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CHARACTERIZATION OF FUMARYLACETOACETATE FUMARYL HYDROLASE

BY

DON J. MAHURAN

A Dissertation
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor

Windsor, Ontario

1975

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ABSTRACT

The modified technique for purification of FAH is simpler, more consistent, gives a better yield, can be done on a larger scale, and results in a stabler enzyme preparation than the original procedure. The resulting enzyme solution is homogeneous based on results from; Ochterlony immunodiffusion, immunoelectrophoresis plates; and polyacrylamide disc gel electrophoresis at pH 9.5, 8.9 and 4.2. The molecular weight of FAH is 80,000 as determined by chromatography on Sephadex G-150. FAH is made up of two polypeptide chains with a molecular weight of 40,000 based on results from polyacrylamide disc gel electrophoresis in mercaptoethanol and sodium dodecyl sulfate, the meniscus depletion technique with the analytical ultracentrifuge, and chromatography on Sepharose 6B of the reduced and alkylated enzyme in 6 M guanidine-HCl. The elution volumes of the reduced and alkylated enzyme and a sample of unreduced FAH on Sepharose 6B in 6 M guanidine-HCl, were approximately the same indicating that there are no inter-polypeptide-chain disulfide bonds. The inactivation of FAH appears to proceed by the conversion of the active dimer to the inactive monomer as shown by the results from the analytical ultracentrifuge and chromatography on Sephadex G-150. The polypeptide chains are probably

identical since they have the same molecular weight, and there is only one amino terminal amino acid, proline, as determined from the dansylated derivative of FAH. The carboxyl terminal sequence of FAH is (Lys-Phe)-Gly-Leu-Ser-Ala-COOH as determined by hydrolysis with Carboxypeptidase A. There are three free sulfhydryl groups per polypeptide chain as determined by the reaction of denatured FAH with 2,2'-dithiobis-5-nitrobenzoic acid in the presence of sodium dodecyl sulfate. Only two of these sulfhydryl groups react when native FAH is reacted (no sodium dodecyl sulfate). The extinction coefficients at 260 nm and 280 nm, and the amino acid composition of FAH are also reported.

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To Isaac Asimov.

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ABBREVIATIONS

BIS	methylenebisacrylamide
BPB	bromophenol blue
DNS-Cl	dimethylaminonaphthalene-5-sulfonyl chloride
DNS-OH	dimethylaminonaphthalene-5-sulfonic acid
DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	ethylenediamine tetraacetate
FAH	fumarylacetoacetate fumarylhydrolase
HMB	p-hydroxymercuribenzoate
OD	optical density
SDS	sodium dodecyl sulfate
TEAE	triethylamino ethyl
Temed	tetramethylenediamine
tris	tris (hydroxymethyl) aminomethane

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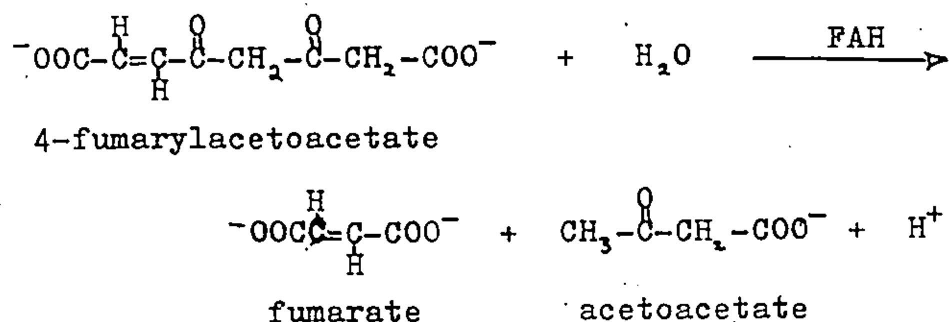
CHAPTER I

INTRODUCTION

Meister and Greenstein(1) in 1948 isolated an enzyme from liver and kidney that cleaved 2,4-diketovalerate to pyruvate and acetate. In the following year Connors and Stotz (2) reported the isolation of a triacetic acid hydrolase from beef liver. Further work (3,4) showed that these two enzymes were probably 4-fumarylacetoacetate fumarylhydrolase (E.C.3.7.1.2). Fumarylacetoacetate fumarylhydrolase (FAH) catalyzes the cleavage of fumarylacetoacetate into fumarate and acetoacetate in the degradation pathway of tyrosine and phenylalanine in mammalian systems.



FAH belongs to a class of enzymes that hydrolyzes carbon-carbon bonds, in particular bonds between a methylene carbon and an adjacent carbonyl carbon. Other examples of enzymes in this class are oxaloacetate acetylhydrolase (E.C.3.7.1.1) from <u>Aspergillus niger</u> which catalyzes the formation of oxalate and acetate from

oxaloacetate (5), and L-kynurenine hydrolase (E.C.3.7.1.3) which catalyzes the formation of anthranilate and L-alanine from kynurenine (6).

Although the mechanism of L-kynurenine hydrolysis is known to involve a Schiff-base formation between the cofactor pyridoxal 5'-phosphate and L-kynureninase (7,8), little work has been done on the general mechanism or physical make-up of this class of enzymes.

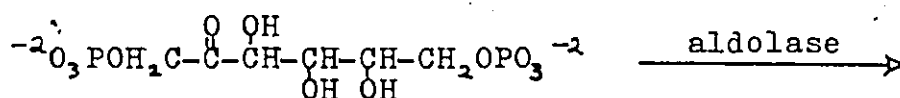
Recent work with FAH suggests that FAH may form a Schiff-base intermediate with the 3-keto group of the substrate which is hydrolyzed to release fumarate, leaving the acetoacetate residue attached in a Schiff-base form with the enzyme. The acetoacetate is then liberated by hydrolysis (9). This scheme is similar to the mechanism proposed for the action of acetoacetate decarboxylase (10). In a second possible scheme, a second nucleophile, rather than water, attacks the 5-carbonyl after Schiff-base formation (9).

It has been shown that there is a free sulfhydryl group at or near the active site of FAH, and that altering it irreversibly inhibits FAH activity. This could be the nucleophile mentioned in the second scheme (9). It has also been suggested that binding of 4-fumarylacetoacetate is through both of the carboxyl groups (11). Thus the active site could have two

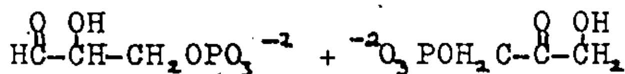
positively charged groups to bind the two end carboxyl groups of the substrate, a sulfhydryl group to attack the 5-carbonyl group, and a lysine amino group to form a Schiff-base with the 3-carbonyl carbon (Figure 1).

Jeffcoat and Dagley (12) have discussed the possibility that decarboxylases, aldolases and hydrolases all evolved from a common gene. They argue that even though the enzymes work on very dissimilar substrates they all cleave carbon-carbon bonds, and the enzymes operate by similar, if not identical mechanisms. Comparing the proposed mechanism for mammalian aldolase with the previously stated mechanism for FAH there does seem to be a great deal of similarity.

Aldolase cleaves fructose 1,6-diphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (13,14).



fructose 1,6-diphosphate

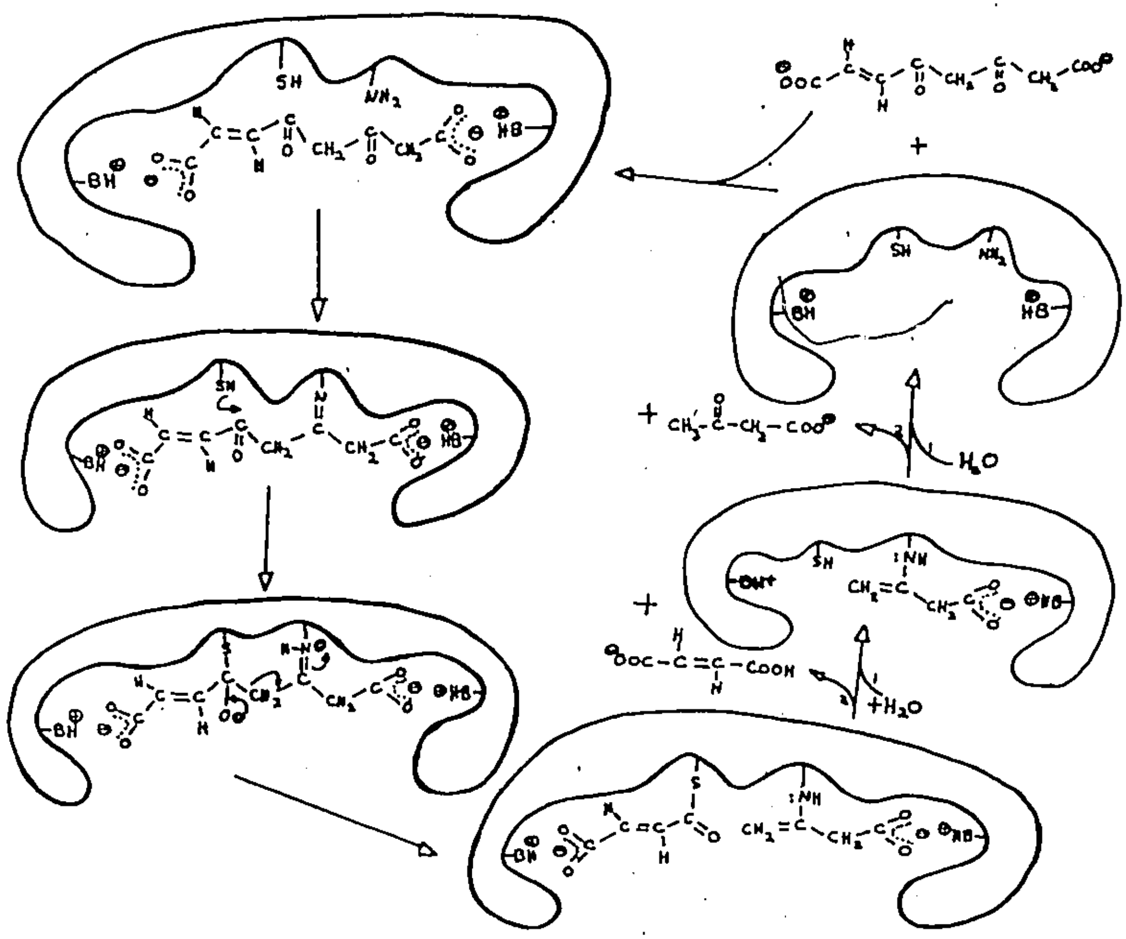


glyceraldehyde 3-phosphate　　dihydroxyacetone phosphate

The open chain form rather than the ring form for fructose 1,6-diphosphate is favored by the presence of

Fig. 1. Proposed mechanism for the cleavage of fumarylacetoacetate into fumarate and acetoacetate by FAH.

