells had enzymatic activity deposited on the cell membrane. The cell membrane enzyme was also sensitive to L-homoarginine (H-arg) and resistant to L-phe. The significance of the localization in these cells is as yet not clear.

While APase activity has been seen at intracellular sites, it can be concluded that the majority of the diverse cell types studied show APase activity in the plasma membrane.

The biological role of APase in the cell is not yet understood and its substrates in vivo are not yet defined. Kay (1926) pointed out fifty years ago that the ability of APase to act upon various substrates did not necessarily indicate the function of this enzyme but might be merely the fortuitous result of a particular type of molecular configuration of fairly frequent occurrence in the cell. Posen et al. (1969) also noted that the function of APase may be unrelated to the hydrolysis of phosphate esters at high pH. In fact, it has been demonstrated that APases have their optimum reaction in an alkaline medium only at high substrate concentrations; at substrate concentrations
approaching the physiological levels of phosphoric esters in the cell, the pH optimum of the enzyme activity is close to the physiological pH values (Morton, 1957). Hence, the so-called APases can display almost their maximum enzyme activity under the conditions of pH and concentrations of phosphate esters in the cell.

The localization of APase suggests a function in transport, possibly of phosphate across the cell membrane. The work of Engström (1961) showed that inorganic phosphate can be incorporated into calf-intestinal APase, since radioactive phosphorylserine was isolated after incubation of the enzyme with radioactive inorganic phosphate. This reaction was inhibited by ethylenediamine tetra-acetic acid (EDTA), indicating that metal is also involved in this process. In 1964 Engström further demonstrated that bovine liver APase incorporated phosphate. Moog and Glazier (1972) also found that phosphate uptake by duodenal ring preparations is inhibited by either beryllium or L-phe, two strong inhibitors of intestinal APase. Other investigators have shown that APase may also function in the transport of fatty acids. For example, Rufo et al. (1973) and Linscheer et al. (1971) found enrichment with APase of the intestinal mucosa and
of the blood in rats ingesting fats. In human studies the thoracic lymph was rich in intestinal APase following the intake of fat (Madsen and Tuba, 1953; Keiding, 1964, 1966).

APase is also implicated in the hydrolysis of dietary sphingomyelin to ceramide and water-soluble compounds (Linscheer et al., 1971).

Several studies have shown that APase from different sources are inseparable from inorganic pyrophosphatase (PPase) activity (Anderson and Nordlie, 1967; Moss et al., 1967; Butterworth, 1968; Woltgens et al., 1971; Vreven et al., 1973). The ability to hydrolyze PPase is also demonstrated in highly purified human APase preparations (Moss et al., 1967; Cox et al., 1967; Harkness, 1968). Since pyrophosphate (PP) is a by-product of several biosynthetic reactions including deoxyribonucleic (DNA) and ribonucleic (RNA) synthesis, it has been suggested that PP might be the natural substrate of APase acting as a PPase. This could provide a mechanism by which the polymerization of nucleic acids would be irreversible (Cox et al., 1967; Slor and Bustan, 1973).

Fishman and Lin (1973) used Streptococcus faecalis as a model system (Thompson, 1971) to explain how APase might act together with other membrane phosphohydrolases and
transferases to regulate the dimensions of the cell membrane. In this system, growth was essentially the result of interdigitation of cell wall synthetase and hydrolase activities with an equilibrium in favor of synthetase. Imbalance in either of these two activities would lead to an increase or a decrease in the size of the cell membrane. By analogy, Fishman and Lin (1973) reasoned that the plasma membrane in mammalian cells has enzymes which function in a similar manner. This model provides a role for membrane enzymes such as APase in some of the basic physiologic functions of the membrane. For instance, the cleavage of membrane phosphate esters could open up the membrane, providing sites for polymer chain elongation catalyzed by the specific synthetase and resulting in the biosynthesis of the membrane. The hydrolase activity causing the opening of the membrane could also allow for the entry and exit of large molecules in and out of the cell.

Studies on the control of APase synthesis in vitro lend themselves to the postulation of a potential function of APase. Nitowsky et al. (1963) demonstrated that cells grown in media with increased osmolarity exhibited increased APase activity which is due to de novo synthesis. Griffin and Cox (1966) studied the mechanism of
prednisolone induction of APase in HeLa cell cultures. Their studies revealed that an elevation in APase activity by prednisolone required both RNA and protein synthesis. Melnykovych et al. (1967), using synchronized cultures, showed an increase in APase activity following completion of mitosis and then a decrease in APase activity during DNA synthesis. In addition, cyclic adenosine monophosphate (cAMP), dibutyryl adenosine monophosphate and 5-bromodeoxyuridine have been shown to induce APase activity requiring synthesis of both RNA and protein (Koyama and Ono, 1971, 1972; Koyama et al., 1972). Immunological methods showed that the amount of enzyme protein was not increased (Ghosh et al., 1972). Therefore, Cox et al. (1971) and Ghosh et al. (1972) postulated that a modifier substance was induced which interacted with APase, resulting in increased catalytic activity. Later, Melnykovych and Bishop (1973) found that prednisolone induction of a heat stable APase in HeLa cells was associated with an increase in glycogen, a phenomenon which could be mimicked by administration of cAMP. Further, cAMP has been shown to be involved in contact inhibition, in suppression of tumor growth, and in the control of the immune response (Otten et al., 1971; Ishizuha et al., 1971). There is, therefore, some indirect
evidence suggesting that APase may be involved with cAMP in the control of cell division.

Several possible functions of APase exists. It could act to transport molecules such as phosphate and oleic acid; as a PPase in nucleic acid synthesis; to regulate the dimensions of the cell membrane; or, in related functions with cAMP, in the control of cell division. It is known that APases from different organs are different. Thus, the function of APase is most likely organ specific. In addition, it is known that there is more than one APase in certain organs. This means that the function of different APases in a particular organ could reflect its histochemical localization in that organ or even its histo-chemical localization in the cells of that organ.

Heterogeneity of Alkaline Phosphatase

The evidence for the existence of species, organ and even tumor specific variants of APase appears to be well established. These isoenzymes differ in electrophoretic mobilities, heat stability, sensitivity to inhibition by amino acids and other chemical agents, pH optimum, $K_m$ values, and immunologic reactivity.

The APase of *E. coli* is a dimer containing two identical subunits, each with a molecular weight of 40,000
to 45,000 daltons (Rothman and Byrne, 1963). The enzyme contains four zinc atoms per molecule (Anderson et al., 1975), two of which are firmly bound and probably have structure preserving properties, whereas the others are important for the catalytic process (Simpson and Vallee, 1968; Reynolds and Schlesinger, 1969; Lazdunski and Lazdunski, 1969). Zinc may be replaced by cobalt, copper, cadmium, nickel, or manganese (Lazdunski et al., 1969; Csopak and Falk, 1970; Ahlers, 1974), but only the cobalt enzyme shows catalytic activity (Ahlers, 1974, LeVine et al., 1975). The enzyme molecule also contains 1.3 ± 0.3 gram atoms of magnesium (Anderson et al., 1975). Since the heat inactivation is decreased when magnesium ions are present in the assay, magnesium ions might have a structure-stabilizing effect (Ahlers, 1974). Anderson et al. (1975) demonstrated that magnesium serves to regulate the mode of binding of zinc and increases the APase activity.

