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INHIBITION OF FUMARYLACETOACETATE

FUMARYL HYDROLASE BY

P-MERCURIBENZOATE AND CYANIDE

BY

MONICA PALCIC NAGAINIS

A Thesis

Submitted to the Faculty of Graduate Studies through the  
Department of Chemistry in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science at the  
University of Windsor

Windsor, Ontario

1975

Canada

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#### ABSTRACT

Fumarylacetoacetate fumarylhydrolase is specifically inactivated by p-mercuribenzoate, a sulfhydryl specific inhibitor. Other sulfhydryl reagents employed, trinitrobenzenesulfonic acid, N-ethylmaleimide, and 5,5'-dithio-bis-(2-nitrobenzoic acid) did not exhibit this selectivity. The inactivation of the enzyme by PMB shows saturation kinetics. Protection against inactivation has been obtained with fluoride and by the substrate propionopyruvate. Cyanide inactivates the enzyme in the presence of substrate but has no effect on the enzyme in the absence of substrate. The cleavage of 2,4-diketo acids by fumarylacetoacetate fumarylhydrolase is postulated to occur by formation of a ketamine between the 2-keto group of the substrate and an amino group on the enzyme. After ketamine formation the 4-keto group of the substrate is attacked by a sulfhydryl group to form a thio ester after cleavage of the carbon-carbon bond.

## ACKNOWLEDGMENTS

I would like to thank my research advisor Dr. D. E. Schmidt Jr. for his inspiring direction and patient encouragement during the course of this research work.

Also, I would like to express my appreciation to Kathy Shuttleworth for her preparation of enzyme and to Don F. Mahuran for his helpful advice. I am indebted to Hamdy Kahlil for his preparation of the Figures. Finally, I would like to thank my fellow graduate students for their interest in this work and for many useful discussions.

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## ABBREVIATIONS

DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
FAH	fumarylacetoacetate fumarylhydrolase
NEM	N-ethylmaleimide
PLP	5'-pyridoxal phosphate
PMB	p-mercuribenzoate
SA	specific activity
TNBS	2,4,6-trinitrobenzenesulfonic acid

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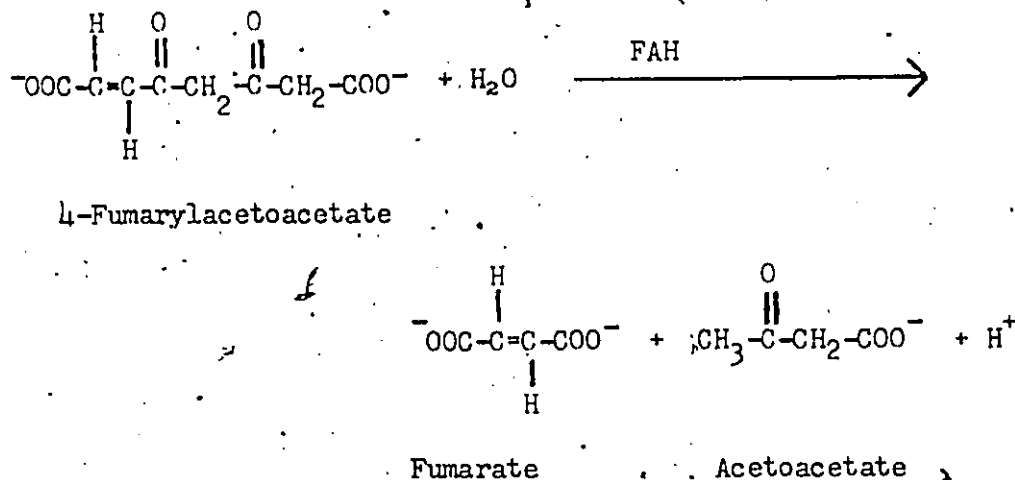
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## CHAPTER I

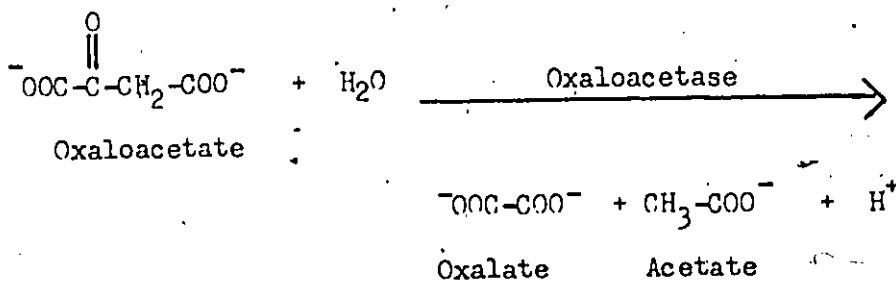
### INTRODUCTION

In 1948 Meister and Greenstein (1) isolated an enzyme from liver and kidney that cleaved 2,4-diketovalerate to pyruvate and acetate. They named it 2,4-diketo acid hydrolase (or acyl pyruvase), since it also cleaved longer-chain 2,4-diketo acids to give pyruvate and the corresponding fatty acid. In the following year Connors and Stotz (2) reported the isolation of a triacetic acid hydrolase from beef liver which cleaved 3,5-diketoheptanoic acid to acetoacetate and acetate. Further work (3,4) showed 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 degradation pathway of tyrosine and phenylalanine in mammalian systems.

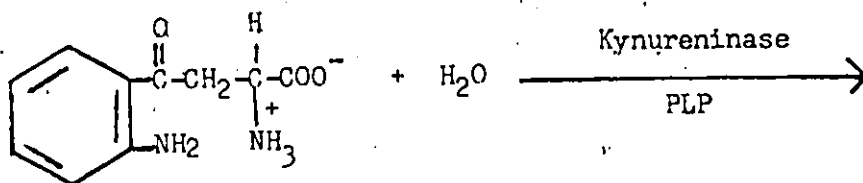


FAH belongs to a class of enzymes that hydrolyze carbon-carbon bonds, in particular bonds between a methylene carbon and an adjacent carbonyl carbon. Other examples of enzymes in this class are oxaloacetate acetylhydrolase (EC 3.7.1.1) from Asperigillus niger

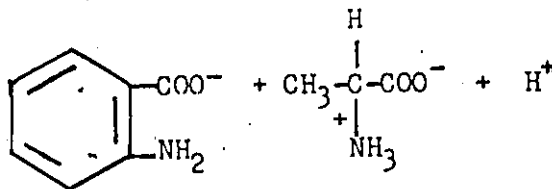
which catalyzes the formation of oxalate and acetate from oxaloacetate(5).



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

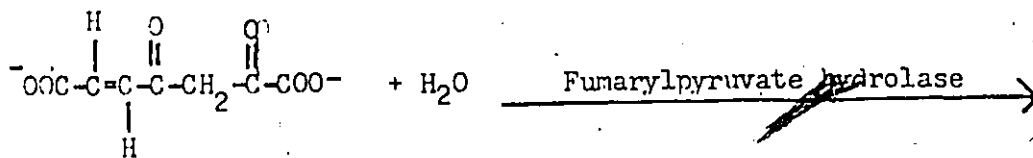


L-Kynurenine

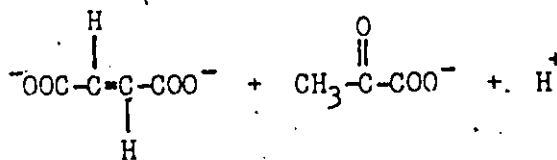


Anthranilate L-Alanine

In a bacterial system the pathway for the degradation of gentisic acid includes the enzyme fumarylpyruvate hydrolase, which hydrolyzes fumarylpyruvate to fumarate and pyruvate (7).



Fumarylpyruvate



Fumarate

Pyruvate

Although the mechanism of L-kynurenine hydrolysis is known to involve a Schiff-base formation between the cofactor pyridoxal-5'-phosphate (PLP) and L-kynureninase (8,9), little work has been done on the general mechanism of these enzymatic reactions.

The mechanism of action of FAH was investigated by further studying the reported inactivation (4) by a series of sulfhydryl-specific reagents and secondly by investigating the possibility of a Schiff-base intermediate as part of the mechanism of hydrolysis.

