ASPARTATE AMINOTRANSFERASE AND ITS ISOZYMES IN CALF AND RAT LENS

by

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DEDICATION

This Doctoral thesis is dedicated to my wife, Barbara, and my daughter, Meredith, who have been inspiring, helpful and most patient.

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I. INTRODUCTION

The present study has investigated the enzyme activity of aspartate aminotransferase (AAT) and its isozymes in normal, aging and sugar-induced cataractous lenses. Enzyme kinetics, immunochemistry, pH, cofactor effects and localization for each of the AAT isozymes have been evaluated in rat and calf lens tissue under various conditions. Anionic and cationic isozymes were partially purified and characterized from calf lens and from rat liver. The following introduction represents a brief review of the current status of lens tissue and of the enzyme, aspartate aminotransferase.

A. LENS TISSUE

The lens is an avascular tissue possessing biconvex and transparent qualities. The lens tissue, which is completely surrounded by a collagenous capsule, consists of an outer, anterior, single layer of epithelial cells which is further subdivided into a central region, a germinative region and a region of cellular elongation; the inner cortex, and a compressed central nucleus (Fig. 1). The ontogeny of the lens cell is such that the epithelial cells near the equatorial region undergo cellular differentiation and elongation to form the lens fiber cells. Fiber cell formation represents the terminal stage of cellular differentiation in the lens. The bulk of the lens is composed of consecutive layers of fiber cells which are continuously laid down throughout the life of the animal. This sequential formation of cells and subsequent displacement into the

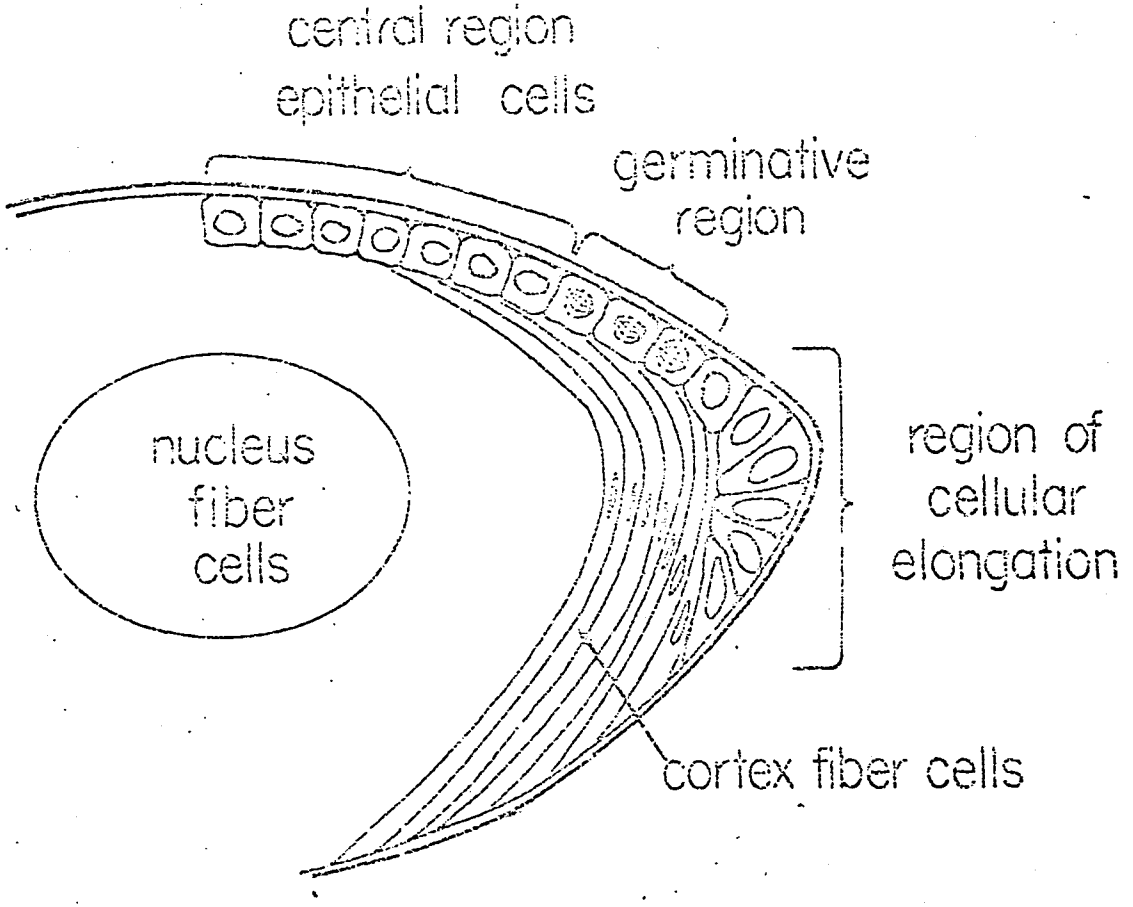

Figure 1. A diagrammatic representation of the structure of the vertebrate lens. (From Papaconstantinou, 1967)

central region accounts for the growth of this tissue. The adult lens therefore would have a compact central or nuclear region composed of those fiber cells formed during embryonic growth, while the most recently formed fiber cells would be found in the peripheral or cortex region. Thus, lens tissue is composed of a single cell type which shows distinct stages of cellular differentiation.

The single layer of epithelial cells has a high mitotic index in the embryonic lens and, as the lens reaches maximal size in the adult, the mitotic index becomes negligible, being restricted primarily to the pre-equatorial region (Hanna and O'Brien, 1961; Cotlier, 1962; Mikulicich and Young, 1963; and Srinivasan and Harding, 1965). After birth the mitotic activity in the central zone of the epithelium decreases rapidly until finally these cells remain in a stationary phase of the cell cycle. This extended G_1 phase appears to be reversible, since the cells can be stimulated out of the G_1 phase into deoxyribonucleic acid (DNA) synthesis (S phase) and mitosis (M phase), by physical or chemical means (Srinivasan and Harding, 1965). In the equatorial region the differentiation of the epithelial cells into elongated fiber cells is associated with major intracellar changes. The nucleus enlarges and both DNA and ribonucleic acid (RNA) have the capacity of being synthesized (Hanna, 1965; Reeder and Bell, 1965). Concommitantly, the number of ribosomes also increased (Eguchi, 1964 and Karasaki, 1964). The mitochondria of the epithelial regions

have been shown to be small in size and moderate in number (Kuwabara, et al., 1969).

Electron microscopic studies have indicated that the nucleus and nucleoli of the differentiated fiber cell were decreased in size and no longer capable of synthesizing DNA and RNA (Eguchi, 1964). The ribosomal population was significantly decreased and the endoplasmic reticulum was observed to take on a smoother appearance as compared to the granular appearance of the epithelial endoplasmic reticulum. Deeper zones of fiber cells lost their nuclei and became more compact (Kuwabara, et al., 1969). Also, the most common microorganelles disappeared with increasing depth of fiber cells.

The anatomy and cytological fine structure of the lens has been described as being very similar in all species of mammals. There appeared to be no significant differences in the epithelial and cortical fiber regions among man (Cohen, 1965), rat, rabbit, monkey, calf and guinea-pig (Wanko and Gavin, 1958, 1959).

The metabolic energy of lens tissue has been reported to be derived primarily from the oxidation of glucose (Hockwin, 1965; Lerman and Zigman, 1965). Although the presence of most of the enzymes of the Krebs cycle has been demonstrated in lens (Ely, 1949; Wortman and Becker, 1956), very little glucose appeared to be metabolized by means of this pathway (Kinoshita and Wachtl, 1958; Kinoshita, 1965). The major metabolic pathway for glucose metabolism

has been shown to be via the Embden-Meyerhof pathway (Green and Solomon, 1956, 1957, 1959; Lerman, et al., 1962, 1965). Substantial hexose monophosphate shunt (Kinoshita, 1955, 1965) and sorbitol pathway (van Heyningen, 1962) activities have also been shown to exist in lens tissue (Fig. 2). In age studies anaerobic glycolysis appeared to be as active in the fetal lens as in the mature or old lens (Pitel and Lerman, 1962; Lerman, et al., 1962; Lerman, 1960, 1961A, 1961B, 1962). Thus glucose oxidation via the hexose monophosphate shunt played a more active role in the rapidly developing lens than in the mature lens. A number of specific enzymes have also been found to have a lower activity in older lenses. These include carbonic anhydrase (Reich and Healy, 1953), hexokinase (Green and Solomon, 1959), glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, isocitric dehydrogenase and lactate dehydrogenase (Kuhlman and Resnik, 1958). On the other hand, slight increases in enzyme activity with lens age have been reported for fructoaldolase (Hockwin, 1965) and methionyl-tRNA synthetase (Weller and Green, 1969).

Mörner (1894) first suggested that lens protein was composed of three soluble fractions: alpha crystallin, beta crystallin and gamma crystallin, as well as insoluble material called the albuminoid fraction. Classically, alpha crystallin was obtained by isoelectric precipitation (Woods and Burky, 1927), beta crystallin by salting-out procedures (Burky and Woods, 1928A), and gamma crystallin comprised

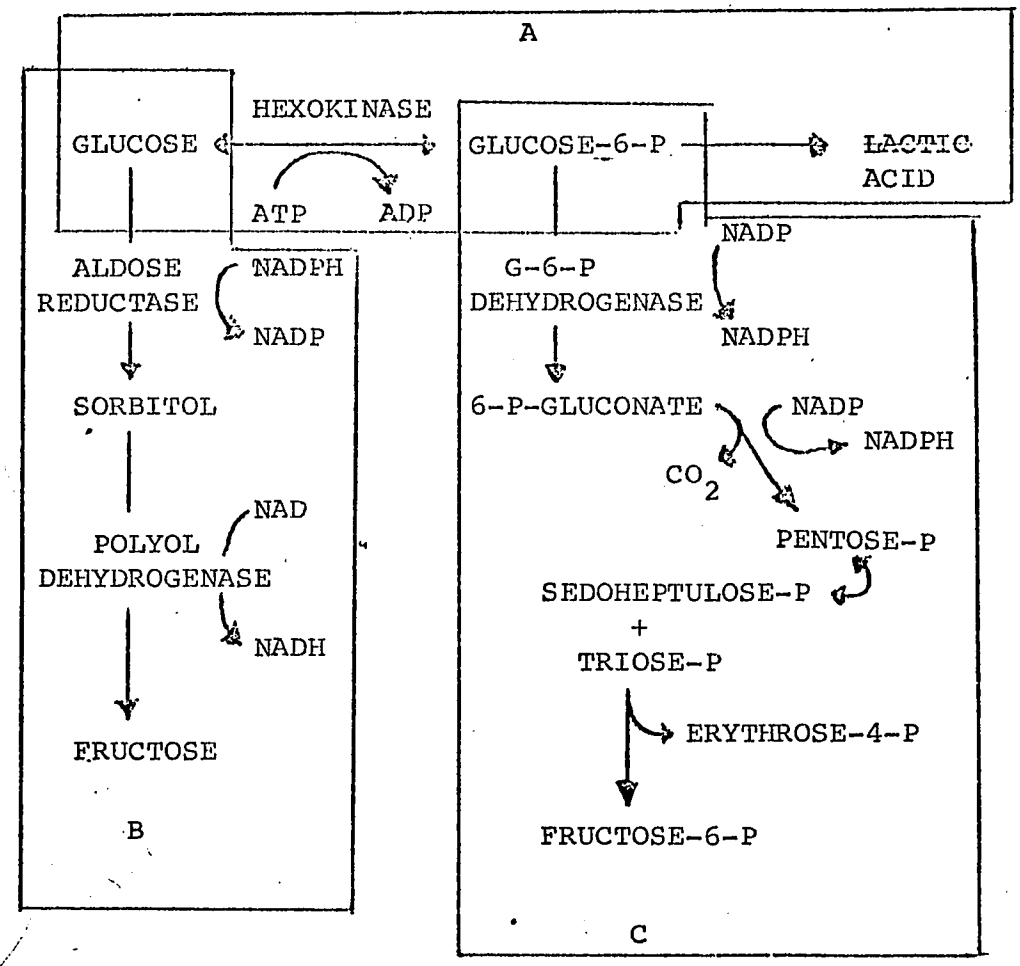

Figure 2. Metabolism of glucose via the Embden Meyerhof Pathway (A), Sorbitol Pathway (B) and the Hexose Monophosphate Shunt (C).

the remaining soluble protein (Burky and Woods, 1928B). Electrophoresis indicated that alpha crystallin had the greatest mobility, gamma crystallin the slowest mobility, and beta crystallin an intermediate mobility (Hesselvik, 1939; Bon and Nobel, 1958). Recent column chromatographic studies have demonstrated that each of the crystallins was composed of subunits (Spector, 1965).

The alpha and beta crystallins have been reported to be structural proteins elaborated by both the epithelial and differentiated fiber cells of the calf lens (Papaconstantinou, 1957). The synthesis of gamma crystallins, on the other hand, has been associated with the differentiation of the lens epithelial cell to a fiber cell (Papaconstantinou, 1964, 1965, 1967; Takata, et al., 1965). Another characteristic of the differentiated fiber cell was the finding that alpha, beta and gamma crystallin protein was synthesized from stabilized messenger-RNA (mRNA) (Stewart and Papaconstantinou, 1967). This increase in the half-life of mRNA in fiber cells appeared at a time during cellular differentiation when nuclear RNA and DNA synthesis decreased and was associated with the loss of mitotic activity. The observation of increased stabilization of mRNA for the production of tissue specific protein during cellular maturation was not unique for lens tissue since it has also been documented in the maturation of the reticulocyte (Reich, et al., 1962; Marks, et al., 1962).

Lenticular opacities (cataracts) have been observed

when animals were exposed to a high galactose diet (Sippel, 1966A, 1966B, 1967), a high xylose diet (Lerman and Heggeness, 1961) or x-irradiation (van Sallmann, 1957). Cataract formation has further been noted with severe diabetes (Pirie, 1965) and senility (ibid; Barber, 1968). In general, induced cataractogenesis was more rapid in younger animals than in older ones (Lerman, 1965). The induction of sugar cataracts has been attributed to the osmotic effects caused by the accumulation of the respective sugar alcohols: dulcitol in the case of galactose, sorbitol from glucose and xylitol in the case of high xylose diets (Kinoshita, et al., 1962). In an in vitro study, Kinoshita, et al. (1968, 1969) reported that opacities did not develop in a high galactose medium when the enzyme, aldose reductase, which produces dulcitol from galactose was specifically inhibited.

Xylose and galactose differ markedly in their cataractogenetic action. Young rats placed on a 50% galactose diet have been shown to develop permanent, dense, nuclear cataracts in 8 to 12 days (Sippel, 1966A, 1966B, 1967). However, lenticular opacities which developed with a 30% xylose diet in 8 to 14 days were neither progressive nor permanent with respect to lens transparency or function even with continued ingestion of xylose (Booth, et al., 1953; van Heyningen, 1959; Lerman and Heggeness, 1961).

Electron microscopic studies of cataracts induced by the feeding of high galactose diets have shown cellular changes as early as the 3rd day (Kuwabara, et al., 1969).

These changes include increased reactivity of the epithelium, edema of lens cells and intercellular vacuolization. With continued galactose ingestion, cellular disruption progressively increased until the lens cells became irreversibly damaged. During this period, the levels of free amino acids in the lens showed marked decreases (Kinoshita, et al., 1969) followed eventually by a 50% loss of soluble lens protein (Sippel, 1966A). By the 2nd day on a galactose diet, the production of lactate (ibid.) and the oxidation of glucose via the hexose monophosphate shunt (Lerman, 1960) showed significant decreases. The adenosine triphosphate (ATP) content of the lens decreased after the 4th day of galactose feeding (ibid.).

The lens activities of several enzymes have been investigated during the time course of galactose-induced cataracts. Lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6-PDH) (Sippel, 1967), 6-phosphogluconic acid dehydrogenase (Lerman, 1960) and creatine phosphokinase (Cotlier, 1964) all retained their initial levels of activity up through the first 15 days of galactose feeding. Shortly thereafter, the level of these enzyme activities in lens tissue substantially decreased. However, aldolase, a sulphydryl-dependent enzyme, showed decreases in activity early in the first week of galactose feeding (Sippel, 1967). This loss in activity has been correlated with the early decreases observed for glutathione levels (Sippel, 1966A).

B. ASPARTATE AMINOTRANSFERASE (AAT)

Aspartate aminotransferase (AAT) is an enzyme which reversibly catalyzes the transfer of the amino group of aspartate to alpha ketoglutarate with the subsequent formation of the corresponding alpha keto acid and amino acid, oxaloacetate and glutamate, respectively (Fig. 3). Braunstein and Kritzmann (1937) were the first to present definite evidence for the general amino group-transfer reaction. Snell (1944) recognized the importance of pyridoxal and pyridoxamine as forms of the coenzyme in transamination. These vitamin B_6 derivatives form Schiff base intermediates in the enzymatic transfer of the amino group. Jenkins and Sizer (1957) in spectrophotometric studies showed the interconversion of the aldehyde and amino forms of the cofactor. Titration of the apoenzyme with the cofactor has indicated that 2 moles of pyridoxal phosphate were bound per mole of the active enzyme for purified preparations of AAT from pig heart (Jenkins, et al., 1954; Banks, et al., 1963; Banks, et al., 1968; Martinez-Carrion, et al., 1967) and from chicken heart (Bertland and Kaplan, 1968).

In recent years, AAT activity has been shown to be ubiquitous in animals (Zimmerman, et al., 1965), plants (Wilson, et al., 1954) and microorganisms (Wada and Snell, 1961). The monitoring of AAT activity has further attracted considerable interest because its concentrations in blood serum and tissues have been found to be altered in a number of disease states (Wroblewski, 1958). Liver damage caused by hepatitis, for example, significantly increased AAT

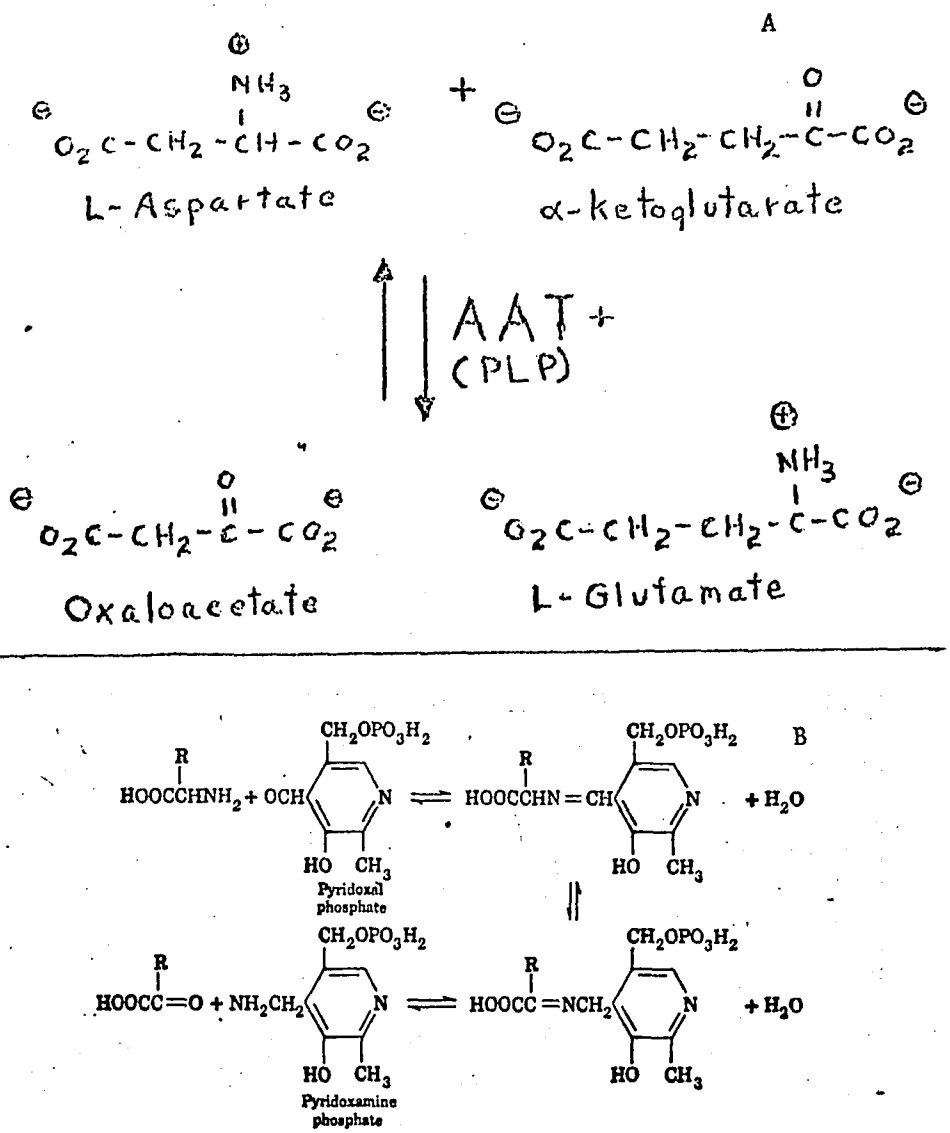

Figure 3. Aspartate aminotransferase reaction (A) and the PLP (pyridoxal phosphate) (Schiff Base) reaction. (B).

activity of blood serum (Bodansky, et al., 1960). Cardiac infarction in humans showed similar increases in blood serum AAT activity (Wroblewski, 1957). Neuromuscular diseases such as muscular dystrophy and dermatomyositis have also been associated with elevated AAT activity in blood serum (La Due and Wroblewski, 1955; Siekert and Fleisher, 1956).

Prior to 1959, because of the methods of AAT purification, there was no indication that this enzyme might occur in more than one active enzymatic form (Jenkins, et al., 1959 and Lis, 1958). Fleisher, Potter, and Wakim (1960) were the first to demonstrate that crude preparations of heart and liver from man, pig, and dog that had previously been thought to be homogeneous were actually composed of two different proteins, both of which possessed AAT activity. The enzyme was separated by paper electrophoresis into a fast moving anionic and a slow moving cationic fraction. Each AAT isozyme showed different substrate affinities in their Michaelis constants for L-aspartate and alpha ketoglutarate (ibid.). These differences have also been reported for highly purified isozymes from human heart and liver (Nisselbaum and Bodansky, 1965). The two AAT isozymes behaved differently with changes in pH. The cationic AAT had a relatively constant enzymatic activity between pH 6.0 and 8.0, while the anionic AAT at pH 6.0 had only about 40% of the activity measured at pH 7.4 (Fleisher, et al., 1959; Boyd, 1961). The available pH profile data indicated that the pre-1960 investigators were in fact studying only the

anionic component (Karmen, et al., 1955A; Jenkins, et al., 1959).

The investigations of Boyd (1961, 1962) revealed why the second AAT isozyme (cationic form) had remained elusive for so long. It was discovered that the cationic isozyme was primarily located in the mitochondria, while the anionic isozyme was a soluble cellular component. The presence of the cationic isozyme in tissue preparations was best observed after the mitochondria was disrupted (generally by water extraction, sonication, or detergents). Serum solutions revealed no cationic isozyme unless a disease state which damaged mitochondria was induced (e.g. CCl_4 poisoning, Fleisher and Wakim, 1961).

In recent years, several reports have indicated that both the cationic and anionic isozymes of AAT existed not as single entities, but that each contained "electrophoretic subforms." Decker and Rau (1963) were the first to present electrophoretic evidence for the presence of AAT subforms. As many as four anionic electrophoretic AAT bands have also been reported for extracts of various mouse tissues (Bhatt and Bajaj, 1969). However, human heart, like human liver and kidney, contained only a fast moving anionic and a slow moving cationic fraction (Boyd and Latner, 1962). Banks, et al. (1968) presented evidence that the anionic AAT purified from pig heart muscle migrated as a single entity when electrophoresis was conducted at pH 8.6. Four anionic subforms became visible when the pH of the starch gel

system was lowered to pH 8.0 and pH 7.5. In contrast to these findings, Bertland and Kaplan (1968) reported that chicken heart extracts and purified anionic AAT yielded six anionic AAT species at pH 8.5. At the same pH, chicken heart extracts and purified cationic AAT from chicken heart gave four cationic electrophoretic AAT subforms. However, the biochemical characteristics of these reported subforms have not been fully resolved and it is possible that some of them may be artifacts.

In 1963 both the anionic and cationic isozymes were obtained in crystalline form from beef liver (Marino, et al., 1963). The cationic AAT was estimated to have a molecular weight (M.W.) of 100,000, while the anionic AAT was reported to be about 120,000. The M.W. of the chicken heart anionic AAT based on sedimentation equilibrium ultracentrifugation was estimated to be about 100,000 (Bertland and Kaplan, 1968). This was slightly lower than the M.W. of 110,000 reported for the pig heart enzyme (Jenkins, et al., 1959) and of the same order as the M.W. of 95,000 of rat liver AAT (Harping, 1965) and 96,000 for ox heart AAT (Marino, et al., 1966). In a recent investigation, Banks, et al. (1968) have reported a substantially lower value (78,600) for the M.W. of the anionic isozyme purified from pig heart muscle as determined by ultracentrifugation and also by gel filtration. Thus, the M.W. of the anionic AAT still remains to be clarified.

Further evidence in support of the view that the

anionic and cationic isozymes of AAT are different from one another, aside from electrophoresis, comes from column chromatography data (Nisselbaum and Bodansky, 1964, 1969). Whether the separation of the isozymes was achieved from a hydroxyl apatite or a diethylaminoethyl cellulose column, each isozyme was eluted from the column with a different concentration of buffer.

Another line of supporting evidence may be derived from the area of immunoenzymology. Specific rabbit antiserum made against each AAT isozyme from human heart inhibited the homologous AAT isozyme specifically and almost completely from human heart and liver (Nisselbaum and Bodansky, 1964). The AAT activity of human erythrocytes, which has been reported to contain only the anionic isozyme, was inhibited by antiserum made specifically against the anionic isozyme from human heart tissue (Nisselbaum, 1965).

The precise role that AAT plays in the metabolic process still remains to be elucidated. Lardy has postulated that AAT isozymes may be intimately involved in gluconeogenesis (1965, 1966) and in the urea cycle (1966). Because of the selective permeability of the mitochondrial membrane, oxaloacetate cannot be directly transported from the mitochondria to the cytoplasm (Lardy, 1966). However, aspartate and alpha ketoglutarate are permeable to the mitochondrial membrane. Aspartate aminotransferase would convert oxaloacetate to aspartate in the mitochondria and would synthesize oxaloacetate from aspartate in the cytoplasm where gluconeogenesis takes place. In addition, the enzymes, glutamate

dehydrogenase (GDH) and AAT in the mitochondria would provide aspartate and ammonium ions which are required for the urea cycle. By this mechanism, large amounts of amino acids may be degraded to supply carbon chains for gluconeogenesis.

C. ASPARTATE AMINOTRANSFERASE IN OCULAR TISSUE

There has been very little work done on the AAT enzyme in lens tissue. To date there have been no AAT isozyme studies reported. In fact, there have been very few isozyme studies in lens tissue (Stewart and Papaconstantinou, 1966; Papaconstantinou, 1967). Most of the references cited in this section were obtained from abstracts of Italian journals. Consequently, some pertinent information is lacking. In rabbit eyes, AAT activity has been reported to be high in the retina and uvea, while the activity in cornea, lens and aqueous was reported as being very low (Muto, 1960). In the ox, the corneal epithelium was stated to contain significant AAT and glutamate-pyruvate transaminase (GPT) activity (Testa, 1960). A constant AAT to GPT ratio of 3:1 was found in ox corneal tissue at three different ages (6 months, 2 and 10 years of age) (Janadet, 1963). Dardenne and Kirsten (1962) reported that both calf and bovine lens epithelium and cortex regions possessed about equal AAT activity. Testa and de Conciliis (1958) reported that small amounts of transferase activity were found in lenses with early cataracts in rabbits. This activity gradually began to disappear when the opacity became complete.

II. MATERIALS AND METHODS

A. ENZYME ASSAY

Aspartate aminotransferase (L-aspartate : 2-oxoglutarate aminotransferase, EC 2.6.1.1.) activity was measured by a modification (Nisselbaum and Bodansky, 1969) of the coupled reaction method of Karmen (1955). L-aspartic acid (Sigma) and α-ketoglutaric acid (Sigma) were both dissolved in water and adjusted to pH 7.4 with 2 N NaOH. Each was then made 10 mM with respect to Tris-HCl buffer, pH 7.4. Malic dehydrogenase (Sigma, 20,000 units per vial) was reconstituted in 10 ml of 100 mM Tris-HCl buffer, pH 7.4 containing 0.15% bovine serum albumin (BSA) (Sigma, Fraction V) and dialyzed against 10 volumes of 100 mM Tris-HCl buffer, pH 7.4 for 2 hours at 4°C to remove ammonium sulfate present in this commercial preparation. Reaction mixtures contained 0.5 ml of appropriately diluted enzyme in 100 mM Tris-HCl buffer, pH 7.4 containing 0.15% BSA; 0.5 ml of 0.1 M L-aspartate; 0.2 ml of 0.1 M α-ketoglutarate; 0.1 ml of malic dehydrogenase, 2,000 units per ml; 0.1 ml of a solution containing 2 mg/ml of reduced sodium nicotinamide adenine dinucleotide ($Na_2NADH \cdot 3H_2O$) (Sigma); 1.6 ml of 100 mM Tris-HCl buffer, pH 7.4, to give a final volume of 3.0 ml. Reaction mixtures containing pyridoxal-5'-phosphate (PLP), were made 0.1 mM with respect to this cofactor by the addition of 0.3 ml of a 1 mM PLP (Calbiochem). The reaction mixture volume was kept constant at 3.0 ml by appropriate changes with Tris-HCl buffer, pH 7.4. Each

reaction was started by the addition of 0.2 ml of 0.1 M ∝-ketoglutarate, after solutions were preincubated at 37°C for 10 min.