There are three loci (pho A, pho R and pho S) involved in the synthesis of APase in E. coli (Bracha and Yagil, 1973). The locus pho A is the structural gene; while the loci pho R and pho S (pho S pho T) are the two regulatory genes (Bracha and Yagil, 1973; Kida, 1974).
Human placenta was shown by Ries (1937) to exhibit APase activity. This was confirmed later by Anagn and Matsudaira according to Aleem (1971) and by Ahmed and King (1960). Human placental APase as well as zebra, llama, alpaca, guanaco, and cow placenta APases are heat stable; whereas the mouse, rat, monkey, baboon, reindeer, hamster, rabbit, and dog placental APases are heat labile. In addition, the goat, saigo, and pig placental APases form a group with intermediate heat stability (Leroux and Perry, 1971).

Human placental APase differs from other human APases in a number of respects. The placental enzyme is more resistant to denaturation by physical or chemical agents. It has its own immunological characteristics (Fishman et al., 1968; Sussman et al., 1968). While placental and intestinal APases are inhibited by L-phe and are resistant to H-arg, they are distinguished by the neuraminidase effect. Intestinal APase is a nonglycoprotein; while placental APase is a glycoprotein (Ghosh and Fishman, 1968).

A number of isoenzymic forms of placental APase exist. Three common phenotypes, fast (F), slow (S) and intermediate (I), have been recognized by the mobilities of the single major enzyme zone characteristic of each (Thomas and
Moss, 1972). Genetic studies suggest that the phenotypes are due to the operation of three autosomal allelic genes controlling the production of F, S, and I phosphatase isoenzymes. F, S, and I appear in individuals homozygous for the respective phosphatase genes and phenotypes FS, FI, and SI (three further phenotypes) correspond to the heterozygous genotypes (Robson and Harris, 1967).

Little differences exist between these phenotypes except for the electrophoretic mobility. In terms of structure, they probably differ no more than a change in a single amino acid residue (Thomas and Moss, 1972). Nitration reduces considerably the degree to which placental APase is inhibited by L-phe (Sokolovsky et al., 1969; Thomas and Moss, 1972).

Harkness (1968) and Ghosh and Fishman (1968) were able to prepare placental APase of high purity. It has been found to be a zinc metalloenzyme. Zinc has a functional role in catalysis. Magnesium or cobalt are also required for activity. Like E. coli APase, placental APase is a dimer with each monomeric subunit having a molecular weight of 58,000 daltons (Gottlieb and Sussman, 1968). The neuraminic acid-containing nature of placental APase has been demonstrated by chemical means and the sialic acid
residues were shown to occupy terminal positions away from the active center of the enzyme. Removal of sialic acid affects the electrophoretic mobility but not the activity of the enzyme (Warnes, 1972). The high molecular variant (200,000 m.w.) can be converted to a lower molecular weight variant (70,000 m.w.) by storage for about four months while the process is reversible by equilibrium dialysis (Ghosh and Fishman, 1968).

Carcinoembryonic Antigens in Human Alkaline Phosphatase

Derepression of the genome of the cancer cell is a mechanism that is frequently used to explain the similarity of new enzymes which are appearing in an ever increasing number of cases of human cancers to enzymes of the embryonic sources. These enzymes have been referred to as carcinoembryonic antigens. There are also reports that the serum, as well as the cancerous tissue of human cancer patients, contain from 4.4 to 15% APase activity which is similar to the human placental APase activity (Fishman et al., 1968, 1968; Stolbach et al., 1969; Nathanson and Fishman, 1971; Cadeau et al., 1974). In order to further differentiate carcinoembryonic antigens, Fishman (1973) has suggested that the APases which resemble the placenta be called carcinoplacental antigens.
The classical carcinoplacental enzyme is the Regan isoenzyme which was first discovered in a male patient with bronchocarcinoma (Fishman et al., 1968). It is a heat stable, L-phe sensitive, neuraminidase cleavable APase with the same electrophoretic mobility as the placenta (Fishman, 1973). In fact, the Regan isoenzyme is considered to be the same as the placental APase isoenzyme. Thus, the name carcinoplacental is appropriate.

Another well documented carcinoplacental APase is the HeLa cell APase. This APase was originally obtained from a black female patient with cervical carcinoma. The TCRC-1 and the TCRC-2 sublines of the HeLa cells are of particular interest. The TCRC-1 produces the Regan isoenzyme and is induced by prednisolone. However, the TCRC-2 cell line produces an APase isoenzyme which differs from the Regan isoenzyme in that it is inhibited by H-arg, is heat labile and is not induced by prednisolone. Studies with these two sublines are providing useful information concerning the relationship between the presence of the Regan isoenzyme and chromosomal variations. Cox and McLeod (1964) suggested that APase production by cultured human cells could provide an enzyme marker which might be useful in studying regulation by mammalian genes. The biggest difference in
the two cell lines occurs in the group of small acrocentric chromosomes. Investigators have found that an increase in the number of small acrocentric chromosomes corresponds to an increase in APase specific activity (DeCarli et al., 1963; Santachiara-Benerecetti et al., 1967). On the basis of isoenzyme profiles of APase in prednisolone-treated human cell populations, Fishman and Singer (1975) predict that the Regan APase has a chromosomal locus different from that of the non-Regan APase.

Still other carcinoplacental APase enzymes include the Regan variant (Warnock and Reisman), the Nagao isoenzyme and the Cha Go cell line. The Regan variant was found in an hepatocellular cancer by Warnock and Reisman (1969). Observations by Warnock and Reisman (1969) revealed that the Regan variant was a moderately heat stable enzyme, inhibited by L-phe, had an electrophoretic mobility faster than the human intestinal APase, and was immunologically distinct from the fetal APase protein. Later Higashino et al. (1972) purified the enzyme and showed that it had some properties similar to the placenta, some similar to the liver and some unique. The unique properties were that it was inhibited by L-leucine and had a lower $K_m$ than the placental enzyme. Nakayama et al. (1970) isolated the
Nagao isoenzyme from a patient with pleural carcinomatosis. The Nagao isoenzyme has also been reported in the metastatic adenocarcinoma of the bile duct (Nakayama et al., 1971; Jacoby and Bagshawe, 1971). This isoenzyme is interesting because while it is identical to the placental isoenzyme immunologically and biochemically, it is inhibited by L-leucine and EDTA. The Cha Go cell line is an epithelial cell line which synthesizes chorionic gonadotropin in vitro. It was derived from the metastatic tissue of a patient with lung carcinoma (Ludueña et al., 1974). It is immunochemically, biochemically, and functionally very similar to the APase synthesized by the original tumor tissue and by the normal placenta.

There is abundant data suggesting that the APase in certain human tumors resembles the human placental APase. Therefore, it appears that the ability to induce placental APase activity is present in all cells whether male or female. Furthermore, this APase is possibly expressed by the activation of a phase of embryonic development.

