IDENTIFICATION AND PARTIAL CHARACTERIZATION

OF

MAMMALIAN ASPARTATE-4-DECARBOXYLASE

by

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A THESIS

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I. SCOPE OF THE THESIS

Aspartic acid is a key amino acid in cell physiology. It functions at the interface between amino acid, carbohydrate and nucleotide metabolism. Numerous enzymes that utilize aspartic acid as a substrate have been purified and extensively studied. Yet, the β-decarboxylation of aspartic acid in mammals and other higher eukaryotes has never been reported.

This thesis deals with the identification of aspartate-4-decarboxylase in rat livers and a description of its chemical and physical properties. A hypothesis for the function of aspartate-4-decarboxylase in mammalian liver is also presented.

Methods and observations related to this research project could be conveniently presented in four major sections. Chapter II deals with the routine methods employed in the course of this study, such as the enzyme-assay systems developed for aspartate-4-decarboxylase and cysteine sulfinate desulfinase and the procedures involved in the preparation of rat liver extracts. Chapters III, IV, and V consist of experimental presentations. Each chapter has its own introduction and discussion section. In Chapter III the circumstances that led to the deduction that aspartate-4-decarboxylase could exist in rat livers are presented. Evidence for the enzymatic activity is also presented in this chapter. The detailed characterization of the physical and chemical properties of rat liver aspartate-4-decarboxylase are presented in Chapter IV. Also there is discussion on the similarities and differences between mammalian and microbial aspartate-4-decarboxylase.

Finally, and perhaps most importantly, the possible significance of aspartate-4-decarboxylase in cell physiology is considered in Chapter V. A hypothesis for the function of the enzyme was erected. Successful predictions of the regulatory properties of the rat liver enzyme were made on the basis of this hypothesis. It is argued that aspartate-4-decarboxylase may be a key enzyme in the regulation of body weight.

The major contributions of this work are summarized in Chapter VI.

II GENERAL METHODOLOGY

II.A Materials

Radiochemicals

L-[4-^{14}C]aspartate (50 μCi/μmol), L-[^{35}S]cystine (250 μCi/μmol), [U-^{14}C]pyruvate, sodium (10 μCi/μmol), 2-Keto [5-^{14}C]glutaric acid, sodium (10 μCi/μmol), and Hyamine hydroxide were purchased from Amersham Corporation. DL-[1-^{14}C]alanine was obtained through ICN Pharmaceuticals, Inc. [^{14}C]carbonate, sodium and omnifluor (premixed chemicals for liquid scintillation counting) were from New England Nuclear. DL-[1-^{14}C]cystine was bought from Research Products International.

L-[^{35}S]cysteine sulfinate and DL-[1-^{14}C]cysteine sulfinate were synthesized from the corresponding radiolabelled cystine, based on the method of Emiliozzi and Pichat (1959). Approximately 50 μCi of radiolabelled cystine ([1-^{14}C] or [^{35}S]) in 0.5 ml was applied to a 0.8 x 4 cm Dowex-1 column equilibrated with H_2O. The resin was washed with 8 ml of H_2O. A small amount of this eluate was subjected to liquid scintillation counting to determine the total radioactivity in the purified cystine. The sample was then diluted with non-radioactive L-cystine to give (0.14 μCi L-[^{35}S]cystine/μmol L-cystine). To solubilize the newly added cystine, 0.5 ml of concentrated HCl was added. The clear solution was rotary evaporated to dryness. The sample was then dissolved in 1.0 ml of 1.5N HCl. To this was added 0.8 ml of 88% formic acid. The mixture was kept on ice and the acidic solution of cystine was treated with 0.02 ml of 30% H_2O_2. After 10 minutes the sample was transferred to room temperature and it was swirled occasionally. After

two hours the sample was dried by rotary evaporation at room temperature. It was dissolved in 2 ml of H_2O; 6 ml of 3% NH_4OH was added, and the alkaline solution (pH 9-11) was kept at room temperature for another 60 minutes. It was then rotary evaporated to dryness, dissolved in 4 ml of 0.5N HCl, and rotary evaporated to dryness once again. At this stage the impure cysteine sulfinate could be stored at -17°C until ready for purification.

Purification of radiolabelled cysteine sulfinate was achieved by ion-exchange chromatography. The sample, prepared as described above, was dissolved in 1 ml of 0.05 N HCl. It was applied to a 0.8 x 16 cm AG50W-X8 column and the amino acids were eluted with 0.01 N HCl. As Figure II-A illustrates, cysteic acid emerged in the excluded fraction, cysteine sulfinate was retarded by the column, and cystine absorbed to the column. The fractions corresponding to the elution pattern of cysteine sulfinate were rotary evaporated to dryness and stored at -17°C.

To monitor purity, representative samples from batches of L-[4-^{14}C]aspartate, DL-[1-^{14}C]cysteine sulfinate and L-[^{35}S]cysteine sulfinate were subjected to descending paper chromatography using 1-butanol:glacial acetic acid:water (12:3:5) as the moving phase. Radioactivity, determined by a Packard 7220/21 radiochromatograph scanner, appeared as a single peak corresponding to the ninhydrin spot of the respective amino acid. This method was also used to verify the identity of amino acid products that eluted off other ion-exchange columns (see Assay Methods).

Other Chemicals

Dowex 1-X8 (200-400 mesh, chloride form), AG50W-X8 (200-400

mesh, hydrogen form), and Bio Rex 5 (100-200 mesh, chloride form) were bought from Bio-Rad Laboratories.

Sigma Chemical Co. supplied the acetyl-coenzyme A, aminooxyacetic acid, coenzyme A, L-cystine, deoxycholate (free acid), histidine (free base), imidazole, and pyridoxal 5'-phosphate. The deoxycholate was recrystallized from 50% ethanol. The crystals were dried and then used to prepare a 10% deoxycholate (sodium salt) solution. Acetyl-coenzyme A was prepared in H_2O less than 10 minutes before it was used.

3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (abbreviated "CHAPS"), and dithiothreitol were purchased from Calbiochem-Behring Corp.

L-Aspartate was bought from Aldrich Chemical Co., Brij 99 was purchased from ICI United States, Inc. DEAE-cellulose was obtained from Carl Schleicher and Schuell Co. After each use the ion-exchange resin was cycled with 0.1 N NaOH, H_2O, 0.1 N HCl, and H_2O again. "Fines" were removed by repeated decanting of less dense cellulose. (Ethylenedinitrilo)tetracetic acid, disodium salt (abbreviated "EDTA"), and semicarbazide hydrochloride were purchased from Matheson, Coleman and Bell. Hydroxylamine hydrochloride, potassium borohydride, Triton X-100, and Ultrodex were obtained from Eastman Kodak Co., Metal Hydrides Inc., Rohm and Haas Co., and LKB Instruments, Inc., respectively.

Sephadex G-150, Phenyl-Sepharose, Pharmalyte (pH 3-10 and pH 4-6.5), and the calibration kit for gel filtration of proteins were all purchased from Pharmacia Fine Chemicals Co.

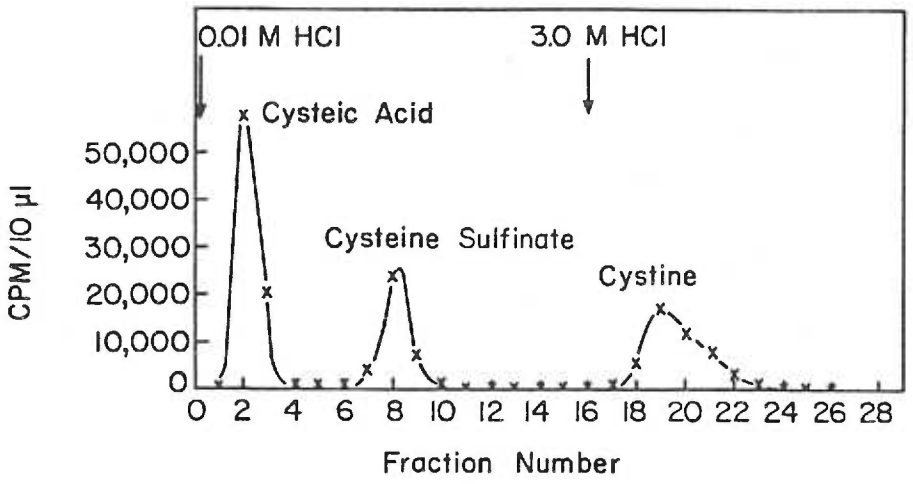

Figure II-A. Purification of radiolabelled cysteine sulfinate. Details in text.

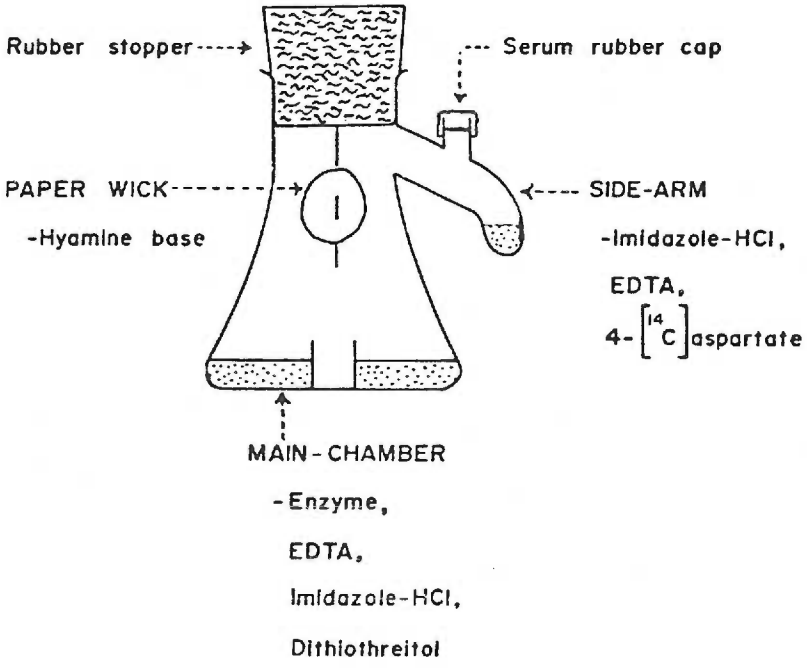

Figure II-B. Reaction Vessel

II.B Assays

Radiochemical Assay System for Aspartate-4-Decarboxylase

This assay system was based on the evolution of $^{14}CO_2$ from L-[4-^{14}C]aspartate incubated with the enzyme. The $^{14}CO_2$ evolved from the reaction was trapped on a hyamine-soaked paper wick suspended by a wire post from the rubber stopper of a reaction vessel, a Warburg flask (Figure II-B). A serum cap was used to seal the opening on the side arm. During routine assay conditions the main chamber of a Warburg flask contained an enzyme fraction, 45 µmol of imidazole HCl (pH 7.0) and 0.5 µmol of EDTA in a total volume of 0.9 ml. The side arm contained 1 µmol of L-[4-^{14}C]aspartate (0.1 µCi), and 5 µmol of imidazole HCl (pH 7.0) in a total volume of 0.1 ml. The reaction was initiated by mixing the contents of the two chambers and was allowed to proceed at 37°C. At a predetermined time 0.5 ml of 20% sulfosalicylic acid, previously injected into a side arm, was tipped into the main chamber. After a 15 minute post-reaction incubation period, the hyamine wick was counted in 5 ml of Buhler's solution (16 g of Omnifluor in 2 liters of toluene and 2 liters of absolute ethanol) by scintillation spectrometry in a Packard 3320 Tri-carb. Boiled-enzyme controls were run to detect any non-enzymatic β-decarboxylation.

When studying the stoichiometry of the β-decarboxylation reaction, the constituents of the assay were doubled and the reaction was terminated with 1 ml of 20% sulfosalicylic acid. $^{14}CO_2$ was trapped on a hyamine wick and counted as described above. After the addition of 1 µmol of valine as an internal standard, aliquots of the deproteinized samples were run on a Component Technicon Automated Analyser to determine the amount of aspartate remaining and alanine formed.

Radiochemical Assay System of Cysteine Sulfinate Desulfinase and Cysteine Sulfinate Decarboxylase

This system was designed to assay cysteine sulfinate desulfinase and cysteine sulfinate decarboxylase simultaneously. A Warburg vessel, fitted with a $^{14}CO_2$ collecting wick as described above, was used as a reaction vessel. During routine assay conditions the main chamber of the flask contained an enzyme fraction, 45 µmol of imidazole HCl (pH 7.0), and 0.5 µmol of EDTA in a total volume of 0.9 ml. The side arm contained 1 µmol of L-cysteine sulfinate, 0.2 µCi of DL-[1-^{14}C]cysteine sulfinate (30-50 µCi/µmol), and 5 µmol of imidazole HCl (pH 7.0) in a total volume of 0.1 ml. The reaction was initiated by mixing the constituents of the assay system, incubated at 37°C for a specified time, and terminated by tipping in 0.5 ml of 20% sulfosalicylic acid. The hyamine wick was counted as described above. The results reflected cysteine sulfinate decarboxylase activity. The remaining contents of the main chamber were centrifuged in a test tube and a 1 ml aliquot of the deproteinized supernatant was layered on a 0.8 x 5 cm Bio-Rex 5 column previously washed with H_2O. After the sample settled, 6 ml of H_2O was passed through the column. An aliquot of the total eluate was counted to determine [1-^{14}C]alanine formed, which reflected cysteine sulfinate desulfinase activity. When the eluant was subjected to paper chromatography (see section II-A-1), the radioactivity appeared as a single peak that co-migrated with authentic alanine. The other metabolites of cysteine sulfinate, such as hypotaurine and taurine, which passed through the anion exchange column were not labelled and thus did not interfere with the desulfinase assay.

When studying the stiochiometry of the reaction, the same apparatus was used except the [1-^{14}C]cysteine sulfinate was replaced, when appropriate, with [^{35}S]cysteine sulfinate. At the end of the reaction no radioactivity was detected on the hyamine wick, suggesting that any SO_2 released was oxidized to sulfate. An aliquot of the deproteinized sample was applied to a 0.8 x 5 cm Bio-Rex 5 column. The radioactivity that eluted off the column with H_2O represented hypotaurine and taurine synthesized by decarboxylation of cysteine sulfinate. When the column was further treated with 0.5 N HCl, unmetabolized cysteine sulfinate was eluted. Finally 2 N HCl was used to elute radiolabelled sulfate (Fellman et al., 1980).

Radiochemical Assay System for Aspartate Aminotransferase

Aspartate aminotransferase activity was assayed by incubating the enzyme, 50 µmol of imidazole HCl, pH 7.0, 1 µmol of dithiothreitol, and 1 µmol of L-aspartate with 1µmol of [5-^{14}C] α-ketoglutarate (0.1µCi) for 30 min at 37°C. The reaction was terminated by adding 0.5ml of sulfosalicylic acid. After centrifuging the proteins down, the supernatant was placed on an AG50W-X8 column and washed extensively with H_2O. Radiolabelled glutamate was eluted with 3N HCl and an aliquot was counted to determine the amount of product formed.

Protein Assays

Normally, protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as a standard protein.

In some studies, where the enzyme preparation had been exposed to Triton X-100, it was important to demonstrate that sufficient dialysis had been performed to keep the aromatic, non-ionic detergent from interfering with the Lowry protein assay. Fraction 2 (see below)

was subjected to nitrogen determination (Di Giorgio, 1974). Assuming 16% nitrogen in the protein, the Lowry method and the micro-Kjeldahl method gave protein values that were in agreement within 10%.

II.C Enzyme Preparations

Two different enzyme preparation methods were used in the course of this study. The first method, which involved solubilization of enzyme using Triton X-100 followed by ammonium sulfate fractionation and extensive dialysis, was used for early studies. These studies involved identification of the enzyme, determining the stoichiometry of the reactions, examining the kinetics of the β-decarboxylase reaction, and characterizing the active site of the enzyme. A second method, which involved extensive purification of the enzyme, was important in studying the physical properties and the regulatory properties of the enzyme.

Earlier Procedure

Adult Sprague-Dawley rats were killed by decapitation and their livers were removed. All subsequent steps were performed at 4°C. The liver was homogenized in five volumes of 10 mM imidazole HCl buffer containing 1.0% Triton X-100 and 1 mM EDTA (pH 7.0). The samples were centrifuged at 100,000 x g for 60 minutes. The resulting supernatants (fraction 1) were subjected to partial purification by ammonium sulfate fractionation. The proteins that precipitated between 35-45% saturation had the highest β-decarboxylase activity. They were suspended in a minimal volume of 50 mM imidazole HCl with 1 mM EDTA (pH 7.0) and were extensively dialyzed against this buffer (at least three times against 200 volumes of buffer). The resulting sample was labelled fraction 2. Whenever fraction 1 had to be assayed for aspartate-4-

decarboxylase activity, it too was extensively dialyzed.