When working with specific inhibitors care must be taken in interpreting inhibition since these compounds can inactivate enzymes by a variety of mechanisms (10). These include:

1. The sulfhydryl group modified is at the active site and functional. It thus may be involved in the binding of substrate, coenzyme or activator or it may participate in the transfer of groups of electrons.
2. The sulfhydryl group reacted is vicinal to the active centre. Thus the reagent introduces a new structure onto the enzyme and it is near enough to modify the reactions at the active centre either sterically or electrostatically.
3. Reactions of the sulfhydryl group alters the enzymes structure by dissociation into non-active subunits.
4. The sulfhydryl reagent interferes in a way unrelated to sulfhydryl groups; it may be structurally similar to the substrate and act as a competitive inhibitor.

The kinetics of inactivation, protection, reversal and molecular weight studies of FAH inhibited by sulfhydryl reagents were carried out to distinguish between the various mechanisms of

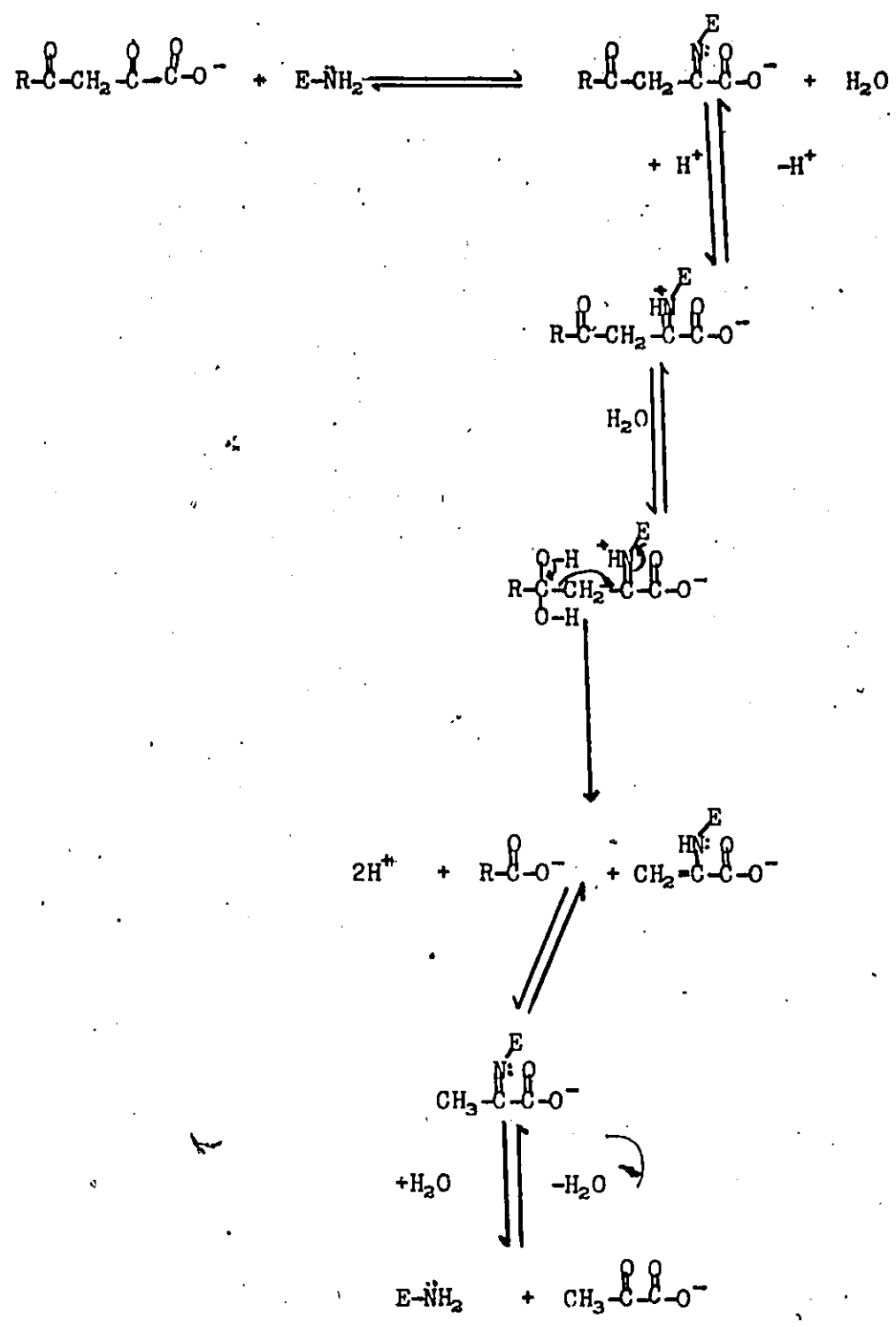
enzyme inactivation by sulfhydryl reagents.

The second part of the work was to investigate the possibility of a Schiff-base formation during FAH action. The enzyme may form a Schiff-base intermediate with the 2-keto group of the substrate which is hydrolyzed to release the fatty acid, leaving the pyruvate residue still in Schiff-base form with the enzyme. The pyruvate is then liberated by hydrolysis (Scheme 1). This scheme is similar to the mechanism proposed for the action of acetoacetate decarboxylase (11). In a second possible scheme, a second nucleophile from the enzyme, rather than the water in Scheme 1, attacks the  $\alpha$ -carbonyl group after Schiff-base formation (Scheme 2).

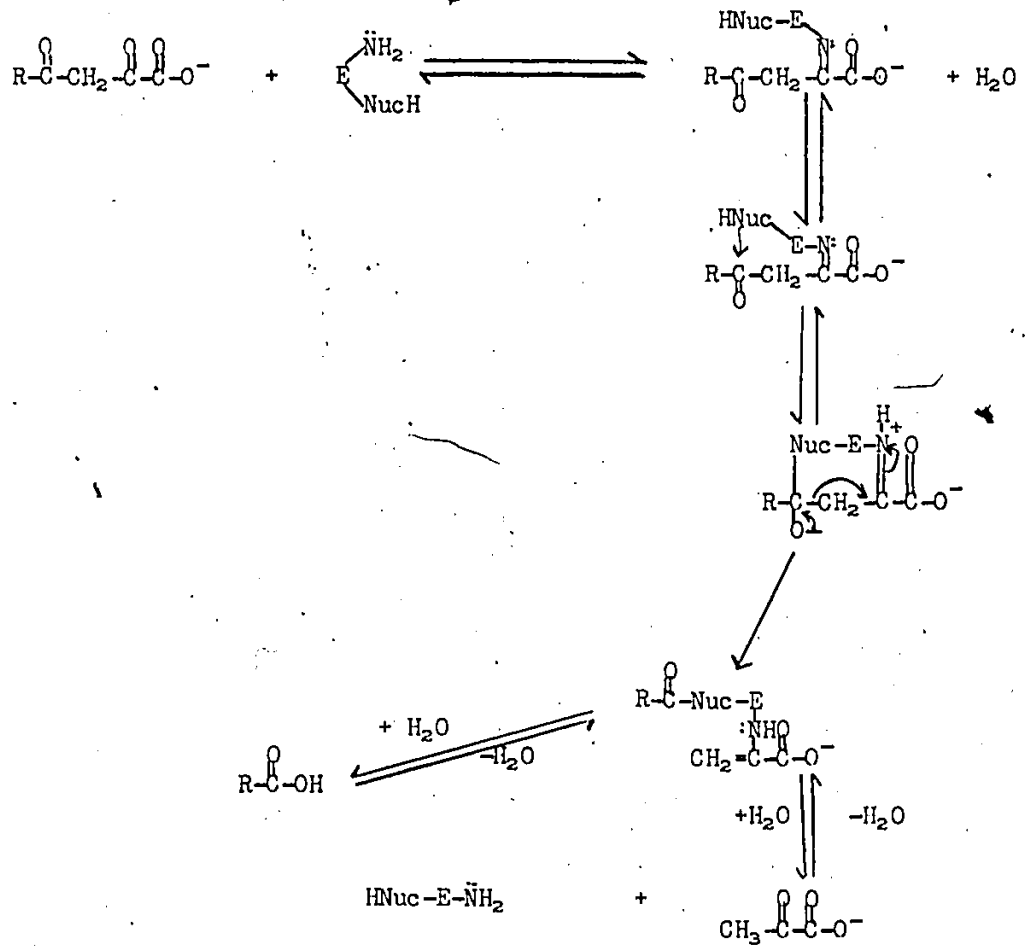
Sodium borohydride has been used to trap intermediates by reducing the Schiff-base formed between enzyme and substrate for a number of enzymes, phosphorylase a (12), transaldolase (13), aldolase (14), acetoacetate decarboxylase (15), 2-keto-4-hydroxyglutarate aldolase (16, 17), and 2-keto-3-deoxy-6-phosphogluconate aldolase (18).

