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Kinetic studies of the hydrolysis of 3,5-diketo acids by fumarylacetoacetate fumarylhydrolase.

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KINETIC STUDIES OF THE HYDROLYSIS OF 3,5-DIKETO ACIDS BY FUMARYLACETOACETATE FUMARYLHYDROLASE

BY

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ABSTRACT

6,6-Dimethyl -3,5-dioxo-heptanoic acid, 3,5-dioxo-hexanoic acid, 5-phenyl -3,5-dioxo-pentanoic acid and fumarylacetone were synthesized. The kinetics of the hydrolysis of these compounds by fumarylacetoacetate fumarylhydrolase were studied. The kinetic parameters of these compounds were compared with the kinetic parameters obtained for 2,4-diketo acids previously. The effect on activity, by fluoride, p-hydroxymecuribenzoate, cyanide, dihydroxytartaric acid, tartronic acid, oxalacetic acid, pyruvic acid and acetoacetic acid was also studied in order to further elucidate the mechanism of FAH.

ACKNOWLEDGEMENTS

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I am indebted to Hamdy Khalil for his preparation of the figures, and Mrs. H. Angus for the typing of this work.

To my parents

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ABBREVIATIONS

6,6-DM	6,6-dimethyl -3,5-dioxo-heptanoic acid
2,4-DNP	2,4-dinitrophenylhydrazone
ϵ max	maximum molar extinction coefficient
FAH	fumarylacetoacetate fumarylhydrolase
3,5-HA	3,5-dioxo-hexanoic acid
p-HMB	p-hydroxymecuribenzoate
O.D.	optical density
5-ϕ	5-phenyl -3,5-dioxo-pentanoic acid
PLP	5'-pyridoxal phosphate
S.A.	specific activity

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CHAPTER I

INTRODUCTION

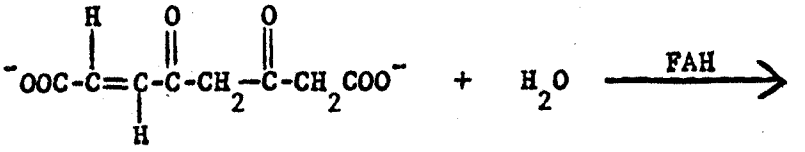
Enzymes catalyze thousands of chemical reactions in biological systems that would be difficult to accomplish otherwise. As a result the study of their mechanisms is a very important area in biochemical and chemical research. One group of enzyme mechanisms that has received little attention is the hydrolysis of carbon-carbon bonds. This type of mechanism is found in the catabolism of amino acids. This breakdown is usually accomplished by the oxidation of the amino acids into a form that can be used by the tricarboxylic acid cycle. Phenylalanine and tyrosine are ultimately metabolized to fumarate and acetyl CoA. In mammalian systems, this last degradation step, unique to the metabolism of tyrosine and phenylalanine, is accomplished by the enzyme fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2) which hydrolyses fumarylacetoacetate to fumarate and acetoacetic acid (Ravdin and Crandall, 1951). The acetoacetic acid can be converted to acetoacetyl CoA which is further degraded to acetyl CoA. Fumarylacetoacetate fumarylhydrolase catalyzes the hydrolysis of a carbon-carbon bond between a methylene carbon and an adjacent carbonyl carbon. Other examples of enzymes which catalyze carbon-carbon bond hydrolysis are oxaloacetate acetyl-hydrolase (EC 3.7.1.1) from Aspergillus niger which catalyzes the formation of oxalate and acetate from oxalacetate (Hayaishi et al.,1956) and L- kynurenine hydrolase (EC 3.7.1.3) which catalyzes the formation of anthranilate and L- alanine from kynurenine (Jakaby and Bonner,1953). Chymotrypsin, in addition to its proteolytic activity, is known to catalyze the hydrolysis of ethyl-(p-hydroxyphenyl)-3-ketovalerate to

3-(p-hydroxyphenyl) propionate and ethyl acetate (Doherty,1955). In an analagous manner, trypsin cleaves ethyl 5-(p-aminophenyl)-3-keto-valerate (Roget and Calvet,1962). In a bacterial system, the pathway for the degradation of gentisic acid includes the enzyme fumarylpyruvate hydrolase, which hydrolyses fumarylpyruvate to fumarate and pyruvate (Lack,1961). Maleylpyruvase, hydrolyses maleylpyruvate to pyruvate and malate in Pseudomonas 2,5 (Hopper et al.,1971). Recently acetyl-pyruvate hydrolase, which is the terminal inducible enzyme of the pathway of orcinol catabolism in Pseudomonas putida, has been isolated(Davey and Ribbons,1975). It catalyzes the quantitative conversion of acetyl-pyruvate into acetate and pyruvate with Mn^{++} as an activator. 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 (Miller and Adelberg,1953; Longenecka and Snell,1955) little work has been done on the mechanism of hydrolysis of carbon-carbon bonds by these enzymes.

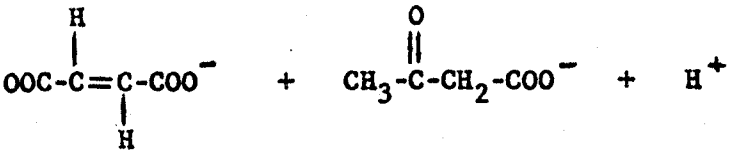
Meister and Greenstein in 1948 isolated an enzyme from liver and kidney that cleaved acetopyruvate to pyruvate and acetate. In the following year Connors and Stotz reported the isolation of triacetic acid hydrolase from beef liver. Further work (Ravdin and Crandall,1951; Hsiang et al.,1972) showed that these two enzymes were probably fumarylacetoacetate fumarylhydrolase. Further purification and characterization studies have been done lately on the enzyme (Mahuran, 1975). Brock and Williamson (1968) have also isolated a diketo hydrolase from rat liver, which is most likely FAH.

The natural substrate for FAH is fumarylacetoacetate (Ravdin

and Crandall,1951; Mahuran,1975).

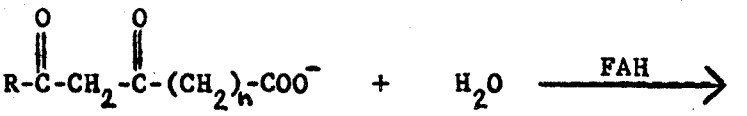


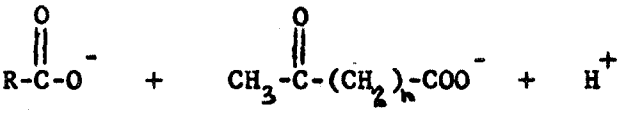
Fumarylacetoacetate



Fumarate Acetoacetate

It has also been shown, that the beef liver enzyme hydrolyses 2,4-diketo acids (Meister and Greenstein,1948; Sim,1971; Braun,1973).





where n = 0,1.

Recent work with FAH suggests that part of the mechanism of hydrolysis involves a Schiff-base intermediate (Nagainis,1975) at the 3-keto group of the substrate which is then liberated by hydrolysis. It has been shown that there is a free sulfhydryl group at or near the active site of FAH, and that altering it irreversibly inhibits FAH activity (Nagainis,1975). From previous work on the mechanism, it has been suggested that the binding of fumarylacetoacetate is through both of the carboxyl groups. So that the active site also has two positively charged groups to bind the two end carboxyl groups of the substrate, a sulfhydryl group to attack the 5-carbonyl group and a possible lysine amino group to form a Schiff-base with the 3-carbonyl group.

The following work was done in order to further previous work on the mechanism of FAH. First by synthesizing 3,5-diketo acids and fumarylacetone, which are closer in structure to the actual substrate, and to study their kinetics with the enzyme.

CHAPTER II

EXPERIMENTAL

A. Methods and Materials

1) General

Infrared (ir) spectra were recorded on Beckman IR-12 instrument in 10% chloroform solution. Nuclear magnetic resonance (nmr) spectra were obtained on a JEOLCO C60 HL and on a Varian EM 360 spectrometers and are reported in parts per million (δ) downfield from tetramethylsilane as internal standard. The splitting pattern of each resonance is codified as follows : s = singlet, q = quartet, m = multiplet and b = broad. The ultraviolet wavelength maxima of the 3,5- diketo acids and fumarylacetone, in 0.025 M sodium phosphate buffer at pH 7.3,were determined using a Beckman ACTA MVI spectrophotometer and the extinction coefficients were determined using a Gilford Model 2000 absorbance recorder attached to a Beckman DU monochromator. A Fisher - Johns melting point apparatus was employed to determine melting points. Analysis on the 6,6- dimethyl- 3,5- dioxo- heptanoic acid was performed by A.B. Gygli Microanalysis Laboratory, Toronto, Ontario. Extinction coefficients of the 3,5- diketo acids and fumarylacetone were obtained from a least squares computer program.

2) Materials

The following materials were commercially available; diisopropylamine, 2,4- pentanedione, 1- phenyl- 1,3- butanedione, dihydroxytartaric acid-sodium salt, (Aldrich); cis-oxalacetic acid, acetoacetic acid, pyruvic acid, p-hydroxymecuribenzoate (Sigma); sodium cyanide, sodium fluoride (Fisher); n-butyl lithium (ALFA). Tartronic acid

was made by the decarboxylation of dihydroxytartaric acid (Aldrich) and re-crystallized from benzene-acetone mp 157-159° [lit. 158-159°, Fenton (1898)]. 5,5- Dimethyl -2,4- hexanedione was made by the method of Adams and Hauser (1944).

3) Generation of Dianion of Diketones

The procedure for the synthesis of the dianion was essentially the same as Huckin and Weiler (1974). A sample of 3.39 g (33.6 m mol.) of diisopropylamine (dried over potassium hydroxide) was weighed into an oven dried 100 ml, 3 neck round bottom flask with two removable glass stoppers and a rubber septum. Tetrahydrofuran (50 ml, dried over lithium aluminum hydride) was distilled directly into the flask. This flask was flushed with nitrogen, stoppered and cooled in ice. n-Butyl lithium (17.55 ml of 2.0 M in hexane, 35.1 m mol.) was added dropwise by syringe through the septum. The resulting faint yellow solution was stirred for 30 min. at 0°. Then a sample of 5.5 m mol. of the diketone was added to the solution by syringe through the septum. The coloured solution was then stirred for 30 min. and kept under a nitrogen atmosphere.

This procedure was used for the generation of the dianions of 5,5- dimethyl -2,4- hexanedione, 2,4- pentanedione and 1- phenyl -1,3- butanedione. The 1- phenyl- 1,3- butanedione was first dissolved in tetrahydrofuran (dried over lithium aluminum hydride) and then added by syringe.

4) Synthesis of 5- Phenyl -3,5- dioxo-pentanoic acid

The solution of the reddish-brown dianion of 1- phenyl -1,3 butanedione was stirred for 30 min. at 0°. Carbon dioxide gas (dried by passing it through calcium chloride) was added by syringe needle through

the septum into the solution for 1.5 hours (the solution's colour had changed to orange). The purification of the acid was similar to that of Harris and Harris (1966) with these variations. After stirring for an additional 30 min, the solution was then added to a mixture of 300g of ice, 15 ml of concentrated hydrochloric acid, 50 ml of ether and 50 ml of tetrahydrofuran. A yellowish slush formed and was removed by filtration to separate the aqueous and organic mixture. The aqueous layer was separated and extracted with 3 x 30 ml of diethyl ether. The organic layers were combined and extracted several times with a cold, 5% solution of sodium bicarbonate. The alkaline extract was then acidified to pH 1 with cold, concentrated hydrochloric acid during which the temperature was maintained at 0°. The light-brown precipitate was then collected and air dried at room temperature. The precipitate was dissolved in anhydrous ether and decolourizing charcoal was added. The mixture was filtered and the solvent removed. The solids were recrystallized from ether-hexane: yield 27%, mp 93-94° [lit mp 93.5-94° (Harris and Harris, 1966)]; ir ($CHCl_3$) 1720, 1610 cm^{-1}; nmr ($CDCl_3$) δ 3.58 (s,2), 6.28 (s,1), 7.42 (m,3), 7.80 (m,2), 11.4 (b,1), 15.6 (b,1).

5) Synthesis of 3,5-Dioxo-hexanoic acid

The solution of the bright yellow dianion of 2,4-pentanedione was stirred for 30 min at 0°. Carbon dioxide gas (dried by passing it through calcium chloride) was added by syringe needle through the septum into the solution for 1.5 hours (a light yellow solid formed). The purification of the acid was similar to that of Harris and Harris (1966) with these variations. After stirring for an additional 30 min, the reaction mixture was added to a mixture of 300g of ice, 15 ml of concentrated hydrochloric acid, 50 ml of ether and 50 ml of tetrahydrofuran. A yellowish slush was formed and was removed by filtration in order

to separate the aqueous and organic mixture . Then the aqueous layer was separated and extracted with 3 x 30 ml of ether. The organic layers were combined and extracted several times with a cold, 5% solution of sodium bicarbonate. The alkaline extract was then acidified to a pH of 1 with cold, concentrated hydrochloric acid during which the temperature was maintained at 0°. This acidified solution was then extracted with 3 x 30 ml of ether. The ethereal extract was washed with water and dried with magnesium sulphate. The ethereal solution was filtered and the solvent removed. The syrupy residue was purified to a crystalline solid by repeated re-crystallizations from ether- petroleum ether in a dry ice- acetone cold bath (-78°). The residue was dissolved in ether- petroleum ether then cooled in a bath until white solids were formed. These solids were decanted off and re- crystallized from the ether- hexane in the dry ice- acetone bath: yield: 35%, mp 29-31° [lit mp 29-31° (Witter and Stotz, 1948)] ; ir ($CHCl_3$) 1720, 1620 cm^{-1}, nmr ($CDCl_3$) δ 2.02 (s,3), 3.33 (s,2), 5.56 (s,1), 10 (b,1).