Later Procedure

Approximately 50 g of livers from adult male Sprague-Dawley rats were minced and frozen at -17°C. After two hours or more they were thawed. All subsequent steps were performed at 4°C. The livers were homogenized in five volumes of 10 mM imidazole HCl buffer with 1 mM EDTA (pH 7.0). The homogenate (fraction A) was centrifuged at 100,000 x g for 60 minutes. The resulting supernatant was labelled fraction B.

Sufficient amounts of 10% deoxycholate, 5% Brij 99 and solid dithiothreitol were added to fracton B to bring the final concentrations to 0.1%, 0.05% and 1 mM respectively. The preparations were stirred for 30 minutes and then mixed with approximately 50 g of DEAE-cellulose equilibrated with H_2O. For thirty minutes the resin and the liver extract were stirred in a 1.5 l beaker.

The mixture was then poured onto a Buchner funnel equipped with a Whatman No. 1 filter paper (15 cm diameter). The unabsorbed and loosely bound proteins were eluted with 500 ml of 50 mM imidazole HCl buffer containing 1 mM EDTA, 0.1% deoxycholate, 0.05% Brij 99 and 1 mM dithiothreitol (pH 7.0) (labelled buffer 1). Next, the anion-exchange resin was washed with 500 ml of buffer 1 containing 70 mM NaCl. The yellowish solution that desorbed with this treatment was concentrated to about 20 ml using an Amicon Model 402 stirred cell (diaflo ultra filtration membrane PM 10). The sample was further concentated to 2 ml using a vacuum dialysis set-up (VWR Scientific, Inc., average pore radius permeability of 24 A). The resulting sample was labelled fraction C.

Fraction C, mixed with 0.1 mg of dithiothreitol, was applied

to a 2.5 x 95 cm Sephadex G-150 column equilibrated with buffer 1 plus 50 mM NaCl. This solution was then pumped at 15 ml/hr through the sieving column and 3 ml fractions were collected. Fractions with the highest aspartate-4-decarboxylase activity were pooled together and concentrated to 2 ml by vacuum dialysis (fraction D).

Fraction D was dialyzed for six hours against one liter of starting buffer of phenyl-sepharose chromatography [50 mM imidazole, HCl, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA (pH 7.0)]. The sample was treated with 1 mM dithiothreitol and applied to a 1.5 x 28 cm phenyl-Sepharose column. Proteins were eluted by running a linear solvent gradient from 250 ml of starting buffer to 250 ml of the same buffer with 80% ethylene glycol. Fractions which had conductivity in the neighborhood of 0.8 millimho were assayed for aspartate-4-decarboxylase activity. Tubes with the highest amount of enzymatic activity were pooled, dialyzed against 50 mM imidazole HCl (pH 7.0), and concentrated to 2 ml by vacuum dialysis (fraction E).

Fraction E was readied for preparative isoelectric focusing by dialysis against 500 ml of 1% histidine. Later, dithiothreitol, Brij 99 and CHAPS were added to a final concentration of 1 mM, 0.05% and 0.1% respectively. Isoelectric focusing was performed on a flat bed of granulated gel based on the method of Randola et al. (1974).

Four grams of Ultrodex was allowed to swell in 100 ml of doubled distilled H_2O containing 0.05% Brij 99 and 0.1% CHAPS. As the resin was stirred gently, 4 ml of ampholytes were added (pharmalyte pH 4-6.5). The slurry was poured onto a glass plate equipped with electrode-wicks at both poles (cathode wick was wetted with 0.1 N NaOH and anode wick was wetted with 0.1 N H_3PO_4). The excess water in the

slurry was allowed to evaporate overnight at room temperature. The next morning an electric fan was used to bring the evaporation to completion (final slurry weight was 65% of the original weight).

The 11 x 23 cm slab was connected to an LKB Multiphore apparatus. Prefocusing was performed for three hours at 1.5 watts (max. volt. 1500 v; max. amp. 10 mA). A sample applicator was used on the cathode end to scoop out a 2 x 9.5 cm section of the gel parallel to the electrode wire. This semi-solid gel was placed in a 10 ml beaker and gently mixed with 3 ml of fraction E which had been dialyzed against 1% histidine and which had been treated with dithiothreitol and the detergents. The slurry was poured back where it came from. The sample applicator was removed and isoelectric focusing was resumed immediately at 15 watts (max. volt. 1500v; max. amp. 10 mA). To retain the maximum amount of enzymatic activity, ice-cold water was circulated under the gel and aluminum foil was wraped around the apparatus to prevent direct exposure to sunlight.

After 13 hours of focusing the power source was turned off. Immediately a 3 x 23 cm strip of Whatman (grade 1) filter paper was layered along the length of the gel to generate a contact print of the separated proteins. After 20 sec. the strip was peeled off and a stainless-steel grid was stabbed through the gel to cut the bed into thirty 0.8 x 11 cm fractions. This prevented extensive diffusion of the separated proteins. The paper strip was dried with an electric hair dryer and subsequently was stained with a solution containing 0.05% Coomassie Brilliant Blue and 4.2% $HClO_4$. Regions of the slab gel, which corresponded to protein bands on the contact print, were scooped out and placed into small plastic chromatography columns. The gels were washed

with 8 ml of 0.1 M imidazole HCl (pH 7.0). The eluant fractions were placed in separate dialysis bags and dialyzed against 50 mM imidazole HCl (pH 7.0) to remove the ampholytes. The dialyzed samples were then assayed for aspartate-4-decarboxylase activity.

III IDENTIFICATION

The identification of aspartate-4-decarboxylase in mammals arose from some earlier work in our laboratory on the metabolism of cysteine sulfinate.

III.A Review of Intermediary Metabolism

Sulfur Amino Acids

Cysteine sulfinate [2-amino-3-sulfinopropionate] is derived from the oxidation of cysteine, a key event in the degradation of sulfur amino acids (Sakakibara et al., 1976). A major fate of the sulfur atom of cysteine sulfinate is desulfination to inorganic sulfite, which is rapidly oxidized to sulfate. In humans 80% of the sulfur from cysteine is excreted as inorganic sulfate (Awapara, 1976).

It is widely accepted that the desulfination of cysteine sulfinate occurs indirectly. The compound is thought to undergo transamination to form beta-sulfinyl pyruvate, an unstable compound that spontaneously degenerates into sulfite and pyruvate (Singer and Kearney, 1956). This transamination of cysteine sulfinate is catalyzed by the mitochondrial and the cytosolic forms of aspartate aminotransferase [E.C. 2.6.1.1] using alpha-ketoglutarate as a cosubstrate (Recasens et al., 1980; Yagi et al., 1979).

There are several reasons to question the alleged monopoly of aspartate aminotransferase in desulfinating cysteine sulfinate. The subcellular distribution, Km, and Vmax of cysteine sulfinate decarboxylase (compared to cysteine sulfinate aminotransferase) would appear to favor the formation of hypotaurine and taurine as final

products of cysteine metabolism (Yu-Chen Lin et al., 1971; Recasens et al., 1980). However, this is clearly not the case. Sulfate is the predominant product. It is, therefore, necessary to consider alternate routes for desulfinating cysteine sulfinate. Figure III-A shows two. First, cysteine sulfinate can be decarboxylated to form hypotaurine, which can form sulfite and acetaldehyde by transamination (Fellman et al., 1980). Alternatively, cysteine sulfinate may be desulfinated directly to alanine and sulfite.

In the literature the direct desulfination of cysteine sulfinate has been mentioned by three groups. Fromageot et al. (1948) used rabbit liver homogenates for converting cysteine sulfinate to equal amounts of alanine and sulfite. At first this reaction was attributed to a "desulfinaticase" but in a following publication (Chatagner et al., 1952), they acknowledged that double transamination could account for the generation of alanine and sulfite from cysteine sulfinate.

cysteine sulfinate + α-ketoglutarate $\rightleftarrows$ β-sulfinyl pyruvate + glutamate

β-sulfinyl pyruvate $\longrightarrow$ pyruvate + sulfite

pyruvate + glutamate $\rightleftarrows$ alanine + α-ketoglutarate

Net reaction: cysteine sulfinate $\longrightarrow$ alanine + sulfite

These investigators felt that the scheme outlined above could satisfactorily explain their data.

Later Sumizu (1961) reported that a partially purified enzyme from rat liver could directly desulfinate cysteine sulfinate. His acetone fractionated preparation had no transaminase activity. The

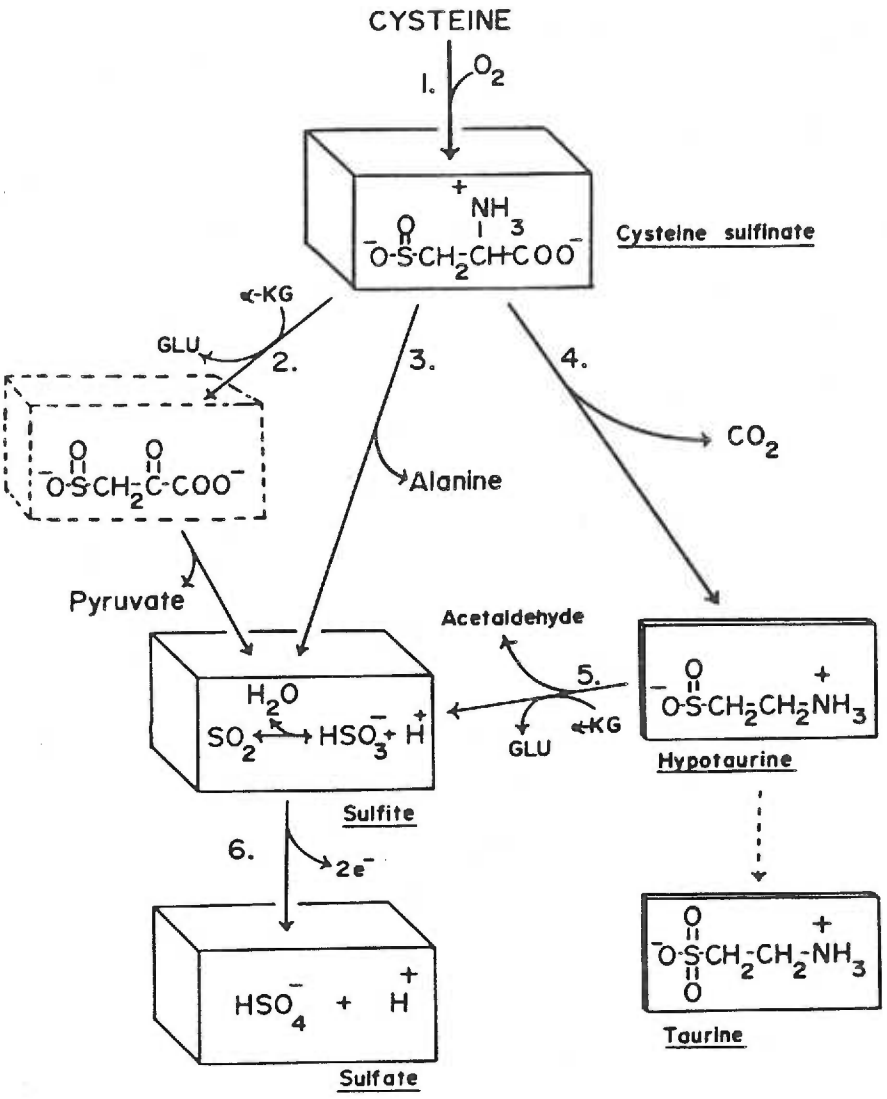

Figure III-A. Cysteine sulfinate metabolism in mammals. The participating enzymes are: 1. cysteine dioxygenase; 2. aspartate aminotransferase; 3. cysteine sulfinate desulfinase; 4. cysteine sulfinate decarboxylase; 5. hypotaurine aminotransferase; and 6. sulfite oxidase.

enzyme was inhibited when dialyzed against 10^{-4} M hydroxylamine and reactivated when pyridoxal 5'-phosphate was added to the preparation. A full report of this finding was never published. Numerous attempts in our laboratory to repeat this work yielded inactive enzyme preparations. The short preliminary note constitutes the only available evidence for the direct desulfination of cysteine sulfinate in eukaryotes.

The direct desulfination of cysteine sulfinate has been reported in microorganisms. Soda et al. (1964) demonstrated that a purified enzyme from Alcaligenes faecalis which catalyzed the beta-decarboxylation of aspartate also catalyzed the desulfination of cysteine sulfinate. The two reactions are analogous (Figure III-B). Detailed properties of microbial aspartate-4-decarboxylase are described in section IV-A.

Evidence for the direct desulfination of cysteine sulfinate in mammals will be presented in a later section. This led us to consider the possibility that mammalian tissues may also be capable of catalyzing the beta-decarboxylation of aspartate.

Aspartic Acid

Free aspartic acid in the cell is known to participate in several metabolic events (Meister, 1965; Lehninger, 1975). A well-known and very important reaction is the transamination of aspartate to oxaloacetate, an intermediate of the tricarboxylic acid cycle. Interestingly, oxaloacetate itself has several possible fates. It can act as a cosubstrate for the oxidative catabolism of acetyl-coenzyme A through the tricarboxylic acid cycle. Alternatively, it can be used as a precursor for the biosynthesis of six-carbon sugars, certain amino acids and fatty acids.

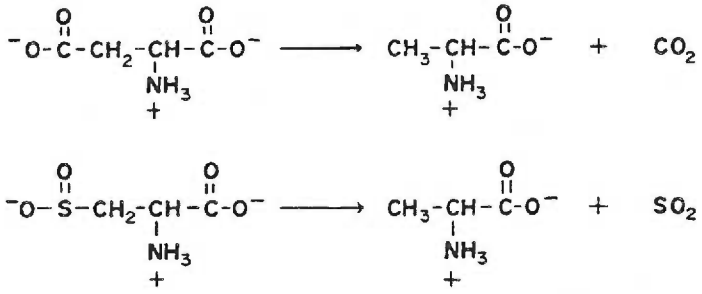

Figure III-B. Analogy between aspartate-4-decarboxylase and cysteine sulfinate desulfinase.

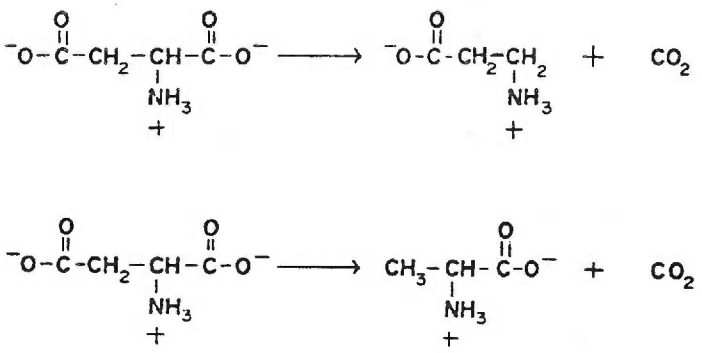

Figure III-C. Decarboxylation of aspartate: Alpha and Beta

Besides its metabolism through oxaloacetate, aspartate is known to participate in the urea cycle and in the biosynthesis of nucleotides.

Unlike several other amino acids (such as L-glutamate, L-histidine, L-arginine, L-dopa, L-tryptophane, L-cysteine sulfinate, L-ornithine, etc.), aspartate in mammals does not undergo α-decarboxylation to form a primary amine. A small amount of β-alanine arises indirectly during the degradation of pyrimidines. In some microorganisms, on the other hand, aspartate can undergo α-decarboxylation to form β-alanine and CO_2 (Williamson and Brown, 1979) and also β-decarboxylation to form L-alanine and CO_2 (Tate and Meister, 1971) (see Figure III-C).

In a following section the β-decarboxylation of aspartate in mammals is examined.

III.B Stoichiometry

Desulfination of Cysteine Sulfinate

Fifteen milligrams of protein from fraction 2 were incubated with 1 μmol of L-cysteine sulfinate and either 0.2 μCi of DL-[1-^{14}C]cysteine sulfinate or L-[^{35}S]cysteine sulfinate. After 60 min at 37°C, the radiolabelled products were analyzed by ion-exchange chromatography (Table III-1). In the presence of DL-[1-^{14}C]cysteine sulfinate the formation of $^{14}CO_2$ from decarboxylation and [1-^{14}C]alanine from desulfination was observed. With L-[^{35}S]cysteine sulfinate, $^{35}SO_4^=$ from desulfination and the decarboxylation products [^{35}S]hypotaurine and [^{35}S]taurine were observed. In calculating pmol of alanine and CO_2 formed, it was assumed that D-[1-^{14}C]cysteine sulfinate was not a

substrate for enzymatic reactions. Alanine and sulfate, the products of desulfination, were formed in equimolar amounts.

Note that a large amount of cysteine sulfinate was consumed by a decarboxylase. When the protein samples were dialyzed against hydroxylamine and then against standard buffer (see section IV-C), the cysteine sulfinate desulfinase remained active. However there was a dramatic decrease in cysteine sulfinate decarboxylase activity (Table III-1).