Reaction velocities were recorded at 30 sec intervals for 5 min by following the decrease in absorbancy at 340 nm in a Beckman model DU-2 spectrophotometer, using 10 mm light path cells. The cuvettes were maintained at 37°C by means of thermospacers connected to a Haake constant temperature circulating bath, maintained at 39°C. Initial velocities were determined during the zero order portion of the reaction. One unit of AAT activity is defined as that amount of enzyme which will cause a decrease of 0.001 in absorbancy in one minute at 37°C. Specific activity was expressed as units per min per mg of protein or per mg of fresh lens weight. Protein was determined by the biuret method of Robinson and Hogden (1940), using crystalline BSA as a standard.

Kinetic studies were carried out in 100 mM Tris-HCl buffer, pH 7.4 containing NADH, L-aspartate, ∝-ketoglutarate, malic dehydrogenase and appropriately diluted enzyme in 100 mM Tris-HCl buffer, pH 7.4 containing 0.15% BSA. The final reaction mixture volume was 3.0 ml. The double reciprocal plot of Lineweaver and Burk (1934) of 1/velocity (1/v) against 1/substrate concentration (1/s) was employed to determine the 1/apparent maximal velocities (1/V'max). In accordance with the procedure of Velick and Vavra (1962) the "true" Michaelis constants, Km, were obtained by replotting 1/V' max for one substrate against the

reciprocal concentrations of the second substrate at which the apparent 1/V'max values were obtained. The method of least squares was used to determine the Km values.

B. LENSES

Spraque-Dawley rats were sacrificed by decapitation. In most experiments 6 to 10 lenses were removed and homogenized in 2.0 ml of 10 mM Tris-HCl buffer, pH 7.4. The homogenizer was washed with a 1.0 ml aliquot of the previous buffer and combined with the original homogenate. The preparation was centrifuged at 12,100 x g for 20 min. and the supernatant was decanted. The pellet was resuspended in an additional 1.0 ml aliquot of the above buffer and centrifuged again at 12,100 x g for 20 min. The wash was combined with the first supernatant to yield the final preparation. The previous steps were carried out at 4°C. Enzymatic assays on single lens preparations were not possible due to insufficient activity.

The dietary studies utilized four-week-old rats weighing approximately 40 gm. The animals were placed on a 50% galactose diet (50% powdered Purina Laboratory Chow by weight, 50% D-galactose (Nutritional Biochemicals)), a 35% xylose diet (65% Purina and 35% D-xylose (Nutritional Biochemicals)), a 50% glucose diet (50% Purina and 50% D-glucose (Nutritional Biochemicals)), or on a control diet (Purina). Galactose-discontinued experiments were conducted where rats were placed on the 50% galactose diet for 3 or 10 days and then were re-fed Purina Chow.

C. PURIFICATION OF RAT LIVER AAT ISOZYMES

Partial purification of the anionic and cationic rat liver isozymes was achieved through the modification of the methods of Nisselbaum (1968) and Nisselbaum and Bodansky (1969), adapted from the procedure of Jenkins, Yphantis and Sizer (1959). 193 gm of rat liver obtained from 20 mature rats was homogenized in 3 volumes of 0.25 M sucrose for 30 seconds in a Waring blender and then centrifuged at 8,000 x g for 30 minutes. The purification of the anionic isozyme from the 8,000 x g supernatant and the cationic isozyme from the 8,000 x g pellet is described below. All steps were carried out at 4°C, except where noted.

1. Anionic Isozyme

The 8,000 x g supernatant was dialyzed overnight against several 4 liter changes of distilled water. The dialyzed supernatant was then made 0.4 mM with respect to α-ketoglutarate by the addition of 100 mM α-ketoglutarate. The solution was heated under constant stirring at 75°C for 10 min in a water bath maintained at 79°C. The solution was rapidly cooled to 5°C in an ice-water bath and the heat-denatured protein was removed by centrifugation for 30 min at 5,860 x g. Fractionation of the supernatant with solid $(NH_4)_2\ SO_4$ between 50% and 67% saturation recovered most of the anionic isozyme. The precipitated enzyme was collected by centrifugation at 5,860 x g for 30 minutes, redissolved in 0.3 M K-maleate buffer, pH 6.0, and dialyzed overnight against 4 liters of 5 mM Tris-acetate buffer, pH 7.5. The dialyzed anionic enzyme was then applied to the top of a

diethylaminoethyl (DEAE)-cellulose (Whatman DE-23) column, 7 cm^2 x 30 cm, previously equilibrated with 5 bed volumes of 5 mM Tris-acetate buffer, pH 7.5. The enzyme preparation was washed into the column with 100 ml of the 5 mM Tris-acetate buffer, pH 7.5. A linear gradient using 500 ml each of 5 mM and 100 mM Tris-acetate buffer, pH 7.5 was used to elute the enzyme from the column. Aliquots of 6.0 ml were collected at a flow rate of 40 ml per hour. Enzymatic assays for AAT localized the peak of enzyme activity. These tubes were pooled and the enzyme precipitated by the slow addition of solid $(NH_4)_2\ SO_4$ to 75% saturation. The enzyme was collected by centrifuging at 5,860 x g for 20 minutes and redissolved in a minimal volume of 0.3 M K-maleate buffer, pH 6.0. The preparation was dialyzed for 48 hours against three, 2 liter changes of distilled water. The dialyzed preparation was clarified by centrifuging at 48,000 x g for 20 min. This partially purified anionic AAT solution was used in enzyme kinetic studies and for the preparation and evaluation of antibodies.

2. Cationic Isozyme

The original 8,000 x g pellet from the liver homogenate was resuspended in 0.3 M K-maleate buffer, pH 6.0 and dialyzed overnight against several 4 liter changes of distilled water. The dialyzed fraction was made 0.4 mM with respect to α-ketoglutarate and heated under constant stirring in a 65°C water bath to 60°C. This temperature was maintained for 10 minutes and the solution was then rapidly

cooled to 5°C in an ice-water bath. The heat-denatured protein was removed by centrifuging at 5,860 x g for 30 minutes. The supernatant volume was adjusted to 500 ml with cold distilled water and fractionated between 60% and 80% with solid $(NH_4)_2\ SO_4$. The precipitated enzyme was recovered by centrifuging at 5,850 x g for 30 minutes and redissolved with a minimal volume of 0.3 M K-maleate buffer, pH 6.0. After dialyzing overnight against 4 liters of 5 mM Tris-acetate buffer, pH 7.5, the cationic enzyme preparation was applied to the top of a DEAE-cellulose column, 7 cm^2 x 30 cm, previously equilibrated with 5 bed volumes of 5 mM Tris-acetate buffer, pH 7.5. Aliquots of 6.0 ml were collected at a flow rate of 40 ml per hour. The cationic AAT was eluted from the column with 5 mM Tris-acetate buffer, pH 7.5. The enzymatically active aliquots were pooled and fractionated with solid $(NH_4)_2\ SO_4$ between the limits of 0% and 75% saturation. The precipitated enzyme was centrifuged at 5,860 x g for 20 minutes and redissolved in a minimal volume of 0.3 M K-maleate buffer, pH 6.0. The solution was dialyzed for 48 hours against three, 2-liter changes of distilled water and the insoluble material discarded by centrifugation at 48,000 x g for 20 minutes. This partially purified cationic AAT preparation was used in enzyme kinetic studies and for the preparation and evaluation of antibodies.

D. PARTIAL PURIFICATION OF CALF LENS AAT ISOZYMES

The anionic and cationic isozymes of AAT from calf lens tissue was prepared by the method of Jenkins, Yphantis

and Sizer (1959), modified by Nisselbaum and Bodansky (1964) and Nisselbaum (1969).

Calf eyes were obtained from the local abattoir and the lenses were removed within two hours after death. The lenses were stored frozed at -20°C until needed. All further purification steps were conducted at 4°C except where noted. Calf lenses (90.5 gm) were homogenized in a Waring blender with 4 volumes of 100 mM Tris-HCl buffer, pH 7.4 for 30 seconds. The homogenate was stirred for 30 minutes and then centrifuged at 8,000 x g for 30 minutes. The 8,000 x g pellet was resuspended in 100 ml of the previous buffer and stirred for 30 minutes. After centrifugation at 8,000 x g, the supernatant wash was recombined with the original supernatant. The combined solution was made 0.4 mM with respect to α-ketoglutarate and then heated to 60°C in a water bath maintained at 65°C for 10 minutes under constant stirring. The solution was cooled to 5°C in an ice-water bath and centrifuged at 5,860 x g for 30 minutes. The resulting supernatant was diluted with an equal volume of 0.01 M Tris-HCl buffer, pH 7.4 and fractionated with solid $(NH_4)_2\ SO_4$. The enzyme activity which precipitated between 40% and 80% saturation, was collected by centrifugation at 6,220 x g for 30 minutes. The precipitate was redissolved with a minimal volume of 0.3 M K-maleate buffer, pH 6.0 and dialyzed overnight against 6 liters of 5 mM Tris-acetate buffer, pH 7.5. Insoluble material was removed by centrifugation at 8,000 x g. The clear supernatant enzyme solution was then applied

to the top of a DEAE-cellulose column, 7 cm^2 x 30 cm, previously equilibrated with 5 mM Tris-acetate buffer, pH 7.5. The enzyme solution was washed into the column with 100 ml of 5 mM Tris-acetate buffer, pH 7.5. The cationic isozyme which was not adsorbed on to the column bed was eluted with an additional 400 ml of the previous buffer. The anionic isozyme was then eluted with a linear gradient using 500 ml each of 5 mM and 100 mM Tris-acetate buffer, pH 7.5. Aliquots of 6.0 ml were collected at a flow rate of 18 ml per hour. The enzymatically active portions of each peak of AAT were pooled separately and fractionated with solid $(NH_4)_2\ SO_4$ between 50% and 75% saturation. Each precipitate was collected by centrifuging at 8,000 x g for 20 minutes and redissolved in 0.3 M K-maleate buffer, pH 6.0. These solutions were made 0.1 mM with respect to PLP and dialyzed overnight against two, 1-liter changes of distilled water. The enzyme preparations were then clarified by centrifuging at 10,800 x g for 30 minutes. The anionic and cationic fractions were then individually rechromatographed on fresh DEAE-cellulose columns. The elution procedure for the individual calf lens AAT isozymes was the same as that used in the column fractionation of the rat liver isozymes, previously described. The individual isozyme column fractions were pooled separately and each precipitated with solid $(NH_4)_2\ SO_4$ between 50% and 75% saturation. Each of the precipitated fractions was redissolved in 0.3 M K-maleate buffer, pH 6.0 and made

0.1 mM with respect to PLP. The separate anionic and cationic solutions were dialyzed overnight against two, 1-liter changes of distilled water and clarified by centrifugation at 10,800 x g for 30 minutes. Lastly, each isozyme fraction was frozen, stored for 36 hours at -20°C and allowed to slowly thaw to a temperature not higher than 4°C. As soon as this temperature was attained and ice crystals were no longer present, each preparation was immediately centrifuged at 48,600 x g for 20 minutes at a temperature of 2°C. These calf lens anionic and cationic isozyme solutions were stored frozen for use in future experiments.

E. ELECTROPHORETIC METHODS

1. Starch Gel Electrophoresis

a. Tris-succinate-Phosphate System

Starch gel electrophoresis was performed according to the methods of Schwartz, et al. (1963) and Nisselbaum (1968) adapted from the procedure of Smithies (1955). Gels contained 12 gm of Connought hydrolyzed starch (Fisher Co.) per 100 ml of 5 mM Tris-succinate buffer, pH 7.2. Electrode vessels each contained 800 ml of 100 mM phosphate buffer, pH 7.2. Each electrophoretic run was for a duration of 18 hours under a constant voltage of 4 V/cm and 12 - 18 ma at 4°C. The isozymes of AAT were located on the sliced gel slabs by the author's modification of the method of Schwartz, et al. (1963). The final concentration of reagents per 100 ml of staining solution were as follows: 20 mg of freshly weighed out Azoene fast

violet B (Alliance Chemical Co.), 10 mM L-aspartate, 2.5 mM α-ketoglutarate, 0.1 mM PLP and 50 mM Tris-HCl buffer, pH 7.4. The starch slab was placed in a plastic tray and the enzymatic staining solution poured in so as to completely cover the gel. Occasionally, the staining solution was incorporated as an agar overlay, according to the method of Schwartz, et al. (1963) with the above modifications of lower dye concentration and added PLP. The enzymatic staining of the gels was allowed to proceed for 45 minutes to 1 hour at 37°C; the staining solution was then poured off or the agar overlay was pealed away. The gels were preserved by fixing them in 5% acetic acid for 3 minutes, washing with distilled water and storing the gels at 4°C wrapped in Saran Wrap.

b. Borate System

Starch gels for electrophoresis, employing a borate buffer system, were prepared according to the procedure of Decker and Rau (1963). A 12% gel was prepared using Connaught hydrolyzed starch in 0.025 M boric acid-NaOH buffer, pH 8.9. Horizontal electrophoresis was conducted between electrode vessels (containing 800 ml of a 0.30 M boric acid-NaOH buffer, pH 8.2) for a period of 18 to 20 hours under a constant voltage of 4 V/cm and 12 - 18 ma at 4°C. AAT isozyme activity was determined by the method of Schwartz, et al. (1963) previously discussed.

2. Polyacrylamide Electrophoresis

Polyacrylamide gels were prepared according to the method of Peacock and Dingman (1967). Four stock solutions

were employed in the preparation of gels: (1) 20% acrylamide (19 gm of acrylamide (Eastman Kodak) and 1 gm of bisacrylamide (Eastman Kodak) in 100 ml of water); (2) 6.4% dimethylaminopropionitrile (DMAPN) (Eastman Kodak) in water; (3) ammonium persulfate, 1.6% in water; and (4) Tris-boric acid - EDTA buffer, pH 8.3 (108 gm of Tris, 55 gm of boric acid, and 9.3 gm of disodium - EDTA made up to 1 liter with water). This buffer was used undiluted in the preparation of gels and was used diluted 1 : 10 for the buffer reservoirs in an E - C polyacrylamide electrophoretic chamber.

A 10% acrylamide gel was prepared by mixing 80 ml of 20% acrylamide, 10 ml of 6.4% DMAPN, 10 ml of undiluted buffer stock, 50 ml of water and lastly 10 ml of 1.6% ammonium persulfate to start the chemical polymerization of the acrylamide. This acrylamide solution was poured into the gel chamber of the electrophoretic unit and allowed to polymerize with the slot former in place for 20 minutes. After gelation, excess gel was removed, diluted buffer was added to both electrode chambers and an anionic pre-run electrophoresis conducted for 45 minutes at 200 volts, with circulating tap water employed for heat dissipation. Enzyme samples were diluted 1 : 2 in 20% sucrose and applied into the sample slots. Bromphenol blue was added in order to visualize anionic migration. Electrophoresis was allowed to proceed for 2.5 hours at 200 volts. AAT activity was then determined as previously described.

F. PREPARATION OF ANTIBODIES

Antisera to the anionic and cationic isozymes of AAT from rat liver were prepared from female rabbits weighing between 2 and 2.5 Kg by an adaptation of the method of Theis and Siskind (1968) and Siskind, et al. (1968). Each partially purified isozyme from rat liver was emulsified in complete Freund's adjuvant (Difco Laboratories). A total volume of 2.5 ml, containing 1 mg of antigen, was injected into three sites on the rabbit (two hind foot pads and subcutaneously into the back of the neck). Four rabbits were used in the production of antisera against each isozyme. Four weeks after the single injection, 35 ml aliquots of blood were withdrawn every 3rd day by heart puncture for a period of two weeks. The rabbit blood was allowed to clot at room temperature. The serum was decanted and centrifuged at 1,000 x g for 20 minutes at $4^{o}C$. The antibody titer of each serum was determined by its ability to inhibit the homologous isozyme of AAT from rat liver. The specific antisera against each isozyme were pooled separately, recentrifuged at 48,000 x g for 20 minutes at $4^{o}C$ and stored frozen.

G. TITRATION OF SPECIFIC ANTISERA AGAINST ANIONIC AND CATIONIC AAT

To evaluate the specific antisera against the homologous and heterologous AAT isozymes of rat liver, a modification of the procedure of Nisselbaum and Bodansky (1964, 1969) was employed. In order to determine a convenient

working dilution of each antiserum, the following experiments were carried out: 0.1 ml of antiserum, either undiluted or diluted 1:5, 1:10 or 1:50 in 0.85% NaCl was mixed with 0.5 ml of homologous isozyme solution (containing about 300 units of AAT activity) in 100 mM Tris-HCl buffer, pH 7.4 containing 0.15% BSA. The final volume of this mixture was brought to 1.0 ml by the addition of 0.4 ml of the previous buffer. Control tubes contained 0.1 ml of normal rabbit serum, either undiluted or diluted 1:5, 1:10 or 1:50. All tubes were then incubated for 90 minutes at 37^{o}C, centrifuged at 1,500 x g for 15 minutes and the AAT activity remaining in 0.5 ml of the clear supernatant was determined.

Once the proper working dilution for each antiserum was determined, a constant amount of each antiserum was then titrated with increasing amounts of homologous and heterologous AAT isozymes. In this titration, 0.1 ml of a 1:10 dilution of each antiserum in 0.85% NaCl was mixed with 0.5 ml of appropriately diluted enzyme of increasing activity. After 90 minutes of incubation at 37^{o}C, the mixtures, along with tubes containing suitable control antiserum and enzyme, were centrifuged at 1,500 x g and decanted. The remaining AAT activity was measured in 0.5 ml of the supernatant.

In all cases involving the use of antiserum, any endogenous AAT activity found in the antiserum alone was suitably subtracted from enzyme assays containing antiserum.

H. DOUBLE AGAR GEL DIFFUSION

The antigen - antibody precipitin reaction in agar gels was determined by the method of Ouchterlony (1948, 1949, 1953). A 1% Ionoagar #2 agar (Oxoid) was prepared and poured into sterile Petri dishes. A series of wells were cut and the agar was removed by suction. Appropriate samples (50ul) were applied to each well and double diffusion was allowed to proceed for 24 hours at ambient temperature. When experiments were conducted for 48 hours, no intensification nor additional bands were observed.

I. DIFFERENTIAL ASSAY OF AAT ISOZYMES

The differential assay of anionic and cationic AAT isozymes in rat lens tissue was determined by using antibodies produced against the specific isozymes from rat liver. Rat lens homogenates were prepared as previously described. Usually 0.5 ml aliquots of a rat lens homogenate were added separately to tubes containing 0.1 ml of a 1:10 dilution of either the antibody made against anionic or cationic rat liver isozymes or of normal rabbit serum. The reaction mixtures were brought to a final volume of 1.0 ml or 1.1 ml with 100 mM Tris-HCl buffer, pH 7.4, containing 0.15% BSA. Suitable control tubes of each of the antiserum and normal rabbit serum alone, were also included. Each tube was then incubated at 37^{o}C for 90 minutes, centrifuged at 1,500 x g for 15 minutes and the remaining activity of a 0.5 ml aliquot of the supernatant determined. Each isozyme activity was calculated by subtracting the enzyme activity recovered

after incubation with each homologous antiserum from the total activity recovered in control tubes containing normal rabbit serum. Suitable corrections were made for endogenous AAT activity in the sera used.

III. RESULTS

A. RAT LIVER AAT ISOZYMES

Table 1 summarizes the various purification steps in the partial purification of the anionic isozymes of AAT from rat liver. The anionic isozyme preparation resulted in an 18 fold purification with a total enzyme activity of 7.4 x 10^6 units (11%, overall yield) and a specific activity of 180,000 (units/mg). Assaying the final preparation in the presence of PLP, yielded an increase in activity of 12%.

The elution profile of this anionic fraction from a DEAE-Cellulose column (Fig. 4) shows a single peak of enzymatic activity emerging from the column during the Tris-acetate buffer gradient elution. When subjected to starch gel electrophoresis (Fig. 5), this preparation migrated as a single anionic component with no visible cationic isozyme ($<0.3\%$) when stained for enzymatic activity.

Also noted in Figure 5 was the predominance of the anionic isozyme in the 8,000 x g supernatant from which this preparation was derived. Examination of the partially purified anionic isozyme by means of polyacrylamide gel electrophoresis (10% gel) also revealed a single anionic migrating band (Fig. 6).

The partial purification of the cationic isozyme from rat liver is summarized in Table 2. The preparation resulted in a 39 fold purification with a total enzyme activity of 5.1 x 10^7 units (overall yield of 18%) and a specific activity of 645,000 (units/mg). This preparation increased 13% in total activity when assayed in the presence of PLP.

Table 1. Purification of Anionic Aspartate Aminotransferase from Rat Liver.

Fraction	Activity (units x 10^{-6})	Specific Activity (units/mg protein)	Step Yield (%)	Overall Yield (%)
8000 x g Supernatant	67	1.07×10^4	(100)	(100)
Heat to 75°C	22	2.9×10^4	33	33
50% - 67% $(NH_4)_2SO_4$	16.7	4.2×10^4	77	25
DEAE Cellulose Effluent	5.9	5.7×10^4	35	9
0 - 75% $(NH_4)_2SO_4$	7.4	18.0×10^4	125	11
with added PLP	8.9			

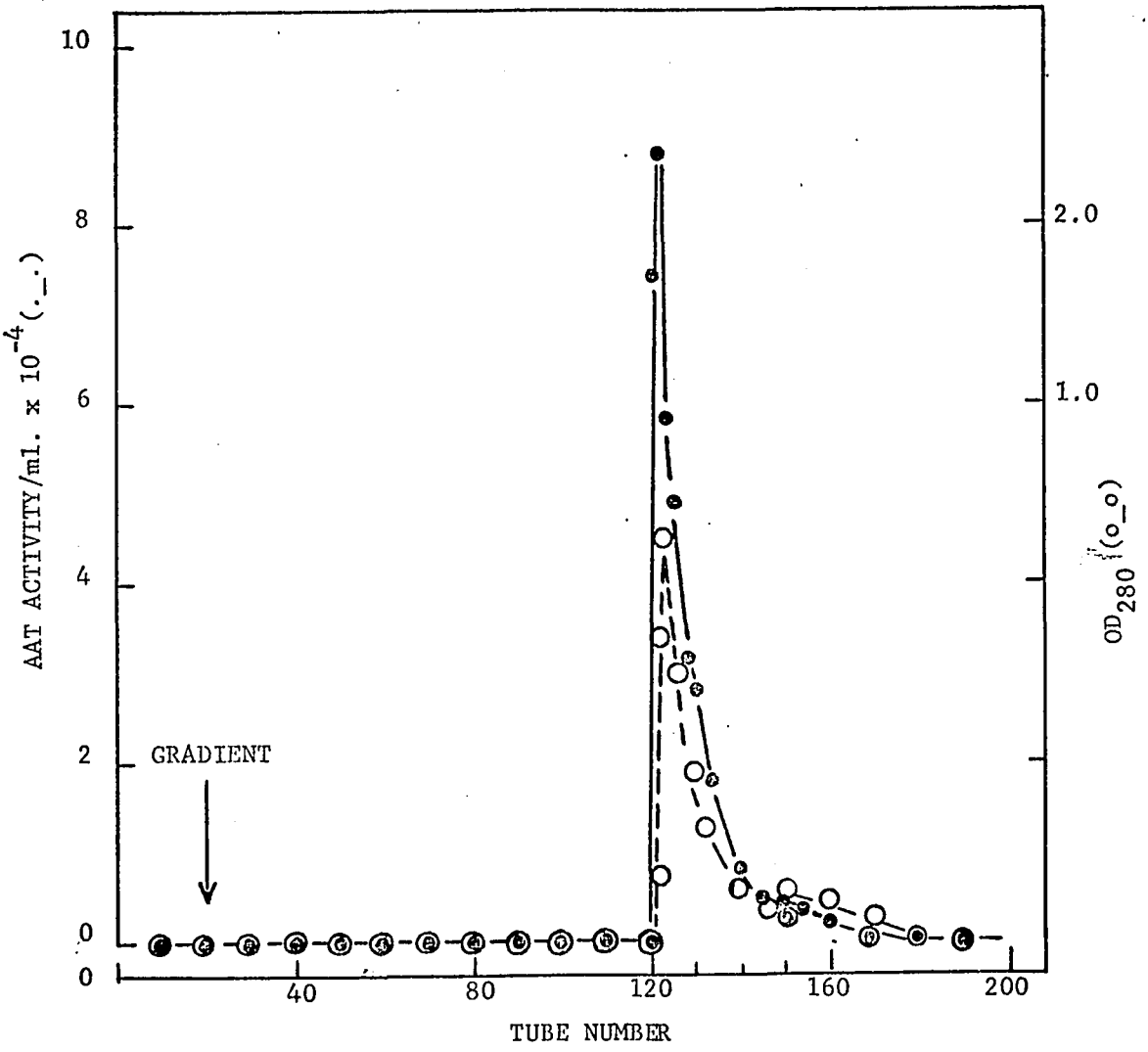

Figure 4. DEAE-cellulose column chromatography of the anionic AAT isozyme from rat liver. Figure shows the elution profile of the anionic AAT isozyme partially purified from the 8,000xg supernatant of a 0.25 M sucrose homogenate of rat liver. Tubes 1-21 were collected by eluting the column with 5mM Tris-acetate buffer, pH 7.5. A linear gradient was started at tube number 22 by mixing equal volumes of 5mM and 100mM Tris-acetate buffers, pH 7.5. All tubes collected approximately 6.0 ml. Tube fractions containing peak AAT activity were pooled and further purified as described in the Methods section.

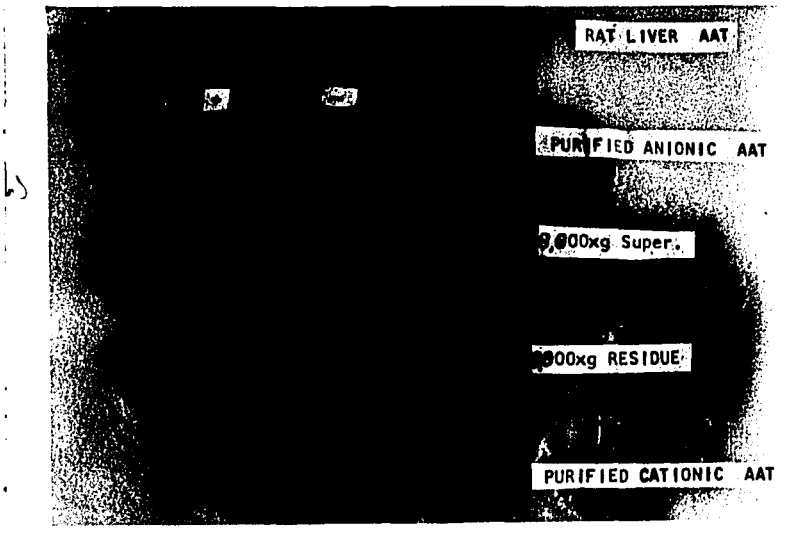

Figure 5. Starch gel electrophoresis of AAT isozymes from rat liver at various stages in the purification procedure. Gels (12%) were made in 5 mM Tris-succinate buffer, pH 7.2. The buffer wells each contained 800 ml of 100 mM phosphate buffer, pH 7.2. AAT enzyme activity was determined as described in the Methods section. Channel (1) purified anionic AAT isozyme from rat liver, (2) 8,000 x g supernatant of 0.25 M sucrose homogenate of rat liver, (3) 8,000 x g residue of 0.25 M sucrose homogenate of rat liver, and (4) purified cationic AAT isozyme from rat liver.

