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Several aspects of the potent inhibition of bovine plasma amine oxidase (PAO) by hydrazines were investigated by kinetic and preparative means. The inhibition was classified as pseudo-irreversible by the method of Ackerman and Potter (Proc. Soc. Exptl. Biol. Med. 72, 1 (1949), and was found to exhibit Zone B kinetic behavior (Straus and Goldstein, J. Gen. Physiol. 26, 559 (1943)). The constancy of the mole ratio of inhibitor to enzyme which produced 50% inhibition, $(I/E)_{50}$, for PAO preparations of different degrees of purity demonstrated the unique specificity of these inhibitors for PAO.

Inhibitor potency as a function of structure was found to parallel the reactivity of these hydrazines towards carbonyls in model systems. The kinetically determined amount of (unsubstituted) hydrazine which produced 100% inhibition was found to correspond

exactly with the reported pyridoxal phosphate (PLP) content of the enzyme. The isolation of a $^{14}C$-labelled EI complex confirmed this stoichiometry. These results, coupled with the spectral observations of Yamada and Yasunobu (J. Biol. Chem. 238, 2669 (1963)) led to the conclusion that the inhibition most likely proceeded from a nucleophilic attack of the hydrazine molecule on the carbonyl of the enzyme's PLP to form a stable azomethine via a transaldiminization reaction.

The kinetic competition observed between hydrazines and substrate indicated that they react with PAO at the same site, PLP, thus confirming the proposal originated by Tabor, Tabor, and Rosenthal (J. Biol. Chem. 208, 645 (1954)) that PLP is involved in the active site.

Inhibitor potency was found to decrease with increasing N-methyl substitution in a manner which could not be related exclusively to either steric or inductive effects of the substituents, but rather, depended on the presence or absence of a hydrogen alpha to the attacking nucleophilic $-NH_2$ on the hydrazine molecule. Thus, binding of hydrazines to the catalytic site of PAO may involve a three-point attachment.

Therefore, the active site of PAO can be visualized to contain two subsites: one which binds the $\alpha$-H of hydrazines or substrates by non-covalent forces, which functions to optimally orient the

molecule for the chemical reaction at the enzyme's primary site, PLP.

The titration of PAO by hydrazines was found to exhibit a biphasic response. Low inhibitor concentrations enhanced PAO activity, but high concentrations inhibited. This apparent homotropic cooperative effect suggested the presence of an allosteric site for the binding of these inhibitors.

PAO was found to exhibit anomalous kinetic order with respect to substrate in the presence of hydrazines; v vs. (S) curves were sigmoidal. Normal Michaelis-Menten kinetics were followed in the absence of these inhibitors, indicating that the binding of a hydrazine molecule by the enzyme potentiated an effect which resulted in the binding of more than substrate molecule. High substrate inhibition of PAO was found to conform to the Haldane mechanism. The dissymmetry of v vs. log (S) plots indicated that at high substrate concentrations PAO binds more than two substrate molecules. Thus, PAO may contain an allosteric site for substrate as well as for hydrazines.

A hypothetical model is presented which accounts for these experimental observations in terms of the nature and interaction of PAO's inhibitor and substrate binding sites.

PAO was found to undergo a time- and concentration-dependent activation in dilute solution at room temperature, pH 7.0, in the

presence or absence of hydrazines which could not be attributed to the presence of an endogenous activator or inhibitor (v vs. (E) plots were linear). Gel-filtration experiments revealed that the activation in the absence of hydrazines was not caused by a shift in the monomer-polymer equilibrium or the dissociation of PAO into subunits. Only one species was eluted from the column (which had a molecular weight corresponding to that of the monomer) whether the enzyme was activated or not. This peak was likewise independent of PAO concentration. These results led to the conclusion that the activation of the enzyme in the absence of inhibitor is most likely due to a conformational change.

The activation of the inhibited enzyme was found to be greater than that of the "enzyme alone" control; in other words, the inhibition appeared to be reversed. This reversal of inhibition was found to follow first order (with respect to (EI) kinetics indicating that it was caused by the catalytic decomposition of the hydrazine inhibitors by the enzyme.

# Studies on the Inhibition of Bovine Plasma Amine Oxidase by Hydrazines

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Typed by Donna L. Olson for Jacqueline Elizabeth Hučko-Haas

"For at the first Wisdom will walk with him by crooked ways and bring fears and dread upon him and torment him by her discipline, until she may trust his soul and try him by her laws. Then will she return the straight way unto him and comfort him and show him her secrets."

Ecclesiasticus 4: 17-18. The Apocrypha.

To my husband, my mother, and the memory of my father. Their unselfish love and constant encouragement made my studies possible. And to Dietrich--who did his best to make them impossible!

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| BH | Benzylhydrazine |
| BOH | β-hydroxyethylhydrazine |
| E | Enzyme |
| EI | Enzyme-inhibitor complex |
| ES | Enzyme-substrate complex |
| ESR | Electron spin resonance |
| FAD | Flavin adenine dinucleotide |
| H | Hydrazine |
| i | Fractional inhibition |
| I | Inhibitor |
| $(I)_{50}$ | Inhibitor concentration giving 50% inhibition |
| $(I/E)_{50}$ | Mole ratio of inhibitor to enzyme at which 50% inhibition is obtained |
| $K_i$ or $K_i'$ | Apparent enzyme-inhibitor dissociation constant |
| $K_m$ | Michaelis-Menten constant |
| $K_m'$ | Enzyme-substrate dissociation constant when S is in the inhibitory position |
| MMH | Monomethylhydrazine |
| PAO | Bovine plasma amine oxidase |
| PLP | Pyridoxal-5'-phosphate |
| S | Substrate |
| $S_o$ | Optimum substrate concentration |

| | |
|---|---|
| S. U. | Spectrophotometric unit (see Methods) |
| UDMH | 1, 1-dimethylhydrazine |
| v | Velocity |
| $V_e$ | Elution volume |
| $V_m$ | Maximum velocity |
| $V_o$ | Void volume of a Sephadex column |
| $V_t$ | Total volume of a Sephadex column |
| ( ) | Symbols in parentheses refer to molar concentrations |

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# STUDIES ON THE INHIBITION OF BOVINE PLASMA AMINE OXIDASE BY HYDRAZINES

## INTRODUCTION

Amine oxidase catalyzes the oxidative deamination of amines to the corresponding aldehydes according to the equation:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2$$

The first mention of an amine oxidase was by Hare (63) in 1928, who described the oxidative deamination of tyramine by liver tissue. Subsequent investigations revealed a wide distribution of this enzyme in animals, plants, insects, and microorganisms.

Amines play major regulatory roles in living systems, from being key functional compounds in the central nervous systems of animals, to serving as possible precursors to the growth hormone, indoleacetic acid, in plants. Thus it is clear that amine oxidases are biologically quite important in potentially controlling the cellular concentrations of all these amines.

The comparative aspects and general properties of these enzymes have been extensively reviewed in recent years (14, 15, 16, 21, 53, 139, 143), hence will not be discussed here except in relation to the central theme of this investigation. It is essential, however, to distinguish between the different classifications of amine

oxidases. The classical distinction until now has been based on substrate specificity; thus, in the literature and according to the system of the Commission on Enzymes of the International Union of Biochemistry, one finds reference to "diamine" or "monoamine" oxidases (EC 1.4.3.6 and EC 1.4.3.4, respectively) depending on whether the enzymes catalyze the oxidative deamination of di- or monoamine substrates. The ever-increasing availability of highly purified amine oxidases from different sources has led to a more meaningful distinction which is based on cellular location and cofactor requirements.

The class of soluble amine oxidases which has been obtained in a highly purified form includes the amine oxidase from pea seedlings (65, 81), bovine plasma (132), pig plasma (18), hog kidney (55, 90), Aspergillus niger (128), rabbit serum (84), and human plasma (82). Although there appear to be minor variations in certain properties of these enzymes which seem to depend on their biological source, common to all are the facts that they contain copper and pyridoxal phosphate (PLP) as essential cofactors[1], have molecular weights which are multiples of 85,000 to 90,000, contain similar

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[1] Although it has not been possible in every case to conclusively establish the identity of PLP, spectral and chemical evidence point to the existence of an aldehyde functional group in the active site with properties very similar to, if not identical with PLP.

quantities of copper (1 g-atom Cu/85, 000-90, 000 g protein), and possess similar absorption spectra which are unlike those of other PLP-containing enzymes. Despite the fact that all other PLP-enzymes which have been examined were found to require free -SH groups for activity (43), Nara, Gomes, and Yasunobu have reported that none of the PLP-containing amine oxidases possess an essential thiol (95). These soluble amine oxidases can oxidize only primary amines (16, 82). This ability to discriminate between substituted and unsubstituted amines is common to other PLP-containing enzymes (17).

In contrast are the mitochondrial amine oxidases which contain flavin and copper, but no PLP. Flavin has been shown to be involved in the catalytic activity (39, 70, 119), but the essentiality of Cu remains open to question (39, 95). ESR studies have shown that the copper to protein bond in these enzymes more closely resembles that in cytochrome oxidase rather than that in the soluble amine oxidases (95). Several workers have reported the existence of an essential thiol group in mitochondrial amine oxidases (34, 37, 39, 57, 95), but the observation that sulfhydryl reagents inhibit non-competitively has led others to conclude that factors beside this are involved in the active site (9, 49). The demonstration that complete inhibition of rat liver mitochondrial amine oxidase by N-methyl-N-benzylpropynylamine did not decrease the -SH group content of the

enzyme ( 52 ) would seem to support the latter opinion. Unlike the soluble amine oxidases, the mitochondrial enzymes react equally well with primary, secondary, or tertiary amines (16, 143). The highly purified particulate amine oxidases that have been reported include those from rat liver (59, 138), bovine liver (95), bovine kidney (39), and pig brain (120).

Forming a class of its own is the hog kidney histaminase of Kapeller-Adler (72, 73), which is reported to contain both PLP and FAD as essential cofactors, but appears to have no heavy metal or active sulfhydryl groups.

In view of the above comparisons, it is obvious that it may be much more significant to classify the amine oxidases according to their essential cofactors, since it is likely that the mechanisms will be more closely dependent on the nature of the cofactors than on the substrate specificity. Since the work presented here is with a soluble, PLP-Cu enzyme, extreme caution must be exerted when comparing the results to the mitochondrial system.

Inhibition of amine oxidase activity by hydrazines was first observed by Zeller and coworkers (140) and by Schuler (109) in 1952. Subsequent to this initial discovery, a great many reports have appeared on the in vitro and in vivo effectiveness of these compounds as amine oxidase inhibitors. Though the pharmacological applications of these compounds have been extensively treated,

unfortunately, there is a dearth of information on how the quantitative characteristics of the inhibition reaction can be extended to elucidate the catalytic mechanism of amine oxidase. Furthermore, relatively few studies on the mechanistic aspects of the hydrazine inhibition of amine oxidases have been reported. Those which have appeared have been based on experiments using the mitochondrial enzyme. Thus, there is a real need to examine the mode of action of these inhibitors with the soluble amine oxidases; this work is directed to that end. Likewise of central interest to the investigations presented here is to use the information obtained from the study of the inhibition of PAO by hydrazines as a tool to contribute to the understanding of what is known about the mechanism of action of the soluble amine oxidases, their active sites, and the physical-chemical properties which relate to the structural-functional aspects of enzyme catalysis.

# GENERAL EXPERIMENTAL PROCEDURES

## Materials

Benzylamine, purchased from Calbiochem Corporation, was redistilled under reduced pressure prior to use in the enzyme assays. The fraction used, which had a boiling point range from 66-68°C at 38 mm Hg appeared free of contaminants when examined spectrally. Putrescine dihydrochloride was obtained from Sigma Chemical Company. Putrescine-1,4-$^{14}$C. 2HCl, specific activity 9.1 millicurie/mmole and UDMH-$^{14}$C of specific activity 1.7 millicurie/mmole were from New England Nuclear Corporation. Monomethylhydrazine (anhydrous) and 1,1-dimethylhydrazine (anhydrous) of 99+% purity were obtained from Matheson, Coleman, and Bell. β-Hydroxyethylhydrazine was a product of K & K Laboratories. Benzylhydrazine hydrochloride was from Aldrich Chemical Company. Hydrazine was used from two sources: Anhydrous hydrazine of 97% purity was obtained from Eastman Chemical Company, and hydrazine sulfate of 99+% purity was a product of Matheson, Coleman, and Bell.

The starting material employed in the PAO purification procedure was either whole steer blood, kindly furnished by the Nebergall Meat Packing Company, or a partially purified preparation of PAO purchased from Sigma. Crystalline amine oxidase from

Aspergillus niger was the kind gift of Dr. Hideaki Yamada.

The reference proteins used in the polymerization studies, of the highest purity available, were obtained from the following sources: Horseheart cytochrome c Type III, twice recrystallized bovine liver catalase, Aspergillus niger glucose oxidase Type III, bovine kidney uricase Type II, and Jack Bean urease Type VI were purchased from Sigma. Bovine serum albumin Fraction V was a product of Mann Research Laboratories.

DEAE-cellulose was purchased from Eastman Chemical Company, and was treated according to the procedure of Peterson and Chiazze (97) prior to use. Hydroxylapatite was prepared by the method of Tiselius (121) or Siegelman (112). Sephadex G-25 and G-200 were obtained from Pharmacia.