6) Synthesis of 6,6- Dimethyl- 3,5- dioxo-Heptanoic acid

The solution of the bright yellow dianion of 5,5- dimethyl- 2,4- heptanadione was stirred for 30 min. at 0°. Carbon dioxide gas (dried by passing it through calcium chloride) was added by syringe needle through the septum into the solution for 1.5hours (the solution turned orange). The purification of the acid was similar to that of Harris and Harris (1966) with these variations. After stirring for an additional 30 min, the solution was then added to a mixture of 300g of ice, 15 ml of concentrated hydrochloric acid, 50 ml of ether and 50 ml of tetrahydro- furan. A yellowish slush was formed and was removed by filtration to separate the aqueous and organic mixture. The aqueous layer was separated and extracted with 3 x 30 ml of ether. The organic layers were combined

and extracted several times with a cold, 5% solution of sodium bicarbonate. The alkaline extract was then acidified to pH 1 with cold, concentrated hydrochloric acid during which the temperature was maintained at 0°. This acidified solution was then extracted with 3 x 30 ml of ether. The ethereal extract was washed with water and dried with magnesium sulphate. The ethereal solution was filtered and the solvent removed. The residue was put in hexane and cooled in a -20° freezer until a white, crystalline solid formed. It was re-crystallized from hexane-ether twice: yield: 30%, mp 43-45°; ir ($CHCl_3$) 1720, 1600 cm^{-1}; nmr ($CDCl_3$) δ 1.16 (s,9), 3.39 (s,2), 5.67 (s,1), 10 (b,1).

Analysis: Calcd: C 58.06% H 7.58%
Found: C 57.80% H 7.80%

7) Synthesis of Fumarylacetone

The procedure for the synthesis of fumarylacetone was essentially the same as the procedure of Fowler and Seltzer (1970) with this variation. The light tan needles were re-crystallized from benzene: yield 35%, mp 162-165° [lit mp 162-166°]; nmr (d_6- acetone) δ 2.24 (s,3), 6.04 (s,1), 6.60 and 7.00 (q,2).

8) Beer-Lambert Plots of 3,5-diketo acids and Fumarylacetone

A plot of absorbance versus concentration was done for the 3,5-diketo acids and fumarylacetone to determine their molar extinction coefficients. Each cuvette (1.0 cm path length, 3.0 ml volume) contained the appropriate volume of the stock solution of 10^{-3} M of the compounds in 0.025 M sodium phosphate buffer, pH 7.3, which was then diluted to volume with 0.025 M sodium phosphate buffer, pH 7.3.

9) Enzyme Assays

The rate of fumarylacetoacetate fumaryl hydrolase- catalyzed hydrolysis of the 3,5— diketo acids was determined by observing the initial rate of decrease in absorption at their wavelength maxima. All kinetic data were obtained on a Gilford Model 2000 absorbance recorder attached to a Beckman DU monochromator. The temperature of all assay systems was maintained at 30.0 ± .5° by circulating an ethylene glycol-water solution from a thermostated bath through the cell compartment. All reagents were pre-incubated at 30.0 ± .5°.

The enzyme was isolated and purified according to the methods of Hsiang et al.(1972) and Mahuran (1975). The enzyme was thawed out, as required, in 1 ml portions and stirred constantly with a magnetic stirrer in an ice bath while the assay proceeded. The volume of enzyme used was either 25 or 50 µl (by syringe) depending on the substrate. The cuvettes used had a .5 cm path length (1.5 ml volume) in all assays to facillitate high substrate concentrations in order to obtain more accurate Vmax and Km values. Substrate solutions were made up fresh each day and the buffers were made up every 3 to 4 weeks. All cuvettes and syringes were siliconized before use in order to make the glass surfaces inert to adsorption of protein from dilute enzyme solutions.

10) Definition of Enzyme Activity

In order to maintain a constant enzyme activity factor in the kinetic data, enzyme controls were run with each experiment. This activity factor will depend on what we define as a unit of enzyme and the purity of the protein, or specific activity.

One unit of enzyme activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 µmole of substrate per minute under standard conditions. Acetopyruvate is the substrate

that is used to determine enzyme activity. Therefore: 1 unit of enzyme = amount of enzyme that hydrolyses 1 μmole of acetopyruvate/min.

$$= \frac{\text{OD/min.}}{7.8\ \text{ml/}\mu\text{mole}} \times (\text{dilution factor})_{\text{ACTIVITY}} \times (\text{vol. of enzyme solution})$$

The specific activity is defined as units of enzyme activity divided by the amount of total protein in solution.

Therefore: Specific Activity $= \frac{\text{units}}{\text{mg}_{\text{ENZYME}}}$

$$= \frac{\text{OD/min.}}{7.8\ \text{ml/}\mu\text{mole}} \times (\text{dilution factor})_{\text{ACTIVITY}} \Big/ (\text{mg/ml})_{\text{PROTEIN}} \times (\text{dilution factor})_{\text{PROTEIN}}$$

From this it has been found that after the final step of purification of the enzyme, a value was obtained for the specific activity of 35×10^{-2} μmole/min/mg protein.

Enzyme activity controls were done before and after each kinetic run. In each case 0.3 ml of 10^{-3} M acetopyruvate was diluted with 2.65 ml of .025 M sodium phosphate buffer pH 7.3. This gave a final acetopyruvate concentration of 1×10^{-4} M. Enzyme (50 μl) was added to the cuvette (1.0 cm path length, 3.0 ml volume) and the rate of decrease in absorbance was measured. All rates were then corrected to a constant control enzyme activity of 25.6×10^{-6} M of acetopyruvate hydrolysed per minute in the presence of 10^{-4} M acetopyruvate by multiplying all rates by the factor:

$$\frac{\text{OD, of } 25.6 \times 10^{-6}\text{ M acetopyruvate}}{\text{OD per minute for control}} \qquad \text{Eq.(1)}$$

to obtain the numerator of Eq.(1), the expression used is:

$A = \epsilon\, c\, l$ $\qquad l = 1.0$ cm. $\epsilon = 7800 \frac{1}{\text{mole} \cdot \text{cm}}$

c = concentration (25.6×10^{-6} M)

A = absorbance (optical density)

$$= 7800 \frac{1}{\text{mole} \cdot \text{cm}} \times 25.6 \times 10^{-6} \frac{\text{moles}}{1} \times 1.0 \text{ cm.}$$

$$= .200$$

Therefore, if OD per minute for control was .014

$$\text{Control factor} = \frac{.200}{.014} = 14.21$$

This constant enzyme activity factor must now be defined in terms of purity of active protein. This is done by correcting the kinetic data to an enzyme of specific activity of 35×10^{-2} μmole/min/$mg_{PROTEIN}$

$$\text{Specific Activity} = \frac{\text{units}}{\text{mg}_{\cdot \text{PROTEIN}}} = \frac{\text{units/l}}{(\text{mg/l})_{\text{PROTEIN}}}$$

$$35 \times 10^{-2} \frac{\text{umole/min}}{\text{mg}} = \frac{25.6 \frac{\text{umole/min}}{1}}{X}$$

$$X = \frac{25.6}{35 \times 10^{-2}} \text{ mg/l}$$

$$= .73 \times 10^{2} \text{mg/l}$$

$$= 73 \text{ mg/l}$$

$$= .073 \text{ mg/ml}$$

Therefore, X represents the mg/l of enzyme of specific activity of 35×10^{-2} μmole/min/mg which will catalyse a hydrolysis rate of 25.6 μmole/l/min. In general, if the standard specific activity chosen is the maximum value recorded the V_{max} = S.A. max. However, due to substrate inhibition, specific activity is defined as $[S_o] \doteq$ Km for fumarylacetoacetate fumarylhydrolase with acetopyruvate as the substrate.

11) Inhibition of FAH - Catalysed Hydrolysis of 6.6 - Dimethyl 3,5-dioxo-heptanoic acid by Fluoride

The fluoride solution was prepared by dissolving sodium fluoride in water. The standard assay procedure was used. Each cuvette (0.5 cm, 1.5 ml) contained 0.05 M sodium phosphate buffer at 7.3. In one set of experiments, substrate concentration was varied while the fluoride concentration was kept constant. In another set of experiments, substrate concentration was kept constant while the fluoride concentration was varied. Hydrolysis was initiated with 25µl of enzyme.

12) Inhibition of FAH Hydrolysis of 3,5 - Diketo acids by Substrate Analogs and by p - Hydroxymecuribenzoate

The substrate analogs oxalacetic acid, tartronic acid, dihydroxytartarate, pyruvate and acetoacetate were dissolved in .025 M sodium phosphate buffer. Dihydroxytartaric acid (sodium salt) was dissolved initially in a minimum amount of 0.2 M hydrochloric acid solution then made up to volume with phosphate buffer. p - Hydroxymecuribenzoate was dissolved initially in 0.2 M sodium hydroxide solution and then made up to volume with buffer. The pH of these solutions were all checked and found to be approximately 7.3. The standard assay method was used. The enzyme (50 µl) was incubated with buffer and inhibitor (controls incubated with buffer) for two minutes and then substrate was added (2×10^{-4} M, final concentration), the absorbance was monitored for 5 minutes. All assays were done at $25.0 \pm .5^{\circ}$.

13) Effect of sodium Cynanide on Enzymatic Activity

6.6 - Dimethyl - 3,5 - dioxo - heptanoic acid and 3,5 - dioxo - hexanoic acid were used as substrates in experiments to trap a Schiff base. The method of assay and control solutions were done

according to Nagainis (1975).

14) Identification of Products

Twenty mg of 6.6 - dimethyl - 3,5 - dioxo - heptanoic acid, 3,5 - dioxo - hexanoic acid and 5 - phenyl - 3,5 - dioxo - pentanoic acid were all dissolved in 10 ml of 0.025 M sodium phosphate buffer. The pH of the resulting solutions were adjusted to 7.3 by addition of .1 M sodium hydroxide. Enzyme was added and the solutions were allowed to stand for 48 hours at room temperature. Then more enzyme was added and the solutions were allowed to stand for another 48 hours. The breakdown of the 3,5 - diketo acids was monitored by spectrophotometric measurement. The solutions were then acidified to pH 2 with a 10% solution of trichloroacetic acid. The precipitated protein was removed by centrifugation.

The protein-free solution was then treated with 3 ml of a 0.15 M solution of 2,4 - dinitrophenylhydrazine dissolved in 20 ml of concentrated sulfuric acid, 30 ml of water and 100 ml of 95% ethanol. An orange suspension of solids formed. This suspension was centrifuged and the solution was decanted off. The solids were allowed to dry and then were dissolved in .2 M sodium phosphate buffer pH 7.2. The solution was centrifuged again to remove any insoluble material and the buffer solution was decanted off. The buffer solutions of the 2,4 - dinitrophenylhydrazone were spotted on thin-layer chromatography plates and on pre-treated filter paper in order to identify the products of the enzyme hydrolysis by thin-layer and paper chromatography. The solid supports for the thin-layer chromatography were Silica Gel G-25 and Cellulose F. The development systems were ethanol : ethyl acetate (70:30) for the silica gel and tert-amyl alcohol : ethanol : water

(50:10:40) for the cellulose. In both cases the solid support was equilibrated with the solvents, overnight. The paper for the paper chromatography was Whatman No. 2 and was pretreated with a glycine-sodium hydroxide buffer pH 8.2 - 8.4 before spotting of the buffer solutions. The solvent system was tert-amyl alcohol : ethanol : water (50:10:40). The stationary phase was equilibrated in the tank with the developing solvents overnight (Altmann et al, 1951). Authentic 2,4- dinitrophenylhydrazone derivatives of pyruvate, acetoacetate and acetone were used as standards. Pyruvate and aceto-acetate derivatives were dissolved in the .2 M sodium phosphate buffer, while the acetone derivative was dissolved in diethyl ether (anyhydrous) for application to the stationary phase. The solution of the derivatives from the enzyme hydrolysis were also mixed with the solution of the acetoacetic acid derivative. The chromatograms were run and the spots were compared.

15) Interpretation of Kinetic Data

a) The preliminary values for Km and V max were obtained from Lineweaver-Burk Plots ($\frac{1}{V_o}$ vs. $\frac{1}{[S]}$).

b) The final values for Km and V max were then calculated by a least-squares non-linear computer program (Wentworth,1965). Errors were reported in terms of standard deviations.

B. Results

1) Spectra of the 3,5- Diketo acids and Fumarylacetone

Fig. 1 to 4 are the spectra obtained for the 3,5-diketo acids and fumarylacetone, in 0.025 M sodium phosphate buffer, pH 7.3.

Fig. 1. Ultraviolet absorption spectrum of 3,5-dioxo-hexanoic acid in 0.025 M sodium phosphate buffer, pH 7.3.

