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An investigation of the mechanism of hydrolysis with 4-fumarylacetoacetate fumaryl hydrolase.

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AN INVESTIGATION OF THE MECHANISM OF HYDROLYSIS WITH 4-FUMARYLACETOACETATE FUMARYL HYDROLASE

BY

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ABSTRACT

The kinetics of 4-fumarylacetoacetate fumaryl hydrolase with a number of 2,4-diketo acids were studied. Substrate inhibition was observed in some cases. The effects of sodium borohydride upon this enzyme were investigated and this reagent was found to cause no inhibition of the enzyme. A large number of sulfhydryl reagents caused inhibition of the enzyme.

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ABBREVIATIONS

AcPy	Acetopyruvic acid
ATP	Adenosine-5'-triphosphate
DDD	2,2'-Dihydroxy-6,6'-dinaphthyldisulfide
DTNB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
E-NH_2	An amino acid residue of the enzyme with a free amino group such as the ε-amino group of lysine
E-NucH	The enzyme with a nucleophilic group such as the sulfhydryl of cysteine
FAA'ase	4-Fumarylacetoacetate fumaryl hydrolase
HMB	Hydroxymercuribenzoate
HNBB	2-Hydroxy-5-nitrobenzyl bromide
NAD	Nicotinamide adenine dinucleotide
NaIA	Sodium iodoacetate
NEM	N-Ethylmaleimide
O. D.	Optical density
PMSF	Phenylmethanesulfonyl fluoride
TNBS	Trinitrobenzenesulfonic acid

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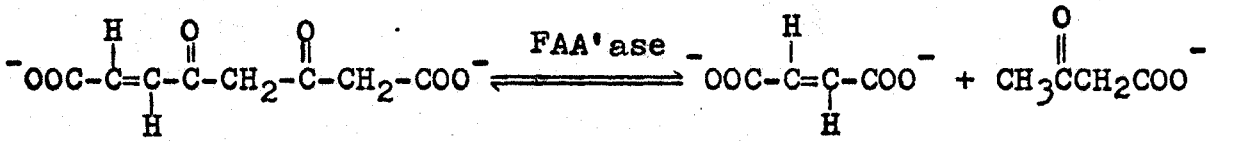
CHAPTER 1

INTRODUCTION

4-Fumarylacetoacetate fumaryl hydrolase (EC 3.7.1.2), or fumarylacetoacetase (FAA'ase), catalyses the cleavage of fumarylacetoacetate into fumarate and acetoacetate (Eq. 1) in the metabolic pathway of tyrosine and phenylalanine degradation in mammalian systems (1).



4-Fumarylacetoacetate Fumarate Aceto-acetate

(Eq.1)

The enzyme was first prepared from beef and rat liver homegenates. It catalyzed the cleavage of a series of 2,4-diketo acids into pyruvic acid and the corresponding fatty acids as well as the hydrolysis of triacetate into acetoacetate and acetate (2,3). It was named as acylpyruvase as well as triacetate hydrolase. The enzyme had no effect on the esters of 2,4-diketo acids, acetylacetone, α-ketoglutaric acid, γ-ketohexanoic acid or levulinic acid. No activation by NAD, ATP or the divalent ions such as Ba^{++}, Ca^{++}, Mg^{++}, and Zn^{++} was found. The optimum pH was between 6.5 and 8.2. This enzyme was later identified with the one that catalyzes the formation of fumarate and acetoacetate from 4-fumarylacetoacetate, a

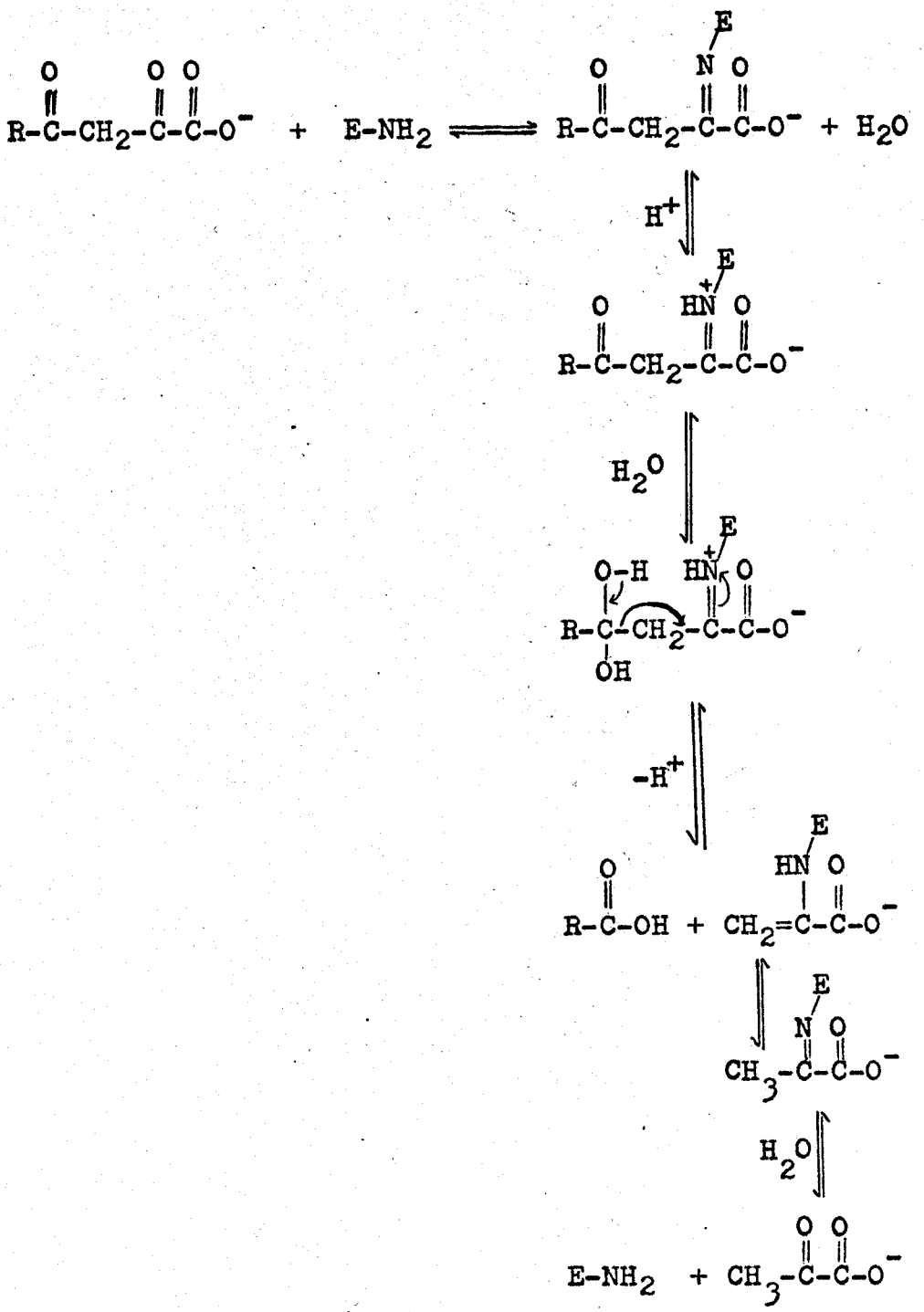
degradation product of homogentisic acid and ultimately of tyrosine or phenylalanine (4,5). It was concluded that the enzyme specifically hydrolyses 2,4- and 3,5-diketo acids.

In a bacterial system where fumarylpyruvate is a degradation product of gentisic acid, an analogous enzyme, fumarylpyruvate hydrolase, which catalyzes the hydrolysis of fumarylpyruvate into fumarate and pyruvate was found (6).

Fumarylacetoacetase and fumarylpyruvate hydrolase constitute a special class of hydrolases which catalyze the hydrolysis of C-C bonds. This type of reaction is exemplified by other enzymes. Oxaloacetase from Aspergillus niger catalyzes the hydrolysis of oxaloacetate to oxalate and acetate (7). Chymotrypsin is known to catalyze the hydrolysis of ethyl 5-(p-hydroxyphenyl)-3-ketovalerate into p-hydroxy-phenylpropionate and ethyl acetate (8). L-Kynurenine hydrolase (EC 3.7.1.3) catalyzes the formation of anthranilate and alanine from L-kynurenine. Although the mechanism of the last named enzyme is known to involve Schiff-base formation with pyridoxal phosphate as a cofactor, and is to be activated by divalent ions such as Cu^{++}, Mg^{++} and Mn^{++} (9,10), the general mechanism for these enzymic reactions has received little attention.

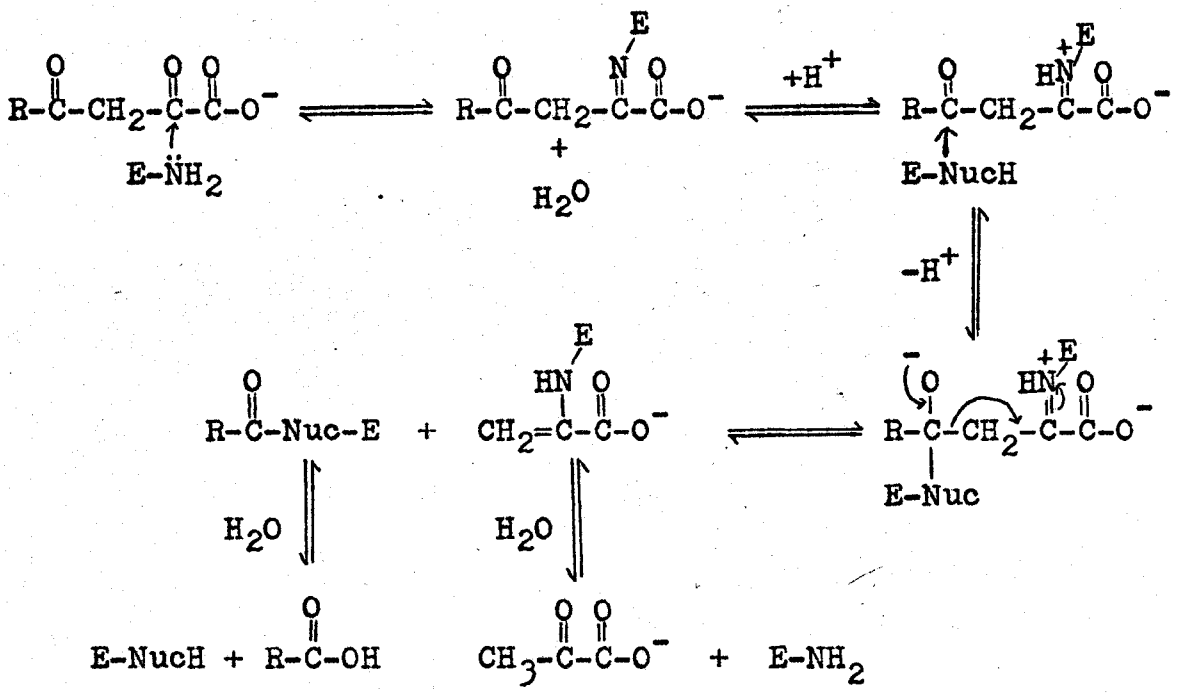
The present study was carried out to elucidate the mechanism of the action of fumarylacetoacetase. Four possible mechanisms are considered, the first two involving a Schiff-

base intermediate, and the third and fourth involving attack by nucleophilic group of the enzyme or of water. Participation of a Schiff-base intermediate might be suspected by analogy with L-kynurenine hydrolase. The first of two possible mechanisms involving Schiff-base formation is simialr to the mechanism of acetoacetate decarboxylase (12). The enzyme may form a Schiff-base intermediate with the 2-keto group of the substrate which is then 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).