Association of Alkaline Phosphatase with Murine Leukemia

The association of APase with murine leukemia has been demonstrated histochemically using a variety of substrates. APase was originally observed in the endothelium
of the arteries and arterial capillaries of the normal thymus in C57Bl mice, using the substrates beta-glycerophosphate (GP) and adenosine triphosphate (ATP) by Smith et al. (1958). Beta-glycerophosphatase (GPase) was also seen as a rim at the surfaces or just inside the cell membranes of lymphocytes and reticular cells in both the cortex and medulla of the thymus; while adenosine triphosphatase (ATPase) was seen in the mesenchymal reticular cells. The endothelial cells of the central and pulp arteries of the spleen had GPase activity and the arteries and arterial capillaries housed the ATPase activity. Yet, Kouvalainen (1971) saw either no GPase activity or only very slight activity in the thymus. Later, Smith (1961, 1962) found no APase activity in the normal thymus using the substrate NP. However, alpha-naphthyl phosphatase (NPase) activity could be seen on the thymocytes in AKR mice, a strain having a high rate of spontaneous thymic lymphoma and x-irradiated induced lymphoma in C57Bl mice, a low leukemic strain. Both the spontaneous lymphoma in the AKR strain and the induced lymphoma in C57Bl mice are known to have a viral etiology (Gross, 1958; Lieberman and Kaplan, 1959; Haran-Ghera, 1967; Doell and Mathieson, 1970; Vredevoe and Harp, 1976). Lagerlöf and Kaplan (1967) and George (1974) found a
low level of NPase activity in the mitotically active cells (lymphoblasts) of the 16-day fetal thymus. Thus, NPase activity is present in the fetal thymus up to 16 days gestation and in the thymic lymphoma, but is absent from normal thymocytes in C57Bl mice.

The NPase activity is on the membrane and in the endothelial cells of vessels in lymphoma cells. Some activity is found in the cells surrounding the germinal follicles of the normal spleen (Lumb and Doell, 1970; Floyd, 1973). In the placenta NPase activity is seen in the syntrophoblast and cytотrophoblast (Floyd, 1973). In the whole fetus NPase appears to be both cytoplasmic and membrane bound in the liver and cartilage and mainly membrane bound in the intestine (Floyd, 1973). APase activity is also found in the envelope of murine leukemia viruses (De Thé, 1966).

In order to follow the chronological appearance of APase in relation to the development of a thymic lymphoma, several investigators used both histochemical and biochemical analysis. Using the Gomori histochemical method, Siegler and Rich (1967) and Wilson et al. (1972) found APase activity in the lymphomatous thymus only after it was at least three times the weight of the normal thymus in
ICR/Ha Swiss mice. Their results suggest that APase elevation is a secondary event occurring after the onset of disease and that it is not a fundamental metabolic alteration concerned with the onset of murine lymphoblastic leukemia. However, using the more sensitive azo dye histochemical method, Lagerlöff and Kaplan (1967) correlated the appearance of APase (NPase) with malignant transformation, ruling out the possibility, therefore, that APase is associated with cellular proliferation. Furthermore, the biochemical studies of Haran-Ghara et al. (1972) and Wilson et al. (1971) demonstrated that the increase in APase (p-nitrophenyl phosphatase) (pNPPase) activity was correlated with actual pathogenesis of murine lymphatic leukemia. Wilson et al. (1971) even showed that APase was not associated with the leukemia virus in the absence of malignant transformation.

Biochemistry of Murine Alkaline Phosphatase

The APase activities of the placenta, fetal, adult and lymphomatous tissues have been characterized using several parameters. Lumb and Doell (1970) showed the liver and duodenum to be different in pH optimum, heat inactivation, ratio of activity toward pNPP and GP and electrophoretic mobility, while the spleen and lymphoma APases
were shown to be similar by these same characteristics. Cory et al. (1971) found normal liver APase activity different from leukemic liver APase activity by its magnesium activation, while acid phosphatase was constant in both. They termed the leukemic APase activity as "Mg\(^{2+}\)-independent" by comparison with normal liver APase which was activated three-fold by magnesium. In comparing leukemic tissue APases with APase in the normal organ source (leukemic spleen versus normal spleen), Neumann et al. (1971) discovered that leukemic APase in the C57Bl/6, SJL/J and AKR mice hydrolyzed the substrate pNPP 2-30 times faster than the substrate cysteamine-S-phosphate (CASP), whereas the normal organ source of APase had a rate of hydrolysis of pNPP/CASP of 1-3. Thus, normal and leukemic tissue APases are able to hydrolyze both substrates pNPP and CASP, with both normal and leukemic tissue APases having a greater affinity for pNPP. While both normal and leukemic tissue APases have a greater affinity for pNPP, leukemic tissue APases (omitting the leukemic thymus comparison with the normal thymus) have a two-fold greater affinity for pNPP over normal tissue APases. By this criterion APase from the normal spleen is different from APase in the leukemic spleen.
While doing a comparative electrophoretic study of normal and leukemic tissues, Floyd (1973) discovered a PPase activity distinct from pNPPase, GPase and NPase activities of placental, whole fetus, spleen and leukemic extracts. She termed the activity of pNPPase, GPase and NPase as non-specific APase activity and the PPase activity as specific APase activity on the basis of electrophoretic separation and neuraminidase sensitivity. She also distinguished the whole fetus from the other tissues on the basis of neuraminidase resistance. Tisdale et al. (1975), in an effort to determine the substrate specificity of the leukemic tissues, further distinguished the PPase activity by its nuclear localization, resistance to L-phe and EDTA, substrate ratio and pH optimum. In addition, ATPase and fructose-1,6-diphosphatase (FDPase) were distinguished from the nonspecific APase by heat inactivation rate. ATPase also had a high level of magnesium activation; while FDPase was resistant to the action of EDTA. While studying the effects of nucleophilic buffers on APase activation, Prioleau (1975) demonstrated that APase could transfer phosphate to nucleophilic buffers. Thus, APase is a phosphotransferase, as well as a phosphohydrolase. It can be concluded from these data that lymphoma APase activity is
similar to normal spleen and placental APase activities and that the nonspecific APase activity is distinct from ATPase, FDPase and PPass activities found in the crude extracts.

The biochemical data obtained, thus far, shows that murine thymic lymphoma APase activity is similar to normal spleen and placental APase activities. Therefore, the murine lymphoma APase may represent a derepressed embryonic function.
CHAPTER III

MATERIALS AND METHODS

Source of Tissues

Inbred C57Bl/Ka mice were originally obtained from Dr. Henry S. Kaplan at Stanford University. They were maintained by brother-sister mating. The spleens were obtained from normal adult C57Bl mice. The 16-day whole fetuses, 16-day placentas, and 16-day fetal thymuses were taken from 16-day pregnant mice.

The lymphomas were induced in the C57Bl mice by urethan or a virus (C57LV) which was isolated from a lymphoma induced with 6-mercaptopurine (Doell and Mathieson, 1971). Some of the lymphomas were pooled, since previous experiments had shown no differences in the lymphomas. Lymphomas used were L-361, L-475, PL1, PL2, PL3, and PL4. L-361 and L-475 were C57LV-induced lymphomas. PL1 contained lymphomas L-410, L-414, and L-435, which were spontaneous. PL2 contained lymphomas L-336, L-346, L-312, L-416, which were C57LV-induced, and L-380 which was urethan-induced. PL3 contained L-165, L-189, L-302, L-304, and L-325, which were C57LV-induced. PL4 contained
lymphomas L-488 and L-493, which were urethan-induced, and L-486, L-487 and L-489, which were C57LV-induced.