FIGURE 1

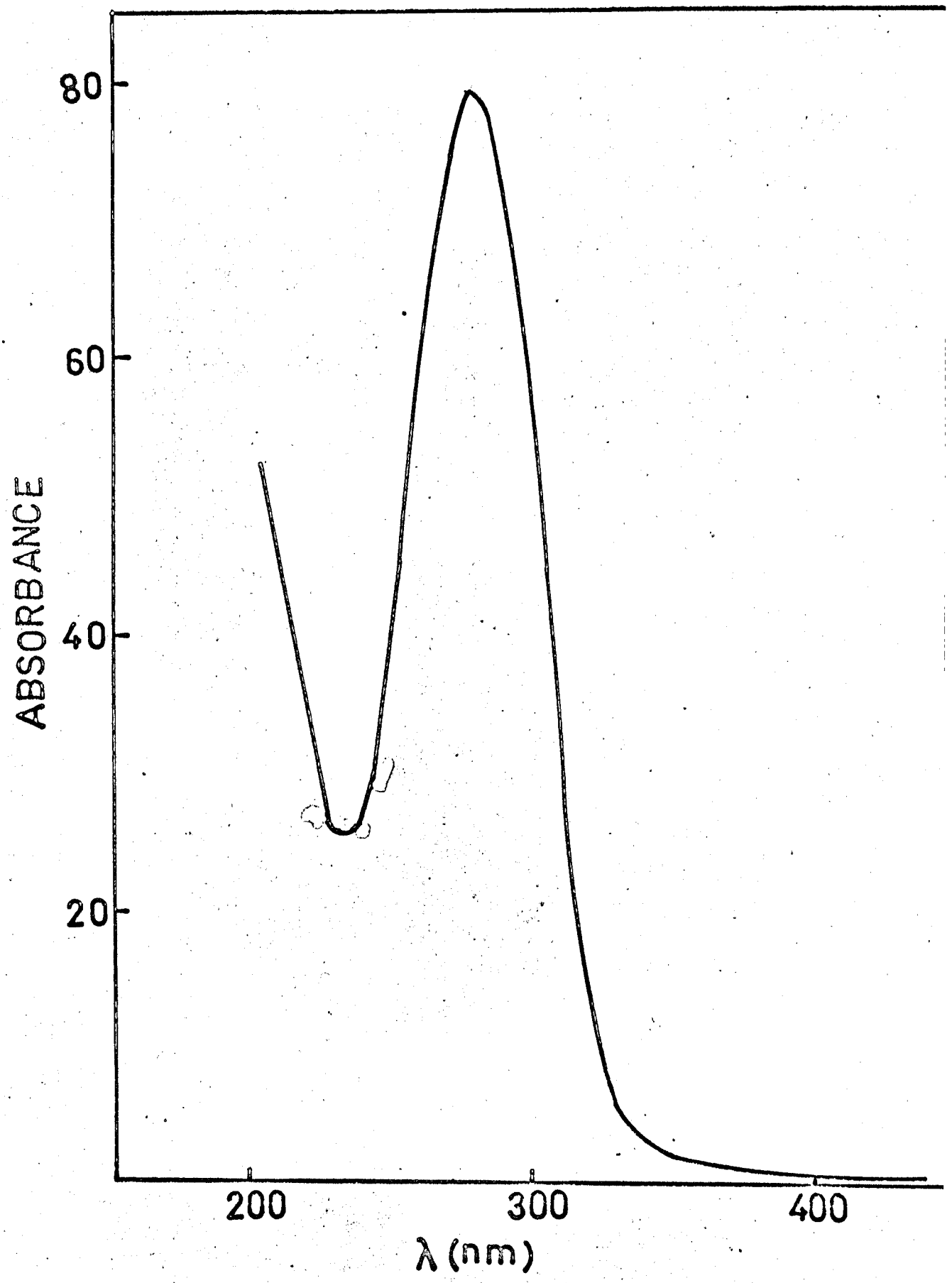


Fig. 2. Ultraviolet absorption spectrum of 6,6-dimethyl-3,5-dioxo-heptanoic acid in 0.025 M sodium phosphate buffer, pH 7.3.

FIGURE 2

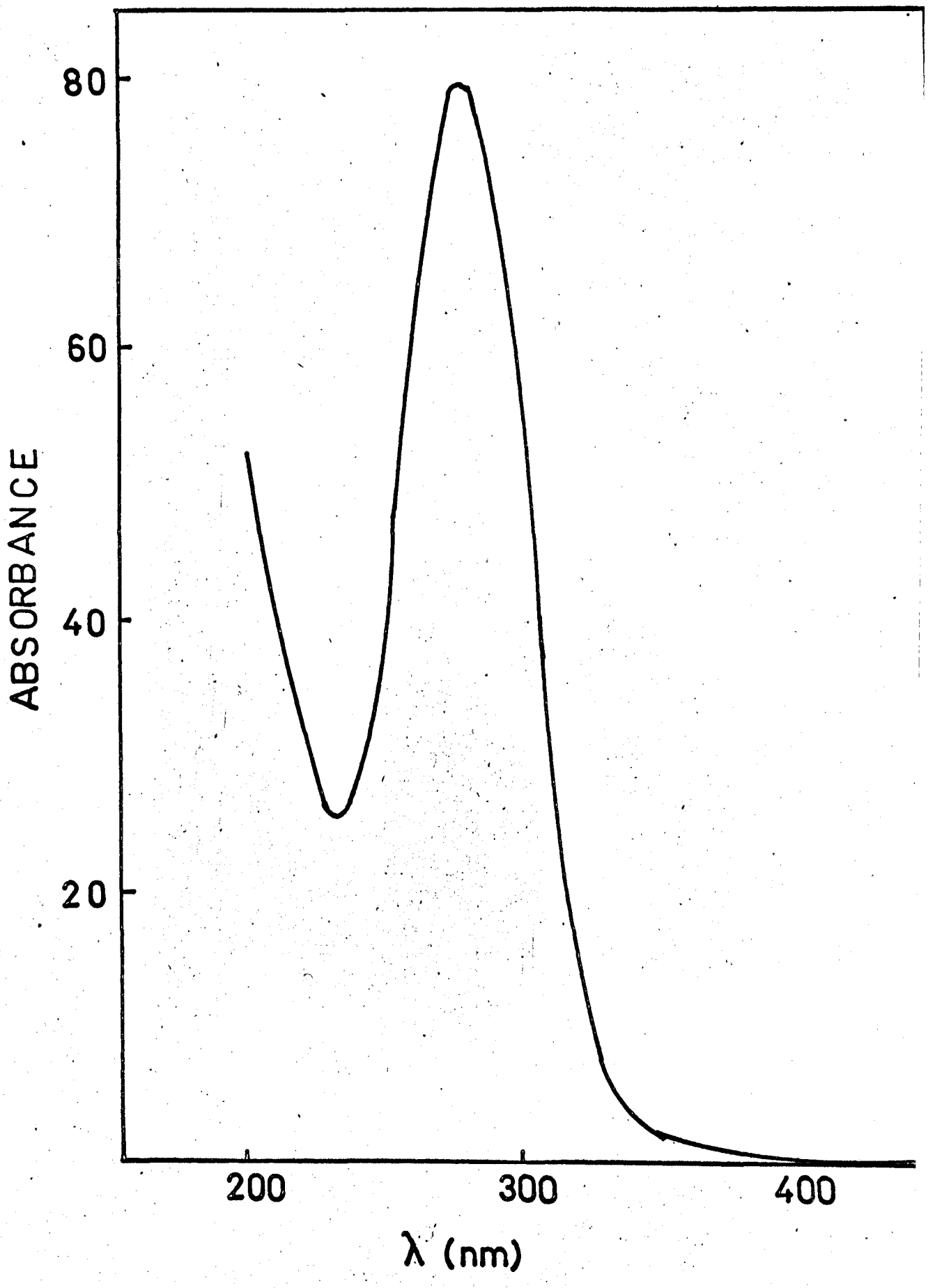


Fig. 3. Ultraviolet absorption spectrum of 5-phenyl-3,5-dioxo-pentanoic acid in 0.025 M sodium phosphate buffer, pH 7.3.

FIGURE 3

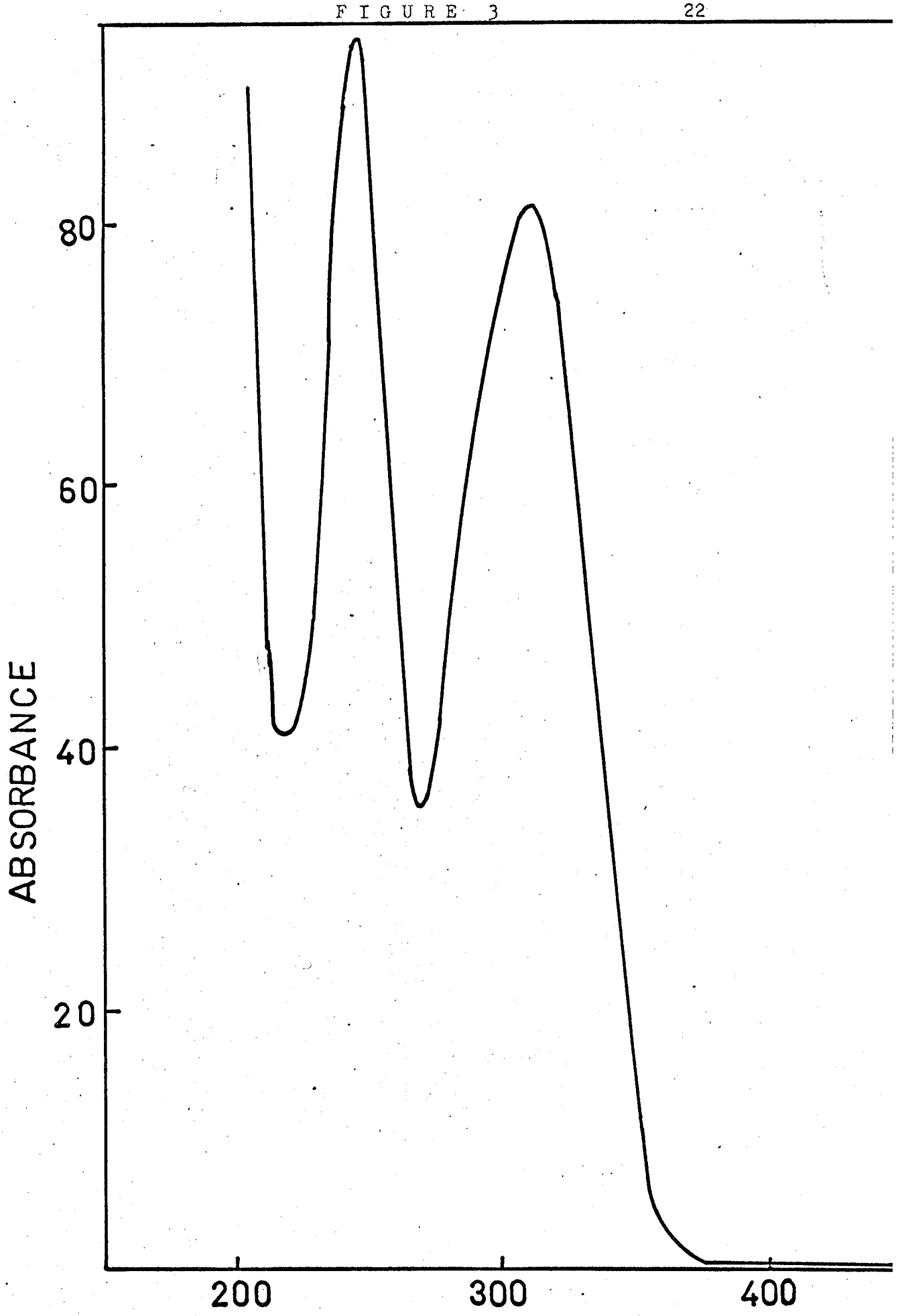
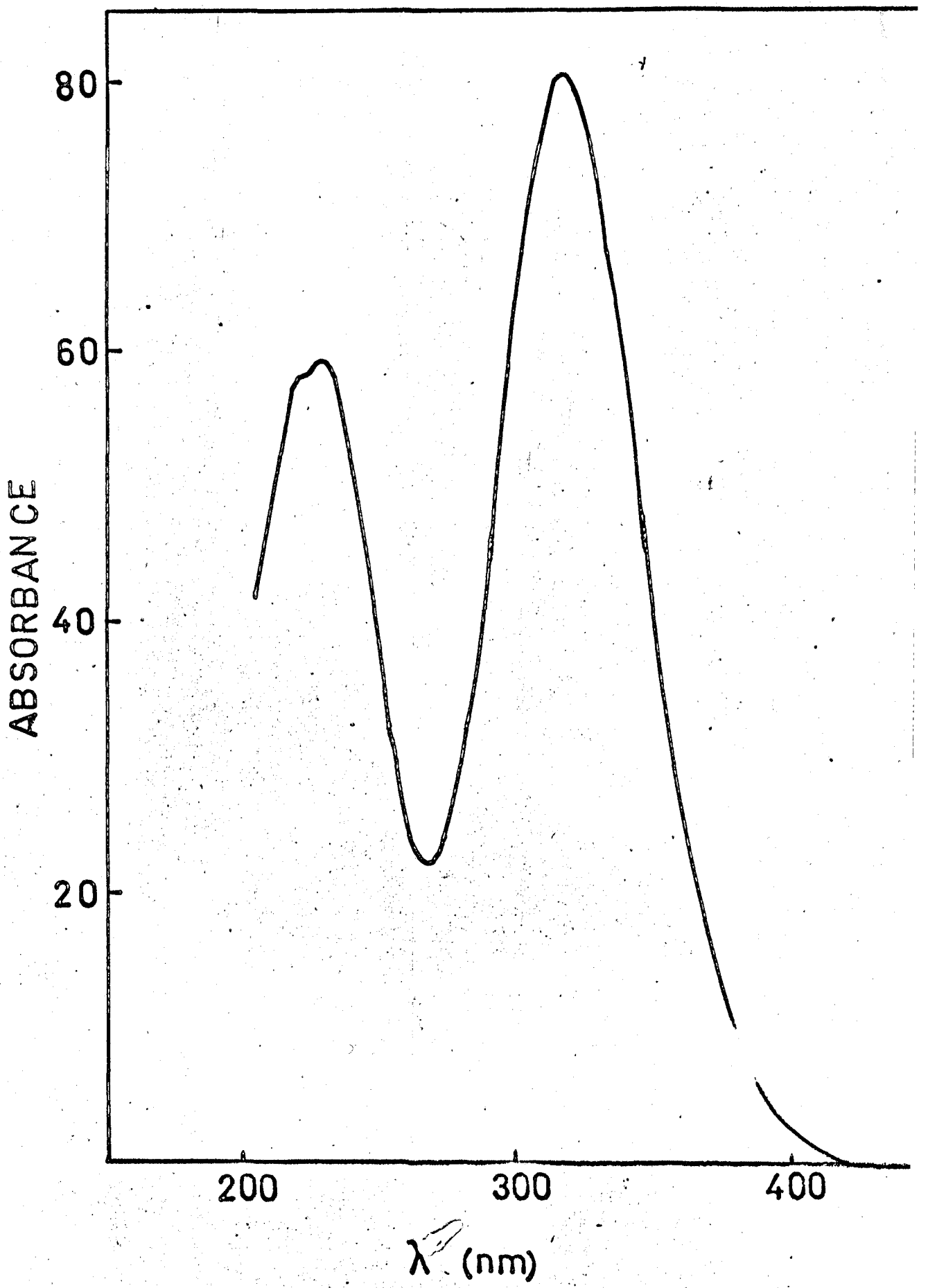


