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# Fumarylacetoacetate fumaryl hydrolase, metal analysis and cyanide inactivation.

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FUMARYLACETOACETATE FUMARYL HYDROLASE,  
METAL ANALYSIS AND CYANIDE  
INACTIVATION

BY

RONALD JOHN EWING

A Thesis

Submitted to the Faculty of Graduate Studies through the  
Department of Chemistry in Partial Fulfillment  
of the Requirements for the Degree of  
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Windsor, Ontario

1977

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## ABSTRACT

Fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2) is not inactivated by cyanide as previously thought. This work shows that the initial inactivation is overcome after prolonged incubation of the assay mixture. The cause of the apparent inactivation has been isolated. After the cyanohydrin that has been formed initially has decomposed the enzymatic activity is unmasked.

Fumarylacetoacetate fumarylhydrolase (FAH) is a metalloenzyme that contains one mole of magnesium per mole of enzyme and one mole of zinc per two moles of enzyme as determined by atomic absorption.

The cleavage of 2,4-diketo acids is postulated to occur by the magnesium holding the substrate and inactivating it followed by a nucleophilic attack. The nucleophile is probably a sulfhydryl group of the enzyme and may form a thioI ester after cleavage of the carbon-carbon bond.

## ACKNOWLEDGEMENTS

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I am forever indebted to my wife Sandra for her help not only with the thesis but with all these years of studies. Also, I would like to thank Hamdy Kahlil for his preparation of the Figures. Finally I would like to thank all of my friends who took an interest in this work.

To Sandra

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## ABBREVIATIONS

DTE	dithioerythritol
ENZ	enzyme
FAH	fumarylacetoacetate fumarylhydrolase
HQSA	8-hydroxyquinoline-5-sulfonic acid
PLP	pyridoxal-5'-phosphate
TCA	trichloroacetic acid
TEAE	triethylamino ethyl
TRIS	tris (hydroxymethyl) amino methane
TRIS-HCl	tris (hydroxymethyl) aminomethane hydrochloride

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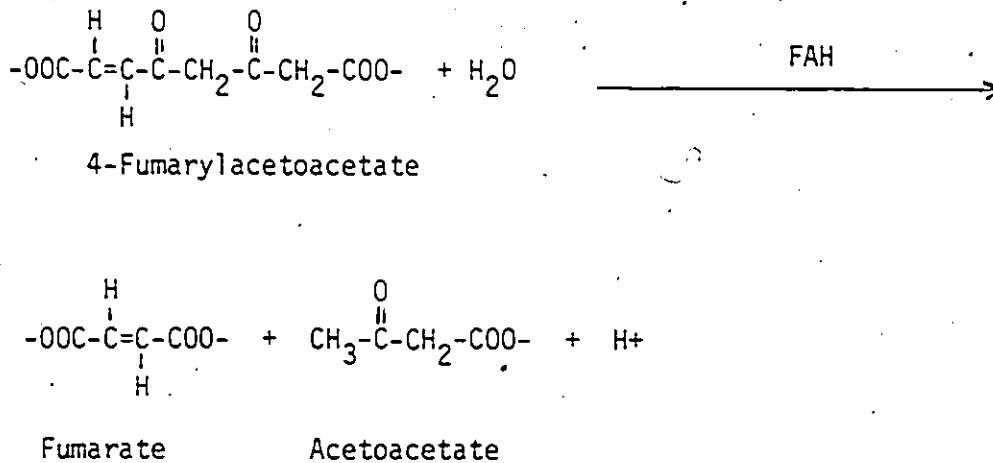
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## CHAPTER I

### INTRODUCTION

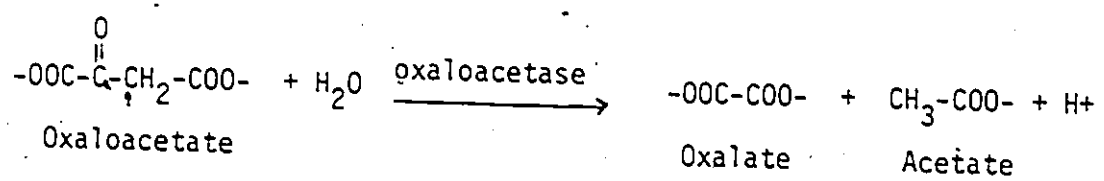
In 1948 Meister and Greenstein (1) isolated an enzyme that catalyzed the cleavage of 2,4-diketovalerate to pyruvate and acetate and was named 2,4-diketoacid hydrolase. Connors and Stotz (2) isolated a triacetic acid hydrolase in 1949. Further work (3,4) has shown that these two enzymes were probably 4-fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2). Fumarylacetoacetate fumarylhydrolase (FAH) catalyzes the cleavage of fumarylacetoacetate into fumarate and acetoacetate in the degradative pathway of tyrosine and phenylalanine in mammalian systems.



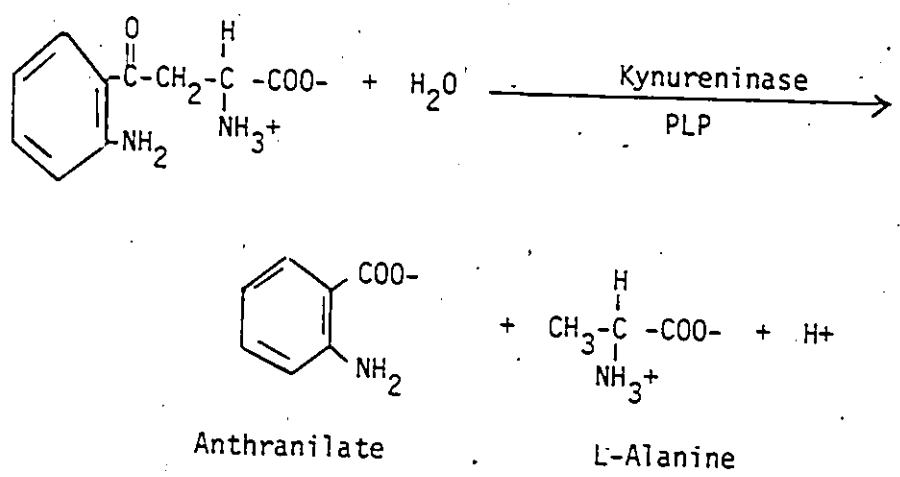
FAH hydrolyses carbon-carbon bonds between a methylene carbon and an adjacent carbonyl carbon and therefore it belongs to a distinct class of enzymes that hydrolyze carbon-carbon bonds. Oxaloacetate

acetylhydrolase, L-kynurenine hydrolase and fumarylpyruvate hydrolase are examples of other enzymes in this class.

Oxaloacetate acetylhydrolase (EC 3.7.1.1) catalyzes the formation of oxalate and acetate from oxaloacetate (5).



L-kynurenine hydrolase (EC 3.7.1.3) catalyzes the formation of anthranilate and L-alanine from L-kynurenine (6).

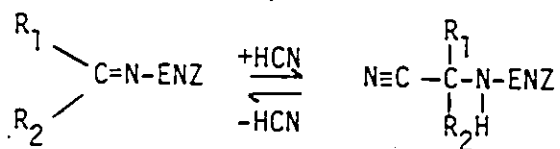


In the gentisic acid degradative pathway, fumarylpyruvate hydrolase catalyzes the cleavage of fumarylpyruvate to fumarate and pyruvate (7). The mechanism of L-kynurenine hydrolysis is known to involve a Schiff-base formation between the cofactor pyridoxal-5'-phosphate (PLP). The enzyme is activated by Cu<sup>++</sup>, Mg<sup>++</sup> and Mn<sup>++</sup> (8.9). These enzymatic reactions have had little research done on them.

FAH was investigated previously and it is known to contain a sulfhydryl group at or near the active site (10,11). The enzyme has a subunit molecular weight of 40,000 gm/mole and requires two subunits for activity (10).

The mechanism of action of FAH was investigated further by examining the inactivation of FAH by potassium cyanide (11) and secondly by an analytical determination of metal content.

