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ISOLATION AND CHARACTERIZATION OF ARGINASE ISOZYMES FROM BOVINE LIVER AND BRAIN

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University of New Hampshire PH.D. 1983

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ISOLATION AND CHARACTERIZATION OF ARGINASE ISOZYMES FROM BOVINE LIVER AND BRAIN

BY

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B. A., Mount Holyoke College, 1974

A DISSERTATION

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This dissertation has been examined and approved.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

ISOLATION AND CHARACTERIZATION OF ARGINASE ISOZYMES FROM BOVINE LIVER AND BRAIN

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A chromatographic method was developed to isolate arginase isozymes from bovine brain and liver. The purified proteins were characterized as to their native molecular weight, subunit structure, amino acid composition, hexose content, behavior on disc gel electrophoresis, and reaction with polyclonal antibodies raised against a liver arginase antigen. An arginase-specific messenger RNA preparation was also isolated from liver polysomes using immunological techniques.

Two liver and four brain arginases were purified, having similar native molecular weights, yet different subunit compositions, and displaying varying affinities for the Mn^{2+} ion that is required for complete enzymatic activity. The amino acid compositions of all six proteins are closely related, although there are some noticeable disparities, particularly with respect to alanine.

When analyzed by disc gel electrophoresis, a bovine liver or brain arginase enzyme will appear as a diffuse band. The carbohydrate moiety

of these molecules may be responsible for this pattern, but a variable loss of Mn^{2+} in the electric field also contributes to the diffuseness, since the migration of each isozyme was observed to change if separated by electrophoresis in the presence of added Mn^{2+} or EDTA.

Several distinct antigenic determinants were recognized by anti-liver arginase antibodies. Two of the bovine brain arginase proteins identified in this work share one of these immunological sites with the liver enzyme, while the other two arginases isolated from brain tissue hold a separate determinant in common with it. Bovine liver arginase antibodies were also observed to cross-react with the arginase enzyme in a mouse liver homogenate.

An arginase enriched mRNA preparation was translated in a cell-free system. After immunoprecipitation and SDS electrophoresis, the translated products migrated to the same positions as authentic liver arginase isozymes.

CHAPTER I

INTRODUCTION

Intermediary metabolism represents the interaction of many related enzymatic pathways. Hence one compound may serve as precursor to several very different products, depending on the location of its production within a cell, and on the metabolic needs of that cell. Isozymic forms of an enzyme frequently exist to initiate the appropriate conversions of such a precursor. Functionally similar enzymes may arise during the several stages of cell differentiation (e.g., muscle pyruvate kinase isozymes M1 and M2, Hance et al., 1982); they may be located in discrete cellular compartments (e.g., cytosolic and mitochondrial chicken heart aspartate aminotransferase, Sonderegger et al., 1982); or they may reside in two separate tissues (e.g., liver and salivary gland α-amylase, Schibler et al., 1980). Similar enzymes may be found in more than one species of animal as well (e.g., rat and cow adrenal phenylethanolamine N-methyltransferase, Park et al., 1982). Characteristics which serve to distinguish these molecules from each other may result from post-translational modifications such as glycosylation, sulfation, or phosphorylation of the proteins. Alternatively, structural and/or functional deviations between isozymes may result from differences in the gene, or, theoretically, differences in the processing of the nuclear messenger RNA precursor. Thus, isoenzymes may originate from closely related genes (Schibler et al., 1980; MacDonald et al., 1982); they may exist because

of gene rearrangements accompanying cell differentiation (Early et al., 1980; Kadowaki and Knox, 1982); or as a consequence of diverse processings of a common mRNA precursor (Alt et al., 1980; Young et al., 1981a).

Any successful characterization and comparison of similar enzyme activities found within subcellular fractions, or located within two dissimilar tissues requires the purification of those proteins to homogeneity. However, the very fact that isoenzymes are molecules that differ in some degree can create difficulties when attempting to apply the same isolation protocol to them. For example, acetylcholinesterase occurs in asymmetrical and globular forms in mammalian brain. These forms exhibit well defined and different solubility properties which call for alternate methods of extraction (Grassi et al., 1982). Problems have also been reported during the separation of the two dopamine β-hydroxylases found in adrenal chromaffin granules. Although the only major distinction between the soluble and membrane-bound enzymes resides in a hydrophobic tail located on the latter protein, this difference necessitates the use of different extraction and affinity chromatography schemes (Fischer-Colbrie et al., 1982).

A number of biochemical parameters are usually employed to evaluate various properties of purified isozymes: native molecular size and subunit structure; reactivity with several substrates, cofactors, or inhibitors; pH optimum; relative electrophoretic mobility; carbohydrate composition; solubility properties, and reaction with antibodies. Post-translational modifications of the polypeptide chain are a frequent cause of isozymic divergence in some of these properties. Glycosylation is a prime example of this phenomenon. Because the addition of sugars to

glycoproteins is not under direct genetic control, but is effected by the sequential action of several glycosyltransferase enzymes, heterogeneity often exists in the carbohydrate portion of these proteins, and manifests itself as apparent differences in isozyme charge, mass, or conformation. Thus, when analyzed by polyacrylamide gel electrophoresis (Sidorowicz et al., 1980), sodium dodecyl sulfate (SDS) electrophoresis (Rangel-Aldao et al., 1979), or two dimensional isoelectric focusing (Hsu and Kingsbury, 1982), isozymes may present very dissimilar migration patterns. Removal of the attached group(s) can restore the nascent polypeptide charge, and hence mobility in an electric field. This is not always true, however, (Chu et al., 1978). Leach et al. (1980) studied the gel chromatographic and electrophoretic behavior of glycoproteins in SDS, and could establish no consistent correlation between carbohydrate content and migratory position. It is therefore not easy to obtain reliable estimations of glycoprotein molecular weight in SDS.

Although sugar residues have been shown to influence substrate binding in human alkaline phosphatase (Komoda and Sakagishi, 1976) and γ-glutamyltransferase (Shaw et al., 1980), carbohydrate is not usually required for effective biological activity in glycoprotein enzymes (Chu et al., 1978; Fujisawa et al., 1978; Olden et al., 1982). Instead, the affixed carbohydrate frequently functions to maintain the protein in a stable conformation (Tashiro and Trevithick, 1977; Chu et al., 1978; Gibson et al., 1979). Heterogeneous sugar moieties are not often the cause of immunological differences between isozymes because they are not usually antigenic (Shaw et al., 1980; Sidorowicz et al., 1980; Young et al., 1981b). The enzyme glucoamylase represents an exception, however,

since Pazur et al. (1981) produced antibodies directed against the single oligosaccharide chain instead of the polypeptide chain of this protein. Park et al. (1982) have also suggested that some of the unique determinants located on bovine adrenal phenylethanolamine N-methyltransferase occur because of the appended carbohydrate.

Alternative polypeptide compositions, with the attendant possibilities for changes in the secondary, tertiary, and quaternary structures of the proteins, contribute more conclusively to distinct molecular properties between isozymes. Although the two types of chicken heart aspartate aminotransferase have similar molecular weights, their amino acid sequence homology is low (46%), and their isoelectric points differ markedly (pH 9.0 vs pH 6.7). This charge difference is thought to be important for the selective integration of the mitochondrial enzyme into the mitochondria. Sonderegger et al. (1982) and Sannia et al. (1982) have further demonstrated that the mitochondrial , but not the cytosolic, isozyme is first synthesized as a higher molecular weight precursor.

Intracellular serine hydroxymethyltransferase proteins compose another pair of isozymes differing in molecular weight, isoelectric point, stability, and reactivity toward a secondary substrate (L-allothreonine) (Ogawa and Fujioka, 1981).

Isoproteins isolated from several tissues display distinctive properties also. Pancreatic and parotid α-amylase show marked differences in electrophoretic mobility, isoelectric point, and reaction with antibodies. Such a result is attributed to variations in the amino acid compositions of the two proteins (Sanders and Rutter, 1972).

Fully active testicular dipeptidyl carboxypeptidase is only two-thirds as large as the pulmonary enzyme, yet it is catalytically and immunologically similar to it. In addition, synthesis of the testicular isozyme is developmentally controlled since its activity is observed to rise sharply at puberty in contrast to the steady production of pulmonary activity. El-Dorry et al. (1982) employed anti-pulmonary-dipeptidyl carboxypeptidase antibodies against in vitro synthesized proteins from both organs to demonstrate that the individual sizes and regulatory properties of the two isozymes are set at the pretranslational level. These two functionally alike molecules are either, as Young et al. (1981a) have suggested for mouse liver and salivary gland α-amylases, the result of tissue-specific differential transcription of a single gene, or else they are the products of separate genes. Gene duplication need not, however, promote an equal divergence of all of the molecular characteristics of the resulting isozymes. Alcohol dehydrogenase proteins show disparate kinetic properties (Holmes et al., 1981), yet they produce similar immunological responses (Talbot et al., 1981).

After the existence of multiple isoenzymes within one or more tissues has been established, and their traits have been investigated, the question of their physiological role(s) may be addressed. Two distinct forms of β-mannosidase have been isolated from goat liver, differing in their pH optima, substrate specificity, and carbohydrate content (Dawson, 1982). The function of the more active acidic isozyme in lysosomes is certain, but the role of the neutral form has not, as yet, been determined. Glutathione S-transferase proteins have been divided into two groups of related macromolecules on the bases of dissimilar kinetic

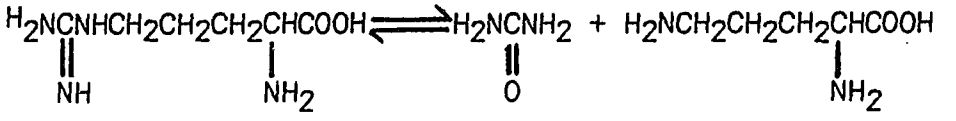
and immunological properties (Mannervik and Jensson, 1982). These enzymes are normally classified as detoxification proteins, but Pattinson (1981) has suggested that they may serve as intercellular transport proteins as well. Arginase (L-arginine amidinohydrolase, E.C. 3.5.3.1) is another biological catalyst which has been assigned several roles. Krebs and Henseleit (1932) originally documented its participation in liver ammonia detoxification via the urea cycle. Since then, arginase activity has been linked, at times, to the synthesis of polyamines, and/or the amino acids proline and glutamic acid (Yip and Knox, 1972; Oka and Perry, 1974; Verma and Boutwell, 1981). Terayama et al. (1982) have recently demonstrated that the arginase in rat liver plasma membranes arrests the growth of mammalian cells in culture.

Because arginase partakes in such a wide range of pathways, isozymic forms of it probably exist, each displaying specialized molecular properties (Kaysen and Strecker, 1973; Farron, 1973; Glass and Knox, 1973). Affinity for substrate and native molecular weight are features that commonly distinguish these several types of enzymes.

Arginase is found primarily in the mammalian liver, although significant amounts are present in numerous other organs which lack a complete urea cycle such as kidney, mammary gland, thyroid, intestine, pancreas, submaxillary gland, epidermis, and brain. This protein is not confined to mammalian sources, moreover, but has been located in non-ureotelic liver (Mora et al., 1965b), ammoniotelic liver (Peiser and Balinsky, 1982), earthworm gut (Reddy and Campbell, 1968), and plant tissue (Legaz and Vicente, 1982) as well.

Since its discovery eighty years ago (Kossel and Dakin, 1904a and

1904b), ureotelic arginase has been studied extensively. It catalyzes the reaction:



L-arginine urea L-ornithine

Arginase is very specific for its substrate because replacement of either the free guanidino, or the free carboxyl group of arginine results in a noticeable loss of activity (Greenberg, 1960). Furthermore, the length of the carbon chain is critical. Decreasing its size to four carbon atoms (γ-guanidinobutyric acid), or increasing it to six carbons (ε-guanidinocaproic acid) prevents the production of urea. Substitution of a carbon by an oxygen atom as in canavanine (α-amino-γ-guanidinoxy-n-butyric acid), on the other hand, does not inhibit enzymatic action entirely. Gross (1921) first observed inhibition of arginase by its end-product ornithine. Hunter and Downs (1945) examined arginase inhibition by amino acids, and found ornithine and lysine to be competitive inhibitors, while glycine, alanine, α-aminobutyric acid, and norvaline were increasingly more effective noncompetitive inhibitors. Greenberg (1960) suggested that citrate buffers could repress arginase activity by chelating the activating ion required by arginase (see below). Although Archibald (1945) noted the ability of borate solutions to adversely influence the hydrolysis of arginine by arginase, and Greenberg (1960) assumed that this phenomenon was related to the carbohydrate component of the molecule, it was only recently that Pace and Landers (1981) proved that this inhibition was noncompetitive.

Mammalian arginase is an oligomeric protein (Hirsch-Kolb and Green-

berg, 1968; Sakai and Murachi, 1969; Vielle-Breitburd and Orth, 1972) which requires activation by a metal ion for complete catalytic activity (Hirsch-Kolb et al., 1970 and 1971). While manganese (Mn^{2+}) is the best metal ion for activating arginase, nickel and cobalt ions have been shown occasionally to enhance (or inhibit) arginase action (Mohamed and Greenberg, 1945; Campbell, 1966; Hirsch-Kolb and Greenberg, 1968; Hirsch et al., 1970). Mohamed and Greenberg (1945) proposed that arginase activation resulted from the reversible conversion of an inactive 'pro-arginase' protein to an active arginase molecule according to the equation:

$$\text{proarginase} + \text{metal}^{2+} \rightleftharpoons \text{arginase - metal}$$

Greenberg (1960) suggested that the cofactor binding site becomes accessible to the activating metal only after a structural rearrangement of the enzyme. Using starch gel electrophoresis, Campbell (1966) obtained evidence for such a transformation of rat liver arginase.

Arginase activation occurs very slowly at room temperature. Archibald (1945) reported that arginase activity could be increased by adding some manganous chloride to a liver mash, and then heating it. Activation of arginase by Mn^{2+} is therefore dependent on temperature, and is optimal at 55°C (Schimke, 1962). Hirsch-Kolb et al. (1971) conducted a series of nuclear magnetic resonance (NMR) studies on Mn^{2+} binding to rat liver arginase, and determined that four moles of metal were bound per mole of fully activated protein. Two moles of Mn^{2+} were easily removed and restored without an irretrievable loss of arginase activity, but withdrawal of the second two ions completely inactivated the enzyme. Because the reactivation by Mn^{2+} was discovered to be pH independent, between pH

7-9, the authors concluded that the metal did not bind to ligands on the surface of the protein, but instead it nestled into a pocket of the molecule. They further theorized that the weak and tight Mn^{2+} binding sites were not fixed, but that after the two easily dissociated ions left the metal - protein complex, the conformation of the enzyme changed to facilitate a more secure binding of the remaining ions. Hence, the rate-limiting step in Mn^{2+} activation was postulated to be a reversal of this structural modification so as to allow all of the ions required for complete activity to return. Raising the temperature would increase the rate of this reorganization, and would explain the observed temperature dependence of the activation process.

A study of rabbit liver arginase (Vielle-Breitburd and Orth, 1972) presented a contrasting scenario, however. Here the catalytic efficiency of the purified enzyme was only marginally enhanced by incubation with Mn^{2+} at 55°C, implying a firm retention of the metal throughout the isolation scheme. Inactivation, resulting from chelation of the metal ions, proceeded very slowly, and was completely reversed by the addition of Mn^{2+} to the preparation without a preincubation period. The authors concluded that changes in protein conformation that occurred as a consequence of activation were minimal because antibodies raised to the native oligomer recognized the ethylenediaminetetraacetic acid (EDTA)-inactivated form equally well. The activation process for liver arginase, therefore, appears to be dependent upon its animal source.

The question of the possible catalytic activity of the arginase monomer has been addressed, and has been recently resolved. Sorof and Kish (1969) used gel filtration to separate soluble rat liver proteins.

Without a prior metal activation of the resulting fractions, most of the arginase activity eluted at a molecular weight of 120,000; when Mn^{2+} was employed as activator, however, a second peak of enzyme activity (assumed to be the arginase monomer) of a molecular weight of 30,000-40,000 appeared. The authors could not establish whether this smaller species of arginase bound Mn^{2+}, or whether it associated in the presence of Mn^{2+} to a catalytically active oligomeric form.

Barańczyk-Kuźma *et al.* (1976) also reported the separation of rat liver arginase into two forms on a Sephadex G-150 column. Monomeric arginase was active after Mn^{2+} had been added to it. The tetramer did not need added Mn^{2+} for its function. The completely inactivated (after EDTA treatment) enzyme moved more rapidly toward the anode on polyacrylamide gel electrophoresis than did the native protein, and it exhibited catalytic activity only with Mn^{2+} present. When the inactive subunit was renatured with Mn^{2+} and reanalyzed by electrophoresis, it migrated to the same position as the oligomer. These results indicate that the EDTA caused a dissociation of the enzyme into inactive subunits which recombined to the active, native molecule when cofactor was added. Vielle-Breitburd and Orth (1972) provided further evidence that formation of the oligomer required Mn^{2+}. However, they reported the additional fact that after dissociation of rabbit liver arginase at low pH or in SDS and 2-mercaptoethanol, and reactivation with Mn^{2+} there existed an enzymatically active molecule which possessed a smaller sedimentation coefficient than that of the native protein. The arginase subunit was therefore postulated to be active upon incubation with its cofactor, Mn^{2+}. Aguirre and Kasche (1983) immobilized rat liver arginase monomers by

coupling them to Sepharose beads, and determined their reactivation properties. The monomer was again seen to catalyze the hydrolysis of arginine after renaturation with Mn^{2+}. The tertiary structure of a single subunit was concluded to be sufficient for the expression of enzymatic activity in oligomeric enzymes, such as rat liver arginase, which appear to be composed of identical monomers. The quaternary organization of arginase was suggested to participate in the regulation of its activity, and to provide a favorable conformation for stable Mn^{2+} binding. Caravajal et al. (1977 and 1978) reported similar results for human liver arginase.

Campbell (1966) remarked on the apparent differences in properties (i.e. stability, specific activity, and electrophoretic mobility) between various types of liver arginase, and postulated that an incomplete saturation of the enzyme with Mn^{2+} might produce several 'arginases', displaying distinctive qualities, while they were in reality distinguishable only by an abnormal metal content. Boutin (1982) described such a phenomenon as it affected the characterization of the arginase protein isolated from iris bulbs. The purified preparation was separated by electrophoresis on a polyacrylamide gradient gel, and sections of the gel were tested thereafter for hydrolytic efficiency. When assayed without Mn^{2+}, only a single band of protein (corresponding to the native enzyme) exhibited arginase activity; several bands were active if cofactor activation was first employed. Boutin (1982) proposed that oligomeric arginase was disassembled during electrophoresis when it lost Mn^{2+} in the electric field. Incubation of the sample at the conclusion of the electrophoretic run with Mn^{2+} allowed the dissociated

subunits to regain catalytic activity by regaining their missing metal.

These type of results emphasize the need for safeguards during purification and characterization of the enzyme(s) against the potential production of artifactual molecules. Previous reports of the arginase enzymes from pig liver, rabbit liver, and human liver have suggested that the multiplicity of arginase-like proteins observed during their isolation could arise from aggregation of the native molecule which would affect its hydrodynamic properties (Sakai and Murachi, 1969), or from oxidation of the normal subunits which would alter their charge characteristics (Sakai and Murachi, 1969; Vielle-Breitburd and Orth, 1972; Berüter et al., 1978). Rat liver arginase is an excellent example of a macromolecule which, because of seemingly minor modifications in the methods utilized to purify it, presents itself either as one protein (Schimke, 1964; Hirsch-Kolb and Greenberg, 1968; Hirsch-Kolb et al., 1970; Herzfeld and Raper, 1976; Peiser and Ballnsky, 1982), or two (Gasiorowska et al., 1970; Porembska et al., 1971; Porembska, 1973; Kaysen and Strecker, 1973; Cheung and Raijman, 1981).

Hirsch-Kolb et al. (1970) compared the physico-chemical properties of liver arginase proteins from several ureotelic species. Mn^{2+} was universally essential for complete catalytic activity, while the molecular weights, Michaelis-Menton constants, and pH optima of all of the proteins that were studied were also similar. Nevertheless, these arginases were divided into two groups on the basis of their variable avidities for Mn^{2+}, and their discrete charge characteristics. Thus, dialysis for one day easily removed a substantial portion of the Mn^{2+} bound to beef, monkey, and rabbit arginases. Prolonged dialysis produced

a permanent inactivation of these proteins. Mouse and rat arginases, in contrast, lost Mn^{2+} much less easily, and the resulting decrease in enzyme activity could be restored almost entirely by incubation with Mn^{2+}. Basic arginases bound to CM-cellulose at pH 7.5, while neutral or slightly acidic proteins would not. The latter enzymes (from beef, rabbit, and monkey liver) were found to be less stable during purification and analysis by isoelectric focusing. This instability was attributed to the looser association between Mn^{2+} and protein in the native enzyme-metal complex.

The behavior of arginases on the anion exchanger DEAE-cellulose has also been investigated (Porembska et al., 1971). Two isozymes were resolved from liver and kidney tissues from several species only if the pH was maintained at 8.3. At other pH values, no arginase activity would bind, and instead, the isozymes would co-elute in the void volume peak. The proportion of the activity in these two forms varied between species. Most of the arginase activity in the livers of man, cat, dog, rabbit, and rat did not adhere to the column (isoenzyme A_1), while a large amount of pig, calf, horse, and ox arginase activity was retained (isoenzyme A_3). The two arginase isozymes from each animal in this study probably were not produced artifactually since no interconversion of the two forms was observed, that is when one of them (A_1) was rechromatographed, a single peak of enzyme was eluted from the column at its original position.

The interaction of a protein with an ion-exchange column is a reflection of its molecular charge. The charge of an enzyme also influences its mobility during disc gel electrophoresis. Basic arginases from

rat (Schimke, 1964; Hirsch-Kolb and Greenberg, 1968) and mouse (Stewart and Caron, 1977) liver have been shown to move toward the cathode. Neutral enzymes from pig (Sakai and Murachi, 1969), rabbit (Vielle-Breitburd and Orth, 1972), beef (Harell and Sokolovsky, 1972), and monkey (Terasaki et al., 1980) were shown to migrate to the anode. Determination of the isoelectric point (pI) of several mammalian arginases confirmed their division into two discrete classes of protein. The pI of the basic rat and dog arginase enzymes is 9.4. Neutral (acidic) arginase proteins have a pI ranging from pH 5.9 (beef) to pH 7.5 (monkey).

Separation of arginases into two classes on the basis of their antigenic properties is not as easily accomplished. Sheep anti-rabbit arginase antibodies partially cross-react with the rat enzyme (Vielle-Breitburd and Orth, 1972), while rabbit anti-rat arginase antiserum reacts with the arginase protein from man, dog, monkey, cow, cat, pig, and ox (Mora et al., 1965b), and rabbit anti-human arginase antibodies precipitate monkey arginase (Terasaki et al., 1980). Alterations in the primordial protein which have modified the charge characteristics of its descendants have therefore not affected its antigenic determinants as significantly. This observation of the cross-reactivity of arginases between species is comparable to studies of the antigenicity of myoglobin, a protein in which all of the antigenic determinants are known to reside in a continuous, conformationally distinct, portion of its surface. The antibodies to sperm-whale myoglobin produced in six different animals will recognize only these sites, and will cross-react and autoreact equally well with myoglobins from many sources (Twining et al., 1980).

Subcellular fractionation by centrifugation in sucrose solutions demonstrates that arginase activity is distributed between nucleus (40%), cytosol (4-7%), microsomes (28-37%), and mitochondria (19-24%) (Mora et al., 1965a; Gasiorowska et al., 1970). Repeated washing with salt has been shown to release 70% of the activity originally associated with microsomes (Mora et al., 1965a), and 90% of the initial mitochondrial activity (Cheung and Raijman, 1981). This would explain the reports (Rosenthal et al., 1956; Schimke, 1962; Porembska et al., 1971) that most of the arginase of rat liver tissue is soluble upon homogenization in electrolytic buffer. Crude mitochondrial and microsomal arginase preparations (prior to salt treatment) are distinguishable by their differential activation by Mn^{2+}: the former fraction is unaffected by preincubation with cofactor, while the activity of the latter preparation increases 60% (Cheung and Raijman, 1981). Both enzymes are more active at pH 9.5 than at pH 7.4. The arginase proteins which remain firmly bound to organelles even after several salt rinses are considered to perform a necessary cellular function either in the excretion of urea (microsomal arginase), or in the transport of ornithine into mitochondria (mitochondrial arginase).

Relatively little is known about arginase proteins in other than liver tissue, and the detailed studies that do exist have dwelt almost exclusively on rat and mouse organs. There is much more arginase activity in liver tissue as compared to other tissues. For example, rat brain (Gasiorowska et al., 1970; Herzfeld and Raper, 1976), mouse brain (Stewart and Caron, 1977), and rat thyroid (Matsuzaki et al., 1981) have one thousand times less arginase activity than the liver, whereas lac-

tating rat mammary gland (Yip and Knox, 1972) has one-fourth of the liver activity. Arginase enzymes from other organs share some general molecular properties with the liver protein. For example, kidney, brain, and submaxillary gland arginases have been shown to be of the same molecular weight as the liver enzyme (Gasiorowska et al., 1970). Rat mammary gland (Glass and Knox, 1973) and rat thyroid (Matsuzaki et al., 1981) arginases require heat activation with Mn^{2+}, while, on the other hand, this procedure is not essential for rat kidney arginase activity (Kaysen and Strecker, 1973). The activity of particulate, but not soluble, mouse epidermal arginase rises by 50% following incubation with Mn^{2+} at 55°C (Verma and Boutwell, 1981).

The fundamentally different nature of the liver proteins becomes apparent when the analysis is on a more sophisticated basis. The two kidney arginase isozymes in man, cat, rat, rabbit, horse, ox, calf, and pig can be separated from the comparable liver proteins by chromatography on DEAE-cellulose at pH 8.3 (Porembska et al., 1971). Most (88-97%) of the kidney arginase activity (in contrast to the liver activity) was retained on the column, and was reported to adhere strongly to subcellular fractions as well. The (minor A_3) liver and (major A_4) kidney isozymes which bound to the anion exchanger were different proteins because they were eluted by different conditions of salt.

Arginase from four rat tissues (liver, kidney, submaxillary gland, and brain), when fractionated on DEAE-cellulose, always presented itself as two peaks of activity (Gasiorowska et al., 1970). Peak II (retarded by the DEAE-cellulose) lost much of its activity during storage for several days at -10°C, whereas the activity in the void volume peak

(peak I) was quite stable to this treatment. The K_m value determined for all of these crude preparations was the same. The electrophoretic mobilities and antigenic properties of arginase proteins from rat liver and submaxillary gland were alike, and distinguished these enzymes from the arginases in rat kidney, intestine, pancreas, and mammary gland (Kaysen and Strecker, 1973; Glass and Knox, 1973; Reddi et al., 1975; Herzfeld and Raper, 1976).

Data such as the above have provided a molecular foundation for the hypothesis that the arginase activity in organs without a true urea cycle is furnished by isozymes of arginase which are likely to participate in a metabolic pathway other than ammonia detoxification. The probability of a second role for arginase is well illustrated by its presence in the brain, a tissue where ammonia is removed largely as glutamine (Berl et al., 1962). Sporn et al. (1959) initially offered evidence of arginase action in this organ by observing the synthesis of urea from radioactive arginine injected into living rat brains. Ratner et al. (1960) tested homogenates directly, and confirmed the existence of arginase protein in the brains of rat, steer, monkey, man, and guinea pig. The activity of the enzyme varied among the animals examined: the specific activity of the rat preparation was five times greater than that of the steer. Homogenates assayed without exogenously added Mn^{2+} expressed 5% of the full activity determined after preincubation with this metal. Rat brain arginase was ten times less active at pH 7.5 than at pH 9.5.

Arginase activity is not distributed uniformly throughout rat brain: cerebellum has twice the activity of cerebral cortex (Sadasivudu and

Rao, 1976). Its assignment in cerebellum may be to furnish ornithine as the precursor to glutamic acid which is needed for controlled brain function.

