HISTOCHEMICAL STUDIES ON THE SUBSTRATE SPECIFICITY OF
ALKALINE PHOSPHATASE IN MURINE TISSUES

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ABSTRACT

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Histochemical Studies on the Substrate Specificity of
Alkaline Phosphatase in Murine Tissues

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Histochemical studies have been performed to determine
the localization of alkaline phosphatase (APase) activity
in several tissues with five different substrates. The
tissues selected for the study were: the kidney, placenta,
spleen, adult and fetal thymus of C57Bl mice. Frozen
sections with alkaline phosphatase activity using Gomori
lead precipitation method and the azo dye method were used.
The substrates used were beta-glycerophosphate (bGP),
adenosine 5'-triphosphate (ATP), fructose-1,6-diphosphate
(FDP) and para-nitrophenyl phosphate (pNPP) for the lead
precipitation method, while alpha-naphthyl phosphate (aNP)
was used for the azo dye method. Using the substrate ATP,
activity was distributed in the glomeruli and vessels in the cortex of the kidney. The placenta showed APase activity in the syntrophoblast and cytotrophoblast cells with all substrates. The spleen showed APase activity in the blood vessels with all the substrates. Using aNP, cell membrane-bound activity was found in cells in the pericortical area, a thymus dependent area of the spleen. The 16-day fetal thymus showed a small amount of cell membrane-bound activity at the periphery of the cortex. This was most pronounced with aNP but was also demonstrated with bGP, ATP, FDP and pNPP. The adult thymus was APase negative except for occasional activity in the blood vessels with all the substrates.

Inhibition studies were done using L-phenylalanine, L-homoarginine, L-tryptophan and ethylene diamine tetra-acetic acid (EDTA). APase activity in the kidney was inhibited by EDTA. Reduced activity in the pericortical area of the spleen was noted with L-homoarginine and EDTA, suggesting two isoenzymes in the spleen. The placenta and the fetal thymus showed no inhibition with L-phenylalanine, L-homoarginine or L-tryptophan. However, reduced activity was demonstrated with EDTA.

These studies show the superiority of the azo dye
method to the lead precipitation method. Using these two methods, two APase isoenzymes were demonstrated in the kidney. One isoenzyme localized in the proximal tubules of the kidney cortex using the substrates bGP, FDP, pNPP and aNP and another localized in the glomeruli and veins using the substrate ATP. Two APase isoenzymes were demonstrated in the spleen using a known inhibitor, L-homoarginine. L-homoarginine inhibited the APase activity in the cells at the periphery of the germinal center, but did not inhibit the APase activity in the blood vessels. These findings suggest a different isoenzyme in the cells at the periphery of the germinal center from that in the blood vessels. In these studies all the substrates used were hydrolyzed by the APase enzyme at pH 10.
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CHAPTER I

INTRODUCTION

In these studies efforts were made to determine the function of APase in the C57B1 mice. The APase (phosphomonoesterases) are nonspecific enzymes that hydrolyze virtually all monoesters of phosphoric acid with the liberation of orthophosphate. These enzymes are active in the alkaline side of neutrality, especially at pH 9 and above. It has been hypothesized that there is a similarity between APase activity of the placenta, fetal thymus, subcapsular cells and the outer perivascular lymphatic sheath cells of the normal adult spleen. The cells with APase activity in the fetal thymus are developing lymphoblast cells. These cells are those that are most immature and localized near the capsule.

The membrane localization of the enzyme suggests active transport as a potential function of APase. It may also be hypothesized that the enzyme functions to provide the energy required for active transport of ions by hydrolyzing ATP. This would account for the energy necessary for splitting ATP. Inhibition by certain amino acids
may also be of relevance to the concept of APase being involved in active transport. The combination of these enzymes of different specificities appears non-specific; even though APase molecules are actually specific. This function for APase could possibly account for its high level in tissues where considerable active transport is involved such as placenta, kidney and intestine.

The possible role of APase in cellular proliferation and differentiation in the fetal thymus may be that membrane-bound activity provides insight into the events which occur during differentiation and dedifferentiation of lymphocytes.

One of the best ways to characterize an enzyme is by doing substrate specificity studies. Several different substrates and tissues were used in this study in an effort to characterize the APase enzyme. Tisdale et al. (1975) did substrate specificity studies in which they found no significant difference in the APase activity of the tissues tested, by the parameters tested in the C57Bl mice. Smith (1961) and Lagerlof and Kaplan (1967) have used tissues from the C57Bl mice to study the histochemical and biochemical activity of APase using different substrates. The histochemical studies will determine if the same enzyme
will split different substrates and localize at the same sites.

The objectives of this study were:

1. To localize the enzyme in the kidney, placenta, spleen, adult and fetal thymus using the substrates bGP, ATP, FDP, pNPP and aNP.