Scintillation grade 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and p-terphenyl were obtained from Packard Instrument Company. Sodium-potassium phosphate buffers were used throughout, except in Step 6 of the enzyme purification. Ammonium sulfate was Mann "special enzyme grade." Other chemicals were obtained from commercial sources in the highest available purity. Deionized glass-distilled water was used in all experiments.

# Methods

## Protein Determination

Protein concentration was determined by the method of Warburg and Christian (122).

## Enzyme Assay

The standard assay procedure employed in all experiments except substrate specificity studies was a modification of the spectrophotometric method of Tabor, Tabor, and Rosenthal (116) using benzylamine as substrate. Unless otherwise indicated, the assay system contained a final concentration of 3.33 mM benzylamine and 67 mM phosphate with variable enzyme and inhibitor concentrations in a final volume of 3.0 ml. The reactions were run at 25°C and pH 7.2. Initial velocities were used to calculate enzyme activity; exceptions are noted in the text.

Calculation of enzyme activity: One spectrophotometric unit (S.U.) is defined as the amount of enzyme which produces an initial rate of change in optical density at 250 mμ of 0.001 per minute at 25°C. Specific Activity (S.A.) is the number of spectrophotometric units per mg of protein. Calculation of the number of moles of PAO in partially purified preparations was based on the specific activity of these fractions and the specific activity of 444 S.U./mg for

crystalline PAO of molecular weight 170,000 (1).

For the enzyme assay with putrescine as substrate, the method of Okuyama and Kobayashi (96) was used.

## Copper Determinations

Copper determinations were carried out after the method of Frey (41) using a Perkin Elmer Model 303 Atomic Absorption Spectrometer. The standard employed was Baker Analyzed Reagent Grade copper wire which was washed with concentrated HCl, rinsed with water, dissolved in concentrated $HNO_3$, and diluted to the desired final volume with deionized glass-distilled water.

## Enzyme Purification

Highly purified bovine PAO was prepared by the method of Yamada and Yasunobu (132) with modifications introduced by Achee (1) and in this laboratory. The modified procedure for a typical purification from steer blood is described below, and the results summarized in Table 1.

When Sigma PAO was used as starting material, the purification was initiated with Step 2.

All operations were carried out at 5°C and all centrifugations were performed in a Sorvall Model RC-2 centrifuge. Ammonium sulfate fractionations were based on saturation at 0°C. Concentrated

ammonia was used to maintain pH 7.0 during the ammonium sulfate additions.

Step 1. Collection of Plasma: Fifteen liters of blood, collected from freshly slaughtered steers, were immediately and vigorously mixed with 3 liters citrate solution (20.44 g sodium citrate and 8.75 g citric acid·$H_2O$ per liter) to prevent clotting. The citrated blood was centrifuged at 16,300 x g for 30 minutes and the plasma was collected.

Step 2. First Ammonium Sulfate Fractionation: To 10,836 ml of plasma, 2129 g of solid ammonium sulfate were added to bring to .35 saturation. After standing six hours, the mixture was centrifuged for 30 minutes at 16,300 x g. Solid ammonium sulfate (1,160 g) was added to the clear red-orange supernatant fluid to bring to .55 saturation, and the precipitate was collected by centrifugation as above after standing overnight. The precipitate was dissolved in a minimum volume of 0.01 M phosphate buffer, pH 7.0, and dialyzed overnight against three changes of 8 liters each of 0.01 M phosphate buffer, pH 7.0. The dialyzed enzyme was centrifuged at 16,300 x g to remove an inactive precipitate.

Step 3. First DEAE-cellulose Column Chromatography: The product of Step 2 (1600 ml of the clear, deep-red dialyzed enzyme solution) was applied to a 6 x 93.5 cm DEAE-cellulose column which had been equilibrated with two column volumes of .01 M phosphate

buffer pH 7.0, and washed into the column with four 5 ml portions of 0.03 M phosphate buffer pH 7.0. The column was first treated with 4.7 bed volumes of 0.03 M phosphate buffer pH 7.0 to elute much inactive and low specific activity protein. The enzyme was subsequently eluted with 3.1 column volumes of 0.07 M phosphate buffer pH 7.0 at a flow rate of 5.3 ml per minute. Fractions of specific activity between 19 and 61 S.U./mg, which were eluted in a peak from 0.9-1.6 bed volumes of the 0.07 M buffer, were combined to give 1680 ml of solution.

Step 3A. Second Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to the pooled active fractions from Step 3 to make .55 saturation. The mixture was centrifuged at 16,300 x g for 30 minutes after standing for six days. The supernatant was adjusted to .75 saturation ammonium sulfate. The active precipitate was collected by centrifugation at 16,300 x g for 30 minutes after standing overnight, dissolved in a small amount of 0.01 M phosphate buffer pH 7.0, and dialyzed overnight against 12 liters (three changes of 4 liters each) of the same.

Step 4. Second DEAE-cellulose Column Chromatography: The product of Step 3A (130 ml) was placed on a 3.1 x 50 cm DEAE-cellulose column which had been equilibrated by passing eight column volumes of 0.01 M phosphate buffer pH 7.0 through it. The active enzyme was eluted with 4.8 bed volumes of 0.07 M phosphate

buffer pH 7.0 at a flow rate of 2.7 ml per minute. Fractions with a range of specific activity from 71-138 S.U./mg, which were eluted in a peak between 1.2-1.7 bed volumes, were pooled.

Step 5. Third Ammonium Sulfate Fractionation: The active eluate from Step 4 was brought to .40 saturation in ammonium sulfate by addition of the solid salt. After standing four hours, the inactive precipitate was removed by centrifugation at 16,300 x g for 30 minutes. The ammonium sulfate saturation of the supernatant was increased to .55 by the addition of solid ammonium sulfate. After standing overnight, the precipitate was collected by centrifuging as above, and dissolved in a minimum volume of 0.06 M potassium phosphate buffer pH 6.8.

Step 6. Hydroxylapatite Column Chromatography: Hydroxylapatite, prepared according to the method of Siegelman (112), was packed into a column 3.1 x 33.5 cm and equilibrated with 16 liters of 0.06 M potassium phosphate buffer pH 6.8. The dialyzed enzyme solution (product of the preceeding step) was applied to the column and washed in with two 2 ml portions of 0.06 M potassium phosphate pH 6.8. The active enzyme was eluted with 0.06 M potassium phosphate buffer pH 6.8 at a flow rate of 0.2 ml per minute. Fractions which were eluted in a peak between 1.3-3.7 column volumes, having a specific activity ranging from 300-500 S.U./mg, were pooled to yield the product of Step 6. A protein peak with specific

activity less than 100 S.U./mg appeared at 0.8 bed volumes, preceeding the elution of the more active peak. A third, inactive protein peak was eluted with 0.2 M potassium phosphate buffer pH 7.0 at 1.0 column volumes.

Step 7. Fourth Ammonium Sulfate Fractionation: Solid ammonium sulfate to .55 saturation was added to the main active eluate from the hydroxylapatite chromatography purification step. The precipitate, removed by centrifugation for 30 minutes at 16,300 x g, was dissolved in a small volume of 0.06 M phosphate buffer pH 7.0. The supernatant was brought to .75 saturation ammonium sulfate by adding more of the solid salt, and after standing overnight was centrifuged as above. The inactive supernatant was discarded and the active precipitate dissolved in 0.06 M phosphate buffer pH 7.0. Both precipitates obtained in this step were dialyzed overnight against 4 liters (four changes of 1 liter each) of 0.06 M phosphate buffer pH 7.0.

## Isolation of Enzyme-Inhibitor Complex

Six g of Sephadex G-25 Fine were allowed to swell for 36 hours at room temperature in 500 ml of glass-distilled water, then packed into a column to final dimensions of 1.3 x 20.5 cm with a total volume of 27.2 $cm^3$. The void volume of the column was determined by passing 1.0 ml of a 0.1% (weight/volume) solution of blue

Table 1. Purification of PAO.

| Step | | Volume ml | Protein mg | Activity S.U. | Specific Activity S.U./mg |
|---|---|---|---|---|---|
| 1. Plasma | | 10,836 | 917,291 | 1,917,318 | 2.1 |
| 2. 1st Ammonium Sulfate Fractionation | | 1,600 | 90,760 | 1,199,847 | 13.2 |
| 3. 1st DEAE-cellulose Chromatography | | 1,680 | 10,761 | 352,944 | 32.8 |
| 3A. 2nd Ammonium Sulfate Fractionation | | 130 | 3,097 | 206,407 | 66.7 |
| 4. 2nd DEAE-cellulose Chromatography | | 191 | 1,740 | 142,948 | 82.2 |
| 5. 3rd Ammonium Sulfate Fractionation | | 33.0 | 1,115 | 160,210 | 143.7 |
| 6. Hydroxylapatite Chromatography | | 584 | 250 | 64,995 | 260.1 |
| 7. 4th Ammonium Sulfate Fractionation | .55 satn. ppt. | 55.5 | 71 | 22,858 | 321.3 |
| | .75 satn. ppt. | 53.0 | 107 | 52,419 | 488.3 |

dextran through the column, and was found to be 10.5-16.1 ml. All elutions were with glass-distilled water and were carried out at room temperature.

UDMH-$^{14}$C ($0.34 \times 10^{-6}$ mole) was added to 38.63 mg of PAO to make a total volume of 1.2 ml. After a 30 minute incubation at room temperature, 1.1 ml of the mixture was applied to the Sephadex column. A single protein peak, which contained the total amount of protein put on the column, was eluted between 9.3-16.0 ml. Flow rate of the column was 0.43 ml per minute.

The protein peak was prepared for $^{14}$C assay by the total combustion method of Schöniger (108) as modified by Kelly (75). The blank for the radioactive determinations was the blue dextran eluate which was prepared for assay in the same way.

Assays for enzyme activity were made immediately prior to the addition of the inhibitor (referred to in the text as zero-time activity), after the 30 minute incubation of enzyme and inhibitor, and subsequent to the elution of the enzyme-inhibitor complex from the Sephadex column. About three hours passed between the addition of the inhibitor and the measurement of activity after elution from the column.

## Polymerization Studies on PAO

The effects of concentration, time in solution, and temperature

on the molecular weight of PAO were determined employing the technique of molecular weight determination by gel-filtration as described by Andrews (4, 5) and Leach and O'Shea (79).

Sephadex G-200 (1.0 g) was allowed to swell in glass-distilled water at 5°C for five days, poured into a column to give dimensions of 1.22 x 26 cm, and was then equilibrated with 0.06 M phosphate buffer, pH 7.0. The void volume ($V_o$) of the column was determined by passing 0.5 ml of a 0.1% (weight/volume) solution of blue dextran through the column. All elutions were at 5°C with 0.06 M phosphate buffer, pH 7.0. Sample size applied to the column was 0.5 ml.

Reference proteins of known molecular weights were eluted from the column and a plot of $V_e/V_o$ versus log molecular weight was used as a standard curve for the molecular weight determinations of the experimental samples.

Samples of PAO varying in concentration, time in solution, and temperature were passed through the column, and the elution pattern plotted as total activity (S.U.) and total mg versus elution volume ($V_e$). The elution volume for each peak was determined from this plot, and the corresponding molecular weight read from the standard curve.

## Spectrophotometric Measurements

Spectrophotometric measurements were carried out in a Gilford Model 2000 Recording Spectrophotometer.

## Radioactivity Determinations

Radioactivity determinations were performed in a Packard Tri-Carb liquid scintillation spectrometer Model 314E using scintillation solution prepared from 30 mg POPOP and 3 g p-terphenyl per liter of toluene. Trapping solution for $CO_2$ consisted of one volume 2-aminoethanol and three volumes absolute ethanol. Five ml trapping solution were used with fifteen ml of scintillation solution in 24 ml counting vials.

# RESULTS AND DISCUSSION

## I. CHARACTERIZATION OF THE INHIBITION REACTION[2]

### Results

#### A. The Locus of Inhibition

It is of utmost importance at the onset of any inhibition study to determine with which component of the enzyme reaction system the inhibitor reacts. Although the literature on amine oxidases abounds with information on the inhibition by hydrazines, the following simple tests have never been reported. They are described here, not because the author has any viable doubt that the inhibition by hydrazines is due to anything but reaction with the enzyme, but rather because of the bearing their results have on conclusions reached in subsequent parts of this thesis.

The experiments described in Table 2 demonstrate that the inhibitor reacts with the enzyme, not the substrate, since for the same mole ratio of inhibitor to enzyme in the assay system, when enzyme and inhibitor were preincubated only two minutes prior to the addition of substrate the inhibition was nearly complete, whereas when substrate and inhibitor were preincubated for the same time

[2]This work was in part reported at the Northwest Regional Meetings of the American Chemical Society, Vancouver, British Columbia, June 16, 1966 (62).

period and the reaction started by the addition of enzyme, only 18% inhibition was observed.

