p-HYDROXYPHENYLPYRUVATE HYDROXYLASE

part 1. Studies on the Chemical and Physical Properties of Avian Liver p-Hydroxyphenylpyruvate Hydroxylase

part 2. The Role of p-Hydroxyphenylpyruvate Hydroxylase in the Liver Metabolism of L-3,4-Dihydroxyphenylalanine

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the
University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
April 1974

APPROVED:

[redacted]

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(Professor in Charge of Thesis)

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(Chairman, Graduate Council)

Acknowledgments:

The author would like to thank D. Rigas and C. Rigas of the University of Oregon Medical School (UOMS) for running the analytical ultracentrifuge studies and instruction in the use of the instrument, H.S. Mason of UOMS for the use of the atomic absorption spectrophotometer, P. McMahill and H.S. Mason for running the EPR study, A. Scott and M. Pollasky of Reed College for performing the activation analyses, J. Joyce and B. Brimhall of UOMS for running amino acid analyses, D. Daves and J. Anderson of the Oregon Graduate Center for performing mass spectral analyses, W. Nyhan of the University of California at San Diego and M. Sandler of the Bernard Baron Memorial Research Institute of London for the use of their gc/ms facilities, and J.H. Fellman for performing gc/ms analyses.

The author is also indebted to E. Roth, T.S. Fujita, N. Campbell, H.S. Mason, T. Kevil, and J. Black for valuable discussions and technical assistance.

Special thanks are given to J.H. Fellman for his guidance and inspiration and to my wife, Donna, for her understanding and encouragement.

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PART 1

STUDIES ON THE CHEMICAL AND PHYSICAL PROPERTIES OF AVIAN LIVER P-HYDROXYPHENYLPYRUVATE HYDROXYLASE

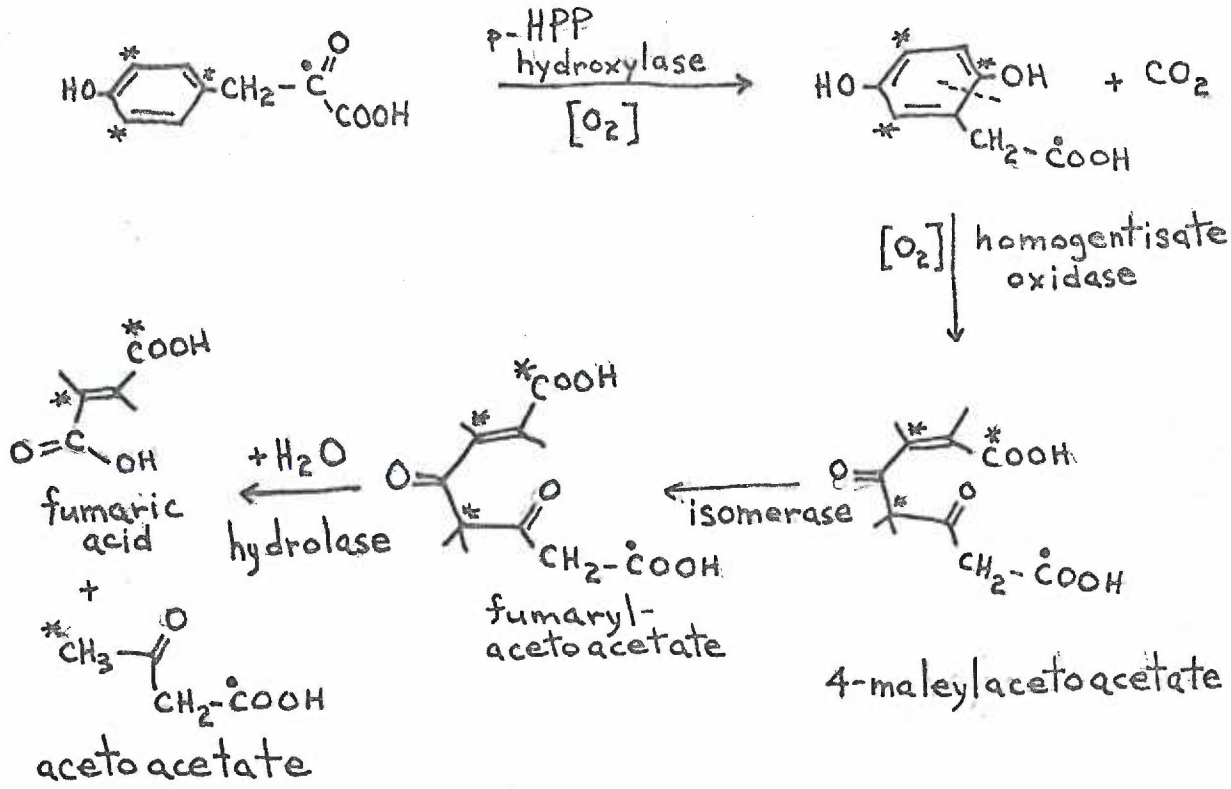
Introduction:

p-Hydroxyphenylpyruvate hydroxylase was noted by Knox and LeMay-Knox (1951) to be one of a series of soluble enzymes in rat liver which oxidize L-tyrosine to acetoacetate and fumarate. Besides its occurance in the livers of hog, rabbit, rat, dog, beef (Mason, 1957), monkey, human, opossum, and salmon and the notable absence in fetal liver tissue (Kretchmer and McNamara, 1956, Goswami and Knox, 1961), the only other tissue possessing the hydroxylase activity in rat, monkey, and human is kidney tissue (Fellman, Fujita, and Roth, 1972). p-HPP[1] hydroxylase is an oxygenase which incorporates one mole of molecular oxygen into one mole of substrate, the natural substrates being p-HPP and phenylpyruvate which are the products of L-tyrosine and L-phenylalanine transamination (for a further discussion of substrate specificity, see part 2, page 69). The reaction catalyzed is thought to be a concerted ring hydroxylation, oxidative decarboxylation, and side-chain migration yielding the

[1] p-HPP is the abbreviation of p-hydroxyphenylpyruvate.

natural products, homogentistic acid and carbon dioxide from p-HPP and o-hydroxyphenylacetic acid and carbon dioxide from phenylpyruvate.

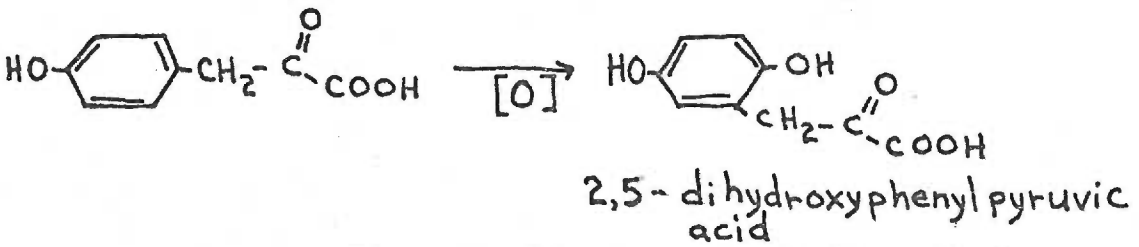
The Reaction Mechanism. Neubauer and Falta (1904) first noted the alkyl side-chain migration when they demonstrated the formation of homogentistic acid from p-HPP in rat liver homogenates. Conclusive evidence for the alkyl migration was presented by Schepartz and Gurin (1949) when they described the use of a carbon-14 ring label to mark the initial position of the alkyl side-chain and its relative position in a metabolite of homogentisic acid, acetoacetate.



The consumption of molecular oxygen was noted in the early studies done on p-HPP hydroxylase. Knox and LeMay-Knox (1951) used a manometric method, following oxygen consumption, to assay for p-HPP hydroxylase activity. The consumption of oxygen and evolution of carbon dioxide was found to be balanced and correlated to substrate consumption and product formation on a mole to mole basis (LaDu and Zannoni, 1955).

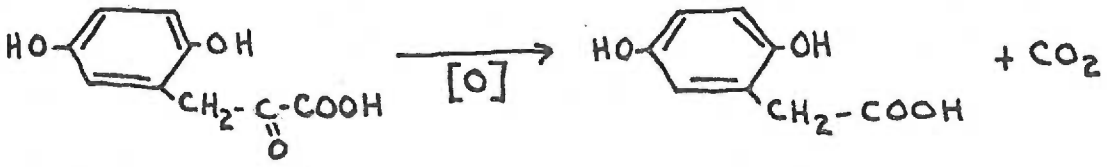
Neubauer originally postulated a two step conversion of p-HPP to homogentisic acid, with 2,5-dihydroxyphenylpyruvic acid as the intermediate:

step 1. The hydroxylation of p-HPP with an accompanying alkyl migration.



2,5-dihydroxyphenylpyruvic acid

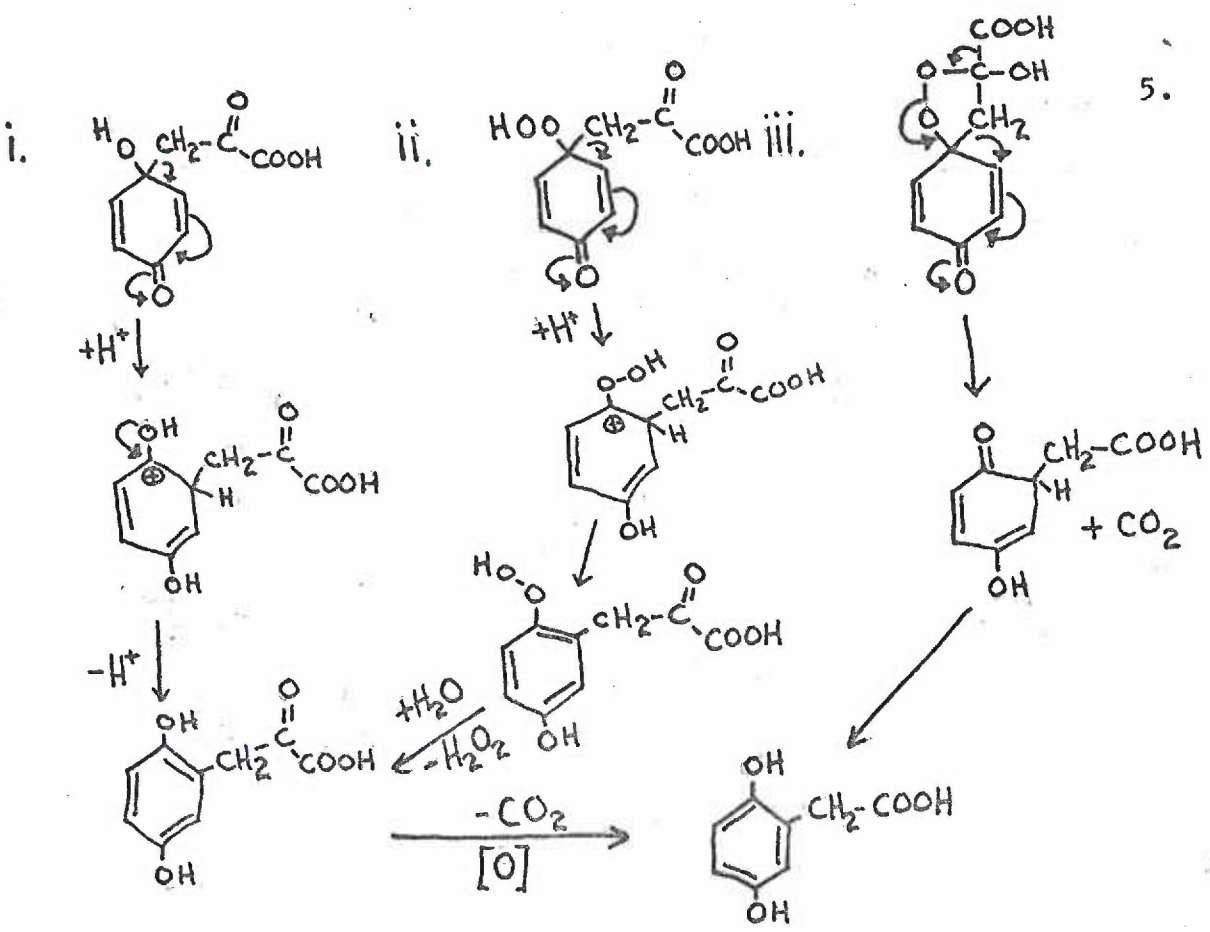
step 2. The oxidative decarboxylation of the resulting 2,5-dihydroxyphenylpyruvate.



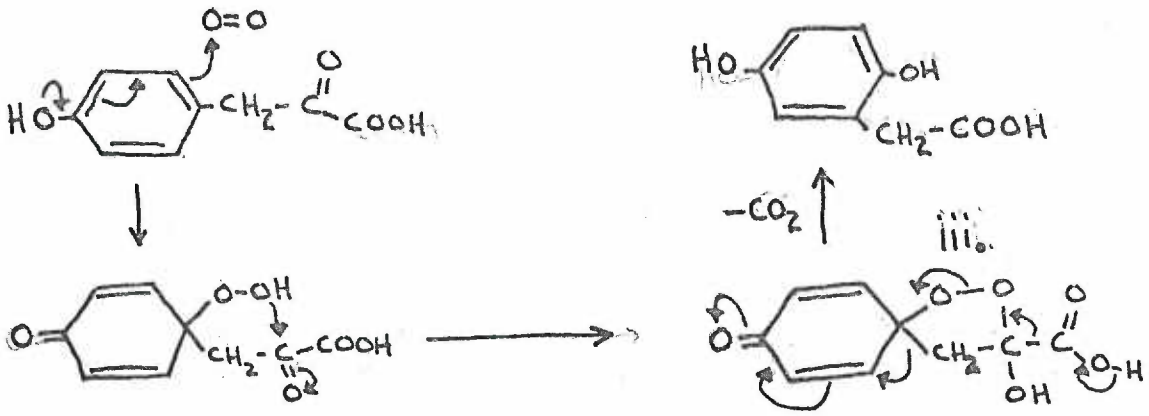
LaDu and Zannoni (1955) later demonstrated that 2,5-dihydroxyphenylpyruvate was not a free intermediate by their finding that it was not a substrate for a partially purified enzyme

preparation which converted p-HPP to homogentisic acid. Edwards, Hsia, and Knox (1955) were the first to report a single enzyme was responsible for catalyzing the conversion. Their conclusion was based on the observation that purification of the enzyme did not separate the enzymatic activities catalyzing the two steps. It was believed that the single enzyme could be catalyzing several separate reactions, as expressed by Mason in a review on the subject (1957). He described a possible mechanism where the intermediates were held at the active site of a single enzyme during a series of reactions. The idea of a concerted hydroxylation, oxidative decarboxylation, and alkyl migration was unprecedented at the time. Mason suggested the use of oxygen-18 tracers to study the mechanism, an idea which, later, was to be of great value.

Goodwin and Witkop (1956), while studying quinol intermediates in the oxidation of phenols, found that alkyl migrations could occur through the participation of the para-hydroxyl group of aromatic rings in a quinoidal intermediate. They proposed three possible intermediates which could account for the alkyl migration found in the conversion of p-HPP to homogentisic acid. One of these intermediates, iii., involved a concerted hydroxylation, alkyl migration, and oxidative decarboxylation for its conversion to product.



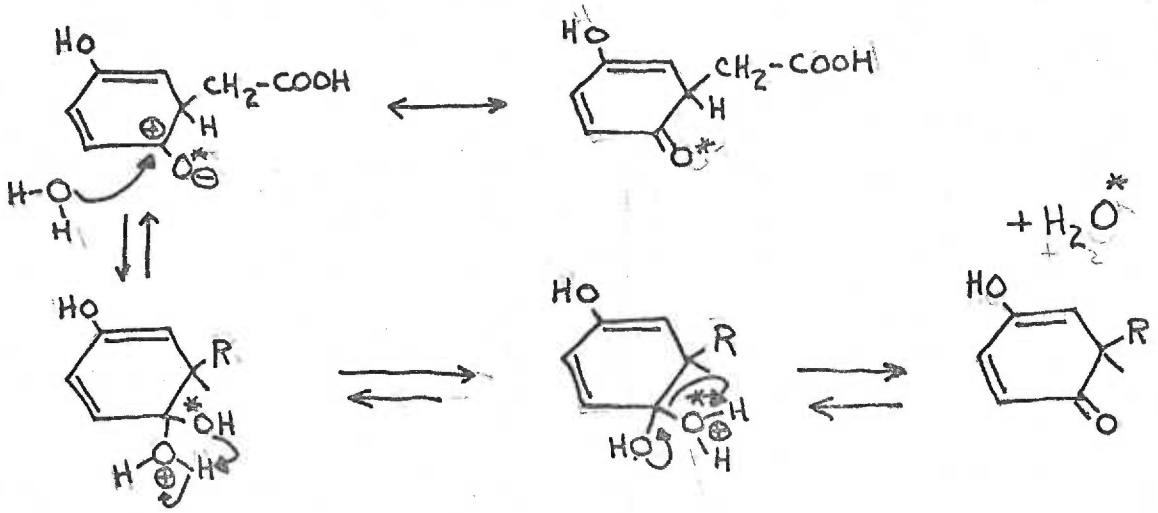
Daly and Witkop (1963) proposed a mechanism for the enzymatic reaction on the basis of the quinoidal intermediate, iii., which involved the electrophilic attack of molecular oxygen on the activated aromatic ring. The resulting hydroperoxide would add intramolecularly to form the intramolecular peroxide-quinoidal intermediate, iii. This intermediate would spontaneously lose carbon dioxide and rearrange to product.



Lindblad, Lindstedt, and Lindstedt (1970) conducted an oxygen-18 tracer study in which they used gas-liquid chromatographic mass spectroscopy to identify the percentage of oxygen labelled with oxygen-18 in the product, homogentisic acid, and its ion fragments. The product was recovered from the incubation of p-HPP hydroxylase, substrate, and either $^{18}O_2$ or $H_2{}^{18}O$. The $^{18}O_2$ donated label was found in the 2-hydroxyl group and in one position of the carboxylate group. The $H_2{}^{18}O$ donated label was found in the other position of the carboxylate group, and it was deduced that this label had come from the exchanging of $H_2{}^{18}O$ with the carbonyl of the substrate before the enzyme catalyzed reaction occurred. These data were consistent with the mechanism of Daly and Witkop in which both atoms from molecular oxygen are incorporated into the substrate.

Lindblad's data was supportable, even in light of Yasunobu et al (1958) who found one atom of oxygen from molecular oxygen and two atoms from water were incorporated into product using the same technique of ^{18}O tracers. Lindblad found that the nuclear hydroxyl at the 2-position of product was exchanging with water after the initial hydroxylation, so that only 30% of the label remained when $^{18}O_2$ was used to label the product. This exchanging was apparent when $H_2{}^{18}O$ was used. 70% of the nuclear hydroxyl was, then, labelled. Lindblad proposed that the oxygen was exchanging through a keto form of the sigma complex after

hydroxylation and alkyl migration.



Lindblad's work did much to clarify the p-HPP hydroxylase reaction mechanism, and new insight into the problem was gained when he recognized the analogy between the reactions catalyzed by p-HPP hydroxylase and the α-ketoglutarate dependent oxygenases[2], such as protocollagen proline and γ-butyrobetaine hydroxylases. The α-ketoglutarate dependent enzymes require α-ketoglutarate as a cosubstrate. The α-ketoglutarate is oxidatively decarboxylated during the reaction in which the substrate is hydroxylated. In essence, this is also what appears to occur during the p-HPP hydroxylation; the α-keto acid moiety of p-HPP is oxidatively decarboxylated during ring hydroxylation. Lindblad's oxygen-18 work

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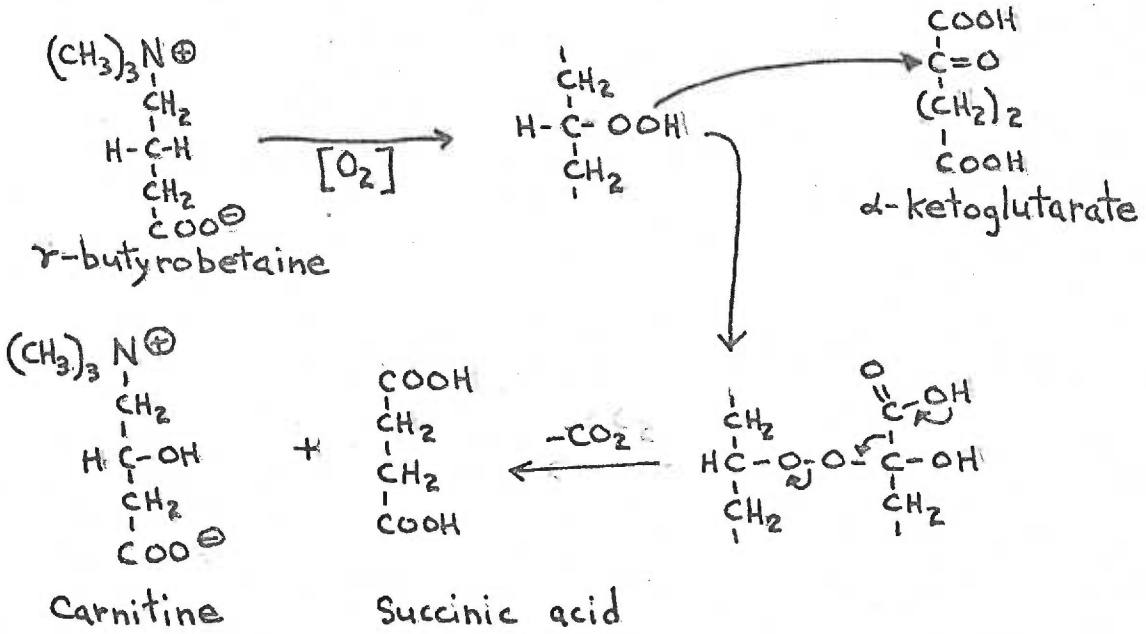
[2]The α-ketoglutarate dependent oxygenases are known to include protocollagen proline hydroxylase (Hutton et al., 1967, Kivirikko and Prockop, 1967), protocollagen lysine hydroxylase (Hausmann, 1967), γ-butyrobetaine hydroxylase (Lindstedt et al., 1968), thymine-7'-hydroxylase (Abbott et al., 1967), and thymidine-2'-hydroxylase (Shaffer et al., 1968).

on γ-butyrobetaine hydroxylase (1969) supported a concerted reaction mechanism very similar to that mechanism proposed by Daly and Witkop for p-HPP hydroxylase. In the case of γ-butyrobetaine hydroxylation, it was proposed that the enzyme-activated molecular oxygen attack is at the unsaturated carbon of γ-butyrobetaine which is to be hydroxylated, forming the hydroperoxide. The hydroperoxide could, then, add to the α-ketoglutarate, cosubstrate, in a reaction analogous to that which forms the intramolecular peroxide-quinoidal intermediate, iii.. The intermolecular peroxide intermediate, thus formed, could rearrange and oxidatively decarboxylate to give the products, carnitine, succinic acid, and carbon dioxide.

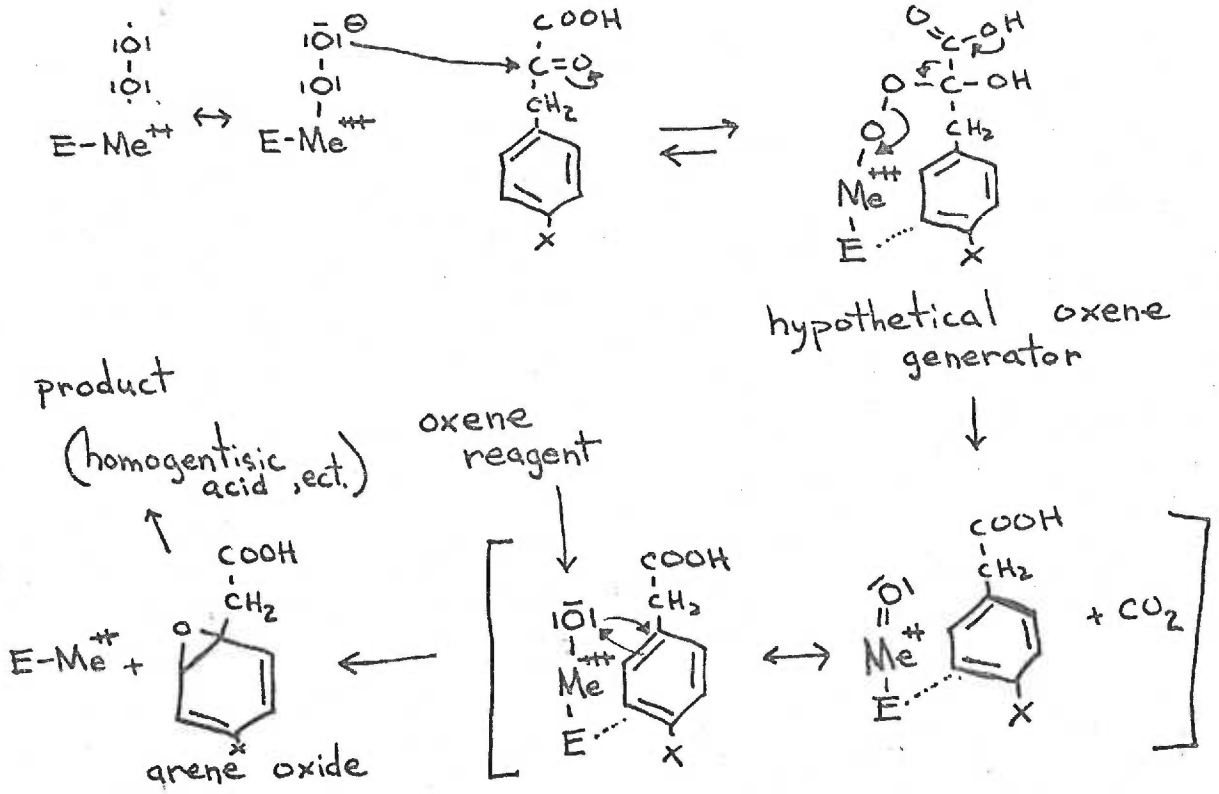
Cardinale and Udenfriend (1971) verified Lindblad's findings when they found incorporation of $^{18}O_2$ donated label into succinic acid and proline during protocollagen proline hydroxylation.



Recently, Hamilton has expressed a dissatisfaction with the proposed mechanisms for the α-ketoglutarate dependent and the p-HPP hydroxylations (1972). Firstly, the requirement for a para-hydroxyl group in the Daly-Witkop mechanism for p-HPP hydroxylase is inconsistent with the fact that the enzyme will hydroxylate substrates with -H, $-CH_3$, -F, and -OH substituents at the para-position of the ring, as demonstrated by Tanaguchi and Armstrong (1963 and 1964). Secondly, the formation of the hydroperoxide on the unactivated proline ring seemed unlikely to Hamilton. He favored the idea of an attack of a molecular oxygen - metal complex at the carbonyl of the α-keto acid moiety forming the peracid. The peracid would, in turn, be directed to the proline ring for an electrophilic attack. Hamilton's mechanism is also applicable to the p-HPP hydroxylation. A peracid could be formed in the same way from the keto acid moiety, and the peracid could attack the aromatic ring. Peracids are known to hydroxylate olefinic and aromatic compounds in non-enzymatic systems, giving the NIH shift with the aromatic compounds (Guroff, et al., 1967). The NIH shift is analogous to an alkyl migration, except that a proton rather than a carbon side-chain migrates. The NIH shift occurs through the arene oxide intermediate (Boyd, Daly, and Jerina, 1972), and the alkyl migration can occur through the same intermediate (Fehnel, 1972). Thus, Hamilton's mechanism for p-HPP hydroxylation entails formation of carbon

dioxide and peracid from α-keto acid and "activated" oxygen, formation of arene oxide by an intramolecular reaction of peracid and aromatic ring, and the final rearrangement of arene oxide to product.

Widman et al. (1973) have recently shown that the para-hydroxyl group of p-HPP does not exchange with water during the enzymatic hydroxylation. This observation tends to support Hamilton's argument against the Daly-Witkop quinoidal intermediate through which water would be expected to exchange with the para-hydroxyl group. Direct formation of the arene oxide by an oxene generating mechanism would not be expected to allow the exchange with water. The oxene generator could be a peracid as in Hamilton's scheme or the hypothetical product of a reaction between enzyme bound metal ($E\text{-}Me^{++}$), O_2, and the α-keto acid moiety of the substrate.



Although the incorporation of both atoms of molecular oxygen into substrate is well established, the nature of the reactive oxygen and the identity of the initial intermediates in the reaction mechanism have not been determined for either p-HPP hydroxylase or the α-ketoglutarate dependent oxygenases. It is, however, clear that the α-keto acid moiety of the p-HPP side chain and of α-ketoglutarate supply the reducing equivalents for the mixed function oxidation occuring during the over-all reactions. In the case of p-HPP hydroxylase, the reaction catalyzed can be classified as an internal mixed-function oxidation and that of the α-ketoglutarate dependent oxygenases as an external mixed-function oxidation (Mason, 1965).

The Putative Metal Prosthetic Group of p-HPP Hydroxylase. The early studies by Knox and LeMay-Knox (1951) revealed a requirement for ascorbic acid as cofactor in the tyrosine oxidizing system of rat liver. It was later determined by LaDu and Zannoni (1955) that the ascorbate was required for the oxidation of p-HPP and that this requirement for ascorbate was a non-specific requirement, fulfilled by catalytic amounts of reduced 2,6-dichloroindolphenol as well as several other reducing agents:

> hydroquinone, isoascorbate (LaDu and Zannoni, 1955), reduced Coenzyme Q_{10}, and a variety of hydroquinoidal compounds (Zannoni, Jacoby, and LaDu,

1962, Zannoni, 1962).

p-HPP hydroxylase was noted to be inhibited by excess substrate, an effect reversed by reducing agents (LaDu and Zannoni, 1955). Partial purification of the enzyme led to the discovery that catalase was necessary for maximal enzyme activity (LaDu and Zannoni, 1956). At first, it was felt that the catalase may be playing an active role in the reaction mechanism, but the work of Hager, Gregermann, and Knox (1957) revealed that neither the reducing agents nor the catalase was required when conditions of low substrate concentration and low oxygen tension were used in the assay medium with fresh enzyme preparations. The reason for substrate inhibition became clearer when studies by Zannoni (1959) demonstrated that substrate generated an inhibitory product in the presence of oxygen. Evidence has been presented by Fellman, Fujita, and Roth (1972) that the inhibitory product exhibits peroxide-like properties. Presumably, catalase protects the enzyme from oxidative inactivation by the substrate and reducing agent generated peroxide. Goswami (1964) has shown that p-HPP hydroxylase can be reversably inactivated by oxidation and can be reactivated by reducing agents. The reverseable inactivation of p-HPP hydroxylase may reflect changes in the oxidation state of a transition metal prosthetic group. The existence of a transition metal prosthetic group has been suspected since the early observation that certain metal chelators,

α, α'-dipyridyl, sodium azide, and diethyldithiocarbamate strongly inhibit the enzyme (LaDu and Zannoni, 1955, Hager, Gregermann and Knox, 1957). The inhibition of p-HPP hydroxylase by DEDTC[3], a strong cupric copper chelator, suggested the participation of cupric copper as the metal prosthetic group. Zannoni noted that copper deficient dogs were also deficient in liver p-HPP hydroxylase, but this low enzyme level did not increase with *in vitro* addition of cupric copper. Evidence supporting ferrous iron as the metal prosthetic group has been presented by Goswami and Knox (1963) and Goswami (1964). Ferrous iron was allegedly removed from crude p-HPP hydroxylase by dialysis against o-phenanthroline and exhaustive dialysis against water, which resulted in a loss of activity. Readdition of ferrous iron resulted in an increase of activity over controls. Contradictory results were obtained by Tanaguchi and Armstrong (1963) who dialyzed against diphenylthiocarbazone, a transition metal chelator which also inhibits the enzyme. They noted that most of the enzyme activity was returned by the removal of the metal chelator by passing the enzyme through a bed of Sephadex G-50. Additions of ferrous iron or cupric copper had no effect on the enzyme activity. Recently, B.L. Goodwin has reported results similar to those of Tanaguchi using DEDTC as the chelator (1972a). Goodwin

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[3]DEDTC is the abbrevation of diethyldithiocarbamate.

(1972b) proposed that Goswami's observation, that ferrous iron stimulated o-Ph[4] treated enzyme, could be explained by its action as an agent with peroxidase activity. By catalyzing the destruction of substrate generated and reducing agent generated peroxides, the ferrous ions may be protecting the intact enzyme from oxidation to an inactive form, rather than acting as a prosthetic group. In my investigation of this possibility, the Goswami experiments were repeated with partially purified enzyme. Both o-Ph and DEDTC were used as chelators in the attempt to remove the putative metal prosthetic group. The results of these experiments indicated that the enzyme activity was not significantly impaired after dialysis against chelators. Thus, if a metal prosthetic group is required for p-HPP hydroxylase activity, it must be tightly associated to the enzyme protein.

Accepting the thesis that a tightly bound metal prosthetic group exists in the case of p-HPP hydroxylase, direct analysis of the purified enzymes for transition metals should reveal the identity of the bound metal. It was proposed that the avian liver p-HPP hydroxylase be purified, several of its important physical characteristics determined, and the purified enzyme preparation subjected to metal analysis by neutron activation and atomic absorption spectroscopy.

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[4] o-Ph is the abbreviation of o-phenanthroline

Experimental Procedure:

Materials. 3,4-Dihydroxyphenylpyruvic acid was prepared by the method of Harley-Mason and Waterfield (1963). 3,4-Dihydroxy (carboxyl-^{14}C) phenylpyruvate (4.1 μcur/mmole), p-hydroxy (carboxyl-^{14}C) phenylpyruvate (9.3 μcur/mmole), and (carboxyl-^{14}C) phenylpyruvate (18 μcur/mmole) were prepared enzymatically from the corresponding amino acids purchased from New England Nuclear. 3-Methoxy-4-hydroxy (carboxyl-^{14}C) phenylpyruvate was chemically synthesized as described by Fellman (paper in preparation, 1973).

o-Hydroxyphenylpyruvic acid lactone was prepared as previously described by Billek (1961). The lactone was converted to the acid by dissolving in 0.1 M KPO_4, pH 7.3.