Differential centrifugation in sucrose showed that most of the ox (Gasiorowska et al., 1969) and rat (Gasiorowska et al., 1970) brain arginase activity is associated with mitochondria. DEAE-cellulose chromatography at pH 8.3 permitted the separation of the two forms of arginase in these brains. Those isozymes that bound to the DEAE-cellulose did so more firmly than the corresponding rat liver protein, and therefore were probably different from it. The two semi-purified ox brain arginases had the same K_m value, but different stabilities (the void volume peak which represented 30% of the recovered activity was inactivated by heat much faster than the other, retained, isozyme). Stewart and Caron (1977) reported that mouse brain contains two arginases which are very stable to heat treatment, and have similar Michaelis-Menton constants for arginine. These enzymes are unlike the single mouse liver arginase protein.

Arginase enzymes from brain tissue have not, as yet, been completely purified and characterized. The investigation detailed herein was undertaken to accomplish this for the arginases in bovine brain, and to compare some of their molecular properties with those of bovine liver proteins isolated concurrently. Preliminary evidence indicates that bovine brain and liver arginases are different molecules (Stewart and Caron, 1977). A homogeneous bovine liver arginase has been analyzed (Harell and Sokolovsky, 1972; Kuchel et al., 1975). This protein is similar to the well studied rat enzyme (Schimke, 1964; Hirsch-Kolb and

Greenberg, 1968) in molecular weight, Svedberg constant, partial specific volume, Mn^{2+} composition when fully activated, and hexose content. It differs from the rat liver arginase in electrophoretic mobility on polyacrylamide gels, isoelectric point, specific activity, and stability after prolonged dialysis.

The latter stages of the purification protocol used by Harell and Sokolovsky (1972) could not be reproduced satisfactorily in the current study, and instead another purification method was developed which yielded two different homogeneous liver isozymes and four distinct brain arginases. These enzymes were named on the basis of their behavior during the isolation scheme: (L) denoted a liver arginase and (B) a brain arginase; 1 or 2 designated nonadherence or adherence, respectively, to a DEAE-cellulose column; A or B described the order of elution from a column of Octyl Sepharose. Thus, for example, B1A was the brain isozyme which came through in the void volume peak of a DEAE-cellulose column and eluted first from an Octyl Sepharose column. Several physico-chemical properties of these bovine arginase proteins were determined, including native molecular weight, subunit size, affinity for Mn^{2+}, electrophoretic mobility, and pH optimum. The liver protein L1B, which corresponded to the arginase described by Harell and Sokolovsky (1972), was used to produce antibodies in rabbits, and immunological similarities between the purified liver and brain arginase isozymes were ascertained. The final step in the study consisted of the isolation of the liver arginase messenger RNA from polysomes by immunological techniques.

CHAPTER II

MATERIALS AND METHODS

Materials

Bovine livers were obtained from the J.T. Trelagan Co., Cambridge, Massachusetts. The fresh tissue was frozen and stored at -20°C.

Mature bovine brains were obtained from Pel-Freez Biologicals, Rogers, Arkansas and stored at -20°C.

New Zealand White rabbits were purchased from Locke Hill Farms, Barrington, New Hampshire. Food (Agway, Inc.) and water were provided *ad libitum*.

All chemicals were reagent grade or the best grade commercially available. All buffers were prepared with water purified by reverse osmosis, deionization and filter sterilization (Milli Q, Millipore Corp., Bedford, Massachusetts).

Arginase Assay

The method of Rüegg and Russell (1980) was employed to assay for arginase activity. Dowex 50W-X8 (200-400 mesh, H+ form) was purchased from Bio-Rad Laboratories, Richmond, California. It was washed with 1 M HCl (5 x wet weight of resin), filtered through a Buchner funnel to remove the acid, and then washed extensively with water. The resin was stored as a 1:1 (w/v) suspension in water. Substrate, L-(guanido-^{14}C) arginine was obtained from New England Nuclear, Boston, Massachusetts,

and stored at -20°C. It was purified by placing a 10 µCi aliquot on a 1.0 ml column of Dowex (see above), followed by 6 ml of water, and then 6 ml of 2 M HCl. The (^{14}C) arginine was eluted with 12 ml of 6 M HCl, then dried on a rotary evaporator, and the residue dissolved in 1 M arginine (Baker) (which had been adjusted to pH 9.7 with 6 M HCl) such that 25 µl of this solution contained approximately 40,000 dpm. Buffer 1 was composed of 750 mM glycine, 0.02% thymol blue, adjusted to pH 9.7 with 4 M NaOH. Buffer 2 consisted of 250 mM acetic acid, 7 M urea, 10 mM arginine, 0.001% methyl red adjusted to pH 4.5 with 4 M NaOH. The solution used to dilute the enzyme to an appropriate concentration was 10 mM Tris-HCl, 10 mM $MnCl_2$, 2 mg/ml BSA, pH 7.4.

Each reaction mixture contained the following components in a final volume of 200 µl: 25 µl buffer 1; 25 µl (^{14}C) arginine (see above); 150 µl water. Liver and/or brain preparations or dilutions thereof were activated at 55°C for ten min. After a ten min preincubation of the reaction mixture at 37°C, 50 µl of the activated enzyme solution was added to it. Control values were obtained by using 50 µl of the enzyme dilution buffer in place of the protein. The initial concentrations the components were: 100 mM arginine; 40,000 dpm (^{14}C) arginine; 75 mM glycine; 2 mM $MnCl_2$; 0.002% thymol blue, pH 9.7.

The reaction was allowed to proceed for 30 min at 37°C in a shaking water bath, and it was terminated by the addition of 0.75 ml of buffer 2. A 0.4 ml portion of the Dowex resin suspension (see above), which was rapidly stirred during pipetting, was added to each tube. The capped tubes were mixed on a Vortex-Genie mixer and centrifuged at 1600 rpm for 1 min. An aliquot (0.7 ml) of the resin supernatant was placed in a

scintillation vial containing 10 ml of either Scinti-Verse II™ (Fisher) or Scint A™ (Packard) scintillation cocktail. Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter, and, using the external standard method, the cpm values were converted to dpm values.

Units of arginase (a unit is defined as the amount of enzyme that produces 1 µmole urea/min at 37°C) were obtained from the equation:

$$m \times \frac{2(b-c)}{a \times t}$$

where m = 25 µmole of arginine

a = total dpm of (^{14}C) arginine in each assay mix

b = dpm in 0.7 ml of resin supernatant of test sample

c = dpm in 0.7 ml of resin supernatant of control

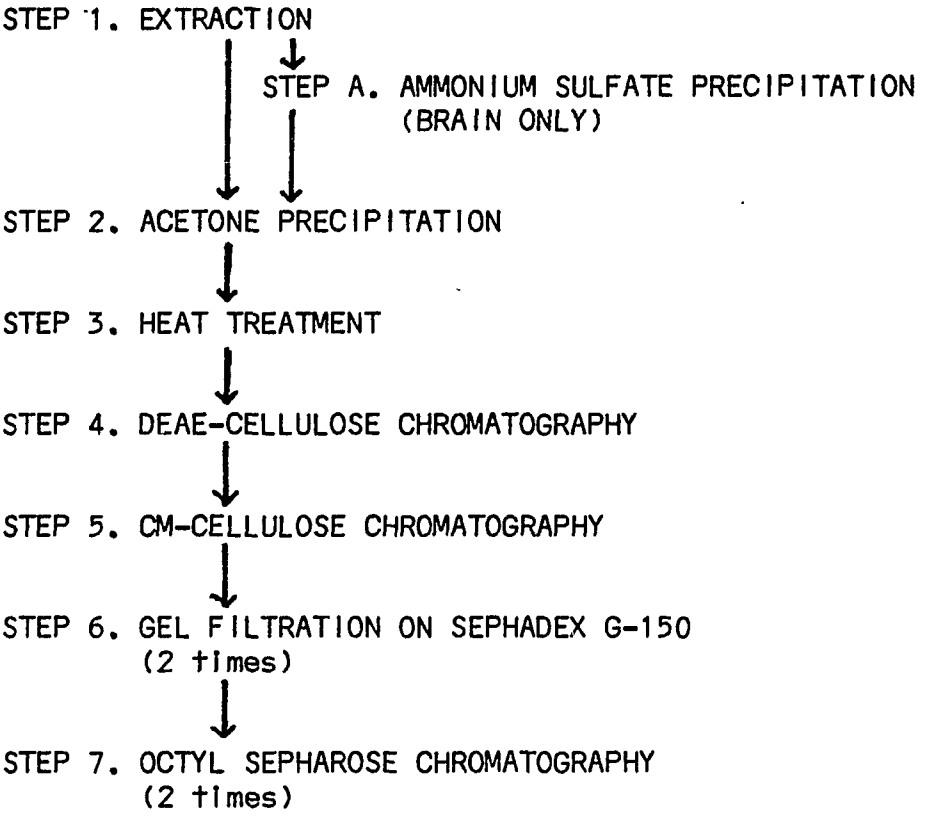
t = time of incubation (min)

Protein concentration was measured by the method of Warburg and Christian (1942) or Bradford (1976).

Purification of Liver and Brain Arginases

All purification procedures were carried out at 4°C unless otherwise noted. A protein determination and an arginase assay were performed at each stage of the protocol with the exception of the ammonium sulfate and acetone precipitation steps. The purification procedure is diagrammed in Fig. 1. The early stages of the isolation of arginase from liver tissue (steps 1-4) were patterned after the method of Harell and Sokolovsky (1972).

Fig. 1. Scheme for purification of bovine liver and brain arginases


STEP 1. EXTRACTION
STEP A. AMMONIUM SULFATE PRECIPITATION
(BRAIN ONLY)
STEP 2. ACETONE PRECIPITATION
STEP 3. HEAT TREATMENT
STEP 4. DEAE-CELLULOSE CHROMATOGRAPHY
STEP 5. CM-CELLULOSE CHROMATOGRAPHY
STEP 6. GEL FILTRATION ON SEPHADEX G-150
(2 times)
STEP 7. OCTYL SEPHAROSE CHROMATOGRAPHY
(2 times)

Step 1. Extraction of Starting Material

Portions of bovine liver or brain were partially thawed, cut into small pieces, and homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, New York) in 0.01 M Tris-HCl + 0.1 M KCl, pH 7.5 containing 0.05 M $MnCl_2$ (3 ml/g wet weight). The homogenate was centrifuged at 13,200 x g for 15 min in a Sorvall RC2-B centrifuge. Lipid material was removed from the supernatant by filtration through glass wool, and the pH of the solution adjusted to 7.5 with 1 M NaOH.

Step A. Ammonium Sulfate Precipitation-Brain Only

The brain extract was made 45% saturated in ammonium sulfate (Baker). It was then refrigerated for 20-25 min prior to centrifugation at 13,200 x g for 15 min. The supernatant was made 70% saturated in ammonium sulfate, stirred for 20 min, and centrifuged as above. The pellet was dissolved in 0.01 M Tris-HCl, pH 7.5 + 0.001 M $MnCl_2$ (10% of the original volume) and dialyzed against 1 liter of this buffer with 3 buffer changes.

Step 2. Precipitation with Acetone

The liver extract (step 1) or the dialyzed product of the ammonium sulfate precipitation of the brain extract (step A) was mixed with 1.5 vol. of -20°C acetone. The acetone was added slowly with constant stirring so that the temperature did not exceed 10-12°C. The mixture was stirred for 10 min at 6-8°C, and then centrifuged at 13,200 x g for 15 min. The pellet was homogenized in 0.01 M Tris-HCl, pH 7.5, containing

0.001 M $MnCl_2$ on a Polytron homogenizer (the volume corresponded to 0.5 vol of the dialyzed ammonium sulfate solution in the case of the brain preparation, and it equaled 0.7 vol of the original weight of the tissue in the case of the liver preparation). Any undissolved material was removed by centrifugation (13,200 x g, 10 min). The supernatant was dialyzed against 0.01 M Tris-HCl, pH 7.5 (10-15 vols) with 2 changes of buffer to remove traces of acetone. The pH of the dialyzed preparation was adjusted to pH 7.5 as needed.

Step 3. Heat Treatment

The solution obtained from step 2 was heated for 20 min in a 60°C water bath. It was then cooled in an ice bath with constant stirring to 4°C, and centrifuged for 10 min at 13,200 x g to remove the precipitated material. The supernatant was concentrated to approximately 10 ml (brain), or 25 ml (liver) under N_2 (30 psi) using a PM-30 Diaflow pressure ultrafiltration membrane (Amicon), and frozen at -20°C.

Step 4. Chromatography on DEAE-Cellulose

A. Liver. 25 ml portions of the material from the preceding step were thawed, dialyzed against 1 liter of 0.05 M Tris-HCl, pH 7.8 with 1 change of buffer, and applied to a 3 x 53 cm DEAE-cellulose column (Schleicher & Schuell, DEAE-20) which had been equilibrated with the above dialysis buffer. A pump was used to maintain a flow rate of 25 ml/hr during sample application and elution. 10 ml fractions were collected, and those containing enzyme activity were pooled (Pool 1) and frozen at -20°C.

b. Brain. 100 ml aliquots of the preparation obtained in step 3 were thawed, and loaded onto a 3 x 50 cm DEAE-cellulose column, pre-equilibrated with 0.01 M Tris-HCl, pH 7.5. Flow rate and fraction size were the same as for the liver samples. The column was washed with equilibration buffer until the absorbance at 280 nm decreased to 0.1, at which time a gradient elution system was initiated consisting of:

300 ml 0.01 M Tris-HCl, pH 7.5

+ 300 ml 0.01 M Tris-HCl + 0.15 M KCl, pH 7.5

The pool of active enzyme which was eluted with the equilibration buffer (Pool 1) was concentrated to 20 ml, and frozen at -20°C. Enzyme fractions eluted with the salt gradient were pooled (Pool 2), dialyzed against 2 liters of equilibration buffer with 1 change, concentrated to 10-15 ml, and frozen.

Step 5. Chromatography on CM-Cellulose

Portions of material from step 4 (100 ml for the brain preparations and 130-140 ml of the liver solution) were thawed, and dialyzed against 1.5 liters of 0.01 M Tris-HCl, pH 7.0 (2 changes). The pH of the final dialyzed solution was adjusted to 7.0, and the sample was centrifuged at 17,300 x g for 15 min. The supernatant was applied to a 3 x 53 cm column of CM-cellulose (Whatman, CF11) which had been equilibrated with 0.01 M Tris-HCl, pH 7.0. A flow rate of 25 ml/hr was kept constant with a pump, and 10 ml fractions were collected. Enzymatically active fractions, eluted with the equilibration buffer, were concentrated to 5-10 ml, and frozen at -20°C. Contaminating proteins which bound to the column were removed with a buffer consisting of 0.01 M Tris-HCl

+ 0.5 M KCl, pH 7.0.

Step 6. Gel Filtration on Sephadex G-150

The enzyme solution from the last procedure was thawed and made 0.05 M in NaCl by the addition of solid NaCl (Baker). It was then loaded onto a 3 x 54 cm Sephadex G-150 column (Pharmacia) in 0.01 M Tris-HCl + 0.05 M NaCl, pH 7.5. 3 ml aliquots were collected at a flow rate of 18 ml/hr. Fractions containing enzyme were pooled, concentrated to 5-6 ml, and reapplied to the Sephadex G-150 column. Active fractions from this second run were also pooled, and concentrated to 5-6 ml before being dialyzed against 1 liter of 10 mM potassium phosphate + 0.2 mM dithiothreitol (DTT), pH 7.0 (buffer A) with 2 changes of dialysis buffer.

Step 7. Hydrophobic Chromatography on Octyl Sepharose

A 0.7 x 13 cm column of Octyl Sepharose (Pharmacia) was equilibrated with 10 mM potassium phosphate + 0.2 mM DTT, pH 7.0 which was 40% saturated with ammonium sulfate at 20°C (buffer B). The dialyzed G-150 sample was made 40% saturated in ammonium sulfate by addition of the solid, and the resulting solution was centrifuged at 17,300 x g for 10 min to remove any undissolved material. The supernatant was placed on the column at a flow rate of 25 ml/hr, and 3 ml fractions were collected. The column was washed with buffer B until the absorbance at 280 nm fell to 0.1. A linear gradient elution system was then initiated which consisted of:

6 column vol buffer B

+ 6 column vol buffer C

Buffer C was composed of 80% ethylene glycol in buffer A. Finally the column was washed with 3-4 column vol of buffer C. Gradient composition was determined by measuring the conductivity of every 10th fraction on a YSI Model 31 conductivity bridge, and comparing their values to those of a set of solutions of known composition. Fractions having enzyme activity were pooled, and dialyzed against 2 liters of buffer A for 9-10 hr with 2 buffer changes. The pools were subsequently concentrated to 3-6 ml, reapplied to a (new) 0.7 x 6.5 cm Octyl Sepharose column, and eluted as above. The active fractions from this second run were dialyzed against buffer A for 9 hr, followed by concentration to 3-5 ml. The pools were then assayed for enzyme activity and protein content before being frozen at -20°C.

Characterization of the Enzymes

A. Disc Gel Electrophoresis

Disc gel electrophoresis of the liver and brain arginase preparations after Octyl Sepharose chromatography was performed according to the method of Davis (1964) on 5% acrylamide gels. The standard anodic gel system that stacks at pH 8.9 and runs at pH 9.5 was used. Separating gels were initially run for at least one hour in a continuous buffer system (a 1:8 dilution of Tris-HCl buffer) to eliminate oxidative contaminants of gel polymerization. Tubes of 13 cm in length and 5 mm in inside diameter were used, and these contained 4.7

cm of separating gel and 0.9 cm of stacking gel.

Duplicate gels of L1B and B1B arginase were run at a constant current of 4 mA/gel. Approximately 30 µg of protein was loaded onto each gel. The migration pattern of arginase was determined by staining one of each pair of gels for protein with 0.5% Buffalo Black NBR, and by enzyme assay of slices from the other unstained gel. After electrophoresis these latter gels were cut into 2 mm slices, each slice was placed in a capped plastic tube holding 0.2 ml of the enzyme assay mixture, crushed into small pieces with a glass rod, and incubated at 37°C for 30 min (liver) or 240 min (brain). Samples were processed thereafter according to the standard assay protocol. Gels were photographed with Polaroid Type 55 P/N film using a Polaroid MP-3 Land camera, and scanned at 620 nm on a Joyce Loebl Chromoscan.

B. Subunit Molecular Weight Determination

1) One Dimensional SDS Gel Electrophoresis in Tube Gels. Purified liver and brain fractions were fractionated by electrophoresis in 7.5% polyacrylamide gels containing SDS according to the method of Weber and Osborn (1969) using 15 cm high tubes with an inside diameter of 5 mm. Samples and standards were denatured at 100°C for 15 min in the presence of SDS:2-mercaptoethanol (2%:2% in water). 6-9 µg of sample protein was applied per gel. Electrophoresis was conducted at a constant current of 8 mA/gel for about 6 hr. The gels were stained for 2 hr at room temperature in 0.25% Coomassie brilliant blue R, destained electrophoretically 2 times for 25 min in 7.5% acetic acid:5% methanol, and left in a diffusion destainer (in 7% acetic acid) until all background

staining was removed. A calibration curve for the gels was constructed from the relative mobilities of the following standard proteins: phosphorylase b (94,000), bovine serum albumin (BSA) (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

2) Two Dimensional Gel Electrophoresis of L1B and L1A Arginase.

The purified liver pools were separated in two dimensions using the procedure of O'Farrell (1975). A 3 µg amount of protein was first subjected to isoelectric focusing (pH 3.5-10) for 6400 V-hr. The second dimension separation was in a 10% acrylamide slab gel containing 0.1% SDS. Electrophoresis was performed at 20 mA/ gel for approximately 3 hr until the bromophenol blue tracking dye had migrated to about 5 mm from the bottom of the slab gel. The gel was fixed for 1 hr in methanol:acetic acid:water (5:1:1), and stained overnight in Coomassie blue. Destaining was accomplished in ethanol:acetic acid:water (3:1:6). Standard proteins used to determine the molecular weight of the arginase subunits were: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α- lactalbumin (14,400).

C. Determination of S Value for the Intact L1B Enzyme By Analytical Ultracentrifugation

Sedimentation velocity of the L1B enzyme (3 mg/ml) was performed in a Beckman Model E analytical ultracentrifuge using an AN-D rotor and Schlieren optics. The centrifugation was at 52,640 rpm and 20°C.

D. Molecular Weight Determination by Gel Filtration On Sephadex G-150 Column

The molecular weights of liver and brain preparations were estimated according to the method of Andrews (1964). 1 ml (containing 0.16-2.0 mg of protein) of each final octyl sepharose fraction was applied to a 1.5 x 98 cm Sephadex G-150 column which had been equilibrated in 0.01 M Tris-HCl + 0.25 M NaCl, pH 7.5. 2 ml fractions were collected at a flow rate of 18 ml/hr. The elution position of the proteins was monitored by absorbance at 235 nm, while the elution of 2-mercaptoethanol was read at 280 nm. Fractions were also assayed for enzyme activity using the standard assay procedure. Calibration of the column was accomplished by measuring the elution position of the following proteins: aldolase (158,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), soybean trypsin inhibitor (20,100), myoglobin (16,900), and ribonuclease A (13,700). Blue Dextran 2000 ($>10^6$) and 2-mercaptoethanol (78) were employed to establish the column void volume and total bed volume, respectively.

E. Hexose Content

Samples of the purified liver and brain proteins were assayed for hexose content according to the procedure of Trevelyan and Harrison (1952). Briefly, 5 ml of anthrone reagent (0.2 g anthrone in 100 ml of 12.85 M H_2SO_4) was placed in test tubes in an ice bath, and then 1 ml of the sample was gently layered on top. After all samples had been applied, the tubes were capped, mixed by swirling, boiled for 10 min,

and then immediately placed in a cold water bath for at least 2 min. Absorbance was measured at 620 nm, and a standard curve was generated from a series of galactose standards (10-100 µg).

F. Amino Acid Composition Analysis

A Beckman model 118CL amino acid analyzer was used to determine the amino acid composition of purified liver and brain arginases. Aliquots of each sample (80 µg of liver arginase and 60-70 µg of brain arginase) were placed in hydrolysis tubes and vacuum desiccated overnight. Samples were hydrolyzed *in vacuo* at 110°C in 1.0 ml of 5.7 M HCl plus one drop of 0.5 M hydrazine, for 24, 48, and 72 hr (liver) or 24 hr (brain). At the end of the hydrolysis period, the preparations were dried, and then dissolved in 0.2 N sodium citrate, pH 2.2 containing 0.5% thiodiglycol and 0.1% phenol.

To determine tryptophan content, liver proteins were hydrolyzed for 24 hr at 110°C in the presence of 0.3 ml of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole. After hydrolysis, the samples were neutralized by adding 0.3 ml of 3.5 N NaOH, and they were then injected into the amino acid analyzer.

Half-cystine was measured in liver samples as cysteic acid following performic acid oxidation using the protocol of Moore (1963).

The compositional homology of the samples was evaluated using the parameter $S\Delta Q$ defined by Marchalonis and Weltman (1971).

$$S\Delta Q = \Sigma_j (X_{i,j} - X_{k,j})^2$$

i,k - identify the two proteins being compared

X_j - number of type j amino acid residues/100 residues

Antibody Production and Immunochemical Analysis

Rabbit Immunization

Two rabbits were immunized with purified L1B arginase. The injection protocol was as follows: 100 μg of protein in 1 ml of Octyl Sepharose buffer A was emulsified with 1 ml Freund's complete adjuvant (Miles Laboratories, Inc.); 0.6 ml of emulsion was injected into each rear thigh muscle, and 0.1-0.2 ml was injected intradermally to 5-6 spots on the back; 10 μg suspensions in Freund's incomplete adjuvant were injected as before on the 14th, 39th, 81st, and 104th day. Rabbits were bled by ear vein periodically to follow the progress of antibody production, and a final bleeding was taken by cardiac puncture one week after the final booster. Blood was allowed to coagulate at room temperature for 2 hr, and then overnight at 4°C; serum was poured off and stored at -20°C until purified.

Analysis of Antiserum

The production of rabbit anti-bovine-L1B arginase antibodies as a result of immunization was investigated on micro double immunodiffusion plates (Hyland, pattern C). Plates were kept for 3 hr at room temperature, followed by overnight incubation at 4°C. Visualization of the precipitin lines was enhanced by using the following procedure: plates were soaked in several changes of 0.9% NaCl, pH 7.7, stained with Coomassie brilliant blue R, and destained in 7.5% acetic acid: 5% methanol. Specificity of the purified (see below) antibodies was tested against purified preparations of bovine liver and brain arginase, as

well as against murine brain and liver homogenates.

Immunoglobulin IgG Purification

Immunoglobulins were separated from other rabbit serum proteins by addition of ammonium sulfate to 40% saturation. The solution was left at 4°C for 30 min, centrifuged at 17,300 x g for 15 min, and the resulting pellet was dissolved in 0.0175 M potassium phosphate, pH 6.8 (buffer A) (1-2 ml/10 ml of starting serum vol). The solution was dialyzed overnight against 4 liters of buffer A. It was next centrifuged at 17,300 x g for 15 min, and the supernatant was applied to a 1.0 x 25 cm DEAE-Sephacel (Pharmacia) column which had been equilibrated with buffer A. This ion-exchange material was used to separate the several immunoglobulin classes (IgG, IgA, IgM) from each other. 5 ml fractions were collected at a flow rate of 40 ml/hr. The column was washed with buffer A until the absorbance at 280 nm was 0.1; elution was then begun with 0.08 M potassium phosphate, pH 6.6 (buffer B), followed by a final elution with 0.3 M potassium phosphate, pH 6.5 (buffer C). The three protein pools were concentrated by precipitation with ammonium sulfate as above; the resulting pellets were dissolved in a minimum volume of buffer A (1 ml/70 ml of eluted pool), and then dialyzed overnight against buffer A before being tested for anti-bovine-L1B arginase activity on immunodiffusion plates.

Removal of Ribonuclease from IgG Fraction of Antiserum

The protocol used was that of Palacios et al. (1972). The IgG fraction isolated on the DEAE-Sephacel column was dialyzed against 4 liters of 10 mM sodium phosphate + 15 mM NaCl, pH 7.2 (buffer D), its protein content determined (Warburg and Christian, 1942), and concentrated as needed to 40-50 mg/ml. The sample was then placed on a sterile 1.5 x 4.5 cm column of DEAE-cellulose overlaid by the same volume of sterile CM-cellulose. Both of the ion-exchangers were equilibrated in buffer D. The sample was eluted with equilibration buffer, and 3 ml fractions were collected. Fractions with the highest absorbance at 280 nm were combined, a protein assay was performed, and 1 ml aliquots were frozen at -80°C in sterile Eppendorf tubes.

Quantitative Immunoprecipitation of L1B Arginase

L1B arginase was titrated against rabbit IgG by the methodology of Snodgrass et al. (1978). 25 µl of antibody or control buffer were incubated with increasing volumes of the liver protein (0.355 mg/ml) and enough enzyme assay buffer to bring the total volume of the solution to 0.2 ml. Samples were kept at 37°C for 45 min, and then overnight at 4°C. Centrifugation at 27,000 x g for 30 min was used to separate the precipitable immune complexes from the free excess antigen. The supernatant was tested in the usual manner for enzyme activity.

Isolation and Characterization of Liver Arginase mRNA

Ribonuclease (RNase) Precautions

All glass and plasticware were soaked in 0.1% diethylpyrocarbonate (DEPC) (Sigma) for 20 min, and baked overnight at 120°C to inhibit RNase activity. Eppendorf tubes used to collect fractions were autoclaved prior to use. Buffers were prepared in glass distilled, deionized water, and they were autoclaved prior to use.

Step 1. Polysome Isolation

Bovine liver polysomes were isolated using the magnesium precipitation method of Palmiter (1974). 60 g portions of frozen bovine liver were cut into small pieces, rinsed 2 times with phosphate buffered saline, and homogenized (0.3 g/1.0 ml buffer) in 25 mM Tris-HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.4, containing 2% Triton X-100, 0.2 mg/ml heparin (Sigma), and 1 µg/ml cycloheximide. The homogenate was centrifuged at 27,000 x g for 30 sec, and 0.1 vol of 1 M $MgCl_2$ was added with swirling to the resulting supernatant. After incubation for 1-1.5 hr at 4°C, a 2x vol of the ribonucleoprotein solution was layered over a 1x vol of 1 M sucrose in 25 mM Tris-HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.5, containing 0.5% Triton X-100, 0.1 mg/ml heparin, and 1 µg/ml cycloheximide, and this preparation was centrifuged at 27,000 x g for 15 min. The supernatant was removed by aspiration, and the glassy-yellow pellet was resuspended by gentle homogenization in 20 ml polyribosome buffer (25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.6, containing 0.1% nonidet P-40 (Sigma), 1 µg/ml cycloheximide, and 0.2 mg/ml heparin). The polysome

supernatant (S1) recovered after centrifugation of the suspension at 34,800 x g for 10 min was held aside, and the pellet was rehomogenized and recentrifuged. Supernatant (S2) from the second centrifigation step was combined with S1. The pellet was discarded.