Cyanide has been reported to inactivate a series of enzymes which function via Schiff-base mechanisms. These include L-kynureninase (6,12), transaldolase (13), acetoacetate decarboxylase (14,15) and fructose 1,6-diphosphate aldolase from rabbit muscle (16). In the latter three cases the inactivation depended upon the presence of a substrate that formed a Schiff-base intermediate with the  $\epsilon$ -amino group of a lysyl residue of the protein. The inhibition was also reversible by dilution or dialysis. It has been theorized that cyanide added reversibly to the azomethine linkage which resulted in the formation of an enzyme bound aminonitrile as shown in Scheme I (17).



This proposed reaction could not be verified by isolation and identification of the adduct because the reaction was reversible.



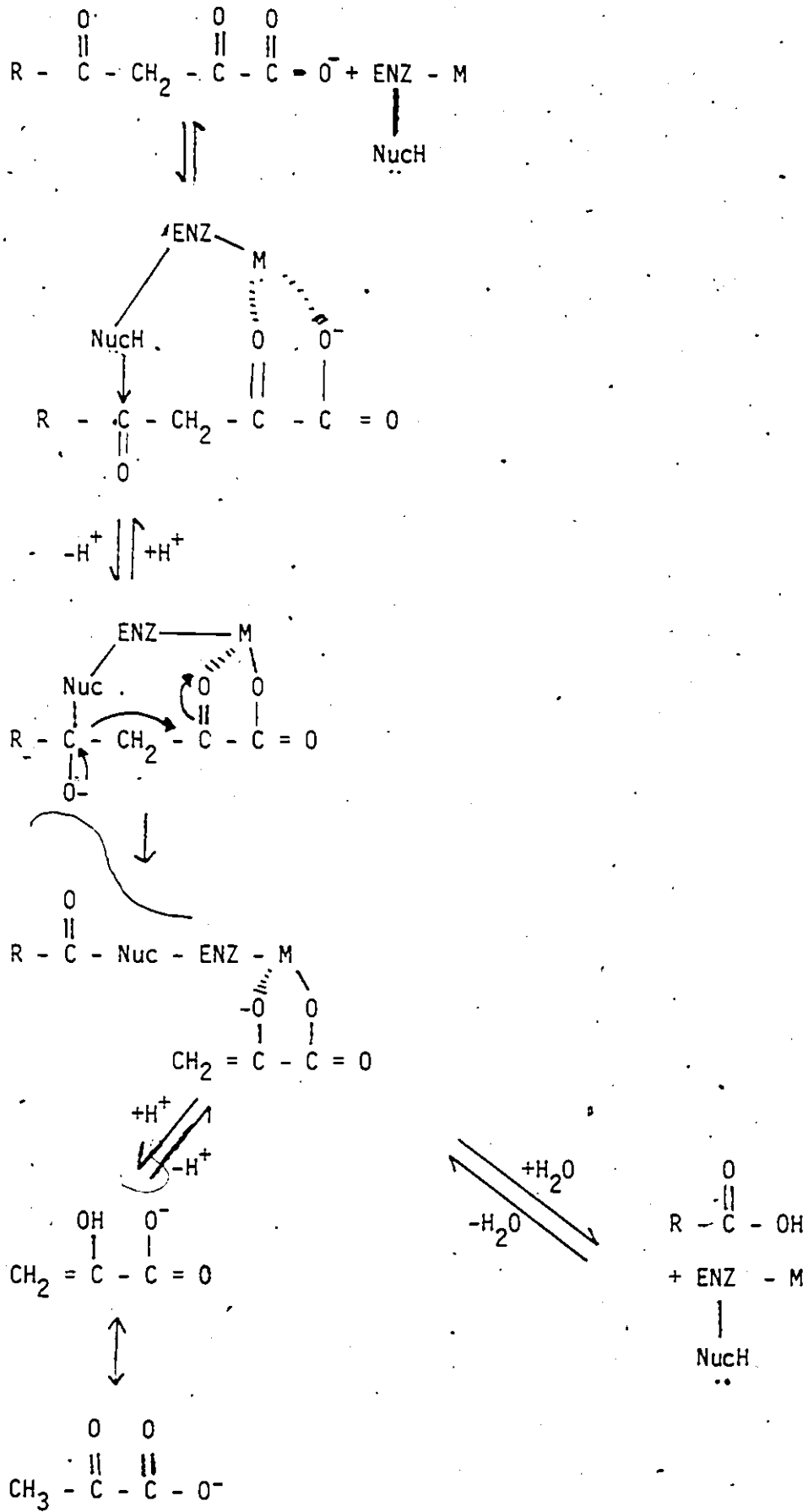
However, irreversible inhibition by cyanide was noted using 2-keto-4-hydroxyglutarate in the presence of aldehydic substrates (18). A labelled fragment was isolated which proved that an amino-nitrile was formed by reaction of cyanide with the enzyme-Schiff-base. This established the use of cyanide for detecting Schiff-base intermediates and therefore cyanide was also used as a reagent to detect a Schiff-base intermediate in the case of FAH (11).

If cyanide does trap a Schiff-base intermediate formed by FAH and substrates, then cyanide may also trap possible aldehyde-adducts with FAH. Cyanide and various aldehydes irreversibly inactivate 2-keto-4-hydroxyglutarate aldolase (18) and the adduct has been isolated as the reduction product of the Schiff-base intermediate. With this in mind the effect of cyanide and selected aldehydes was investigated.

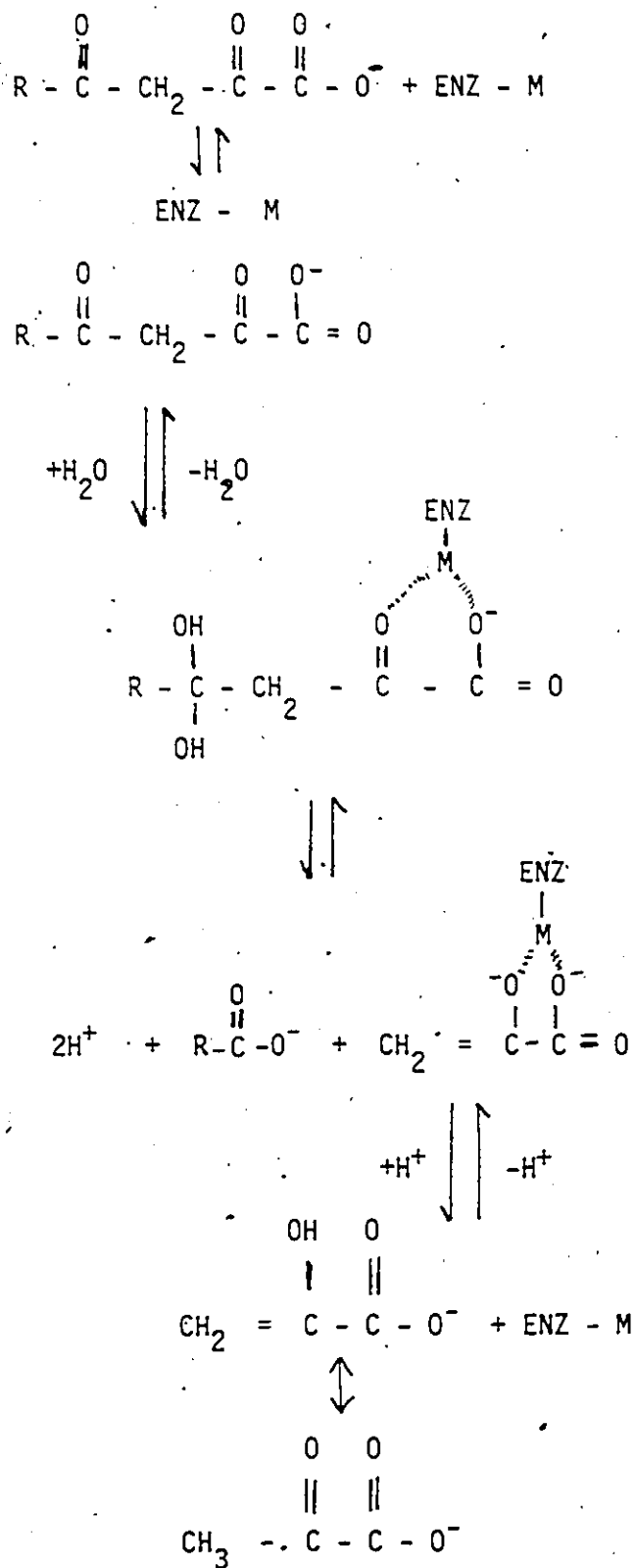
Boyer (19) reviewed the case of  $\beta$ -decarboxylation of  $\beta$ -keto acid and stated two types of enzymes are known. One type involves imine formation between the substrate and a lysine residue of the enzyme. The second type requires metal ions and does not involve imine formation.