Preparation of Extracts

Extracts were prepared by homogenizing the tissue in a glass homogenizer in 0.01 M tris(hydroxymethyl)aminomethane (tris) at pH 7. For electrophoresis experiments, the homogenized extract was centrifuged at 75,000 x g in a Beckman L3-50 ultracentrifuge for 30 minutes. The precipitate was treated with 1% Noniodet P-40 (Shell Oil, Inc.) (NP40), incubated at 37 C for 15 minutes, and centrifuged at 75,000 x g for 30 minutes. The NP40 treatment was repeated twice on the precipitates and the three resulting supernatants were pooled. The pooled supernatants and the remaining precipitate were then treated with 30% butanol and centrifuged at 75,000 x g for 30 minutes. The resulting aqueous layer was dialyzed to remove the butanol.

For electrophoresis experiments, the fetal thymus and lymphoma extracts were treated with 1.0% NP40 and incubated at 37 C for 15 minutes. One of the fetal thymus extracts had to be further concentrated changing the detergent concentration from 1.0% to 10%.

Buffers

Ammediol (2-amino-2-methyl-1,3-propanediol), tris,
borate, and diethanolamine (DEA) were used at varying concentrations in order to find the best buffer to use for maximum sample activity.

Fluorimetric Assay for Alkaline Phosphatase

The Turner model 111 was used for fluorimetric assays. The fluorimetric assay was employed when using the substrate NP (Moss, 1960). The excitation of NP was at 336 nm and the emission of NP was at 464 nm. The buffer was pre-incubated at 37 C for five minutes in a 4 ml reaction volume. The substrate (0.052 mM) and the extract were added to the buffer and the formation of alpha-naphthol was recorded by fluorescence for one to five minutes at constant temperature. For the experiments to test the level of inhibition with ethylenediamine tetra-acetic acid (EDTA), the extract was preincubated with the buffer but no magnesium chloride and the reaction initiated by the addition of NP.

Spectrophotometric Assay for Alkaline Phosphatase

The Hitachi model 102 digital spectrophotometer was used for the spectrophotometric assay for APase. The Nissei Sangyo n-14A printer was used to record the enzyme activity. The reaction was run for four minutes at 37 C in buffer at pH 10. When using the substrate pNPP, the enzyme
and 0.1 ml of 33.8 mM substrate were added to the buffer in a total reaction volume of 1 ml. When using the substrate CASP, the enzyme and CASP (0.36 mg) were added to the buffer with 0.5 mg 5,5-dithiobis(2-nitrobenzoic acid) in a total reaction volume of 1 ml (Neumann, 1968). The p-nitrophenol and cysteamine complex were measured at 400 nm.

Optimum pH

To determine optimum pH, the extracts were incubated with the appropriate substrate with the pH varying from 9 to 10.5.

Heat Inactivation

Heat inactivation rate was determined by heating tubes prepared for assay, including 5 mM magnesium chloride, for varying times at 55 C prior to assay. The heat inactivation rate is described in the equation

$$ A = A_0 e^{-Kt} $$

where $A_0$ is the unheated activity, $A$ is the activity remaining after time $t$ and $K$ is the heat inactivation rate constant. The rate was determined by Least Square's analysis of the data expressed as the natural log of activity versus time at 55 C.
Activation and Inhibition

Experiments were performed to determine the level of activation or inhibition by 5 mM magnesium chloride (Mg), 0.1 mM EDTA, 10 mM H-arg, 10 mM L-phe, 0.5 M ammediol buffer and 1 M DEA buffer. Percent activation or inhibition was determined by comparison with the appropriate control.

Magnesium Determination

Magnesium ions were chelated by 8-hydroxyquinoline-5-sulfonic acid. The resulting chelate exhibited fluorescence at 500 nm when excited by the light of 360 nm (Schachter, 1961). The working reagent contained 0.1 M tris and 0.0025 M 8-hydroxyquinoline-5-sulfonic acid with varying concentrations of Mg for the standard curve in a total reaction volume of 4 ml. For Mg determination in the tissue extracts, 1 microliter was added to 4 ml reaction volume and fluorescence was measured. Magnesium content was determined by referring to the standard curve (Fig. 1).

Kinetics

The enzyme reaction was carried out as indicated above, using 0.104, 0.052, 0.026, and 0.013 mM NP as substrate. A Lineweaver-Burke plot of the results was used to determine the Michaelis constant ($K_m$).
Fig. 1. Magnesium standard curve showing the fluorescence versus the magnesium concentration.
Electrophoresis

Preparation of the Sample. The extracts had a final concentration of 10% sucrose, 0.5% Triton X 100 (Fishman, 1974) and 0.017% bromphenol blue tracking dye. Enzyme activity was at least 0.40 micromoles NP per minute per ml. Samples were treated with equal volumes of 10 units neuraminidase (Cl. perfringens Type V) per 0.05 ml de-ionized water for 30 minutes at 37 C. The migration of neuraminidase-treated enzyme was compared with that of an untreated control.

Gel Casting. The running gel contained 8% acrylamide, 0.22% N,N'-methylenediacrylamide, 1.64% tris, 0.056% N,N,N',N'-tetramethylenediamine, 8.8% 1 M hydrochloric acid (HCl) and 0.25% ammonium persulfate. The running gel was pipetted into electrophoresis tubes, water-layered immediately and allowed to polymerize for 15 minutes. After polymerization, the water was removed and the concentrating gel was added. The concentrating gel contained 2.5% acrylamide, 0.625% N,N'-methylenediacrylamide, 2.19% tris, 11.7% 1 M HCl, 0.75% N,N,N',N'-tetramethylethylenediamine, 0.0005% riboflavin, and 10% sucrose. The concentrating gel was layered with the upper buffer and allowed to polymerize by exposure to ultra-violet light for 30 minutes.
Buffers. The upper and lower buffers contained 0.10 M tris-borate at pH 9.5. The upper buffer also contained 0.5% Triton X 100.

Electrophoretic Separation. Current was applied using a Vokan power supply. For the first 5 minutes, constant current was applied at 1 mA per tube and then for the next 35 minutes at 4 mA per tube. The power was switched to constant voltage at 200 V for 80 minutes.

Gel Removal. Gels were removed from the tubes by submerging them in a pan of cold water and ringing them with a cannula.