Table 2. Effect of Order of Addition of the Components of the Enzyme-Substrate-Inhibitor Assay System on the Amount of Inhibition.

| Experiment | Preincubation | Reaction started by | % Inhibition |
|---|---|---|---|
| A | E+I | S | 96.6 |
| | E | S | 0 |
| B | S+I | E | 18.0 |
| | S | E | 0 |

UDMH was the inhibitor employed. The mole ratio, UDMH/E, was 9.66 in both experiments and substrate concentration was 3.33 mM. In Experiment A, partially purified PAO with specific activity 8.4 S.U./mg was used at a final concentration of 65 mμM. Purified PAO with specific activity 317.9 S.U./mg at a final concentration of 100 mμM was used in Experiment B. Concentrations refer to a final volume of 3 ml. Preincubation was for two minutes at room temperature in 0.06 M phosphate buffer, pH 7.0.

The standard enzyme assay employed in this investigation is based on the spectrophotometric measurement of the rate of appearance of the aldehyde product. In view of the fact that hydrazines are well-known carbonyl reagents, it seemed feasible that their observed inhibition of the amine oxidase reaction might be an artifact due to product removal rather than interaction with the enzyme. To test this possibility, the reaction of PAO with benzylamine was allowed to proceed for five minutes with product formation measured by

recording the increase with time in optical density at 250 mμ. Subsequently, 0.05 ml of either 5 μM or 25 μM BOH was added to the 3 ml assay system to give a mole ratio of BOH/E of 0.667 or 3.33, respectively. Optical density readings were initiated immediately. Neither concentration of inhibitor produced a decrease with time in optical density at 250 mμ which was greater than the control, to which 0.05 ml of 0.06 M phosphate buffer, pH 7.0 had been added. It was therefore concluded that the observed inhibition of amine oxidase by hydrazines is not due to reaction of the latter with the aldehyde product.

B. Potency of Hydrazine Inhibitors

1. Classification of the Inhibition by the Ackerman-Potter Treatment. Ackerman and Potter (2) have devised a simple graphical test to determine the degree of reversibility of an enzyme-inhibitor reaction. When the velocity of the reaction is measured at different enzyme concentrations in the presence and absence of inhibitor, it should be found that for the control (no inhibitor) the rate is proportional to the enzyme concentration, so that a straight line passing through the origin is obtained when velocity is plotted against enzyme concentration. In the presence of a reversible inhibitor, a straight line through the origin also results, but with slope less than that of the control. With an irreversible inhibitor, the slope of the

line is the same as that of the control but it will intercept the X-axis to the right of the origin by an amount that is proportional to the amount of enzyme inactivated by the inhibitor. When the $K_i$ has an intermediate value, the line will pass through the origin, being parabolic at lower enzyme concentrations and assuming linearity at higher enzyme concentrations with a slope which is less than that of the control. The basis for this classification of inhibitor potency is independent of any assumptions about the mechanism of the inhibition and depends solely upon the relation between the $K_i$ and the enzyme concentration.

Variable amounts of purified PAO with a specific activity of 496 S. U./mg and a protein concentration of 13.85 mg/ml were incubated at room temperature in 0.06 M phosphate buffer pH 7.0 with $0.2 \times 10^{-9}$ moles of H, MMH, or UDMH in a constant volume of 0.2 ml. At the end of 30 minutes when the inhibition was complete, a 0.1 ml aliquot of the incubation mixture was assayed for enzyme activity. The control consisted of incubating the enzyme in the absence of inhibitor under the same conditions. The data were subjected to the Ackerman-Potter analysis and plotted in Figure 1. The results indicate that all three inhibitors can be classified as pseudo-irreversible, and the apparent potency is H > MMH > UDMH.

Pseudo-irreversible inhibitors are those which have such a high affinity for the enzyme that they give the appearance of

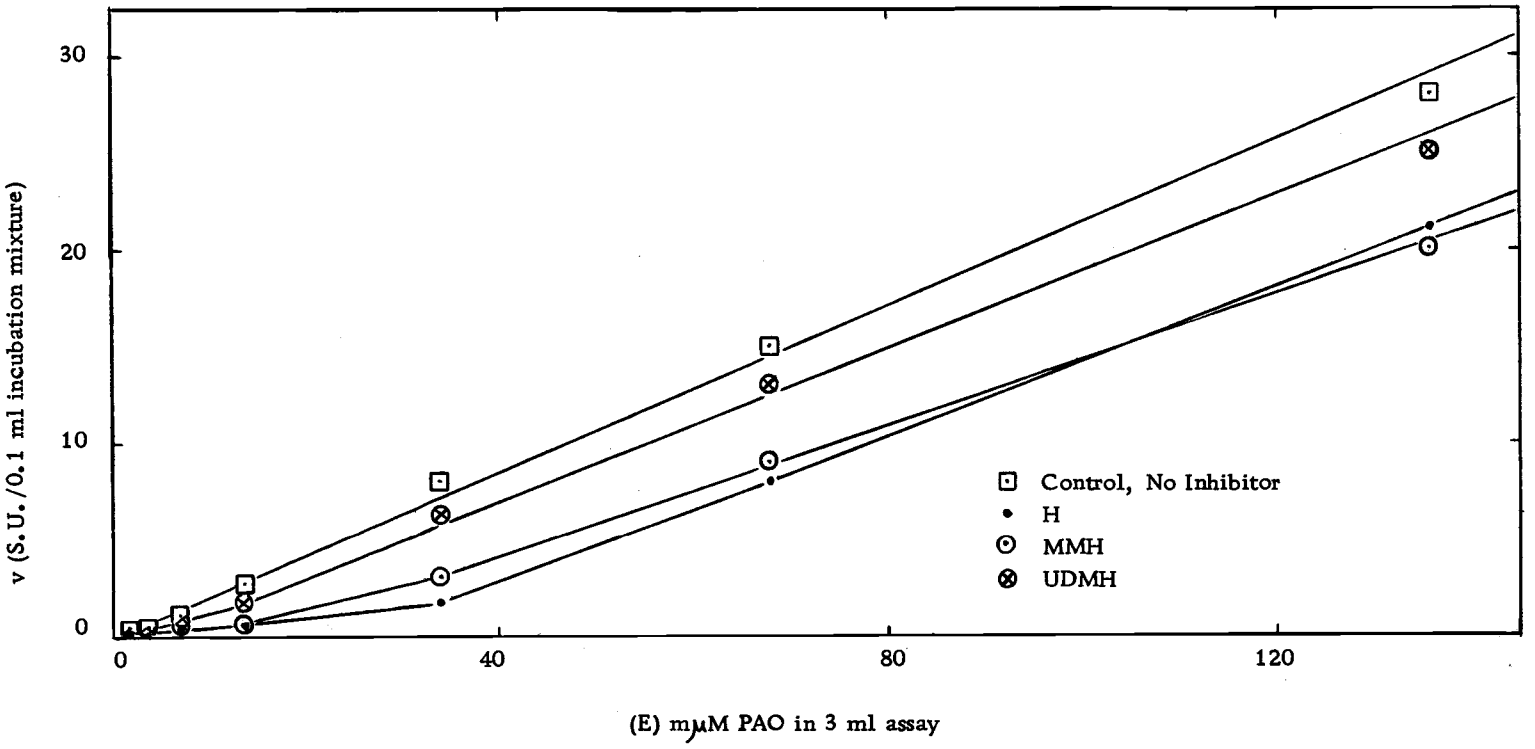


Figure 1. Ackerman-Potter analysis of PAO activity in the presence and absence of H, MMH, or UDMH. Experimental conditions are described in the text.

irreversibility. A major consequence of the fact that the hydrazine inhibitors fall into this category is that the amount of inhibition depends on both enzyme and inhibitor concentrations and the classical Michaelis-Menten treatment cannot be applied to determine kinetic constants without modifications that take into account the mutual depletion of enzyme and inhibitor. Michaelis-Menten kinetics involve the assumption that the combination of enzyme with inhibitor or substrate does not appreciably reduce the inhibitor or substrate concentrations. When inhibitor is not present in excess, or when the $K_i$ is very small, one must then include in the kinetic expressions the reduction of inhibitor concentration in systems where conditions lead to mutual depletion.

2. Time Course of the Inhibition. Irreversible inhibition is characterized by a progressive increase of the inhibition with time, ultimately reaching completion even with very low inhibitor concentrations provided that the inhibitor is present in an amount greater than or equal to the number of sites with which it reacts. The calculation of constants and potency relationships based on equilibrium conditions for pseudo-irreversible, time-dependent inhibitors thus have little meaning unless it is demonstrated that equilibrium has been reached and the inhibition under the specified conditions is complete.

Davison (34) demonstrated that the reaction of iproniazid with

rat liver monoamine oxidase in the absence of substrate was progressive with time and showed kinetics characteristic of a bimolecular reaction with one component (presumably the inhibitor) in excess (pseudo-first order). A careful time study by Yamasaki (136) on the inhibition of pea-seedling diamine oxidase by hydrazine, UDMH, and BOH likewise revealed the time-dependency of the inhibition and showed the reaction to be second order with respect to enzyme and inhibitor concentrations which were approximately equal.

For the inhibition studies with PAO, when enzyme and inhibitor were incubated prior to the addition of substrate, the usual incubation time employed was 30 minutes. Since the reaction between PAO and hydrazines has been classified as pseudo-irreversible, and because it is a mutual depletion system, it is necessary to demonstrate that the inhibition is complete if one is to justify the use of $K_i$ values or inhibitor concentrations which produce a given amount of inhibition as a measure of potency.

The data summarized in Table 3 represent the time course of the inhibition for the least potent (UDMH) and one of the most potent (BOH) hydrazine inhibitors employed in these studies, covering the range of enzyme concentrations used. It is seen that the inhibition was complete at 30 minutes for both inhibitors as well as for both extremes of the range of enzyme concentrations used, thus

Table 3. Time Course of the Inhibition of PAO.

| Experiment | Inhibitor | μM Concentration in Incubation Mixture (E) | (I) | I/E | % Inhibition after incubation of E+I for: 2 min. | 15 min. | 30 min. | 45 min. | 60 min. | 75 min. |
|---|---|---|---|---|---|---|---|---|---|---|
| A | BOH | 2.18 | 0.625 | 0.29 | 35 | 38 | 35 | 32 | 32 | - |
| | | | 6.25 | 2.86 | 94 | 97 | 97 | 96 | 97 | - |
| | | | 12.50 | 5.73 | 94 | 100 | 97 | 97 | 95 | - |
| B | UDMH | 2.16 | 3.13 | 1.45 | 39 | 40 | 40 | 35 | 30 | - |
| | | | 12.50 | 5.80 | 77 | 79 | 79 | 73 | 70 | - |
| C | BOH | 0.22 | 0.117 | 0.53 | 16 | 50 | 60 | 63 | 65 | 62 |

A constant amount of enzyme was incubated with variable amounts of inhibitor at room temperature in 0.06 M phosphate buffer, pH 7.0. At the indicated time intervals a 0.1 ml (for Experiments A and B) or a 0.3 ml (for Experiment C) aliquot of the incubation mixture was assayed for enzyme activity. The percent inhibition was referred to a control of enzyme in the absence of inhibitor incubated under identical conditions. Partially-purified PAO was used in all three experiments. In Experiment A the enzyme used had a specific activity of 8.95 S.U./mg with a protein concentration of 36.9 mg/ml. The enzyme used in Experiment B had a specific activity of 8.95 S.U./mg with protein concentration of 36.4 mg/ml. Enzyme with a specific activity of 89.9 S.U./mg and protein concentration of 0.267 mg/ml was used in Experiment C.

verifying the validity of the equilibrium assumption employed in the following sections.

3. Determination of $K_i'$ Values for Hydrazine Inhibitors. Straus-Goldstein Zone Behavior. A constant amount of enzyme (0.40 μmoles) was incubated with varying amounts of inhibitor at room temperature in 0.06 M phosphate buffer, pH 7.0 in a total volume of 0.2 ml. At the end of a 30-minute incubation a 0.1 ml aliquot was pipetted into a cuvette containing the standard assay system and the enzyme activity was determined. The control (100% remaining activity) consisted of incubating the enzyme in the absence of inhibitor under the same conditions. Titration curves were constructed which are plots of percent remaining activity versus inhibitor concentration (I), or the mole ratio of inhibitor to enzyme (I/E) (see Figure 5 for a representative plot). The amount of inhibitor giving 50% inhibition (expressed either in terms of the inhibitor concentration or the mole ratio, $(I/E)_{50}$) was determined from the plots. The $(I/E)_{50}$ values are listed in Table 4.

The apparent enzyme-inhibitor complex dissociation constants ($K_i'$) listed in Table 4 were calculated from Equation (5) below, which was derived from the following relationships, where ($E_t$) is the total enzyme concentration, ($I_t$) the total inhibitor concentration which gives i fractional inhibition, (E) and (I) represent the concentrations of free enzyme and inhibitor, and (EI) is the concentration

Table 4. $K_i'$ and $(I/E)_{50}$ Values for the Inhibition of PAO by Hydrazines. Straus-Goldstein Zone Behavior.

| Inhibitor | $(I/E)_{50}$ | $K_i'$ (mμM) | $E'=(E_t)/K_i'$ | Zone |
|---|---|---|---|---|
| H | 0.53 | 1.9 | 35.8 | B |
| MMH | 0.71 | 14.1 | 4.8 | B |
| UDMH | 1.96 | 98.8 | 0.7 | B |
| BOH | 0.63 | 8.6 | 7.9 | B |
| BH | 0.59 | 5.7 | 11.8 | B |

PAO samples of varying degrees of purity were titrated with hydrazine inhibitors by procedures described in the text. Enzyme concentration was constant at 67.5 mμM in the 3 ml assay. The values listed here represent an average of five to eleven determinations on different PAO fractions.

of the enzyme-inhibitor complex.