TABLE III-1

Stoichiometry of Cysteine Sulfinate Catabolism

	pmol of products			
	From Desulfinase Activity		From Decarboxylase Activity	
Enzyme Source	Alanine	Sulfate	CO_2	Hypotaurine and Taurine
Fraction 2	110 ± 3	119 ± 6	997 ± 27	809 ± 66
Fraction 2 after NH_2OH treatment	252 ± 10	217 ± 18	13 ± 1	11 ± 1

Beta-Decarboxylation of Aspartate

Twenty-six milligrams of protein from fraction 2 were incubated with 2 µmol of L-aspartate containing 0.18 µCi of L-[4-^{14}C]aspartate and with 2 µmol of dithiothreitol in a total volume of 2 ml. At the end of the reaction (60 min, 37°C), as described in section II-B, $^{14}CO_2$ release was quantitated by liquid scintillation spectrometry

and the amount of alanine formed and aspartate consumed were quantitated by an automated amino acid analyzer. The results shown in Table III-2 demonstrate that for each mole of aspartate consumed, one mole of alanine and one mole of CO_2 were generated. A boiled preparation showed no enzymatic activity.

TABLE III-2

Stoichiometry of Aspartate-4-Decarboxylase

Enzyme Preparation	Aspartate Consumed	Alanine Synthesized	CO_2 Synthesized
Fraction 2	0.96	1.03	1.00
Fraction 2 (Boiled)	0	0	0

III.C Solubilization

Subcellular distribution of aspartate-4-decarboxylase could not be conclusively ascertained. A liver homogenate prepared in 0.15 M KCl or 0.25 M sucrose showed 40-70% less enzymatic activity than a homogenate prepared in low ionic strength buffer (0.01 M imidazole HCl, pH 7.0). Furthermore, after differential centrifugation (deDuve et al., 1955), aspartate-4-decarboxylase activity from all the subcellular fractions amounted to less than 30% of the activity found in whole homogenates. For this reason, although the mitochondria and the soluble fractions had relatively higher aspartate-4-decarboxylase activity, it is premature to say that rat liver aspartate-4-decarboxylase is localized primarily in the mitochondria and the cytosol.

TABLE III-3

Distribution of Rat Liver Aspartate-4-Decarboxylase in Fractions Enriched in Subcellular Organelles

Fraction	Specific Activity (pmol/min/mg protein)	Total Activity (pmol/min/g tissue)
Whole	117	10,200
"Nuclear" (180 x g, 10')	17	370
"Mitochondrial and Lysosomal" (20,000 x g, 10')	29	570
"Microsomal" (100,000 x g, 45')	4	50
"Soluble"	52	1,900

*The individual fractions were prepared in 250 mM sucrose with 5 mM imidazole HCl (pH 7.0). After isolation, the fractions were treated with Triton X-100 (to 1%) and finally dialyzed against 50 mM imidazole HCl (pH 7.0) with 1 mM EDTA before enzyme assay.

In order to press on with other studies on mammalian aspartate-4-decarboxylase, it was necessary to develop solubilization procedure(s) which would yield all the enzyme activity in an accessible form.

Several approaches were initiated, including extraction using buffers of varying ionic strength, extraction from an acetone powder, extraction from a freeze-thawed preparation, and detergent treatment. Only the latter two methods provided quantitative enzymatic activity in

the supernatant. When rat livers were homogenized with a Teflon-pestle glass homogenizer in a buffer with 10 mM imidazole HCl (pH 7.0), 1 mM EDTA, and 1% Triton X-100, maximum solubilization and specific activity were achieved as determined by recovery of enzymatic activity in the supernatant after centrifugation at 100,000 x g for 60 min (Figure III-D). Control experiments confirmed that the predominant effect of using the detergent was indeed solubilization rather than the mere stimulation of a latent soluble enzyme.

An alternate approach, which proved to be useful during the purification of rat liver aspartate-4-decarboxylase, involved freeze thawing. Rat livers frozen at -17°C for at least two hours could be thawed and homogenized in a buffer with 10 mM imidazole HCl (pH 7.0) and 1 mM EDTA and centrifuged at 100,000 x g for 60 minutes to give 100% recovery of aspartate-4-decarboxylase in the supernatant along with a two-fold increase in specific activity.

III.D Tissue Distribution Amongst Mammals

To survey the occurrence of aspartate-4-decarboxylase in mammals, various organs from rats and livers from various mammals were studied. Fresh tissues were homogenized in three volumes of 10 mM imidazole HCl (pH 7.0), 1 mM EDTA using a Brinkman polytron homogenizer. To this was added two volumes of the same buffer containing 3% Triton X-100 to bring the final detergent concentration to 1%. After 20 min of gentle stirring the homogenates were centrifuged at 100,000 x g for 60 min and the supernatants were dialyzed against 50 mM imidazole HCl (pH 7.0), 1 mM EDTA before assaying.

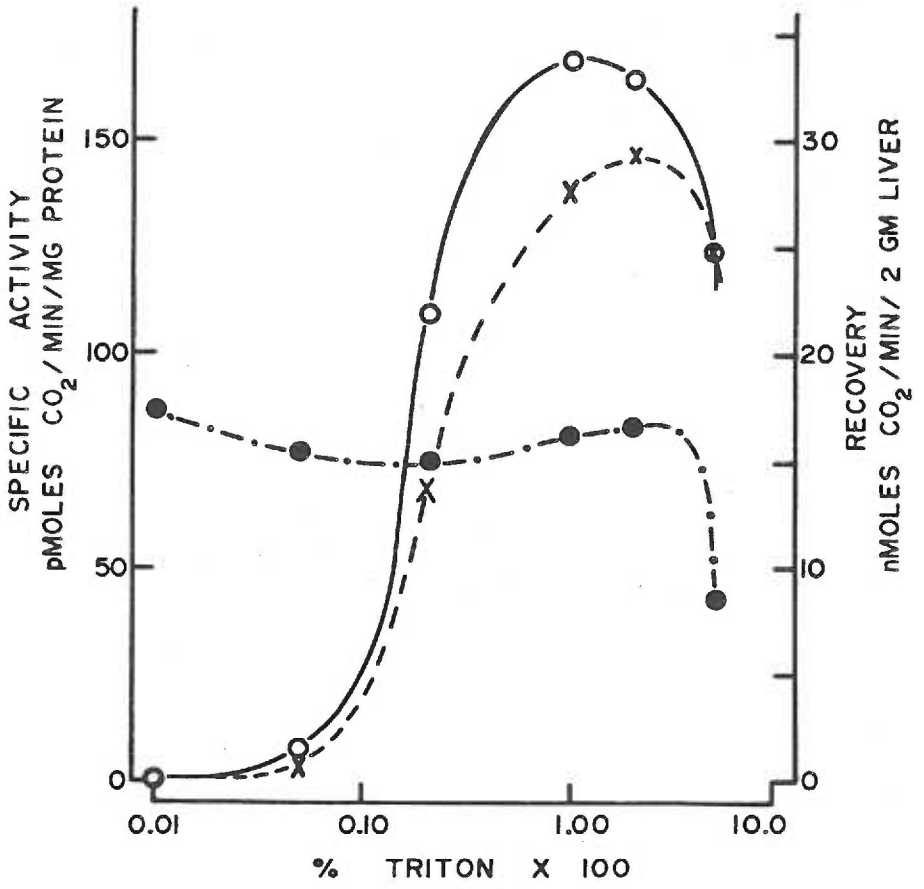

Figure III-D. Release of aspartate-4-decarboxylase into 100,000 x g supernatant by Triton X-100 treatment. (—·—●—·—) Absolute activity in whole homogenates, (— —X— —) Absolute activity in supernatants, (——O——) specific activity in supernatants

By far the highest amount of activity was observed in rat and mouse livers (Table III-4). Cat liver and rat kidney had appreciable amounts of enzymatic activity too. Curiously, aspartate-4-decarboxylase activity in all the other tissues was negligible.

TABLE III-4

Distribution of Aspartate-4-Decarboxylase in Vertebrate Tissues

Tissue	Aspartate-4-Decarboxylase Activity (nmol of CO_2/hr/g of wet tissue)
Bovine liver	15 ± 2
Chicken liver	18 ± 1
Pig liver	47 ± 8
Cat liver*	785 ± 20
Mouse liver	1950 ± 84
Rat liver	1049 ± 46
Rat kidney	242 ± 12
Rat heart	21 ± 1
Rat pancreas	21 ± 2
Rat brain	18 ± 1
Rat spleen	≤10
Rat muscle	≤10

*This tissue was not fresh. It had been frozen at -17°C for six months before homogenization and assay.

III.E DISCUSSION

In the course of some studies on cysteine metabolism it was observed that rat liver homogenates, which had been depleted of α-ketoacids, continued to catalyze the desulfination of cysteine sulfinate. This observation contradicted the popular belief that the formation of sulfate from cysteine sulfinate was dependent on a transaminase reaction that used α-ketoglutarate as a cosubstrate.

The direct desulfination of cysteine sulfinate in mammals had been proposed twenty years ago by Sumizu (1961). His observations have been ignored in the literature. This may be because he failed to follow up on his preliminary findings and also because other investigators (including myself) could not repeat his work.

Using independent methods, the possible occurrence of a cysteine sulfinate desulfinase in rat livers was re-examined in our laboratory. A rat liver preparation that had undergone ammonium sulfate fractionation and extensive dialysis (fraction 2) was assayed for pyruvate content and found to be essentially void in this alpha-keto acid. (The protein fraction contributed less than 1 nmole of pyruvate to a typical desulfinase assay vessel.) This preparation, then, could be used to assay for the desulfinase without interference from any transaminase activity.

Fraction 2 catalyzed the conversion of cysteine sulfinate to alanine and sulfate, the latter because sulfite can be readily oxidized to sulfate by rat liver sulfite oxidase (Cohen and Fridovich, 1971).

These preparations also catalyzed the α-decarboxylation of cysteine sulfinate. However unlike the desulfinase, cysteine sulfinate

decarboxylase was found to be very sensitive to hydroxylamine treatment. The decarboxylase is known to be a pyridoxal 5'-phosphate dependent enzyme (Jacobsen et al., 1964) and dialysis against hydroxylamine presumably removed most of this coenzyme from the holoenzyme to leave an inactive apoenzyme. This idea was supported by the observation that hydroxylamine-treated fraction 2 could regain 60% of its cysteine sulfinate decarboxylase activity when incubated with 10^{-5} M pyridoxal 5'-phosphate. For practical purposes this difference in susceptibility to hydroxylamine treatment could be utilized to assay cysteine sulfinate desulfinase in a crude enzyme preparation without interference from the decarboxylase.

In Alcaligenes faecalis the desulfination of cysteine sulfinate is catalyzed by the same enzyme that catalyzes the β-decarboxylation of aspartate (Soda et al., 1964). Having demonstrated cysteine sulfinate desulfinase activity in rat livers, it was logical to look for aspartate-4-decarboxylase activity in mammals. Using fraction 2 it could be demonstrated that the β-decarboxylase was also present in mammals.

For reasons that can only be speculated upon, the occurrence of aspartate-4-decarboxylase in mammals was overlooked by other biochemists. This is rather surprising considering the central role that aspartate plays in intermediary metabolism. One would expect that in the course of studies on aspartate aminotransferase, argininosuccinate synthetase, aspartate transcarbamylase, or one of several other aspartate-utilizing enzymes involved in nucleotide biosynthesis, a careful measure of the stoichiometry of these reactions should have led to the discovery of aspartate-4-decarboxylase.

Apparently, investigators must have been using assay methods that were best suited to study reactions of interest to them; methods that were not suited for detecting aspartate-4-decarboxylase. For example, rat liver aspartate-4-decarboxylase is about three-fold more active in 50 mM imidazole-HCl (pH 7.0) buffer than in 50 mM potassium-phosphate buffer (pH 7.0). Beyond this, it is not uncommon for investigators to dismiss small discrepancies in data (say, aspartate consumed versus oxaloacetate generated in the transminase assay) as due to limitations of their analytical methods.

This thesis attempts to characterize the molecular properties of the newly discovered mammalian aspartate-4-decarboxylase and advances a hypothesis for its physiological function. However, before this can be done, it is important to correctly choose a tissue with which to work. Rat livers were used for the initial studies because they were readily available and the biochemistry of rat liver cells was better understood than that of most other mammalian cells. This, as we will see in Chapter V, becomes important when results obtained from our laboratory are compared with related observations in other areas of biochemistry.

Having decided on studying aspartate-4-decarboxylase from rat livers, it would be useful to determine its subcellular localization. Such knowledge usually facilitates purification of the enzyme and it also is an important consideration when one is proposing a function for the enzyme.

Several attempts at obtaining a subcellular fraction of rat liver cells which was enriched in aspartate-4-decarboxylase were unsuccessful. It was decided, at this point, to proceed with detergent-

solubilized aspartate-4-decarboxylase. This preparation accounted for 100% of the activity in rat livers. It was recognized that in the course of purifying this enzyme, more than one physically distinct form of aspartate-4-decarboxylase may be isolated. The strategy was to wait until such an event occurred before seriously considering the possibility of multiple isozymes originating from various subcellular fractions. This was true in spite of an additional recognition that physically indistinguishable enzymes may be expressed in different subcellular organelles (Meldolesi et al., 1980).

Using solubilization procedures and assay conditions that were optimum for detecting rat liver aspartate-4-decarboxylase, various other vertebrate tissues were examined for this activity. The most impressive finding was that livers from such animals as chickens, cows and pigs had an almost undetectable amount of aspartate-4-decarboxylase activity. The low β-decarboxylase activity in these animals may be attributed to the assay procedures and enzyme-preparation techniques which were developed to optimize rat liver aspartate-4-decarboxylase. An alternate and more provocative explanation is considered in Chapter V; an explanation based on a hypothesis for the function of aspartate-4-decarboxylase in mammalian cells.

IV PARTIAL CHARACTERIZATION

IV.A Microbial Enzyme: A Review

Since aspartate-4-decarboxylase from Alkaligenes faecalis has been purified and extensively studied, a review of its physicochemical and catalytic properties serves as a fulcrum for this chapter. This is so, even though the properties of the mammalian enzyme, as we will see, are considerably different from those of the microbial enzyme.

Aspartate-4-decarboxylase has been demonstrated, not only in several microorganisms (Tate and Meister, 1971), but also in silkworms (Bheemeswar, 1955), in crayfish and in lobsters (Gilles and Schoffeniels, 1966). Highly purified preparations have been obtained from Alkaligenes faecalis (Tate et al., 1970), Achromobacter sp. (Miles, 1970), and Pseudomonas docunhae (Kakinoto et al., 1969). Essentially, the enzyme preparations from these different sources share identical properties. Since most of the work has been done using aspartate-4-decarboxylase from Alkaligenes faecalis, the properties described below were derived largely from studies on this microorganism.

Microbial aspartate-4-decarboxylase could be purified to homogeneity relatively easily using conventional protein purification techniques such as ammonium sulfate fractionation, anion-exchange chromatography, gel-permeation chromatography, and hydroxyapatite chromatography. Yields were in the order of 10-30% with approximately 100-fold purification.

Gel permeation chromatography was a useful step in the purification because microbial aspartate-4-decarboxylase had an unusually high molecular weight. The enzyme was composed of 12

apparently identical subunits with a total molecular weight of 680,000. For every subunit, the enzyme contained a molecule of pyridoxal 5'-phosphate. This coenzyme appeared to be important in at least three aspects: (1) the stability of the quaternary structure of the enzyme; (2) its mechanism of catalysis; and (3) regulation of aspartate-4-decarboxylase by α-keto acids.

Removal of pyridoxal 5'-phosphate of microbial aspartate-4-decarboxylase from the protein moiety had interesting effects on the stability of the quaternary structure of the enzyme (Tate and Meister, 1970). The apoenzyme, at pH 6, existed as a dodecamer like the holoenzyme but when the pH was raised to 8 it disassociated into dimers. Addition of pyridoxal 5'-phosphate to the apoenzyme at pH 8 resulted in spontaneous reassociation of the subunits to give a dodecameric structure.

Pyridoxal 5'-phosphate was also considered to be important in the catalytic mechanism of rat liver aspartate-4-decarboxylase and in its regulation by α-keto acids. The proposed mechanism of these phenomena, based on the accumulated evidence, is shown in figure IV-A. The amino acid first forms a Schiff base with the cofactor on the enzyme, then undergoes decarboxylation, and finally alanine is released.