Staining Procedures

Azo-stain. The azo-dye method used was according to Manheimer and Seligman (1948) as modified by McKay et al. (1953) with the following modifications: 1 mg of Fast Blue RR salt and 1 mg maphthol AS-MX phosphoric acid disodium salt were ground in 1 ml of 0.2 M tris buffer (pH 10) with 5 mM MgCl₂. This mixture was poured on the gel and placed in the refrigerator for 40 minutes. The stain was rinsed off the gel and more stain was applied if necessary.

pNPP. A 33.8 mM pNPP solution in 1 M DEA was applied on the gel.
Statistical Analysis

All data are reported as the mean and standard deviation of at least three experiments. A student's t-test was performed to determine significance, using the facilities of the Atlanta University Center Computing Center.
CHAPTER IV

EXPERIMENTAL RESULTS

Optimum Fluorometric Conditions

Table 1 shows that increasing the molarity of borate buffer inhibits the APase activity. It was thought that borate might be a neutral buffer which could be used as a control for the experiments with nucleophilic buffers. However, this was not the case, for borate definitely inhibited the APase activities with the concentrations used. To achieve the maximum efficiency on the fluorometer, a number of nucleophilic buffers were tried to determine their sensitivity level, as well as to compare their ability to activate APase. The results show that increasing the molarity of the nucleophilic buffers increases the APase activity (Table 2). In comparing the nucleophilic buffers, tris, ammediol and DEA, it was found that DEA was the best activator of APase (Table 3). In addition, DEA would zero at 30 X sensitivity on the fluorometer, making it 61.63-fold more sensitive than ammediol which is 1.90-fold more sensitive than tris.

The NPase activities of all four tissues were
Table 1. The effect of varying concentrations of borate buffer on alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0.05 M Borate</th>
<th>0.50 M Borate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>68.0 ± 9.2a</td>
<td>89.1 ± 1.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>51.8 ± 6.1</td>
<td>86.9 ± 1.7</td>
</tr>
<tr>
<td>Placenta</td>
<td>54.3 ± 10.9</td>
<td>82.9 ± 6.3</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>65.1 ± 5.3</td>
<td>82.6 ± 3.9</td>
</tr>
</tbody>
</table>

*The percent inhibition of alkaline phosphatase activity by borate buffer compared to activity in 0.05 M ammediol buffer expressed as the mean and standard deviation of at least three experiments.*
Table 2. The effect of various concentrations of buffers on alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0.05 M&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0.10 M</th>
<th>0.50 M</th>
<th>1.00 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris(hydroxymethyl)aminomethane</td>
<td>1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.16</td>
<td>4.71</td>
<td>4.65</td>
</tr>
<tr>
<td>2-amino-2-methyl-1,3-propanediol</td>
<td>1.00</td>
<td>1.71</td>
<td>2.71</td>
<td>np&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>1.00</td>
<td>1.25</td>
<td>2.60</td>
<td>2.69</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of the buffer.

<sup>b</sup> The alkaline phosphatase activity in each buffer is compared with the 0.05 M buffer as the control. The numbers given represent the ratio of APase activity in each buffer divided by the APase activity in 0.05 M of buffer indicated.

<sup>c</sup> np means that experiment was not performed because the particular buffer would not zero at the sensitivity (3X) used.
Table 3. Comparison of each buffer concentration with ability to activate alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0.05 M&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0.10 M</th>
<th>0.50 M</th>
<th>1.00 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris(hydroxymethyl)aminomethane</td>
<td>1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2-amino-2-methyl-1,3-propanediol</td>
<td>1.90</td>
<td>1.03</td>
<td>1.10</td>
<td>np&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>2.88</td>
<td>1.11</td>
<td>1.59</td>
<td>1.66</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration of the buffer.

<sup>b</sup>The figures given represent the ratio of APase activity in buffer at particular molarity divided by tris(hydroxymethyl)aminomethane at same molarity indicated.

<sup>c</sup>np means that the experiment was not performed because the particular buffer would not zero at the sensitivity (3X) used.
activated by ammediol and DEA (Table 4).

Biochemical Characterization

Figure 2 shows the pH optima for the tissues tested. As shown in the Fig., lymphoma, spleen, placenta, and fetal thymus extracts all have an APase activity with a pH optimum of 10 using the substrate NP.

Figure 3 shows the heat inactivation of all APase activities. It is clear that all tissues have a similar pattern of inactivation. A statistical analysis of the heat inactivation rates verifies this conclusion (Table 5).

Table 6 shows that magnesium activated all APase activities in 0.05 M buffer. However, in some cases, magnesium was inhibitory (up to 20%) in high molarity buffers.

Table 7 shows that EDTA inhibited the APase activities approximately 31% using the substrate NP. Previous experiments showed that EDTA inhibited the APase activities 96-100% using the substrate pNPP. In performing the spectrophotometric assay, the enzyme with the buffer containing EDTA was preincubated, the substrate was added, and the reaction was run for 30 minutes. At the end of the reaction, the product that formed was measured. When the fluorometer was used, the buffer containing EDTA was preincubated and the enzyme
Table 4. The level of activation of alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ammediol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diethanolamine&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>1.24 ± 0.29</td>
<td>2.88 ± 0.32</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.91 ± 0.14</td>
<td>2.81 ± 0.11</td>
</tr>
<tr>
<td>Placenta</td>
<td>1.65 ± 0.26</td>
<td>2.78 ± 0.18</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>1.63 ± 0.64</td>
<td>2.87 ± 0.43</td>
</tr>
</tbody>
</table>

<sup>a</sup>The activity of 0.5 M ammediol divided by the activity of 0.05 M ammediol expressed as the mean and standard deviation of at least three experiments.

<sup>b</sup>The activity of 1 M diethanolamine divided by the activity of 0.05 M diethanolamine expressed as the mean and standard deviation of at least three experiments.
Fig. 2. The pH optimum of alkaline phosphatase activities of placenta, spleen, lymphoma and fetal thymus using the substrate alpha-naphthyl phosphate.
alpha-naphthyl phosphate in pM

- Lymphoma
- Spleen
- Placenta
- Fetal Thymus

pH

alpha-naphthyl phosphate in pM
Fig. 3. Heat inactivation of alkaline phosphatase activities of placenta, spleen, lymphoma, and fetal thymus. The natural logarithm of the activity remaining is plotted against the time incubated at 55 C in order to determine the heat inactivation rate constant.
Table 5. Heat inactivation rate of alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Heat Inactivation Rate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>$0.15 \pm 0.01^b$</td>
</tr>
<tr>
<td>Spleen</td>
<td>$0.17 \pm 0.01$</td>
</tr>
<tr>
<td>Placenta</td>
<td>$0.15 \pm 0.02$</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>$0.15 \pm 0.02$</td>
</tr>
</tbody>
</table>

$^a$The rate of heat inactivation is determined by plotting the slope of the natural logarithm of the activity remaining versus the time heated at 55 C.

$^b$The mean and standard deviation were obtained from at least three experiments.
Table 6. The effect of magnesium on alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Magnesium Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 M^a</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1.46 ± 0.21^b</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.46 ± 0.16</td>
</tr>
<tr>
<td>Placenta</td>
<td>1.46 ± 0.31</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>1.47 ± 0.17</td>
</tr>
</tbody>
</table>

^a Concentration of buffer.

^b The activity of 0.05 M diethanolamine with 5 mM magnesium divided by the activity of 0.05 M diethanolamine without magnesium expressed as the mean and standard deviation of at least three experiments.