It has been reported that sodium borohydride showed no effect on the activity of FAH in the presence of substrate (19). Since borohydride is a powerful reducing agent it may have reduced the substrate in solution before the enzyme-substrate Schiff-base complex could form. A more controlled reduction was attempted by using sodium cyanoborohydride. Cyanoborohydride is reported to be more specific for the reduction of imines at pH 6-8 with negligible reduction of aldehydes and ketones (20). Cyanoborohydride has been used to reduce the Schiff-base between aldolase and





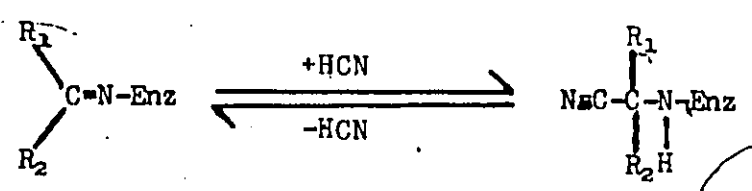
Scheme 1.



Scheme 2.

its substrate (21).

Cyanide has also been reported to inactivate a series of enzymes which function via Schiff-base mechanisms. These include fructose 1,6-diphosphate aldolase from rabbit muscle (22), transaldolase (23), and acetoacetate decarboxylase (24,25); work with the latter enzyme was the first reported instance of this type of inhibition (26). In all these cases, however, the observed inactivation was dependant upon the presence of a substrate which formed a Schiff-base intermediate with the ε-amino group of a lysyl residue of the protein, and the observed inhibition was reversible by dilution or dialysis. By chemical analogy (27), it was proposed that cyanide added to the azomethine linkage resulting in the formation of an enzyme-bound aminonitrile, as shown in Scheme 3:



Scheme 3.

The reversibility of all the previously noted systems precluded the possibility of verifying this proposed reaction by isolating and identifying the adduct.

Recently however an irreversible inhibition by cyanide of 2-keto-4-hydroxyglutarate aldolase in the presence of aldehydic substrates has been reported (28). The isolation of a labelled fragment positively identified that an aminonitrile was formed by reaction of cyanide with enzyme-substrate Schiff-base. With the existence of the enzyme-bound aminonitrile established a

basis exists for the use of cyanide for detecting Schiff-base intermediates and thus cyanide was also used as a reagent to detect a Schiff-base intermediate.

## CHAPTER II

### EXPERIMENTAL

#### A. METHODS AND MATERIALS

##### 1) Materials

The following materials were commercially available, 5,5'-dithio-bis-(2-nitrobenzoic acid), (DTNB) and sodium cyanoborohydride, (Aldrich); N-ethylmaleimide (NEM) and 2,4,6-Trinitrobenzenesulfonic acid (TNBS), (Pierce Chemical Company); sodium p-hydroxymercuribenzoate (PMB), glutathione, cysteine, aldolase, chymotrypsinogen, ribonuclease A, potassium cyanide, dithioerythritol (DTE), (Sigma), 2,4-dinitro-phenyl-L-aspartic acid and Blue Dextran 2000 (Pharmacia).

##### 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 enzyme solution on the column, 200 ml of 0.02 M Tris-hydrochloride buffer pH 8.5 was passed through the column. Then the enzyme was eluted with Tris-hydrochloride buffer (0.08 M, pH 8.5,  $1 \times 10^{-4}$  M DTE). All fractions with specific activity of over 36 were combined and precipitated with 40% (W/V) ammonium sulfate. Packages of enzyme were prepared by dissolving the precipitate in a small volume of 0.1 M sodium phosphate buffer pH 7.3. No glycerol or dithioerythritol was added. These were kept at  $-20^{\circ}$  until used.

##### 3) Standard Assay

Enzyme activity was determined in a standard reaction mixture containing 0.2 mM propionopyruvate in 3.0 ml of 0.025 M

sodium phosphate buffer (pH 7.3). Propionopyruvate has an absorption maxima at 290 nm due to the enol form. The reaction was initiated by addition of 50  $\mu$ l of enzyme solution. It was followed by observing the decrease in absorption at 290 nm and was linear for at least the first five minutes. The initial rates were calculated from the slopes on the chart paper.

All kinetic data were obtained on a Gilford Model 2000 Absorbance Recorder attached to a Beckman DU monochromator or a Beckman ACTA MVI spectrophotometer with cell compartments thermostated to  $25.0 \pm 0.5^\circ$ . Data was analyzed by methods of least square analysis.

#### 4) Inactivation of Enzyme by Sulfhydryl Reagents

Enzyme in a 1.0 ml volume of 0.1 M sodium phosphate buffer, pH 7.3 was treated with various sulfhydryl reagents. Protection against inhibition was studied by adding potentially protective substances to the enzyme before addition of modifying reagents. The solution was constantly stirred at  $25.0 \pm 0.5^\circ$  before addition of inhibitor. In all cases 30  $\mu$ l of inhibitor were added. Aliquots were taken from the reaction mixture at appropriate time intervals and tested for activity by the standard assay. Incubation under identical conditions in the absence of inhibitor served as controls. Enzyme solutions were stable for 20 minutes under these conditions. All glassware, cuvettes and pipettes were silicon treated before use to make surfaces inert to adsorption of protein from dilute enzyme solutions. Reagents were incubated before use to  $25.0 \pm 0.5^\circ$ .

In cases where the modifier acted as a competitive inhibitor 3.0 ml cuvettes (1.0 cm path length) containing 0.025 M sodium

phosphate buffer, inhibitor and varying propionopyruvate concentrations were used. The hydrolysis was initiated by the addition of 50  $\mu$ l of enzyme solution.

#### 5) PMB Solutions

Fresh solutions were prepared by dissolving 8-20 mg PMB in 25 ml  $1 \times 10^{-3}$  M sodium hydroxide. The concentration of PMB in this stock solution was determined spectrophotometrically based on a molar extinction coefficient of  $1.6 \times 10^4$  at 232 nm at pH 7 as determined by Boyer (29) and by spectrophotometric titration with glutathione at 250 nm (30).

#### 6) Determination of Molecular Weight

Samples of control and enzyme inactivated by PMB at 0° were run through a Sephadex G-150 column at 4.0°. The markers used for estimation of molecular weight were aldolase (mol wt 158,000), chymotrypsinogen (mol wt 22,500), ribonuclease A (mol wt 13,700), 2,4-dinitrophenyl-L-aspartic acid (mol wt 299) and Blue Dextran 2000 (mol wt  $> 10^6$ ). A linear plot for  $(K_d)^{2/3}$  against  $(\text{Molecular Weight})^{1/2}$  was obtained.

#### 7) Effect of Sodium Cyanoborohydride and Potassium Cyanide on Enzymatic Activity

Acetopyruvate and propionopyruvate were used as substrates in experiments to trap a Schiff-base. A fresh solution of cyanoborohydride was prepared by dissolving an appropriate amount of sodium cyanoborohydride in 10 ml of 0.1 M sodium phosphate buffer pH 7.3. Potassium cyanide was dissolved in water. The enzyme concentration was approximately 0.2 mg/ml, determined by measuring spectrophotometrically at 260 and 280 nm by the method of Warburg

and Christian (31). In each case 0.1 ml substrate and 0.1 ml of cyanoborohydride or cyanide solution were added to 0.3 ml enzyme and incubated at  $25 \pm 0.5^\circ$ . Then 50  $\mu$ l aliquots were removed after appropriate time intervals and tested for activity by the standard assay. In control reactions 0.2 ml buffer, or 0.1 ml buffer plus 0.1 ml substrate, cyanoborohydride or cyanide were used. For protection studies 0.1 ml competitive inhibitor was added to 0.2 ml enzyme solution before addition of reducing agent or substrate.



## B: RESULTS

1) Effect of Specific Inhibitors on Enzyme Activity

TNBS and NEM showed little effect on enzymatic activity in the concentration ranges studied (Table I). FAH is inhibited by DTNB. Although DTNB is usually found to be a sulfhydryl specific inhibitor a plot of the reciprocal of the initial velocity ( $v_0^{-1}$ ) against the reciprocal of substrate concentration ( $[PPA]^{-1}$ ), indicate DTNB is acting as a competitive inhibitor (Fig. 1).  $K_m$  was calculated directly from the reciprocal of the X-axis intercept of the Lineweaver-Burke plot for no added DTNB and was found to be  $9.8 \times 10^{-4}$  M. The  $V_{max}$  was obtained from the reciprocal of the Y-axis intercept and found to be  $19.2 \mu\text{M}/\text{min}$ .