Based on the chemistry of pyridoxal 5'-phosphate, it is known that this compound, by itself, can catalyze a number of reactions involving amino acids (Braunstein, 1973; Davies and Metzler, 1972). Most pyridoxal 5'-phosphate-containing enzymes, however, have stringent substrate and reaction specificities. These specificities are attributed to functional groups at the active site of these enzymes. Alkaligenes faecalis aspartate-4-decarboxylase was found to be more

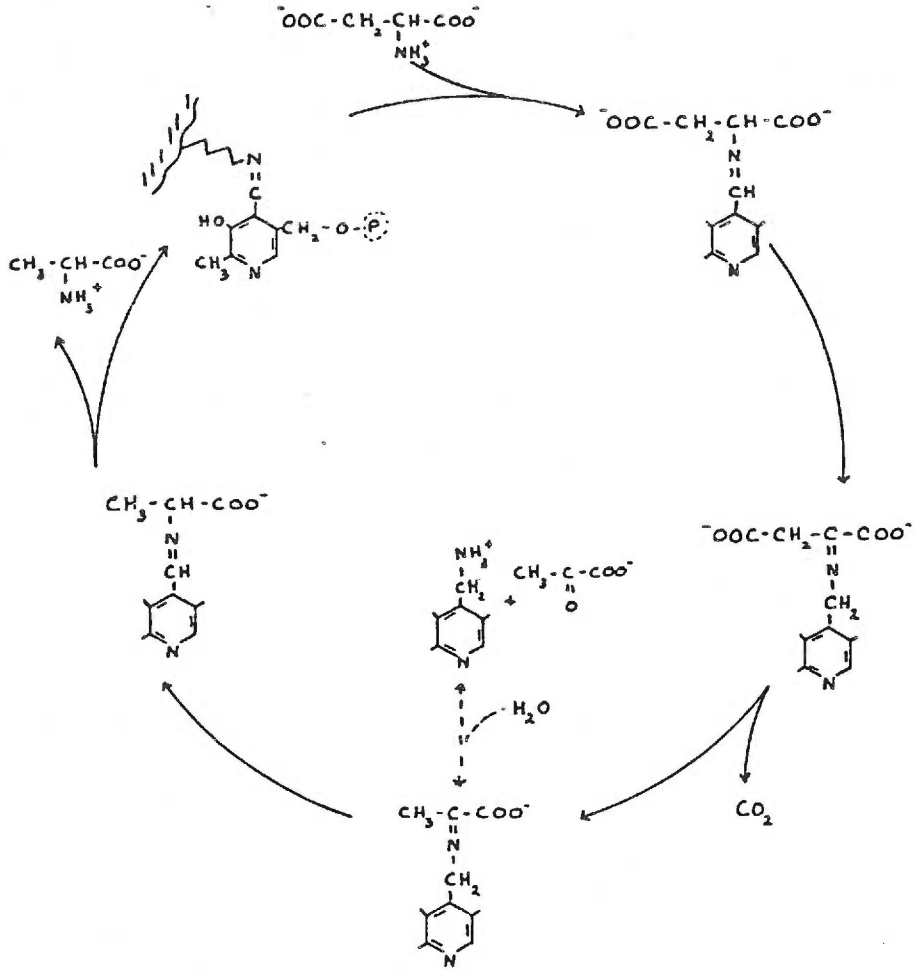

Figure IV-A. Proposed mechanism for microbial aspartate-4-decarboxylase. Catalytic cycle and regulation by α-keto acids.

versatile than most other pyridoxal 5'-phosphate containing enzymes.

The purified enzyme was shown to catalyze not only the β-decarboxylation of aspartate and the desulfination of cysteine sulfinate, but also a slow transamination of aspartate (dotted lines on figure IV-A). When the enzyme was incubated with aspartate, it slowly lost β-decarboxylase activity (Tate and Meister, 1969). A molecule of aspartate had reacted with the enzyme to form a molecule of pyruvate, a molecule of CO_2, and a molecule of pyridoxylamine 5'-phosphate form of the enzyme. This derivative of the enzyme could not catalyze the β-decarboxylation of aspartate. Small but sufficient (10^{-6}M to 10^{-5}M) concentrations of α-keto acids in the medium reverted most of the enzyme into its active pyridoxal 5'-phosphate form. Either pyruvate or α-ketoglutarate could satisfy the requirements for such a modulator. Pyridoxal 5'-phosphate added to the inactivated enzyme could also reactivate it. However, simultaneous addition of pyridoxal 5'-phosphate and α-ketoglutarate did not have additive effects.

The essence of these studies is that microbial aspartate-4-decarboxylase, a pyridoxal 5'-phosphate-requiring enzyme, can be activated or inactivated by an endogenous regulatory activity. The degree of activation can be regulated by fluctuations in catalytic quantities of α-keto acids. Although Meister and his colleagues were confident that this regulation of microbial aspartate-4-decarboxylase was physiologically significant, they made no suggetions on its possible cellular function. A useful attribute of this slow transaminase activity in the laboratory was that incubation of aspartate-4-decarboxylase with its substrate, over a prolonged period of time, resulted in complete conversion of the enzyme into its pyridoxamine 5'-

phosphate form. This compound could be readily dialyzed away from the enzyme to prepare the apoenzyme.

IV.B Kinetics of Rat Liver Enzyme

Aspartate-4-decarboxylase was demonstrated in rat livers in the previous chapter. In anticipation of studies on the purification of this enzyme and the determination of its regulatory properties, the optimum conditions for assaying this enzyme were evaluated.

Early studies, using a variety of buffers (Tris, phosphate, Bis-Tris, imidazole, citrate, acetate, and their various combinations), showed that aspartate-4-decarboxylase was about 50% more active in 50 mM imidazole HCl than in Tris buffer or phosphate buffer of equivalent strength. The other buffers tried were even less effective. From this point on, imidazole HCl was embraced as the buffer of choice for studies on rat liver aspartate-4-decarboxylase.

Alterations of activity with changing pH showed that rat liver aspartate-4-decarboxylase was most active at pH 7.0 (figure IV-B). A pH change of half a unit in either direction resulted in approximately 50% decrease in aspartate-4-decarboxylase activity. In the course of this work, the stability of the enzyme in media of different pH was also examined. Fraction 2 could be exposed to a pH as high as 9.5 without any loss of aspartate-4-decarboxylase activity. However exposure to solutions with a pH lower than 5.5 was associated with an irreversible loss of activity.

Release of $^{14}CO_2$ from L-[4-^{14}C]aspartate incubated with fraction 2 was linear with respect to time until 20% or more of the substrate was consumed (figure IV-B). However, the rate of CO_2 release was not directly proportional to the amount of protein used. The

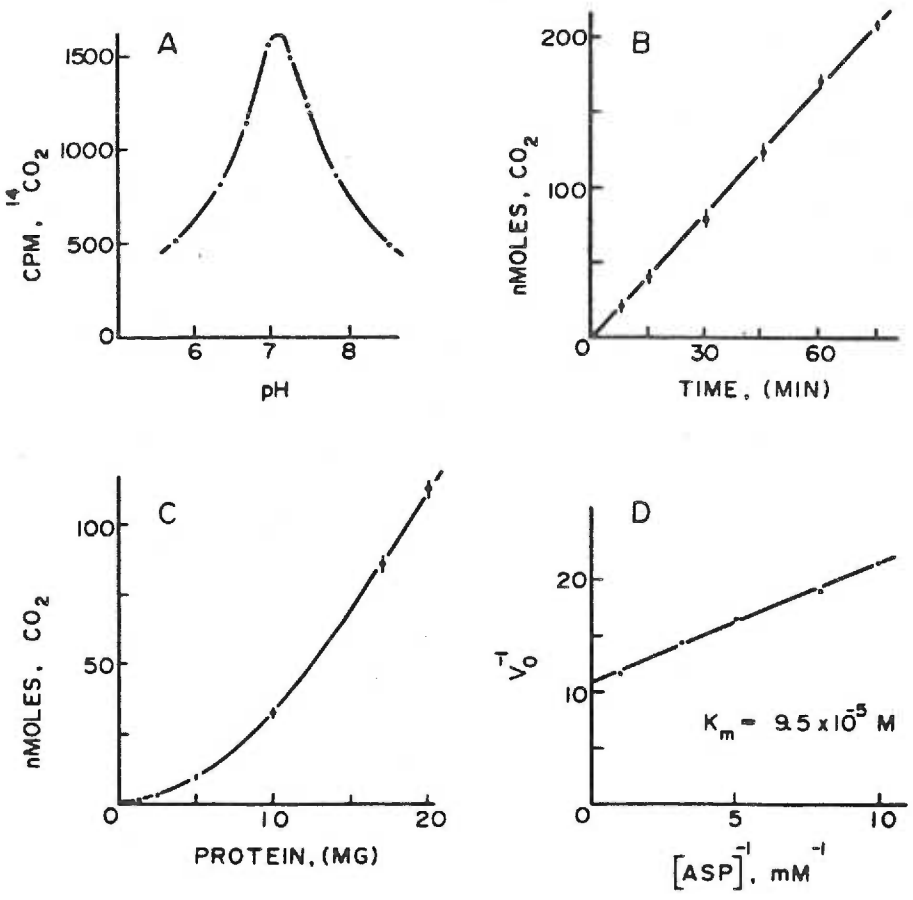

Figure IV-B. Kinetics of rat liver aspartate-4-decarboxylase

apparent specific activity of the enzyme increased with higher protein concentrations (figure IV-B). Several explanations were considered to account for this behavior. Exponential increase in activity with increasing protein concentration can be associated with such phenomena as substrate inhibition, product activation, a two (or more) step reaction involving a free intermediate, or the activation of one enzyme by a second enzyme (e.g., by dephosphorylation). For the enzymatic activity under consideration, these possibilities can be discounted since the amount of CO_2 formed at any protein concentration was directly proportional to the time of incubation.

One is forced to consider alternate explanations such as instability imposed on the enzyme at low protein concentration. However, when bovine serum albumin or gelatin were added to keep the total protein concentration constant while the amount of fraction 2 was varied, the activity response was still nonlinear.

The hypothesis that the loss of "specific activity" upon dilution of the enzyme was due to a concentration-dependent disassociation of an active polymeric enzyme into smaller enzymatically inactive or less active subunits was examined. If this were the case, the loss of specific activity of the diluted enzyme should be recovered upon reconcentration. When 40 mg/ml of fraction 2 was diluted to 2 mg/ml and then concentrated back to 40 mg/ml by vacuum dialysis, more than 95% of the enzyme activity was recovered, consistent with the subunit dissociation hypothesis.

For practical purposes and ease of interpretation, the nature of the preparation and the quantity of protein used for enzyme assays in this investigation have been specified. Unless otherwise mentioned, all

assays were performed using 6-7 mg of protein from fraction 2.

The effect of substrate concentration on enzyme activity was also studied. The rate of CO_2 release with increasing L-aspartate in the assay medium demonstrated simple saturation kinetics with an apparent K_m of 9.5×10^{-5} M for aspartate (figure IV-B).

III.C Active Site of Rat Liver Enzyme

Since microbial aspartate-4-decarboxylase required pyridoxal 5'-phosphate as a coenzyme, it was expected that rat liver aspartate-4-decarboxylase would also employ this cofactor.

When fraction 2 was assayed with and without pyridoxal 5'-phosphate, the difference in aspartate-4-decarboxylase activity was insignificant (Table IV-1). This suggested that either aspartate-4-decarboxylase from rat livers does not use pyridoxal 5'-phosphate, or the protein moiety is bound to the native coenzyme so firmly that there is no free apoenzyme in the preparation which can be activated by exogenous pyridoxal 5'-phosphate.

Since pyridoxal 5'-phosphate has an aldehyde group that is generally essential for its biological activity, addition of compounds that react with carbonyl groups have inhibitory effects on pyridoxal 5'-phosphate-utilizing enzymes. Indeed, hydroxylamine, amino-oxyacetic acid, and potassium borohydride inhibited rat liver aspartate-4-decarboxylase (Table IV-1).

The inhibition of rat liver aspartate-4-decarboxylase by reduction by potassium borohydride was irreversible (Table IV-1). This observation is not difficult to rationalize. At the active sites of many enzymes pyridoxal 5'-phosphate molecules react with ε-amino groups

of lysine residues to give stable aldimine linkages which can be reduced, irreversibly, to leave catalytically inactive derivatives of the enzymes (Fisher et al., 1958).

The inhibition by hydroxylamine provided some surprising results. It was anticipated that a coenzyme-reagent complex (such as the oxime that results from a reaction between pyridoxal 5'-phosphate and hydroxylamine) could be removed by dialyzing the enzyme against such a carbonyl-reagent. When the enzyme was dialyzed against hydroxylamine, followed by dialysis against 50 mM imidazole HCl (pH 7.0) and 1 mM EDTA, only the reagent was displaced and the enzymatic activity was completely recovered (Table IV-1). Again, addition of pyridoxal 5'-phosphate to such a preparation caused an insignificant increase in activity. Similar results were obtained with other carbonyl-reagents such as hydrazine, phenylhydrazine, semicarbazide, and phenylsemicarbazide.

TABLE IV-1

Characterization of the Active Site of Rat Liver Aspartate-4-Decarboxylase

Additions	Enzyme Activity (% of Control) Before Dialysis	After Dialysis *
Control	100	100 (102)
NH_2OH (10^{-4}M, 15 min)	3	97 (98)
Aminooxyacetic Acid (10^{-4}M, 15 min)	0	11 (16)
(10^{-3}M, 30 min)	0	7 (10)
KBH_4 (1.5 mg/mg protein, 15 min)	---	1 (3)
Aspartate (10^{-3}M, 30 min)	---	97 (99)

*Activities determined in the presence of 10^{-5}M pyridoxal 5'-phosphate are shown in parenthesis.

The fact that hydroxylamine (added to an assay vessel) inhibits aspartate-4-decarboxylase suggests that a carbonyl-group is indeed necessary for its enzymatic activity. When the enzyme is dialyzed against standard buffer, the concentration of hydroxylamine is reduced. If there is a strong nucleophile at the active site of rat liver aspartate-4-decarboxylase, it could displace any residual hydroxylamine to reactivate the enzyme. This may be the most plausible explanation to account for the reversibility of inhibition by hydroxylamine. A reasonable candidate for this nucleophile may be an ε-amino group of a lysine residue which normally forms a Schiff base with the carboxyl-group.

Aminooxyacetic acid-induced inhibition of rat liver aspartate-4-decarboxylase could not be reverted by dialysis against standard buffer. Furthermore, addition of pyridoxal 5'-phosphate failed to resurrect the enzyme (Table IV-1). This observation is puzzling. Possibly aminooxyacetic acid succeeded in generating an apoenzyme, but the protein moiety was so unstable in the absence of its cofactor that it underwent irreversible denaturation. Alternatively, the reagent may have reacted with pyridoxal 5'-phosphate at the active site to generate a stable product that was difficult to dialyze out or to exchange with added pyridoxal 5'-phosphate. Indeed, the product between aminooxyacetic acid and a carbonyl-group would closely resemble the predicted transition state intermediates of the β-decarboxylation reaction. The possibility that rat liver aspartate-4-decarboxylase may utilize a carbonyl-containing coenzyme other than pyridoxal 5'-phosphate could not be dismissed.

As mentioned earlier in this chapter, the slow transaminase

activity of microbial aspartate-4-decarboxylase was exploited by Meister (1970) to prepare its apoenzyme. The strategy involved incubation of the enzyme with aspartate to convert it to its pyridoxamine form, followed by resolution of the protein moiety from the pyridoxamine 5'-phosphate by dialysis. When fraction 2 was incubated with aspartate at 37°C for 60', there was no appreciable change in aspartate-4-decarboxylase activity (Table IV-1). A reasonable interpretation of this data is that mammalian aspartate-4-decarboxylase does not catalyze a slow transamination-reaction which is characteristic of the microbial enzyme.

In summary then, rat liver aspartate-4-decarboxylase could be inhibited by reagents which react with aldehyde groups. However, in no instance do we see an unambiguous reactivation of the enzyme by pyridoxal 5'-phosphate.

Physical Properties of Rat Liver Enzyme

As we have seen, dialysis of fraction 1 and 2 to remove α-ketoacids resulted in a preparation that provided some useful information about rat liver aspartate-4-decarboxylase. However, numerous other questions are not easy to answer using this crude enzyme preparation. For instance, is the nonlinear response in enzymatic activity with decreasing protein concentration solely due to disassociation of a polymeric enzyme into small subunits? Does the active-site have a carbonyl-containing coenzyme other than pyridoxal 5'-phosphate? Can the rat liver enzyme be activated by α-ketoglutarate by a mechanism other than transamination? What is the molecular weight of rat liver aspartate-4-decarboxylase and how many subunits does the

enzyme contain? Although all of these questions cannot be answered within the scope of this thesis, it was recognized that development of a method to purify rat liver aspartate-4-decarboxylase would be very valuable to investigators who will pursue this investigation. A pure enzyme preparation would also be useful to those who wish to prepare antibodies against this enzyme for studying such phenomena as the *in vivo* turnover of aspartate-4-decarboxylase. On these bases, a study was launched to determine a means for purifying rat liver aspartate-4-decarboxylase, if possible, to apparent homogeneity. The study revealed some interesting features of the enzyme.

Dispersion by Detergents

It was found that aspartate-4-decarboxylase from fraction 2 could not be separated from the bulk of other proteins using standard protein purification techniques (gel filtration, ion-exchange chromatography, isoelectric focusing, and hydroxyapatite chromatography). At first it was hypothesized that this was due to a continual disassociation of the enzyme during purification. This explanation became increasingly improbable as more protein purification systems were tried unsuccessfully.