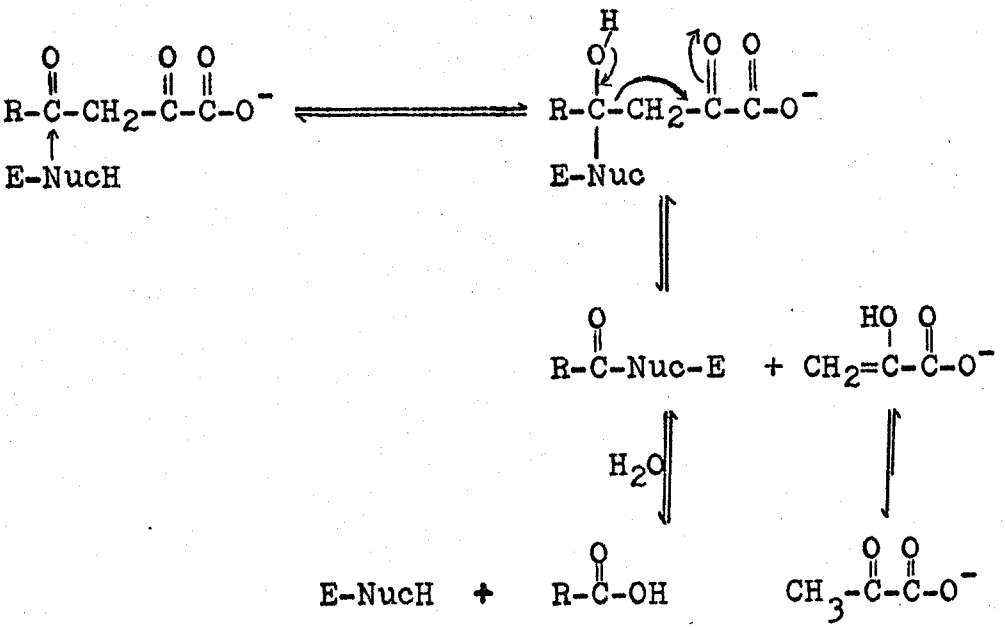
Scheme 1.

In the second possible scheme, a second nucleophile from the enzyme, in contrast to water in Scheme 1, attacks the 4-carbonyl group after Schiff-base formation (Scheme 2). After substrate cleavage, the two fragments remain covalently bonded to the enzyme until they are each released as free acid following hydrolysis.



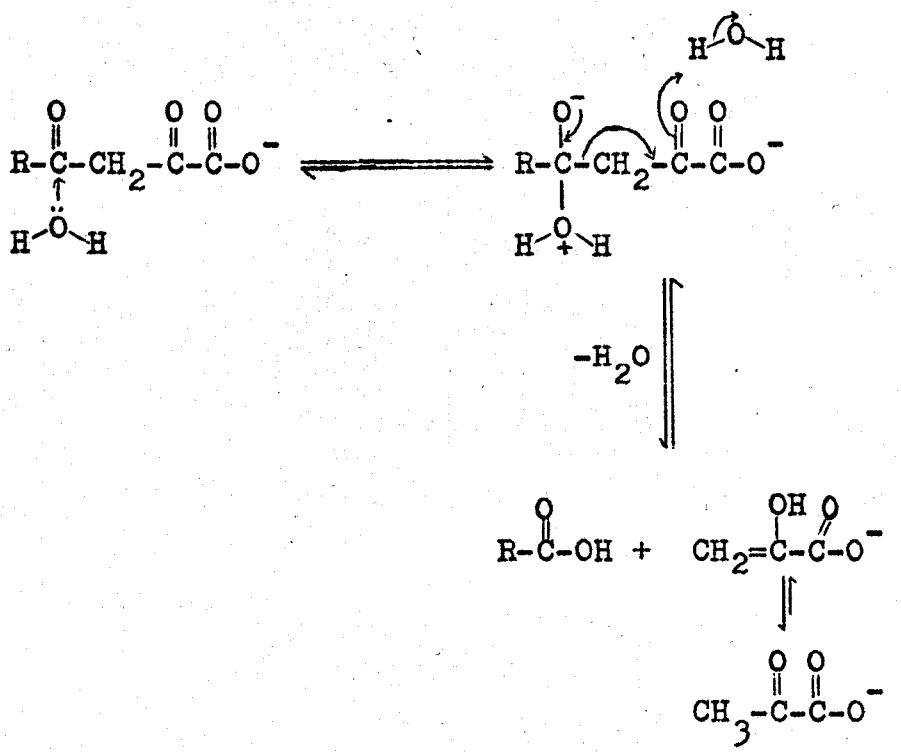
Scheme 2.

Two mechanisms involving substrate attack by nucleophilic groups are postulated. Nucleophilic groups of the enzyme such as the hydroxyl from serine or sulfhydryl from cysteine may attack the carbonyl group of the substrate. In Scheme 3, a 4-keto group attack is followed by the formation of an acyl enzyme with liberation of the enolpyruvate. This mechanism is similar to the normal chymotrypsin action with esters and amides where an alcohol or amine is the leaving group (13, 14) and is most probably the mechanism by which chymotrypsin cleaves 5-(p-hydroxyphenyl)-3-ketovalerate.



Scheme 3.

In Scheme 4, a water molecule attacks the 4-keto group. The acid and enolpyruvate are then released through a concerted mechanism. The enzyme may facilitate the reaction by polarizing the carbonyl group or the water molecule so as to increase the lability of the carbonyl group or the nucleophilic character of the water molecule.

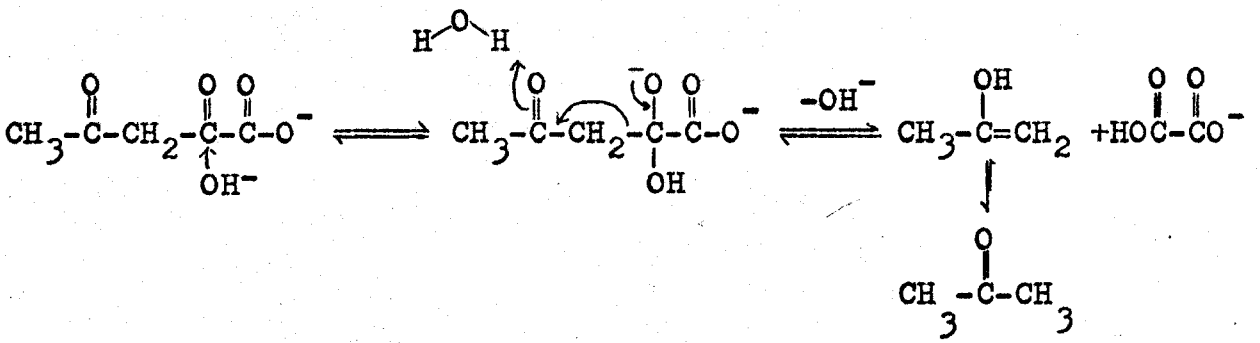


Scheme 4.

Schemes 1 & 2, involving a Schiff-base intermediate, can readily be differentiated from the other two schemes by trapping any Schiff-base intermediate with sodium borohydride. This reagent has been successfully used in trapping Schiff-

bases from pyridoxal and phosphorylase a (15), transaldolase (16), aldolase (17), acetoacetate decarboxylase (18), 2-keto-4-hydroxy glutarate aldolase (19,20) and 2-keto-3-deoxy-6-phosphogluconate aldolase (21).

Finally, it may be of interest to contrast the enzyme-catalyzed reaction with non-enzymatic hydrolysis of acetopyruvic acid which is a model substrate for this enzyme. This substrate, perfectly stable at physiological temperature and pH, is hydrolyzed by strong base to acetone and oxalate (22). The base-catalyzed reaction mechanism probably is the reverse of Claisen condensation (Eq. 2).



(Eq. 2)

CHAPTER 2

EXPERIMENTAL

A. METHODS AND MATERIALS

1) Preparation of 2,4-Diketo Acids Substrates

The general procedure of Claisen condensation was used in the synthesis of the sodium ethyl esters or the ethyl esters of 2,4-diketo acids by condensing ethyl oxalate with an appropriate methyl ketone in the presence of sodium ethoxide. Except for succinylpyruvic acid, the free acids listed in Table I were obtained by hydrolysis with base of the sodium ethyl ester rather than the ethyl ester.

Acetopyruvic Acid A mixture of 135 ml (1 mole) of ethyl oxalate and 73 ml (1 mole) of acetone was added slowly to a 1-liter round-bottomed flask containing 25 gm of sodium metal dissolved in 560 ml of absolute alcohol as described by Marvel (23). A white precipitate appeared which turned yellow as the reaction proceeded. Towards the end, the mixture became very thick and stirring was difficult. The yellow precipitate, sodium ethyl acetopyruvate, was separated by centrifugation as suggested by Lehninger (22). To obtain the free acid, 2 gm of the dry powdered sodium ethyl acetopyruvate was dissolved in a minimum volume of water (about 60 ml). One equivalent of 4N sodium hydroxide was added dropwise while maintaining the pH below 12, until the rate

of pH decrease became negligible. The solution was acidified with 3N sulfuric acid followed by overnight continuous ether extraction. The ethereal solution was dried over anhydrous magnesium sulfate and the solvent was removed by a rotary evaporator. The crude crystals which formed slowly after the ether was removed were recrystallized from carbon tetrachloride. The compound was sublimed in vacuo. The melting point of the dry colorless sublimate was 98-99°C (reported 98°(22)).