Schemes 1 and 2 propose a metal activated mechanism for FAH (11). Nucleophile represents a nucleophile and ENZ-M represents the metallo-enzyme.

Scheme 1.



Scheme 2.

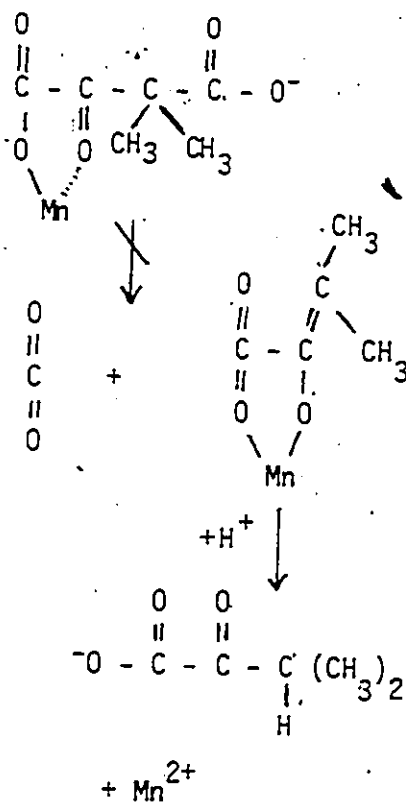


The nucleophile of Scheme 1 may be a sulfhydryl group which is known to be at or near the active site of FAH (10, 11).

Other enzymes known to follow a similar reaction mechanism are pyruvate carboxylase and oxaloacetate decarboxylase (19).

Scheme 3 presents the mechanism for the decarboxylation of dimethyloxaloacetate using oxaloacetate decarboxylase.

Scheme 3



## CHAPTER II

### EXPERIMENTAL

#### A. METHODS AND MATERIALS

##### 1) Materials

The following materials were commercially available; dithioerythritol (DTE), glyoxylic acid, tris (hydroxymethyl) aminomethane (TRIS) and 8-hydroxyquinoline-5-sulfonic acid (HQSA) from Sigma; potassium cyanide, formaldehyde, manganese, magnesium standard for atomic absorption, egg albumin and primary grade iron were from Fisher; nitric acid, hydrochloric acid and sulfuric acid from G.F. Smith; tris (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl) and ammonium sulfate from Schwartz Mann; trichloroacetic acid (TCA) from Baker; Chelex 100 from Biorad. All reagents used were of A.C.S. quality or better.

##### 2) Enzyme Isolation

FAH was isolated and purified by the method of Hsiang *et al.* (4) with the following modifications. The linear gradient on the TEAE-cellulose column was replaced by a stepwise elution. After placing the enzyme solution on the column, 300 ml of 0.02 M TRIS-HCl buffer pH 8.5 was passed through the column. Then the enzyme was eluted with 0.08 M TRIS-HCl buffer pH 8.5 with  $1 \times 10^{-4}$  M DTE. All fractions with specific activity over 0.26  $\mu$ moles of acetopyruvate hydrolyzed/min were combined and precipitated with 40% (w/v) ammonium sulfate (4). Packages of enzymes were prepared by dissolving

the precipitate in a small volume of 0.1M sodium phosphate buffer at pH 7.3. No glycerol or DTE was added. The enzyme was stored at  $-20^{\circ}$  until used.

### 3) Assay of Incubation Mixtures

Into a 3 ml. cuvette were pipetted 2.6 ml of 0.1M sodium phosphate buffer pH 7.3 and 0.3 ml of 2 mM propionopyruvate (in sodium phosphate buffer). Propionopyruvate has an absorption maximum at 290 nm due to the enol form. The reaction was initiated by addition of 50  $\mu$ l. of solutions. The rate of reaction was monitored by observing the decrease in absorption at 290 nm. The initial rates were calculated from slopes on the chart paper. FAH concentrations of pure enzyme solutions were determined by using the extinction coefficient of the enzyme,

$$\epsilon_{280} = 1.31(OD_{280}) \text{ (ml)/(mg FAH) (cm)} \quad (10).$$

All kinetic data were obtained on a Gilford Model 2000 Absorbance Recorder attached to a Beckman DU monochromator with all compartments thermostated to  $25.0 \pm 0.5^{\circ}$ .

### 4) Effect of Potassium Cyanide and Aldehydes on Enzymatic Activity

Propionopyruvate was used as the substrate to form a Schiff-base with FAH. An appropriate weight of potassium cyanide was added to water to make a 2.7 mM solution of cyanide. The enzyme concentration was approximately 0.2 mg/ml. The aldehydes were dissolved in 0.1M sodium phosphate buffer pH 7.3.

For the first experiment 0.1 ml substrate and 0.1 ml cyanide solution were added to 0.3 ml enzyme and incubated at  $25.0 \pm 0.5^\circ$ . Then 50  $\mu$ l aliquots were removed after appropriate time intervals and tested for activity by standard assay. The control reactions were done by deleting the enzyme, substrate or cyanide and replacing the volume with the appropriate volume of buffer.

A second type of experiment required separate incubation of 0.3 ml FAH with 0.2 ml buffer and another tube with or without either cyanide or substrate. After incubation of the components separately, a 50  $\mu$ l enzyme aliquot and a 150  $\mu$ l aliquot from the other incubation mixture were placed in the same cuvette for the standard assay i.e. 2.5 ml buffer plus 0.3 ml substrate.

Another set of experiments consisted of adding 0.1 ml of an aldehyde, either formaldehyde or glyoxylic acid to 0.1 ml cyanide solution and 0.3 ml enzyme. At appropriate time intervals 50  $\mu$ l aliquots were removed and assayed for enzymatic activity using the standard assay. The aldehydes were made up in 0.9 M sodium phosphate buffer pH 7.3. Control reactions were done by omitting the aldehydes and or cyanide from the incubation mixture and replacement with the appropriate volume of buffer.

##### 5) Metal-Free Buffers, Glassware and Dialysis Membranes

The 0.1 M sodium phosphate buffer pH 7.3 was made using distilled deionized water and no further treatment. However

the preparation of metal-free TRIS-HCl was a necessity.

The 0.025 M TRIS-HCl buffer pH 8.5 was prepared by blending the appropriate weights of TRIS-HCl and TRIS then dissolving into distilled deionized water. Metal extraction was effected by letting the buffer stand at 0° over Chelex 100 (100-200 mesh) in the hydrogen form (20). One volume of Chelex 100 was used per five volumes of buffer (20). This buffer was filtered before use to remove the Chelex 100 resin. This buffer was not shaken with dithizone prior to the Chelex treatment as was done by Anderson and Vallee (21). Dithizone was very soluble at pH.8.5.

All glassware and Nalgene ware used was soaked overnight in nitric-sulphuric acid, then rinsed with copious volumes of distilled deionized water as described by Thiers (22).

All cellulose membranes used for dialysis were rendered metal-free by the following treatment: membranes were boiled twice in 10 mM HQSA, then three times in distilled deionized water, rinsed twice in metal-free buffer and stored in metal-free buffer kept over Chelex 100.

#### 6) Sample Ashing for Metal Analysis

All samples were ashed in quartz beakers with quartz watch glasses. The method of Middleton and Stuckey (23) was used for ashing and was done with slight modifications.