FIGURE 1

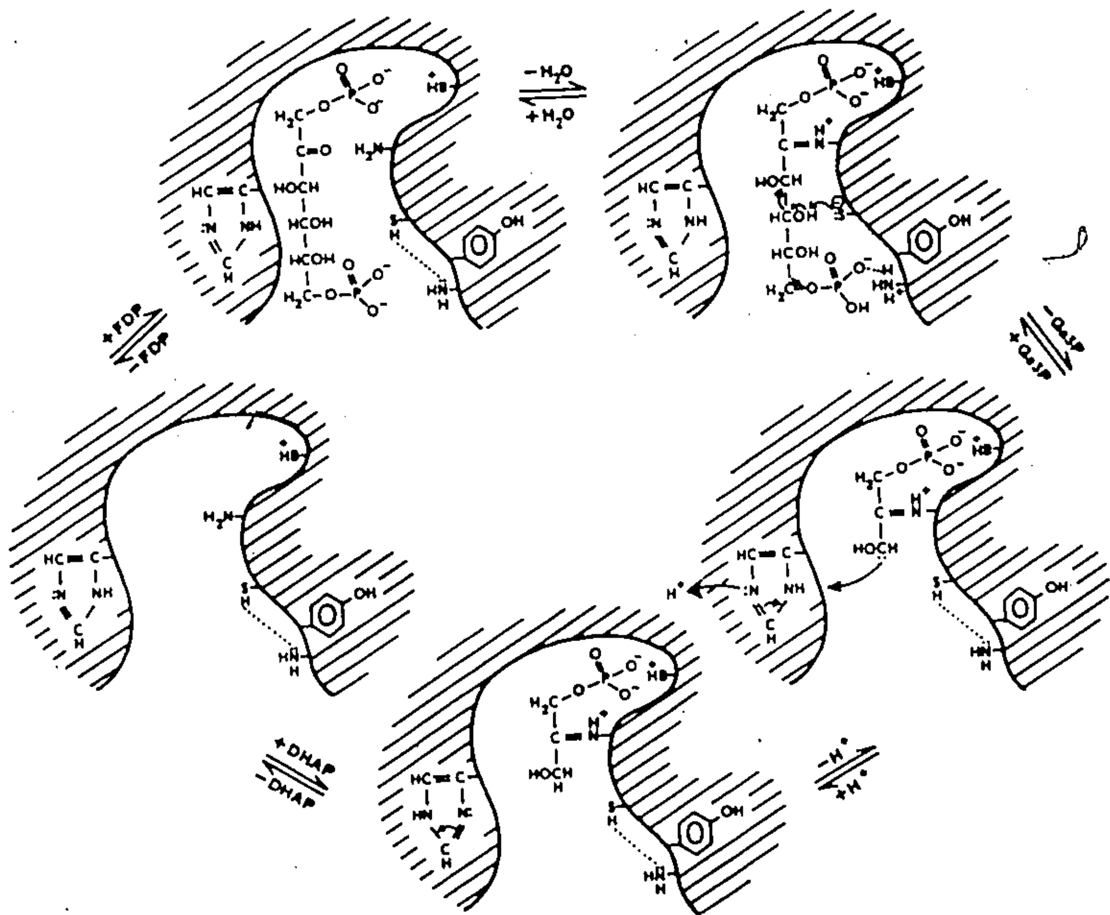


two positively charged sites at either end of the aldolase active site which bind the two phosphate groups of the substrate (15). Similarly the two end carboxyl groups of acetoacetate may bind at either end of the active site of FAH. A lysine amino group forms a Schiff-base with the 2-carbonyl group (16) and an ionized sulfhydryl group enhances the dealdolization reaction by abstracting the proton of the alcohol group on carbon-4 (14,17). Glyceraldehyde 3-phosphate is then freed, leaving dihydroxyacetone phosphate still in the Schiff-base form (14). Following the discharge of the carbanion by a proton from the external environment, the dihydroxyacetone phosphate group dissociates to complete the reaction (14) (Figure 2).

The following work was done in order to elucidate some of the physical characteristics of FAH and to determine if they, like the proposed mode of action, bear any similarity to aldolase.

Fig. 2. Proposed mechanism for the cleavage of fructose 1,6 diphosphate (FDP) into glyceraldehyde 3-phosphate (Ga3P) and dihydroxyacetone phosphate (DHAP) by muscle aldolase.(Reproduced from Horecker (13) without the permission of the publisher).

FIGURE 2



CHAPTER II

EXPERIMENTAL

A. MATERIALS

The following materials were commercially available; ribonuclease, chymotrypsinogen, aldolase, bovine serum albumin, alkaline phosphatase, malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, carboxypeptidase A, p-hydroxymercuribenzoate, and DNP-aspartic acid from Sigma; blue dextran 2000, Sephadex G-150, Sephadex G-25, and Sepharose 6B from Pharmacia; and anhydrous dimethyl formamide, dimethylaminonaphthalene-5-sulfonyl chloride, sequanal grade N-ethylmorpholine, and 2,2'dithiobis-(2-nitrobenzoic acid) from Pierce; and polyamide T.L.C. plates from Brinkmann.

B. METHODS

1) Preparation of FAH (4)

a) Preliminary Treatment of the Beef Liver

Fresh beef liver can be cut into sections and frozen for a year at -20° without much loss of FAH activity. When ready for FAH preparation the liver sections were thawed and the liver cubed while removing as much of the white connective tissue as possible. The cubed liver was passed through a meat grinder. This and all future steps were done at 4°.

To each 200g of ground tissue, 400 ml of cold sodium chloride (0.85%) made 10^{-4} M dithioerythritol (DTE) was added in a blender jar. The suspension was homogenized for 1 min and stored in 2 l flasks for 4 h.

b) First Alcohol Precipitation

Ethyl alcohol (95%) was added to the homogenate (890 ml/800 ml of homogenate). The solution was thoroughly and quickly mixed to give a final ethanol concentration of 50% (v/v). The mixture was allowed to stand overnight. The solid material was discarded after centrifugation at 1600g for 15 min.

c) Second Alcohol Precipitation

To each 100 ml of clear yellow or red supernatant was added 800 ml of ethanol (95%). This brings the alcohol concentration to 70% (v/v). After

standing overnight most of the precipitate settled to the bottom of the flasks. The major amount of supernatant can then be removed with a siphon. The bottom suspension was then centrifuged at 13200g for 5-10 min and the supernatant discarded. The resulting paste was then washed once in sodium chloride solution (0.85%, 10^{-4} M DTE) made 70% in ethanol (v/v).

The paste was stirred in 0.025 M sodium phosphate buffer (pH 7.3, 10^{-4} M DTE). About 10 ml of buffer was used for every 4g of paste. The resulting suspension was centrifuged at 13200g for 10-15 min and the solid residue discarded.

The solution was dialyzed at least 3 h against 4 l of 0.025 M phosphate buffer (pH 7.3, 10^{-4} M DTE), to remove any remaining ethanol.

d) First Ammonium Sulfate Precipitation

Enough solid ammonium sulfate was added to the enzyme solution to give a final concentration of 25% (w/v). The solution was stirred gently for at least 1 h. The solution was centrifuged at 1800g for 10-15 min and any precipitate was discarded. The ammonium sulfate concentration was then increased to 35% (w/v). The precipitate this time contained 95% of the FAH activity. The precipitate was separated by centrifugation and dissolved in 20-40 ml of 0.025 M

tris-HCl buffer (pH 8.5, 10^{-4} M DTE). The resulting solution was dialyzed overnight against 4 l of 0.025 M tris-HCl buffer (pH 8.5, 10^{-4} M DTE). The dialyzed solution, when made 10% (v/v) with glycerol can be stored for very long periods of time at -20° with little loss of FAH activity.

e) TEAE Column Chromatography

About 100g of TEAE-cellulose was prepared by the method of Srere and Kosicki (18). The resulting slurry was packed in a 50 x 4.5 cm column with a 3 metre head pressure after equilibration in 0.025 M tris-HCl buffer (pH 8.5, 10^{-4} M DTE). About 25 ml of enzyme solution containing about 40 OD_{280}/cm was placed on the column. Then 1 l of tris-HCl buffer (0.025 M, pH 8.5, 10^{-4} M DTE) was passed through the column with a head pressure of 1 metre. Next, 1.5 l of tris-HCl buffer (0.08 M, pH 8.5, 10^{-4} M DTE) was passed through and 15 ml fractions collected. Every fifth fraction was assayed for FAH activity and the active fractions combined.

f) Second Ammonium Sulfate Precipitation

To the FAH-containing fractions from the TEAE-cellulose column were added sufficient ammonium sulfate to give a final concentration of 45% (w/v). The solution was stirred gently for at least 1 h. The

white precipitate formed was compacted by centrifugation at 100000g in an I.E.C. preparative ultracentrifuge. The precipitate was dissolved in a small amount of 0.025 M sodium phosphate buffer (5-15 ml) (pH 7.3, 10^{-4} M DTE). For storage over a long period this solution should be dialyzed against 1 l of the phosphate buffer made 10% (w/v) with glycerol.

g) Spectrophotometric Method for FAH Assay

Into a 3 ml cuvette were pipetted 2.6 ml of 0.025 M sodium phosphate buffer (pH 7.3) and 0.3 ml of 1.22×10^{-3} M acetopyruvic acid solution. To this solution was added from 0.01-0.10 ml of enzyme solution. The disappearance of substrate was monitored at 295 nm for 5 min using a Beckman spectrophotometer Model DU with a Gilford recorder.

Protein concentration (mg/ml) was determined using a nomograph of Warburg and Christian (19) for OD readings at 260 nm and 280 nm of any enzyme solution. Thus

Specific Activity (S.A.) = ΔOD/5 min/mg/ml of protein

and

Units = (ΔOD/5 min) (total volume of solution/ volume of solution used in assay)

2) Determination of FAH Purity by Immunological Methods

a) Antibody Preparation

A rabbit was injected twice over a one month period with freshly prepared FAH (S.A. = 11 and 23). To 1 ml of FAH solution, 1 ml of adjuvent was added. Two weeks after the last injection the rabbit was bled and the anti-FAH serum separated.

b) Ouchterlony Immunodiffusion Plate

The steps for preparing the slide were as follows: 1) To a microscope slide were added 4.5 ml of 1.5% agar in 0.8% sodium chloride. 2) Holes were punched in the agar and the samples were added (Figure 3). 3) The slide was incubated at 4° for 2 days. 4) The slide was washed twice in 0.8% sodium chloride over a two day period. 5) The slide was washed twice in water over two days. 6) The holes were filled with 1.5% agar in water and the slide dried overnight in air. 7) The slide was dyed for 15 min in a solution of amido black (0.1%) in methanol:acetic acid:water (5:1:4). 8) The slide was washed in the methanol:acetic acid:water solution until it cleared, and then was left to dry.

c) Immunoelectrophoresis

The steps in preparing the slide were as follows: 1) To a microscope slide was added 4.5 ml of

2% agar in 0.5 M barbitol buffer (pH 8.6). 2) Two holes were cut in the slide for the test samples and a well added after electrophoresis for the anti-FAH (Figure 4). 3) The test samples were added to the holes and 1.6 milliamperes per inch were applied to the slide for 90 min. The same barbitol buffer was placed between the electrodes. 4) The well was cut and anti-FAH was added. 5) The plate was treated in the same manner as previously described in 2b), from step number 3.