Propionopyruvic and Butyropyruvic Acids To prepare the sodium ethyl esters of propionopyruvic acid and butyropyruvic acid, a mixture of 14.7 gm (13.5 ml, 0.1 mole) diethyl oxalate with 7.2 gm (8.9 ml, 0.1 mole) 2-butanone and with 8.6 gm (10.6 ml, 0.1 mole) 2-pentanone respectively was added slowly with stirring to a 100-ml round-bottomed flask containing 2.3 gm sodium dissolved in 50 ml of absolute alcohol (24). The solution turned gradually from yellow to brown as the reaction proceeded. A yellow solid precipitate appeared after two days in both cases. The precipitates were collected by filtration and dried. To obtain the free acids, the yellow sodium salts were dissolved in a minimum amount of water and then hydrolyzed with 4N sodium hydroxide as in the case of acetopyruvic acid. The solution was then acidified with 25% hydrochloric acid and extracted into ether. After drying with anhydrous sodium sulfate, ether was removed by a rotary evaporator. The crude crystals of propionopyruvic

acid were recrystallized from chloroform; m.p. 78-80°. Elemental analysis showed: C, 50.00; H, 5.67; calculation for $C_6H_8O_4$: C, 50.00; H, 5.59. The butyropyruvic acid was recrystallized from carbon tetrachloride; m.p. 53-55°. Elemental analysis showed: C, 52.91; H, 6.40; calculation for $C_7H_{10}O_4$: C, 53.16; H, 6.37.

Pivalopyruvic Acid The sodium ethyl ester of pivalopyruvic acid was prepared similarly. Sodium metal, 2.3 gm, was cut into thin slices and dissolved in 30 ml absolute alcohol. After cooling, a mixture of 10 gm (12.4 ml, 0.1 mole) 3,3-dimethyl-2-butanone and 14.7 gm (13.5 ml, 0.1 mole) diethyl oxalate was added slowly with stirring. The stirring was continued overnight. A yellow precipitate formed when a small amount of water was added. The precipitate was then collected by filtration and dried. The free acid was obtained in the usual manner. The crude product was recrystallized from a chloroform-petroleum ether mixture (25). The melting point of the pure pivalopyruvic acid was 57-58° (reported m.p. 57-58.5° (25)).

Benzoylpyruvic Acid The benzoylpyruvic acid was synthesized according to the method of Cattelain (26). A mixture of acetophenone (60 gm, 58.3 ml, 0.5 mole) and diethyl oxalate (74 gm, 68 ml, 0.5 mole) was added slowly with stirring to 23 gm (1 mole) of sodium in 250 ml absolute alcohol. The solution rapidly became yellow and viscous. The

mass was poured into an evaporating dish, dried overnight and then pulverized. This material (17.3 gm) was then stirred into 170 ml water. The solution was filtered and the filtrate was neutralized to phenolphthalein end point with 25% acetic acid. Sodium acetate, 4 gm, in a little water was then added to form the sodium salt of benzoylpyruvic acid. The solution was acidified with 25% hydrochloric acid and a precipitate was obtained which was recrystallized from ether, m.p.158-160° (reported m.p. 150° (26), 156-158° (27), 157° (2)).

p-Methylbenzoylpyruvic Acid To prepare p-methylbenzoylpyruvic acid, 34.5 gm (33.8 ml. 0.25 mole) of p-methylacetophenone and 34 ml (0.25 mole) of diethyl oxalate was added slowly with stirring to a solution of 5.8 gm sodium in 125 ml absolute alcohol. The yellow precipitate was collected and dried soon after the addition. The precipitate, 13 gm, was dissolved in about 300 ml water. The clear solution after filtration was then hydrolyzed with 4N sodium hydroxide. The free acid was obtained after acidification and it was recrystallized from ether, m.p. 143-144° (reported m.p. 142° (2)).

3-Phenylpropionopyruvic Acid The ethyl ester of 3-phenylpropionopyruvic acid was made by adding a mixture of benzylacetone (7.4 gm, 0.05 mole) and diethyl oxalate (7.3 gm, 0.05 mole) to a solution of 1.2 gm of sodium in 30 ml absolute alcohol. The solution first turned to yellow and

then later to brown. After stirring for 1 hour, the yellow precipitate was collected by centrifugation. To obtain the free acid, 5 gm of the dried precipitate was dissolved in water, hydrolyzed with 4N sodium hydroxide and acidified. The precipitate was taken up with ether and recrystallized from carbon tetrachloride. It had a melting point of 86-87° (reported m.p. 86.5-87.5° (27)).

Cinnamoylpyruvic Acid The cinnamoylpyruvic acid was prepared according to the method of Keskin (27). A mixture of benzalacetone (13 gm) and diethyl oxalate (13 gm) was added to a cooled solution of 2 gm of sodium dissolved in 25 ml absolute alcohol. The sodium salt was collected by centrifugation and then hydrolyzed with 4N sodium hydroxide. A precipitate, formed on acidifying the hydrolysate, was collected and recrystallized from chloroform, m.p. 139-141° (reported m.p. 139-140° (27)).

Succinylpyruvic Acid The procedure of Wislicenus (28,29) was employed with slight modification. Sodium, 2.3 gm, was covered with xylene which was heated until the sodium melted and the whole solution was stirred with a magnetic stirring bar. The xylene was decanted and the finely dispersed sodium was washed three times with anhydrous ether. It was then covered with about 75 ml anhydrous ether. To this was added 10 ml anhydrous ethanol and allowed to stir overnight in a flask fitted with a calcium chloride drying

tube. Diethyl oxalate (about 17 ml) was then added dropwise. The sodium ethoxide went into solution and the solution turned brown. After 2-3 hours, 15 ml ethyl levulinate (0.1 mole) was added dropwise and the solution was allowed to stir overnight. Then 100 gm of ice was put into the mixture and the turbid ethereal solution which contained the excess oxalate was separated from the aqueous layer. The aqueous solution was then acidified. The diethyl succinylpyruvate separated as an oil which was taken up with ether. The ethereal solution was washed with 50 ml of 5% sodium bicarbonate solution and the ether removed by evaporation. The crude ester was then put into 10 ml of water and hydrolyzed by adding 4N sodium hydroxide until pH change became very slow. The solution was extracted once with ether, then it was acidified. It was extracted with 6 portions of ether. The combined ethereal solution was dried and evaporated to dryness. The residue was recrystallized in hot benzene, m.p. 128-130° (reported m.p. 100-125° (29), 127-129° (6)).

Structures of all the 2,4-diketo acids synthesized have been confirmed by N.M.R. and these compounds were dried under vacuum before use.

2) Enzyme Assay

All the 2,4-diketo acids studied, except the aromatic ones, exhibit an absorption maximum (λmax) at 295 mμ at pH 7.3 due to the enol structure. The benzoylpyruvic acid and p-methylbenzoylpyruvic acid have a λmax at

355 mμ.

The rate of enzyme-catalyzed reaction was followed by observing the rate of decrease in absorption at the λmax (2).

In a typical experiment, a measured amount of 2,4-diketo acid was dissolved in 0.025M sodium phosphate buffer at pH 7.3. The absorbance of the compound was determined with a Beckman DU monochromator by putting 1 ml of the solution in spectrophotometer cell with a 0.5 cm light path.

The enzyme, purified 200-fold from beef-liver, and identified as homogenous by polyacrylamide gel electrophoresis and molecular sieve chromatography (30), was a gift from Miss Holly Hsiang. The enzyme concentration in 0.025M sodium phosphate buffer at pH 7.3 with 1 X 10^{-4} M dithioerythritol was adjusted with the same buffer so that 0.02 ml would give an initial rate of decrease in absorbance within the range of 0.1 to 0.25 O.D. units per 5 minutes with 1 ml of 5 X 10^{-4}M acetopyruvic acid as substrate.

Solutions of the compounds to be studied were equilibrated in a water bath at 37°C before putting them into the spectrophotometer cell. The reaction was initiated by the addition of 0.02 ml of enzyme solution to 1 ml substrate solution in the cell and the rate was recorded on a Gilford Model 2000 Absorbance Recorder attached to a Beckman DU monochromator. The temperature of the reaction chamber was held constant at 37°C. The rate of reaction was constant for

at least the first three minutes and the initial rates (V_o), expressed as ΔO.D./5 min , were calculated from the slopes of the chart paper data. The relative activity of the enzyme was determined by measuring the activity of 0.02 ml enzyme solution with 1 ml of a standard 5 X 10^{-4}M acetopyruvic acid solution.

3) Effect of Sodium Borohydride on Enzyme Activity

Acetopyruvic acid, propionopyruvic acid, benzoylpyruvic acid, 2,4-pentanedione and 1-phenyl-1,3-butane-dione were used as substrates in experiments to determine whether a Schiff-base intermediate was involved in the enzyme reaction.

A fresh sodium borohydride solution (2 X 10^{-3}M) was prepared by dissolving 0.0040 gm in 50 ml of 1 X 10^{-3} M sodium hydroxide. The enzyme concentration used in this study was about 0.6 mg/ml. In each case, 0.1 ml of the substrate solution and 0.1 ml of sodium borohydride solution were added to 0.3 ml enzyme solution and incubated at 0 ^{o}C for 30 minutes. An aliquot (0.02 ml) of the above mixture was then put into a spectrophotometer cell containing 1 ml of 5 X 10^{-4}M acetopyruvic acid pre-equilibrated at 37^{o}C. The rate of the reaction was then measured immediately. In the control reaction, 0.2 ml buffer replaced the 0.1 ml of the substrate solution and the 0.1 ml of sodium borohydride solution.

4) Effect of Other Specific Reagents on Enzyme Activity.

In an attempt to study the active center of the enzyme, eight specific reagents (listed in Table V) were used. In each case, 2 ml of 0.025M sodium phosphate at pH 7.3 containing the requisite amount of the specific reagents was mixed with 0.08 ml enzyme solution at 0°C. After incubating the mixture at 37°C for 30 minutes, a 0.5 ml aliquot was transferred to a spectrophotometer cell containing 0.5 m moles acetopyruvic acid or propionopyruvic acid in 0.5 ml buffer solution. The rate measurement was then made immediately.

5) Materials

The following compounds were commercially available: benzylacetone and 5,5'-dithio-bis-(2-nitrobenzoic acid) (Aldrich); dithioerythritol and sodium iodoacetate (Calbiochem); 3-benzoylacrylic acid, 3,3-dimethyl-2-butanone, 2,4-pentanedione, 2-pentanone, 1-phenyl-1,3-butanedione (Eastman); acetone, acetophenone, 2-butanone, diethyl oxalate and sodium borohydride (Fisher); 2,2'-dihydroxy-6,6'-dinaphthyldisulfide, N-ethylmaleimide, 2-hydroxy-5-nitrobenzyl bromide, phenylmethanesulfonyl fluoride and 2,4,6-trinitrobenzenesulfonic acid (Pierce Chemical Company); hydroxymercuribenzoate (Polysciences Inc.)