(1) $$(E) + (I) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (EI)$$

(2) $$(E_t) = (E) + (EI)$$

(3) $$(I_t) = (I) + (EI)$$

(4) $$i = (EI)/(E_t)$$

(5) $$K_i' = k_{-1}/k_1 = \frac{(E)(I)}{(EI)} = \frac{[(E_t) - (EI)]\,[(I_t) - (EI)]}{(EI)}$$

$$= \frac{[(E_t) - i(E_t)]\,[(I_t) - i(E_t)]}{i(E_t)}$$

This method for calculating $K_i'$ is valid for mutual depletion systems where $(I_t)$ approximates $(E_t)$ under non-competitive (with respect to substrate) conditions (123). Figure 9 illustrates that the hydrazine-PAO-benzylamine system demonstrates apparent non-competitive behavior under the conditions when enzyme and inhibitor are incubated until the inhibition is complete prior to the addition of substrate. Mutual depletion classification of this system was determined in Section IB-1. When these data were subjected to the diagnostic treatments of Dixon (35), Straus and

Goldstein (114), and Webb (123), constants were obtained which were in good agreement with those calculated from Equation (5).

Straus and Goldstein (114) have shown that enzyme-inhibitor and enzyme-substrate systems behave in three distinct ways depending on the concentrations of the reactants and the dissociation constants of the system. They have made a detailed analysis of the kinetics for each situation and devised experimental methods to distinguish between three zones of behavior.

The zones for an enzyme-inhibitor system are defined as follows (where E' is the ratio of the total concentration of active centers to the apparent dissociation constant for the EI complex): when E' has a value less than 0.1 the system lies in Zone A, when E' is greater than 100 the system lies in Zone C, and when intermediate values are obtained, Zone B kinetics should be employed. This is a convenient method of classification which tells at a glance which kinetic methods are valid. For example, in Zone A one can ignore the concentration of bound inhibitor compared to free inhibitor (classical Michaelis-Menten kinetics), in Zone C one can ignore the concentration of free inhibitor because the inhibitor is mainly combined with the enzyme, and in Zone B both bound and unbound inhibitor must be considered. Mutual depletion systems can lie either in Zone B or C, and the relative potencies of inhibitors are Zone C > Zone B > Zone A for a constant enzyme concentration.

When the PAO-hydrazine inhibitor system was analyzed by this method it was found that the five inhibitors tested all lie in Zone B, thus substantiating the conclusions reached from the Ackerman-Potter treatment of the same data.

4. Specificity. Reed, Dost, and Wang (99) have shown that injections of hydrazines into rats at levels as low as 10 μmoles/kg body weight completely inhibit methylamine and putrescine conversion to respiratory $CO_2$, thus demonstrating the potent effectiveness of these compounds in vivo. The treatment described in the preceeding section has demonstrated that hydrazines may be classified as pseudo-irreversible inhibitors of PAO in vitro. If such an inhibitor is specific for a certain enzyme, being bound to nothing else, it may be said to titrate the enzyme in the sense that the amount of enzyme inactivated will be proportional to the amount of inhibitor added.

The specificity of hydrazine inhibitors for PAO was tested by titrating the enzyme at various stages of purification with the inhibitors. If the interaction between the hydrazine inhibitors and PAO is indeed specific, a correspondence between the concentration of inhibitor to produce a given amount of inhibition and the degree of purity of the enzyme is a necessary consequence. The results of the titrations are summarized in Table 5. The general trend is clear: as the purity of PAO increases, the number of moles of enzyme per mg total protein of course increases, and it is seen that the number

of moles of inhibitor at which 50% inhibition is obtained per mg total protein likewise increases. Note that amine oxidase from Aspergillus niger, though from a different source, fits the general pattern. It can thus be concluded from these results that the PAO-hydrazine interaction is specific.

Table 5. Titration of Amine Oxidases of Different Degrees of Purity with Hydrazine Inhibitors.

| Purification Step | Percent Purity | mμM (E) in assay | Inhibitor | | | | |
|---|---|---|---|---|---|---|---|
| | | | H | MMH | UDMH | BOH | BH |
| | | | μμmoles inhibitor per mg protein at which 50% inhibition is obtained | | | | |
| 1 | 1.4 | 55.8 | 51 | 96 | 127 | 69 | 49 |
| 2 | 2.5 | 70.0 | 80 | 80 | 164 | 74 | 85 |
| 3 | 5.9 | 63.3 | 187 | 185 | 370 | 289 | - |
| 6 | 21.1 | 14.7 | 339 | 1700 | 3300 | 1160 | 1980 |
| 6-II | 42.0 | 39.7 | - | - | - | 1230 | - |
| DJR-PAO | 100.0 | 67.9 | 2942 | 2764 | 23536 | - | - |
| Aspergillus niger | 100.0 | 13.7 | - | - | - | 7930 | - |

Titration procedure is described in IB-3. Enzyme concentration was held constant and inhibitor concentration varied.

When the data of Table 5 were recalculated on a molar basis (described in Methods), it was found that the ratio of the number of moles of inhibitor to the number of moles of enzyme at which 50% inhibition is obtained, $(I/E)_{50}$, remained constant throughout the purification for any one inhibitor. (The values were within the range of the averages calculated for the data of Table 4.) The

deviations obtained did not follow any trend corresponding to the degree of purity of the enzyme, which indicated that any variability observed was not due to a non-specific reaction of the inhibitor with other components present in the crude enzyme. This leads to the conclusion that the reaction between PAO and hydrazine inhibitors is both stoichiometric and specific. This finding validates the comparisons made throughout this thesis of results obtained from PAO preparations of varying degrees of purity.

5. Effect of Substrate Concentration and Preincubation of Enzyme and Inhibitor on the Potency and Type of Inhibition. Pseudo-irreversible inhibitors may be competitive or non-competitive, but if truly the former, it may not be obvious since little change in enzyme activity will be observed upon adding substrate to the inhibited enzyme due to the slow dissociation of the inhibitor, or due to the fact that the inhibitor is bound so much more tightly to the enzyme than is the substrate that competition at equilibrium cannot be demonstrated. Thus, though the inhibition is truly competitive in that inhibitor and substrate compete for the same enzyme site, apparent non-competitive behavior will be observed when enzyme and inhibitor are incubated until the inhibition is complete prior to the addition of substrate.

Table 6 shows the effect of varied concentrations of benzylamine on the inhibition of PAO by H, MMH, and UDMH when

Table 6. Effect of Substrate Concentration and Preincubation of Enzyme with Inhibitor on the Inhibition of PAO by H, MMH, and UDMH.

| A. Preincubation of E+I (E) = 65 mμM | | | | B. No Preincubation (E) = 100 mμM | | | |
|---|---|---|---|---|---|---|---|
| | $(I/E)_{50}$ | | | | $(I/E)_{50}$ | | |
| mM (S) | H | MMH | UDMH | mM (S) | H | MMH | UDMH |
| 0.3 | 0.60 | 1.0 | 2.4 | 0.13 | 0.26 | - | - |
| 0.5 | 0.69 | 1.25 | 2.0 | 0.33 | - | 0.55 | - |
| 1.0 | 0.62 | 1.0 | 2.6 | 0.66 | 0.50 | 0.95 | - |
| 3.0 | 0.59 | 1.0 | 2.2 | 1.33 | 1.4 | 2.1 | 52 |
| 5.0 | 0.63 | 1.1 | - | 3.33 | 1.6 | 3.5 | 92 |
| | | | | 5.0 | 2.9 | 3.7 | - |

In Experiment A, enzyme and inhibitor were preincubated 30 minutes prior to the addition of substrate according to the procedure described in Section IB-3. Partially purified PAO with specific activity of 8.95 S.U./mg at a final concentration of 65 mμM in the 3 ml assay system was used. In Experiment B, inhibitor was added to the 3 ml assay cuvette less than two minutes before 300 μμmoles of PAO with specific activity of 318 S.U./mg were added to start the reaction. Inhibitor concentrations varied from 0.01-10.0 μM and $(I/E)_{50}$ values were calculated from titration curves as described in Section IB-3. Substrate was benzylamine.

added simultaneously or after a 30 minute preincubation of the enzyme with inhibitor. When both substrate and inhibitor were added simultaneously to the enzyme, the degree of inhibition decreased progressively with increasing concentration of benzylamine. No such protective action of the substrate could be observed when the enzyme was preincubated with the inhibitor prior to the addition of substrate; variation of the substrate concentration by more than sixteen-fold had no effect on the inhibitor potency even for very low inhibitor concentrations. Also, for any one substrate concentration, a striking difference in inhibitor potency was observed between samples in which enzyme and inhibitor were preincubated or not prior to the addition of substrate. Without preincubation, the hydrazine inhibitors were much less potent, as indicated by their greater $(I/E)_{50}$ values. One possible explanation for this observed difference in potency is that a reaction between substrate and inhibitor occurs to reduce the effective inhibitor concentration. Besides the fact that such a reaction is improbable chemically, this can be ruled out by the experiments described in Section IA. Another possibility is that the presence of substrate masks inhibitor binding sites on the enzyme, implying that the substrate and inhibitor react at the same enzyme site. The third possible explanation to be considered is that when the enzyme and inhibitor were incubated for thirty minutes before adding substrate, the inhibition was complete

and equilibrium was reached (Table 3); however, when substrate and inhibitor were added simultaneously to the enzyme and initial velocities were used to calculate the activities, the inhibition was not yet complete and a kinetic competition was observed.

From the experiments discussed here, it can be concluded that the amine substrate can protect against but not reverse the inhibition of PAO by hydrazines, implying that inhibitor and substrate combine at the same enzyme site. The substrate could exert its protective action by decreasing the rate of reaction between enzyme and inhibitor or by masking inhibitor binding sites on the enzyme. The turnover number of PAO, calculated from the data of Figure 1, was found to be 17.0 moles substrate/min/mole enzyme. This extremely low value would certainly augment the substrate's protective effect.

These results provide evidence that substrate and hydrazine inhibitors react at the same enzyme site, yet this inhibition is not truly competitive in the classical sense because when enzyme and inhibitor were preincubated in the absence of substrate until maximum inhibition was achieved, the addition of substrate even at high concentrations could not reduce or reverse the inhibition. One must then distinguish between kinetic competition, where the rate of inhibition is reduced in the presence of substrate but the final degree of inhibition is not affected, and equilibrium competition where both the rate and final inhibition are decreased by substrate. In the

former case (the PAO-hydrazine-benzylamine system is of this type) the standard plotting procedures will indicate non-competitive behavior when equilibrium inhibitions are measured (see Figure 9). However, when enzyme and inhibitor are not incubated before substrate is added, the inhibition will not have reached its final equilibrium value. Under these conditions, kinetic competition is being measured.

The non-competitive plot obtained here (Figure 9) disagrees with the results of Yamada and Yasunobu (134), who reported competitive behavior of the PAO-hydrazine-benzylamine system under identical conditions. However, the data are in accord with the findings of various workers (9, 19, 34, 56, 82, 113, 145) who made similar studies on amine oxidases from other sources.

6. Effect of Inhibitor Structure. If one is attempting to correlate inhibition with chemical structure, the relative affinities of the active forms of the inhibitors are desired, not merely the fractions of the inhibitors in the active form at a chosen pH. Therefore, it is extremely important, especially for ionizable inhibitors, that the true $K_i$ values be determined from a study of the effect of pH on the inhibition reaction because the apparent $K_i$ values ($K_i'$) determined at any one pH may relate principally to the ionization constants of the inhibitors rather than to their relative binding strength to the enzyme. Such studies have not been carried out for PAO, yet in the

discussion which follows, inhibitor potency is related to structure. This may be justified because for pseudo-irreversible inhibitors, if the inhibitor is not all in the active ionic form that binds to the enzyme at the pH of the experiment, the strong binding will shift the equilibrium of the ionization until all possible inhibitor molecules are combined with the enzyme. Also, for an inhibition mechanism that involves chemical combination between enzyme and inhibitor (as opposed to an electrostatic mechanism), it is possible that the inhibition rate may be affected by changing the pH, but the final level of inhibition reached remains unchanged by pH if the $K_i$ is sufficiently small so that the inhibition is pseudo-irreversible (123).

Most (pseudo-) irreversible inhibitors have been shown to react chemically with the enzyme, for example, the arylation of amino groups with dinitrophenyl derivatives, the alkylation of thiol groups with iodoacetate, and the phosphorylation of serine residues with phosphofluoridates. Davison (34) determined the energy of activation for the inhibition of mitochondrial monoamine oxidase by hydrazine derivatives and found it to have a high value which is characteristic of a chemical combination. In addition, the fact demonstrated in the preceding section that once the inhibition has been produced it cannot be reversed by high substrate concentrations likewise implies that the inhibition reaction involves the formation of a strong covalent bond between the enzyme and inhibitor. Thus it

appears that the irreversible inhibition of amine oxidase by hydrazines can be added to the preceding list.