An alternate hypothesis considered was that rat liver aspartate-4-decarboxylase was a hydrophobic protein which, under hydrophilic conditions, aggregated rather randomly with other hydrophobic proteins. In such a situation, protein purification systems would separate aggregates of proteins on the basis of their cumulative physical properties rather than the properties of the component proteins. Indeed, proteins solubilized with non-ionic detergents are notorious for such behavior (Tanford and Reynolds, 1976). Ionic

detergents such as sodium dodecylsulfate are very effective at disaggregating hydrophobic protein but they tend to denature proteins (Helenius and Simons, 1975). One class of detergents, bile salts such as sodium deoxycholate or sodium cholate, tend to be both effective dispersers of membrane proteins and also nondenaturing. This is particularly true if they are used in combination with non-ionic detergents (Hjelmeland et al., 1979). One of the drawbacks of common ionic detergents is that on binding hydrophobic proteins they add a net charge to the protein. This makes it virtually impossible to use such techniques as isoelectric focusing in combination with detergents such as deoxycholate. Recently, in an attempt to get around this problem, Hjelmeland (1980) designed and synthesized a non-denaturing, zwitterionic detergent which is a derivative of cholic acid. The compound, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, usually abbreviated "CHAPS", can now be purchased commercially.

Figure IV-C shows the effect of several detergents on rat liver aspartate-4-decarboxylase. Non-ionic detergents such as Triton X-100 and Brij 99 did not inhibit the enzyme. Neither did CHAPS. Deoxycholate inhibited rat liver aspartate-4-decarboxylase but dialyzing the detergent away resulted in complete recovery of enzymatic activity.

When supernatant (Fraction B) from freeze-thawed livers was subjected to gel-filtration chromatography (sephadex G-150), aspartate-4-decarboxylase appeared as a single, sharp, symmetric peak only when detergents were added to the elution buffers (figure IV-D). A combination of 0.1% deoxycholate and 0.05% Brij 99 in 50 mM imidazole HCl (pH 7.0), 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol was ideal. Brij 99 was used because, unlike Triton X-100, it was a non-

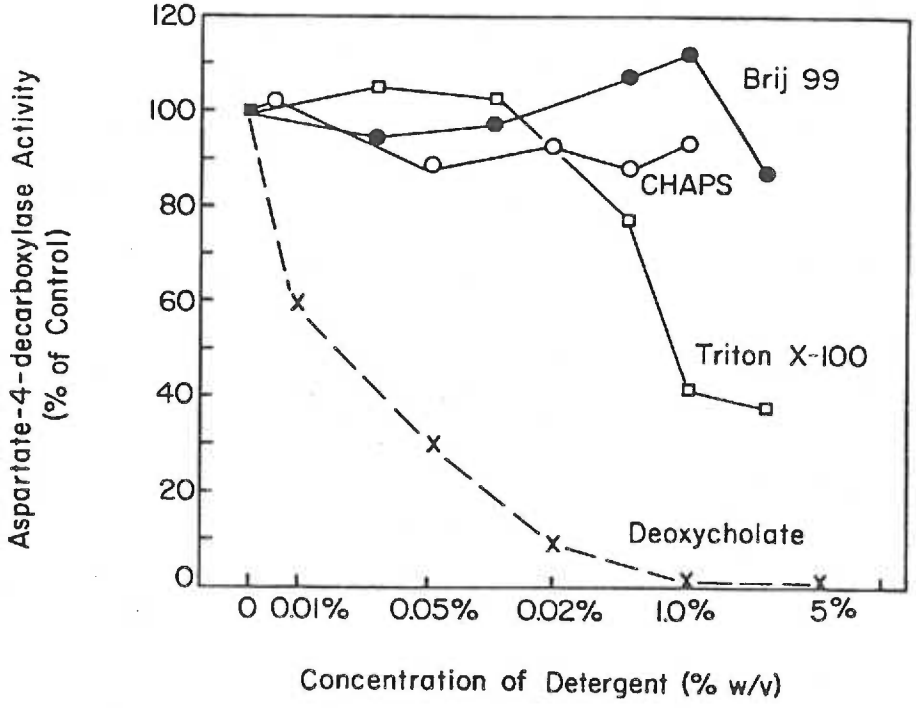

Figure IV-C. Effect of detergents on the activity of aspartate-4-decarboxylase.

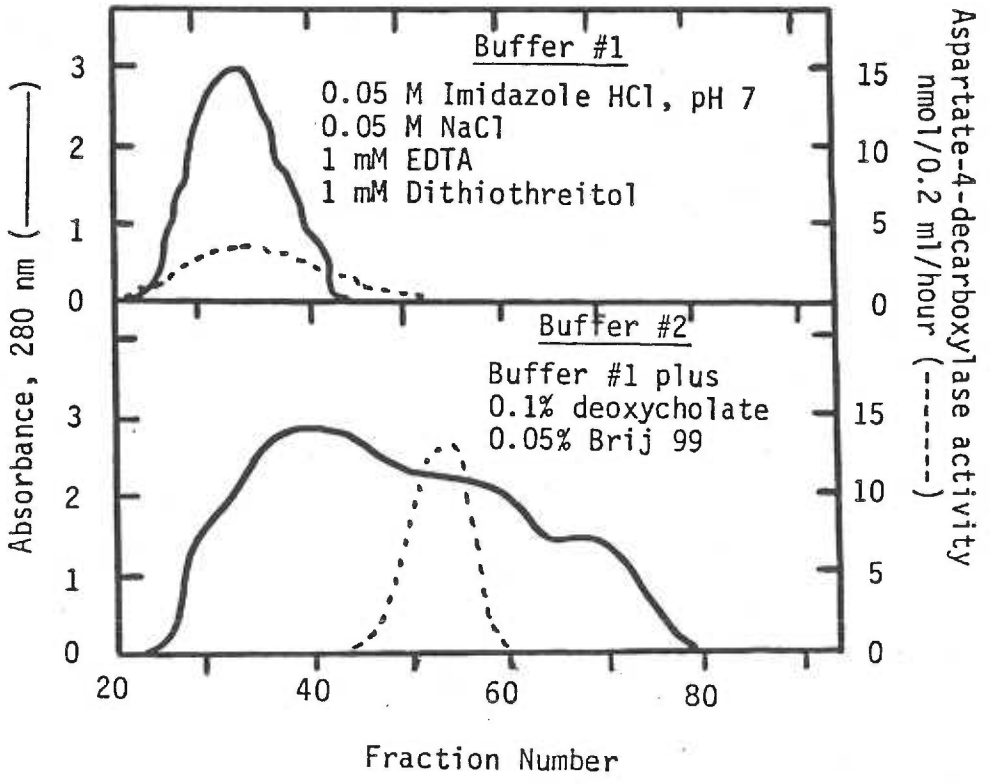

Figure IV-D. Effect of detergents on gel-filtration of aspartate-4-decarboxylase.

aromatic detergent which did not interfere with detection of proteins by UV spectrometry at 280 nm. CHAPS could replace deoxycholate but its prohibitive cost did not justify its use except under special circumstances.

Purification Strategy

On the basis of these studies it was decided that rat liver aspartate-4-decarboxylase solubilized by freeze-thawing would be used for enzyme purification. Freeze-thawing is a milder solubilization technique than homogenization in 1% Triton X-100. The latter treatment could solubilize a majority of the integral membrane proteins and increase the number of hydrophobic proteins with which aspartate-4-decarboxylase could potentially aggregate.

It was decided that 0.1% deoxycholate and 0.05% Brij 99 would be added to all buffers used in the early stages of enzyme purification. During isoelectric focusing, 0.1% CHAPS would replace 0.1% deoxycholate. Presumably, after most hydrophobic proteins have been separated away from aspartate-4-decarboxylase, there would be no need for maintaining detergents in the buffers.

Ion-Exchange Chromatography

With or without detergents, rat liver fractions which were bound to anion-exchange resins (such as DEAE-sephadex or DEAE-cellulose) contained aspartate-4-decarboxylase. The enzyme could be selectively desorbed with increasing NaCl in the elution buffer. As figure IV-E shows, rat liver aspartate-4-decarboxylase adsorbed to DEAE-cellulose could not be released with 50 mM imidazole HCl (pH 7.0) with 1 mM EDTA, 1 mM DTT, 0.1% deoxycholate, and 0.05% Brij 99. When the resin was washed with the above buffer containing 70 mM NaCl, the enzyme was

desorbed. The ion-exchange step of the purification scheme was performed on a Buchner funnel because large amounts of proteins could be handled easily and quickly. Homogenization of freeze-thawed rat liver, its centrifugation at 100,000 x g for 60 min, and absorption and desorption of the resulting supernatant to an anion-exchange resin were all performed on the same day.

In a separate experiment involving a column of DEAE-Sephadex which was eluted with a linear gradient of NaCl, it could be shown that cysteine sulfinate desulfinase and aspartate-4-decarboxylase activities co-migrated. It should be noted that, depending upon the assay conditions, the two activities may not appear to overlap. This is because cysteine sulfinate decarboxylase, which competes with the cysteine sulfinate desulfinase for the common substrate, overlaps with the desulfinate and appears to suppress the desulfinase activity. This effect can be overcome by dialyzing each sample against hydroxylamine and then against 50 mM imidazole HCl (pH 7.0). Under these conditions the cysteine sulfinate decarboxylase is selectively inhibited (see section III.B). The desulfinase peak and aspartate-4-decarboxylase peak now overlap.

When the active fraction from DEAE-cellulose was applied to Carboxyl-methyl-Sephadex, Sulfopropyl-Sephadex, or hydroxyapatite, all the enzymatic activity appeared in the void volume and no additional purification was achieved.

Gel Filtration Chromatography

A sample of fraction C concentrated to approximately 5 ml was applied to a 2.5 x 98 cm sephadex G-150 column which had been equilibrated with the elution buffer [50 mM imidazole HCl (pH 7.0), 50

mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% deoxycholate, 0.05% Brij 99]. This same buffer was then pumped through the column. Aspartate-4-decarboxylase and cysteine sulfinate desulfinase again appeared as single peaks which overlapped. The elution position of aspartate-4-decarboxylase is shown in figure IV-F. Tubes which contained active aspartate-4-decarboxylase were pooled and prepared for the next step (see section II.C).

Hydrophobic Interaction Chromatography

This technique involved binding of proteins to Sepharose beads that have hydrophobic side chains. The proteins then may be eluted by decreasing the polarity of the buffer. Aspartate-4-decarboxylase would bind to Octyl-Sepharose only when it was prepared in high salt concentration (1 M ammonium sulfate). In contrast, it absorbed to Phenyl-Sepharose relatively easily [50 mM imidazole HCl, 50 mM NaCl (pH 7.0)]. Elution of the enzyme off Phenyl-Sepharose could be achieved by increasing the concentration of ethylene glycol in the buffer (figure IV-G). This purification step was associated with a disproportionally large loss in total activity (see Table IV-2). However, a large number of adventitious proteins are also removed in this step (see SDS-polyacrylamide gel pattern in Figure IV-H). More importantly, hydrophobic interaction chromatography successfully resolved aspartate-4-decarboxylase activity from aspartate aminotransferase activity and, suprisingly, cysteine sulfinate desulfinase activity (see Figure IV-G and Table IV-3).

TABLE IV-2

Summary of Partial Purification of Rat Liver Aspartate-4-Decarboxylase

Fraction	Total Protein	Total Activity (pmol/hr)	Specific Activity (pmol/mg/hr)	Purification	Recovery (%)
A Whole homogenate	11,100	26,800	2.4	1.00	100
B Supernatant	5,200	18,600	3.6	1.48	69
C DEAE-Cellulose	1,500	17,318	11.7	4.84	65
D Sephadex G-150	250	9,375	37.5	15.60	35
E Phenyl-Sepharose	12	450	37.5	15.60	2

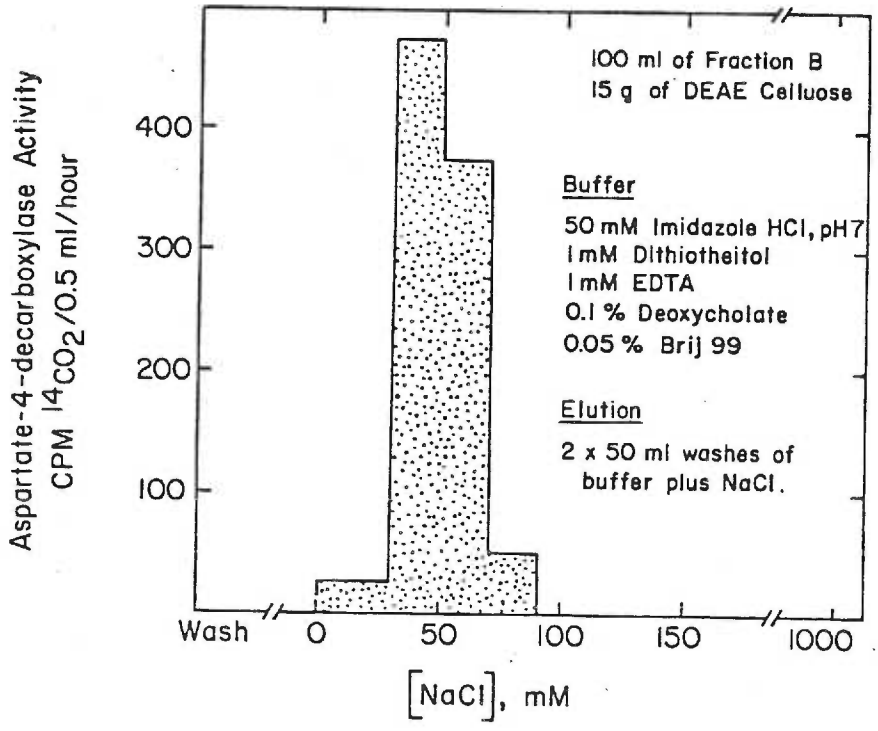

Figure VI-E. Elution off DEAE-Cellulose

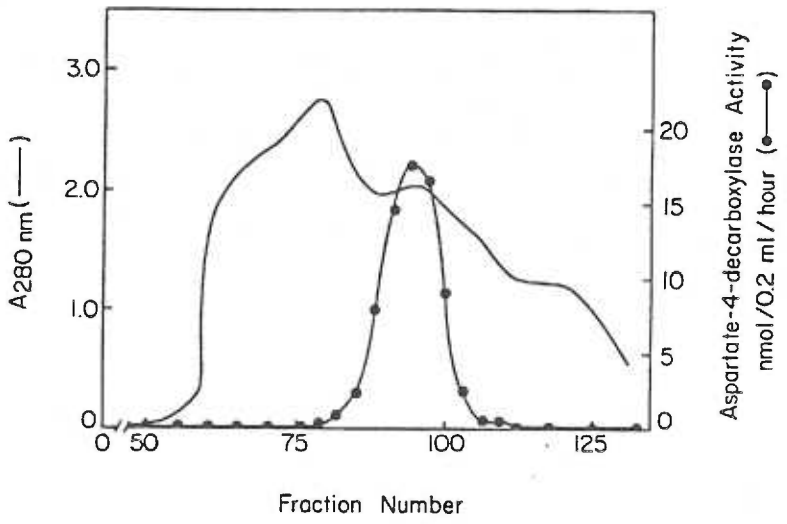

Figure IV-F. Elution off Sephadex G-150

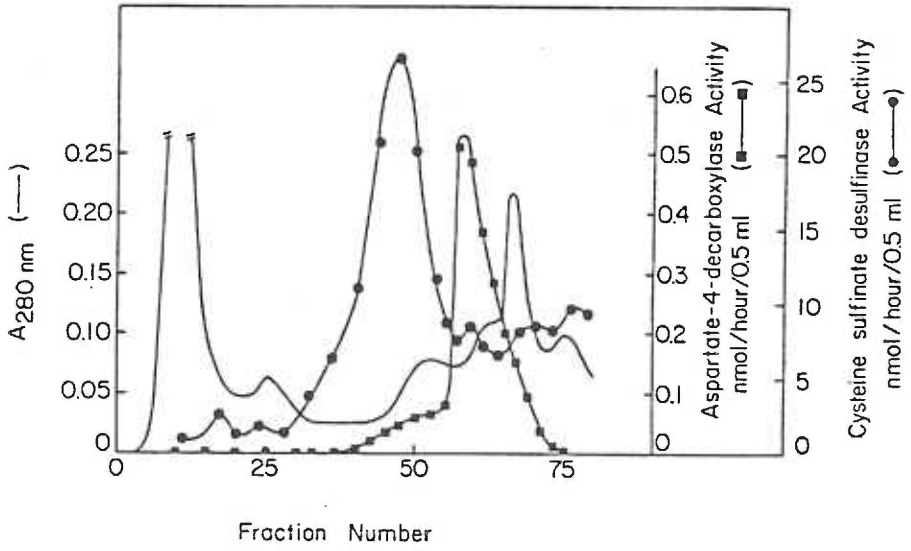

Figure IV-G. Elution off phenyl-Sepharose

Table IV-3

Relative Purification of Aspartate-4-Decarboxylase, Cysteine Sulfinate Desulfinase, and Aspartate Aminotransferse

Fraction	Aspartate-4-Decarboxylase	Cysteine Sulfinate Desulfinase	Aspartate Aminotransferase
A Whole homogenate	100%	100%	100%
B Supernatant	148%	274%	100%
C DEAE-Cellulose	484%	221%	603%
D Sephadex G-150	1560%	760%	1876%
E Phenyl-Sepharose	1560%	0%	27%

100% Aspartate-4-decarboxylase = 2.41 nmol/mg/hr

100% Cysteine sulfinate desulfinase = 6.64 nmol/mg/hr

100% Aspartate aminotransferase = 57.7 nmol/mg/hr

Isoelectric-Focusing

The active fractions from the previous run were pooled and prepared for preparative isoelectric focusing as described in section II.C. Isoelectric focusing was performed on a flat bed of granulated gel in the presence of CHAPS and Brij 99. A contact print of the focused protein showed 3 bands. However, none of the fractions contained aspartate-4-decarboxylase.