Benzalacetone was synthesized according to Drake (31) by condensing benzaldahyde (British Drug House) with acetone in sodium hydroxide. The potassium salt of benzyli-

denepyruvic acid was synthesized according to Stecher (32) by condensing pyruvic acid (Matheson Coleman & Bell) and benzaldehyde with potassium hydroxide in methanol.

B. RESULTS

1) Kinetic Studies of FAA'ase-catalyzed Hydrolysis of 2,4-Diketo Acids

The molar extinction coefficients of the 2,4-diketo acids (Table I) were obtained from the slope of a plot of absorbance at λmax against concentration covering a range from 5×10^{-5} M to 1×10^{-3} M. Deviation from linearity were observed in most cases at concentrations of 5×10^{-4} M. K_m values (Table II) were found directly from the reciprocal of the X-axis intercept of the Lineweaver-Burk plots. The V_{max} values were obtained by converting the reciprocal of the Y-axis intercept into mM/min and have been corrected for changes in enzyme activity. The Lineweaver-Burk plots (Fig.1-Fig.7) have been treated by the method of least-squares. The X-axis and Y-axis intercepts were obtained by computer calculation. Correlation coefficients of all curves were better than 0.97.

Substrate inhibition was observed when succinylpyruvic acid and 3-phenylpropionopyruvic acid were used as substrates. In these two cases, the reciprocal of the initial rate was plotted against substrate concentration (Fig. 8,9) according to Eq. 5 (see discussion). The reciprocal of the Y-axis intercept gave V_{max} while the X-axis intercept gave -Ks' which is the dissociation constant of ES_2 ($ES + S \rightleftharpoons ES_2$), a non-reactive enzyme-substrate complex.

Table I. Some Physical Properties of 2,4-Diketo Acids

Compound	Structure[a]	Melting Point (°C)	Maximum Molar Extinction Coefficient
Acetopyruvic Acid	$CH_3\overset{O}{\overset{\|}{C}}-X$	98-99	6400[b]
Propionopyruvic Acid	$CH_3CH_2\overset{O}{\overset{\|}{C}}-X$	78-80	4828[b]
Butyropyruvic Acid	$CH_3CH_2CH_2\overset{O}{\overset{\|}{C}}-X$	53-55	5712[b]
Pivalopyruvic Acid	$CH_3-\overset{CH_3}{\overset{\|}{\underset{CH_3}{\underset{\|}{C}}}}-\overset{O}{\overset{\|}{C}}-X$	57-58	4666[b]
Benzoylpyruvic Acid	$C_6H_5-\overset{O}{\overset{\|}{C}}-X$	158-160	7568[c]
p-Methylbenzoyl- Acid	$CH_3-C_6H_4-\overset{O}{\overset{\|}{C}}-X$	143-144	8000[c]
3-Phenylpropiono- pyruvic Acid	$C_6H_5-CH_2CH_2\overset{O}{\overset{\|}{C}}-X$	86-87	6500[b]
Cinnamoylpyruvic Acid	$C_6H_5-CH=CH-\overset{O}{\overset{\|}{C}}-X$	139-141	14194[d]
Succinylpyruvic Acid	$HO-\overset{O}{\overset{\|}{C}}CH_2CH_2\overset{O}{\overset{\|}{C}}-X$	128-130	4102[b]

[a] -X represents the pyruvic acid group, $-CH_2-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-OH$

[b] at 295 mμ in 0.025M sodium phosphate buffer at pH 7.3

[c] at 310 mμ in 0.025M sodium phosphate buffer at pH 7.3

[d] at 355 mμ in 0.025M sodium phosphate buffer at pH 7.3

Table II. Values of K_m & V_{max} of FAA'ase-catalyzed Hydrolysis of 2,4-Diketo Acids

Compound	K_m (moles/liter)	V_{max} (mM/min)
Acetopyruvate	1.066×10^{-3}	0.037
Propionopyruvate	5.855×10^{-4}	0.079
Butyropyruvate	2.307×10^{-4}	0.017
Pivalopyruvate	8.217×10^{-4}	0.019
Benzoylpyruvate	1.967×10^{-4}	0.001
p-Methylbenzoyl-pyruvate	4.107×10^{-4}	0.003
Cinnamoylpyruvate	2.880×10^{-5}	0.002
3-Phenylpropiono-pyruvate	2.932×10^{-4} *	0.023
Succinylpyruvate	3.137×10^{-4} *	0.025

* The two are Ks'.

Fig. 1. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Acetopyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO. D. / 5 min.

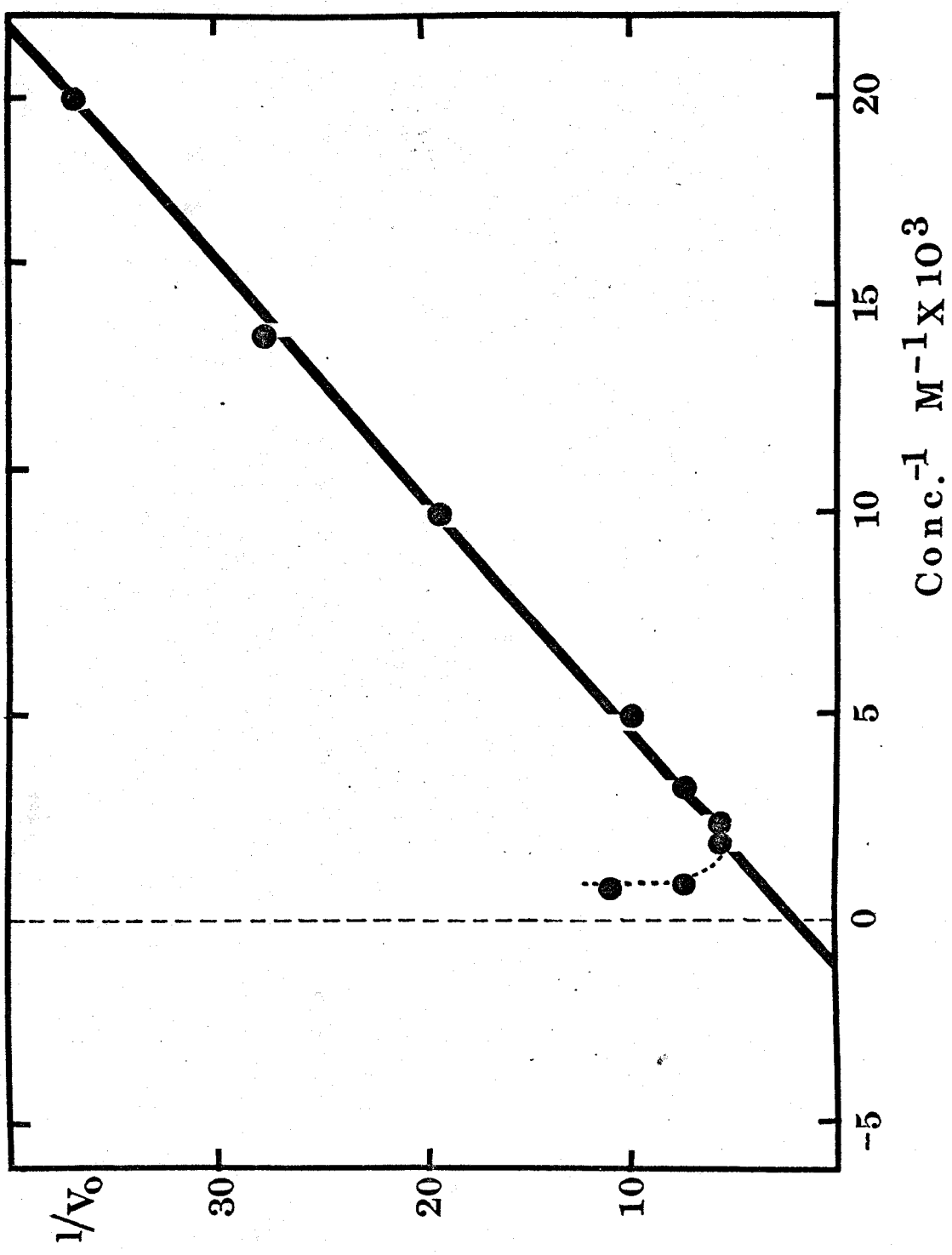


Fig. 1

Fig. 2. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Propionopyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO. D. / 5 min.

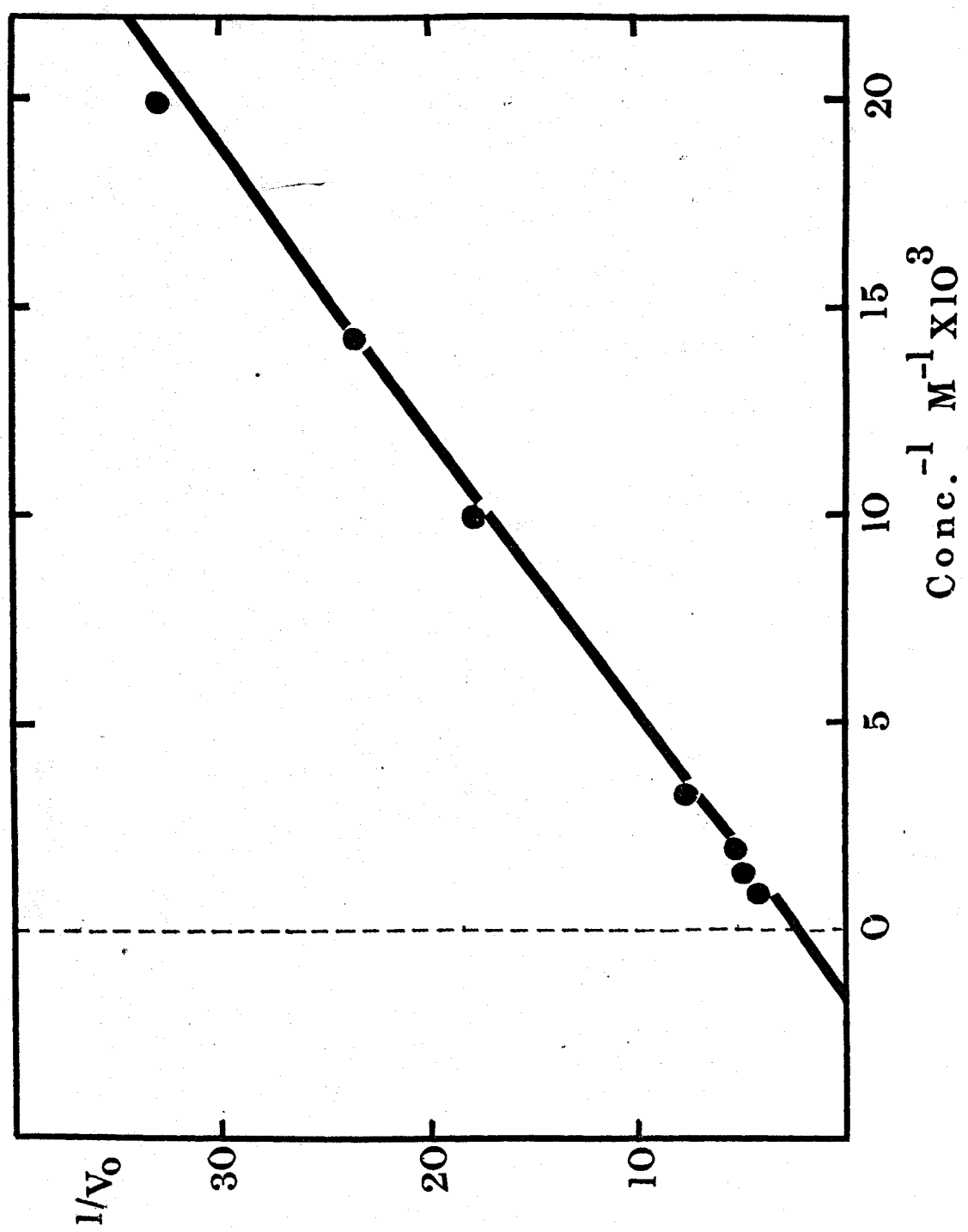


Fig. 2

Fig. 3. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Butyropyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO. D. / 5 min.