Since the inhibition of PAO by hydrazines was demonstrated to be pseudo-irreversible, and the mechanism of inhibition most likely involves a chemical combination, the $K_i'$ or $(I/E)_{50}$ values at any given pH will reflect a true measure of potency when the inhibition reaction is complete.

Inhibitor studies by Tabor, Tabor, and Rosenthal (116) on two hundred-fold purified beef PAO revealed its sensitivity to carbonyl reagents. These results led these workers to be the first to suggest that a carbonyl group (possibly PLP) is present at the enzyme's active site. This initial proposal was substantiated by the work of Yamada and Yasunobu on their more highly purified preparation of beef PAO. They identified both PLP (1.0 mole/mole enzyme)[3] and Cu (II) (2 g atoms/mole enzyme)[4] as essential cofactors (131, 133, 134, 135). Spectral studies on PAO and its Cu-free apoenzyme have provided evidence which indicates that the enzyme's active site very likely involves a Cu(II)-PLP complex (18, 131, 133) and that the carbonyl functional group of PLP most probably is not free but exists as a Schiff base with an amino group in the enzyme (18, 22,

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[3] The original data (131, 133, 134, 135) was recalculated on the basis of PAO having a molecular weight of 170,000 (1).

[4] Ibid.

134).

The spectrophotometric behavior of beef PAO in the presence of hydrazine compounds suggested the formation of hydrazone derivatives involving a carbonyl functional group on the enzyme (134). Studies with the pig plasma (18, 21), the pig kidney (55, 89) and the pea seedling (65, 81) enzymes yielded similar results. The possibility that this functional group is the carbonyl of PLP would not be diminished by the fact that it may already exist as a Schiff base with an amino group of the enzyme because it has been demonstrated that azomethines are even more reactive towards carbonyl reagents than free carbonyls (31, 125). Thus, the inhibition of PAO by hydrazines could be visualized as hydrazone formation between the hydrazine nitrogen and the carbonyl carbon of the enzyme's PLP via a transaldiminization reaction. This interpretation is one of many that are feasible. Other proposed mechanisms that have been reported in the literature (all of which are based on studies on the mitochondrial enzyme) are reviewed in the discussion which follows.

An investigation of inhibitor potency as a function of structure was undertaken to obtain insight into the mechanism of hydrazine inhibition of PAO and to determine the chemical nature of the site(s) on the enzyme with which these inhibitors combine. The structures of the hydrazine inhibitors used in these studies are shown in Table 7. Their comparative potency is reflected in their $(I/E)_{50}$ values

obtained from titration experiments as described in Section IB-3.

Table 7. Structure and Potency of Hydrazine Inhibitors.

| | Inhibitor | $(I/E)_{50}$ |
|---|---|---|
| $H_2N\text{-}NH_2$ | Hydrazine (H) | 0.53 |
| $H_3C\text{-}NH\text{-}NH_2$ | Monomethylhydrazine (MMH) | 0.71 |
| $(H_3C)_2\text{-}N\text{-}NH_2$ | 1,1-Dimethylhydrazine (UDMH) | 1.96 |
| $HOCH_2CH_2\text{-}NH\text{-}NH_2$ | β-Hydroxyethylhydrazine (BOH) | 0.63 |
| $C_6H_5CH_2\text{-}NH\text{-}NH_2$ | Benzylhydrazine (BH) | 0.59 |

Legend is the same as Table 4.

Comparing H, MMH, and UDMH, a general trend is observed for decreasing potency with increasing N-methyl substitution. Since the larger molecules BOH and BH do not fall into this pattern, it is unlikely that steric hindrance alone is the cause. Neither can an inductive effect be solely responsible, since there is no trend in inhibitor potency corresponding to the presence or absence of electron-releasing substituents. It is apparent that the availability of the hydrogen atom on the substituted nitrogen is important because of the striking difference in potency between UDMH (which lacks this hydrogen atom) and the other hydrazines.

Hinman (66, 67) made a detailed study of the reactivity of

hydrazines towards carbonyls as a function of structure. He found that substitution of an alkyl group for a hydrogen atom in the hydrazine molecule was accompanied by a decrease in reactivity. This result was quite unexpected in view of the electron-donor characteristics usually ascribed to alkyl groups; therefore it can be most reasonably explained in terms of steric effects. Hinman's results demonstrated that both nitrogens of the hydrazine molecule are potential nucleophiles, thus should be equally reactive. However, when a hydrogen atom on one of the nitrogens is replaced by a larger group, the nucleophilic reactivity of the molecule is decreased because the attack by the substituted nitrogen atom is sterically hindered. The unsubstituted nitrogen is then the favored attacking species. In addition, it was found that the presence of a hydrogen atom on the nitrogen adjacent to the one making the nucleophilic attack strikingly increased the reactivity of the hydrazine molecule towards carbonyls. This observation has been interpreted in terms of the hydrogen lowering the energy of the transition state by hydrogen bonding with the carbonyl oxygen, and so enhancing the reaction. Thus, the main determinants of the reactivity of hydrazines towards carbonyls in model systems are: inductive effects, where electron-releasing substituents augment the nucleophilicity by increasing the availability of the electron pair on the attacking nitrogen atom, steric factors, where any substituent

decreases the reactivity by hindering the nucleophilic attack, and the presence of a hydrogen atom alpha to the attacking nitrogen, which may function to lower the energy of the transition state. From Hinman's experimental data, the latter two seem to be the most important.

When the hydrazines shown in Table 7 are considered in the light of the foregoing information, unsubstituted hydrazine would be expected to be the most reactive, UDMH the least reactive, with the others having intermediate reactivities towards carbonyls which are probably determined by the electronic effects of their substituents. It was found that the potency of the hydrazines as PAO inhibitors followed a corresponding trend: unsubstituted hydrazine was the most effective and UDMH the least effective inhibitor. This could be taken as evidence that the inhibition involves a reaction with a carbonyl functional group on the enzyme, thus supporting the conclusions reached from the spectral observations reported by Yamada and Yasunobu (134).

The potency of an inhibitor can be related to the stability of the enzyme-inhibitor complex as well as to its reactivity towards the functional group on the enzyme with which it combines. The stability of the enzyme-inhibitor complex, in turn, depends on the nature and number of bonds joining the enzyme and inhibitor. For the studies of PAO inhibition by hydrazines, the nature of the

(primary) enzyme-inhibitor bond should be identical because all inhibitors have the same functional group; the $(I/E)_{50}$ values reported here should then reflect a difference in the number of bonds.

Studies by Bernstein et al. (13) on the inhibition of mouse brain amine oxidase by stereoisomers of hydrazine inhibitors demonstrated the stereospecificity of the reaction. These results imply that the hydrazines are bound to the enzyme by a multipoint attachment, which would necessitate the presence of more than one binding locus. One of these could possibly involve the hydrogen on the substituted nitrogen of the inhibitor molecule. This would be in accord with Hinman's interpretation of the role of this hydrogen atom as well as with the suggestion of Sarkar et al. (104), Barsky et al. (10) and Zeller (140, 145) that a hydrogen which is alpha to the $-NH_2$ group of the hydrazine moiety is involved in the binding of the inhibitor to the enzyme. The participation of this alpha hydrogen in the binding of hydrazines to PAO could explain the great difference in potency between UDMH and the other inhibitors studied. For a hydrazine to exert its maximum inhibitory power, it must be optimally oriented so that the chemical reaction which produces the inhibition can take place. This could be accomplished through the binding of this alpha hydrogen. To produce a given amount of inhibition, UDMH, which lacks such a hydrogen atom, must be present in amounts much greater than the other inhibitors (H, MMH, BOH,

and BH) which possess an alpha hydrogen. The diverse effectiveness of the latter class of hydrazines most likely is determined by steric and electronic effects of the substituents.

If the inhibition mechanism does indeed involve the nucleophilic attack of the hydrazine molecule on a carbonyl functional group of the enzyme (or its Schiff base) to form a stable azomethine, as was implied in the preceding discussion and suggested by the spectral results of Yamada and Yasunobu, then another inhibitor binding site could well be this carbonyl moiety. Because the combination of an inhibitor molecule with this locus results in the formation of a stable covalent bond which is most likely responsible for the potent and irreversible nature of the inhibition by hydrazines, this carbonyl group (or its Schiff base) may be considered to be the primary binding site for the hydrazine inhibitors. Since unsubstituted hydrazine was found to be the most effective inhibitor in the series studied, the molar stoichiometry of its combination with PAO might then be taken as the best measure of the number of primary binding sites present on the enzyme molecule.

Titration experiments (Tables 4 and 7) showed that hydrazine produced 50% inhibition when the inhibitor to enzyme mole ratio was 0.53; 100% inhibition would then occur when 1.06 moles of H combined with one mole of PAO, which corresponds exactly to the PLP content of the enzyme. This stoichiometry between the number of

moles of hydrazine required for complete inhibition and the PLP content of the enzyme suggests that the carbonyl functional group of PLP is the primary inhibitor binding site. A more conclusive proof of this hypothesis would come from the isolation of a stable enzyme-inhibitor complex from which the number of moles of enzyme-bound inhibitor could be determined by other than kinetic means. Preliminary studies of this nature which have been carried out (see Section IC) support the foregoing conclusions.

## C. Isolation of an Enzyme-Inhibitor Complex

In an attempt to verify the kinetically determined stoichiometry of the inhibition of PAO by hydrazines, the enzyme was reacted with radioactive inhibitor according to the procedure outlined in Table 8. If the kinetic results are valid, the amount of protein-bound inhibitor obtained in this experiment should be the same as the amount of UDMH needed to give 100% inhibition determined from the titration experiments. The $(I/E)_{50}$ value for UDMH was found to be 1.96 (Tables 4 and 7), so for complete inhibition of one mole of PAO, 3.92 moles UDMH should be necessary. The results of the experiments described in Table 8 show that 0.42 mole of UDMH-$^{14}$C was bound per mole of PAO. This is considerably less than the amount predicted from the titration experiments.

This apparent discrepancy can be explained as follows. The

Table 8. Binding of UDMH-$^{14}$C to PAO.

| | | | Time (hours) | Enzyme Activity (S.U./mg) | Percent Inhibition |
|---|---|---|---|---|---|
| | UDMH-$^{14}$C | + PAO | 0 | 5.1 | 0 |
| Mole Ratio | 85.4 | 1 | | | |
| | | Incubation | 0.5 | 1.2 | 76.5 |
| | | Sephadex G-25 | | | |
| | (UDMH-$^{14}$C-PAO Complex) | | 3 | 2.8 | 45.1 |

40 μμmoles UDMH-$^{14}$C bound/mg protein
0.42 mole UDMH-$^{14}$C bound/mole PAO

Random labelled UDMH-$^{14}$C (0.34 μmole) was incubated for 30 minutes at room temperature with 38.6 mg of partially purified PAO in a total volume of 1.2 ml, then passed through a Sephadex G-25 column. The amount of protein-bound radioactivity eluted from the column was determined by liquid scintillation methods. Enzyme activity was assayed before adding the inhibitor (0-time), after the 30 minute incubation of enzyme and inhibitor, and subsequent to elution from the column. Results are an average of two determinations. Additional experimental details are described in Methods.

treatment of the data in the preceding section led to the conclusion that PAO probably contained two types of inhibitor binding sites. One of these (involving the carbonyl of PLP) participated in the formation of a stable covalent bond between the enzyme and the hydrazine inhibitor, and the other functioned through non-covalent forces. From structural considerations, the stoichiometry obtained from the inhibition by unsubstituted hydrazine was taken to be the best measure of the number of primary (covalent) binding sites on the enzyme. One might then expect that the amount of protein-bound UDMH determined by physical methods would better correspond to the value predicted from the titration of PAO with unsubstituted hydrazine.

Hydrazine was found to produce 50% inhibition when the inhibitor to enzyme mole ratio was 0.53; 100% inhibition should then occur when 1.06 moles of inhibitor are bound per mole of PAO, but the amount of enzyme-bound UDMH-$^{14}$C found in this experiment is still too low. This disparity can be resolved by a consideration of the enzyme activity at the different stages of the experiment.

The time studies of the inhibition reaction presented in Section 1B-2 demonstrated that the inhibition had attained its maximum level after a thirty minute incubation of enzyme and inhibitor prior to the addition of substrate. In this experiment, quite unexpectedly, some of the enzyme activity was regained after passing the

enzyme-inhibitor mixture through the Sephadex column (Table 8). This observation may be explainable in terms of the apparent reversal of the inhibition which occurs when enzyme and inhibitor in solution are incubated at room temperature (Section IIIC).

About three hours elapsed between the addition of the UDMH-$^{14}$C to PAO and the measurement of enzyme activity after elution from Sephadex. Studies of the reversal of inhibition showed that about half of the enzyme activity is regained when excess UDMH is incubated with PAO at room temperature for a corresponding time period. Although the causes of this phenomenon are not yet completely clear, it may result from the inhibitor no longer being irreversibly bound to the enzyme (Section III). If this were true, the degree of inhibition would then be a measure of the amount of inhibitor that is irreversibly bound to the enzyme.