In the past, isoelectric focusing performed exactly as described yielded active aspartate-4-decarboxylase around pI 5.3 to 5.8. It is only when the Phenyl-Sepharose-treated fraction was added to the system that enzymatic activity was lost. The possible causes for the inactivation of aspartate-4-decarboxylase during these later stages

of purifcation are discussed in the next section.

The methods used to focus the enzyme were discussed in detail to allow future investigators to improve on the technique.

Degree of Purification Achieved

Response of aspartate-4-decarboxylase activity to increasing amounts of fraction 2 was exponential (see section IV-B). This property of the enzyme persisted through each purification stage and so the specific activity of aspartate-4-decarboxylase in any given fraction depended on the protein concentration at which the assay was performed. If a constant amount of protein had been assayed (say 2 mg) after each purification step, the increase in specific activity would not be directly proportional to the actual amount of purification achieved. A more conservative and accurate means of evaluating purification was as follows:

1. Each fraction was assayed for 30' at several different protein concentrations. Activity versus protein concentration was graphed.
2. The amount of protein necessary to get 6 nmol CO_2/hr was determined.
3. The specific activity (nmol CO_2/mg/hr protein) was calculated using the protein value determined above (2).

On the basis of such evaluation, the complete purification procedure resulted in aspartate-4-decarboxylase preparation fifteen-fold more pure than fraction A, with a total recovery of 2% (Table IV-2).

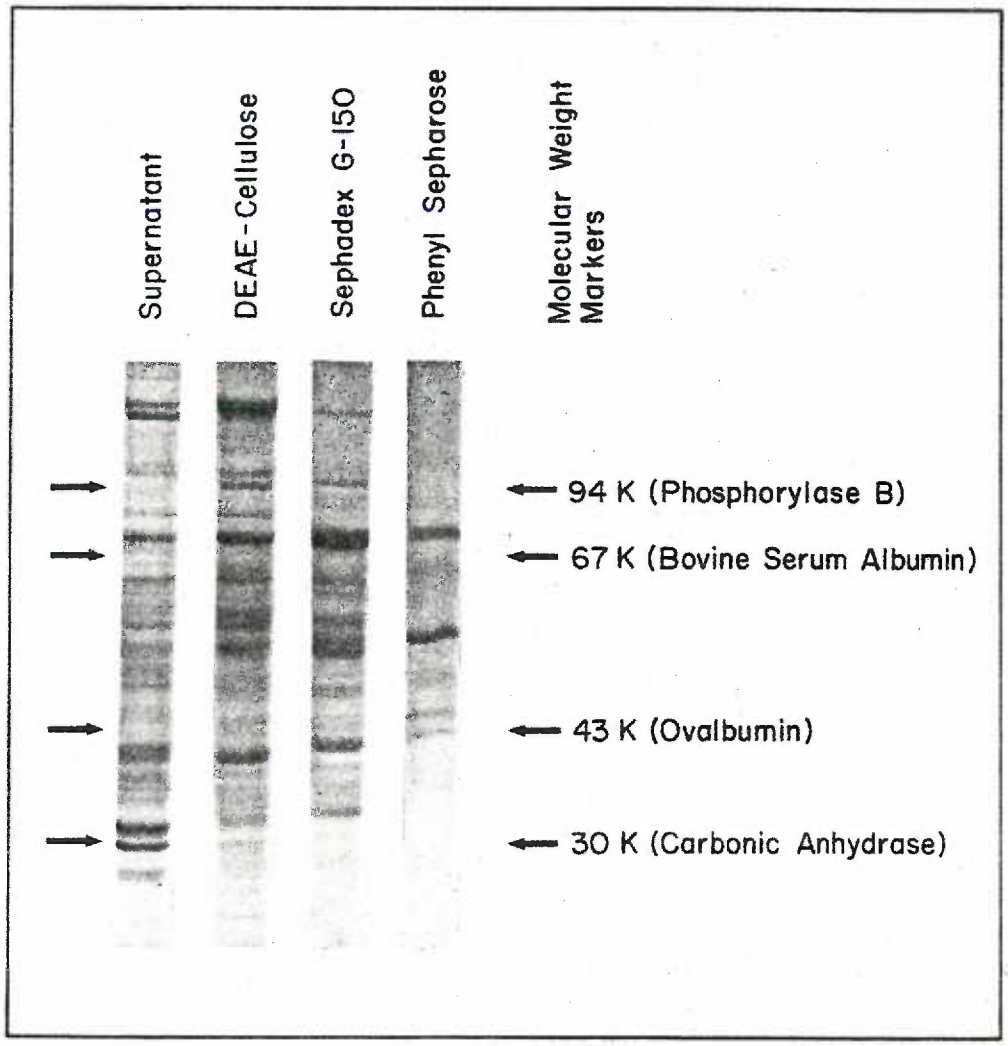

Figure IV-H. SDS-polyacrylamide gel electrophoresis of the purified proteins. Electrophoresis was performed in 12.5% gels with Tris-HCl and the Tris/glycine buffer system of Laemmli (1970) according to the method of Weber, Pringle and Osborn (1972).

DISCUSSION

Initial studies on the physical and chemical properties of rat liver aspartate-4-decarboxylase revealed several differences between the mammalian enzyme and the well-studied microbial enzyme.

The rat liver enzyme, assayed in imidazole HCl buffer, had an optimum pH of 7.0 in contrast to the microbial enzyme which is most active in phosphate buffer between pH 5 and 5.5. At these acidic conditions the mammalian enzyme showed an irreversible loss of activity. In addition, the rat enzyme showed a loss of specific activity when diluted. This loss of activity could be reversed when the proteins were reconcentrated. It is hypothesized that this observation may be due to the reversable disassociation of a polymeric enzyme into its subunits which are catalytically less active than the whole enzyme. Such a phenomenon has not been reported with the microbial enzyme.

Further differences between the microbial and mammalian enzyme were observed during studies on the active site of the rat liver enzyme. Although the rat liver enzyme was inhibited by compounds which react with carbonyl-groups, there was no evidence for stimulation of mammalian aspartate-4-decarboxylase by pyridoxal 5'-phosphate. The two most likely explanations for these observations are:

(i) The mammalian enzyme is pyridoxal 5'-phosphate-dependent, but the nature of the binding between the apoenzyme and the coenzyme is unusually strong.

(ii) The mammalian enzyme, in contrast to the microbial enzyme, uses a carbonyl-containing cofactor other than

> pyridoxal 5'-phosphate. [Recently, several decarboxylases have been shown to utilize a covalently linked pyruvoyl residue as a prosthetic group rather than pyridoxal 5'-phosphate (Riley and Snell, 1968; Dermetrion et al., 1978; Satre and Kennedy, 1978), including aspartate-1-decarboxylase from Escherichia Coli (Williamson and Brown, 1979)].

There are other observations to suggest that the active-site of mammalian aspartate-4-decarboxylase differed from that of the microbial enzyme. Incubation of the mammalian enzyme with aspartate did not result in loss of β-decarboxylase activity presumably because, unlike the microbial enzyme, the mammalian enzyme had no aspartate aminotransferase activity. Purification of the mammalian enzyme to apparent homogeneity will allow unambiguous characterization of its active site. For example, aspartate aminotransferase activity, if present, could be assayed directly. Chemical analyses of the protein would confirm the presence or absence of pyridoxal 5'-phosphate at the active site (UV spectroscopy, reduction with radiolabelled borohydride followed by separation of a pyridoxal-derivative, etc.).

Purification studies on rat liver aspartate-4-decarboxylase revealed even more differences between the mammalian and the microbial enzyme. First, the mammalian enzyme was found to be very hydrophobic. Its purification could be achieved only in the presence of detergents. Second, during Phenyl-sepharose chromatography it was possible to separate the majority of cysteine sulfinate desulfinase activity from the aspartate-4-decarboxylase activity. In microorganisms the two enzymatic activities co-purify until an apparently homogeneous protein

is achieved. It is reasonable to conclude that rat liver contains a protein which catalyses the desulfination of cysteine sulfinate but not the β-decarboxylation of aspartate. This interpretation is supported by studies on the regulatory properties of mammalian aspartate-4-decarboxylase and cysteine sulfinate desulfinase (section V.C).

Using 0.1% deoxycholate (an ionic bile salt derivative) and 0.05% Brij 99 (polyoxyethylene (20) oleyl ether, a non-ionic detergent) in the buffers, ion-exchange chromatogrphy and gel-filtration chromatography yielded a preparation that was at least fifteen-fold purer than aspartate-4-decarboxylase in whole homogenate. The estimation of purification of rat liver aspartate-4-decarboxylase was not easy. First, in any given fraction the specific activity of the enzyme varied with the amount of protein used to assay the enzyme. This problem was overcome by using an unusual method to determine specific activity. The amount of protein necessary to attain 6 nmol CO_2 release/hour was determined and this value was used to calculate the usual expression of specific activity, "nmol CO_2/mg protein/hr." The second problem in determining net purification had to do with an increasing amount of aspartate-4-decarboxylase lost after each purification step. For example, with Phenyl-Sepharose chromatography it is apparent that a great many proteins which do not have aspartate-4-decarboxylase activity were removed (see figure IV-G and IV-H), yet the resulting preparation, fraction E, did not have a specific activity much higher than fraction D.

Although the source of this loss of activity was not determined, the most likely candidates included one or more of the following:

(i) instability of the highly purified protein.

(ii) loss of vital membrane components with increasing exposure to surfactant materials.

(iii) separation of unidentical protein components of an intact enzyme to yield inactive subunits.

Once the cause of this loss of enzymatic ativity is determined, it should be simple to purify mammalian aspartate-4-decarboxylase to apparent homogeneity. Inspection of SDS-PAGE pattern (figure IV-H) of fraction E shows the presence of 5 to 6 protein bands. Preparative isoelectric focusing (non-denaturing) can resolve at least 3 protein bands. If necessary an affinity column, using acetyl-coenzyme A as a ligand, may be utilized as a last step in the purification procedure (see section V.C for the effect of acetyl-coenzyme A on rat liver aspartate-4-decarboxylase).

V FUNCTION IN CELL PHYSIOLOGY

Evidence for the existence of aspartate-4-decarboxylase in mammals was presented in Chapter III. Purification of the enzyme to homogeneity will reveal a great deal about its physico-chemical properties. Ultimately of course, we should be interested in the function of this enzyme in mammalian cell physiology. It is not possible to turn to studies on the Alcaligenes faecalis enzyme for insights. After several decades of work on the molecular properties of microbial aspartate-4-decarboxylase, we know as little about its function in cell physiology as we did when it was first discovered (Mardashev, 1947).

In this chapter a hypothesis is presented for the physiological function of aspartate-4-decarboxylase in mammals. Later it is shown that the regulatory properties of rat liver aspartate-4-decarboxylase are consistent with this hypothesis.

V.A Development of the Hypothesis

Nature of Reaction

Often the reaction that an enzyme catalyzes can provide useful clues regarding its function in the cell. For instance, consider the well known enzyme phenylalanine hydroxylase which converts phenylalanine, an essential amino acid, to tyrosine (Kaufman and Fisher, 1974). It is implicit in this reaction that whenever adequate phenylalanine is available in the diet, tyrosine is not an essential amino acid. Furthermore, the hydroxylase must be an important reaction in the degradation of phenylalanine because there are several enzymes

which vigorously degrade tyrosine but not phenylalanine.

On first examination similar insights on the possible function of aspartate-4-decarboxylase are not self-evident. It is highly unlikely that the enzyme serves as an important means for the conversion of aspartate to alanine. There are enzymes in rat livers (and many other tissues) which will degrade aspartate and synthesize alanine more vigorously than aspartate-4-decarboxylase. More importantly, these alternate reactions couple the decarboxylation step to an energy conserving reaction. For instance, aspartate can be converted to alanine using aspartate-aminotransferase (Berthland and Kaplan, 1968) to form oxaloacetate; this can undergo decarboxylation to pyruvate using pyruvate-carboxylase (Utter and Keech, 1963), an enzyme which couples the decarboxylation to the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. The pyruvate generated from this reaction may then be converted to alanine by the action of alanine aminotransferase (Green et al., 1945). The combined effect of these reactions is the β-decarboxylation of aspartate and the simultaneous phosphorylation of ADP.

In contrast to the three actions described above, the β-decarboxylation of aspartate is an essentially irreversible reaction (calculated $\Delta G^{o'}$ = -62 Kcal/mol).

Potential Importance in Metabolic Regulation

The exothermic nature of aspartate-4-decarboxylase suggests that it could play an important role in the regulation of intracellular concentrations of aspartate and indirectly, all metabolites that are in equilibrium with aspartate. It is well recognized that regulation of an enzyme which catalyzes a reaction far from equilibrium can have a

profound effect on the steady-state concentrations of the reactant(s) (Newsholme and Start, 1973).

Potential Danger: Energy Drain

The fact that aspartate-4-decarboxylase is not coupled to an energy conserving step adds another aspect that should be taken into consideration in a hypothesis for its physiological function. Aspartate-4-decarboxylase, pyruvate carboxylase and the two transaminases may be aligned to form a potential substrate cycle (also called futile cycle) (Figure V-A). If not carefully regulated, the combined reactions simply degrade and resynthesize aspartate. In accordance with the second law of thermodynamics, such cycling can be maintained only at the expense of ATP hydrolysis.

Substrate cycles have been implicated in the regulation of metabolic pathways and in thermogenesis (Newsholme, 1980; Belfiore and Iannello, 1978). However, it is difficult to prove that these cycles are physiologically important. A common objection to these proposals is that for most anabolic reactions in biochemistry (synthesis of six-carbon sugars, proteins from amino acids, fatty acids from acetyl-coenzyme A, etc.), there are corresponding catabolic reactions (glycolysis, hydrolysis of protein, fatty acid oxidation, etc.). Therefore, the number of potential futile cycles are almost endless. Yet, it is argued, all of these reactions are unlikely to be important sites for futile cycling. This is particularly true because usually there exist regulatory control mechanisms that prevent simultaneous synthesis and degradation of biological compounds such as glucose (Bioteux et al., 1980).