Step 2. Immune Isolation of Arginase-Synthesizing Polyribosomes and Elution of Specific mRNA

Arginase-synthesizing polysomes were separated from the polysomes isolated in the previous step according to the methodology of Shapiro and Young (1981). S1 and S2 were centrifuged at 34,800 x g for 10 min to remove any aggregated polysomes. RNase free IgG from rabbit #2 was also centrifuged prior to use. A 0.6 ml volume of IgG (3.1 mg/ml) was added to 37.5 ml of polyribosomes. The mixture was left at room temperature for 1 hr, and then overnight at 4°C before application to a Protein A Sepharose (Pharmacia) column (0.7 x 11.1 cm) equilibrated in polyribosome buffer. Flow rate was 4-5 ml/hr. The column was washed overnight with buffer (85 ml). mRNA from specifically bound polysomes was eluted with 11-12 ml of 25 mM Tris-HCl, pH 7.6, containing 20 mM EDTA, 0.2 mg/ml heparin. Flow rate during elution was 10 ml/hr. Fractions of 1 ml were collected, and those containing the highest absorbance at 260 nm were pooled.

Step 3. Purification of Polyadenylated RNA

The mRNA pool removed from the Protein A column was heated at 65°C for 5 min, quick cooled on ice, and brought back to room temperature. It was adjusted to 10 mM Tris-HCl, 500 mM NaCl and 0.1% SDS and loaded onto

a 1 ml Pasteur pipet column of oligo-dT cellulose (Sigma) equilibrated at room temperature with 10 mM Tris-HCl, pH 7.6, containing 500 mM NaCl, 0.1% SDS, 1 mM EDTA. Flow rate was 9.5 ml/hr. The column was washed with 13 ml of equilibration buffer, and the bound RNA was eluted with 9 ml of the same buffer lacking NaCl. Fractions showing the greatest absorbance at 260 nm were combined, brought to 0.2 M potassium acetate, and precipitated with 2.5 vol of 95% ethanol. After overnight storage at -20°C, the RNA was centrifuged at 17,300 x g for 20 min, the supernatant was decanted, and the pellet was washed 3 times with 175 µl of 70% ethanol. The pellet was dried in a desiccator, brought up in 20 µl of sterile water, and transferred to an Eppendorf tube for storage at -80°C.

Cell-Free Translation of Liver mRNA

Polyadenylated RNA was translated using a nuclease treated lysate prepared from rabbit reticulocytes (New England Nuclear) according to the protocol of Pelham and Jackson (1976). (^{35}S)-methionine (1170 µCi/mmol) and RNA in a total volume of 10 µl were incubated at 37°C for 90 min. Control translations were run using water in place of the mRNA preparation. Reactions were terminated by the addition of 40 µl of electrophoresis treatment buffer (62.5 mM Tris-HCl, pH 6.8, 0.2% SDS, 10% glycerol, 5% 2-mercaptoethanol), followed by denaturation at 100°C for 15 min.

Immune Precipitation of Translated Products

Newly synthesized proteins were also prepared for immune precipitation after the 90 min translation period by adding 10 µl L1B

arginase (0.355 mg/ml) and 25 µl of either rabbit #1 IgG (24.1 mg/ml), or rabbit #2 IgG (7.6 mg/ml) to them. These mixtures were incubated at 37°C for 45 min, then left overnight at 4°C. After a 30 min centrifugation step in a microfuge, the supernatant was poured off, and the immune precipitate was washed 3 times with 0.9% NaCl, pH 7.7. Electrophoresis treatment buffer (50 µl) was added, and the protein mixture was boiled for 15 min.

Electrophoresis and Fluorography of Translated Products

Electrophoresis of translated proteins was accomplished as described by Laemmli (1970) on a 7.5% acrylamide slab gel containing 0.1% SDS. The gel was stained overnight in Coomassie blue, and destained in 7% acetic acid:5% methanol. It was soaked in several changes of water to remove the acid and methanol, and then it was incubated in 1 M salicylic acid for 30 min to enhance fluorography. The gel was mounted on a piece of 3MM paper (Whatman), and dried in a Hoeffer slab gel dryer. Kodak XAR-5 film was exposed at -80°C for 90 hr. Standard proteins used to determine the size of the translated proteins were: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

CHAPTER III

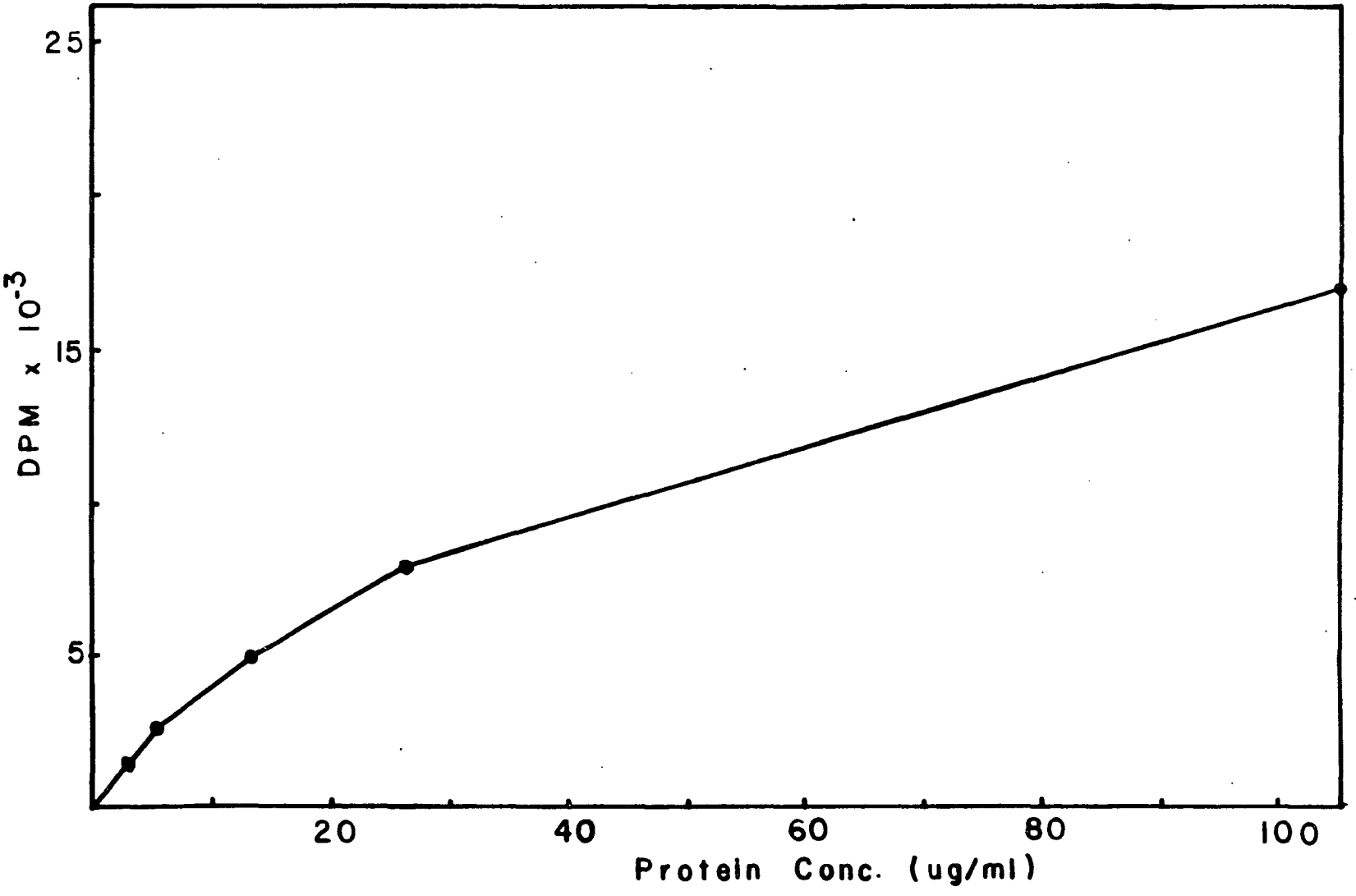
RESULTS

Standard Assay

The arginase enzyme activity assay was based on the formation of ^{14}C urea from L-(guanido-^{14}C) arginine (Rüegg and Russell, 1980). To obtain accurate values of enzyme activity at each stage of the purification protocol, appropriate dilutions of the sample had to be made. This need arose because of the well documented inhibition of the reaction by the other end product, ornithine (Hunter and Downs, 1945; Glass and Knox, 1973; Pace and Landers, 1981). This is illustrated in Fig. 2 which shows the effect on ^{14}C urea production of incubating a fixed concentration of substrate with increasing amounts of partially purified bovine liver arginase. A linear response occurred only for the first 5-6% of hydrolysis.

Using a commercial preparation of calf liver arginase, Rüegg and Russell (1980) found that the 10 min treatment at 55°C caused a 5% loss of activity. In the present work, just the opposite result was observed: activity was enhanced 3.5-fold by the heat activation step. There has been disagreement as to the efficacy of this procedure, depending on tissue and animal source (Schimke, 1962; Glass and Knox, 1973; Herzfeld and Raper, 1976).

Fig. 2. Arginase enzyme activity with respect to protein concentration of a partially purified bovine liver preparation starting with 41,866 dpm of ^{14}C-arginine in the assay mix.


25
15
5
DPM x 10^{-3}
20
40
60
80
100
Protein Conc. (ug/ml)

Purification of Bovine Liver and Brain Arginases

In a typical extraction, 300-350 g of frozen bovine liver or brain tissue was extracted. The arginase specific activity in liver samples ranged from 1.96-3.74 units/mg protein (mean was 2.79), while that in the brain samples was between 0.010 and 0.033 units/mg protein (mean was 0.026). The wide range of activities arises from differences in enzyme activities between animals (multiple bovine brains and livers were needed, see Tables 1 and 2).

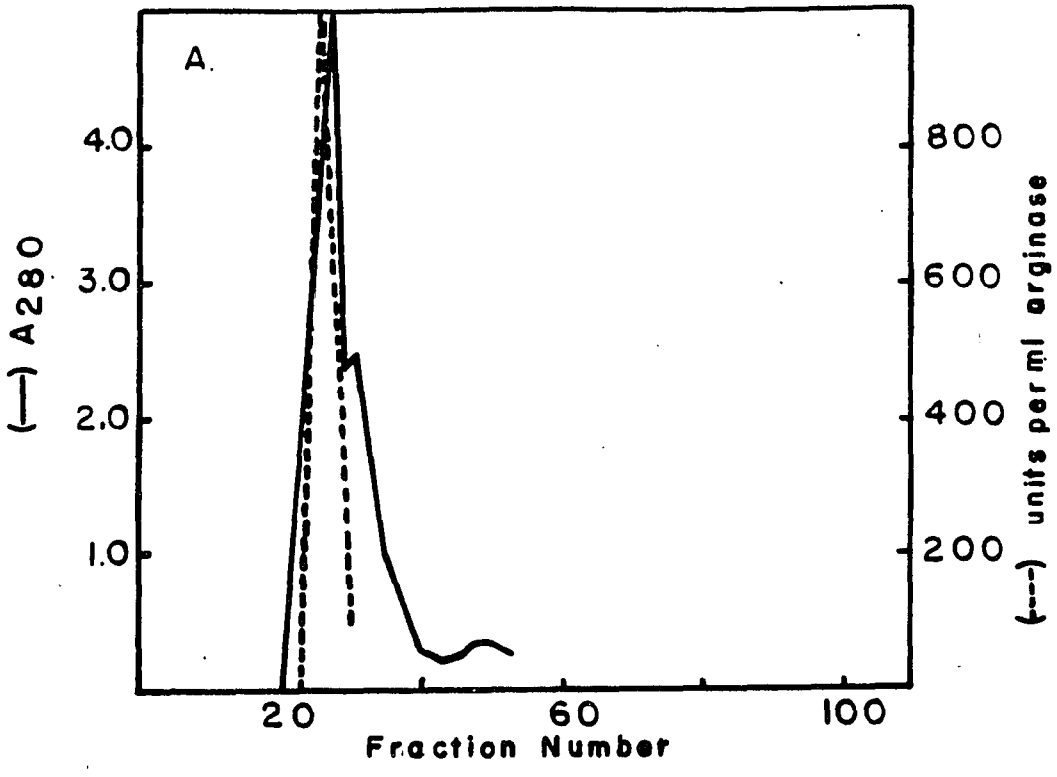
During the first attempts at purification of the brain enzyme, samples were tested for activity and protein content after the ammonium sulfate and acetone precipitation steps. The ammonium sulfate precipitation step generally led to a 3-fold rise in specific activity while the use of acetone aided the purification only minimally. In contrast, treatment of liver samples with acetone increased the specific activity 3-4-fold. Later, as the need to apply the purification scheme to larger quantities of tissue became apparent, aliquots were no longer assayed for protein and arginase activity after these steps. Thus in Tables 1 and 2A, values are shown only for the extraction and heat procedures. The purity of the enzyme had increased 13.0-fold and 15.8-fold, respectively, for liver and brain samples after heat treatment.

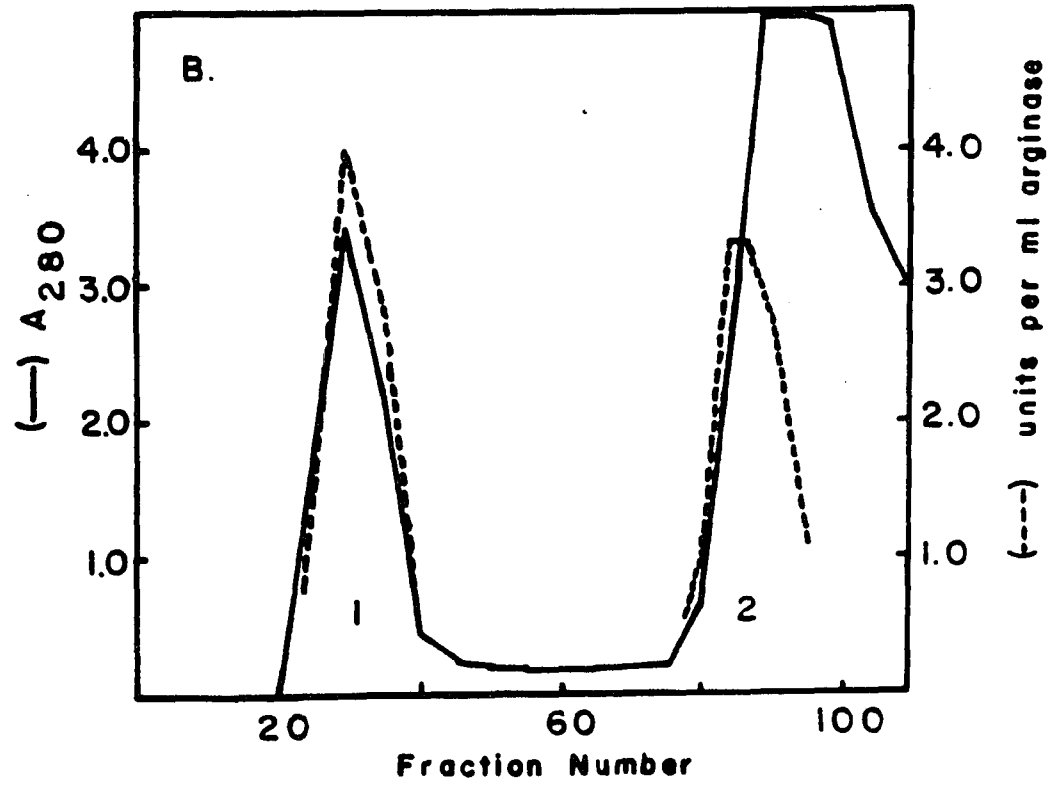
After dialysis, the heat treated preparation was applied to a DEAE-cellulose column. Figs. 3A and 3B illustrate the A_{280} and arginase activity of the eluant from these columns. Liver arginase activity did not bind to a DEAE-cellulose column at pH 7.8, while brain arginase yielded two peaks of activity when eluted from the column: pool B1 (the

Fig. 3. Elution profiles of bovine liver and brain arginase preparations from DEAE-cellulose column.

(A). 25 ml of dialyzed bovine liver sample after heat treatment was put onto a DEAE-cellulose column (3 x 53 cm). The column was eluted with 0.05 M Tris-HCl, pH 7.8 at a flow rate of 25 ml/hr and 10 ml fractions were collected. A_{280} (——); units/ml arginase (- - -).

(B). 100 ml of heat treated bovine brain sample was loaded onto a DEAE-cellulose column (3 x 50 cm). The column was washed with 400 ml of 0.01 M Tris-HCl, pH 7.5, followed by a linear gradient between 0 and 0.15 M KCl in Tris buffer. Flow rate and fraction size were as in 3A. A_{280} (——); units/ml arginase (- - -).


A.
4.0
3.0
2.0
1.0
(—) A280
800
600
400
200
(---) units per ml arginase
20
60
100
Fraction Number


B.
4.0
3.0
2.0
1.0
(—) A280
(---) units per ml arginase
1
2
20
60
100
Fraction Number

fraction of activity that is comparable to the liver arginase activity in that it did not bind to DEAE-cellulose) and pool B2 which eluted between 0.025-0.085 M KCl. The recovery of liver enzyme units loaded onto this first ion exchange column ranged from 31-63%. Comparable results for the brain were: 13-41% in pool B1 and 18-48% in pool B2. The application of liver material to DEAE-cellulose equilibrated in 10 mM Tris-HCl, pH 7.5 instead of 50 mM Tris-HCl, pH 7.8 caused a major portion of the arginase activity to be retained on the column. This activity could be removed with high salt, but because this strategy did not yield as great a purification of the enzyme as the elution system described in Methods, it was not retained as an isolation protocol.

After dialysis, the pool(s) collected from the DEAE-cellulose column was loaded onto a cation exchange column of CM-cellulose. Fig. 4 contains the elution profile of a liver preparation. Placement of either brain pool onto this column gave an elution profile similar to the liver sample. Some purification was achieved in this step, as seen in Tables 1, 2B, and 2C, because contaminating proteins did bind to the CM-cellulose, and could be subsequently removed with high salt (0.5 M KCl, data not shown). The fractions containing arginase activity were pooled, concentrated and stored at -20°C.

The concentrated enzyme solution from the CM-cellulose column (5-10 ml) was thawed and put onto a Sephadex G-150 column (3 x 54 cm). Fig. 5 illustrates the A_{280} and arginase activity elution patterns after one round of gel filtration. The center of the liver arginase activity peak (Fig. 5A) was at fraction 57. Reapplication of active fractions to the column yielded enzyme activity localized at the leading edge of the

Fig. 4. Elution profile of bovine liver arginase on CM-cellulose column.

130 ml of the dialyzed pool from the DEAE-cellulose column were placed on a CM-cellulose column (3 x 53 cm). The sample was washed through the column with 0.01 M Tris-HCl, pH 7.0 at a flow rate of 25 ml/hr and 10 ml fractions were collected. A_{280} (——); units/ml arginase (- - -).

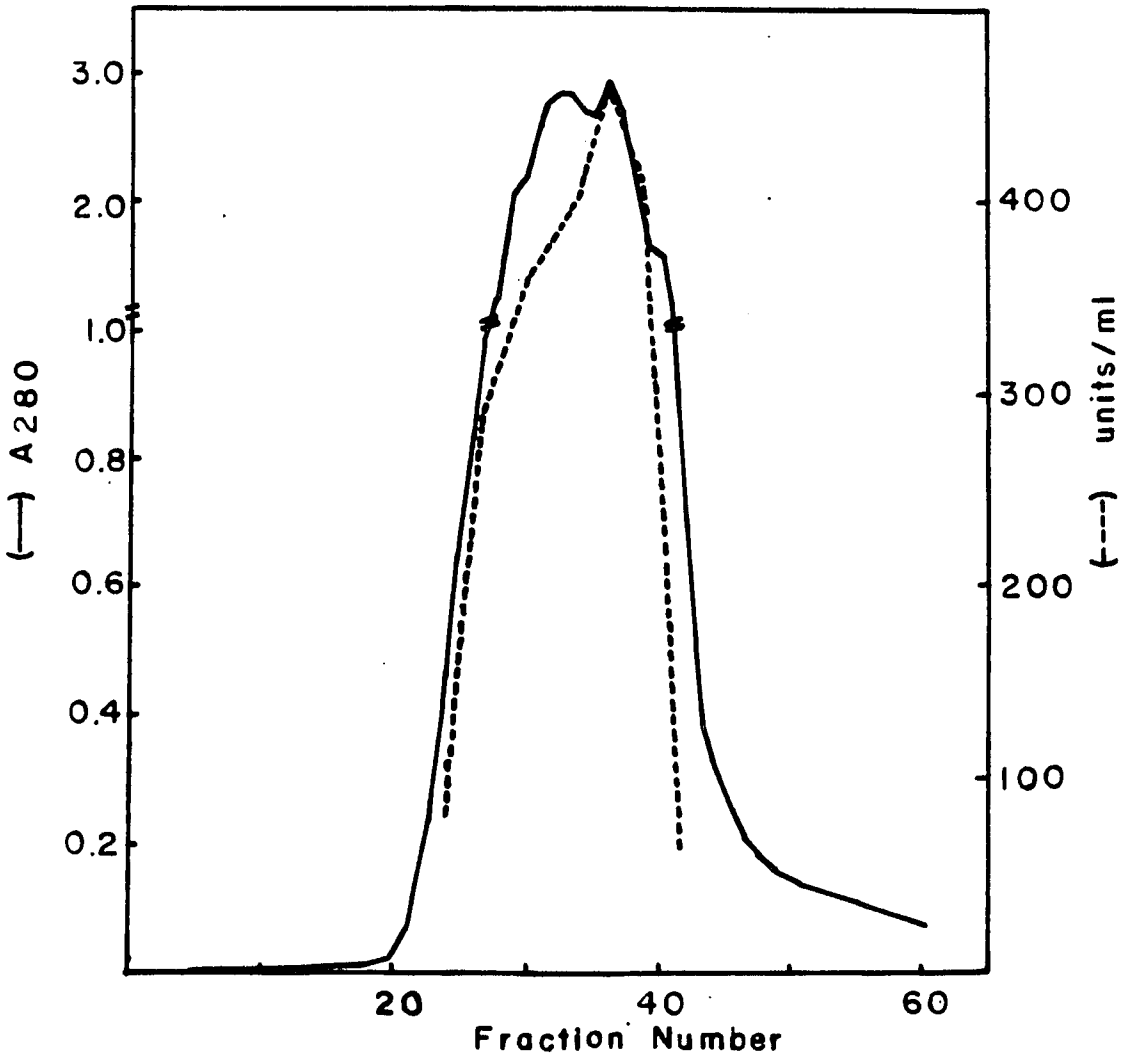

3.0
2.0
1.0
0.8
0.6
0.4
0.2
(—) A280
400
300
200
100
(---) units/ml
20
40
60
Fraction Number

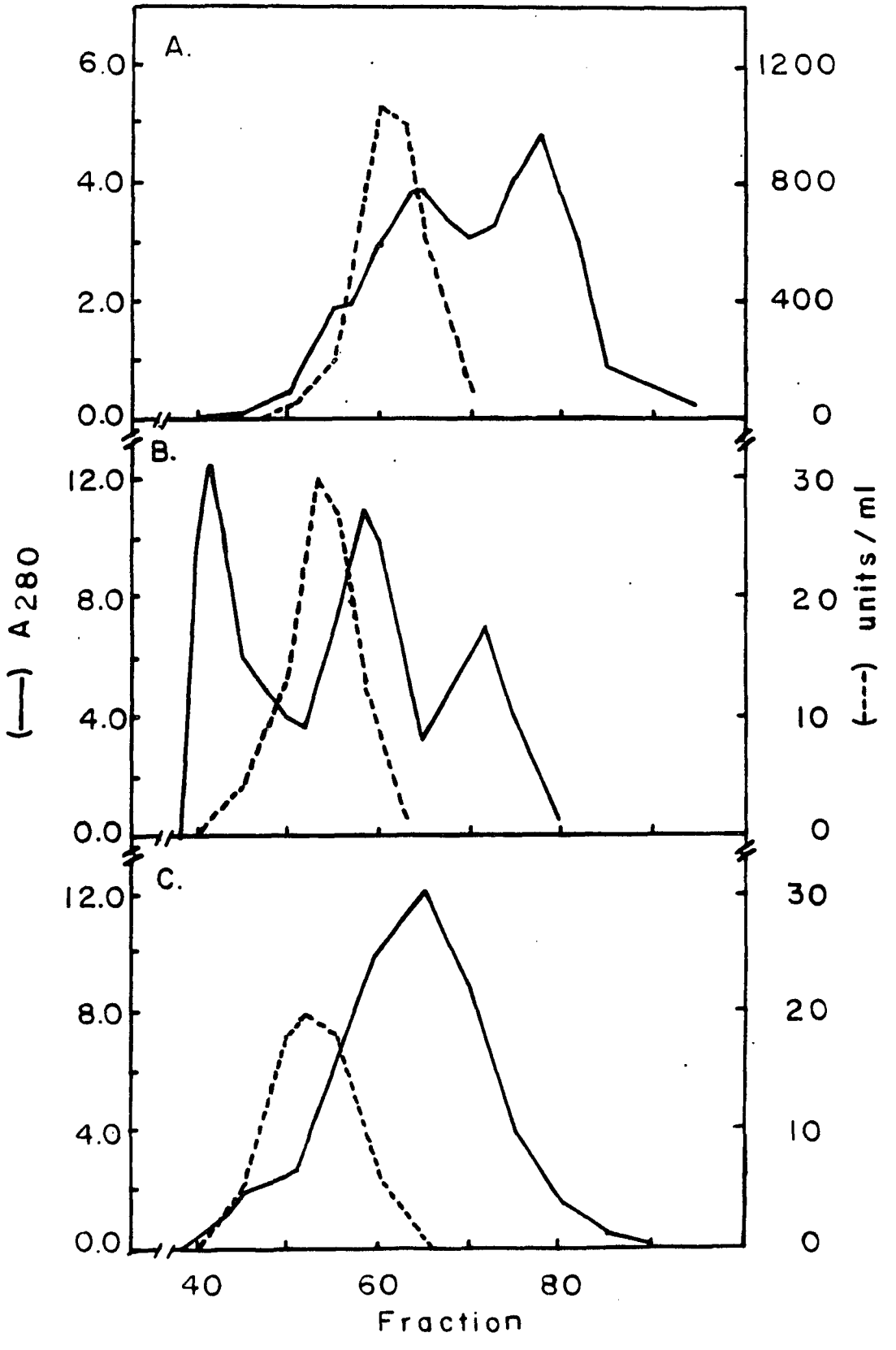
Fig. 5. Elution profiles of bovine liver and brain arginase pools on Sephadex G-150 column

The several preparations recovered from a CM-cellulose column were, after concentration using PM-30 Diaflow pressure ultrafiltration, applied to a Sephadex G-150 column (3 x 54 cm). The column was eluted with 0.01 M Tris-HCl + 0.05 M NaCl, pH 7.5 at a flow rate of 18 ml/hr, and 3 ml fractions were collected. A_{280} (——); units/ml arginase (- - -).

A. 5 ml of bovine liver pool L1.

B. 6.5 ml of bovine brain pool B1.

C. 4.7 ml of bovine brain pool B2.


A.
B.
C.
6.0
4.0
2.0
0.0
1200
800
400
0
12.0
8.0
4.0
0.0
30
20
10
0
(—) A280
(---) units / ml
40
60
80
Fraction

buffer front (data not shown).

The elution profile for brain pool B1 on Sephadex G-150 is shown in Fig. 5B. Arginase activity was found between the first two protein peaks (the greatest activity was noted at fraction 53). On the second run through the gel matrix, arginase eluted as one peak centered again at fraction 53 (data not shown).

Fig. 5C shows the elution pattern of brain pool B2 after one pass through the G-150 column: the single peak of arginase activity was centered at fraction 52 between the weak shoulder and the major protein band. Reapplication of pool B2 to the Sephadex (data not shown) produced similar profiles of A_{280} and enzyme material, although the greatest arginase activity was found at fraction 56.