2,6-Dichlorophenolindolphenol from Eastman Organic Chemicals, reduced glutathione from Sigma Chemical Co., Hyamine in methanol (1 M) from Packard, Catalase (2x crystallized) from Calbiochem were all purchased for the enzyme assay system. Omniflour scintillator was from New England Nuclear.

Sephadex G-25, G-150, SP-sephadex C-50, QAE-sephadex A-50 were all purchased from Pharmacia Fine Chemicals Inc.

The hydroxyapatite (in powder form), 100 ml beaker dialyzer with cellulose fibers, Chelex 100 and AP 50w x 8 resins were purchased from Biorad Laboratories.

Acrylamide, N,'N-methylene-bisacrylamide, N,N,'N,'N-tetramethylethylenediamine for polyacrylamide gels were

purchased from Eastman Organic Chemicals.

Ampholine ampholytes, pH range 6-8, 40% solution was purchased from LKB Produkter AB.

The Calibration Kit of protein standards (ribonuclease A, α-chymotrypsinogen, ovalbumin, and aldolase) was purchased from Pharmacia Fine Chemicals. Hemoglobin (human) from the laboratory of Dr. G.V.F. Seaman (Departments of Neurology and Biochemistry, University of Oregon Medical School) and trysin (2x crystallized, Worthington) from the laboratory of Dr. J.A. Black (Department of Medical Genetics and Biochemistry) were gifts.

Dansyl-Cl[5] from Pierce Chemical Co., commercially prepared silica gel G TLC[6] plates and silica gel G from E. Merck, Darmstadt, and Ultra Pure Urea and Guanidine-HCl from Schwartz/Mann were purchase for the fingerprinting of Dansyl-peptides and thiol determination.

All other reagents were of analytical grade unless otherwise specified.

Methods. Purification of Avian Liver p-HPP. Lindblad et al. (1971) have recently reported a purification of human liver p-HPP hydroxylase from liver acetone powders.

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[5]Dansyl-Cl is the trivial name for 5-dimethylamino-napthalene sulfonylchloride.

[6]TLC is the abbreviation of thin-layer chromatography.

Their preparation gave a single protein band during polyacrylamide gel electrophoresis and a molecular weight of (90-100) 10^3 daltons by sedimentation equilibrium methods. The isoelectric point, determined by isoelectric focusing, was approximately pH 7. Using a radiochemical assay system for p-HPP hydroxylase activity which has been previously described by Fellman, Fujita, and Roth (1971), Lindblad (1971), and Raheja et al. (1973), chicken liver hydroxylase was purified following Lindblad's procedure.

The Radiochemical Assay System for p-HPP Hydroxylase. This assay system is based on the evolution of $^{14}CO_2$ from carboxyl-^{14}C-labelled substrate incubated with p-HPP hydroxylase. The $^{14}CO_2$ evolved from the reaction was collected on a hyamine soaked filter paper wick suspended from the rubber stopper of the reaction vessel, a Warburg flask, by a nichrome wire post. During routine assay conditions, the main chamber of a Warburg flask contained enzyme, 1000 international units of catalase, and 0.1 ml of 1:1 mixture of 32 mg/ml freshly neutralized reduced glutathione and 1.63 mg/ml of 2,6-dichlorophenolindolphenol in 0.1 M KPO_4 buffer, pH 7.3, with a total volume of 2.0 ml. 0.5 ml of p-hydroxy (carboxyl-^{14}C) phenylpyruvate in the side arm (.32 mg/ml) was tipped into the main chamber after a 5 min. activation period in a 37° shaker bath. The reaction was allowed to proceed for 6 or 10 min after which 0.3 ml of

2 M H_2SO_4, previously injected into the side arm during the reaction, was tipped-in to stop the reaction and release the $^{14}CO_2$ for collection on the hyamine wick. After a 15 min post-reaction incubation period, the hyamine wick was counted in 19 ml of Buhler's solution[7] by scintillation spectrometry in the Packard Tricarb. Boiled enzyme controls were run to account for any non-enzymatic decarboxylation. During the purification of p-HPP hydroxylase, 0.025 ml aliquots of enzyme were assayed. The Lowry-Phenol method of Lowry et al (1951) was used to determine protein concentrations for specific activity calculations.

Preparation of Chicken Liver Acetone Powders. Livers were kept on ice after being taken. Extraneous tissue was dissected away and 2000 gm was homogenized in a Waring blender with 2 L of ice cold 0.1 M KPO_4, pH 7.3. The homogenate was filtered through two layers of cheese cloth in the cold room at 4°, and the filtrate was spun at 9000 rpm in the Sorval GSA-3 head for 40 min. The resulting crude supernate had a volume of 1850 ml. 500 ml of supernate was stirred into 3.5 L of acetone at -15°, forming a fine precipitate.

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[7]Buhler's solution consists of 4 gm Omniflour and 2 L of toluene and 2 L of absolute ethanol. 62% counting efficiency for hyamine soaked wicks and 59% efficiency for substrate in 0.1 ml of KPO_4 buffer were obtained in Buhler's solution.

The precipitate was filtered in a large buchner funnel, and the resulting cake was resuspended in cold acetone and filtered. The cake was then washed with diethyl ether, crumbled, and air dried. The resulting powder was dessicated over phosphorous pentoxide in the refrigerator overnight, yielding 220 gm. The powder was stored at -15° until needed.

Extraction of the Acetone Powder. 109 gm of powder was extracted with 800 ml of .1 M KPO_4, pH 7.3, stirred at 4° for 2 hr. The suspension was spun at 9000 rpm in the Sorval GSA-3 for 30 min, yielding 700 ml of acetone powder extract.

Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to the stirred acetone powder extract at 4°. The precipitate collected between 35% (24.7 gm/100 ml) and 55% (38.8 gm/100 ml) saturation by centrifugation was saved and stored at -15°. When needed, half of the precipitate was redissolved with 25 ml of cold H_2O, giving a total volume of approximately 60 ml.

Beaker Dialysis to Remove Ammonium Sulfate. The beaker dialyzer from BioRad Laboratories was filled with the dissolved ammonium sulfate precipitate and the apparatus was capped with pasteur pipette bulbs to allow for osmotic pressure. This solution was stirred in the dialyzer by a

magnetic stirrer at 4° while 6 L of 25 mM KPO_4, pH 6.1, was allowed to flow through the dialyzer fibers by gravity feed. The desalting process was usually completed within 4 hr.

SP-Sephadex Chromatography. A 2.5 x 40 cm column of SP-Sephadex C-50 equilibrated with 25 mM KPO_4, pH 6.1, was loaded with the desalted ammonium sulfate fraction. The column was then washed for 5 hr with starting buffer (25 mM KPO_4, pH 6.1, 25 mM KCl). The p-HPP hydroxylase activity was then eluted with a 25 to 150 KCl linear gradient with a total volume of 500 ml. The protein elution profile was monitored by an ISCO UV monitor. Figure 1. The fractions containing enzyme activity were pooled and applied directly to the hydroxyapatite column.

Hydroxyapatite Chromatography. After the sample was loaded on a 2.5 x 40 cm hydroxyapatite column, equilibrated with 100 mM KPO_4, pH 6.8, a 100 to 500 mM KPO_4 linear gradient with a total volume of 500 ml was used to elute the enzyme. The p-HPP hydroxylase eluted late in the gradient, essentially free of yellow colored contaminants which eluted earlier in the gradient. Figure 2. The active fractions were pooled and applied to the QAE-sephadex column, utilizing in-line dialysis to desalt the sample. The beaker dialyzer was set-up so that the sample flows through the fibers and the dialysis buffer (10 mM Tris-HCl, pH 8.1,

35 mM KCl) was allowed to flow through the stirred main chamber of the apparatus.

QAE-Sephadex Chromatography. A 1 x 20 cm column of QAE-sephadex A-50 equilibrated with 10 mM Tris-HCl, pH 8.1, 35 mM KCl was eluted with a linear 35 to 200 mM KCl gradient of 250 ml, total volume, after the sample application. The enzyme activity eluted in several peaks, observed in the optical density 280 nm profile as peaks superimposed on a backround of o-HPP[8], a buffer constituent added in later runs. Figure 3. The active fractions were pooled and concentrated to a small volume, 2 ml, by vacuum dialysis. This was the final enzyme preparation which was stored at -15° as an ammonium sulfate precipitate under nitrogen.

Results:

Purification of p-HPP hydroxylase. The yields and specific activities of the purification steps were found to be low as a result of the instability of the enzyme activity. Testing various methods of enzyme stabilization, it was found that storage under nitrogen gas in the presence of a substrate analogue, o-HPP, significantly increased the stability of the enzyme. Furthermore, inclusion of 0.4 mM o-HPP in the buffer systems of the purification process resulted in increased

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[8] o-HPP is the abbreviation of o-hydroxyphenylpyruvate.

yields and a 16 fold increase in the specific activity of the final preparation. Table 1. The overall purification increased to approximately 1000 fold over the crude supernate. The yield and specific activity of each step in the purification are detailed in table 2.

Criteria of Homogeneity. Gel filtration in Sephadex G-150 equilibrated with 100 mM NaCl, 50 mM KPO_4, pH 7.3, 0.4 mM o-HPP was performed to determine the degree of homogeneity of the purified enzyme with respect to molecular weight. The 2.5 x 40 cm colum was calibrated with globular protein standards, ribonuclease A, α-chymotrypsinogen, ovalbumin, and aldolase, for an estimation of molecular weight. Figure 4. The p-HPP hydroxylase activity eluted with the protein peak at a constant ratio, with no other protein peaks observed by optical density at 280 nm. Figure 5. Plotting Kav $\left(\text{Kav} = \frac{\text{elution volume - void volume}}{\text{total column volumn - void volume}}\right)$ versus log molecular weight, according to the method of Whitaker (1963), the elution volume of p-HPP hydroxylase corresponded to a molecular weight of 81,000. Figure 6. This value is approximate since the elution volume of a molecule in gel filtration is a function of the Stoke's radius of the molecule rather than a direct function of the molecular weight (Ackers, 1968).

A sedimentation velocity experiment on the purified

enzyme was conducted in the Beckman analytical ultracentrifuge equipped with schlieren optics. A 8.7 mg/ml sample of p-HPP hydroxylase was dialyzed against several changes of 100 mM NaCl, 0.4 mM o-HPP at 4^{o} for 24 hr, before the sedimentation velocity experiment was conducted at 20^{o}. A symetrical boundary was observed to sediment at a velocity corresponding to a $s_{20,w}$ value of 5.2835 S at 59,780 rpm which was consistent with the homogeneity of the purified enzyme with respect to molecular weight and shape. Figure 7.

The molecular weight of the purified enzyme was determined by the conventional slow speed sedimentation equilibrium method in a Beckman Model E analytical ultracentrifuge equipped with Raleigh interference optics. The enzyme was dialyzed at 4^{o} under nitrogen for 24 hr against 0.1 M KPO_4, pH 7.2, 0.4 mM o-HPP. The final concentration of protein was 0.384 mg/ml, as assayed by the Lowry-Phenol method. When the rotor, run at 20^{o}, reached speed, 6903 rpm, a zero time picture was taken. Equilibrium was reached within 72 hr, when the final picture of the interference pattern was taken. Figure 8. The deflection of the fringes was measured for the zero and 72 hr times, and the difference between these values was defined as ΔY. A linear plot of log ΔY versus r^2 (r = distance from the rotor center to the point where ΔY is measured) indicated a homogeneous solute with respect to molecular weight. Figure 9.

The density of the solvent, (ρ), 0.1 M KPO_4, pH 7.2, was calculated as 1.0097 at 20° and 463 mm Hg from values published in the International Critical Tables (1928). The inclusion of 0.4 mM o-HPP in the solvent caused a negligible error in the solvent density, estimated at 0.007% error. The partial volume ($\bar{v}$) of p-HPP hydroxylase was estimated at 0.734 from its amino acid composition by the method of Cohn and Edsall (1943) (see appendix, page 54). This method has been shown by McMeekin and Marshall (1952) to be an accurate estimation of protein partial specific volume for several proteins, even though it does not consider the three dimensional structure of native proteins. The molecular weight was calculated from the following formula according to DeCamelli et al (1970):

$$\bar{M}\ \text{apparent} = \frac{d(\log \Delta Y)}{d\,(r^2)} \cdot \frac{4.606\ RT}{(1-\bar{v}\rho)\,w^2}$$

$$= 97{,}320 \pm 4{,}900$$

Polyacrylamide gel electrophoresis conducted according to Davis (1967)[9] revealed an underlying heterogeneity of the purified enzyme preparation. Approximately 40 μg of protein were applied per gel in 40% sucrose to the top of the stacking gel. The heterogeneity of the sample became clear when the electrophoresis was run at 5 ma per gel at pH 8.9 for longer times, 2 and 3 hr. Four bands of protein were

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[9]The procedure followed was that published in the Buchler Polyanalyst Instruction Booklet, 1966.

observed after staining of the gels with amido black and destaining. Figure 10. An attempt at elution of enzyme activity from the gels by rapid freezing of the gels by rolling them on a block of dry ice, slicing the frozen gels into 1 mm slices, and incubating the slices in assay medium, was frustrated by the instability of the enzyme during electrophoresis. This problem was overcome by inclusion of 0.4 mM o-HPP in the upper buffer, prerunning the gels for 15 min before application of the sample, and running the gels at 4°, utilizing an electrophoresis apparatus with a cooling jacket to maintain the low temperature. By assaying one gel and staining the other of a pair of gels, it was possible to demonstrate that all four protein bands possessed enzyme activity. It was important to compensate for gel swelling during the staining and destaining process by multiplying the migration distance of the protein bands in stained gels by the gel length $\frac{\text{before}}{\text{after}}$ staining. In figure 11, the optical density profile obtained by a scan in the Gilford gel scanner was correlated with the enzyme activity in gel slices. The multiple banding of purified p-HPP hydroxylase was observed consistently, in several different enzyme preparations. Furthermore, preincubation of the enzyme with 1% β-mercaptoethanol and running the polyacrylamide gel electrophoresis with 0.1% β-mercaptoethanol in the upper buffer did not alter the multiple banding of the enzyme.

Polyacrylamide gel electrophoresis in sodium dodecyl-

sulfate was performed on the purified enzyme by the method of Weber and Osborn (1969) and Neville (1971). 5 μg of purified protein standards (hemoglobin, α-chymotrypsinogen, ovalbumin, catalase, and bovine serum albumin) and p-HPP hydroxylase were applied to gels together and in separate gels. Only one protomer was observed for the purified enzyme preparation, with some minor contamination. Figure 12. The protomer had an estimated molecular weight of 49,000 ± 3,000 daltons as determined from a plot of relative mobility versus log molecular weight. Figure 13. From the proceeding information and the molecular weight of the holoenzyme, 98,000, it appears that p-HPP hydroxylase is a dimer composed of two subunits of the same molecular weight, 49,000.

Studies on the Multiple Molecular Forms of p-HPP Hydroxylase. Isoelectric focusing in a sucrose density gradient was performed on the purified enzyme as described by Vesterburg and Svensson (1966), using ampholytes from pH 6.0 to 8.0 and 0.4 mM o-HPP in the light solution of the gradient to stabilize the enzyme. The o-HPP was found to be essential for the recovery of enzyme activity from the focused column. A 10 ml cushion of heavy sucrose solution (2.2 ml of 40% ampholytes, 32.7 g of sucrose made up to 70 ml) and 0.1 ml of phosphoric acid was placed at the bottom of a 120 ml electrolysis column with cooling mantel and a 20% sucrose solution

plus 0.2 ml phosphoric acid was used to fill the inner electrode. The top electrode was covered with 5 ml of light solution (0.7 ml of 40% ampholytes made up to 70 ml with 0.4 mM o-HPP) plus 0.2 ml ethylenediamine. The sample, 10 mg of p-HPP hydroxylase, was applied in one of the middle fractions of the gradient after it was dialyzed against 1% glycine, 0.4 mM o-HPP for 24 hr, according to Ellfork and Siever (1969). The column was focused for 26 hr at 4° with the anode at the bottom, starting at 300 volts and slowly increasing the voltage to 750 volts in the first 3 hr. After focusing, the final current was 0.7 ma, dropping from an initial current of 2 ma. The gradient was drained and collected in 1 ml fractions. The pH was measured in every fifth tube, and the enzyme activity was assayed in every third tube, using both p-HPP and phenylpyruvate as substrates. Optical density at 280 nm was measured in several fractions which were dialysed to remove the o-HPP. The purified enzyme preparation was resolved into four components with isoelectric points of I - 6.0, II - 6.2, III - 6.4, and small amounts of IV - 6.7. Figures 14 and 15. The ratio of p-HPP/phenylpyruvate activities were very similar for all components, approximately 45. The fractions containing I, II, and III were separately pooled and dialyzed for 30 hr against three changes of 50 mM Tris-HCl, pH 7.6, to remove the ampholytes and buffer glycine.

Amino acid compositions of the three components of

p-HPP hydroxylase were determined, using the Beckman automatic amino acid analyzer to analyze the 20 hr hydrolysate (6 N HCl at 110°) by the method of Stein and Moore (1963). The total of ½ cystine plus cysteine was determined by performic acid oxidation of the enzyme before hydrolysis and automatic amino acid analysis (Moore, 1963). The amino acid compositions were expressed as ratios between component I and the other two components. Table 3. The ratios, II/I and III/I, were very similar and approximately equal to 1 for most of the amino acids, indicating very similar compositions for the three components. The total amino acid composition of p-HPP hydroxylase was determined by hydrolysis of the enzyme with 3 M p-toluenesulfonic acid, 0.2% 2-aminoethyl-5-indole, at 110° for 20, 48, and 72 hr and automatic amino acid analysis by the method of Liu and Chang (1971). The basic amino acids, including tryptophan, were separated on a short column (0.9 x 16 cm) of Aminex-5 eluted with 0.12 M sodium citrate, pH 5.2, preserved with a trace of octanoic acid (1 ml/16 L). Table 4. It was noted that no glucosamine or galactosamine, which both elute before tryptophan on the short column, were found in the hydrolysates of the enzyme. This reaffirmed the suspicion that this cytosol enzyme is not a glycoprotein, since glucosamine and galactosamine are common hexosamines found in glycoproteins (Gottschalk and Graham, 1966).

Tryptic peptide mapping was performed on the three

components of p-HPP hydroxylase isolated by isoelectric focusing, I, II, and III, in order to obtain some criteria by which the extent of their structural differences could be assessed. Because of the small amount of these components available, 1 mg of each component, a method utilizing dansylation of the tryptic peptides and separation of these dansylated peptides by two dimensional TLC was attempted. The Dansyl-peptides can be detected by their flourescence under long wave ultraviolet light, making it possible to detect peptides representing as little as 1 nmole of protein during peptide mapping (Seiler, 1970). The procedure described by Zanetta et al (1970) was followed since their chromatographic solvent systems gave the best resolution of Dansyl-peptides (see appendix, page 54 , for a detailed description of the Dansyl-peptide mapping procedure). The peptide maps of components I and II were extremely similar, and only one spot differences were detectable. The peptide map of III was also similar, but more than one spot differences were noted. Figure 16 . A control map of tryptic peptides from bovine pancreatic ribonuclease A, gave a much different map with fewer spots. The numbers of peptides discernable on the maps of p-HPP hydroxylase I, II, and III were less than the sum of arginines and lysines per subunit of enzyme, 50. This result is consistant with the hypothesis that two identical subunits exist per holoenzyme molecule. If more than 50 tryptic peptides were obtained on the maps, dissimilar

subunits would be indicated.

It was suspected that non-enzymic deamidation of the nascent p-HPP hydroxylase could be the cause of the observed microheterogeneity. Flatmark observed that the process of non-enzymic deamidation was accelerated by high pH and high ionic strength (1966); therefore, the effect of these conditions on p-HPP hydroxylase was examined. Three 0.75 mg portions of lyophylized p-HPP hydroxylase was dissolved with 2.6 ml of 0.5 M K_2PO_4, pH 9.0, in 5 ml tubes. The tubes were then gassed with nitrogen and sealed with rubber stoppers. One tube was immediately frozen and stored at -15°. The other two tubes were allowed to stand at room temperature, 23°, for 40 hr, after which one tube was frozen and stored at -15°. The remaining tube was incubated for an additional 30 hr at 37°. Polyacrylamide gel electrophoresis was utilized to characterize the enzyme from each of the tubes. A mixture of 0.1 ml, approximately 30 μg of protein, of dialyzed enzyme solution and one drop of glycerol was applied to the tops of stacking gels and electrophoresis was conducted as previously described on page 24. The time of electrophoresis was 3 hr, with the current set at 4 ma per gel. After electrophoresis, the gels were removed from the tubes, fixed in 10% trichloroacetic acid for 30 min, and stained with Coomassie Brilliant Blue for two hr. The patterns obtained after 7 days of destaining in 7.5% acetic acid, 0.5% methanol solution are illustrated in figure 17. A

progressive loss of protein from the bands of lower mobility and a relative increase of protein in the bands of higher mobility was observed in the stained gels. From the enzyme incubated for a total of 70 hr, the gels revealed the appearance of an additional protein band of higher mobility than those observed in the unincubated control. This new band was labelled f in figure 17. Preincubation of the treated enzyme with β-mercaptoethanol and inclusion of β-mercaptoethanol in buffers during electrophoresis did not alter the results.

Studies on the Putative Metal Prosthetic Group of p-HPP Hydroxylase. An attempt was made to remove the alledged transition metal prosthetic group from p-HPP hydroxylase by dialysis against buffers containing either o-Ph, a ferrous iron chelator, or DEDTC, a cupric copper chelator. Partially purified enzyme eluted from hydroxyapatite, was used in this study. The enzyme was in 100 mM KPO_4, pH 6.8, 0.4 mM o-HPP at a concentration of 1.07 mg/ml and an activity of 44 units/ml. 5 ml of enzyme was placed in a 25 ml flask with 0.3 ml neutralized ascorbate (88 mg/ml in 100 mM KPO_4, pH 7.3) and 50 μg of catalase. After addition of 5 mg of o-Ph or 5.6 mg of DEDTC in 0.25 ml of 40% ethanol, the enzyme was incubated for 10 min at 37°. The enzyme was then dialyzed for 20 hr against three changes of buffer, 1.0 mM chelator, 10 mM Tris-HCl, pH 7.6, 0.4 mM o-HPP, in a

chamber gassed with nitrogen at 4°. Inclusion of o-HPP in the buffer and anaerobic conditions were two precaustions taken to prevent enzyme denaturation. The inhibitory effects of o-Ph and DEDTC on p-HPP hyroxylase specific activity after the preceding treatment are presented in table 5. Treatment with o-Ph resulted in enzyme with 34% of the control specific activity. The control was subjected to the same treatment without inclusion of chelator in the buffer. The observed inhibition was partially reversed with $Fe(NH_4)_2(SO_4)_2$ at 0.4 mM in the assay medium, increasing the enzyme activity to 55% of control. Treatment with DEDTC resulted in enzyme with 12% of control specific activity which was activated to 58% of control by 0.4 mM $CuSO_4$.

1 ml of chelator treated enzyme was then run through a bed of Sephadex G-25 (1 x 20 cm) equilibrated with 10 mM Tris-HCl, pH 7.6, 0.4 mM o-HPP. It was then assayed with or without ferrous iron or cupric copper additions to 0.4 mM. The results, presented in table 6, indicated that removal of the chelator from the enzyme by gel filtration was sufficient to reverse the inhibition of the enzyme almost completely, and the addition of ferrous iron and cupric copper to the assay medium had no activating effect over controls. The activation of chelator treated enzyme, before the removal of the chelator, by the iron and copper ions was probably the result of competition of these ions with the enzyme binding site for chelator. Thus, the preceding results are consistant with

the conclusion that a metal prosthetic group is tightly bound to the enzyme; it is not removed by dialysis against metal chelators under the conditions described in these experiments.

The steady-state kinetics of o-Ph and DEDTC inhibition of p-HPP hydroxylase were determined by the Lineweaver-Burke method of plotting reciprocal reaction velocity versus reciprocal substrate concentration. The plots were done for experiments in which the inhibitor concentrations were 0, 0.01 mM for DEDTC, or 0.08 mM for o-Ph. The inhibitor was added simultaneously with substrate by inclusion in the same sidearm of the Warburg reaction vessel. The enzymic reaction was assayed by the standard radio-chemical method described earlier on page 16. The results, depicted in figures 18 and 19, demonstrated the competitive inhibitory kinetics, with respect to substrate, exhibited with DEDTC and o-Ph inhibition.

Analysis of p-HPP Hydroxylase for associated transition metals was performed by neutron activation analysis. A purified fraction of p-HPP hydroxylase, eluted from QAE-sephadex, and a fraction of lower purity, a fraction eluted from SP-sephadex, were dialyzed for 24 hr against two changes of 50 mM Tris-HCl, pH 7.8, at 4^{o} in a nitrogen gassed chamber. Both of the samples, designated QAE (3.9 ml, 1 mg of protein/ml, 463 units/mg protein) and SP (2.75 ml, 2.6 mg of protein/ml, 17 units/mg protein), were irradiated along with

separate aliquots of their dialysates in the Triga Mark I reactor at Reed College (Portland, Oregon). The samples were then analyzed for their gamma emission energy spectrum using a Kicksort 4096 channel pulse height analyzer equipped with lithium drifted germanium semiconductor detector from Nuclear Diode. On the basis of the gamma emission spectrum, it was possible to semiquantitatively determine the transition metal content of each sample (Lederer, Hollander, and Perlman, 1967). Zinc, cadmium, gold, and arsenic were present in trace amounts, at the nanogram level. Only copper was found to be present in amounts greater than the dialysate level which approached a stoichiometric relationship to the amount of enzyme in the samples, at the microgram level. Activation analysis proved inadequate for the detection of iron at the microgram level; thus, no information on the iron content of the samples was obtained from this method.

The copper and iron content of p-HPP hydroxylase was quantitated by atomic absorption spectroscopy. The Varian-tectron AA-5 atomic absorption spectrophotometer was employed for the metal analyses utilizing an acetylene flame for atomization of samples. The procedure used for sample preparation was that used for the analysis of iron and copper in blood serum (Berman, 1965, and Zettner et al., 1966), since the atomic absorption analysis of the samples from the activation analysis studies required the separation of metals from precipitated protein. 1 ml of sample, 1 ml

of 10% trichloroacetic acid, and 0.25 ml of 0.05% o-Ph and 0.25 ml of 0.05% DEDTC were stirred together for 20 min at room temperature. The precipitated protein was centrifuged down and washed with 0.5 ml of 10% trichloroacetic acid. The total sample dilution was 1 to 3. For the copper and iron analyses, the spectrophotometer was calibrated with standard solutions of $CuSO_4 \cdot 5H_2O$ and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ from 5 to 15 μM. The samples were aspirated into the spectrophotometer without extraction into an organic phase. The results are presented in table 7 where they are expressed as the difference in metal content between the dialysate and the enzyme samples. Based on the concept of equilibrium dialysis, unbound metal ions should be distributed randomly between the dialysis buffer and the enzyme sample. Protein bound metal ions should be restricted to the enzyme sample by the dialysis membrane. Thus, the dialysate buffer was the control for unbound metal in the enzyme sample. The amount of bound iron found in the QAE fraction was 12 times the amount of the bound copper found in the same fraction. The iron/protein mole ratio was 0.88. The mole ratio calculation was based on the subunit molecular weight of 50,000 daltons; thus, the iron/protein mole ratio of the QAE fraction approached one iron atom per enzyme subunit molecule. As depicted in figure 20, the increase in the iron/protein mole ratio correlated well with the purification of the enzyme specific activity. The copper/protein mole ratio,

however, remained unchanged.

When special precautions were taken to eliminate non-specifically bound metal ions from the enzyme samples, a decrease in the iron/protein mole ratio was noted with an accompanying decrease in the enzyme specific activity. The QAE fraction was dialyzed against 0.1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.4 mM o-HPP for 24 hr, and 2 ml of both this QAE fraction and 2 ml of the SP fraction were run through separate beds of Sephadex G-25 (1 x 20 cm), equilibrated with 10 mM Tris-HCl (pH 7.6), 0.4 mM O-HPP to remove the chelator and chelated metal. The collected QAE fraction was 8 ml in volume, 1.01 mg protein/ml, and 267 units/mg protein in specific activity. The collected SP fraction was 6.3 ml in volume, 1.05 mg protein/ml, and 2.8 units/mg protein. These enzyme samples were then passed through Chelex 100 deionizing resin columns (0.5 x 6 cm), equilibrated with the same buffer as that used during gel-filtration. Buffer samples were collected off of the Chelex columns to give backround controls for iron and copper. The QAE fraction collected off of the Chelex column was 8 ml, 0.96 mg protein/ml, and 231 units/mg protein. 6.3 ml of the SP fraction was collected off of the Chelex column which was 1.0 mg protein/ml and 2.65 units/mg protein. These samples were aspirated directly into the atomic absorption spectrophotometer without precipitating the protein, since these samples were not irradiated and were clear solutions. The data, presented in table 8, demon-

strated that iron was again copurified with the enzyme activity. There was a dramatic 88 fold purification of the iron from the SP to the QAE fraction and a corresponding 90 fold purification of specific activity. Figure 21. The iron/protein mole ratio was lower for this QAE fraction, 0.55, as compared with the equilibrium dialyzed QAE fraction, 0.88.

An EPR spectrum was run on the purified p-HPP hydroxylase. The enzyme was concentrated to 6.6 mg protein/ml in 10 mM Tris-HCl (pH 8.1), 0.4 mM o-HPP, 100 mM KCl by vacuum dialysis. 0.3 ml of the dialyzed enzyme was carefully frozen in a sample tube, and the EPR spectrum was scanned. Figure 22. The only distinct signal was observed at the g = 4 region of the spectrum. The exact g-value of the signal was calculated from an integration of the signal. Figure 23. The g-value was found to be 4.33.

Discussion:

The study of p-HPP hydroxylase has been hindered by the inability to obtain highly purified enzyme preparations which retain their enzymic activity. The most recent report of a purification of p-HPP hydroxylase was made by Lindblad et al. (1971). They purified the enzyme to a high degree of purity, to one band in polyacrylamide gel electrophoresis; yet, the purification of the specific activity was no better than 100 fold, an achievement previously reported by Edwards, Hsia, and Knox (1955) and Zannoni and LaDu (1959). In my preparation of avian liver p-HPP hydroxylase, the inclusion of a substrate analogue, o-HPP, in buffers during the purification process resulted in a 16 fold increase in specific activity over the purification in which no o-HPP was present. The use of o-HPP to stabilize the enzyme was found to be of primary importance for the preparation of highly active, purified enzyme which possessed a specific activity increased 1000 fold over crude avian liver supernatant. o-HPP was also found to be a required constituent of buffers used in electrophoretic procedures which depend upon the preservation of enzyme activity to mark the position of the enzyme. Without the inclusion of o-HPP, electrophoretic procedures such as polyacrylamide gel electrophoresis and isoelectric focusing resulted in proteins which were distributed in the same patterns but without enzyme activity. The use of o-HPP has allowed the application of these techniques of protein separation to the

study of p-HPP hydroxylase.

My final preparation of avian liver p-HPP hydroxylase, the QAE fraction, was found to be homogeneous with respect to molecular weight by the criteria of gel-filtration in Sephadex G-150, sedimentation velocity and sedimentation equilibrium in the analytical ultracentrifuge. The more sensitive method of SDS polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue revealed a single, principle protomer and some minor contamination. A conservative estimate of the contamination was less than 5% of the total protein, and, by this criteria, the purity of the preparation was estimated at greater than 95% pure. A more serious challenge to the homogeneity of the enzyme preparation was raised when polyacrylamide gel electrophoresis demonstrated an underlying microheterogeneity, denoted by multiple, close banding of protein in stained gels. Since each of these bands possessed enzyme activity, as demonstrated by elution of the activity from sliced gels, the microheterogeneity was that of p-HPP hydroxylase and not that of contamination. The initial finding of the microheterogeneity of avian liver p-HPP hydroxylase made in polyacrylamide gels was confirmed by the separation of the purified p-HPP hydroxylase into at least four active forms by isoelectric focusing in a sucrose gradient. It was also noted that, during the purification of the enzyme, QAE-Sephadex chromatography resolved the enzyme activity into multiple peaks, an observation that had been

previously puzzling. These peaks can now be interpreted as representing different forms of the enzyme.

The nature of the difference distinguishing each of the multiple molecular forms of p-HPP hydroxylase must be that of charge, since this is the basic physical property by which electrophoretic and ion-exchange chromatographic methods separate proteins of the same molecular weight. The possibility of subunit polymerization through dissulfide bonds was obviated by the lack of β-mercaptoethanol dissassociation of the microheterogeneity and by the homogeneity of the enzyme with respect to molecular weight. The possibility of grossly different primary structure of the multiple molecular forms of the enzyme was ruled out by the similarity of the amino acid compositions and tryptic peptide maps of three forms isolated by isoelectric focusing. N-terminal analysis by the method of Gros and Labouesse (1969) failed to detect a free N-terminal amino acid in the three forms of enzyme, while the method gave the expected aspartic acid as the N-terminal amino acid for bovine serum albumin. This finding was also consistent with the other data indicating similar primary structure for the multiple molecular forms of p-HPP hydroxylase. Since amino acid analysis by the method of Liu and Chang (1971) revealed that the enzyme contained no hexosamine, the possibility that the microheterogeneity of p-HPP hydroxylase was due to a heterogeneous carbohydrate moiety was ruled out. Almost all glycoproteins contain at least

one residue of hexosamine (Gottschalk and Graham, 1966). The existence of multiple alleles for p-HPP hydroxylase was deemed unlikely to occur in the small population of chickens from whom the liver enzyme was taken, especially in the high proportions found for the multiple molecular forms. An alternative explanation was that a single nascent enzyme is synthesized and the progressive non-enzymic deamidation of the asparagine and glutamine residues results in multiple molecular forms of the enzyme. Deamidation of asparagine and glutamine groups has been noted to occur for many proteins (Robinson, McKerrow, and Cary, 1970) resulting in the microheterogeneity of these proteins. The phenomena has been thoroughly studied by Flatmark in the case of beef heart cytochrome C (1966). Flatmark's observation that high ionic strength and high pH accelerate the process of non-enzymic deamidation was utilized as a test to determine whether this phenomena was occuring in the case of p-HPP hydroxylase. It was found that the conditions of high ionic strength and high pH caused an interconversion of the multiple molecular forms of p-HPP dyroxylase. There seemed to be a conversion of forms of lower mobility in polyacrylamide gel electrophoresis at pH 8.9 to forms of higher mobility. This conversion of lower to higher mobility corresponds to an increase in the net negative charge of the protein, neglecting possible changes in protein size. Non-enzymic deamidation of the enzyme could account for the increase in nega-

tive charge, as was noted in the case of cytochrome C by Flatmark. The increase in mobility could not be explained by the formation of intramolecular dissulfide bridges since β-mercaptoethanol had no effect on the mobility of the treated enzyme forms. Thus, the available evidence and precedent indicate non-enzymic deamidation as the cause of the multiple molecular forms of p-HPP hydroxylase; the significance of this phenomena is unclear, since it may be occuring both in vivo and as a purification artefact.