The sample was added to a quartz beaker and an equal volume of nitric acid with 5% sulfuric acid was added. After heating gently to disperse the sample it was then evaporated to dryness. The



sample was left uncovered and heated at 320° until no further change. After cooling, the beakers were covered and nitric acid, about 1 ml, was added to each sample. The samples were allowed to digest until all acid was gone. The samples were then heated to 320° until no further change was observed. The addition of nitric acid followed by digestion was continued until a white ash was obtained.

#### 7) Manganese Analysis

Manganese was analyzed by the formaldoxime method (24). A stock manganese solution was prepared by dissolving 0.1 gm of 99.9% manganese metal in 50 ml of 1.6 N nitric acid, then making up to a volume of 1 l. using distilled deionized water. This solution was stored in Nalgene containers at 0° to avoid losses due to absorption on glassware. Working standards were prepared from this stock solution on the day of analysis.

A known amount of FAH in 0.025 M sodium phosphate buffer pH 7.3 was added to a quartz beaker. A volume of buffer equivalent to the volume of FAH solution was placed in a second quartz beaker. This served as a blank. Into a third quartz beaker was placed an appropriate volume of buffer containing the same weight of egg albumin as FAH, plus a known amount of manganese. Another beaker with buffer and a known amount of manganese was also prepared as a suitable blank. The samples were then ashed, dissolved in a minimal amount of nitric acid and then analyzed for manganese content.

All readings were taken on a Hitachi Perkin-Elmer spectrophotometer using 100 mm cells at 450 nm. Data obtained were analyzed by the method of least squares.

#### 8) Zinc Analysis

Atomic absorption spectrophotometry is an established method for determining zinc in protein samples (25, 26, 27, 28). Metal quantitation using 10% TCA is well documented (29, 30) and thus was utilized.

For all analyses enzyme solutions in sodium phosphate buffer were dialyzed against a 1000 fold excess of metal-free 0.025M TRIS-HCl buffer pH 8.5 for 12 hours at 0°. One sample was dialyzed for twenty four hours to determine the effect of denaturation. Samples were ashed then dissolved in 12.5% TCA. The blank used was 0.025 M TRIS-HCl buffer pH 8.5. A sample containing appropriate amounts of TRIS-HCl buffer with or without egg albumin and a known amount of zinc was also analyzed. A stock solution of 200 mg zinc per ml was made up from zinc sulphate acidified with sulfuric acid. This solution was stored in Nalgene ware at 0°. The working standards for atomic absorption were made up in 12.5% TCA.

All atomic absorption analyses for zinc were done on a Unicam SP90A atomic absorption spectrophotometer at 213.9 nm. Data were analyzed by the method of least squares.

### 9) Magnesium Analysis

FAH dialyzed against a 1000 fold excess of metal-free TRIS-HCl buffer was analyzed by atomic absorption by two methods. Applying the method of Henry (31) an FAH solution was treated with 25% TCA and then analyzed for metal content. A blank was tested in the same manner. Other FAH samples were ashed and dissolved in 12.5% TCA. The blank was metal free buffer that was treated in the same manner. The recovery of magnesium was checked by the addition of a known amount of magnesium to a metal-free buffer. The sample was then ashed and analyzed.

The magnesium standards were prepared using a Fisher certified standard of 1000 mg magnesium per ml. The final working standards contained 12.5% TCA and were made the day of the analysis.

The analyses for magnesium were done on a Varian 175 atomic absorption spectrophotometer by monitoring 285.2 nm. All data were analyzed by the method of least squares.

### 10) Iron Analysis

The FAH solution used was dialyzed against metal-free TRIS-HCl buffer before use. A 1000 fold excess of the buffer was used.

The protein sample was ashed then taken up in 0.1 N HCl. A blank using metal-free buffer and a sample containing a known amount iron and metal-free buffer were treated in the same manner.

A stock solution of 1  $\mu$ g/ml was made from primary grade iron wire. This was stored in Nalgene at 0°. Working standards were prepared from the stock solution and were made 0.1 N hydrochloric acid.

All work was done on a Varian 175 atomic absorption spectrophotometer by monitoring absorption at 248.3 nm. Data obtained were analyzed by the method of least squares.

## B. RESULTS

### 1) Effect of Potassium Cyanide and Aldehydes on FAH Activity

FAH appears to be inactivated for only a few minutes, as seen in Table I. After some time partial activity is regained. Cyanide causes an apparent increase in activity. This is due to formation of the cyanohydrin that causes a disappearance of substrate (32). Substrate and cyanide alone cause an increase in absorbance that may be due to decomposition of the cyanohydrin in the standard assay cuvette. Table II shows that FAH and cyanide with substrate incubated separately and aliquots taken from each and assayed for activity yields the same pattern obtained for incubation of these materials together.

The aldehydes, glyoxylic acid and formaldehyde, incubated with enzyme only caused inhibition. The addition of cyanide caused no further inhibition as seen in Table III.

Table I  
 Effect of Potassium Cyanide on  
 Apparent Enzymatic Activity

Additions <sup>a</sup>	% Change in Optical Density After	
	5 min	10 min
FAH	100	100
FAH + 2.7 mM Cyanide	200	175
FAH + 4.0 mM Propionopyruvate	100	100
FAH + 2.7 mM Cyanide + 4.0 mM Propionopyruvate	0 (to 67) <sup>b</sup>	0 (to 50) <sup>b</sup>
2.7 mM Cyanide	75 (to 0) <sup>b</sup>	-
2.7 mM Cyanide + 4.0 mM Propionopyruvate	-75 (to 0) <sup>b</sup>	-

<sup>a</sup>All incubations were in 0.1 M phosphate buffer pH 7.3 at 25.0 ± 0.5°. Aliquots were removed after 5 or 10 minutes and assayed.

<sup>b</sup>After observation for a few minutes.

Table II

Effect of Cyanide on Apparent Enzymatic  
Activity using a Separate Incubation of FAH

Additions <sup>a</sup>	% Change in Optical Density After	
	5 min	10 min
None	100	100
4.0 mM Propionopyruvate	150	108
2.7 mM Cyanide	183	200
2.7 mM Cyanide + 4.0 mM Propionopyruvate	0 (to 67) <sup>b</sup>	-100 (to 100) <sup>b</sup>

<sup>a</sup>All incubations were in 0.1 M phosphate buffer pH 7.3 at  $25.0 \pm 0.5^\circ$ .  $150 \mu\text{l}$  aliquots were removed at 5 and 10 minutes, combined with  $50 \mu\text{l}$  of a FAH incubation mixture and then assayed immediately for enzyme activity.

<sup>b</sup>After observation for a few minutes

Table III  
 Effect of Aldehydes and Cyanide on  
 Apparent Enzymatic Activity

Additions <sup>a</sup>	% Change in Optical Density after 5 min
FAH	100
FAH + 2.7 mM Cyanide	119
FAH + 2.7 mM Cyanide + 4.0 mM Formaldehyde	75
FAH + 4.0 mM Formaldehyde	81
FAH + 2.7 mM Cyanide + 4.0 mM Glyoxylic Acid	106
FAH + 4.0 mM Glyoxylic Acid	88

<sup>a</sup>All incubations were done in 0.1 M sodium phosphate buffer pH 7.3 at  $25.0 \pm 0.5$ . Aliquots of  $50 \mu\text{l}$  were removed after 5 minutes and assayed.



## 2) Manganese Analysis

Figure 1 is the standard curve obtained for this analysis. From Table IV there are no significant amounts of manganese native to FAH. There was a small amount of manganese in egg albumin so that the buffer was used as a blank for the enzyme analysis.