The three main types of competitive inhibition are: simple, hyperbolic and parabolic (32,33). Analysis of these three types of competitive inhibition shows that a plot of  $v_0/v_1$  vs [DTNB] should be fit by an equation of the form for simple competitive

$$\frac{v_0}{v_1} = 1 + \frac{K_m}{K_1([S_0] + K_m)} [\text{DTNB}] \quad (1)$$

for hyperbolic competitive

$$\frac{v_0}{v_1} = \frac{[S_0]}{K_m + [S_0]} + \frac{K_m(1 + a[\text{DTNB}])}{K_m + [S_0]} \quad (2)$$

and for parabolic competitive

$$\frac{v_0}{v_1} = \frac{[S_0]}{K_m + [S_0]} + \frac{K_m(1 + c[\text{DTNB}] + d[\text{DTNB}]^2)}{K_m + [S_0]} \quad (3)$$

where  $v_0$  = initial velocity in the absence of inhibitor,  $v_1$  = initial velocity in the presence of inhibitor,  $K_m$  = dissociation constant of the enzyme-substrate complexes  $K_1$  = dissociation

TABLE I: Effect of Specific Reagents on FAH Activity

Reagent	Concentration mM	% Activity <sup>c</sup> Remaining 2 min	% Activity Remaining 15 min	% Activity Remaining 20 min
TNBS <sup>a</sup>	5	100	99	-
	25	100	80	-
	100	Solution Precipitated		
NEM <sup>b</sup>	5	100	-	100
	50	100	-	90
	87	100	-	88

<sup>a</sup> A stock solution was prepared in acetonitrile and a small aliquot was added to incubation mixture in 0.1 M sodium phosphate buffer pH 7.3.

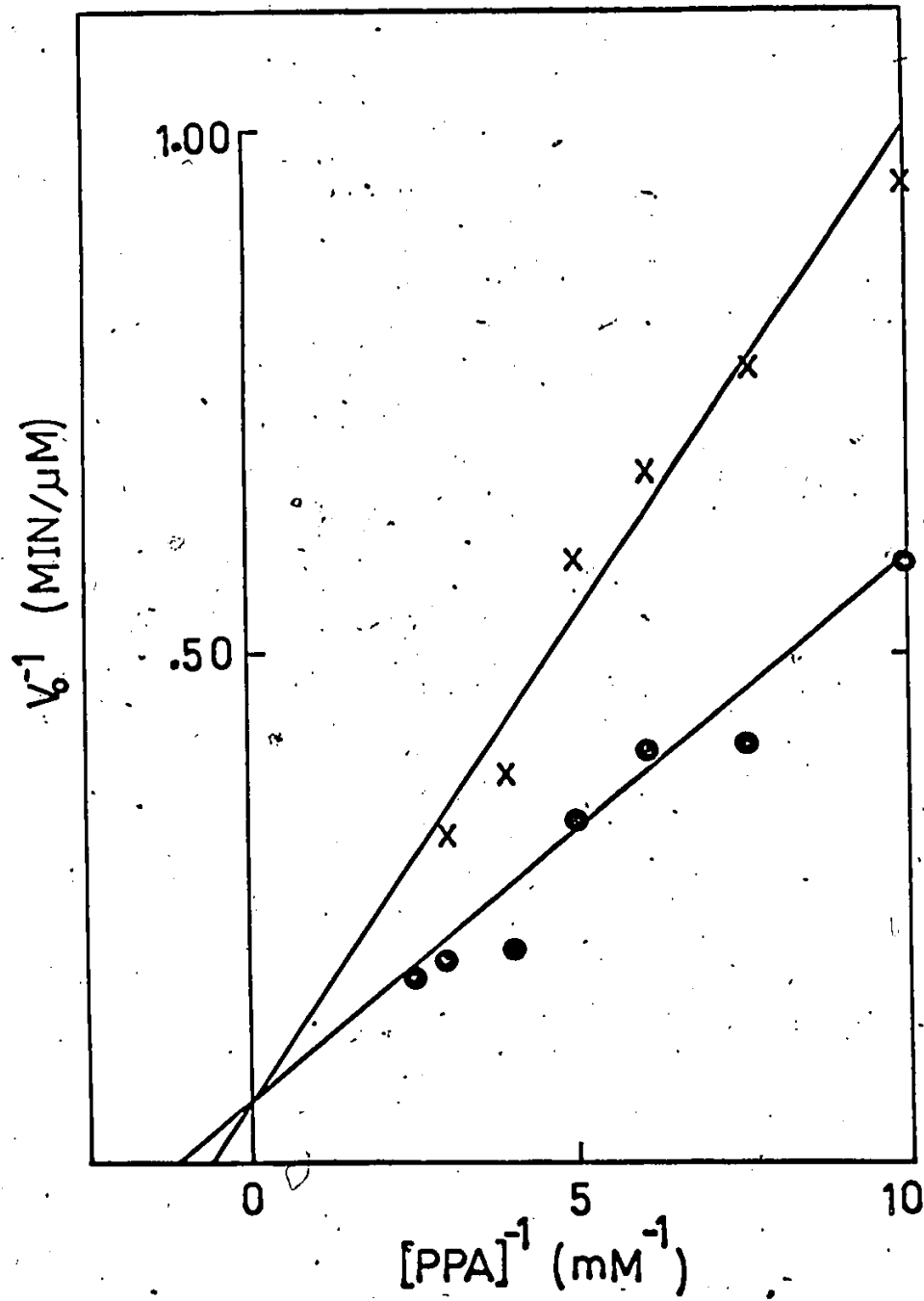
<sup>b</sup> NEM was studied in 0.1 M sodium phosphate buffer, pH 7.0 to prevent hydrolysis to N-ethylmaleimic acid.

<sup>c</sup> The concentration of substrate was 0.20 mM which allows detection of competitive inhibition if present.

Fig. 1. Competitive Inhibition by DTNB of FAH-catalyzed hydrolysis of propionopyruvate.

The standard assay procedure was used. Each cuvette (1.0-cm light path, 3.0 ml volume) contained 0.025 M sodium phosphate buffer pH 7.3, and propionopyruvate as indicated: (X) 16.6 M DTNB; (●) 0.0 M DTNB.

FIGURE 1



constant for the enzyme-inhibitor complex,  $[S_0]$  = initial substrate concentration,  $[DTNB]$  = concentration of DTNB, and the constants  $a$ ,  $b$ ,  $c$ , and  $d$  are complex functions of constants. Inspection of eq 1-3 indicates that a plot of  $v_0/v_1$  vs  $[DTNB]$  at constant substrate concentration will be linear for simple competitive inhibition. When this plot was done (Fig. 2), the function  $v_0/v_1$  was directly proportional to DTNB concentration and the intercept was unity indicating DTNB probably inhibits FAH by simple competitive inhibition. For hyperbolic and parabolic competitive inhibition  $v_0/v_1$  would not be a linear function of inhibitor concentration and the Y-intercept would be equal to  $\frac{[S_0]}{K_m + [S_0]}$  which in this case is equal to .17.