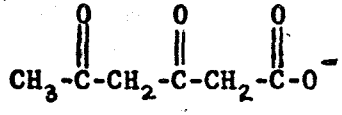
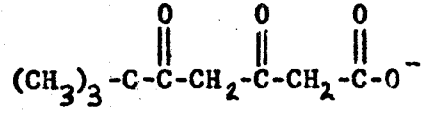
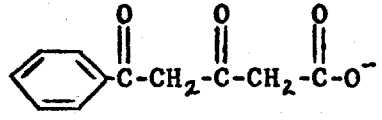
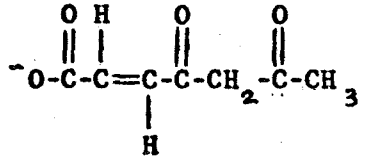
Fig. 4. Ultraviolet absorption spectrum of fumarylacetone in 0.025 M sodium phosphate buffer, pH 7.3.



2) Determination of Extinction Coefficient

The Beer-Lambert plots for the three 3,5- diketo acids and fumarylacetone were used to obtain the molar extinction coefficient (ϵ max) listed in Table I. These plots were analysed by a least-squares computer program which calculates the slope (ϵ max) of the best fitting line of absorbance versus concentration data.

TABLE I: Some Physical Properties of 3,5-Diketo Acids and Fumarylacetone

Compound	Structure	Wavelength Maximum	Maximum Molar Extinction Coefficient [a]
3,5-Dioxo-hexanoic acid		277	3184
6,6-Dimethyl-3,5-dioxo-heptanoic acid		280	3214
5-Phenyl-3,5-dioxo-pentanoic acid		318	7430
Fumarylacetone		314	9610

[a] Determined in 0.025 M sodium phosphate buffer at pH 7.3.

3) Kinetic Parameters for FAH - Catalyzed Hydrolysis of the 3,5- Diketo Acids

Fig. 5 to 7 are the Lineweaver-Burk plots for the 3,5- diketo acids. Substrate inhibition was observed at high concentrations of 3,5- dioxo-hexanoic acid as in some of the 2,4- diketo acids (Braun, 1973). For 6,6- dimethyl - 3,5- dioxo-heptanoic and 5-phenyl 3,5- dioxo-pentanoic acids the Lineweaver-Burk plots were linear throughout the concentration ranges employed . This saturation of the enzyme can be seen more strikingly in a regular rectangular hyperbole plot of initial velocity, V_o versus substrate concentration, [S]; Fig. 8 to 10.

Table II lists the Km and V max values of the 3,5- diketo acids and the 2,4- diketo acids (Braun, 1973) obtained from the least-squares non-linear computer program, which gives a best fit of the regular rectangular hyperbole plot of V_o vs. [S] . The V max values for the 3,5 and 2,4- diketo acids are defined in terms of purity of active protein. That value is 73 mg/ml of enzyme of specific activity of 35 x 10^{-2} µmole/min/mg which will catalyse a hydrolysis rate of 25.6 µmole/l/min. In general, all the Km values of the 2,4-and 3,5- diketo acids are similar.

The V max values of the 2,4-and 3,5- diketo acids are quite different. The best substrate, propionopyruvate, has a V max value 120 times larger than the worst, benzoylpyruvate.Fumarylacetone was not hydrolysed by the enzyme.

Table III lists the Rf values of the 2,4- dinitrophenyl-hydrazone derivatives of the product isolated from the enzymatic

hydrolysis of the 3,5- diketo acids and that of authentic samples of 2,4- dinitrophenylhydrazone derivatives of acetoacetic acid, pyruvic acid and acetone. The hydrolysis derivatives and acetoacetate derivatives gave two spots for the silica gel and paper chromatography. One of these spots corresponds to the acetone derivative, which was also noted by Altmann et al. (1951) and Turnock (1953) when this type of chromatography was used. This would be caused by a decarboxylation of acetoacetate derivative during chromatography. When the 2,4- DNP derivatives of the hydrolysis were spotted with the 2,4- DNP derivatives of acetoacetate, two spots were seen again as in the standard acetoacetate derivative. Cellulose F showed only one spot for the 2,4- DNP derivatives of the enzyme hydrolysis and acetoacetic acid. These spots also corresponded to the spot of the acetone derivative. This is due probably to the longer time of development for this system (48 hrs.), as opposed to the paper and silica gel (12 and 3 hrs. respectively), which allows the derivative of acetoacetate to totally decarboxylate.

Fig. 5. Lineweaver-Burk plot for 3,5-dioxo-hexanoic acid.

The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and the concentration of 3,5-dioxo-hexanoic acid indicated. The hydrolysis was initiated by the addition of .050 ml of FAH.

O- substrate inhibition

FIGURE 5

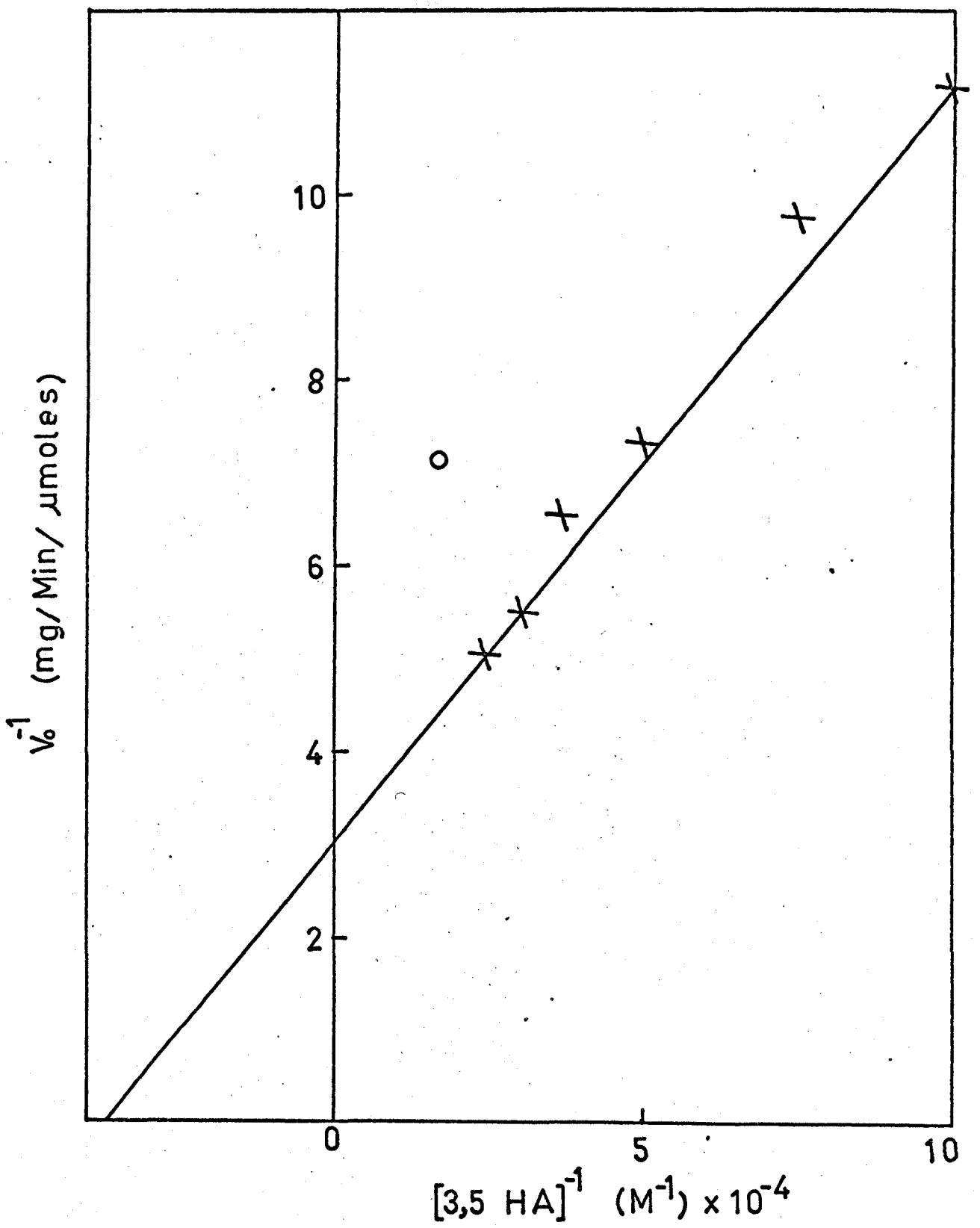


Fig. 6. Lineweaver-Burk plot for 6,6-dimethyl-3,5-dioxo-heptanoic acid. The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and the concentration of 6,6-dimethyl-3,5-dioxo-heptanoic acid indicated. The hydrolysis was initiated by the addition of .050 ml FAH.

FIGURE 6

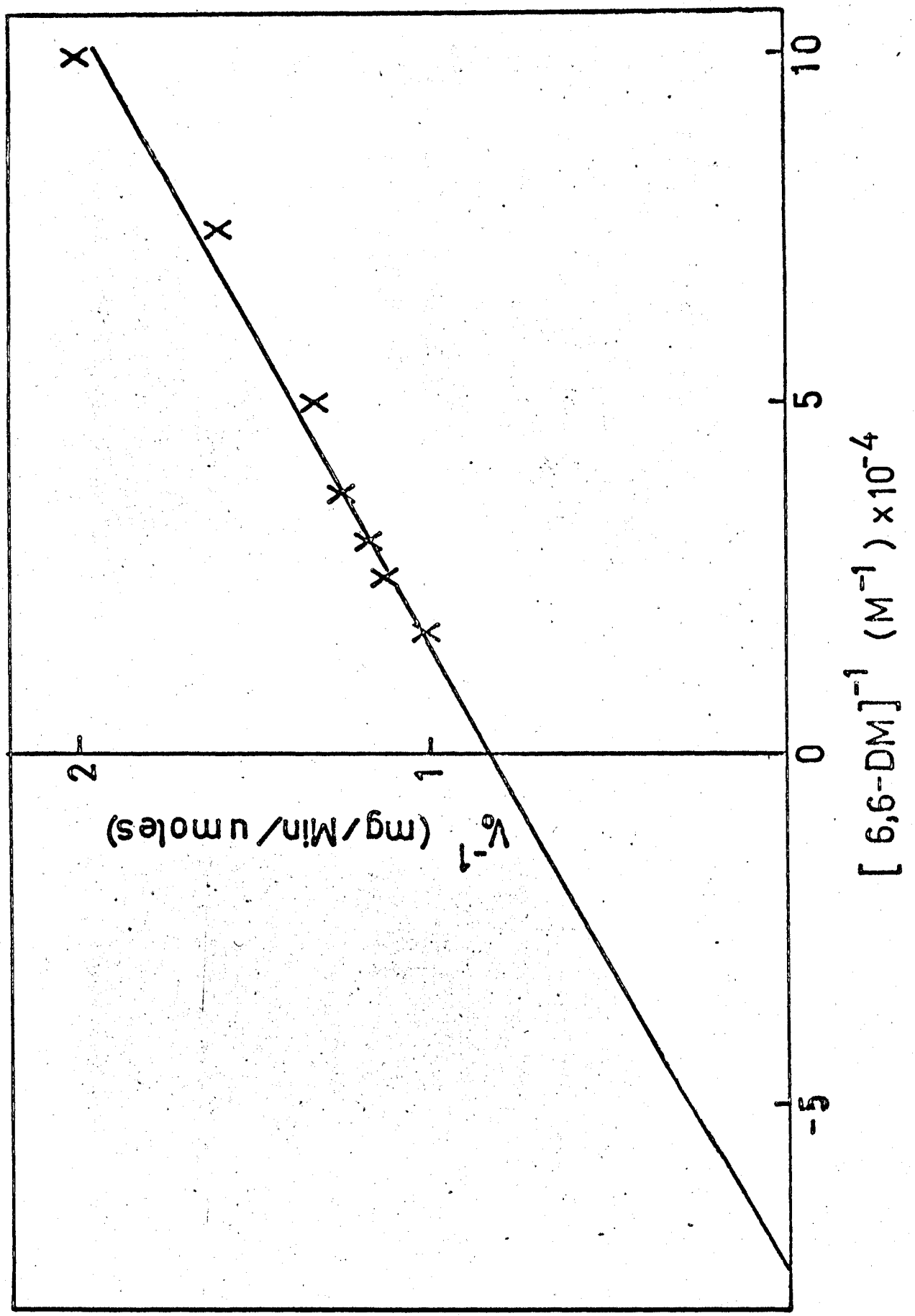


Fig. 7. Lineweaver-Burk Plot for 5-phenyl-3,5-dioxo-pentanoic acid. The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer,pH 7.3, and the concentration of 5-phenyl-3,5-dioxo-pentanoic acid indicated. The hydrolysis was initiated by .050 ml of FAH.

