PARTIAL PURIFICATION OF PHENYLALANINE HYDROXYLASE FROM DOG LIVER

A Thesis

Presented to

the Faculty of the Department of Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Peggy L. Ross

December 1975

To My Husband

Larry L. Ross

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ABSTRACT

Phenylalanine hydroxylase from dog liver was purified ten fold. The physical and kinetic properties of phenylalanine hydroxylase from dog liver in many respects closely resemble physical and kinetic properties of the same enzyme from other organism reported in the literature. The Km value determined for phenylalanine hydroxylase from dog liver for phenylalanine is 3.7 ± 1.1 mM; for 6,7-dimethyltetrahydropterin is 0.17 ± 0.10 mM; and for tetrahydrobiopterin is 0.34 ± 0.03 mM.

A new assay developed in this study is especially suited for detection of phenylalanine hydroxylase from dog liver and it makes possible the detection of enzyme activity at low concentration of enzyme.

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INTRODUCTION

Phenylalanine hydroxylase (EC 1.14.3.) converts phenylalanine to tyrosine. Hydroxylases or mixed function oxygenases insert one of the two oxygen atoms of O_2 and the other oxygen atom is utilized to oxidize NADH (Lehninger, 1971). In higher organisms this conversion serves two purposes: the catabolism of phenylalanine to CO_2 and water, and the provision of an endogenous source for the amino acid, tyrosine, and indirectly for such tyrosine-derived metabolities as melanin, norepinephrine, and epinephrine (Kaufman, 1962).

History

The first *in vitro* demonstration of the enzymatic conversion of phenylalanine to tyrosine was reported by Embden in 1913. In 1952 Undenfriend and Cooper reported that a crude extract from rat liver catalyzed the hydroxylation reaction. Mitoma in 1956 found that two fractions existed in the extract and both were necessary for activity. Liver extract from sheep was found to be a better source for one of the fractions. Therefore two enzymes were purified, one from sheep and one from rat, and these were referred to as rat liver and sheep liver enzymes. In 1957 Kaufman did balance studies and proposed the overall reaction as follows (Kaufman, 1971):

$$NADPH + H^+ + \text{Phenylalanine} + O_2 \rightarrow NADP^+ + \text{Tyrosine} + H_2O$$

Kaufman's (1958) kinetic experiments indicated there was another cofactor besides NADPH that was necessary for enzyme activity. The cofactor structure was found to be dihydrobiopterin (Kaufman, 1963). Other components which were isolated from the rat liver crude extract of

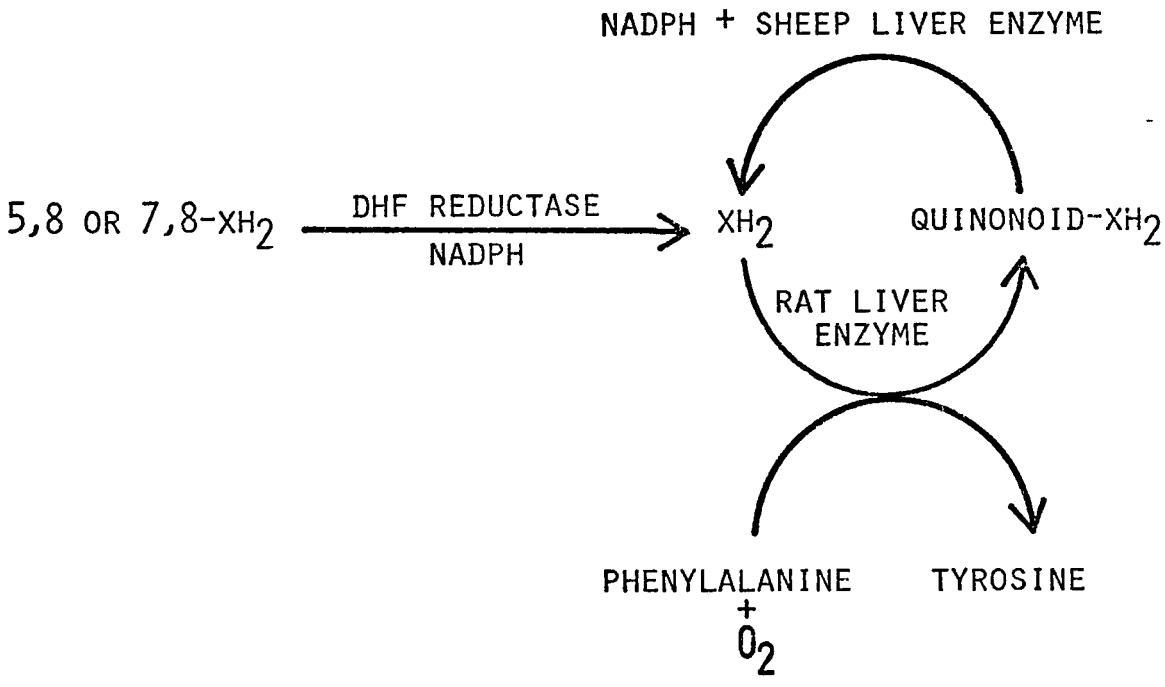
phenylalanine hydroxylase were: another enzyme, dihydrofolate reductase (Kaufman, 1967), quinonoid dihydropterin (Kaufman, 1964), and phenylalanine hydroxylase stimulator (Kaufman, 1963). Kaufman (1963) proposed the scheme shown in Fig. 1 to explain the role of each component found in the crude extract.

Phenylalanine hydroxylase (rat liver enzyme) catalyzes the reaction of phenylalanine to tyrosine. Dihydropteridine reductase (sheep liver enzyme) catalyzes the NADPH-mediated reduction of the quinoid dihydrobiopterin back to the tetrahydro form, and dihydrofolate reductase is necessary to catalyze the NADPH-mediated reduction of dihydrobiopteridine to the tetrahydro form. After this the dihydrofolate reductase is no longer needed in the remainder of the hydroxylase system (Kaufman, 1964). The structure of the cofactor was determined by Kaufman (1967) to be 7,8-dihydro-2-amino-4-hydroxy-6-[1,2-dihydroxypropyl]-pteridine.

As the purification of phenylalanine hydroxylase from rat liver became more refined, Kaufman and Fisher (1970) found the enzyme had two isozymes having the same molecular weight, 210,000, but differing in electrophoretic mobility. Barranger et al. (1972) reported three isozymes of similar molecular weights (200,000), and Stokes radii, but differing in electrophoretic mobility. The three isozymes were called pi, kappa, and upsilon. The ratio of total activity in pi, kappa, and upsilon was 1:8:6, pi having only 5 to 10 per cent of the total activity. The pi isozyme was found to be in an ethanol extract which had been discarded by Kaufman and Fisher in their earlier extraction procedure of phenylalanine hydroxylase.

FIGURE 1

A scheme proposed by Kaufman (1963) to explain the role of each component in the phenylalanine hydroxylase enzyme complex.


NADPH + SHEEP LIVER ENZYME
5,8 OR 7,8-XH2
DHF REDUCTASE
NADPH
XH2
QUINONOID-XH2
RAT LIVER
ENZYME
PHENYLALANINE
+
O2
TYROSINE

Fisher et al. (1972) reported that phenylalanine hydroxylase had 1 to 2 moles of iron per mole of enzyme (assuming 100,000 molecular weight), and five cysteines per 50,000 molecular weight. Gillam et al. (1974) reported the enzyme, from rat liver, had 2 atoms of iron, 1 atom of copper, 4-5 free SH groups and one molecule of FAD per molecule of enzyme (assuming 110,000 molecular weight).

Kaufman and Fisher (1970) and Barranger (1972) extracted multiple forms (100,000 and 200,000 molecular weight) of phenylalanine hydroxylase from the livers of rats and humans. Gillam et al. (1974) also found two forms in rat liver (110,000 and 210,000 molecular weight), but Woo et al. (1974) found that by changing the homogenization step and, in particular, filtering through glass wool after centrifugation, resulted in all of the phenylalanine hydroxylase activity of the human liver appearing in a single molecular species of 108,000 molecular weight with respect to both Sephadex-gel filtration and sucrose-density-gradient contrifugation.

Upon further studies, Woo et al. (1974) found that phenylalanine partly protects the enzyme against thiol-binding reagents; since phenylalanine has no known effect in protecting free thiol groups, it was suggested that either one or more essential SH groups are located close to the binding site for phenylalanine and are included when phenylalanine binds to the enzyme, or the binding of phenylalanine induces an allosteric conformational change of the enzyme, such that the essential SH groups and the metal ions are no longer available for the blocking agents.

In the course of their studies Fisher and Kaufman have proposed

many reaction schemes and intermediates for the phenylalanine hydroxylase reaction. Due to the work of McCord and Fridovich (Kaufman and Fisher, 1973), who demonstrated that the xanthine oxidase-catalyzed reduction of cytochrome c or oxidation of epinephrine is mediated by a superoxide anion O_2^-, as an intermediate. Kaufman and Fisher (1973) used erythrocuprein which has the superoxide dismutase activity, $2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2$, to test whether the superoxide anion, O_2^-, was an intermediate in the phenylalanine hydroxylase reaction. It was found that erythrocuprein did not inhibit the hydroxylation of phenylalanine or p-tyrosine-dependent NADPH oxidation by phenylalanine hydroxylase. These results implied that either the superoxide anion was not an intermediate in the phenylalanine hydroxylase catalyzed reaction or that the superoxide anion was not available to the dismutase. If the superoxide anion were an intermediate in the hydroxylase reaction, it might appear in solution during the uncoupled reaction. However, under uncoupled conditions phenylalanine hydroxylase did not reduce cytochrome c or oxidize epinephrine. These results suggest that the superoxide anion is not an intermediate in the phenylalanine hydroxlase reaction, although superoxide dismutase did inhibit by 50 per cent the autoxidation of the tetrahydropterin. Even though the hydroxylase-catalyzed oxidation of tetrahydropterin does not seem to involve the superoxide anion, the nonenzymatic oxidation seems to involve the superoxide anion as an intermediate. The work of Nishikimi (1975) also indicates O_2^- plays an important role in the overall process of tetrahydropteridine oxidation by O_2. He found that the reduction of nitro blue tetrazolium with tetrahydropteridine under aerobic conditions take place

spontaneously, and superoxide dismutase inhibited the reduction of nitro blue tetrazolium.

Assay Methods

Many variations in kinetic data for phenylalanine hydroxylase appear in the literature, as outlined in Table I. This is a result of many things, such as different assay methods, purification procedures, purity of chemicals used, and animals used. Table II lists some of the major assay procedures used. The reasons for the selection of different procedures and conditions are discussed.

The assay for phenylalanine hydroxylase can be either a continuous or fixed point assay. The continuous assay is a spectrophotometric method in which the disappearance of the reduced cofactor, pterin, or NADPH can be recorded at 340nm. This method can not be used with crude extract, so the fixed point assay is usually used to monitor the purification of phenylalanine hydroxylase, or with crude preparations. The product tyrosine is measured in the fixed point assay.

A good assay meets the following requirements: velocity versus amount of enzyme is linear, saturating amounts of substrate are used, and optimum conditions such as pH, salt concentration, temperature, and cofactor concentration should be used.

There is a great need for a standard assay, to enable researchers to compare their results. From Table II it is clear that none of the assay methods are the same. Although each research has done studies to determine the optimum conditions, they obtained different results.

Fisher and Kaufman (1973a), Woo et al. (1974) and Bublitz (1969)

TABLE I

COMPARISON OF APPARENT Km VALUES FOR SUBSTRATES

	Animal	Km			
		L-Phenylalanine	Tetrahydro-biopterin	6,7-Dimethyltetra-hydropterin	6-Methyltetra-hydropterin
Fisher & Kaufman (1972)	Rat	1.3mM	----	0.07mM	----
	Rat	0.3mM	.0027mM	----	----
Friedman & Kaufman (1973)	Human	0.04mM	0.003mM	----	----
	Human	1.6mM	----	0.05mM	----
	Human	0.9mM	----	----	0.04mM
(1972)	Human (hyper-phenylalaninaemic)	0.67mM	----	0.005mM	----
Cotton (1971)	Monkey	0.4mM, 0.5mM, 0.8mM	----	0.08mM, 0.09mM	----
Gillam et al. (1974)	Rat (250,000 M.W.)	1.42mM ± 0.02mM	----	0.028mM ± 0.0046mM	----
	Rat (110,000 M.W.)	1.22mM ± 0.014mM	----	0.055mM ± 0.004mM	----
Woo & Gillam (1974)	Human (108,000 M.W.)	.318mM	----	0.0702mM	----

TABLE I--continued

	Animal	Km			
		L-Phenylalanine	Tetrahydro-biopterin	6,7-Dimethyltetra-hydropterin	6-Methyltetra-hydropterin
Barranger (1972)	Rat (Pi)	0.87mM	----	----	----
	(Kappa)	0.78mM	----	----	----
	(Upsilon)	0.64mM	----	----	----
Jakubovic (1971)	Human Fetus (11 weeks)	0.4mM	----	0.0375mM	----
	(17 weeks)	0.49mM	----	0.078mM	----
	(20 weeks)	0.6mM	----	----	----
Tourian (1971)	Rat	1.75mM ± 0.08mM	----	----	----
McGee (1972)	Rat	0.125mM	----	----	----

TABLE II

COMPARISON OF ASSAY METHODS

	Components of Assay	Total Vol.	Time Period	Temp. °C	pH
McGee (1972)	0.1 or 0.2 ml phenylalanine hydroxylase (supernatant) 100 mM potassium phosphate 10 mM L-phenylalanine 0.75 mM 6,7-dimethyltetrahydropterin 5 mM dithiothreitol	1 ml	20 min	25	6.8
Jakubovic (1971)	3.97 mg phenylalanine hydroxylase 0.2 M phosphate 8 µmoles L-phenylalanine 0.8 µmole NADH 5 µmoles nicotinamide 5 µmoles dithiothreitol 0.5 µmoles 6,7-dimethyltetrahydropterin 1830 units catalase	1 ml	30 min	25	6.8
Connellan (1973)	0.1 ml phenylalanine hydroxylase 2 µmoles L-phenylalanine 0.25 µmole 6,7-dimethyltetrahydropterin 1 µmole dithiothreitol 50 µmoles Tris-HCl	0.5 ml	10 min	37	6.8
Cotton (1971)	10 µmoles Tris-HCl 2 µmoles L-phenylalanine 0.25 µmole 6,7-dimethyltetrahydropterin 1 µmole dithiothreitol phenylalanine hydroxylase	0.5 ml	5 min	37	7.5
Tourian (1971)	100 µmoles potassium phosphate 8 µmoles L-phenylalanine 1 µmole 6,7-dimethyltetrahydropterin 10 µmoles dithiothreitol phenylalanine hydroxylase	1 ml	1 min	25	7.2

TABLE II--continued

	Components of Assay	Total Vol.	Time Period	Temp. °C	pH
Ayling (1974)	100 µmoles Tris-HCl 1 µmole phenylalanine 0.2 µmole 6,7-dimethyl-tetrahydropterin 5 µmoles dithiothreitol 0.25 mg catalase	1 ml	----	27	7.4
Spectrophotometric method (same as above except delete dithiothreitol, read at 330 nm)					
Kaufman (1970)	100 µmoles potassium phosphate 2 µmoles L-phenylalanine 0.25 µmole NADP 250 µmoles glucose glucose dehydrogenase (in excess) dihydropterin reductase (in excess) 0.1 µmole 6,7-dimethyl-tetrahydropterin 7800 units catalase	1 ml	30 min	25	6.8
Present	100 µmoles Tris-HCl 10 µmoles L-phenylalanine 0.25 mg catalase 5 µmoles dithiothreitol 0.1 ml phenylalanine hydroxylase 1.0 µmole 6,7-dimethyl-tetrahydropterin	1 ml	5 min	37	7.4
Biopterin	100 µmoles Tris-HCl 1 µmole L-phenylalanine 0.25 mg catalase 5 µmoles dithiothreitol 0.1 ml phenylalanine hydroxylase 0.08 µmole biopterin	1 ml	5 min	37	7.4

observed linearity of the enzymic activity with respect to time for the initial 30 minutes. Jakubovic (1971) and Cotton (1971) reported the reaction rate not to be linear for long periods of time, which is reflected in Cotton's assay. The nonlinear effect seen by Cotton could be due to inhibition of phenylalanine hydroxylase by H_2O_2 or an intermediate produced in the reaction between dimethyltetrahydropterin and oxygen, or the presence of an organic peroxide in the extract (Jakubovic, 1971). Jakubovic and Woolf (1971) reported hydroxylation of phenylalanine with fresh mouse liver supernatant was inhibited if the concentration of added cofactor was higher than 0.25 mM. However, addition of catalase prevented this inhibition. Kaufman (1962, 1970) and Bublitz (1969) also reported catalase had a protective effect on phenylalanine hydroxylase. McGee et al. (1972) reported addition of purified catalase was without effect on the assay. This could be due to catalase in the crude homogenate. The inhibition could not be due to the inactivation of the cofactor since addition of more liver extract with phenylalanine and catalase caused the expected formation of tyrosine from phenylalanine. Only in the presence of sufficient amounts of catalase is there a direct relationship between the amount of tyrosine formed and concentration of the cofactor (Jakubovic, 1971).

Catalase, however, was ineffective in preserving the cofactor. If dithiothreitol was present from the start of preincubation, dimethyltetrahydropterin remained fully active even after 45 minutes. Dimethyltetrahydropterin dissolved in phosphate buffer, pH 6.8 for 45 minutes of preincubation at 25°C in air lost 100 per cent of the cofactor activity. Dithiothreitol may act in several ways: as a very effective

reducing agent it regenerates the active cofactor from the quinonoid dihydropterin formed in the hydroxylation of phenylalanine (Bublitz, 1969 and Jakubovic, 1971) and also protects the cofactor from rapid oxidation by air (Jakubovic, 1971).