The inhibitor constant  $K_i$  was calculated from the slope of the line in Fig. 2 according to the equation:

$$\text{Slope} = \frac{K_m}{K_i([S_0] + K_m)} \quad (4)$$

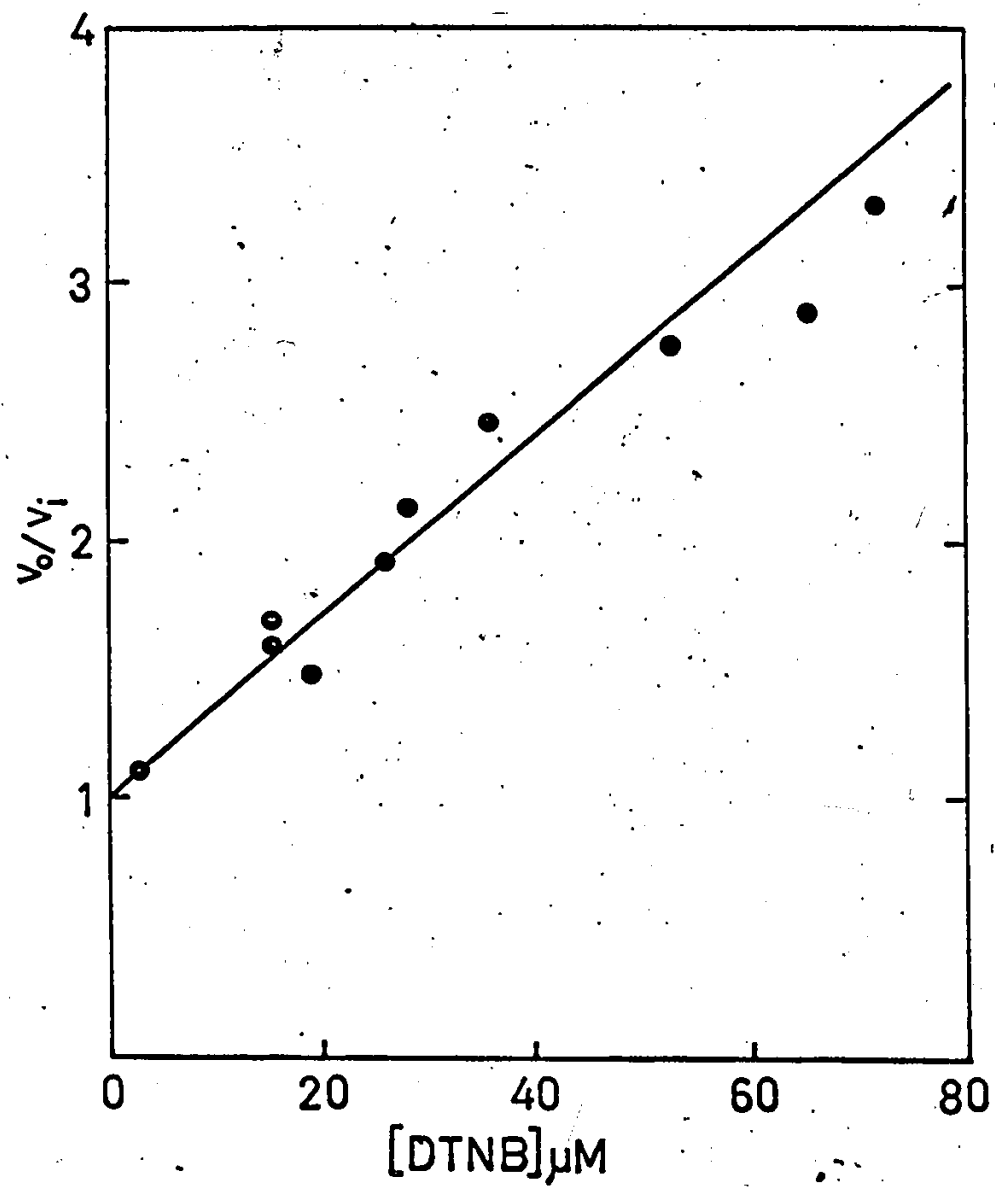
and was found to be  $29 \mu\text{M}$ .

Absorption by DTNB at 290 nm prevented the utilization of higher concentrations of this inhibitor.

Fig. 2. Inhibition of FAH by DTNB.

The standard assay procedure was used. Each cuvette (1.0 cm light path, 3.0 ml volume) contained  $2 \times 10^{-4}$  M propionopyruvate, 0.025 M phosphate buffer, pH 7.3 and DTNB at the concentration indicated. The hydrolysis was initiated by the addition of 0.05 ml of FAH.

FIGURE 2



2) Inactivation of FAH by PMB

FAH is very susceptible to inhibition by PMB.

On incubation with PMB, the enzyme was progressively and irreversibly inactivated. Fig. 3 shows that the increasing pseudo-first-order rate constants ( $k_{app}$ ) of enzyme inactivation were not linear with increasing PMB concentrations. A possible reaction scheme for the inactivation of enzyme is the formation of a dissociable complex (EM) from enzyme (E) and PMB (M) which is converted to an irreversible  $EM_I$  derivative:



By analogy with Kitz and Wilson (34) the apparent first-order rate constant for inactivation ( $k_{app}$ ),  $K_I$  and  $k_3$  are related by the equation:

$$k_{app} = \frac{k_3(M)}{K_I + (M)} \quad (6)$$

where  $K_I$  is the apparent half-saturation constant or when  $k_3$  is rate limiting,  $K_I$  is equal to the dissociation constant of the EM complex.

Equation 6 has the form of the Michaelis-Menton equation (35) and a plot of the reciprocal of the rate of inactivation ( $k_{app}^{-1}$ ) against reciprocal PMB concentration, ( $[PMB]^{-1}$ ) gives a straight line (Fig. 4) as predicted in equation 7.

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_3} \frac{1}{(M)} \quad (7)$$

Values of  $k_3$  and  $K_I$  were estimated to be  $0.37 \text{ min}^{-1}$  and  $0.041 \text{ mM}$  respectively.



Fig. 3. The effect of PMB concentration on enzyme inactivation. Enzyme was incubated with 0.014-0.14 mM PMB in 0.1 M sodium phosphate buffer at pH 7.3 and  $25.0 \pm 0.5^\circ$ . Aliquots were removed and assayed after varying time intervals and pseudo-first-order rate constants of inactivation ( $k_{app}$ ) were plotted against concentration of PMB.