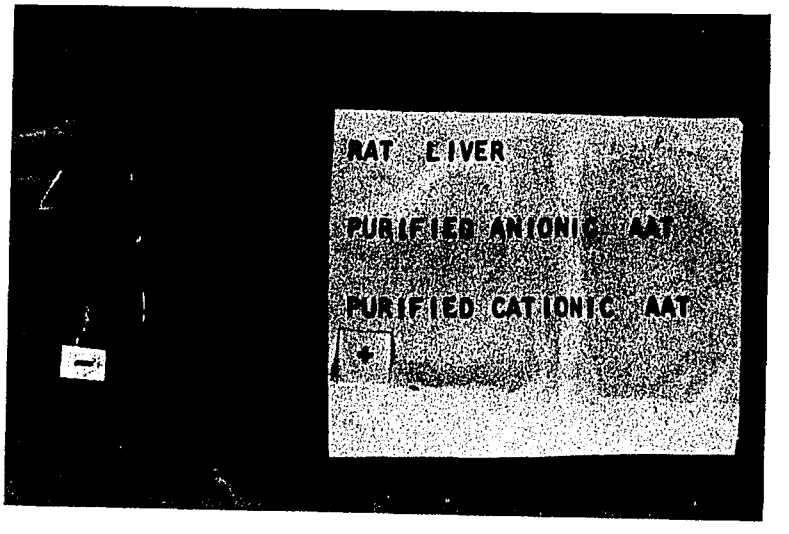

Figure 6. Polyacrylamide gel electrophoresis of partially purified anionic and cationic AAT isozymes from rat liver. Polyacrylamide gels (10%) were prepared as described in the Methods section. Buffer wells each contained 89 mM Tris-borate-EDTA buffer, pH 8.3. AAT enzyme activity was determined as described in the Methods section. Channel (1) purified anionic AAT isozyme from rat liver, (2) purified cationic AAT isozyme from rat liver.

Table 2. Purification of Cationic Aspartate Amiontransferase from Rat Liver

Fraction	Activity (units x 10^{-7})	Specific Activity (units/mg protein)	Step Yield (%)	Overall Yield (%)
8000 x g Pellet	27.4	1.66×10^4	(100)	(100)
Heat to 60°C	17.0	13.0×10^4	62	62
60% - 80% $(NH_4)_2SO_4$	10.8	27.4×10^4	63	39
DEAE Cellulose Effluent	5.7	54.0×10^4	53	21
0 - 75% $(NH_4)_2SO_4$	5.1	64.5×10^4	90	18
with added PLP	6.7			

Figure 7 shows the elution profile of this fraction from a DEAE-cellulose column. The single enzymatic peak emerged from the column with the 5 mM Tris-acetate buffer.

This peak of enzymatic activity coincided with a single protein peak measured at 280 nm. Starch gel electrophoresis (Fig. 5) of the fractionated pooled peak revealed the sample to be composed of a single cationic migrating component, containing less than 0.1% of the anionic isozyme. Figure 5 shows that the 8,000 x g pellet, from which the cationic preparation was derived, predominantly contained the cationic isozyme. On polyacrylamide gel electrophoresis (10%) the cationic isozyme appeared to remain at the origin (Fig. 6).

The isozymes maintained 96-98% of their original activity when stored at -15^{o}C over a period of 12 months (Table 3). Similar stabilities were noted when the isozymes were assayed in the presence of added PLP.

The DEAE-cellulose column chromatography elution profiles for each of the isozymes from rat liver were very similar to the pattern previously reported (Nisselbaum, 1968 and Nisselbaum and Bodansky, 1969). Although the specific activity and purification was not as high as previously reported (ibid.), these parameters for each of the isozymes from rat liver were substantial and resulted in preparations reasonably devoid of the other contaminating isozyme.

B. CALF LENS AAT ISOZYMES

Table 4 outlines the various purification steps in

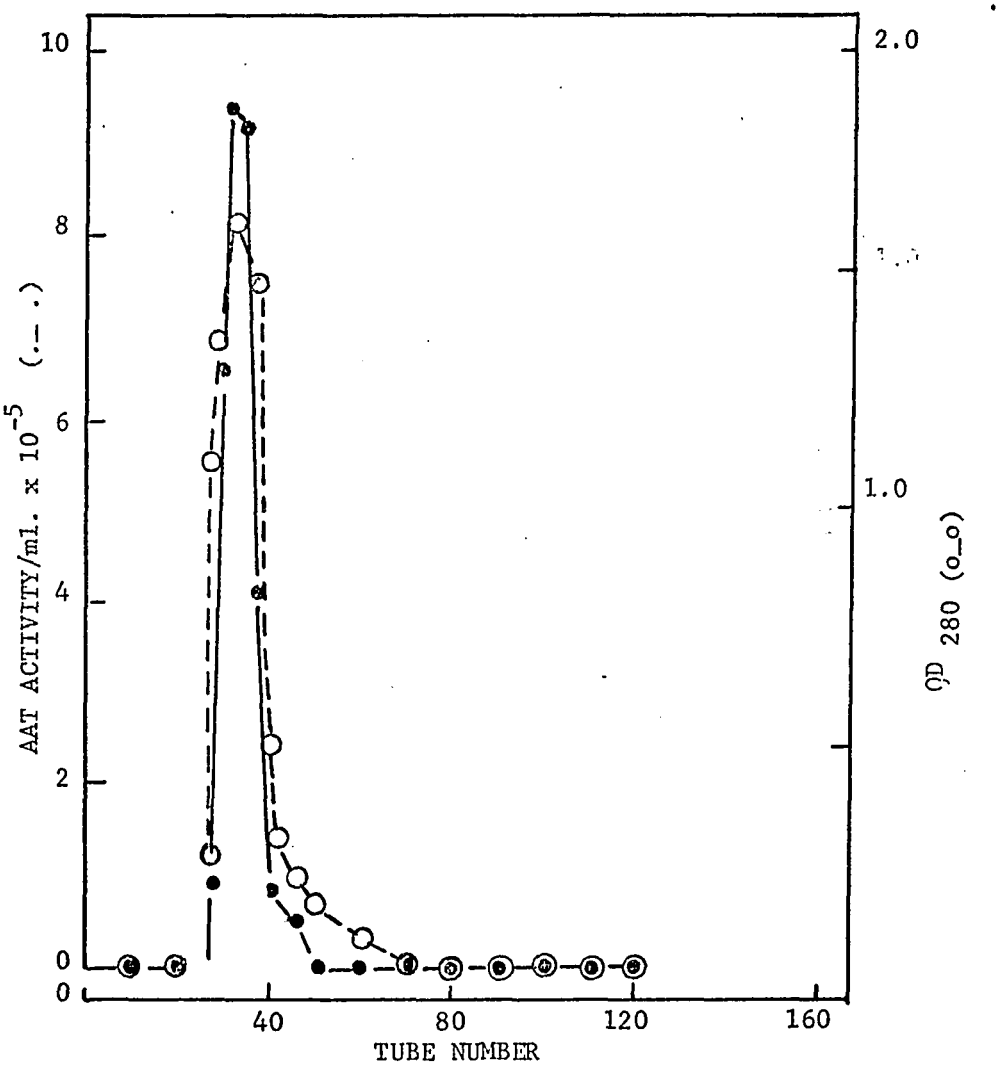

Figure 7. DEAE-cellulose column chromatography of the cationic isozyme from rat liver. Figure shows elution profile of the cationic AAT isozyme partially purified from the 8,000xg residue of a 0.25 M sucrose homogenate of rat liver. The enzymatic peak was collected by eluting the column with 5mM Tris-acetate buffer, pH 7.5. All tubes collected approximately 6.0 ml. Tube fractions containing peak AAT activity were pooled and further purified as described in the Methods section.

Table 3. Enzymatic Stability of the Anionic and Cationic Isozymes of Aspartate Aminotransferase from Rat Liver

		Original Activity (units/ml)	Activity 12 Months Later (units/ml)	Percent of Original Activity
Anionic	no PLP	1.94×10^5	1.90×10^5	98%
	with PLP	2.44×10^5	2.4×10^5	98%
Cationic	no PLP	1.95×10^6	1.87×10^6	96%
	with PLP	2.56×10^6	2.5×10^6	98%

Table 4. Purification of Anionic and Cationic Aspartate Aminotransferase from Calf Lens Tissue

Fraction	Activity (units x 10^{-5})	Specific Activity (units/mg protein)	Step Yield (%)	Overall Yield (%)
Homogenate	13.0	46	(100)	(100)
60°C Heat Treatment	13.0	46	100	100
40% - 80% $(NH_4)_2SO_4$	20.0	347	153	153
		Cationic Fraction		
5 mM Tris-Acetate DEAE Cellulose Effluent	5.4	1250	27	41
50% - 75% $(NH_4)_2SO_4$	5.2	2630	96	40
2nd DEAE	0.24	-	-	-
0 - 75% $(NH_4)_2SO_4$	0.22	500	4.2	1.7
Freeze Thaw	0.22	550	100	1.7
		Anionic Fraction		
Gradient Elution from DEAE Cellulose Column	5.5	466	27	42
0 - 75% $(NH_4)_2SO_4$	5.3	638	96	41
2nd DEAE	3.8	-	-	-
0 - 75% $(NH_4)_2SO_4$	3.7	5200	70	28
Freeze Thaw	3.7	5600	100	28

the partial purification of the anionic and cationic isozymes of AAT from calf lens. The final anionic isozyme showed a 122 fold purification with a total activity of 3.7×10^5 units (28% overall yield) and a specific activity of 5600. The cationic isozyme from calf lens had a final total activity of 2.2×10^4 units representing an overall yield of 1.7%. This represented a 7.5 fold purification, having a specific activity of 550.

The elution profiles of the cationic (Fig. 8) and anionic (Fig. 9) isozymes from calf lens from a DEAE-cellulose column were similar to the elution profiles of the respective isozymes from rat liver previously described (Figure 7 and Figure 6, respectively). The calf lens cationic isozyme was eluted from the column with 5 mM Tris-acetate buffer, pH 7.4, while the anionic isozyme from calf lens had an enzymatic peak emerging during the Tris-acetate buffer gradient.

Starch gel electrophoresis showed that each isozyme fraction contained less than 0.5% of the other isozyme, when stained for enzymatic activity. The anionic isozyme from calf lens moved more slowly than the rat liver anionic isozyme. Figure 10 shows that calf lens anionic isozyme (40-80% $(NH_4)_2SO_4$ fraction) moved only 1.0-2.5 cm while the rat liver anionic isozyme moved 3.5 cm. The concentration of cationic isozyme from calf lens in Figure 10 was too low for detection. Separate electrophoretic experiments of more concentrated cationic isozyme from calf lens showed

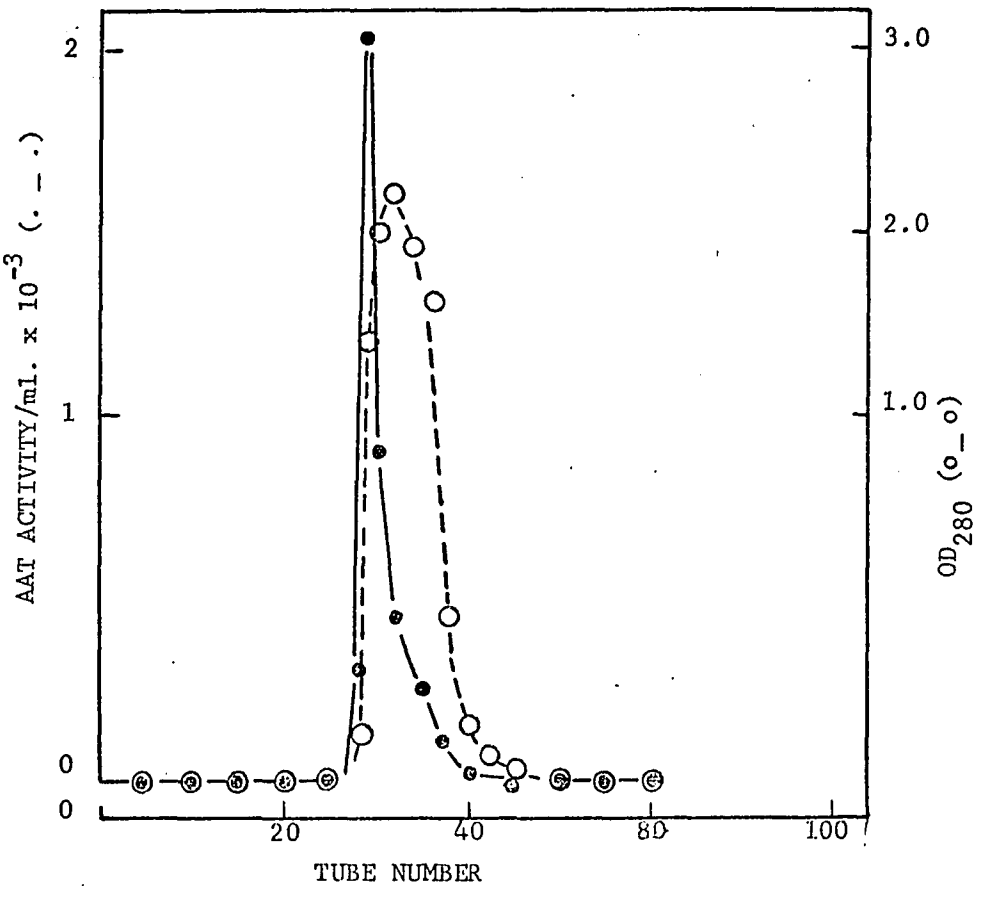

Figure 8. DEAE-cellulose column re-chromatography of the cationic AAT isozyme from calf lens. Figure shows the elution profile of the cationic AAT isozyme partially purified from the 8,000xg supernatant of calf lenses homogenized in 10mM Tris-HCl buffer, pH 7.4. The enzymatic peak was collected by eluting the column with 5mM Tris-acetate buffer, pH 7.5. All tubes collected approximately 6.0 ml. Tube fractions containing peak AAT activity were pooled and further purified as described in the Methods section.

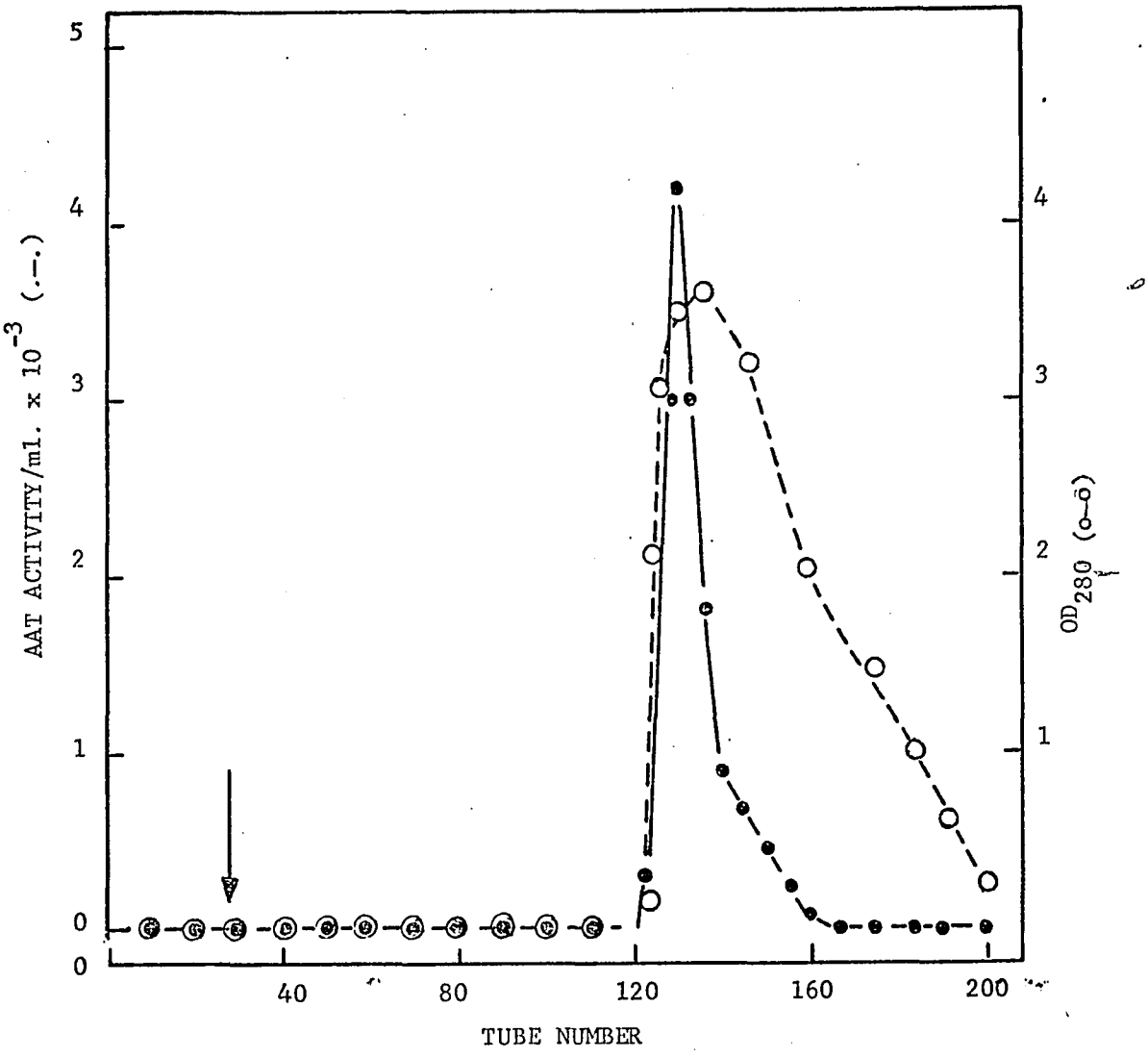

Figure 9. DEAE-cellulose column re-chromatography of the anionic AAT isozyme from calf lens. Figure shows the elution profile of the anionic AAT isozyme of calf lenses homogenized in 10mM Tris-HCl buffer, pH 7.4. Tubes 1-25 were collected by eluting the column with 5mM Tris-acetate buffer, pH 7.5. A linear gradient was started at tube number 26 by mixing equal volumes of 5mM and 100mM Tris-acetate buffers, pH 7.5. All tubes collected approximately 6.0 ml. Tube fractions containing peak AAT activity were pooled and further purified as described in the Methods section.

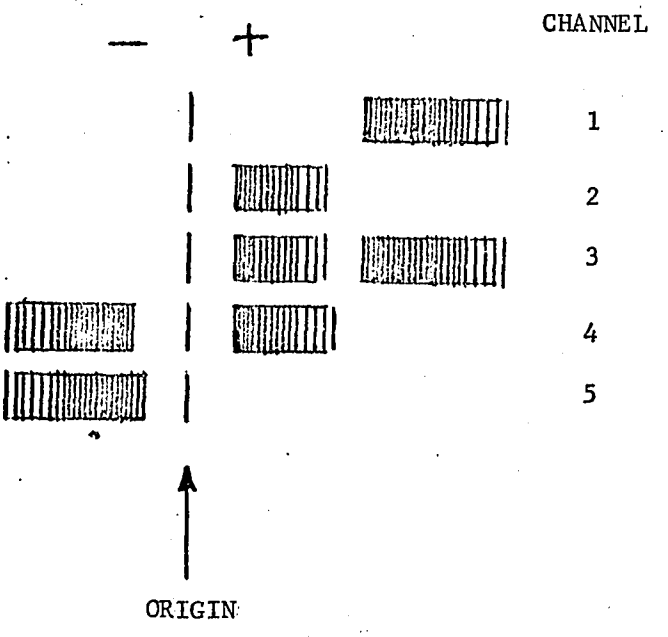

Figure 10. Starch gel electrophoresis of AAT isozymes from rat liver and calf lens. Gels (12%) were made in 25mM boric acid-NaOH buffer, pH 8.9. The buffer wells each contained 800 ml of 300mM boric acid-NaOH buffer, pH 8.2. AAT enzyme activity was determined as described in the Methods section. Channel (1) purified anionic AAT from rat liver; (2) calf lens preparation (40-80% $(NH_4)_2SO_4$ fraction); (3) purified anionic AAT from rat liver plus calf lens preparation (40-80% $(NH_4)_2SO_4$ fraction); (4) purified cationic AAT from rat liver plus calf lens preparation (40-80% $(NH_4)_2SO_4$ fraction); (5) purified cationic AAT from rat liver.

it to have similar cationic mobility to rat liver.

In a Tris-succinate pH 7.2 electrophoretic system, the calf lens anionic isozyme (as a crude homogenate or a partially purified fraction) still appeared to have a mobility less than that of the rat liver anionic isozyme. This distinctness was maintained even when the calf lens preparation was subjected to electrophoresis (channel 3, Fig. 10).

C. EVALUATION OF ANTISERA MADE AGAINST RAT LIVER AAT ISOZYMES

Unfractionated rabbit antisera made against the individual anionic and cationic isozymes from rat liver were evaluated by titration against each of the antigens. Figure 11 shows the titration data of the rat liver anionic AAT isozyme with the homologous and heterologous rabbit antisera and unfractionated rabbit control serum. Under these titration conditions, between 96% and 100% of the anionic AAT rat liver activity was inhibited by the homologous antiserum when 2000 units of activity or less were tested. Beyond this activity, the amount of anionic isozyme activity recovered in the assayed supernatant increased up to about 80% when 10,000 units per ml were tested. The titration was not followed further. Throughout the above anionic isozyme concentration range and under the same conditions, the heterologous antiserum caused no appreciable loss in enzyme activity. This lack of inhibition with the heterologous antiserum is of importance especially at the lower enzyme concentrations. This same lack of

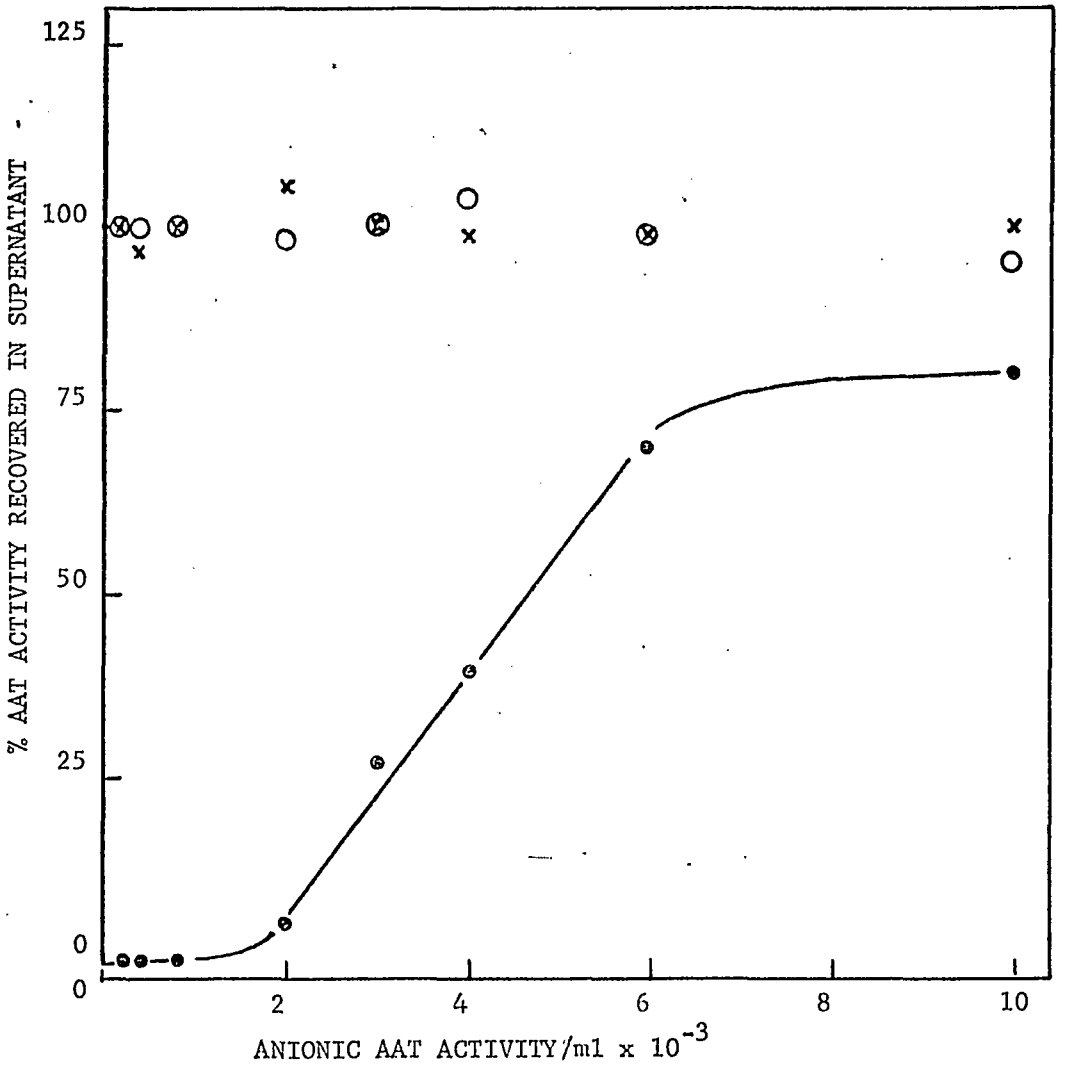

Figure 11. Titration curve of the anionic AAT isozyme purified from rat liver with rabbit antisera made against the anionic and cationic AAT isozymes from rat liver. Incubation mixtures contained 0.5 ml of appropriately filuted enzyme in 100mM Tris-HCl buffer, pH 7.4 containing 0.15% BSA and 0.1 ml of a 1:10 dilution of antiserum or normal rabbit serum. The final volume of each mixture was brought to 1.0 ml with the above buffer. After incubation for 90 minutes at 37°C, the mixtures were centrifuged. The remaining AAT activity in the decanted supernatants was measured for each mixture. Percentage recovery was calculated from values obtained with normal rabbit serum along with suitable antisera controls. Each point represents the average of duplicate determinations.
●—● , antiserum made against anionic AAT from rat liver;
○ , antiserum made against cationic AAT from rat liver;
× , normal rabbit serum.

inhibition was observed when anionic enzyme was reacted with normal rabbit control serum.

The titration data of the cationic AAT isozyme from rat liver with homologous and heterologous rabbit antisera and rabbit control serum are illustrated in Figure 12. Between 97% and 100% of the cationic rat liver activity was inhibited when incubated with its homologous antiserum when 3600 units per ml or less activity were assayed. As the cationic isozyme concentration was increased, the recoverable supernatant activity increased. The titration curve was followed up to approximately 14,400 units/ml, at which point 87% of the activity was recovered. The anionic antiserum or normal rabbit control serum did not inhibit the cationic isozyme activity over the enzyme concentrations employed.

The technique of double agar diffusion, utilizing Ouchterlony plates, revealed three specific precipitin bands (A, A^1 & B, Fig. 13) when rat liver anionic isozyme (well 1) diffused against its homologous antiserum (center well). Band A or A^1, or possibly both, probably represented the antigen-antibody complex of the anionic isozyme. Precipitin band B probably represents a contaminating (non-AAT-enzymatic) protein present in both isozyme preparations, since band B formed a continuous precipitin arc with the rat liver cationic isozyme fraction (well 2). The common antigen visualized as the antigen-antibody complex (band B) cannot have AAT activity since no enzymatic cross-

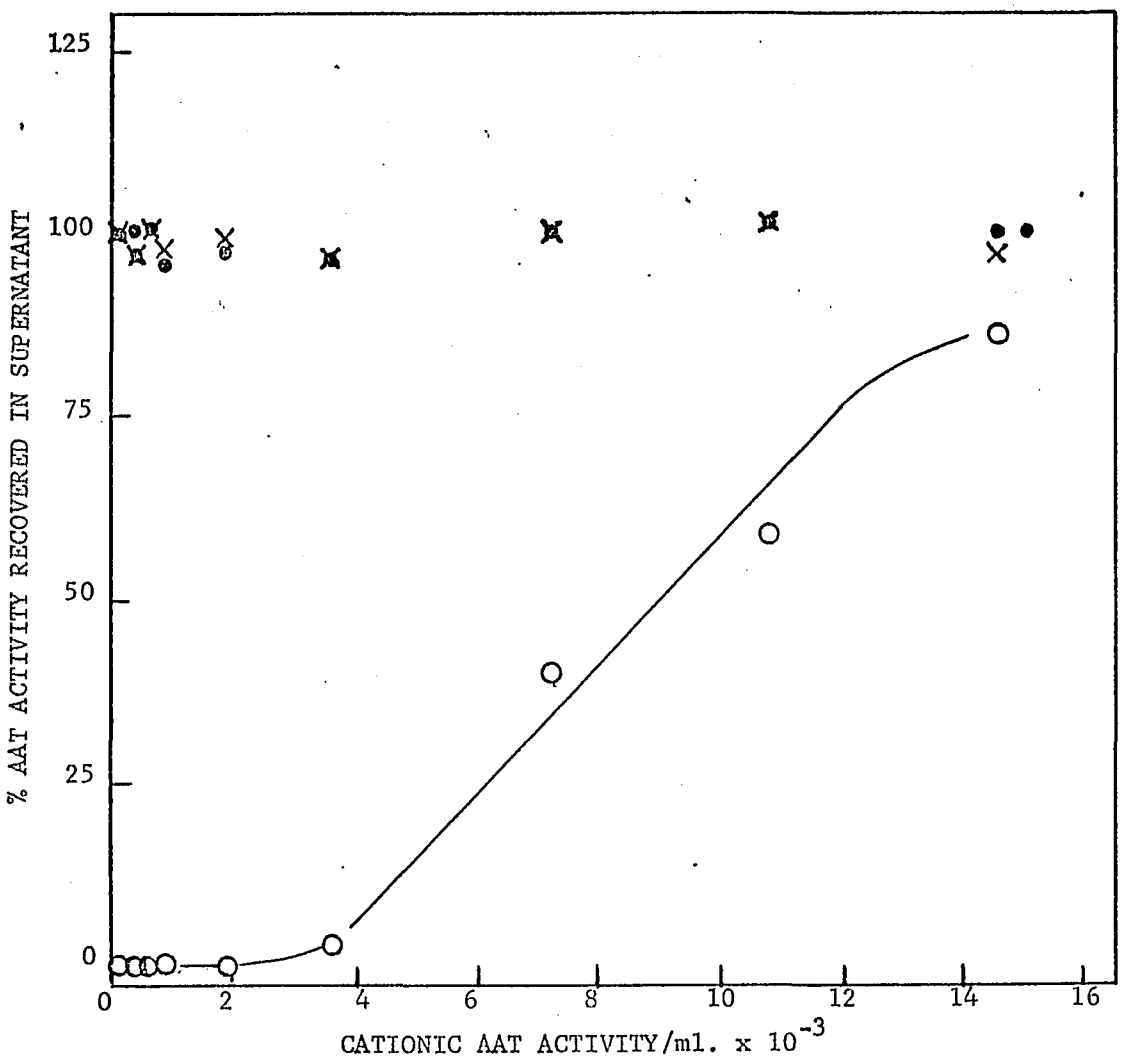

Figure 12. Titration curve of the cationic AAT isozyme purified from rat liver with rabbit antisera made against the anionic and cationic AAT isozymes from rat liver. Incubation mixtures contained 0.5 ml of appropriately diluted enzyme in 100mM Tris-HCl buffer, pH 7.4 containing 0.15% BSA and 0.1 ml of a 1:10 dilution of antiserum or normal rabbit serum. The final volume of each mixture was brought to 1.0 ml with the above buffer. After incubation for 90 minutes at 37°C, the mixtures were centrifuged and the remaining AAT activity in the supernatants was measured. Percentage recovery was calculated from values obtained with normal rabbit serum along with suitable antisera controls. Each point represents the average of duplicate determinations.
O—O , antiserum made against cationic AAT from rat liver;
• , antiserum made against anionic AAT from rat liver;
X , normal rabbit serum.