2. To compare the azo dye method with the lead precipitation method for demonstrating APase activity.

3. To do histochemical demonstrations of isoenzymes using certain known inhibitors (L-phenylalanine, L-homo-arginine, L-tryptophan and EDTA).
CHAPTER II

REVIEW OF LITERATURE

The enzymes make up the largest and one of the most highly specialized class of protein molecules. They can be considered instruments for the expression of gene action, because they catalyze many of the chemical reactions that collectively constitute the intermediary metabolism of cells.

The discovery and early studies concerning enzymatic digestion in the stomach in the period 1760 to 1825 started the important experiment on chemical catalysis. Pasteur postulated in 1860 a link between enzyme and life and structure of yeast cells. In 1897, E. Buchner succeeded in extracting the enzymes engaged in alcoholic fermentation from yeast cells. J. B. Sumner, in 1926, was the first to isolate an enzyme, urease, which was extracted from jack bean in pure crystalline form.

The fact that enzyme activity could liberate phosphoric acid from certain compounds has been known since the beginning of this century. In 1923 Robison performed the first macroscopic histochemical test for the visualization of APase by incubating pieces of bone from rachitie rats in a
calcium hexose monophosphatase substrate solution followed by treatment of the specimen with silver nitrate. Gomori (1941) modified this technique for microscopic visualization of sites where phosphatase was associated with the passage of metabolites across the cell membrane. This hypothesis provides some support for the histochemical localization of APase in the cell membrane of many tissues. It has been found that the placenta exhibits high APase activity which increases as pregnancy progresses. This has been related to the absorption of maternal blood glucose by the placenta (Anagnostopoulos and Matsudaira, 1958). Moog (1962) has reviewed literature with reference to the possible functional role of APase activity and suggests that it may be related to the "sodium pump" which is coupled to the active intake of sugar molecules.

The general type of substrates for phosphatase included esters and amides of phosphoric acids of these monoesters of alcohols, phenols and diesters, have been utilized for the most part. Some of the early substrate specificity studies were done by Belfanti et al. (1935) and Fleury, et al. (1950) in which they compared the substrate specificities of phosphatase from kidney, bone, intestine, prostrate and urine. They concluded that the enzymes were broad in their
action upon various substrates but could be characterized to a certain extent according to the pH optimum and the effect of inhibitors. Lin and Fishman (1972) used L-homoarginine as an inhibitor of certain APases. These investigators found that L-homoarginine is an organ-specific, uncompetitive inhibitor of human liver and bone alkaline phosphohydrolases. Thus, the inhibition by L-homoarginine was independent of pH, but dependent on the substrate concentration up to 5 mM.

Gomori (1949) tested the histochemical specificity of a large number of phosphate esters by employing his metal salt method for the demonstration of phosphatase at varying pH. He introduced the use of lead nitrate which has been modified and often used by investigators today. Almost all the metal salt methods for demonstrating enzymes possesses some inherent defects. Palade (1951) analyzed the quantitative aspects of precipitation in the histochemical procedure and concluded that calcium phosphate exhibited a marked tendency to form supersaturated solutions. Then, by the time the critical concentration of phosphate ions sufficient to result in precipitation at center of the enzymatic activity is reached, diffusion of phosphate from the site of production will have caused a condition of supersaturation
to prevail in a wide zone around the centers. It is believed that the presence of preformed crystal nuclei within this zone will initiate the precipitation of calcium phosphate, even in the absence of any local enzymatic activity. Thus, a positive reaction need not be related to the presence of the enzyme at that particular site. Gomori (1949, 1950) indicated that two primary factors which regulate the deposition of soluble phosphate in tissue sections are rates of phosphate production and rates of diffusion. Feign and Wolf (1957) published a review dealing with artifactual staining that may occur preferentially in the immediate vicinity of the site of high activity where transient high concentrations of phosphate ions may occur.

The non-enzymatic impregnation of tissue structures by Pb is well known (Palade, 1951). Clarkson and Kench (1958) evaluated physical and physio-chemical mechanisms of lead absorption to tissue components. The use of naphthyl phosphates has improved the localization of APase by simultaneous azo dye technique. Menten et al. (1944) devised the first azo dye method by which APase could be demonstrated by the incubation of a tissue section in a substrate solution containing b-naphthyl phosphate and diazotized 2-naphthylamine.

In 1946, Daniella modified the forementioned technique of
Menten et al. and used b-naphthyl phosphate as a substrate in conjunction with a number of diazotized amines including b-naphthylamine, p-nitroaniline, amino anthraquinone and benzedine. Manheimer and Seligman (1948) modified the Menten et al. method by employing a stabilized diazotate of a-naphthylamine.

Although 2-naphthyl phosphate had been synthesized as early as 1894, Gomori was the first to show the distinct superiority of this compound in an improved azo dye technique for the demonstration of APase. Specialized naphthol AS phosphate compounds have been developed for improvement in the azo dye method. These compounds are capable of giving exceedingly sharp localization over a wide range of pH. Burstone (1958) has stated that almost any primary naphthyl phosphate is susceptible to enzyme hydrolysis.