The search for a metal prosthetic group for the oxygen metabolizing enzyme, p-HPP hydroxylase, has both historical and theoretical roots. Otto Warburg (1949) took the position that cells must contain "ferments", enzymes, capable of "activating" oxygen for respiratory processes and that these enzymes must require a heavy metal for their catalytic function. His theory was based on the observation that certain metal ions can catalyze the air oxidation of a number of organic compounds such as amino acids, thiols, etc., at physiological temperature. Since Warburg first presented his proposal, it has been found that almost every known oxygen metabolizing process requires an enzyme with a heavy metal prosthetic group to catalyze the terminal reaction with oxygen. The theoretical need for oxygen activation lies with the ground state configuration of the valence electrons in the oxygen molecule. The oxygen molecule has a diradical nature in the ground state with two unpaired electrons. The

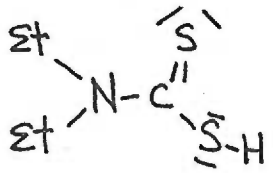
reaction of a diradical with a singlet substrate such as a ground state organic compound is, energetically, highly unfavorable. The quantum physical law requiring the "conservation of spin" dictates that the product of such a reaction be a radical, which is a high energy species. The formation of radicals usually occur only at high temperatures or under the influence of high energy radiation. At physiological temperature and conditions, catalysts are necessary to allow the reaction of molecular oxygen to occur, and the catalysts are thought to "activate" the oxygen molecule. The empty and partially filled d-orbitals of the metal prosthetic group can overlap with orbitals of the oxygen molecule, forming a new configuration for the valence electrons around the oxygen molecule metal complex and destroying the diradical nature of the molecule. In this way, the metal prosthetic group of an enzyme can lower the energy barrier for oxygen reaction, "activating" the oxygen molecule.

Crandall (1965) had classified p-HPP hydroxylase with a group of iron activated oxygenases. Crandall wrote, "(p-HPP hydroxylase) ... gives no evidence of either requiring iron or containing it. Nevertheless, it appears to belong in this group, because of the nature of the substrate reaction." - The similarity in substrate reaction lies with the observation that both atoms of molecular oxygen participate in the reaction with substrate without the apparent need for external reducing equivalents. This is also true for other iron

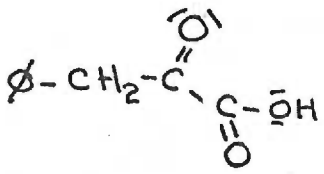
activated oxygenases: Homogentisate oxidase, 3-hydroxyanthranilate oxygenase, tryptophan oxygenase, heme α-methylene oxygenase, and inositol oxygenase. There has since been evidence presented which indicates that the α-ketoglutarate dependent oxygenases, i.e. collagen proline hydroxylase and γ-butyrobetaine hydroxylase, may also fit into this same group. The α-ketoglutarate dependent oxygenases are also ferrous iron activated enzymes.

The information available on other oxygenases indicate that iron should be the prosthetic group for p-HPP hydroxylase but the experimental evidence for this proposal has been fraught with ambiguity. Goswami's evidence for ferrous iron activation of p-HPP hydroxylase was dependent on the preparation of apo-enzyme by the dialysis of active enzyme with o-Ph (1964). One weakness in his data is that he assumed that dialysis against o-Ph removes the putative iron prosthetic group. My studies, which were done on the effect of dialyzing p-HPP hydroxylase with buffers containing o-Ph or DEDTC, indicate that the inhibition caused by these chelators is removed by the removal of the chelator by gel-filtration. There appeared to be no loss of any component required for enzyme activity, and the addition of ferrous iron to the treated enzyme had no activating effect over controls, which were dialyzed against buffers not containing any chelator. Furthermore, the steady-state kinetics of both o-Ph and DEDTC inhibition were found to be strictly

competitive with respect to substrate, a finding made earlier by LaDu and Zannoni, 1954. The competitive kinetics are consistant with a totally reversible inhibition where the chelators are binding at the active site, at or near the substrate binding site. The inhibitor binding site may or may not be the putative metal prosthetic group. It is very possible that the chelator, DEDTC acts as a substrate analogue since the thiocarbamate moiety of the chelator is chemically and sterically analogous with the pyruvate moiety of the substrate.

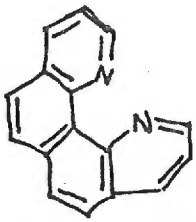


DEDTC



Phenylpyruvate

o-Ph is less likely to be acting in this same manner, as a substrate analogue, because of its bulky aromatic rings, but the competitive kinetics by which o-Ph inhibits the enzyme indicates that there must be some overlap with the substrate binding site.



o-Ph

It, thus, appears that o-Ph and DEDTC do not remove the putative metal prosthetic group from avian liver p-HPP hydroxylase as reported by Goswami for the crude rat liver enzyme.

Attempts at identifying the putative metal prosthetic group of p-HPP hydroxylase by neutron activation analysis of the purified enzyme revealed that copper and iron were the only transition metals which could be present in significant amounts. The quantitation of the iron and copper by atomic absorption analysis demonstrated that only iron was present in amounts which approached a mole to mole relationship with enzyme protein. 1.8 moles of iron were found per 1 mole of enzyme (100,000 daltons), or approximately 1 mole of iron per 1 mole of enzyme subunit (50,000 daltons). The specific association of iron to p-HPP hydroxylase was demonstrated by the correlation of the purification of enzyme activity with the purification of iron content. It was also found that the loss of specific activity incurred during treatment of the enzyme with EDTA and Chelex 100, a deionizing resin, was accompanied by a corresponding loss of iron content. This observation may prove to be of importance when further work is done on the preparation of an apo-enzyme. On the other hand, the loss of enzyme activity and iron may be the result of non-specific enzyme denaturation and, subsequent, removal of the iron from buffer by the EDTA and Chelex 100 resin. The proof that iron plays the catalyic role of the metal prosthetic group is yet to be unambiguously accomp-

lished, but the evidence for the specific association of iron to p-HPP hydroxylase is quite direct and unambiguous.

A confirmation of the atomic absorption data supporting an iron prosthetic group for p-HPP hydroxylase was obtained by EPR spectroscopy. A single strong signal was obtained at g = 4.33 for scans of the purified preparation of enzyme. The signal obtained in the region of g = 4.3 is a common ferric iron EPR characteristic of high spin ferric iron in a rhombic ligand field (Blumberg, 1967). A similar signal has been observed for iron associated to transferrin, g = 4.14 (Aasa et al., 1963), and for pyrrocatechase which is an iron activated oxygenase, g = 4.2 (Hayaishi, 1969). Ferric-EDTA is a model system which also has been noted to give the 4.3 EPR. The EPR of p-HPP hydroxylase was probably due to the ferric form of the enzyme, since either the high or low spin configuration of ferrous iron would be expected to be diamagnetic giving no EPR. Activation of the enzyme with ascorbic acid or reduced dichlorophenolindolphenol would result in reduction of the enzyme to the ferrous form, which is, apparently, the active form of the enzyme. Thus, the form of the enzyme observed by EPR spectroscopy is probably the inactive form of the enzyme and only a portion of the total iron present in the sample of enzyme.

The identity of metal ligands in metallo-enzymes is a question which has been satisfactorily answered in only a few cases. The enzymes having an iron-heme prosthetic group are

some of these cases. Many of the non-heme iron activated oxygenases are thought to have thiol ligands. The evidence for this proposal comes from the lability of these enzymes to thiol reagents such as p-CMB[10] and the need for glutathione during the reactivation of apo-enzyme with iron ions. Unlike these iron activated oxygenases, p-HPP hydroxylase was not found to be inhibited by thiol reagents (Tanaguchi and Armstrong, 1963, and Goswami and Knox, 1963). My own investigation of this question was consistent with previous findings. p-CMB, N-ethylmaleimide, and mercuric chloride had no inhibitory effect on p-HPP hydroxylase activity. Figure 24. Silver nitrate had an inhibitory effect, but the mode of action of this reagent may be that of a general oxidizing agent rather than a specific thiol reagent. It was possible that the thiol groups of p-HPP hydroxylase are sequestered in a region of the enzyme molecule inacessible to the thiol reagents. A spectrophotometric method for the determination of the extent of the reaction between p-CMB and protein associated thiol groups (Boyer, 1954) was used to determine the acessibility of the thiols in p-HPP hydroxylase. A control of ovalbumin was run to check the quanitation of the method. As depicted in figure 25, the enhancement of p-CMB absorption at 250 nm by p-HPP hydroxylase was followed over a time course which indicated that approximately four

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[10] p-CMB is the abbreviation of p-chloromercuribenzoate.

equivalents of thiol were reactive per subunit of enzyme. Since the enzyme subunit contains only 3 equivalents of thiol, it appears that the lack of p-CMB inhibition could not be due to a lack of reaction.

In an attempt to obtain some information on the nature of the metal ligands of p-HPP hydroxylase, some preliminary experiments were done on the effect of carboxyl, amine, arginine, and histidine reagents[11] on p-HPP hydroxylase activity. The effect of EDAC[12], a water soluble carbodiimide, was tested on the enzyme. EDAC is a reagent which reacts with carboxyl groups, forming a reactive ester. The reactive ester can, in turn, react with any nucleophile; in this case, the principle nucleophile would be water. p-HPP hydroxylase, partially purified to the hydroxyapatite stage, was preincubated with 0.2 mM EDAC in 100 mM KPO_4, pH 6.0, 1 mg/ml bovine serum albumin from 0 to 60 min at 37°. The enzyme was then activated with ascorbic acid and assayed as described in figure 26. There was no detectable inhibition of p-HPP hydroxylase by EDAC.

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[11]The selectivity and reaction conditions of the carboxyl, amine, arginine, and histidine reagents have been compiled by Means, G.E., and Feeney, R.E., 1971.

[12]EDAC in the abbreviation for 1-ethyl-3 (3'-dimethylaminopropyl)-carbodiimide.

The reaction of an amine and histidine reagent, 0.2 mM diethylpyrocarbonate, with the enzyme in the same way as that described for EDAC resulted in an entirely inactive enzyme. The lack of any inhibition by the amine reagent, N-ethylmaleimide (20 mM) at pH 7.5, seems to indicate that there are histidine residues involved in the mechanism of p-HPP hydroxylase catalysis. Figure 26. The arginine and amine reagent, phenylglyoxal (33 mM), strongly inhibited the enzyme. Steady-state kinetics of the phenylglyoxal inhibition indicated non-competative inhibition when the inhibitor was preincubated with the enzyme. With simultaneous addition of substrate and inhibitor, weak competative inhibition was observed. Figure 27. Although phenylglyoxal is structurally similar to p-HPP, the principle mechanism of inhibition appears to be noncompetitive, as an arginine reagent.

Of the reagents tested, those which react with histidine or arginine were the reagents which inhibited p-HPP hydroxylase activity. Notably, the thiol reagents and a carboxyl reagent, EDAC, did not at all inhibit the enzyme. If either of these two, thiol and carboxyl, groups were metal ligands, reaction of the enzyme with reagents attacking these groups would be expected to inactivate the enzyme. Since it was demonstrated spectrophotometrically that the thiol reagent, p-CMB, does react with the thiol groups, they cannot be ligands. It is still possible that the carboxyl is involved

as a ligand and is unreactive or protected from the EDAC by inacessibility to the reagent. The probability that histidine and arginine are involved with p-HPP hydroxylase catalysis is high, but the involvement of either of these residues as ligands is still speculative. It is, however, an interesting consideration since two histidine residues appear to serve as ligands in transferrin, with the participation of three tyrosine residues (Line, Grolich, and Bezkorovainy, 1967). Some valuable information on the identity of the iron ligands in p-HPP hydroxylase may be obtained by determining the effect of specific histidine and arginine reagents on the iron content of the enzyme.

Summary and Conclusions:

Avian liver p-HPP hydroxylase was purified to a 1000 fold increase in specific activity over crude supernatant, utilizing a substrate analogue, o-HPP, to stabilize the enzyme. The preparation was found to be homogeneous with respect to molecular weight. The molecular weight of the holo-enzyme was determined to be 97,350 $\pm$ 4,900 by sedimentation equilibrium, and the molecular weight of the subunits was determined to be 49,000 $\pm$ 3,000 by sodium dodecylsulfate polyacrylamide gel electrophoresis. It was concluded that theenzyme is a dimer consisting of subunits of the same molecular weight. Tryptic peptide maps of the enzyme gave a number of peptides consistant with tryptic cleavage of the enzyme at each arginine and lysine in 49,000 daltons of enzyme, based on the amino acid composition; thus, the maps were consistant with an identical subunit model for the enzyme.

Polyacrylamide gel electrophoresis revealed an underlying micro-heterogeneity of the p-HPP hydroxylase. This micro-heterogeneity was probably due to charge differences between the multiple molecular forms of the enzyme since the enzyme was homogeneous with respect to molecular weight. The multiple molecular forms were separable by isoelectric focusing, and their isoelectric points ranged from pH 6.7 to 6.0. After it was observed that conditions which are known to accelerate the non-enzymic deamidation of other proteins

caused the conversion of enzyme with lower electrophoretic mobility into enzyme with higher mobility, it was concluded that the microheterogeneity of p-HPP hydroxylase appears to be due to non-enzymic deamidation of a single nascent enzyme.

The only transition metal present in the purified p-HPP hydroxylase at significant levels was iron. The amount of iron present in equilibrium dialyzed samples was equivalent to 1 atom of iron per enzyme subunit molecule or 2 atoms of iron per holo-enzyme molecule. The purification of enzyme activity correlated with the purification of protein bound iron. It was also observed that the decrease in iron content of the enzyme, caused by EDTA and Chelex 100 treatment, was accompanied by a corresponding decrease in the specific activity of the enzyme. It was concluded that the iron was specifically associated with p-HPP hydroxylase, perhaps, as a prosthetic group involved in oxygen "activation". An EPR scan of the enzyme gave a signal at $g = 4.33$ which is characteristic of high spin ferric iron in a rhombic ligand field, supporting the preceding conclusions.

The inhibition of p-HPP hydroxylase activity by histidine and arginine reagents suggested that these two residues participate in the catalysis of p-HPP hydroxylation. The lack of inhibition by thiol, carboxyl, and amine reagents suggested that they do not participate. It was concluded that the question of the identity of p-HPP hydroxylase iron ligands requires further investigation in order to arrive

at a meaningful conclusion.

Appendix:

Calculation of the Partial Specific Volume of p-HPP Hydroxylase by the method of Cohn and Edsall (1943). The method of Cohn and Edsall is based on the assumption that the partial specific volume of a protein is approximately equal to the summation of the partial specific volumes of the residues of which it is composed times their weight fractional contribution to the total weight of the protein. Thus, if the partial specific volumes of the amino acid residues are known, the partial specific volume of a protein can be calculated from its residues and from the partial specific volumes of the amino acids, which can be experimentally determined. Using the following formula,

$$\bar{v}p = \frac{\sum_i \bar{v}_i w_i}{\sum_i w_i}$$

where $\bar{v}p$ = partial specific volume of the protein
$\bar{v}_i$ = partial specific volume of the amino acid residue i
w_i = percent weight of residue i in protein p
$\sum_i w_i = 100\%$

the partial specific volumes of the amino acid residues, and the amino acid composition of p-HPP hydroxylase, the $\bar{v}$ of p-HPP hydroxylase was calculated to be 0.734 cc/g.

Procedure for Dansyl-peptide Mapping of p-HPP Hydroxylase. Each component off of the isoelectric focusing column

was reduced and s-carboxymethylated by the method of Crestfield, Moore, and Stein (1963) to prevent disulfide bridging in the protein to be trypsinized. After it was dialyzed for 24 hr against water and lyophylized, 1 mg of protein was placed in a 5 ml vial gassed with nitrogen. The protein was dissolved with 0.06 ml of EDTA solution (50 mg/ml, disodium ethylenediaminetetraacetic acid) and 0.6 ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1 N HCl to 30 ml total volume). Deionized urea, 0.480 g was added and the solution made up to 1.0 ml with water. The nitrogen gassing was monentarily discontinued while 0.02 ml of β-mercaptoethanol was added to the solution. The space above the solution was gassed with nitrogen for a short period of time, and the vial was sealed with parafilm. The reduction was allowed to proceed for 4 hr at room temperature. The solution was again gassed with nitrogen, and 0.2 ml of a solution of 0.269 g of iodoacetic acid in 1 ml of 1 N NaOH was added. The thiol groups react rapidly with iodoacetic acid, and within 15 min the reaction should be complete. The reaction was stopped by the addition of 4.0 ml of acetone - 1 N HCl (39:1) at 4^{o} which precipitated the protein (Jirgensons and Ikenaka, 1959). The precipitate was washed three times with the same solvent and, finally, with cold ether. Evaporation of the ether leaves the powdered, reduced S-carboxymethylated p-HPP hydroxylase, free of urea. This form of the protein was found by Crestfield, Moore, and Stein (1963) to be a better

substrate for trypsin than the preparation in which cystine and cysteine are oxidized to cysteic acid.

Trypsin digestion was performed according to Hirs (1956). 1 mg of reduced S-carboxymethylated p-HPP hydroxylase was dissolved in 0.5 ml of 0.2 $NaPO_4$, pH 8.2. 0.05 ml of trypsin (Worthington, 2x crystallized, 1.2 mg/ml in 0.001 N HCl) was added to the solution which was incubated at 37° for 20 hr. 0.05 ml aliquots of the digest were placed in 5 ml ampoules for dansylation, and the remaining digest was acidified to pH 3.5 with formic acid to stop the digestion before being frozen for storage.

The tryptic peptides were dansylated according to Gray and Hartley (1963). To the 0.05 ml aliquots of protein, were added 300 μL of 0.2 M $NaPO_4$, pH 8.85, buffer and 300 μL of Dansyl-Cl[12] in acetone (1 mg/ml). After 2 hr, 4 drops of 1 M KOH was added to hydrolyze excess Dansyl-Cl, allowing at least 10 min for complete hydrolysis. The pH was then adjusted to 3.5 with glacial acetic acid. The Dansyl-peptides were then desalted and the Dansyl-OH removed by absorption of the peptides onto Dowex 50w x 8 (0.5 x 2.5 cm) equilibrated with 0.01 M acetic acid. The blue-green fluorescence of the Dansyl-OH was washed from the column with at least 100 ml of 0.01 M acetic acid. The Dansyl-peptides

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[12]Dansyl-OH is the trivial name for 5-dimethylaminonapthalene sulfonic acid.

were then eluted with water-acetone-25% ammonia (80:20:4) until all yellow fluorescence was washed off. The eluate was lyophylized, and the residue was redissolved in a small volume of acetone-water (1:1) for spotting on 20 x 20 cm silic gel G plates. The silica gel plates were developed in the first dimension by methylacetate-isopropanol-25% ammonia (9:6:4) for 60 min, and in the second demension by isobutanol-acetic acid-water (15:4:2) for 140 min with a 10 min activation at 110° in between the two runs. All three forms of the p-HPP hydroxylase and a control of bovine pancreatic ribonuclease A were chromatographed simultaneously in the same tank to insure comparable chromatographs. Acceptable resolution of the Dansyl-peptides during two-dimensional TLC was difficult to achieve, and conditions for analytical TLC should be strictly followed (Stahl, E., 1965). Commercially prepared silica gel G plates were found to require excessive running times for solvent fronts to reach the ends of plates. Thin layers prepared on glass plates according to Zanetta et al. (1970) proved satisfactory.

Bibliography:

Aasa, R., Malmström, B.G., Saltman, P., and Vänngard, T.
The specific Binding of Iron (III) and Copper (II) to Transferrin and Conalbumin.
Biochim. Biophys. Acta, 1963. 75, 203-222.

Abbott, M.T., Schandl, E.K., Lee, R.F., Parker, T.S., and Midgett, R.J.,
Cofactor Requirements of Thymine 7-hydroxylase.
Biochim. Biophys. Acta, 1967. 132, 525-528.

Ackers, G.K.
Molecular Sieve Studies of Interacting Protein Systems.
J. Biol. Chem., 1967. 242, 3026-3034.

Berman, E.
Serum Copper Determination by Atomic Absorption Spectroscopy.
Atomic Absorption Newsletter, 1965. 4, 296.

Billek, G.
Synthesis of Phenylpyruvic Acaids. IV. Condensation of Aromatic Aldehydes with Hydantoin.
Monatsh. Chem., 1961. 92, 352-360.

Blumberg, W.E.
The EPR of High Spin Fe^{3+} in Rhombic fields.
In A. Ehrenberg, B.G. Malmström, and T. Vänngärd (Eds.)
Magnetic Resonance in Biological Systems.
Werner-Gren Center Internat. Symp. Series,
Oxford, Pergammon Press, 1967. Vol. 9, pp. 119-133.

Boyd, D.R., Daly, J.W., and Jerina, D.M.
Rearrangement of (1-^{2}H) and (2-^{2}H) Naphthalene 1,2-oxides to Napthol.
Mechanism of the NIH Shift.
Biochem., 1972. 11, 1961-1966.

Boyder, P.D.
Spectrophotometric Study of Reaction of Sulfhydryl Groups with Organic Mercurials.
Biochem., 1972. 11, 1961-1966.

Cardinale, G.J., Rhoads, R.E., and Udenfriend, S.
Simultaneous Incorporation of ^{18}O into Succinate and Hydroxyproline Catalyzed by Collagen Proline Hydroxylase.
Biophys. Res. Commun., 1971. 43, 537-543.

Cohn, E.J. and Edsal, J.T.
Density and Apparent Specific Volume of Proteins. In H. Neurath (Ed.) Proteins, Amino Acids and Peptides. New York, N.Y., Reinhold, 1943. Chapter 16, pp. 370-381.

Crandall, D.I.
Molecular Oxygenation by the Iron Activated Enzymes in Mammalian Metabolism. In T.E. King, H.S. Mason, and M. Morrison (Eds.) Oxidases and Related Redox Systems. New York, John Wiley & Sons, Inc., 1965. Vol. 1, pp. 263-285.

Crestfield, A.M., Moore, S., and Stein, W.H.
The Preparation and Enzymatic Hydrolysis of Reduced and S-Carboxymethylated Proteins.
J. Bio. Chem., 1963. 238, 622-627.

Daly, J.W., and Witkop, B.
Recent Studies on the Centrally Active Endogenous Amines.
Angewandte Chemie, Internat. Ed., 1963. 2,421-440.

Davis, B.J.
Disc Electrophoresis - II method and application to human serum proteins.
Ann. N.Y. Acad. Sci., 1964. 121, 404-427.

DeCamelli, R.F., Holomam, P.D., Basinger, S.F., LeBowitz, J.
Molecular Weight by Low-Speed Sedimentation Equilibrium. A computation of the Nazarian Equation and the Griffith Rapid Equilibrium Technique.
Analyt. Biochem., 1970. 36, 470-494.

Edwards, S.W., Hsia, D.Y.-Y., and Knox, W.E.
The First Oxidative Enzyme of Tyrosine Metabolism, p-Hydroxyphenylpyruvate Oxidase.
Fed. Proc. Fedn. Am. Soc. exp. Biol., 1955. 14, 204 (abstract).

Ellfork, N. and Sievers, G.
Cytochrome C Peroxidase: Isoelectric Focusing and Analysis of the Crystalline Enzyme in an Acid pH Gradient.
Acta Chem. Scand., 1969. 23, 2550-2551.

Fehnel, E.A.
Methyl Migration in Aromatization of para-Xylene 1,2-oxide.
J. Am. Chem. Soc., 1972. 94, 3961-3963.

Fellman, J.H., Fujita, T.S., and Roth, E.S.
Substrate Specificity of p-Hydroxyphenylpyruvate Hydroxylase.
Biochim. Biophys. Acta, 1971. 268, 601-604.

Fellman, J.H., Fujita, T.S., and Roth, E.S.
Assay, Properties, and Tissue Distribution of p-Hydroxyphenylpyruvate Hydroxylase.
Biochim. Biophys. Acta, 1972. 284, 90-100.

Flatmark, T.
On the Heterogeneity of Beef Heart Cytochrome C.
II. Some Physico-chemical Properties of the Main Subfractions (CyI-CyIII).
III. A Kinetic Study of the Non-enzymic Deamidation of the Main Subfractions (CyI-CyIII).
IV. Isoelectric Fractionation by Electrolysis in a Natural pH Gradient.
Acta Chem. Scand., 1966. 20, 1487-1503.

Goodwin, B.L.
Tyrosine Catabolism; the biological, physiological and clinical significance of p-hydroxyphenylpyruvate oxidase.
Oxford, Clarendon Press. 1972a. (pp. 21-23)

Goodwin, B.L.
A Potential Source of Error in the Assay of p-Hydroxyphenylpyruvate Oxidase.
Analyt. Biochem., 1972b. 47, 302-305.

Goodwin, S. and Witkop, B.
Quinol Intermediates in the Oxidation of Phenols and their Rearrangements.
J. Am. Chem. Soc., 1956. 79, 179-185.

Goswami, M.N.D.
Activation and Inactivation of p-Hydroxyphenylpyruvate Hydroxylase.
Biochim. Biophys. Acta, 1964. 85, 390-399.

Goswami, M.N.D. and Knox, W.E.
Developmental Changes of p-Hydroxyphenylpyruvate Oxidase Activity in Mammalian Liver.
Biochim. Biophys. Acta, 1961. 50, 35-40.

Goswami, M.N.D. and Knox, W.E.
An Evaluation of the Role of Ascorbic Acid in the Regulation of Tyrosine Metabolism.
J. Chron. Dis., 1963. 16, 363-371.

Gottschalk, A. and Graham, E.R.B.
The Basic Structure of Glycoproteins. In H. Neurath (Ed.) The Proteins. Composition, structure and function.
2nd ed., New York, Academic Press, 1966. Vol. 4, pp. 95-149.

Gray, W.R. and Hartley, B.S.
The Structure of a Chymotryptic Peptide from Pseudomonas Cytochrome C-551.
Biochem. J., 1963. 89, 379-380.

Gros, G. and Labouesse, B.
Study of the Dansylation Reaction of Amino Acids, Peptides and Proteins.
Europ. J. Biochem., 1969. 7, 463-470.

Guroff, G., Daly, J.W., Jerina, D.M., Renson, J., Witkop, B., and Udenfriend, S.
Hydroxylation-induced Migration: The NIH Shift.
Science, 1967. 157, 1524-1530.

Hager, S.E., Gregerman, R.I., and Knox, W.E.
p-Hydroxyphenylpyruvate Oxidase of Liver.
J. Biol. Chem., 1957. 225, 935-947.

Hamilton, G.A.
The Proton in Biological Redox Reactions.
Bioorganic Chem., 1972. 1, 83-157.

Harley-Mason, J. and Waterfield, W.R.
The Synthesis, Rearrangement and Certain Reactions of 1-(3,4-Dihydroxybenzyl)-1,2,3,4-tetrahydro -β -Carboline and its 9-methyl Analogue.
Tetrahedron, 1963. 19, 65-76.

Hausmann, E.
Cofactor Requirements for the Enzymatic Hydroxylation of Lysine in a Polypeptide Precursor of Collagen.
Biochim. Biophys. Acta, 1967. 133, 591-593.

Hayaishi, O.
Electronic Aspects of Catalysis of the Oxygenases.
Ann. N.Y. Acad. Sci., 1969. 158 art. 1, 318-335.

Hirs, C.H.W., Moore, S., and Stein, W.H.
Peptides Obtained by Tryptic Hydrolysis of Performic Acid Oxidized Ribonuclease.
J. Biol. Chem., 1956. 219, 623-642.

Hutton, J.J., Tappel, A.L., and Udenfriend, S.
Cofactor and Substrate Requirements of Collagen Proline Hydroxylase.
Biochim. Biophys. Acta, 1967. 118, 231-240.

Internat. Critical Tables of Numerical Data, Physics, Chemistry, and Technology. E.W. Washburn (Ed.).
New York, McGraw Hill, Inc., 1928. Vol. III, pp. 89.

Jirgensons, B. and Ikenaka, T.
Disulfide Bridges and Configuration of Human Serum Albumin. Relation between viscosity, optical rotation, dispersion, and configuration.
Makromol. Chemie, 1959. 31, 112-120.

Kivirikko, K.J. and Prockop, D.J.
Purification and Partial Characterization of the Enzyme for the Hydroxylation of Proline in Protocollagen.
Arch. Biochem. Biophys., 1967. 118, 611-618.

Knox, W.E. and LeMay-Knox, M.
The Oxidation in Liver of L-Tyrosine to Acetoacetate through p-Hydroxyphenylpyruvate and Homogentisic Acid.
Biochem. J., 1951. 49, 686-693.

Kretchmer, N. and McNamara, H.
Certain Aspects of Tyrosine Metabolism in the Young. II. The tyrosine oxidizing system of fetal rat liver.
J. Clin. Invest., 1956. 35, 1089-1093.

LaDu, B.N. and Zannoni, V.G.
Intermediary Metabolism of Tyrosine in Liver: oxidation of p-hydroxyphenylpyruvic acid to homogentisic acid.
Fed. Proc. Fedn. Am. Soc. exp. Biol., 1954. 13, 246. (abstract)

LaDu, B.N. and Zonnoni, V.G.
The Tyrosine Oxidation System of Liver. II. Oxidation of p-hydroxyphenylpyruvic acid to homogentisic acid.
J. Biol. Chem., 1955. 217, 777-787.

LaDu, B.N. and Zannoni, V.G.
A requirement for Catalase in Tyrosine Metabolism: the oxidation of p-hydroxyphenylpyruvic acid to homogentisic acid.
Nature, 1956. 177, 574-575.

Lederer, C.M., Hollander, J.M., and Perlman, I.
Table of the Isotopes. 6th ed., New York, Wiley, 1967.

Lindblad, B.
Radiochemical Assay for p-Hydroxyphenylpyruvate Hydroxylase Activity in Human Liver.
Clin. Chim. Acta, 1971. 34, 113-121.

Lindblad, B., Lindstedt, G., Tofft, M., and Lindstedt, S.
The Mechanism of α-Ketoglutarate Oxidation in Coupled Enzymic Oxygenations.
J. Am. Chem. Soc., 1969. 91, 4604-4606.

Lindblad, B., Lindstedt, G., and Lindstedt, S.
The Mechanism of Enzymic Formation of Homogentisate from p-Hydroxyphenylpyruvic Acid.
J. Am. Chem. Soc., 1970. 92, 7446-7449.

Lindblad, B., Lindstedt, G., Orlanger, B., and Omfeldt, M.
Purification of p-Hydroxyphenylpyruvate Hydroxylase from Human Liver.
Acta Chem. Scand., 1971. 25, 329-330.

Lindstedt, G., Lindstedt, S., Orlander, B., and Tofft, M.
α-Ketoglutarate and Hydroxylation of γ-Butyrobetaine.
Biochim. Biophys, Acta, 1969. 158, 503-505.

Line, W.F., Grohlich, D., and Berzkorovainy, A.
The Effect of Chemical Modification on the Iron Binding Properties of Human Transferrin.
Biochem., 1967. 6, 3393-3402.

Liu, T.Y. and Chang, Y.H.
Hydrolysis of Proteins with p-Toluenesulfonic Acid.
J. Biol. Chem., 1971. 246, 2842-2848.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.
Protein Measurement with the Folin Phenol Reagent.
J. Biol. Chem. 1951. 193, 265-275.

McMeekin, T.L. and Marshall, K.
Specific Volumes of Proteins and the Relationship to their Amino Acid Contents.
Science, 1952. 116, 142-143.

Mason, H.S.
Mechanisms of Oxygen Metabolism.
Adv. in Enzymology, 1957. 19, 79-233.

Mason, H.S.
Oxidases. In the Annual Review of Biochemistry, 1965. 34, 595-635.

Means, G.E. and Feeney, R.E.
Chemical Modification of Proteins.
San Francisco, Holden-Day, Inc., 1971. (pp. 11-20)

Moore, W.H.
On the Determination of Cystine as Cysteic Acid.
J. Biol. Chem., 1963. 238, 235-237.

Neubauer, O. and Falta, W.
Uber das Schicksal einiger aromatischer Sauren bei der Alkaptonurie.
Z. Physiol. Chem., 1904. 42, 81-101.

Neville, D.M., Jr.
Molecular Weight Determination of Protein-Dodecyl Sulfate Complexes by Gel Electrophoresis in a Discontinuous Buffer System.
J. Biol. Chem., 1971. 246, 6328-6334.

Raheja, M.C., Dylenski, I., and Crawhall, J.C.
Estimation of p-Hydroxyphenylpyruvic Acid Hydroxylase Activity based on $^{14}CO_2$ Evolution from the 1-^{14}C-labeled Substrate.
Canad. J. Biochem., 1973. 51, 172-177.

Robinson, A.B., McKerrow, J.H., and Cary, P.
Controlled Deamidation of Peptides and Proteins: an experimental hazard and a possible biological timer.
Proc. Natl. Acad. Sci. U.S.A., 1970. 66, 753-757.

Schepartz, B. and Gurin, S.
The Intermediary Metabolism of Phenylalanine Labeled with Radioactive Carbon.
J. Biol. Chem., 1949. 180, 663-673.