The potential aspartate-alanine cycle is unique. Unlike

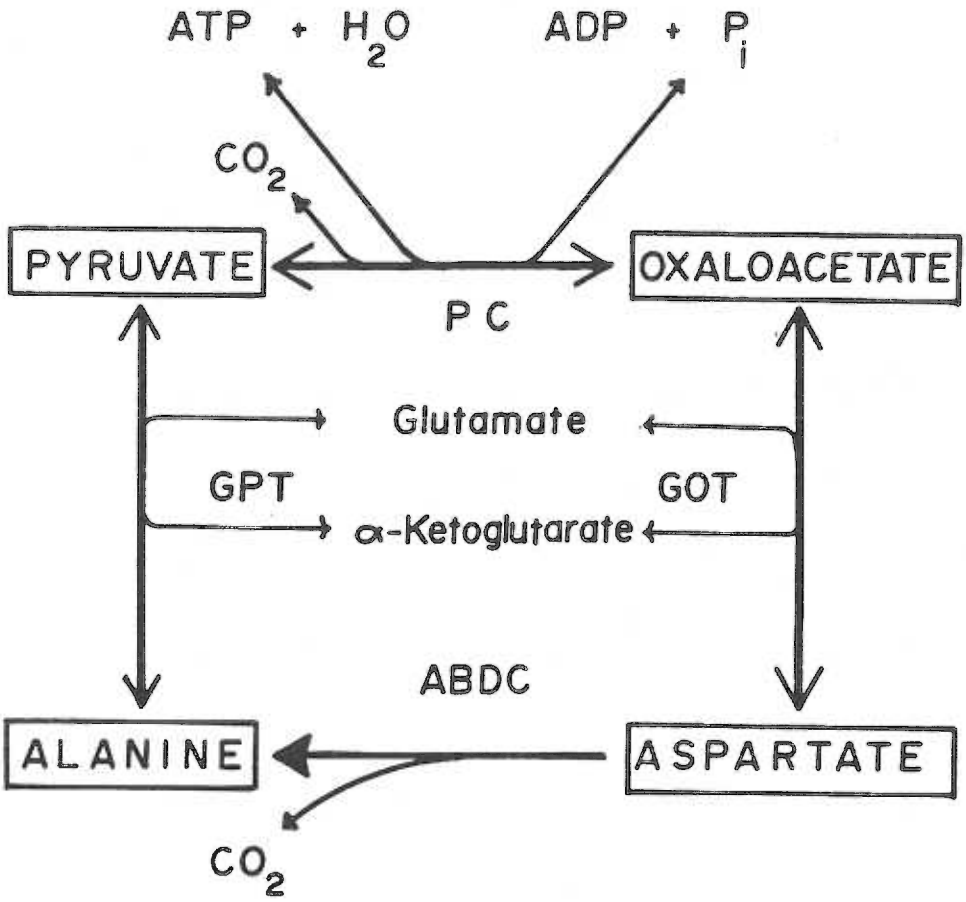

Figure V-A. A potential futile cycle

enzymes of other futile cycles, aspartate-4-decarboxylase does not appear to be part of any important metabolic pathway. Whether it participates in a futile cycle with pyruvate-carboxylase depends on how freely the substrates may diffuse between the component enzymes, and on the possibility that the enzymes are expressed simultaneously.

V.B Statement of Hypothesis

Regulation of Oxalocetate

Taking all of these points into consideration, a hypothesis for the function of aspartate-4-decarboxylase in rat livers is considered. It is proposed that the enzyme is not an important means for the conversion of aspartate to alanine. Instead, it is a key site in the regulation of steady-state concentration of aspartate, and therefore oxaloacetate. Aspartate and oxaloacetate are in equilibrium by the action of aspartate aminotransferase. Shifts in intracellular aspartate, then, should have a proportional effect on the intracellular oxaloacetate levels.

Oxaloacetate is a key metabolite of the tricarboxylic acid (TCA) cycle. It reacts with acetyl-coenzyme A (from carbohydrates or fatty acids) to form citrate (Shepherd and Garland, 1969). In the past, numerous enzymes have been advanced as possible regulators of intracellular oxaloacetate. The most widely accepted is pyruvate carboxylase (EC 6.4.1.1) which synthesizes oxaloacetate from pyruvate and CO_2 and simultaneously hydrolyses ATP to ADP (Utter and Keech, 1963). A key piece of evidence in considering pyruvate carboxylase in this regard is that it has an absolute requirement for acetyl-coenzyme A. This compond exerts its stimulatory effects on pyruvate carboxylase

by allosteric mechanisms (Scrutton and White, 1973). Apparently when energy demands are high and the cell is degrading stored lipids, acetyl-coenzyme A levels rise and pyruvate carboxylase is stimulated (Wieland et al., 1964). This, in turn, raises the concentrations of oxaloacetate and fuels the TCA cycle for the production of useful energy.

Thermogenesis and Weight Control

The proposal that aspartate-4-decarboxylase is an important enzyme in the regulation of oxaloacetate has important implications regarding energy metabolism. Since the β-decarboxylation of aspartate by the enzyme is not coupled to an energy-conserving step, the energy released during the reaction is lost as heat. Furthermore, since the reaction is irreversible, every time oxaloacetate is metabolized through aspartate-4-decarboxylase, less of it is available for processing through enzymes that salvage energy.

It is further hypothesized that aspartate-4-decarboxylase would be used to catabolize oxaloacetate (and, therefore, other TCA cycle intermediates) whenever fuel sources are abundant and carbohydrates are to be oxidized inefficiently. This "inefficient" metabolism may be important in regulating thermogenesis and, perhaps, body weight (see Discussion).

V.C Regulatory Properties

Predictions

Aspartate-4-decarboxylase and pyruvate carboxylase have been proposed to be regulators of steady-state concentrations of oxaloacetate; it was expected that the two enzymatic activities could be modulated in a coordinated manner. Since aspartate-4-decarboxylase

tends to decrease oxaloacetate levels and pyruvate carboxylase tends to increase oxaloacetate levels, it was predicted that the modulators would have "reciprocal" effects on the two activities.

It is well known that acetyl-coenzyme A is an essential positive modulator of rat liver pyruvate carboxylase. Coordinated regulation can be effectively achieved if aspartate-4-decarboxylase is inhibited by acetyl-coenzyme A. This way, when acetyl-coenzyme A accumulates, the degradation of oxaloacetate can be decreased by inhibiting aspartate-4-decarboxylase and the synthesis of oxaloacetate can be increased by stimulating pyruvate carboxylase. The reverse can occur when acetyl-coenzyme A levels drop back to normal.

Pyruvate carboxylase, in addition to being stimulated by acetyl-coenzyme A, is inhibited by compounds which reflect the steady-state concentrations of TCA cycle intermediates. For instance, chicken liver and rat liver pyruvate carboxylase are inhibited by α-keto-glutarate and glutamate (Scrutton and White, 1974). Yeast pyruvate carboxylase is inhibited by aspartate (Cazzulo and Stoppani, 1968). According to our hypothesis, aspartate-4-decarboxylase should be stimulated by such compounds. Indeed, the stimulation of microbial aspartate-4-decarboxylase by α-ketoglutarate is very well known (see section IV-A). However, it is also known that this stimulation of microbial aspartate-4-decarboxylase is mediated by a slow transaminase activity which the rat liver enzyme appears to lack (see section IV-C). It is predicted then that rat liver aspartate-4-decarboxylase would be stimulated by α-ketoglutarate using alternate mechanisms or the enzyme may be stimulated by compounds other than α-ketoglutarate which reflect the intracellular concentrations of TCA cycle intermediates.

Obsevations

When 7 mg of fraction B was assayed for rat liver aspartate-4-decarboxylase activity in the presence of increasing amounts of acetyl-coenzyme A, the enzyme was inhibited (Figure V-B), as predicted by the hypothesis. The sensitivity of rat liver aspartate-4-decarboxylase to acetyl-coenzyme A (apparent K_i = 5 x 10-^{6}M) was within the range of physiological variations in acetyl-coenzyme A (Soboll et al 1976; Wieland et al, 1964). Furthermore, the modulation appeared to be very specific in nature. Coenzyme A, the unacetylated compound, had no appreciable effect on rat liver aspartate-4-decarboxylase activity at concentrations as high as 100 µM. Neither did nucleotides such as ATP and ADP. Inhibition of rat liver aspartate-4-decarboxylase by acetyl-coenzyme A did not increase but decreased with time (Figure V-C). This suggests that the effect is mediated by an allosteric mechanism rather than covalent modification of the enzyme. The decrease was attributed to instability of the thioester bond of acetyl-coenzyme A.

It was predicted that compounds which reflect the concentrations of TCA cycle intermediates would stimulate rat liver aspartate-4-decarboxylase. Aspartate, itself, is one such compound. Since it is in equilibrium with oxaloacetate, its intracellular concentrations would increase as TCA cycle intermediates accumulated. Rat liver aspartate-4-decarboxylase displayed simple saturation kinetics with respect to its substrate (Km = 9.5 x 10-^{5}, Figure IV-B), and would be stimulated by increases in aspartate concentrations.

The effect of α-ketoglutarate on rat liver aspartate-4-decarboxylase could not be evaluated unambiguously since the enzyme was not purified away from glutatmate aspartate aminotransferase.

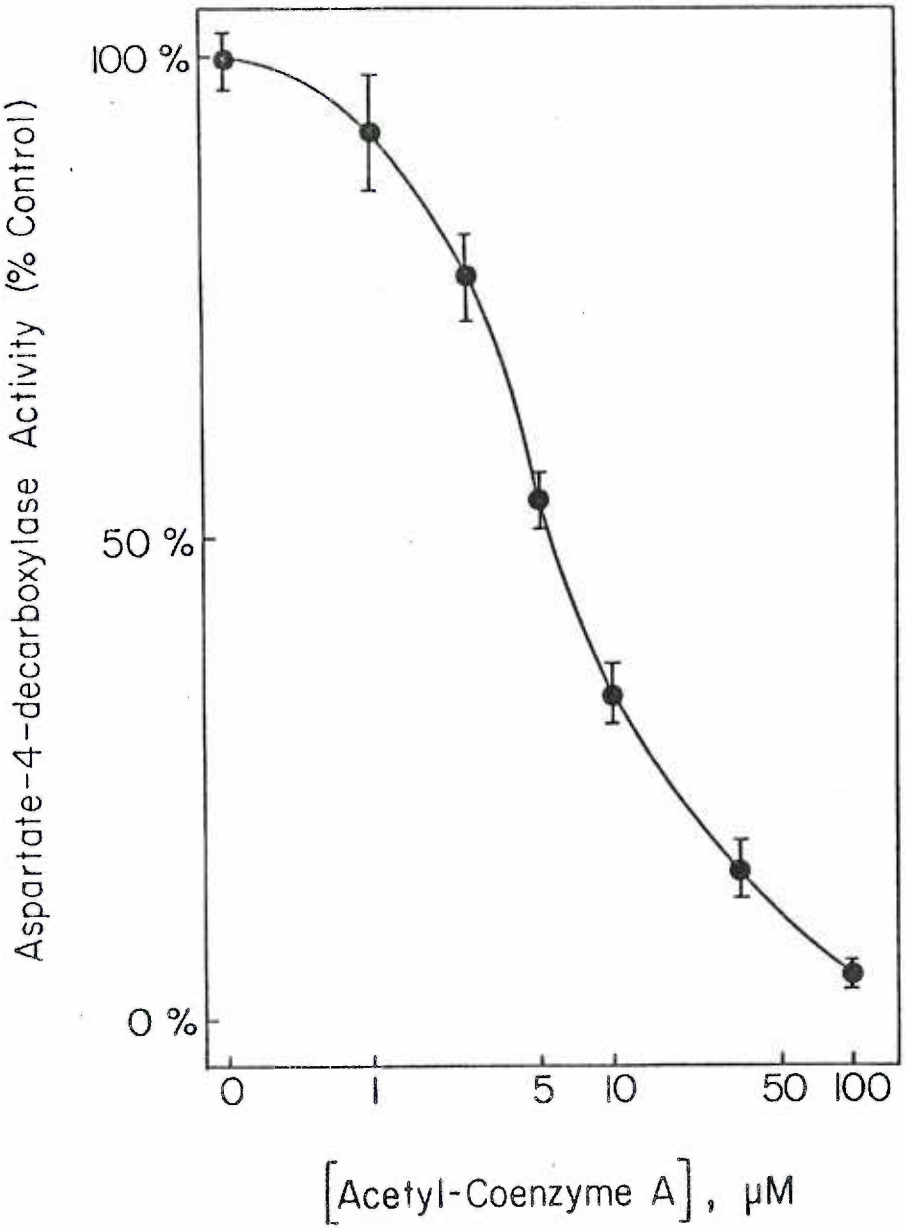

Figure V-B. Effect of acetyl-coenzyme A on rat liver aspartate-4-decarboxylase.

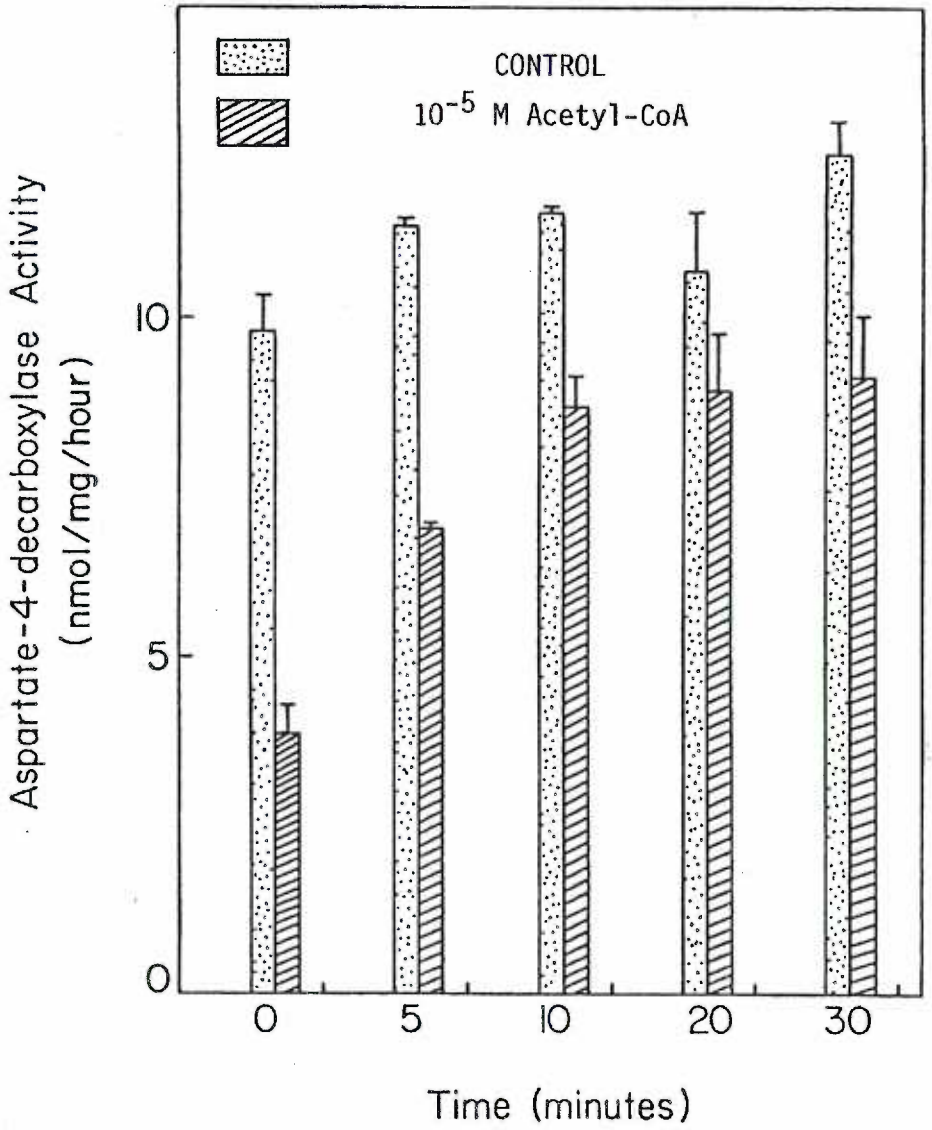

Figure V-C. Time dependency of inhibition by acetyl-coenzyme A.

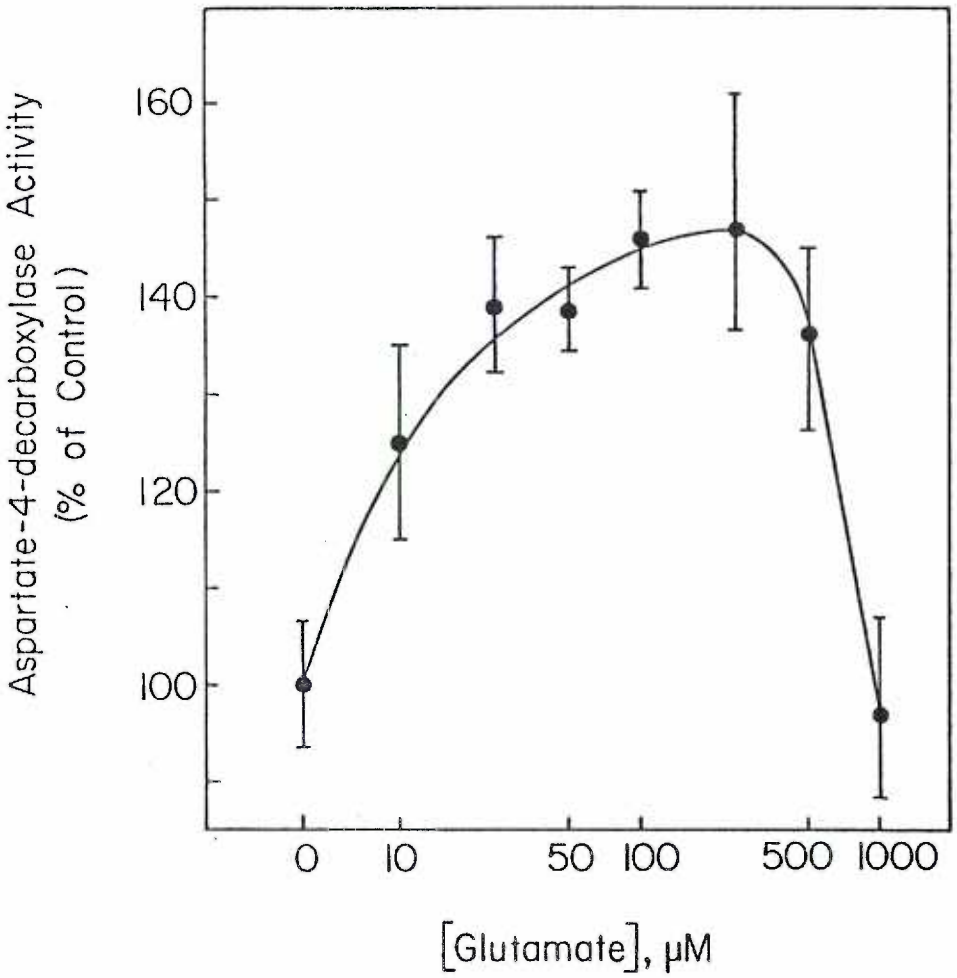

Figure V-D. Effect of glutamate on rat liver aspartate-4-decarboxylase.