Kaufman does not use dithiothreoitol to reduce the cofactor. An enzyme system is used to reduce the cofactor. Dihydropterin reductase catalyzes the NADPH-medicated reduction of the cofactor. Glucose dehydrogenase supplies NADPH by oxidizing β-D-glucose to D-glucono-δ-lactone (Kaufman and Fisher, 1970). This is a very complex system and if there is not enough glucose, glucose dehydrogenase, or dihydropterin reductase present, they can act as the limiting factor and the true activity of the hydroxylase will not be observed.

The pH used for the assays in Table II range from 6.8 to 7.5. Woo et al. (1974) reported a broad pH optimum around pH 7. Bublitz (1969) found the rate of the reaction to increase from pH 6.0 to 7.2 and falls as the pH is increased beyond 7.2. Cotton (1971) reported phenylalanine hydroxylase from monkey liver showed more activity at pH 8.7 than pH 6.8, the optimum pH being 7.6. Bublitz (1969) and McGee et al. (1972) both reported that the activity at optimum pH was the same in phosphate and Tris buffer. The reported pH range varies even within the same species of animal. The optimum pH can not be determined from the literature.

Temperature also varies in the assay from 25°C to 37°C. Undenfriend and Cooper (1952) report the optimum temperature to be about 35°C, but the destruction of tyrosine occurred at this temperature, so 25°C was used since little destruction of tyrosine occurred. Kaufman

(1971) also reported that the ratio of NADPH oxidized to tyrosine formed is higher at 35°C. The disadvantages, destruction of tyrosine and uncoupling due to the higher temperature will have to be compared to the advantages, of higher tyrosine production before a conclusion can be reached on what temperature to use.

The molar concentrations of phenylalanine and pterin differ greatly from assay to assay. The concentration used for cofactor and substrate is usually the one which gives the optimum activity. This is not always possible if the enzyme is inhibited by the cofactor or substrate. Phenylalanine hydroxylase is inhibited by both the cofactor and phenylalanine. The synthetic cofactor is less inhibitory than tetrahydrobiopterin (biopterin). Kaufman and Fisher (1970) used 0.1 mM of 6,7-dimethyltetrahydropterin and 2 mM of phenylalanine in the assay, but when biopterin was used, the concentration of the cofactor was lowered to 3 μM and phenylalanine was lowered to 1 mM (Fisher and Kaufman, 1973). Kaufman does not use the highest concentration of substrate or cofactor to give the maximum velocity, but velocity versus amount of enzyme is linear (LaDu and Zannoni, 1967). With purified enzyme from human liver, Woo et al. (1974) observed virtually no substrate inhibition with synthetic cofactor even at phenylalanine concentrations of 20 mM. Jakubovic et al. (1971) and Cotton (1970) found no inhibition even at 1.0 mM pterin concentration. Cotton (1971) and McGee et al. (1972) found no inhibition up to or higher than 10 mM phenylalanine concentration respectively. The amount of substrate and cofactor to use to give an accurate picture of activity is still in question. Both low and high concentrations of substrate and cofactor are linear for the plot of

substrate versus amount of enzyme. Is it better to use low concentrations of substrate and cofactor which during purification is accurate, or is it better to use the optimum amounts of substrate and cofactor to make the detection of enzyme activity more sensitive for liver extract with low activity such as in liver extract from hyperphenylalaninaemic patients?

Another problem in assaying liver extract with low activity is the nonenzymatic production of tyrosine. Woolf et al. (1971b) reported the rate of formation of tyrosine in a standard assay procedure is only about eight per cent of the total tyrosine produced nonenzymatically and enzymatically. The rate of formation is 1.5 times greater in 0.2 M sodium phosphate buffer than 0.01 M sodium phosphate buffer pH 6.8. Catalase completely prevents the hydroxylation if present in the assay. In the presence of trichloroacetic acid, pH 2, tyrosine was formed more slowly than at pH 6.8. If the deproteinized solution was stored overnight a substantial amount of tyrosine would be produced to make the assay inaccurate, especially if the enzyme had very low activity or no activity. To eliminate this problem catalase should be used in the incubation, and the deproteinized solution should be analyzed for tyrosine immediately.

The different results in pH, temperature, and molar concentration of substrate and cofactor reported by different researchers may be a result of their purification procedure, as Fisher and Kaufman (1973b) stated. Rat liver phenylalanine hydroxylase, isolated by our method, is in a low activity form with regard to natural cofactor, tetrahydrobiopterin. Woo et al. (1974) suggested the high molecular weight

(210,000) form of the enzyme was due to the purification procedure and caused the enzyme to be less active.

Purification

The liver is homogenized in a variety of solutions: 0.15 M KCl (Barranger et al., 1972, Jakubovic et al., 1971, Friedman and Kaufman, 1973); 0.01 M acetic acid (Kaufman and Fisher, 1970); 0.1 M Tris HCl, pH 8.7, containing 10^{-2} M L-phenylalanine, 10^{-4} M EDTA (Cotton, 1971); 0.05 M potassium phosphate buffer, pH 7.0, containing 0.15 M KCl, 1 mM dithiothreitol, 10 mM phenylalanine; and 5 per cent (v/v) glycerol (Woo et al., 1974). Phenylalanine has a definite effect on the active state of the enzyme as reported by Ayling et al. (1974) and Tourian (1971). Tourian also stated the degree of activation and time required to arrive at the fully activated form of the enzyme was directly proportional to the phenylalanine concentration. After homogenization the enzyme may be in different active states and respond differently to the purification procedures to follow, depending on the solution used for homogenization. The most desirable homogenizing solution would be one which gives the most stability to phenylalanine hydroxylase and results in the most active form of the enzyme.

McGee et al. (1972) and LaDu and Zannoni (1967) reported the 16,000 x g supernatant fraction was more stable than the crude liver homogenate. Addition of the nucleo-mitochondrial preparation from the 16,000 x g precipitation inhibited the 16,000 x g supernatant fraction. This inhibition could not be reversed by increasing the concentration of 6,7-dimethyltetrahydropterin, dithiothreitol, and ascorbate. The microsomal fraction, however, was devoid of inhibitory activity. The

centrifugation speed used to obtain the crude extract should be 16,000 x g or greater to remove the inhibitory nucleo-mitochondrial fraction from the supernatant.

Most of the enzyme is found in the soluble fraction, but Connellan and Danks (1973) found activity in the particulate fraction. The enzyme could not be removed by repeated washing with buffer, but could be extracted with sodium deoxycholate. The per cent of activity in the particulate fraction is so small that it is probably not necessary to include it in the purification, but further studies on the properties of the phenylalanine hydroxylase from the particulate fraction would be of value.

There are a variety of different procedures that can be used to purify phenylalanine hydroxylase, but it seems many of the researchers used some form of calcium phosphate in the purification procedure. The major problem is enzyme stability during purification. Both Kaufman and Fisher (1970) and Woo et al. (1974) claim 400-fold purification, but their purification procedure shows only 174- and 292-fold purification, respectively. The loss of activity is responsible for the difference.

Enzyme Activity and Kinetics

A unit of activity for phenylalanine hydroxylase is defined as one μmole of tyrosine produced per minute under the conditions of the assay. Phenylalanine hydroxylase is very easily affected by the conditions of the assay and other factors which cause the kinetics and activity of the enzyme to differ greatly. To get a better insight into the enzyme's activity and its kinetic properties, a review of many of

the researchers and their results is presented.

Variations in catalytic activity of the hydroxylase vary with varying conditions. For example, the initial rate of the hydroxylation reaction varies with order of addition to the hydroxylase of phenylalanine and tetrahydropterin. When phenylalanine is added before tetrahydrobiopterin, the reaction is characterized by a high initial rate that declines during the first five to six minutes and then becomes essentially constant. The initial burst of activity is not seen when the order of addition is reversed (Kaufman, 1971).

A protein with molecular weight of 56,000 to 59,000 was reported to affect the activity of the enzyme. It was found in a purified preparation of sepiapterin reductase. It was thought that sepiapterin reductase might be necessary for phenylalanine hydroxylase stimulator (PHS) activity, but when acetate was added to sepiapterin reductase extract, sepiapterin reductase lost its activity, but PHS remained active. The natural occurring cofactor, 7,8-dihydrobiopterin, shows a 200-300 per cent stimulation. The synthetic cofactor, 6-methyltetrahydropteridine, shows only a 20-30 per cent stimulation, and 6,7-dimethyltetrahydropteridine shows no stimulating effect.

Under the usual conditions of the assay, there is only small stimulation by adding PHS. When the pH is high (pH 8) and the phosphate concentration is low, the stimulating effect of PHS is large. To gain more information about the PHS mechanism, it was incubated with tetrahydrobiopterin. If the PHS affected the cofactor instead of the enzyme itself, the activity of the assay with the incubated cofactor and PHS should be greater than the unincubated assay. This was not found when

the assays were compared. The stimulation by PHS might be due to its ability to protect the enzyme from inactivation, but the experimental data did not support this. The possibility was examined that PHS stimulates the rate of hydroxylation by decreasing the Km of the hydroxylase for its substrate or coenzyme. It was found that PHS had no significant effect on the apparent Km for the tetrahydrobiopterin and only a slight effect on the apparent Km for phenylalanine. Although PHS has only a slight effect on the apparent Km for phenylalanine, it does markedly increase the inhibition by excess phenylalanine. PHS had little effect on the NADPH to tyrosine ratio (Kaufman, 1970).

Tourian (1971) and Ayling et al. (1971) have reported that phenylalanine hydroxylase exists in a non-activated and activated state, in which phenylalanine shifts the sedimentation constant of the non-activated state (6.1 S) to the activated state (8.1 S). It is believed activation shifts the equilibrium from a dimer of 110,000 molecular weight to a tetramer of 210,000 molecular weight.

The fully activated enzyme gives a classical Michaelis Menten hyperbolic curve and the double reciprocal plot of 1/v versus 1/[S] is a straight line for an 80-fold change in phenylalanine from 0.1 to 8 μmoles. The double reciprocal plot for the enzyme in the process of activation approaches a parabola (Tourian, 1971).

Fisher and Kaufman (1973b) found that when 6,7-dimethyltetrahydropterin is used as cofactor, the initial rate versus phenylalanine concentration curve is biphasic at 25°C and 37°C. At low phenylalanine concentrations the curve is hyperbolic, at concentrations above 0.1 mM the curve is sigmoidal. A Hill plot of the results obtained at 37°C

gives two straight line portions. At low phenylalanine concentrations the Hill coefficient is 0.9, which is not significantly different from 1.0. This value indicates no positive cooperativity. At higher phenylalanine concentrations the Hill coefficient is 2.1 indicating a high degree of cooperativity. These results indicate that at 30°C phenylalanine hydroxylase exists in two kinetically distinguishable forms. About 25 per cent of the activity is due to a form having a low Km (0.04 mM) for phenylalanine and having noncooperative, hyperbolic kinetics. The other 75 per cent of the activity appears to be due to a form with higher Km (0.17 mM) and displaying positive cooperativity.

If the two forms of the hydroxylase suggested by the above results were interconvertible, it should be possible to chemically convert the enzyme from one form to another. It was found that lysolecithin, in the presence of the natural cofactor, increases the maximum velocity 20-fold and decreases the Km of the hydroxylase for phenylalanine. Without lysolecithin, the phenylalanine saturation curve is sigmoidal and the Km was about 0.2 mM. In the presence of lysolecithin, the kinetics of phenylalanine saturation curve become hyperbolic and the Km was 0.09 mM. When the synthetic cofactor, 6,7-dimethyltetrahydropterin, was used, lysolecithin increased the maximum velocity only 1.15-fold and decreased the Km for phenylalanine from 1.3 to 0.8 mM (Fisher and Kaufman, 1972). The stimulation of lysolecithin can be reversed by diluting out the lysolecithin. Chymotrypsin treatment increases the maximum velocity to the same extent as lysolecithin, and decreases the Km of the hydroxylase for phenylalanine to 0.06 mM and enhances substrate inhibition by phenyl-

alanine in the same manner as lysolecithin. Chymotryspin stimulation, however, is irreversible (Fisher and Kaufman, 1973b).

The effect of lysolecithin on phenylalanine hydroxylase is accompanied by the exposure of a sulfhydryl group and therefore appears to alter the conformation of the enzyme. In contrast, chymotrypsin partially hydrolyzes the hydroxylase, reducing its size from a dimer with a molecular weight of 100,000 (composed of two 50,000 molecular weight subunits) to a dimer with molecular weight of 67,000 (composed of two 35,000 molecular weight subunits) (Fisher and Kaufman, 1973b).

The stimulating properties of phenylalanine hydroxylase stimulator (PHS) is also affected by the presence of lysolecithin. PHS stimulates the hydroxylase very little at pH 6.9 in the absence of lysolecithin. In the presence of lysolecithin, however, PHS increases the hydroxylase activity greatly at high enzyme concentrations (Fisher and Kaufman, 1973b).

However, Woo et al. (1974) reported lysolecithin, in the presence of synthetic cofactor, with a concentration of 0.1 to 2 mg/ml was slightly inhibitory to phenylalanine hydroxylase from human liver. Higher concentrations (from 0.5 to 10 mg/ml dispersed in water by sonication) showed more marked and highly significant inhibition.

The reason for the difference in the results between Woo and Kaufman is not clear. Woo's enzyme could be in a different form due to his purification procedure, resulting in different data. The enzyme definitely seems to exist in different states of activity. What brings the change about, and what actually is occurring to the structure of the enzyme is not known. Tourian (1971) and Ayling et al. (1974)

reported a definite change in the sedimentation constant, and suggest the enzyme aggregates and shifts from a dimer to a tetramer. Woo et al. (1974) on the other hand believes the change to the higher molecular state is due to the aggregation of phenylalanine hydroxylase with foreign material, some of it of a lipid nature.

Fisher and Kaufman (1973b) found in the presence of lysolecithin or α-chymotrypsin, rat liver phenylalanine hydroxylase catalyses the p-tyrosine-dependent oxidation of tetrahydropterin. p-tyrosine is not hydroxylated during this reaction. Thur, tetrahydropterin oxidation occurs in the absence of hydroxylation of the substrate. For each equivalent of NADPH (or tetrahydropterin) oxidized, one equivalent of oxygen is reduced to hydrogen peroxide. It has been shown that hydrogen peroxide is formed under conditions of partial or complete uncoupling (the hydroxylase reaction when there is more tetrahydropterin oxidized than tyrosine formed). Oxygen at the reduction level of a hydroperoxide may be the intermediate in enzymatic hydroxylation of phenylalanine. Fisher and Kaufman (1973b) suggest the pterin hydroperoxide might be the intermediate and Mager et al. (1967) suggest pterin or flavin hydroperoxide as an intermediate. Woo et al. (1974) report that there is one mole of FAD associated with one mole of enzyme, which might make the flavin hydroperoxide a more probable candidate for the intermediate.

Purpose of Study

The purpose of this study was to purify phenylalanine hydroxylase from dog liver and covalently attach the enzyme to glass beads. The enzyme would then be insoluble and the same enzyme could be used many times for different studies, to eliminate some of the variables. The

phenylalanine hydroxylase complex itself could be studied by attaching the cofactor, biopterin, dihydropteridine reductase, dihydrofolate, and NADPH.

Phenylalanine hydroxylase attached to some type of inorganic or organic support might be a useful therapeutic tool to lower the phenylalanine level in the blood of phenylketouric patients. Mosbach (1973) proposed that if the enzyme could be trapped in a gel, the immunological reaction would be prevented. The enzyme could be enclosed in tiny semipermable polymer beads that would allow the substrate to diffuse in and the product to diffuse out. The beads could be placed directly into the bloodstream of the patient or packed in a shunt chamber connected to the circulatory system. Other substances such as nylon tubing or mesh or synthetic membrane could be used.

There is no assurance the enzyme can be attached or if attached that it will remain active. If the attachment is possible, there is still the problem of stability of the enzyme. The soluble enzyme is unstable. Also the cofactor, biopterin, and dihydropteridine reductase must be available to phenylalanine hydroxylase. However the enzyme must first be purified and attached before these potential problems can be examined.

During the course of the study it was rapidly learned that, as others have found, purification of the enzyme is difficult. The goal, therefore, has been modified to a study of ten-fold purification of phenylalanine hydroxylase and improving of the assay procedure.

MATERIALS AND METHODS

When this research was begun the purification procedure of Kaufman and Fisher (1970) for rat enzyme, as shown in Table III was used. Due to experimental problems with the ethanol and calcium phosphate gel fractionation steps with dog liver, other approaches for purification were tried. The following procedure resulted.

Purification Procedure

Preparation of Liver

Materials:

Dog liver

Scissors

Ice tray

Saran Wrap

Foil

-80°C Freezer ---------- Revco Ultra Low

Method:

The dog livers were donated by Dr. David S. Mailman's laboratory. The livers were from mongrels weighing 11 to 18 kgs and represented a random sample with respect to physiological condition and sex of the animals. Sodium pentobarbital was used to anesthetize the animals before removal of the liver. The livers were received fresh and immediately placed in cold deionized water, then cut into small pieces with scissors. The small pieces were then placed on Saran Wrap in thin layers in the ice trays. This helps facilitate fast freezing and thawing. The trays are wrapped as air tight as possible with Saran Wrap and

TABLE III

COMPARISON OF PURIFICATION PROCEDURES FOR PHENYLALANINE HYDROXYLASE

Author	Species	Purification Steps	Specific Activity (units/mg)	Yield %
Kaufman (1970)	Rat	Liver Extract	0.0034	100
		Ethanol Fraction (10-21%	0.011	112
		First Ammonium Sulfate (29-37%)	0.036	101
		Calcium Phosphate Gel Elute	0.083	51
		Second Ammonium Sulfate & Sephadex G-25	0.096	31
		DEAE - Cellulose	0.029	11
		Sephadex G-25	0.59	5
(Degree of Purification 173.5-fold)				
Woo et al. (1974)	Human	Liver Homogenate	0.00028	100
		Liver Extract	0.00077	98.5
		Ammonium Sulfate (26-44%	0.00584	94.6
		Sephadex G-25 Fraction	0.00586	86.8
		DEAE - Cellulose	0.0228	52
		G-200 Sephadex	0.0843	19.1
		Hydroxyapatite	0.2251	5.2
(Degree of Purification 292.2-fold)				

TABLE III--continued

Author	Species	Purification Steps	Specific Activity (units/mg)	Yield %
Cotton (1971)	Monkey	Crude Extract	0.0022	
		Protamine Sulfate	0.0032	
		Ethanol (35%)	0.005	
		Ammonium Sulfate (50%)	0.01	
		Sephadex G-200	0.03	
		DEAE - Cellulose	0.058	
		Brushite Batch	0.012	
		Brushite (Gradient)	0.6	
(Degree of Purification 272.2-fold)				

foil and kept at -80°C.