Seiler, N.
Use of the Dansyl Reaction in Biochemical Analysis.
Methods of Biochem. Analysis, 1970. 18, 259-337.

Shaffer, P.M., McCroskey, R.P., Palmatier, R.D., and Abbott, M.T.
Substrate Specificity of the Hydroxylase Reaction in which Thymidine is converted to Thymine Ribonucleoside.
Biochem. Biophys. Res. Commun., 1968. 33, 806-811.

Stahl, E.
Instruments used in Thin-Layer Chromatography and their Operation.
In E. Stahl (Ed.), Thin-Layer Chromatography; a laboratory handbook.
Berlin, New York, Springe-Verlag, 1965. (pp. 5-27)

Stein, S., and Moore, W.H.
Chromatographic Determination of Amino Acid by the Use of Automatic Recording Equipment.
Methods in Enzymology, 1963. VI, 819-831.

Tanaguchi, K. and Armstrong, M.D.
The Enzymic Formation of o-Hydroxyphenylacetic Acid.
J. Biol. Chem., 1963. 238, 4091-4097.

Tanaguchi, K., Kappe, T., and Armstrong, M.D.
Further Studies on Phenylpyruvate Oxidase.
J. Biol. Chem., 1964. 238, 3389-3395.

Vesterburg, O. and Svensson, H.
Isoelectric Fractionation, Analysis, and Characterization of Amphelytes in Natural pH Gradients. IV. Further studies on the resolving power in connection with separation of myoglobins.
Acta Chem. Scand., 1966. 20, 820-834.

Warburg, O.
Heavy Metal Prosthetic Groups and Enzyme Action.
London, Oxford University Press, 1949. (pp. 28-45)

Weber, K. and Osborn, M.
The Reliability of Molecular Weight Determination by Dodecyl Sulfate-polyacrylamide Gel Electrophoresis.
J. Biol. Chem., 1969. 244, 4406-4412.

Whitaker, J.R.
Determination of Molecular Weights of Proteins by Gel Filtration on Sephadex.
Analyt. Chem., 1963. 35, 1950-1953.

Widman, A.S., Soloway, A.H., Stern, R.L., and Bursey, M.N.
Lack of Exchange of the Phenolic Functions in the Enzymatic Conversion of p-HPP to Homogentisic Acid.
Bioorganic Chem., 1973. 2, 176-178.

Yasunobu, K., Tanaka, T., Knox, W.E., and Mason, H.S.
Oxygen Transfer by p-Hydroxyphenyl Pyruvate Oxidase.
Fed. Proc. Fedn. Am. Soc. exp. Biol., 1958. 17, 340. (abstract)

Zanetta, J.P., Vincendon, G., Mandel, P., and Gombos, G.
The Utilization of 1-Dimethylaminonapthalene-5-sulphonyl Chloride for Quantitative Determination of free Amino Acids and Partial Analysis of Primary Structure of Proteins.
J. Chromatography, 1970. 51, 441-458.

Zannoni, V.G.
Tyrosine Oxidation System of Liver. V. The ability of various quinones to reactivate inhibited pHPP-acid oxidase.
J. Biol. Chem., 1962. 237, 1172-1176.

Zannoni, V.G. and LaDu, B.N.
The Tyrosine Oxidation System of Liver. IV. Studies of the inhibition of pHPP oxidase.
J. Biol. Chem., 1959. 234, 2925-2931.

Zannoni, V.G., Jacoby, G.A., and LaDu, B.N.
Effect of Coenzyme Q_{10} on Tyrosine Metabolism in Guinea Pigs.
Biochem. Biophys. Res. Commun., 1962. 7, 220-224.

Zettner, A., Sylvia, L.C., and Capacho, Delgado, L.
The Determination of Serum Iron and Iron Binding Capacity by Atomic Absorption Spectroscopy.
Am. J. Clin. Path., 1966. 45, 533-540.

Stabilization of p-Hydroxyphenylpyruvate Hydroxylase during Purification by o-Hydroxyphenylpyruvate

	Specific Activity (units/mg protein)		
	0.0	4.0 (10^{-4})M	o-HPP
Step I Ammonium Sulfate	1.86	1.5	
Step II SP-Sephadex	7.50	19.2	
Step III Hydroxyapatite	15.90	179.8	
Step IV QAE-Sephadex	27.90	510.0	

Table 1. Stabilization of p-HPP Hydroxylase[a]

[a]The assay of enzyme samples containing o-HPP required adequate dilution (1 into 100) to reduce the o-HPP inhibition to an insignificant level. A unit of enzyme activity was defined as 1 μ mole of product formed per hr at 37°.

Purification of Avian Liver p-HPP-hydroxylase

Isolation Step	Total Enzyme (units, umole/hr)	Yield (%)	Total Protein (mg)	Specific Activity (units/mg protein)	Purification (over crude supernate)
1. Crude Supernatant	24,188	100	49,025	0.5	1.0
2. Acetone Powder Extract	13,608	56	15,225	0.9	1.8
3. 35-55% Ammonium Sulfate precipitation	10,192	44	5,880	1.5	3.0
4. SP-Sephadex C-50 column	9,245	37	481.7	19.2	39.2
5. Hydroxyapatite column	7,500	30	41.8	179.8	367.0
6. QAE-Sephadex A-50 column	4,200	17	8.25	510.0	1040.0

Table 2. Purification of Avian Liver p-HPP Hydroxylase

	II/I	III/I
Lysine	.94	.94
Histidine	1.13	1.23
Amide-N	1.26	1.07
Arginine	1.02	.92
Aspartic acid	1.01	1.06
Threonine	.92	1.04
Serine	.95	1.02
Glutamic acid	0.89	.97
Proline	1.12	0.92
Glycine	.97	1.27
Alanine	.94	.99
1/2 Cystine[a]	0.76	0.79
Valine	1.10	1.06
Methionine	.83	.83
Isoleucine	0.92	.92
Leucine	1.01	.92
Tyrosine	1.10	1.05
Phenylalanine	1.10	.96

a. Value from oxidation of cystine and cysteine to cysteic acid by performic acid and hydrolysis in 6 N HCl at 110° for 20 hr (Moore, S., 1963)

Table 3. Comparison of Amino Acid Compositions of p-HPP Hydroxylase Fractions I, II, and III.

	μ moles/mg protein	Moles/49,000 gm protein
Lys	0.630	30.8
His	0.285	14.0
Arg	0.392	19.2
Trp[b]	0.102	4.9
Amide-N[a]	1.201	58.9
Asp	0.890	43.6
Thr[b]	0.458	22.4
Ser[b]	0.504	24.7
Glu	1.157	56.7
Pro	0.375	18.8
Gly	0.663	32.5
Ala	0.489	26.8
Val[c]	0.627	30.7
Met	0.159	7.8
Ile	0.370	18.1
Leu[c]	0.743	36.4
Tyr	0.352	17.3
Phe	0.492	24.0
1/2 Cystine[d]	0.064	3.1
Cysteine[e]	----	≃4.0

a. Value estimated from 22 hr hydrolysis
b. Values extrapolated to zero time from 22, 48 and 72 hr hydrolyses in 3 N p-toluenesulfonic acid, 0.2% 3-(2-aminoethyl)-indole at 110° (Liu & Chang, 1971)
c. Values from 72 hr hydrolysis
d. Value from oxidation of cystine and cysteine to cysteic acid by performic acid and hydrolysis in 6 N HCl at 110° for 20 hr (Moore, S., 1963).
e. Value estimated from enhancement of p-CMB absorption at 250 nm by thiol (Boyer, P., 1959).

Table 4. Amino Acid Composition of Avian Liver p-HPP Hydroxylase.

INHIBITION OF p-HPP-HYDROXYLASE BY

DIETHYLDITHIOCARBAMATE AND o-PHENANTHROLINE

	$Fe^{++}4(10^{-4})M$	Spec. Act. (units/mg)	(%) Control
Enzyme Control	+	22.9	--
	-	19.1	--
Enzyme + DEDTC ($10^{-3}M$)	+	2.6	11
	-	2.7	14
Enzyme + o-Phen ($10^{-3}M$)	+	12.6	55
	-	6.4	34
	$Cu^{++}4(10^{-4})M$		
Enzyme Control	+	26.6	--
	-	20.3	--
Enzyme + DEDTC ($10^{-3}M$)	+	15.4	58
	-	2.37	12
Enzyme + o-Phen ($10^{-3}M$)	+	8.5	32
	-	6.0	29

Table 5. Inhibition of p-HPP Hydroxylase by Diethyldithiocarbamate and o-Phenanthroline[b]

[b]The addition of ferrous iron, cupric copper, and chelators to the assay medium was made prior to the 10 min preincubation period with enzyme.

ATTEMPTS AT REMOVAL AND REPLACEMENT

OF Fe^{++} AND Cu^{++} BY DIALYSIS AND GEL-FILTRATION

	$Fe^{++}4(10^{-4})M$	Spec. Act. (units/mg prot.)	% Control
Enzyme Control	+	25.6	--
	-	24.2	--
Enzyme, DEDTC treated	+	20.9	82
	-	21.6	89
Enzyme, o-Phen treated	+	20.5	80
	-	20.0	82
	$Cu^{++}4(10^{-4})M$	Spec. Act.	% Control
Enzyme Control	+	28.2	--
	-	22.0	--
Enzyme DEDTC treated	+	23.3	82
	-	20.2	91.8
Enzyme o-Phen treated	+	19.3	68
	-	17.4	79

Table 6. Attempt at Removal of Ferrous Iron and Cupric Copper from p-HPP Hydroxylase by Dialysis against chelators and gel filtration[c].

[c]The addition of ferrous iron or cupric copper to the assay medium was made prior to the 10 min preincubation period with enzyme. No additional chelator was added to the assay medium.

Table 7. Atomic Absorption Analysis of Equilibrium Dialyzed p-HPP Hydroxylase Fractions.

Atomic Absorption Analysis of Equilibrium Dialyzed p-HPP Hydroxylase Fractions

	Volume (ml)	Cu (μM)	Bound Cu (μM)	Total Bound Cu (n mole)	Total Protein (n mole)	Cu/Protein (mole ratio)
QAE-Fraction	11.7	3.75	0.5	5.85	78	0.075
Dialysate	—	3.25	—	—		-----
SP-Fraction	5.25	3.75	0.75	3.94	91	0.043
Dialysate	—	3.00				
		Fe (μM)	Bound Fe (μM)	Total Bound Fe (n mole)		Fe/Protein (mole ratio)
QAE-Fraction	11.7	7.2	5.9	69.03	78	0.88
-Dialysate	—	1.3				
SP-Fraction	5.25	3.25	0.74	3.88	91	0.04
-Dialysate	—	2.4				

Atomic Absorption Analysis of EDTA and Chelex 100-Treated p-HPP Hydroxylase Fractions

	Volume (ml)	Bound Cu (μM)	Total Bound Cu (n mole)	Total Protein (n mole)	Cu/Protein (mole ratio)
QAE-Fraction	3.5	0.9	3.15	67	0.047
SP-Fraction	3.0	1.0	3.0	60	0.045
		Bound Fe (μM)	Total Bound Fe (n mole)		Fe/Protein (mole ratio)
QAE-Fraction	3.5	10.5	36.8	67	0.55
SP-Fraction	3.0	0.12	0.38	60	0.006

Table 8. Atomic Absorption Analysis of EDTA and Chelex-100 treated p-HPP Hydroxylase Fractions.

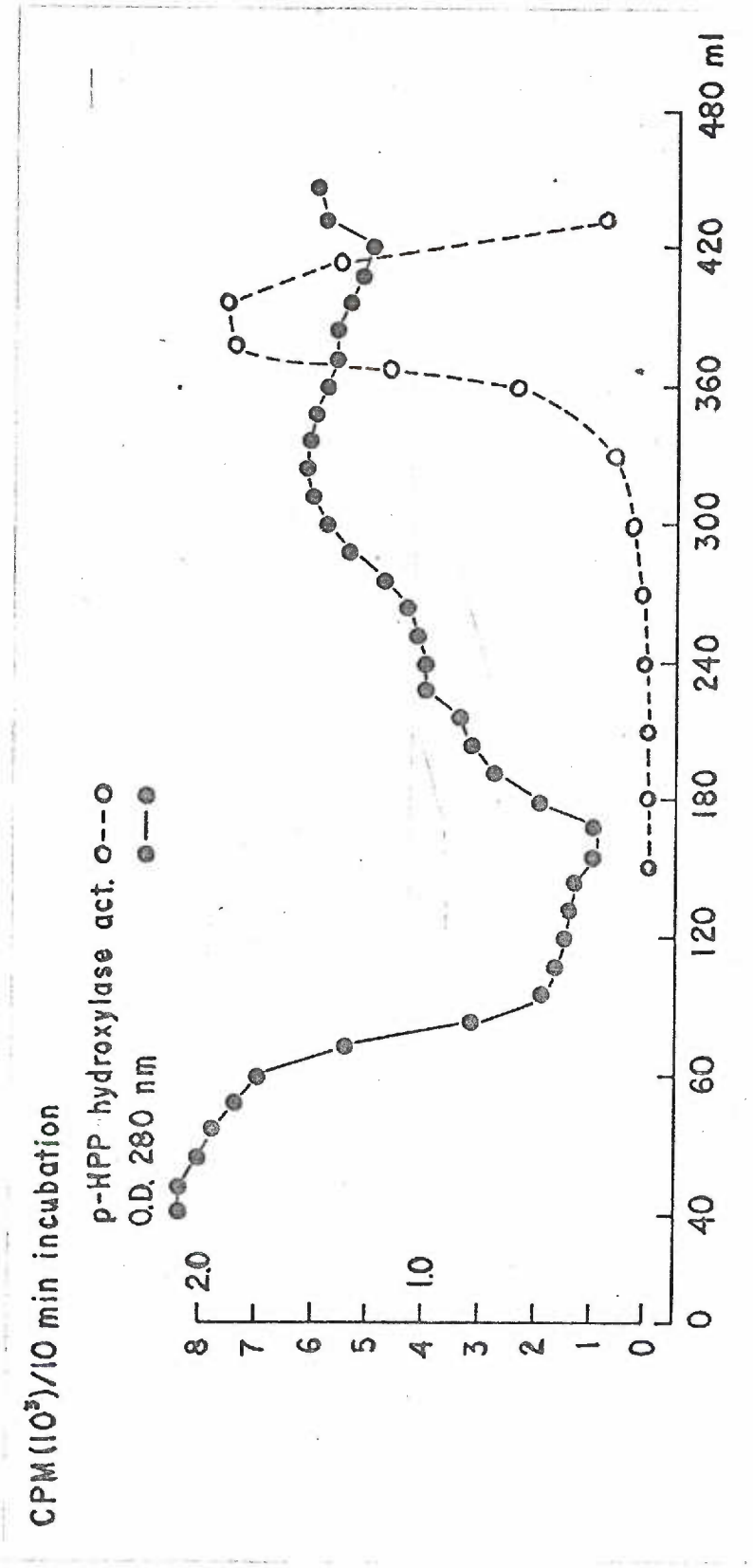


Figure 1 SP-Sephadex Elution Profile.

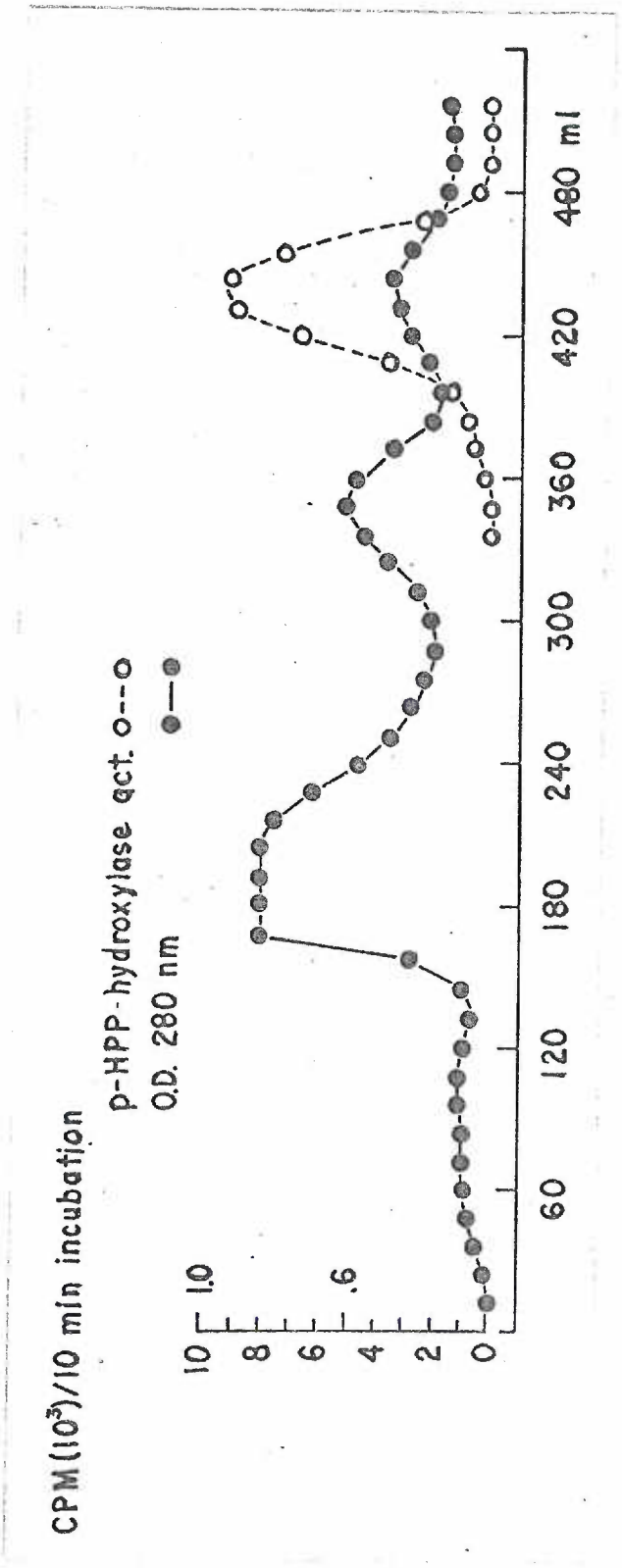


Figure 2. Hydroxyapatite Elution Profile.

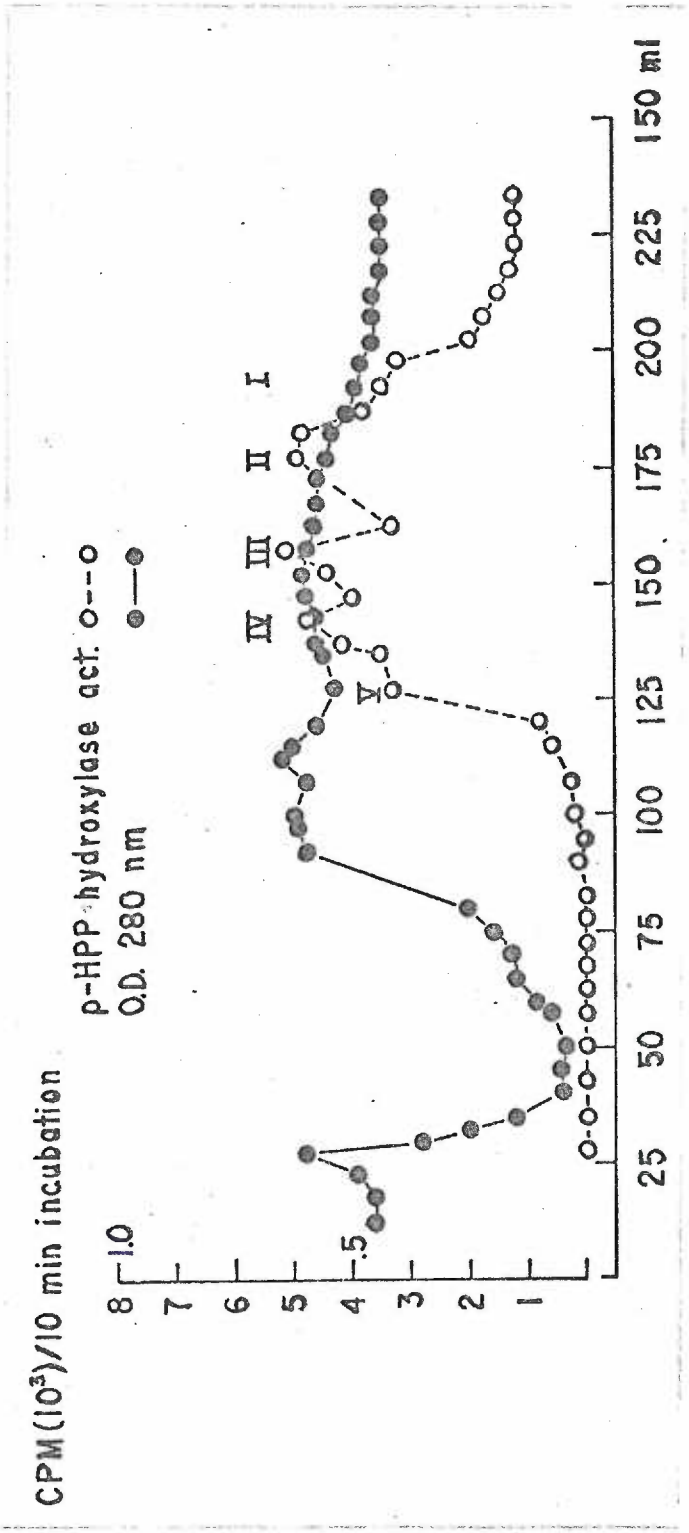


Figure 3. QAE-Sephadex Elution Profile.

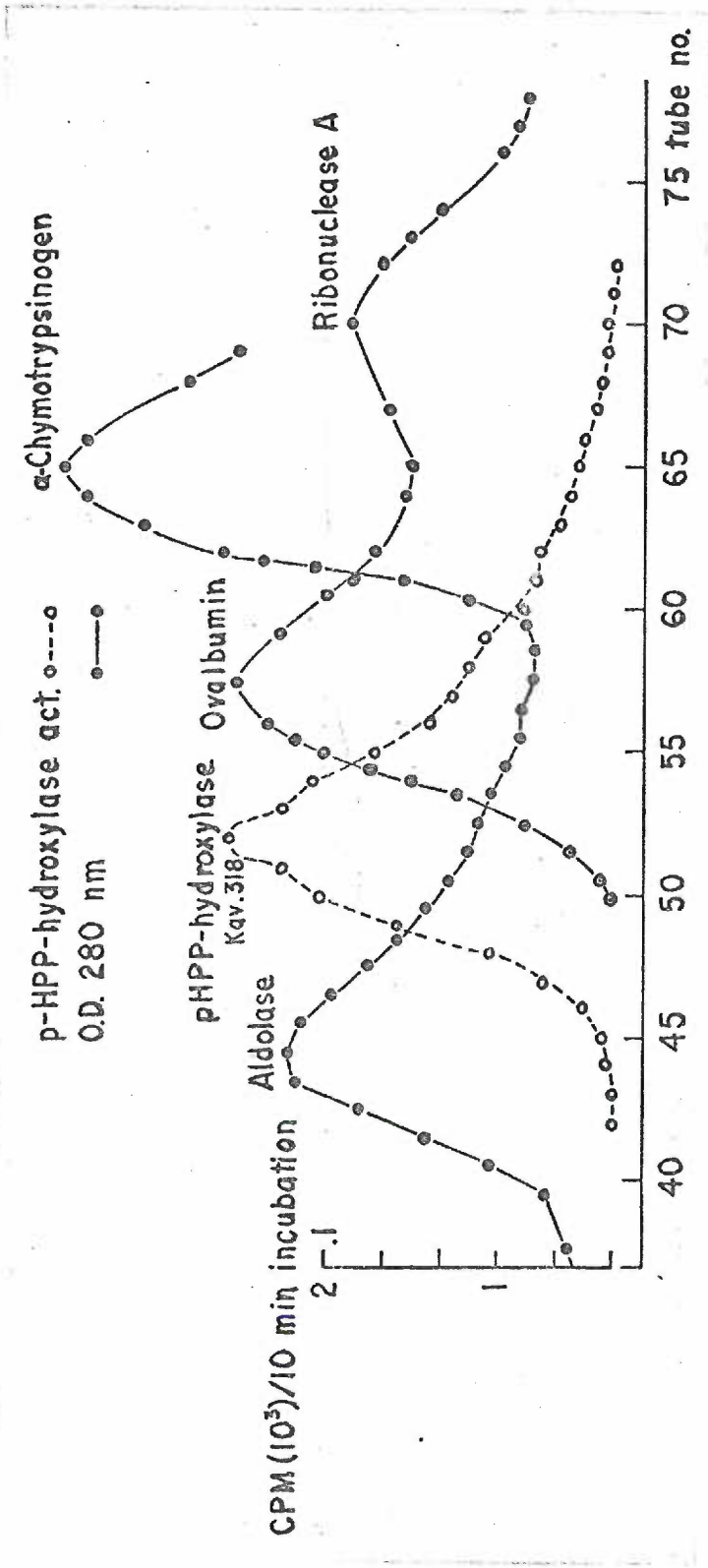


Figure 4. Calibration of Sephadex G-150 Column with Protein Standards.

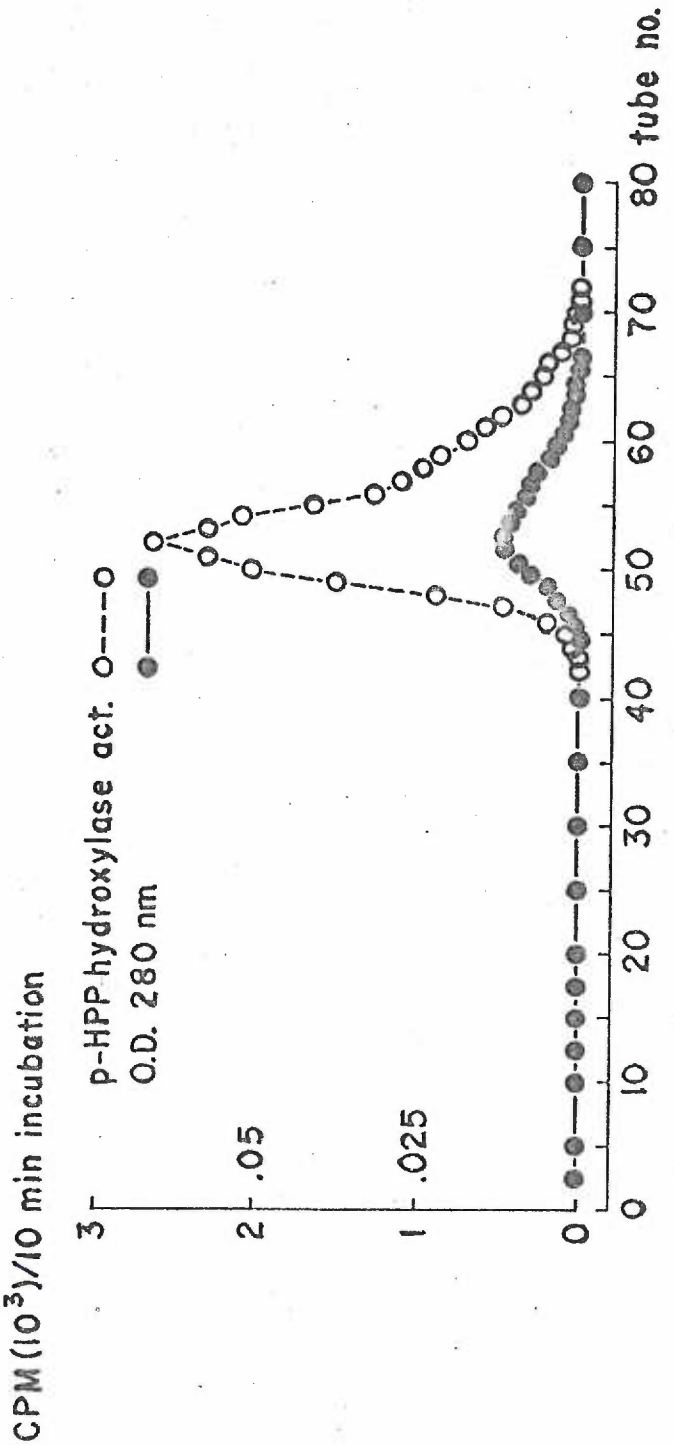


Figure 5 Sephadex G-150 Elution Profile of p-HPP Hydroxylase.

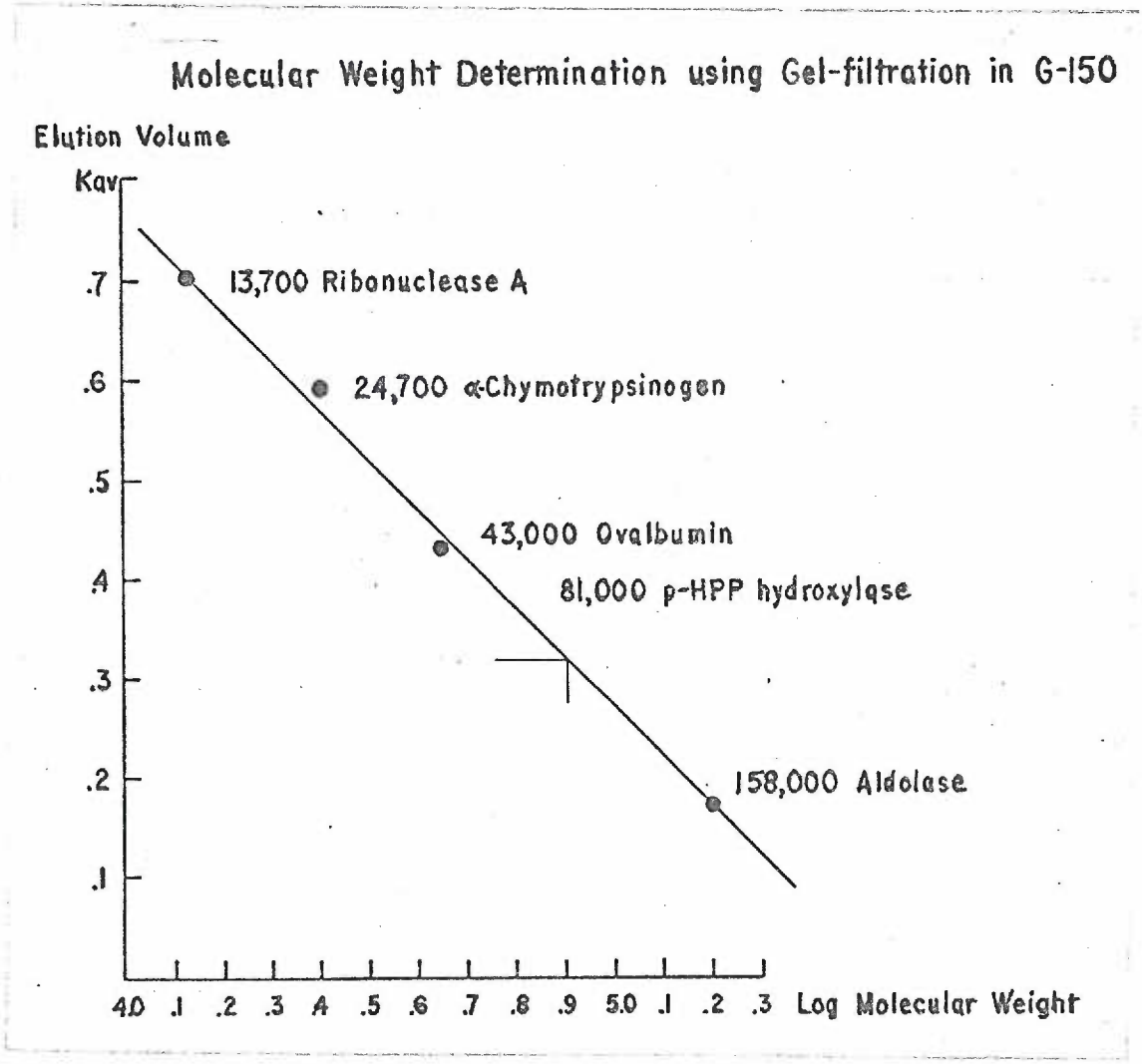


Figure 6. Molecular Weight Determination using Gel-filtration in G-150.