A 140-180 mg amount of protein from the Sephadex G-150 column was placed on a hydrophobic column of Octyl Sepharose. Fig. 6 shows the resulting A_{280} and arginase activities of the eluant. Liver arginase eluted as a broad, ill-defined peak (Fig. 6A). Nevertheless, the enzyme activity was divided into two pools (designated L1A and L1B) which were concentrated after a dialysis step which removed the ethylene glycol that interferes with concentration. Pool L1B was made 40% saturated in ammonium sulfate, and then was reapplied to a second, smaller column of Octyl Sepharose. The A_{280} and enzyme activity profiles from this column run are presented in Fig. 7A. Although arginase was broadly distributed throughout the elution profile, only one pool, again called L1B, was processed for analysis as detailed in Methods. It eluted between approximately 52-80% ethylene glycol.

In a preliminary experiment, a small portion of pool L1B (from

Fig. 6. Elution profiles of bovine liver and brain arginase pools on the first Octyl Sepharose column.

The dialyzed preparations recovered from two runs on G-150 were made 40% saturated in ammonium sulfate, and were loaded onto an Octyl Sepharose column. The column was washed with 10 mM potassium phosphate, pH 7.0, 0.2 mM DTT, 40% saturated with ammonium sulfate. Elution was accomplished by a linear gradient of decreasing ammonium sulfate and increasing ethylene glycol concentration (final concentrations 0% and 80%, respectively), followed by 3-4 column volumes of 80% ethylene glycol in buffer. Flow rate was 25 ml/hr, and 3 ml fractions were collected. A_{280} (——); units/ml arginase (- - -); % ethylene glycol (...).

A. 140 mg pool L1 was placed on a 1 x 30 cm column.

B. 142 mg pool B1 was placed on a 0.7 x 13 cm column.

C. 184 mg pool B2 was placed on a 0.7 x 13 cm column.

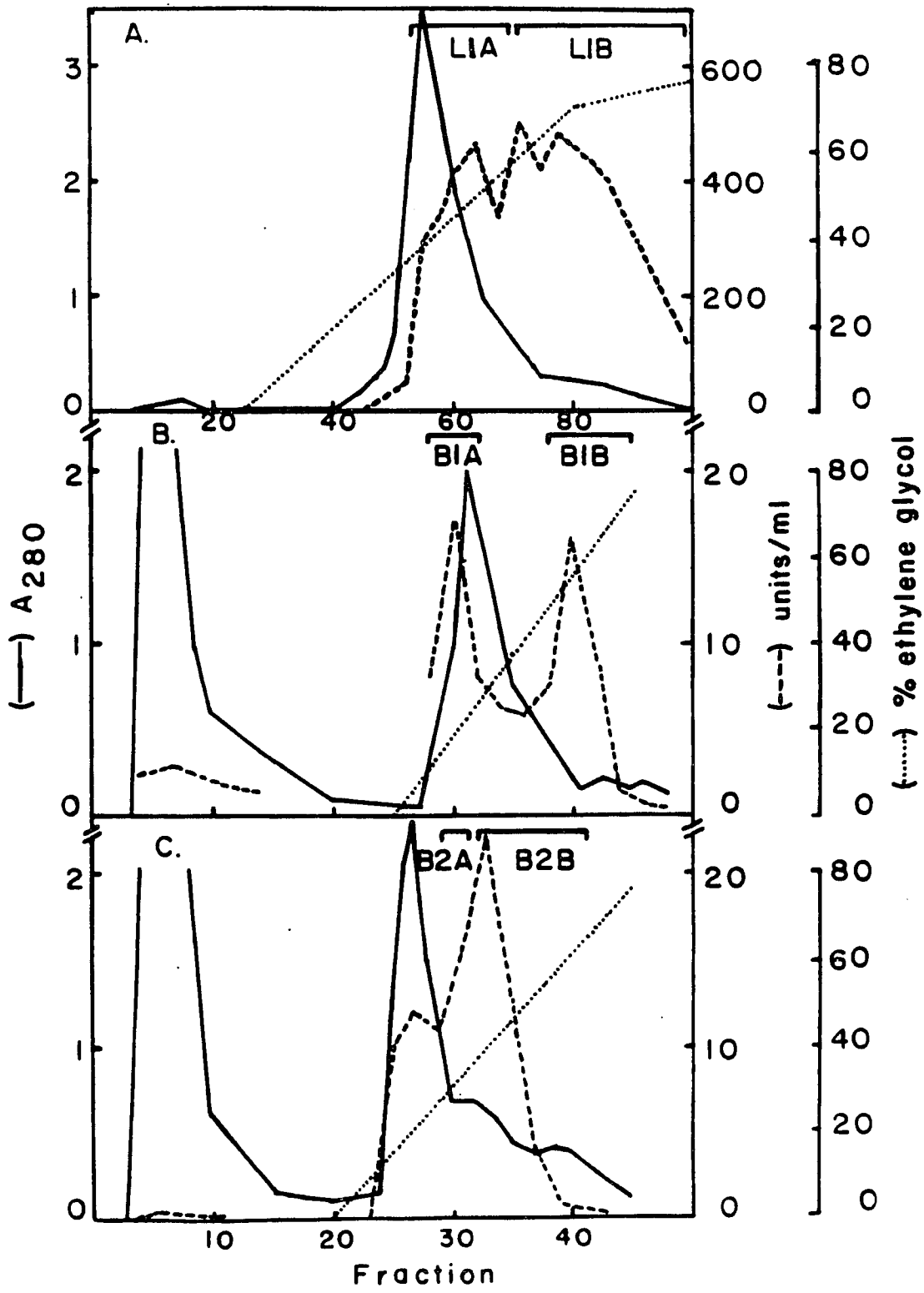

A.
LIA
LIB
B.
BIA
BIB
C.
B2A
B2B
(——) A280
(---) units/ml
(·····) % ethylene glycol
Fraction

Fig. 7. Elution profiles of bovine liver and brain arginase pools on second Octyl Sepharose column.

Liver and brain pools collected from the first Octyl Sepharose column were loaded onto a second Octyl Sepharose column (0.7 x 13 cm (liver)) or (0.7 x 6.5 cm (brain)). Elution was the same as in figure 6. 2 ml fractions were collected at a flow rate of 25 ml/hr. A_{280} (——); units/ml arginase (- - -); estimated % ethylene glycol (...).

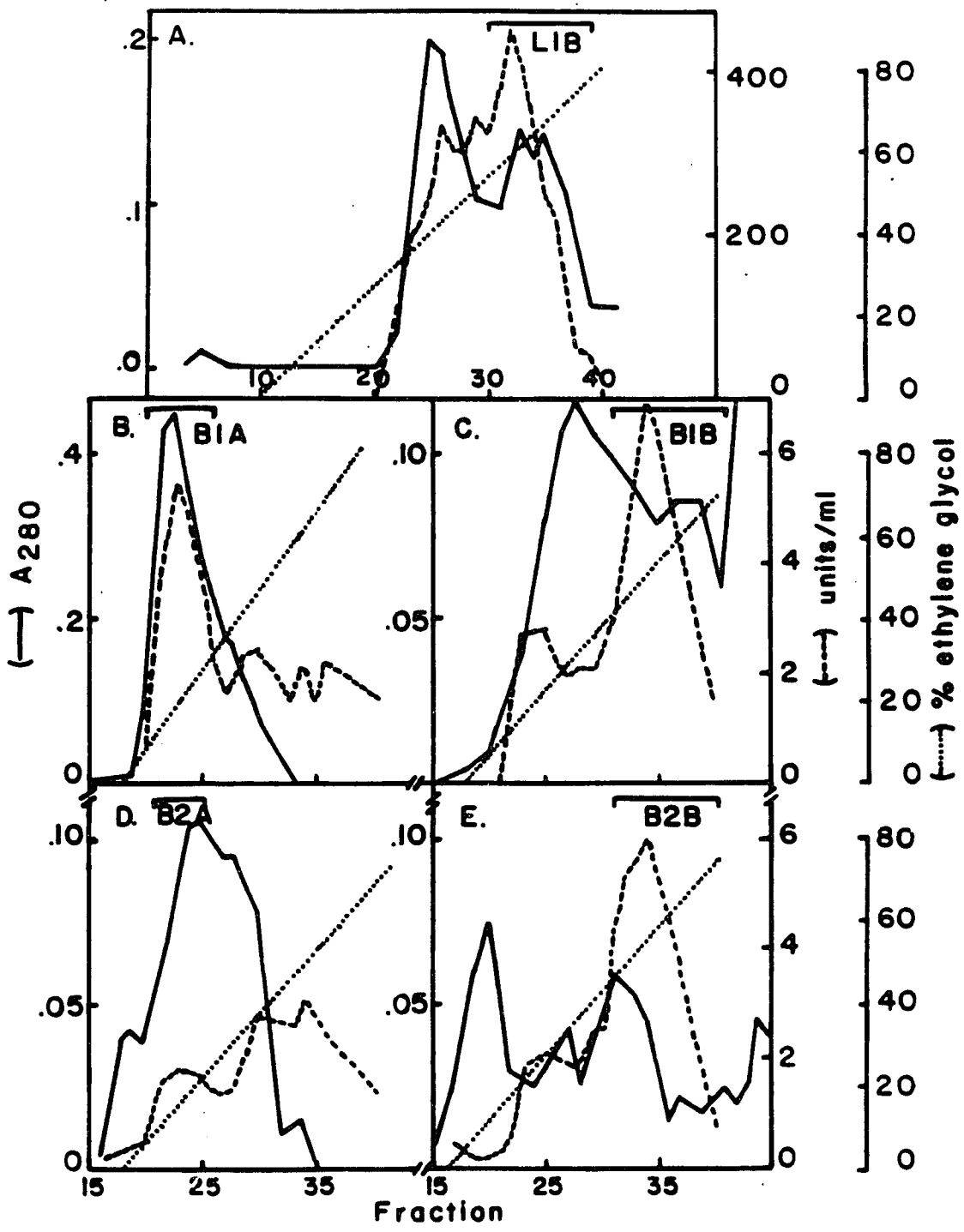
A. 12 mg liver pool L1B

B. 13 mg brain pool B1A

C. 6 mg brain pool B1B

D. 6.5 mg brain pool B2A

E. 4.3 mg brain pool B2B


A.
L1B
B.
B1A
C.
B1B
D.
B2A
E.
B2B
A280
units/ml
% ethylene glycol
Fraction

Octyl Sepharose run 1) was tested for its ability to bind to a Mātrex Gel PBA-30 (Amicon) column. This gel contains m-aminophenyl boronic acid covalently coupled to agarose which interacts with cis-diol containing molecules such as carbohydrates and glycoproteins. The arginase in the liver sample bound to the gel, and was released by 25 mM mannitol as a single peak of activity and protein. This result implies the presence of cis-diol groups on arginase which compete for binding to the PBA with mannitol. Alternatively, attachment may arise from hydrophobic or ionic interactions between certain amino acids in the protein and the phenyl rings bound to the gel matrix. The interaction, whatever its nature, is weak and easily broken. The utility of this method as a late step in the purification of arginase was not pursued, but probably should be investigated in greater depth because the elution conditions are not as severe as those required by hydrophobic chromatography on Octyl Sepharose.

Fig. 6B illustrates the result of applying brain pool B1 to Octyl Sepharose. Some arginase activity appeared in the flow-through peak, but the majority of it was recovered among the gradient fractions as two clearly defined species whose peaks were located at fractions 30 and 40, respectively. Pool B1A reapplied to an Octyl Sepharose column (Fig. 7B) showed one large protein peak which contained the arginase activity as well. It was eluted from the Octyl Sepharose in 10-33% of ethylene glycol. When pool B1B was put back onto the hydrophobic column (Fig.7C), A_{280} material and enzyme activity did not correspond. The existence of two distinct areas of arginase activity, as was the case in Fig. 6B, indicated that complete separation of the two isozymes was not effected

by a single application of the brain material to the column. The use of 43-74% ethylene glycol in buffer could elute the major area of enzyme activity (pool B1B).

The placement and subsequent removal of pool B2 from Octyl Sepharose is seen in Fig. 6C. Two peaks of arginase activity were seen as with brain B1, but the resolution into two separate species was not as sharp. Pool B2A (which lay midway between the two activity peaks and so presumably contained material from both) and pool B2B were separately reloaded onto a second Octyl Sepharose column. The results of their elution from these columns appear in Figs. 7D and 7E, and are comparable to those of B1A and B1B (Figs. 7B and 7C). Pool B2A came off between 15-29% ethylene glycol, while pool B2B eluted in 45-70% ethylene glycol.

All preparations isolated after the second Octyl Sepharose step were labeled as 'purified' and maintained at -20°C until analyzed. Some loss of activity was noted after several months storage.

Table 1 presents the results of the bovine liver arginase purification protocol starting from 1.5 kg wet weight. The purification of enzyme L1B was about 300-fold with a 0.7% recovery of starting arginase activity. A second isozyme, pool L1A, was purified 26-fold at a recovery of 0.25%.

Tables 2A, 2B, and 2C show the purification of bovine brain arginases (see Introduction for a complete description of the arginase nomenclature used in this study) from more than 22 kg of tissue. The pools corresponding to L1B (as shown by their similar elution from Octyl Sepharose), named B1B and B2B, were recovered at a yield of 0.6% and 0.5%, respectively. The purification for both of these preparations was

TABLE 1 PURIFICATION OF ARGINASES FROM BOVINE LIVER (1)

Procedure	ml	Total mg (2)	Total units $\times 10^5$	unit/mg	Yield %	Fold Purification
Extraction	4625	1.67×10^5	4.65	2.79	100	
Heat	125	5925	2.15	36.26	46.2	13.0
DEAE-cellulose	273	1262	1.06	83.91	22.8	30.1
CM-cellulose	17.3	598	0.86	143.17	18.4	51.3
Sephadex G-150 after 2 runs	7.5	51.5	0.20	389.23	4.3	139.5
Octyl Sepharose after 2 runs						
L1A	2.0	16	0.011	73.41	0.25	26.3
L1B	4.1	3.99	0.033	833.54	0.70	298.7

(1) Starting material was 1530 g frozen bovine liver

(2) Protein determined by Warburg and Christian (1942) method for all procedures except octyl sepharose chromatography. Protein assayed in that step by Bradford (1976) reagent.

TABLE 2A INITIAL STEPS IN PURIFICATION OF ARGINASES FROM BOVINE BRAIN (1)

Procedure	ml	Total mg (2)	Total units	unit/mg	Yield %	Fold Purification
Extraction (3)	65000	338500	9013	0.026	100	
Heat	606	13900	5774	0.410	64.1	15.8
DEAE-cellulose						
Pool 1	116.5	1739	1462	0.84	16.2	32.3
Pool 2	72.8	2289	2106	0.92	23.4	35.4

(1) Starting material was approximately 22.5 kg frozen bovine brain. Each brain weighs 350-400 g.

(2) Protein determined by Warburg and Christian (1942) method.

(3) Values shown for this step are averages obtained after assaying 5-7 different brain extracts.

TABLE 2B CONTINUED PURIFICATION OF BOVINE BRAIN 1 ARGINASE AFTER DEAE-CELLULOSE

Procedure	ml	Total mg	Total units	unit/mg	Yield %	Fold Purification
CM-cellulose (1)	6.5	653	1056.3	1.62	11.7	62.3
Sephadex G-150 after 2 runs (1)	5.1	266	732	2.75	8.1	105.8
Octyl Sepharose after 2 runs (2)						
Pool B1A	3.2	3.9	33.1	8.54	0.4	328.5
Pool B1B	2.6	0.83	52.0	62.53	0.6	2405

(1) Protein determined by Warburg and Christian (1942) method.

(2) Protein determined by Bradford (1976) method.

TABLE 2C CONTINUED PURIFICATION OF BOVINE BRAIN 2 ARGINASE AFTER DEAE-CELLULOSE

Procedure	ml	Total mg	Total units	unit/mg	Yield %	Fold Purification
CM-cellulose (1)	9.2	1537	1342	0.87	14.9	33.6
Sephadex G-150 after 2 runs (1)	5.2	184	728	3.95	8.1	151.9
Octyl Sepharose after 2 runs (2)						
Pool B2A	2.6	0.95	15.1	15.85	0.2	609.6
Pool B2B	2.3	0.59	44.5	75.29	0.5	2896

(1) Protein determined by Warburg and Christian (1942) method.

(2) Protein determined by Bradford (1976) method.

greater than 2000-fold. Pools B1A and B2A (which are comparable to L1A by the criterion of elution position from a hydrophobic column) were purified 320-fold and 610-fold, respectively, and their respective yields were approximately 0.4% and 0.2%.

Because the isolation scheme gave low recoveries of enzyme, the purity analysis of the isolated proteins was difficult. All of the methods described in Methods were employed to characterize L1B arginase, but only some of them were applied to L1A and the several brain arginases.

Characterization of the Enzymes

Activation Properties

The pH optimum of both L1A and L1B arginase was pH 10.0. These two preparations, as well as the brain arginases that were purified in this study, were 14-20 times more active at pH 9.5 than at pH 7.0. Diluted samples of L1B and L1A (at a concentration of 0.25 µg/ml and 3.0 µg/ml, respectively) were tested for activation by exogeneously added Mn^{2+} and Co^{2+}. Mn^{2+} increased the activity of L1A 3-fold, and it enhanced the activity of L1B 1.6-fold. Co^{2+} appeared to have a minor inhibitory effect on the activity of both samples (data not shown).

A. Disc Gel Electrophoresis

The liver and brain arginase preparations isolated after two rounds of hydrophobic chromatography on Octyl Sepharose were analyzed by polyacrylamide disc gel electrophoresis. 5% acrylamide was used because it

allowed arginase to migrate to a midposition in the gel. Electrophoresis was performed as described in Methods. Although none of the pools except B1A showed a well-defined sharp disc of protein (see below for a discussion of this problem), pre-electrophoresis of the separating gel to remove oxidative contaminants was found to sharpen the broad band of stainable material, and so pre-electrophoresis was routinely performed before applying samples to the top of the stacking gel.

Disc gels were removed from their tubes, and either stained with Buffalo Black, or immediately sliced into 2 mm pieces which were assayed individually for enzyme activity as reported in Methods.

Fig. 8 shows the stained gels obtained with 5% acrylamide at pH 9.5. An examination of gels 1-3 (L1B, B1B, B2B, respectively) reveals wide expanses of lightly stained protein. The center of each of these areas was located approximately 2.1 cm from the origin. This is a misleading comparison, however, as is apparent from the densitometer scans shown in Fig. 9. The gel containing L1B arginase (Fig. 9A) has a single symmetrical band of protein and enzyme activity, as does that containing B2B protein (Fig. 9C). The most intensely stained protein in B1B (Fig. 9B) migrates to the same location as L1B, and in addition, this preparation has a shoulder of protein on the lagging edge of the protein peak. This is a consistent result with arginase B1B. These data may indicate some differences in size and/or charge between arginase enzymes in the two tissues. While the migration of arginase B2B through the polyacrylamide gel is grossly similar to that of the other two preparations, it does differ in that some of the stained material is closer to the anode

Fig. 8. Disc gel electrophoresis of purified brain and liver arginases.

Approximately 30 µg of each pool from the second Octyl Sepharose column was loaded onto a 5% acrylamide gel, pH 9.5. Gels are shown with the origin at the top, the dye front at the bottom. Full details for electrophoresis are given in Methods. From left to right: (1) L1B; (2) B1B; (3) B2B; (4) L1A; (5) B1A; (6) B2A.

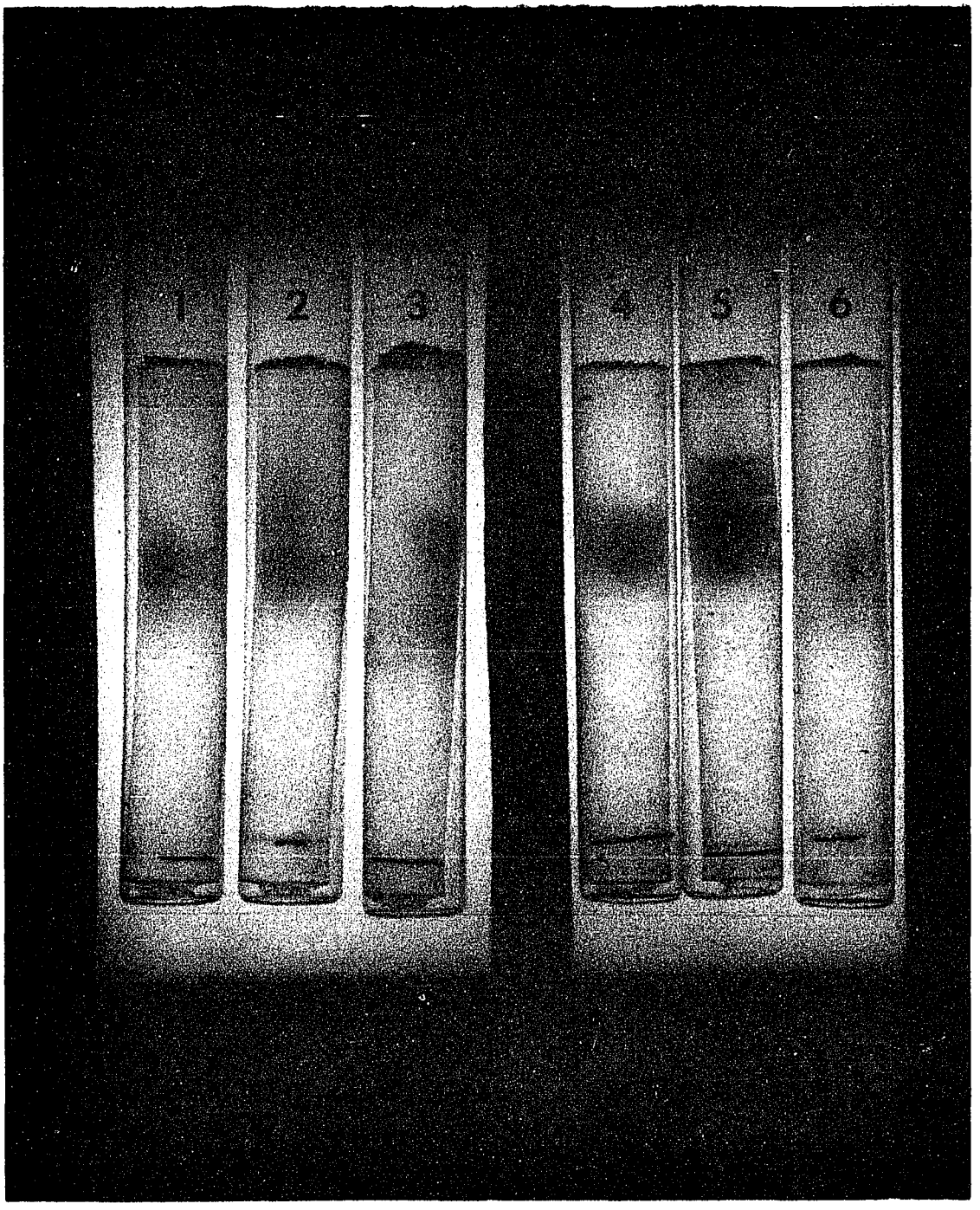

1
2
3
4
5
6

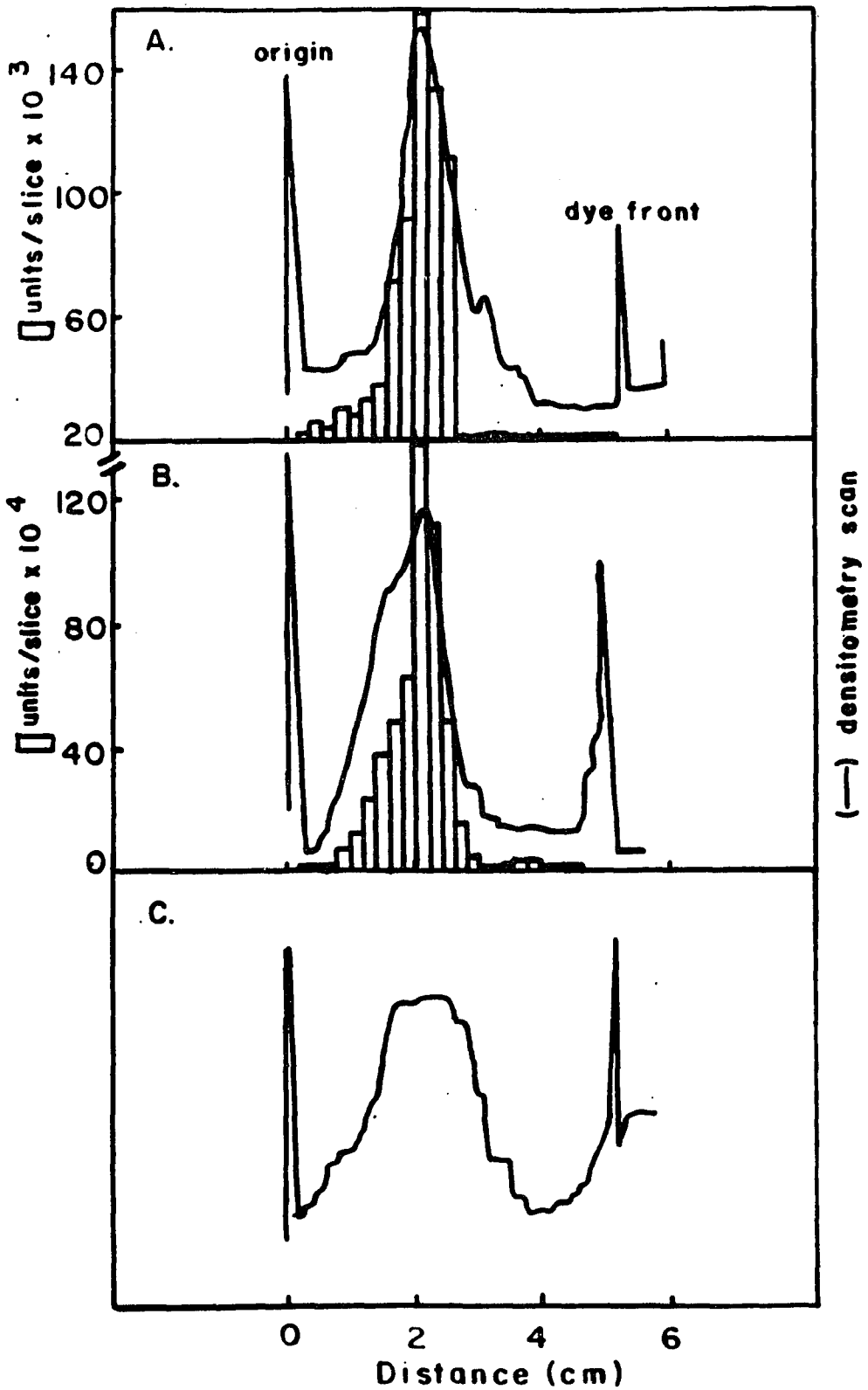
Fig. 9. Comparison of densitometry scans at 620 nm of 5% acrylamide disc gels of L1B and B1B arginase with the enzyme activity profile.

Duplicate gels of 5% acrylamide, pH 9.5 were loaded with 30 µg of the appropriate protein pool. After electrophoresis, one gel from each pair was stained for protein, and the other was assayed for arginase activity. The gel is shown with the origin to the left, the dye front to the right. Full details for electrophoresis and arginase assay are given in Methods.

A. L1B

B. B1B

C. B2B (densitometry scan only)


A.
origin
dye front
140
100
60
20
units/slice x 10 3
B.
120
80
40
0
units/slice x 10 4
(—) densitometry scan
C.
0
2
4
6
Distance (cm)

(Figs. 8 and 9). This fact correlates with its behavior on DEAE-cellulose. B2B arginase binds to the DEAE-cellulose whereas the other two arginases do not, and so the B2B protein must be more negatively charged.

Figs. 9A and 9B show the results of assaying the sliced gels of L1B and B1B for arginase activity. There is an excellent correspondance between the staining pattern and the enzymatic activity profile. The shoulder of slower migrating protein material in the brain preparation contains a significant amount of arginase activity, perhaps indicative of a greater heterogeneity in the charge and/or size of the active enzyme molecules. The 10-fold higher activity of the liver slices should be noted as well because it agrees with the data in Tables 1 and 2B showing the greater specific activity of the purified liver fraction.