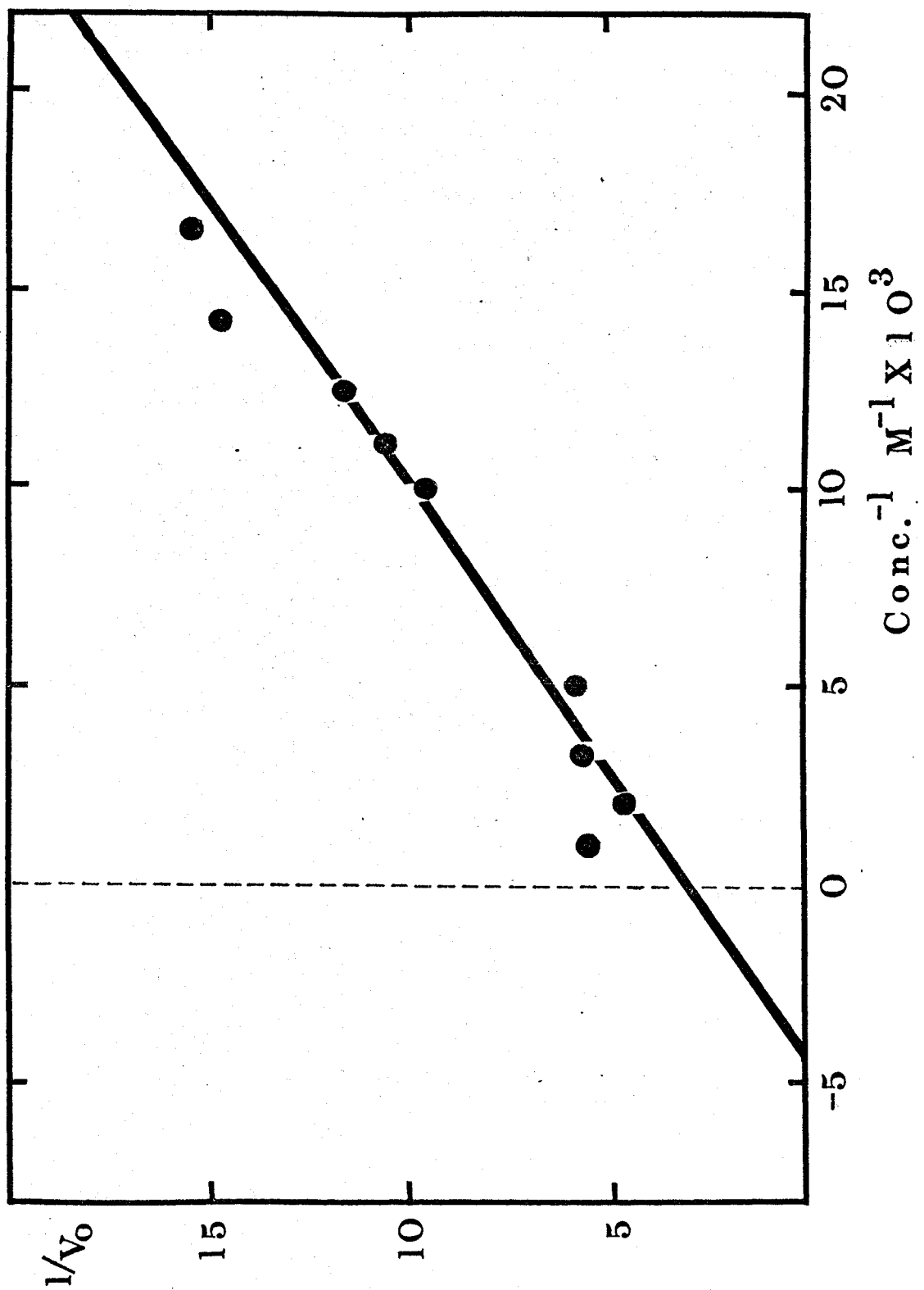


Fig. 3

Fig. 4. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Pivalopyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D./ 5 min.

Fig. 4

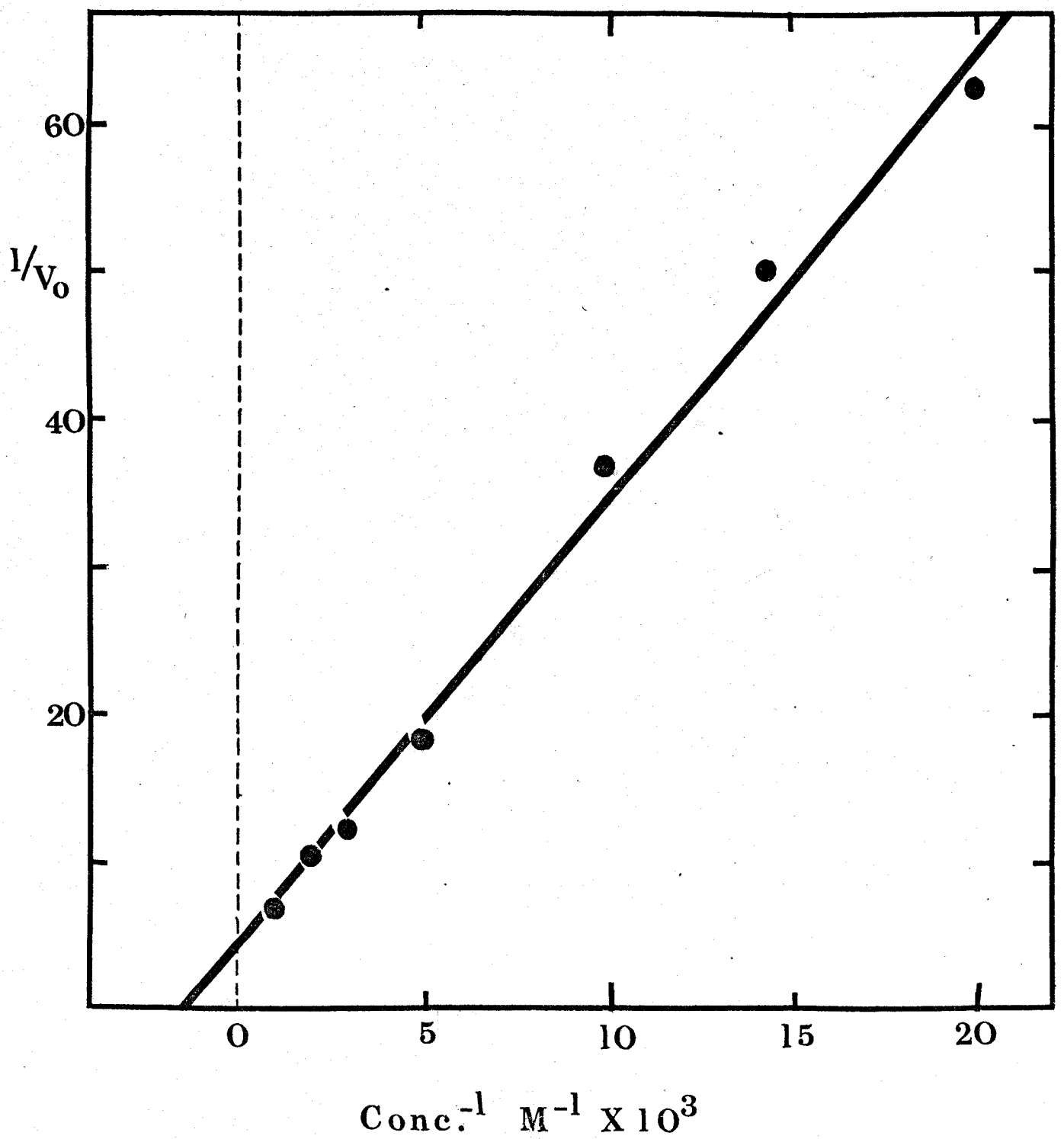


Fig. 5. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Benzoylpyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D. / 5 min.