3) Purity and Molecular Weight Determination with Polyacrylamide Disc Electrophoresis

a) Determination of Purity of FAH (20)

Three different buffer systems were used, the first being a continuous buffer i.e. where the same components are at the same concentration in the gel and the electrode vessel reservoir, and the last two being discontinuous i.e. using different buffers and a spacer gel between the reservoir and the running gel. In all three cases the running gel monomer solution and the catalyst solution were the same.

System I: The monomer stock solution (monomer 1) contained; 28.0g acrylamide and 0.735g methylenebis-acrylamide (BIS) in 100 ml of water. The buffer used was 0.377 M tris-glycine (pH 9.5) with 1.2 ml/l of NNN'N'-tetramethylethylenediamine (Temed) added as the initiator

for polymerization of the monomer solution (buffer 1). The catalyst solution was 20g/100 ml of ammonium persulfate in water. The running gel contained 1 volume of buffer, 1 volume of monomer 1 solution, and 2 volumes of catalyst solution. Buffer 1 was diluted 1 to 3 with water and added to the reservoir.

System 2: Running gel buffer (buffer 2) contained 36.6g tris, 0.23 ml Temed titrated to pH 8.9 with about 48 ml of 1 M hydrochloric acid and diluted with water to 100 ml. The spacer gel buffer (buffer 3) contained 5.98g tris, 0.46 ml Temed, titrated to pH 6.7 with about 48 ml 1 M hydrochloric acid and diluted with water to 100 ml. The reservoir buffer contained 28.8g glycine in 900 ml water, titrated to pH 8.3 with 6.0g tris in 100 ml water and then diluted ten times for use. The monomer solution for the spacer gel contained 10g of acrylamide and 2.5g of BIS in 100 ml of water (monomer 2). The running gel contained 1 volume of buffer 2, 2 volumes of monomer 1 solution, 1 volume of water, and 4 volumes of catalyst solution. The spacer gel contained 1 volume of buffer 3, 2 volumes of monomer 2, and 1 volume of catalyst solution.

System 3: The only difference between this system and system 2 was in the buffers. The running gel buffer which contained 22.4 ml of glacial acetic acid and

4.6 ml of Temed was titrated to pH 4.2 with 48 ml of 1 M potassium hydroxide and then was diluted with water to 100 ml (buffer 2'). The spacer gel which contained 3.1g of glacial acetic acid and 0.46 ml of Temed was titrated to pH 5.8 with 48 ml of 1 M potassium hydroxide and then was diluted to 100 ml with water (buffer 3'). The reservoir buffer which contained 17.25g glycine in 900 ml water was titrated to pH 4.0 with glacial acetic acid and then was diluted to 100 ml with water. As before, this buffer was diluted ten times for use.

From 5-25 µl of FAH solution containing about 1 mg/ml was taken up in a 100 µl syringe, then double that volume of 1% bromophenol blue (BPB) in a 40% sucrose solution was taken up in the syringe on top of the enzyme solution. The sample was then applied under the reservoir buffer on top of the gel.

At the beginning of the experiment a current of 1-2 milliamperes per gel tube was used. After the BPB entered the gel, this was increased to 5 milliamperes per tube. The runs took from 1-2 h.

The gels were stained in 0.1% naphthalene black in 7% acetic acid for 30-60 min. The gels were destained by soaking in 7% acetic acid.

b) Molecular Weight Determination (21)

Aldolase from rabbit muscle (MW 40,000),

ribonuclease (MW 13,700), chymotrypsinogen (MW 25,000) and bovine serum albumin (MW 67,000) were used as molecular weight standards. The proteins, including FAH, were incubated at 37° for 2 h in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. The protein concentration was normally between 0.2 and 0.6 mg/ml. After incubation the protein solution was dialyzed overnight at 4° against 2 l of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS and 0.1% β-mercaptoethanol.

Gel buffer contained 7.8g sodium phosphate monobasic, 38.6g sodium phosphate dibasic, and 2g SDS per litre. The monomer solution contained 22.2g acrylamide and 0.6g BIS in 100 ml of water.

The glass gel tubes were 7 x 0.5 cm. For a typical run of 12 gels, 15 ml of gel buffer and 13.5 ml of monomer solution were deaerated and mixed with 1.5 ml of fresh ammonium persulfate solution and 0.045 ml Temed. Each tube was filled to within 1 cm of the top and a few drops of water layered on it to prevent the formation of a meniscus. Gel buffer was diluted 1:1 with water and added to the reservoir. From 5-25 µl of protein solution was added with BPB in 40% sucrose as previously described in 3a).

Electrophoresis was performed at constant current of 8 milliamperes per gel tube. The BPB band moves about 75% of the way down in 3-4 h. Staining and destaining were done as previously described in 3a).

The length of the gel was measured before and after staining. The BPB distance of migration was measured before staining and the distance of migration of the protein band after staining. Mobility was then defined as: [(distance of protein migration) x (length of gel before staining)] / [(length of gel after destaining) x (distance of BPB migration)] . The mobilities were then plotted versus the natural logarithm of the molecular weight (Figure 6).

4) Molecular Weight Determination by Gel Filtration (22,23)

a) Sephadex G-150

Sephadex G-150 (12g) was suspended in 800 ml of a solution of 0.05 M tris-HCl buffer (pH 8.5) made 0.1 M with sodium chloride. The gel was allowed to swell for at least 72 h at room temperature. The suspension was deaerated overnight at 4°, the column's running temperature. The G-150 suspension was then poured into a 50 x 1.5 cm column with a settling-bulb attached, and allowed to settle overnight. Excess buffer and gel were removed and buffer run through the column

with a 5 cm head pressure overnight. The head pressure was then increased to 15 cm, its normal running pressure, and buffer was run through the column for 18 h.

Blue dextran and DNP-aspartic acid were used to determine the void volume (V_0) and the volume within the gel pores (V_i) respectively. The protein markers used were; chymotrypsinogen (MW 25,000), aldolase (MW 158,000), ribonuclease (MW 13,700), alkaline phosphatase (MW 86,000), and malate dehydrogenase (MW 70,000).

The protein samples were adjusted to 6 OD_{280}/ml per protein in solution i.e. some samples contained more than one marker protein. Then 0.7 ml of the protein solution was placed on the column.

Test tubes were weighed before and after fractions were collected. The difference in weight was treated as the volume of eluate in the fractions i.e. assuming a density of 1 g/ml. The volumes were then summed over the series of test tubes collected. A Gilson fraction collector equipped with a constant volume attachment was used to collect between 1.2 and 1.5 ml per tube. The sum of each fraction volume and all preceeding fraction volumes were plotted against the fraction's

OD_{280}. The volume to the nearest 0.1 ml corresponding to the centres of each protein peak was taken as the elution volume (V_e). Chymotrypsinogen, aldolase, and ribonuclease's V_e's were determined twice, once before and once after the V_e for FAH (which itself was run twice in succession). The V_e's for alkaline phosphatase and malate dehydrogenase were determined once each. A plot was made of $(Kd)^{1/3}$ against $(\text{Molecular Weight})^{0.555}$ where the Kd for each protein equals $(V_e - V_o)/V_i$ (Figure 7).

b) Sepharose 6B in 6 M Guanidine-HCl

Sepharose 6B is supplied in the hydrated form. Guanidine hydrochloride (286g) was added to a graduated cylinder to which the sepharose 6B suspension was added with stirring to the 500 ml mark. The suspension was then titrated to pH 5 with hydrochloric acid. A 6 M solution of guanidine hydrochloride was used as the elutant (pH 5).

A sepharose 6B column (50 x 1.5 cm) was prepared as described in 4a), except all operations were performed at room temperature.

Six proteins were used as molecular weight markers. Each protein was reduced with β-mercaptoethanol and alkylated with iodoacetic acid (24). The protein solutions were dialyzed against distilled water and freeze-dried. Then each protein (6 mg) was dissolved

in 1 ml of 6 M guanidine-HCl (pH 5). From this solution 0.7 ml were added to the column.

The protein markers and the respective subunit molecular weights were; bovine serum albumin, 68,000; aldolase, 40,000; alcohol dehydrogenase, 37,000; lactate dehydrogenase, 36,000; chymotrypsinogen, 25,000; and ribonuclease, 13,700. V_o and V_i were determined using blue dextran and bromophenol blue respectively.

The elution volumes of each protein were determined in the same manner as previously described in 4a). The V_e of each protein was determined twice. The V_e for FAH was determined three times. Then $(Kd)^{1/3}$ was plotted against $(\text{Molecular Weight})^{0.555}$ (Figure 9).

5) Molecular Weight Determination with the Ultracentrifuge

Meniscus depletion equilibrium studies were carried out in a Beckman Model E Ultracentrifuge equipped with temperature control and interference optics. The molecular weight was calculated using the method for meniscus depletion described in A Manual of Methods for the Analytical Ultracentrifuge (25). Three different rotor speeds (20,410; 23,150; and 25,980 rpm) were used. FAH was dissolved in 0.025 M sodium phosphate buffer (pH 7.3). The temperature was held at 12° during all three runs.

Ultracentrifuge plates were measured on a Nikon Profile Projector Model 6C.

6) Amino Terminal Amino Acid Determination

The general method of Gray (26) for making dansylated derivatives of FAH and various amino acid markers was used.

Freeze-dried FAH (5 mg) and 0.1 ml of 1% SDS were placed in a three ml test tube. The suspension was dissolved by heating the test tube in a boiling water bath for 5-10 min. After the solution cooled, 0.1 ml of N-ethylmorpholine was added. Dimethylamino-naphthalene-5-sulfonyl chloride (dansyl chloride, DNS-Cl) in anhydrous dimethyl formamide (0.15 ml, 25 mg/ml) was added to the FAH solution. The solution was left to incubate at room temperature for 3-4 h. The labelled protein was precipitated by the addition of 1 ml of acetone.

The protein suspension was centrifuged on a desk top centrifuge. The protein pellet was washed twice in 80% acetone and then dried. Hydrochloric acid (0.5 ml, 6 M) was added, and the tube sealed. Hydrolysis was carried out for 6 h at 105°. The hydrochloric acid was then evaporated and the sample dissolved in 0.03 ml of acetone:glacial acetic acid (3:2, v/v). This solution was spotted in the lower

right hand corner of a 0.1 mm polyamide sheet. Chromatography in one direction was carried out for 50 min with water:90% formic acid (200:3, v/v). The plate was dried, turned through 90° and chromatographed the second time for 1 h with benzene:glacial acetic acid (9:1, v/v). The plate was dried and the spot visualized with a "black ray" fluorescent lamp.

The small changes in this procedure from that of Gray (26) were due to proline being the N-terminal amino acid. The proline dansyl derivative is unstable to prolonged acid hydrolysis. For normal amounts of FAH (50-250 µg) and 18 h hydrolysis no DNS-proline spot could be detected.