Smith (1961) observed that lymphoid cells of the thymic cortex of adult C57Bl mice were completely devoid of histochemically demonstrable APase activity; the only structures stained were the vascular endothelium. Lagerlöf and Kaplan (1967) revealed faint APase in the thymus of 17-day fetuses but none in the thymic lymphoid cells of newborn C57Bl mice or in those of older mice up to 180 days of age.

Lumb and Doell (1970) determined characteristics such
as pH optimum, heat inactivation, the ratio of activity toward two substrates and electrophoretic mobility of the APase of lymphoma and normal tissues. The APase of lymphoma was shown to be different from that of liver and duodenum but similar to that of the normal spleen and, in preliminary experiments, to that of the fetal thymus. Floyd (1976) did more extensive chemical studies and reported no significant differences among the APase activity of the placenta, lymphoma, adult spleen and fetal thymus. These reports gave support that murine lymphoma APase is a carcinoembryonic enzyme.
CHAPTER III

MATERIALS AND METHODS

Source of Tissues

Animals used in this study were C57Bl mice which were originally obtained from Kaplan at Stanford University. They were maintained in the animal room under standard conditions and fed Purina mouse chow and water at will. The spleen, kidney and adult thymus were taken from adult males. The placentas were taken from 16-day pregnant mice and the fetal thymus from 16-day fetuses. Animals were sacrificed by cervical dislocation and the desired tissues were excised, cut into small sections and placed on the cryostat (Ames Model II) using OCT (Ames Company) as the embedding medium. Tissues were frozen within five minutes after removal from the animal and sectioned within a twenty-four hour period. After allowing 2 hours at -20 C for the proper freezing, the sections were cut at 5μ and placed on glass cover slips.

Histology

Sections for general histology were stained with Paragon (a multiple dye of hematoxylin and eosin). Sections from all the tissues were placed in a dilute solution of
Paragon 1:2 (one part Paragon and two parts distilled water) for thirty seconds and then rinsed in distilled water, dried, mounted and observed.

Buffers

Tris buffer was used for the localization of APase activity with the substrate aNP AS MX at a concentration of 0.2M with 1% MgCl₂ at pH 10. The buffers used in the inhibition studies were 0.2M Tris with 1% MgCl₂ at pH 10 containing the different inhibitors. The inhibitors were used in the following concentrations: 40 mM L-phenylalanine, L-homoarginine or L-tryptophan and 10 mM of EDTA. For the lead precipitation method, the buffer was 0.1M Tris (pH10.0) with 5mM CaCl₂ and 0.5% MgCl₂.

Substrates

The substrates beta-glycerophosphate, adenosine 5′-triphosphate, fructose-1,6-diphosphate and para nitrophenyl phosphate were used in the lead precipitation method. Alpha-naphthyl phosphate AS MX was used in the azo dye method for the localization of APase.

Histochemical Assay for APase using the Lead Precipitation Method

For the lead precipitation method, the incubation mixture contained 100 mg of the desired substrate in 20 ml
of buffer. The incubation time used was the same as that used for the azo dye method. If the mixture was cloudy it was filtered before incubation with the tissue section. After the proper incubation time at 37 C, the sections were rinsed in distilled water for one minute and treated with 2% lead nitrate for one minute. The sections were rinsed again and treated with 1% ammonium sulfide for five minutes, rinsed in distilled water twice, dried, mounted and observed. Sites of APase activity were stained black or brownish black.

Histochemical Assay for APase using the Azo Dye Method

The azo dye method described by Manheimer and Seligman (1948) as modified by McKay et al. (1953) was used for the histochemical demonstration of APase activity. The incubation mixture contained the substrate aNP AS MX 10 mg, 25 mg of fast blue RR dye and 25 ml of 0.2 M Tris buffer with 1% MgCl₂ at pH 10. Frozen sections were incubated in this mixture in the cold (4 C) and dark at varying times. The spleen sections and the adult thymus sections were incubated in the mixture for 40 minutes, 30 minutes for the fetal thymus, 10 minutes for the kidney and 5 minutes for the placenta sections. Control sections were incubated for
10 minutes in a 1% HCl solution, rinsed and incubated along with experimental sections. After the required incubation time the sections were then rinsed with distilled water, dried, mounted and observed.

Histochemical Assay for Isoenzymes

For the demonstration of isoenzymes, sections were incubated in the buffers containing L-phenylalanine, L-homoarginine, L-tryptophan or EDTA. Sections were preincubated with the buffer containing the inhibitor in the refrigerator for 20 minutes prior to staining for APase activity. Control sections were preincubated in the same buffer without inhibitor. In order to test for nonenzymatic precipitation, sections were incubated for 10 minutes in a 1% HCl solution, rinsed and incubated along with the experimental section in each of the incubation mixtures with inhibitor. The sections were then stained by the azo dye method and observed.