Analysis of gels 4-6 in Fig. 8 reveals that the same kind of relationships exist between the migration patterns of L1A, B1A, and B2A as between those of L1B, B1B, and B2B. There are slower migrating proteins in the B1A preparation than are found in L1A. There was a very slight indication of proteins banding as sharp, compact discs in the arginase L1A sample at 1.8 cm and 2.1 cm from the origin. Because arginase characteristically presents itself after disc gel electrophoresis as a homogeneous, but diffuse band (Schimke, 1964; Hirsch-Kolb and Greenberg, 1968), the existence of these sharp bands may indicate contamination of the L1A arginase (however, see below for a further discussion of this unusual appearance of liver arginase during electrophoresis). As might be predicted, B2A, overall, has some components which run more quickly than the other two samples.

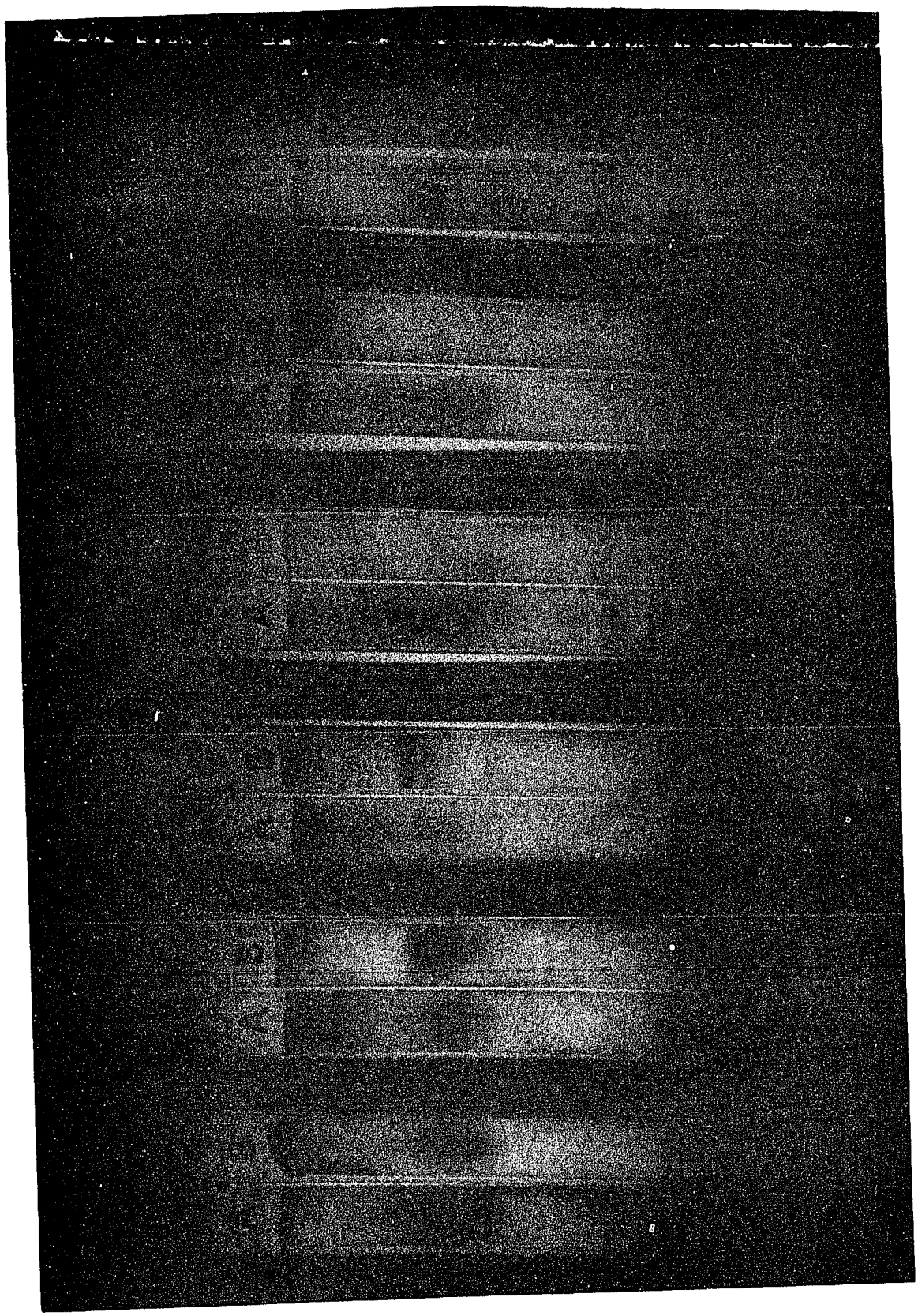
A comparison between isozymes distinguishable by their hydrophobic interactions with Octyl Sepharose (Fig.8: compare gels 1 and 4; 2 and 5; 3 and 6) reveals no striking differences. A general trend toward greater anodic migration was observed, however, for those preparations which bind more firmly to the hydrophobic support.

Staining of the gels with Coomassie brilliant blue R in methanol:acetic acid, or trichloroacetic acid did not sharpen the banding pattern of the samples.

Because proteins are separated on disc gel electrophoresis on the bases of differences in molecular weight and/or charge, the diffuse bands observed for arginase (Fig. 8) might arise from the overlap in migration of a number of proteins whose size and charge differed only minimally. The fact that most of the procedures used in the purification of arginase relied for their success on differentials in protein charge and size supports this explanation. The loss of Mn^{2+} from the enzyme during electrophoresis might also alter the protein's charge characteristics, causing a single native polypeptide to appear as a diffuse band. Such an effect has been reported for iris bulb arginase (Boutin, 1982). To determine whether the same phenomenon applies to bovine liver and brain arginase proteins, 5% acrylamide gels were run as before, except that all of the gel and buffer solutions were first made 1 mM in EDTA or 1 mM in manganous chloride. Enzyme pools were also made 10 mM in these compounds, and they were incubated at 37°C for 15 min before electrophoresis to ensure the removal or addition of sufficient metal to saturate the cofactor binding sites (Aguirre and Kasche, 1983). The outcome appears in Fig. 10. Inclusion of Mn^{2+} led to a consistent

Fig. 10. Disc gel electrophoresis of purified brain and liver arginases run in the presence of 1 mM EDTA or 1 mM $MnCl_2$.

Approximately 30 µg of each pool from the second Octyl Sepharose column was loaded onto a 5% acrylamide gel, pH 9.5. The first gel in each pair (A) contained 1 mM EDTA, and the second gel (B) contained 1 mM $MnCl_2$. The gels are shown with the origin at the top, the dye front at the bottom. From left to right: (1A & B) L1B; (2A & B) L1A; (3A & B) B1A; (4A & B) B1B; (5A & B) B2A; (6A & B) B2B.



sharpening of the protein band (compare Fig. 8 and gel B in each pair of gels in Fig. 10). This result was most pronounced for pool B1A. Use of EDTA, in contrast, caused a broadening of the protein band. Chelation of the arginase cofactor also resulted in the aggregation of L1A and B1A protein at the origin of the gel (Fig. 10, gels 2A and 3A). This effect was not as noticeable for the B1B and B2A arginases, and it probably did not occur with the L1B and B2B preparations. A further consequence of EDTA treatment was the release of a highly charged protein which migrated to a position just above the dye front. This sharp, discoid band was very prominent in B1B arginase (Fig. 10, gel 4A), but it was present in the other preparations as well. EDTA had a unique influence on the electrophoretic migration of the B1B pool in that a distinct component which moved to within 1 cm of the dye front was apparent in this sample alone. These experiments indicate that the removal of metal ions does modify the behavior of arginase during electrophoresis, and therefore electrophoresis should routinely be run in the presence of Mn^{2+}.

Pools L1A and L1B were also subjected to electrophoresis on disc gels which contained 6 M urea. Each of them appeared as a narrow, although diffuse band of lightly stained material. They must be different proteins, however, because L1A migrated 7 mm from the origin of the gel, and L1B moved 15 mm from the origin (data not shown). This reinforces the information determined during the purification of the liver isozymes where it was demonstrated (Fig. 6A) that the two proteins could be removed from Octyl Sepharose using different amounts of ethylene glycol.

Because of the lack of sharp, disc-like bands on electrophoresis of the denatured and native protein (even in the presence of added Mn^{2+}), both the L1B and B1B arginases were analyzed by electrophoresis at pH 2.3. Harell and Sokolovsky (1972) reported that acid treatment of bovine liver arginase at pH2.6 causes dissociation into its subunits. Although electrophoresis at pH 2.3 should substantiate this result, this methodology was not successful. Broad bands were again observed. The composition of the enzyme from the two tissues is different, nonetheless, because the liver protein moved to the midpoint of the gel while the brain preparation remained close to the origin (data not shown)

B. Subunit Molecular Weight Determination

1). One Dimensional SDS Gel Electrophoresis. The several pools of liver and brain arginase from Octyl Sepharose fractionation were separated by electrophoresis on 7.5% acrylamide gels containing SDS (SDS-PAGE) in order to determine the number and molecular weights of their subunits. Electrophoresis was conducted as described in Methods. Fig. 11 shows the result of the analysis. A single band was observed for each of the liver preparations, while the brain samples contained multiple proteins.

A standard curve was made using the relative mobilities of the proteins in the Pharmacia mixture, and this curve is presented in Fig. 12. The molecular weights (M_r) of the subunit(s) for each preparation estimated by this method are found in Table 3. The less hydrophobic liver and brain pools (pools L1A, B1A, B2A) have the same M_r. B1A has a

Fig. 11. SDS-polyacrylamide gel electrophoresis of liver and brain arginases.

5-10 µg of sample protein was applied to a 7.5% SDS polyacrylamide gel. Gels are shown with the origin at the top, the dye front at the bottom. Full details for electrophoresis are given in Methods. From left to right: (1) L1B; (2) B1B; (3) B2B; (4) L1A; (5) B1A; (6) B2A; (7) low molecular weight standard mixture (Pharmacia). See legend to Fig. 12 for the composition of the standard mix.

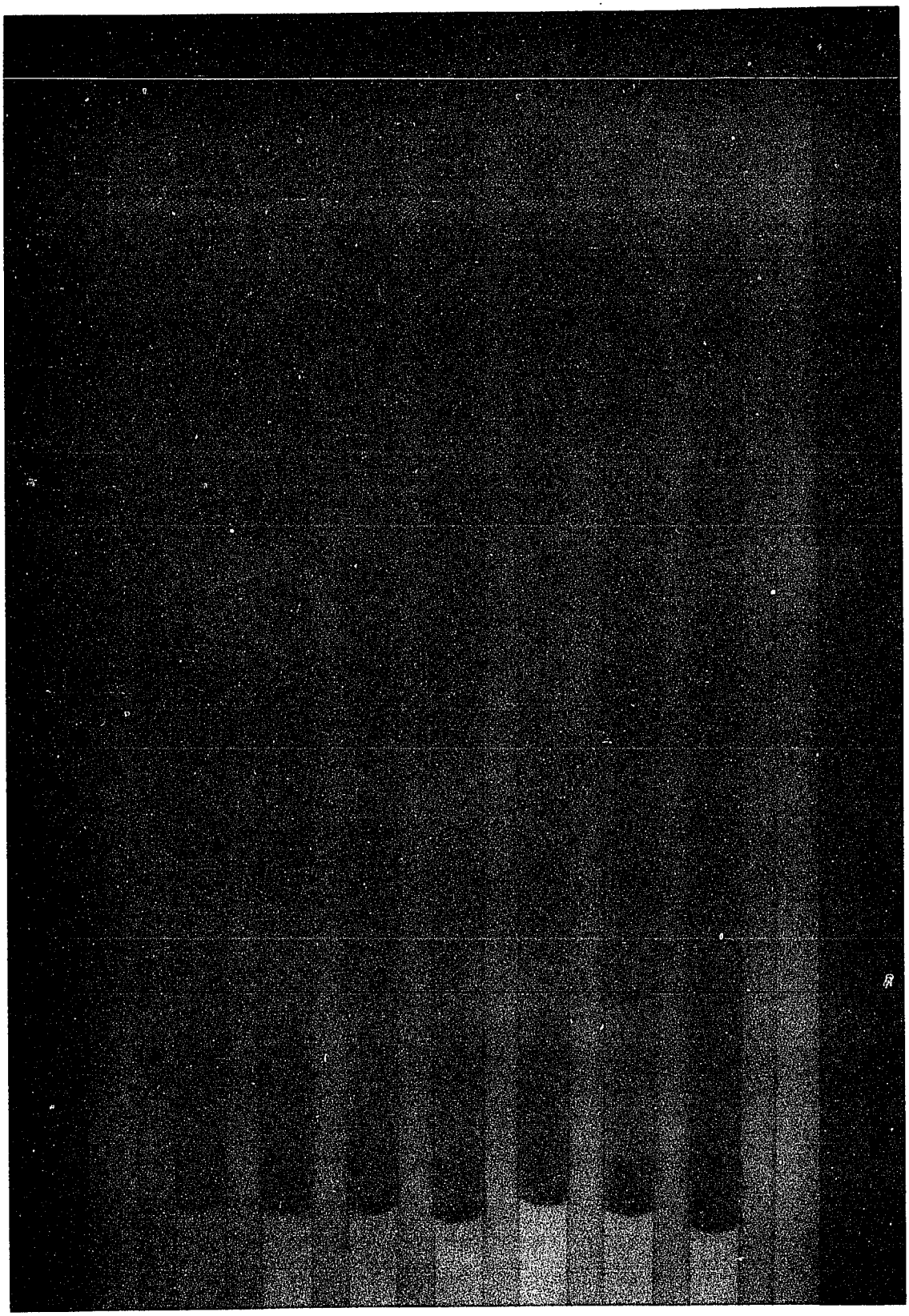


Fig. 12. Calibration curve for SDS-polyacrylamide gel electrophoresis.

The standards included the following proteins: phosphorylase b (p, 94,000), bovine serum albumin (a, 67,000), ovalbumin (o, 43,000), carbonic anhydrase (c, 30,000), trypsin inhibitor (t, 20,100), and α-lactalbumin (l, 14,400).

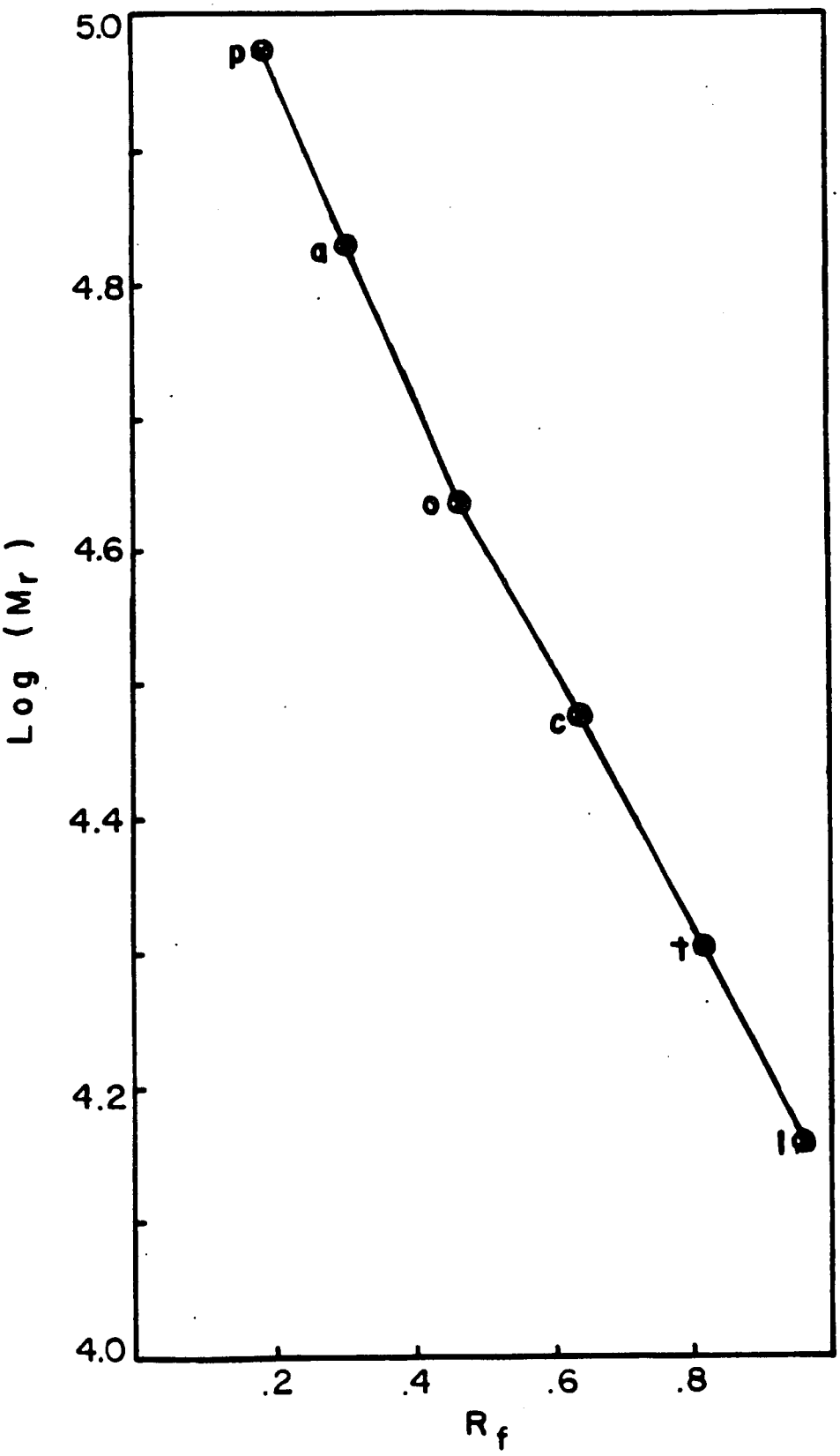

5.0
4.8
4.6
4.4
4.2
4.0
Log (M_r)
p
a
o
c
t
l
.2
.4
.6
.8
R_f

TABLE 3

COMPARISON OF SUBUNIT MOLECULAR WEIGHTS OF BOVINE ARGINASE ISOZYMES DETERMINED BY SDS POLYACRYLAMIDE ELECTROPHORESIS

Enzyme Source	Octyl Sepharose Pool A	Octyl Sepharose Pool B
Liver L1	49,900	37,800
Brain B1	61,100 50,600	69,300 49,400
Brain B2	49,400	79,100 70,300

second larger subunit as well (Fig. 11, gel 5). This represents either a contaminant, or a distinctive subunit composition for this particular isozyme.

The more hydrophobic brain arginases (pools B1B and B2B) do not share a common sized subunit with the comparable liver enzyme (pool L1B). B1B and B2B appear to be at least 80-85% pure on the basis of densitometry scans. They are composed of two subunits that are distinctly larger than the single L1B subunit (69,300 and 49,400 for B1B vs L1B 37,800; 79,100 and 70,300 for B2B vs 37,800 in L1B).

2) Two Dimensional Gel Electrophoresis of L1B and L1A. The result of fractionating the L1B arginase by two dimensional electrophoresis where isoelectric focusing was the first dimension appears in Fig. 13A. There are two major spots whose M_r was determined to be 37,800, in agreement with the value obtained on one dimensional SDS gels (Table 3). The migration of the more basic spot in SDS was marginally slower than that of the other molecule, possibly indicating a slightly greater molecular weight. Fig. 13B shows a similar two dimensional separation of the L1A protein. Three separate spots are observed. A trail of basic material is also seen to the left of the first spot. These proteins have a M_r of 51,300 which corresponds to the value obtained on one dimensional SDS gels (Table 3). In data not shown, two dimensional separation of B1B arginase also resolved each subunit (Table 3) into at least two spots. Separation by isoelectric point showed the larger M_r subunit to be a distinctly more basic protein than the other subunit.

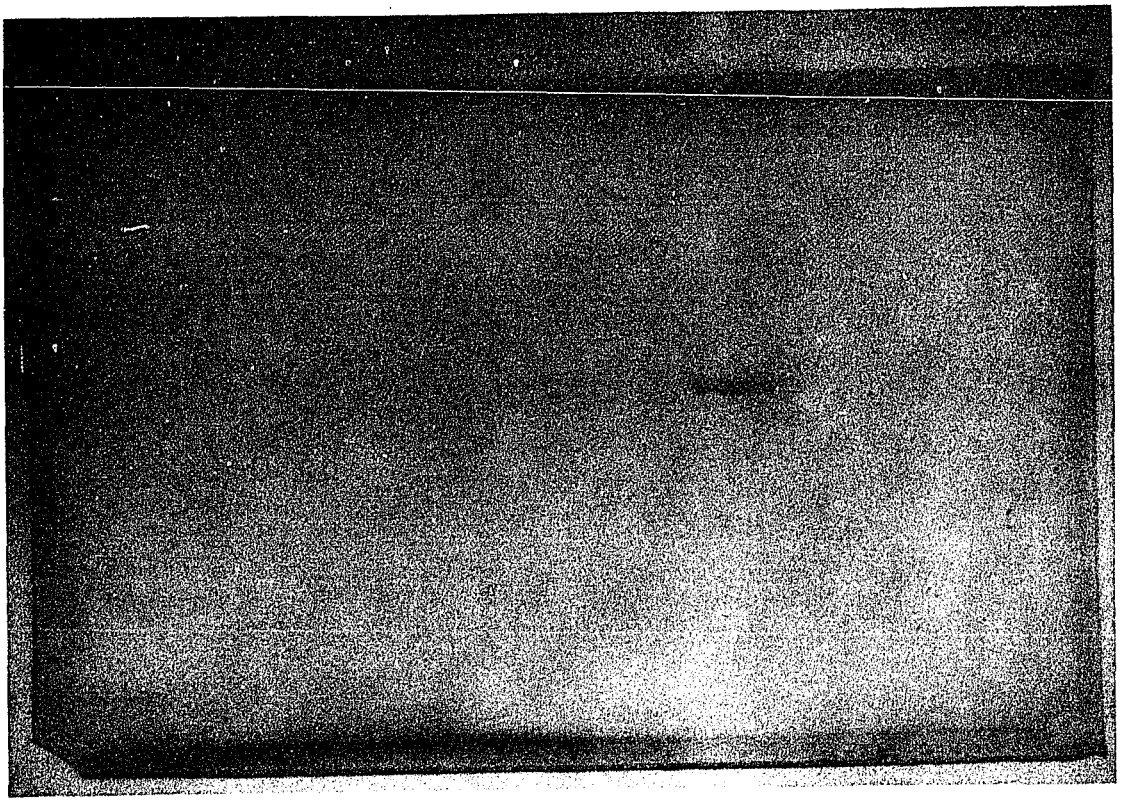
The influence of Mn^{2+} on the two dimensional separation of the

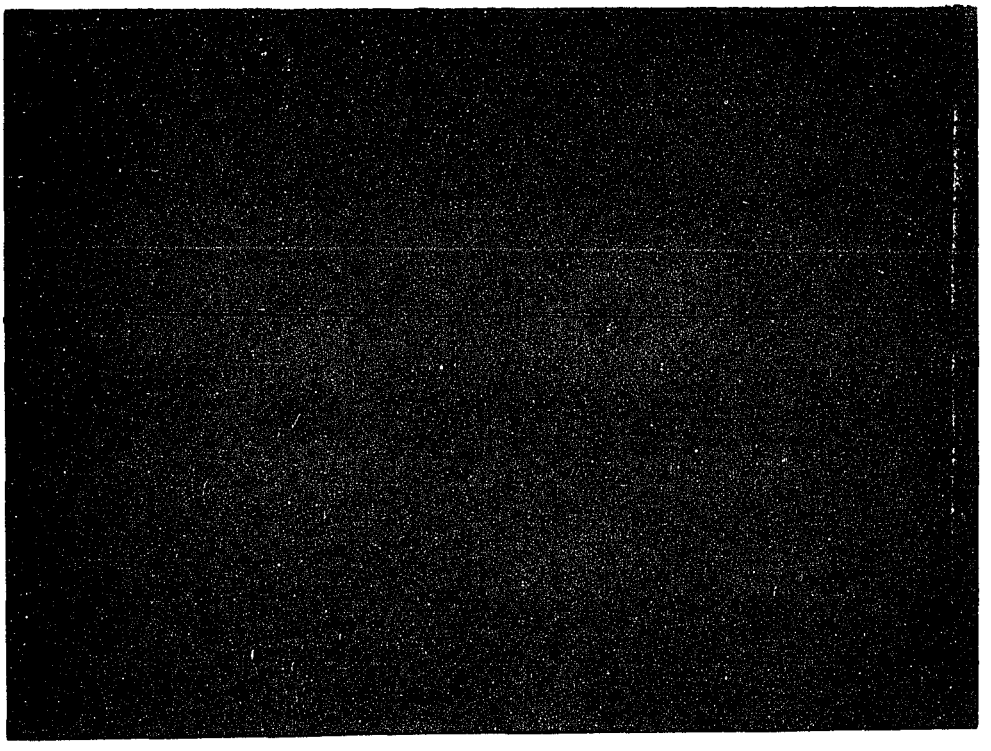
Fig. 13. Two dimensional separation of L1B and L1A arginases.

3 µg of sample protein was separated by electrophoresis in two dimensions. The direction of migration in SDS was from top to bottom, and the separation by pH was from left to right (basic to acidic pH).A complete description of the electrophoretic conditions is given in Methods.

A. L1B

B. L1A





liver preparations was also investigated. Protein samples, gel solutions, and buffers for the first dimensional separation were all made 1 mM in either EDTA or Mn^{2+}, and electrophoresis was run thereafter as described in Methods. Separation in SDS was accomplished without the addition of EDTA or Mn^{2+}. Fig. 14 reflects the effect of adding Mn^{2+} to L1B and L1A, while Fig. 15 shows the influence of EDTA on their mobilities. As was true for the native gel electrophoretic results (Figs. 8 and 10), the addition of EDTA or Mn^{2+} changed the overall spot pattern of L1B only slightly (compare Fig. 13A with Figs. 14A and 15A). EDTA may create some minor broadening of the spots, but the experiment would need to be repeated to confirm this result. Manganese or the lack thereof has a decided effect on the charge of the L1A subunit(s). Although a trail of basic material still remains after Mn^{2+} is added to L1A, the major spot focuses closer to the anode, and the other two spots that were originally observed are present in only trace amounts (compare Figs. 13B and 14B). EDTA treatment, in contrast, abolishes the trail (Fig. 15B). Three well-defined spots are instead visible in a ratio of roughly 3:2:1.

It is difficult to precisely determine the isoelectric point of a protein using the O'Farrell (1975) methodology, but the L1B subunit(s) appears to be slightly more acidic than the major (in terms of intensity of staining with Coomassie blue) L1A subunit.

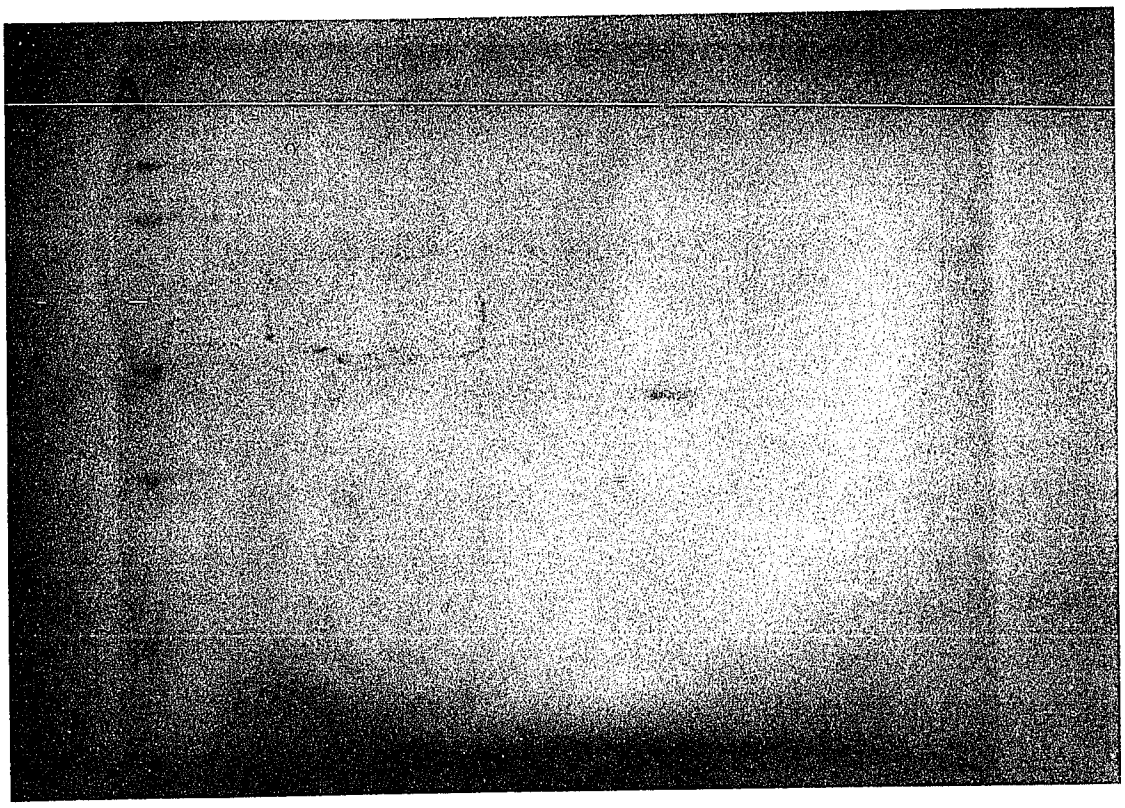
These experiments confirm the evidence from the one dimensional analysis in SDS that L1A and L1B are pure proteins. The M_r of L1B was consistently calculated to be 37,200, and the M_r of L1A was found to be 50,500. This analysis furthermore proves that L1A arginase loses Mn^{2+}

Fig. 14. Two dimensional separation of L1B and L1A arginases in the presence of 1 mM $MnCl_2$.