The percent recoveries were determined by correction for the appropriate blank, then calculating the percentage of manganese added initially. For example, 8  $\mu$ g manganese plus buffer the percent recovery is determined as follows:

$$\begin{aligned} \text{Recovery} &= \frac{\mu\text{g manganese determined} - \mu\text{g manganese in buffer}}{\mu\text{g manganese expected}} \times 100\% \\ &= \frac{8.08 - .68}{8.00} \times 100\% \\ &= 92.5\% \end{aligned}$$

The molar ratio of metal to enzyme is determined as follows:

$$\begin{aligned} \frac{\text{moles Mn}}{\text{moles FAH}} &= \frac{\text{weight of Mn determined in FAH} - \text{weight of Mn in buffer}}{\text{atomic weight of Mn}} \\ &\quad \frac{\text{weight of FAH used}}{\text{molecular weight}} \\ &= \frac{\frac{1.31 \mu\text{g} - 0.68 \mu\text{g}}{54.94 \text{ gm/mole}}}{\frac{5.35 \text{ mg}}{40,000 \text{ gm/mole}}} \\ &= .09 \end{aligned}$$

## Figure 1

Standard Curve for Manganese by the  
Formaloxime Method

An analysis of standards prepared from pure manganese metal using the formaloxime method (24). Standards were measured using a 100 mm light path cells in a Hitachi Perkin-Elmer spectrophotometer at 450 nm.

Figure 1

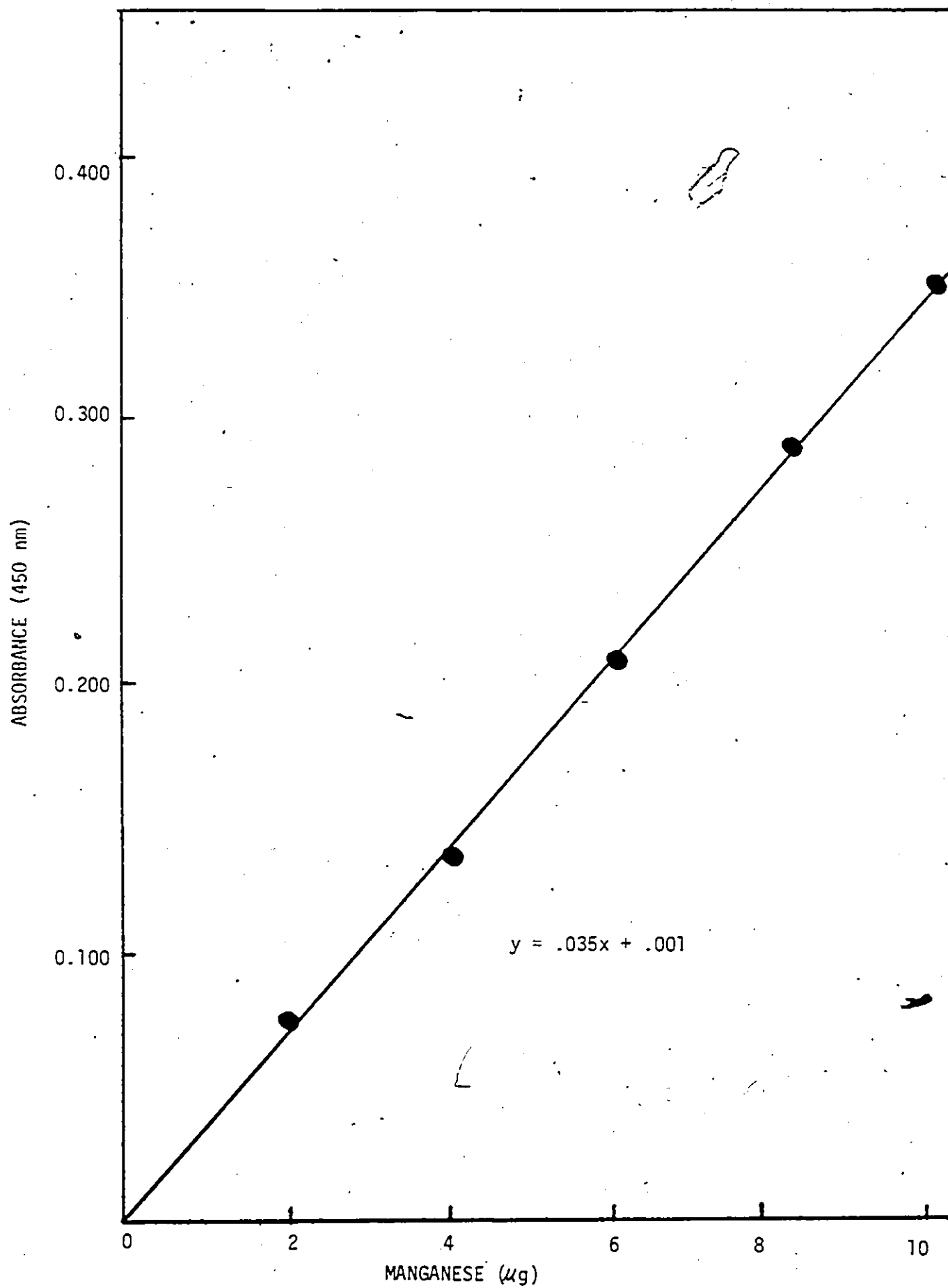


Table IV  
Manganese Analysis

Additions <sup>a</sup>	Absorbance	Manganese ( $\mu$ g)	$\frac{\text{Moles Manganese}}{\text{Mole FAH}}$	% Recovery
None	0.025	0.68		
8 $\mu$ g Manganese	0.285	8.08		92.5 $\pm$ 10
8 $\mu$ g Manganese + 5 mg Egg Albumin	0.347	9.84		110.9 $\pm$ 10
Egg Albumin 5 mg	0.035	0.97		
5.35 mg FAH <sup>b</sup>	0.047	1.31	0.09	

<sup>a</sup>All samples contained 5 ml of 0.1M sodium phosphate buffer pH 7.3 prior to digestion

<sup>b</sup>The blank for FAH was 5 mg Egg Albumin

### 3) Zinc Analysis

An example of a standard curve obtained for an analysis is given by Figure 2. The molar ratio of zinc to enzymes and percent recovery were calculated in the same manner as in 2. FAH dialyzed for twelve hours, was analyzed and it contained an average of 0.46 moles of zinc per mole of enzyme. FAH analyzed after a twenty four hour dialysis contained 0.19 moles of zinc per mole of enzyme.

From Table V the average recovery was 105%.

Figure 2

## Standard Curve for Zinc by Atomic Absorption

This is the standard curve for run number one in Table II.

The standards were in 12.5% TCA. All samples were acid digested at the same time and taken up in 12.5% TCA. The blank contained TRIS - HCl buffer, the enzyme sample contained 3.89 mg FAH in buffer, and the standard contained 4 mg egg albumin and 1.5  $\mu$ g of zinc in buffer. The final volumes were 2 ml for blank and standard and 4 ml for the enzyme. All readings were taken on a Unicam SP90A atomic absorption spectrophotometer at 213.9 nm.