Controls for Histochemical Assays

The localization of APase employed the lead precipitation method and azo dye method. With the lead precipitation method, precipitation often occurred around the site of activity. Therefore to determine artifact staining, two controls were used, the control section minus the substrate
and the control section treated with 1% HCl to inactivate the enzyme. A small amount of artifact staining was often present in both controls. For the azo dye method, controls were made by inactivating the enzyme by using 1% HCl. No artifact staining was noted in these controls.
CHAPTER IV

EXPERIMENTAL RESULTS

Histochemical Localization of APase in the Kidney

The histology of the kidney section was observed as shown in Fig. 1. It was stained with Paragon which is a multiple stain. The control sections (Figs. 2 and 3) showed no APase activity. The kidney sections using the substrate bGP had APase activity in the proximal tubules in the cortex of the kidney as seen in Fig. 4. The kidney section using the substrate ATP had APase activity in the glomeruli and capillaries as seen in Fig. 5. With the substrate FDP and pNPP APase activity localized in the proximal tubules in the cortex of the kidney (Figs. 6 and 7). Figure 8 is a control section for the azo dye method using HCl to inactivate the enzyme; therefore no activity was demonstrated. Using the substrate aNP following the azo dye method, activity was localized in the proximal tubules (Fig. 9). A control kidney section for the inhibition study showed no APase activity using HCl to inactivate the APase enzyme (Fig. 10). A kidney section showing APase activity in the proximal tubules was a control for the demonstration
Fig. 1. A kidney section stained with Paragon (x 60).
Fig. 2. A control kidney section using the lead precipitation method in which the substrate was left out (x 60). No APase activity was present in the control.

Fig. 3. A control kidney section using the lead precipitation method using 1% HCL to inactivate the enzyme (x 60).

Fig. 4. Histochemical localization of APase activity in the proximal tubules of the kidney using bGP as the substrate (x 60).

Fig. 5. Histochemical localization of APase activity in the proximal tubules of the kidney cortex using the substrate ATP (x 60).

Fig. 6. Histochemical localization of APase activity in the proximal tubules of the kidney using the substrate FDP (x 60).

Fig. 7. Histochemical localization of APase activity in the proximal tubules of the kidney using pNPP as the substrate (x 60).

Fig. 8. A control kidney section using 1% HCl to inactivate the enzyme (x 60).

Fig. 9. Histochemical localization of APase activity in the proximal tubules of the kidney using the substrate aNP (x 60).
of APase activity (Fig. 11). In the inhibition study no inhibition of APase activity was noted in the proximal tubules of the kidney using L-phenylalanine, L-homoarginine or L-tryptophan (Figs. 12-14) as compared to the control section demonstrating APase activity (Fig. 11). Using EDTA as the inhibitor, inhibition of APase activity was demonstrated (Fig. 15).

Histochemical Localization of APase in the 16-day Placenta

A placenta section stain with Paragon shows the trophoblast cells with characteristic intervillious spaces (Fig. 16). The placenta had slight artefact staining in the control sections (Figs. 17 and 18) for the lead precipitation method. With the substrates bGP, ATP, FDP and pNPP (Figs. 19-22) activity was localized in the trophoblast cells. No artefact staining was present in the control section of the placenta using the azo dye method and the substrate aNP (Fig. 23). With the substrate aNP, APase activity localized in the trophoblast cells of the placenta (Fig. 24). A control section was made for the inhibition study treated with 1% HCl to inactivate the enzyme; no APase activity was demonstrated (Fig. 25). A placenta section showing APase activity without the presence of an
Fig. 10. A control kidney section for the inhibition study using 1% HCl to inactivate the enzyme (x 60).

Fig. 11. Histochemical localization of APase activity in the proximal tubules of the kidney without an inhibitor, thereby serving as a control for the demonstration of APase activity (x 60).

Fig. 12. Histochemical localization of APase activity in the proximal tubules of the kidney using L-phenylalanine as the inhibitor. No inhibition of APase activity was demonstrated (x 60).

Fig. 13. Histochemical localization of APase activity in the proximal tubules of the kidney using L-homoarginine as the inhibitor. No inhibition of APase activity was demonstrated (x 60).

Fig. 14. Histochemical localization of APase activity in the proximal tubules of the kidney using L-tryptophan as the inhibitor. No inhibition of APase activity was demonstrated (x 60).

Fig. 15. Histochemical localization of APase activity in the proximal tubules of the kidney using EDTA as the inhibitor. EDTA partially inhibited the APase enzyme in the proximal tubules of the kidney (x 60).
Fig. 16. A placenta section stained with Paragon (x 60).
Fig. 17. A control placenta section for the lead precipitation method in which the substrate was left out (x 60). No APase activity was demonstrated.