6 µg of sample protein was separated by isoelectric focusing in the presence of 1 mM $MnCl_2$. Fractionation in SDS was accomplished as described in Methods. The direction of migration in both dimensions was as in Fig. 13.

A. L1B

B. L1A



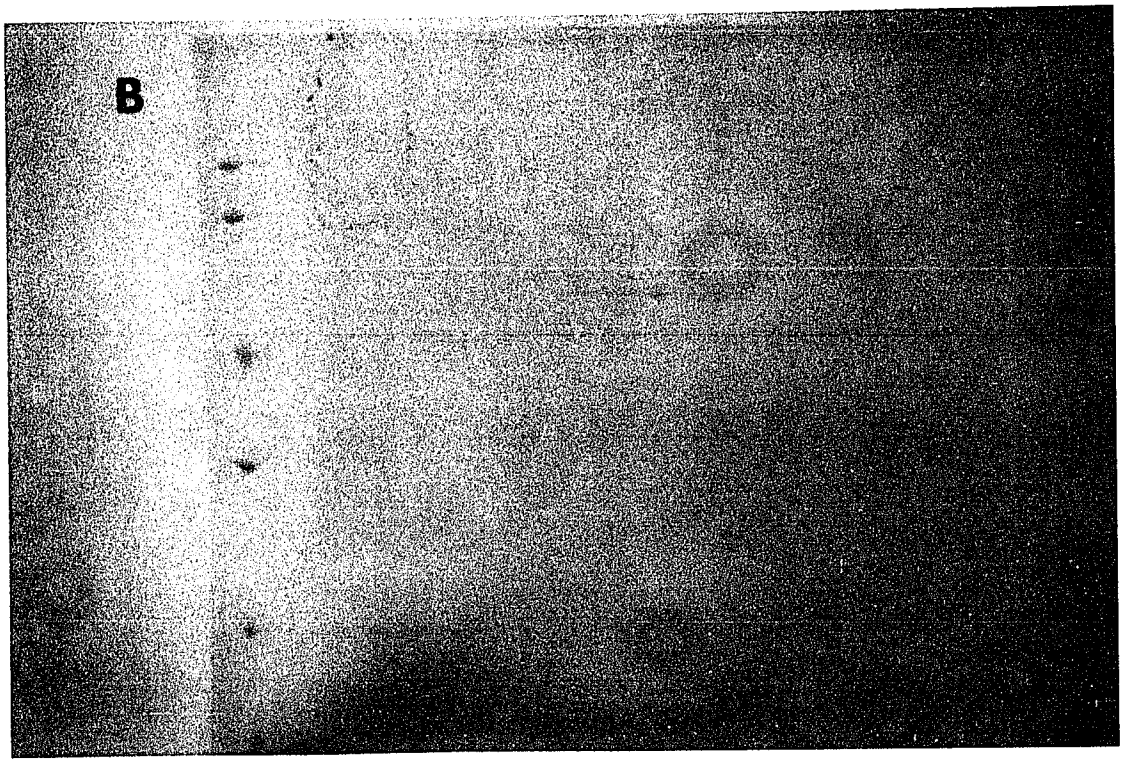
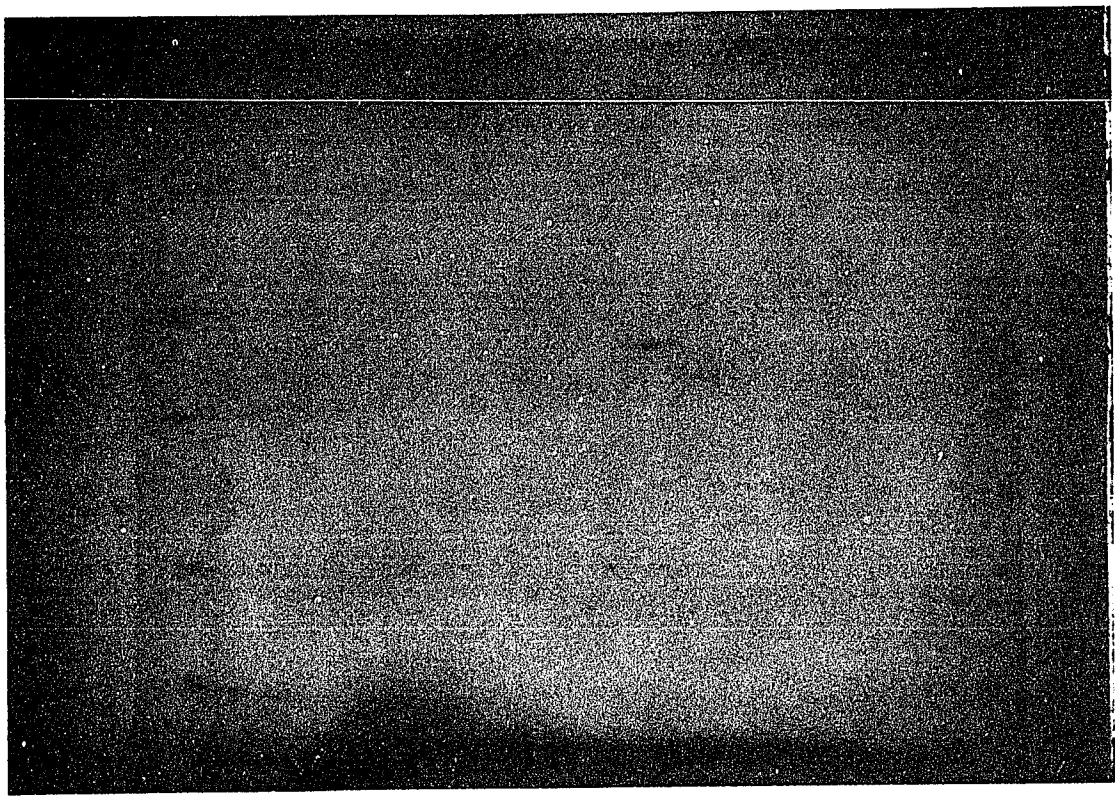

B

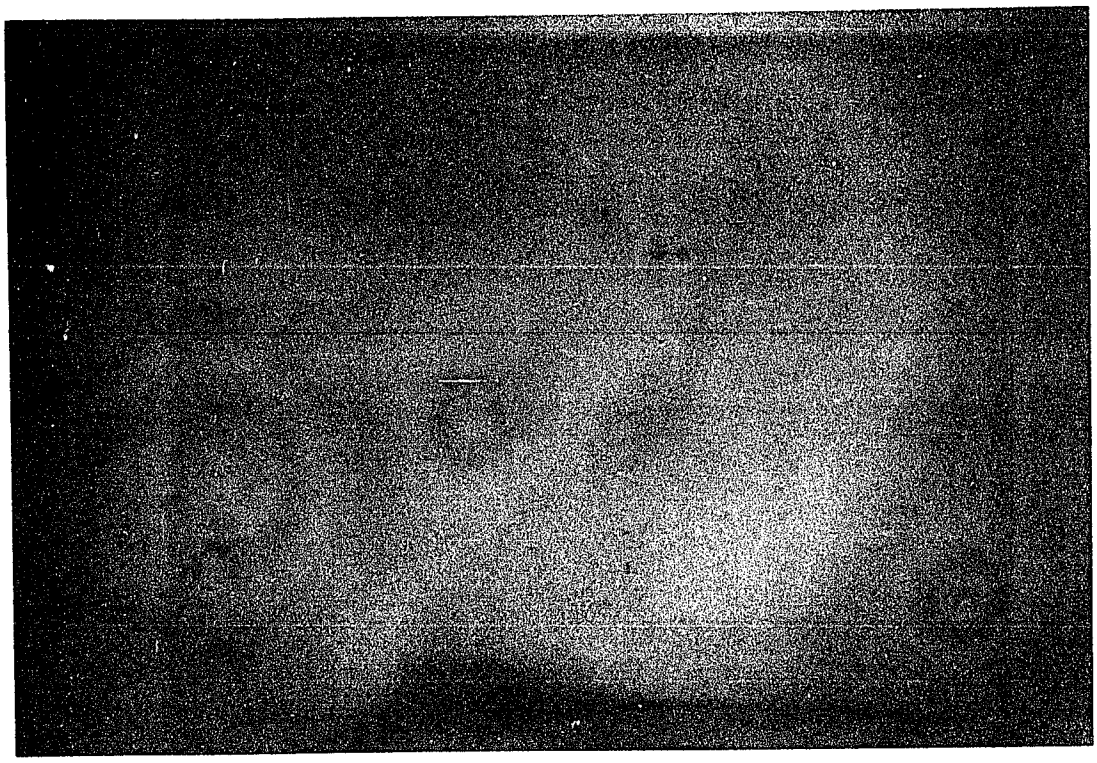
Fig. 15. Two dimensional separation of L1B and L1A arginases in the presence of 1 mMM EDTA.

6 µg of sample protein was separated by isoelectric focusing in the presence of 1 mM EDTA. Fractionation in SDS was accomplished as described in Methods. The direction of migration in both dimensions was as in Fig. 13.

A. L1B

B. L1A





much more easily than L1B arginase. To preclude anomalous findings, Mn^{2+} should therefore always be included during the purification and characterization of bovine liver arginases.

The number of different subunits in each sample is still not certain. The three spots shown for L1A may represent truly different proteins, or they be the same subunit which has lost variable quantities of metal ion. The consistent appearance of two spots in the L1B sample strongly implies that this protein is composed of two diverse subunits. However substantiation of this conclusion must await analysis of the preparation under a wider range of conditions which can clearly distinguish between potential sources of charge diversity such as amino acid substitutions, carbohydrate additions, and metal ion retention or loss.

C. Determination of S Value for the Intact L1B Enzyme By Analytical Ultracentrifugation

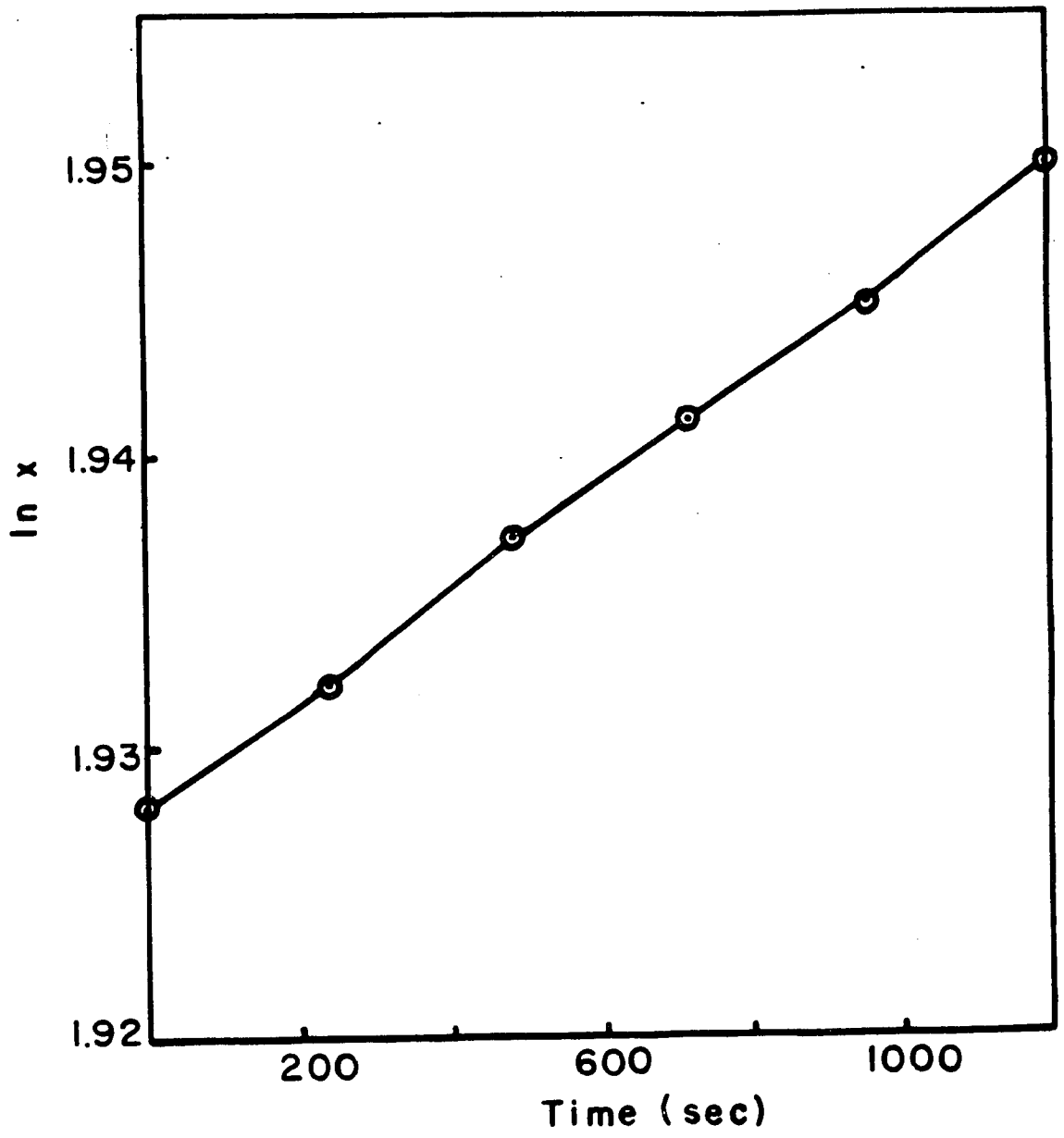
A sedimentation coefficient ($S_{20,w}$) of 6.0 S at a protein concentration of 3 mg/ml was obtained from a sedimentation velocity experiment using an analytical ultracentrifuge. A single Schlieren peak was observed. The S value was calculated by monitoring the distance between the protein boundary and the center of rotation as a function of sedimentation time (Fig. 16), and by using the formula

$$\ln x = s\omega^2 t$$

where x = distance from the center of rotation to the Schlieren peak

s = sedimentation coefficient (sec)

Fig. 16. Calibration curve for determination of the sedimentation coefficient of L1B arginase.


1.95
1.94
1.93
1.92
ln x
200
600
1000
Time (sec)

ω = angular velocity (radians/sec)

t = time (sec)

The observed sedimentation coefficient was corrected to the value obtained in water at 20°C ($S_{20,w}$).

D. Molecular Weight Determination by Gel Filtration On a Sephadex G-150 Column

The molecular weights of the two liver arginases and several brain enzymes were estimated by gel filtration on a column of Sephacryl S-200 (Pharmacia) or Altex Spherogel TSK-3000. These gels yielded anomalous results. L1B was eluted from a column of Sephacryl S-200 (1.5 x 95 cm) as a single symmetrical peak whose A_{235} and enzyme activity profiles matched exactly (data not shown). The molecular weight of this protein was estimated to be 97,700 which is 10-20% lower than any published figures. A similar application of L1A to the S-200 column gave a M_r of 93,400. These low estimates of molecular weight may have been caused by the adsorption of the proteins onto the gel. Sephacryl S-200 has been reported to act as a cation exchanger at pH 8, but the use of 0.2 M NaCl in the buffer lessens this effect (Belew et al., 1978). This explanation is not applicable to bovine liver arginases for two reasons: 1) 0.2 M NaCl was included in the elution buffer, and 2) at the pH at which the experiment was run (pH 7), liver arginase does not bind to cation exchangers (i.e., step 5 of the purification scheme, chromatography on CM-cellulose). Some other feature(s) of liver arginases must induce their abnormal retention on S-200. The sugar attached to arginase (see following section) is such a potential source of error in molecular

weight determination by gel filtration (Andrews, 1965; Smith and Winkler, 1967; Leach et al., 1980).

Because of the problems associated with the use of S-200, the molecular weights of the brain arginases were not examined by this method. Instead both liver and brain arginases were analyzed on a high performance liquid chromatography (HPLC) gel exclusion column. The molecular weights calculated for the samples using this technique were consistently low. The HPLC column separation of brain arginases gave multiple protein peaks. However when these peaks were analyzed for arginase activity and subunit composition on SDS gels, they were found not only to be enzymatically active, but also to have approximately the same subunit sizes originally determined. Clearly the proteins were being retained on the column for abnormally long periods of time.

Arginase preparations were next run in buffer containing a high salt concentration (0.5 M vs 0.25 M KCl) to prevent this binding. The increase in salt did not substantially diminish the elution time of a liver preparation (L1B). It also did not alter the pattern of elution of the standard proteins. Because arginase is capable of strong hydrophobic interactions, it was possible that the enzyme was interacting in this manner with the hydroxylated polyether of the column. To preclude this problem, 60% ethylene glycol was included in the eluant buffer in place of salt. However, this caused a build up of column back pressure which necessitated a 50% reduction in elution flow rate so as to prevent the build up of too great a pressure. In addition, the elution characteristics of the standard proteins became anomalous: ferritin (440,000) eluted with Blue Dextran ($>10^6$), while ovalbumin (43,000) migrated with

aldolase (158,000). Use of 40% ethylene glycol relieved the pressure problem, but it failed to eliminate the abnormal elution of the standards. Although sensitive and rapid, analytical gel exclusion chromatography is an unsatisfactory method for estimating the molecular size of proteins such as arginase.

The molecular weight and Stokes' radius calibration curves for the Sephadex G-150 column eventually adopted for these determinations are presented in Figs. 17 and 18. The parameters determined for liver and brain arginases from these experiments are listed in Table 4. In each case, the more hydrophobic enzyme (pool B) was larger, although this difference was more pronounced for the liver proteins. There was an excellent concurrence between protein and enzyme activity for L1B arginase as eluted from the G-150 column. This exact coincidence did not occur for B1B arginase: the peak of enzyme activity was at a somewhat higher M_r than that for protein. To ascertain whether this result effected an increase in purification of the enzyme, active enzyme fractions were pooled, dialyzed to remove salt, concentrated, and analyzed by SDS-PAGE. There was only one subunit present in this B1B arginase pool which had a M_r of 68,200. This was comparable to the value of 69,300 shown in Table 3. In contrast, when assayed for enzyme activity, this pool had a noticeably lowered specific activity of 12.8 unit/mg compared to the 62.53 unit/mg listed in Table 2B. When L1A and B1A arginases were eluted from G-150, their protein and enzyme activity profiles were consistently out of step with each other. A substantial amount of the protein material in these preparations (of lower M_r) eluted after the active enzyme fractions. This result also repeatedly

Fig. 17. Calibration curve for molecular weight determination of liver and brain arginase isozymes by gel filtration on a Sephadex G-150 column.

The protein standards included the following: aldolase (a, 158,000), phosphorylase b (p, 94,000), bovine serum albumin (b, 67,000), ovalbumin (o, 43,000), trypsin inhibitor (t, 20,100), myoglobin (m, 16,900), ribonuclease A (r, 13,700).

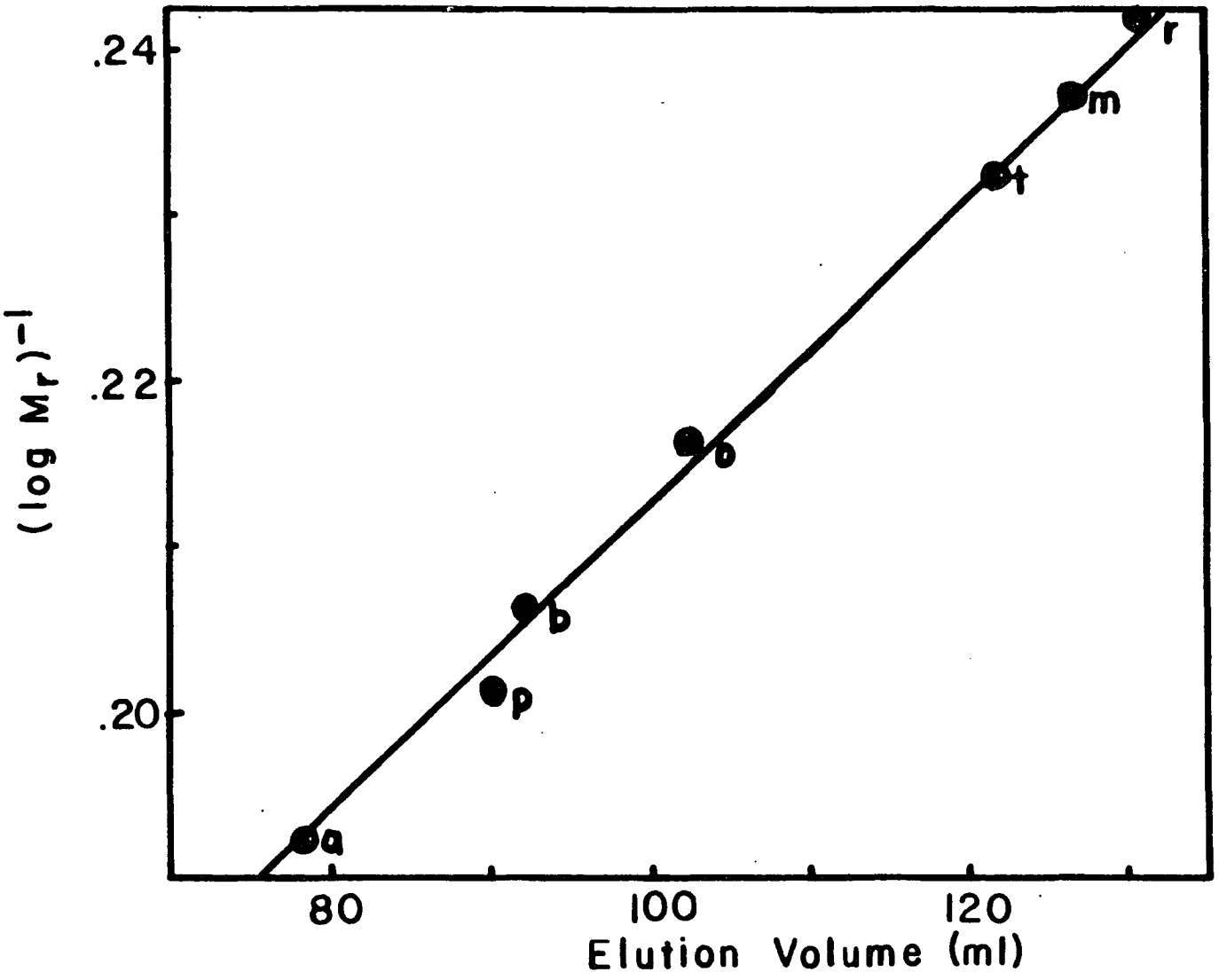

.24
.22
.20
(log M_r)$^{-1}$
80
100
120
Elution Volume (ml)
r
m
t
b
b
p
a

Fig. 18. Calibration curve for Stokes' radius determination of liver and brain arginase isozymes by gel filtration on a Sephadex G-150 column.

The protein standards consisted of: aldolase (a, 48.1); albumin (b, 35.5), ovalbumin (o, 30.5), trypsin inhibitor (t, 20.8), ribonuclease A (r,16.4).

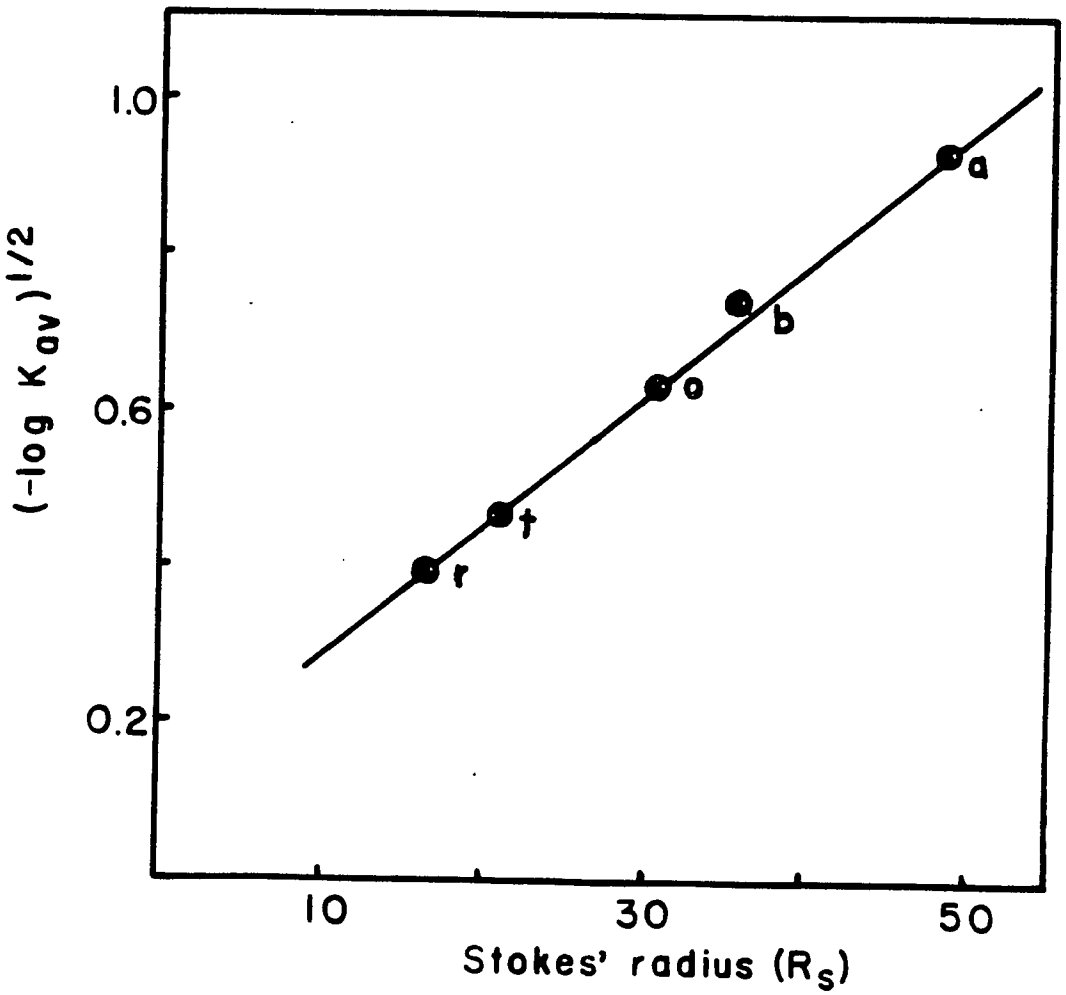

1.0
0.6
0.2
$(-\log K_{av})^{1/2}$
a
b
o
t
r
10
30
50
Stokes' radius (R_S)

TABLE 4

MOLECULAR WEIGHT AND STOKES' RADIUS OF LIVER AND BRAIN ARGINASES DETERMINED ON SEPHADEX G-150

Pool	Molecular Weight	Stokes' Radius (R_s)
Liver		
L1A	105,600	41.4
L1B	119,600	43.2
Brain		
B1A	110,400	41.7
B1B	114,300	42.5

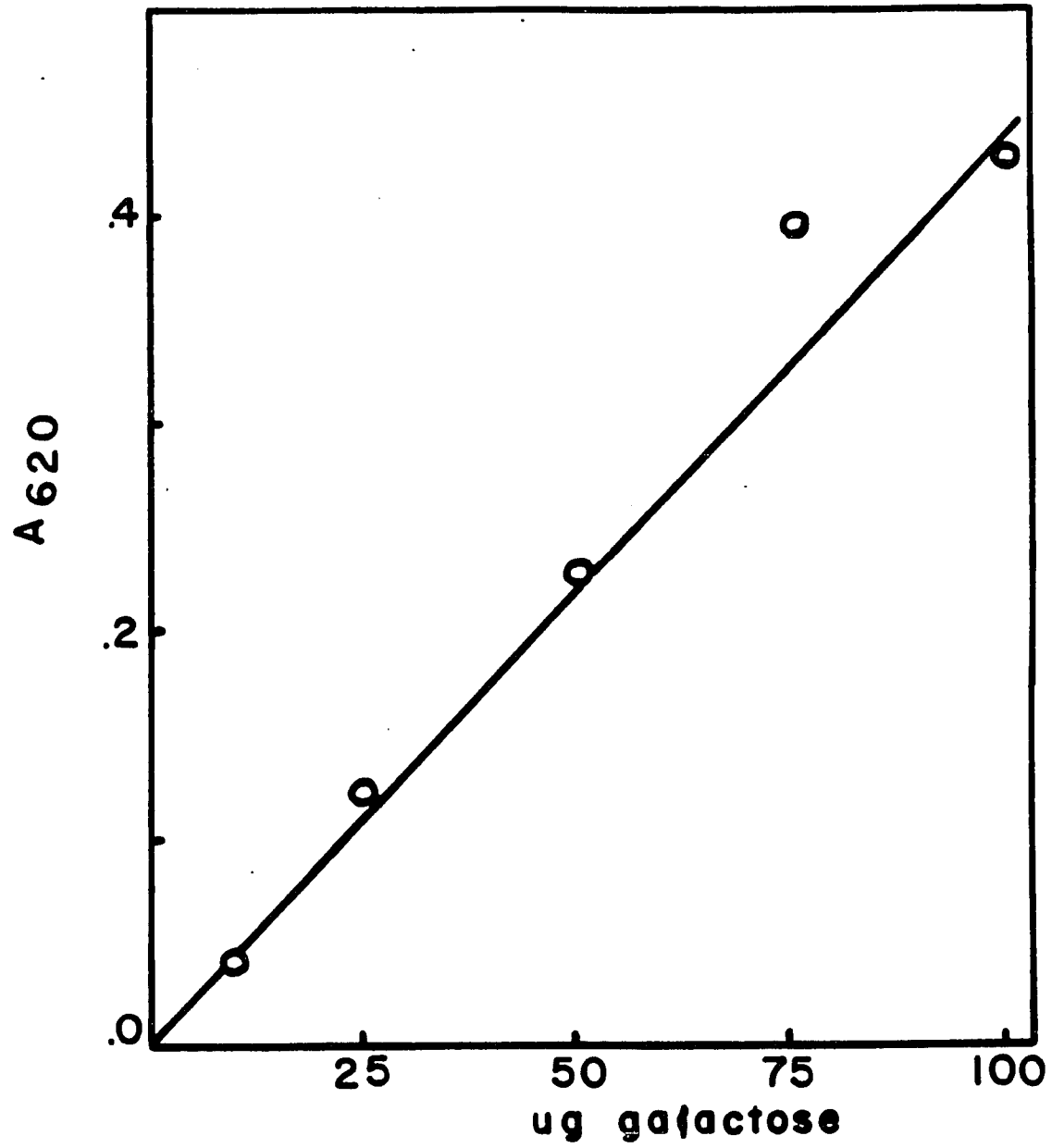
occurred when L1A was chromatographed on Sephacryl S-200. SDS-PAGE was run on the active L1A and B1A enzymes recovered from the G-150 column. No change in the electrophoretic patterns of the samples was observed relative to those shown in Fig. 11.