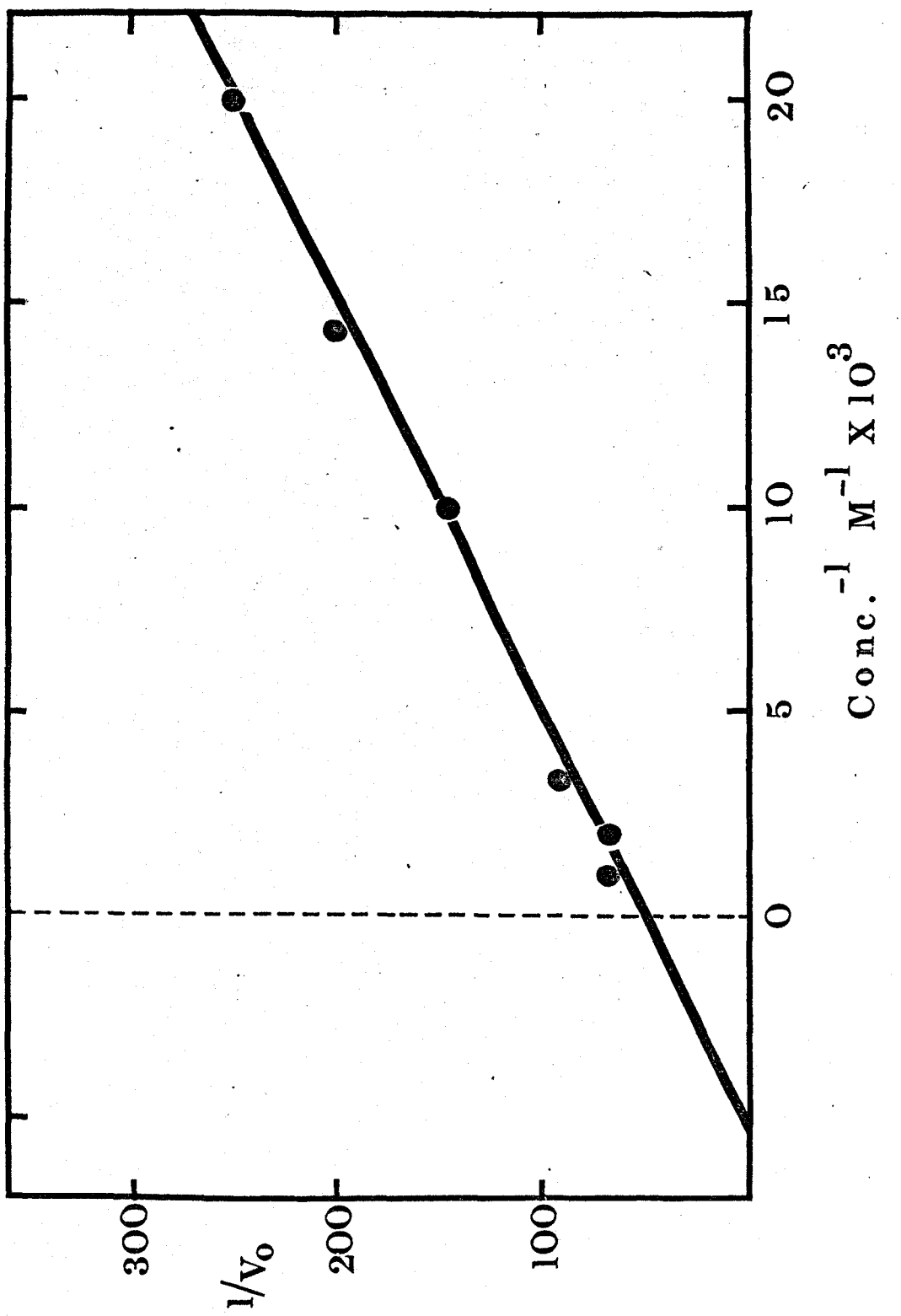


Fig. 5

Fig. 6. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of p-Methylbenzoylpyruvic Acid.
Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D./ 5 min.

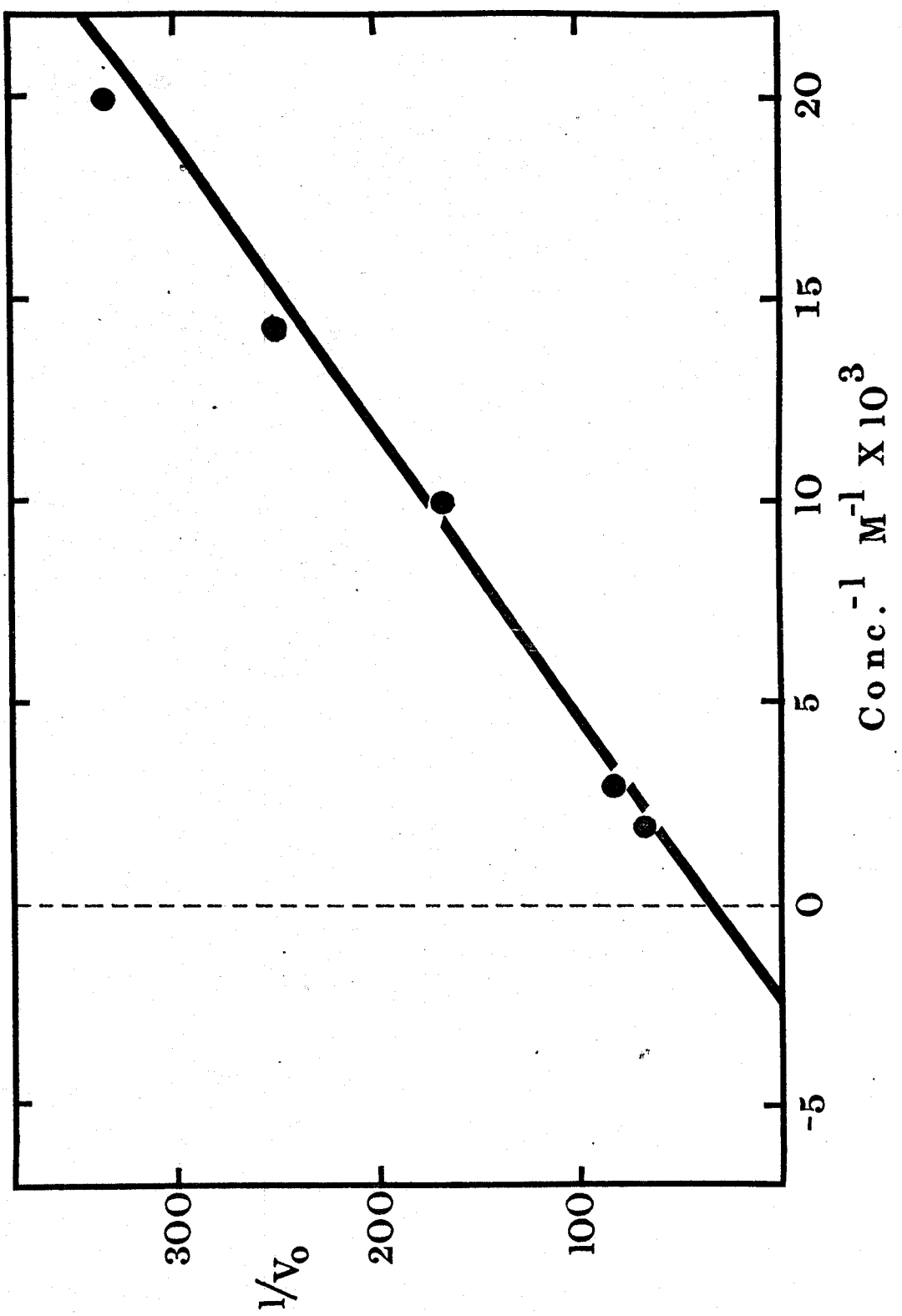


Fig. 6

Fig. 7. Lineweaver-Burk Plot of FAA'ase-catalyzed Hydrolysis of Cinnamoylpyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025 M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D./ 5 min.

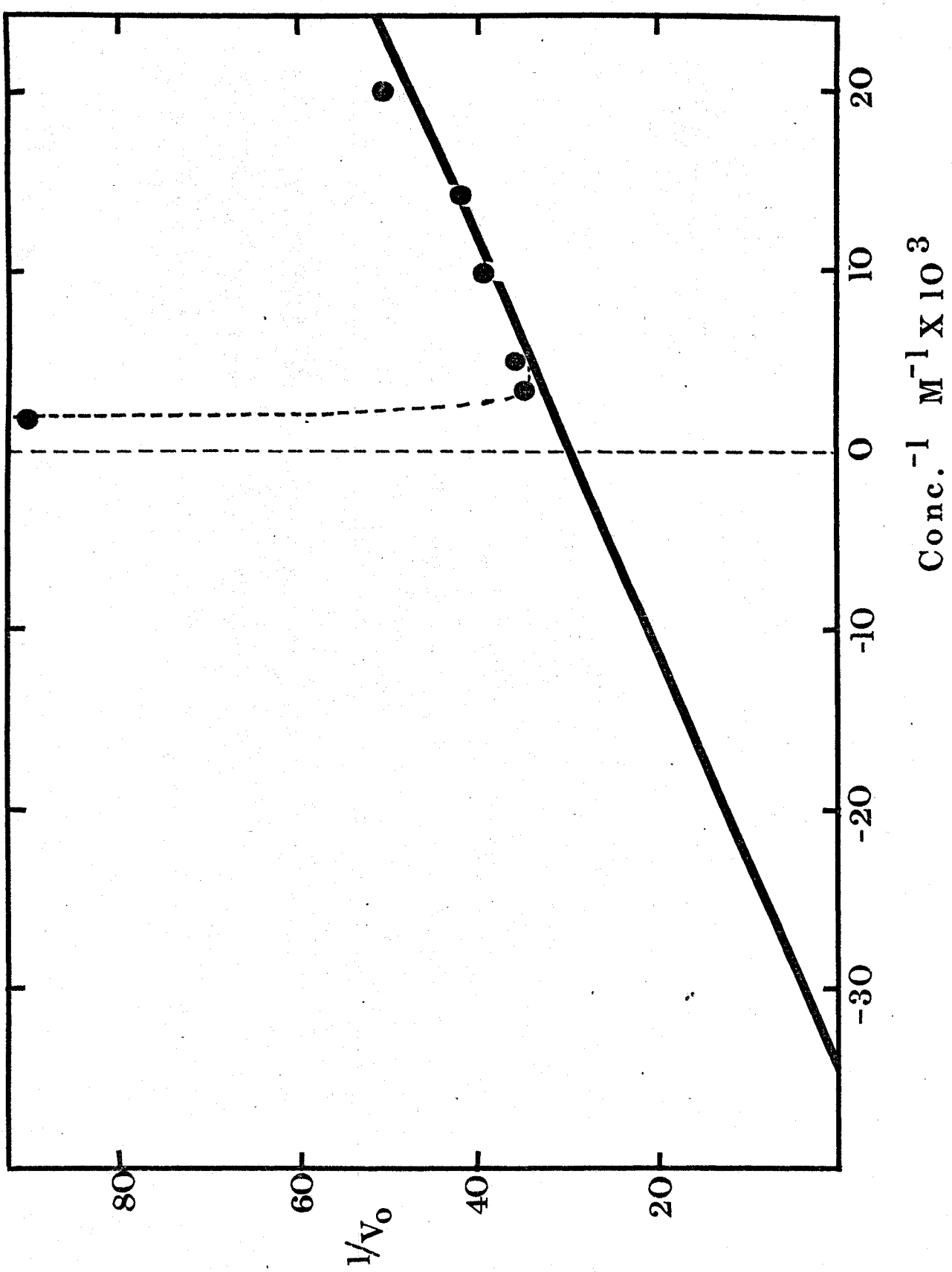


Fig. 7

Fig. 8. Plot of Reciprocal of Initial Rates against Concentration of 3-Phenylpropionopyruvic Acid.
Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025 M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D./ 5 min.