^c The activity of 1.0 M diethanolamine with 5 mM magnesium divided by the activity of 1.0 M diethanolamine without magnesium expressed as the mean and standard deviation of at least three experiments.
Table 7. The level of alkaline phosphatase inhibition by ethylenediamine tetra acetic acid (EDTA)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>EDTA Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>36.4 ± 18.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>31.5 ± 6.2</td>
</tr>
<tr>
<td>Placenta</td>
<td>30.1 ± 14.9</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>30.5 ± 15.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mean and standard deviation were obtained from at least three experiments.
and substrate were added at the same time. The reaction was measured for one to five minutes. In order to determine if the same enzyme hydrolyzed the substrates NP and pNPP or if the difference in EDTA inhibition was a reflection of the assay system, a time curve of the EDTA inhibition was performed using the substrate NP. Figure 4 shows that the APase activity rapidly decreases with increased preincubation of the enzyme with EDTA. After 20 minutes preincubation, 97-100% of the APase activity is lost. Therefore, the difference in EDTA effect shown in Table 7 is due to a difference in the assay system rather than indicating that the substrates pNPP and NP are hydrolyzed by different enzymes.

The level of APase inhibition by the amino acids L-phe and H-arg is shown in Table 8. In low molarity nucleophilic buffer, the APase activities are inhibited 100% by H-arg and approximately 46% by L-phe; whereas in high molarity buffer the APase activities are inhibited approximately 81% by H-arg and 7.5% by L-phe.

Substrate ratios indicate that the pH 10 APase activities of lymphoma and fetal thymus hydrolyze pNPP and NP with approximately the same level of activity (Table 9). Michaelis constants (K_m) for the NPase activities of all
Fig. 4. A time curve of ethylenediamine tetra-acetic acid (EDTA) inhibition showing the effect of EDTA on alkaline phosphatase activity when the preincubation times of the enzyme was varied with EDTA. The substrate used was alpha-naphthyl phosphate.
Table 8. The level of alkaline phosphatase inhibition by amino acids in low and high molarity nucleophilic buffers

<table>
<thead>
<tr>
<th>Tissue</th>
<th>L-homoarginine</th>
<th>L-phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.05 M&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 M</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>81.91 ± 3.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.82 ± 7.08</td>
</tr>
<tr>
<td>Spleen</td>
<td>80.01 ± 5.14</td>
<td>49.20 ± 5.01</td>
</tr>
<tr>
<td>Placenta</td>
<td>81.44 ± 6.95</td>
<td>45.66 ± 4.63</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>81.22 ± 2.22</td>
<td>49.36 ± 6.93</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration of the buffer diethanolamine.

<sup>b</sup>The mean and standard deviation were obtained from at least three experiments.
Table 9. Substrate ratio of alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Substrate Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>1.03 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.76 ± 0.17</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.91 ± 0.01</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>1.01 ± 0.16</td>
</tr>
</tbody>
</table>

<sup>a</sup>Substrate ratio is the ratio of activity using the substrate alpha-naphthyl phosphate divided by the activity using the substrate p-nitrophenyl phosphate.

<sup>b</sup>The mean and standard deviation were obtained from at least three experiments.
four tissues in Table 10 show that all APases have a similar affinity for the substrate NP. Student's t-test showed that the substrate ratio (Table 9) and the $K_m$ (Table 10) values were not significantly different for the different APases (>0.05).

Electrophoretic Analysis

In attempting to find the best conditions for APase localization after electrophoresis, several experiments were performed. Using different concentrations of tris, ammediol, and DEA, it was decided that, if post-coupling (incubating the gel in the substrate for the appropriate time, then adding the coupling reagent) was performed, tris was the buffer of choice. The low molarity buffers were just as good as the high molarity buffers when looking at alpha-naphthol fluorescence under ultra-violet light. High molarity buffers coupled to the dye themselves. This prevented the dye from coupling with the alpha-naphthol. Surprisingly, 1 M DEA was the worst buffer to use for electrophoretic staining. Figure 5 shows the APase activities in the placenta and whole fetus. In this particular electrophoretic run many bands of APase activity were found in the placenta and two bands of APase activity were found in the whole fetus. Figure 6 shows the importance of small
Table 10. The Michaelis constant for alkaline phosphatase

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Michaelis Constant (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>0.024 ± 0.007(^a)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.034 ± 0.004</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.030 ± 0.016</td>
</tr>
<tr>
<td>Fetal Thymus</td>
<td>0.021 ± 0.007</td>
</tr>
</tbody>
</table>

\(^a\)The mean and standard deviation were obtained from at least three experiments.
Fig. 5. Electrophoresis of alkaline phosphatase showing isoenzymes of NPase activity. P represents the placental extract; W represents the whole fetal extract; * represents the tracking dye, bromphenol blue.
Fig. 6. Electrophoresis of alkaline phosphatase showing the effect of varying the volume of placental extract. A-10 microliters of placental extract. B-100 microliters of placenta extract; * represents the tracking dye, bromphenol blue. Both volumes of placental extract have the same APase activity.
initial volumes (10λ) versus larger volumes (100λ) of the placenta extract. While both volumes contain equivalent APase activity, the smaller volume has sharper resolution. Both bands of APase activity were in the larger volume, but they were fainter and more diffuse. Figure 7 shows the electrophoretic patterns of APases from all five tissues using the substrate NP. The placenta, spleen, lymphoma and fetal thymus APases have a similar electrophoretic mobility. The lymphoma and fetal thymus APases have identical electrophoretic mobilities. The whole fetus APase definitely has a distinct electrophoretic mobility.

In comparing the effect of neuraminidase (Figure 8) the lymphoma APase and faster migrating APase of the whole fetus are slowed by neuraminidase. The neuraminidase treatment significantly retards the tailing in the placenta and spleen extracts. Such retardation may represent an action on the APases in these extracts, although the total migration of APase is not retarded by these extracts. The fetal thymus APase is significantly slowed by the neuraminidase treatment (Figure 9), as is the lymphoma APase (Figure 8). All tissues have pNPPase activity (Figure 10). Figure 11 compares the electrophoretic mobility of the NPase and pNPPase activities of all tissues.
Fig. 7. Electrophoresis of alkaline phosphatase showing NPase activity. P represents the placental extract; S represents the spleen extract; W represents the whole fetal extract; L represents the lymphoma extract; F represents the fetal thymus extract; * represents the tracking dye, bromphenol blue.
Fig. 8. Electrophoresis of alkaline phosphatase showing NPase activity in the presence and absence of neuraminidase. P represents the placenta extract; S represents the spleen extract; W represents the whole fetus extract; L represents the lymphoma extract; n indicates that extract was treated with neuraminidase; * represents the tracking dye, bromphenol blue.
Fig. 9. Electrophoresis of alkaline phosphatase showing NPase activity of the fetal thymus in the presence and absence of neuraminidase. F represents the fetal extract; n indicates that extract was treated with neuraminidase; * represents the tracking dye, bromphenol blue.
Fig. 10. Electrophoresis of alkaline phosphatase showing pNPPase activity. P represents the placenta extract; S represents the spleen extract; W represents the whole fetus extract; L represents the lymphoma extract; F represents the fetal thymus extract; * represents the tracking dye, bromphenol blue.
Fig. 11. Electrophoresis of alkaline phosphatase comparing pNPPase and NPase activities. P represents the placental extract; S represents the spleen extract; W represents the whole fetus extract; L represents the lymphoma extract; F represents the fetal thymus extract; a indicates pNPPase activity; b indicates NPase activity; * represents the tracking dye, bromphenol blue.
Each tissue APase activity appears to have NPase and pNPPase activities with identical electrophoretic mobilities. The fetal thymus APase appears to have a different mobility from the lymphoma APase in Figures 9, 10 and 11. This difference in mobility could be due to a higher detergent content in this fetal thymus extract.
CHAPTER V

DISCUSSION AND CONCLUSION

This biochemical comparison was performed to determine if the APase of murine lymphoma represents a derepressed embryonic function. No significant differences among the lymphoma, spleen, placenta, and fetal thymus APases have been shown by the pH optimum, heat inactivation, activation by magnesium, inhibition by EDTA, L-phe and H-arg or substrate ratios. The mobility of the APase of the lymphoma and fetal thymus extracts was significantly slowed by neuraminidase. The placenta and spleen APase activities were slowed to the level of the top of the broad migrating band of APase activity. All tissue APases have both NPase and pNPPase activities with identical mobilities, indicating that NPase and pNPPase are activities of the same enzyme.