Glycine, phenylalanine, leucine, aspartic acid, and proline were used as standard amino acid markers. A 0.1 ml sample of a solution containing 10 m*M* of each amino acid, in 0.5 *M* sodium bicarbonate, was dansylated by adding 0.1 ml of DNS-Cl in acetone (7 µmoles/0.1 ml). The reaction solution was incubated at room temperature for 2-3 h. The solution was then diluted to 1 ml with 10% formic acid and stored in a stoppered test tube. The dansylated amino acid solution (2-4 µl) was spotted on a polyamide plate, and chromatographed as previously described.

7) Carboxyl Terminal Amino Acid Determination (27)

Freeze-dried FAH (0.077g) was performic acid oxidized (28) and dissolved in 7.5 ml of 0.112 M SDS by heating in a boiling water bath for 5-10 min. After the solution cooled, 7.5 ml of 0.4 M N-ethylmorpholine acetate (pH 8.5) was added. This solution and a 'blank' solution containing no FAH were placed in a 25° water bath. At time '0' 0.30 mg of Carboxypeptidase A prepared by the following method, was added to each solution.

Commercially obtained diisopropyl phosphoro-fluoridated-treated Carboxypeptidase A (1.0 mg) was washed with 2 ml of distilled water. The suspension was centrifuged for 5 min at 2000g and the supernatant discarded. The crystals were dissolved in 1.5 ml sodium bicarbonate, 1% (w/v) and cooled in an ice bath until needed.

At time intervals of 0.25, 0.5, 1, 2, 4, 8, and 14 h, 2 ml of the reaction mixture and the blank were removed and diluted with sufficient acetic acid to lower the pH of the solution to 2.5-3. Any protein that precipitated was removed by centrifugation, and the supernatant dried (vacuum desiccator). The samples were then dissolved in 0.6 ml of 0.2 M sodium citrate buffer (pH 2.2) for application to the amino acid analyzer.

8) Amino Acid Analysis

Freeze-dried FAH (5 mg) was dissolved in 3.5 ml of 6 M hydrochloric acid. The solution was divided into three 1 ml portions and placed in three hydrolysis tubes. The tubes were sealed under vacuum and placed in a 105° oil bath. The first tube was removed after 24 h, the second after 48 h, and the third after 72 h. This enabled correction to be made for the destruction of serine and threonine, and the slow release of valine and isoleucine.

Tryptophan was determined by hydrolysis in p-toluenesulfonic acid (29). Amino acid determinations were made on a Beckman Amino Acid Analyzer Model 120C.

9) Free Sulfhydryl Group Determination (30)

The number of free sulfhydryl groups was determined by reacting FAH with 2,2'-dithiobis-5-nitrobenzoic acid (DTNB) in 0.08 M sodium phosphate buffer (pH 8.0) made 2% SDS and 0.5 mg/ml EDTA. The number of free sulfhydryls can then be determined from the absorbance of the 2 nitro-5-thiobenzoate anion formed at 412 nm. The anion has a molar absorbance of 13,600 $M^{-1} cm^{-1}$.

One ml of FAH, approximately 7 mg/ml was passed through a 29 x 1 cm column containing Sephadex G-25 medium, in 0.08 M sodium phosphate buffer (pH 8.0) to

remove the 10^{-4} M dithioerythritol in which the enzyme is normally stored. The resulting 2.5 ml contained 2.8 mg/ml of FAH as determined by its OD_{280}. The solution (1 ml) was diluted 1:1 with 0.08 M sodium phosphate buffer (pH 8.0) made 4% SDS and 1.0 mg/ml EDTA. The resulting 2 ml was diluted 1:1 with 0.08 M phosphate buffer made 2% SDS and 0.5 mg/ml EDTA. The absorbances at 280 nm and 412 nm were read. From the OD_{280} the protein concentration was determined (0.70 mg/ml). The FAH solution (3 ml) was incubated for 15 min with 0.1 ml DTNB solution (40 mg DTNB in 10 ml of 0.08 M sodium phosphate buffer (pH 8)). The absorbance was read at 412 nm against a reagent blank. The absorbance of the protein solution alone at 412 nm was subtracted from this absorbance to give the net absorbance.

The experiment was repeated as described above, but in the absence of the denaturing agent (SDS). The absorbance was followed at 412 nm as a function of time until the absorbance ceased to increase.

10) <u>Determination of the Extinction Coefficient at 280 nm and 260 nm</u>

FAH, after dialyzing in distilled water and freeze-drying, is irreversibly denatured and cannot be redissolved in normal buffer. To determine the ϵ_{280} and ϵ_{260} the amount of nitrogen in a weighed sample of

freeze-dried FAH was compared to the amount of nitrogen in a volume of FAH in a solution of known absorbance at 280 nm and 260 nm.

Nitrogen was determined by the Kjeldahl procedure (31). Two FAH freeze-dried samples of 5.75 and 5.85 mg; one FAH sample in 0.08 M sodium phosphate buffer (pH 8.0) with an absorbance of 2.87 OD/ml at 280 nm, and 1.81 OD/ml at 260 nm, 1.50 ml; and one blank containing 1.5 ml of the phosphate buffer; were digested at 400° in 1.5 ml of concentrated sulfuric acid with 1.2g potassium sulfate and 0.13g mercuric oxide (red) for 1 h. The solutions were cooled and diluted with 20 ml of water. Each solution was then quantitatively transferred to a steam distillation apparatus. Ten millilitres of 40% sodium hydroxide containing 5% anhydrous sodium thiosulfate, were added and the solution distilled into 10 ml of 4% boric acid. A few drops of an indicator made from five parts of a 0.1% solution of bromocresol green, with one part of a 0.1% solution of methyl red, in ethanol, were added to the boric acid. The boric acid was titrated with 0.0243 M hydrochloric acid to a slightly pink color with a 5 ml buret.

11) Preparation of Fumarylacetoacetate

Homogentisic acid was prepared from

p-dimethoxybenzene using the method described by Abbott and Smith (32).

Homogentisate oxidase was isolated from rat liver by the method of Knox and Edwards (33). Using this enzyme, homogentesic acid was converted to maleylacetoacetate in Warburg vessels with oxygen as the gas phase, and allowed to spontaneously isomerize to fumarylacetoacetate in an acid solution (34).

The isomerization was followed daily by the absorbance at 316 nm in a solution at pH 1 (34). The solution was also tested by adding 0.05 ml to a 3 ml solution containing FAH (pH 7.3). The change in absorbance at 330 nm was monitored for 5 min.

After 4 days the isomerization was complete and the effect on the change in absorbance at 330 nm in the presence of FAH alone and FAH incubated with 10^{-3} to 10^{-5} M p-hydroxymercuribenzoate (HMB) was determined.

12) Least Squares Analysis (35)

All linear plots were analyzed by the least squares method. Assuming the set of experimental points fit into the general linear equation

$$y = a + bx$$

where 'x' and 'y' are the dependent and independent variables, 'a' is the 'y' intercept and 'b' is the slope of the resulting line. This method chooses an

'a' and 'b' so that the average sum of the squares of the difference between the experimental 'y' values (y exp) and the calculated 'y' values (y calc) is at a minimum. The two final equations used to calculate 'a' and 'b' are

$$a = \frac{\left(\sum_{n=1}^{k} x_n^2 \sum_{n=1}^{k} y_n - \sum_{n=1}^{k} x_n \sum_{n=1}^{k} (x_n y_n)\right)}{k \sum_{n=1}^{k} x_n^2 - \left(\sum_{n=1}^{k} x_n\right)^2}$$

Where 'k' is the number of experimental points. For simplicity the denominator will be called 'z' and the indices on the summation signs will be omitted. Thus

$$b = (k\sum(x_n y_n) - \sum x_n \sum y_n)/z$$

To find the standard deviation of the slope and intercept, the values of 'r' are first computed. When

$$r = (y_n \text{ cal}) - (y_n \text{ exp})$$

the standard deviation of the 'y' values (S_y) would be

$$S_y = \sqrt{\sum r_n^2/(k-1)}.$$

The standard deviation of the intercept (S_a) would be

$$S_a = S_y\sqrt{(k\sum x_n^2)/((k-1)z)}$$

and the standard deviation of the slope (S_b) would be

$$S_b = S_y\sqrt{k^2/((k-1)z)}$$

The resulting linear equation could then be expressed by

$$y = (a \pm S_a) + (b \pm S_b)x.$$

If the appropriate positive or negative values for 'S_a' and 'S_b' are substituted in the equation to

make $|x|$ maximum it can be said that the calculated value of 'x' for a fixed 'y' can be as great as

$$x_{cal} + (|x_{max}| - |x_{cal}|)$$

or as small as

$$x_{cal} - (|x_{max}| - |x_{cal}|).$$

The percent error can be expressed as

$$((|x_{max}| - |x_{cal}|)/|x_{cal}|) \cdot (100).$$

This least squares method was incorporated into a Fortran computer program which reported the best fit 'a' and 'b' plus S_a and S_b for each linear plot reported.

C. RESULTS

1) Preparation of FAH

The purification of FAH from beef liver is summarized in Table I.

This modified technique is simpler, gives much more consistent results, and can be adapted to a much larger scale of FAH production than the original procedure (4). The introduction of dialyzing the enzyme solution after the second ethanol and ammonium sulfate precipitation, plus the addition of 10% (v/v) of glycerol for storage, results in larger and more stable yields of the enzyme.

It was also discovered that beef liver obtained during the summer months contained much less stable FAH than those obtained during the rest of the year. All liver homogenates yield approximately equal specific activities and number of units. As the purification procedure progressed, however, the number of units and the specific activity of the summer livers were much less than those for the winter livers.