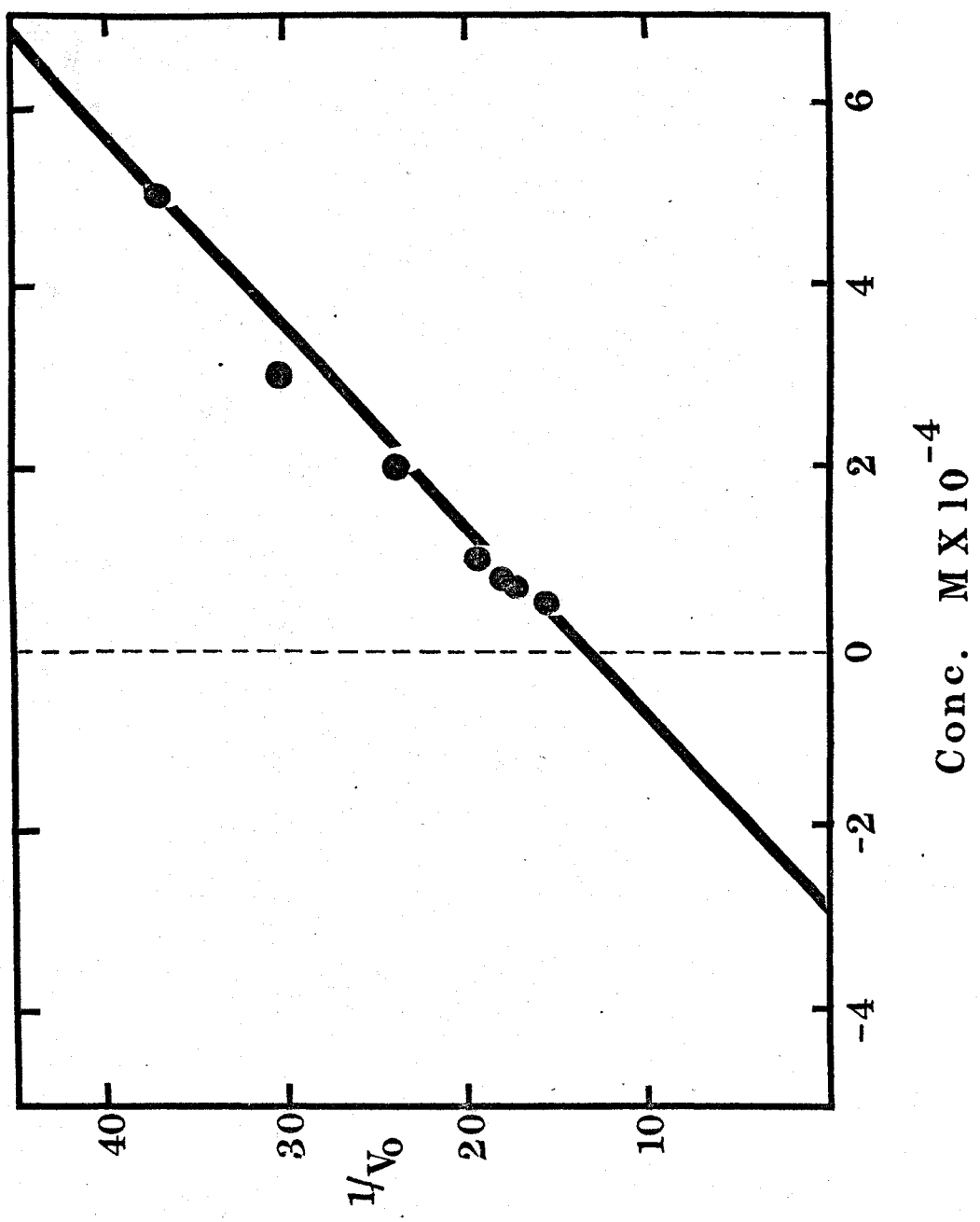


Fig. 8

Fig. 9. Plot of Reciprocal of Initial Rates against Concentration of Succinylpyruvic Acid.

Enzyme assay procedure followed that described in Methods and Materials. A Beckman DU monochromator with a spectrophotometer cell of 0.5 cm light path, 1 ml solution of 0.025M sodium phosphate buffer at pH 7.3 at 37°C were used. V_o is expressed as ΔO.D. / 5 min.

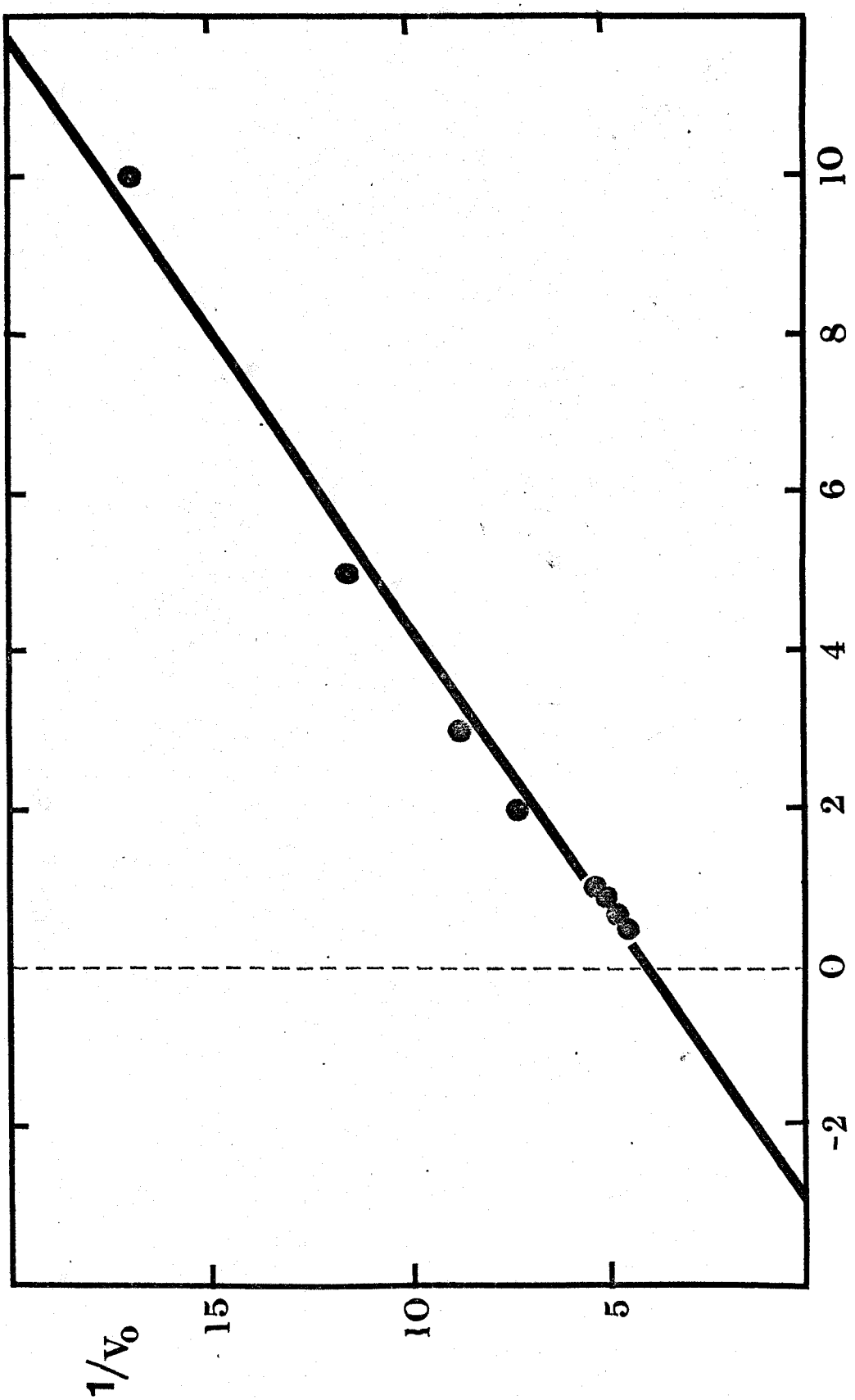


Fig. 9

Compounds which failed to serve as substrate in the enzymatic reaction include 2,4-pentanedione, 1-phenyl-1,3-butanedione, benzoacrylic acid and potassium salt of benzylidenepyruvic acid. The first two inhibited the enzymatic hydrolysis of acetopyruvic acid (Table III).

Table III. Effect of Some Compounds on the Rate of FAA'ase-catalyzed Hydrolysis of Acetopyruvic Acid

	1-Phenyl-1,3-butanedione		1,4-Pentanedione		Benzoacrylic Acid		Potassium Benzylidenepyruvate
	a	b	a	b	a	b	
Conc. (m moles)	0.5	0.25	0.5	0.5	0.5	0.25	0.15
AcPy (m moles)	0.5	1.0	0.5	1.0	0.5	0.5	0.5
Rate (ΔOD/5min)	0.018	0.026	0.048	0.054	0.051	0.052	0.043
Rate of Control (ΔOD/5min)	0.038	0.067	0.065	0.081	0.053	0.048	0.047
Activity as % of Control	46	36	74	67	96	100	92

The standard assay method stated in Methods and Materials was used. The concentration of AcPy was 5×10^{-3}M. Enzyme solution, 0.02 ml, was incubated with 1 ml buffer solution containing the stated amount of compound for 30 mins at 37°C. Rate was measured immediately following addition of acetopyruvic acid. The rate of control was measured under the same condition in the absence of compound .

2) Effect of Sodium Borohydride on Enzyme Activity

Results from the study with acetopyruvic acid, propionopyruvic acid, benzoylpyruvic acid, 2,4-pentanedione, and 1-phenyl-1,3-butanedione as substrate showed that sodium borohydride did not inhibit the fumarylacetoacetate fumaryl hydrolase (Table IV).

Table IV. Effect of Sodium Borohydride on FAA'ase Activity

	Control[a]	Pre-Incubating Substrates[b]				
		Aceto-pyruvic Acid	Propiono-pyruvic Acid	Benzoyl-pyruvic Acid	2,4-Pentane-dione	1-Phenyl-1,3-butane-dione
Concentration of pre-incubating substrate	--------	4×10^{-3}M	4×10^{-3}M	4×10^{-3}M	4×10^{-3}M	2.5×10^{-3}M[c]
Enzymatic Activity (O.D./5min)	0.324	0.294	0.313	0.306	0.315	0.329
Activity as % of Control	100	90.7	96.9	94.4	97.2	100

a FAA'ase preincubated without substrate or sodium borohydride as described in Methods and Materials.

b FAA'ase , sodium borohydride and substrate preincubated as described in Methods and Materials.

c Lower concentration of this compound was used because of the limit of its solubility in buffer solution.