Previous experiments showed that NPase and pNPPase were non-specific APase activities of the same enzyme. Furthermore, these experiments showed that neuraminidase inhibited the mobility of the placenta, spleen and lymphoma APases using the substrates NP and pNPP (Floyd, 1973). The difference in the treatment of extracts could account
for the differences seen in these APases. From Figures 5 and 6 it can be seen that the isoenzymes are varying in the placental extracts. It appears that there are two isoenzymes present in the placenta, a feature not noted previous in the work of Floyd (1973). Better resolution of the placenta APase isoenzyme is due to the use of the detergent Triton X 100 in the electrophoretic separation. In electrophoresing the same placental extract (freezing and thawing several times), it appears that the number of bands in the placenta varies, while the difference in mobilities between the slower and faster APase decreases. It appears that either the placental extract has a low level of neuraminidase present which is cleaving some carbohydrate away from the enzyme or that a difference in buffer ionic strength coupled with using the same voltage caused the observed differences in pattern (Figures 6 and 7). The spleen and lymphoma also had two bands of APase originally (Figure not shown) and the inhibition of migration by neuraminidase was apparent. What appears obvious from these observations is that the carbohydrate content in the lymphoma and fetal thymus APases may not be the same as the carbohydrate content in the placenta and spleen APases. This would not affect the stability or
catalytic properties of the enzyme (Warnes, 1972), but it might change the antigenic properties of the enzyme (Usategui-Gomez et al., 1969). Usategui-Gomez et al. (1969) found that removal of the carbohydrate from APase changed the ability of the enzyme to cause agglutination of the H-1 virus. Numerous investigators report no effect on catalytic activity of APase by removing the carbohydrate from the enzyme. Another very obvious effect of neuraminidase treatment is that tailing is significantly reduced. The differences in carbohydrate content, even in the tissue extracts, could reflect the difference in membrane location of the APases.

While it was known that the whole fetus was different from the lymphoma APase, since it contains APases from several tissues, its electrophoretic profile was re-examined with the other tissue APases. This experiment clearly shows that the faster moving band of the whole fetus is slowed by neuraminidase. Floyd (1973) showed no effect on neuraminidase, but from looking carefully at the photographs, it appears that the faster migrating band of APase in the whole fetus was fainter than the control.

From these results, it can be concluded that the lymphoma, fetal thymus, placenta and spleen APases are
similar. It is not surprising that the fetal thymus, adult spleen and placenta APases resemble the lymphoma APase. The APase activity is seen in the cell membrane of the fetal thymus up to 16 days gestation (Lagerlöf and Kaplan, 1967). The APase of the adult spleen is in the thymic-dependent areas of the spleen (Floyd, 1973). It is known that lymphocytes must undergo dedifferentiation in certain immunological functions. The placenta APase activity is in the cell membrane of the fetal portion of the placenta (Hulstaert et al., 1973). Thus, histochemical localization of APase and association of APase with dedifferentiation help make it clear why these APases might be similar.

Because all the murine APases tested are similar, the lymphoma APase may represent a derepressed embryonic function. The idea that one of the mechanisms of malignant transformation is the derepression of embryonic functions is not unique. The phenomenon of embryonic antigens and isoenzymes appearing in human and experimental tumors is well established. In fact, cancer and differentiation have been closely linked through much of the history of cancer. The data thus far suggest that a library of genes exist which are concerned solely with the early
embryogenesis and that some of these genes may be re-expressed in cancer (derepression of embryonic gene functions). Indeed the mechanisms which allow the embryo to escape immune destruction may also allow the tumor to escape immune destruction. If this is the case, then, in cancer one may be fighting his own genes.

Although the function of APase is not known with certainty, it is possible to speculate on a possible function from these experiments coupled with information obtained from the literature. The APase of murine lymphoma has several known characteristics which may be important in the consideration of a potential function. APase is activated by sodium, potassium (Williams, 1971), magnesium ions, high molarity nucleophilic buffers and is inhibited by EDTA, L-phe and H-arg. APase has been shown to transfer phosphate to high molarity nucleophilic buffers (Prioleau, 1975). One suggestion was that APase may be functioning as a PPase which removes one of the products of DNA and RNA polymerase activity and, therefore, shifts the equilibrium in favor of synthesis. This possibility has been ruled out by the work of Floyd (1973) and Tisdale et al. (1975) because PPase activity was demonstrated to be clearly distinct from APase activity. The APase activity
associated with murine lymphoma hydrolyzes pNPP, NP and GP, indicating a range of phosphate donors. It appears that the specificity of the APase is probably with the acceptor of the phosphate rather than the donor of phosphate. A very interesting possibility is that APase acts as a protein kinase in cAMP-related cell membrane events. The amino acid inhibition patterns support this contention. L-phe inhibits APase in low molarity nucleophilic buffer but to a markedly less extent in high nucleophilic buffer, hinting that it competes with the nucleophilic buffer which possibly acts as a natural acceptor of phosphate in vivo. It is possible that H-arg works at this site also, since its level of inhibition is slightly reduced. So, the function of APase may have something to do with its ability to act as a transphosphorylase.

An interesting observation was that magnesium is inhibitory at high nucleophilic buffer concentrations. Magnesium probably works at the catalytic site rather than aiding in the stability or regulating the access to the active site of the enzyme.

These results are in general agreement with other reports of biochemical characterization of murine leukemic APase. Lumb and Doell (1970) found the lymphoma APase
activity to be similar to the low level of APase in the normal spleen. Cory et al. (1971) have identified a new APase activity in leukemic livers which was "Mg\textsuperscript{2+}-independent" when compared to the normal liver APase which was activated three-fold by magnesium. However, their "Mg\textsuperscript{2+}-independent" isoenzyme was activated 46%, the same level of activation that was observed in these experiments. Likewise, Neumann et al. (1971) stated in the summary that magnesium was inhibitory but showed inhibition with 60 mM magnesium and variable (30% to 80%) activation by 1 mM magnesium. Lumb and Doell (1970), Floyd (1973) and Tisdale et al (1975) found the electrophoretic mobilities of the lymphoma, spleen and placenta APases were similar using the substrates NP. Floyd (1973) further states that the APases were similar electrophoretically using the substrates pNPP, GP and PP. However, she found the PPase activity clearly distinct from the GPase, NPase and pNPPase activities which were similar. The whole fetus was found to have a similar electrophoretic mobility but was distinguished from the other APases by its resistance to neuraminidase. Moore (1975) and Tisdale et al. (1975) further distinguished APase activity from the PPase, ATPase and FDPase activities by certain biochemical criteria. Thus, they have an APase
activity which hydrolyzes the substrates NP, pNPP and GP but is clearly not associated with activity of PPase, ATPase, or FDPase. Because these studies were performed on crude extracts, purified APase enzyme may have a low level of affinity for these substrates as a function of its hydrolase activity. All reports show a similar heat inactivation pattern which is in agreement with these data, that is, approximately half the activity is destroyed by 55°C in 5 minutes.