E. Hexose Content

Liver and brain preparations were tested for their carbohydrate composition using the anthrone reagent which reacts with diols to produce a green-blue color. The curve generated using galactose as a standard is shown in Fig. 19. L1B arginase contained 1-2% carbohydrate by weight, as did the B1B protein. Sugar was not detected in L1A arginase, while it represented only 0.2-0.5% of the B1A protein. These estimates are not quantitatively reliable because the absorbances observed were near the lower detection limit of the spectrophotometer. Restrictions on the quantity of protein available for this analysis prevented a confirmation of the findings. In addition, because the specific carbohydrate(s) attached to the protein is unknown, the system may not have been optimized. For example, mannose standards produce only 80% of the absorbance at 620 nm of galactose solutions. Glycoproteins often contain a significant number of attached mannose residues. If arginase belongs to this class of proteins, then its actual carbohydrate moiety may be larger than reported here.

Fig. 19. Calibration curve for estimation of carbohydrate composition of liver and brain arginases.

Increasing amounts of a galactose standard solution (100 µg/ml) were used.


A620
.4
.2
.0
25
50
75
100
ug galactose

F. Amino Acid Compositional Analysis

Table 5 lists the amino acid compositions of the L1A and L1B arginases. These calculations are based on the native enzyme sizes presented in Table 4. Both of these proteins contain more acidic than basic amino acids, although L1A has a greater excess of acidic residues. This finding is at variance with their behavior in 6 M urea disc gels: L1B is less negatively charged in such an environment, yet it migrates further toward the anode. The unknown contribution of asparagine and glutamine residues may account for the discrepancy.

Table 6 presents amino acid compositional data for all of the arginase enzymes isolated from bovine brain and liver tissues. The data are given as number of amino acid residues/100 residues. The degree of homology between the proteins was analyzed by applying the statistical method of Marchalonis and Weltman (1971) to the data (Table 7). Values of $S\Delta Q$ are all less than 75, ranging from 4-72. This reflects a high degree of relatedness. L1B and B2B arginases are the most dissimilar of the arginases as judged by the larger $S\Delta Q$ values calculated for these two proteins.

The amino acid data in Table 6 were also analyzed to establish the cellular location of the proteins, i.e. soluble or membrane-bound. The parameters required for this determination (Barrantes, 1975) are listed in Table 8. In no case did an arginase enzyme meet the expectations of an integral membrane protein. All of the arginases, except L1B, fell within the ranges allowed for a soluble or peripheral membrane protein. The R_3 ratio for L1B (0.883) clearly placed it within the non-membrane

TABLE 5

AMINO ACID COMPOSITION OF BOVINE LIVER ARGINASES

Amino Acid	Residues/mole Protein			
	L1A	nearest integer	L1B	nearest integer
Aspartic Acid	106.0	106	89.6	90
Threonine (1)	39.8	40	72.7	73
Serine (1)	63.8	64	78.2	78
Glutamic Acid	96.8	97	96.5	97
Proline	40.1	40	69.1	69
Glycine	81.5	82	111.1	111
Alanine	100.7	101	66.1	66
Half Cystine (2)	12.1	12	12.5	13
Valine	74.7	75	86.7	87
Methionine	9.8	10	21.9	22
Isoleucine (3)	62.1	62	68.1	68
Leucine (3)	87.7	88	115.2	115
Tyrosine	26.3	26	35.6	36
Phenylalanine	36.9	37	37.0	37
Histidine	14.5	15	22.1	22
Lysine	72.2	72	77.2	77
Arginine	42.2	42	39.1	39
Tryptophan (4)	5.6	6	5.3	5
Total	972.8	975	1104.0	1105

(1) Extrapolated to zero time

(2) Determined as cysteic acid

(3) Extrapolated to infinite hydrolysis time

(4) Determined after hydrolysis with methanesulfonic acid and 3(2-aminoethyl)indole

TABLE 6

A COMPARISON OF THE AMINO ACID COMPOSITION

OF BOVINE LIVER AND BRAIN ARGINASES

Amino Acid	Residues/100 residues					
	Liver (1)		Brain (2)			
	L1A	L1B	B1A	B1B	B2A	B2B
Aspartic Acid	11.0	8.2	11.1	11.0	10.2	10.8
Threonine	4.1	6.7	4.3	4.7	5.0	4.4
Serine	6.6	7.2	5.4	5.7	5.0	4.4
Glutamic Acid	10.1	8.8	10.3	10.4	10.0	12.6
Proline	4.1	6.3	4.5	4.8	5.6	5.8
Glycine	8.4	10.2	8.4	9.1	9.2	7.6
Alanine	10.5	6.0	9.4	10.1	9.4	10.2
Half-cystine	1.1	1.0	0.8	0.9	0.8	0.3
Valine	7.8	7.9	8.1	6.8	8.3	6.7
Methionine	1.0	2.0	1.9	1.7	1.7	1.8
Isoleucine	6.4	6.3	6.0	6.2	6.0	5.3
Leucine	9.1	10.5	8.8	8.3	8.6	8.8
Tyrosine	2.7	3.3	2.4	2.6	2.5	1.8
Phenylalanine	3.8	3.4	4.1	3.9	3.8	3.8
Histidine	1.5	2.0	2.1	1.8	2.1	2.3
Lysine	7.5	7.0	7.9	7.9	7.9	9.1
Arginine	4.4	3.5	4.5	4.4	4.2	4.4

(1) Based on native enzyme composition in Table 5

(2) Based on values obtained after a single 24 hr hydrolysis.

TABLE 7

AN ESTIMATE OF BOVINE LIVER

AND BRAIN ARGINASE RELATEDNESS

(S Δ Q)

	L1B	B1A	B2A	B1B	B2B
L1A	49.79	4.76	9.87	4.84	22.00
L1B		43.85	32.28	45.47	72.07
B1A			3.71	3.54	13.76
B2A				5.05	16.19
B1B					13.34

Calculations are based on values in Table 6. Half-cystine values were not used in these calculations

TABLE 8

PARAMETERS NECESSARY FOR

CLASSIFICATION OF PROTEIN AS

SOLUBLE OR MEMBRANE BOUND

Protein	Hydrophilic Residues (1)	Hydrophobic Residues (2)	R_3 (3)
L1A	34.5	30.8	1.120
L1B	29.5	33.4	0.883
B1A	35.9	31.3	1.147
B1B	35.5	29.5	1.203
B2A	34.4	30.9	1.113
B2B	39.2	28.2	1.390

(1) Hydrophilic residues = Lys+Arg+His+Asx+Glx/100 residues

(2) Hydrophobic residues = Ile+Tyr+Phe+Leu+Val+Met/100 residues

(3) R_3 = Hydrophilic/hydrophobic

Calculations are based on values listed in Table 6

class. B2B protein may be peripherally attached to a membrane because the R_3 ratio estimated for it lay closer to that of the average peripheral protein than to that of the average non-membrane protein (Barrantes, 1975).

The elution profile of the amino acids frequently showed the presence of another (non amino acid) ninhydrin-positive component, eluting shortly after ammonia. The identification of this extra (sometimes very large) peak was not attempted, but might be of interest in later work.

Antibody Production and Immunochemical Analysis

Immunoglobulin IgG Purification

After the homogeneity of L1B arginase had been established, a total of 140 µg was injected into each of two rabbits as detailed in Methods. Serum recovered after cardiac puncture of the animals was purified to obtain the immunoglobulin fraction. Immunoglobulins were precipitated from serum with 40% ammonium sulfate, and separated into classes by applying them to a DEAE-Sephacel column. Three pools were made, concentrated by ammonium sulfate precipitation, and tested on micro double immunodiffusion plates for reaction with the L1B arginase antigen. Only the immunoglobulin IgG fraction reacted. This fraction was employed in all subsequent immunochemical analyses.

Quantitative Immunoprecipitation of L1B Arginase

The ability of a given amount of purified immunoglobulin to inhibit enzyme activity was evaluated by incubating it with L1B protein. The results appear in Fig. 20. Antibodies obtained from both animals were capable of inhibiting at least 88% of the arginase activity. The inhibition was greater than that calculated, however, because excess arginase was present in control supernatants to the extent that product inhibition by ornithine prevented an accurate assessment of the enzymatic activity originally present. A more representative titration curve of enzyme inhibition by immunoglobins would necessitate starting with lesser quantities of enzyme. Fig. 20 nevertheless does illustrate differences in affinity of the two IgG fractions for arginase. On a g/g basis three times as much IgG from rabbit #1 (Fig. 20A) was required to inhibit 70% of the available arginase activity.

Analysis of Structural Similarities Between Arginases By Double Immunodiffusion Plates

Immunoglobulin IgG fractions isolated from sera of two rabbits were tested for their specificity by incubation in micro double immunodiffusion plates with the purified bovine liver and brain arginase preparations. The results of the experiment are to be found in Figs. 21 and 22. Serum removed from the rabbits prior to immunization showed no precipitin line on immunodiffusion plates (data not shown). Possible cross-reactivity of the immunoglobulins with arginase from another species was determined by including mouse liver and brain homogenates in

Fig. 20. Quantitative immunoprecipitation of L1B enzyme activity by rabbit anti-L1B IgG.

Inhibition of arginase activity by anti-L1B arginase IgG was performed as described in Methods. (●—●) % inhibition; (x-x-x) arginase activity using 36,000 dpm of ^{14}C-arginine per assay.

A. 0.60 mg rabbit #1 IgG was incubated with L1B arginase

B. 0.19 mg rabbit #2 IgG was incubated with L1B arginase

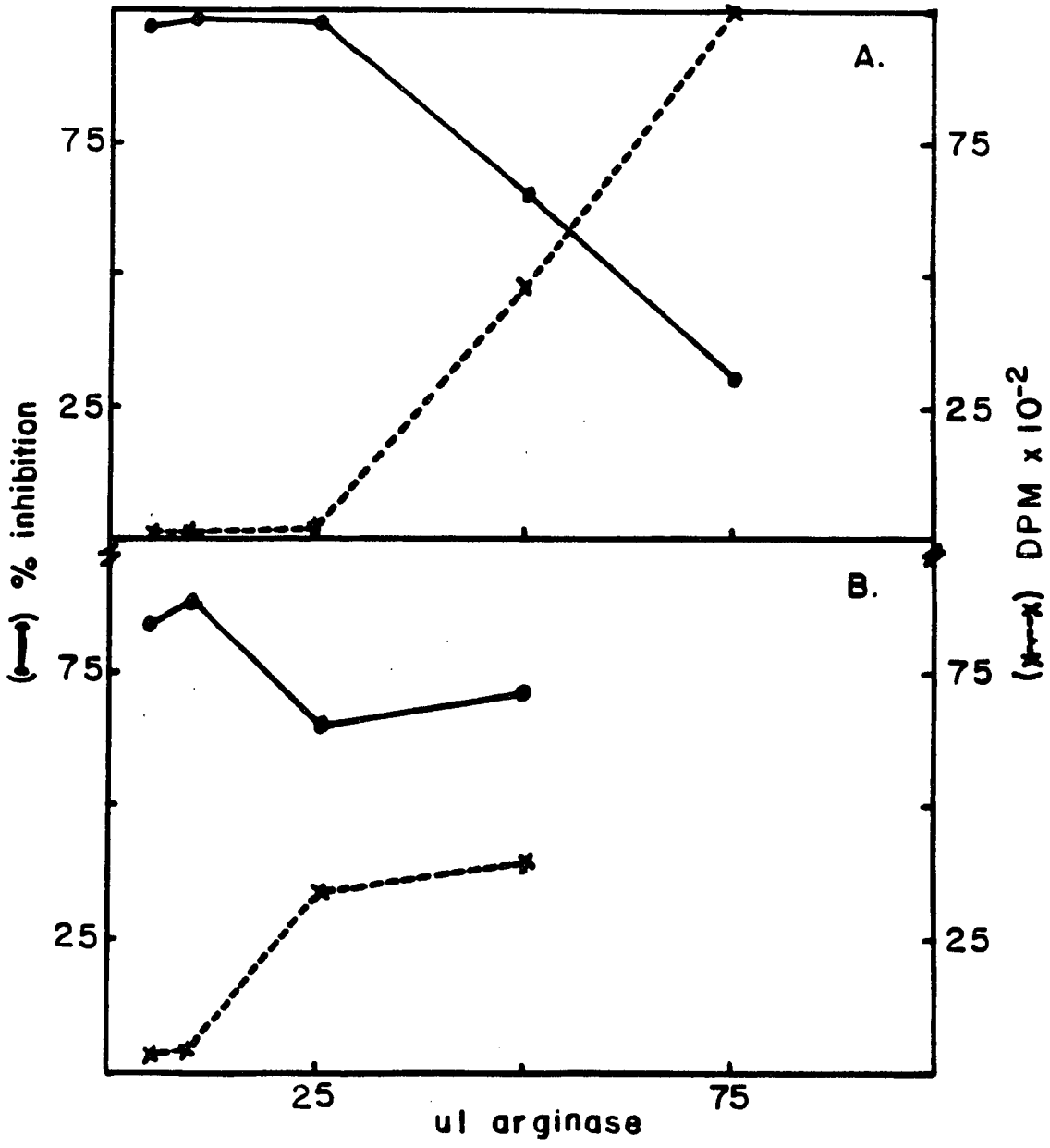
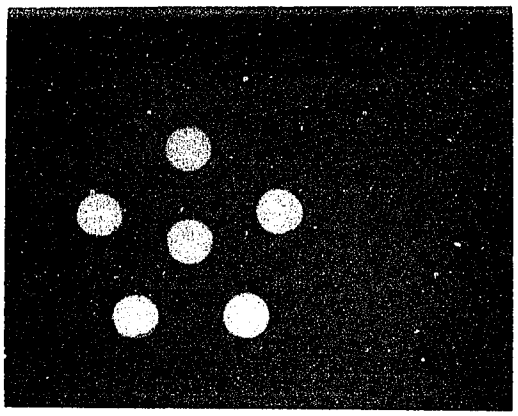

A.
B.
75
25
75
25
75
25
25
75
(—●) % inhibition
(x--x) DPM x 10⁻²
ul arginase

Fig. 21. Comparison of specificity between immunoglobulins from rabbit #1 and #2 on a micro immunodiffusion plate.

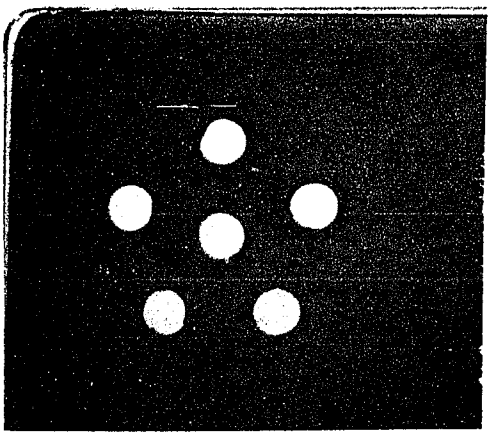
The protein concentrations of bovine brain and liver samples were 0.26-0.36 mg/ml; mouse liver protein concentration was 6 mg/ml.

A. The center well contained rabbit #1 IgG at a concentration of 24.1 mg/ml. The outer wells contained, clockwise from top: L1B; B1B; B2B; mouse brain homogenate; mouse liver homogenate.

B. The center well contained rabbit #2 IgG at a concentration of 7.6 mg/ml. The outer wells were as in A.



B
Rabbit 2 IgG



Rabbit 1 IgG

Fig. 22. Comparison of specificity between immunoglobulins from rabbits #1 and #2 on a micro immunodiffusion plate.

The antigen concentrations were as in Fig. 21.

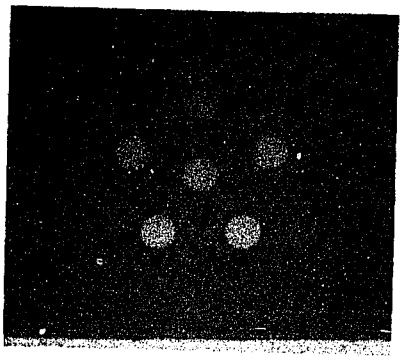
A. The center well contained rabbit #2 IgG at a 7.6 mg/ml concentration. The outer wells held, clockwise from top: L1B; L1A; B1A; B2B; B1B.

The center well in B-D contained rabbit #1 IgG at 7.3 mg/ml.

B. The outer wells were, clockwise from top: L1B; B1B; B2B; semi-pure B2B; L1A.

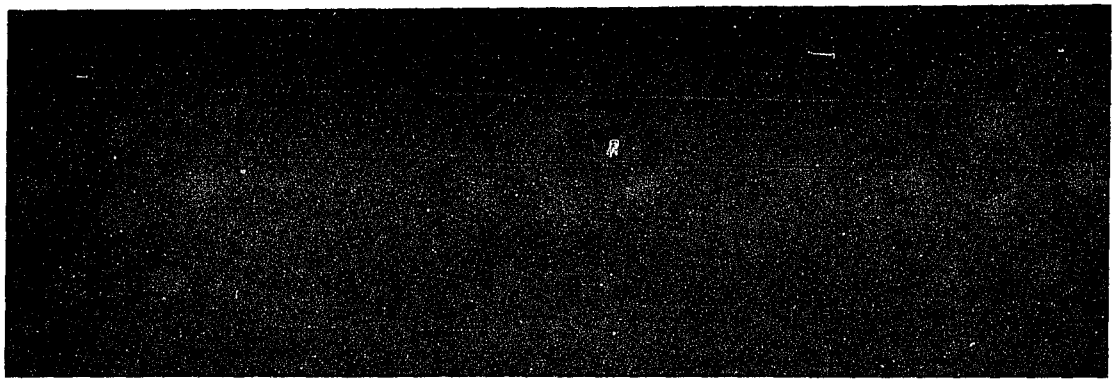
C. Clockwise from top, the outer wells contained: L1B; B1B; B2A; B1A; L1A.

D. The top outer well held L1B; the other well contained L1A.



A

Rabbit 2 IgG



B C D

Rabbit 1 IgG

the assay (Fig. 21). Neither IgG reacted with a mouse brain homogenate. While the IgG from rabbit #1 did form a precipitin line with a mouse liver preparation, the IgG from rabbit #2 did not (Fig. 21). The IgG from rabbit #2 also did not precipitate B1A (Fig. 22A).

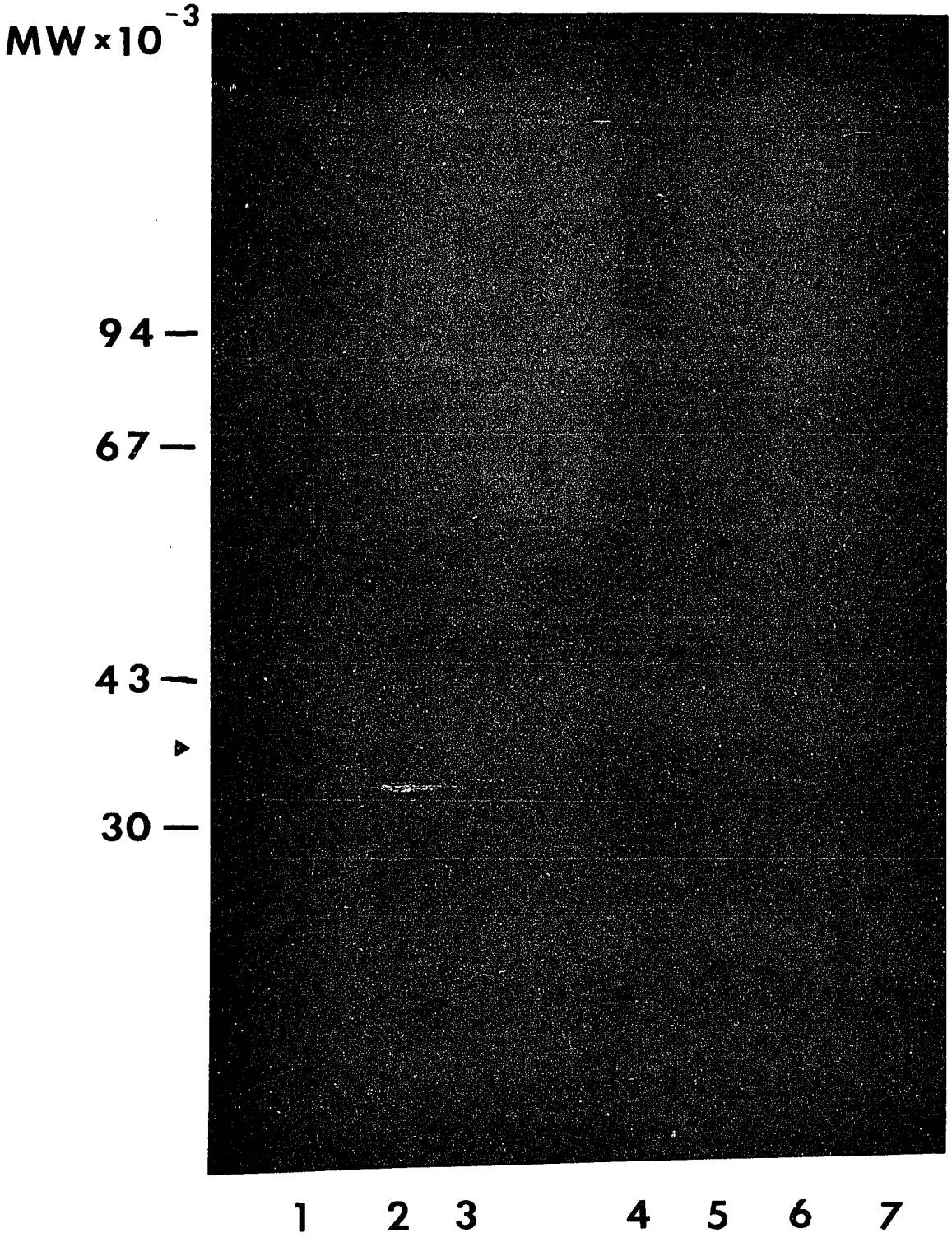
The polyclonal nature of the antibodies produced in this experiment was revealed after a close examination of the precipitin lines seen in Figs. 21 and 22. The predominant IgG family recognized similar determinants located on L1A, L1B, B1B, and B2B arginases. A second class of antibody reacted with determinants shared by L1B, B1B, L1A, B1A, and B2A proteins. A final type of IgG molecule detected a specificity found on L1B arginase, mouse liver arginase, and possibly L1A arginase.

Electrophoresis and Fluorography of Translated Products

Polyadenylated RNA specifically enriched for the arginase messenger(s) was isolated from bovine liver polysomes as described in Methods. A portion of the preparation was translated in a reticulocyte lysate system, and the translated products were separated by SDS-PAGE. Fig. 23 shows the result. A polypeptide having the same M_r as authentic L1B was immune precipitated from the total proteins translated from the mRNA preparation. This protein was not evident in the control immunoprecipitate (compare lanes 5 and 1, Fig. 23). Another polypeptide of lower M_r (29,800) was also uniquely translated from the liver mRNA. This protein is probably the translation product of a prematurely terminated L1B arginase messenger. The L1A messenger may have been isolated from the polysomes as well because a protein of 50,400 M_r was immune precipitated from the total polypeptides synthesized from the sample mRNA. Lane 7

Fig. 23. Electrophoretic analysis of mRNA-dependent rabbit reticulocyte lysate translation products.

Electrophoresis of ^{35}S-labeled proteins was performed in a 7.5% polyacrylamide slab gel in the presence of 0.1% SDS. Fluorography of the dried slab gel was performed with Kodak XAR-5 film. From left to right:
(1) total control translation products; (2) control translation products immune precipitated with rabbit #1 IgG; (3) control translation products immune precipitated with rabbit #2 IgG; (4) total translation products of purified arginase-specific mRNA; (5) arginase-specific mRNA translation products immune precipitated with rabbit #1 IgG; (6) arginase-specific mRNA translation products immune precipitated with rabbit #2 IgG; (7) total translation products of a second isolation of arginase specific mRNA. The left lane contains marker proteins for molecular weight estimation. The arrow indicates the migration position of authentic L1B arginase.


MW x 10 -3
94
67
43
30
1
2
3
4
5
6
7

(Fig. 23) shows the result of an unsuccessful attempt to purify the L1B messenger. None of the polypeptides migrated to the location predicted for L1B.

CHAPTER IV

DISCUSSION

Although all mammalian arginase enzymes catalyze the same reaction, that is, the conversion of arginine to urea and ornithine, these proteins differ in a number of molecular properties such as specific activity, isoelectric point, stability during dialysis and activation by Mn^{2+}. The diversity in these characteristics is usually noted when arginase is isolated from different animals (Hirsch-Kolb et al., 1970; Porembska et al., 1971), but discrete traits are also observed among the enzymes extracted from several tissues of the same animal (e.g. rat (Gasiorowska et al., 1970; Reddi et al., 1975; Herzfeld and Raper, 1976); mouse (Stewart and Caron, 1977); and humans (Berüter et al., 1978)). Different properties may even be seen in the several arginase proteins which are located within a single tissue (Herzfeld and Raper, 1976; Stewart and Caron, 1977; Cheung and Raijman, 1981).