Purification of Phenylalanine Hydroxylase

Materials:

Magnetic stirrer

0.15 M KCl

2 per cent Protamine sulfate in 0.1 M potassium phosphate, pH 7.5

Ammonium sulfate ---------- Swarz/Mann, Lot #AZ1971

-80°C Freezer ---------- Ultra Low Revco

50 ml Polypropylene, round bottom, beaded rim, centrifuge tubes

Water bath

Waring blender with rheostat control

Beckman J-21 B Model centrifuge, JA14 rotar

250 ml Polypropylene bottle

Methods:

All steps are carried out at 4°C unless otherwise stated.

Step 1. Crude Extract. The livers are partially thawed, weighed and homogenized in a Waring blender for 30 seconds with 1.5 volumes (v/w of liver) of cold 0.15 M KCl. The blender is at one-third speed, controlled by a rheostat. Another 1.5 volumes of cold 0.15 M KCl is added and blending is continued for another 30 seconds. The homogenate is centrifuged at 18,000 x g for 45 minutes, and the sediment is discarded. A 30 minute period of stirring the homogenate before centrifugation was tried, to observe if more enzyme would go into solution, but no significant increase in activity in the supernatant was observed due to additional stirring.

Step 2. Protamine Sulfate. The supernatant from step one is treated with protamine sulfate (one part of 2 per cent solution of protamine sulfate in 0.1 M potassium phosphate, pH 7.5, to five parts of supernatant) to remove nucleic acids. The enzyme extract is stirred continuously with a magnetic stirring bar while the protamine sulfate solution is added by pouring it down a glass rod over a ten minute time period. After the addition of protamine sulfate, stirring is continued for 20 minutes. The solution is centrifuged at 18,000 x g for 20 minutes. The pellet is discarded.

Step 3. Ammonium Sulfate Fraction. The protamine sulfate supernatant was fractionated with ammonium sulfate (4°C). A saturated ammonium sulfate solution was added by pouring the solution down a glass rod over a ten minute period; to give a solution of 35 per cent saturation, the ammonium sulfate fraction is stirred slowly with a magnetic stirring bar for 30 minutes. It then is poured into 250 ml centrifuge tubes and centrifuged for 20 minutes at 18,000 x g. The pellet is discarded and a 45 per cent saturated (v/v) fraction is made by adding saturated ammonium sulfate to the supernatant in the same manner as the 35 per cent fractionation. The 45 per cent fraction is centrifuged and the pellet is resuspended in 0.005 M Tris-HCl, pH 7.0 buffer. The supernatant is discarded.

Step 4. Heat Treatment. The resuspended enzyme extract is divided into 12 ml portions. To each 12 ml portion, 2 ml portions of 1.0 Tris-HCl, pH 7.4, 0.1 M phenylalanine, 0.005 M dithiothreitol (freshly prepared), and catalase (25 mg/ml) to give a total volume of 20 ml. The enzyme solution is placed into a 125 ml Erlenmeyer flask.

The solution is kept at 4°C until it is placed in a 55°C water bath for seven minutes, with constant shaking. After seven minutes the flask is placed in an ice water bath and cooled to 4°C. The heat treated solution is then centrifuged at 18,000 x g for 20 minutes. The pellet is discarded and the supernatant is precipitated with two volumes of saturated ammonium sulfate (4°C). The ammonium sulfate is done in the same manner as the earlier ammonium sulfate fractions. The pellet is resuspended in 0.005 M Tris-HCl, pH 7.0, and stored in the -80°C freezer.

Protein Determination

The spectrophotometric method of Warburg and Christian (1941-42) was used in which the absorbance was read at 260 nm and 280 nm.

$$\text{Protein concentration (mg/ml)} = 1.55A_{280} - 0.760A_{260}$$

Preliminary studies of protein determination showed no significant difference between the procedures of Lowry *et al*. (1951) and Warburg and Christian (1941-42).

Assay Method

In the course of this study four different assay methods were used: that of Kaufman and Fisher (1970); that of Ayling *et al*. (1974); the assay developed in this study; and a modification of the new assay when biopterin is used as a cofactor. In each of the tables of data the assay used in obtaining the data will be denoted by the first author's name in the reference in which the assay is found. The Kaufman and Fisher (1970) assay, shown in Table II, was the first assay used. To use this assay dihydropteridine reductase had to be purified from dog liver. In the beginning the dihydropteridine reductase was purified

through the ammonium sulfate step (Kaufman, 1962) which left the enzyme in a crude condition and could be a limiting factor in the assay. It was decided a different assay should be used. Ayling's assay (Ayling et al., 1974), shown in Table II, which uses a chemical means to reduce the cofactor, was chosen, the further purification of dihydropteridine reductase was not necessary

The Ayling et al. (1974) assay worked very well, but it was found that by increasing the concentration of both phenylalanine and cofactor, tyrosine production was much higher. An assay, therefore, was developed to give more optimum tyrosine production.

Materials:

Dubnoff metabolic shaking incubator

50 ml Polypropylene, round bottom, beaded rim, centrifuge tubes

0.2 ml and 1.0 ml Biopette ---------- Schwarz/Mann

Beckman J-21 B model centrifuge, JA-20 rotar

Deionized water

Mettler H15 balance

12% Trichloracetic acid

Incubation Mixture

Tris-HCl, pH 7.4 ---------- 100 µmoles ---------- Sigma

Phenylalanine ---------- 10 µmoles ---------- Sigma

Catalase ---------- 0.25 mg ---------- Sigma

Dithiothreitol ---------- 5 µmoles ---------- Sigma

Phenylalanine hydroxylase ----- 0.1 ml

6.7-Dimethyltetrahydropterin -- 1.0 µmole in 0.005 M HCl --- Aldrich

Methods:

The complete reaction mixture contains the following components in a final volume of 1.0 ml: 100 µmoles of Tris-HCl, pH 7.4; 10 µmoles of phenylalanine; 0.25 mg of catalase; 5 µmoles of dithiothreitol; 0.1 ml of phenylalanine hydroxylase and 1.0 µmole of 6,7-dimethyltetrahydropterin (pterin). The components are added in a 0.1 ml volume in the order they are listed. Deionized water is used to bring the volume up to 1 ml. Pterin is not added until after a five minute preincubation. Both dithiothreitol and pterin solutions are made immediately before addition. Dithiothreitol is put into solution with cold deionized water and pterin is put into solution with cold 0.005 M HCl. The incubation mixture is added to 50 ml centrifuge tubes in an ice bath. For a blank two ml of tricholroacetic acid is added before the enzymes are added. A tyrosine standard prepared in the same manner as the blank, except that it contains a known amount of tyrosine, is carried through each assay. All the components of the assay are added except pterin after which the tubes are placed in a metabolic shaker at 37°C for five minutes, shaking at 130 revolutions per minute. After the five minutes, pterin is added at ten second intervals to each tube. The mixture is incubated for five minutes, then the reaction is stopped by addition of two ml of 12 per cent trichloroacetic acid at ten second intervals. The deproteinized solution is centrifuged in the Beckman J-21 B centrifuge at 7,800 x g for ten minutes, and the supernatant was analysed for tyrosine by the nitrosonaphthol method.

Nitrosonaphthol Procedure

Materials:

15 ml Conical centrifuge tubes

Thelco Model 83 water bath

Clay Adams Inc. safety head table top centrifuge

Beckman Mocel 25 spectrophotometer

Cuvettes, 10 mm path length, 165-2500 nm, quartz -------- Curtin #30-300

1-Nitroso-2-naphthol ---------- 0.1 per cent 1-nitroso-2-naphthol in 95 per cent alcohol

Nitric acid reagent ---------- 1:5 nitric acid containing 0.5 mg per ml of $NaNO_2$

1,2-Dichloroethane

Method:

Two ml aliquots of the deproteinized solution from each tube is added to glass centrifuge tubes. One ml each of the nitroso-naphthol and nitric acid mixtures was added to each centrifuge tube, mixing after each addition. The tubes are loosely capped, placed in a water bath at 55°C for 30 minutes, and then removed and cooled to room temperature. Ten ml of dichloroethane are added to each tube and the tubes are shaken to extract the unchanged nitroso-naphthol. The tube is then centrifuged at low speed for five minutes and the supernatant aqueous layer is transferred to a cuvette. The optical density is determined at 450 nm in a spectrophotometer. A standard curve for tyrosine is used to determine the amount of tyrosine present. The optical densities are almost proportional to the concentration at least up to 0.8 µM of tyrosine (Undenfriend and Cooper, 1952b).

Solubilization of the Particulate Enzyme

Materials:

Waring blender with speed control

One per cent desoxycholate

0.15 M KCl

0.01 M Tris-HCl, pH 7.5

Beckman J-21 B model centrifuge, JA 14 rotor

250 ml Polyproplene centrifuge bottles

Method:

The liver was partially thawed, weighed, and homogenized in 3 volumes (v/w of liver) of 0.15 M KCl. The procedure for homogenization and centrifugation were the same as in step one of the purification procedure. The homogenate is centrifuged, the pellet from the centrifugation is resuspended in three volumes of 0.005 M Tris-HCl, pH 7.0, buffer and an equal volume of one per cent desoxycholate is added to the mixture and homogenized gently for one minute. The homogenate is centrifuged and the supernatant was tested for enzyme activity. The supernatant from the crude homogenate, and the supernatant of the Tris-HCl wash are also checked for enzyme activity.

The Effect of Tris-HCl, pH 8.7, on the Extraction of Phenylalanine Hydroxylase

Materials:

Waring blender with rheostat

0.1 M Tris-HCl, pH 8.7, with 0.15 M KCl

0.15 M KCl

Method:

The liver is partially thawed and weighed, then divided into two equal portions. One portion is homogenized in 0.1 M Tris-HCl, pH 8.7, with 0.15 M KCl and the other portion is homogenized in 0.15 M KCl. The same procedure for homogenization and centrifugation are used as in step one of the purification procedure. The supernatants from the centrifugation are tested for mg of protein per ml and enzyme activity per mg of protein.

The Effect of Dialysis on Phenylalanine Hydroxylase Activity

Materials:

0.005 M Tris-HCl, pH 7.0

Dialysis tubing

Magnetic stirrer

Two liter beaker

Method:

Phenylalanine hydroxylase extract (33-45 per cent ammonium sulfate fraction) which had been frozen is divided into two portions. One portion was stored at 4°C and the other portion is divided into five ml aliquots and put into 10 mm dialysis tubing which had been boiled in 10^{-4} M EDTA and rinsed with deionized water. The five ml aliquots of phenylalanine hydroxylase extract is dialyzed against two liter volumes of cold 0.005 M Tris-HCl, pH 7.0, 4°C, buffer every two hours for twelve hours. The dialyzing buffer is stirred with a magnetic stirring bar. The aliquots, both stored and dialyzed were removed after twelve hours and assayed for phenylalanine hydroxylase activity.

The Effects of Additional Mechanical Stirring

Method:

After the homogenation step in the purification procedure the crude liver extract is divided into two aliquots. One is centrifuged immediately and the other aliquot is stirred slowly with a magnetic stirring bar for three hours. Then the stirred aliquot is centrifuged, both aliquots are centrifuged for 45 minutes at 18,000 x g. The supernatants of both aliquots are tested for enzyme activity and mg of protein per ml.

Kinetic Studies

Materials:

Assay components as stated in Table II

Olivetti Underwood Programma 101

Least Square Analysis Program

Ayling Assay (Ayling et al., 1974)

Present Assay (Materials and Methods)

Methods:

The components of the assay are the same except when stated in each experiment. To determine the Km values for phenylalanine, pterin, and biopterin, the Liveweaver Burk plot was used. Both graphing 1/V versus 1/[S] and the program for least square analysis was used to achieve greater accuracy.

G-25 Sephadex Column Experiment

Materials:

G-25 Sephadex (20-80 μm particle size) ------- Pharmacia Fine Chem. Inc.

Pasteur pipet

Glass wool

0.005 M Tris-HCl, pH 7.0

Method:

Small columns are used due to loss of enzyme activity in the large column. The G-25 Sephadex is allowed to stand in 0.005 M Tris-HCl (pH 7.0) buffer overnight with occasional stirring, to allow the Sephadex to swell. Glass wool is packed in the bottom of the Pasteur pipet giving a flat surface for the Sephadex to rest on. The column was then poured in the usual manner. The volume of the column is approximately 1.98 ml. Approximately 0.6 ml (1/3 volume of column) of enzyme extract is placed on the column and 0.5 ml fractions were collected.

RESULTS AND DISCUSSION

The attempts to purify phenylalanine hydroxylase from dog liver has been handicapped by the lack of a simple and reliable method to measure enzyme activity. The methods reported in the literature are tedious and involve a complex reaction mixture. Furthermore, relatively crude extracts may easily contain substances which either might deactivate the enzyme or influence the amount of tyrosine available for assay. Early attempts at purification depended upon the assay of Kaufman and Fisher (1970), and other assay methods were examined to determine whether or not they were better for this study. Finally, a modification was developed in this study which proved to be more sensitive and better suited for work with dog liver extracts.

Therefore, different assay methods were used for different parts of this study. There are:

1. Kaufman and Fisher (1970)
2. Ayling et al. (1974)
3. Present assay
4. Present assay with biopterin

Some of the variation probably is attributable to differences in assay reliability and conditions. As shown in Table II the incubation period of the assays varies greatly from researcher to researcher, and the assay as reported by Ayling et al. (1974) does not state the period of time for incubation. Therefore, for this study measurement of tyrosine production as a function of the time of reaction was done. It was found that tyrosine production was essentially linear for about seven minutes,

as shown in Fig. 2. The points at three and nine minutes do not fall directly on the line; however, this variation is probably a result of technique in the assay procedure. It was also found that the tyrosine production decreases after ten minutes of incubation. To insure the initial velocity was used in the incubation, a five minute period for incubation was chosen. A preincubation period also was used since it increased tyrosine production as shown in Fig. 3. This could be due to stimulation of phenylalanine hydroxylase by phenylalanine as reported by Tourian (1971). He concluded phenylalanine hydroxylase has a second activating site for phenylalanine distinct from its catalytic site. The time it takes to fully activate the enzyme is directly proportional to the concentration of phenylalanine. With the concentration used in the present assay a five minute preincubation was decided on.

In doing preliminary kinetic studies it was learned that tyrosine production was increased greatly if the phenylalanine concentration was increased over the concentration used in the assay mixture of Ayling et al. (1974). The assays of Kaufman and Fisher (1970) and Ayling et al. (1974) both give linear plots of velocity versus protein concentration. The assay developed in this study also shows linear behavior of velocity versus protein concentration, as shown in Fig. 4. The assay developed in the present study is very helpful with low activity or to detect tyrosine production after the enzyme has been diluted after separation on a column. Enzyme activity can be detected with the present assay when no activity is measured by the assay of Ayling et al. (1974). After finding increased phenylalanine concentration increased tyrosine production, it was decided that each component

FIGURE 2

Velocity (μmoles of tyrosine/5 minutes) versus time for phenylalanine hydroxylase.