2) FAH Purity Determination by Immunological Methods

a) Ouchterlony Immunodiffusion Plate

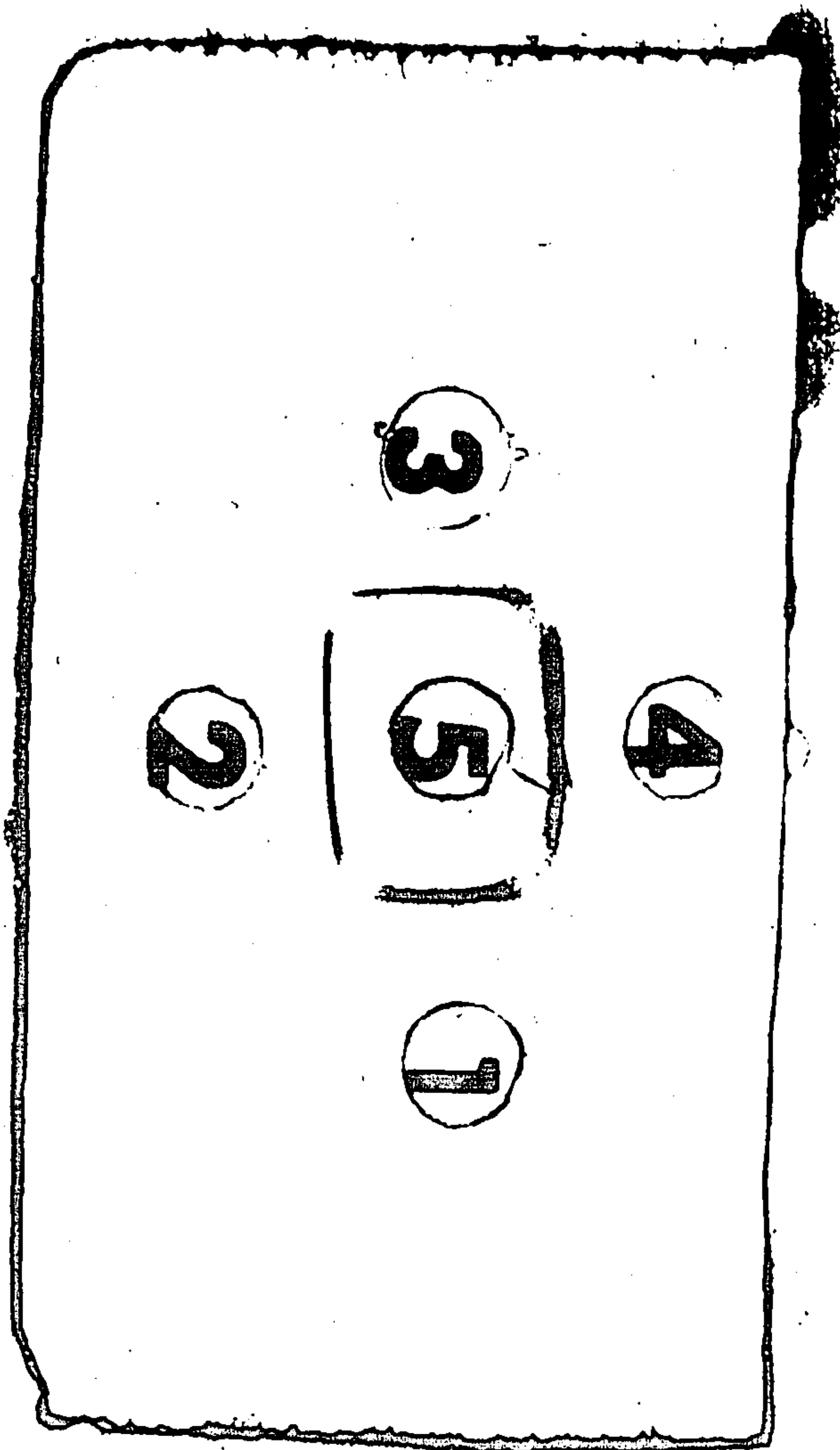
A serial dilution of purified FAH gave a one line identity reaction with anti-FAH serum (Figure 3).

TABLE I: Purification of Fumarylacetoacetate Fumaryl Hydrolase

Fractionation Steps	Enzyme Activity (units)	Yield (%)	Specific Activity	Purification (fold)
a) NaCl extraction	7,000	(100)	0.10	(1)
b) 50% ethanol precipitation	3,000	(43)	0.44	(4.4)
c) 70% ethanol precipitation	900	(13)	1.1	(11)
d) 1st ammonium sulfate precipitation	650	(9)	2.4	(24)
e) TEAE-cellulose chromatography	300	(4)	20	(200)

Fig. 3. Ouchterlony immunodiffusion plate showing the precipitation of FAH by FAH antisera. In the centre well (5) was placed FAH antisera. Wells 1-4 contained purified FAH solutions at various concentration (5mg/ml (4), 4mg/ml (1), 3mg/ml (2), and 2mg/ml (3)).

FIGURE 3



b) Immunoelectrophoresis

For two different concentrations of FAH only one line was seen on the plate (Figure 4).

3) Purity and Molecular Weight Determination with Polyacrylamide Disc Gel Electrophoresis

a) Determination of Purity of FAH

Polyacrylamide gel electrophoresis at pH 8.9 and pH 9.5 indicated that the purified enzyme has a protein component which migrates as a single diffuse band (Figure 5). All attempts at measuring any enzymatic activity in the protein band or in any other portion of the gel were unsuccessful. The enzyme preparation before the TEAE-cellulose chromatography step showed at least seven bands.

Gel electrophoresis at pH 4.2 indicated a single homogeneous band which appeared to be precipitated protein and could not be stained. When the step before the TEAE-cellulose chromatography step was analyzed there appeared in the gel at least three bands of what again seemed to be precipitated protein.

b) Molecular Weight Determination

The graph of mobilities versus the natural logarithm of the molecular weight resulted in a straight line with the equation (Figure 6):

$$y = -2.81(\pm 0.15)\ x + 11.80(\pm 0.08)$$

Fig. 4. Immunoelectrophoresis plate showing the precipitation of FAH at 5 mg/ml (A) and 1 mg/ml (B) after 90 min of electrophoresis at 1.6 milliamperes per inch, by FAH antisera placed in the centre trough.

FIGURE 4

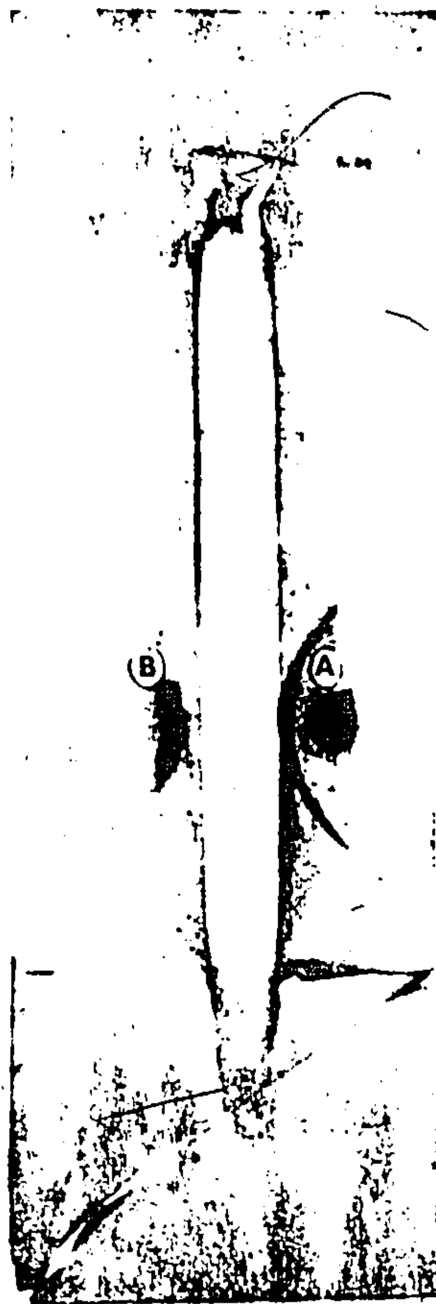
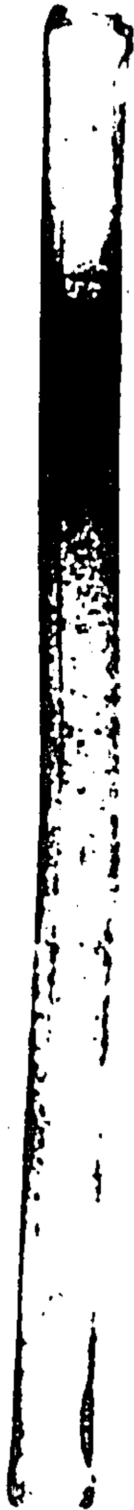


Fig. 5. Acrylamide gel disc electrophoresis of purified FAH at pH 9.5 and pH 8.9 in 5% gels.

FIGURE 5



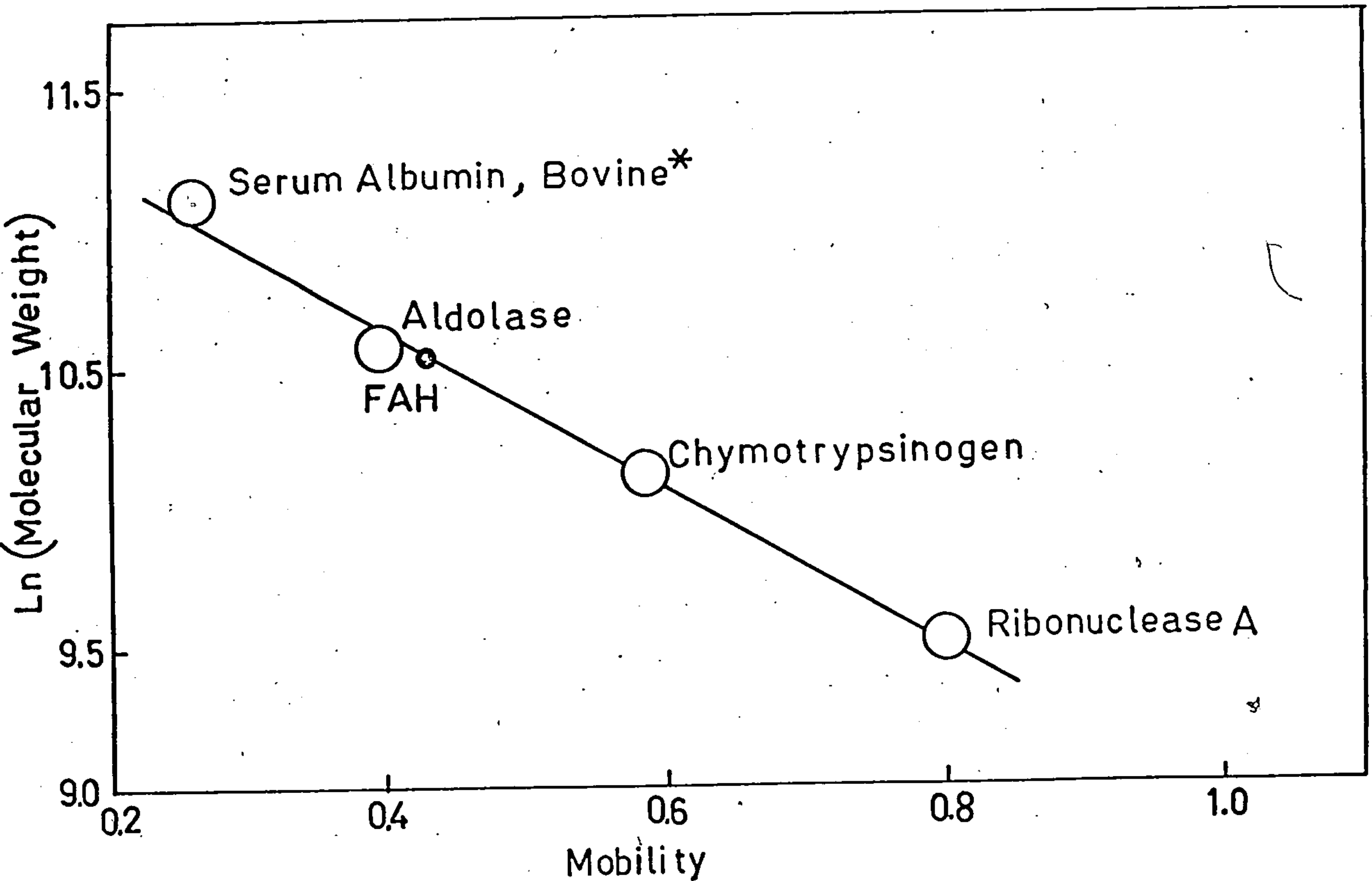
pH 8.9

pH 9.5

Fig. 6. Graph of mobilities versus the natural logarithm of the molecular weights of various standard proteins and FAH as determined by polyacrylamide disc gel electrophoresis in 0.1% SDS and 0.1% β-mercaptoethanol.