The UDMH-$^{14}$C-PAO complex eluted from the column is 45.1% inhibited. The mole ratio of hydrazine to PAO which gives 45.1% inhibition (determined from a titration curve as described in Section IB-3) was found to be 0.465, which is in good agreement with the amount of labelled inhibitor bound in the enzyme-inhibitor complex isolated in this experiment.

Both the fact that the amount of enzyme-bound UDMH-$^{14}$C is less than is expected from the kinetic determinations, as well as the decreased inhibition observed subsequent to passing the

enzyme-inhibitor mixture through the Sephadex column might possibly be explained in terms of the behavior of Zone B inhibitors towards dilution (48, 114). However, the six-fold dilution obtained when the enzyme-inhibitor mixture was passed through the column is not sufficient to account for the observed degree of reversal of inhibition (114). These dilution effects therefore would also be inadequate to fully resolve the discordance between the results obtained from the kinetic and radio-tracer methods.

Although it is likely that both the dilution effects and the time-dependent apparent reversal of inhibition in solution (Section IID) play a role in explaining the results obtained here, it is not yet clear to what extent. Regardless of which explanation is employed, it is possible to conclude that the number of moles of UDMH-$^{14}C$ bound per mole of enzyme is in agreement with the stoichiometry of enzyme-inhibitor combination obtained kinetically. The unique specificity of hydrazine inhibitors for PAO is likewise verified.

## Discussion

### A. General Properties of the Inhibition of PAO by Hydrazines

The data presented in the preceding sections have demonstrated that hydrazines are potent, pseudo-irreversible inhibitors of PAO. It was shown kinetically and by radiotracer methods that the

interaction between PAO and hydrazine inhibitors is extremely specific, exhibiting a molar stoichiometry even in crude systems.

Although the effectiveness of hydrazines as amine oxidase inhibitors is a well-documented fact, little, if any, attention has been drawn to the stoichiometric nature of the inhibition and possible applications of this knowledge. One of the few investigations along this line was made by Smith, Weissbach, and Udenfriend (113) on the inhibition of partially purified guinea pig liver mitochondrial amine oxidase by iproniazid. They demonstrated the inhibition to be irreversible (to dialysis and treatment with 8 M urea) and specific (the inhibitor was found to bind only to amine oxidase even when present in a mixture of several other proteins), and showed that about twice as much iproniazid-$^{14}C$ was bound than was required for complete inhibition.

The number of known stoichiometric enzyme inhibitors is small; diisopropylfluorophosphate, reacting specifically with the serine at the active site of cholinesterase (38) and succinoxidase inhibition by antimycin A are two classical examples. Inhibitors of this type can be used to estimate the relative amounts of enzyme in crude preparations, tissues, or even intact organisms; in addition, the principle of enzyme titration can be applied to chemotherapy. However, the most significant consequence relevant to the studies reported here is that the stoichiometric inhibition of PAO by

hydrazines could possibly serve as a useful tool to determine the nature and number of the sites on the enzyme which will bind inhibitor molecules.

The inhibition of PAO by hydrazines, like other irreversible enzyme-inhibitor systems, is time-dependent. An incubation of the enzyme with inhibitor prior to adding substrate is required to produce inhibition at a maximum rate. Substrate, even at high concentrations, could not reverse the inhibition under these conditions. When inhibitor and substrate are added simultaneously to the enzyme, the inhibitor potency is markedly decreased with increasing substrate concentration. This protective effect of the substrate can be interpreted to mean that the inhibitor and substrate compete for the same enzyme site. This explanation is in agreement with the data obtained for similar studies with both soluble and particulate amine oxidases of various sources (9, 19, 34, 52, 56, 57, 82, 113, 134, 145).

## B. Chemical Nature of Inhibitor Binding Sites

The finding that the potency of hydrazine inhibitors as a function of structure parallels their reactivity towards carbonyls in model systems led to the conclusion that the inhibition reaction involves a carbonyl functional group on the enzyme. The stoichiometry between the kinetically determined amount of hydrazine

needed to give 100% inhibition and the PLP content of the enzyme suggests that the carbonyl of PLP is the one with which these inhibitors react.

Spectral data reported in the literature, substantiate these conclusions. The soluble amine oxidases have a characteristic absorption maximum in the 480-500 mμ region associated with the catalytic activity and attributed to the Cu (II)-PLP complex presumably at the active site. This peak, which is responsible for the pink color of these enzymes, is reversibly bleached by substrate (23, 55, 64, 84, 91, 130, 131, 132, 129). When hydrazines are added anaerobically, the pink color disappears and cannot be restored by the addition of oxygen (134). Although the results indicate a carbonyl type of reaction, the spectra obtained are not typical of the products of the reaction between these reagents and PLP. However, when Cu was removed from the enzyme, the spectral changes agreed with the spectra of the corresponding PLP-hydrazones (18, 21, 65, 89, 134). Further support comes from the studies of Davison (33), who found that the kinetics of the inhibition of pig kidney amine oxidase by the hydrazine derivative, isoniazid, parallelled those of this compound's combination with PLP. Moreover, the high value obtained for the energy of activation of the inhibition reaction (33, 34) and the fact that the inhibition was not reversed by high substrate concentration (which suggest the formation of covalent bonds between

the enzyme and inhibitor), give additional credence to the proposal that the inhibition takes place by a reaction between the hydrazine and the carbonyl of the cofactor, PLP, to form a stable hydrazone. Since it was found that the carbonyl functional group of PLP very likely exists as a Schiff base with an amino group of the enzyme, the azomethine formed between it and a hydrazine inhibitor would occur via a transaldiminization reaction.

Most PLP enzymes are strongly inhibited by carbonyl reagents such as cyanide, bisulfite, hydroxylamine, and hydrazine derivatives (20). The most potent inhibitors are those which can form stable azomethines (89, 118, 134). This has been taken as evidence for the generally accepted mechanism to be that of Schiff base formation between the inhibitor and PLP (32, 118, 124). Since it has been demonstrated that the cofactor is bound to many of these enzymes through an aldimine linkage involving the carbonyl of PLP and an $\epsilon$-amino group of the enzyme protein (3, 40, 71, 86, 107), the most effective inhibitors are thought to act as $-NH_2$ donors to displace the protein's amino group. Thus, the mode of action proposed for the PAO inhibition by hydrazines would be in agreement with that thought to be operative in the inhibition of other PLP-containing enzymes by these compounds.

In the light of the above considerations, it appears that PLP is one of the sites of hydrazine binding. The stereospecificity of

the amine oxidase reaction with hydrazines demonstrated by Bernstein (13) led to the conclusion that there is more than one inhibitor binding site. The great difference in potency between UDMH and the other inhibitors studied was related to the presence or absence of a hydrogen atom on the substituted nitrogen. From this interpretation came the idea that another of the inhibitor binding loci on the enzyme might then involve this hydrogen atom, which concurs with the interpretations advanced by Zeller and coworkers (10, 104, 145, 146).

## C. Possible Mechanisms of Inhibition

### 1. Zeller's Carbonyl Mechanism.

The data and conclusions presented in the preceding discussion favor the hypothesis that a carbonyl-type interaction is responsible for the inhibition of PAO by hydrazines. Zeller's laboratory has proposed a similar mechanism for the hydrazine inhibition of the mitochondrial enzyme. They suggest that hydrazines react with the enzyme by virtue of their nucleophilic nature to form a hydrazone with a carbonyl group present on the enzyme. Since mitochondrial amine oxidase contains neither PLP nor any other readily detectable aldehyde or ketone, Zeller proposed that the carbonyl residue could be part of an amide, peptide, or ester linkage. Results of inhibitor specificity studies on alpha-substituted hydrazines have been explained by the

assumption that one alpha hydrogen is essential for the formation of the enzyme-inhibitor complex, while a second alpha hydrogen, if available, participates in the process of dehydrogenation (the step subsequent to the initial nucleophilic attack), resulting in a thermodynamically stable enzyme-inhibitor complex (10, 25, 104, 145, 146).

2. Davison's Dehydrogenation Scheme. Davison's (34) interpretation of the mechanism of inhibition is illustrated in Figure 2. He postulated that the initial interaction of amine oxidase with a hydrazine derivative results in the formation of a reversible enzyme-inhibitor complex (I). Then in the presence of $O_2$, a dehydrogenation occurs to form the -ylidine derivative (II), which combines irreversibly with the enzyme by an addition reaction across the new double bond to give (III).

3. Alkyl Free Radical Interpretation. Another school of thought is that the inhibition occurs by a free radical mechanism. This opinion, likewise, has arisen from studies on mitochondrial amine oxidase.

On the basis of the many similarities between the hydrazine inhibition of amine oxidase and the autoxidation of hydrazines which is catalyzed by Cu (II), Eberson and Persson (37) and Green (57) have concluded that the two reactions occur by analogous mechanisms. The scheme in Figure 3 outlines the sequence of

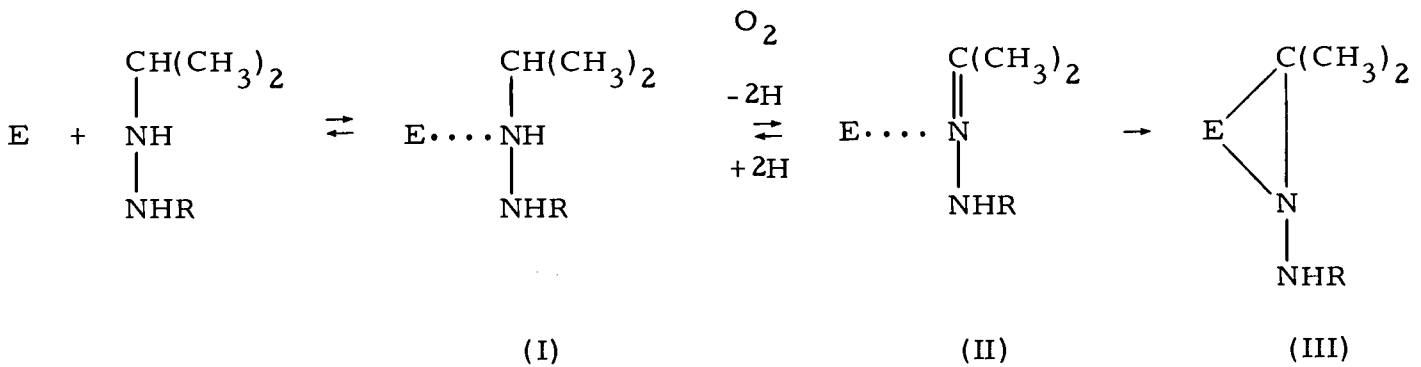


Figure 2. Mechanism of hydrazine inhibition of rat liver mitochondrial amine oxidase proposed by Davison.

Illustration is for iproniazid, where R = isonicotinoyl.

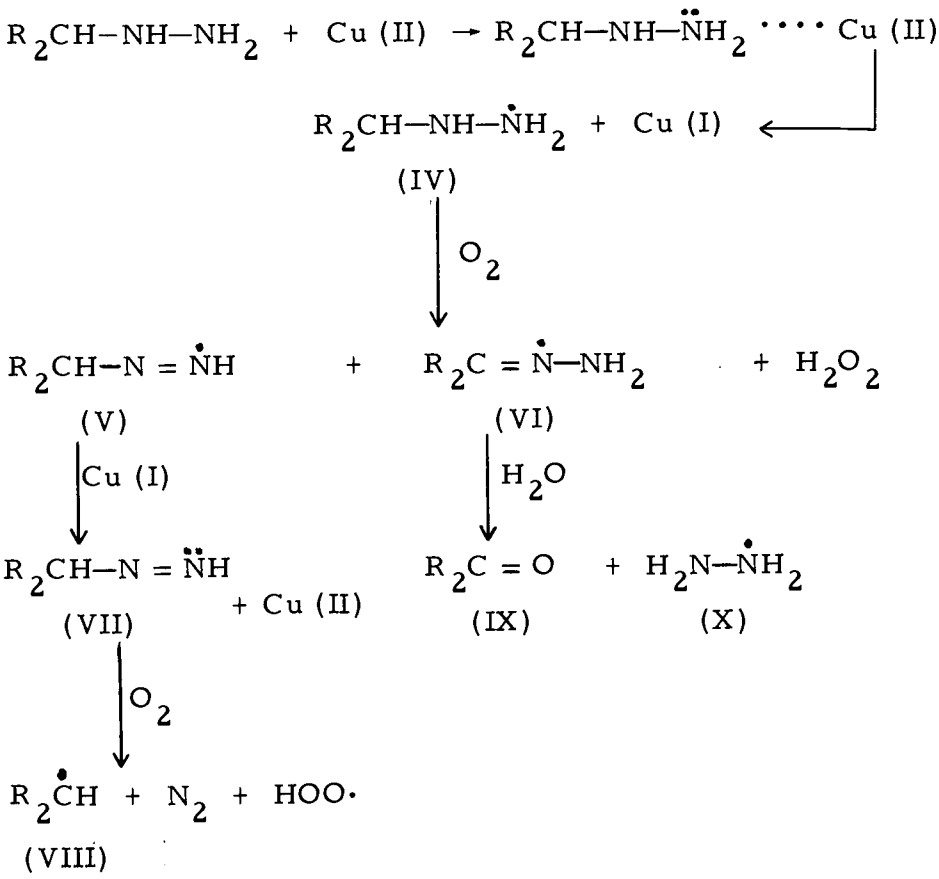


Figure 3. Mechanism for the oxidative decomposition of hydrazines catalyzed by Cu (II) postulated by Eberson and Persson.

reactions which Eberson and Persson have proposed to account for the catalytic decomposition of hydrazines they studied in model systems. Initially a complex is formed between the hydrazine and Cu (II). In the decomposition of this complex (which was found to be the rate-determining step), a single electron is transferred from the hydrazine to Cu (II) to form the radical (IV) and Cu (I). An oxidation by molecular $O_2$ produces the unsaturated species (V) and (VI) which react further as shown to give other radicals, regenerating Cu (II) in the process. These workers suggest that in the enzymatic reaction, in the presence of $O_2$, the Cu (II) of the enzyme could effect the catalysis of hydrazine decomposition, liberating free radicals in the vicinity of the active site. These radicals could then combine with the enzyme to produce irreversible inhibition. Their results indicated that alkyl or aryl radicals are the inhibiting species because unsubstituted hydrazine itself does not inhibit. Since it is well known that thiols are readily susceptible to free radical alkylation, it was proposed that the locus of attack of these radicals is a sulfhydryl group at the enzyme's active site (37). This conjecture could well explain the observations that the inhibitor potency is decreased by oxidzed glutathione (34, 57) and that dimercaptopropanol partially reactivated the inhibited enzyme (34).