(Present Assay)

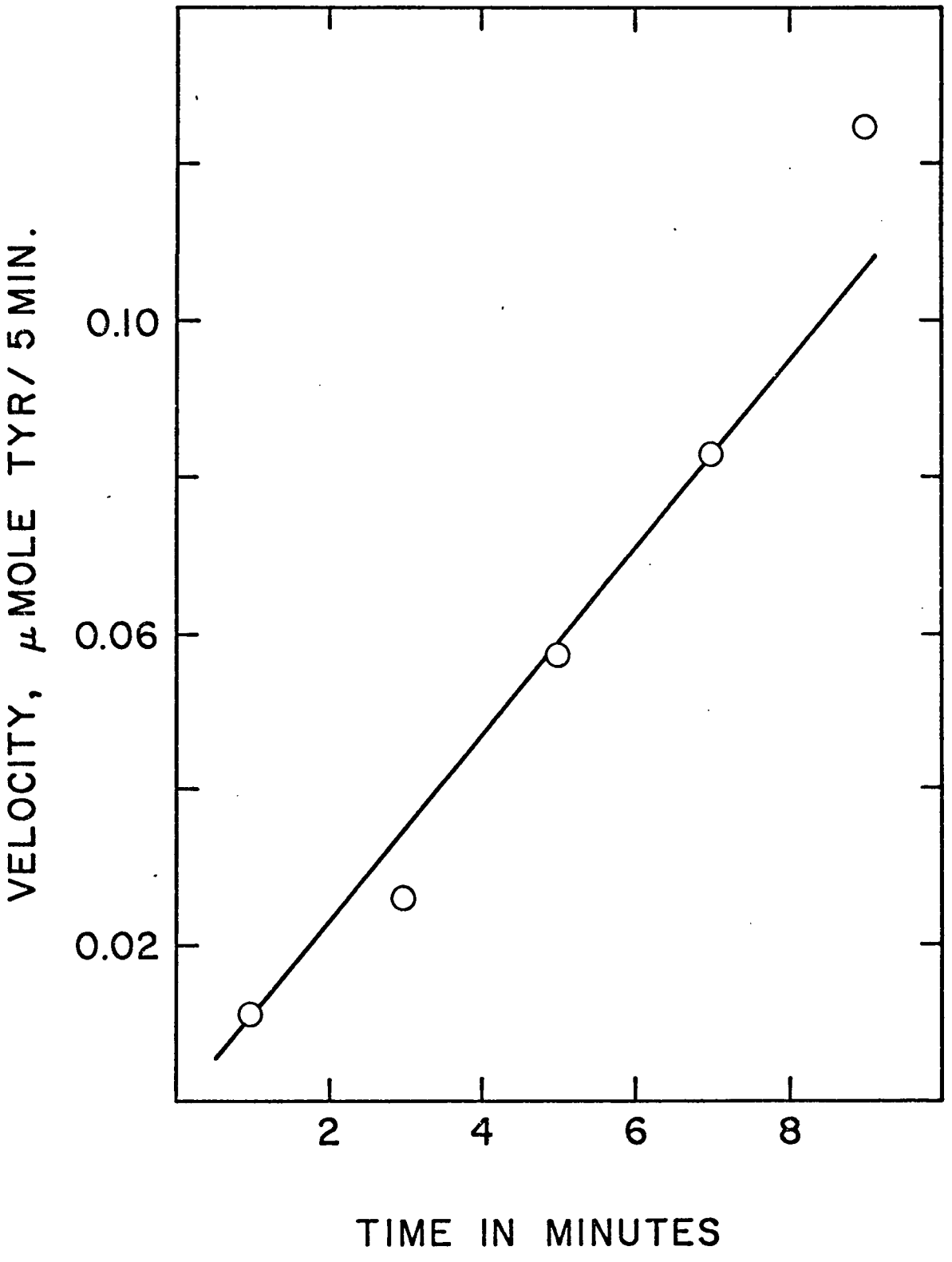

VELOCITY, μ MOLE TYR/5 MIN.
0.10
0.06
0.02
2
4
6
8
TIME IN MINUTES

FIGURE 3

Velocity (μmoles of tyrosine/5 minutes) versus phenylalanine concentration for dog liver phenylalanine hydroxylase.

preincubation o

no preincubation Δ

(Ayling Assay)

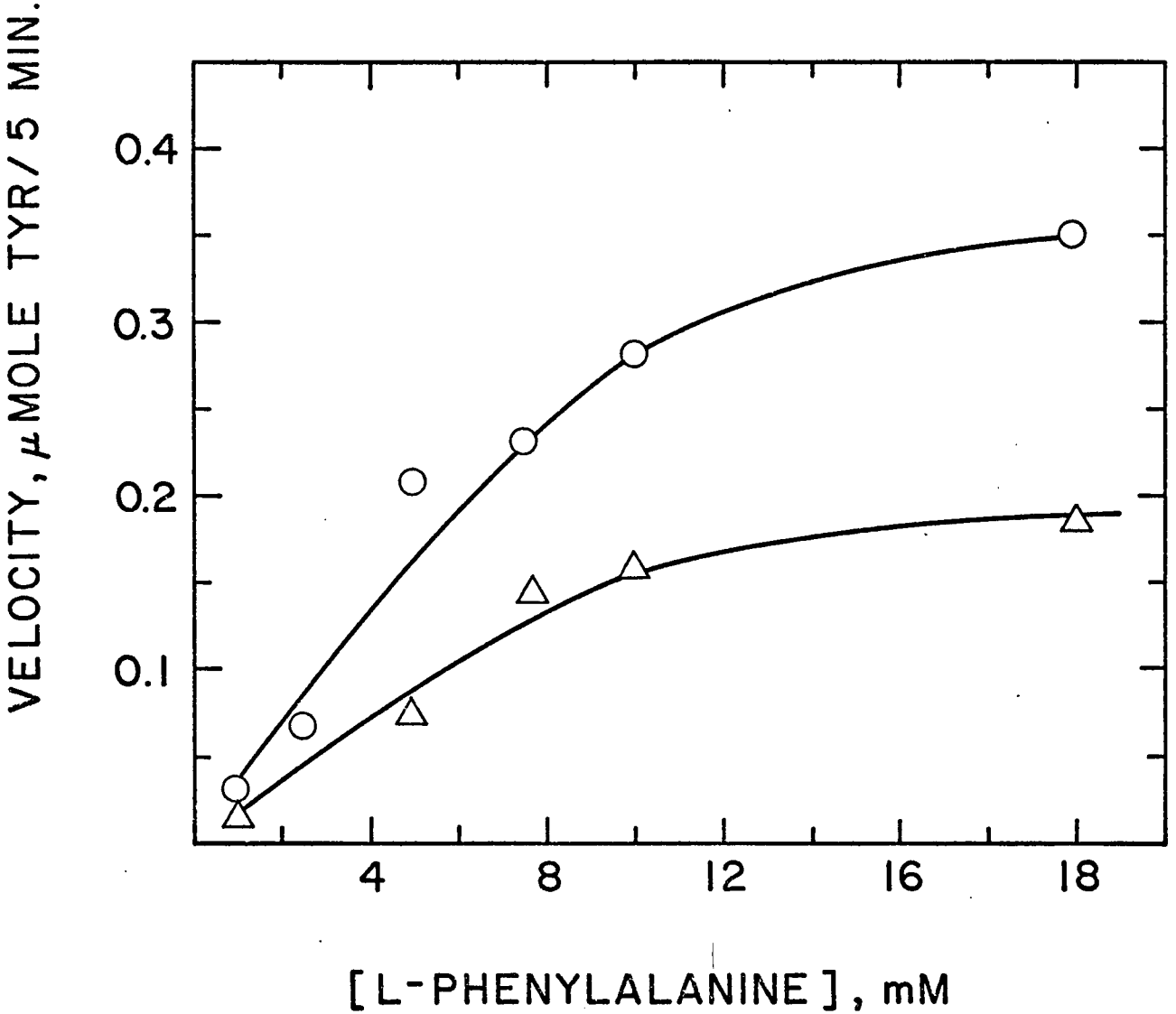
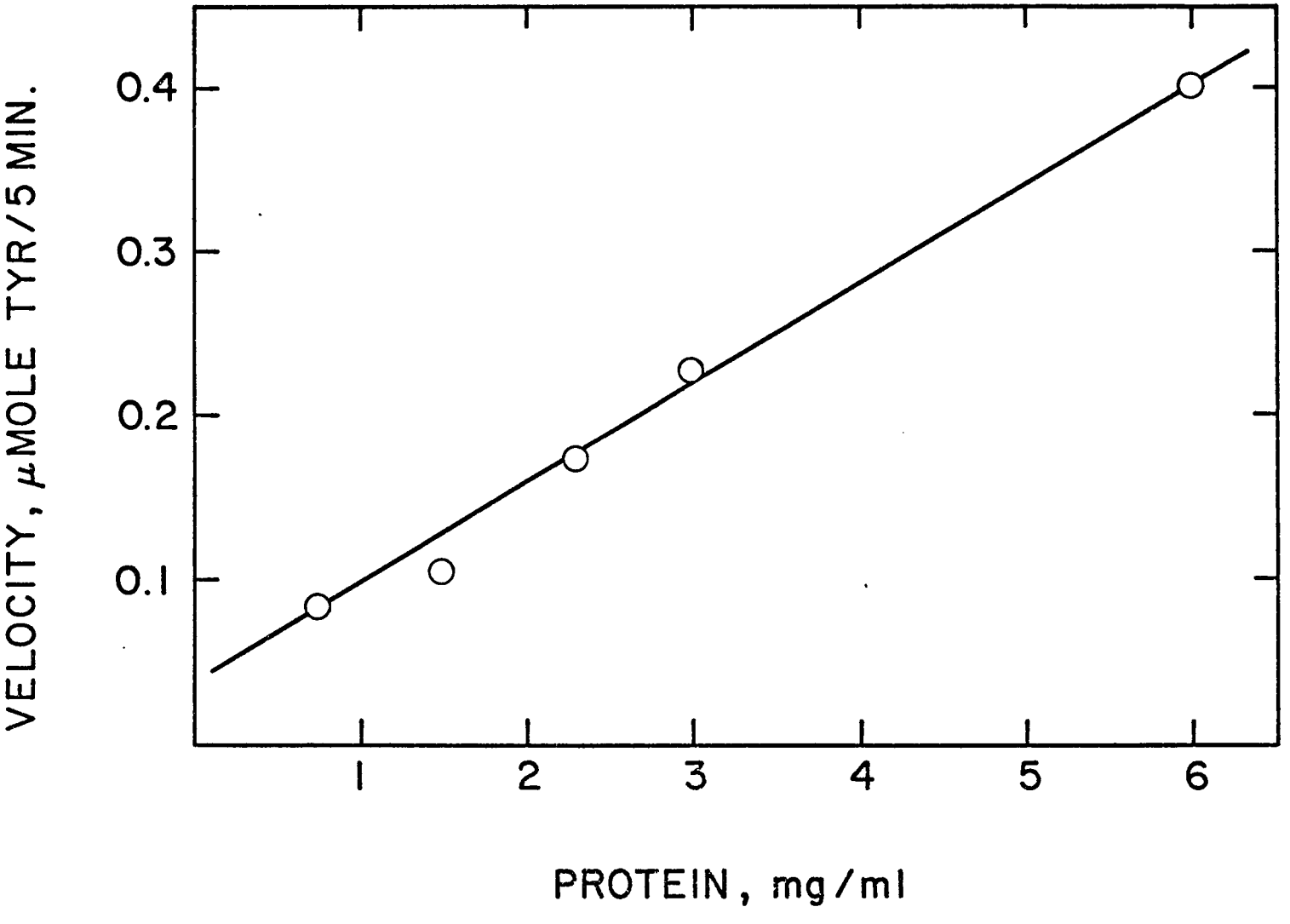

VELOCITY, μMOLE TYR/5 MIN.
0.4
0.3
0.2
0.1
4
8
12
16
18
[L-PHENYLALANINE], mM

FIGURE 4

Velocity versus mg/ml of protein for dog liver phenylalanine hydroxylase.

(Present Assay)


VELOCITY, μMOLE TYR/5 MIN.
0.4
0.3
0.2
0.1
1
2
3
4
5
6
PROTEIN, mg/ml

of the assay mixture would be studied to develop an assay with optimum tyrosine production.

When phenylalanine was increased to 90 mM, inhibition occurred somewhere above 20 mM, as shown in Table IV. Inhibition also occurs when pterin concentration is increased to approximately 3 mM as shown in Table V. When the natural cofactor, tetrahydrobiopterin (biopterin), is used the inhibition of phenylalanine hydroxylase by both phenylalanine and biopterin occurs at a much lower concentration of both substrate and cofactor. As shown in Fig. 5 phenylalanine inhibits phenylalanine hydroxylase when the concentration is above 2.5 mM. Inhibition due to biopterin occurs in the approximate range of 0.1 mM as shown in Table VI. The reason for the phenomenon is not known, but Fisher and Kaufman (1973a) report that when pterin is used as cofactor the enzyme is less efficient in the production of tyrosine. When the natural cofactor, biopterin, is used the ratio of NADPH used to tyrosine produced is one. If the mechanism for production of tyrosine is affected, then it is likely that the mechanism for inhibition is also affected.

The effect of catalase on tyrosine production was studied and it was found that the tyrosine production was not significantly greater in the presence of catalase (Fig. 6). McGee et al. (1972) also reported addition of catalase was without effect on the assay. It is possible that in the crude enzyme extracts there is enough catalase present to protect phenylalanine hydroxylase from H_2O_2 inhibition.

The optimum temperature and pH for dog liver enzyme were determined. The pH at which the maximum tyrosine is produced is

TABLE IV

INHIBITION OF PHENYLALANINE HYDROXYLASE BY PHENYLALANINE

Phenylalanine mM	µMoles of Tyrosine/5 Minutes		
	Exp. 1	Exp. 2	Exp. 3
0.5	--	--	0.0185
1.0	0.0405	--	0.025
2.5	--	0.008	0.0605
5.0	0.102	0.028	0.1595
7.5	--	0.028	0.1675
10.0	0.150	0.036	0.203
20.0	0.203	0.040	0.1955
30.0	0.236	0.0435	0.1795
40.0	0.235	0.037	0.1765
90.0	--	0.0345 (low act.)	0.143

Ayling Assay

TABLE V

INHIBITION OF PHENYLALANINE HYDROXYLASE
BY 6,7-DIMETHYLTETRAHYDROPTERIN

6,7-Dimethyltetrahydropterin mM	µMoles of Tyrosine/5 Minutes
0.1	0.1755
0.2	0.2355
0.5	0.2600
1.0	0.2655
1.5	0.2915
2.0	0.2980
3.0	0.2960
5.0	0.2320

FIGURE 5

Velocity (μmoles of tyrosine/5 minutes) versus phenylalanine concentration for dog liver phenylalanine hydroxylase.

(Present Assay with biopterin modification)

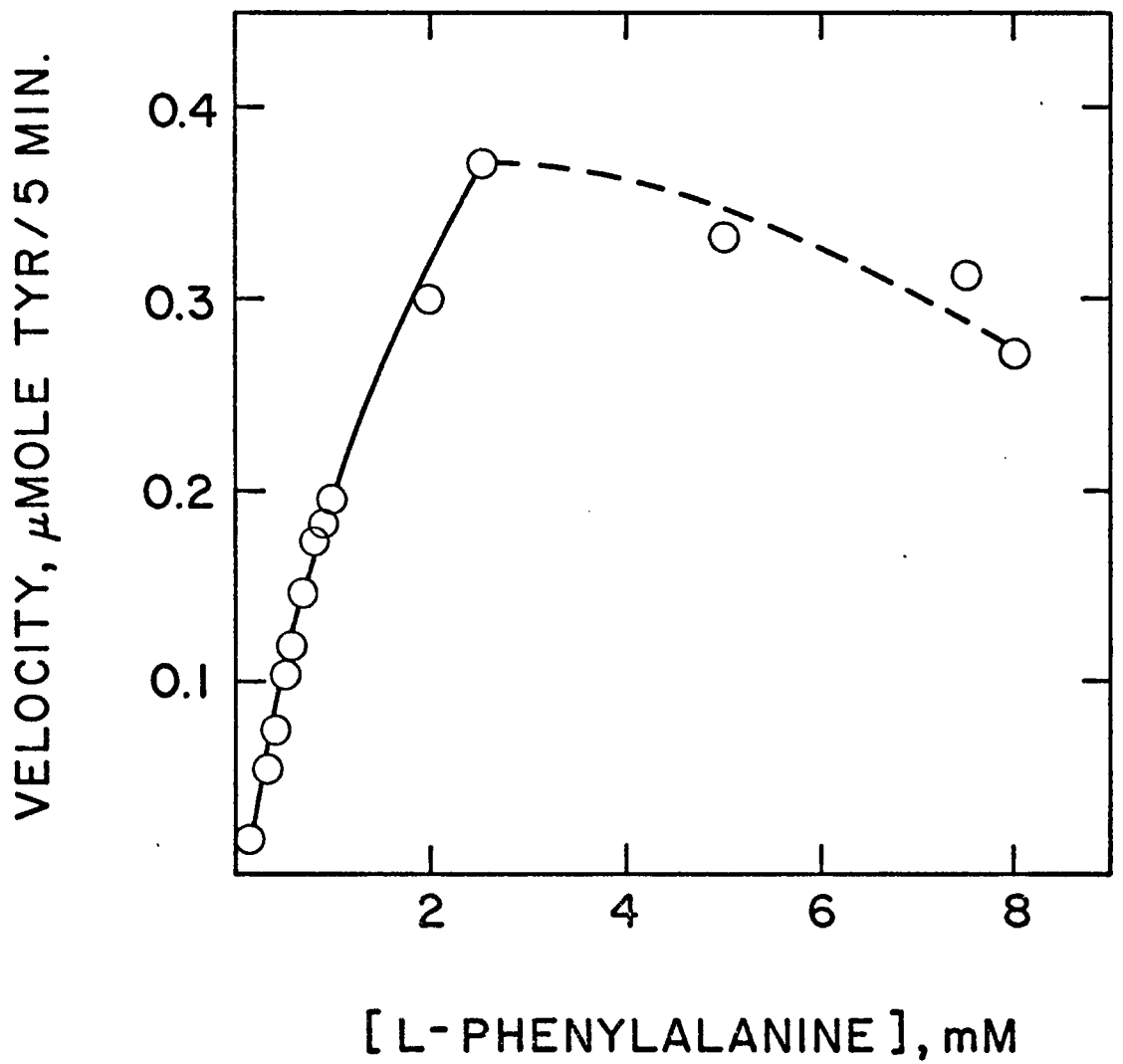

VELOCITY, μMOLE TYR/5 MIN.
0.4
0.3
0.2
0.1
2
4
6
8
[L-PHENYLALANINE], mM

TABLE VI

EFFECT OF CONCENTRATION OF TETRAHYDROBIOPTERIN ON PHENYLALANINE HYDROXYLASE ACTIVITY

Tetrahydrobiopterin mM	µMoles of Tyrosine/5 Minutes	
	Exp. 1	Exp. 2
0.010	--	0.0425
0.020	--	0.1030
0.025	0.1055	--
0.030	--	0.1280
0.040	--	0.1415
0.050	0.177	0.1845
0.060	--	0.1775
0.070	--	0.1915
0.080	--	0.2030
0.090	--	0.2295
0.100	0.2215	0.1885
0.250	0.1740	--
0.500	0.2030	--