Figure 2

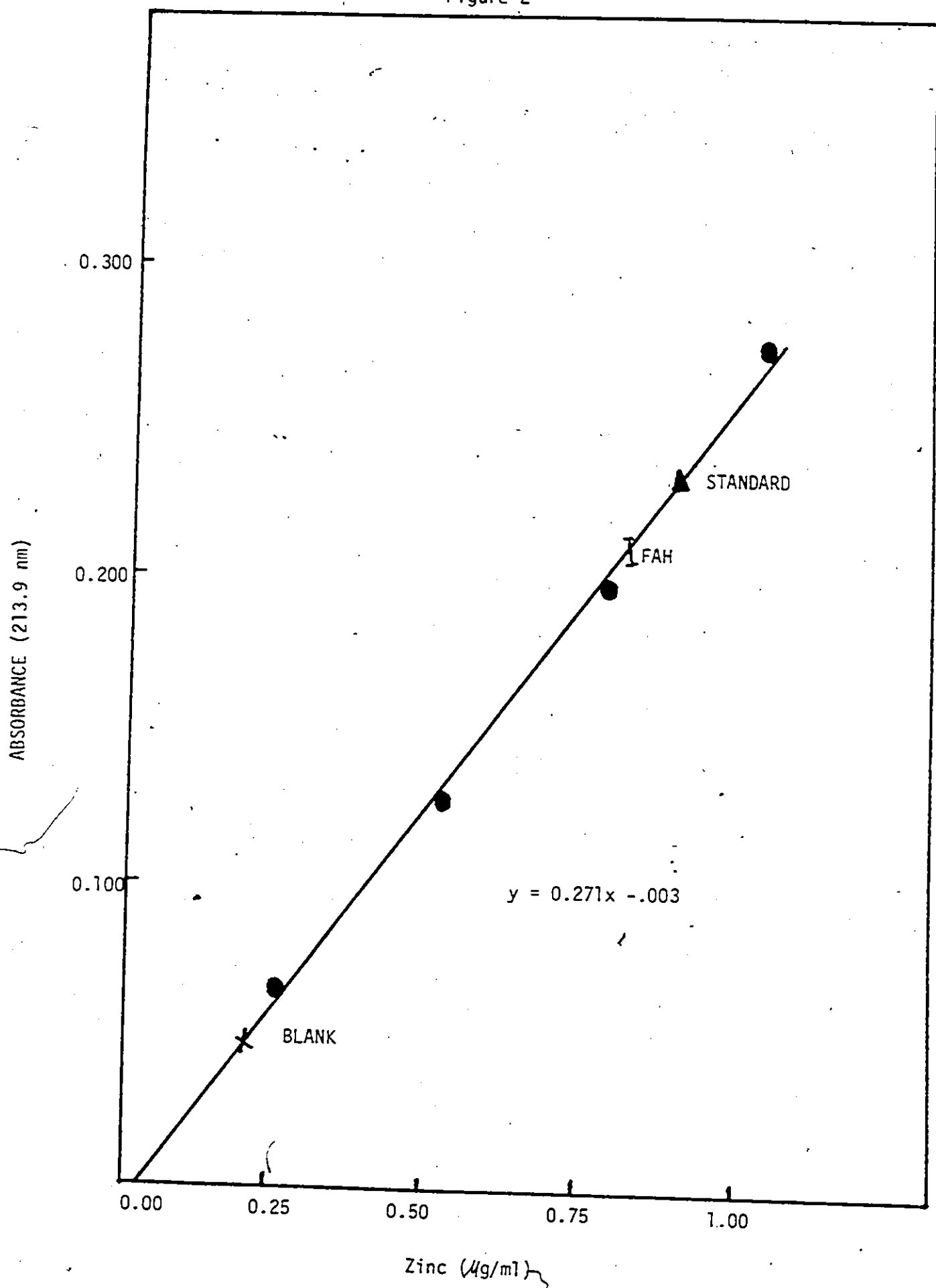


Table V

## Zinc Analysis

Run #1	Additions <sup>a</sup>	Final Volume <sup>b</sup> (ml)	Zinc ( $\mu$ g/ml)	Total Zinc <sup>c</sup> ( $\mu$ g)	Moles Zinc Mole FAH	% Recovery
	A 12 hr FAH Dialysts					
None		2	0.181			
3.89 mg FAH		4	0.793	2.85	0.45	
4 mg Egg Albumin						
+ 1.5 $\mu$ g Zinc		2	0.876	1.39		92.7 $\pm$ 9
	Run #2 A 12 hr FAH Dialysts					
None		2	0.198			
1 $\mu$ g Zinc		2	0.768	1.14		114 $\pm$ 9
0.89 mg FAH		2	0.538	0.68	0.47	

Continued ...



Table V  
Zinc Analysis (Continued)

Run #3	Additions <sup>a</sup>	Final Volume <sup>b</sup> (ml)	Zinc ( $\mu$ g/ml)	Total Zinc ( $\mu$ g)	$\frac{\text{Moles Zinc}}{\text{Mole FAH}}$	% Recovery
	A 24 hr FAH Dialysis					
	None	2	0.165			
	1.5 $\mu$ g Zinc	2	0.981	1.63		108.7 $\pm$ 9
	3.4 mg FAH	2	0.744	1.16	0.21	

<sup>a</sup>All samples contained 0.025 M TRIS - HCl pH 8.5

<sup>b</sup>After acid digestion samples were taken up in 12.5% TCA

<sup>c</sup>Corrected for the blank

#### 4) Magnesium Analysis

Figure 3 represents a typical standard curve obtained for an analysis. All calculations for Table VI were done as in 2.

The FAH that was dialyzed for twelve hours contained 0.97 and 1.28 moles of magnesium per mole of FAH (Table VI) or an average of 1.13 moles of magnesium per mole. Run number three of Table VI shows that after a twenty four hour dialysis, 0.42 moles of magnesium per mole of FAH were found. The substantial loss of metal may have been due to denaturation and subsequent release of metal.

The recovery of the method was 91.0% and 97.5%. An average would be 94.7%. The amount of variance in reading a standard was less than  $\pm 5\%$  of the absorbance reading.

## Figure 3

## Magnesium Analysis by Atomic Absorption

The standard curve for run number 4 of Table 2 is presented. All standards contained 12.5% TCA and were prepared the day of the run.

The samples were acid digested then taken up in 2 ml of 12.5% TCA. The blank contained TRIS - HCl buffer and the standards 0.4  $\mu$ g magnesium in TRIS - HCl buffer. All data was obtained using a Varian 175 atomic absorption spectrophotometer monitoring 285.2 nm.