* Represents standard points that were determined only once. Other standards were repeated twice.

FIGURE 6



The molecular weight of FAH based on a mobility of 0.43 was 40,000 (± 6,000).

4) Molecular Weight Determination by Gel Filtration.

a) Sephadex G-150

A graph of $(Kd)^{1/3}$ versus $(\text{Molecular Weight})^{0.555}$ resulted in a straight line with the equation (Figure 7):

$$y = -6.52 \times 10^{-4} (\pm 0.20 \times 10^{-4})\, x + 0.969 (\pm 0.010)$$

The molecular weight of FAH based on a $(Kd)^{1/3}$ of 0.611 was 85,000 (± 8,500)

When FAH was chromatographed on Sephadex G-150, a plot of A_{280} versus volume showed a large protein peak with a shoulder on the higher elution volume (lower molecular weight) side (Figure 8). On the other hand, a plot of FAH activity versus volume shows only one peak which falls in the same area as the large protein peak. A sample from the shoulder which showed no FAH activity, and a sample taken from the peak of FAH activity were analyzed by Ouchterlony immunodiffusion with anti-FAH serum (2b). Both samples gave one line. The intersection of the two lines demonstrated an identity reaction.

b) Sepharose 6B in 6 M Guanidine-HCl

A graph of $(Kd)^{1/3}$ versus $(\text{Molecular Weight})^{0.555}$ resulted in a straight line with the equation (Figure 9):

$$y = -1.12 \times 10^{-3} (\pm 0.07 \times 10^{-3})\, x + 0.972 (\pm 0.025)$$

Fig. 7. Graph of $(Kd)^{1/3}$ versus the molecular weights of various standard proteins and FAH as determined on a 50 x 1.5 cm Sephadex G-150 column in 0.05 M tris-HCl buffer (pH 8.5) made 0.1 M with sodium chloride. * Represents standard points that were only determined once. Other standards were repeated twice.

FIGURE 7

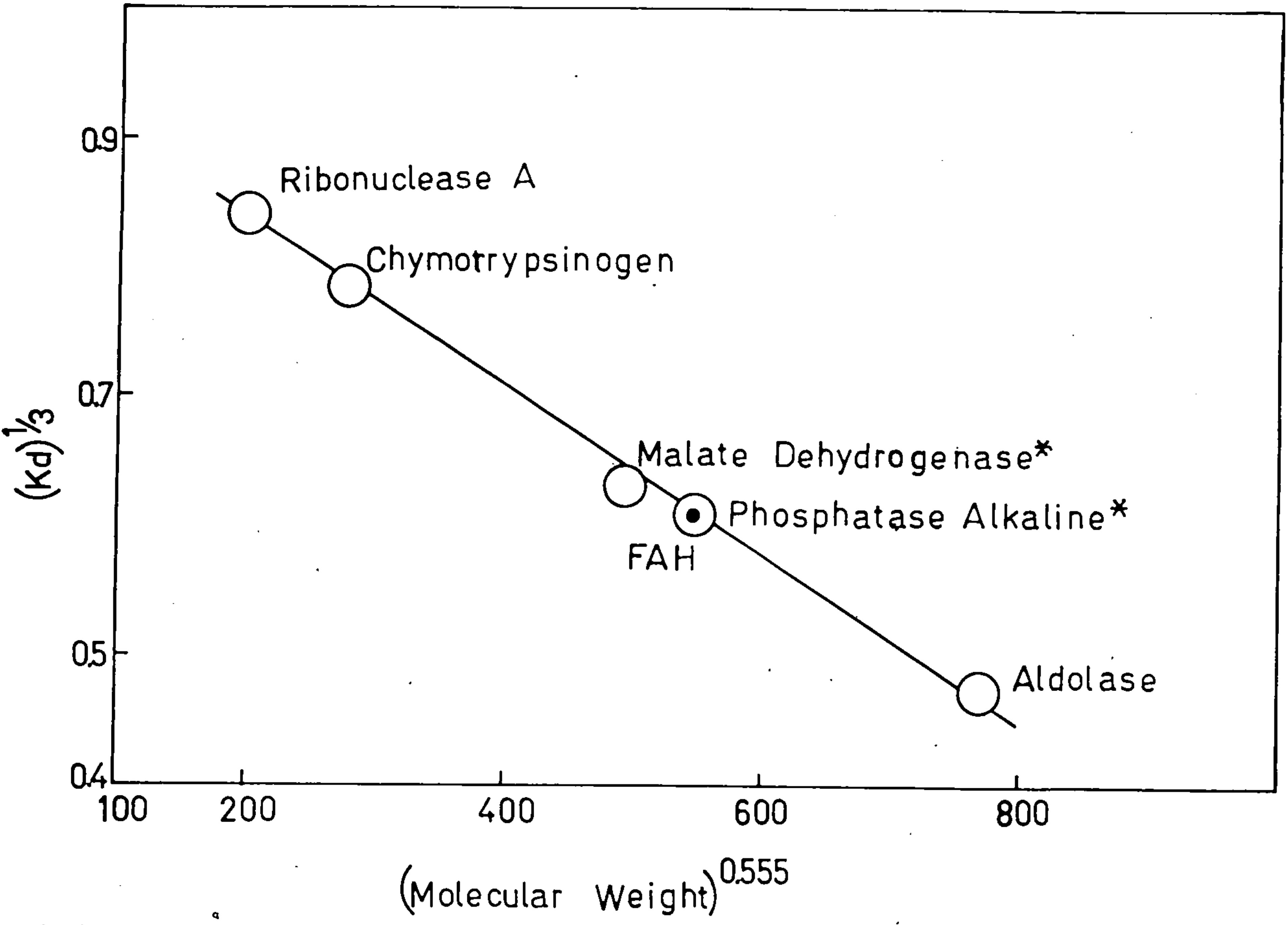


Fig. 8. Graph of the elution volumes versus OD_{280} and ΔOD_{335}/5 min for FAH from a 50 x 1.5 cm Sephadex G-150 column.

FIGURE 8

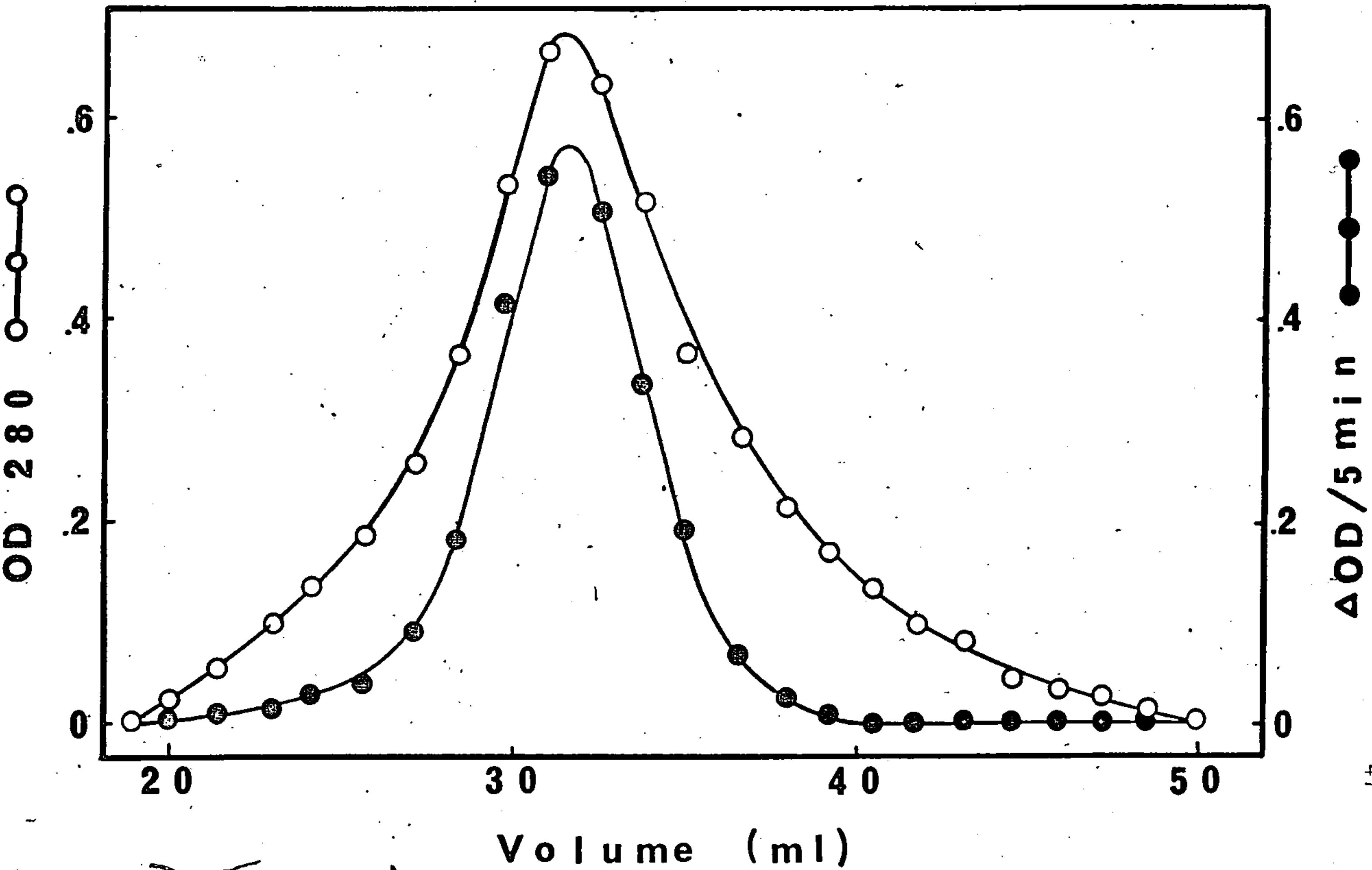
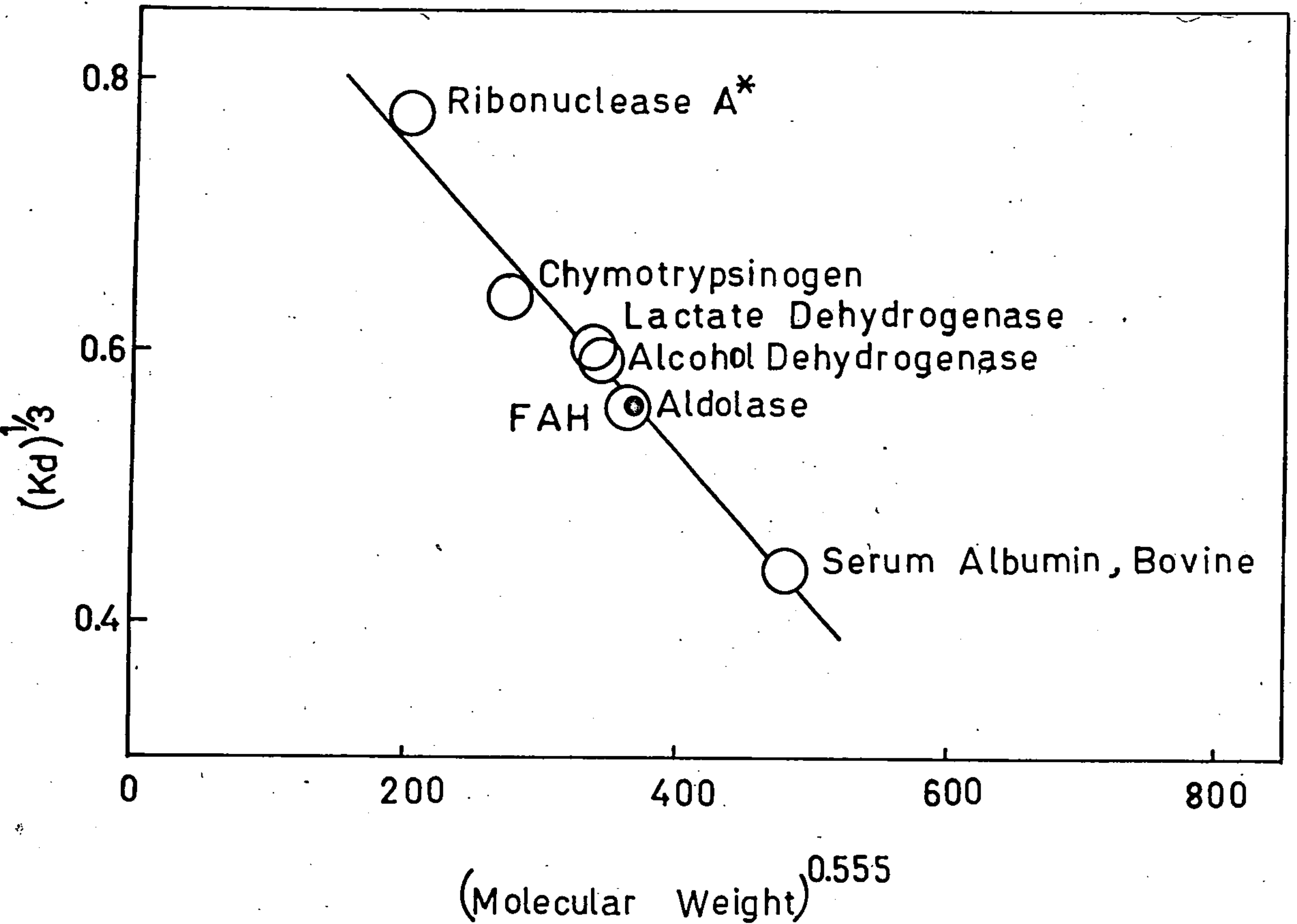