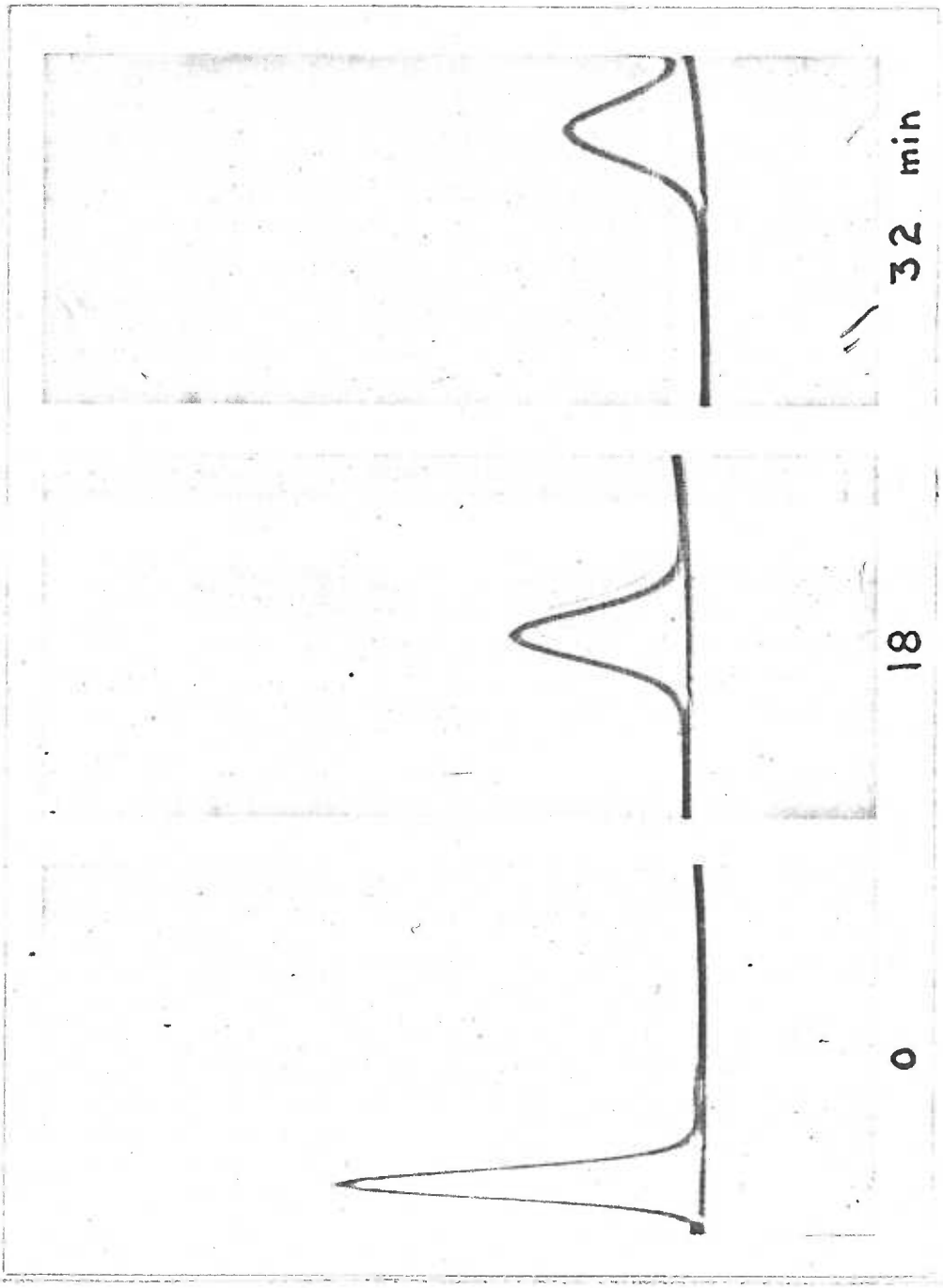


Figure 7. Sedimenting Boundary of p-HPP Hydroxylase Observed through Schlieren Optics. The photographs of the boundary were taken at 0, 18, and 32 min.

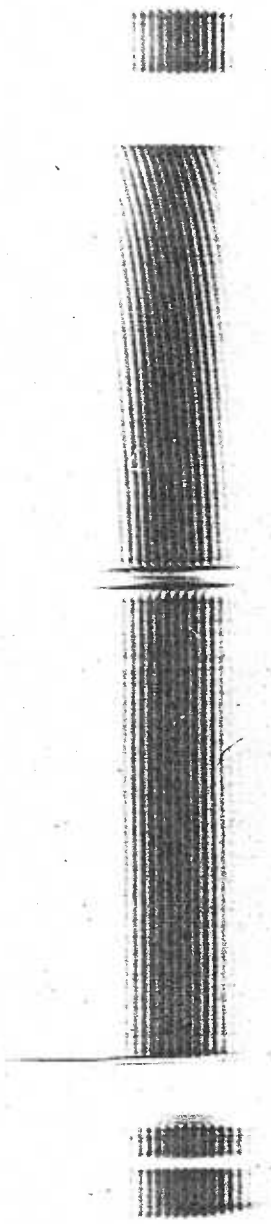


Figure 8. Sedimentation Equilibrium of p-HPP Hydroxylase Observed through Raleigh Interference Optics. The final photograph of the sedimentation run was taken at 72 hr.

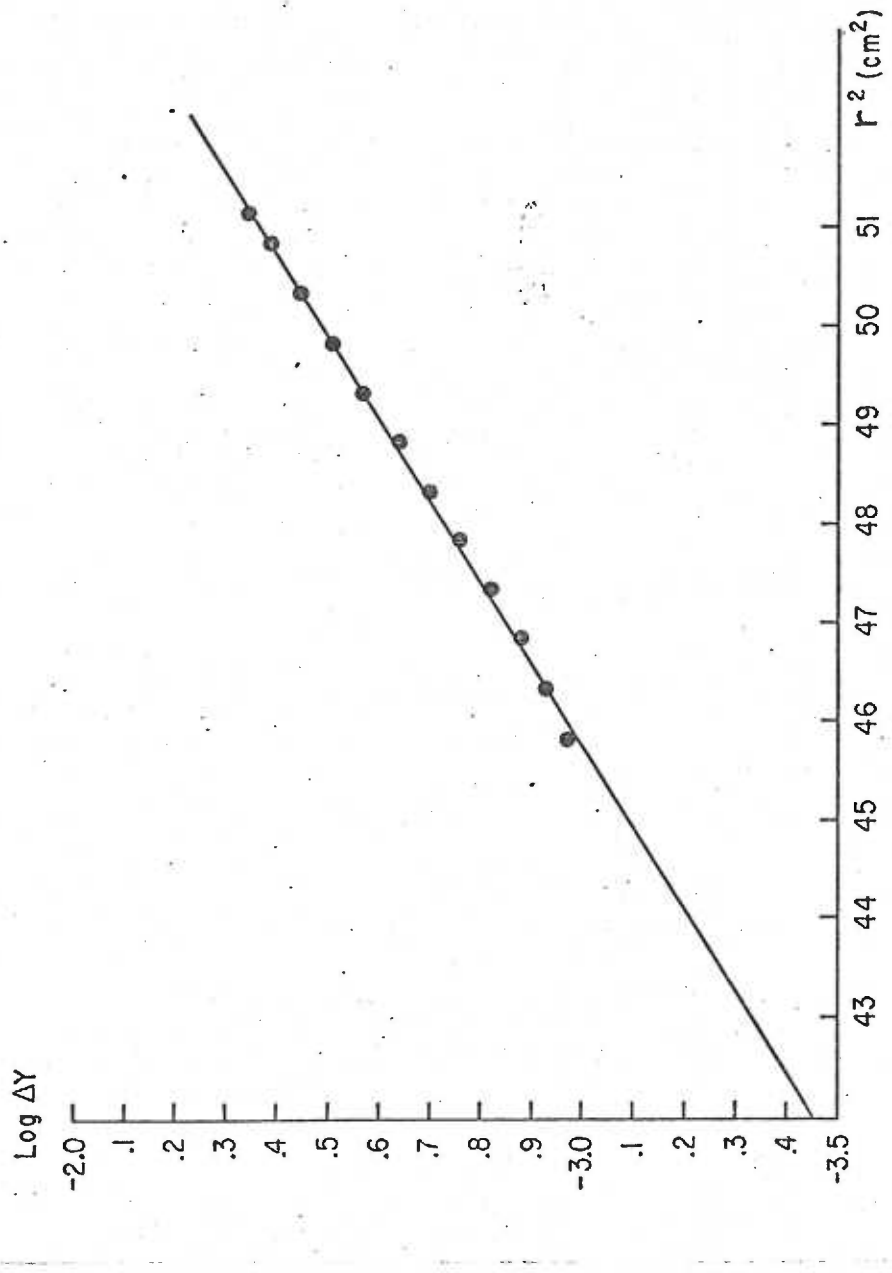


Figure 9. Sedimentation Equilibrium Plot of Log ΔY versus r^2. The slope of the Plot of log ΔY versus r^2 was determined by the method of least squares

$\frac{d \log \Delta Y}{d\ r^2}$ = 0.1173 and the Y intercept = -8.374

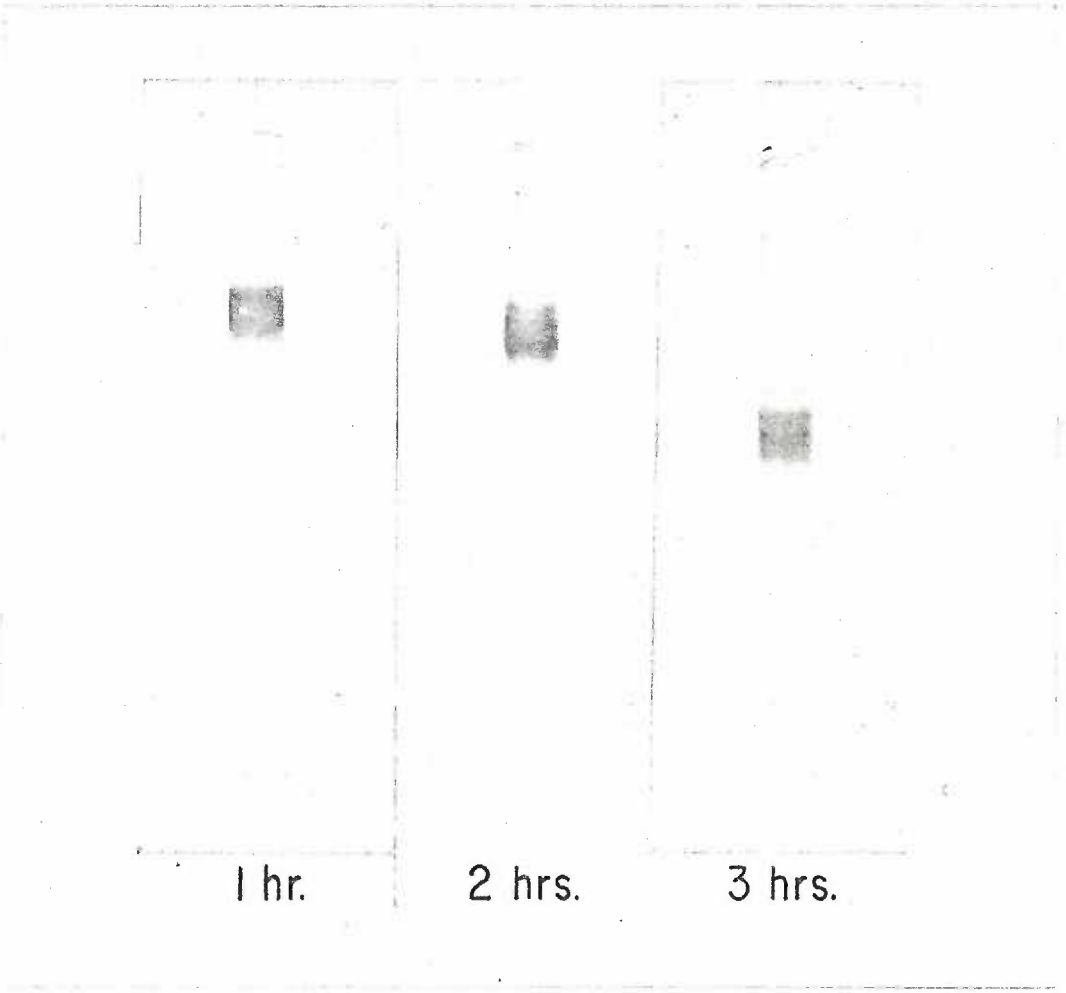


Figure 10. Polyacrylamide Gel Electrophoresis of p-HPP Hydroxylase.

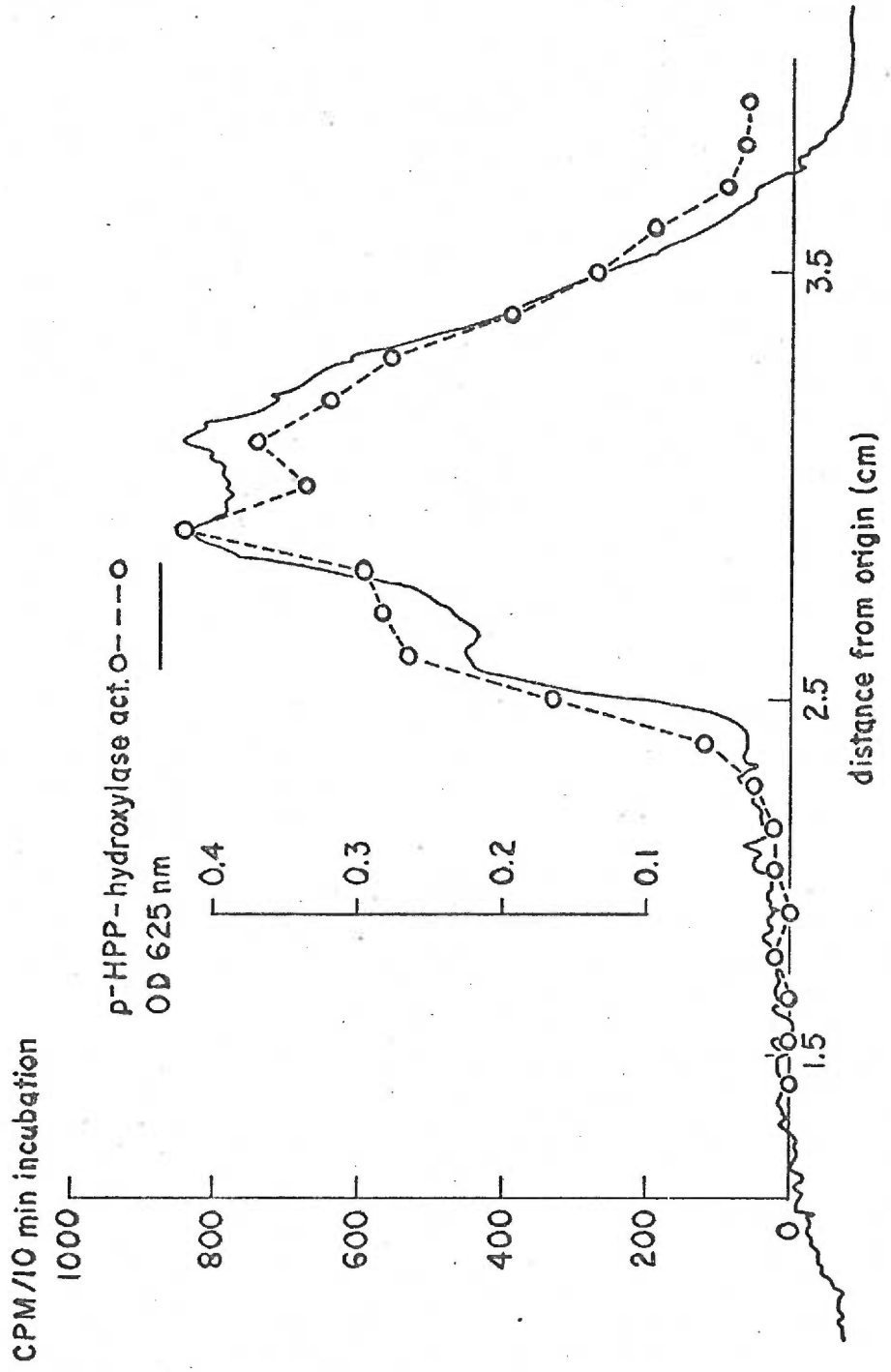


Figure 11. Elution of p-HPP Hydroxylase Activity from Polyacrylamide Gels. The polyacrylamide gels were sliced into 1 mm slices, and the individual slices were incubated in the assay medium for the 10 min preincubation period.

Figure 12. SDS Polyacrylamide Gel Electrophoresis of p-HPP Hydroxylase. The SDS polyacrylamide gel electrophoresis was conducted according to the method of Weber and Osborn (1969).

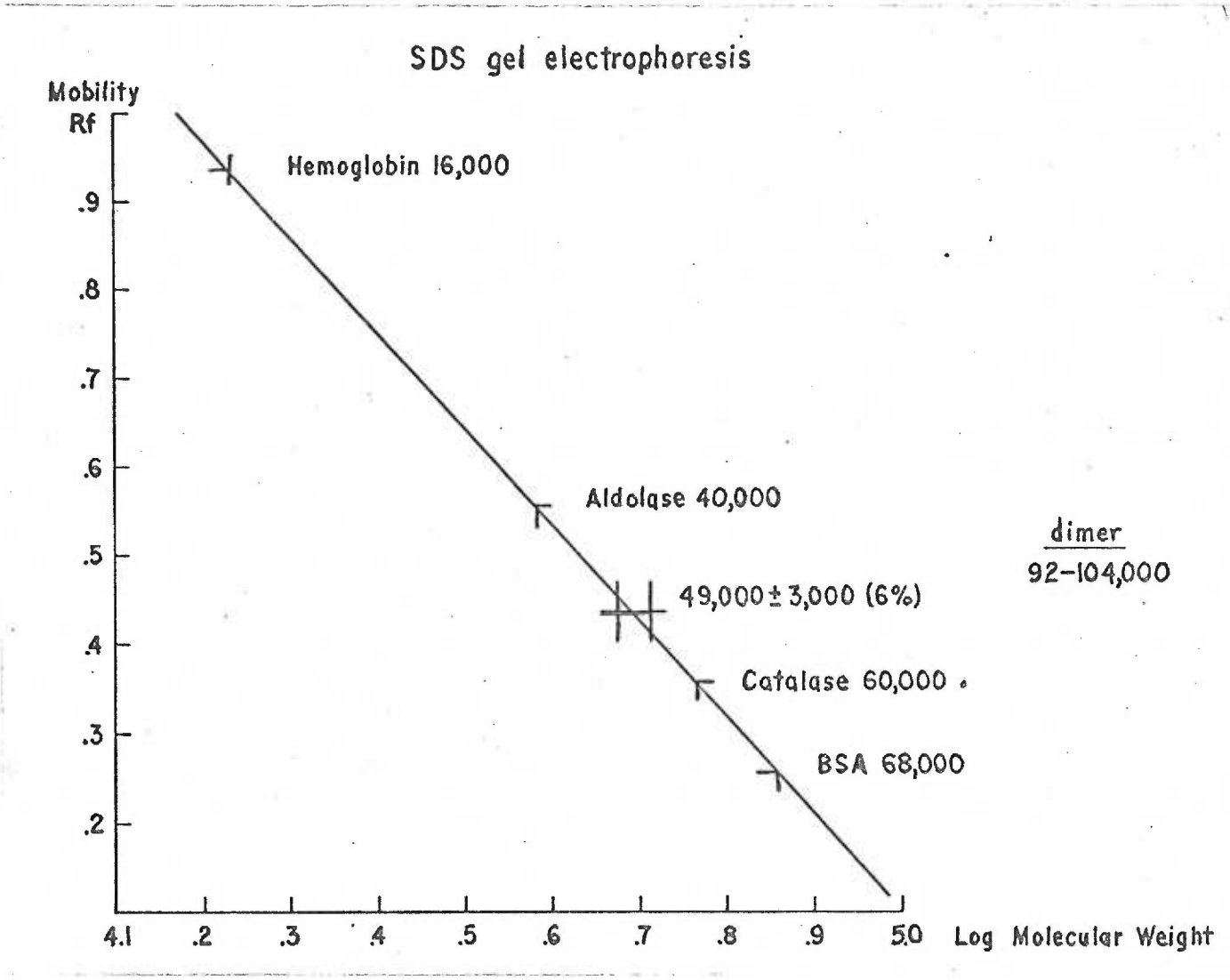


Figure 13. Molecular Weight Determination of p-HPP Hydroxylase Subunits by SDS Polyacrylamide Gel Electrophoresis. The method utilized was that of Neville (1971). The relative mobility of the various proteins was determined by marking the bromophenol blue, dye, migration distance by insertion of a nichrome wire and measuring the protein and dye migration distance in the stained gels. The relative protein mobilities were expressed as Rf values (protein migration distance/dye migration distance) and plotted versus log molecular weight.

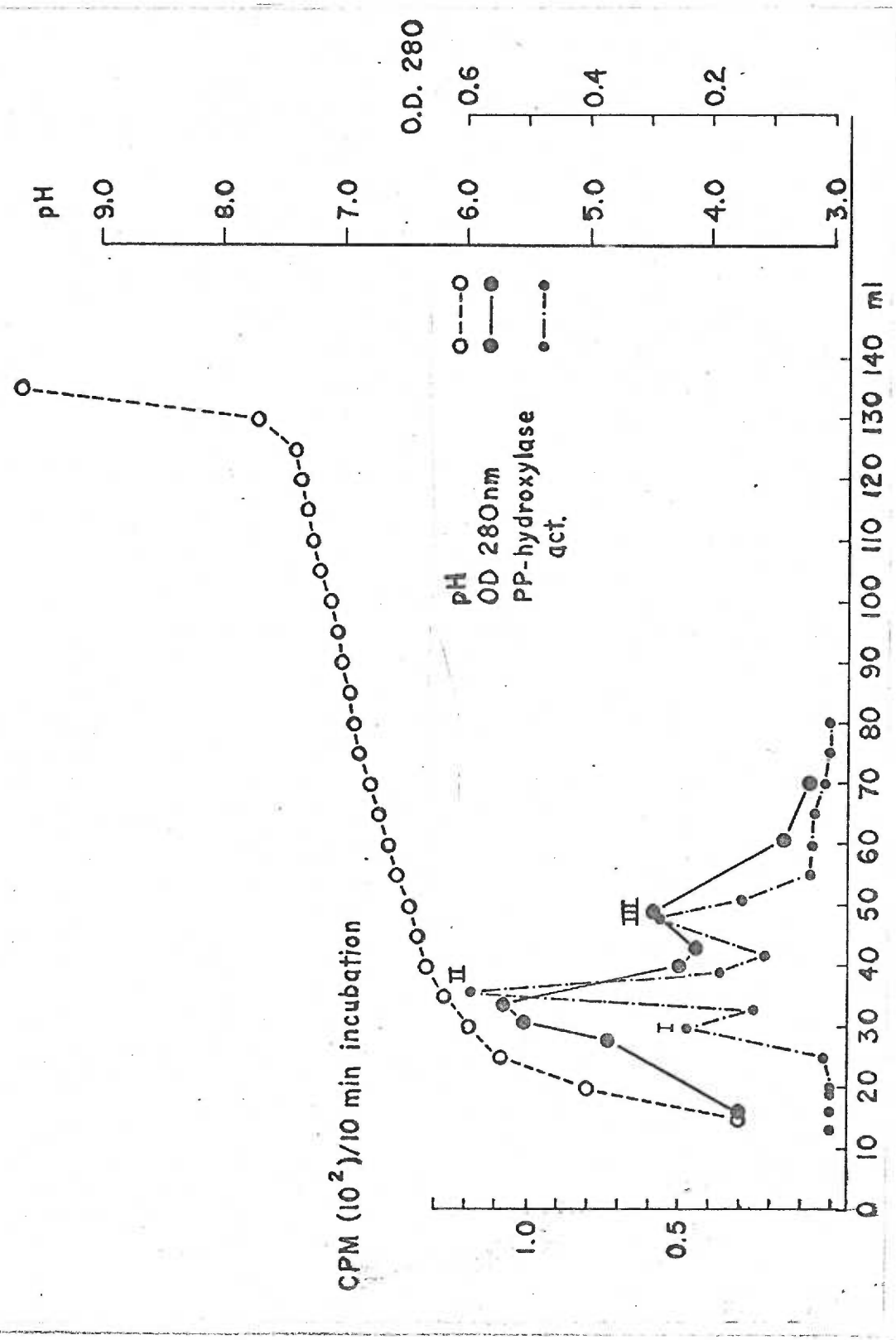


Figure 14. Isoelectric Focusing of Phenylpyruvate Hydroxylase Activity in a Sucrose Gradient. pH and phenylpyruvate hydroxylase activity were measured at regular intervals in the sucrose gradient. O.D. 280 nm was measured in selected fractions which were dialyzed free of o-HPP, a UV absorbing compound.

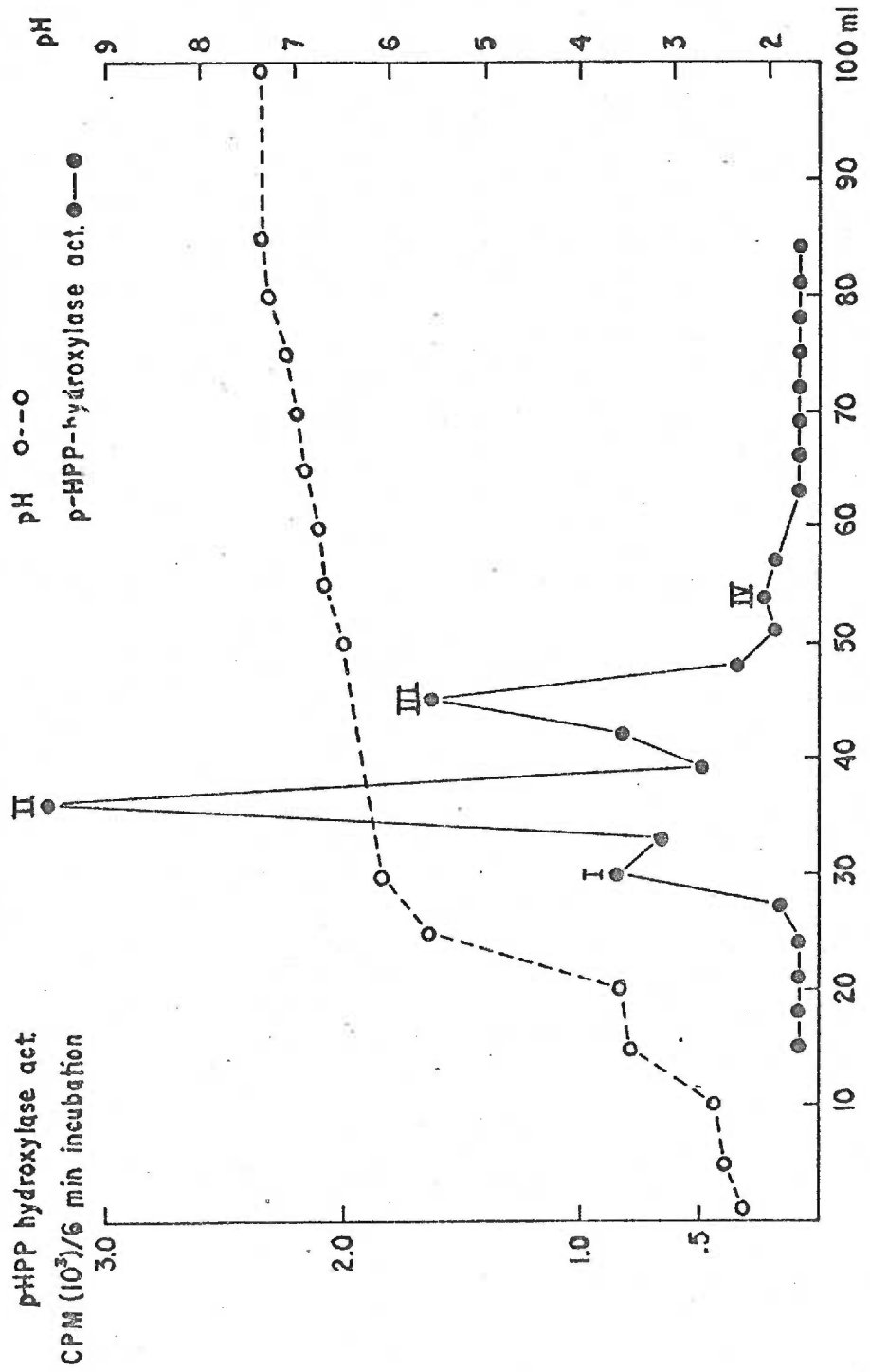


Figure 15 Isoelectric Focusing of p-HPP Hydroxylase Activity in a Sucrose Gradient.

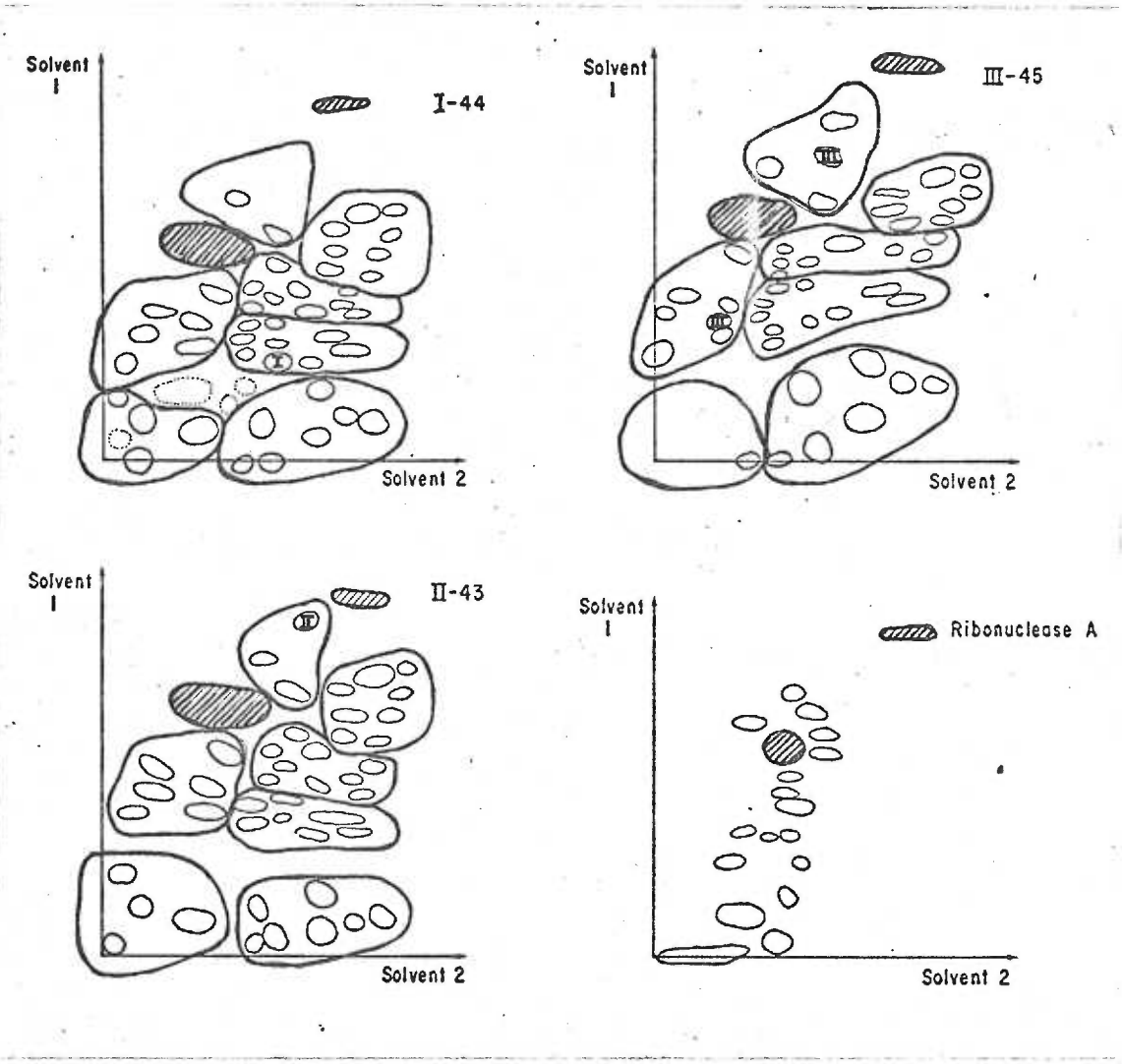


Figure 16. Dansyl-Peptide Maps of Reduced, Carboxymethylated p-HPP Hydroxylase, Fractions I, II, and III. The two dimensional TLC was run 60 min in solvent I, methyl acetate - isopropanol - 25% ammonia (9:6:4, v/v/v) and 140 min in solvent 2, isobutanol-acetic acid - water (15:4:2, v/v/v) with a 10 min activation at 110° in between runs.

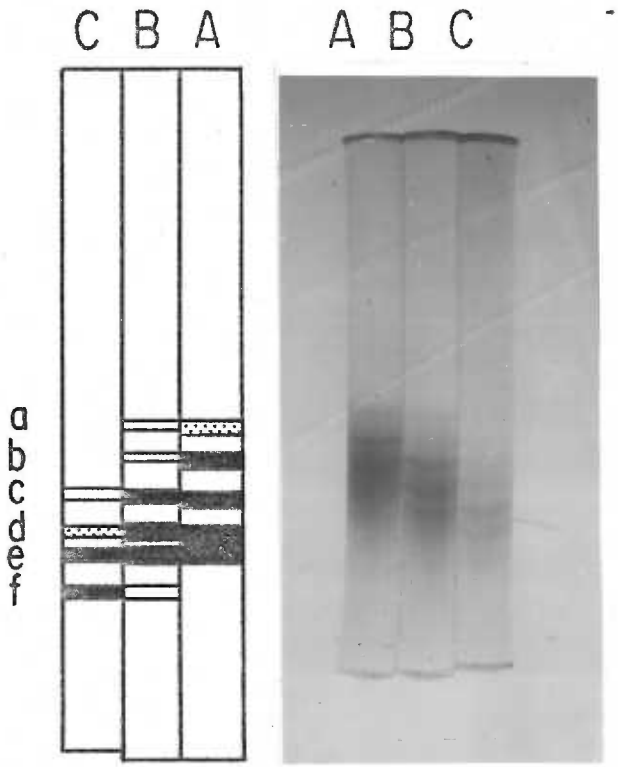


Figure 17. Attempt at Chemical Deamidation of p-HPP Hydroxylase.
A. stored at -15° for 70 hr.
B. incubated at 23° for 40 hr.
C. incubated at 23° for 40 hr and 37° for 30 hr.
The protein bands in the stained gels were labeled a to f in order of increasing mobility.