Fig. 18. A control placenta section for the lead precipitation method in which the APase activity was inactivated with 1% HCl (x 60).

Fig. 19. Histochemical localization of APase activity in the syntrophoblast cells using the substrate ATP (x 60).

Fig. 20. Histochemical localization of APase activity in the syntrophoblast cells using the substrate ATP (x 60).

Fig. 21. Histochemical localization of APase activity in the syntrophoblast cells using the substrate FDP (x 60).

Fig. 22. Histochemical localization of APase activity in the syntrophoblast and cytotrophoblast cells using the substrate pNPP (x 60).

Fig. 23. A control placenta section in which the enzyme was inactivated by treating the section with 1% HCl (x 60).

Fig. 24. Histochemical localization of APase activity in the syntrophoblast and cytotrophoblast cells of the placenta using aNP as the substrate (x 60).
inhibitor served as a control for the demonstration of APase activity for the inhibition study (Fig. 26). No inhibition of APase activity was demonstrated in Figs. 27-29 using L-phenylalanine, L-homoarginine or L-tryptophan respectively. When EDTA was used as the inhibitor, APase activity was inhibited in the placenta (Fig. 30) as compared to Fig. 26.

Histochemical Localization of APase in the Spleen

The general histology of the spleen shows that it is well vascularized and has red and white pulp (Fig. 31). The spleen required a longer incubation time for the demonstration of APase activity and artifact was noted in the control sections (Figs. 32 and 33) using the lead precipitation method. APase activity was localized in the blood vessels of the spleen using the substrates bGP, ATP, FDP and pNPP (Figs. 34-37) following the lead precipitation method. A control section (Fig. 38) was treated with 1% HCl and showed no APase activity using the azo dye method. With the substrate aNP, using the azo dye method, activity was localized in the blood vessels and in the pericortical region (Fig. 39). A control was made for the inhibition study by treating the section with 1% HCl after preincubation to inactivate the APase enzyme. No APase activity was demonstrated in the
Fig. 25. A control placenta section for the inhibition study treated with 1% HCl to inactivate the enzyme (x 60).

Fig. 26. Histochemical demonstration of APase activity in the trophoblast cells of the placenta without an inhibitor, thereby serving as a control for the demonstration of APase activity (x 60).

Fig. 27. Histochemical localization of APase activity in the trophoblast cells of the placenta using L-phenylalanine as the inhibitor (x 60).

Fig. 28. Histochemical localization of APase activity in the trophoblast cells of the placenta using L-homoarginine as the inhibitor (x 60).

Fig. 29. Histochemical localization of APase activity in the trophoblast cells of the placenta using L-tryptophan as the inhibitor (x 60).

Fig. 30. Histochemical demonstration of reduced APase activity in the trophoblast cells of the placenta using EDTA (x 60).
Fig. 31. A spleen section stained with Paragon (x 60).
Fig. 32. A control spleen section for the lead precipitation method without the substrate (x 60). No APase activity was demonstrated in this control section.

Fig. 33. A control spleen section for the lead precipitation method using 1% HCl to inactivate the enzyme (x 60).

Fig. 34. Histochemical localization of APase activity in a blood vessel of the spleen using the substrate bGP (x 240).

Fig. 35. Histochemical localization of APase activity in a blood vessel of the spleen using the substrate ATP (x 240).

Fig. 36. Histochemical localization of APase activity in a blood vessel of the spleen using the substrate FDP (x 60).

Fig. 37. Histochemical localization of APase activity in a blood vessel of the spleen using the substrate pNPP (x 60).

Fig. 38. A control spleen section for the azo dye method showing no APase activity because the enzyme was inactivated with 1% HCl (x 60).

Fig. 39. Histochemical localization of the APase activity in a blood vessel and in the cells at the periphery of the germinal center in the spleen using the substrate aNP (x 60).
section (Fig. 40). A spleen section (Fig. 41) showing APase activity in the blood vessel and in cells at the periphery of the germinal center was used as a control for the demonstration of APase activity. Using the known inhibitor L-phenylalanine, no reduction in APase activity was noted (Fig. 42). When L-homoarginine was used it inhibited the APase activity in the cells at the periphery of the germinal center but did not inhibit blood vessel activity therefore demonstrating two different isoenzymes of the APase enzyme in the spleen. One isoenzyme in the cells at the periphery of the germinal center was inhibited by L-homoarginine; another isoenzyme in the blood vessel was not inhibited by L-homoarginine (Fig. 43). L-tryptophan did not cause inhibition of APase activity in the blood vessels or in cells at the periphery of the germinal center (Fig. 44). EDTA inhibited the APase activity in the cell at the periphery of the germinal center and caused a reduction of APase activity in the blood vessels (Fig. 45).