Fig. 9. Graph of $(Kd)^{1/3}$ versus the molecular weights of various reduced and alkylated standard proteins and FAH on a 50 x 1.5 cm Sepharose 6B column in 6 M guanidine-HCl (pH 5).

* Represent standard points that were only determined once. Other standards were repeated twice.

FIGURE 9



The molecular weight of reduced and alkylated FAH based on a $(Kd)^{1/3}$ of 0.557 was 43,000 (± 9,000).

FAH which was not reduced or alkylated gave a $(Kd)^{1/3}$ of 0.579 or a molecular weight of 38,000.

5) <u>Molecular Weight Determination by Ultracentrifugation</u>

A sample of FAH was centrifuged for 48 h; at 16 h, 28 h, and 47 h pictures were taken. In order to be reasonably sure that the condition of zero concentration at the meniscus is attained, the fringes should be straight over approximately the upper half of the cell. If this condition is not met the rotor speed is increased (25). Thus in order to assure zero concentration the rotor speed had to be increased from the initial 20,410 rpm at 16 h to 23,150 at 28 h, and finally to 25,980 rpm at 47 h. Each set of plates gave horizontal lines over approximately half the cell, but only the last plate showed no change in the interference fringe pattern over a few hours time. Calculation of the molecular weight of each of the three plates showed a decreasing molecular weight.

A plot of the logarithm of $[y(r) - y(o)]$, a value proportional to the protein concentration, versus $(r)^2$, the distance to the centre of rotation, resulted in straight lines in all cases. The average slope of five of these lines was used to calculate the molecular weight. A graph

of $y(r) - y(o)$ versus (r^2) for one line of the 47 h plate is shown in Figure 10. The slope of the line is equal to:

$$d \log(y(r) - y(o))/d(r^2) = 0.670$$

To calculate the weight average molecular weight, which for a homogeneous solution is the molecular weight, the equation

$$M.W. = RT/(1 - vP)w^2 \ (2) \ 2.303 \log(y(r) - y(o))/d(r^2)$$

is used. Where R is the gas constant (8.313 x 10 erg $(g \text{ mole})^{-1} \deg^{-1}$), T is the absolute temperature, v is the partial specific volume, P is the density, and w is the rotor speed.

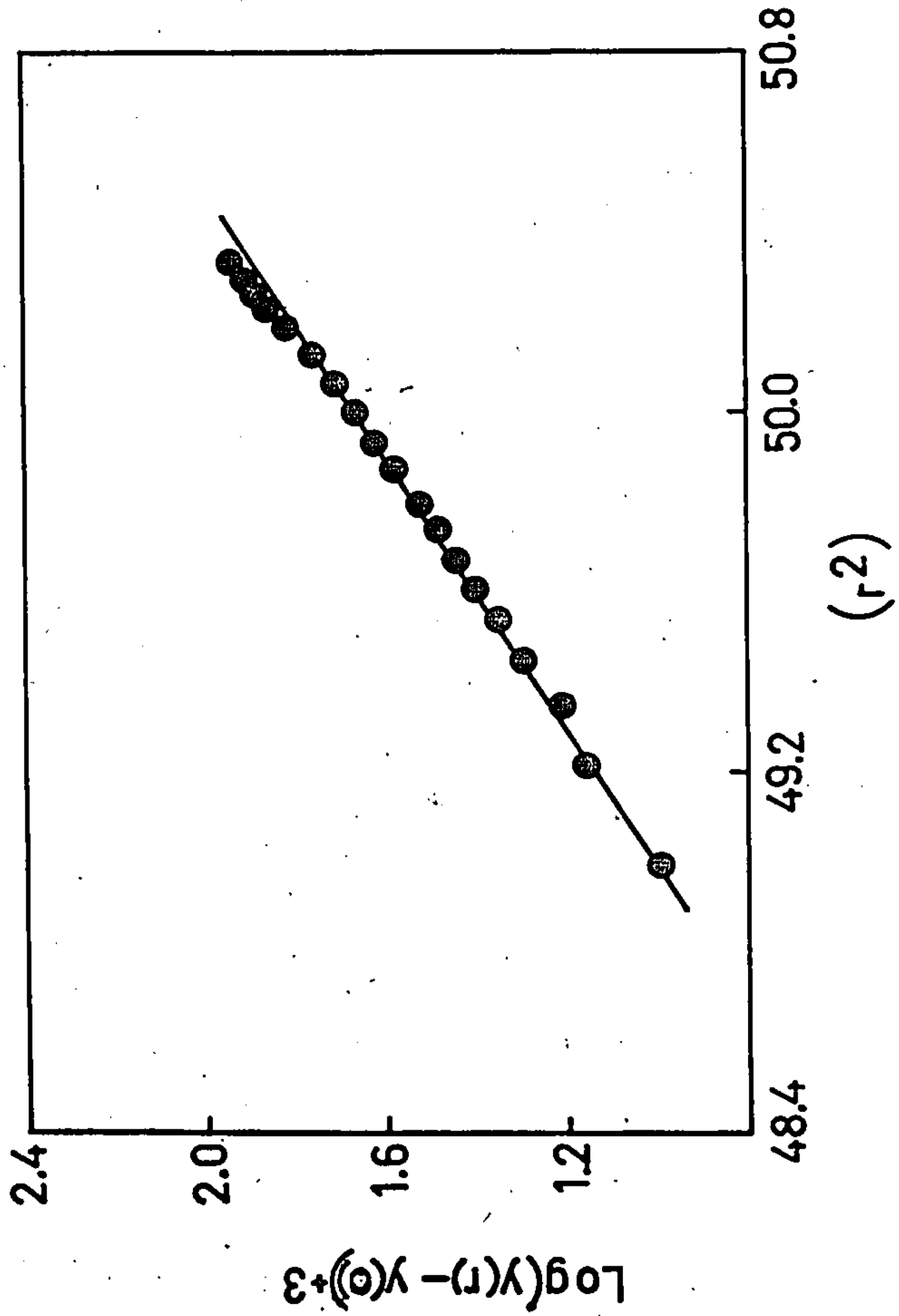
If (1 - vP) is assumed to be 0.25 (25), the weight average molecular weight drops from 59,000 at 16 h, to 45,000 at 28 h, and finally to 40,000 at 47 h.

6) Amino Terminal Amino Acid Determination

Hydrolysis of 100 µg of DNS-FAH for 18 h resulted in no spot that would correspond to any DNS-amino acid, however large amounts of DNS-OH were seen. On hydrolysis for 6 h of 5 mg of DNS-FAH, a spot with an Rf in the water-90% formic acid direction of 0.43, and an Rf in the benzene-acetic acid direction of 0.70 was seen. DNS-proline results in Rf's of 0.44 and 0.70 respectively. When DNS-proline and DNS-FAH were spotted together on a TLC plate, only one spot was seen.

Fig. 10. Graph of $\log[y(r') - y(o)] + 3$ versus r^2 for one line of the interference-optic-system-plate from the ultracentrifugation of FAH after 47 h at 25,980 rpm and 12°.

FIGURE 10



7) Carboxyl Terminal Amino Acid Determination

The hydrolysis of FAH by carboxypeptidase A resulted in the release of six major amino acids (Lys, Phe, Gly, Leu, Ser, and Ala). The reaction proceeded very rapidly. There was no further change in the ratios of the amino acids released, per mole of FAH, after the first hour of the reaction. It can be concluded that the last four amino acids are -Gly-Leu-Ser-Ala-COOH and that the fifth and sixth amino acids in the series are Lys and Phe, though the order of appearance is not certain. The difference between Ser and Ala was small and should be confirmed by hydrazinolysis (27), (Table II)

8) Amino Acid Analysis

The results obtained from analyses of different preparations of FAH subjected to acid hydrolysis are presented in Table III

9) Free Sulfhydryl Group Determination

The reaction of 0.70 mg/ml of FAH with DTNB in the presence of 2% SDS resulted in a net absorbance at 412 nm of 0.78. This would correspond to 3.2 moles of free sulfhydryl groups per mole of FAH.