Figure 3

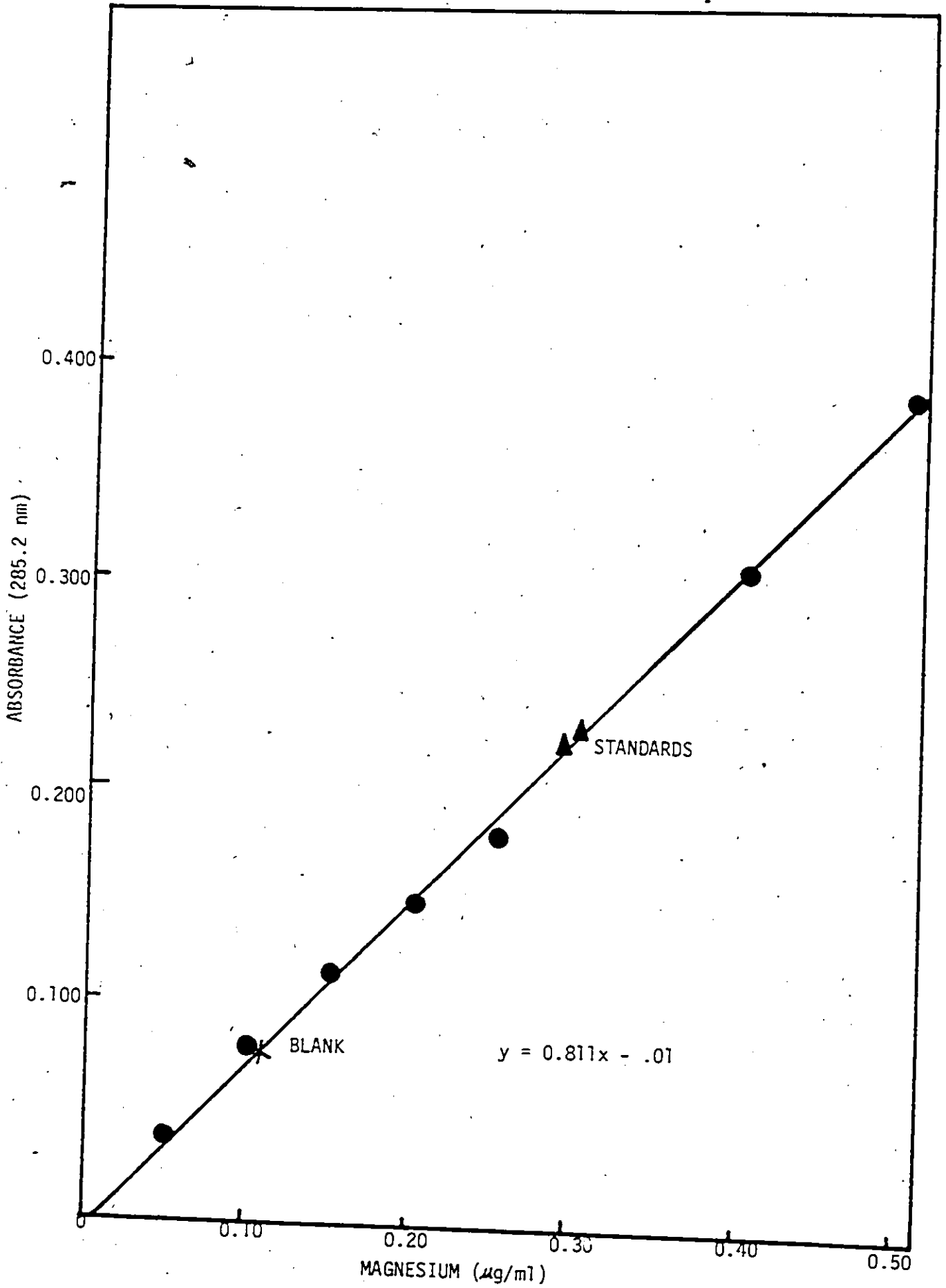


Table VI

## Magnesium Analysis

Additions <sup>a</sup>	Final Volume <sup>b</sup> (ml)	Magnesium ( $\mu$ g/ml)	Total Metal ( $\mu$ g)	$\frac{\text{Moles Metal}}{\text{Mole FAH}}$	% Recovery
Run #1 A 12 hr FAH Dialysis					
None	2	0.140			
0.93 mg FAH	4	0.216	.544	.97	
Run #2 A 12 hr FAH Dialysis					
None	2	0.049			
0.93 mg FAH <sup>c</sup>	4	0.206	.723	1.28	
Run #3 A 24 hr FAH Dialysis					
None	4	0.025			
2.05 mg FAH	4	0.155	0.520	0.42	

Continued ...

Table VI  
Magnesium Analysis (Continued)

Run #4	Additions <sup>a</sup>	Final Volume <sup>b</sup> (ml)	Magnesium ( $\mu$ g/ml)	Total Metal ( $\mu$ g)	$\frac{\text{Moles Metal}}{\text{Mole FAH}}$	% Recovery
	None	2	0.105			
	0.4 $\mu$ g Magnesium	2	0.278	.367		91.0 $\pm$ 4
	0.4 $\mu$ g Magnesium	2	0.300	.39		97.5 $\pm$ 4

<sup>a</sup>All samples contained the same initial volume of .025 M TRIS-HCl pH 8.5

<sup>b</sup>After dissolution in 12.5% TCA

<sup>c</sup>A TCA extraction of metal and no digestion, standards contained buffer.

5) Iron Analysis

The standard curve for the iron analysis is presented as Figure 4.

All calculations for Table VII were done as in 2. From Table VII the iron recovery was 98% and the molar ratio of iron to FAH was 0.16. This level of iron is too low to be meaningful.

Figure 4

## Standard Curve for Iron Analysis

All standards were made 0.1N HCl and were prepared the day of the analysis. The samples were acid digested then taken up in 2 ml of 0.1N HCl. See Table VII for more information. All analyses were done on a Varian 175 atomic absorption spectrophotometer by monitoring 248.3 nm.



Figure 4

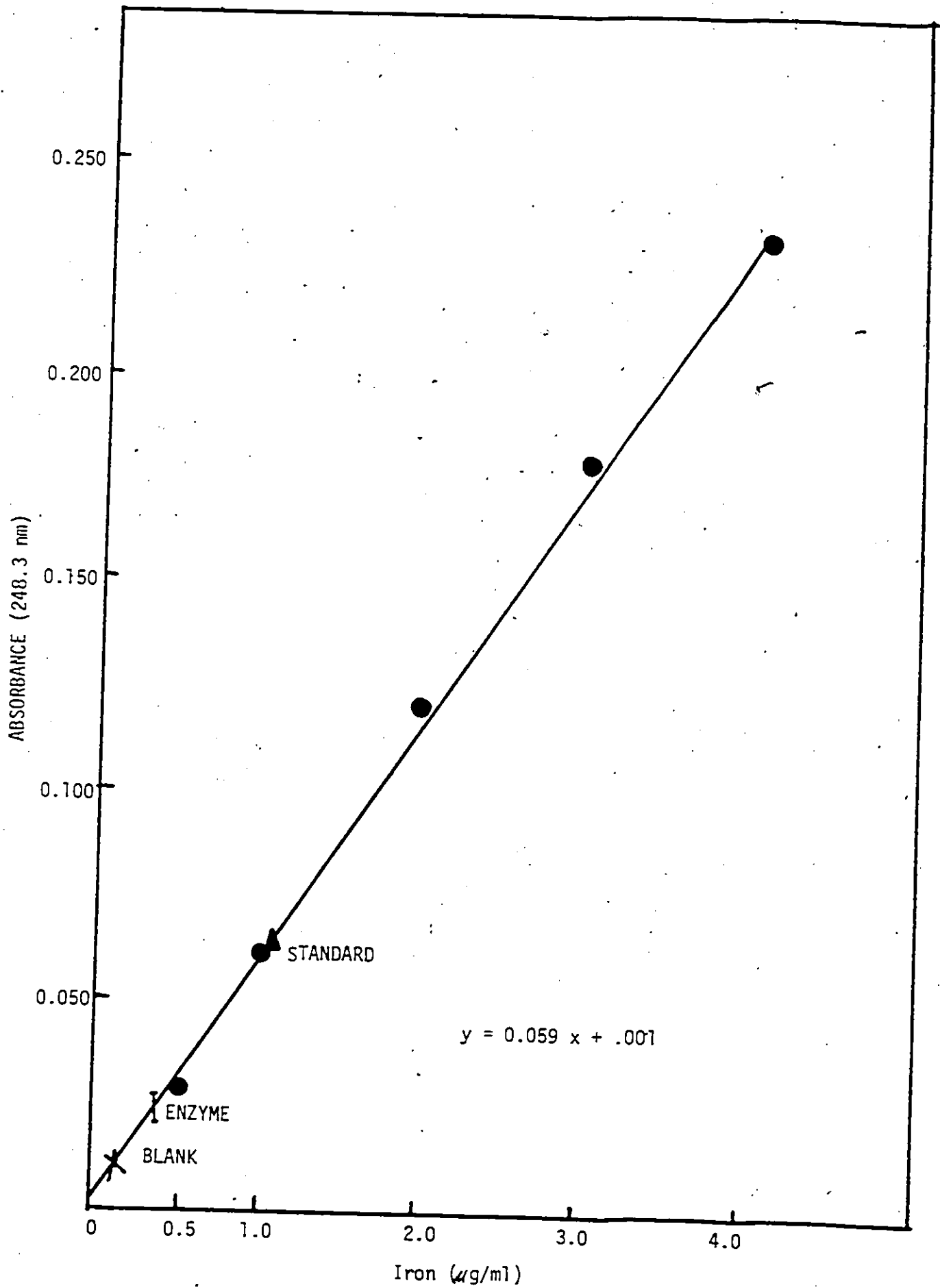


Table VII

Iron Analysis

Additions <sup>a</sup>	Final Volume <sup>b</sup> (ml)	Iron ( $\mu$ g/ml)	Total Iron <sup>c</sup> ( $\mu$ g)	Moles Iron Mole FAH	% Recovery
None	2	.12			
2 $\mu$ g Iron	2	1.07	.95		95% $\pm$ 5
1.89 mg FAH	2	.34	.44	.17	

<sup>a</sup>All samples contained 1 ml 0.025 M TRIS - HCl buffer pH 8.5 before analysis

<sup>b</sup>After acid digestion

<sup>c</sup>For entire sample

## CHAPTER III

### DISCUSSION

The Schiff-base mechanism postulated for FAH (11) was based upon cyanide inactivation of the enzyme; my experiments show the effect of cyanide was artificial. A cyanohydrin was formed when the substrate and cyanide were incubated together. When an aliquot of the cyanohydrin is added to the assay it dissociated into substrate and cyanide. If no FAH was present there was an increase in substrate concentration (Table I). If FAH was used in the appropriate concentration there was no net change in a substrate concentration initially. This was interpreted as inhibition by Nagainis (11). After prolonged incubation of the assay mixture the enzyme activity approached the control level because substrate released from the cyanohydrin form stopped.