Present Assay

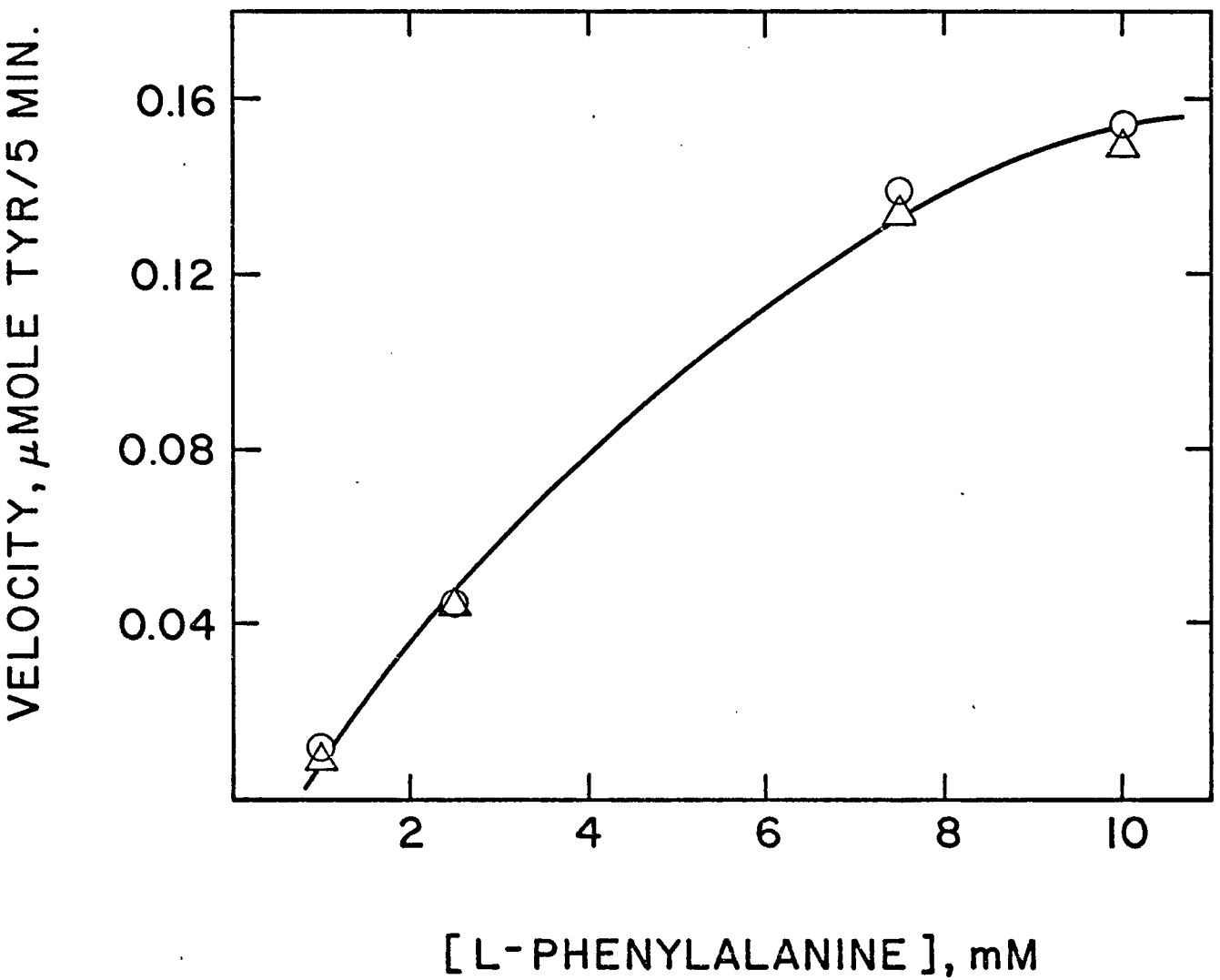
FIGURE 6

Velocity (μmoles of tyrosine/5 minutes) versus phenylalanine concentration for dog liver phenylalanine hydroxylase.

Catalase present in assay mixture o

Catalase absence in assay mixture Δ

(Present Assay)


VELOCITY, μMOLE TYR/5 MIN.
0.16
0.12
0.08
0.04
2
4
6
8
10
[L-PHENYLALANINE], mM

approximately 7.5, as shown in Fig. 7. Bublitz (1969) reported the reaction rate to increase from pH 6.0 to 7.2 and fall as the pH is increased beyond 7.2. Cotton (1971) reported the optimum pH to be pH 7.6. Others such as Kaufman and Fisher (1970) prefer a much lower pH of 6.8. The present study was done in both Tris-HCl and potassium phasphate buffer. It was found that the potassium phosphate buffer gave higher tyrosine production at all values of pH. This could be due to the stimulation of the enzyme by phosphate as reported by Tourian (1971). The optimum temperature was in the range of 35°C-45°C as shown in Fig. 8. However, Kaufman (1971) reported that the ratio of NADPH oxidized to tyrosine formed is higher at 35°C than 25°C. Undenfriend and Cooper (1952) reported the optimum temperature to be 35°C but the destruction of tyrosine was higher at 35°C than 25°C. Both groups of researchers came to the conclusion that 25°C would give a more valid picture of the enzyme activity. Since the assay developed in this study is not for use in kinetic studies, but only for detection of enzyme activity, it was decided the temperature of 37°C would be used since it is physiological temperature. The temperature study showed that the enzyme was stable up to 45°C and lost little activity at 55°C over a 15 minute period. Due to these results a heat treatment was tried as part of the purification procedure, and it proved successful.

When the assay of Kaufman and Fisher (1970) was compared to the assay of Ayling et al. (1970) in this laboratory, it was found the tyrosine production was one-tenth of the Ayling et al. (1974) assay as shown in Table VII. The low production in the Kaufman and Fisher (1970) assay could be due to the crude preparation of dihydropteridine

FIGURE 7

Velocity (μmoles of tyrosine/5 minutes) versus pH for dog liver phenylalanine hydroxylase.

100 mM Tris-HCl o

100 mM Potassium phosphate Δ

(Ayling Assay)

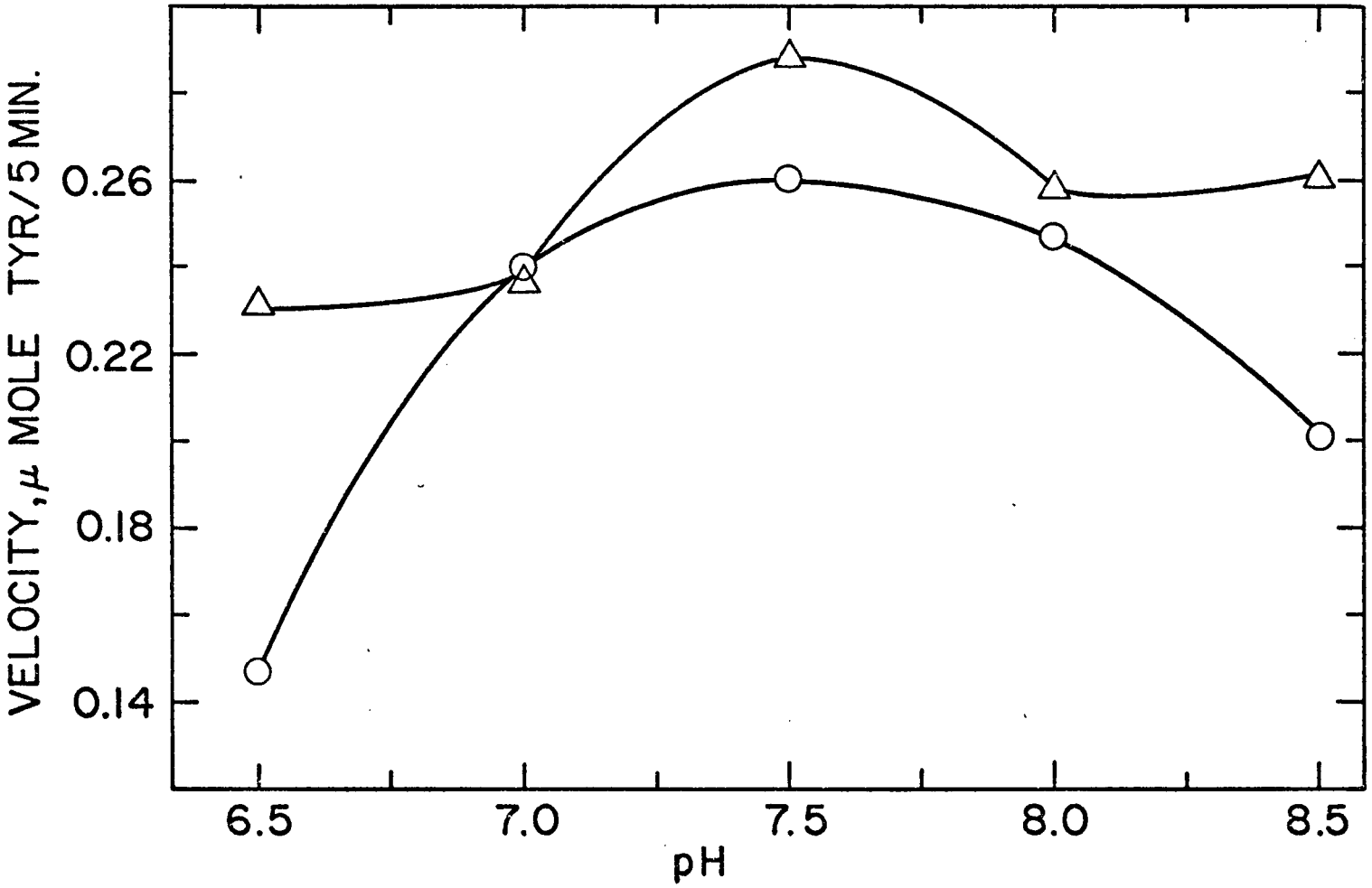
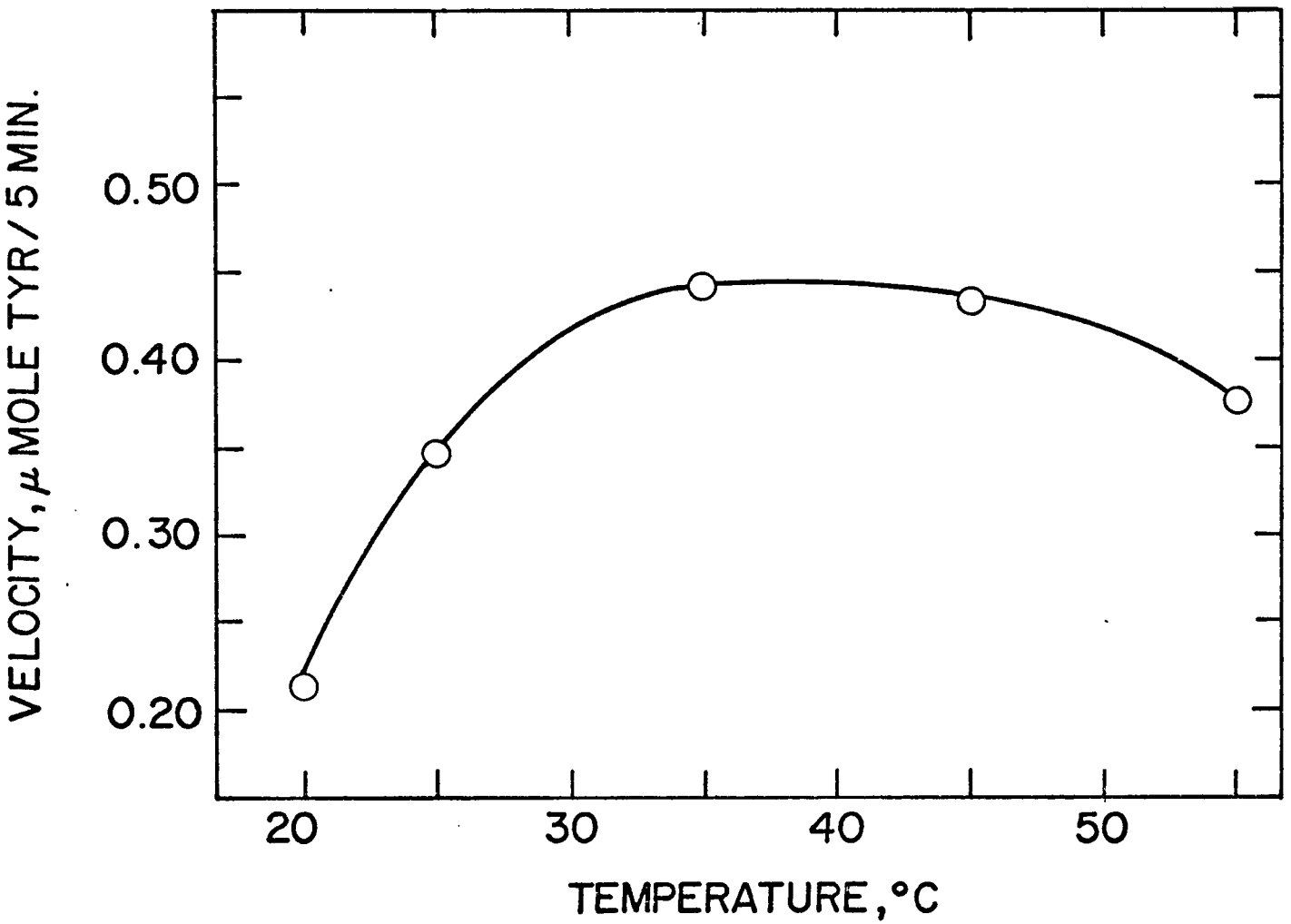

VELOCITY, μ MOLE TYR/5 MIN.
0.26
0.22
0.18
0.14
6.5
7.0
7.5
8.0
8.5
pH

FIGURE 8

Velocity (μmoles of tyrosine/5 minutes) versus temperature, °C, for dog liver phenylalanine hydroxylase.

(Ayling Assay)


VELOCITY, μ MOLE TYR/5 MIN.
0.50
0.40
0.30
0.20
20
30
40
50
TEMPERATURE, °C

reductase used in this comparison.

Ayling's et al. (1974) assay was also compared to the assay developed in the present study and it was found to have one-tenth the tyrosine production of the developed assay as can be seen in Table VII. Most of the results reported were obtained using either Ayling et al. (1974) or the newly developed assay.

TABLE VII

COMPARISON OF ASSAYS

Assay	μMoles of Tyrosine/minute
Kaufman and Fisher (1970)	0.001
Ayling et al. (1974)	0.010
Ayling et al. (1974)	0.010
Assay from study	0.123

Purification

There are many obstacles to the purification of phenylalanine hydroxylase from dog liver. Time is the biggest problem, since the enzyme loses activity quickly at 4°C. When the enzyme is purified only ten fold, as shown in Table VIII, the procedure is rapid (taking only one day to complete) and little activity is lost. The assay procedure itself was a major problem. The assay procedure takes at least three hours to complete and if there are many samples as in a column, the completion time was much longer.

When phenylalanine hydroxylase is placed on a column of

TABLE VIII

PURIFICATION PROCEDURE

Fractionation Steps	Volume	Total Enzyme Units	Total Protein	Specific Activity	Purification
	ml	μmoles tyrosine/min.	mg	units/mg	
Crude Extract	100	35.7	14,877	0.0024	1.00
Protamine Sulfate	110	34.9	7,130	0.0049	2.04
Ammonium Sulfate (30-45%)	25	14.7	1,775	0.0083	3.45
Heat Treatment	33	10.3	398	0.02589	10.79

substantial size, the enzyme loses so much activity that an increase in specific activity is not seen. Many different types of columns were tried, as shown in Table IX, but none proved successful.

TABLE IX

COLUMNS WHICH DID NOT IMPROVE PURIFICATION OF PHENYLALANINE HYDROXYLASE

Column	Reference	Type
Polyphenylalanine Agarose		Affinity
Phenylalanine Agarose	Cuatrecasas et al. (1968)	Affinity
High Ionic Strength Hydrophobic	Rimerman and Hatfield (1973)	Salt-Mediated Hydrophobic Chromatography
Blue Dextran Agarose	Thompson et al. (1975)	Affinity
DEAE	Kaufman and Fisher (1970)	Ion Exchange

The DEAE column shows the most promise. The enzyme is held on the column while some of the protein is eluted off in the void volume. Phenylalanine hydroxylase can be eluted off with KCl above 1.5 M, while other protein is eluted off at 0.22 M. Due to the instability of the enzyme, it was not possible to detect any increase in purification. If the enzyme can be stabilized, the DEAE column could be used in further purification of phenylalanine hydroxylase. Other columns were also tried such as affinity columns with both phenylalanine and polyphenylalanine, but the enzyme was eluted in the void volume. This could be due

to the Km of the phenylalanine and polyphenylalanine being too high or there may not be enough substrate attached to the column to hold enough enzyme that the tyrosine production could be detected. An affinity column with a substrate or analog of the substrate with a low Km might prove successful.

High ionic strength hydrophobic chromatography (Rimerman and Hatfield, 1973) was tried in which phenylalanine was attached to an agarose column. The protein was added to the column in the presence of high salt concentration. The most soluble, least hydrophobic, proteins are weakly attached to the column. As the salt concentration is reduced the least hydrophobic proteins are eluted. In the present study phenylalanine hydroxylase was eluted in the void volume.

A blue dextran-agarose column that binds enzymes with a dinucleotide fold was used (Thompson et al. (1975). The dinucleotide find is known to form the NAD-binding site in lactate, malate, and glyceraldehydephosphate dehydrogenases, to form the ATP-binding site in phosphoglycerate kinase, and to be present in the structures of alcohol dehydrogenase, adenylate kinase, and phosphoglycerate mutase. Since biopterin is close in structure to NAD, it was thought that phenylalanine hydroxylase might be bound to the column, but this was not found to be true, since the enzyme was eluted in the void volume.

A column similar to the columns above would be of great use if successful, but first and of greatest importance is the stabilizing of the enzyme so longer purification steps can be used.