FIGURE 3

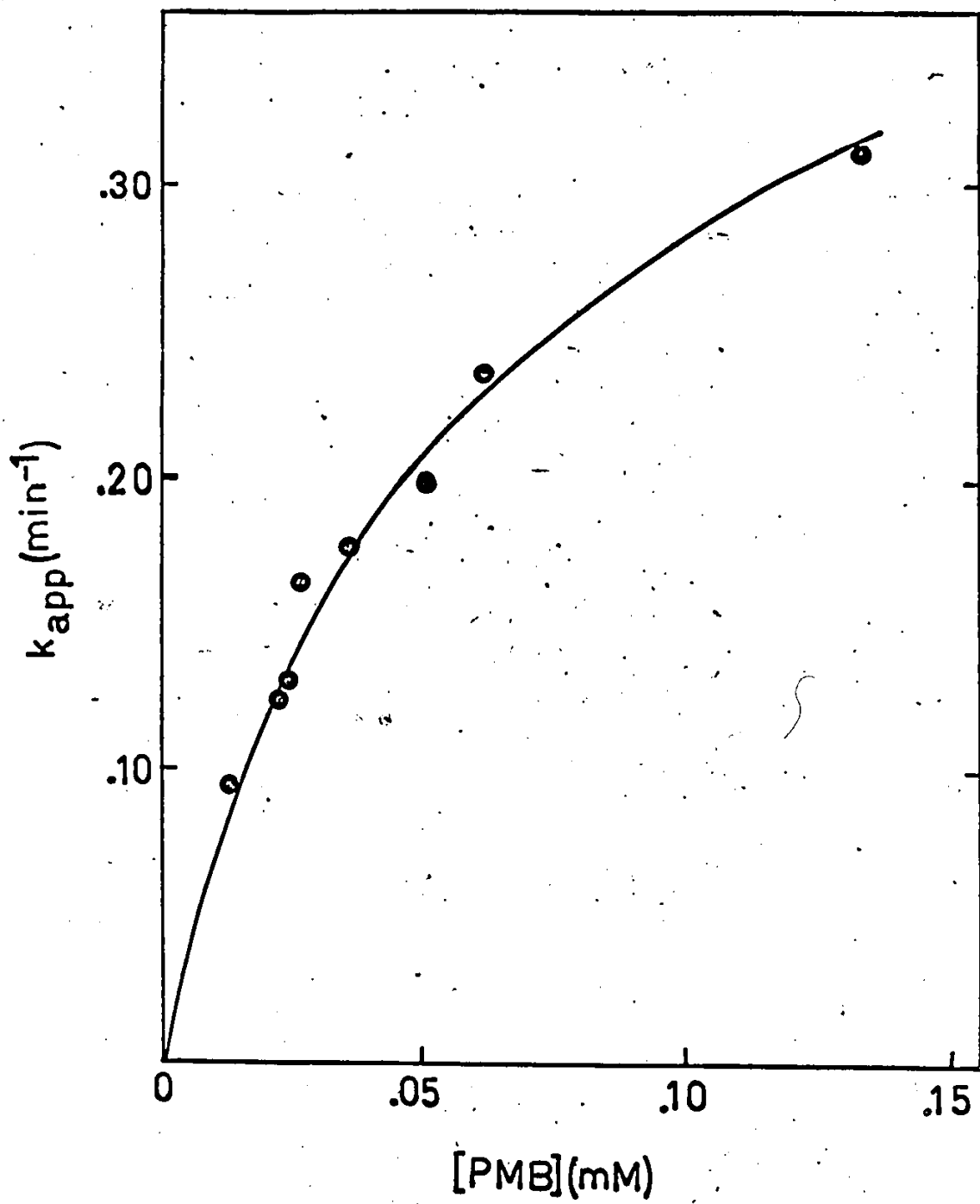
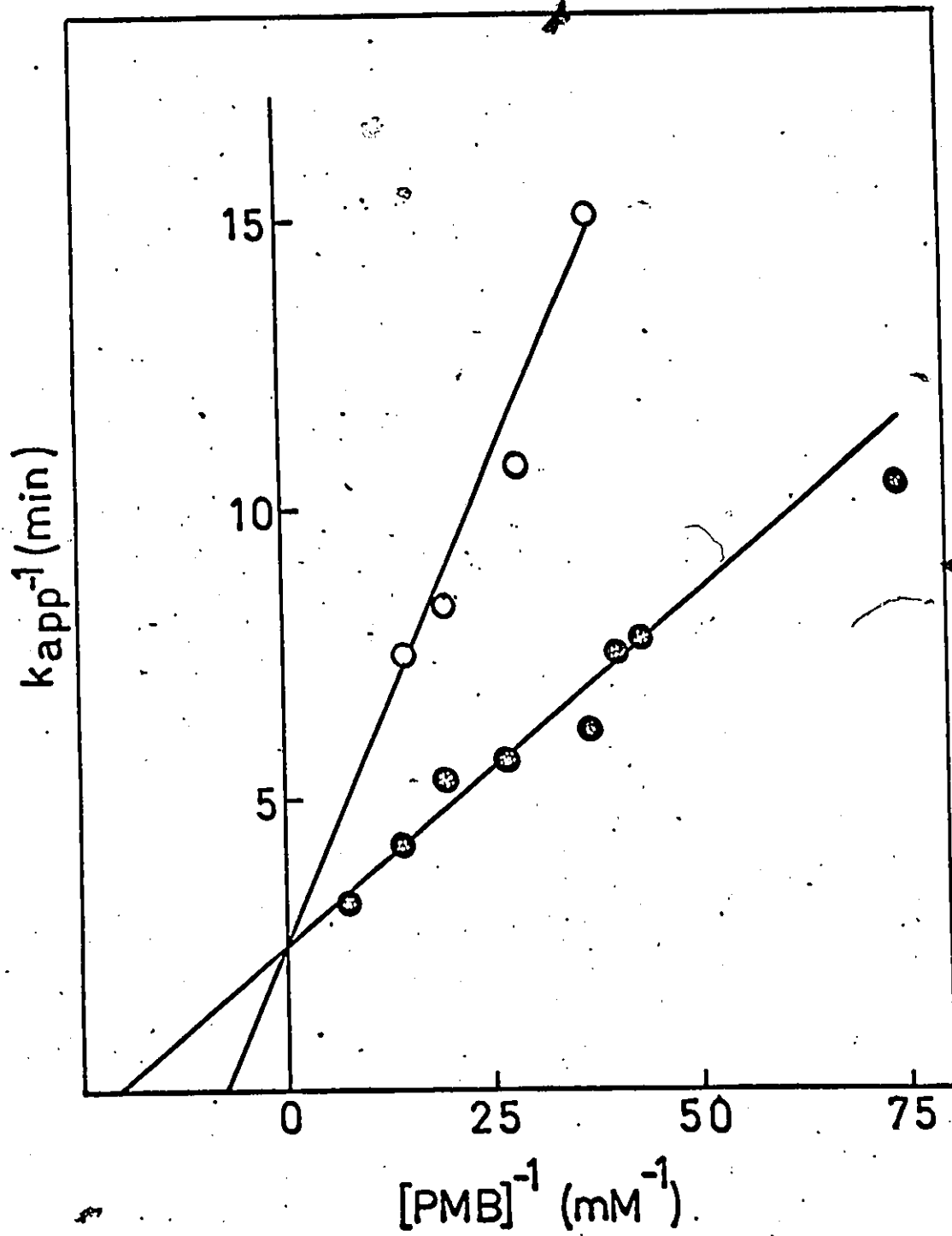


Fig. 4. Reciprocal plot of  $k_{app}$  vs PMB concentration for inactivation of FAH by PMB (●) and PMB + 0.02 M fluoride (○).

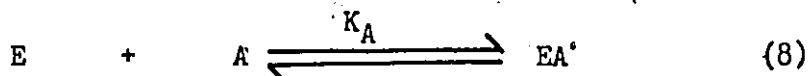
FIGURE 4



3) Influence of Fluoride and Propionopyruvate on Inactivation of FAH by PMB

If PMB is inactivating FAH by binding at the enzymes active site then it would be expected that the presence of the substrate propionopyruvate or a competitive inhibitor should slow inactivation. Fig. 5 shows that propionopyruvate affords protection against inactivation by PMB indicating that this agent is probably active-site directed.

The development of irreversible inhibition was slowed, as would be expected if the active site were involved. Adding to eq 5



where A (either substrate or competitive inhibitor) binds at the active site to form an EA complex, we obtain eq 9

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_T}{k_3} \left( 1 + \frac{(A)}{K_A} \right) \cdot \frac{1}{(M)} \quad (9)$$

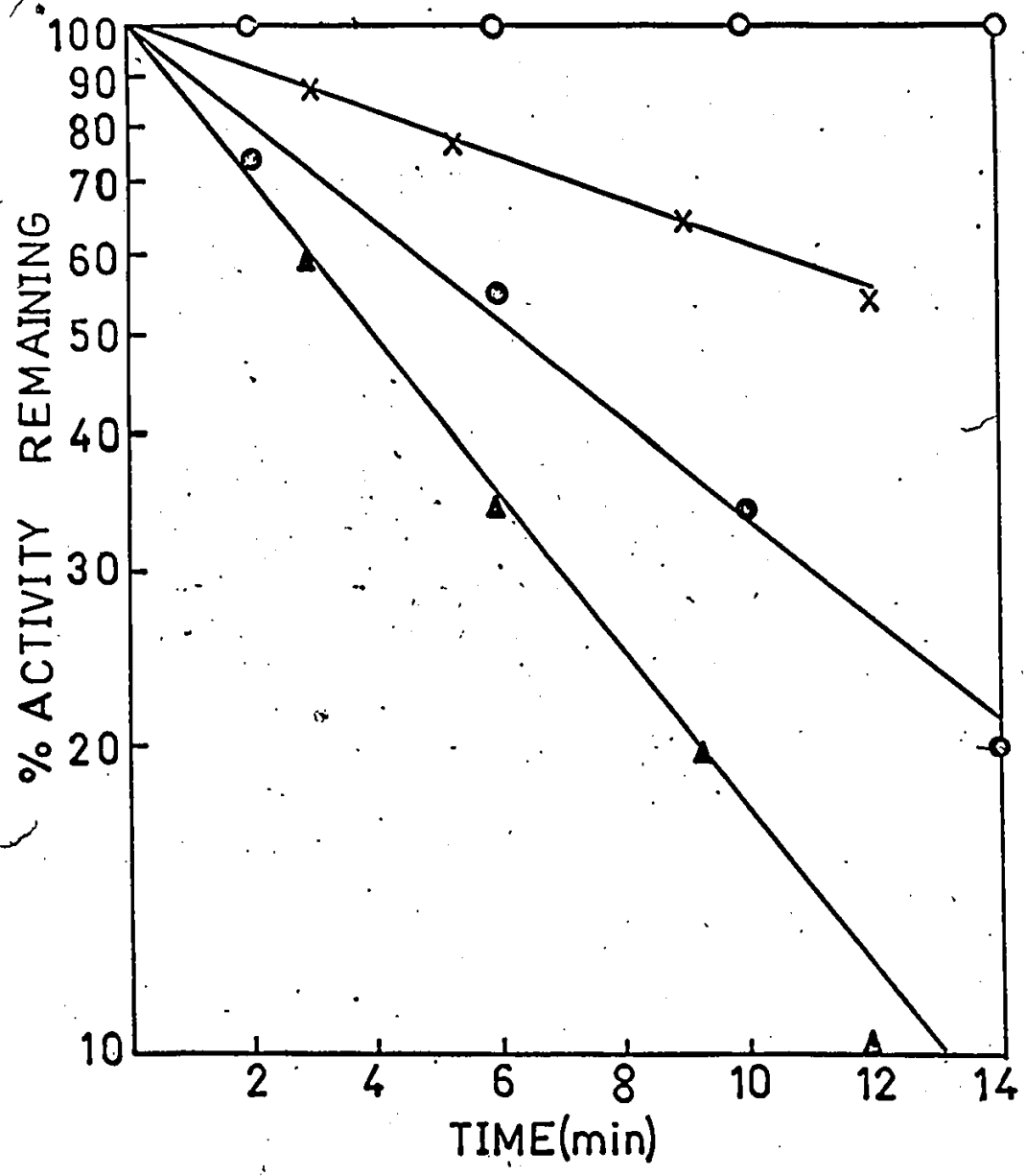
From data plotted in accordance with this equation (Fig. 4),  $K_A$  (the dissociation constant of the EA complex) for fluoride was found to be 9.86 mM.