Separate incubation of the components for trapping a Schiff-base intermediate (Table II) indicated that the "inactivation" of FAH is duplicated even when FAH is not present in the same incubation mixture initially. Aldehydes and cyanide had no effect on enzyme activity (Table III). If FAH functioned via a Schiff-base mechanism one might have expected some inhibition (18). It is also known that sodium borohydride and sodium cyanoborohydride do not inactivate FAH (11,33). Since the Schiff-base mechanism has been refuted, a new mechanism involving the two carbonyl groups of

the substrate was investigated.

Manganese analyses of FAH indicate no significant amount of this metal is bound to the enzyme (0.09 moles per mole of FAH). It is likely that the amount detected is due to non-specific binding and may be attributed to contamination from metal blades and equipment used in the isolation of FAH.

Iron analyses revealed a negative answer as well. Only 0.17 moles of iron per mole of FAH was found and this level of iron is again probably a contaminant from steel utensils used during enzyme isolation.

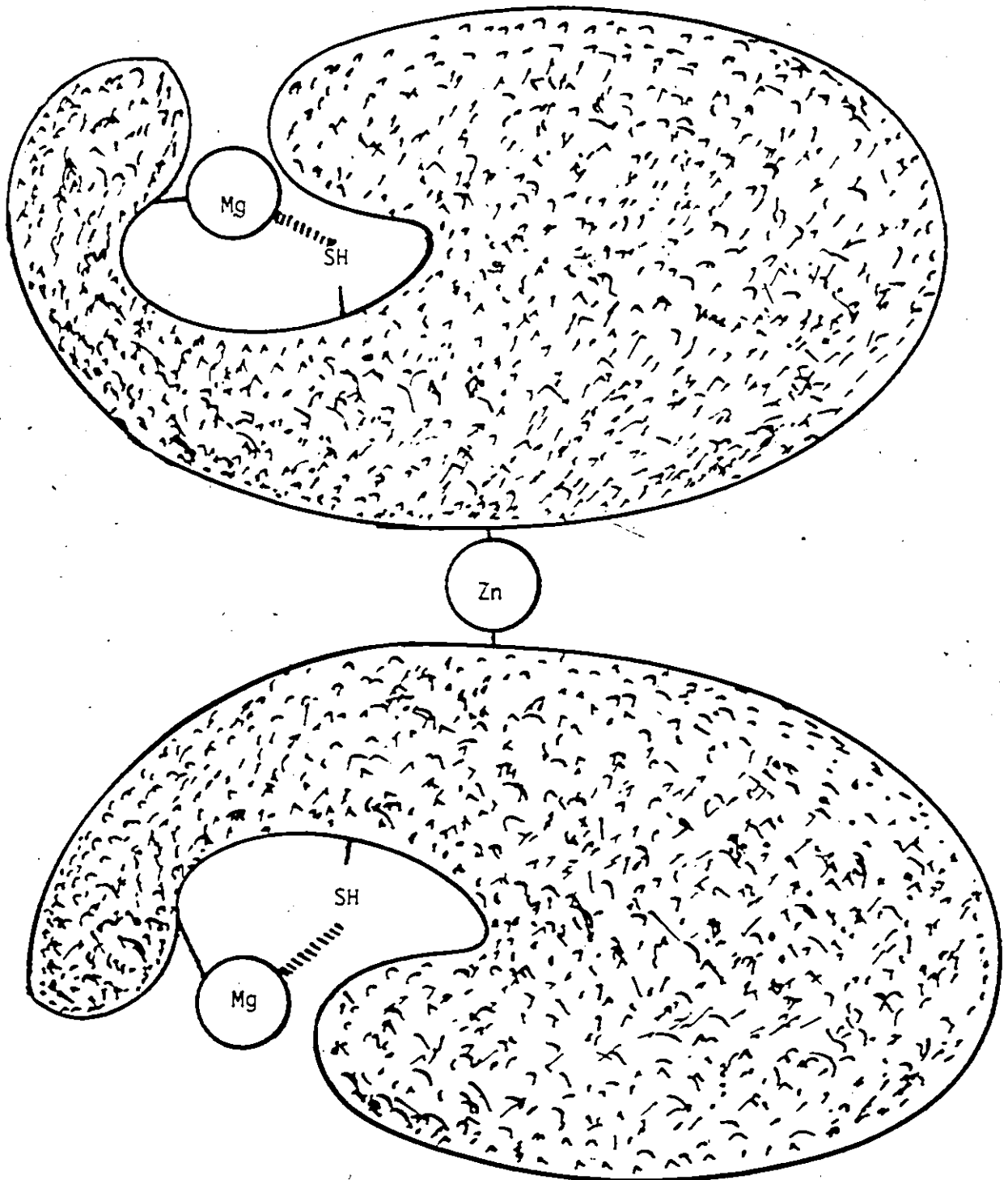
FAH probably contains one mole of zinc per two moles (or subunits) of FAH. Table V summarized reveals there is an average of 0.46 moles of zinc per mole of enzyme. There are two identical subunits of FAH (10). It is not likely that one molecule of zinc would be associated with only one subunit and not with the other subunit of the active FAH complex. Figure 5 proposes that zinc is used as a structural requirement. Upon denaturation zinc is lost to the solution. After a twenty four hour dialysis only 0.21 moles of zinc per mole of FAH remained bound to enzyme (Table V). The remaining zinc is probably directly related to the amount of active enzyme that remained. During enzyme isolation it was noticed that substantial losses of enzyme activity occurred after prolonged dialysis. Zinc may be bound to the subunits using one thiol group. Cheng (34) has discovered that ethylenediaminetetraacetic acid treatment releases an additional, hitherto

## Figure 5

Proposed Model of Fumarylacetoacetate  
Fumarylhydrolase

This proposal incorporates previous information on  
sulfhydryl groups (10) and present knowledge of metal content.

Figure 5



unknown thiol group per subunit of FAH. This is strong evidence that zinc may be indeed bound to FAH as suggested.

The magnesium analysis suggests there is probably one mole of magnesium per mole of FAH. Experimentally there was found to be an average molar ratio of 1.13 of magnesium to FAH. When FAH is denatured magnesium is lost. This is reflected by the lower level of 0.42 moles of magnesium per mole of enzyme found after a sample of partially denatured dialyzed FAH was analyzed (Table VI).

A ratio of one bound magnesium per FAH molecule indicates that this metal may be involved as more than a structural entity. Scheme 1 proposes that this metal may be found at the active site of the FAH molecule. The free sulfhydryl group per FAH molecule may be involved in chelating the magnesium. An enzyme-metal-enzyme intermolecular bridge may be formed to stabilize the metal when no substrate is available. When substrate is in the active site magnesium may act as proposed in either Scheme 1 or 2. The major difference between the two schemes is that Scheme 1 features a nucleophilic attack on the carbonyl carbon atom from within the enzyme while Scheme 2 features an attack initiated by water. The first scheme is favoured because it allows the use of a free sulfhydryl group to initiate the nucleophilic attack on the substrate.

According to Boyer (19), FAH is not a type I metalloenzyme but rather a type II or III metalloenzyme. Type I metalloenzymes

are metal activated enzymes that do not retain their metals during purification and employ an enzyme-substrate-metal bridge. Type II enzymes use an enzyme-metal-substrate bridge. Type III enzymes utilize the metal as a structural unit with no interactions with the substrate. FAH can be tentatively classified as a type II enzyme with magnesium as the metal required for catalytic activity. FAH can also be regarded as a type III enzyme with zinc as a structural unit.



## CHAPTER IV

### SUMMARY

Fumarylacetoacetate fumarylhydrolase does not catalyze cleavage via a Schiff's base mechanism. The enzyme is a metalloenzyme and contains both magnesium and zinc. The possible role of each metal was discussed. Zinc may be a structural entity, and magnesium may hold and activate the 2,4-diketo acid so that the enzyme's sulfhydryl group can make a nucleophilic attack on the 4 - keto carbon.

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