The multiple arginase enzymes, which may be isozymes, present in any one animal or tissue should perform distinctive roles, depending on the variable metabolic demands of the organ of concern. For example, one arginase isozyme may exist to complete the conversion of toxic ammonia into urea in tissues undergoing rapid amino acid catabolism, while a second isozyme may be required during protein synthesis to furnish ornithine as the precursor to proline and glutamic acid. The most abundant and most studied arginase is located in liver tissue where it

functions primarily to complete the detoxification of ammonia by incorporating it into urea (Krebs and Henseleit, 1932). Submaxillary gland arginase - unlike the arginase(s) found in other organs - is similar to the liver enzyme with respect to its electrophoretic mobility and immunological reactivity, and so the role ascribed to liver arginase might reasonably be assigned to this protein as well. However, the submaxillary gland has an incomplete urea cycle since it lacks ornithine transcarbamoylase (Herzfeld and Raper, 1976). The arginase present in this tissue (and by extension the liver enzyme as well) must therefore be involved in some other aspect of ornithine catabolism (Herzfeld and Raper, 1976). Indeed, because a substantial increase in liver arginase activity was observed during lactation in the rat, Folley and Greenbaum (1947) concluded that liver arginase must have a second metabolic role. Yip and Knox (1972) suggested that some of the arginase protein in the liver could be required for proline and/or glutamic acid production. Such a function is postulated for at least some of the arginase activity located in mammary gland (Yip and Knox, 1972), kidney (Kaysen and Strecker, 1973), brain (Sadasivudu and Rao, 1976), and epidermis (Verma and Boutwell, 1981). The arginase protein found in lymphocytes (Klein and Morris, 1978), and thyroid (Matsuzaki et al., 1981) is thought to contribute to the synthesis of polyamines, as may a portion of the arginase activity associated with mammary gland (Oka and Perry, 1974), and epidermis (Verma and Boutwell, 1981).

The existence of two bovine liver arginases with different physicochemical properties indicates that each of these proteins probably operates in different metabolic pathways. The L1B arginase characterized

in this study is surely the urea cycle enzyme, while the L1A protein may be comparable to the mitochondrial arginase of rat liver, described by Cheung and Raijman (1981), which helps to move ornithine into the mitochondrial matrix. The presence of four arginases in bovine brain tissue may, at first, appear to be excessive, particularly because the brain has an incomplete urea cycle (Ratner et al., 1960; Stewart and Caron, 1977), and so should not require arginase, at least for ammonia detoxification. Sadasivudu and Rao (1976) have suggested a different role for brain arginase, that of promoting glutamic acid production, to explain its location within this organ. The multiplicity of enzymes exhibiting arginase activity in the current study implies, perhaps, more than one function for this protein in brain tissue. Based entirely on the similarity in several of their properties, B1B and B2B may be essential components of one pathway, while B1A and B2A may be participants in a second series of reactions. The ability to distinguish an apparent difference in charge between B1B and B2B, or B1A and B2A, by placement on a DEAE-cellulose column, could be a reflection of their residence in different brain cell types (e.g. glial and neuronal cells) which demand slightly different molecular properties for efficient arginase activity. Isolation of arginase from brain cells separated by type would be instructive in this regard.

One question arising from the repeated observation of well-defined differences in the molecular properties of arginase isozymes concerns their genetic origins. Are these isoproteins processed from the same mRNA precursor, or are they the products of separate genes? The arginase proteins found in liver and erythrocytes are assumed to be specified by

a single gene locus, yet differences in their regulation have been reported for several Macaca fascicularis monkeys (Terasaki et al., 1980), since normal arginase activity could be detected in the livers of these monkeys, whereas no arginase activity could be found in their red blood cells. Terasaki et al. (1980) proposed several hypotheses to explain these findings, but were unable to favor one theory over the others on the basis of the available data.

Stewart (1981) described a 45% increase in the activity of the two arginases found in the brains of genetically spastic mice as compared to those activities determined for the corresponding enzymes located in normal mouse brains. Because a partial analysis of the 'normal' and 'spastic' brain arginase properties revealed no differences in their molecular characteristics, it was concluded that an alteration in a regulatory element, rather than in the proteins themselves, was responsible for the observed rise in activity. A similar modification in the regulation of the liver arginase protein in spastic mice was not observed since the enzyme activities in the livers of normal and spastic mice were the same. There is no current theory as to the genes coding for mouse liver and brain arginases, thus these observations could reflect either a tissue-specific regulation of a single mRNA precursor, or the differential regulation of duplicate genes.

Schimke (1962; 1964) studied the liver arginase protein isolated from groups of rats fed different diets, and determined that the increase in activity associated with a high casein diet corresponded to the presence of more arginase protein, rather than an alteration in the specific properties of the enzyme itself. The adaptation of arginase

activity to diet was achieved through suitable modifications in the rates of both the synthesis and the degradation of the protein (Schimke, 1964).

Aperia et al. (1979) studied the arginase protein in rat kidney, and found that, in contrast to the liver arginase examined by Schimke (1962), arginase activity rises when the animal is fed a protein-deprived diet. An adaptation of the activity of arginase to an environmental change was again proposed, in this case (because urea is known to be necessary for the formation of concentrated urine) allowing the animal to conserve water during starvation. Since, like many tissues, kidney contains two arginase activities (Gasiorowska et al., 1970; Porembska et al., 1971; Herzfeld and Raper, 1976), it would be instructive to know whether both enzymes show altered activity following protein deprivation. Snellman et al. (1979) demonstrated a differential distribution of modified arginase activity in the rat kidney: the increase in activity on a low protein diet was greatest in the kidney cortex.

The discrete regulation of the two kidney arginase proteins and the one liver arginase enzyme which was described in these studies could be explained on the basis of separate regulators of a single gene, but the existence of two arginase genes is as likely an explanation for the data, and indeed, Spector et al. (1980) have presented preliminary evidence for the presence of two arginase genes in man. One of these genes is expressed in liver and kidney, and a second, an inducible gene, is expressed only in the kidney. Induction of the second arginase protein, with decidedly different molecular properties than the

noninducible enzyme, has also been demonstrated in plant tissue (Legaz and Vicente, 1982).

Several liver arginases from ureotelic animals have been purified to homogeneity in the last twenty years. The initial isolation procedures commonly involve acetone precipitation, ammonium sulfate precipitation, and heat treatment of the homogenate. These protocols are followed by an ion-exchange chromatography step on DEAE-cellulose or CM-cellulose. Depending on the animal source of the enzyme, the purification scheme may require gel filtration chromatography or isoelectric focusing as a final step.

The adsorption of arginase protein to an ion-exchange column differs between species. For example, the rat (Schimke,1964; Hirsch-Kolb and Greenberg, 1968), human (Bascur et al., 1966), and mouse (Stewart and Caron, 1977) liver arginases bind to CM-cellulose, whereas the enzymes from rabbit (Vielle-Breitburd and Orth, 1972) and bovine (Stewart and Caron, 1977) tissues do not bind. The behavior of arginases on an anion exchanger (DEAE-cellulose) is also disparate : rat (Schimke, 1964), bovine (Harell and Sokolovsky, 1972), and mouse (Stewart and Caron, 1977) arginases are not retained, but the rabbit (Vielle-Breitburd and Orth, 1972), and human (Berüter et al., 1978) proteins are bound. In the current study, bovine liver arginases did not become attached to either DEAE-cellulose (Fig. 3) or CM-cellulose (Fig. 4).

Some of the apparent diversity in the charge characteristics of these enzymes arises from the experimental conditions themselves. The choice of pH has been found to be critical for the successful binding of some arginase isozymes to DEAE-cellulose (Porembska et al., 1971), and

the use of sulfhydryl reagents has been shown to prevent the appearance of multiple pig (Sakai and Murachi, 1969) and rabbit (Vielle-Breitburd and Orth, 1972) arginases on an ion-exchanger. The behavior of rat kidney arginase on DEAE-cellulose has been observed to depend on the previous use or non-use of acetone (Kaysen and Strecker, 1973). Berüter et al. (1978) reported an alteration in the electrophoretic pattern of purified human liver arginase as a consequence of its incubation with Mn^{2+} at 37°C and pH 8.0.

Bovine liver arginase shares several physicochemical properties with the enzyme in rabbit, horse, ox, monkey, and pig tissues (Hirsch-Kolb et al., 1970; Porembska et al., 1971). However, neither Harell and Sokolovsky (1972) nor Kuchel et al. (1975), during previous isolations of bovine liver arginase, reported any of the problems of aggregation or oxidation which were demonstrated for the pig (Sakai and Murachi, 1969) and rabbit (Vielle-Breitburd and Orth, 1972) proteins. These difficulties were not encountered in the current study either. There was no indication of a separation of the enzyme activity into several peaks until hydrophobic chromatography was performed (compare Figs. 3-5 with Fig. 6). Although brain arginase activity was an exception to this rule in that it did elute from a DEAE-cellulose column as two well separated peaks, a preliminary study showed that the addition of 2-mercaptoethanol to the eluting buffers had no effect on this two peak pattern. This sulfhydryl compound was, therefore, not routinely used. Two brain arginase activities from ox (Gasiorowska et al., 1969), and mouse (Stewart and Caron, 1977) tissues have also been shown to elute from DEAE-cellulose columns.

Following the suggestion of Kuchel et al. (1975), a Sephadex gel was routinely used during gel filtration of the liver and brain preparations (step 6 of the purification scheme) in place of the Bio-Gel P-150 employed by Harell and Sokolovsky (1972). This dextran material did indeed achieve a better purification of the enzyme than was obtained in several preliminary runs on P-150 (data not shown).

The major liver arginase isozyme (L1B) was purified to a specific activity of 833 unit/mg (Table 1), in agreement with the results of Grassman et al. (1958), Harell and Sokolovsky (1972), and Kuchel et al. (1975). The specific activity of the second liver protein (L1A) was one tenth that of L1B, as were the specific activities of the more hydrophobic brain pools, B1B and B2B (Tables 2B and 2C). Brain pools B1A and B2A were very inactive compared to L1B, having specific activities of 8.54 unit/mg and 15.85 unit/mg, respectively. These data imply that these other arginases are different proteins from L1B.

Given the disparity in the activities of arginase proteins separable only by hydrophobic chromatography, and the knowledge that the arginase enzyme from many sources (e.g. rat kidney, human liver, rabbit liver, pig liver) is easily transformed by the experimental conditions themselves, it is reasonable to propose that the less hydrophobic enzymes (L1A, B1A, B2A) were artifactually made from their more active counterparts (L1B, B1B, B2B, respectively). However, the later characterization of the bovine liver and brain arginases purified in this investigation proved that this was not so, since these proteins differed in a number of physicochemical criteria, not all of which would be predicted to be altered to the same extent by the elution conditions

imposed on the enzymes by their application to Octyl Sepharose columns.

A single pH optimum for both the L1B and the L1A arginase proteins was determined, in contrast to the study of Robbins and Shields (1956) on semi-pure bovine liver arginase, in which two optima at pH 7.0 and pH 9.2 were demonstrated. Greenberg (1960) reported that the pH optimum of arginase depended on the metal used for its activation: Mn^{2+} activated arginase had an optimum at pH 10, whereas the Co^{2+} and Ni^{2+} activated enzyme was most efficient at pH 7. Furthermore, the Co^{2+} activated protein was said to be less stable that the Mn^{2+} activated arginase. An attempt was made to establish whether L1A was the Co^{2+} activated arginase. Both L1B and L1A protein were assayed at pH 7 and pH 9.5 following activation with Co^{2+}. Neither of these arginases was more active at pH 7 than it had been when activated with Mn^{2+}, and, in fact, L1A and L1B activities were decreased at both pH values in the presence of Co^{2+} in place of the usual Mn^{2+}.

All of the isolated brain arginases were many times more active at pH 9.5 than at pH 7.0. This conflicts with the literature on semi-pure ox (Gasiorowska et al., 1969) and rat (Gasiorowska et al., 1970) brain arginases which reported two optima for these proteins, and a relative ratio of activity at pH 9.5:pH 7.0 of only 8:5. Differences between species or distinctions in assay protocol may account for the discrepancy in the results.

The amino acid compositions of the L1B and L1A proteins which are presented in Table 5 may provide a molecular foundation for understanding their discriminate behavior on an Octyl Sepharose column (Figs. 6A and 7A). L1B arginase contains more of the hydrophobic amino

acids proline, leucine, valine, and methionine than does the L1A molecule. The presence of large numbers of these amino acids in L1B may, then, account for its strong attachment to the hydrophobic support.

Although only one hydrolysis time point was taken for all of the brain isozymes purified in this study, the same type of compositional relationship appears to exist between the B1B and B1A proteins: B1B has more hydrophobic residues than does B1A. The correlation between the compositions of B2A and B2B, and their elution from Octyl Sepharose, in contrast, is not as well-defined. Furthermore, it may not be correct to relate the strong binding of arginases to Octyl Sepharose solely to their amino acid compositions. Other experiments need to be conducted to delineate the binding forces between arginase proteins and Octyl Sepharose.

The hydrophobicity of a protein is often an indication that it is a peripheral or integral membrane protein. Therefore, it would be reasonable to assume that L1B arginase, being more hydrophobic than L1A (on the basis of adhesion to Octyl Sepharose), is likely to be attached in some way to a cellular membrane. The values in Table 8, together with the data presented in Barrantes (1975), belie such a theory. Until something is known of the actual amino acid sequence of the L1B protein, and its attendant tertiary and quaternary structures, the exact location of this enzyme within the cell cannot accurately be predicted from amino acid data alone. In addition, no special extraction methods were required to solubilize this protein, implying, at best, a weak binding to a membrane.

What does seem to be consistently clear from the information in

Tables 7 and 8, however, is that the L1A, B1A, B1B, and B2A arginases are closely related to each other, irrespective of which compartment of the cell they reside in. The L1B and B2B arginases are noticeably distinct from them and from each other, yet these two enzymes remain, relatively speaking, closely related to the other arginases described in this study. Interestingly enough, when the statistical method of Marchalonis and Weltman (1971) is used to determine the degree of relatedness of L1A to the bovine liver arginase isolated by Harell and Sokolovsky (1972), the value is 51.63. This figure may indicate that the Harell and Sokolovsky enzyme bears the same relationship to L1A that L1B does, since the corresponding number for these two proteins is 49.79 (Table 7). When L1B is compared to the Harell and Sokolovsky protein, the S Δ Q value is 11.72, chiefly because of differences in their threonine, serine, and lysine contents. Such alterations in the amino acid composition of the two enzymes may be responsible for the current lack of success in reproducing the earlier bovine liver arginase isolation scheme.

The high degree of relatedness between four of the arginases purified in this investigation suggests that they could have a similar subunit structure. The data in Table 3 tend to verify this hypothesis: L1A, B1A, B1B, and B2A arginases seem to share a subunit having a M_r value of about 50,000. Table 3 also provides additional evidence that L1B and B2B are proteins distinct from the other bovine arginases. Each of these enzymes has a unique subunit whose M_r value is 37,800 (L1B), or 79,100 (B2B). B1A alone has a subunit of M_r 61,100 which could, conceivably, be a breakdown product of the 70,000 M_r subunit that B1B and B2B

arginase appear to have in common. The true extent of the similarity between brain and liver arginase subunits can be evaluated most successfully by sequencing the appropriate proteins. Severe limitations on the availability of sufficient amounts of each protein prevent the realization of such a scheme at the present time.

Some information on the structural properties of the proteins can also be derived from immunological tests. Figs. 21 and 22 reveal that L1B holds one immunological determinant (#1) in common with the L1A, B1A, B2A, and B1B arginases, while it shares a second, separate determinant (#2) with the B1B, B2B and L1A proteins. A portion of the L1B molecule is also similar to a determinant (#3) on mouse liver arginase, indicating partial similarity between arginase molecules found in different species.

The exact location of these antigenic moieties within each molecule is, at present, unknown, but it is tempting to speculate that the first named specificity (#1) is located on the 50,000 M_r subunit that all of the concerned enzymes appear to contain, and that the other structure (#2) resides on the 70,000 M_r subunit that B1B and B2B both seem to have. In such a scenario, the B1A subunit of 61,100 M_r could be derived from the 70,000 M_r subunit by proteolysis, or by a differential processing of the mRNA precursor. This modification of the subunit would remove the amino acids which allow the B1B and B2B arginases to bind firmly to Octyl Sepharose as well as deleting determinant #2 from the B1A molecule. However, since the 50,000 M_r L1A enzyme also seems to share determinant #2 in common with B1B and B2B, it might be postulated that the 50,000, 60,000, and 70,000 M_r liver and brain arginase subunits

are all ultimately derived from the 79,100 M_r protein seen in the B2B preparation, and that this molecule itself is a dimer of the 37,800 M_r subunit of L1B arginase. Such a hierarchy of subunits could account for the fact that all of these preparations are cross-reacting with antibodies raised to the L1B arginase molecule. As before, however, confirmation of the theory awaits the primary sequence determination of all of the bovine enzymes.

A preliminary estimation of the oligomeric structure of the B1A, B1B, L1A and L1B arginase proteins can be attempted by combining the information presented in Tables 3 and 4. Thus, L1A may be a dimeric molecule composed of two identical monomers, while B1B and B1A may be dimers formed from dissimilar subunits. The L1B enzyme has a native molecular weight of 119,600 which agrees with the values reported by Harell and Sokolovsky (1972) and Kuchel et al. (1975). Given a subunit of 37,800 M_r, as estimated by SDS-PAGE, this implies a (trimeric) structure for the protein, similar to that of the rabbit enzyme (Vielle-Breitburd and Orth, 1972). Trimeric proteins are not very common. Indeed, despite the values determined for the rabbit arginase (a native M_r of 110,000 and a subunit M_r of 36,500), Vielle-Breitburd and Orth (1972) suggested that the enzyme was actually a tetramer like rat arginase (Hirsch-Kolb and Greenberg, 1968).

The oligomeric structure of a protein is suggested from a knowledge of its hydrodynamic properties (native molecular weight) and its relative mobility in an electric field (subunit size). Both of these techniques are subject to error (see Introduction and Results for more complete discussions of these problems). One way to discover which of

the two measurements is more likely to contribute to misleading values is to employ a second independent measurement of the parameter in question. Researchers frequently calculate the native size of a protein by determining its sedimentation coefficient at high centrifugal speeds. The arginase literature consistently describes a gel filtration M_r estimate of 110,000-120,000, and a corresponding sedimentation coefficient of 5.7 S - 6.1 S. The values determined herein for L1B fall well within these ranges, and imply that these measurements are the correct ones.

The Stokes' radius of a protein is also often determined because it is actually the effective hydrodynamic radius of a macromolecule rather than the molecular weight itself that defines its elution position from a Sephadex column (Smith and Winkler, 1967). If the partial specific volume of a protein is known (and it can be calculated from amino acid data), the molecular weight estimated from a gel filtration calibration curve (e.g. Fig. 17) can be checked by combining Stokes' radius and sedimentation coefficient data. When such a determination was made for the L1B enzyme, its molecular weight was found to be 117,600. Again, the hydrodynamic information on L1B is consistent. In this regard, it should be noted that the partial specific volume of L1B is calculated to be 0.745 ml/g (in agreement with Harell and Sokolovsky, 1972). This value is characteristic of a globular protein with little attached carbohydrate, and so it confirms the reported low hexose content for this enzyme.

Values in the literature for the arginase subunit M_r vary greatly: the rat liver monomer has been reported to be 26,000 (Peiser and

Balinsky, 1982) and 30,000 (Hirsch-Kolb and Greenberg, 1968; Barańczyk-Kuźma et al., 1976); the rat liver plasma membrane arginase which inhibits the growth of cells in culture has a M_r of 40,000 (Kawakama and Terayama, 1981); the human liver monomer is 35,000 (Berüter et al., 1978); and the rabbit liver subunit is 36,500 (Vielle-Breitburd and Orth, 1972). In ammoniotelic Xenopus laevis liver, the arginase monomer has a M_r of 18,000 (Peiser and Balinsky, 1982), and in earthworm gut, the native enzyme is a monomer whose size is 27,000 (Reddy and Campbell, 1968). The disparity in these values indicates that any error in judging the oligomeric structure of L1B may arise from the SDS-PAGE measurement.

As discussed in the introduction, glycoproteins behave anomalously in SDS. This is particularly apparent at low percentages of acrylamide (Gahmberg and Anderson, 1982). Since L1B is a glycoprotein (1-2% by weight) (Harell and Sokolovsky (1972) reported a similar content for their preparation of bovine liver arginase), its carbohydrate moiety may be responsible for a slower migration through the acrylamide than is actually mandated by its size. This retention would cause the subunit size to be overestimated, and the subunit number to be underestimated. Possible problems of this nature may be averted by running a glycoprotein sample on SDS-PAGE in several buffer systems, under both reduced and unreduced conditions (Poduslo, 1981). In this study of bovine liver arginases, however, the effect of carbohydrate on protein mobility through an SDS acrylamide gel has been assessed by analyzing the immune precipitated translation products of arginase-specific mRNAs by electrophoresis so as to be able to compare their migrations to those of authentic liver arginase proteins. If sugar residues do contribute to

the sluggish migration of L1B, then, because carbohydrates cannot be added to newly synthesized proteins in a reticulocyte lysate translation system, the de novo synthesized enzyme should move closer to the anode than the arginase protein purified from bovine liver. The results shown in Fig. 23 indicate this does not occur because a band with a mobility expected for the L1B protein appears in lane 5. The migration of L1A is not influenced by post-translational modifications either, because a 50,000 M_r band appears in lane 5 (Fig. 23) as well.

Single amino acid substitutions have been demonstrated to affect mobility in SDS (deJong et al., 1978). For example, replacement of threonine or alanine by proline decreases migration, as does replacement of leucine and alanine by glutamine and threonine, respectively. Thus, differences in the charge or hydrophobicity of proteins may influence their electrophoretic mobility. Makino and Niki (1977) reported on a protein whose M_r value determined by SDS-PAGE was greater than predicted. The authors suggested that an unusual interaction of the hydrophobic region of the protein with SDS was responsible for the atypical migration observed. Since L1B is capable of strong interactions with Octyl Sepharose, it is conceivable that a similar process is occurring as this enzyme complexes with SDS. An examination of the data in Table 6 indicates that, overall, L1B contains more threonine, glycine, leucine, and proline than the other bovine arginase proteins, while it has less aspartic acid, glutamic acid, and alanine. The lack of alanine is particularly striking. Perhaps somewhere within this information lies a key to understanding the behavior of L1B on SDS-PAGE, as well as an explanation for the apparent diversity in the size of the

arginase subunit.

Single point mutations in the (liver) gene may have contributed to this divergence in properties because changing a nucleotide in the codon of one of the above mentioned amino acids will convert it into another. For example, if the alanine codon, GCU, is changed to ACU, the amino acid threonine will be put into the polypeptide chain instead of alanine. Depending on the location and the role of this new amino acid within the protein, such a substitution could conceivably modify the processing of the molecule such that a longer, but still functional, arginase subunit would be made. Sequencing of the bovine arginase gene(s) may resolve this point.

The electrophoretic migration of bovine liver and brain arginases on disc gels is presented in Fig. 8. Because the native molecular weights of these proteins are very similar (Table 4), the diffuse staining patterns which are observed must arise from charge differences between the molecules within each preparation. The two dimensional separation of L1B and L1A arginases (Fig. 13) confirms this conclusion since the net charge of the uniformly sized subunit in each sample is dispersed over a range of values.

One widely recognized source of charge heterogeneity between similar molecules is their variable carbohydrate content (Sanders and Rutter, 1972; Sidorowicz et al., 1980; Baumann and Held, 1981; Carlsson and Stigbrand, 1982). At least two of the bovine arginase preparations (L1B and B1B) contain a detectable sugar component, and therefore this moiety may partially be responsible for the lack of a sharp banding pattern which is consistently observed during analysis by disc gel

electrophoresis.

The influence of carbohydrate on the electrophoretic migration of these enzymes might be determined by altering the sugar, but not the amino acid, content of the polypeptide. Such a modification of the enzymes could be accomplished by incubating the preparations with sugar cleaving enzymes like β-mannosidase or neuraminidase which would remove mannose or sialic acid residues, respectively. If carbohydrate does affect mobility, then the protein staining pattern should be changed by this treatment. Because the exact composition of the sugars attached to arginase proteins is unknown, a variety of glycosidases should be employed in such an experiment to ensure that at least one or more of the carbohydrate residues is removed. Without a wide sampling of cleaving enzymes, a negative result could mean simply that an ineffective enzyme had been chosen for the experiment.

Another potential source of arginase charge heterogeneity was actually investigated in this study. As can be seen by comparing Fig. 10 with Fig. 8, and Figs. 14 and 15 with Fig. 13, the Mn^{2+} content of the proteins contributes significantly to their charges. The loss of Mn^{2+} during electrophoresis is well documented (Hirsch-Kolb et al., 1971; Boutin, 1982), so that this finding for bovine arginases is not surprising. What should be noted about these experiments, however, is the fact that they reveal differences in the binding of Mn^{2+} to arginase proteins. L1B holds onto the metal quite firmly because its staining pattern after disc gel electrophoresis is not influenced to any extent by the inclusion of Mn^{2+} or EDTA in the gel. The two dimensional separation of L1B protein under a variety of conditions does not change

noticeably either. In contrast, the electrophoretic mobility of L1A depends strongly on its metal content (compare Figs. 13B, 14B, 15B), as does the migration of the brain arginase proteins (Figs. 8 and 10). Clearly, some singular feature of the L1B enzyme (e.g. a unique conformation, a particular amino acid composition, etc.) prevents a significant loss of its Mn^{2+} activator during analysis.

The greater stability of L1B simplifies its physicochemical characterization, and may also explain the coincident elution of its protein and activity profiles from gel filtration columns as opposed to the noncoincidence observed in the patterns of the other bovine arginases tested. Gel filtration is known to remove Mn^{2+} from arginase (Hirsch-Kolb et al., 1970; 1971). The S-200 and the G-150 columns used in this study were not eluted with Mn^{2+}-containing buffer, so that it is likely that some metal was lost from the preparations as they ran through these columns. The consequence of a lowered Mn^{2+} content should be a decrease in the catalytic efficiency of the enzymes, but because the column fractions were always assayed in buffer that contained Mn^{2+}, some enzyme activity could be detected. The lack of concurrent protein and activity profiles for the L1A, B1A, and B1B pools applied to gel filtration columns might, theoretically, be caused by an irreversible loss of metal ion in those fractions eluting at the lagging edge of the protein peak. Such a loss would prevent the expression of enzyme activity in these aliquots although the arginase molecules are, in fact, present.

The unusual elution pattern observed when brain arginase preparations are loaded onto a HPLC column may also be a Mn^{2+} effect. Under conditions of high pressure, these proteins may both combine into macro-

molecular aggregates and dissociate into subunits. Such an effect occurs when these enzymes are subjected to electrophoresis in the presence of EDTA (Fig. 10). Although possibly inactive in these several configurations, if tested in Mn^{2+}-containing assay buffer, the multiple protein peaks apparent in each sample may (and indeed, do) express arginase activity. This theory should be verified by observing the effect on the elution profile of adding Mn^{2+} to the eluant. Alternatively, samples could be run as before, and then they could be assayed in buffer which lacked Mn^{2+} so as to determine which of the peaks is the active form of the enzyme.

In summary, this research has consisted of a investigation of the physicochemical properties of the arginase proteins found in bovine liver and brain tissues. A purification scheme has been devised which yields homogeneous preparations of two distinct liver arginases. Four separate, relatively pure (at least 80-85% pure as judged by SDS-PAGE), brain arginase proteins have also been isolated. All of these proteins require Mn^{2+} for activation, and they share a pH optimum at pH 10. Their electrophoretic mobilities during disc gel electrophoresis are similar, as are their native molecular weights. The overall amino acid compositions of bovine arginases are alike, but there are noticeable differences which probably account for the diversity in the stability and the hydrophobicity of the several proteins. The subunit sizes and oligomeric structures also vary between arginase isozymes.

Future studies should be aimed at the relationship between the amino acid composition and the functional properties of these arginase isozymes. Also genetic studies should be undertaken, using a cDNA probe,

to determine the number of arginase genes within the bovine genome, and the regulation of their expression.

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