In the beginning of the study, the purification procedure of Kaufman and Fisher (1970) for phenylalanine hydroxylase from rat liver

was used as a guide. There were problems with the ethanol fraction and the calcium phosphate fraction. Phenylalanine hydroxylase lost almost all of its activity during the ethanol fraction in the beginning, so a new apparatus for adding the ethanol was built and tried. Loss of activity was less, but there was still a 50% loss in enzyme activity. The loss of activity was no problem in the calcium phosphate fraction. The first problem encountered was the finding that the phenylalanine hydroxylase would not absorb to the gel. After adjusting the pH of the gel, the enzyme would absorb, but when eluted with buffer all the other protein which had been absorbed also was eluted and no purification resulted. Due to these problems other techniques were tried.

Since activity was low in the crude extract, it was thought that more stirring to enable more enzyme to go into solution might increase the specific activity. This was not found to be true as can be seen in Table X. Altering the pH with a Tris-HCl buffer (pH 8.7) did not affect the solubility of the enzyme as reported by Cotton (1971). As mentioned earlier the enzyme lost much of its activity when placed on columns. It was thought that dilution of the enzyme might be affecting the activity. Kaufman (1970) reported enzyme activity was affected by enzyme concentration. In the dilution study the specific activity was higher than expected for each dilution as shown in Table XI. As a result dilution was not the problem with the columns.

McGee et al. (1972) reported crude extract centrifuged at 16,000 x g had the same specific activity as crude extract centrifuged at 100,000 x g, which agrees with this study. Specific activity of the crude extract centrifuged at 18,000 x g was 0.00115 units/mg and

TABLE X

EFFECT OF ADDITIONAL MECHANICAL STIRRING AND TRIS-HCl, pH 8.7, ON ENZYME SOLUBILITY

Exp. No.	Tris-HCl vs. KCl	Time Extract Stirred	Spec. Activity (units/mg)	Protein (mg/ml)
1.	Tris-HCl	0	0.00192	60.4
	KCl	0	0.00282	42.6
2.	Tris-HCl	0	0.000969	36.6
	KCl	0	0.001	41.7
3.	KCl	0	0.0025	30.0
	KCl	3	0.0026	41.2

(Ayling Assay)

TABLE XI

DILUTION STUDIES

Protein Concentration (mg/ml)	μMoles Tyr/ml/min.	Specific Activity (units/mg)
6.00	0.0802	0.01335
3.00	0.0452	0.01505
2.30	0.0310	0.01499
1.50	0.0210	0.01393
0.75	0.0168	0.02225

(Present Assay)

0.0012 units/mg at 80,000 x g. McGee et al. (1972) also found if the sediment of the 16,000 x g was added to the supernatant of 16,000 or 100,000 x g the specific activity was lower. LaDu and Zannoni (1967) also reported this effect. McGee et al. (1972) found that the addition of the nucleo-mitochondrial preparation of liver inhibited the particle free fraction. As a result of the present study and reports from the literature, 18,000 x g was used for the centrifugation of the enzyme homogenate.

Protamine sulfate was used to precipitate the nucleic acids present in the crude extract.

A solution of saturated ammonium sulfate was used, because it was much faster than adding the solid ammonium sulfate. As can be seen in Table XII the protamine sulfate fraction and ammonium sulfate fraction were the most unstable over a 16 hour period at 4°C. The heat

TABLE XII

STABILITY OF ENZYME AT EACH PURIFICATION STEP
OVER 16 HOUR PERIOD AT 4°C

Step	Specific Activity (units/mg)		% Activity Loss
	Zero Time	After 16 Hours	
Crude	0.0024	0.001	58.4
Protamine Sulfate	0.0049	0.002	75.88
Ammonium Sulfate (30-45%)	0.084	0.0012	82.36
Heat Treatment (Present Assay)	0.0259	0.017	34.36

treatment step was the most stable of all the steps in the purification procedure losing only 34 per cent of its activity.

To reduce the concentration of ammonium sulfate in the enzyme extract, dialysis is usually used. It was found, however, that up to 83 per cent of the activity was lost in dialysis, as shown in Table XIII. The dilution which occurs in dialysis is not the reason for the loss of activity as can be seen in Table XI. It is possible that some component necessary for activity is lost in dialysis. If the reason for this loss of activity can be determined, one of the problems of enzyme stability may be solved. To desalt, a G-25 Sephadex column can be used and little activity is lost as shown in Table XIV.

As mentioned earlier Connellan and Danks (1973) reported activity in the particulate fraction of the crude extract, which could only be removed by deoxycholate. In this study it is difficult to determine if the activity from the particulate fraction is covalently linked to the particulate matter or adsorbed, as shown in Table V. If the activity is linked in some way it is only about five per cent of the total enzyme activity present in both soluble and insoluble fraction and as a result not important in increasing the total amount of enzyme in the purification procedure. However it could be of great importance in the study of its properties as related to the enzyme. It could increase the understanding of phenylketonuria and its various conditions.

TABLE XIII

EFFECT OF DIALYSIS ON ENZYME ACTIVITY

Exp. No.	Buffer	Fraction	Specific Activity (units/mg)		% Activity Lost
			Before Dialysis	After Dialysis	
1.	0.005 M Tris-HCl pH 7.0	50% S.A.S.	0.00057	0.0004	29.8
	0.005 M Tris-HCl pH 7.0	66% S.A.S.	0.00020	0.00005	75.0
2.	0.005 M Tris-HCl pH 7.0	50% S.A.S.	0.00100	0.0002	80.0
	0.005 M Tris-HCl pH 7.0	50% S.A.S. of Na-desoxycholate treated pellet	0.0009	0.0005	44.4
3.	0.005 M Tris-HCl pH 7.0	33-43% S.A.S. Centrifuged 80,000 x g	0.0012	0.0002	83.0
4.	0.1 M Tris-HCl pH 7.4, 10^{-6} phe, 10^{-3} EDTA	35-65% S.A.S.	0.0008	0.0006	25.0
5.	0.1 M Tris-HCl pH 7.3	30-45% S.A.S.	0.00034	0.00011	67.7

TABLE XIV

EFFECT OF A G-25 SEPHADEX COLUMN ON PHENYLALANINE HYDROXYLASE

Exp. No.	Fraction	Protein (mg/ml)		Specific Activity (units/mg)	
		Before G-25	After G-25	Before G-25	After G-25
1.	After Heat Treatment	47	25.9	0.0252	0.013
2.	After 30-45% S.A.S.	23.4	20.1	0.0035	0.003
3.	After Heat Treatment (Present Assay)	25.6	24.8	0.011	0.012

TABLE XV

SOLUBILIZATION OF THE PARTICULATE ENZYME

Fraction	Protein (mg/ml)	Specific Activity (units/mg)
Exp. 1		
Supernatant of Crude Extract	35.36	0.0026
A 300 ml 0.15 M KCl wash of pellet	6.79	0.00065
A homogenate of pellet in 300 ml Na-desoxycholate (0.5%) in 0.1 M Tris, ph 7.0	23.38	0.0004
Exp. 2		
Supernatant of Crude Extract	148.77	0.0024
A 150 ml 0.15 M KCl wash of pellet	55.60	0.0022
A homogenate of pellet in 150 ml Na-desoxycholate (0.5%) in 0.01 M Tris-HCl, pH 7.0	178.07	0.00095

Kinetic Studies

In this research only a crude phenylalanine hydroxylase preparation could be used in the kinetic studies. For this reason the results can only be used as a guide and not as accurate kinetic data. Therefore, the Km values may vary from experiment to experiment as shown in Table XVI. When low concentrations of phenylalanine are used, the Km values are comparable to the Km values reported in the literature for phenylalanine, pterin and biopterin as shown in Table I. As shown in Fig. 9 the lower concentrations of phenylalanine in a Lineweaver Burk plot give one straight line.

However, if the concentration range for phenylalanine is over ten fold and if pterin is used as the cofactor, the 1/velocity versus 1/phenylalanine M^{-1} plot (Fig. 10) gives two straight lines instead of one. When a Hill plot of the same data is made, two lines resulted, one with a slope of 0.714 and the other with a slope of 3.3 (Fig. 11). The reason for the two lines which result when the data is plotted in a Lineweaver Burk plot and the two straight line portions in the Hill plot are not evident from the data obtained. Fisher and Kaufman (1973b) reported the same phenomenon when the natural cofactor, biopterin, was used. Fisher and Kaufman (1973b) believe the enzyme exists in two kinetically distinguishable forms which were interconvertible. Tourian (1971) also reported phenylalanine hydroxylase existed in two kinetically distinguishable forms. He referred to the two forms as the non-activated phenylalanine hydroxylase and the activated phenylalanine hydroxylase. However, Tourian (1971) did not observe the presence of two lines in the Lineweaver Burk plot found in this study or reported by Fisher and

TABLE XVI

KINETIC STUDIES ON CRUDE PHENYLALANINE HYDROXYLASE

Exp. No.	Compound	Range of Conc. (mM)	Km	Vm	Assay
1.	Phenylalanine	(0.5-10)	2.6 mM	--	Ayling
2.	Phenylalanine	(1.0-40)	4.87 mM	0.2354	Ayling
3.	Phenylalanine	(5.0-30)	4.045 mM	0.048	Ayling (low act.)
4.	Phenylalanine	(1.0-20)	3.675 mM	0.46	Ayling
	(divided into two lines)	(1.0-5.0)	2.13 mM	0.3165	
		(7.5-20)	14.93 mM	0.9191	
5.	Phenylalanine (divided into two lines)	(0.5-2.5)	1.55 mM	0.0716	Ayling
		(2.5-20)	51.1 mM	1.206	
6.	Phenylalanine with 1 mM Pterin	(1.0-10)	23.08 mM	1.1961	Present
7.	Phenylalanine with 2 mM Pterin	(2.5-10)	71.4 mM	1.335	Present Modified
8.	Phenylalanine with 0.1 mM Biopterin	(0.1-2.0)	0.72 mM	0.3	Biopterin Modified

TABLE XVI--continued

Exp. No.	Compound	Range of Conc. (mM)	Km	Vm	Assay
9.	Phenylalanine with 0.01 mM Biopterin	(0.5-10)	3.76 mM	1.02266	Biopterin
10.	6,7-Dimethyltetrahydropterin	(0.1-2.0)	0.2719 mM	0.2854	Ayling
11.	6,7-Dimethyltetrahydropterin	(0.1-2.0)	0.06899 mM	3.3275	Ayling (3 points)
12.	Tetrahydrobiopterin with 1 mM Phenylalanine	(0.025-0.1)	0.06578 mM	0.38392	Biopterin
	Tetrahydrobiopterin with 10 mM Phenylalanine	(0.025-0.1)	0.024 mM	0.3508	Biopterin Modified
13.	Tetrahydrobiopterin with 1 mM Phenylalanine	(0.01-0.1)	0.04 mM	0.3117	Biopterin

FIGURE 9

A double reciprocal Lineweaver-Burk plot of 1/V [(μmoles of tyrosine/ 5 minutes)$^{-1}$] versus 1/phenylalanine concentration (mM^{-1}) for dog liver phenylalanine hydroxylase.

(Ayling Assay)

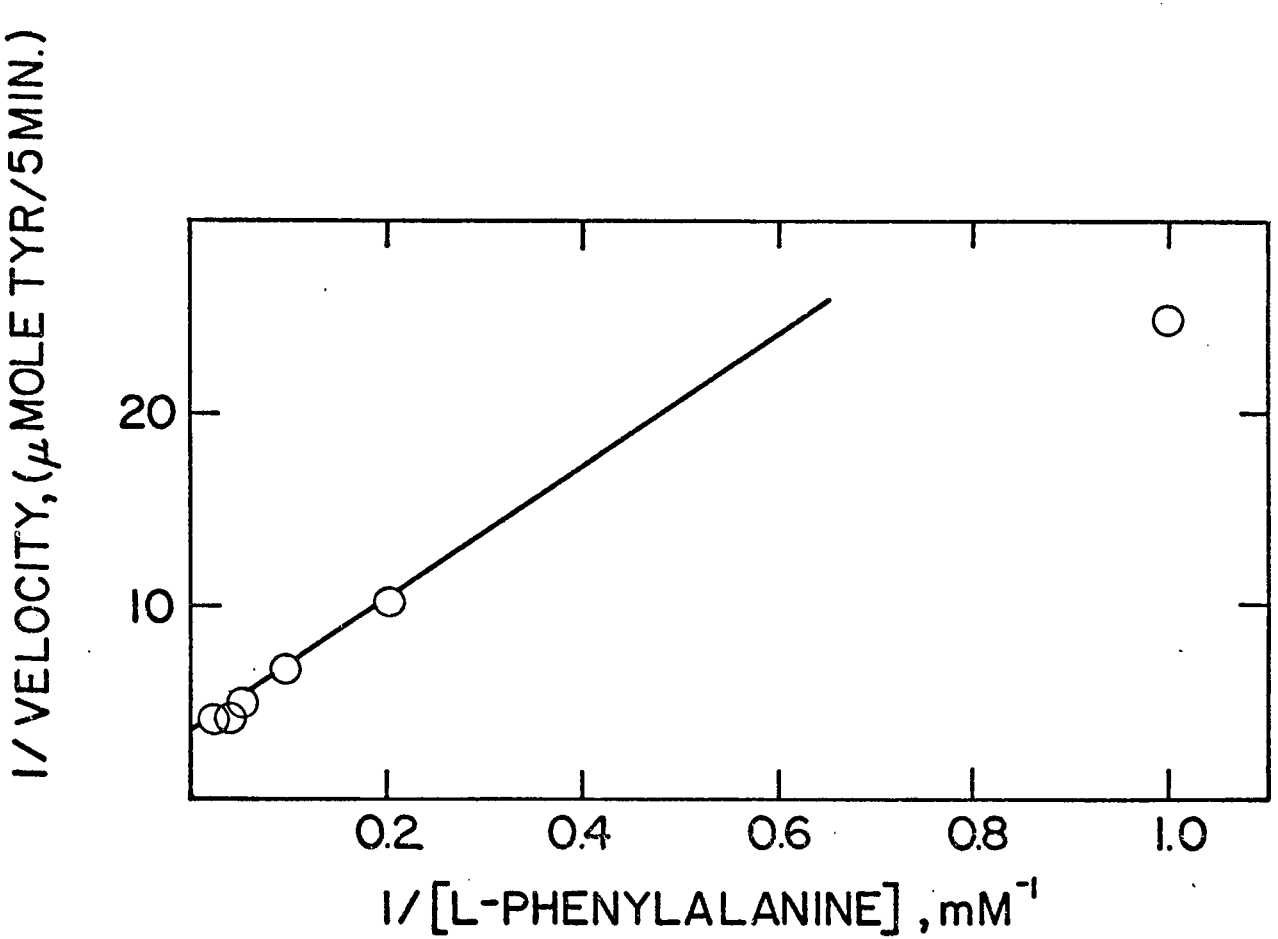

1/ VELOCITY, (μ MOLE TYR/5 MIN.)$^{-1}$
20
10
0.2
0.4
0.6
0.8
1.0
1/ [L-PHENYLALANINE] , mM^{-1}

FIGURE 10

A double reciprocal Lineweaver-Burk plot of 1/V [(μmoles of tyrosine/ 5 minutes)$^{-1}$] versus 1/phenylalanine concentration (mM^{-1}) for dog liver phenylalanine hydroxylase.

(Ayling Assay)