FIGURE 7

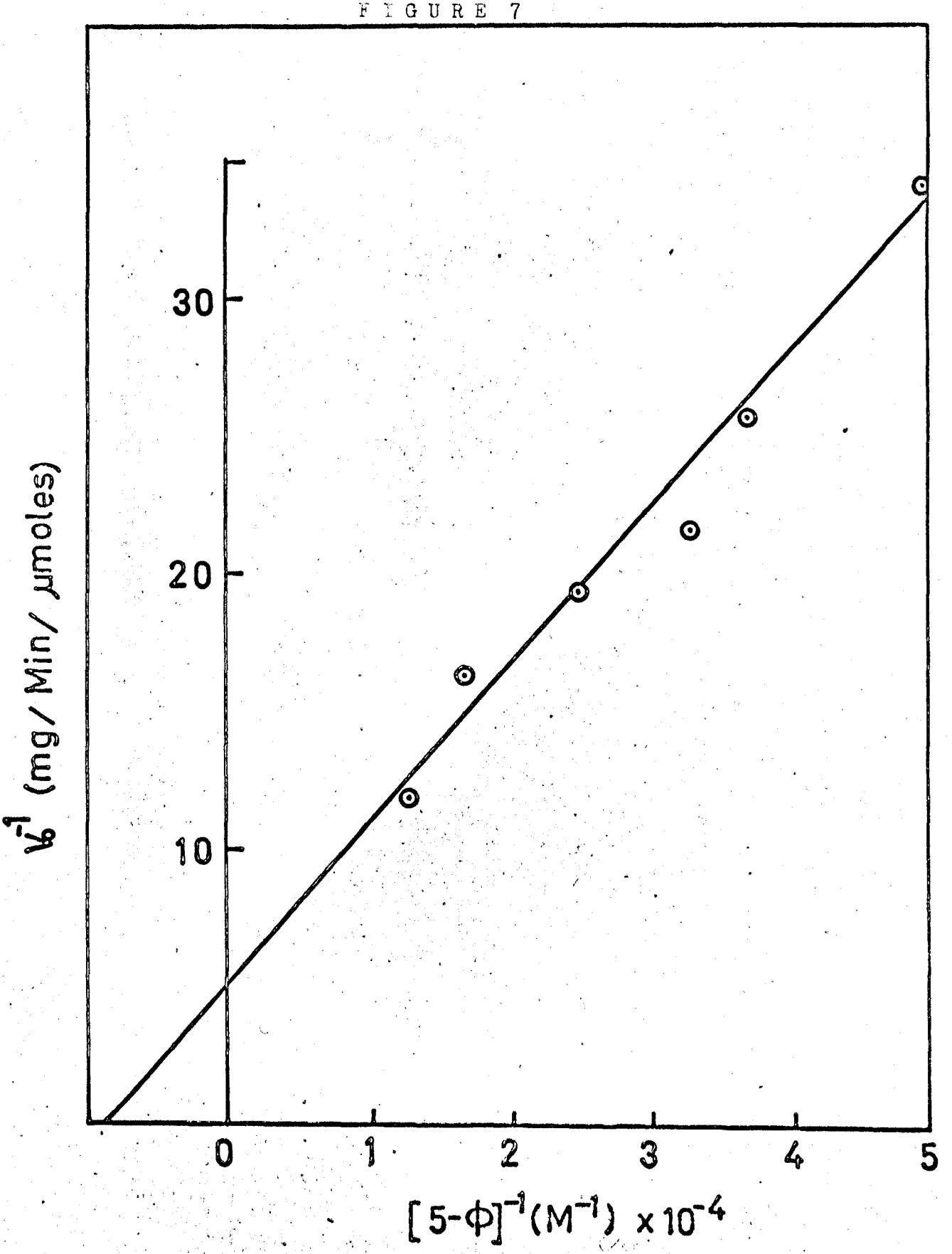


Fig. 8. Regular rectangular hyperbole plot for 3,5-dioxo-hexanoic acid. The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and the concentration of 3,5-dioxo-hexanoic acid indicated. The hydrolysis was initiated by the addition of .050 ml of FAH.

X - substrate inhibition

FIGURE 8

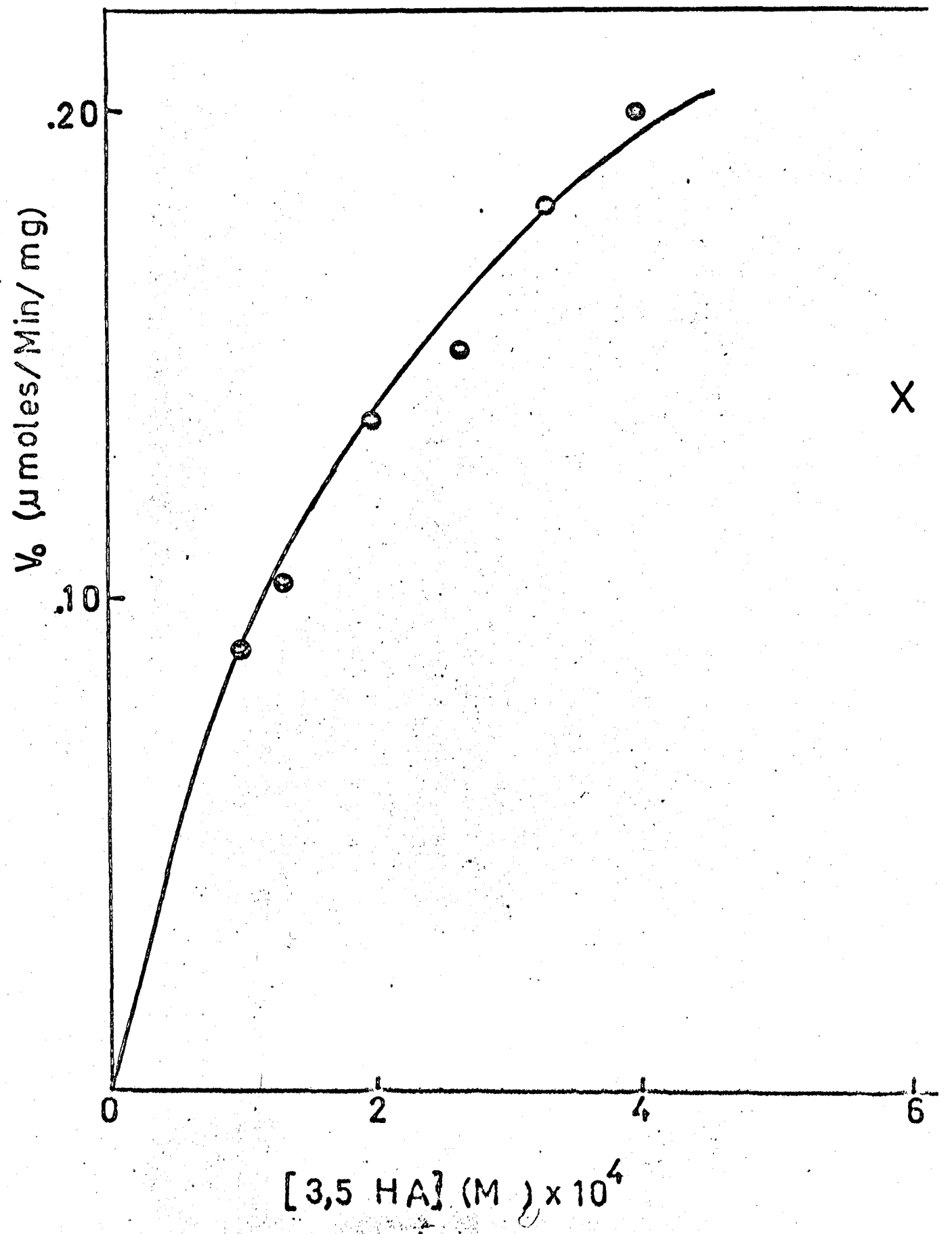


Fig. 9. Regular rectangular hyperbole plot for 6,6-dimethyl-3,5-dioxo-heptanoic acid.

The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and the concentration of 6,6-dimethyl-3,5-dioxo-heptanoic acid indicated. The hydrolysis was initiated by .025 ml of FAH.

FIGURE 9

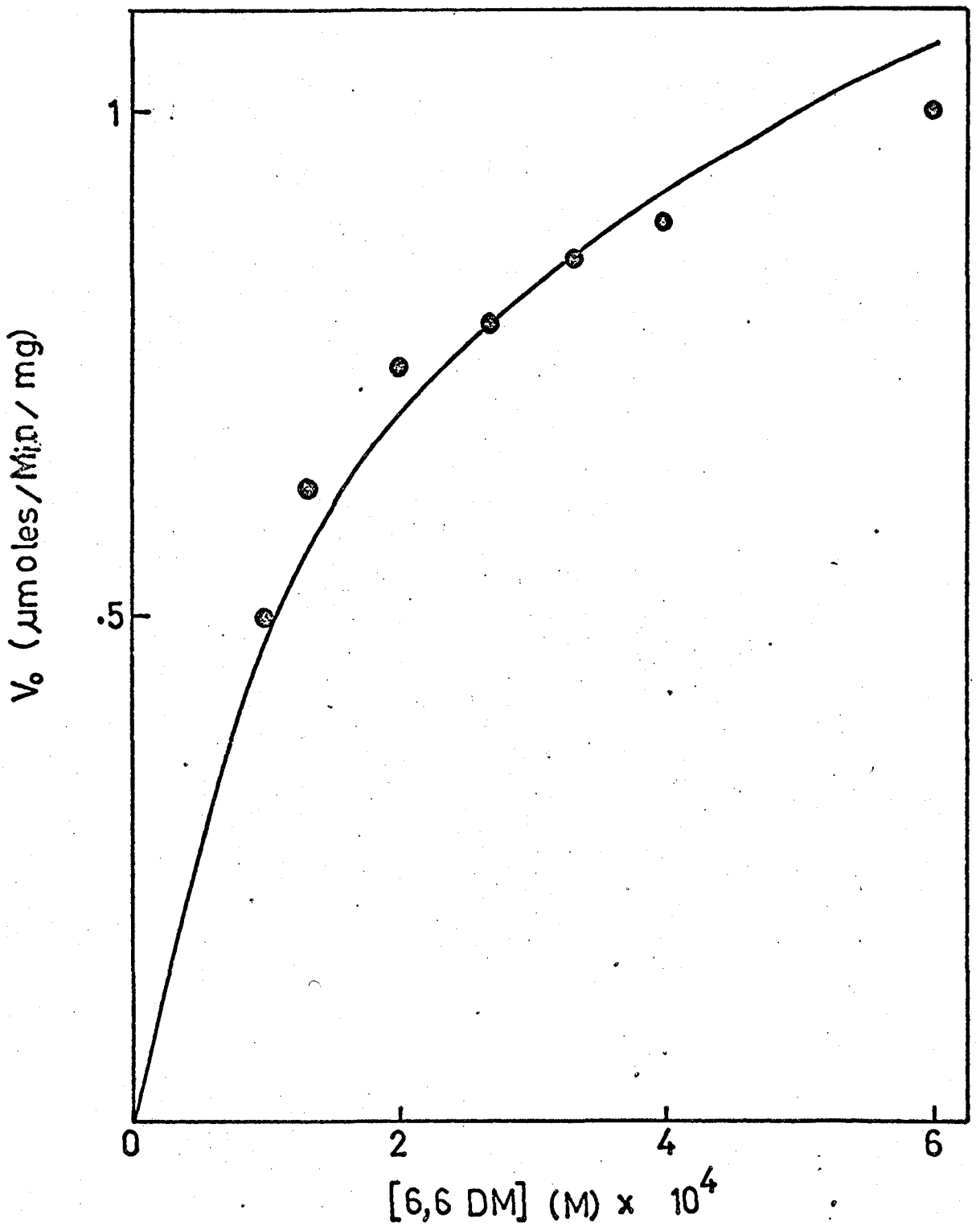


Fig. 10. Regular rectangular hyperbole plot for 5-phenyl-3,5-dioxo-pentanoic acid.

The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and the concentration of 5-phenyl-3,5-dioxo-pentanoic acid indicated. The hydrolysis was initiated by the addition of .050 ml of FAH.