Fig. 5. Effect of propionopyruvate on inactivation of FAH by PMB.

The rate of inactivation was measured under the following conditions:

(▲) 0.027 mM PMB; (●) 0.025 mM PMB; (X) 0.025 mM PMB and 0.4mM propionopyruvate; (○) 0.0 mM PMB and 0.0 mM propionopyruvate.

FIGURE 5



#### 4) Reversibility of PMB Inactivation of FAH

Enzymes inhibited by PMB can often be reactivated by nucleophilic agents such as cysteine. The reversal of PMB inhibition is much more rapid than its inactivation. This was demonstrated by the addition of 8.0 mM cysteine to an enzyme mixture that retained 12% of its activity after treatment with 13.5 mM PMB. After two minutes 74% of control activity was restored. Longer incubation times with cysteine did not further increase the amount of activity restored.

#### 5) Molecular Weight Determinations

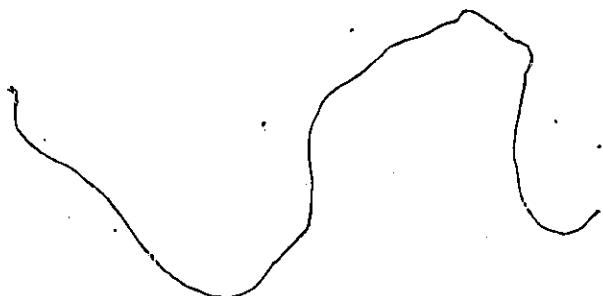
To determine whether PMB inactivated FAH by dissociating it into subunits, native and PMB-treated enzyme was passed through a calibrated G-150 column. The molecular weights obtained correspond to 87,000 and 78,400 respectively (Fig. 6), indicating no dissociation had occurred. This is in agreement with a molecular weight of 80,000 determined in a series of other studies (57).

Cysteine (80 mM) was added to PMB treated and native enzyme from the column. PMB treated enzyme showed an increase in activity from 30-400%, native enzyme showed little increase (Table II).



Fig.6. Estimation of the molecular weights of native and PMB treated FAH from elution data by gel filtration.

The Sephadex G-150 column ( 50 X 2 cm) was equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.3 at 4.0 °. The cube root of the partition coefficient between the mobile liquid phase and the stationary liquid phase ( $K_D$ )<sup>1/3</sup> was plotted against the square root of the molecular weights of the indicated marker proteins..



2

FIGURE 6

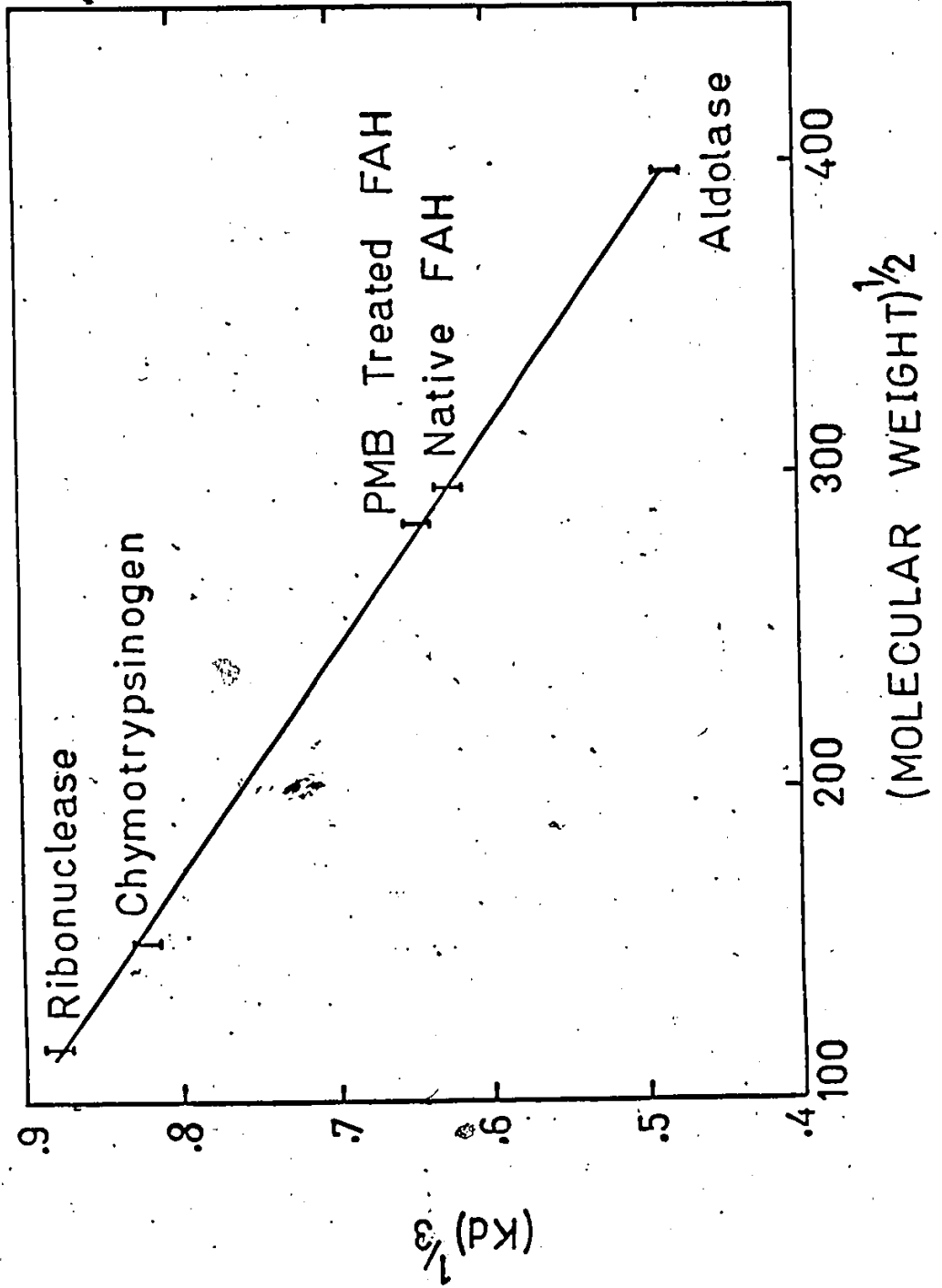


TABLE II: Reversal of PMB Inactivation of Enzyme

Enzyme Treatment <sup>a</sup>	Additions		% Increase
	0.0 mM Cysteine Activity <sup>b</sup>	80 mM Cysteine Activity	
Native	.045	.047	4
	.018	.023	30
PMB Treated	.019	.025	31
	.010	.027	170
	.009	.049	440

<sup>a</sup> Tubes were taken from fractions emerging from column and assayed before and after addition of cysteine.

<sup>b</sup> Activity expressed as OD/5 min.

6) Effect of Sodium Cyanoborohydride on FAH Activity

FAH is inactivated by sodium cyanoborohydride alone in a concentration dependant, time independant way. The addition of propionopyruvate does not increase this inactivation as seen in Table III.