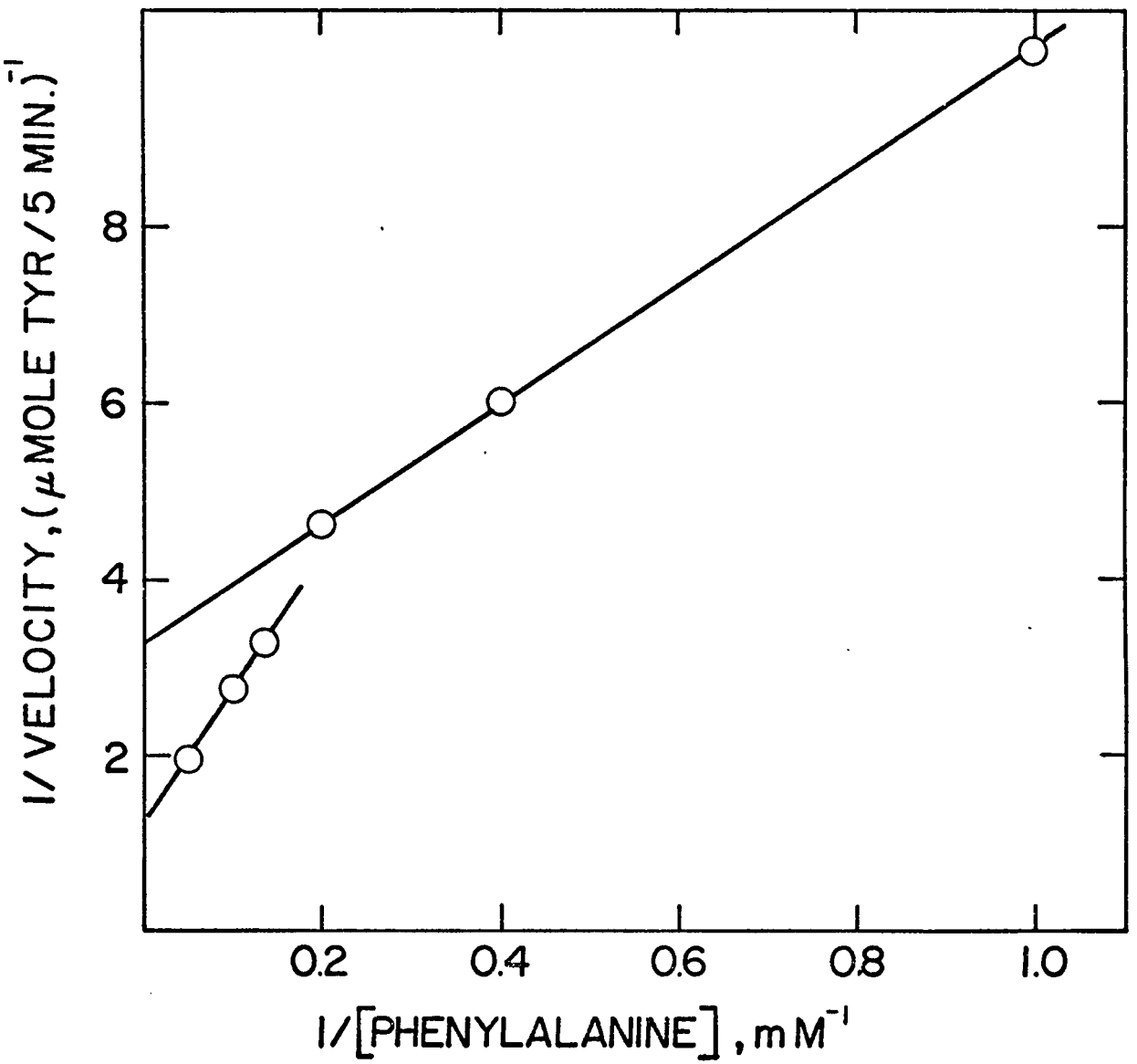
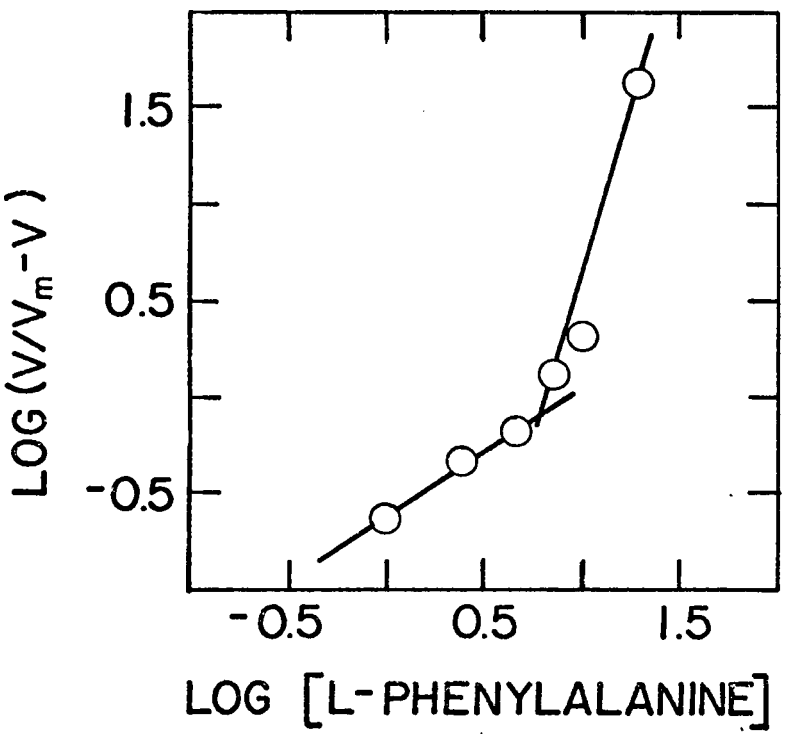

1/ VELOCITY, (μMOLE TYR/5 MIN.)$^{-1}$
8
6
4
2
0.2
0.4
0.6
0.8
1.0
1/[PHENYLALANINE], m M^{-1}

FIGURE 11

A Hill plot of log (V/Vm-V) versus log of phenylalanine concentration for dog liver phenylalanine hydroxylase.

(Ayling Assay)


1.5
0.5
-0.5
LOG (V/Vm-V)
-0.5
0.5
1.5
LOG [L-PHENYLALANINE]

Kaufman (1973b).

When the natural cofactor, biopterin, is used the Vm is extrapolated because the curve is sigmoidal as shown in Fig. 12, and the Km value is only an estimate. The Lineweaver Burk plot gives a curve which approaches a parabola as shown in Fig. 13. If the Vm is 0.3, the Km is approximately 0.72 mM. In experiment nine, as shown in Table XVI, the Km for phenylalanine was found to be 3.76 mM, but this measurement had only three good points so its validity is questionable.

Both cofactors pterin and biopterin give a standard rate curve when velocity versus concentration of cofactor is plotted, as shown in Fig. 14. When the data is plotted in a Lineweaver Burk plot a straight line results as shown in Fig. 16 and 17.

Fisher and Kaufman (1973b) reported phenylalanine hydroxylase was stimulated 20-fold by lysolecithin in the presence of biopterin. In this study there was no significant stimulation and possibly some inhibition as shown in Fig. 18. Woo et al. (1974) reported inhibition of phenylalanine hydroxylase by lysolecithin in the presence of pterin.

In future studies, the assay developed in this study could be improved so it could be used for kinetic studies. The uncoupling reported by Kaufman and Fisher (1971) due to high temperature, and the destruction of tyrosine at the higher temperatures reported by Cooper and Undenfriend (1952) need to be studied. If the assay is to be used in kinetic studies, the uncoupling of the enzyme and destruction of tyrosine at high temperature will have to be eliminated. The mechanism of the enzyme at different pH's needs to be studied. As Kaufman and Fisher (1971) reported at pH 6.8 as the concentration of the enzyme is

FIGURE 12

Velocity (μmoles of tyrosine/5 minutes) versus phenylalanine concentration with biopterin as cofactor.

(Biopterin Assay)

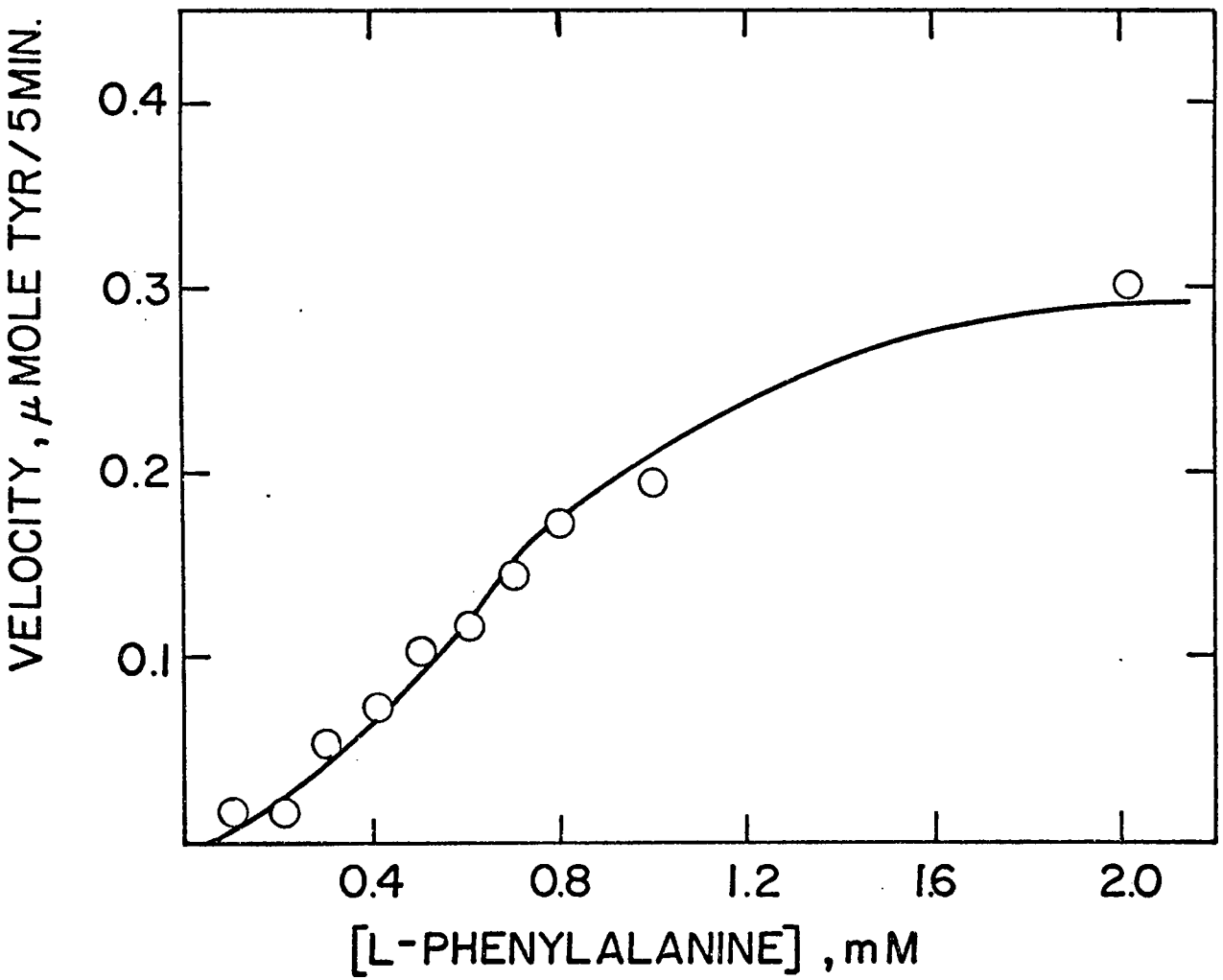

VELOCITY, μ MOLE TYR/5MIN.
0.4
0.3
0.2
0.1
0.4
0.8
1.2
1.6
2.0
[L-PHENYLALANINE], mM

FIGURE 13

A double reciprocal Lineweaver-Burk plot of 1/V [(μmoles of tyrosine/ 5 minutes)$^{-1}$] versus 1/phenylalanine concentration (mM^{-1}) for dog liver phenylalanine hydroxylase with biopterin as cofactor. (Biopterin Assay)

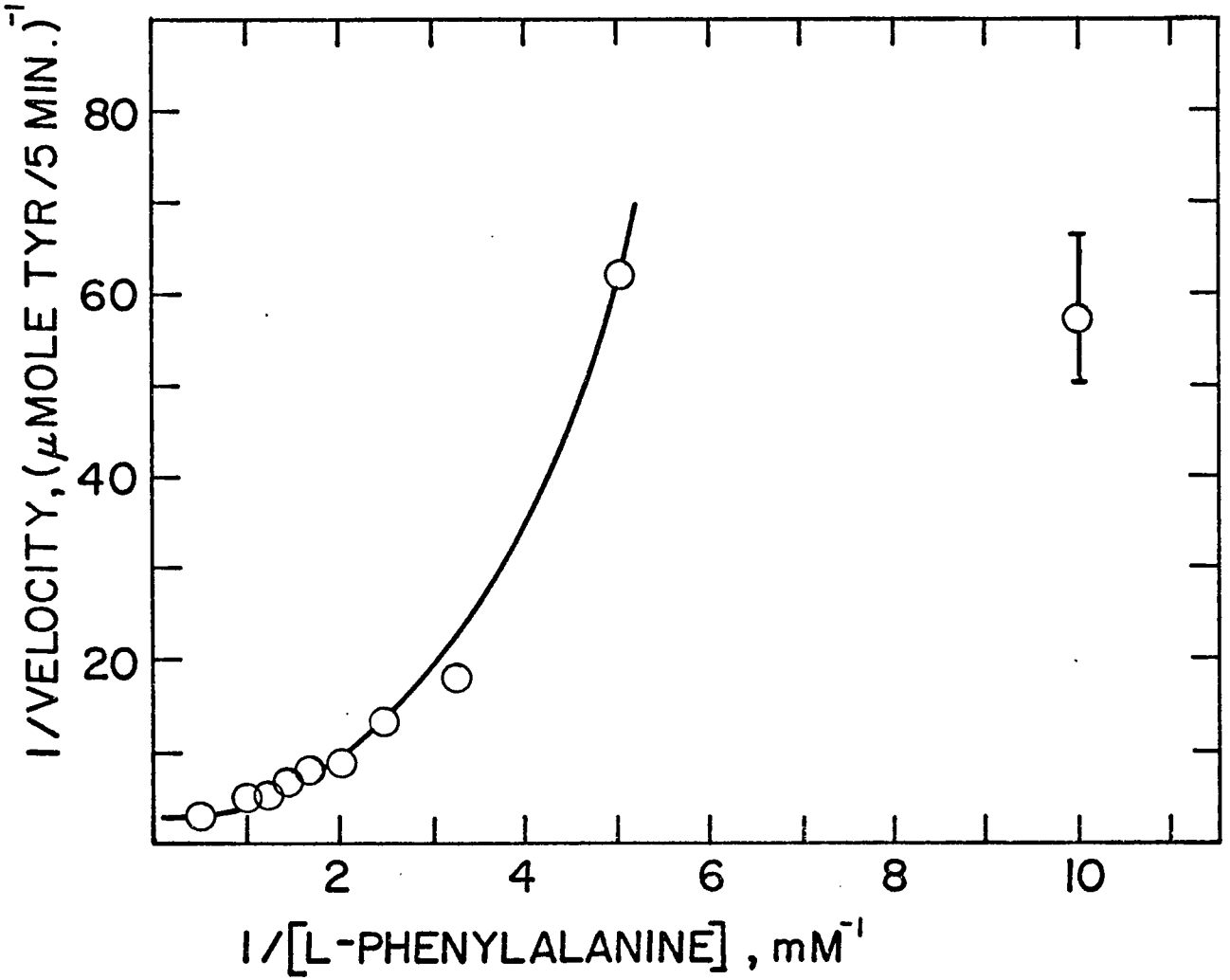

1/VELOCITY, (μMOLE TYR/5 MIN.)⁻¹
80
60
40
20
2
4
6
8
10
1/[L-PHENYLALANINE], mM⁻¹

FIGURE 14

Velocity (μmoles of tyrosine/5 minutes) versus 6,7-dimethyltetrahydropterin concentration for dog liver phenylalanine hydroxylase. (Ayling Assay)

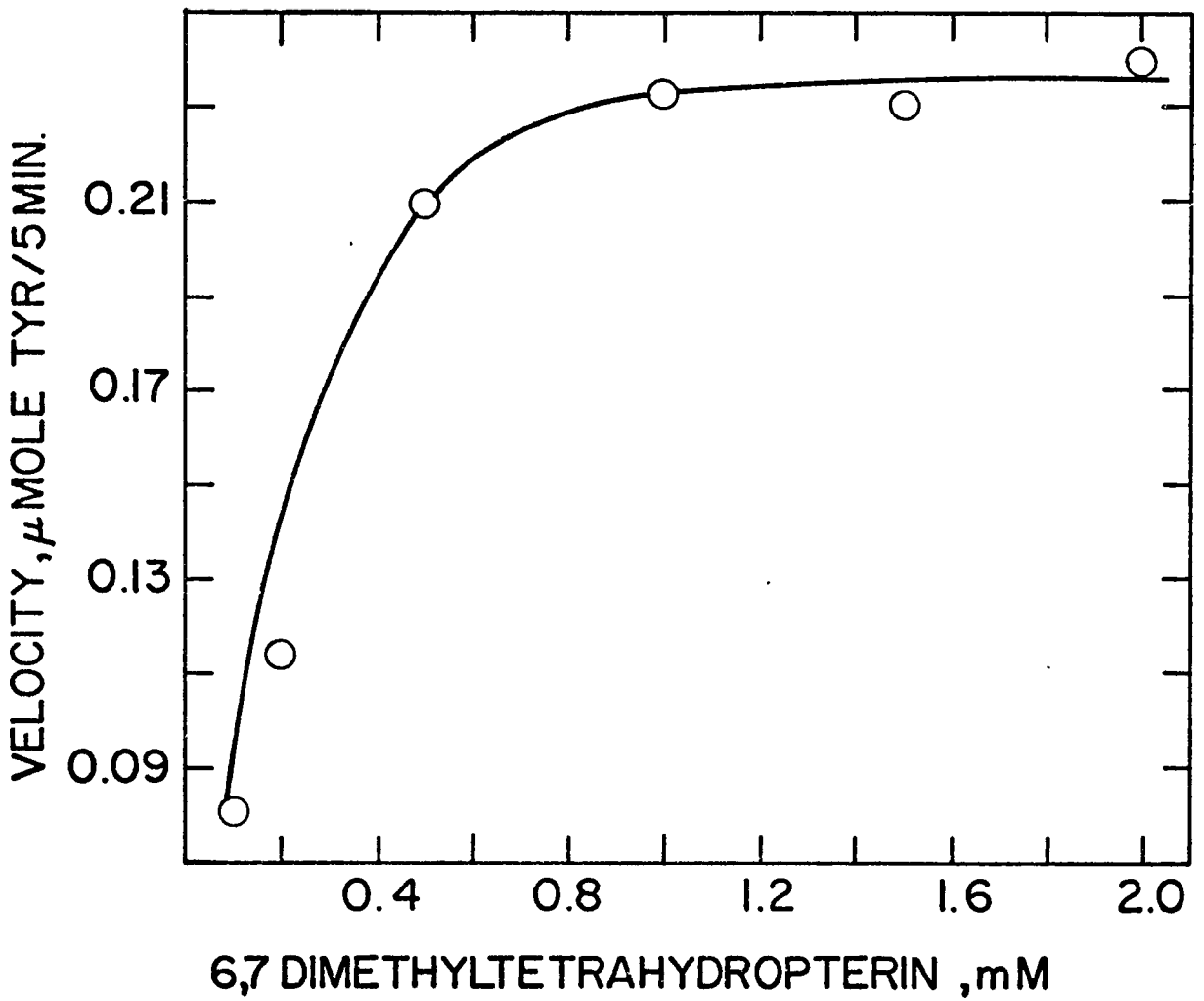

VELOCITY, μMOLE TYR/5MIN.
0.21
0.17
0.13
0.09
0.4
0.8
1.2
1.6
2.0
6,7 DIMETHYLTETRAHYDROPTERIN ,mM

FIGURE 15

Velocity (μmoles of tyrosine/5 minutes) versus tetrahydrobiopterin concentration for dog liver phenylalanine hydroxylase.