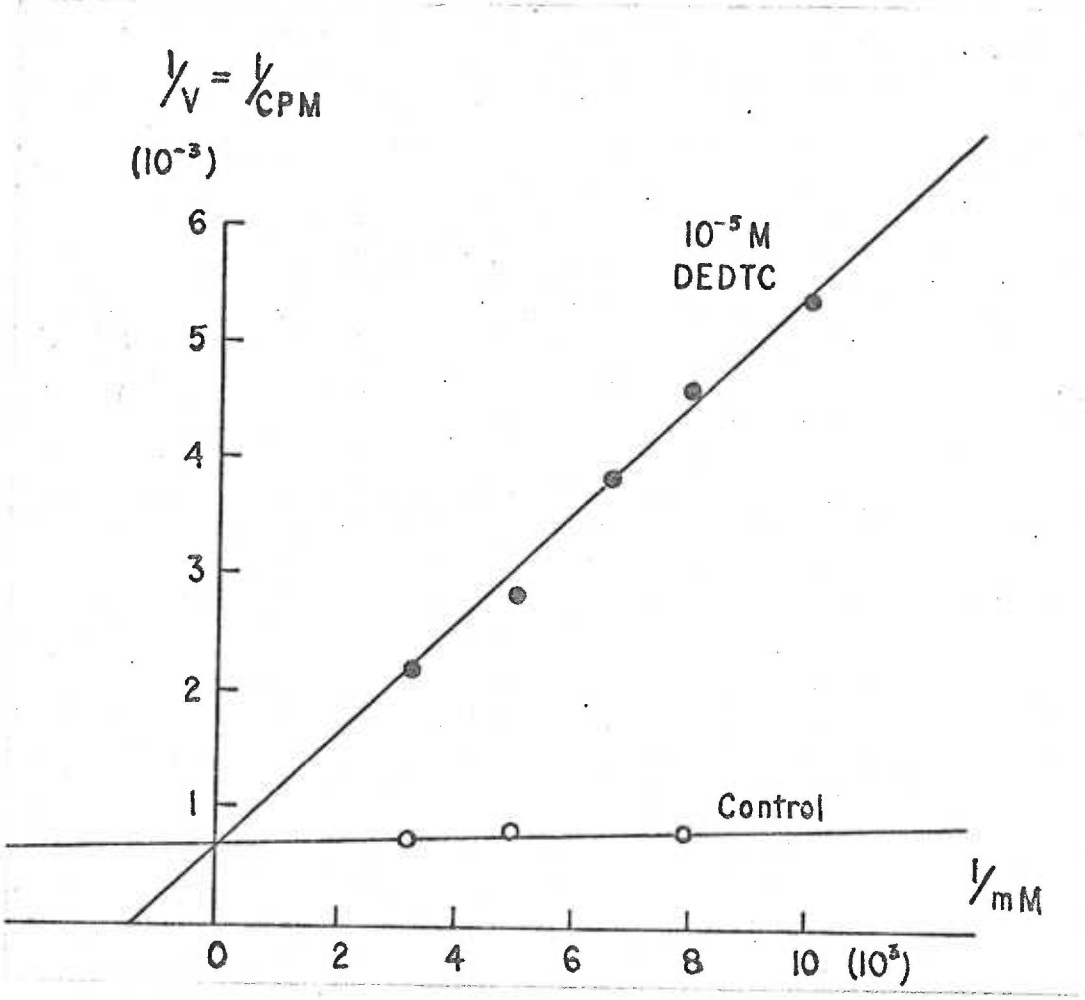


Figure 18. Lineweaver-Burke Plot of Diethyldithiocarbamate Inhibition of p-HPP Hydroxylase. The enzyme was activated for 5 min with 0.44 mg/ml of neutralized ascorbic acid instead of dichlorophenolindolphenol and glutathione. The standard assay procedure was, then, followed. The addition of DEDTC to the assay medium was simultaneous with substrate tip-in.

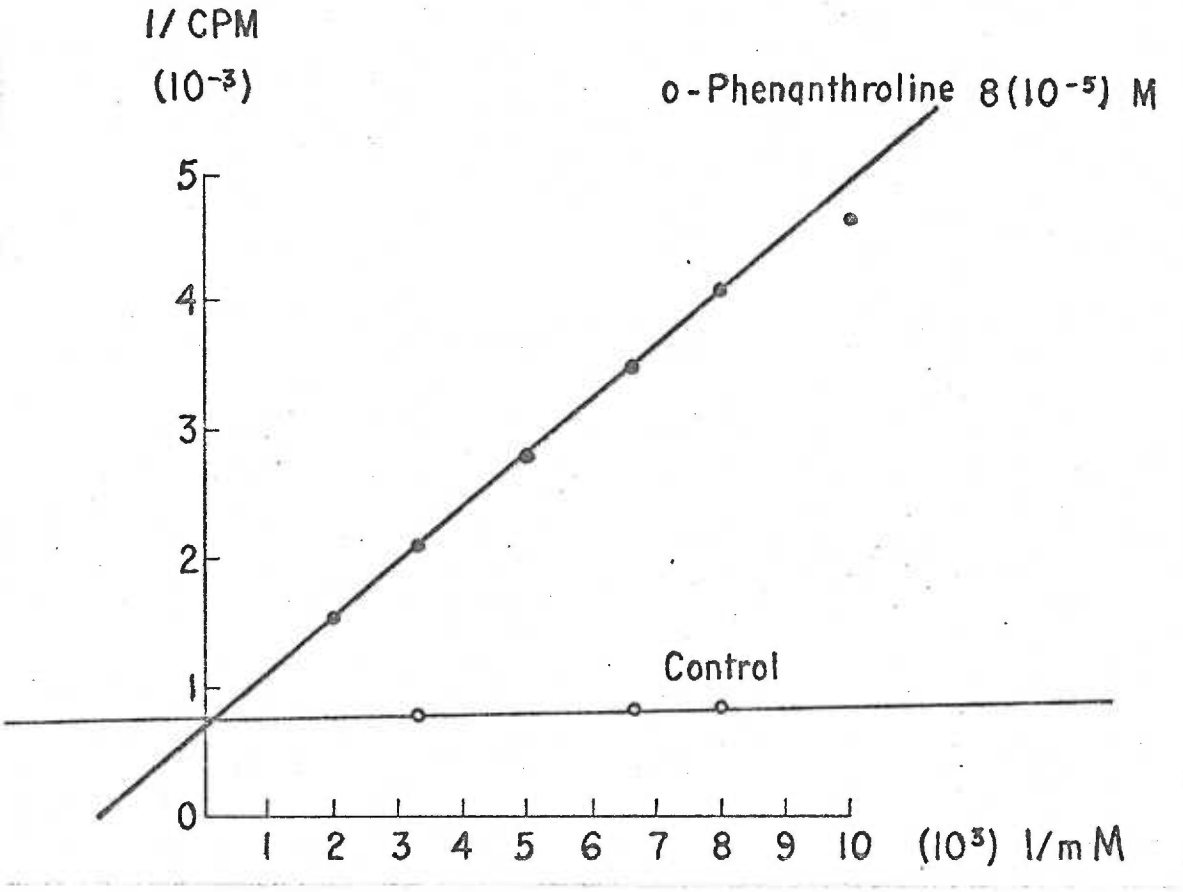


Figure 19. Lineweaver-Burke Plot of o-Phenanthroline Inhibition of p-HPP Hydroxylase. The o-Ph was added simultaneously with substrate tip-in.

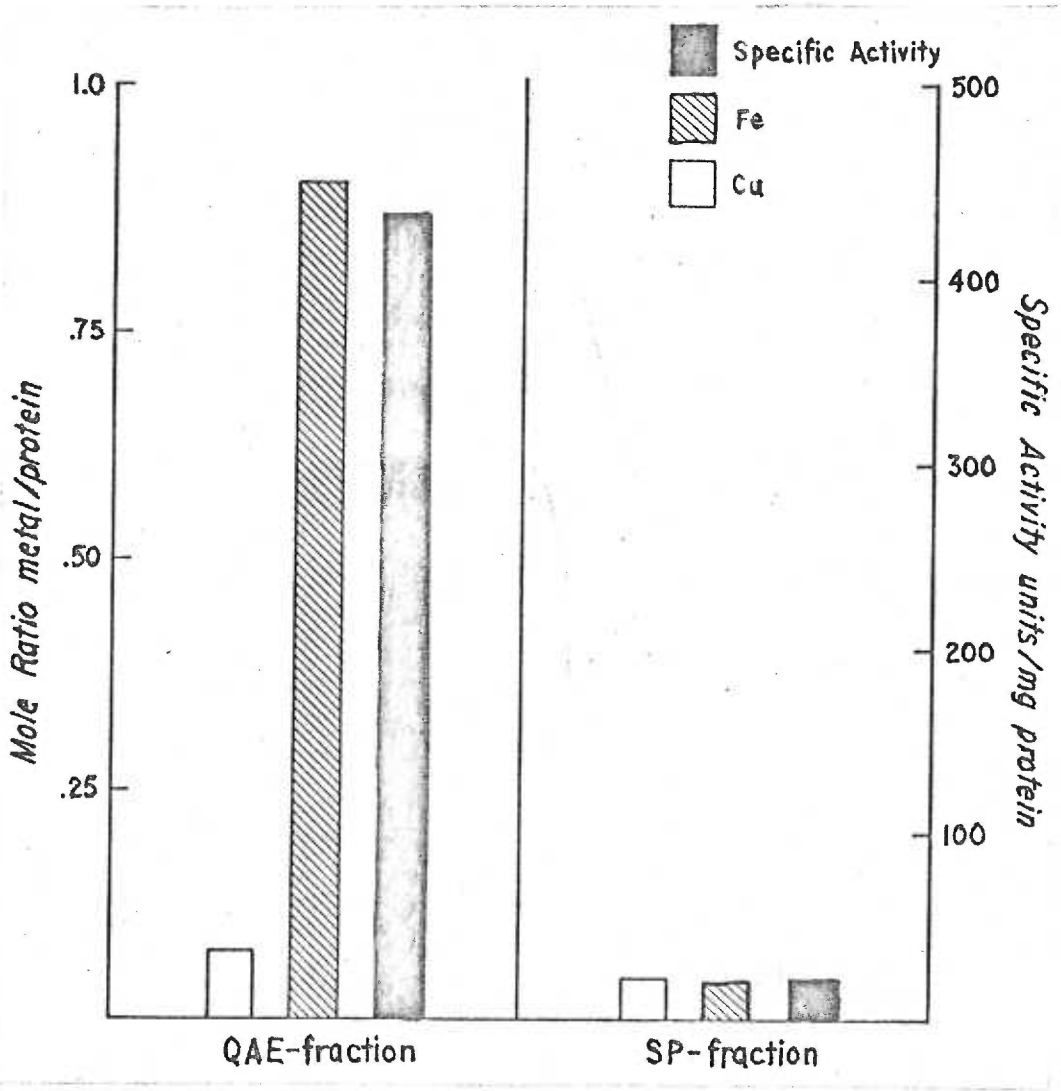


Figure 20. The Iron and Copper Content of Equilibrium Dialyzed p-HPP Hydroxylase.

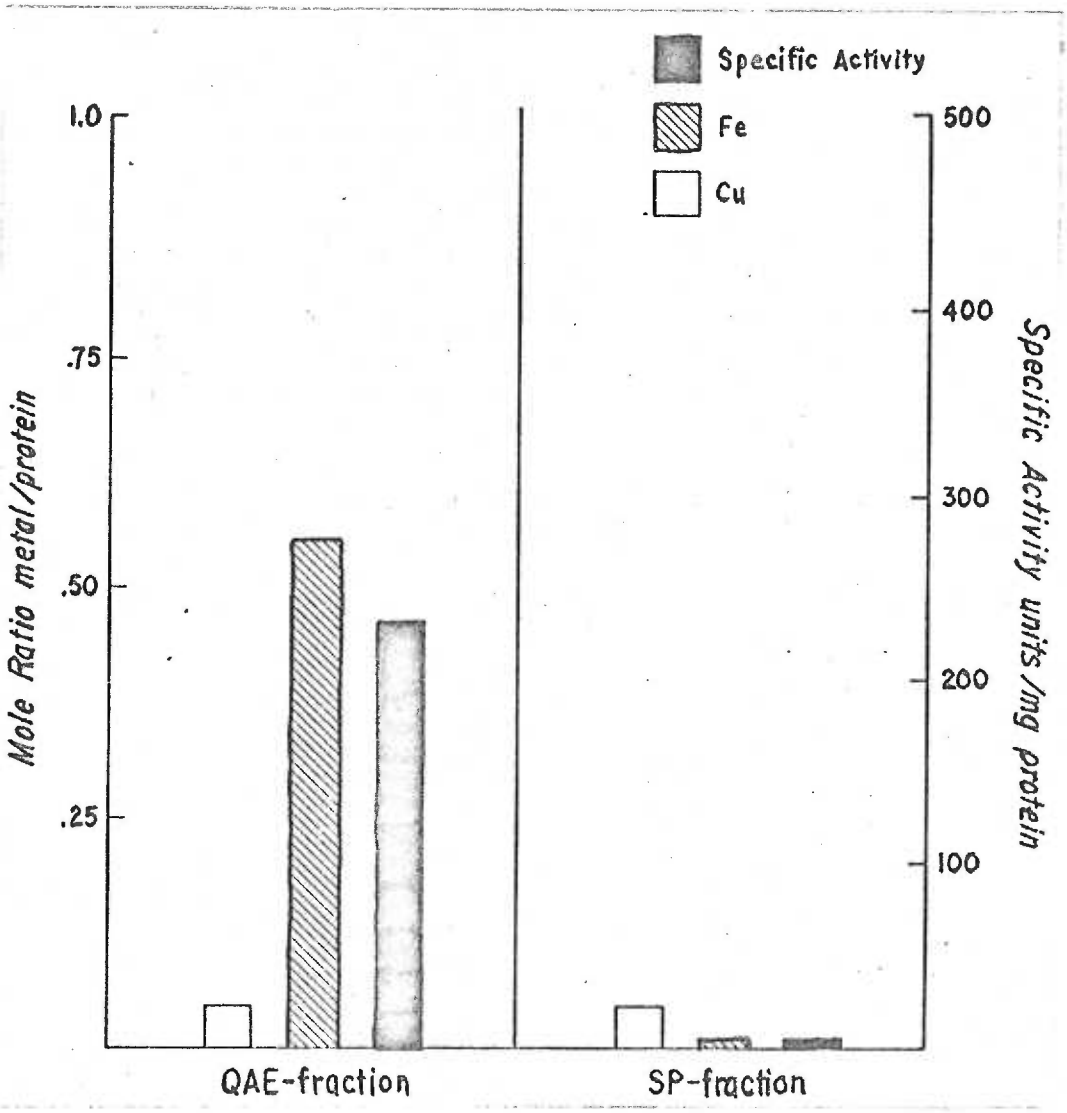


Figure 21. The Iron and Copper Content of EDTA and Chelex 100 Treated p-HPP Hydroxylase.

Figure 22. The EPR Spectrum of p-HPP Hydroxylase.
instrument settings:
modulation amplitude - 3200
time constant - 1
gain - 100
field range - 1000 gauss
scan time - 5 min
klystron freq. (Kv) - 9.143 Kmegacycles
p-HPP Hydroxylase concentration - 6.6 mg/ml
in pH 8.0, Tris-HCl 10 mM
o-HPP 0.4 mM
KCl 100 mM

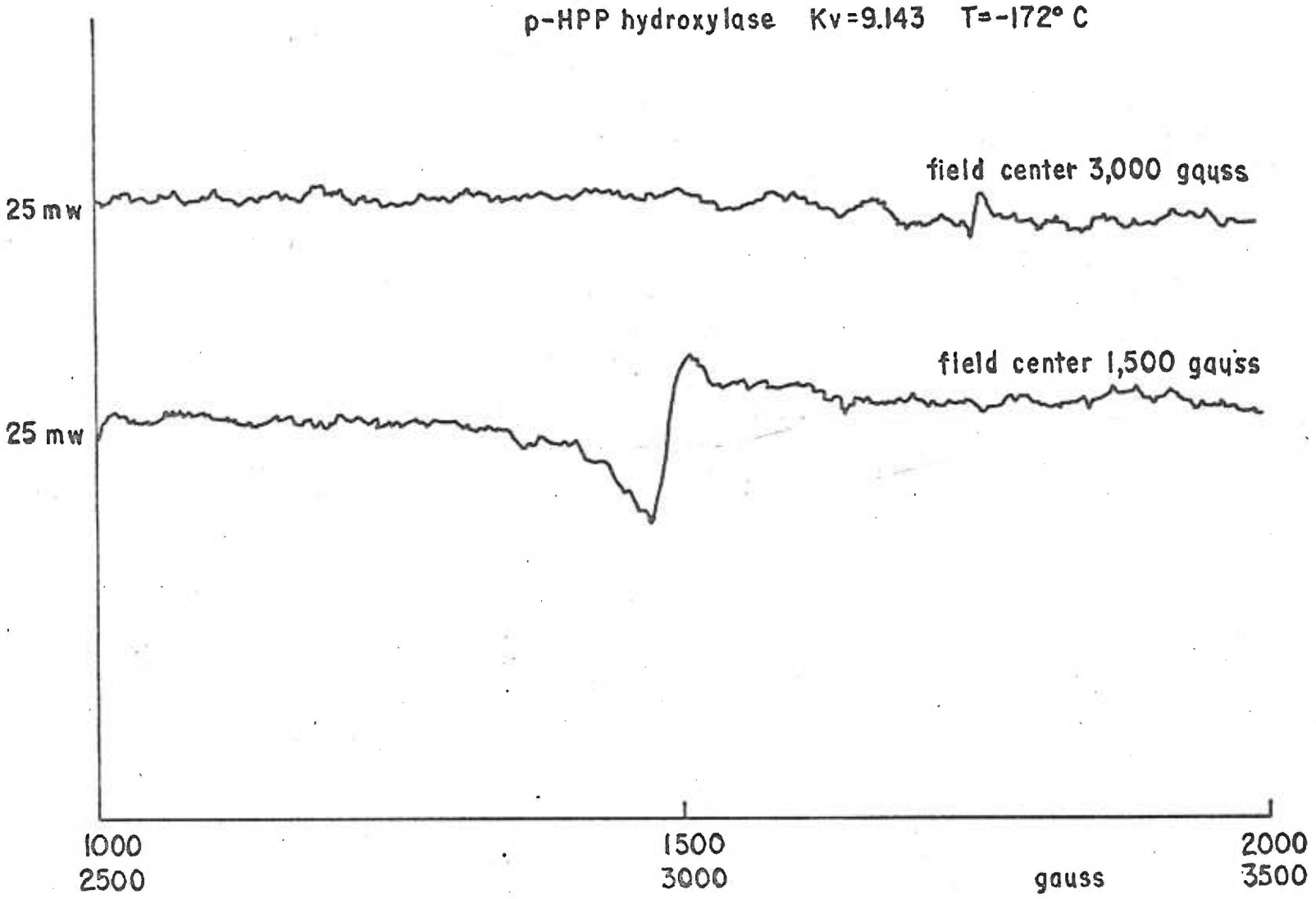
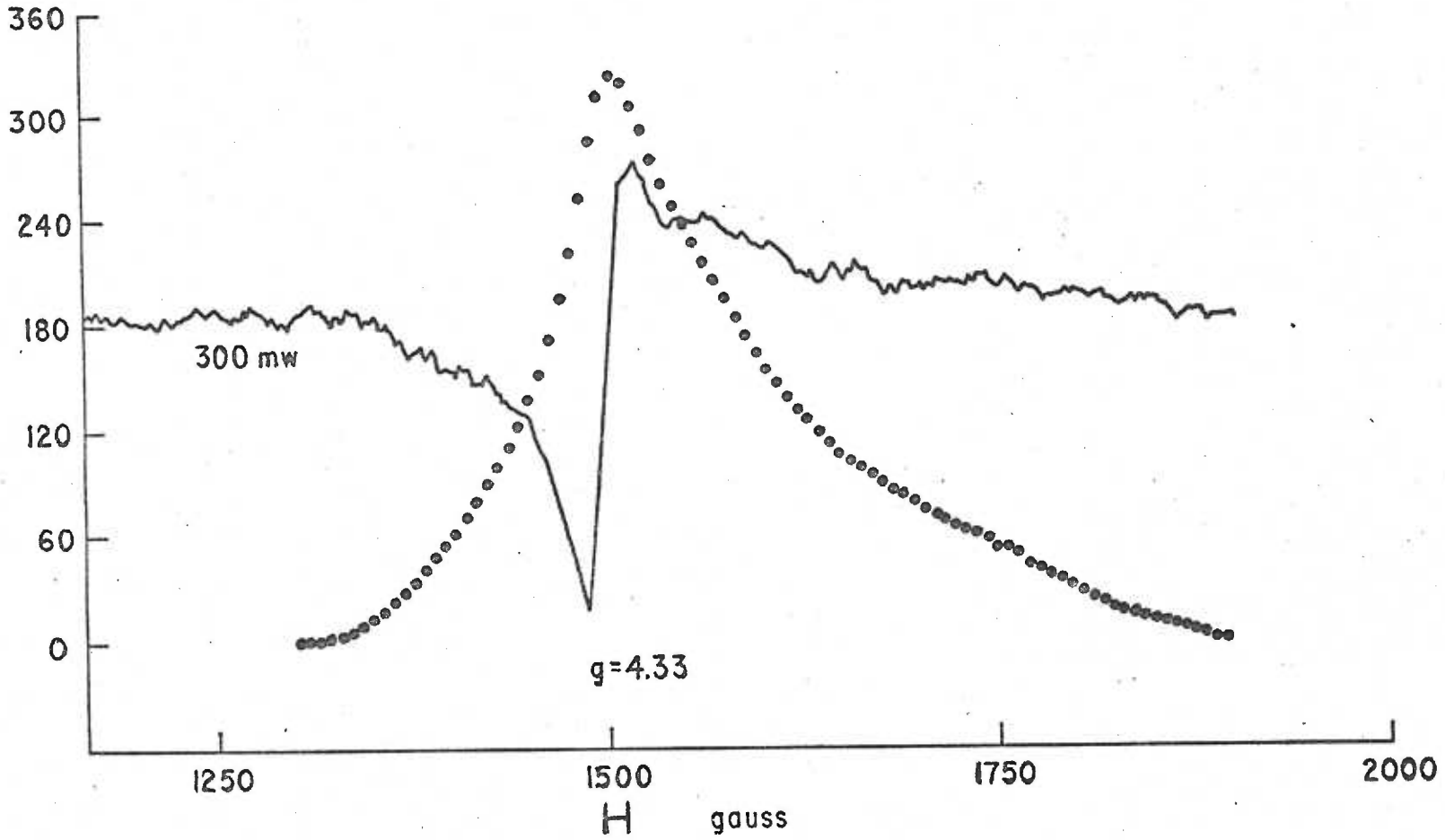

p-HPP hydroxylase Kv=9.143 T=-172° C
field center 3,000 gauss
25 mw
field center 1,500 gauss
25 mw
1000
2500
1500
3000
gauss
2000
3500

Figure 23. Integration of p-HPP Hydroxylase EPR Signal.

$$g = \frac{0.71379 \times Kv}{\text{signal H in K gauss}}$$

where signal H is the value of H at the peak of the integration.


360
300
240
180
120
60
0
300 mw
g=4.33
1250
1500
1750
2000
H gauss

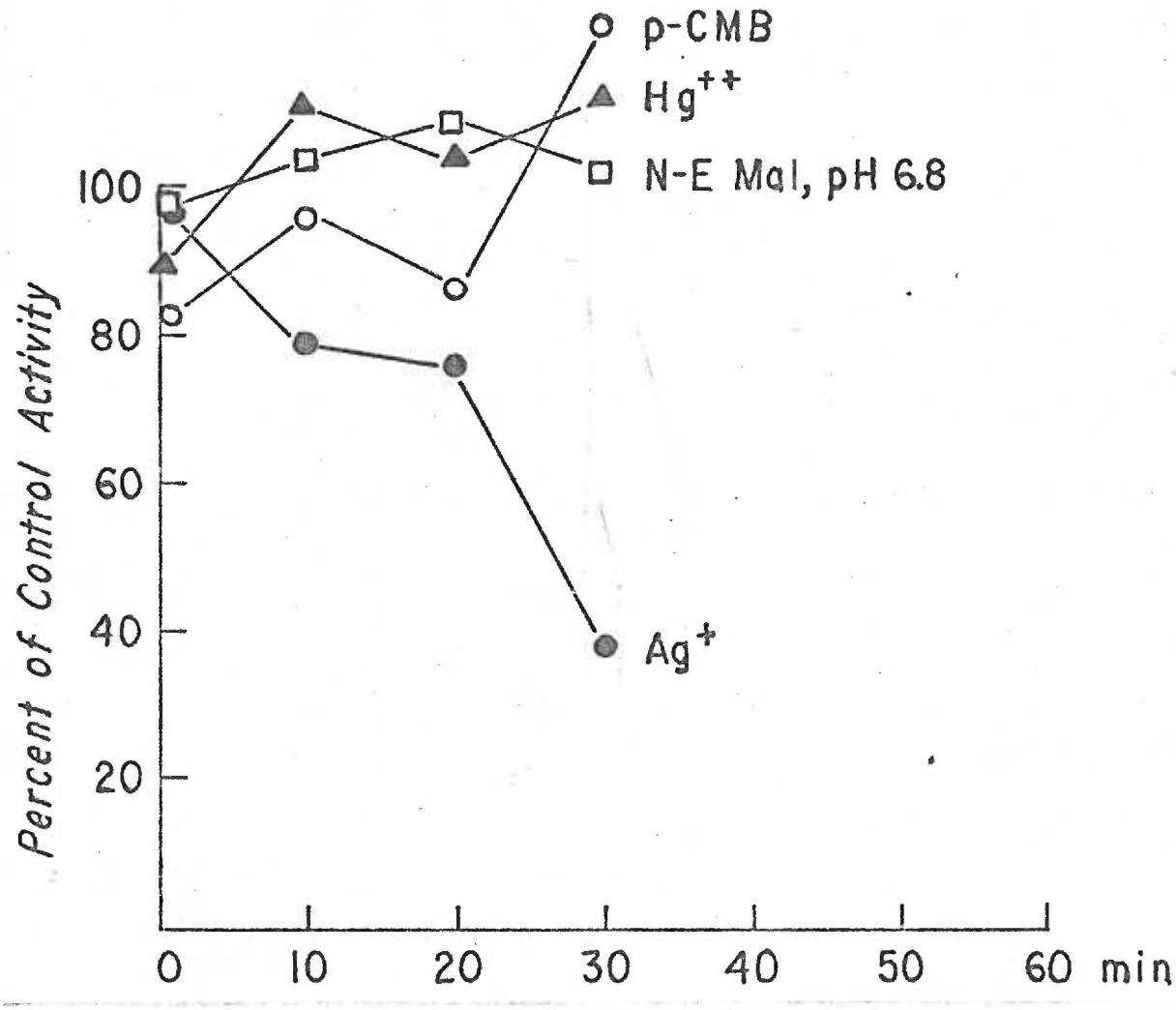
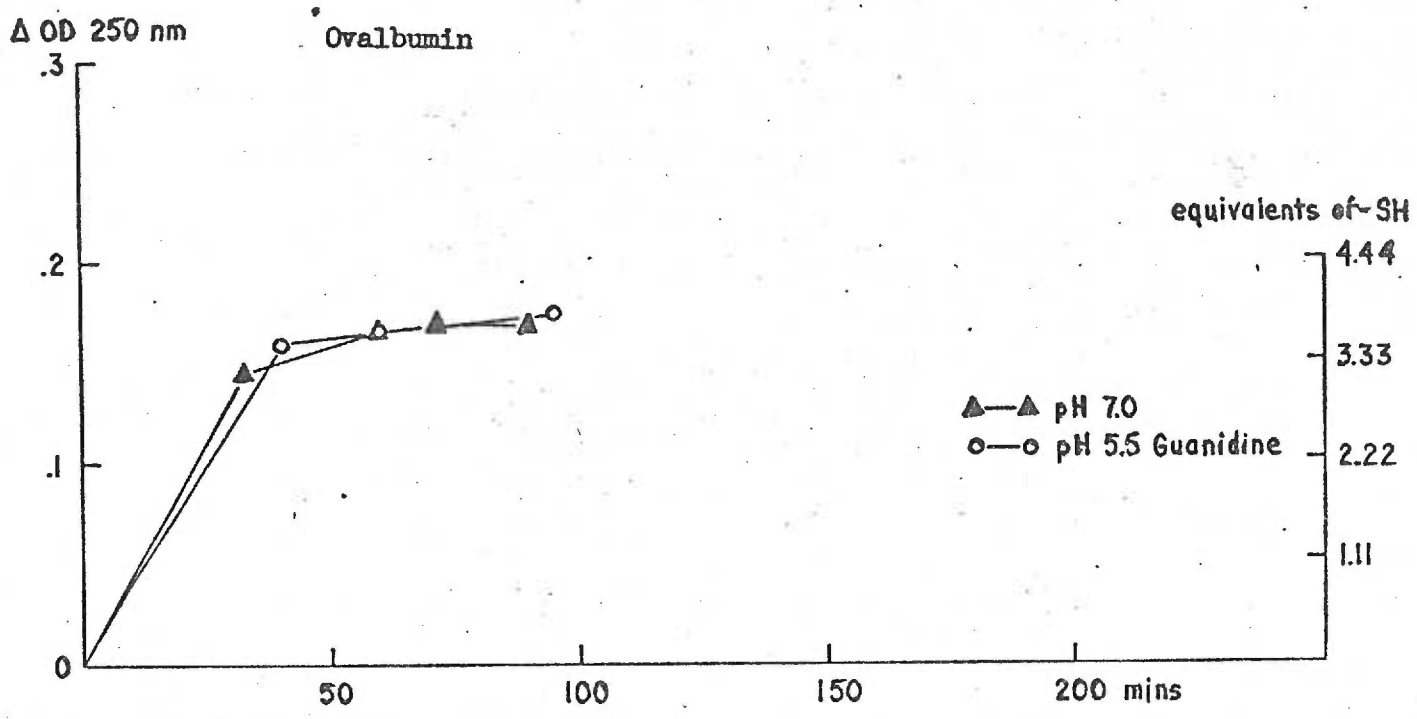


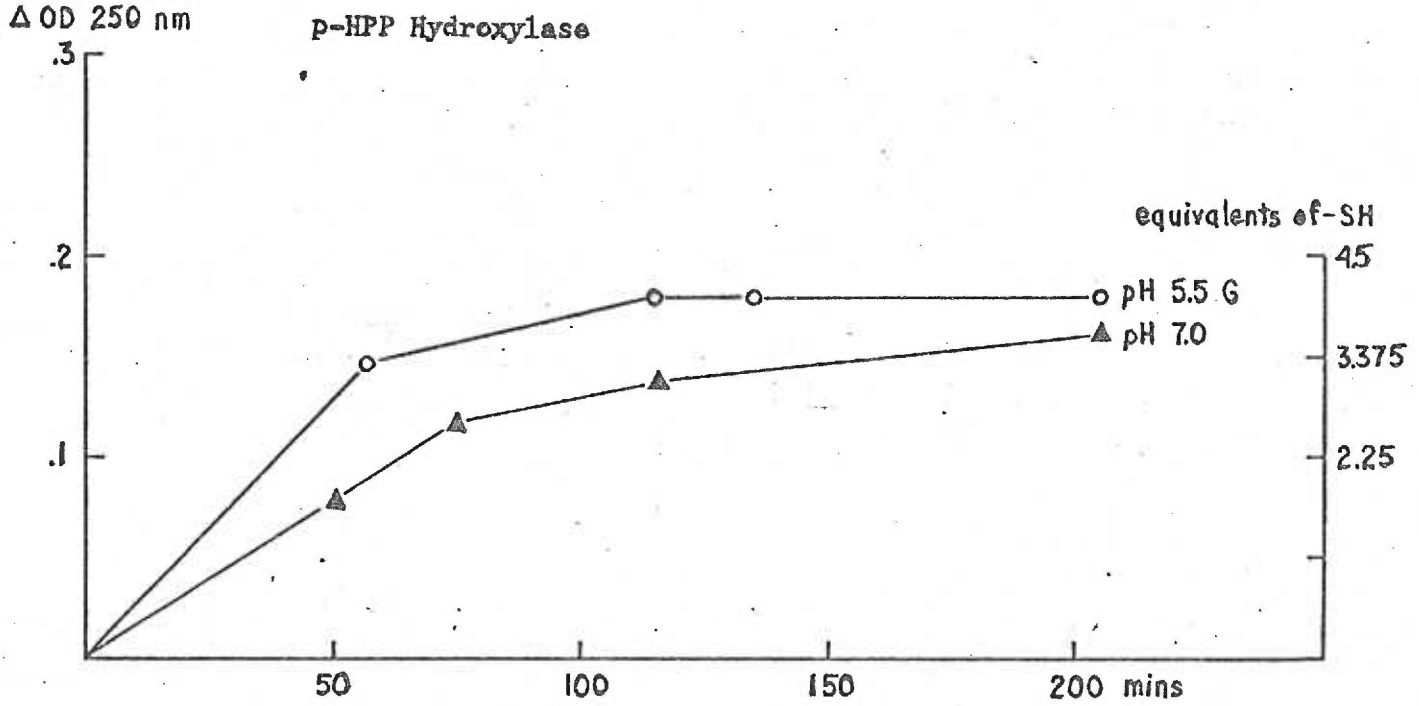
Figure 24. Effect of Thiol Reagents on p-HPP Hydroxylase Activity. The enzyme was incubated at 37° in 50 mM KPO_4 buffer, pH 6.8, 2% glycerol with inclusion of either p-chloromercuribenzoate (p-CMB) 5 mM, N-ethylmalimide (N-EMal) 20 mM, silver nitrate 0.01 mM, mercuric chloride 1 mM, or nothing (control) for times of 0, 10, 20, and 30 min. The p-HPP hydroxylase assay was then initiated by activation with 0.44 mg/ml neutralized ascorbic acid for 5 min, followed by substrate tip-in.

Figure 25. Enhancement of p-CMB Absorption at 250 nm by p-HPP Hydroxylase and Ovalbumin. To p-HPP hydroxylase, 0.31 mg/ml, and Ovalbumin, 0.33 mg/ml, in 50 mM KPO_4, pH 7.0, 0.1 ml of p-CMB, 3.22 mg/ml, was added, and the reaction was allowed to procede at room temperature. O.D. 250 nm was followed over a time course. Controls of buffer plus p-CMB and enzyme plus buffer were also run. Control values were substrated from reaction values, and the representative equivalents of thiol reacting with the p-CMB were calcutated from the O.D. 250 nm using molar extinction coefficient given by Boyer (1954) for the thiol-p-CMB complex $\varepsilon M = 7.6\ (10^3)$.

p-CMB was reacted with ovalbumin and p-HPP hydroxylase in the presence of guanidine-HCl, 1.1 g added to 3 ml of protein plus p-CMB solution. The addition of the guanidine- HCl resulted in a new pH of 5.5 The controls were protein plus guanidine and p-CMB plus guanidine and guanidine, all in buffer.