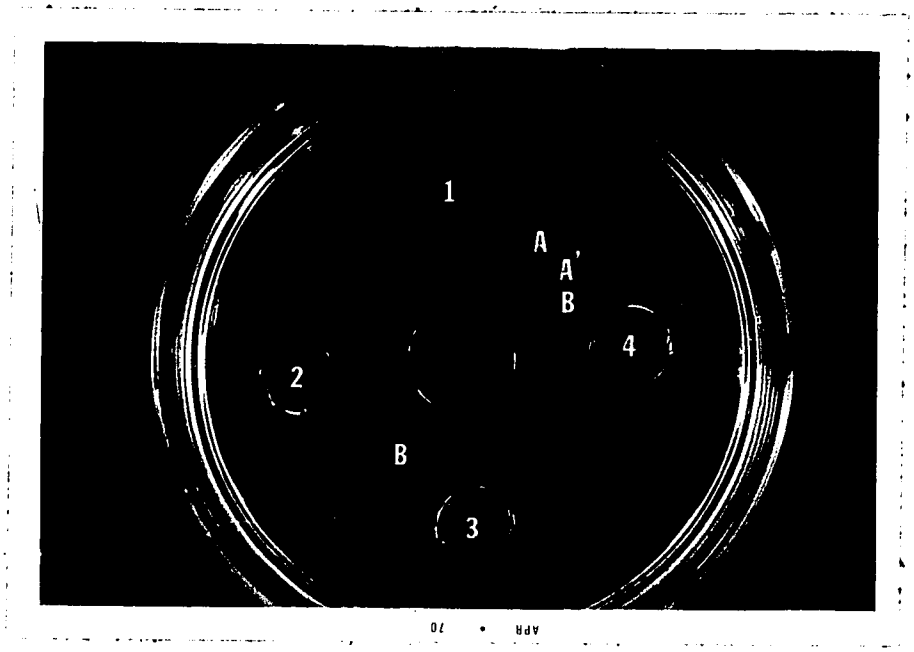

Figure 13. Ouchterlony plate of the anionic rabbit antiserum against various AAT enzyme preparations. Double agar diffusion was allowed to proceed for about 24 hours in 1% agar gels. Center well contained 0.05 ml of undiluted anionic AAT rabbit antiserum. Well (1) 0.05 ml purified anionic AAT isozyme from rat liver; (2) 0.05 ml purified cationic AAT isozyme from rat liver; (3) 0.05 ml rat lens homogenate; (4) 0.05 ml calf lens homogenate.

reactivity was observed when each isozyme was incubated with its heterologous antiserum. A similar continuous precipitin arc (band B) was also seen when the center well contained cationic antiserum (Fig. 14). In support of this view, experiments were conducted where the center well contained antiserum mixed with its heterologous antigen. With both situations, band B was not visible when run against either anionic or cationic antigens. Band A was probably the precipitin band due to the complex formed between cationic AAT isozyme and its homologous antiserum.

Wells #3 and #4 in both Figures 13 and 14 contained rat lens homogenate and calf lens homogenate, respectively. No precipitin bands appeared between these wells and each center well, containing each antiserum. The AAT activity of each homogenate was extremely low in comparison with the activity present in the partially purified preparations of AAT isozymes from rat liver. The lens homogenates had an activity per ml of from 4.5×10^2 to 2.4×10^3 units, whereas the purified anionic isozyme from rat liver contained 2.0×10^5 units per ml and the cationic isozyme contained 2.0×10^6 units per ml. When either anionic or cationic isozyme purified from rat liver was applied at a 1:10 dilution, no precipitin bands were observed. Thus no precipitin bands could be expected from either homogenate.

In electrophoretic studies, it was found that each AAT isozyme was effectively inhibited by its homologous

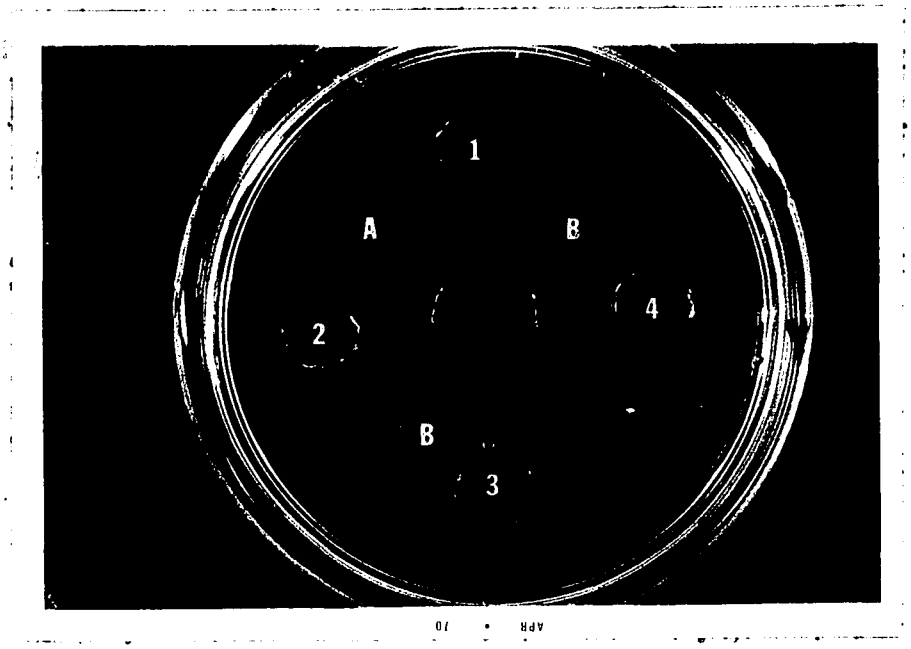

Figure 14. Ouchterlony plate of cationic AAT rabbit antiserum against various AAT enzyme preparations. Double agar diffusion was allowed to proceed for about 24 hours in 1% agar gels. Center well contained 0.05 ml of undiluted cationic AAT rabbit antiserum. Well(1) 0.05 ml purified anionic AAT isozyme from rat liver; (2) 0.05 ml purified cationic AAT isozyme from rat liver; (3) 0.05 ml rat lens homogenate; (4) 0.05 ml calf lens homogenate.

antiserum (Fig. 15). Channels 2 and 5 containing the individual isozymes and their respective antiserum showed no enzymatic staining. The electrophoretic pattern of a mixed isozyme sample is shown in channel 3. The same mixture containing rat liver isozymes plus the antiserum to each of the isozymes showed no enzymatic staining of either isozyme (channel 4).

D. ISOZYME ACTIVITY IN RAT LENS HOMOGENATES

Rat lens homogenates were initially tested for the presence of possible inhibitors of AAT activity. After rat lens homogenates had been dialyzed for 8 hours at $4^{o}C$, the amount of AAT activity recovered was essentially the same as the assayed activity prior to dialysis. Further, when the homogenates were diluted up to a factor of 10, the calculated activity per ml remained relatively constant throughout the dilution range. Thus, there appeared to be no change in AAT activity in rat lens homogenates due to release from either a dialyzable or a dissociable factor.

The antibodies made specifically against rat liver anionic and cationic AAT isozymes were evaluated in the rat lens homogenate system. Table 5 represents results from a typical experiment where aliquots of lens homogenate were incubated separately with either normal rabbit serum, or anti-anionic rat liver AAT isozyme, or anti-cationic rat liver AAT isozyme, or both antisera. When both anionic and cationic antisera were incubated with lens homogenate, 100% of the AAT activity was inhibited. The calculated

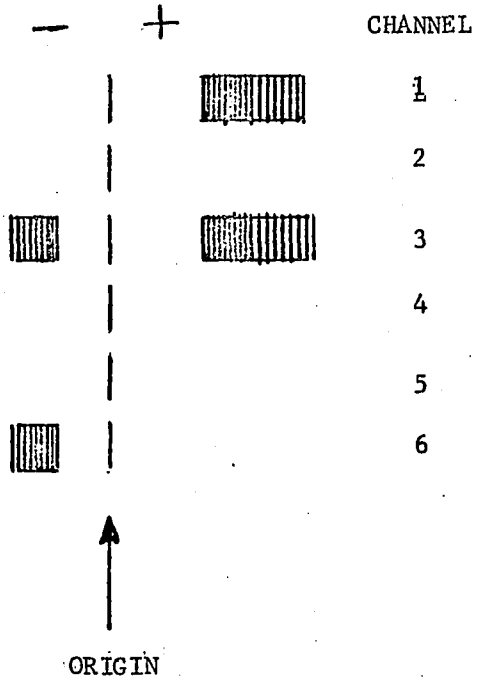

Figure 15. Starch gel electrophoresis of purified anionic and cationic AAT isozymes from rat liver mixed with anionic and cationic AAT rabbit antisera. Gels (12%) were made in 5mM Tris-succinate buffer, pH 7.2. The buffer wells contained 100mM phosphate buffer, pH 7.2. AAT enzyme activity was determined as described in the methods section. Channel (1) purified anionic AAt isozyme from rat liver; (2) purified anionic AAT isozyme from rat liver plus anionic AAT rabbit antiserum; (3) purified anionic and cationic AAT isozymes from rat liver; (4) purified anionic and cationic AAT isozymes from rat liver plus anionic and cationic AAT rabbit antiserum; (5) purified cationic AAT isozyme from rat liver plus cationic AAT rabbit antiserum; (6) purified cationic AAT isozyme from rat liver.

Table 5. Rat Lens Homogenate plus Rat Liver AAT Isozyme Antisera

	Calculated Activity (units per ml)	Recovery (%)	Inhibition (%)
Homogenate aliquot	270	(100)	
Homogenate and Normal Rabbit Serum	256	95	
Homogenate and Anionic Antiserum	198	77	23
Homogenate and Cationic Antiserum	64	25	75
Homogenate and Anionic and Cationic Antisera	0	0	100

per cent inhibition for each of the antiserum was also in excellent agreement, yielding on the average 100±3% total inhibition. Lens homogenates consistently contained more cationic AAT isozyme than anionic AAT isozyme in the ratio of about 3:1.

The inhibition of anionic and cationic AAT isozymes in rat lens homogenates by specific antisera was further tested in enriched rat lens homogenate systems. Aliquots of a rat lens homogenate (0.4 ml) containing 675 AAT units per ml were enriched with either 0.15 ml of cationic rat liver AAT containing 2000 units per ml or 0.15 ml of anionic rat liver AAT containing 2000 units per ml. Then 0.1 ml of a 1:10 dilution of antiserum made against cationic or anionic AAT from rat liver or 0.1 ml of a 1:10 dilution of normal rabbit serum was added along with 0.35 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15% BSA. Each series of tubes was incubated at 37°C for 90 minutes. After centrifugation, 0.5 ml of each supernatant was assayed for remaining AAT activity. Table 6 demonstrates that inhibition by specific antiserum against the anionic and cationic AAT isozymes of enriched rat lens homogenates was in excellent agreement with calculated values.

This methodology of inhibiting each specific AAT isozyme of rat lens homogenates by its homologous antiserum made against rat liver isozyme, represented the means by which the anionic and cationic AAT isozymes of rat lens systems were evaluated.

Table 6. Rat Lens Homogenate Fortified with AAT Isozymes from Rat Liver

Rat lens homogenate (0.4 ml) containing 675 AAT units per ml was enriched with either 0.15 ml of anionic rat liver AAT containing 2000 units per ml or 0.15 ml of cationic rat liver AAT containing 2000 units per ml. Specific antisera (0.1 ml of 1:10 dilution) and normal rabbit serum (0.1 ml of 1:10 dilution) were added to separate tubes and 0.35 ml of Tris buffer was added. After incubation at 37°C for 1 hour, the solutions were centrifuged and 0.5 of each supernatant was assayed for remaining AAT activity.

Units of Activity Remaining in Supernatant After Incubation with:

	Anionic Antiserum		Cationic Antiserum		Normal Rabbit Serum	
	Calculated	Found	Calculated	Found	Calculated	Found
Homogenate		202		64	270	256
Homogenate and Anionic Isozyme	202	200	364	360	570	555
Homogenate and Cationic Isozyme	502	505	64	62	570	565

E. SUBSTRATE KINETICS OF RAT LIVER, CALF LENS AND RAT LENS AAT ISOZYMES.

The "true" Km values for L-aspartate and ∝-ketoglutarate of the anionic and cationic AAT isozymes of partially purified rat liver and calf lens and rat lens homogenates were determined by the procedure of Velick and Varva (1962). Individual isozyme substrate kinetics were evaluated in rat lens homogenates after the other isozyme was inhibited by the addition of its homologous antiserum. The AAT isozyme that remained in the lens homogenate was then assessed for its kinetics. Each antiserum at dilutions used contributed no AAT activity.

The Km (L-aspartate) values (Table 7) for the anionic AAT isozymes of rat liver, calf lens and rat lens homogenate were 2.07 mM, 2.76 mM and 2.33 mM, respectively, and were not significantly different from each other (p values being greater than 0.05). These values were significantly higher than the corresponding Km (L-aspartate) values of 0.57 mM, 0.65 mM and 0.64 mM for the respective sources of cationic AAT isozymes (in all cases, p value $<.001$). The cationic Km (L-aspartate) mean values were not significantly different from each other ($p>0.05$).

Conversely, the Km (∝-ketoglutarate) mean values for the anionic preparations of rat liver, calf lens and rat lens homogenate (0.076 mM, 0.102 mM and 0.096 mM, respectively) were significantly lower than the Km (∝-ketoglutarate) values for the respective cationic isozymes ($p<0.001$). However,

Table 7. Km Values for AAT Isozymes.

Anionic AAT Isozymes

	Km Aspartate x 10^3 moles/liter	Km ∝-ketoglutarate x 10^4 moles/liter
Rat Liver	2.1 ± 0.24 (5)	0.76 ± 0.13 (5)
Calf Lens	2.8 ± 0.22 (5)	1.02 ± 0.03 (5)
Rat Lens Homogenate	2.3 ± 0.16 (4)	0.96 ± 0.04 (4)

Cationic AAT Isozymes

	Km Aspartate x 10^4 moles/liter	Km ∝-ketoglutarate x 10^3 moles/liter
Rat Liver	5.70 ± 0.23 (5)	1.07 ± 0.08 (5)
Calf Lens	6.50 ± 0.36 (5)	1.38 ± 0.03 (5)
Rat Lens Homogenate	6.40 ± 0.29 (5)	1.35 ± 0.09 (5)

Kinetic studies were carried out at 37°C in 100 mM Tris-HCl buffer pH 7.4 containing 0.15% BSA, 8.7 x 10^{-5}M NADH and 200 optical density units of MDH in a final volume of 3.0 ml. The anionic isozyme Km values were determined by varying L-aspartate from 1.67 mM to 20 mM and ∝-ketoglutarate from 0.066 mM to 0.2 mM. The cationic isozyme Km values were determined by varying L-aspartate from 0.66 mM to 20 mM and ∝-ketoglutarate from 1.0 mM to 6.66 mM. Replotting the apparent maximum velocities according to Velick and Varva (1962) as described in Methods yielded Km values. The averages ± the standard error of the mean and the number of determinations (in parenthesis) are presented.

the Km (α-ketoglutarate) values within each of the anionic and cationic groups were not significantly different from one another ($p > 0.01$ for rat liver and calf lens; all other comparisons were $p > 0.02$). Furthermore, there was a significant difference between Km (L-aspartate) mean values and Km (alpha ketoglutarate) mean values in each of the anionic and cationic preparations (in all cases, $p < 0.001$).

F. THE EFFECT OF pH ON THE RELATIVE ACTIVITIES OF AAT ISOZYMES

The relative activities of the AAT isozymes of various preparations were determined as a function of pH. Figure 16 reveals that each AAT isozyme (partially purified from rat liver, partially purified from calf lens and rat lens homogenates) behaved similarly under various pH conditions. All three cationic AAT isozyme preparations showed a constant activity between pH 6.0 to 8.0. On the other hand, the anionic AAT isozymes showed significant decreases below pH 7.0. The anionic AAT isozyme activities for all the tissues were only 40% as active at pH 6.0 as compared to their pH 7.4 values. At around pH 6.0, the cationic isozymes essentially retained 100% of their pH 7.4 activity.

G. AAT LOCALIZATION IN RAT LIVER, CALF AND RAT LENS HOMOGENATES

In a single experiment, the total AAT lens activity was determined in isolated calf cortex and epithelium regions. The cortex contained 1.88×10^4 units per lens while the epithelium contained 265 units per lens. The

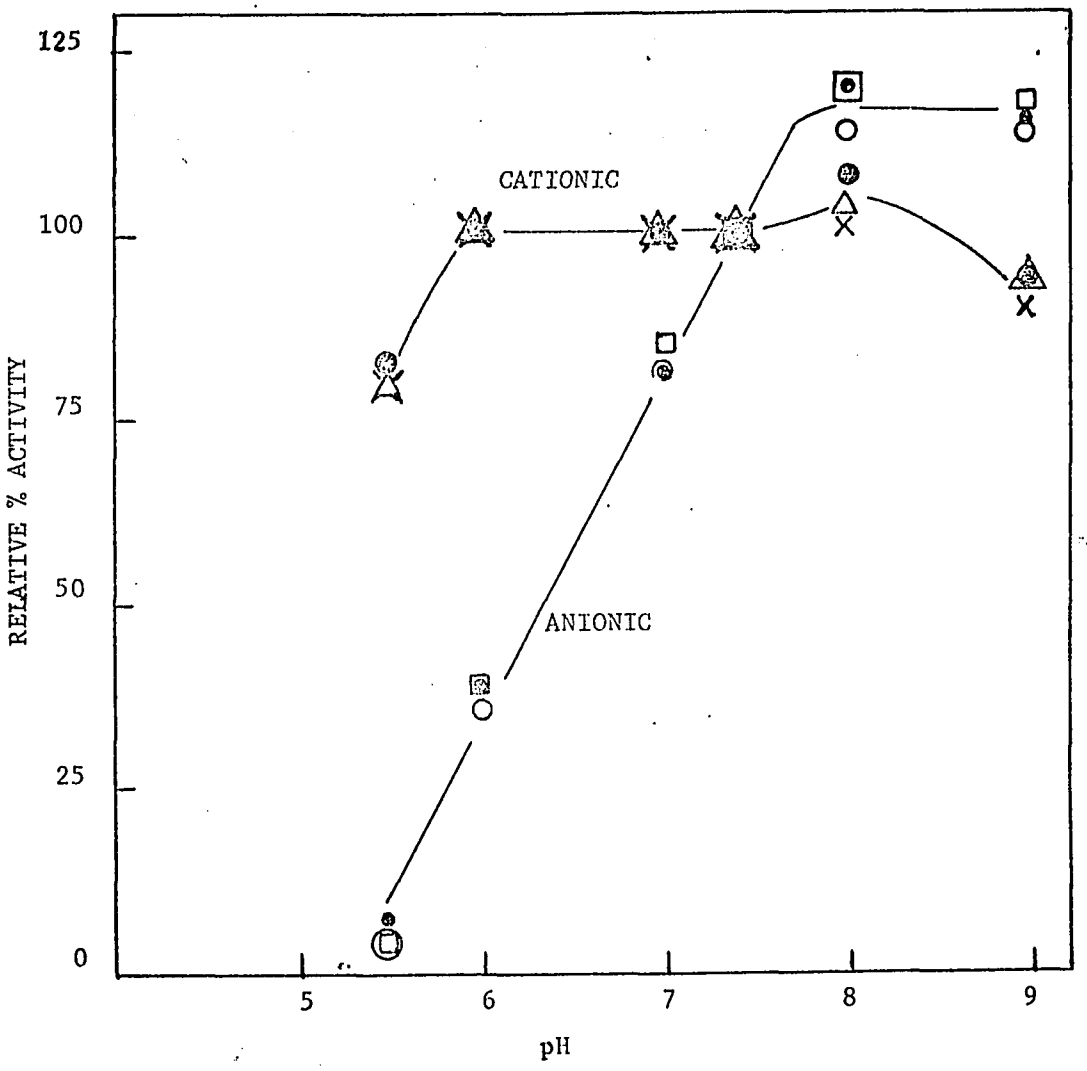

Figure 16. AAT isozyme activity of rat lens homogenate and of partially purified isozymes from rat liver and calf lens as a function of pH. Relative per cent activity was expressed as AAT activity recovered at each pH value based on the activity as assayed at pH 7.4. Each AAT isozyme from rat lens was obtained by incubating lens homogenates with heterologous AAT rabbit antiserum as described in the Methods section. Each point represents the average of assays performed in duplicate. Anionic AAT isozymes: from rat liver, ●—● ; rat lens homogenate, ○—○ ; calf lens, □—□ . Cationic AAT isozymes from rat liver, ●—● ; rat lens homogenate, ×—× ; calf lens, △—△ .

specific activity (units per mg fresh lens weight) was 15.8 for cortex and 8.0 for epithelium. These values are not very different from those values extrapolated from the data of Dardenne and Kirsten (1962).

Homogenates of rat liver, calf lens and rat lens in 0.01M Tris-HCl buffer, pH 7.4, containing 0.25M sucrose and 0.5% BSA (Lehninger, 1959; Balboni, 1965, 1968, personal communication) were subjected to differential centrifugation. Table 8 shows that about 75% of the total AAT present in rat liver lies latent within the mitochondrial pellet and about 92% of this value was the cationic isozyme. On the other hand, about 78% of the 10,000 x g supernatant was the anionic isozyme.

When rat lens homogenates were assayed under the same conditions, over 90% of the AAT activity was found in the 10,000 x g supernatant fraction, while only about 4% was detected in the lysed 10,000 x g pellet (Table 9). No further increase in AAT activity of the lysed mitochondrial pellet fraction was observed even after an overnight dialysis. Furthermore, about 71% of the soluble activity was composed of the cationic AAT isozyme, while 27% was the anionic isozyme. Thus, the AAT isozyme distribution in the 10,000 x g supernatant of rat lens (or of the rat lens homogenate) appeared to be reversed from that of the rat liver system. In no case was it possible to demonstrate rat lens activity in the epithelium and as previously discussed, calf lens epithelium contained about 1.5% of the total activity.

Table 8. AAT Localization and Distribution in Rat Liver Tissue.

	Total AAT Activity	Percent Anionic AAT Isozyme*	Percent Cationic AAT Isozyme**
Homogenate	5.3×10^4		
10,000 x g Supernatant	5.5×10^4	78	22
10,000 x g Lysed Pellet	16.7×10^4	8	92

* Calculated from the percent activity remaining after incubation with anionic AAT rat liver antibody.

** Calculated from the percent activity remaining after incubation with cationic AAT rat liver antibody.

Table 9. AAT Localization and Distribution in Rat Lens Tissue.

	Total AAT Activity/lens	Percent Anionic AAT Isozyme*	Percent Cationic AAT Isozyme**
Homogenate	1.8×10^2	27	72
10,000 x g Supernatant	1.8×10^2	27	70
10,000 x g Lysed Pellet	$.07 \times 10^2$	***	***

* Calculated from the percent activity remaining after incubation with anionic AAT rat liver antibody.

** Calculated from the percent activity remaining after incubation with cationic AAT rat liver antibody.

*** Insufficient activity present.

Calf lens homogenates contained about 95% of the AAT activity in the 10,000 x g supernatant, while only 4.5% could be demonstrated in the 10,000 x g lysed pellet (Table 10). Furthermore, if the epithelial layer of both rat and calf lenses were stripped away before homogenization, similar large differences in activity were obtained for the supernatant and pellet fractions. In contrast to the rat lens but similar to rat liver, about 82% of the 10,000 x g supernatant activity was demonstrated to be anionic AAT isozyme, while about 15% was cationic.

Since calf lens isozyme studies required a ten-fold increase in the amount of both antibodies used, a second-independent method was employed to substantiate the reversed isozyme patterns in calf and rat lens homogenates. The relative activities of the isozymes were now measured by performing the assays at pH 7.4 and 6.0 (see Fig. 16). Table 11 summarizes the percentage distribution for the AAT isozymes, as determined by the pH method, in calf and rat lens homogenates. Seventy percent of the total calf lens AAT activity was anionic, while the rat lens contained only about 23% of the same isozyme. Thus the reversed percent distribution of the isozymes in calf and rat lens homogenates was obtained by both immunological and pH methods.

It was recognized that the pH method was neither specific nor definitive and was subject to large variations in values obtained. Thus this method was not utilized as

Table 10. AAT Localization and Distribution in Calf Lens Tissue.

	Total AAT Activity/lens	Percent Anionic AAT Isozyme*	Percent Cationic AAT Isozyme**
Homogenate	10.9×10^3	-	-
10,000 x g Supernatant	11.0×10^3	82	15
10,000 x g Lysed Pellet	0.5×10^3	-	-

* Calculated from the percent activity remaining after incubation with anionic AAT rat liver antibody.

** Calculated from the percent activity remaining after incubation with cationic AAT rat liver antibody.

Table 11. AAT Distribution in Calf and Rat Lens Homogenates Determined by the pH Method.

	Percent AAT Anionic Isozyme* Average	Percent AAT Cationic Isozyme* Average
Calf Lens Homogenate	73 ± 2.7	27 ± 2.7
Rat Lens Homogenate	23 ± 2.8	77 ± 2.8

* Percent Isozyme calculated from activities determined at pH 6.0 and pH 7.4. The percentage represents the average ± the standard error.

a general quantitative technique, but rather to grossly substantiate the reversed percentages obtained for the anionic and cationic AAT isozymes in rat and calf lenses.

H. TOTAL AAT ACTIVITY IN RAT LENS AS A FUNCTION OF AGE

Figure 17 shows the total AAT activity in normal rat lens as a function of animal age. From about 28 through 460 day old rats, the total AAT activity per lens appeared to increase only slightly. The overall increase was about 15% over the entire age range (a correlation coefficient of 0.52). When the AAT assays contained PLP, a substantial increase in total activity was observed in lenses from about 50 to 120 day old rats, with a maximum increase in 80 day old animals (Fig. 17). Beyond about 100 days of age, the total activity (assayed with PLP) decreased slightly and then appeared to level off. At 460 days, the total activity (assayed with PLP) represented an increase of about 15% over that of the youngest rat lenses. Figure 18 presents these data as per cent increase in total AAT activity per lens when assayed in the presence of 0.1 mM PLP from those assayed without PLP. This "PLP effect" (representing increased AAT activity when assayed in the presence of PLP) again appeared most pronounced in lenses from rats between 55 and 80 days of age.