(Biopterin Assay)

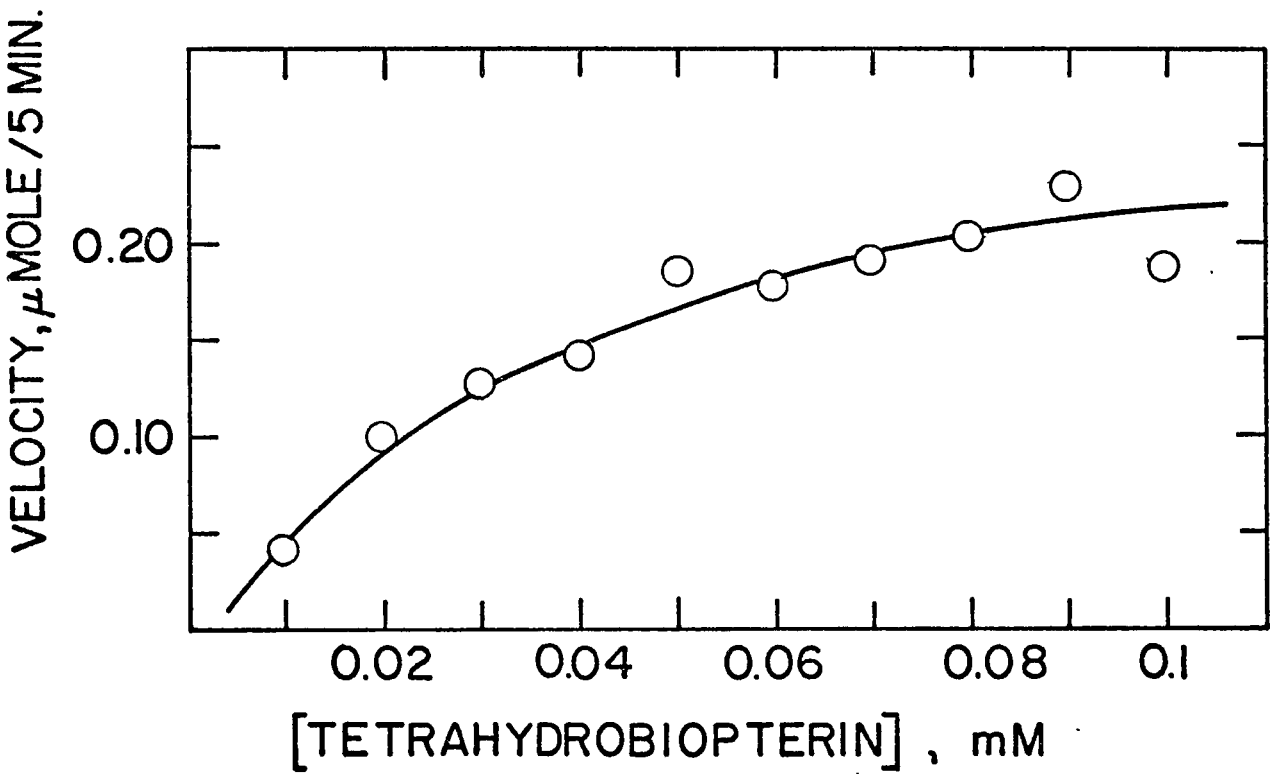

VELOCITY, μMOLE/5 MIN.
0.20
0.10
0.02
0.04
0.06
0.08
0.1
[TETRAHYDROBIOPTERIN], mM

FIGURE 16

A double reciprocal Lineweaver-Burk plot of 1/V [(μmoles of tyrosine/ 5 minutes)$^{-1}$] versus 6,7-dimethyltetrahydropterin concentration (mM) for dog liver phenylalanine hydroxylase.

(Ayling Assay)

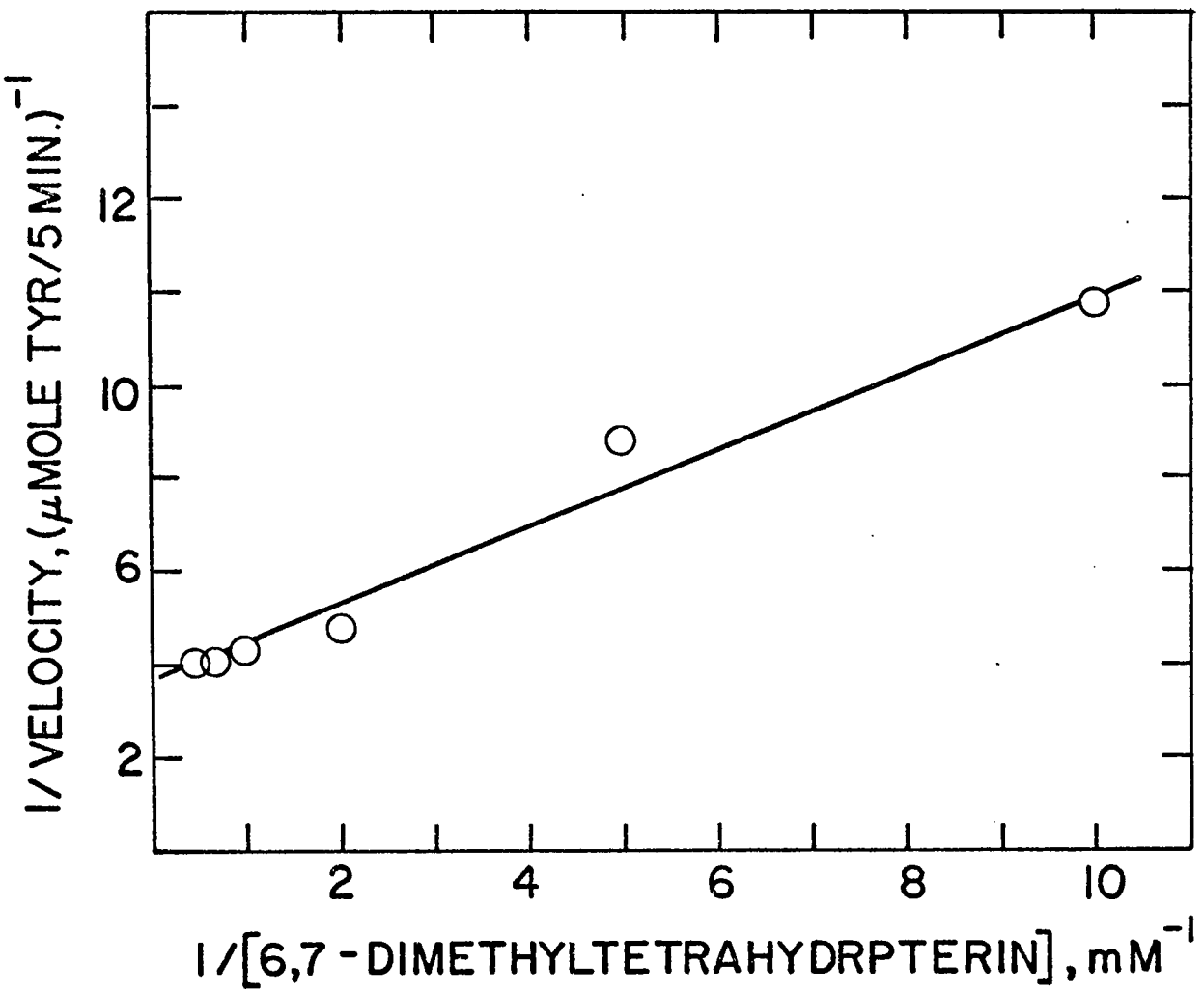

1/VELOCITY, (μMOLE TYR/5 MIN.)$^{-1}$
12
10
6
2
2
4
6
8
10
1/[6,7-DIMETHYLTETRAHYDRPTERIN], mM^{-1}

FIGURE 17

A double reciprocal Lineweaver-Burk plot of 1/V [(μmoles of tyrosine/ 5 minutes)$^{-1}$] versus tetrahydrobiopterin concentration (mM) for dog liver phenylalanine hydroxylase.

(Biopterin Assay)

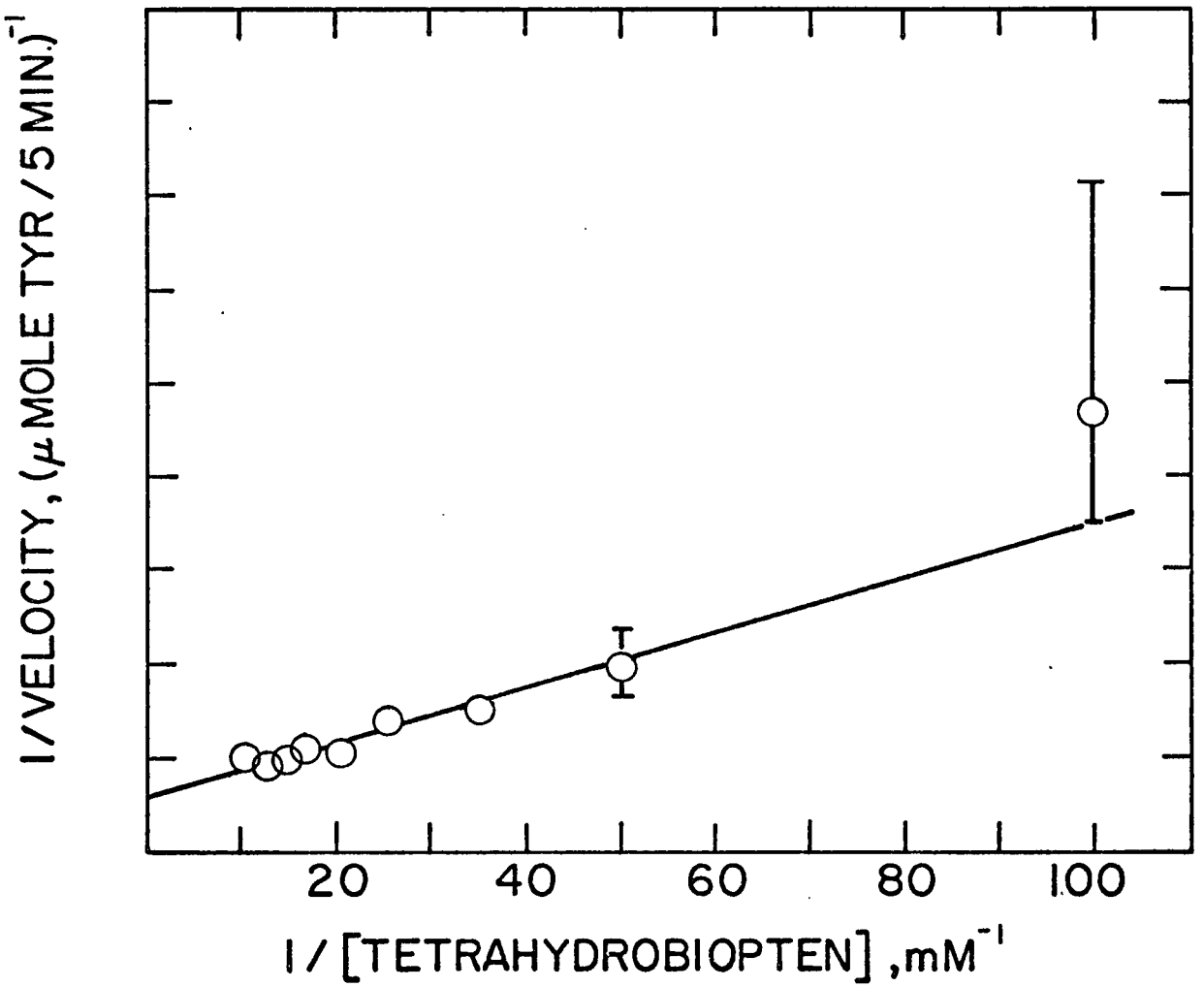

1/VELOCITY, (μ MOLE TYR/5 MIN.)$^{-1}$
20
40
60
80
100
1/[TETRAHYDROBIOPTEN], mM^{-1}

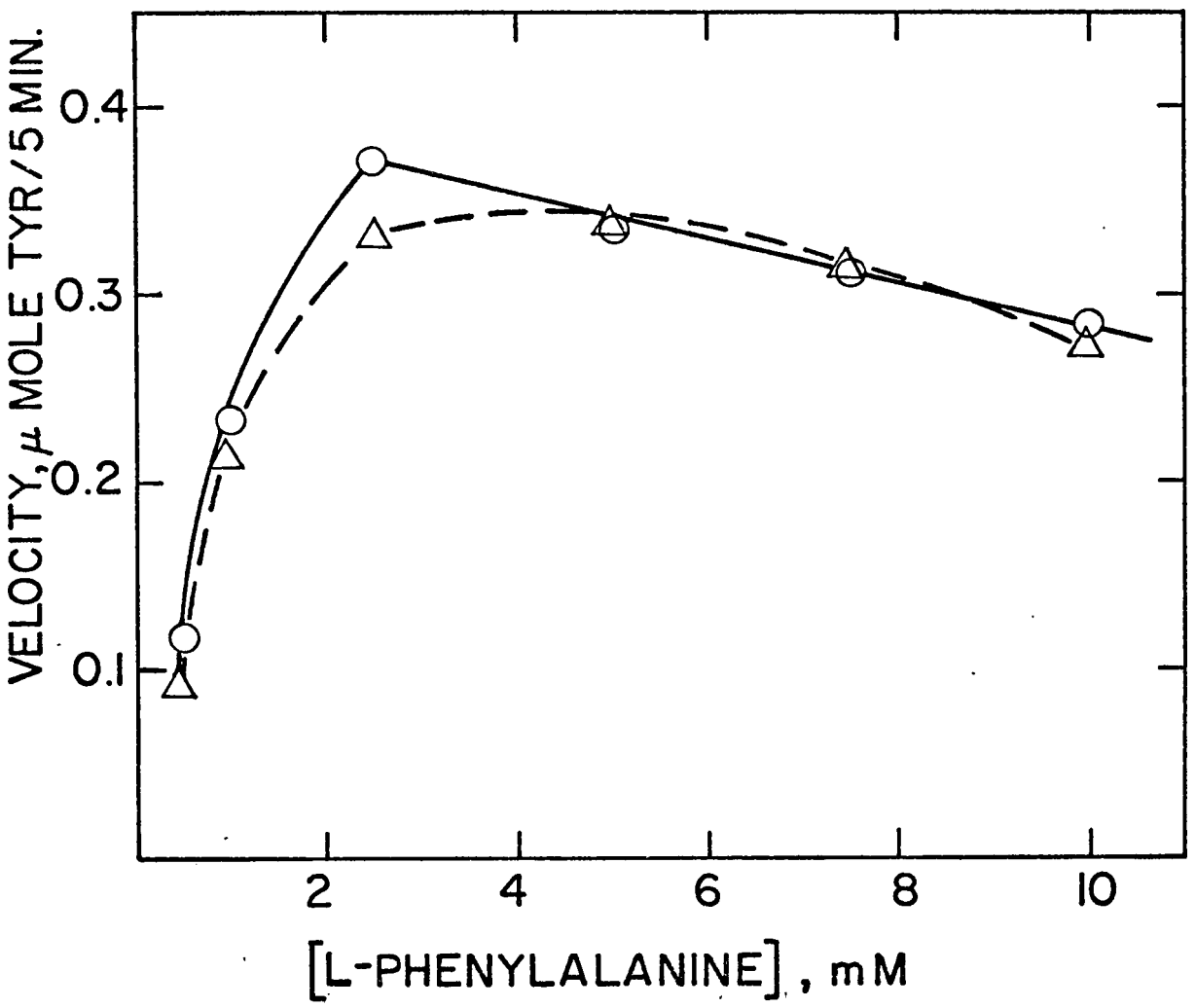
FIGURE 18

Velocity (μmoles of tyrosine/5 minutes versus phenylalanine concentration (mM) for dog liver phenylalanine hydroxylase with 0.1 mM biopterin as cofactor.

0.1 mM lysolecithin present in assay mixture Δ

0.1 mM lysolecithin absent in assay mixture o

(Biopterin Modified Assay)


VELOCITY, μ MOLE TYR/5 MIN.
0.4
0.3
0.2
0.1
2
4
6
8
10
[L-PHENYLALANINE], mM

greater the enzyme activity increases, but at pH 8.0 the activity of the enzyme decreases as the concentration is increased.

It would also be interesting to test the assay with other animals besides the dog. It is possible that inhibition may make the use of high concentrations of phenylalanine and pterin impossible.

There is much more work necessary in the purification of the enzyme. The first thing necessary is to stabilize the enzyme either by further purification or by adding some stabilizing compound or compounds. Further purification would have to be a quick process so little activity would be lost. Since it has been found that the assay mixture, except for the cofactor, stabilizes the enzyme to heat, it is possible this mixture may stabilize the enzyme at 4°C. The stabilizing effect of the substrate at both 55°C and 4°C has been studied and phenylalanine alone did not stabilize the enzyme. Phenylalanine is not completely responsible for the stabilizing effect at 55°C. A study of each compound and combination of compounds and their effect on the stability of the enzyme at both 4°C and 55°C could prove very helpful in understanding how the assay mixture stabilizes the enzyme at 55°C and if this stabilizing effect is functional at 4°C.

If the enzyme could be stabilized, an affinity column might prove very successful in purification, since it would be fairly quick and should give a high degree of purification in one step. The enzyme always may be fairly unstable during purification, and the fewer the steps in the purification procedure the lower the loss of activity. At the present time phenylalanine hydroxylase has been purified 400 fold, but the yield is only five per cent. Ayling and Helfand (1974) reported that in

the presence of the natural cofactor, biopterin, p-chlorophenylalanine has an apparent Ki of 0.03 mM. It is possible that this compound could be attached to an insoluble compound and be used as an affinity column. The problem with this, however, is that biopterin would have to be present and in the reduced form when the enzyme is placed on the column. To elute phenylalanine from the column, the cofactor could be removed. There is also another problem with using p-chlorophenylalanine: it is not very soluble in water or saline. Even with the problems associated with p-chlorophenylalanine, it seems to be the best compound available for an affinity column.

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