Histochemical Localization of APase in the Adult Thymus

The adult thymus was highly involuted from the histology and contained lymphocytes (Fig. 46). Controls were made by leaving out the substrate and treating another
Fig. 40. A control spleen section for the inhibition study treated with 1% HCl to inactivate the APase enzyme (x 60).

Fig. 41. Histochemical localization of APase activity in a blood vessel and in cells at the periphery of the germinal center of the spleen without an inhibitor serving as a control for the demonstration of APase activity (x 60).

Fig. 42. Histochemical localization of APase activity in the blood vessels and in cells at the periphery of the germinal center in the spleen using L-phenylalanine (x 60). No APase activity was demonstrated.

Fig. 43. Histochemical demonstration of an isoenzyme of the APase enzyme in a spleen section using L-homoarginine (x 60). The APase activity of the cell at the periphery of the germinal center was inhibited while the APase activity of the blood vessel was not inhibited.

Fig. 44. Histochemical localization of APase activity in the cells at the periphery of the germinal center and in a blood vessel of the spleen using L-tryptophan (x 60).

Fig. 45. Histochemical localization of complete inhibition of APase activity in the terminal center and reduced APase activity in a blood vessel of the spleen using EDTA as the inhibitor (x 240).
Fig. 46. An adult thymus section stained with Paragon (x 60).
section with 1% HCl to inactivate the APase enzyme following the lead precipitation method (Figs. 47 and 48). No APase activity was noted in the control sections. Using the substrates bGP, ATP, FDP and pNPP, no APase activity was demonstrated in the adult thymus (Figs. 49-52). A control section for the azo dye method was made by inactivating the enzyme with 1% HCl (Fig. 53). Using the substrate aNP no APase activity was demonstrated except in a blood vessel (Fig. 54).

Histochemical Localization of APase in the 16-day Fetal Thymus

The histology of the fetal thymus showed the thymic cortex to be rich in lymphocytes (Fig. 55). Two controls were made following the lead precipitation method, one without the substrate and the other treated with 1% HCl to inactivate the enzyme (Figs. 56 and 57). No APase activity was demonstrated in the control sections. The 16-day fetal thymus using the substrates bGP, ATP, FDP and pNPP demonstrated APase activity in the outer cortex region of the fetal thymus (Figs. 58-61). A control section for the azo dye method treated with 1% HCl showed no APase activity (Fig. 62). Using the substrate aNP, APase activity was localized in the outer cortex region of the 16-day fetal
Fig. 47. A control adult thymus section in which the substrate was left out (x 60). No APase activity was demonstrated.

Fig. 48. A control adult thymus section treated with 1% HCl to inactivate the enzyme (x 60).

Fig. 49. An adult thymus section showing no APase activity using the substrate bGP (x 60).

Fig. 50. An adult thymus section showing no APase activity using the substrate ATP (x 60).

Fig. 51. An adult thymus section showing no APase activity using the substrate FDP (x 60).

Fig. 52. An adult thymus section showing no APase activity using the substrate pNPP (x 60).

Fig. 53. A control adult thymus section treated with HCl to inactivate the APase enzyme (x 60).

Fig. 54. An adult thymus section showing no APase activity except in a blood vessel using aNP as the substrate (x 60).
Fig. 55. A fetal thymus section stained with Paragon (x 60).
Fig. 64. A control fetal thymus section treated with HCl to inactivate the APase enzyme for the inhibition study (x 60).

Fig. 65. A fetal thymus section showing APase activity in the subcapsular area without an inhibitor thereby serving as a control for the demonstration of APase activity (x 240).

Fig. 66. A fetal thymus section showing no reduction of APase activity using L-phenylalanine as the inhibitor (x 60).

Fig. 67. A histochemical demonstration of APase activity in the fetal thymus using L-homoarginine as the inhibitor (x 60). No reduction of APase activity was demonstrated.

Fig. 68. A histochemical demonstration of APase activity in the fetal thymus using the inhibitor L-tryptophan (x 60). No inhibition of APase activity was demonstrated.

Fig. 69. A histochemical demonstration of reduced APase activity in the fetal thymus using EDTA as the inhibitor (x 60).
thymus (Fig. 63). A control section was made for the inhibition study using 1% HCl to inactivate the APase enzyme (Fig. 64) and no APase activity was noted. Figure 65 shows a 16-day fetal thymus section for the demonstration of APase activity. No inhibition of APase activity in the 16-day fetal thymus was demonstrated using L-phenylalanine, L-homoarginine or L-tryptophan (Figs. 66-68). EDTA caused a reduction in APase activity in the cortex region of the thymus (Fig. 69).
Fig. 56. A control fetal thymus section following the lead precipitation method in which the substrate was left out (x 60). No APase activity was demonstrated in the section.

Fig. 57. A control fetal thymus section for the lead precipitation method treated with HCl to inactivate the enzyme (x 60).