FIGURE 10

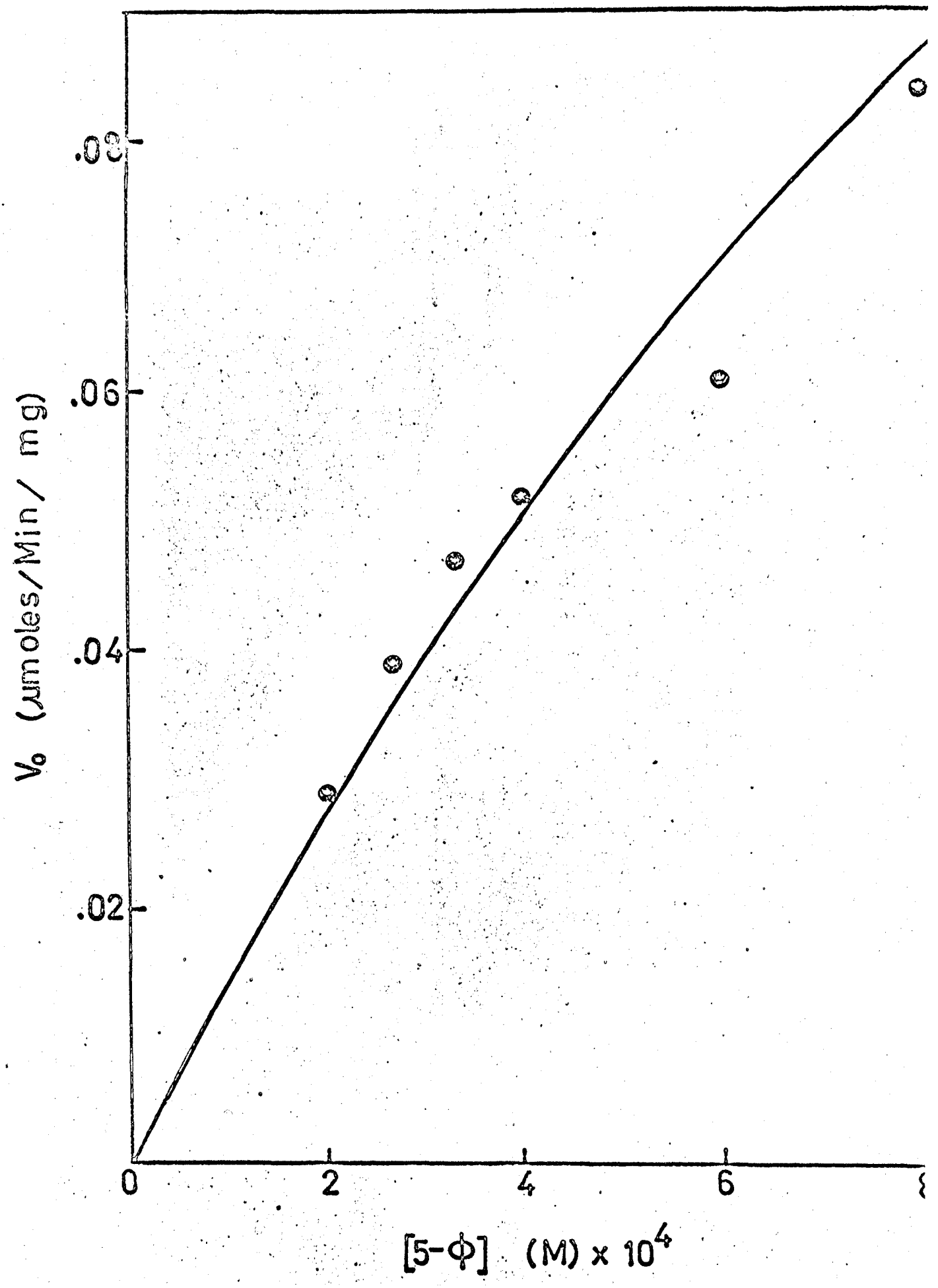


TABLE II: Kinetic Parameters for FAH-Catalyzed Hydrolysis of Selected Diketo Acids

Substrate [a]	V max (umole/min/mg)	Km x 10^4 (mole/l)
2,4-Dioxo-pentanoic acid	4.7 ± .3 [b]	4.7 ± .5
2,4-Dioxo-hexanoic acid	8.4 ± .6	4.7 ± 1.7
2,4-Dioxo-heptanoic acid	1.9 ± .3	3.1 ± .8
5,5-Dimethyl-2,4-dioxo-hexanoic acid	1.1 ± .1	6.4 ± .8
6-Phenyl-2,4-dioxo-hexanoic acid	6.1 ± 1.5	2.2 ± .9
4-Phenyl-2,4-dioxo-butanoic acid	0.072 ± .006	1.2 ± .3
3,5-Dioxo-hexanoic acid	.34 ± .2	2.7 ± .5
6,6-Dimethyl-3,5-dioxo-heptanoic acid	1.2 ± .1	1.4 ± .1
5-Phenyl-3,5-dioxo-pentanoic acid	.16 ± .04	8.9 ± 3.1

[a] Studies carried out in 0.025 M sodium phosphate buffer pH 7.3 at 30.0 ±.5°

[b] Errors are standard deviations.

TABLE III: R_f [a] Values from the Paper and Thin Layer Chromatography

Chromatography	R_f Values for 2,4-DNP Derivatives					
Solid support	Products of Enzyme Hydrolysis			Authentic Samples		
(Solvent system)	 [b]	CH_3-X	t-Bu-X	Acetone	Pyruvate	Acetoacetate
Silica Gel G-25	.58	.59	.57	.59	.40	.59
(Ethanol: Ethyl acetate) (70:30)	.42	.43	.43			.44
Cellulose F (t-Amyl alcohol: Ethanol: water) (50:10:40)	.96	.97	.96	.97	.86	.97
Paper (Whatman No.2)	.91	.89	.92	.91	.50	.90
(t-Amyl alcohol: Ethanol: water) (50:10:40)	.62	.62	.64			.63

[a] R_f = distance compound has travelled from origin / distance developing solvent has travelled from origin

[b] X = $-C(=O)-CH_2-C(=O)-CH_2-COO^-$

4) Inhibition of FAH Activity by Fluoride

Fumarylacetoacetate fumarylhydrolase catalyzed hydrolysis of 6,6- dimethyl - 3,5- dioxo-heptanoic acid (2×10^{-4} M) is competitively inhibited by sodium fluoride (Fig. 11). There are three main types of competitive inhibitors (Plowman, 1972; Cleland, 1963).

a) simple linear competitive inhibition:

$$\frac{Vo}{Vi} = 1 + \frac{Km}{Ki\ ([S_o] + Km)}\ [F^-] \quad (1)$$

b) hyperbolic competitive inhibition

$$\frac{Vo}{Vi} = \frac{[S_o]}{Km + [S_o]} + \frac{Km\left[\frac{1 + a[F^-]}{1 + b[F^-]}\right]}{Km + [S_o]} \quad (2)$$

c) parabolic competitive inhibition

$$\frac{Vo}{Vi} = \frac{[S_o]}{Km + [S_o]} + Km\ (1 + a\ [F^-] + b\ [F^-]^2) \quad (3)$$

where Vo is the uninhibited initial velocity, Vi is the initial velocity of the inhibited reaction, Km is the observed dissociation constant of the enzyme-substrate complex, $[F^-]$ is the inhibitor concentration, S_o is the initial substrate concentration, Ki is the inhibitor constant and the constants a and b are complex functions of constants. In order to differentiate among these three types of competitive inhibition, Vo/Vi was plotted against the inhibitor concentration (Fig. 12). The Y - intercept was one and Vo/Vi was a linear function of the inhibitor concentration, thus conforming to the equation for simple linear competitive inhibition (eq. 1). If the inhibition were hyperbolic or parabolic, Vo/Vi would not be a linear function of inhibitor concentration and the Y- intercept would be equal to $\frac{[S_o]}{Km + [S_o]}$ which in

this case is equal to 0.59.

The fluoride concentration required to cause 50% inhibition under the standard assay conditions for 6,6- dimethyl- 3,5- dioxo-heptanoic acid is 5.0 m M. The inhibitor constant, Ki, is 2.15 m M calculated from the slope of the line in Fig. 12 according to the equation:

$$\text{slope} = \frac{Km}{Ki\ (\ [S_o] + Km)} \qquad (4)$$

The number of inhibitor molecules, 'r', in an enzyme inhibitor complex:

$$E + r\ I \rightleftharpoons EIr$$

is related to various kinetic coefficients as follows (Ebersole et al.,1944; Bergman and Segal,1954):

$$\log\left(\frac{Vo}{Vi} - 1\right) = r \log I + \log\left(\frac{Km}{Km + [S_o]\ Ki}\right) \qquad (5)$$

The value of ' r ' is readily determined by plotting $\log\left(\frac{Vo}{Vi} - 1\right)$ against log I and estimating the slope of the resulting line. In the case of fluoride inhibition of 6,6- dimethyl -3,5- dioxo-heptanoic acid, the slope was .90. Therefore, the inhibition appears to be of the EI type, that is, one molecule of inhibitor reacts with each active site.

Fig. 11. Competitive Inhibition by Fluoride of FAH-catalyzed hydrolysis of 6,6-dimethyl-3,5-dioxo-heptanoic acid. The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 0.025 M sodium phosphate buffer, pH 7.3, and 6,6-dimethyl-3,5-dioxo-heptanoic acid as indicated.

(●) .004 M NaF

(○) 0.0 M NaF

FIGURE 11

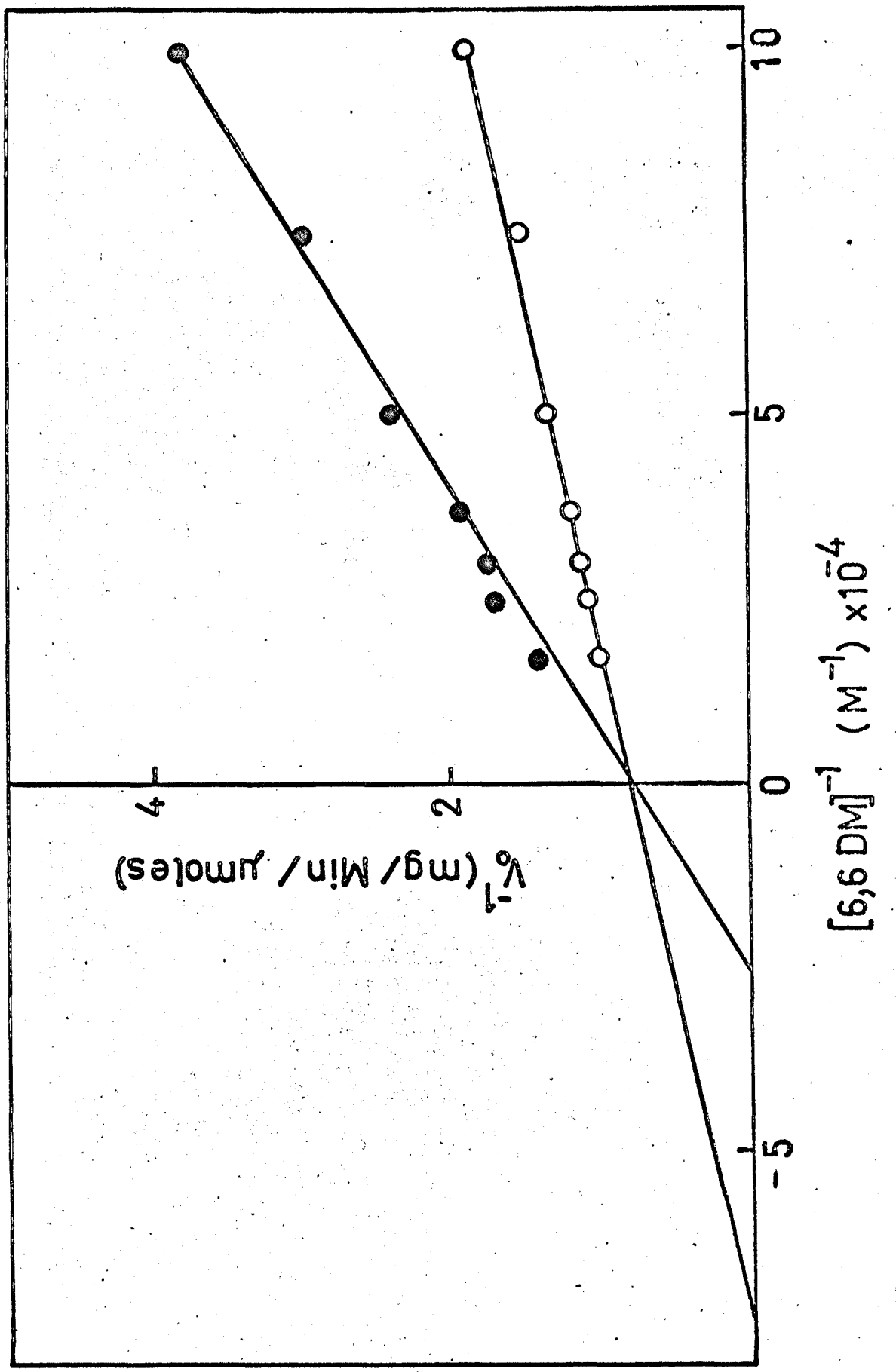
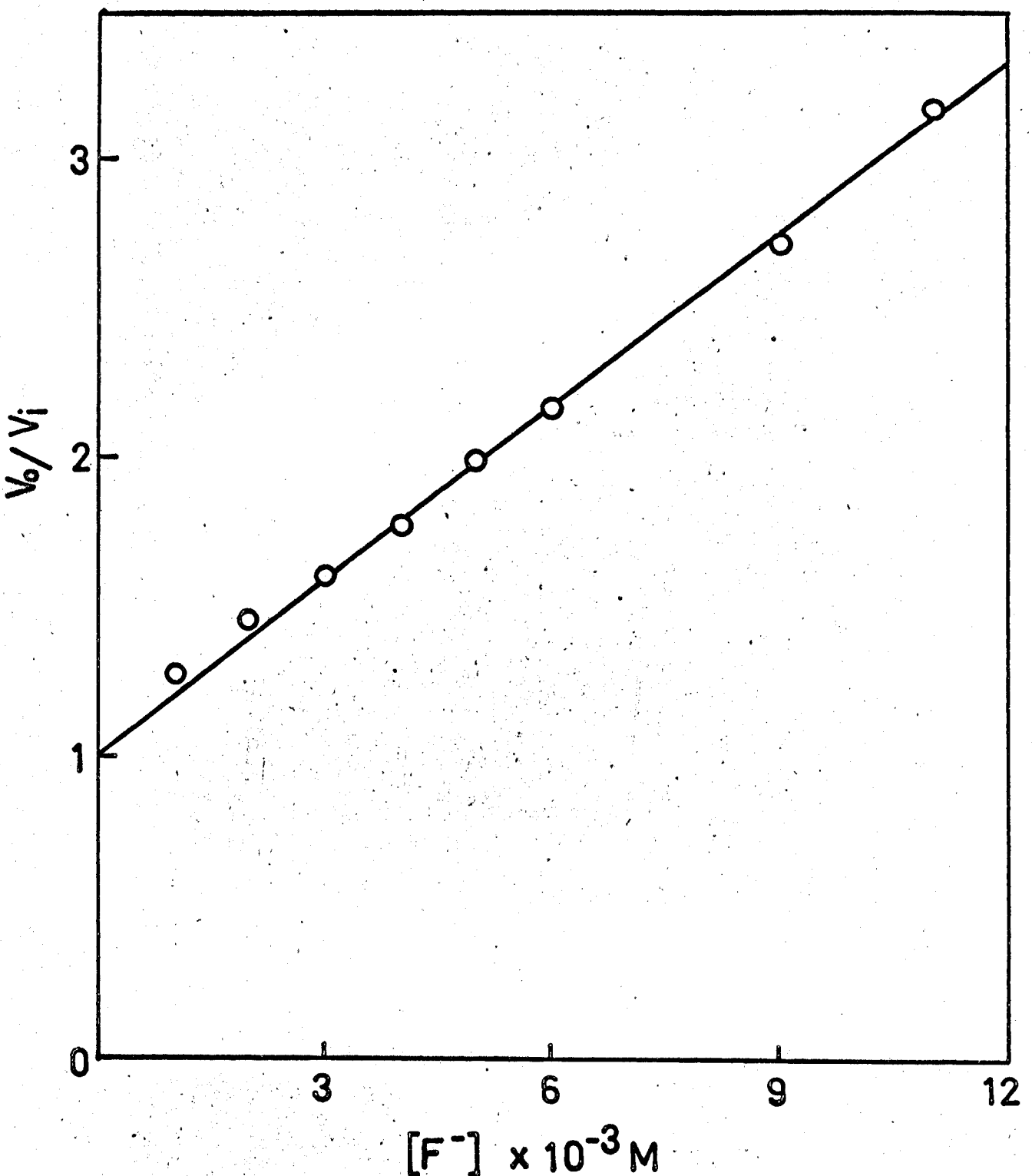