Bloom (19) likewise attributed the inhibition to an alkylation

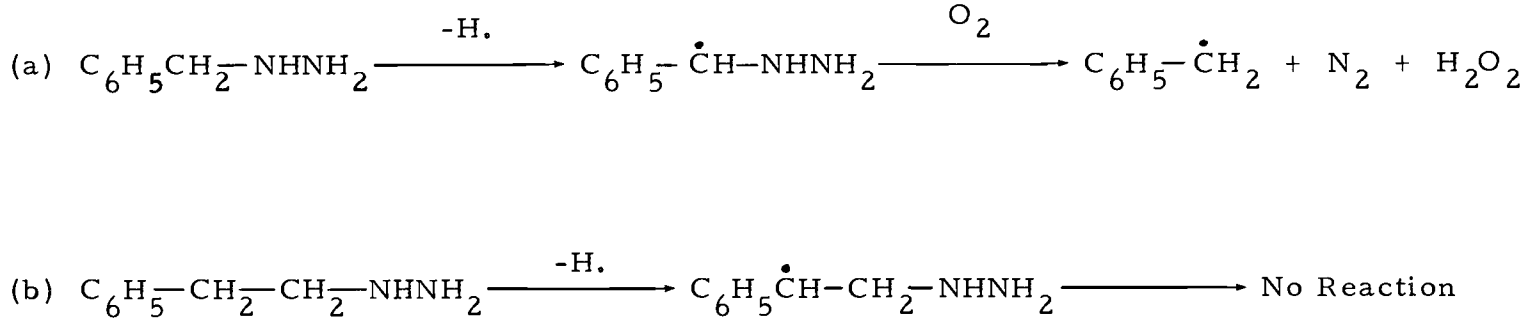


Figure 4. Reaction sequence for the generation of alkyl radicals from hydrazines as proposed by Bloom.

Illustrated for (a) benzylhydrazine
(b) β-phenylethylhydrazine

of the enzyme's catalytic site via a free radical mechanism, but his interpretation differs from that of Green and Eberson and Persson in that he postulates an oxygenative attack by an unspecified component of the enzyme molecule to produce a hydrazine radical in which the lone electron is located on a carbon rather than a nitrogen atom. This intermediate subsequently decomposes to $N_2$, $H_2O_2$, and an alkyl radical--the inhibiting species (see Figure 4).

4. Copper Reduction Theory. Since hydrazines are powerful reducing agents, another possibility to be considered is that their inhibition of amine oxidase activity is caused by their reduction of the enzyme's Cu (II) to Cu (I). Copper has been shown to be an essential amine oxidase cofactor (18, 21, 22, 23, 24, 27, 55, 57, 61, 65, 81, 82, 87, 90, 91, 95, 115, 129, 130, 131, 133, 134, 135) and it has been demonstrated by both chemical and physical means to be present entirely in the cupric state (23, 24, 55, 64, 95, 130, 133). In addition, both chemical determinations and ESR studies have shown that it does not change valence during the catalytic reaction in the presence of substrate (18, 21, 24, 55, 61, 90, 91, 95, 129, 130, 133, 135). Thus, it would not be difficult to visualize how a reduction of the enzyme's cupric copper to the cuprous state might be deemed responsible for the observed loss of activity in the presence of hydrazines.

5. Copper Chelation Hypothesis. In conjunction with the facts

that Cu (II) is essential to amine oxidase activity and that hydrazines are known to readily chelate metal ions, another feasible conception of the nature of the inhibition is that it could be due to an irreversible complex formation between the hydrazine and the copper present in the enzyme.

## D. Differences Between the Hydrazine Inhibition of Mitochondrial and Soluble Amine Oxidases

Thus there appear to be five different theories on how the hydrazine inhibition of amine oxidases could take place. The salient features of each of these have been summarized above. Before proceeding with the attempt to assign the mechanism which best explains the experimental results obtained both in the literature and in the studies presented here, it is well to recall the differences between the properties of the mitochondrial and soluble amine oxidases which were pointed out in the Introduction.

With respect to their inhibition by hydrazines, the two classes of enzymes differ in that inhibition of the particulate amine oxidases requires oxygen (9, 19, 34, 37, 57, 113, 145) and is potentiated by cyanide (34, 36, 57, 113), whereas the spectral effects observed when PAO and hydrazines were incubated anaerobically would seem to indicate that the inhibition of the soluble enzymes can proceed in the absence of oxygen (134), and no effect of cyanide has been noted.

In addition, the structural requirements of the hydrazine inhibitors differ markedly for the two types of enzymes: mitochondrial amine oxidases can be inhibited only by substituted hydrazines (17, 25, 57, 104, 140, 144, 145, 147), but the soluble enzymes can be inhibited by unsubstituted hydrazine as well (17, 68, 89, 91, 129, 130, 134).

One must then bear in mind that because hydrazine derivatives inhibit both particulate and soluble amine oxidase activities, the similarity in the mechanisms of inhibition or the sites on the enzymes with which the inhibitors combine is not a necessary requisite. In view of the foregoing considerations it indeed seems likely that one or both of these would be different.

## E. Assignment of an Inhibition Mechanism to the Mitochondrial Amine Oxidases

Eberson and Persson (37) and Green (57) have pointed out several experimental observations which weaken Zeller's idea that the inhibition of mitochondrial amine oxidase is caused by the nucleophilic attack of the hydrazine moiety on a carbonyl group of a peptide, amide, or ester bond in the enzyme in a reaction analogous to that occurring with an ordinary aldehyde or ketone group. The inhibition requires the presence of oxygen, is potentiated by cyanide, and decreased by oxidized glutathione, which this theory does not predict. Also, it is difficult to explain why simple alkyl hydrazines

are potent inhibitors but conventional carbonyl reagents such as hydroxylamine and unsubstituted hydrazine are inert. In addition, the concept of an activated peptide, amide, or ester carbonyl function capable of reacting with a hydrazine group in the way indicated above is entirely hypothetical and does not correspond to any known reaction of these groups. The observation that no decrease in inhibition resulted when benzylhydrazine was incubated with a large excess of sucrose or PLP prior to its addition to the enzyme (56) would seem to further disprove Zeller's theory.

Green (57) has argued against the mechanism proposed by Davison (Figure 2) on the basis of the finding that the unsaturated intermediates (II) did not themselves inhibit (34). This could be explained if the dehydrogenation and addition steps were to occur simultaneously without the incipient double bond ever fully forming, or if it were assumed that the energy of activation for the formation of the enzyme-inhibitor complex is too high for the unsaturated compounds, which would require the dehydrogenation to occur while the inhibitor is bound to the enzyme. If this were the case, there must be a hydrogen atom on the alpha carbon for the dehydrogenation to take place; but Green's data showed that the presence of such a hydrogen was not essential for inhibitory activity ( 57 ). The alternate possibility, that the double bond is formed between the two nitrogen atoms, fails to explain the lack of inhibitory power of

unsubstituted hydrazine. This mechanism can also be ruled out for PAO because of its oxygen requirement.

The proposal that the inhibition occurs by an alkyl radical mechanism analogous to the copper-catalyzed decomposition of hydrazines finds its support in the many similarities between the two reactions. Oxygen is required in the model system as it is for the inhibition of (mitochondrial) amine oxidases (37, 57). Both are enhanced by cyanide (34, 37, 56, 57, 113) and retarded or suppressed by compounds which can chelate or reduce cupric copper (57). Moreover, the oxidation-reduction potentials of amine oxidase, hydrazines, and the cupric-cuprous couple are favorable for electron transfer between hydrazines and the enzyme analogous to the transfer of an electron from a hydrazine to Cu (II) in the model reaction (37). Although the catalytic decomposition (Figure 3) occurs for all hydrazines, it appears to be necessary to have an alkyl or aryl substituent for the inhibition reaction, since hydrazine itself and unsubstituted hydrazides do not inhibit; also, N-benzylhydroxylamine (which would decompose like benzylhydrazine) is an inhibitor but hydroxylamine is not. This theory has one apparent weakness. In a study of inhibitor potency as a function of structure on the guinea pig liver mitochondrial enzyme, Green (57) observed that increasing N-substitution of the hydrazines decreased inhibitor potency; however the results of Eberson and Persson (37) showed

that this same structural variation had no effect on the rate of the Cu (II)-catalyzed decomposition. Green ruled out steric hindrance as the cause since the isosteric amines were equally reactive as reversible competitive inhibitors, and offered as an explanation that perhaps the Cu (II)-catalyzed decomposition of disubstituted hydrazines could give rise to two free radicals which combine more readily with each other than with the enzyme. Bloom's mechanism (Figure 4) is in close agreement with that postulated by Eberson, Persson, and Green except that he does not speculate on how the copper of the enzyme might be responsible for the generation of the inhibiting alkyl radicals.

Thus, of the three mechanisms described in the literature based on studies of mitochondrial amine oxidases, the alkyl radical theory seems best to explain the hydrazine inhibition of this enzyme. The suggestion that the locus of radical attack is an -SH group in the active site is open to question, as was noted earlier. The detection of semiquinone formation during catalytic activity (70) and the fact that an FAD peptide is an essential cofactor of the mitochondrial enzymes make the flavin moiety another likely candidate. The possibility that the inhibition results from hydrazine reduction of the enzyme's copper is not compatible with the fact that oxygen is required for the inhibition since $O_2$ can oxidize Cu (I) to Cu (II) The copper-chelation hypothesis cannot be ruled out, but since

copper does not seem to be an absolute requirement for the catalytic activity of these enzymes, this explanation is not as satisfactory.

## F. Assignment of an Inhibition Mechanism to the Soluble Amine Oxidases

In contrast to the mitochondrial enzymes, the hydrazine inhibition of the soluble amine oxidases[5] cannot be adequately explained by the alkyl radical mechanism.

The rate-determining step in Bloom's mechanism is the decomposition of the hydrazine radical to the alkyl radical, $N_2$, and $H_2O_2$. He proposed that this decomposition results from the instability of the heteropolar $-\dot{C}-N$ bond. Characteristic of all radical mechanisms, the initial hydrogen abstraction favors the formation of the most stable radical. If, as in the case of reaction (a) of Figure 4, the only radical possible is on a carbon adjacent to the hydrazine moiety, the resulting $-\dot{C}-N$ bond is particularly unstable and decomposes very readily. Bloom's study of the relation between chain length in arylalkylhydrazines and inhibitor potency revealed that the most effective amine oxidase inhibitors had bonds of this type. (Comparing reactions (a) and (b) in Figure 4, benzylhydrazine

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[5] No other reports have appeared to date on the mechanism of inhibition of these enzymes, so the conclusions based on the results of studies with PAO presented in this thesis may not be valid for the entire class of soluble amine oxidases.

was found to be a far more potent inhibitor than β-phenylethylhydrazine). In the light of these considerations, BH, BOH, MMH, and UDMH would then be expected to exhibit approximately equal effectiveness as amine oxidase inhibitors since the initial oxygenative attack would give rise to radicals which contained the $-\dot{C}-N$ bond for all four compounds. This clearly is <u>not</u> the case for their inhibition of PAO (Table 7).

Perhaps the most conclusive evidence against the alkyl radical mechanism's being operative in the hydrazine inhibition of PAO is that unsubstituted hydrazine was found to be the most potent inhibitor of all. Nevertheless, a radical mechanism analogous to the Cu (II)-catalyzed decomposition of hydrazines cannot be ruled out on this basis, because unsubstituted hydrazine can undergo this reaction. However, since radicals are highly reactive species and can react rather unspecifically in a number of ways giving rise to irreversible changes by virtue of their ability to act as oxidizing agents, to form covalent bonds, or to initiate chain reactions, the high degree of specificity displayed by the interaction of hydrazines with PAO would be difficult to explain in terms of a radical mechanism. Evidence which further diminishes the likelihood of this theory is that oxygen is required for the autoxidation of hydrazines catalyzed by Cu (II) but is not essential for inhibition to occur. In addition, it would be difficult to account for the fact that increased

N-substitution of hydrazines strikingly decreases the inhibitor potency but has no effect on their Cu (II)-catalyzed decomposition.