Enhancement of p-CMB Absorption at 250 nm by -SH in Ovalbumin and p-HPP Hydroxylase





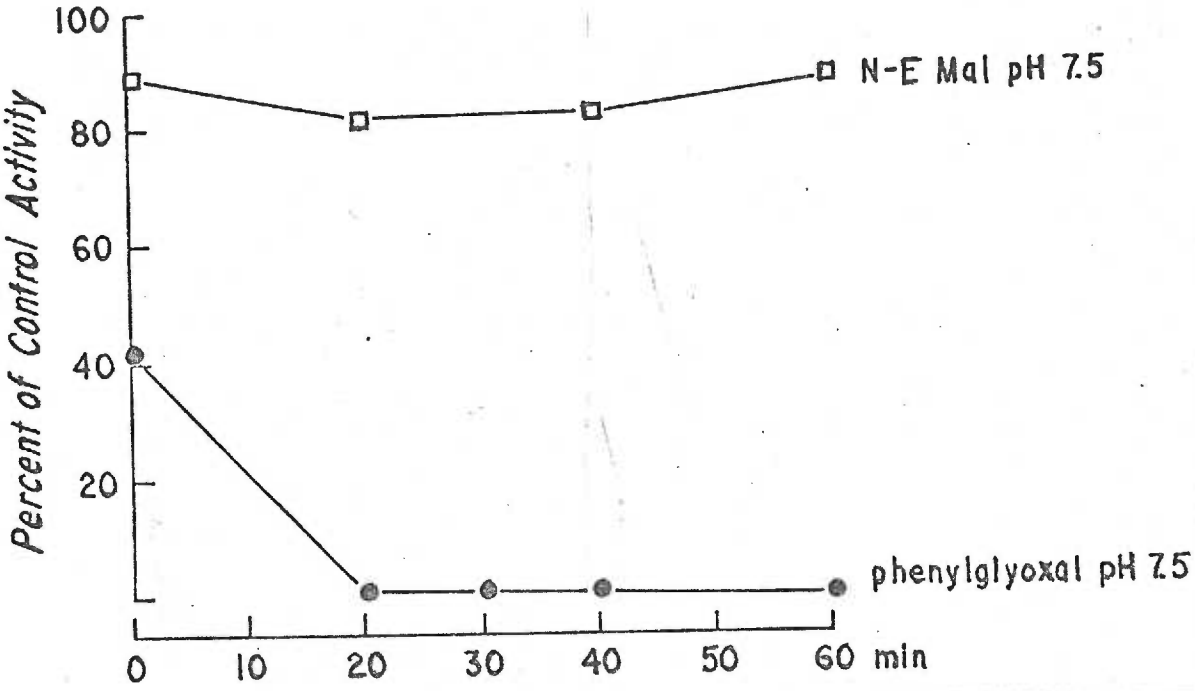


Figure 26. Effect of Amine and Arginine Reagents on p-HPP Hydroxylase Activity. The enzyme was incubated at 37° in 50 mM KPO, pH 7.5, with the inclusion of either N-ethylmalimide (N)EMal) 20 mM, phenylglyoxal 33 mM, or nothing (control) for times of 0, 20, 40, and 60 min. The p-HPP hydroxylase assay was then initiated by activation with 0.44 mg/ml neutralized ascorbic acid for 5 min followed by substrate tip-in.

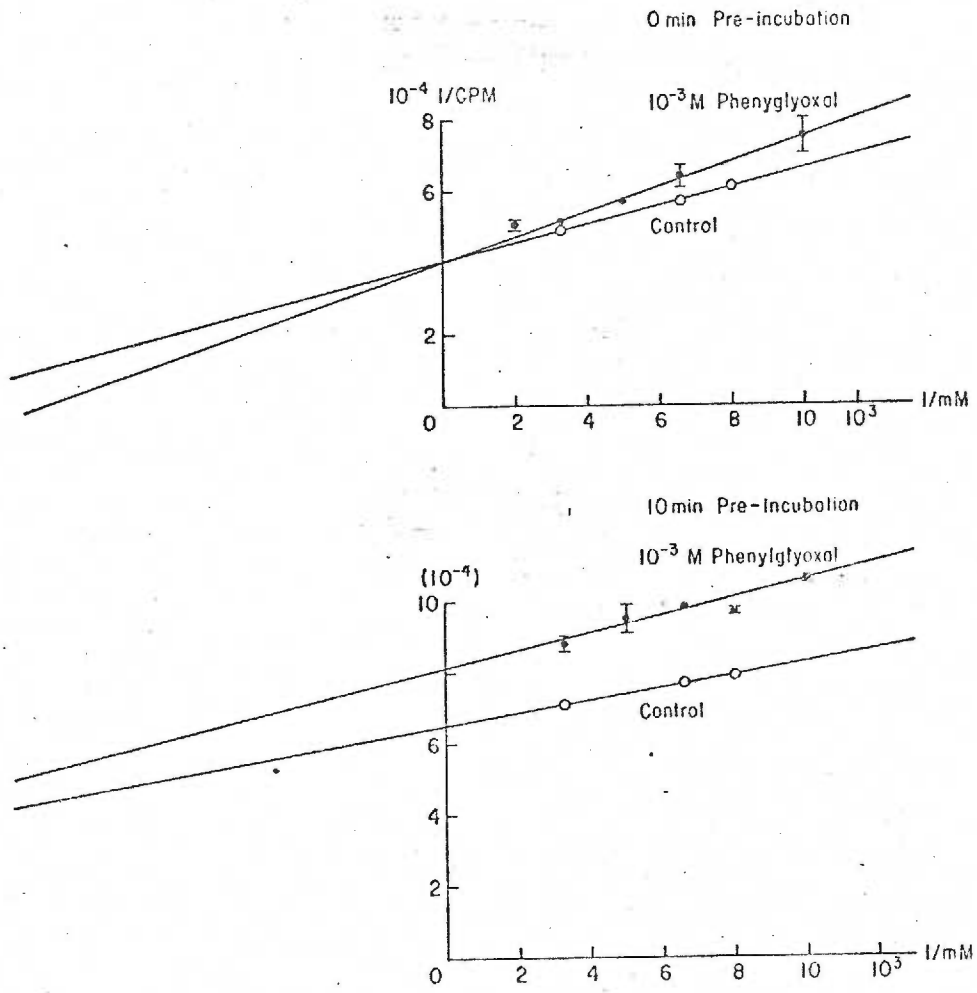


Figure 27. Lineweaver-Burke Plots of Phenylgloxal Inhibition of p-HPP Hydroxylase. The enzyme assay was conducted as in figure 26 with phenylglyoxal added simultaneous with substrate tip-in and preincubated with enzyme for 10 min at 37° before activation by ascorbic acid.

PART 2

THE ROLE OF P-HYDROXYPHENYLPYRUVATE HYDROXYLASE IN THE LIVER METABOLISM OF L-3,4-DIHYDROXYPHENYLALANINE

Introduction:

Interest in the metabolic fate of L-DOPA[1] has expanded since the introduction of this amino acid as a therapeutic agent for the treatment of Parkinson's disease (Cotzias et al., 1969; Yahr et al., Mawdsley, 1970). A review of L-DOPA metabolism reveals 3,4-dihydroxyphenylpyruvic acid as an intermediary metabolite of L-DOPA. The initial contributions to this subject come from Shaw et al. (1957). They administered 0.5 grams of L-DOPA, orally, to human volunteers and observed the major urinary metabolites to be:

1. DOPAmine (3,4-dihydroxyphenylethylamine) from the decarboxylation of L-DOPA by DOPA decarboxylase.

2. DOPA acetic acid (3,4-dihydroxyphenylacetic acid) from the oxidative deamination of dopamine by monamine oxidase and subsequent oxidation to the acetic acid by aldehyde dehydrogenase.

3. Homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) from the O-methylation of the DOPA acetic acid by catechol O-methyl-transferase,

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[1] DOPA is the abbreviation for 3,4-dihydroxyphenylalanine.

which can also O-methylate L-DOPA to form L-3-methoxytyrosine (Axelrod, 1962).

Under the heavier loading of L-DOPA which is orally administered to patients suffering from Parkinson's disease, up to 9 gm/day, Calne et al (1969) were able to identify a minor urinary metabolite, vanillactic acid (3-methoxy-4-hydroxyphenyllactic acid). Their observation indicated that L-DOPA was being transaminated to 3,4-DHPP[2], reduced by a lactic dehydrogenase to DOPA lactic acid, and O-methylated by COMT[3] to the vanillactic acid. Calne's finding was consistent with the findings of Gjessing (1963) who observed vanillactic acid in the urines of a patient suffering from a neuroblastoma of the sympathetic nervous system. These tumors tend to overproduce DOPA and its metabolites, as well as those of norepinephrine, which spill over into the urine. Gjessing and Borud (1965) later reported the appearance of 3,4-DHPP and vanilpyruvic acid in the urines of a patient with a similar affection. These collected findings affirmed the suspicion that L-DOPA is transaminated in vivo.

L-DOPA was demonstrated to be a substrate for tyrosine aminotransferase, a soluble liver enzyme, by Cammarta and Cohen (1950) and Fellman and Roth (1971). They found that

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[2]DHPP is the abbreviation of dihydroxyphenylpyruvate.

[3]COMT is the abbreviation of catechol O-methyltransferase.

L-DOPA was a better substrate than L-phenylalanine and that L-3-methoxytyrosine was also a substrate. The transamination products are 3,4-DHPP and vanilpyruvic acid. The presence of these transamination products in liver presents the possibility that they are further metabolized by the enzyme, p-HPP[4] hydroxylase, which normally handles the product of tyrosine transamination.

Gjessing (1966) predicted that a trihydroxyphenylacetic acid could be formed from 3,4-DHPP through the forementioned system.

p-HPP hydroxylase is an enzyme of moderate substrate specificity which is capable of oxidizing a number of substituted phenylpyruvic acids, besides p-HPP:

> phenylpyruvic acid (Tanaguchi and Armstrong, 1963) to o-hydroxyphenylacetic acid,
>
> p-fluorophenylpyruvic acid and p-methylphenylpyruvic acid to 2-hydroxy-5-fluorophenylacetic acid and 2-hydroxy-5-methylphenylacetic acid (Tanaguchi, Kappe, and Armstrong, 1964)

The pattern of ring substitution for substrates is more flexible than side-chain structure. The pyruvate moiety is an essential component of substrate structure, and any modification of the side-chain seems to destroy the enzyme's ability to oxidize the compound for reasons that become apparent when the reaction mechanism is considered (see Part 1, pg. 4).

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[4] p-HPP is the abbreviation of p-hydroxyphenylpyruvate.

The structural requirements for competitive inhibitors are less restrictive. o- and m-HPP (Hager et al., 1957, Tanaguchi and Armstrong, 1963), 2,4-DHPP and p-hydroxybenzaldehyde (Hager et al, 1957) and salicylaldehyde (Zannoni and LaDu, 1959) are strong competitive inhibitors.

3,4-DHPP was found to be a substrate for p-HPP hydroxylase by Fellman et al (1972). They co-purified the p-HPP and DHPP hydroxylase activities, measuring the DHPP/p-HPP ratio of activities at each step of the purification. A new method of radiochemically assaying the activity was utilized. 1-carbon 14-labelled substrates release $^{14}CO_2$ when incubated with p-HPP hydroxylase. This $^{14}CO_2$ evolution and homogentisic acid formation correlated on a mole to mole basis for the 1-^{14}C-p-HPP substrate, after the appropriate boiled enzyme controls were subtracted. The DHPP/p-HPP ratio of activities remained constant through the purification. Lineweaver-Burke plots indicated that DHPP was a competitive inhibitor of the hydroxylation of p-HPP when added to the reaction mixture simultaneously with the substrate. It was concluded that DHPP and p-HPP were mutually competitive substrates for p-HPP hydroxylase. Vanilpyruvic acid was tested as a substrate by Fellman and Wada (in preparation, 1973) using the chemically synthesized 1-^{14}C-vanilpyruvic acid. This compound was not a substrate, but it was a competitive inhibitor of slightly lower apparent affinity, judging from its Ki.

	substrate activity (relative to p-HPP)		
p-HPP	1.0	5.0 (10^{-5}) M	Km
3,4-DHPP	0.125	2.5 (10^{-5}) M	Ki
vanil-pyruvate	0.0	10.65(10^{-5}) M	Ki

The putative product of the hydroxylation of 3,4-DHPP is 2,4,5-THPAA[5] by analogy with the established formation of homogentisic acid from the enzymic oxidation of p-HPP. However, since the metabolism of 3,4-DHPP by p-HPP hydroxylase was demonstrated only by following the rate of decarboxylation of the substrate, there is no evidence for ring hydroxylation and side-chain migration. The following work was undertaken to provide evidence for the thesis that the THPAA is, indeed, the product of this enzymatic event: (1) the chemical synthesis and properties of 2,4,5-THPAA, (2) evidence for its formation from 3,4-DHPP by the enzyme p-HPP hydroxylase.

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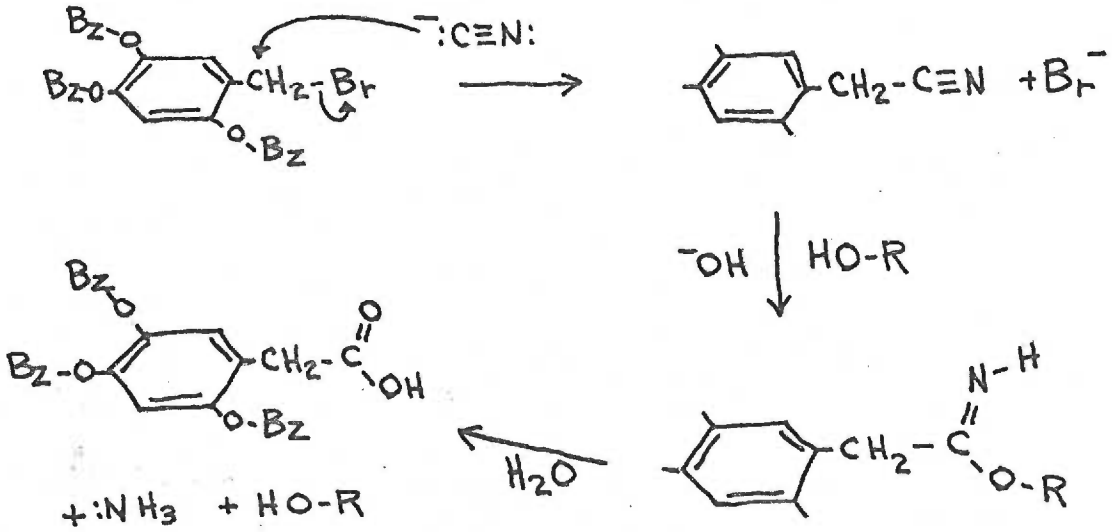
[5]THPAA is the abbreviation for trihydroxyphenylacetic acid.

Materials: see part 1.

Methods: Synthesis of 2,4,5-Trihydroxyphenylacetic Acid. An attempt was made to synthesize the 2,4,5-THPAA through the hydrolysis and reduction of the corresponding benzyloxy-aceto nitrile. The nitrile was prepared by a nucleophilic substitution reaction with 2,4,5-tribenzyloxybenzylbromide as the substrate (prepared according to Ong, Creveling, and Daly, 1969) and NaCN. The procedure followed was adapted from Daly's method for the synthesis of 2,5-dihydroxy-4-methoxyphenylaceto nitrile (Daly, 1965).



2,4,5-tribenzyloxyphenylaceto nitrile. 3 gms of 2,4,5-tribenzyloxybenzylbromide (0.012 mole) is added to 3.25 gms of NaCN (0.12 mole) in 60 mls of dimethylsulfoxide. The mixture is stirred without heating for 2.5 hrs and then poured into 300 mls of water. The reaction vessel is washed

with a small volume of DMSO. The water slurry is refrigerated overnight. The solid is separated by filtration and is air dried. The solid is dissolved in a small volume of benzene and treated with activated charcoal to decolorize. After the charcoal is filtered off onto Whatman No. 2 filter paper, the filtrate is titrated with hexanes. Long tan needles were obtained, with a melting point of 93-95°. The yield was 1.64 gm, 63% of theoretical yield.

2,4,5-tribenzyloxyphenylacetic acid. The method of Buchler and Pearson (1971) is used to saponify the nitrile. 435 mgs of 2,4,5-tribenzyloxyphenylaceto nitrile (10 nmoles), 100 mls of diethylene glycol, and 285 mgs of KOH (5 mmoles) is heated together for 3 hrs under N_2 at 190°. After cooling to room temperature, the emulsion is diluted with water and extracted four times with half volumes of dichloromethane. The aqueous phase is titrated to pH 2 with 6 N HCl and extracted twice with half volumes of dichloromethane. Both fractions are pooled and dried over anhyd. Na_2SO_4, filtered, and evaporated down to an oily residue by rotary evaporation. The residue was redissolved in water and reextracted using three half volumes of ethylacetate. This was done to remove diethylene glycol which partitioned into the organic phase during the first extraction. The organic phase was dried over Na_2SO_4 and treated with activated charcoal to decolorize the dark red extract. The mixture was filtered through Whatman No. 2 and evaporated down to a solid on the rotary

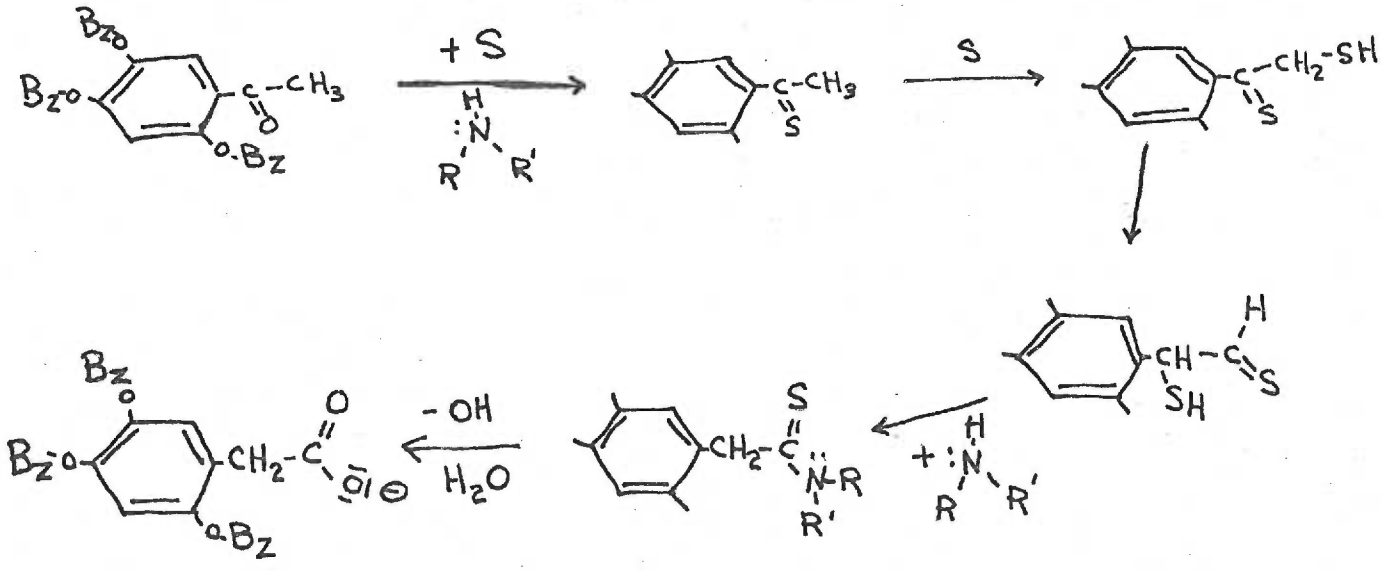
evaporator. This procedure yielded a tan solid containing the 2,4,5-tribenzyloxyphenylacetic acid and the 2,4,5-tribenzyloxybenzaldehyde. The benzaldehyde was separated off using silica gel column chromatography.

A 3 cm column of silica gel was poured into a 4 cm scintered glass funnel. The silica was carried in hexane. The products of saponification were dissolved in a small volume of ethylacetate, and hexane was titrated in until the solution became cloudy. The solution was then applied to the column and eluted with diethyl ether:hexane (70:30). 7 ml fractions were collected, and the phenylacetic acid and benzaldehyde forms of the desired product were monitored using TLC (silic gel support with ether:hexane, 70:30). The fractions containing 2,4,5-tribenzyloxyphenylacetic acid were pooled, and the solvent was evaporated on the rotary evaporator. A white solid was obtained with a melting range of 148-154°. The yield was approximately 30 mg, 7%. The yield was less than satisfactory for a preparative method. Identity of the product was established by mass spectrometry done on the methyl esterified product.

Methyl-(2,4,5-tribenzyloxyphenyl)-acetate. Etherial diazomethane is added in excess to an ice cooled solution of 2,4,5-tribenzyloxyphenylacetic acid, 10 mgs/4 mls of ethyl-acetate. After two minutes, a stream of N_2 is used to blow off the diazomethane while the reaction vessel is warmed in

a water bath. The methyl esterified product had to be separated from impurities due to the low purity of the starting material. Separation was achieved by chromatography on Alumina with ethylacetate as the solvent. Mass spectral analysis of the product gave parent mass ions of 468 and 346, and fragments of 453, 255, 181, and a base of 91. This fragmentation and parent masses are consistent with methyl-(2,4,5-tribenzyloxyphenyl)-acetate and small amounts of the lactone of 2-hydroxy-3,4-dibenzyloxyphenylacetic acid.

A Willgerodt rearrangement of 2,4,5-tribenzyloxyacetophenone by a modified method of Wolkowitz and Dunn (1955) was found to be a shorter and more effective scheme for the synthesis of 2,4,5-tribenzyloxyphenylacetic acid. The latter was reductively debenzylated with H_2/Pd on carbon to the desired 2,4,5-trihydroxyphenylacetic acid.



2,4,5-Tribenzyloxyphenylacetic acid. To two gms (2.28 mmole) of 2,4,5-tribenzyloxyacetophenone, synthesized by the method of Daly et al (1965), was added 0.22 gm of sulfur and 1.2 gm of redistilled morpholine. The reaction mixture was refluxed gently for 7 hours and cooled to room temperature. At this point 3.8 ml ethanol, followed by 1.6 ml of 50% NaOH was added. The mixture was shaken vigorously and then refluxed for 42 hours. The ethanol (3.5 ml) was distilled off and 10 ml of water was added to the residue. The cooled residue was extracted with three volumes of ethyl acetate. The extract was dried over anhydrous Na_2SO_4, and the solvent removed. The brownish residue was recrystallized from ethylacetatehexane and yielded 1 gm of product (48% yield): Mp=152-154°.

The product, when examined with TLC on Silica gel (Kodak 6060) in anhydrous ethylether-hexane (70:30), exhibited a major component Rf=0.15 which gave a methyl ester with etherealdiazomethane Rf=0.72. 2,4,5-Tribenzyloxyphenylacetophenone under the same conditions had an Rf=0.61. The esterified product was examined with TLC on Silica gel with a benzene, methanol, acetic acid (45:8:4) solvent system. Two components were resolved. The major component Rf=.93 was scraped from the plate and a mass spectrum was obtained on Dupont 21-110B high resolution mass spectrometer. Its spectrum was consistent with the expected 2,4,5-tribenzyloxyphenylacetate methyl ester. A minor component of Rf=0.78

exhibited a mass spectrum consistent with the lactone of 2-hydroxy-4, 5-dibenzyloxyphenylacetic acid.

2,4,5-Trihydroxyphenylacetic Acid. 10 mg of 2,4,5-tribenzyloxyphenylacetic acid was dissolved in 8 ml of anhydrous ethanol in a 10 ml serum bottle. 15 mg of 5% palladium on carbon and 0.025 ml of 2 N HCl was added and the suspension stirred with a small magnetic stirring bar. The vial was stoppered with a rubber serum bottle cap and purified hydrogen was bubbled through the mixture. A second needle, just penetrating the rubber cap, provided a vent for the slow stream of hydrogen. The reduction was allowed to proceed for 3.5 hours. The mixture was filtered and the solvent evaporated under a stream of nitrogen. The residue gave a single TMS[6] derivative when treated with BSTFA[7] for 30 min at 60° and analyzed with an LKB 9000 GC/MS[8] (see results for details).

Avian p-hydroxyphenylpyruvate hydroxylase was prepared from chicken liver acetone powder by the method of Lindblad et al (1971).

The enzyme activity assay was carried out using condi-

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[6]TMS is the abbreviation for trimethylsilyl.

[7]BSTFA is the abbreviation for N,O-bis-(trimethylsilyl) trifluoroacetamide.

[8]GC/MS is the abbreviation for gas chromatographic/mass spectrometry.

tions employed in radiochemical procedures of Fellman et al (1971).

Results.

Enzymatic Formation of 2,4,5-Trihydroxyphenylacetic Acid.

Hydroxyquinone product. From our observations of the behavior of 2,4,5-THPAA in buffer solutions at pH 7.3 (the pH at which the enzymatic assays are conducted), we concluded the the THPAA is unstable and oxidizes rapidly to the hydroxyquinone. This property has been described for the trihydroxyphenyl compounds, 2,4,5-trihydroxyphenylethylamine (Senoh and Witkop, 1959, Adams et al, 1972), and 2,4,5-trihydroxyphenylacetic acid as an intermediary metabolite of p-hydroxyphenylacetic acid in Penicillium chrysogenum (Isono, 1958). Figure 1 illustrates these properties. At pH 4.5 the 2,4,5-THPAA has an absorption maximum of 290 nm. When the pH of the solution was adjusted to 7.3, the colorless solution rapidly turned pink and the spectrum exhibited a maximum at 278 nm and the characteristic hydroxyquinone absorption peak at 480 nm. The addition of acid to this solution suppressed the dissociation of the hydroxyl group and the absorption spectrum shifted to peaks at 262 nm and 375 nm.

These absorption spectra were used to identify the enzymatic formation of trihydroxyphenylacetic acid from 3,4-DHPP. The enzymatic reaction mixture contained 0.05 mg

of 3,4-DHPP, 0.88 mg of freshly neutralized ascorbic acid, 5000 units of catalase, 3 units[9] of purified avian p-HPP hydroxylase in a final volume of 2.0 ml (0.1 M phosphate buffer, pH 7.3 was routinely employed). A boiled enzyme control was run with all experiments. The substrate was tipped in at zero time and the reaction mixture incubated in air at 37° for 30 min. The visible absorption spectra of these reaction mixtures were determined with a Cary 15 spectrophotometer. 0.3 ml of 2 N H_2SO_4 was added to bring to pH down to 2.0 and the precipitated protein separated by centrifugation. The visible absorption spectra were identical to those obtained with synthetic THPAA. The broad absorption maximum at 480 nm appeared only in the reaction vessels containing active enzyme. No change in optical density in the visible region was observed in the reaction vessels containing boiled enzyme. Again on adding acid, the 480 nm peak shifted to 375 nm, as did the synthetic hydroxyquinone of THPAA.

In another series of experiments, we varied the amount of enzyme which we added to the reaction mixture and demonstrated that the appearance of the peak at 480 nm was linearly related at the amount of enzyme added. Figure 2.

Finally, we studied the effect of a known inhibitor of p-HPP hydroxylase, o-hydroxyphenylpyruvate (Tanaguchi and

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[9]Unit of enzyme activity is defined as 1 μmole of p-HPP oxidized/hr at 37°.

Armstrong, 1963, and Fellman et al, 1971), on the rate of formation of the product which exhibited the 480 nm absorption peak and $^{14}CO_2$ evolution. The results of these experiments shown in Table 1 clearly demonstrate inhibition of product formation by this inhibitor.

Isolation of the product and identification by GC/MS. An attempt was made to unequivocally identify the formation of THPAA by GC/MS. The reaction mixture contained 8 units of purified p-HPP hydroxylase, 0.15 mg of 3,4-DHPP, 5000 units of catalase, 1.8 mg neutralized ascorbic acid in a final volume of 4.0 ml containing 0.1 M phosphate buffer pH 7.3. A boiled enzyme control was run identically as above, save the addition of a heat inactivated enzyme. The incubation at 37° in air was allowed to proceed for 30 min; 0.6 of 2 N H_2SO_4 was added, followed by a small amount of zinc dust. The pink solution turned yellow with the addition of acid and colorless after shaking with the zinc dust, indicating reduction of the quinone.

The solution was extracted 3 times with an equal volume of ethyl acetate, after saturation with NaCl. The combined ethyl acetate extracts were dried briefly over anhydrous Na_2SO_4, and the solvent evaporated under a stream of nitrogen. The dried residue was treated with 200 µl BSTFA silylating agent at 60° for 30 min. The material was injected into the inlet port of a LKB 9000 GC/MS instrument.

A 1% OV-1, 12 foot column with helium flow 38 ml/min, programmed at a 3°/min temperature rise was employed. A single product was observed to emerge at 195° whose column retention and mass spectrum was identical to that exhibited by the synthetic 2,4,5-THPAA silylated under identical conditions. These results are diagrammed in Figure 3. The boiled enzyme did not contain any product. Similar results were obtained with a 1% OV-25, 12 foot column. The product from the enzyme reaction emerged from the column at approximately 200° and precisely 20.04 methylene units, as did the synthetic standard. Their mass spectra were identical.

Discussion:

Earlier studies of 3,4-DHPP as a substrate for liver p-HPP hydroxylase employed an assay procedure that left doubt as to the nature of the product. This doubt has been eliminated by the results of the present studies. I have clearly established by two means, the identity of 2,4,5-THPAA as the enzymic product of the action of p-HPP hydroxylase on the substrate 3,4-DHPP, *in vitro*.

The possibility that the rearrangement proceeded to the alternative product, 2,3,6-THPAA was set aside when the mass spectrum and gas chromatographic methylene units of this isomer (prepared by alkaline oxidation of homogentisic acid in a manner similar to that described for the alkaline oxidation of 3,4-DHPP by Wada and Fellman, 1973, and illustrated

in figure 6) was compared to those of the enzymic product. Comparison of their respective behavior with GC/MS revealed many similarities, but some distinguishing fragment ions, diagrammed in figure 4, and the different gas chromatographic properties (2,4,5-isomer 20.04 methylene units, 2,3,6-isomer 20.27 methylene units) led to the conclusion that the enzymic product was the 2,4,5-THPAA isomer.

Urine Studies. The urines of three patients with Parkinson's disease who were being treated with large amounts of L-DOPA were examined by Fellman for the presence of 2,4,5-THPAA (Wada and Fellman, 1973). At the time of the urine collections, these patients were ingesting a total of 5-8 gms of L-DOPA per day. The urines were collected in acid, and a sample was treated with zinc dust and extracted with ethylacetate as described for the in vitro studies. The silylated residue was injected into an LKB 9000 GC/MS, using an OV-25, 12 foot column with a He flow of 18 ml/min and a 4 deg/min temperature rise. A number of DOPA-derived products were observed including, prominently, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylpyruvate, 3-methoxy-4-hydroxyphenyllactic acid, and the 2,4,5-THPAA. Figure 5. The latter product emerged at approximately 200 deg, as did the synthesized standard. It too was identified by its mass spectrum.

A number of attempts were made to demonstrate the

formation of 2,4,5-THPAA from 2,4,5-trihydroxyphenylethylamine, using mitochondrial monaminoxidase preparations. In no instance was any THPAA observed to be formed in this system (unpublished communication from Fellman and Roth, 1973). The observation was made that 3,4-dihydroxyphenylacetic acid, a prominent DOPA metabolite, could spontaneously oxidize and rearrange to 2,4,5-THPAA at pH 12. Figure 6. However, it appears that this process occurs to an insignificant extent at physiological pH or in acidified urine samples (Wada and Fellman, 1973). Thus, the principle source of the 2,4,5-THPAA found in the urine of patients undergoing L-DOPA therapy is most likely to be that formed by metabolism of L-DOPA through the liver and kidney tyrosine aminotransferase and p-HPP hydroxylase enzyme systems.

In support of this conclusion, it is noted that urinary levels of p-hydroxyphenyllactic acid (Calne et al, 1969) and p-HPP (Honos et al, 1970) were elevated by L-DOPA therapy, suggesting *in vivo* inhibition of p-HPP hydroxylase by a DOPA metabolite. Since past (Fellman et al, 1971) and present findings show that 3,4-DHPP is a co-substrate with p-HPP for the enzyme, the inhibitory metabolite could be the DHPP.

Relative to other L-DOPA metabolites, there were small amounts of 2,4,5-THPAA detected in urine samples from patients undergoing L-DOPA therapy. The quantitation of these amounts

of THPAA and its corresponding hydroxyquinone from urine samples would be of questionable significance, because of the instability of these products. Even in frozen samples, it was noted that storage resulted in loss of the THPAA. Using rat liver tissues in an _in vitro_ system, Fellman and Roth (in preparation) have estimated the relative amount of 1-^{14}C-L-DOPA metabolized through the tyrosine aminotransferase and p-HPP hydroxylase pathway versus the total L-DOPA decarboxylated as 14% of the total. This work was based on the relative amounts of decarboxylation taking place in the presence and absence of α-ketoglutarate, a cosubstrate for the transamination of L-DOPA, in liver homogenates.

The establishment of 2,4,5-THPAA as a metabolic product of DOPA metabolism in man is a matter of some consequence, since the trihydroxy moiety is known to be quite reactive. 2,4,5-Trihydroxyphenylethylamine (6-hydroxydopamine) has been shown to be toxic either as a result of its capacity to undergo oxidation to a reactive p-quinone (Saner and Thoenen, 1970) or by _in situ_ oxidative generation of semiquinone and superoxide radicals (Adams et al, 1972, and Heikkila and Cohen, 1973) and hydrogen peroxide (Heikkila and Cohen, 1971). The identical capacity exists for the THPAA. Elevation of blood urea nitrogen and serum transaminases have been reported in patients treated with L-DOPA (M^{c}Dowell et al, 1970). Furthermore, there have been reports of decreases in aromatic L-amino acid decarboxylase activity

of rat liver (Dairman et al, 1971) and catechol-o-methyl-transferase activity of rat liver and kidney (Sharpless et al, 1973) during chronic adminstration of L-DOPA. These effects are attributed to lower enzyme levels rather than the presence of inhibitors or lack of adequate levels of co-factors. The same enzymes in other tissues were unaffected by the same treatment. These phenomena could be explained by an increase in enzyme degradation, caused by a toxic metabolite, such as THPAA. The 2,4,5-THPAA, generated by liver and kidney p-HPP hydroxylase during L-DOPA metabolism, could explain the selective inactivation of the enzymes in these two tissues.