Between 28 and 460 days of age, the mg fresh weight per lens increased about 3.5 fold (Fig. 19A). The fresh lens weight increases (approximately) linearly until about the 200th day. Beyond this age, lens weight increased at a

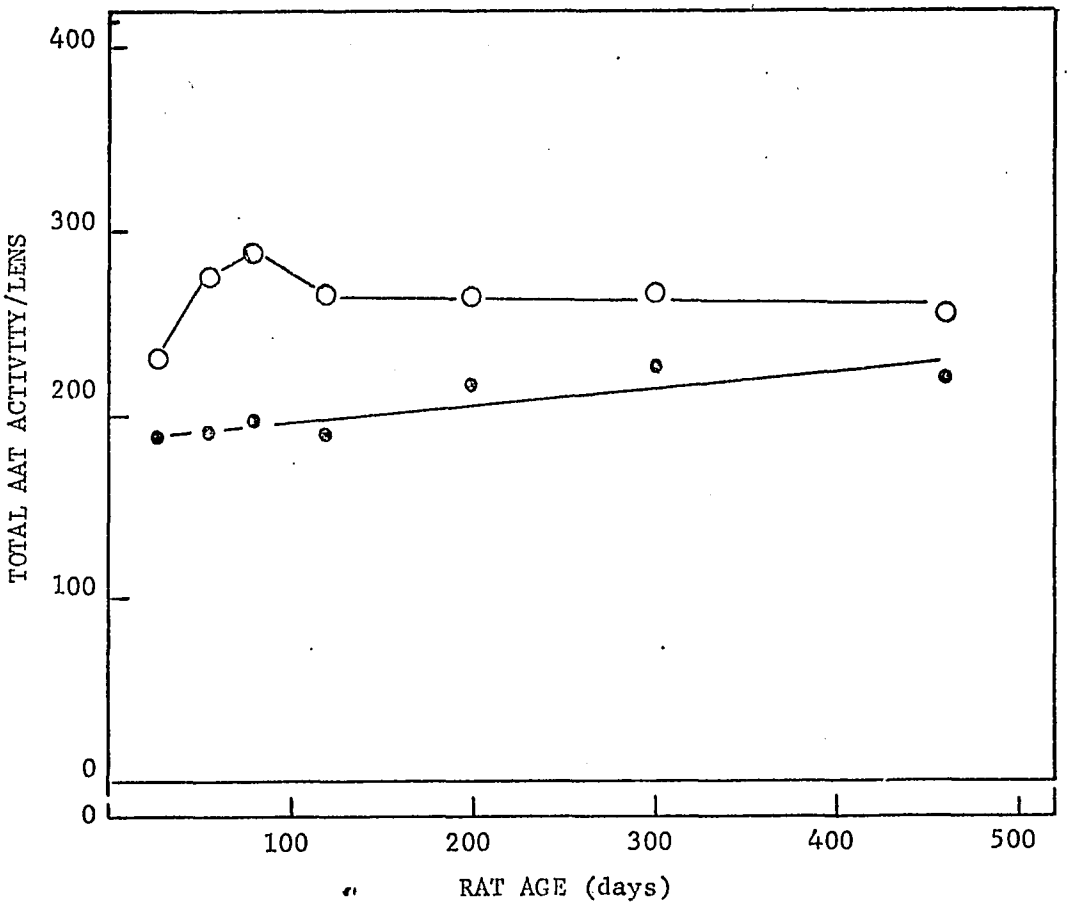

Figure 17. Total AAT activity per lens in rats ranging from 28 to 460 days of age.
O—O , assays contained 0.1mM PLP; ●—● , no PLP in assays.

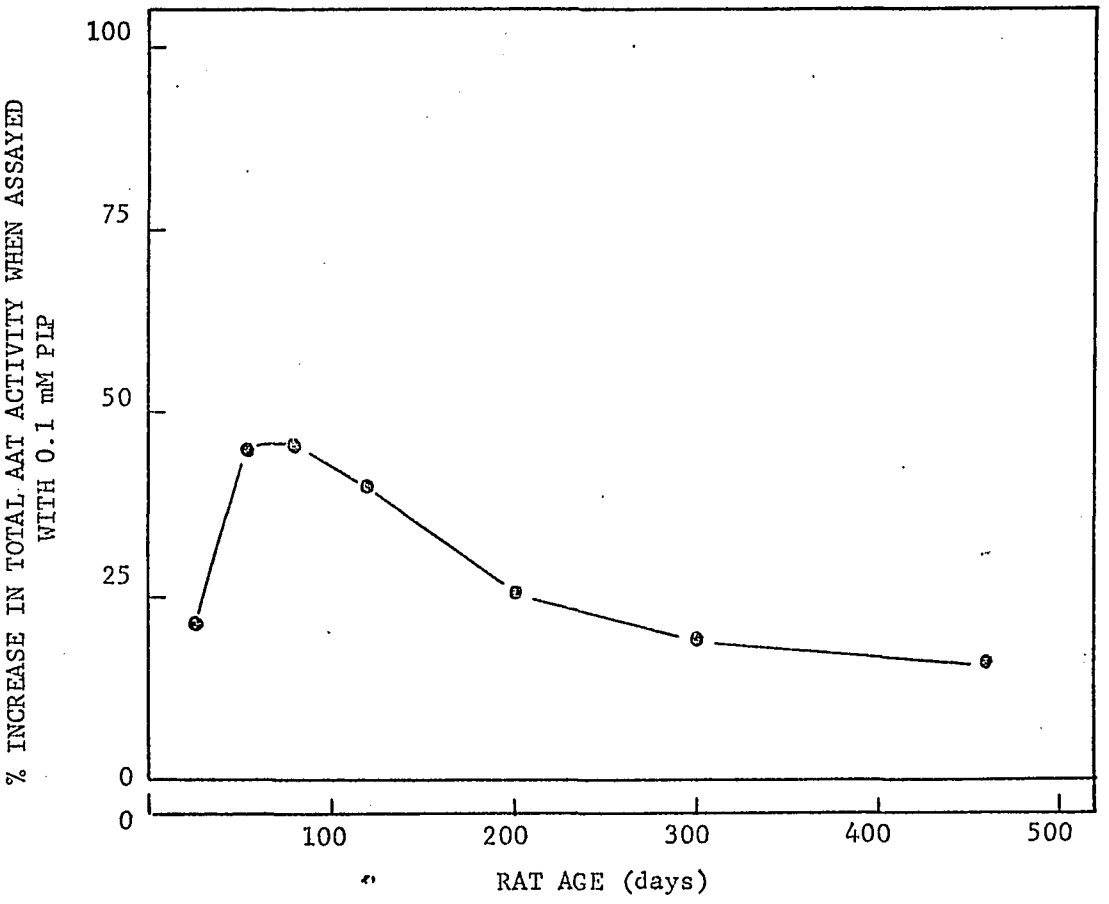

Figure 18. Per cent increase in lens total AAT activity when assays contained added PLP in rats ranging from 28 to 460 days of age.

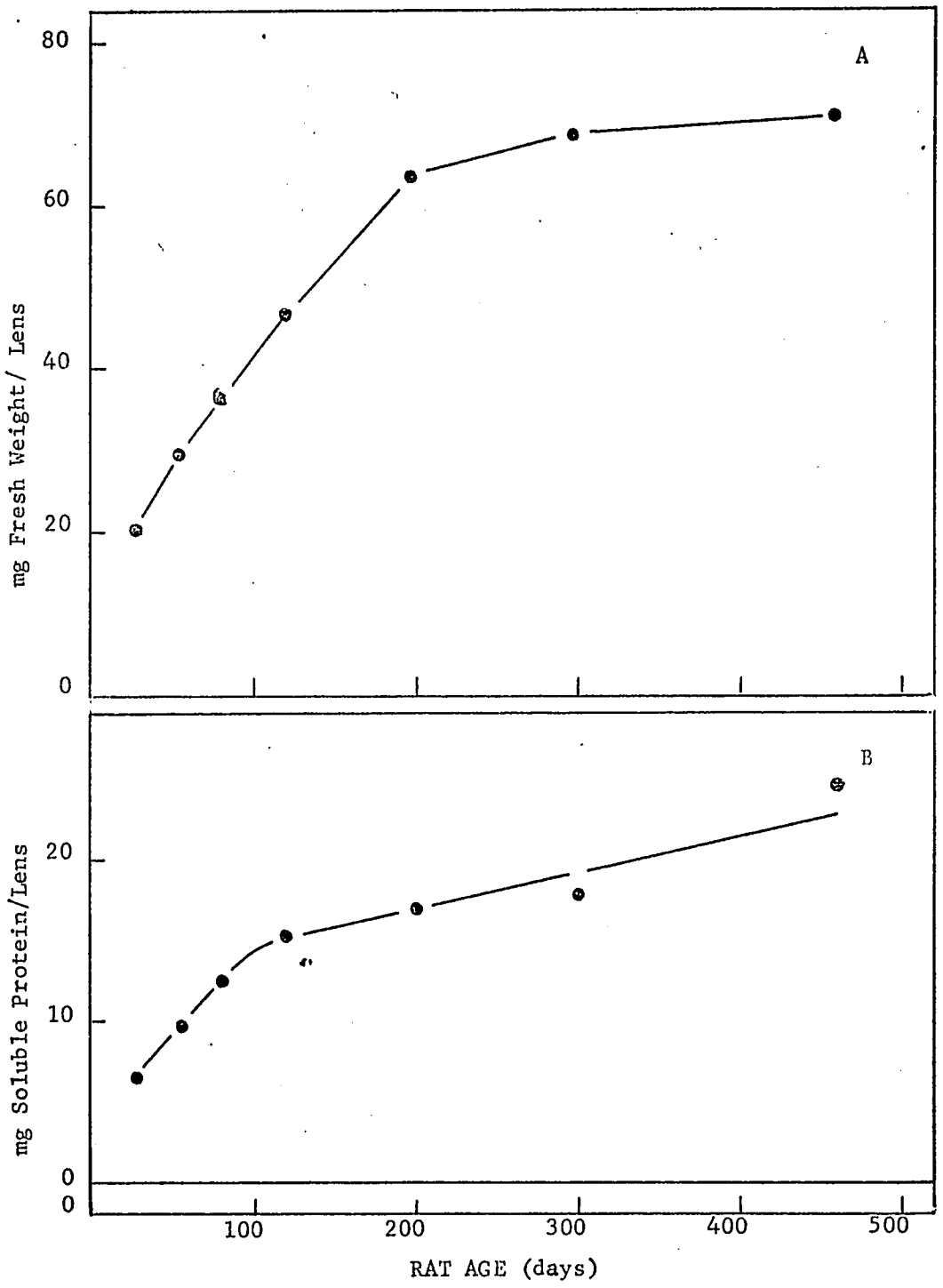

Figure 19. Lens fresh weight (A) and lens soluble protein (B) in rats ranging from 28 to 460 days of age.

slower rate. Similar lens weight values have been reported for the Wistar strain of rat (Sippel, 1965). When the total AAT activity per lens was expressed as per mg of lens fresh weight (Fig. 20), the specific activity decreased by a little more than 50% up through the first 120 days of age. Thereafter, it appeared to level off and remained constant up through 460 days.

Figure 19B shows the mg of soluble lens protein per lens with age. Here, again, a 3.5 fold increase was observed over the entire age period studied. Of this, about 75% of the increase occurred during the first 120 days. These values are in good agreement with the extrapolated values from the data of Lerman and Zigman (1965). The total AAT activity expressed per mg of soluble lens protein is shown in Figure 21. These specific activity values decreased by about 50% during the first 120 days of age and then remained constant up through 460 days. When assayed with added PLP the specific activity values obtained, though higher, revealed a similar pattern.

I. AAT ISOZYMES IN RAT LENS WITH AGE

The AAT anionic and cationic isozyme activities in rat lens was investigated through the application of specific AAT isozyme antibodies. The per cent inhibition obtained with each antibody for each of the isozymes was directly correlated with the per cent that each isozyme represented in the total activity of the lens homogenate system. Figure 22 compares the per cent distribution of

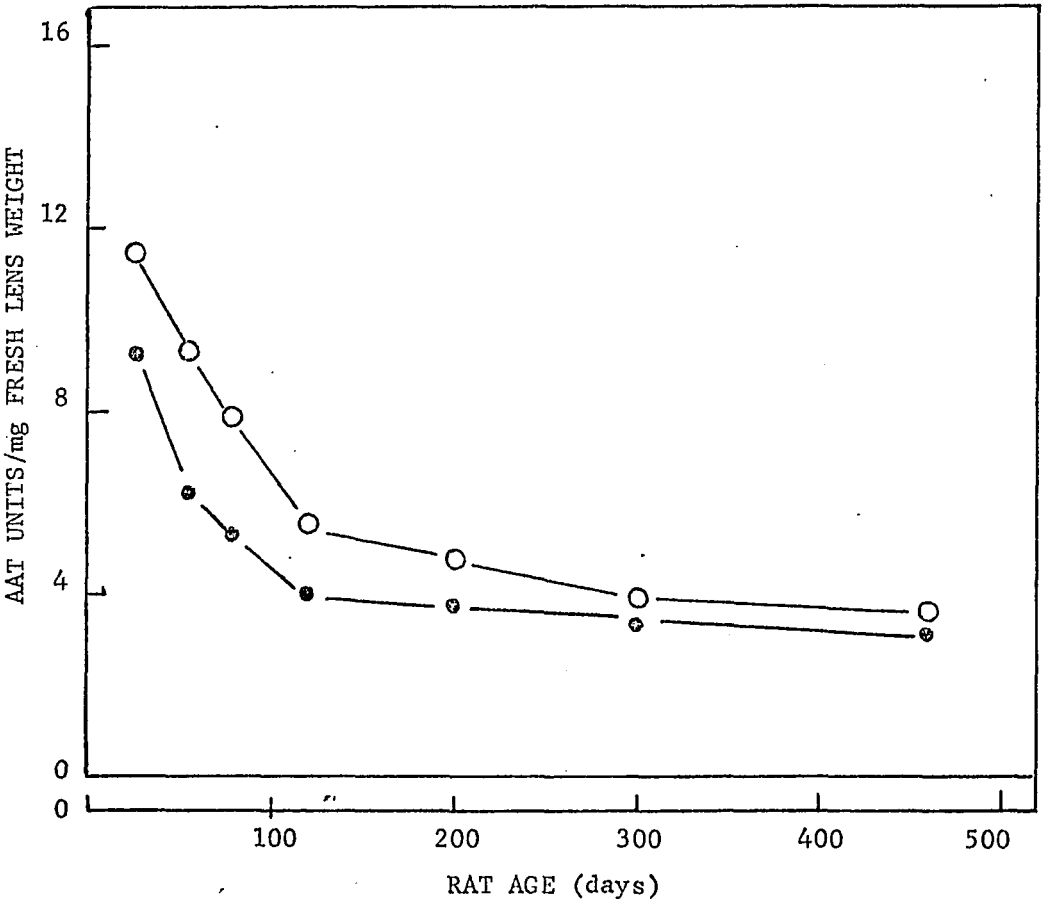

Figure 20. Lens AAT activity expressed per mg fresh lens weight in rats ranging from 28 to 460 days of age. O—O , assays contained 0.1mM PLP, ●—● , no added PLP in assays.

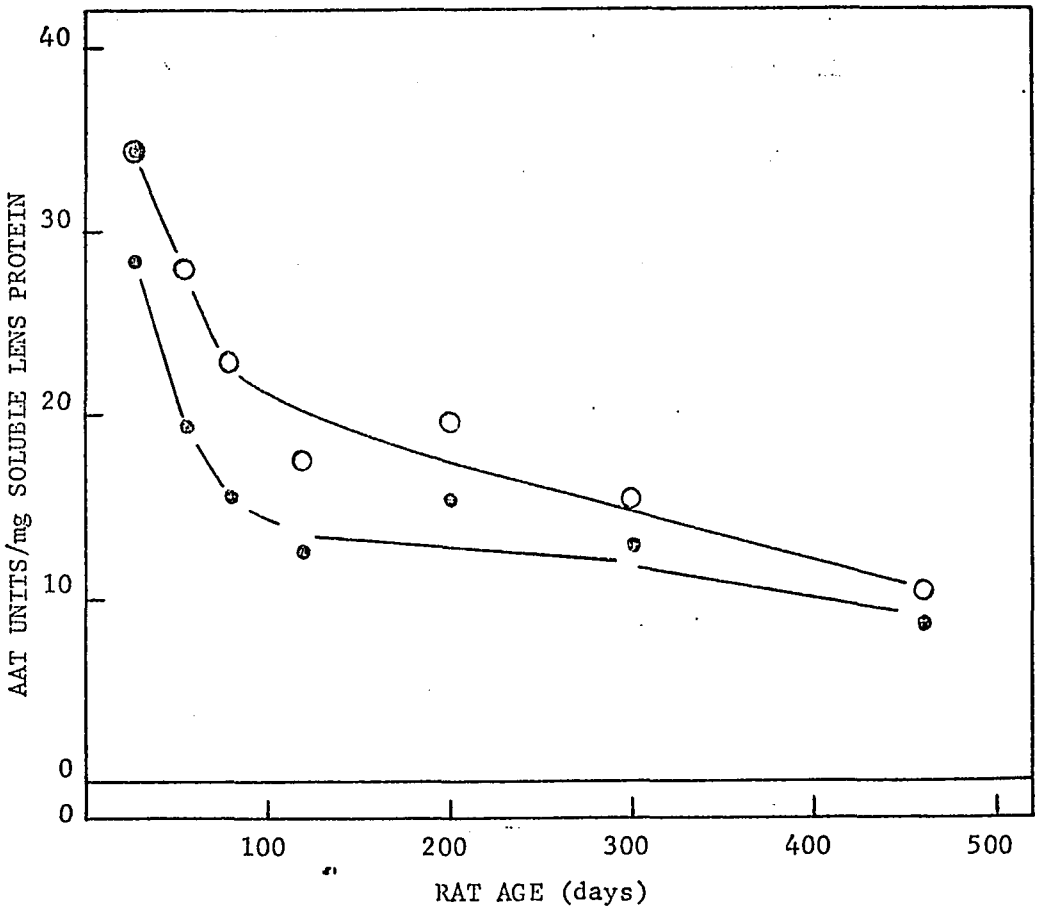

Figure 21. Lens AAT activity expressed per mg of soluble lens protein in rats ranging from 28 to 460 days of age. O—O , assays contained 0.1mM PLP; ●—● , no added PLP in assays.

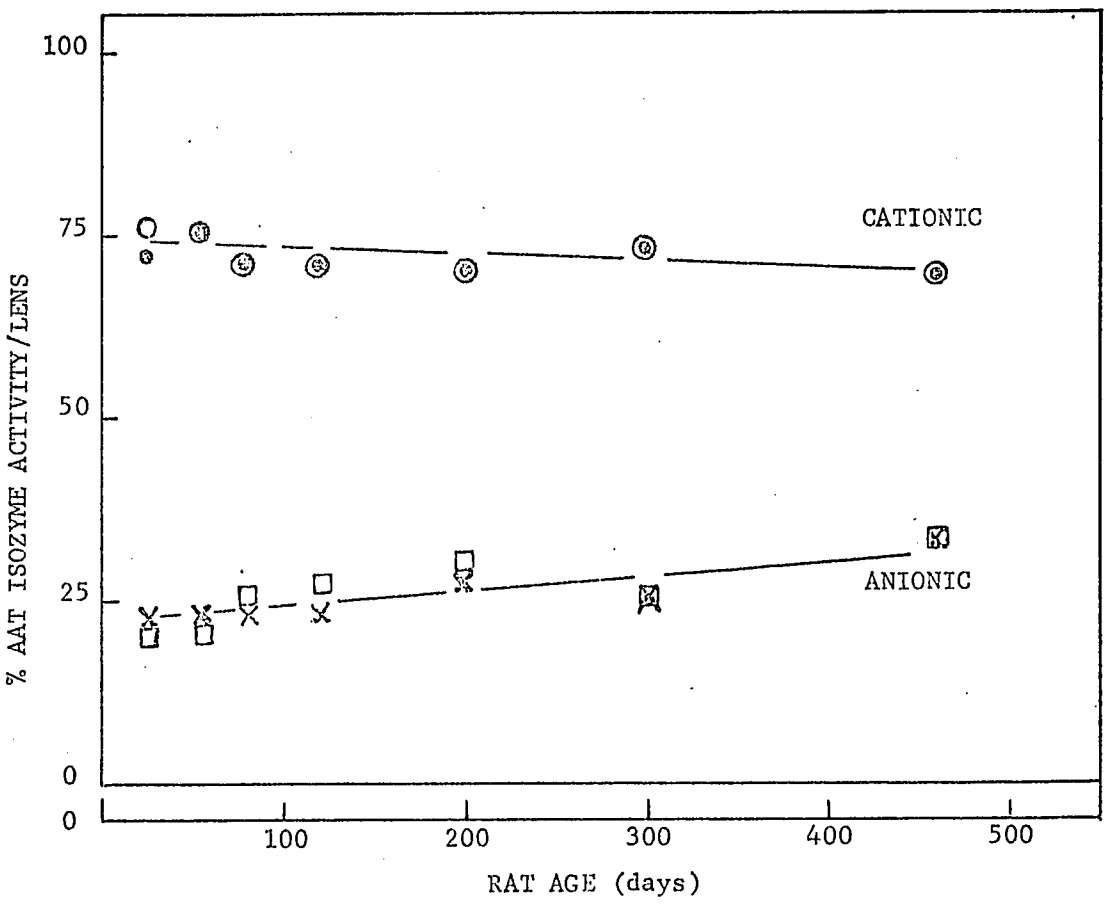

Figure 22. Per cent AAT isozyme activity in lenses of rats ranging from 28 to 460 days of age. Each AAT isozyme was independently determined with heterologous and homologous AAT rabbit antiserum as described in the Methods section. Cationic AAT isozyme assayed with added PLP (0.1mM), O—O ; no added PLP in assays, ●—● . Anionic AAT isozyme assayed with added PLP (0.1mM), □—□ ; no added PLP in assays, X—X .

the anionic and cationic AAT isozymes in rat lens with age. Initially, about 75% of the total AAT activity in the lenses of young rats was contributed by the cationic isozyme, while about 21% was attributed to the anionic isozyme. With aging the cationic isozyme percentage decreased to about 69% while the anionic isozyme percentage increased to about 35% in lenses of 460 day old rats. Over the entire age range there was a significant difference ($p < 0.001$) between the anionic and cationic per cent distribution. Figure 22 also shows that the per cent distribution of the isozymes remained virtually the same as those values obtained in the presence of PLP even though there was a substantial increase in the total activity when assayed in the presence of added PLP (see Fig. 17). Even in the 80 day old rat lens which had the greatest "PLP effect," there was no significant difference in the per cent distribution of either isozyme when assayed with or without PLP. (p value > 0.1 for both anionic and cationic isozymes.)

Each isozyme total activity (Fig. 23) per lens was determined immunologically. The overall shapes of the total isozyme activity curves in rat lens were similar to the total AAT activity curves seen in Figure 17. The slopes of the anionic and cationic total isozyme activities, assayed without added PLP, were not significantly different. The total anionic isozyme activity over the age period studied increased by about 85%, while the total cationic isozyme activity increased by about 10%.

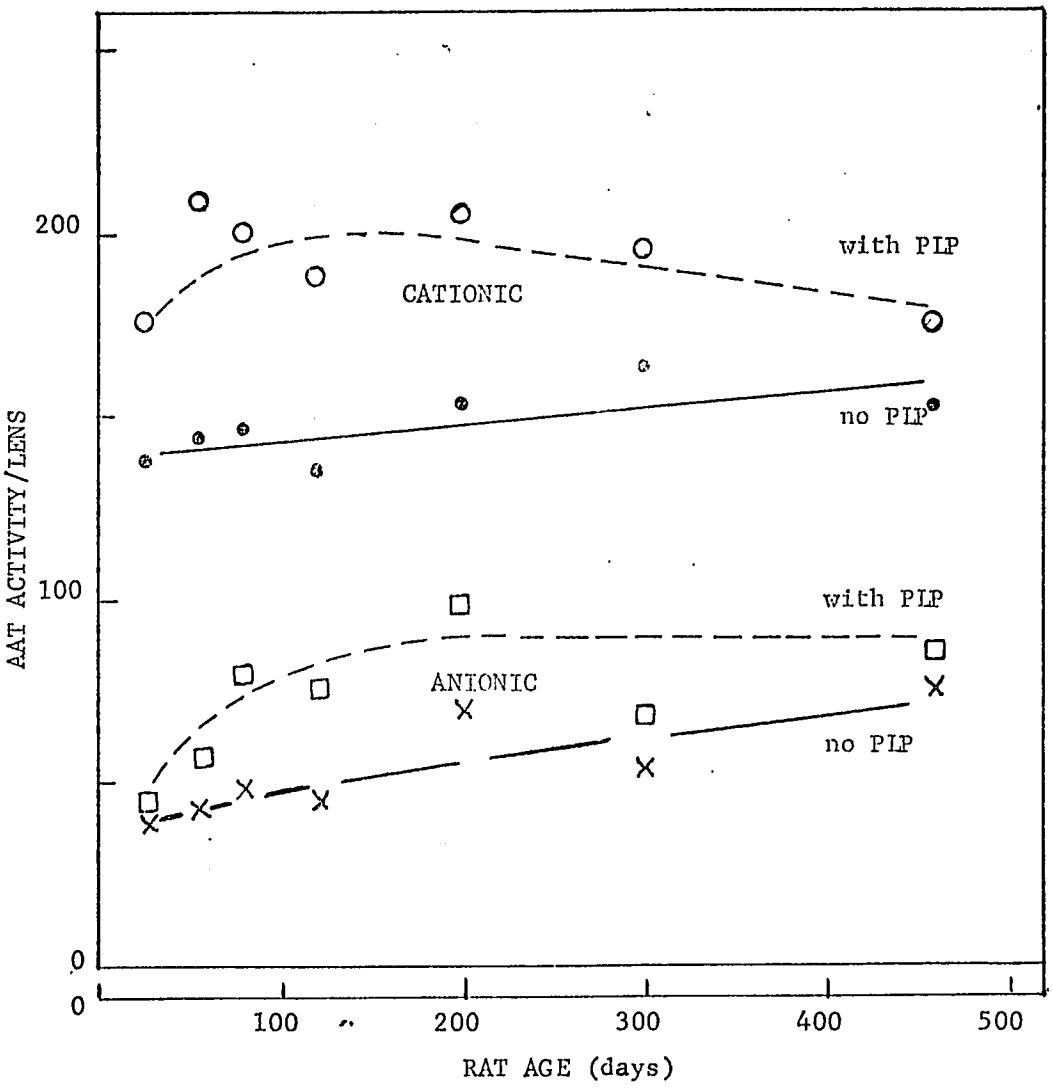

Figure 23. Lens AAT isozyme activity in rats ranging from 28 to 460 days of age. Each AAT isozyme was independently determined by incubating lens homogenates with heterologous and homologous AAT rabbit antisera as described in the Methods section. Cationic AAT isozyme assayed with added PLP (0.1mM), O--O; no added PLP in assays, ●—● . Anionic AAT isozyme assayed with added PLP (0.1mM), □--□; no added PLP in assays, X—X .

Figures 24 and 25 show the isozyme specific activities expressed as AAT units per mg lens weight and AAT units per mg soluble lens protein, respectively. In both figures, each isozyme specific activity decreased rapidly and then leveled off. Similar specific activity patterns were observed when assayed in the presence of added PLP.

J. LENS AAT ACTIVITY IN RATS FED A HIGH SUGAR DIET: GALACTOSE STUDIES

The total AAT activity and AAT isozyme activities were determined in lenses from rats fed 50% galactose, 50% glucose, 35% xylose, or a normal diet for various periods of time.