Fig. 12. Inhibition by Fluoride of FAH catalyzed hydrolysis of 6,6-dimethyl-3,5-dioxo-heptanoic acid.

The standard assay procedure was used. Each cuvette (.5 cm path length, 1.5 ml volume) contained 2 x 10 M 6,6-dimethyl-3,5-dioxo-heptanoic acid, 0.025 M sodium phosphate buffer, pH 7.3, and fluoride at the concentration indicated. The hydrolysis was initiated by the addition of 0.05 ml FAH.

FIGURE 12



5) Inhibition of FAH Activity by Substrate Analogs and p-Hydroxymecuribenzoate

Table IV shows the effect of structural analogs of fumarylacetoacetate fumarylhydrolase substrates on the hydrolysis of 6,6-dimethyl-3,5-dioxo-heptanoic acid. At a concentration of 10^{-3}M, dihydroxytartrate and tartronic acid are the most potent inhibitors.

Table V shows the effect of p-hydroxymecuribenzoate on enzymatic activity using the 3,5-diketo acids. It can be seen that for 3,5-dioxo-hexanoic acid and 6,6-dimethyl-heptanoic acid are inhibited approximately 80% at 10^{-5} M p-HMB, while 5-phenyl-3,5-dioxo-pentanoic acid is only inhibited 17% under the same incubation conditions. At 10^{-4}M p-HMB, there is complete inhibition of the enzyme for all the substrates.

TABLE IV: Inhibition of Fumarylacetoacetate Fumarylhydrolase Activity by Substrate Analogs [a]

Inhibitor	% Inhibition		
	10^{-3} M	10^{-4} M	10^{-5} M
Dihydroxytartaric acid	91.9	21.4	6.0
Tartronic acid	99.7	24.6	---
Oxalacetic acid	36.8	25.7	17.1
Acetoacetic acid	32.8	25.5	---
Pyruvic acid	29.2	21.2	---

[a] The inhibition studies were carried out in 0.025 M sodium phosphate buffer pH 7.3 at 25.0 $\pm$ .5 , using 2 x 10 M 6,6-dimethyl-3,5-dioxo-heptanoic acid as substrate. Incubation time was 2 min.

TABLE V: Inhibition of FAH-Activity by p-Hydroxymecuribenzoate

Substrate[a]	% Inhibition		
	10^{-4} M p-HMB	10^{-5} M p-HMB	10^{-6} M p-HMB
3,5-Dioxo-hexanoic acid	100	79.5	9.0
6,6-Dimethyl-3,5-dioxo-heptanoic acid	100	83.5	8.2
5-Phenyl-3,5-dioxo-pentanoic acid	100	16.7	---

[a] Substrate concentration was 2 x 10 M in 0.025 M sodium phosphate buffer, pH 7.3 at 25.0 ± .5 . Incubation time was 2 min.

6) Effect of Sodium Cyanide on FAH Activity

FAH activity is unaffected by cyanide in the presence of 6,6- dimethyl - 3,5- dioxo-heptanoic acid and 3,5- dioxo-hexanoic acid . Cyanide alone causes a slight increase in the activity, although not as great as reported by Nagainis (1975). The increase is probably due to the formation of a cyanohydrin with substrate which appears as a slight increase in activity.

CHAPTER III

DISCUSSION

As previously reported (Braun,1973) fumarylacetoacetate fumarylhydrolase from beef liver also catalyzes the hydrolysis of 3,5-diketo acids. The natural substrate, fumarylacetoacetate, is a 3,5-diketo acid which has two carboxyl groups separated by a six carbon chain. Contrary to our expectations, the 3,5-diketo acids only bound as well to the enzyme as the 2,4-diketo acids and were hydrolysed at about the same rate or more slowly than their 2,4-diketo analogs (Table II). 6,6-Dimethyl-3,5-dioxo-heptanoic acid and 5,5-dimethyl-dioxo-hexanoic acid have the same V max while 3,5-dioxo-hexanoic acid has a maximal velocity, at high concentrations, 10 times less than its analog, acetopyruvate. Benzoylpyruvate has half the V max of its 3,5-analog.

Unfortunately, the problems in synthesizing other 3,5-diketo acids make any comparisons about the differences between 2,4- and 3,5-diketo acids difficult. The basic problems was in purification of the 3,5-diketo acids. Low melting points and the difficulty of purification at temperatures, which should be at or below room temperature to avoid decarboxylation (Swain et al.,1961), reduced the possible number of 3,5-acids that could be used for kinetics. Some conclusions about mechanism can be drawn however, from the results of hydrolysis of diketo acids and the inactivity of FAH toward fumarylacetone.

The Km values for the 3,5- and 2,4-diketo acids only vary by a factor of 7, while the V max values vary by a factor of 120. If Km is a measure of binding, this indicates that these substrates are bound

equally well to the enzyme and yet they are hydrolysed at various rates, some faster, some slower.

In enzymic catalysis, as opposed to chemical catalysis, the energy from the binding interactions between enzyme and the nonreacting portions of specific substrates provides more available energy. Therefore, looking at catalysis in terms of stabilizing the transition state of the enzyme-substrate complex, this available energy makes the enzyme more effective. In looking at these interactions we will be mainly concerned with Gibbs free energies arising from them. The term "binding energy" refers to a standard free-energy change. Therefore, we can say that the enzyme , because it can use the free-energy from the interaction of non-reacting portions of a substrate, is able to stabilize the transition state and effect catalysis. All the energy available from the non-covalent interactions of substrate and the active site of the enzyme is the intrinsic binding energy of the enzyme-substrate complex. This is an ideal quantity which is usually not reflected in a low Km. Most enzymes have a Km between 10^{-3} and 10^{-5}M, so most of the intrinsic binding energy is not used in binding. The amount of energy available due to interactions between substrate and enzyme can be better seen in antibody-antigen reactions. In multivalent antigen-antibody interactions (Hornick and Karush,1972), which have a Km between 10^{-7} and 10^{-9}M, more binding energy is used, manifested in a low Km, because of low concentrations of reactants. Also, because of low concentrations, the required energy needed for reaction is very great, more than obtained from the interaction of a monovalent antigen with the site of an antibody molecule. However, by utilizing multivalent

antibodies that react with multivalent antigens, the intrinsic binding of several sites may be utilized to make the free energy of interaction more favorable. The intrinsic binding energy over that implemented in binding is then utilized to provide the driving force for catalysis. Therefore, the addition of a substituent group to a substrate may cause little change in the observed binding energy, as measured by Km, but may be used up in increasing the reaction rate by destabilizing a substrate or causing a loss of entropy in the enzyme-substrate complex (Jencks,1975).

Destabilization of a substrate by strain, desolvation, electrostatic repulsion and hydrogen-bond breaking help the mechanism of catalysis to be more effective if the destabilization is relieved in the transition state. Therefore, the free energy of activation that is required to reach the transition state can be reduced. The intrinsic binding energy may be used for this, by destabilizing the substrate which in turn decreases the observed free energy of reaction.

The loss of entropy may also be very important in the mechanism of catalysis. In order that the substrate molecule and the enzyme come together in the correct position for a reaction to occur, these molecules must lose their freedom to move and rotate freely in solution. Another way the intrinsic binding energy can be used to overcome a favourable entropy situation is to provide the necessary energy to "freeze" the substrate in the enzyme-substrate complex. The amount by which the difference in free energy between the ground state enzyme-substrate complex and transition state is reduced, is important for reaction. This can be done by decreasing

the entropy, using intrinsic binding energy from the enzyme-substrate complex. By using binding energy, to make attainment of the transition state more favourable, the enzyme can decrease the amount of entropy that need be lost, which is unfavourable, during catalysis.

Therefore, the effectiveness of these energy using processes (destabilization and loss of entropy) will depend on how much intrinsic binding energy will be gained by a particular substrate and how much of this intrinsic binding energy is used to decrease the change in free energy between the ground state enzyme-substrate complex and the transition state. Therefore, if there is a large amount of favourable free energy available due to the interaction between substrate and enzyme, much of this may be used to increase the rate of reaction and still leave enough to provide binding (Fig. 13). This increase in rate may be due in part to the small amount of intrinsic binding energy used to decrease the difference between the ground state and the transition state (Fersht, 1974).

In the case of the 2,4-and 3,5-diketo acids, any effect on the intrinsic binding energy and its utilization in decreasing the free energy difference between the enzyme-substrate complex and the transition state may be due to the various R groups on the substrates. This may occur because of an adjacent subsite near the catalytic groups in the active site which helps in the binding of the substrate and the subsequent distortion, by intrinsic binding energy, of the substrate to a transition state. So, if the binding is incorrect, due to the R groups, loss of entropy and destabilization will not occur and a slower rate of catalysis will be observed (Jencks and Page,1972). This is one possible explaination for varying V max values with similar Km values.

Fig.13. Free Energy Diagram of the Effects of Intrinsic Binding Energy in Enzyme Catalysis.