7) Effect of Potassium Cyanide on FAH Activity

FAH is inactivated by potassium cyanide in the presence of propionopyruvate and acetopyruvate as seen in Table IV. Cyanide alone causes an increase in activity. The increase above control is presumably due to the formation of a cyanohydrin with substrate which would appear as an increase in activity(36). Propionopyruvate alone also shows an increase in activity. This may be due to carrying over of substrate from incubation mixture to assay mixture, thereby increasing the concentration of substrate and hence, its rate of hydrolysis (activity). Incubation with the competitive inhibitors fluoride and DTNB did not protect the enzyme from inactivation. Fluoride and DTNB alone showed little effect on activity in the concentrations used.

TABLE III: Effect of Sodium Cyanoborohydride on Enzyme Activity

Additions <sup>a</sup>	% Activity Remaining 2 min	% Activity Remaining 5 min	% Activity Remaining 10 min
None	-	100	100
25 mM Cyanoborohydride	-	18	18
25 mM Cyanoborohydride + 4.0 mM Propionopyruvate	-	16	16
5.0 mM Cyanoborohydride	53	51	-
5.0 mM Cyanoborohydride + 4.0 mM Propionopyruvate	-	65	-

a

All incubation mixtures were in 0.1 M phosphate buffer pH 7.3 at 25.0±0.5°. Aliquots were removed at indicated times and assayed.

TABLE IV: Effect of Potassium Cyanide on Enzyme Activity

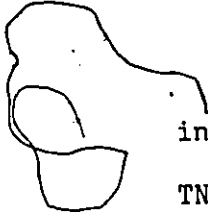
Additions <sup>a</sup>	% Activity Remaining after 5 min
None	100
2.7 mM Cyanide	130
4.0 mM Propionopyruvate	120
2.7 mM Cyanide + 4.0 mM Propionopyruvate	0
2.7 mM Cyanide + 4.0 mM Propionopyruvate + 20 mM Fluoride	0
4.0 mM Acetopyruvate	95
2.7 mM Cyanide + 4.0 mM Acetopyruvate	0
2.7 mM Cyanide + 4.0 mM Acetopyruvate + 20 mM Fluoride	0
0.20 mM DTNB	75
0.20 mM DTNB + 4.0 mM Acetopyruvate +	100
0.20 mM DTNB + 4.0 mM Acetopyruvate + 2.7 mM Cyanide	0

a

All incubation mixtures were in 0.1 M phosphate buffer pH 7.3 at 25.0±0.5°. Aliquots were removed after 5 min and assayed.

## CHAPTER III

### DISCUSSION



The results demonstrate that PMB is a potent and specific inactivating agent of FAH. The other sulfhydryl reagents employed, TNBS, NEM, and DTNB showed little irreversible effect on the activity of the enzyme even in large concentrations. PMB however, in very small amounts inactivates FAH, presumably by binding at a critical sulfhydryl group which the other sulfhydryl specific inhibitors were unable to attack. The presence of substrate or competitive inhibitor protects the enzyme from inactivation. This suggests that the binding of these ligands directly involves amino acid residues with sulfhydryl groups or they bind to the enzyme in such a way as to propagate a conformational change so that these groups are buried and thus protected from PMB attack. The conformational change is not in the nature of dissociation into subunits as has been reported for a number of PMB treated enzymes. These include phosphorylase (36), aspartate transcarbamylase (37), human hemoglobin (38), aspartase (39), glyceraldehyde-3-phosphate dehydrogenase (40), and formyltetrahydrofolate synthetase (41).

The observation that cyanide inactivates the enzyme in the presence of propionopyruvate and acetopyruvate suggests that an amine group on the enzyme is involved and the catalytic reaction proceeds through a ketamine intermediate. This inactivation could not be prevented by the competitive inhibitors fluoride or DTNB. This suggests that these competitive inhibitors act on a different portion of the enzyme than is involved with Schiff-base formation

between enzyme and substrate.

Cyanoborohydride was found to be ineffective as an agent to trap a Schiff-base intermediate between substrate and enzyme. It may be too large a molecule to enter and interact with the enzyme-substrate complex. Previous work (42), has shown that monovalent anions act as competitive inhibitors of FAH. The inactivation of FAH by cyanoborohydride alone may be due to its acting as a competitive inhibitor.

It is not possible to distinguish between the two catalytic mechanisms proposed. They both involve Schiff-base formation and attack by a second nucleophile, either water or a second group on the enzyme.

One might speculate that the second nucleophile is the sulfhydryl of a cysteine residue on the enzyme which is blocked by the action of PMB. The competitive inhibitor fluoride and substrate which afford protection against inactivation act in the region of the second nucleophile and prevent inactivation by masking this sulfhydryl group from the mercurial.

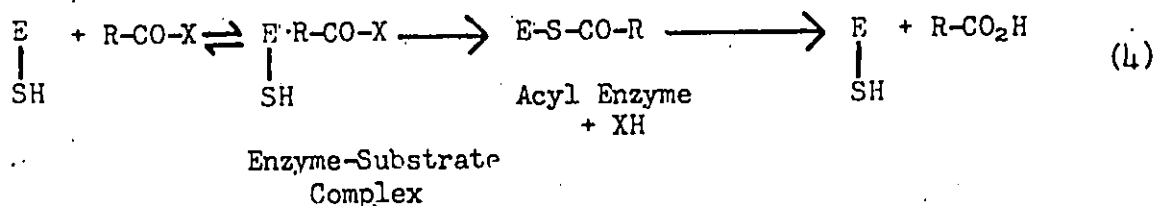
This evidence is all indirect and a more direct method of identifying the sulfhydryl group should be employed. This has been done for a number of enzymes with a sulfhydryl in the active site. In general the group has been labelled with substrate, pseudosubstrates and/or specific inhibitors. This approach is taken when a covalent bond is expected to form between substrate and sulfhydryl group. The stable enzyme-substrate complex is isolated, the protein is hydrolyzed and the amino acid residue that formed the covalent bond with substrate or inhibitor is



isolated and identified.

The identification of a sulfhydryl group in the active site by this method has been reported for acetyl-Co A thiolase (43), liver transglutaminase (44, 45), glyceraldehyde-3-phosphate dehydrogenase (46-49), yeast pyruvate decarboxylase (50,51), and ribulose-1,5-diphosphate carboxylase (52,53).

The mechanism employing a sulfhydryl group as a nucleophile is analogous to that of papain. The action of papain can be represented by the following scheme:



where X = OR, NHR, SR

The formation of an intermediate thioester between the sulfhydryl group of papain and the acyl residue of a pseudosubstrate has been confirmed spectrophotometrically (54-56). For FAH where X =  $\text{CH}_2-\overset{\text{E}}{\underset{\text{NH}}{\text{C}}}-\text{COO}^-$  the hydrolysis of the bond is facilitated by the Schiff-base intermediate formed prior to nucleophilic attack by the sulfhydryl group.

The protonated Schiff-base acts as an electron sink to accelerate the rupture of a carbon-carbon bond. The use of Schiff-base intermediates to facilitate carbon-carbon bond cleavage has also been postulated for aldolase (14), acetoacetate decarboxylase (15), 2-keto-3-deoxy-6-phosphogluconate aldolase (18) and 2-keto-4-hydroxyglutarate aldolase (16, 17).

## SUMMARY

Fumarylacetoacetate fumarylhydrolase is specifically inhibited by p-mercuribenzoate. This inactivation shows saturation kinetics and substrate or the competitive inhibitor fluoride protects the enzyme. Cyanide inhibits the enzyme only in the presence of substrate. The enzymic mechanism of 2,4-diketo acid hydrolysis is discussed in terms of ketamine formation between enzyme and the 2-keto carbon of substrate and nucleophilic attack of the enzyme sulfhydryl group on the 4-keto carbon.

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VITA AUCTORIS

- 1950: Born in Hamilton, Ontario, November 7, 1950.
- 1956- 1964: Attended Holy Family School,  
Hamilton, Ontario.
- 1964- 1969: Attended Bishop Ryan Secondary School,  
Hamilton, Ontario.
- 1969- 1973: Attended University of Windsor, Windsor,  
Ontario. Graduated in 1973 with degree  
of Bachelor of Science.
- 1973: Accepted into the Faculty of Graduate Studies  
at the University of Windsor, Windsor, Ontario.