The lenses of animals on a 50% galactose diet for a 21 day period showed a progressive decrease in the amount of soluble lens protein after about the 6th day on the diet (Fig. 26B). On the 21st day, the lenses of rats fed 50% galactose contained only about 35% of the soluble lens protein of control lenses. The values for the mg soluble lens protein during the 21 day study for both control and cataractous lenses were in agreement with reported values (Sippel, 1966A). The lenses of rats on the diet for only 3 days and then returned to a normal control diet, had a soluble lens protein content similar to that of control animals throughout the 21 day period. However, when rats were on a 50% galactose diet for 10 days and then returned to a normal diet, the soluble lens protein per lens showed similar decreases to that of animals still on the 50%

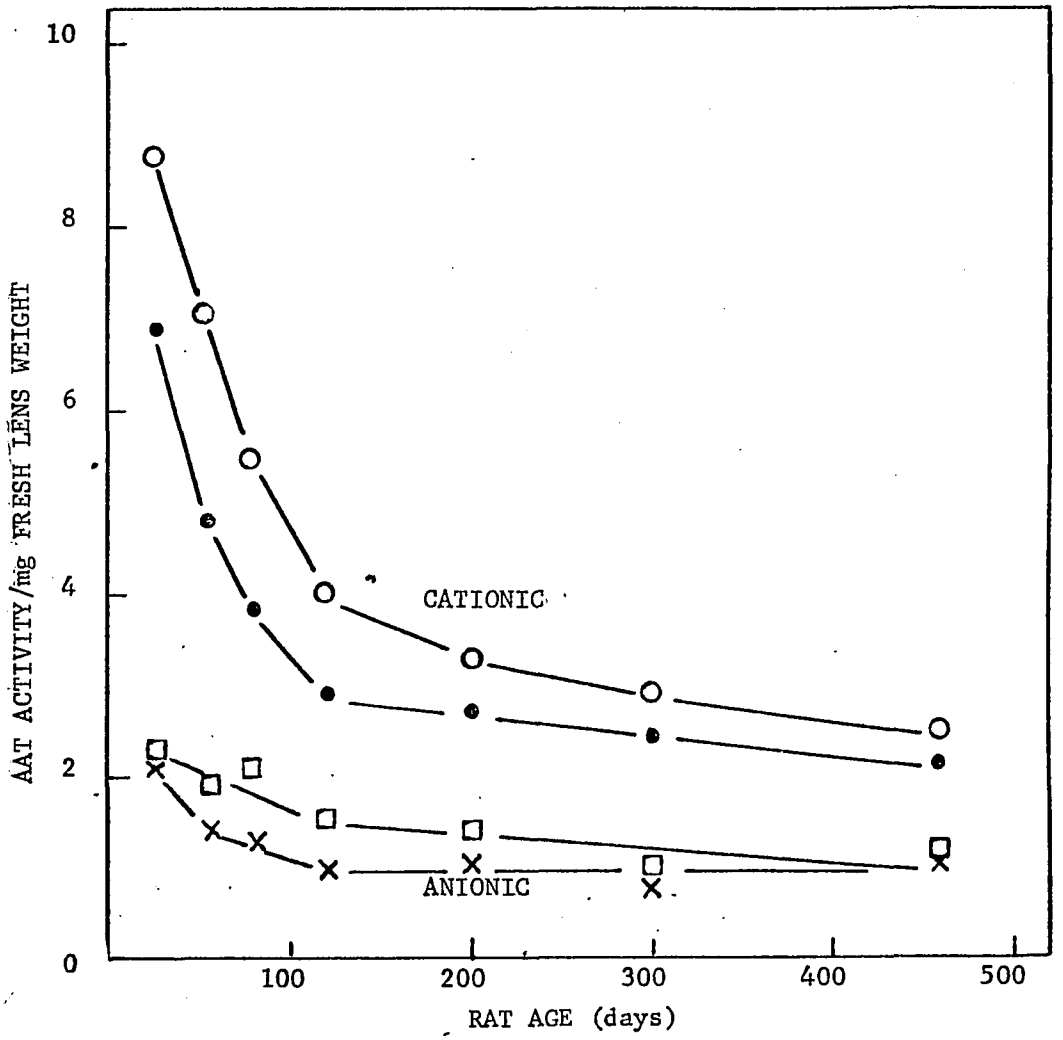

Figure 24. Lens AAT isozyme activity expressed per mg fresh lens weight in rats ranging from 28 to 460 days of age. Each AAT isozyme activity was independently determined by incubating lens homogenates with homologous AAT rabbit antiserum as described in the Methods section. Cationic AAT isozyme assayed with added PLP (0.1mM), O—O ; no added PLP in assays, ●—● . Anionic AAT isozymes assayed with added PLP (0.1mM), □—□ ; no added PLP in assays, ×—×.

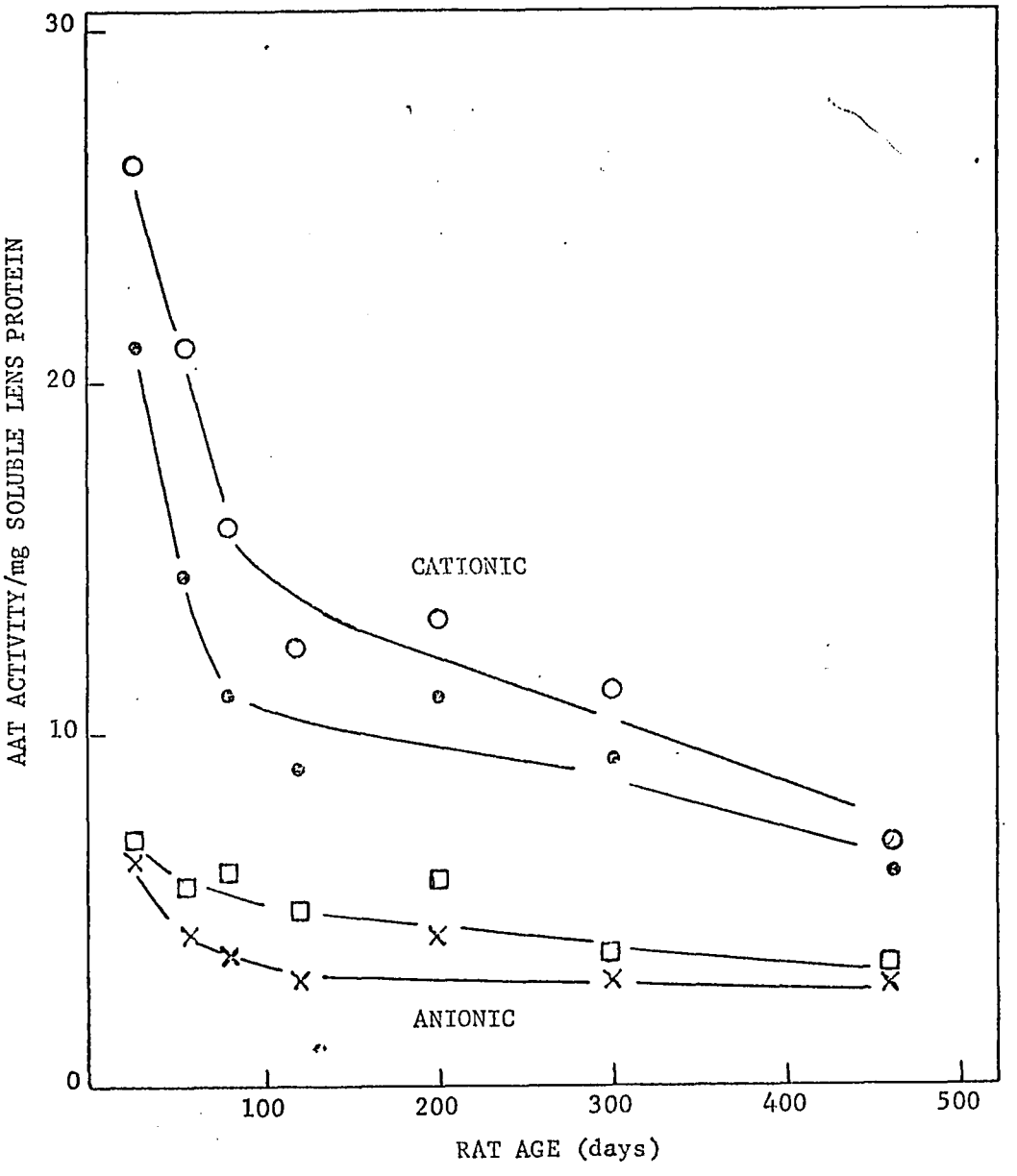

Figure 25. Lens AAT isozyme activity expressed per mg soluble lens protein in rats ranging from 28 to 460 days of age. Each AAT isozyme activity was independently determined by incubating lens homogenates with homologous AAT rabbit antiserum as described in the Methods section. Cationic AAT isozyme assayed with added PLP (0.1mM), O—O ; no added PLP in assays, ●—● . Anionic AAT isozyme assayed with added PLP (0.1mM), □—□ ; no added PLP in assays, X—X.

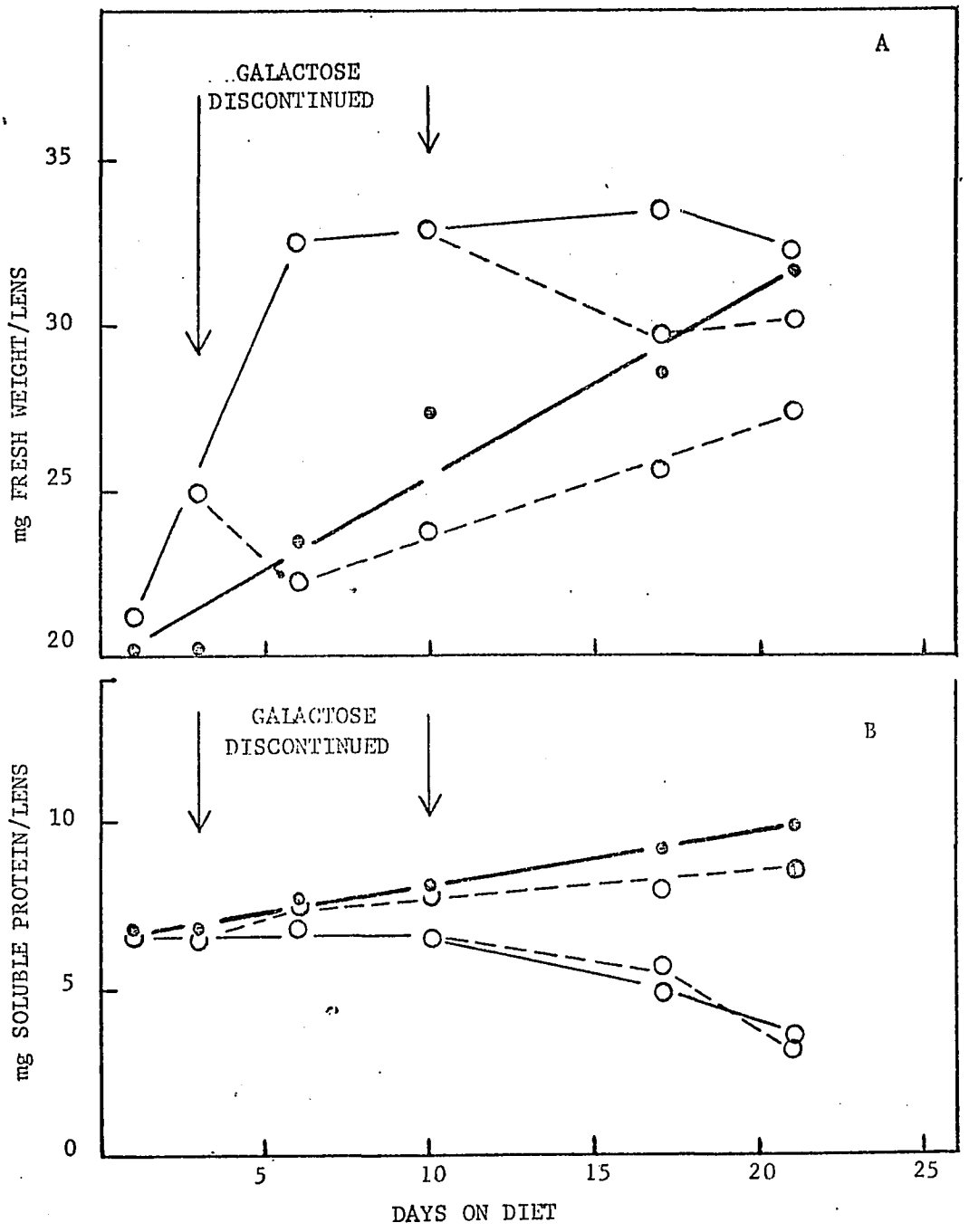

Figure 26. Lens weight expressed as mg fresh weight (A) and mg soluble protein (B) per lens of rats fed control (●——●) and 50% galactose (O——O) diets. Two groups of rats were returned to a control diet after 3 and 10 days of 50% galactose feeding (O- -O).

galactose diet.

The fresh weight of lenses from animals fed a 50% galactose diet showed a progressive increase over that of control lenses and reached a maximum on about the 6th day (Fig. 26A). On the 21st day of galactose feeding the fresh lens weight approached that of control lenses. The lens fresh weight values from animals fed control and galactose diets are in agreement with the values of Sippel (1966A). This increase in lens fresh weight has been attributed to the osmotic effect caused by the accumulation of galactitol (Kinoshita, et al., 1962). Lenses of rats showed a substantial decrease in fresh weight when they were returned to a normal diet after being on 50% galactose for either 3 or 10 days.

The total AAT activity per lens (without added PLP), for control and galactose fed animals are presented in Figure 27A. The control lenses showed a small increase in activity over the 21 day period studied. Total activity of lenses from animals fed galactose for 17 and 21 days was significantly lower than control values ($p < 0.001$). The total activities for each of the isozymes is illustrated in Figure 27B and the overall patterns were similar to that found for the total AAT activity (Fig. 27A).

The Km of the cationic isozyme of lenses from animals fed galactose for 17 days was determined. Although the activity was significantly lower, the Km values for L-aspartate (0.63 mM) and α-ketoglutarate (1.44 mM) were

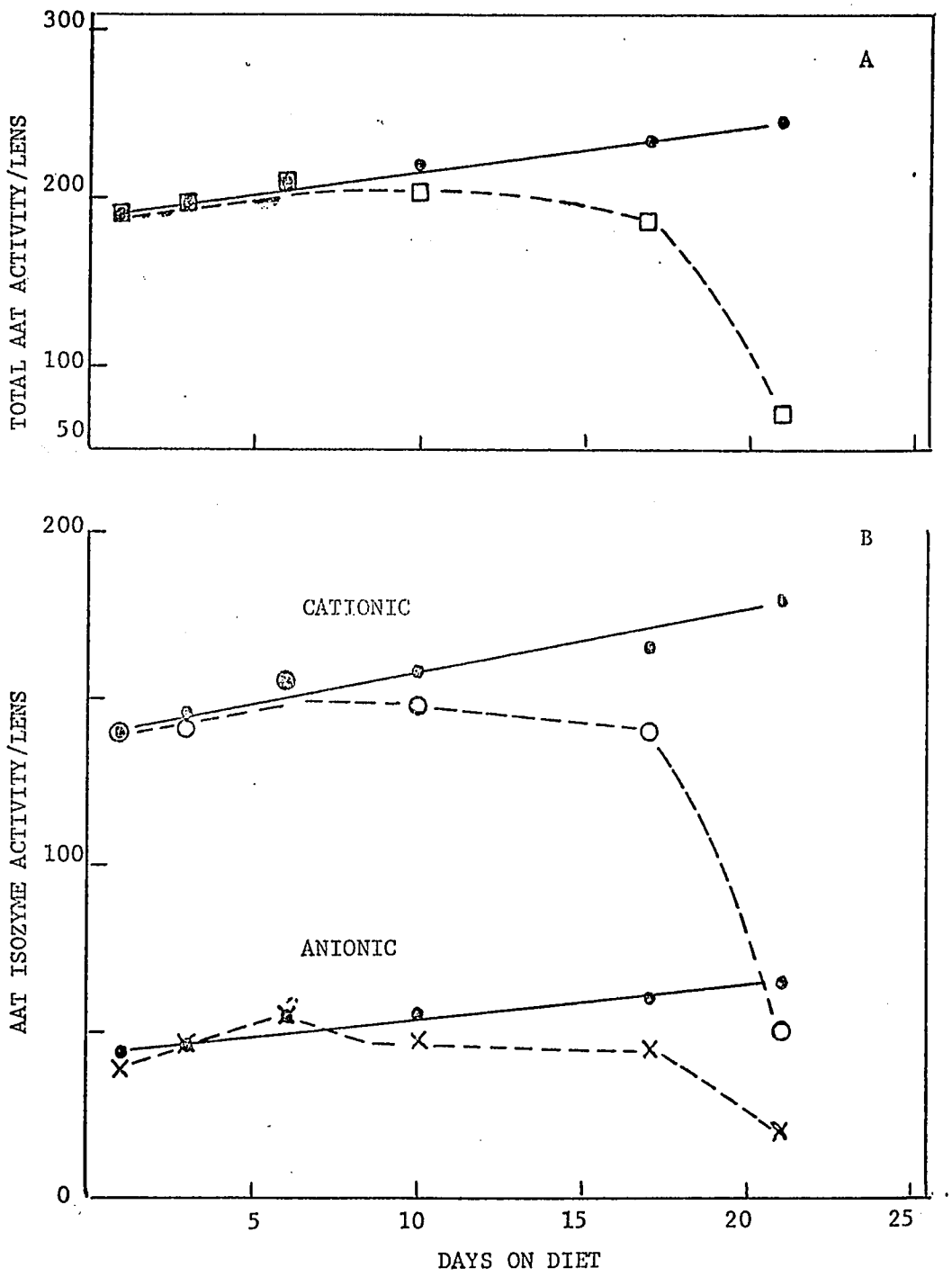

Figure 27. Total AAT activity (A) and AAT isozyme activity (B) per lens of rats fed control and 50% galactose diets. Control diet AAT activity, ●——● ; 50% galactose dieted rats: total AAT activity, □--□ ; cationic AAT isozyme activity, O--O ; anionic AAT isozyme activity, ×--× . Assays did not contain added PLP.

similar to those values found in normal rat lens homogenates.

The activity values in the presence of added PLP were higher than the values without added PLP. However, the overall patterns for the total activity or individual isozyme activities were generally similar to those patterns obtained without added PLP (Fig. 28A and 28B).

The total AAT activity per lens, now expressed as percent of control values, for galactose fed animals, is shown in Figure 29 (without added PLP). The total activity in lenses from rats fed galactose decreased around the 10th day and was about 30% of control on the 21st day (p value <0.01).

It was further noted that the total AAT activity of lenses from rats (expressed as percent of control values) on the diet for only 3 days showed a significant increase when returned to a normal diet (Fig. 29). A 25% increase over that of the normal control value was observed in animals that were refed the normal diet for 7 days. Significant increases were observed on the 10th and 17th day (p values <0.01). Similarly, overcompensation of AAT activity values on the 6th and 10th day (p values <0.01) after refeeding a normal diet were obtained when assayed in the presence of 0.1 mM PLP (Fig. 30). By the 18th day on a normal diet, the AAT activity had returned to the control value. However, when rats were fed a galactose diet for 10 days and then returned to a normal diet, the AAT activity per lens did not differ from animals maintained on a 50% galactose diet.

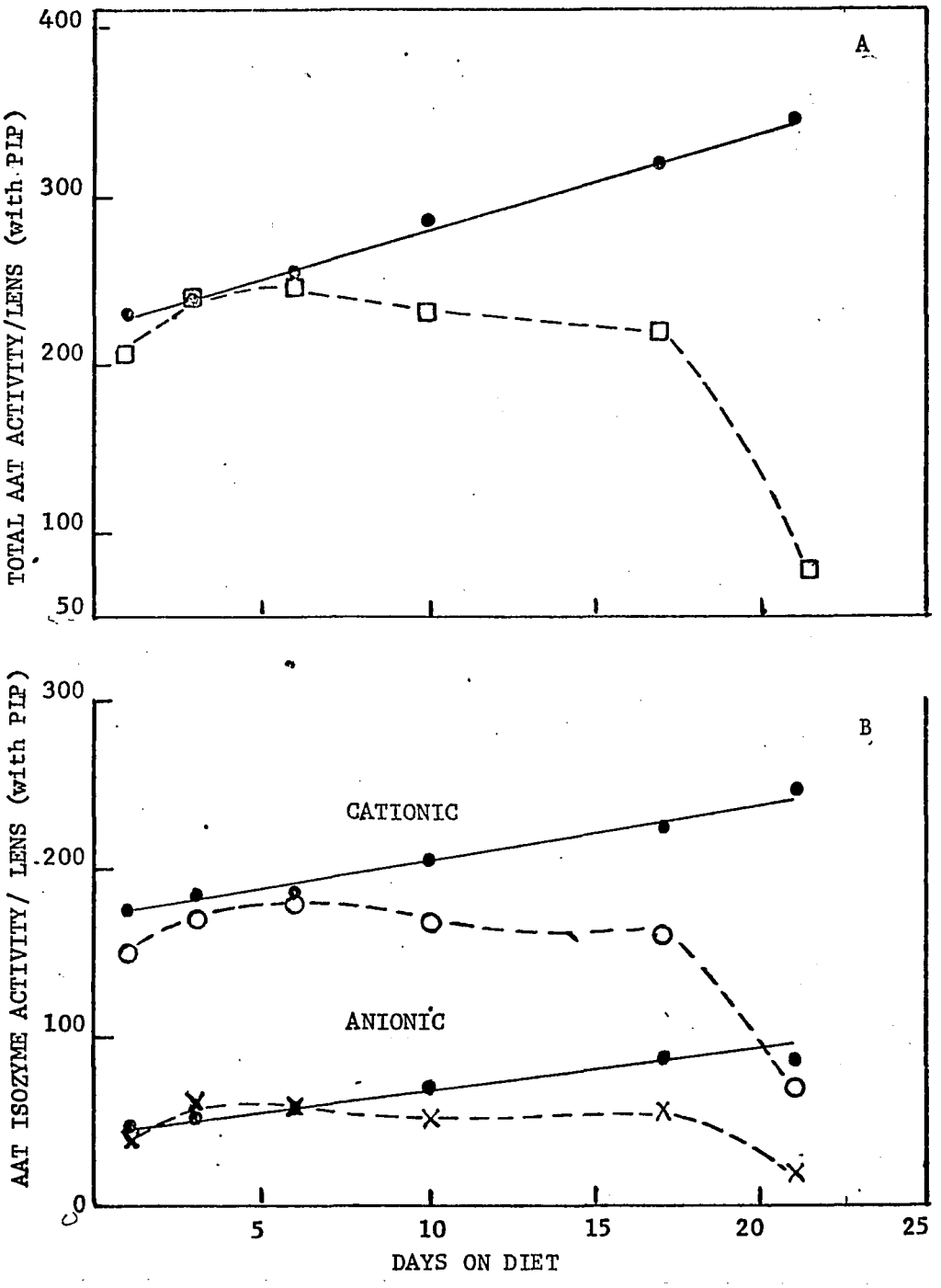

Figure 28. Total AAT activity (A) and AAT isozyme activity (B) per lens of rats fed control and 50% galactose diets when assayed in the presence of added PLP. Control diet AAT activity, ●—●; 50% galactose dieted rats: total AAT activity, □--□; cationic AAT isozyme activity, O--O ; anionic AAT isozyme activity, ×--× .

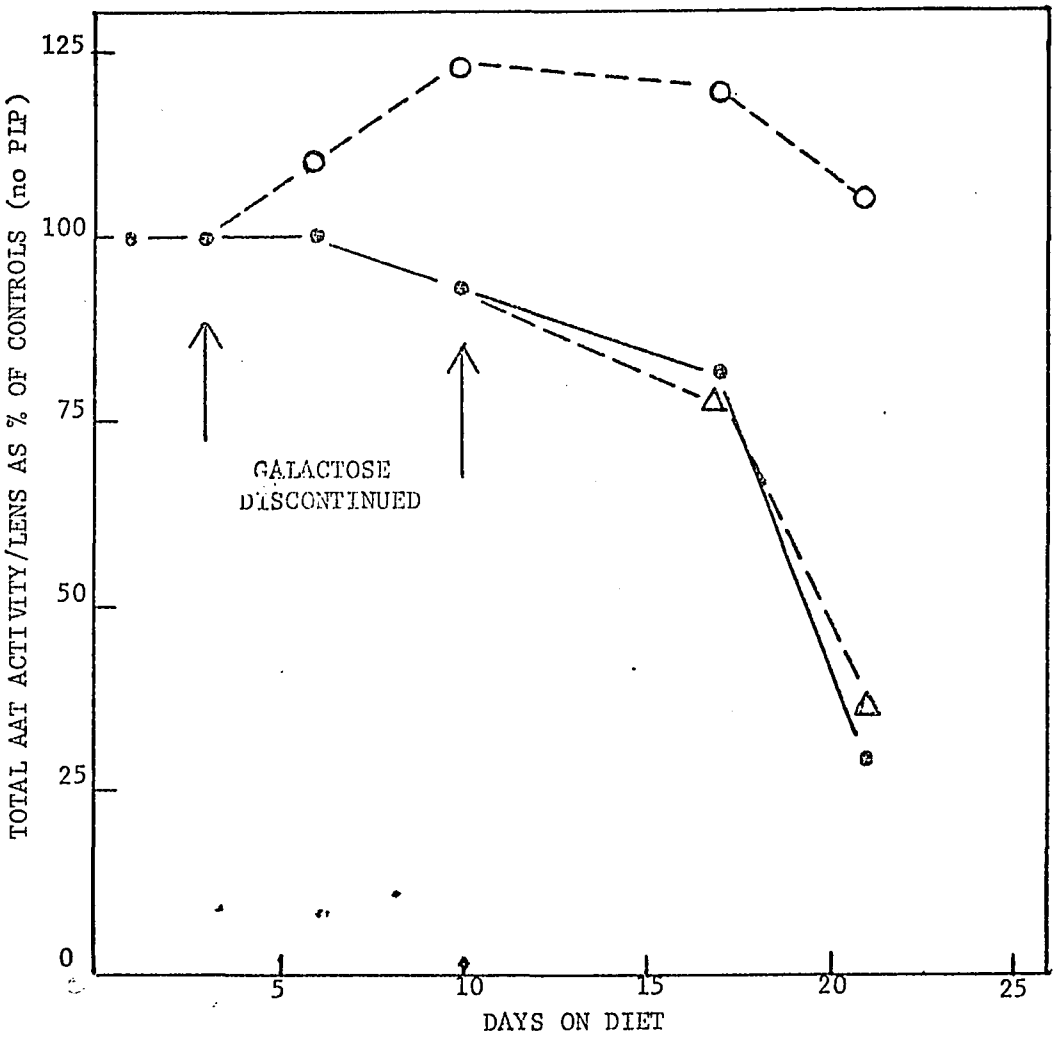

Figure 29. Total AAT lens activity of rats fed a 50% galactose (●—●) diet expressed as per cent of control diet values. Two groups of rats were returned to a normal diet after being fed 50% galactose for 3 (O--O) and 10 days (Δ--Δ). AAT assays did not contain added PLP.

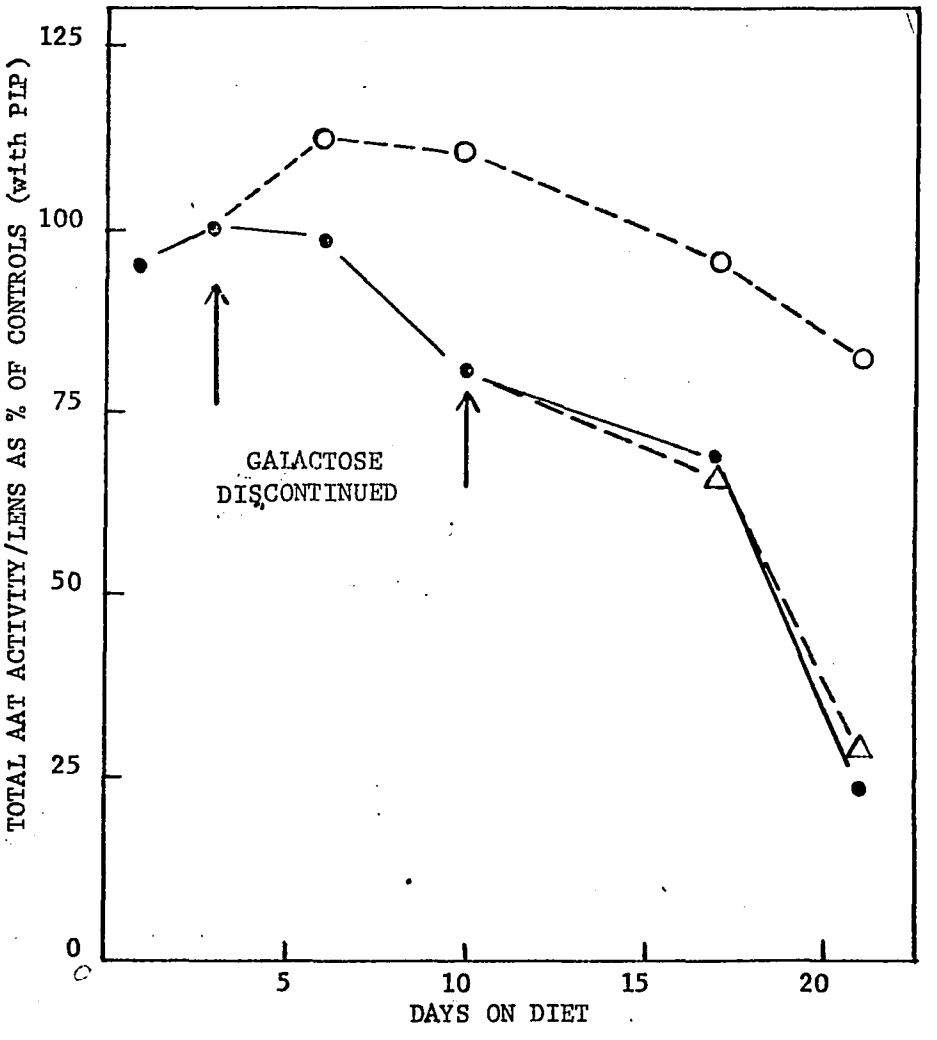

Figure 30. Total AAT activity per lens (assayed with added PLP) of rats fed a 50% galactose (o——o) diet expressed as per cent of control diet values. Two groups of rats were returned to a normal diet after feeding on 50% galactose for 3 (O--O) and 10 days (Δ--Δ).

Total AAT activity values when assayed in the presence of added PLP showed similar relationships.