Fig. 58. A fetal thymus section showing APase activity in the subcapsular area of the cortex region of the fetal thymus using the substrate bGP (x 240).

Fig. 59. Histochemical demonstration of APase activity in the subcapsular area of the cortex region of the fetal thymus using the substrate ATP (x 60).

Fig. 60. A histochemical demonstration of APase activity in the subcapsular area of the cortex region of the fetal thymus using the substrate FDP (x 60).

Fig. 61. A histochemical demonstration of APase activity in the subcapsular area of the fetal thymus using the substrate pNPP (x 60).

Fig. 62. A control fetal thymus section treated with HCl to inactivate the APase enzyme using the azo dye method (x 60).

Fig. 63. A histochemical demonstration of APase activity in the subcapsular area of the fetal thymus using the substrate aNP (x 240).
CHAPTER V

DISCUSSION AND CONCLUSIONS

Alkaline phosphatase is an enzyme that is found in tissues that are involved in active transport. The study of the mechanism of enzyme action begins with the substrate specificity of the enzyme. It is an old and working hypothesis that the substrate molecule, or a portion of it, fits the active site in a lock and key relationship. APases hydrolyze many different esters of phosphoric acid. From the research of substrate specificity studies two distinct structural features determine the specificity of an enzyme for its substrate. First, the substrate must possess the specific chemical bond or linkage that can be attacked by the enzyme. Second, the substrate usually possesses some other functional group (or groups) which binds to the enzyme and positions the substrate molecule properly on the catalytic site.

The lead precipitation method and the azo dye method were used. Using the lead precipitation method artifact was often noted which is caused by the non-enzymatic hydrolysis of the substrates. The report of artifact is not new. Many
researchers have had to deal with this problem. A positive reaction of the enzyme at site can only be verified if the proper controls are used. Feign and Wolf (1957) reported that they found that artifactual staining occurs preferentially in the immediate vicinity of site of high activity where high concentration of the phosphate ion may be present. In these studies the azo dye method was found to be a superior method as compared to the lead precipitation method. Using the azo dye method, localization was much more precise and sensitive. Artifact staining is very easy to notice using this method along with a control. There are many different naphthyl phosphate derivatives that can be used and almost any primary naphthyl phosphate is susceptible to enzymatic hydrolysis. Therefore the azo dye method using the substrate aNP was chosen over the lead precipitation method for the inhibition studies.

The kidney was used in this study because of its high alkaline phosphatase activity and served as an excellent control for the demonstration of the enzyme in the substrate specificity studies. Much work has been done using the kidney to demonstrate the APase enzyme but not using the C57B1 mice. The localization of APase using bGP, FDP, pNPP, and aNP was restricted to the proximal tubules which are
located in the cortex of the kidney. With ATP as the substrate, activity seemed to be localized in the glomeruli and veins and was not restricted to the brush border of the proximal tubules. Padykula and Herman (1955) studied factors affecting the activity of adenosine 5'-triphosphate (ATPase) and other phosphatases as measured by histochemical techniques. They found ATPase activity in the kidney cortex and the outer edge of the medulla using the substrate ATP.

Wachstein and Meisel (1957) did histochemical studies using a variety of substrates: sodium glycerophosphate, glucose-6-phosphate, ATP, and muscle adenylic acid and showed frozen sections from a normal rat kidney stained for APase activity showing activity in the proximal tubules using bGP. They also found that frozen sections from a formalin fixed rat kidney stained for ATPase activity showed prominent staining of glomeruli, capillaries, ascending limb of Henle's loop and distal convoluted tubules, while strongest activity in proximal convoluted tubules was seen, and the activity was somewhat less distinct in the terminal segments and almost absent in the median part of the proximal convoluted tubules. The data presented here suggest that there may be two different enzymes present in the kidney, since ATP differed in its localization as compared to bGP, FDP, pNPP and aNP.
The inhibition study employed the use of several amino acids and a metal chelating agent (EDTA). A preincubation time of 20 minutes was used for the tissue sections. Fishman (1975) suggested using a two hour preincubation time in which they were able to obtain better inhibition results with human placenta. The two hour preincubation time was tried which resulted in deterioration of the tissue and 20 minutes was found to be a suitable time without causing too much destruction. L-phenylalanine, L-homoarginine and L-tryptophan at 40 mM did not cause any inhibition in the brush border of the proximal tubules of the kidney using the azo dye method. With 10 mM of EDTA, APase activity in the proximal tubules of the kidney was inhibited. Alkaline phosphate has many metals that are associated with it, such as magnesium and zinc. EDTA was used because it can chelate metals thereby causing the inhibition of alkaline phosphatase. This was demonstrated in the slight inhibition of APase activity in the kidney suggesting two isoenzymes of the APase enzyme.