Detoxification of the THPAA might occur through o-methylation by COMT in the same way that 2,4,5-trihydroxy-phenylethylamine is converted to 2,4-dihydroxy-5-methoxy-phenylethylamine (Daly et al, 1961). However, the failure of O'Gorman's effort at finding the 2,4-dihydroxy-5-methoxyphenylacetic acid in urines of patients receiving L-DOPA therapy (O'Gorman et al, 1970) may indicate the inability of COMT to o-methylate this substrate before it oxidizes to the hydroxyquinone. Rapid autooxidation to the hydroxyquinone may also explain the *in vitro* evidence that homogentisate oxidase cannot further metabolize 2,4,5- THPAA (Wada and Fellman, 1973).

Summary and Conclusions:

The product of p-HPP hydroxylase oxidation of 3,4-DHPP exhibited a UV spectra identical to the chemically synthesized 2,4,5-THPAA. The product underwent rapid autooxidation at neutral pH to a secondary product which exhibited the UV spectral properties of the hydroxyquinone of 2,4,5-THPAA. The possibility of 2,3,6-THPAA being the enzymic product was ruled out. Mass spectral and gas chromatographic analysis positively identified the product of the hydroxylation of 3,4-DHPP as 2,4,5-THPAA.

It was concluded that the hydroxylation of 3,4-DHPP by p-HPP hydroxylase results in oxidative decarboxylation and alkyl migration, yielding 2,4,5-THPAA. Since 3,4-DHPP is an intermediary metabolite of L-DOPA, it was concluded that p-HPP hydroxylase participates in the metabolism of L-DOPA, and 2,4,5-THPAA is a L-DOPA metabolite. The finding that 2,4,5-THPAA was present in the urines of human patients ingesting large amounts of L-DOPA supported this conclusion.

Bibliography:

Adams, R.N., Murrill, E., McCreery, R., and Blank, L.
6-Hydroxydopamine, a New Oxidation Mechanism.
Europ. J. Pharmacol., 1972. 17, 287-292.

Axelrod, J.
Catechol-O-methyl Transferase from Rat Liver.
Methods in Enzymology, 1962. V, 748-751.

Calne, D.B., Karoum, F., Ruthven, C.R.J., and Sandler, M.
The Metabolism of Orally Administered L-DOPA in Parkinsonism.
Brit. J. Pharmacol., 1969. 37, 57-68.

Cammarata, P.S., Cohen, P.P.
The Scope of the Transamination Reaction in Animal Tissues.
J. Biol. Chem., 1950. 187, 439-452.

Cotzias, G.C., Papavasiliou, P.S., and Gellene, R.
Modification of Parkinsonism -- chronic treatment with L-DOPA.
New Eng. J. Med., 1969. 280, 337-345.

Dairman, W., Christenson, J.G., and Udenfriend, S.
Decrease in Liver Aromatic L-amino-acid Decarboxylase Produced by Chronic Administration of L-DOPA.
Proc. Natl. Acad. Sci. USA, 1971. 68, 2117-2120.

Daly, J., Horner, L., and Witkop, B.
Chemical and Enzymatic Routes to Methoxy Dopamines.
J. Amer. Chem. Soc., 1961. 83, 4787-4792.

Daly, J., Benigui, J., Minnis, R., Kanaoku, Y., and Witkop, B.
Synthesis and Metabolism of 6-Hydroxycatecholamines.
Biochem., 1965. 4, 2513-2525.

Fellman, J.H., Fujita, T.S., and Roth, E.S.
Substrate Specificity of p-Hydroxyphenylpyruvate Hydroxylase.
Biochim. Biophys. Acta, 1971. 268, 601-604.

Fellman, J.H., Fujita, T.S., and Roth, E.S.
Assay, Properties, and Tissue Distribution of p-Hydroxyphenylpyruvate Hydroxylase.
Biochim. Biophys. Acta, 1972. 284, 90-100.

Fellman, J.H. and Roth, E.S.
Inhibition of Tyrosine Aminotranferase Activity by L-3,4-dihydroxyphenylalnine.
Biochem., 1971. 10, 408-414.

Gjessing, L.R.
Studies of Functional Neural Tumors. V. Urinary Excretion of 3-Methoxy-4-hydroxyphenyl-lactic Acid.
Scand. J. Clin. Lab. Invest., 1963. 15, 649-653.

Gjessing, L.R.
In L.R. Gjessing (Ed.), Symposium on Tyrosinesis, Norwegian Monographs on Medical Science. Oslo, Norway, Universitatsforlagets, 1966. (pp. 62)

Gjessing, L.R. and Borud, O.
Studies of Functional Neural Tumors. VII. Urinary Excretion of Phenolic Pyruvic Acids.
Scand. J. Clin. Lab. Invest., 1965. 17, 80-84.

Heikkila, R. and Cohen, G.
Inhibition of Biogenic Amine Uptake by Hydrogen Peroxide: a mechanism for toxic effects of 6-hydroxydopamine.
Science, 1971. 172, 1257-1258.

Heikkila, R. and Cohen, G.
6-Hydroxydopamine: Evidence for Superoxide Radical as an Oxidative Intermediate.
Science, 1973. 181, 456-457.

Isono, M.
Oxidative Metabolism of Phenylacetic Acid by Penicillium chrysogenum Q 176. XIII. Isolation and Identification of an Intermediate of the Metabolism of p-Hydroxyphenylacetic Acid.
Nippon Nogei-kagaku Kaishi, 1958. 32, 256-259.

Lindblad, B.
Radiochemical Assay for p-Hydroxyphenylpyruvate Hydroxylase Activity in Human Liver.
Clin. Chem. Acta, 1971. 34, 113-121.

Lindblad, B., Lindstedt, S., Orlanger, B., and Omfeldt, M.
Purification of p-Hydroxyphenylpyruvate Hydroxylase from Human Liver.
Acta Chimica Scand., 1971. 25, 329-330.

Mawdsley, C.
Treatment of Parkinsonism with Levo-DOPA.
Brit. Med. J., 1970. 1, 331-337.

McDowell, F., Lee, J.E., Swift, T., Sweet, R.D., Ogsbury, J.S., and Kessler, J.T.
Treatment of Parkinson's Syndrome with L-dihydroxyphenylalanine (Levodopa).
Ann. Intern. Med., 1970. 72, 29-35.

O'Gorman, L.P., Borud, O., Khan, I.A., and Gjessing, L.R.
The Metabolism of L-3,4-dihydroxyphenylalanine in Man.
Clin. Chem. Acta, 1970. 29, 11-119

Raheja, M.C., Dylewski, I., and Crawhall, J.C.
Estimation of p-Hydroxyphenylpyruvic Acid Hydroxylase Activity based on $^{14}CO_2$ Evolution from the 1-^{14}C-labeled Substrate.
Canad. J. Biochem., 1973. 51, 172-177.

Saner, A. and Thoenen, H.
Model Experiments on the Molecular Mechanism of Action of 6-Hydroxydopamine.
Mol. Pharmacol., 1971. 7, 147-154.

Senoh, S. and Witkop, B.
Formation and Rearrangements of Aminochromes from a New Metabolite of Dopamine and Some of its Derivatives.
J. Amer. Chem. Soc., 1959. 81, 6231-6235.

Sharpless, N.S., Tyce, G.M., and Owen, C.A., Jr.
Effect of Chronic Administration of L-DOPA on Catechol-O-methyltransferase in Rat Tissues.
Life Sci. (I), 1973. 12, 97-106.

Shaw, K.N.F., McMillan, A., and Armstrong, M.D.
The Metabolism of 3,4-Dihydroxyphenylalanine.
J. Biol. Chem., 1957. 226, 255-266.

Wada, G.M. and Fellman, J.H.
2,4,5-Trihydroxyphenylacetic Acid. A Metabolite of L-3,4-Dihydroxyphenylalanine.
Biochem., 1973. 12, 5212-5217.

Wolkowitz, H. and Dunn, M.S.
Homogentisic Acid. B. 2,5-Dimethoxyphenylacetic Acid (III).
Biochem. Prepn., 1955. 4, 8.

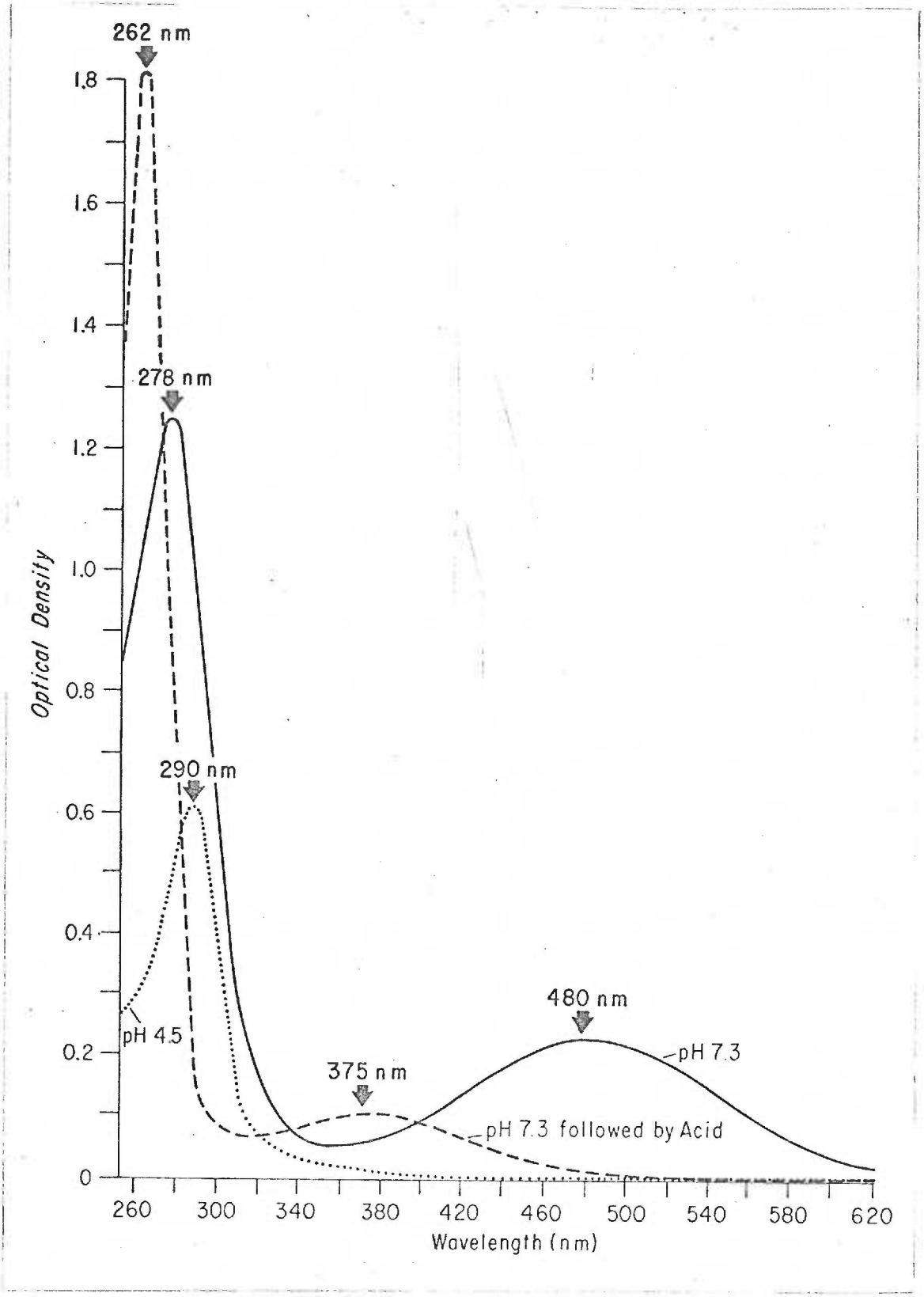
Yahr, M.D., Duvoisin, R.C., Schear, M.N., Barnett, R.E., and Hoehn, M.M.
Treatment of Parkinsonism with Levodopa.
Arch. Neurol., 1969. 21, 343-354.

TABLE I: Inhibition of 3,4-dihydroxyphenylpyruvate hydroxylase activity by o-hydroxyphenylpyruvate. Enzyme activity assayed by $^{14}CO_2$ evolution and O.D. at 480 nm.[a]

	o-hydroxyphenylpyruvate		
assay system	0	10^{-6} M	10^{-5} M
$^{14}CO_2$/5 mins evolution	413 cpm	276 cpm	92 cpm
% inhibition	0	33%	78%
ΔOD 480/5 mins	0.055 ΔAbs.	0.034 ΔAbs.	0.013 ΔAbs.
% inhibition	0	38%	76%

[a]The hydroxylation of 3,4-dihydroxyphenylpyruvic acid was followed by two assay systems: a) $^{14}CO_2$ evolution-concentration of assay components and assay conditions were the same as in Figure 2 with substitution of 1-^{14}C-labelled substrate and twice the total volume. $^{14}CO_2$ was captured on a hyamine wick as previously described (Feliman et al., 1971) b) O.D. 480 nm - same conditions as in Figure 2. Additions of inhibitor were made simultaneously with substrate in both systems.

Figure 1 The absorbance spectra of 2,4,5-trihydroxyphenylacetic acid and the hydroxyquinone of 2,4,5-trihydroxyphenylacetic acid. 2,4,5-Trihydroxyphenylacetic acid (2.94 μ mole) was dissolved in 20 ml of 0.1 M KPO_4 buffer at pH 4.5 and at pH 7.3. Then visible and UV absorbance spectra were scanned in a Cary 15 spectrophotometer. The pH 7.3 solution was acidified by 0.06 ml 6 N HCl/ml to give a final solution of pH 2.0. Under these conditions, molar extinction coefficients found were: 2,4,5-trihydroxyphenylacetic acid pH 4.5 290 = 4,014; hydroxyquinone pH 7.3 278 = 9,435; 480 = 1,497; pH 2.0 262 = 12,245; 375 = 680


262 nm
278 nm
290 nm
480 nm
375 nm
pH 4.5
pH 7.3
pH 7.3 followed by Acid
Optical Density
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
260
300
340
380
420
460
500
540
580
620
Wavelength (nm)

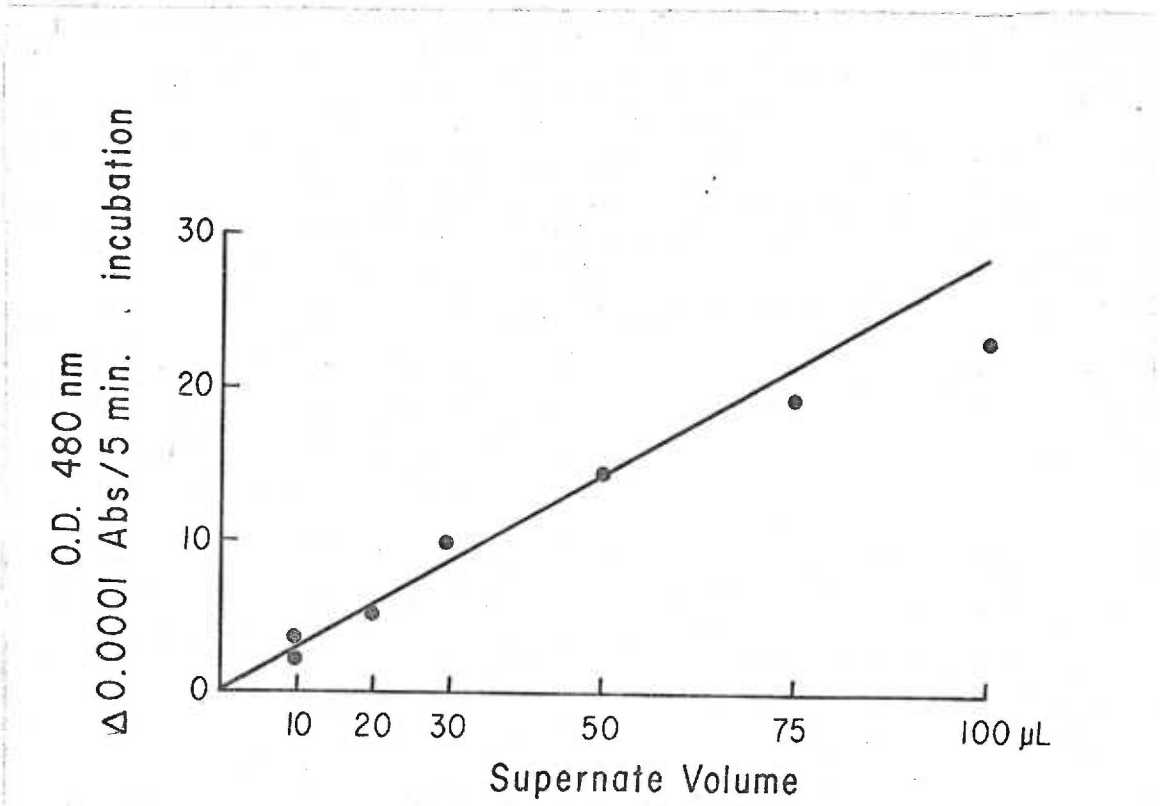


Figure 2 The enzyme catalyzed formation of 480 nm absorbing product. Assay system (1.0 ml): 44 g/ml neutralized ascorbic acid, 35 g/ml dihydroxyphenylpyruvate, and 10 - 100 L chicken liver supernatant, dialyzed overnight against 0.1 M KPO_4, pH 7.3. The reaction was started by addition of substrate, and a Gilford Recording spectrophotometer at 37° was used to follow optical density at 480 nm. A supernate protein control in addition to a control containing all components of the assay system except the supernate protein was run to compensate for increased turbidity during incubation. O.D. 480 = Δ Abs/5 min (reaction) - {Δ Abs/5 min (control) + Δ Abs/5 min (protein)}.

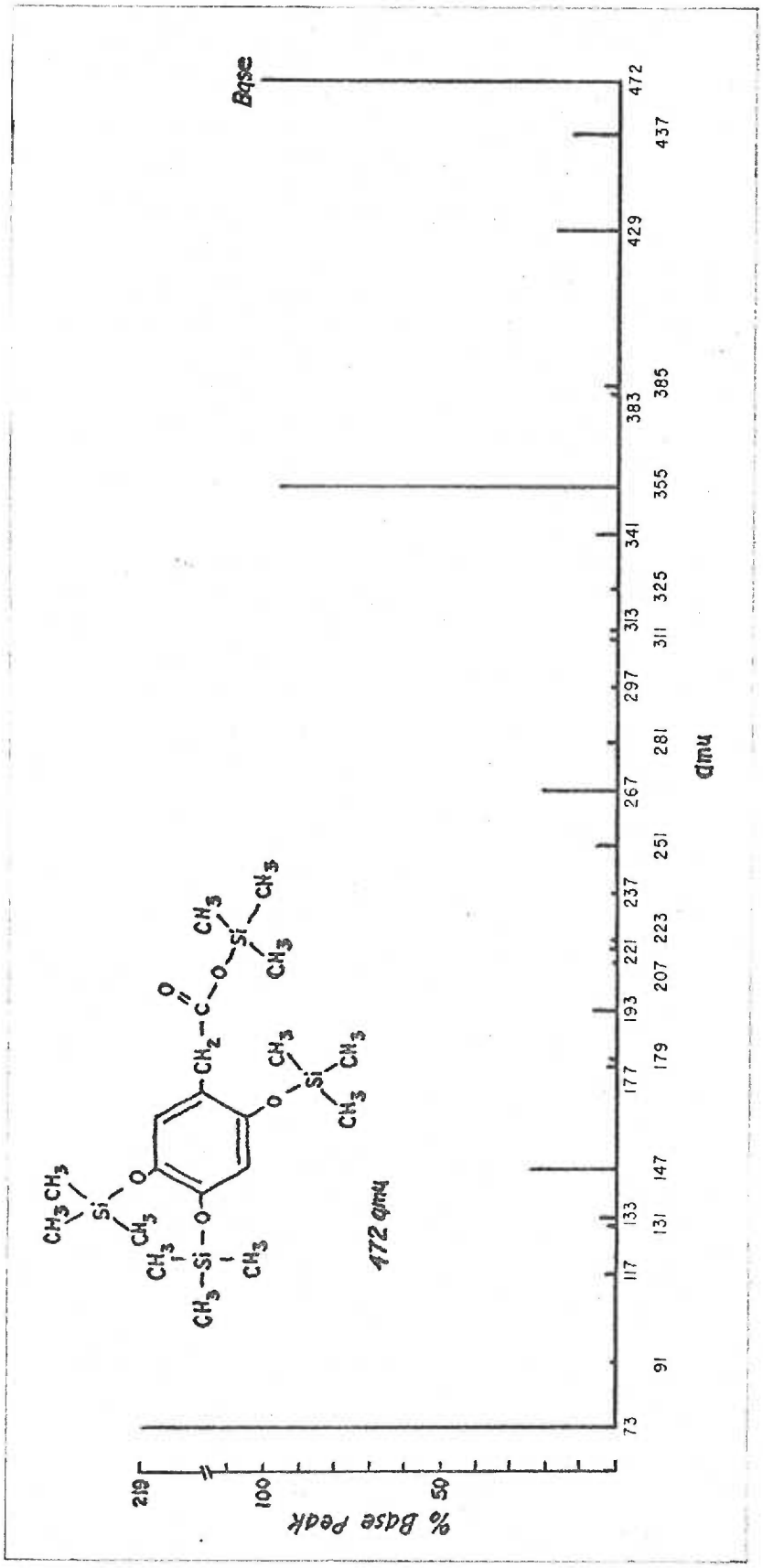


Figure 3 Mass spectrum of 2,4,5-trihydroxyphenylacetic acid - TMS derivative.

Figure 4 Mass spectral ion fragmentation patterns distinguishing 2,4,5- and 2,3,6-trihydroxyphenylacetic acid. A. 2,3,6-isomer (from homogentisate oxidation and rearrangement). B. Product from hydroxylation of 3,4-dihydroxyphenylpyruvate. C. 2,4,5-isomer (synthesized standard).

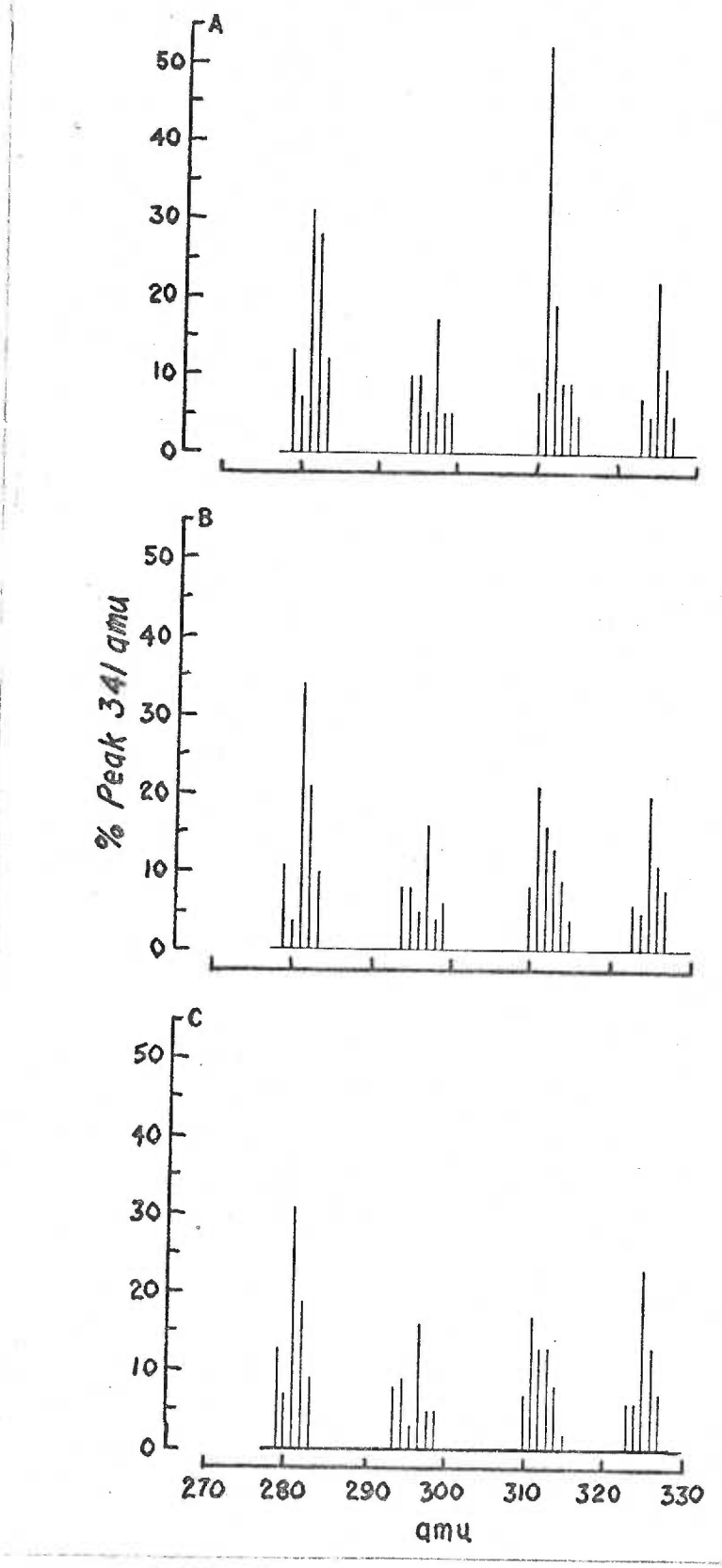
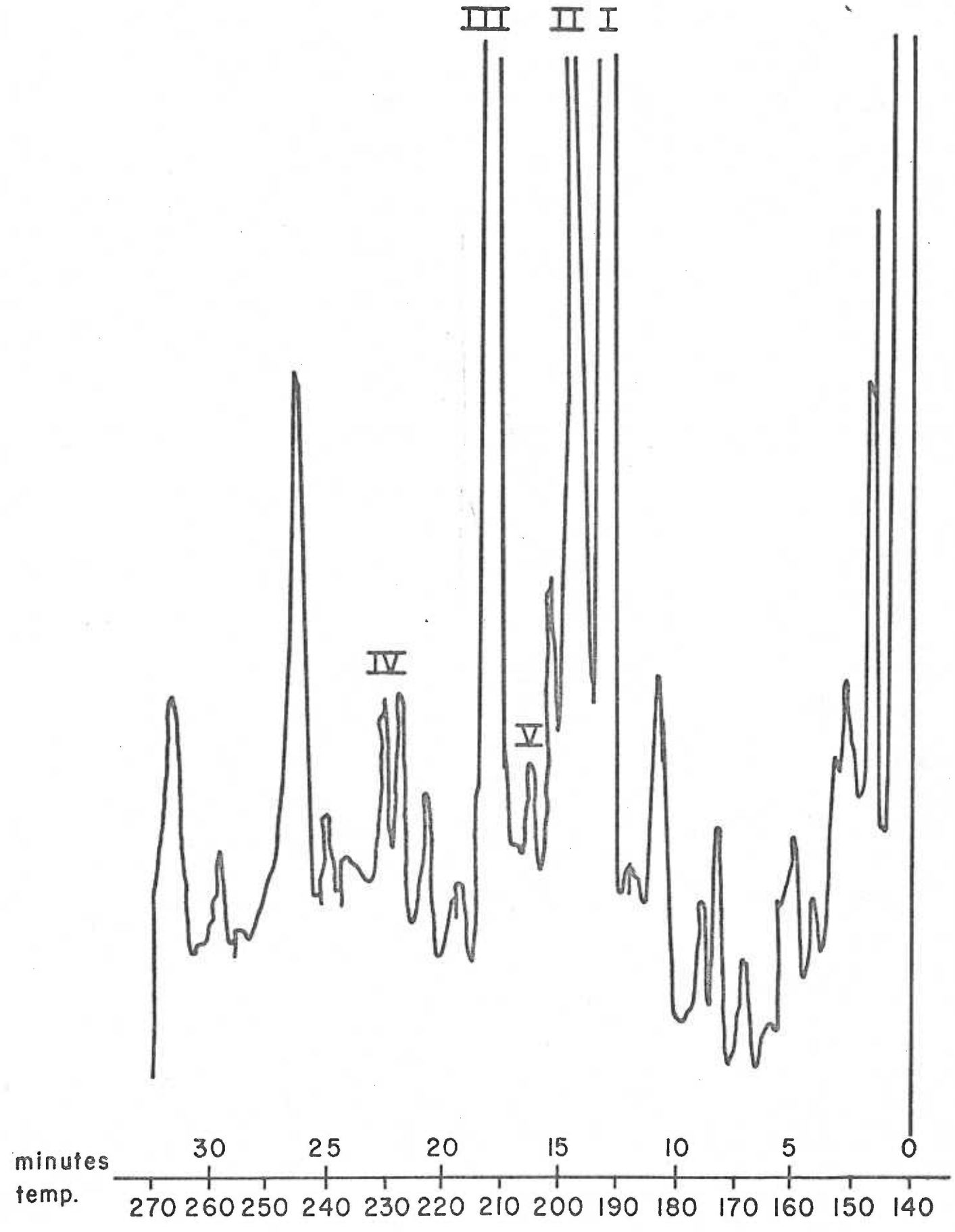

A
B
C
50
40
30
20
10
0
% Peak 341 amu
270
280
290
300
310
320
330
amu

Figure 5 Gas chromatographic elution profile from a patient undergoing L-DOPA therapy. TMS-derivatives of:
I. 3-methoxy-4-hydroxyphenylacetic acid, P=326;
II. 3,4-dihydroxyphenylacetic acid, P=384;
III. 3-methoxy-4-hydroxyphenyllactic acid, P=428;
IV. 3-methoxy-4-hydroxyphenylpyruvic acid, P=428; metastable 396-400; V. 2,4,5-trihydroxyphenylacetic acid, P=472.


III
II
I
IV
V
minutes
30
25
20
15
10
5
0
temp.
270 260 250 240 230 220 210 200 190 180 170 160 150 140

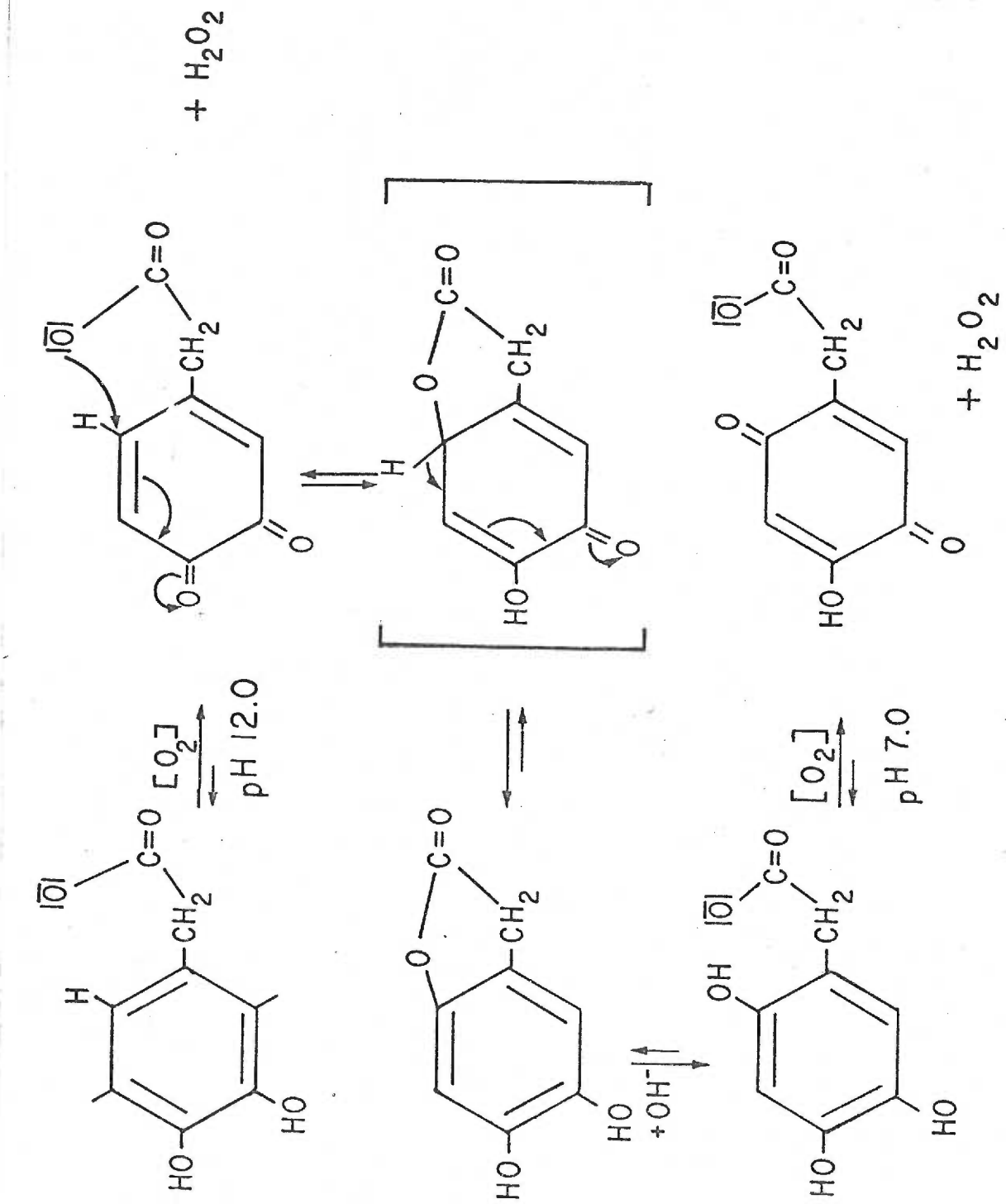


Figure 6 Oxidation and intramolecular addition of 3,4-dihydroxyphenylacetic acid.