An interesting "PLP effect" was noted in lenses from animals fed galactose. The percent increase in total AAT activity per lens when assayed in the presence of 0.1 mM PLP over the 21 day galactose diet period remained relatively constant (only about a 15% increase) (Fig. 31). Even after animals were returned to normal diets after 3 and 10 days of galactose feeding, the percent increase was similar to values obtained from rats maintained on the 50% galactose diet. In contrast, control animals showed a progressive increase in the "PLP effect" as a function of age. During the 21 days of this experiment, the total activity increased from 20% to 40% when assays contained added PLP. This coincides well with the normal PLP increase with age (Fig. 18).

Figure 32B shows the specific activity (total AAT activity per mg fresh lens weight) for the galactose diet experiment. The control curve showed a consistent linear decrease having a significant negative slope (correlation coefficient of 0.88). The specific activity curve for galactose fed animals also showed a decrease, having a steeper negative slope than that of control animals. The intercepts for the specific activity of the galactose and control animals were not significantly different from each other (p value >0.3). However, the slope for the lenses of galactose fed rats was significantly different from that of

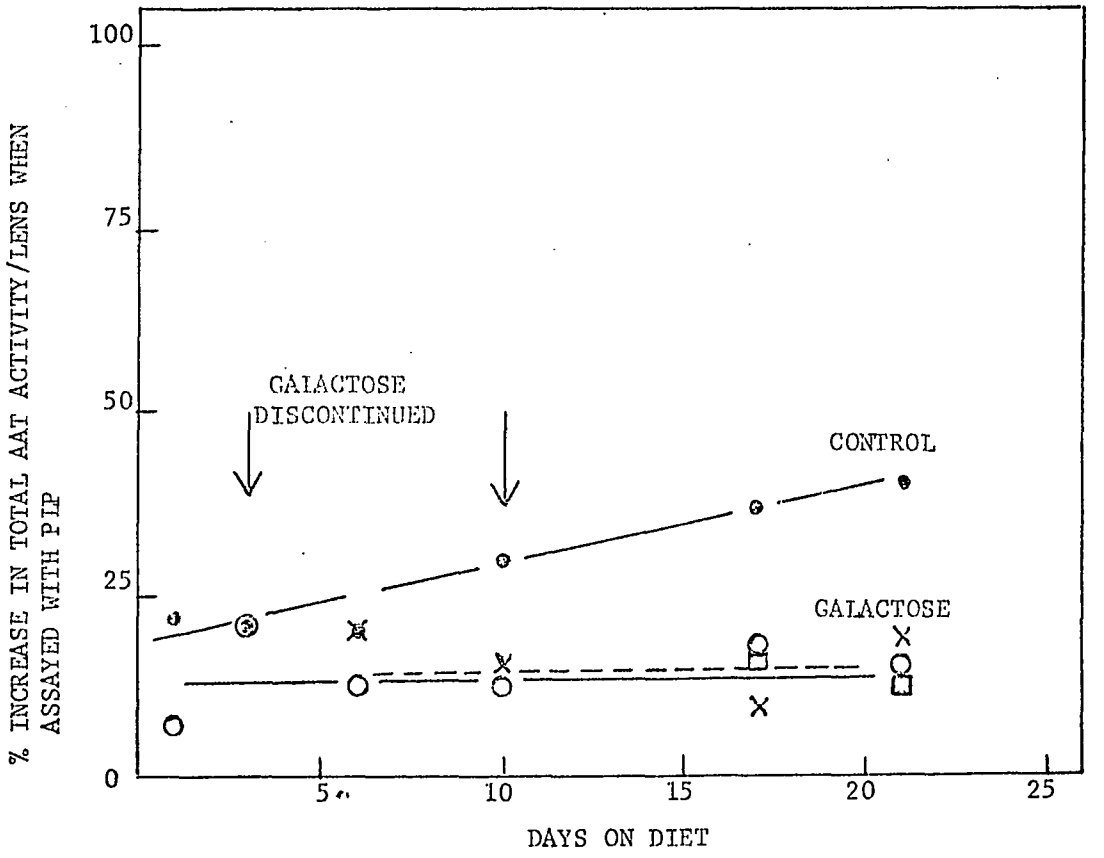

Figure 31. Per cent increase in total AAT lens activity in those assays that contained added PLP (0.1mM) from rats fed control (●—●) and 50% galactose (O—O) diets. Two groups of rats were returned to a control diet after being fed 50% galactose for 3 (X— —X) and 10 days (□— —□).

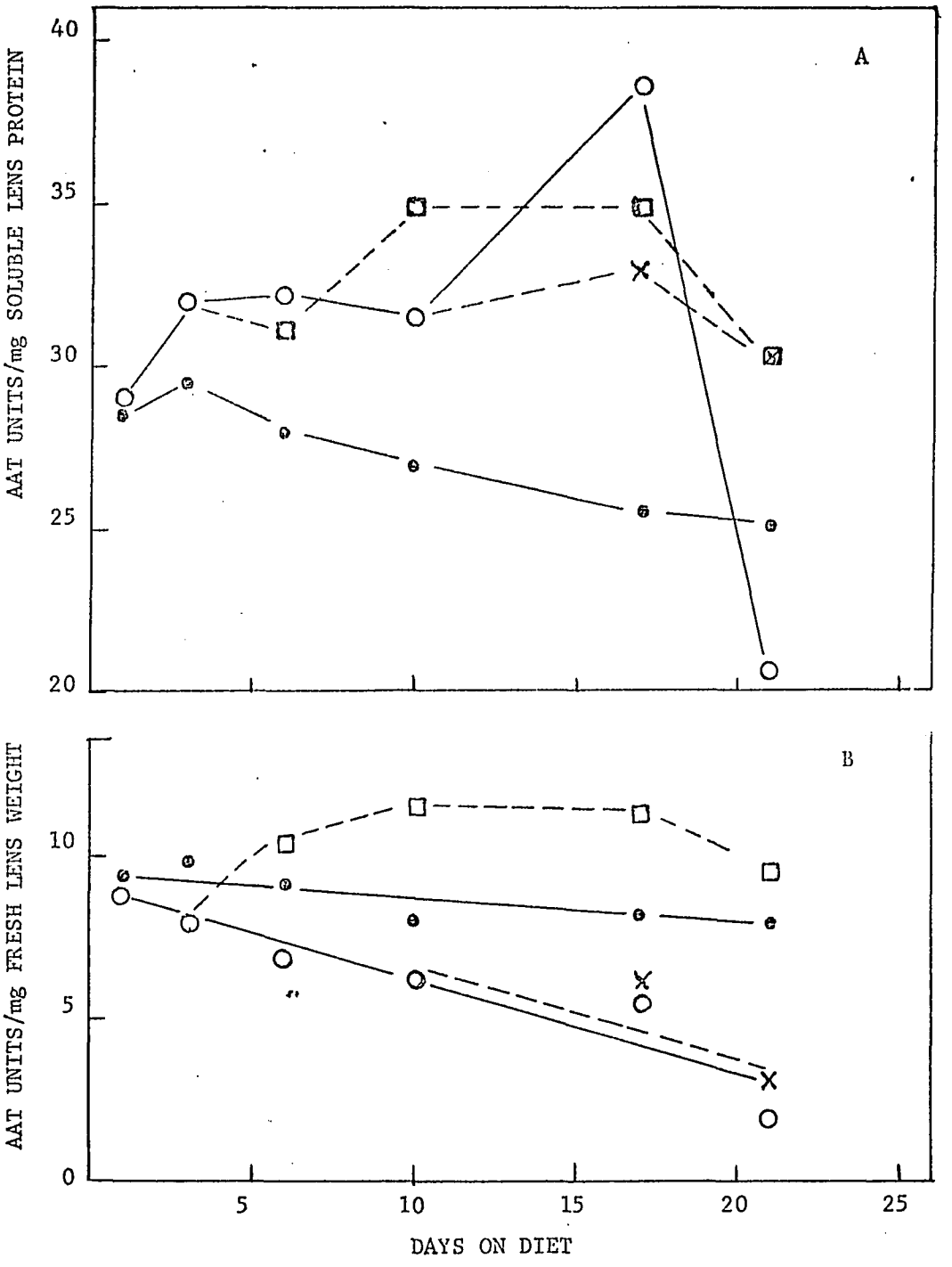

Figure 32. AAT activity expressed as mg soluble lens protein (A) and mg fresh lens weight (B) of rats fed control (●——●) and 50% galactose (O——O) diets. Two groups of rats for each specific activity were returned to a control diet after feeding on 50% galactose for 3 (□--□) and 10 days (×--×). Assays did not contain added PLP.

control rat lenses (p value < 0.01). When animals were returned to a normal diet after 3 days of 50% galactose feeding, the lens specific activity increased to values greater than that of controls, reflecting both the loss of lens fresh weight (see Fig. 26A) and the increase in total AAT activity per lens (see Fig. 27A). Lens specific activities of rats returned to a normal diet after 10 days on a 50% galactose diet had values similar to those from rats maintained on the galactose diet. Similar results were obtained when the specific activity was determined in the presence of added PLP.

The specific activity when expressed as activity per mg of soluble lens protein for control and galactose fed rats can be seen in Figure 32A. Control values assumed a slight negative slope throughout the 21 day study. The specific activity values of lenses from galactose fed animals were elevated above controls, rose sharply on the 17th day and then fell below control values on the 21st day. When rats were returned to a normal diet after being on the 50% galactose diet for either 3 or 10 days, the AAT activity per mg of soluble lens protein by the 21st day was similar for both groups and were above control values.

The percent distribution of each of the AAT isozymes was determined in rat lens homogenates from normal and galactose fed animals (Fig. 33). During the 21 day study all groups (controls, galactose fed and galactose-discontinued) had the same percent distribution of each of the AAT isozymes.

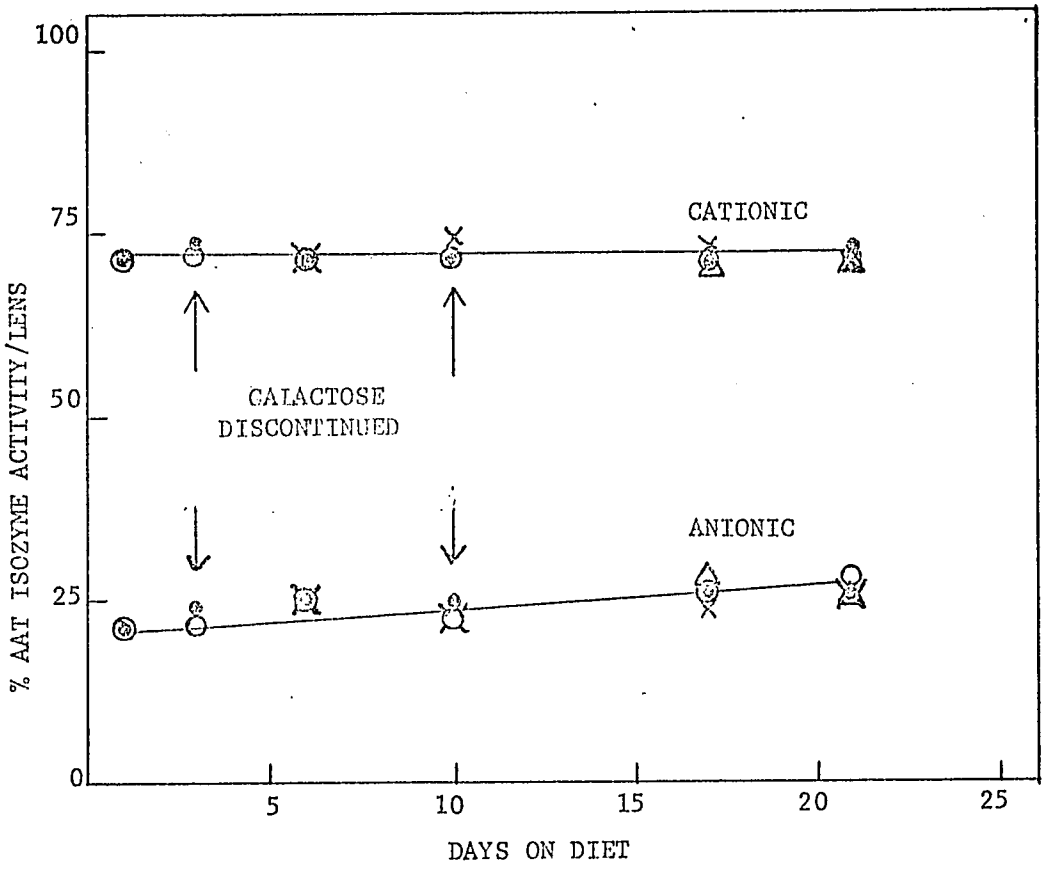

Figure 33. Per cent distribution of lens AAT isozymes of rats fed control (●—●) and 50% galactose (O—O) diets. Two groups of rats were returned to a control diet after feeding on 50% galactose for 3 (×—×) and 10 days (△—△). Each AAT isozyme was independently determined by incubating lens homogenates with AAT rabbit antisera as described in the Methods section. Assays did not contain added PLP.

The percent distribution of each isozyme obtained when PLP was added to the assays was not significantly different from those values obtained without added PLP (Table 12). The overall average for the anionic isozyme in lenses from rats maintained on galactose was 23.9% (control value was 24.6%) and the cationic isozyme average was 72.2% (control value was 73.0%). In all cases the percent anionic distribution (in control and in galactose diet studies) was always significantly different from the cationic percentage (p value less than 0.001). Since the percentage distribution for each of the AAT isozymes remained the same in all the diet studies, the individual isozyme activity per mg fresh lens or per mg soluble protein may be obtained easily from Figure 32. The overall patterns for each of the isozyme specific activities would be similar to that previously shown for the total AAT specific activities.

K. LENS AAT ACTIVITY IN RATS FED A HIGH SUGAR DIET: XYLOSE AND GLUCOSE STUDIES

Young rats (about 40g) were maintained on a 35% xylose diet, a 50% glucose diet, or a powdered Purina Chow diet for a period of 31 days. Figures 34A and 34B show the mg of fresh weight per lens and the mg of soluble lens protein per lens, respectively, over the 31 day period. Lenses from animals fed xylose, glucose or normal diets, all showed the same increase in fresh weight or in soluble protein over the period studied.

Animals from all of the diet groups had the same

Table 12. Percent AAT Isozyme Distribution in Lenses from Rats fed a 50% Galactose Diet.

	Percent Anionic Activity*	Percent Cationic Activity*
All Controls	24.6 ± .54 (27)	73.0 ± .48 (27)
with added PLP	23.4 ± .71 (19)	73.4 ± .65 (19)
All Galactose	23.9 ± .53 (27)	72.2 ± .45 (27)
with added PLP	23.3 ± .61 (19)	73.5 ± .74 (19)
Galactose Discontinued after 3 days	24.9 ± .65 (16)	73.1 ± .75 (16)
with added PLP	24.3 ± .73 (16)	74.3 ± .53 (16)
Galactose Discontinued after 10 days	26.1 ± .96 (8)	71.3 ± .42 (8)
with added PLP	27.4 ± 1.23 (8)	73.1 ± 1.03 (8)

* Values represent the mean value ± the standard error of the mean. The number in parenthesis represents the number of determinations.

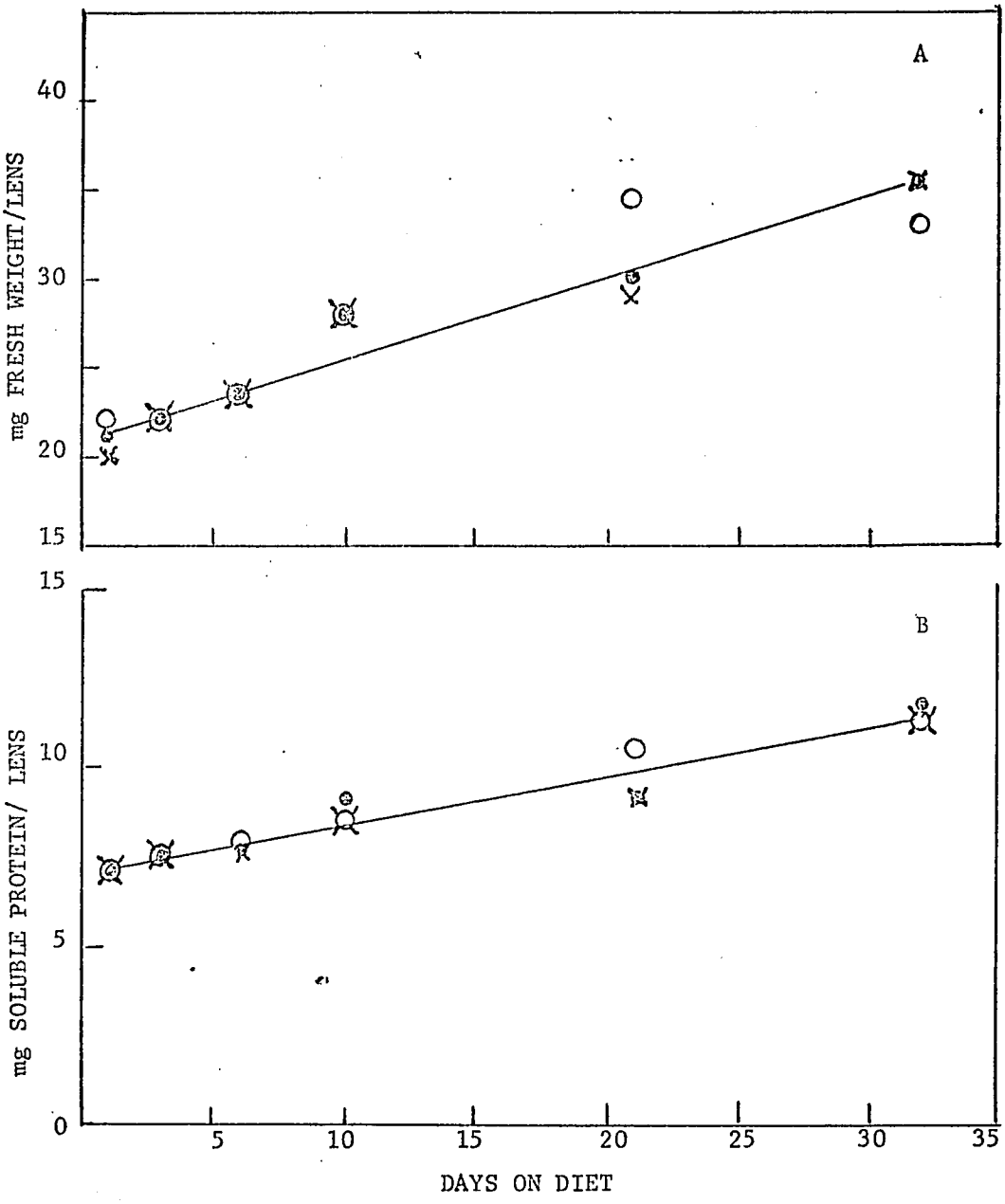

Figure 34. Lens fresh weight (A) and soluble lens protein (B) of rats fed control (●—— ●), 35% xylose (O —— O) and 50% glucose (×—×) diets.

AAT activity per lens. The total activity per lens expressed as percent of control (assayed without added PLP) is shown in Figure 35. Similar results were obtained when the assays contained added PLP. The percent increase in total AAT activity per lens due to the presence of added PLP for animals on either of the sugar diets was not significantly different from normal diet control values (Fig. 36). On the first day of xylose, glucose, and control diets, the average percent increase in the presence of added PLP in activity per lens was about 17%. This value steadily increased and on the 31st day was about 40%. These results with xylose and glucose were in contrast to the constant percent increase of about 15% obtained when rats were fed a 50% galactose diet (Fig. 31).

The specific activities (total activity per mg fresh lens weight and total AAT activity per mg soluble lens protein) for lenses from xylose, glucose and control fed rats showed similar decreases (Fig. 37A and 37B). Similar patterns were obtained when values represented assays containing 0.1 mM PLP.

The percent AAT distribution for each of the isozymes of lenses from rats maintained on a 35% xylose or a 50% glucose diet was not significantly different than control values (Fig. 38). Also, the percentage distribution for each of the AAT isozymes was not significantly different when assays were performed in the presence of added PLP. The average anionic AAT isozyme percentage in lenses from rats

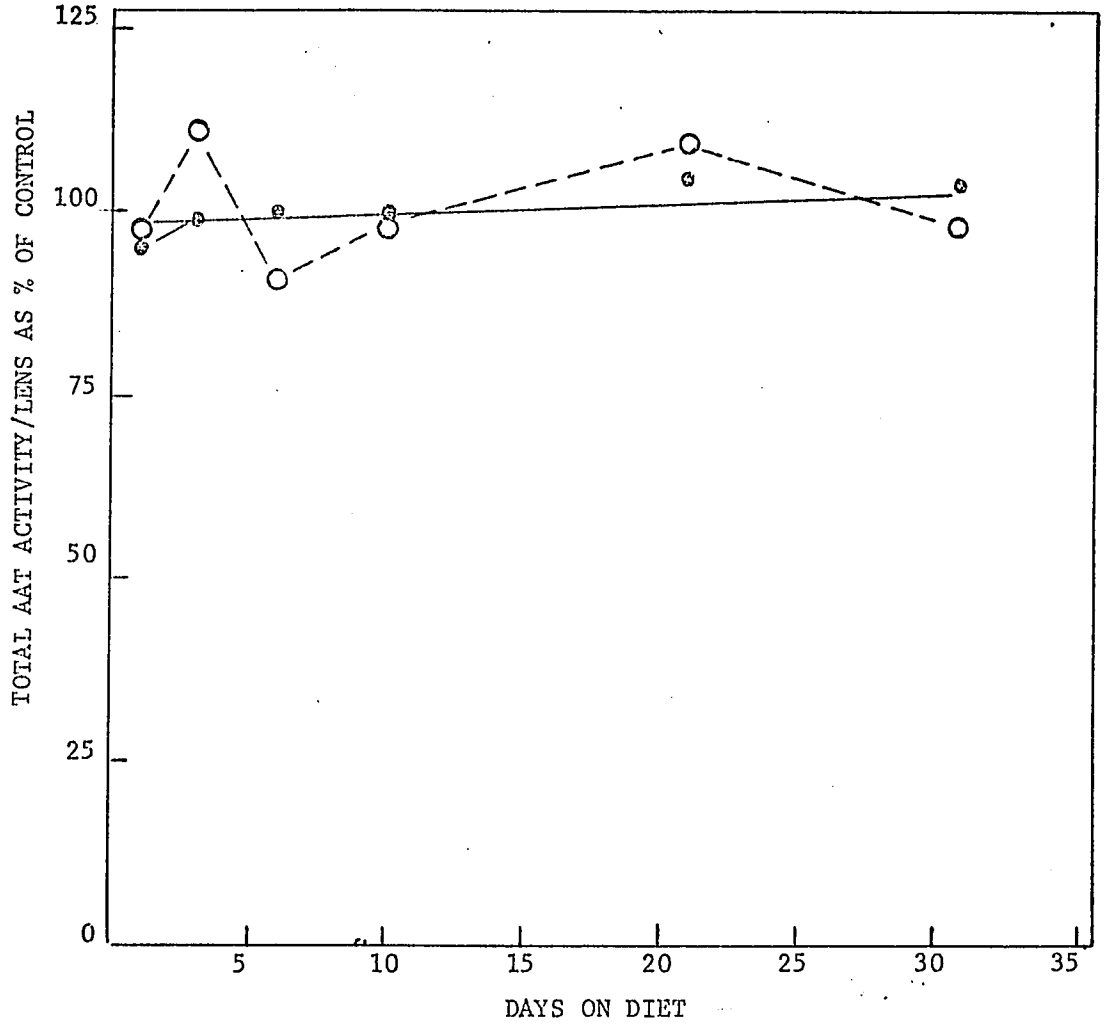

Figure 35. Total AAT lens activity in rats fed 35% xylose (O--O) and 50% glucose (•——•) diets expressed as per cent of control diet values. AAT assays did not contain added PLP.

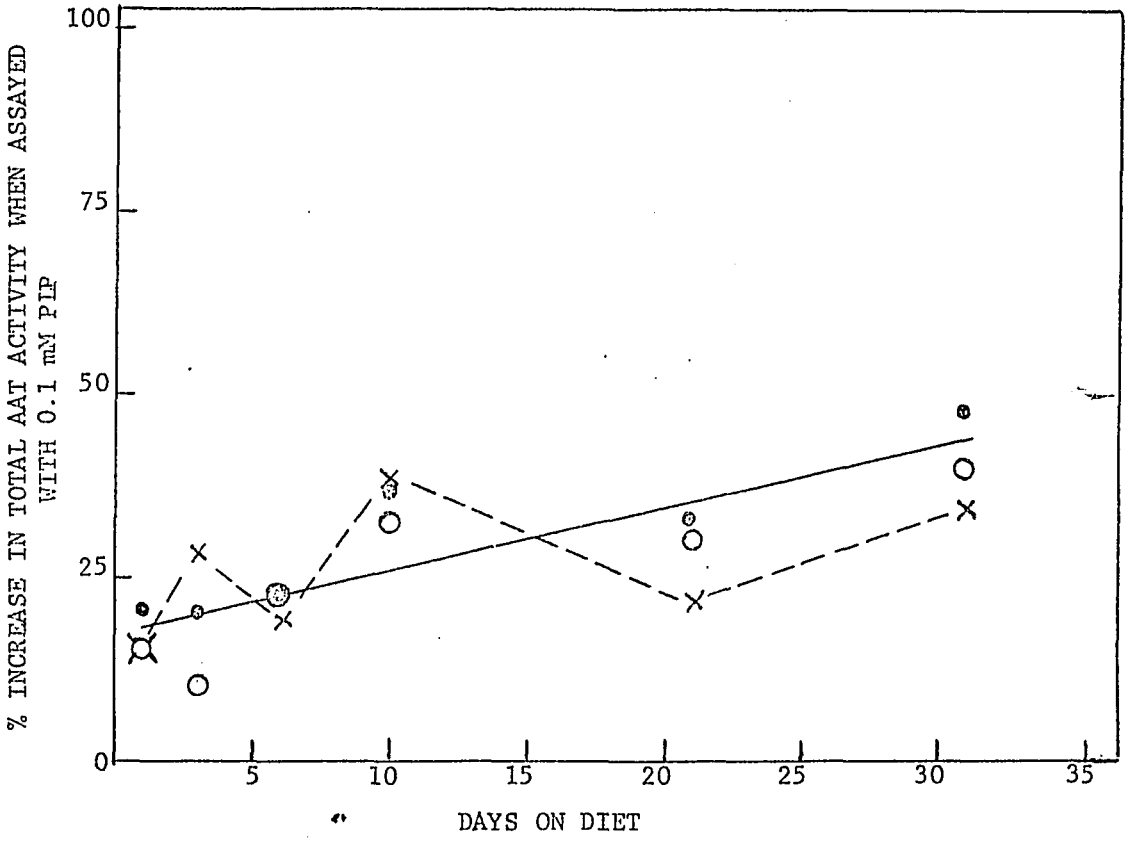

Figure 36. Per cent increase in total AAT lens activity in those assays that contained added PLP (0.1mM) in rats fed control (•——•), 35% xylose (O——O) and 50% glucose (x----x) diets.

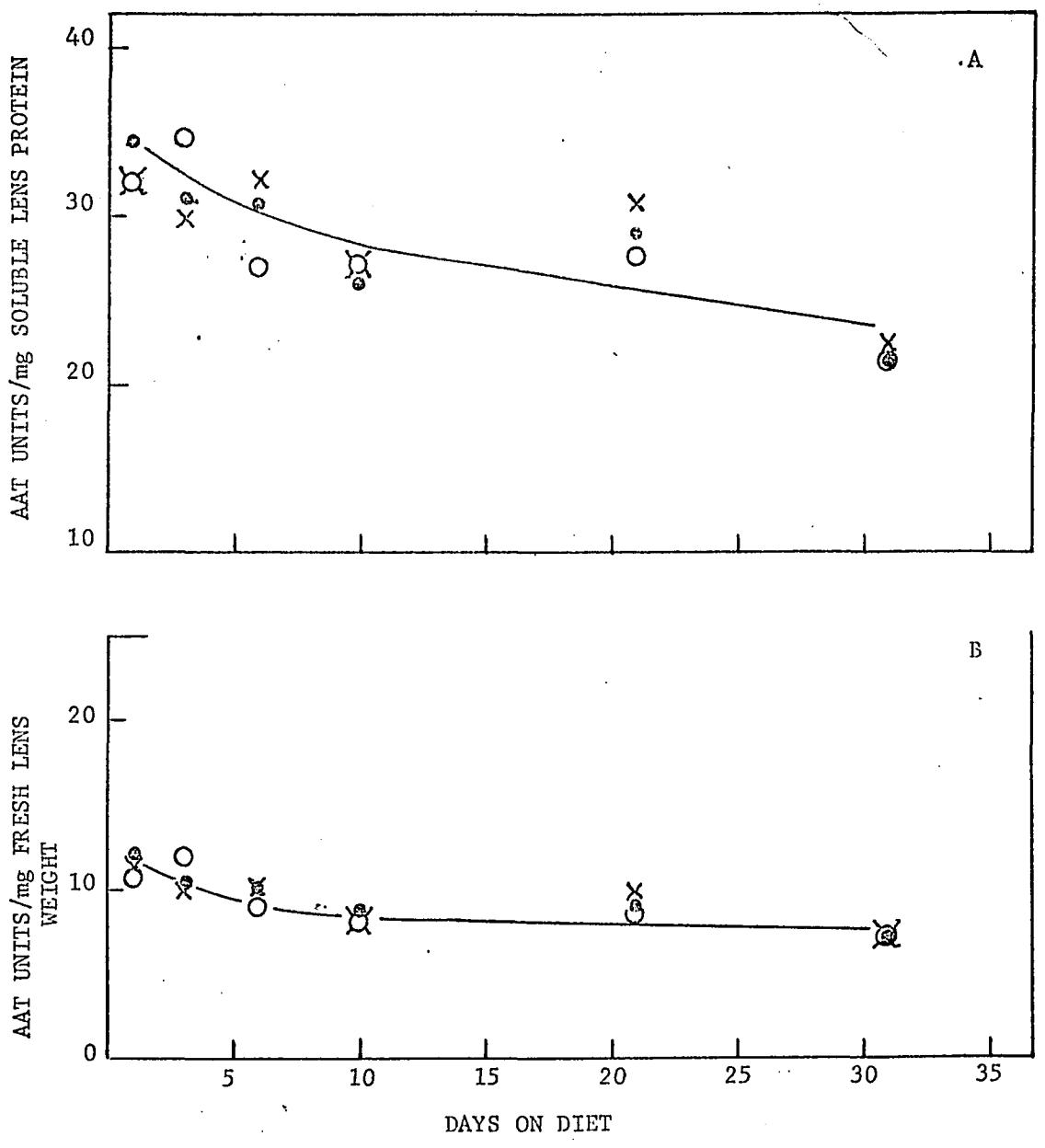

Figure 37. AAT activity expressed as mg soluble lens protein (A) and mg fresh lens weight (B) of rats fed control (•——•), 35% xylose (O——O) and 50% glocose(x——x) diets. AAT assays did not contain added PLP.