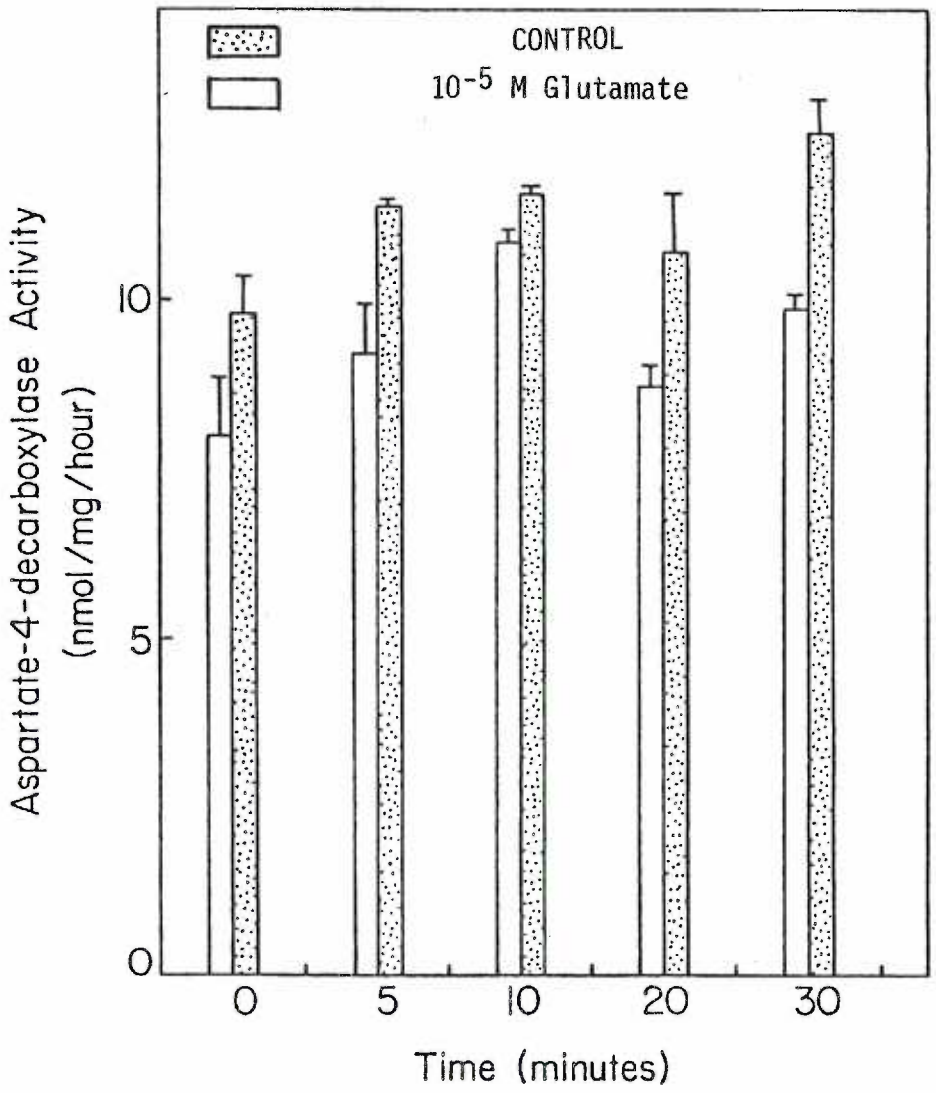

Figure V-E. Time dependency of stimulation by glutamate.

Glutamate is in equilibrium with intracellular α-ketoglutarate and therefore is as good an indicator of TCA cycle intermediates as α-ketoglutarate. The effect of glutamate on rat liver aspartate-4-decarboxylase was examined (Figure V-D). Normally glutamate, which is a homologue of aspartate, would be expected to act as a competitive inhibitor of rat liver aspartate-4-decarboxylase. Instead, as predicted, at physiological concentrations it was found to activate the enzyme. At higher concentrations it was less effective as an activator. Presumably at these concentrations glutamate not only bound a regulatory site on the enzyme, but also competed for the active site. Preincubation of fraction 2 with glutamic acid had an insignificant effect on aspartate-4-decarboxylase activity (Figure V-E).

It was pointed out in section IV.E that rat liver aspartate-4-decarboxylase could be resolved from a majority of cysteine sulfinate desulfinase activity, suggesting that in mammals the two enzyme activities are catalysed by different proteins. The effect of acetyl-coenzyme A (a potent inhibitor of mammalian aspartate-4-decarboxylase) on rat liver cysteine sulfinate desulfinase was examined. At 10^{-5}M acetyl-coenzyme A, the desulfinase was inhibited by less than 15%.

V.E Discussion

One of the central health problems in affluent societies is obesity. The role of excessive food consumption in the development of obesity cannot be disputed. However, there is increasing evidence to suggest that the ability to regulate metabolism of ingested foods may be equally important. It is well known that the rate of weight gain from a given caloric intake varies from one normal person to another (Sims et

al, 1973). Furthermore for a given subject, it can be demonstrated that the efficiency of carbohydrate utilization is higher when food intake is below levels required for maintenance than when it is above that level (Blaxter, 1970). This variation in efficiency of metabolism has been called "luxus consumption."

Numerous investigators now accept the idea that the regulation of body weight and the regulation of intermediary metabolism are related (Newsholme, 1980; Belfiore and Iannello, 1978; Bray, 1977). It is thought that when energy demands are low and the supply of fuels high, the body promotes chemical events which do not conserve energy but produce heat. Conversely, when energy demands are high, such processes must be inhibited. The specific tissues and reactions which may be responsible for the regulation of thermogenesis are the subject of intense study.

Brown adipose tissue is found in many cold-adapted animals, hibernators and the neonates of many species, including man. These animals show increased heat production which is not associated with muscular activity (Lindberg, 1970). It is felt that brown adipose tissue may account for up to 60% of non-shivering thermogenesis in the cold-adapted rat (Foster et al, 1978). More recently, it has been shown that heat production may be induced in experimental rats who voluntarily overeat (Rothwell and Stock, 1979). Like cold-induced thermogenesis, diet-induced thermogenesis also involves changes in brown adipose tissue. In general then, brown adiose tissue is thought to be an important site for thermogenesis. The quantitative significance of energy expenditure of this tissue in man awaits the development of direct methods for studying local tissue heat production and blood flow

(Elliott, 1980).

The possible importance of brown adipose tissue in thermogenesis has stimulated many biochemical studies on this tissue. Interesting biochemical differences were found between brown adiose tissue metochondria and mitochondria from other sources (Nicholls, 1979). However, our understanding of biochemical events underlying the regulation of thermogenesis in brown adipose tissue is, at best, superficial.

Aside from work on brown adipose tissue, numerous investigators examined genetic models of obseity for altered enzymatic changes. The general hypothesis was that the absence of a substrate cycle (see section IV.A) which was critical in "burning off" energy would lead to an inborn error of metabolism which manifested itself as an abnormality in the control of body weight. Available data obtained from genetically obese mice, fatty rats and obese humans indicated that the key enzymes and substrate turnover related to several substrate cycles were increased rather than decreased in obesity (Belfiore and Iannello, 1979). The only possible exception is the decrease in sodium-potassium dependent adenosinetriphosphatase activity [$(Na^+ + K^+)$-ATPase] in liver of genetically obese mice (York et al, 1978; Lin et al, 1978) and in erythrocytes of obese human subjects (DeLuise et al, 1980). As important as these observatios are in our understanding of the biochemical basis of weight control, they raise many unanswered questions. Is the decrease in $(Na^+ + K^+)$-ATPase activity the cause of obesity or simply one of its effects? If it is a cause, how does it impair normal regulation of weight control?

The "bottom line" to all these studies is that there is little

doubt that patterns of energy metabolism may effect the control of thermogenesis and body weight. The precise biochemical events that are responsible for the generation of heat and which are subject to regulation have not been conclusively demonstrated.

A hypothesis which allows one to make unexpected predictions successfully is usually taken seriously. Such is the case with the idea that, in coordination with pyruvate carboxylase, aspartate-4-decarboxylase is important in the regulation of oxaloacetate. It led to an a priori prediction, which later was experimentally confirmed, that the enzyme would be inhibited by acetyl-coenzyme A and that it would be stimulated by compounds such as glutamate. Reciprocal regulation of pyruvate carboxylase and aspartate-4-decarboxylase has two effects. First, it is a convenient way for coordinated regulation of the two enzymes which eventually affect oxaloacetate. Second, since acetyl-coenzyme A is an essential positive-modulator of pyruvate carboxylase and a potent inhibitor of aspartate-4-decarboxylase, the cell is assured that these two enzymes may not operate at full capacity simultaneously. This places severe restrictions on the operation of a potential futile cycle (Figure V-A) and prevents a potential catastrophic drain on the cell's energy reserves.

Taking into account the regulatory and catalytic properties of aspartate-4-decarboxylase, it is possible to construct a model which ties the regulation of oxaloacetate metabolism through aspartate-4-decarboxylase to the efficiency with which ingested foods are metabolized. This, as was discussed earlier, is thought to be important in the regulation of thermogenesis and thus, obesity.

After a meal rich in carbohydrates and amino acids, the liver

funnels carbon-backbones into the TCA cycle (Figure V-F). Under these conditions the levels of TCA cycle intermediates would rise and the level of acetyl-coenzyme A would drop as it is utilized for fatty-acid biosynthesis (Wieland, et al, 1964). These changes in intracellular metabolites would cause a greater proportion of oxaloacetate to be "short-circuited" through aspartate-4-decarboxylase instead of going through the usual pathways that harness decarboxylation to energy-conserving reactions. When fuel sources are depleted, the concentrations of key metabolites that regulate aspartate-4-decarboxylase would change so as to favor more efficient metabolism. This self-regulating model for "luxus consumption" is based on the modulation of aspartate-4-decarboxylase by common intracellular metabolites.

At this point it may be noted that the three animals with lowest enzyme activity in Table III-3 were all obtained from three local meat vendors. In view of the hypothesis for the function of aspartate-4-decarboxylase, is it not possible that the selective breeding patterns and feeding patterns that are widely used to raise "meaty" cows, pigs and chickens involve, among other things, lowering of aspartate-4-decarboxylase activity so as to promote efficient energy metabolism and the associated anabolic events?

Future studies on the control of aspartate-4-decarboxylase expression by hormones and diet (particularly in specialized organelles such as brown adipose tissue) should present more insights into the physiological function of the enzyme.

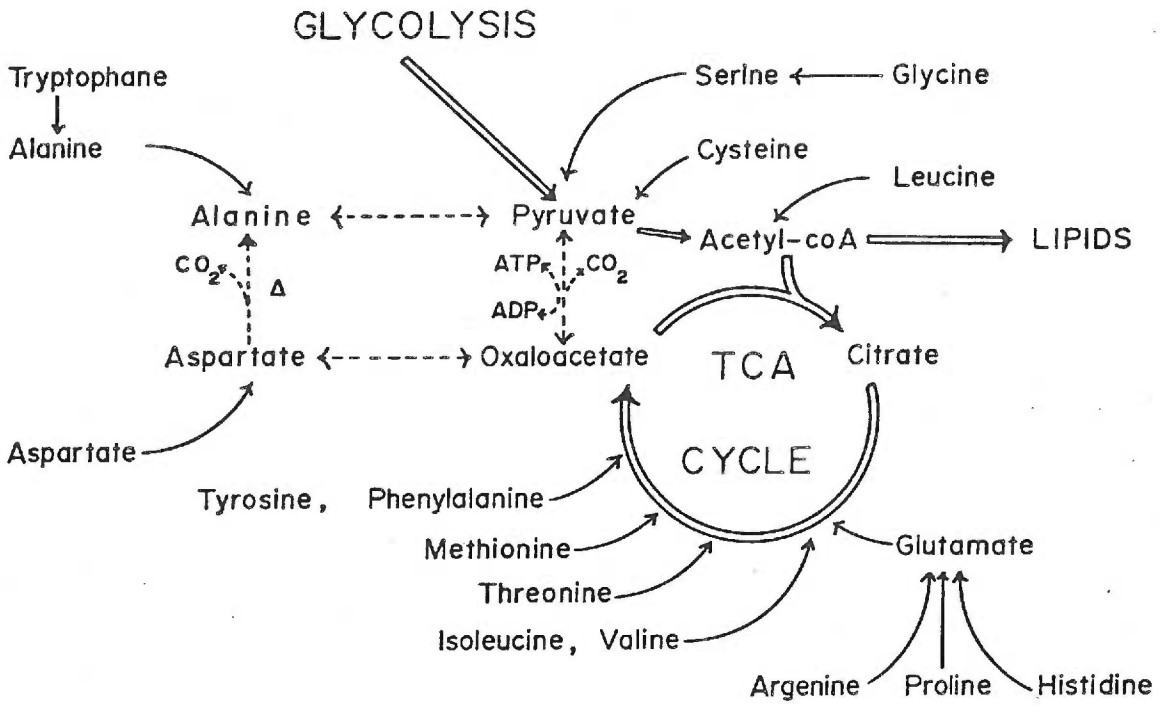

Figure V-F. Aspartate-4-decarboxylase and its relationship to overall energy metabolism.

SUMMARY AND CONCLUSIONS

Inception of the idea that aspartate-4-decarboxylase was present in rat livers led to numerous studies on its biochemical properties. These studies were detailed in previous chapters. The major conclusions of this thesis may be summarized as follows.

1) ASPARTATE-4-DECARBOXYLASE IS PRESENT IN MAMMALS:
   - The occurrance of this enzyme was deduced from earlier work in our laboratory on the metabolism of cysteine sulfinate.
   - The stoicheometry of the reaction was demonstrated.
   - A simple radiometric assay method was devised to assay aspartate-4-decarboxylase.
   - Although abundant activity was seen in rat and mouse liver, the enzymatic activity in agriculturally important animals was conspicously low.

2) THE RAT LIVER ENZYME WAS DIFFERENT FROM THE MICROBIAL ENZYME STUDIED BY OTHER INVESTIGATORS:
   - The mammalian enzyme was most active at pH 7.0 as opposed to pH 5 for the bacterial enzyme.
   - The mammalian enzyme lost activity on dilution in a reversible manner. Such a phenomenon has not been observed with the microbial enzyme.
   - The mammalian aspartate-4-decarboxylase, unlike its microbial counterpart, could not be stimulated by pyridoxal 5'-phosphate nor could it be inactivated on incubation with its substrate. Inhibition of rat liver aspartate-4-decarboxylase by carbonyl-reagents such as hydroxylamine,

could be reversed by dialysis against standard buffer which did not contain pyridoxal 5'-phosphate.

- The rat liver enzyme was very hydrophobic. The use of detergents in buffers facilitated its partial purification. The enzyme adsorbed to Phenyl-sepharose, a hydrophobic resin, and it could be eluted by decreasing the solvent polarity.
- Unlike microbial aspartate-4-decarboxylase, the mammalian enzyme does not copurify with cysteine sulfinate desulfinase and aspartate aminotransferase.

3) THE RAT LIVER ENZYME HAS UNFORESEEN ALLOSTERIC REGULATORY PROPERTIES WHICH MAY PLAY A CRUCIAL ROLE IN ENERGY METABOLISM:

- It was hypothesised that rat liver aspartate-4-decarboxylase had the potential to influence the steady state concentrations of oxaloacetate and to regulate its metabolic fate.
- Aspartate-4-decarboxylase was expected to have regulatory properties that would allow "reciprocal" modulation of this enzyme and pyruvate carboxylase.
- It was demonstrated, in accordance with this hypothesis, that the enzyme was inhibited by micromolar amounts of acetyl-coenzyme A and it was stimulated by less than millimolar amounts of glutamate.
- For the first time, it was possible to rationalize the physiological function of the regulation of microbial aspartate-4-decarboxylase by α-ketoglutarate.
- Together these observations support the notion that

aspartate-4-decarboxylase may be a key enzyme in the biological regulation of heat production from ingested metabolic fuels. This has important implications with regard to our understanding of biochemical events underlying the regulation of body weight.

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