E-Enzyme

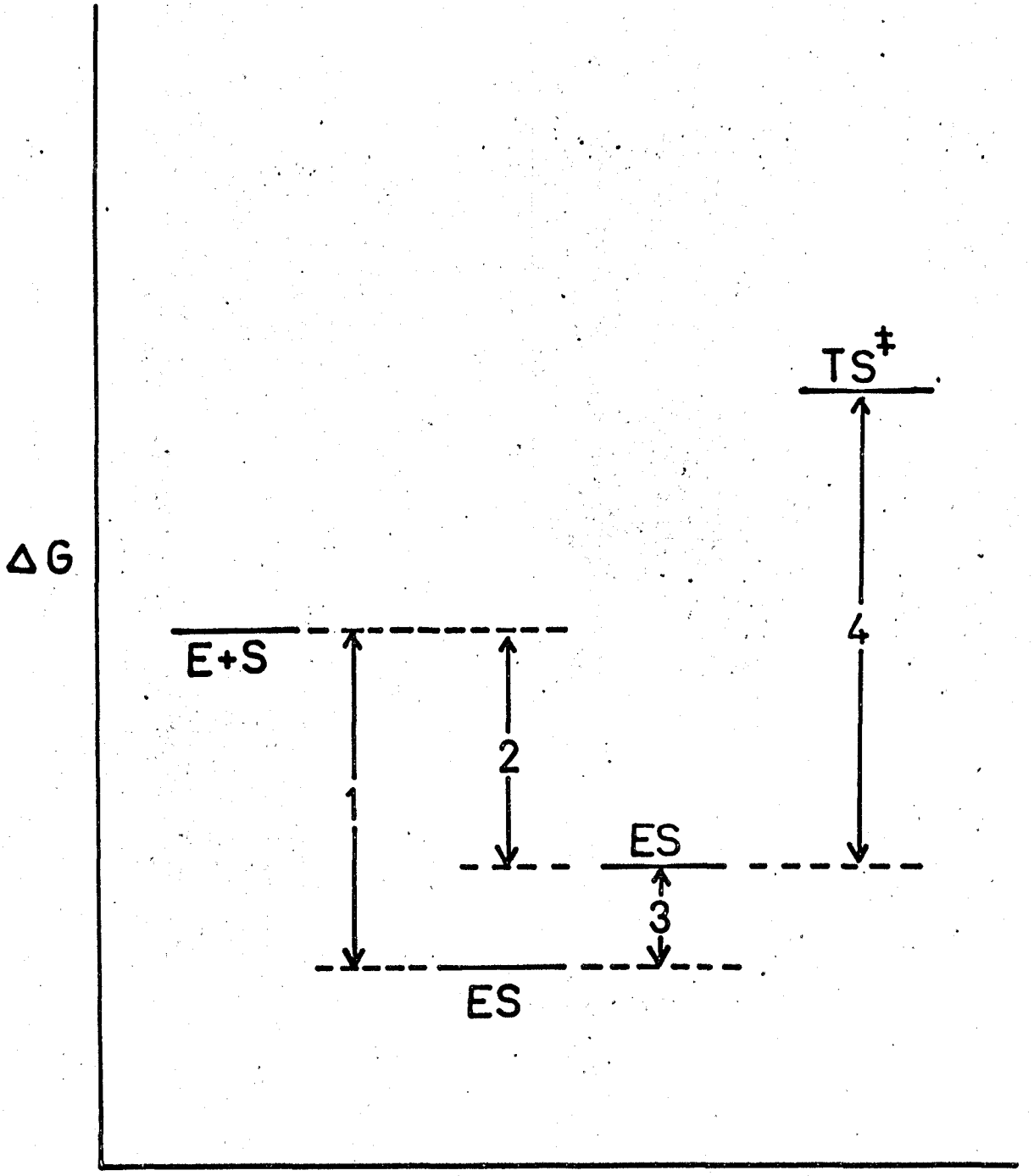
S-Substrate

ES-Enzyme-Substrate Complex

ES'-Enzyme-Substrate Complex affected by Destabilization and/or Entropy Loss.

$TS^{\ddagger}$-Transition State

FIGURE 13



1 INTRINSIC BINDING ENERGY

2 BINDING ENERGY EXPRESSED AS Km

3 POTENTIAL INTRINSIC BINDING ENERGY USED FOR DESTABILIZATION AND/ OR ENTROPY LOSS

4 Δ G cat

5-Phenyl-3,5-dioxo-pentanoic acid and 4-phenyl-2,4-dioxo-butanoic acid have very low catalytic rates. They are bound as well as any of the other substrates in the enzyme-substrate complex, but because of their phenyl groups they may not be able to gain as much intrinsic binding energy or they require more energy than is available to decrease the free-energy difference between the enzyme-substrate complex and the transition state. As a result any destabilization, which would put strain on the substrate, may not occur due to lack of binding energy. The substrate may also not have enough intrinsic binding energy to cause a loss of entropy which would "freeze" the substrate thus causing an improper alignment in the transition state, so that the active groups on the substrate would be incorrectly aligned in the active site. Yet, 6-phenyl-2,4-dioxo-hexanoic acid has a very high rate of hydrolysis. This is also probably due to its structure which is similar to propionopyruvate. It is probably better oriented in the active site so that the cost in free energy is probably smaller. Therefore, any available binding energy is used to increase catalysis. 3,5-Dioxo-hexanoic acid is hydrolysed 10 times slower than acetopyruvate although it has a slight better binding constant and is closer in structure to fumarylacetoacetate. Its hydrolysis rate may be hampered because it fits too well in the active site, due to its small methyl group and it may not provide much intrinsic binding energy. As a result the substrate may require too much energy to get to the transition state complex by using destabilization or decreasing entropy of the substrate. While, aceto-pyruvate because it is one carbon less may be more subject to strain which could be released in the transition state. 6,6-Dimethyl-3,5-

dioxo-heptanoic acid is the best substrate of the 3,5-diketo acids made, but in general, it and its 2,4-analog are not hydrolysed as fast as other substrates. The large t-butyl groups may make available more intrinsic binding energy from their interactions with the enzyme, but they may also hamper catalysis by making it harder to decrease the entropy in the active site. They are bound well in the enzyme-substrate complex, so there may be an adjacent subsite to the active site favourable to the t-butyl groups. The t-butyl groups may cause destabilization in the enzyme-substrate complex, which could be partially relieved in the transition state therefore decreasing the free-energy difference and making catalysis more favourable. Fumarylacetone did not prove to be a substrate although it is similar to the natural substrate except for the missing carboxyl group at the 1-position of fumarylacetone. This seems to be a very important group in the hydrolysis of these compounds. In inhibitor studies done on propiono-pyruvate, the most inhibitory compounds were hydroxy acids (Braun and Schmidt,1973). The two neighbouring groups to the active site which are positively charged seem to be very important to catalysis. Therefore, fumarylacetone would fit into one part of the active site but not the other. Therefore, the molecule might not be oriented well for effective catalysis. The carboxyl group position on the substrate may also cause a change in the intrinsic binding energy available for catalysis and changing its position may have an effect.

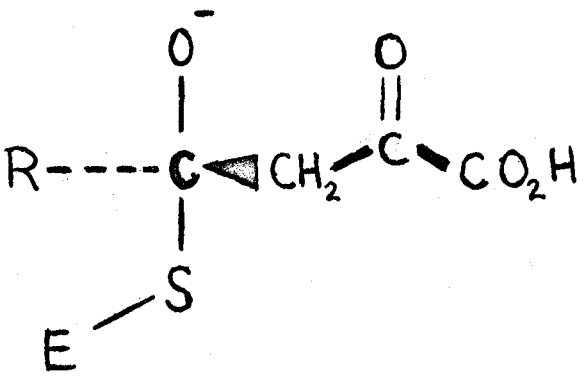
It has been suggested that the hydrolysis of the natural substrate in the active site involves the carboxyl groups bound to two positively charged groups, a sulfhydryl group to attack the 5-carbonyl group and and a possible lysine amino group to form a Schiff-base intermediate

with the 3-carbonyl group. As a result, these possibilities were further investigated with fluoride (positive sites), p-hydroxy-mecuribenzoate (sulfhydryl group) and cyanide (Schiff-base). Substrate analogs were used to study possible transition state structures of the catalytic mechanism (Wolfenden,1972;Lienhard,1973) and reaction products were studied to confirm the possible mechanism.

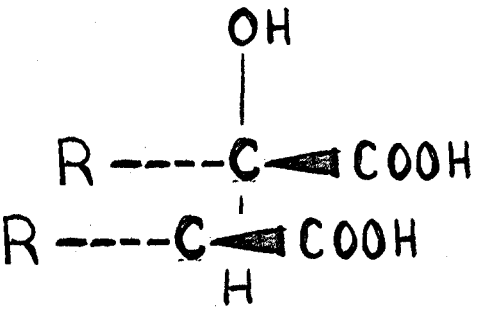
The mechanism of catalysis has often been studied using transition state analogs for various enzymes. Therefore, by using substrate analogs one can first find which are inhibitory to the enzyme and then what part of their structure may be causing the inhibition. Since an inhibitor with a structure that resembles a possible transition state may fit the active site better than does a normal substrate, the inhibitors observed binding will be tighter. Also it does not have to use binding energy to overcome destabilization. Therefore, the destabilization mechanism of the enzyme-substrate complex to the transition state,and the transition state can be studied using these analogs (Jencks,1975).

Some substrate analogs of fumarylacetoacetate fumaryl-hydrolase substrates have been studied before and found to be inhibitory at high concentrations and in some cases not as inhibitory as some of the monovalent anions studied (Braun and Schmidt,1973). Hydroxy acids are the most inhibitory, so this type of inhibition of FAH was further investigated for the FAH hydrolysis of 6,6-dimethyl -3,5-dioxo-heptanoic acid with the most inhibitory analogs of the 2,4-diketo acids at lower concentrations (Table IV). At 10^{-4}M all the compounds were of equal inhibitory strength. At 10^{-3}M the most inhibitory were dihydroxytartaric acid and tartronic acid which

are α-hydroxy acids. Further work, is required in this area, but it can be seen that the transition state may be close to an hydroxy acid in structure for a nucleophilic attack by a sulfhydryl group. The transition state of the enzyme-substrate complex would be an unstable tetrahedral intermediate.



possible transition state



inhibitor structure

The hydroxy acids, because they could fit into the active site in the form of the transition state, would inhibit the enzyme in the presence of a non-transition state shaped substrate. The inhibitor is ready made to fit into the active site and would require very little energy to do so. The substrate on the other hand, requires the use of intrinsic binding energy to destabilize towards the transition state geometry. These differences would make the inhibitor more favourable for binding than the substrate.

Pyruvate and acetoacetate were slightly inhibitory which is probably due to product inhibition. They also use available binding energy to bind to the enzyme. Oxalacetic acid which was a potent inhibitor of propionopyruvate was not as inhibitory with 6,6-dimethyl -3,5-dioxo-heptanoic acid. The possible reason being that the structure of oxalacetic acid is more involved in binding with the enzyme than in any transition state. As a result it is probably more easily displaced by the 3,5-diketo acid which is a better binder than the 2,4-diketo acid.

As expected acetoacetate was identified as one of the reaction products of the FAH-catalyzed hydrolysis of 3,5-dioxo-hexanoic acid, 6,6-dimethyl -3,5-dioxo-heptanoic acid and 5-phenyl -3,5-dioxo-pentanoic acid. Thus a possible mechanism for the hydrolysis of the 3,5-diketo acids might involve the formation of a Schiff-base intermediate with the 3-keto group and subsequent attack by a second nucleophile, a sulfhydryl group of cysteine on the 5-keto group followed by hydrolysis regenerating the free enzyme, acetoacetate and an acid.

Anion inhibition of FAH activity has been investigated with the 2,4-diketo acids (Braun and Schmidt, 1973) and were found to be simple competitive inhibitors, with fluoride being the most inhibitory. In this study, FAH activity in the hydrolysis of 6,6-dimethyl -3,5-dioxo-heptanoic acid, fluoride also showed inhibitory qualities. For propionopyruvate, the Ki for fluoride was .61 mM and the concentration required for 50% inhibition was 1.2 mM. While for 6,6-dimethyl -3,5-dioxo-heptanoic acid, the Ki for fluoride was 2.15 mM and the concentration required for 50% inhibition was 5.0 mM. These

are higher numbers, but the Ki is still lower than other anions studied for propionopyruvate and the 50% inhibition concentration is close to thiocyanate, the second most inhibitory. However, experimental error may be the only reason for the slight difference. As a result, fluoride seems to be the best anion inhibitor of FAH activity. It also shows simple competitive inhibition for the hydrolysis of 6,6-dimethyl -3,5-dioxo-heptanoic acid as seen from Fig. 12 . Fluoride may fit in well at the positive sites in the active site, making them unavailable for use in the catalysis of substrates or fluoride may bind at another site.

Table V shows that 10^{-4} M p-hydroxymecuribenzoate totally inhibits the FAH catalyzed hydrolysis of all 3,5-diketo acids tested. Therefore, as previously found (Braun,1973) with the 2,4-diketo acids and further studies (Nagainis,1975) with p-hydroxymecuribenzoate, there is a possibility that the second nucleophile is the sulfhydryl group of cysteine. So as in fluoride the sulfhydryl inhibitor either makes catalytic groups in the active site unavailable or alters the structure of the active site inactivating it. In any case, interacting thus, fluoride and p-HMB inhibition of 2,4-and 3,5-diketo acid hydrolysis by FAH occurs at the same active site.

It was previously observed that cyanide inactivated the enzyme in the presence of propionopyruvate and acetopyruvate suggesting that an amine group on the enzyme is involved in a Schiff-base mechanism to facillitate catalysis (Nagainis,1975). However, studies with 3,5-dioxo-hexanoic acid and 6,6-dimethyl-

3,5-dioxo-heptanoic acid showed no such inactivation of enzyme. Since cyanohydrins were easily formed with the 2,4-diketo acids and not with the 3,5-diketo acids, investigation of the inhibition by cyanohydrins were carried out (Ewing,1976). These studies indicated that there may not have been an inactivation of enzyme by "trapping" the Schiff-base (Stewart and Li,1938) as found in other enzymes (Cash and Wilsom,1966; Brand and Horecker,1968; Westheimer,1963; Autor and Fridovich,1970; Fridovich and Westheimer,1962) but an interaction of the cyanohydrin in the hydrolysis reaction. Thus a Schiff-base mechanism for catalysis is doubtful.

CHAPTER IV

SUMMARY

The kinetics of fumarylacetoacetate fumarylhydrolase-catalyzed hydrolysis of 3,5-diketo acids and previous results on 2,4-diketo acids were reported. The binding of these substrates, as reflected in the Km (factor of 7) are the same, while the V max (factor of 120) varies greatly. These results are discussed in terms of utilization of intrinsic binding energy from non-covalent interactions in the enzyme-substrate complex which may increase the effectiveness of catalysis. p-Hydroxymecuribenzoate causes inhibition of the FAH-catalyzed hydrolysis of 3,5-diketo acids by inactivating a catalytic group in the active site. Fluoride is a simple competitive inhibitor of FAH activity. Substrate analogs were used in an effort to determine the structure of a possible transition state complex and the findings were discussed. The enzyme was not inactivated by cyanide in the presence of 3,5-dioxo-hexanoic and 6,6-dimethyl -3,5-dioxo-heptanoic acids.

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