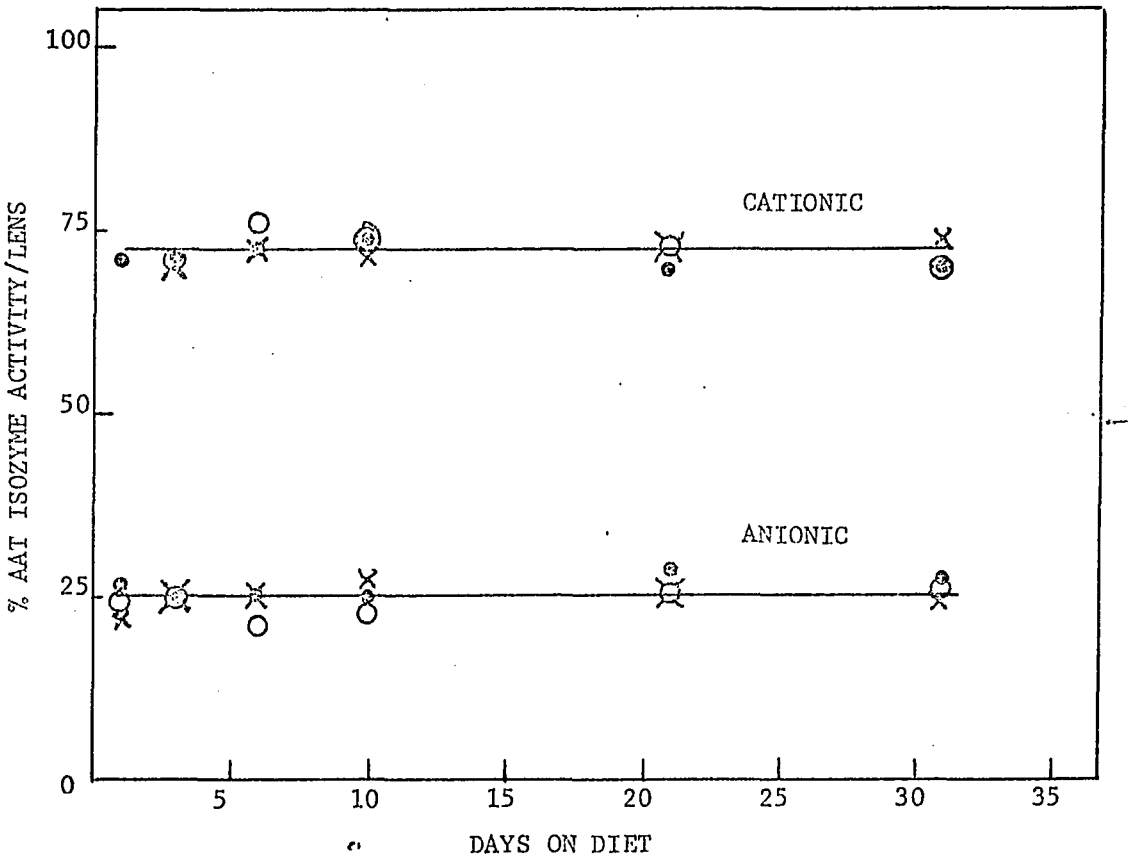

Figure 38. Lens AAT isozymes of rats fed control (•——•), 35% xylose (O——O) and 50% glucose (X——X) diets. Each AAT isozyme was independently determined by incubating lens homogenates with AAT rabbit antisera as described in the Methods section. AAT assays did not contain added PLP.

on 35% xylose and 50% glucose was 24.1% and 24.9%, respectively (control value was 26.1%), while the average cationic percentage was 73.4% and 72.5% for xylose and glucose fed animals, respectively (control value was 71.6%). The percent anionic AAT isozyme distribution in lenses from rats on normal, xylose or glucose diets was in all cases significantly different from the cationic percentage.

IV. DISCUSSION

The anionic and cationic isozymes of aspartate aminotransferase (AAT) have been evaluated with respect to their kinetics, immunochemistry and localization in normal rat and calf lens tissue. This enzyme and its isozymes were further investigated in normal rat lens tissue with regard to aging and in sugar-induced cataractogenesis.

The anionic and cationic isozymes from calf lens were partially purified utilizing a DEAE-cellulose column. The calf lens elution profile for each isozyme was similar to that obtained from rat liver as noted in the present study and as reported previously (Nisselbaum, 1968 and Nisselbaum and Bodansky, 1969). The isozymes of AAT have also been substantially purified from pig heart (Banks, et al., 1968; Green, et al., 1945; Jenkins, et al., 1967 and Nisselbaum and Bodansky, 1966), ox heart (Marino, et al., 1966), beef liver (Morino, et al., 1963), chicken heart (Bertland and Kaplan, 1968) and from human heart and liver (Nisselbaum and Bodansky, 1964).

The calf lens anionic AAT isozyme was purified 122 fold with a final specific activity of 5,600 (units per mg protein). The cationic AAT isozyme preparation represented a 7.5 fold purification with a specific activity of 347. These specific activity values are low compared to isozymes purified from more active tissue sources (e.g., rat liver isozymes: 180,000 for anionic, 645,000 for cationic). Vergee and Evered (1969) noted that the low specific activity

obtained in their purification of anionic AAT isozyme from wheat germ may be related to a tissue source variation and not to the purification procedures. Fowlkes, et al. (1964) have also reported that LDH isozymes purified from bovine, rabbit and dog lenses had low specific activities.

The anionic and cationic isozymes partially purified from calf lens were homogenous for the isozyme isolated as determined by starch gel electrophoresis. The electrophoretic distinctness of the partially purified anionic and cationic isozymes from pig heart (Nisselbaum and Bodansky, 1966), human heart and liver (Nisselbaum and Bodansky, 1964) and rat liver (Nisselbaum and Bodansky, 1969) has been described previously. The individual isozymes from calf lens homogenates showed the same electrophoretic separation. The cationic isozymes from calf lens and from rat liver had similar mobilities. However, the anionic isozyme from calf lens had a slower migration than the anionic preparation from rat liver. Similar differences in the anionic isozyme electrophoretic migration have been noted in extracts of heart tissue of several species (pig > human > rat > dog) (Decker and Rau, 1963). The species variation in electrophoretic mobility of the anionic AAT isozyme suggest molecular diversity similar to those reported for the isozymes of alkaline phosphatase, peroxidase and esterases (Paul and Fottrell, 1961).

In the present study, rabbit antisera made against partially purified anionic and cationic isozymes from rat

liver almost completely inhibited both AAT isozymes in calf lens homogenates (96%). In individual assays the anionic antibody inhibited the total AAT activity of calf lens homogenates 82%. This was interpreted to mean that 82% of the total calf lens activity was due to anionic isozyme. Similarly, using the cationic antibody, 15% of the total calf lens activity was attributed to the cationic isozyme (see Table 10).

The Km values were obtained for the partially purified preparations of the anionic and cationic AAT isozymes from calf lens and rat liver. The Km values of each isozyme from unfractioned whole rat lens homogenates were also determined by inhibiting the second isozyme with its homologous antiserum. To the author's knowledge, this is the first report to evaluate isozyme Km values from homogenates. The Km (L-aspartate) was significantly different from the Km (alpha ketoglutarate) for the anionic AAT isozyme in all the tissues evaluated (all p values $<$ 0.001). The Km (L-aspartate) values for the anionic isozyme from rat liver (2.1 mM), calf lens (2.8 mM) and rat lens homogenates (2.3 mM) were not significantly different from each other (all p values $>$ 0.05). These results were in general agreement with the values reported for rat liver (2.15 mM) (Boyd, 1961), pig heart (4.4 mM) (Henson and Cleland, 1964), beef liver (4.3 mM) (Morino, et al., 1963), human heart (5.8 mM) and human liver (4.3 mM) (Nisselbaum and Bodansky, 1964). These values were substantially lower than 11.9 mM reported for

dog heart (Fleisher, et al., 1960). It should be noted that Fleisher, et al. (1960) measured and reported Km'. The Km (alpha ketoglutarate) values for the anionic rat liver (0.076 mM), calf lens (0.10 mM) and rat lens homogenates (0.096 mM) showed no significant difference (all p values $>$ 0.05). The value previously reported of 0.2 mM for rat liver (Boyd, 1961) was very similar, while values of 0.38 mM for pig heart (Henson and Cleland, 1964), 0.30 mM for beef liver (Morino, et al., 1963), 0.69 mM for human heart and 0.60 mM for human liver (Nisselbaum and Bodansky, 1964) and 0.54 mM for canine heart (Fleisher, et al., 1960) were slightly higher.

The Km (L-aspartate) and Km (Alpha ketoglutarate) for cationic isozymes of rat liver, calf lens and rat lens homogenates were significantly different from each other (p values $<$ 0.001). The cationic isozyme Km (L-aspartate) for rat liver, calf lens and rat lens homogenates were 0.57 mM, 0.65 mM and 0.64 mM respectively, and were not significantly different from each other (all p values $>$ 0.05). The reported values of 0.47 mM for rat liver (Boyd, 1961), 0.40 mM for beef liver (Morino, et al., 1963), 0.70 mM for canine heart (Fleisher, et al., 1960) were in the same range. All of these values were substantially lower than the 3.9 mM value reported for pig heart (Henson and Cleland, 1964). The Km values reported by Henson and Cleland (1964) were actually for the anionic isozyme and not the cationic isozyme as shown by Nisselbaum and Bodansky (1966). The Km

(alpha ketoglutarate) values for the cationic isozymes were 1.07 mM for rat liver, 1.38 mM for calf lens and 1.35 mM for rat lens homogenates. These values were not significantly different from each other (all p values>0.02). The cationic AAT isozyme values of 1.0 mM for rat liver (Boyd, 1961), 2.0 mM for beef liver (Morino, et al., 1963) and 1.18 mM for canine heart (Fleisher, et al., 1960) were in a similar range with the present values. The values of 2.9 mM for human heart and 3.3 mM for human liver (Nisselbaum and Bodansky, 1964) were slightly higher, while the value of 0.43 mM for pig heart was somewhat below the values of other tissues from other species (Henson and Cleland, 1964).

The Km (L-aspartate) and Km (alpha ketoglutarate) values for rat liver, calf lens and rat lens homogenates, were generally in good agreement with published values. The noted differences in Km values may be related to species variation and/or differences in assay conditions (Boyd, 1968 and Nisselbaum, 1968).

Analyses of young rat lenses with rabbit antibodies prepared against anionic and cationic isozymes from rat liver showed that about 73% of the total activity was the cationic isozyme while about 23% was anionic. This ratio of about 3:1 (cationic to anionic) in rat lens was reversed from that found in calf lens (1:3, cationic to anionic). The reasons for these observed reversed ratios in rat and calf lens is not yet clear. In both species the same antibodies were used to determine the isozyme ratios.

These rabbit antibodies were prepared against the partially purified isozymes from rat liver and each showed a high degree of specificity against its homologous antigen. No cross reactivity with its heterologous antigen was observed at any of the antigen-antibody concentrations studied. The addition of both antisera, either combined or separately, to either calf or rat lens homogenates yielded about 100% inhibition. It was not surprising, however, that 10 times more antibody (prepared against rat liver isozymes) was required in the calf lens as compared to the rat lens system. These results indicate that the antigens had similar specificities but were not immunologically identical.

The application of specific antibodies to evaluate isozymes has been reported for lactate dehydrogenase (Nisselbaum and Bodansky, 1959, 1961, 1963; Kaplan and White, 1963; Ng and Gregory, 1969), creatine kinase (Samuels, 1963 and Bulcke and Sherwin, 1969), alkaline phosphatase (Boyer, 1963; Stolback, et al., 1969 and Fishman, 1969) and AAT (Morino, et al., 1964; Nisselbaum and Bodansky, 1964, 1966 and 1969).

An independent method was employed to ascertain the isozyme distributions reported here for calf and rat lens. Previous studies (Fleisher, et al., 1959 and Boyd, 1961) and present studies on the isozymes partially purified from rat liver and calf lens clearly showed a distinct isozyme pH profile. The cationic isozyme was relatively stable between the pH range 6.0 to 8.0, while the anionic

isozyme showed decreased AAT activity below pH 7.0. At pH 6, the anionic isozyme had only 40% of its pH 7.4 activity. The percent distribution of the AAT isozymes in homogenates was evaluated after determining the enzyme activities at pH 6.0 and 7.4. The pH experiment substantiated the lens isozyme distribution values obtained immunologically.

The isozyme localization in lens cells was determined and compared with the literature. The compartmentalization of the AAT isozymes within rat liver tissue was first observed by Boyd (1961). The cationic isozyme was demonstrated to be localized in the mitochondria, while the anionic was found primarily in the supernatant fraction. Recent electron microscopic evidence further substantiated that AAT activity was largely located in the mitochondria of rat liver (Lee and Torak, 1968A and 1968B) and rat cardiac tissue (Lee, 1969). In the present study, at least 75% of the total AAT activity of rat liver was within the mitochondrial fraction (10,000 x g pellet, lysed in distilled water, frozen and thawed) and of this activity, 92% was cationic. The 10,000 x g supernatant fraction contained 25% of the total AAT rat liver homogenate activity and 78% of this was anionic.

On the other hand, rat lens tissue was demonstrated to possess 96% of its AAT activity in the 10,000 x g supernatant fraction of an isotonic sucrose homogenate. In contrast to the rat liver experiments, about 70% of the rat lens 10,000 x g supernatant was the cationic isozyme. The 10,000 x g pellet when lysed in distilled water, and

subsequently frozen and thawed, did not contain substantial AAT activity (about 4% of the total activity).

Similarly, about 96% of the total AAT activity of calf lens homogenates in isotonic sucrose was found in the 10,000 x g supernatant fraction with about 4% of the activity in the disrupted 10,000 x g pellet. In contrast to the rat lens tissue, the 10,000 x g supernatant fraction contained about 82% of the anionic AAT isozyme and only 15% of the cationic isozyme.

Furthermore there was almost no AAT activity in the epithelial layer (stripped away) of calf or rat lens. This highly metabolic layer has been shown to contain almost all the mitochondria of the lens (Cohen, 1965 and Kuwabara, et al., 1969). Thus the high cationic isozyme activity in the rat lens was not associated with mitochondria or with the epithelial layer. The significance of the high cationic isozyme distribution ratio in rat lens (or of the reverse ratio in calf lens) has not yet been elucidated.

The total AAT activity in rat lens tissue was determined in animals ranging from 28 days to 460 days of age. Throughout this age study, no significant increase in the total AAT activity per lens was observed although the lens weight increased from 20 to 70 mg. Similar results were recently reported for methionyl-tRNA synthetase (Weller and Green, 1969).

In the presence of added PLP, there was a substantial increase in the total activity per lens over the

values obtained in the absence of PLP. This "PLP effect" was most pronounced in lenses of rats between 55 and 80 days of age. Rat lenses during this period of time were still in a major growth period as evidenced by the increases in fresh weight and mg of soluble protein per lens (Figures 16A and 16B, Sippel, 1965; Lerman and Zigman, 1965 and Dische, et al., 1956A). An important characteristic of this age range may be that it corresponded to the period of pubescence. At the present time, nothing is known of the metabolism and distribution of this cofactor in lens tissue.

Throughout the age range studied, the percentage of the cationic AAT isozyme was significantly higher than the percentage of the anionic isozyme, as determined immunologically. Between 28 and 460 days of age, there was a decrease in the percent distribution of the cationic isozyme from 75% to 69% (correlation coefficient 0.73). Conversely the anionic isozyme percent distribution increased from 21% to 35% (correlation coefficient 0.82). Thus it appeared that with aging the observed increase in total activity was due to anionic AAT. There was no significant difference in each of the isozyme percentage values when assayed with PLP.

In the present study, lenticular opacities developed in rat lenses between 8 to 17 days when maintained on 35% xylose diet (Lerman and Heggeness, 1961). Beyond this period, the opacities disappeared and by the 31st day

on this diet, opacities were no longer present. Animals that were maintained on a 50% glucose diet never developed lenticular opacities. The total AAT activities per lens and specific activities (expressed per mg of lens fresh weight and per mg of soluble lens proteins) in lenses from rats maintained on either 35% xylose or 50% glucose were not significantly different from control values. These enzyme activities for both sugar diets, when evaluated in the presence of added PLP, were also not significantly different from control AAT activity assayed in the presence of PLP. The percent distributions for the anionic and cationic AAT isozymes in rat lenses for each of the diets were also not significantly different from control values, either in the presence or absence of added cofactor. Even at the time when opacities were produced in lenses by the continual feeding of 35% xylose, none of the AAT values studied were different from control values. Similarly normal values for NAD and NADP xylitol dehydrogenase activities were reported for lenses of rats maintained on a xylose diet (van Heyningen, 1959).

Young rats fed a galactose-rich diet developed lens opacities as early as the 3rd day. These lens opacities progressively intensified until dense white nuclear cataracts were visible between 8 and 12 days of galactose feeding (Sippel, 1966A, 1966B, 1967; Weller and Green, 1969). The total AAT activities per lens for galactose fed rats were similar to control values for the first 10 days. After

this time, AAT activities significantly decreased until on the 21st day the cataractous lenses contained only 30% of control activity. A similar relationship was observed between cataractous and control lens activities when AAT assays contained added PLP. Other lenticular enzymes have been reported to retain their initial levels of activity up through the 15th day of galactose feeding. These enzymes include 6-phosphogluconic acid dehydrogenase (Lerman, 1960), creatine phosphokinase (Cotlier, 1964), lactate dehydrogenase (LDH), alpha glycerophosphate dehydrogenase (alpha GPD), malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) (Sippel, 1967). The enzymes, LDH, MDH and G-6-PDH, decreased 50% in activity soon after the 15th day on a galactose diet. In contrast to these enzymes, aldolase activity was noted to decrease in the lens early in the first week of galactose feeding.

It was possible that profound changes in substrate affinities could be associated with the observed loss of enzyme activity. The Km values of the cationic isozyme of lenses from rats fed galactose for 17 days were similar to values obtained from normal lenses. At this time the enzyme activity was falling rapidly, but the affinities for L-aspartate and alpha ketoglutarate were unaffected.

At the present time, it is not clear whether the loss of AAT activity is due to protein leakage from cataractous lenses, increased enzyme catabolism, decreased

AAT synthesis and/or the presence of an inhibitor. The aspects of enzyme synthesis and catabolism were not investigated. The presence of an inhibitor cannot be ruled out entirely, however dialysis, dilution and mixing experiments failed to show the presence of any inhibitor. Much of the enzyme loss may be attributed to protein leakage from cataractous lenses. The decrease in AAT activity corresponded to a time in the galactose induced cataractous lens when half of the soluble lens protein had been lost (Patterson and Bunting, 1965; Sippel, 1966A and present study). The AAT isozyme percent distribution in lens tissue remained constant and similar to control values, even when the total AAT activity decreased to 30% of the control activity. The increased AAT specific activity (expressed per mg of soluble lens protein) for the galactose lenses (especially on the 17th day) may be explained by simply assuming that the percentage loss of the total protein exceeded that of the enzymatic protein.

When rats were put back on a normal diet after feeding on a 50% galactose diet for the first 3 days, the total AAT activity per lens was observed to significantly increase as much as 25% above control values. Significantly increased AAT values (above controls) were also noted when lens homogenates were assayed in the presence of added PLP. The percent distribution of the isozymes (measured with or without added PLP) remained the same as control values over the entire period studied. By the 18th day on a normal

diet, the total AAT activity per rat lens had returned to the control value. Weller and Green (1969) have studied the activity of methionyl-tRNA synthetase and did not observe any significant increase in enzyme activity when galactose was discontinued after 3 days.

Animals refed a normal diet after feeding on a 50% galactose diet for the first 10 days, did not show any increased AAT activity. The AAT activities per lens on the 7th and 11th day of normal refeeding were the same as those values found in lenses from animals fed galactose for the entire study (see Fig. 29). The percent isozyme distribution did not change in any group (with or without added PLP).

The percent increase in total AAT activity when assayed in the presence of added cofactor was constant (about 15%) in lenses from animals fed galactose for the total 21 days or in the 3- or 10-day galactose discontinued experiments. This is in contrast to control lenses which showed as much as a 40% increase after 21 days of the study.

The galactose discontinued studies showed that permanent alterations appeared to have occurred in lenses from rats fed galactose for 10 days. Furthermore, the increase in lens AAT activity in the 3-day galactose discontinued studies may indicate new anionic and cationic isozyme synthesis or decreased catabolism. The increased metabolic and mitotic activities observed after 3 days of galactose feeding (van Sallmann, 1957; Hanna and O'Brien, 1960 and Grimes and van Sallmann, 1968) may now be evident

because the animals were removed from the galactose diet. Such increases in enzyme activity in a recovery state have been well documented in bone (Balogh Jr. and Hajek, 1965), liver (Weiss, 1961; Bucher, 1963 and Schmidt, 1968) and corneal tissue (Kaufman, et al., 1964). To the author's knowledge, this is the first report indicating substantial increases of a specific lens enzyme in a recovery or healing situation.

V. SUMMARY

The anionic and cationic isozymes of aspartate aminotransferase (AAT) were partially purified from calf lens and were compared with the AAT isozymes partially purified from normal rat liver. These isozymes, as well as the isozymes in rat lens homogenates, were evaluated with regard to their substrate kinetics, pH profiles, partial tissue localization and immunochemistry. AAT and its isozymes were further studied in normal rat lens tissue in relation to aging and in sugar-induced cataractogenesis.

The DEAE cellulose column chromotography elution profile of the anionic AAT isozyme from calf lens was distinct from the cationic AAT isozyme. The elution profiles for the calf lens isozymes were similar to the elution profiles from a rat liver preparation. Starch gel electrophoresis of the cationic AAT isozymes partially purified from calf lens and rat liver showed similar mobilities, while the anionic isozyme from calf lens had a slower anionic electrophoretic mobility than the homologous liver isozyme.

Rabbit antisera made against the anionic or cationic AAT isozyme partially purified from rat liver completely inhibited the homologous AAT isozymes from rat liver and rat lens. Similar inhibitions of AAT activity in calf lens homogenates required the use of higher concentrations of antisera.

The Km (L-aspartate) and Km (alpha ketoglutarate)

values obtained for the anionic AAT isozymes partially purified from rat liver and calf lens were significantly different from the values obtained with the cationic AAT isozymes from these tissues. The homologous AAT isozymes from rat liver and calf lens showed similar kinetic characteristics. The Km values of the isozymes in rat lens homogenates (determined after antibody inhibition of the heterologous isozyme) were similar to values found for the partially purified isozymes from rat liver and calf lens.

The activities of the cationic isozyme preparations from rat liver, calf lens and rat lens homogenates showed a marked constancy between pH 6.0 and 8.0. On the other hand, the anionic isozyme activities from these tissues were only 40% as active at pH 6.0 as compared to their pH 7.4 values.

AAT enzyme activity in both calf lens and rat lens homogenates was found predominantly in the 10,000 x g supernatant fraction of 0.25 M sucrose homogenates (96% for both tissues). This was in contrast with rat liver tissue, where most of the AAT activity was found in the lysed 10,000 x g pellet of a 0.25 M sucrose homogenate (75%). As expected, the 10,000 x g pellet of rat liver homogenates contained predominantly the cationic isozyme while the 10,000 x g supernatant activity was predominantly anionic. The rat lens activity was predominantly due to the cationic isozyme (70%) as determined immunologically. On the other hand, in calf lens homogenates 82% of the total activity

was determined to be the anionic isozyme. The reversed isozyme distribution in calf and rat lenses was substantiated by differential pH studies.

Total AAT activity per rat lens appeared to remain relatively constant between 28 and 460 days of age. When enzyme assays were performed in the presence of added co-factor, pyridoxal phosphate (PLP), a significant increase in the total activity per rat lens was observed between 55 and 80 days of age. Thereafter the activity per lens decreased slightly and leveled off. The total AAT activity when expressed per mg of fresh lens weight or per mg of soluble lens protein decreased until approximately 120 days and then appeared to plateau through 460 days. Between 28 and 460 days of age, there was a slight decrease in the percent distribution of the cationic isozyme and a corresponding increase in the anionic isozyme percentage. A similar isozyme distribution pattern was obtained when assay mixtures contained added PLP.

The total AAT activity, percent AAT isozyme distribution and AAT activities expressed per mg of fresh lens weight or per mg of soluble lens protein of lenses from rats maintained on either 35% xylose or 50% glucose diets did not significantly differ from the control values. However, the AAT activity in lenses from rats maintained on a 50% galactose diet was significantly lower than control values by the 17th day of galactose feeding. On the 21st day, the total AAT activity had declined even further to

about 30% of the control value. The Km values of the cationic isozyme in lenses from rats fed galactose for 17 days were similar to control values.

The percent distribution of the AAT isozymes during the entire galactose study remained constant and similar to control values (anionic: galactose, 23.9% and control, 24.6%; cationic: galactose, 72.2% and control, 73.0%). The percent increase in total AAT activity when assayed in the presence of added PLP was constant throughout the galactose dietary study (about a 15% increase), while control values showed as much as a 40% increase in activity after 21 days.

Rats fed 50% galactose for 3 days and then returned to a normal diet showed increased lens AAT activity on the 3rd and 7th day. By the 18th day on a normal diet, the total AAT activity per rat lens had returned to the control value. When galactose was discontinued after 10 days, AAT lens activities on the 7th and 11th day of normal refeeding of standard diet were the same as that found in lenses from animals fed galactose for the entire study. In both galactose discontinued studies, the percent distribution of the AAT lens isozymes was similar to control values.

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VII. AUTOBIOGRAPHICAL STATEMENT

Donald E. Packer

Born 3/19/35

Married with 2 children

Honorable discharge from USNR (1961)

Born and raised in New York City, I graduated from W. Cullen Bryant H.S., Queens, N.Y. in 1952. After graduating from Oglethorpe University, Atlanta, Ga. in 1956 with a B.S. degree in Biology, I obtained a position with Sloan-Kettering Institute for Cancer Research, first working in the Cancer Chemotherapy Division and subsequently transferring into the Department of Enzymology and Intermediary Metabolism under Dr. Nisselbaum. While working in the latter position, I completed my Masters Degree in Biology at Long Island University as an evening student (1961 - 1964) and was awarded the Phi Sigma award for scholarship. Following enrollment in the Doctoral Program at The City University of New York, I spent the next year and a half in the laboratory of Dr. Samuels at Hunter College investigating muscle enzymes. Subsequent positions included working under Dr. Cavalieri at Sloan-Kettering Institute studying the physical nature of DNA and with Schwartz Bio Research as a research enzymologist. My doctoral research was finalized during the next year and a half on a full time residency basis at Hunter

College. Presently, I am employed by Pfizer and Co. as a staff member of their Diagnostics Research Division where I am working on better and more efficient means by which clinically important enzymes and health states may be evaluated.

Masters Thesis: Kinetics of Inhibition by Oxalate and Oxamate of Lactate Dehydrogenase Variants from Human Heart, Liver and Brain.

Publications:

1. Nisselbaum, J.S., Packer, D.E. and Bodansky, O., 1964, J. Biol. Chem. 239: 2830. Comparison of the Actions of Human Brain, Liver and Heart Lactate Dehydrogenase Variants on Nucleotide Analogues and on Substrate Analogues in the Absence and Presence of Oxalate and Oxamate.

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