3) Effect of Other Specific Reagents on Enzyme Activity

Results of the studies of the effect of specific reagents on the activity of the enzyme with acetopyruvic acid and propionopyruvic acid as substrates are shown in Table V. It is clear that all inhibit the enzyme markedly.

Table V. Effect of Specific Reagents on FAA'ase Activity

a All solution except those specified in below were prepared in 0.025M sodium phosphate buffer solution.

DDD: A 2.5 X 10^{-3}M solution was prepared in 50% alcohol. In this study, 0.4 ml of the prepared solution was diluted to 2 ml with buffer and the solution became cloudy.

HMB: A 0.02M solution was prepared according to Thibert (33). HMB, 7 gm, was dissolved in 200 ml 1N NaOH and then diluted to 1 liter. The solution was diluted ten fold with 0.2N NaOH to make the concentration to 0.002N.

HNBB: Solubility of this compound was less than 0.2 mg/ml. A saturated solution was prepared in phosphate buffer solution and the saturated solution was diluted 5 times for use in this study.

PMSF: A 5 X 10^{-3}M solution was prepared by dissolving 0.0087 gm PMSF in 3 ml 2-propanol and 7 ml buffer. 2 ml of this solution was used directly in this study.

b ΔO.D./5 min.

c Activity as % of control.

Table V. Effect of Specific Reagents on FAA'ase Activity

Reagent[a]	Conc. (m moles/2ml)	Acetopyruvate		Propionopyruvate	
		Rate[b]	Activity[c]	Rate[b]	Activity[c]
DDD	1.0	0.074	72	0.069	70
DTNB	0.4	0.051	49	0.061	65
HMB	0.4	0	0	0.023	24
HNBB	< 0.4	0.020	19	0.067	71
NaIA	60.0	0.069	67	0.069	73
NEM	12.0	0.062	60	0.054	57
PMSF	10.0	0.017	16	0.027	33
TNBS	4.0	0.015	14	0.043	46
Control	-	0.104	100	0.094	100

CHAPTER 3

DISCUSSION

A. SUBSTRATE INHIBITION

Substrate inhibition on FAA'ase was observed when succinylpyruvate and 3-phenylpropionopyruvate were used. The reaction rate decreased with increasing substrate concentration. In these cases, the Lineweaver-Burk plots were replaced by plots of the reciprocal of velocity against substrate concentration (Fig. 8,9) according to the model as follows (34):

$$E + S \overset{Ks}{\rightleftharpoons} ES$$

$$ES + S \overset{Ks'}{\rightleftharpoons} ES_2$$

$$ES \overset{k}{\rightleftharpoons} E + \text{Product}$$

The rate of reaction can be expressed as:

$$v = \frac{ke}{1 + \frac{Ks}{S} + \frac{S}{Ks'}} \qquad \text{(Eq. 3)}$$

where e is concentration of the total enzyme; S, concentration of the substrate; k, the rate constant of the break down of ES; and Ks, Ks', the dissociation constant of ES and ES_2 respectively. At high substrate concentrations, S much much greater than Ks, the term Ks/S becomes very small and the equation becomes:

$$v = \frac{V_{max}}{1 + \frac{S}{Ks'}} \qquad \text{(Eq. 4)}$$

where $ke = V_{max}$. Thus,

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{S}{Ks' V_{max}} \qquad \text{(Eq. 5)}$$

A plot of 1/v against S will give $1/V_{max}$ at the Y-axis intercept and $-Ks'$ at the X-axis intercept.

It is possible that substrate inhibition may occur at higher concentration of other substrates tested. It was indeed observed in cinnamoylpyruvic and acetopyruvic acids at concentrations higher than $5\text{-}7 \times 10^{-4}$ M (Fig. 1 & 7). Other substrates were not tested at higher concentration because of the high absorption reading which is out of the allowed range of the instrument. Similarly, limitation of the spectroscopic method used made it infeasible to use lower concentration of the inhibiting substrate, succinylpyruvate or 3-phenylpropionopyruvate, to attain a Lineweaver-Burk plot from which Ks could be calculated.

Substrate inhibition might be visualized by assuming that the enzyme may have two positively charged groups to bind with the two carboxyl groups of the natural substrate (Fig. 10). Only when one substrate molecule is correctly aligned with both these groups will the enzyme-substrate complex become effective. It is possible that a binding

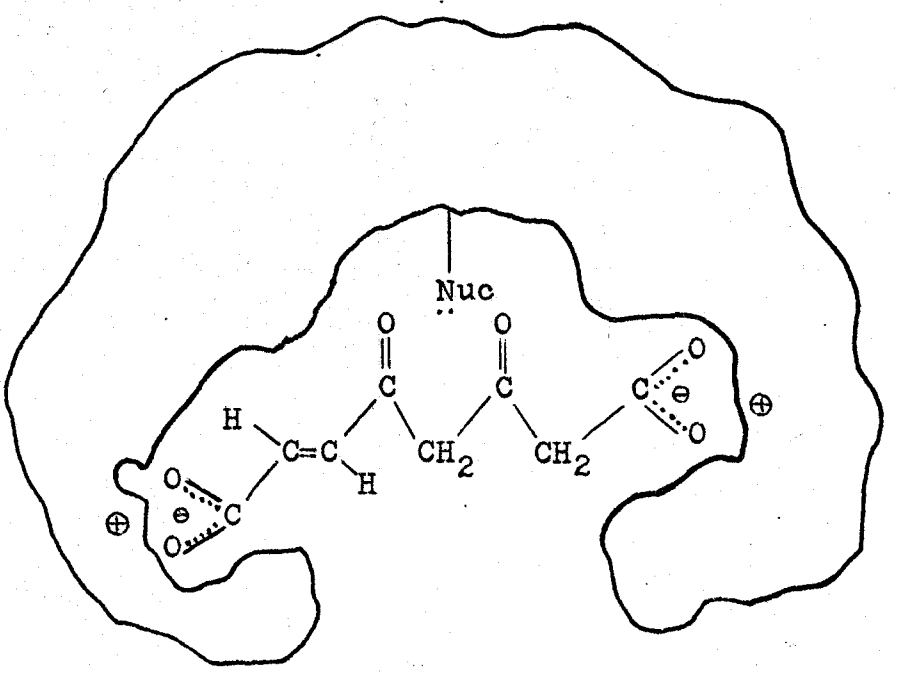


Fig. 10. Proposed Model for Binding Between Enzyme and Substrate.

site of an enzyme may be occupied by one substrate molecule, and the other site by another. Such an enzyme-substrate complex would be ineffective. At high substrate concentrations, where the substrate molecules tend to crowd onto the enzyme, the chance of formation of ineffective complexes with two substrate molecules combined with the active centre increases and hence the reaction rate may decrease correspondingly.

Since substrate inhibition for succinylpyruvate and 3-phenylpropionopyruvate is observed at concentrations as low as 5×10^{-5} M and even the Ks' is about 3×10^{-4}M, the Ks for these two must be less than 10^{-6}M. In other words, the binding between the substrate and the enzyme must be very strong. This is reasonable for succinylpyruvate because the structure of this molecule is very similar to the natural substrate, fumarylacetoacetate. However, the strong binding of 3-phenylpropionopyruvate is difficult to account for.

The behavior of cinnamoylpyruvic acid is quite unexpected. Substrate inhibition was observed from 3×10^{-4}M to 5×10^{-4}M (Fig. 7) and practically no reaction was observed when cinnamoylpyruvic acid concentration reached 7×10^{-4} M. Furthermore, the rate of reaction was much lower than that of 3-phenylpropionopyruvic acid under the same condition. Since 3-phenylpropionopyruvic acid is a relatively good substrate, cinnamoylpyruvic acid, with the presence of a double bond being more similar to the natural substrate, would be expected

to be an even better substrate in terms of chain length alone. Thus the observed effect cannot be accounted for by simple explanations. It can be speculated that the possible cis and trans structures of cinnamoylpyruvic acid do not fit the specific stero requirement of the enzyme yet its chain length is long enough to occupy both binding sites. Thus substrate inhibition occurs.

Other compounds having their chain lengths too short to occupy both of the binding sites do fit into the catalytic site, hence reaction takes place. The rate reaches a maximum with a carbon chain six units long. The relative V_{max} observed is in agreement with the results obtained by Meister (2).

B. MECHANISM

Of the site-sepcific reagents studied, all showed inhibitory effects. 2,2'-Dihydroxy-6,6'-dinaphthyl disulfide (35,36), 5,5'-dithio-bis-(2-nitrobenzoic acid) (37), hydroxymercuribenzoate (38), 2-hydroxy-5-nitrobenzyl bromide (39), sodium iodoacetate (40) and N-ethylmaleimide (40) are all known to be specific for sulfhydryl groups. Trinitrobenzenesulfonic acid is suggested as a specific alkylating reagent of amino and sulfhydryl groups (41,42), while phenylmethanesulfonyl fluoride is thought to be specific for serine (42). With the present data, one might suggest that an amino acid residue with nucleophilic character such as a sulfhydryl, hydroxyl or an amino group may be in the active site of the enzyme. Quantitative conclusions regarding the number and nature of groups at the active center cannot be derived from this study, although one might strongly suspect that one of the active site catalytic groups may be a sulfhydryl group.

Since sodium borohydride showed no effect on any of the reactions studied, formation of a Schiff-base intermediate between substrate and enzyme (Schemes 1 & 2) is unlikely. Schemes 3 and 4 remain as possible mechanisms with some preference given to Scheme 3 due to the effect of the sulfhydryl reagents. Isolation of an acyl enzyme intermediate would provide a stronger support for Scheme 3.

CHAPTER 4

SUMMARY

Acetopyruvic, propionopyruvic, butyropyruvic, pivalopyruvic, benzopyruvic, p-methylbenzoylpyruvic, 3-phenylpropionopyruvic, cinnamoylpyruvic and succinylpyruvic acids were synthesized by the condensation of ethyl oxalate with an appropriate methyl ketone in the presence of sodium ethoxide followed by base hydrolysis. Reactions of these compounds with the enzyme, 4-fumarylacetoacetate fumaryl hydrolase isolated from beef-liver, was studied by a spectrophotometric method. Substrate inhibition was demonstrated with succinylpyruvic and 3-phenylpropionopyruvic acid as substrates. Two binding sites with positively charged groups were proposed to be present in the active center of the enzyme. The enzyme was not inhibited by sodium borohydride but was affected by sulfhydryl reagents and certain other specific reagents. It was suggested that the mechanism of the enzyme-catalyzed hydrolysis involves a nucleophilic group such as a sulfhydryl or a hydroxyl on the enzyme which may form an acyl intermediate with the 2,4-diketo acids in the manner similar to the α-chymotrypsin-catalyzed hydrolysis of esters or amides.

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