In an electrophoretic characterization of extracts from normal, leukemic and fetal mouse tissues, Tyndall et al. (1971) showed that esterase and lactose dehydrogenase profile changes were similar in the leukemic and fetal thymus and fetal spleen extracts. Microdensitometric analyses showed a decrease of prealbumin and haptoglobin esterase activity with concomitant increase of postalbumin esterase in both fetal and leukemic thymuses and spleen tissues. So while Tyndall et al. (1971) did not study the APase profiles, they did find from their studies that the leukemic tissues were similar to the fetal spleen and thymus tissues.

A comparison of the murine lymphoma with certain human tumors APases is shown in Table 11. The murine lymphoma
Table 11. Properties of human tumors and murine lymphoma alkaline phosphatases

<table>
<thead>
<tr>
<th></th>
<th>L-phe&lt;sup&gt;a&lt;/sup&gt;</th>
<th>H-arg&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Heat In.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>EDTA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>AntiPf</th>
<th>Prednisolone</th>
<th>Cellular Localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine Lymphoma</td>
<td>9%</td>
<td>82%</td>
<td>labile</td>
<td>100%</td>
<td>.03</td>
<td>+</td>
<td></td>
<td>cell membrane</td>
<td></td>
</tr>
<tr>
<td>Regan</td>
<td>75%</td>
<td>5%</td>
<td>stable</td>
<td>10%</td>
<td>2.2</td>
<td>+</td>
<td></td>
<td>intra membrane space of mitochondria</td>
<td>1,2,3, 5,6,7</td>
</tr>
<tr>
<td>Nagao</td>
<td>90%</td>
<td>81%</td>
<td>stable</td>
<td>50%</td>
<td>.26</td>
<td>+</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Regan Variant</td>
<td>75%</td>
<td>0%</td>
<td>moderate</td>
<td>34%</td>
<td>1.1</td>
<td>+</td>
<td></td>
<td>controversial</td>
<td>4</td>
</tr>
<tr>
<td>HeLa Parent</td>
<td>77%</td>
<td>37%</td>
<td>stable</td>
<td>+</td>
<td>500%</td>
<td></td>
<td></td>
<td>controversal</td>
<td>7</td>
</tr>
<tr>
<td>TCRC-1</td>
<td>73%</td>
<td>11%</td>
<td>stable</td>
<td>+</td>
<td>175%</td>
<td></td>
<td></td>
<td>mitochondria membrane</td>
<td>7</td>
</tr>
<tr>
<td>TCRC-2</td>
<td>0%</td>
<td>78%</td>
<td>labile</td>
<td>-</td>
<td>0%</td>
<td></td>
<td></td>
<td>cell membrane</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup>L-phe represents L-phenylalanine.

<sup>b</sup>H-arg represents L-homoarginine.

<sup>c</sup>Heat In. represents heat inactivation. Heat inactivation for human tissues is at 65 C for 10 minutes; for murine lymphoma 55 C for 20 minutes.

<sup>d</sup>EDTA represents ethylenediamine tetra-acetic acid.
Table 11 (Continued).

$eK_m$ represents Michaelis constant in mM. Substrate for human tumors is phenylphosphate. Substrate for murine lymphoma is alpha-naphthyl phosphate.

$^f$Antip represents Antiplacental isoenzyme. Human tumors were treated with human placenta antiserum. Murine lymphoma was treated with murine placenta antiserum. Information comes from Flanigan (1974).

$^g$This localization assumed from the work of Sasaki and Fishman (1973).

1Fishman et al. (1968)

2Fishman et al. (1968)

3Nakayama (1970)

4Higashino et al. (1972)

5Sasaki and Fishman (1973)

6Singer and Fishman (1974)

7Higashino et al. (1975)
differs from the human Regan isoenzyme in its heat
stability, inhibition by L-phe, H-arg and EDTA, $K_m$, and
histochemical localization. More interesting information
is obtained by comparing the murine lymphoma APase to the
APase of the HeLa cell sublines. The TCRC-1 cell line
APase appears very similar to the Regan isoenzyme while
the TCRC-2 cell line APase is surprisingly similar to the
murine lymphoma APase. Impressive are the similarities of
L-phe and H-arg inhibition, heat lability and histochemical
localization of the two APases. If these two APases from
different systems are similar then one would suspect that
prednisolone would not enhance APase activity of the murine
lymphoma cells. A final test is to perform an ouchterlony
test with the murine lymphoma APase and purified human
placenta APase against anti-TCRC-2 APase antibody to see if
they are immunologically distinct. If the murine placenta
APase is distinct from the human placenta APase but shows
identity with the TCRC-2 APase by this criterion, then the
murine placenta APase may be an appropriate alternate to
study human ovarian cancer as well as human lymphoma.

Noteworthy is the fact that while the placenta is not
similar in all parameters with certain tumors listed
(Table 11), Fishman (1973) calls all the tumors listed
carcinoplacental enzymes (APases) in an effort to distinguish them from carcinofetal (tumors which resemble the fetal tissue source) enzymes.

Despite the detailed differences between the murine lymphoma APase and the human placenta and tumor APases, the analogy between the two systems is evident. In both systems an enzyme which is normally found in the placenta is also found in the tumor.

The inescapable conclusion is that the APase represents the reactivation of a gene which is usually only active during fetal stages. Mounting evidence in many systems which indicates that embryonic and fetal proteins, isoenzymes and antigens appear in malignant cells has been recently reviewed (Coggin and Anderson, 1973). In the murine lymphoma system this relationship is made clearer by the appearance of a similar isoenzyme in the fetal thymus and in a few cells in the thymic-dependent areas of the spleen. This provides a system in which a normal dedifferentiation process, the immune response, can be compared to the abnormal dedifferentiation process, malignant transformation.
CHAPTER VI

SUMMARY

1. The purpose of this study was to determine if the APase (NPase) of the lymphoma induced in C57B1 mice is similar to the APase activities of the normal 16-day fetal thymus, 16-day placenta and adult spleen.

2. To achieve this purpose a biochemical characterization including pH optimum, heat inactivation, substrate ratio, $K_m$, activation and inhibition and electrophoretic profiles was performed.

3. The lymphoma APase appeared similar to the fetal thymus, placenta and spleen APases by all criteria.

4. Therefore, the lymphoma APase may represent a derepressed embryonic function. The murine lymphoma APase can be called a cell membrane carcinofetal enzyme.


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