When the SDS was omitted, the reaction of FAH with DTNB resulted in the precipitation of the FAH. This prevented the following of the reaction as a

TABLE II: Mole A.A./Mole FAH (M.W.= 40,000) Released by Carboxypetidase A Hydrolysis

Amino Acid	0.25 h	0.5 h	1 h	2 h	Relative Rate of Release (27)
Alanine	0.60	0.66	0.70	0.70	Rapid
Serine	0.48	0.62	0.64	0.72	Slow
Leucine	0.46	0.54	0.62	0.66	Rapid
Glycine	0.36	0.40	0.42	0.42	Very Slow
Phenyl-alanine	0.32	0.36	0.40	0.38	Rapid
Lysine	0.32	0.38	0.40	0.44	Slow

TABLE III: Amino Acid Composition of FAH

Amino Acid	Moles a.a./mole FAH (40,000g/mole)
Lysine	14.6
Histidine	7.3
Arginine	14.0
Tryptophan	4.5
Aspartic Acid	28.6
Threonine	15.8
Serine	24.2
Glutamic Acid	29.4
Proline	23.6
Glycine	27.9
Alanine	22.7
Half Cystine	4.8
Valine	21.1
Methionine	6.0
Isoleucine	14.7
Leucine	32.9
Tyrosine	8.9
Phenylalanine	13.2

function of time. At the end of 1.5 h there appeared to be no further increase in the absorbance at 412 nm. The solution was centrifuged to remove the precipitated protein and the absorbance taken at 412 nm. The resulting calculations showed that 1.8 moles of free sulfhydryl groups reacted per mole of FAH.

10) Determination of the Extinction Coefficient

The percent by weight of nitrogen in a freeze-dried sample of FAH determined by the Kjeldahl method was 16.2%. A sample of 5.75 mg gave off 0.0666 mmoles of ammonia or 0.932 mg of nitrogen. A 1.5 ml solution of FAH with an absorbance of 2.87 OD_{280}/cm and 1.81 OD_{260}/cm gave off 0.0379 mmoles of ammonia or 0.531 mg of nitrogen. Thus the ϵ_{280} would be

$$[0.932/5.75(\text{mg N/mg FAH})] \cdot [(2.87)(1.5)/0.531(OD_{280}\text{ml/cm/mg N})]$$
$$= 1.31\ (OD_{280})(\text{ml})/(\text{mg FAH})(\text{cm}).$$

In a similar manner the ϵ_{260} was calculated to be 0.829 (OD_{260})(ml)/(mg FAH)(cm).

These results compare closely with the extinction coefficients found for FAH in a 2% SDS solution by a Beer-Lambert plot of

ϵ_{280} = 1.43 OD ml/mg cm and ϵ_{260} = 0.954 OD ml/mg cm.

11) Preparation of Fumarylacetoacetate

The isomerization of maleylacetoacetate to fumarylacetoacetate was followed over a 7 day period by the increase in absorbance at 316 nm in a pH 1

solution. The ability of FAH to hydrolyze this solution was determined by the ΔOD at 330 nm at pH 7.3. It was seen that as the amount of fumarylacetoacetate increased so did the ΔOD at 330 nm.

HMB has been shown to be an inhibitor of FAH activity towards several substrates (11). HMB was found to inhibit FAH activity towards its natural substrate, fumarylacetoacetate, as well. After 30 min incubation with 10^{-5}, 10^{-4}, and 10^{-3} $\underline{M}$ HMB the activity dropped from 100% to 40.6%, 2.2%, and 0% respectively.

CHAPTER III

DISCUSSION

Since the enzyme isolated does hydrolyze fumarylacetoacetate, the assumption made previously (4) that the enzyme is FAH was correct.

The sulfhydryl specific inhibitor, p-mercuribenzoate, also inhibits FAH hydrolysis of fumarylacetoacetate much to the same extent as it has been shown to inhibit FAH hydrolysis of propionopyruvate, acetopyruvate, butyropyruvate, pivalopyruvate, 5-phenyl-3,5-pentanedionoate, and cinnamoylpyruvate (11). Evidence has been presented that p-hydroxymercuribenzoate irreversibly modifies a sulfhydryl group at or near the active site of FAH which hydrolyzes propionopyruvate (11). Apparently FAH, whose natural substrate is fumarylacetoacetate, has the ability to hydrolyze a large number of diketoacids all at the same active site.

FAH, after chromatography on TEAE-cellulose, is homogeneous based on several criteria for homogeneity. First, the elution profile from the Sepharose 6B column gave only one symmetrical peak within the separation range of the gel (10^3 - 10^5 g/mole). Second, only one band was seen on the polyacrylamide gels at pH 8.9, 9.5, and 4.5. Third, the immunodiffusion and

immunoelectrophoresis plates gave only one line. Fourth, the amino terminal amino acid determination gave only one DNS-amino acid (proline) on hydrolysis for 6 h and showed no DNS-amino acid spot after 18 h of hydrolysis (DNS-proline is the only DNS-amino acid that is unstable towards prolonged acid hydrolysis, while an 18 h hydrolysis is necessary to hydrolyse the peptide bonds containing either DNS-isoleucine or DNS-valine (26)).

The molecular weight for FAH, determined by the various methods outlined in the experimental section are summarized in Table IV.From these values, FAH appears to be a dimer of molecular weight 77,000 - 95,000 with subunits of molecular weight 37,000 - 43,000.

The two subunits of FAH probably are identical since only one symmetrical peak was seen on the Sepharose 6B column in 6 M guanidine-hydrochloride, only one primary band was seen in the polyacrylamide gel done in sodium dodecylsulfate, and only one amino terminal amino acid was found.

The fact that little difference is seen in the elution volume of reduced and alkylated FAH and non-reduced, unalkylated FAH in 6 M guanidine hydrochloride indicates two physical characteristics of FAH. First, each of the two subunits consists of only one major

TABLE IV: Summary of the Molecular Weight Studies

Method	Conformation of FAH	Molecular Weight	Error Limits
I. Gel Filtration			
A. Sephadex G-150	Native	85,000	± 8,500
B. Sepharose 6B	Random Coil	43,000	± 9,000
II. Polyacrylamide Disc Gel Electrophoresis	Random Coil	40,000	± 6,000
III. Ultracentrifugation	Native	40,000	± 4,000

polypeptide chain, and second, there are no disulfide bonds joining the subunits together. If these two conditions are not met, one would expect to see units of much larger molecular weight than 40,000 in the non-reduced, unalkylated protein.

The inactive shoulder on the elution profile of FAH from the G-150 column is probably dissociated subunits. The protein in the shoulder is immunologically identical to the protein in the main peak, which exhibits FAH activity. This shoulder appears around the elution volume that would correspond to a protein of molecular weight 40,000, the weight of the FAH monomer. In addition, the specific activity of the main peak was about twice as great as the original FAH sample placed on the column, which by all previously described criteria was pure FAH. This could only be explained by the separation of inactive FAH subunits from the active FAH dimer. The fact that the dimer does dissociate in normal buffer solution was demonstrated by the ultracentrifuge results, where the weight average molecular weight of purified FAH dropped from 59,000 after 16 h to 45,000 after 28 h and finally to 40,000 after 47 h. This does not, however, mean 100% dissociation since the speed of the centrifuge was also increased over the 47 h period. The increased speed forces the larger molecular weight molecules to the

bottom of the cell and hence are not measurable. This does, however, mean that the molecular weight of the smallest molecule in the solution was 40,000 since the rotor speed for the 47 h determination (26,000 rpm) is suitable to detect molecules of 50,000 or less molecular weight (25). The rotor speed for the 16 h determination (20,500 rpm) is suitable to detect molecules of 90,000 or less (25). Thus enough of the dimer was in solution at 16 h and 20,500 rpm to give a weight average molecular weight of 59,000.

The main method of inactivation for FAH is probably the dissociation of the dimer. Thus there is probably a monomer ⇄ dimer equilibrium in normal buffer solutions. This would help explain why FAH is unstable in solutions containing ethanol or neutral salts such as the 0.1 M sodium chloride in the Sephadex G-150 elution buffer or the residual ammonium sulfate in the precipitate after ammonium sulfate precipitation, since both ethanol (36) and neutral salts (37) tend to decrease hydrophobic bonding. Glycerol (10% v/v) on the other hand, seems to prevent much of the loss of FAH activity, it must then increase hydrophobic bonding and thus help to keep the dimer together.

It has been shown that there is a sulfhydryl group at or near the active site of FAH. DTNB, however, is

only a simple competitive inhibitor of FAH, while p-mercuribenzoate, another sulfhydryl inhibitor, irreversibly inactivates FAH. This would indicate that DTNB cannot "reach" the sulfhydryl group in the active site, though it is reversibly bound at some point near enough to the active site to block entry by the substrate (9). Using DTNB to determine the total number of free sulfhydryl groups in FAH by "unwinding" the protein with a 2% solution of sodium dodecyl sulfate so that all the sulfhydryl groups were available to the DTNB for reaction (30), FAH was found to have three free sulfhydryls per subunit. When native FAH was reacted with DTNB in sodium phosphate buffer only, two of the three sulfhydryl groups reacted per subunit. This would indicate, since the subunits are identical, that there is one active sulfhydryl group per subunit. If the sulfhydryl group is in the active site then there appears to be one active site per subunit.

From this work, a few physical similarities are apparent between rabbit muscle aldolase and FAH. Although aldolase is a tetramer and FAH is a dimer, their monomers seem to be of the same molecular weight, 40,000 (14). The assembly of the aldolase tetramer appears to occur as a dimerization of the dimers (38).

The four subunits are chemically identical except for the deamination of a single asparagine in two of the subunits. Like FAH there are no disulfide bonds between subunits, however, unlike FAH the formation of the oligomer is irreversible under normal conditions and there is no evidence of a monomer ⇌ oligomer equilibrium (38).

FAH and aldolase also have the same amino terminal amino acid, proline.

Unlike muscle aldolase, little work has been done on liver aldolase, however, it is known that the active sites of liver and muscle aldolase are basically identical (14). Liver aldolase does differ from muscle aldolase slightly in amino acid composition, and in its carboxyl terminal sequence ending in (ala-val-ser)-gly rather than (asn-his-ala)-tyr as found in muscle aldolase (39). Liver aldolase also will not react with anti-muscle-aldolase-serum (39) thus there probably is some structural difference. FAH differs from both liver and muscle aldolase in its carboxy terminal sequence, -Gly-Leu-Ser-Ala-COOH, and there is no cross reaction between FAH antisera and muscle aldolase.

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