The placenta is an organ in which the physiological exchange of nutrients and waste products takes place between the embryonic and the maternal circulation. The placenta has high APase activity in the syntrophoblast and
cytotrophoblast cells. The APase in the trophoblast cells of the placenta hydrolyzed all the substrates tested (bGP, ATP, FDP, pNPP and aNP). The APase activity was not inhibited with L-phenylalanine, L-homoarginine or L-tryptophan and only slight inhibition occurred with EDTA. This suggests the presence of two isozymes in the placenta. Floyd (1976) did biochemical studies using L-phenylalanine, L-homoarginine and EDTA in which she was able to show inhibition of the APase enzyme with different inhibitors.

The APase activity in the spleen was localized in the blood vessels with all the substrates tested. With the substrate aNP AS MX using the azo dye method activity was also present on the membrane of the cells at the periphery of the germinal center. APase activity in the spleen using the lead precipitation method had slight artifact staining in both controls. The artifactual staining was more pronounced here because the spleen had to be incubated for a longer period of time than all of the other tissues used. Longer incubation times often causes artifactual staining and diffusion at the site of activity. The azo dye method was used to localize the enzyme in the spleen and no artifactual staining was seen in the controls. With the azo dye method using the substrates aNP APase activity could be seen in
blood vessels and in the cells at the periphery of the germinal centers.

The thymic organ has been shown to have a role in the process of leukemogenesis. Kaplan (1961) found that radiation-induced lymphoid tumor did not develop in the C57B1 mice that had been thymectomized but did develop in mice that had spleen or gonads removed. In these studies all the substrates, bGP, ATP, FDP, pNPP and aNP were hydrolyzed by the APase enzyme in the 16-day fetal thymus. Activity was localized in the outer part of the cortex region of the fetal thymus. The adult thymus was used, and no activity was demonstrated in the cortex or medulla area with any of the substrates except in an occasional blood vessel. These results agree with Smith (1961) who found no activity in the adult thymus. The adult thymus was eliminated from the inhibition studies since it is generally APase negative. There was no inhibition of APase activity using L-phenylalanine, L-homoarginine or L-tryptophan. Reduced APase activity was shown with EDTA in the fetal thymus.

From these substrate specificity studies it seems evident that the kidney may have two different isoenzymes since localization of activity was different with ATP than with bGP, FDP, pNPP and aNP. These studies have shown
APase to be present in the fetal thymus up to 16 days using the substrate bGP, ATP, FDP, pNPP, and aNP in the outer cortex region. The fetal thymus APase activity was similar to the APase activity in the placenta and spleen in that it did hydrolyze all the substrates used. In the inhibition studied with L-homoarginine and EDTA, APase activity in cells at the periphery of the germinal center was inhibited suggesting the presence of two different isoenzymes in the germinal center. APase activity in the tissues used in the inhibition studies was also inhibited by EDTA. L-homoarginine inhibited the APase isoenzyme in the cells at the periphery of the germinal center but did not inhibit blood vessel activity, again suggesting the presence of two isoenzymes.

The findings showed that APase hydrolyzed all the substrates tested in all the tissues used and that the azo dye method gave a more precise localization as opposed to the lead precipitation method. APase activity in the kidney was slightly inhibited by EDTA. In the spleen APase activity in the cells at the periphery of the germinal center was inhibited by L-homoarginine and EDTA. However, the EDTA also caused inhibition of the APase activity in the blood vessels of the spleen, also suggesting two
isoenzymes. The adult thymus from the histology was highly involuted and was APase negative except in blood vessels, while the 16-day fetal thymus had APase activity in the cortex region which was slightly inhibited by EDTA.

The evidence here suggests a possible role of APase in transport of materials across the membrane since APase activity is found in abundance in kidney tubules and trophoblast cells of the placenta. A possible fetal function is evident from this study since APase activity was found in the 16-day fetal thymus up to 16-day gestation and was absent in the adult thymus.

Further studies to characterize the APase enzyme utilizing the substrates and tissues used in this study should be done at the electron microscopical level. This will show more precise localization of the enzyme thereby adding to the pool of knowledge about the character of the APase enzyme.
CHAPTER VI

SUMMARY

1. The azo dye method is superior to the lead precipitation method for the localization of APase in fresh frozen sections.

2. All substrates, aNP, bGP, ATP, FDP and pNPP, were hydrolyzed by the APase of the kidney, fetal thymus, spleen and placenta.

3. Using the substrates aNP, bGP, FDP and pNPP, localization of the kidney APase was in the proximal tubules in the cortex. However, using ATP as the substrate, APase was localized in the glomeruli and veins. These data suggest the presence of two isoenzymes of APase in the kidney.

4. The fetal thymus had APase activity in the outer cortex region; whereas the adult thymus was APase negative.

5. From the inhibition studies using different amino acids and EDTA, it seems evident that the spleen may have two isoenzymes. L-homoarginine and EDTA inhibit the cell membrane-bound activity in the pericortical areas while only causing slight inhibition of the blood vessel activity with EDTA.


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