Hydrazines, besides being carbonyl reagents, possess powerful reducing agent and metal chelation properties. Since cupric copper has been demonstrated to be essential to PAO activity, it is not unreasonable to suspect that the inhibition could be ascribed to the hydrazines' interaction with this metal cofactor. Supporting evidence comes from the observations that for several of the Cu (II)-PLP-containing amine oxidases, including PAO, enzyme activity is lost when the Cu (II) is reduced by such agents as dithionite or hydroxylamine or when certain chelating compounds are added (18, 22, 24, 55, 64, 65, 81, 82, 90, 91, 95, 131, 133, 135). The demonstration that inhibition of hog kidney amine oxidase by hydrazine derivatives can be reversed by ferricyanide, a strong oxidizing agent, is compatible with the assumption that the inhibition might be caused by a reduction of some component of the enzyme (77). However, it would be most difficult to account for the structural effects of the inhibitors, the stereospecificity of the inhibition reaction, and the spectral observations in the presence of hydrazines if the inhibition is due solely to a chelation or an oxidation-reduction reaction.

The data presented in this thesis (Section IB-6) point to the conclusion that hydrazines react with PAO by virtue of their

nucleophilic nature, with the electrophilic counterpart on the enzyme involving the aldehyde functional group of PLP or its Schiff base. However, there are several observations in the literature which indicate that the Cu (II) cofactor of the soluble amine oxidases may also play a role in the inhibition reaction.

Yamada and Yasunobu reported that the inhibition of PAO by hydroxylamine (which, like the hydrazines, is both a good carbonyl reagent and a potent reducing agent) was accompanied by a reduction of the enzyme's cupric copper to the cuprous state. Partial restoration of enzyme activity required the addition of both Cu (II) and PLP (131, 133). Spectral observations of the pig plasma enzyme in the presence of hydroxylamine (22) indicate that an alteration of both the protein-bound Cu (II) and the enzyme-PLP Schiff base occurs. Allied to these results are the findings that the copper-free apoenzymes of both the pig kidney and pea seedling amine oxidases could be reactivated only by a mixture of Cu (II) and PLP (55, 115).

The participation of both Cu (II) and PLP in the inhibition of soluble amine oxidases by hydrazines is indeed an attractive consideration, especially since several lines of evidence point to the intimate association of these cofactors in the active sites of these enzymes (18, 22, 40, 55, 61, 65, 89, 90, 91, 131, 133, 134). The possibility that hydrazines owe their inhibitory action to their

reducing ability can be ruled out on the basis of Goryachenkova's recent report (55) that the Cu (II) signal in the ESR spectrum of pig kidney amine oxidase does not change in the presence of 0.01 M phenylhydrazine, but the involvement of copper in some other manner is still feasible. Indeed, studies with Cu-PLP-valine model compounds (55) showed that these results would only discard the four coordination sites of the copper through which it is bound to the enzyme, but the possibilities that hydrazines interact with the enzyme's copper through the remaining two coordination sites or with such groups of the ligands that are not directly involved in the chelation with copper cannot be excluded.

Mondovi's observation that the anaerobic addition of substrate does not diminish the Cu (II) signal in the ESR spectrum of hog kidney amine oxidase provides evidence that substrate does not react directly with copper. However, the observed alteration of the hyperfine structure in a manner which was characteristic of the particular substrate used, would seem to indicate that the environment of copper is sensitive to the presence of substrate and the type of substrate added. Experiments using $^{14}N$- and $^{15}N$-putrescine produced no change in the ESR spectrum. The conclusion reached from these results was that the amine substrate was not a direct ligand of Cu (II), but rather, produced subtle changes in the environment of the copper (90, 91).

In view of the analogies (pointed out below) between the substrate and hydrazine reactions, an extrapolation of Mondovi's results to the inhibition reaction seems justified. On the basis of such a projection, one could eliminate the possibility (which was suggested by Goryachenkova's work with the Cu-PLP-valine model system) that hydrazine interaction with the enzyme's copper is through coordination sites which are not involved in its binding to the enzyme. If copper is involved in the inhibition reaction, then it would be most likely through groups of its ligands that are not directly involved with its chelation.

## G. Possible Application of Knowledge Obtained from the Enzyme-Inhibitor Reaction to the Enzyme-Substrate Mechanism

The numerous similarities between the substrate and inhibition reactions offer strong evidence that amine substrates and hydrazine inhibitors react at the same enzyme site(s) by analogous mechanisms. The same pH-activity curve is obtained for both the inhibition reaction and substrate oxidation (9, 19, 34). The sensitivity of the enzyme to inhibition is not changed after partial heat inactivation (34). Hydrazine inhibitors, like substrates, alter the 480-500 mμ peak associated with the catalytic activity of the soluble amine oxidases (18, 21, 55, 89, 64, 134). Both reactions appear to involve PLP as the primary binding site (Section IB-6) (18, 21, 22, 33, 55, 65, 82,

83, 84, 85, 89, 132, 133, 134), but the existence of more than one site has been suggested from structure-activity relationships of substrates (21, 83, 84, 85) and inhibitors (Section IB-6), the stereospecificity of the substrate (11, 12) and inhibitor (13) reactions, and kinetic behavior (Section II). In addition, the protective effect of substrate demonstrated in this thesis (Section IB-5) and elsewhere (9, 19, 34, 52, 56, 57, 82, 113, 134, 145) and the fact that the most potent hydrazine inhibitors are isosteric with the best amine substrates (9, 34, 37, 104, 145, 146) substantiate this proposal. Thus, it would seem possible to extend many of the conclusions obtained from the studies of the quantitative aspects of the inhibition reaction to further the knowledge of the catalytic mechanism of amine oxidase.

# II. POSSIBLE ALLOSTERIC PROPERTIES OF THE PAO-BENZYLAMINE-HYDRAZINE SYSTEM[6]

## Results

### A. Hydrazines as Homotropic Cooperative Effectors

The titration of PAO by H, MMH, and UDMH (where enzyme and inhibitor were preincubated prior to the addition of substrate) is shown in Figure 5. Note that the inhibitors exhibit a biphasic response: they activate at low concentrations and inhibit at high concentrations. The amount of activation is inversely related to inhibitor potency, and both phenomena were found to be independent of substrate concentrations ranging from 0.3 to 5.0 mM (Figures 5-8). This type of behavior is characteristic of a cooperative effect, where the attachment of one inhibitor molecule to the enzyme facilitates the binding of others. Although unsubstituted hydrazine did not produce an actual increase in activity over that of the control, the sigmoidicity of its titration curve apparent at low inhibitor concentrations is likewise indicative of the cooperative binding of H molecules by PAO.

When PAO was not preincubated with the hydrazines before substrate was added, UDMH was the only inhibitor which exhibited

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[6] This work was in part presented at the Seventh International Congress of Biochemistry, Tokyo, August 21, 1967 (69).

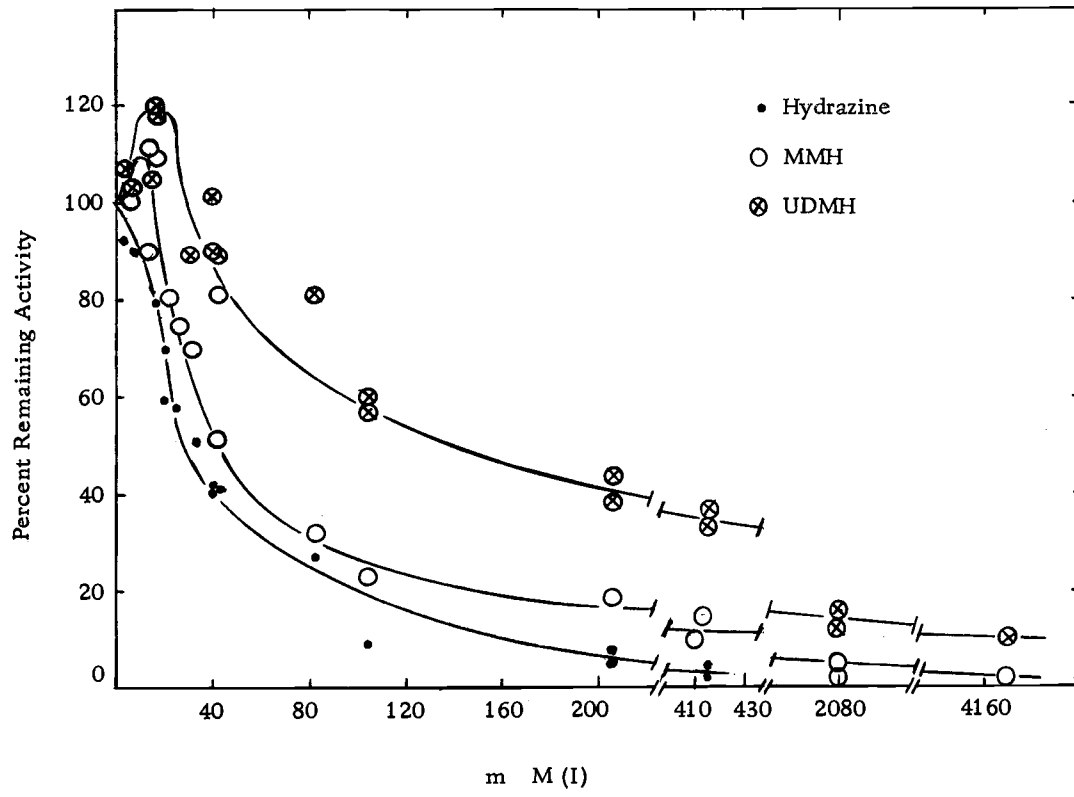


Figure 5. Titration of PAO with hydrazines.

PAO of specific activity 8.95 S.U./mg and protein concentration of 33.01 mg/ml was titrated with the indicated hydrazines according to the procedure described in Section IB-3. Enzyme and inhibitor were incubated at room temperature for 30 minutes prior to the addition of substrate. Enzyme concentration was constant at 65 mμM in the 3 ml assay system. Inhibitor concentration was varied as indicated. Each point on the curve represents the determination of enzyme activity at five different benzylamine concentrations ranging from 0.3 to 5.0 mM.

activation at low concentrations (Figure 12). This stimulation was, like the inhibition (Table 6), found to be substrate dependent. The maximum activation (for mole ratios of UDMH/PAO $\leq$ 6.67) was 26% at a substrate concentration of 0.133 mM, and decreased with increasing substrate concentration to a value of 4% at 5.0 mM benzylamine.

These sigmoidal saturation curves exhibited by hydrazines in their titration of PAO are indicative of cooperative interactions between identical ligand molecules (27, 93). This behavior is characteristic of many allosteric inhibitors (7, 92, 93, 100, 101, 103, 105, 106, 117). The biphasic titration curves are particularly typical to allosteric effectors which are substrate analogues (7, 27, 47, 93, 102, 103, 105, 106). These observations initiated the investigation into the possibility that PAO might be an allosteric protein.

Because hydrazines are substrate analogues, the apparent activation perhaps could be ascribed to the inhibitors merely acting as poor substrates. If this were the case, hydrazines might exert their inhibitory effect by preventing the binding of the better substrate, benzylamine, but might themselves be oxidized by the enzyme, thus resulting in an apparent increase in activity. This interpretation can be discarded since oxidation products of these hydrazines would not be expected to absorb at 250 mμ, the wavelength which is used to measure product formation in the standard enzyme

assay.

Another potential explanation for this observed stimulation of PAO activity at low inhibitor concentrations originates from a consideration of the effect of dilution on the zone behavior of enzyme-inhibitor systems. The amount an inhibition can be reversed by dilution is Zone A > B > C (2, 48, 114). The experimental procedure for the titrations of PAO with hydrazines employs a thirty-fold dilution in the assay of the enzyme-inhibitor incubation mixture. The inhibition of PAO by all hydrazines tested was found to exhibit Zone B kinetics, but the proximity to Zone A (hence, the susceptibility to reversal by dilution) followed the order UDMH > MMH > H (Table 4). Since this parallels the amount of activation demonstrated by these inhibitors (Figure 5), the question then arises if the observed activation might not be an artifact caused by a dilution-induced reversal of inhibition. This possibility can be eliminated because such a dilution effect could not produce a specific activity of the inhibited enzyme which is greater than that of the control. For this same reason, inhibition reversal (Section III-C) and the decomposition of the inhibitor in dilute solution can also be ruled out.

Thus it can be concluded with reasonable certainty that the biphasic response found in the hydrazine titration of PAO is indeed due to a cooperative effect. This implies that PAO contains more than one inhibitor binding site.

## B. Heterotropic Cooperative Effects between Hydrazines and Benzylamine

The series of curves which follow (Figures 6-8) show the effect of substrate concentration on PAO activity in the presence and absence of different concentrations of hydrazine inhibitors. In these experiments the enzyme was preincubated with the inhibitor until the inhibition was complete before substrate was added. There are no cooperative effects; the curves are hyperbolic at all inhibitor concentrations used, indicating that only one substrate molecule reacts.. Lineweaver-Burk plots of the same data are linear (Figure 9).

PAO was found to exhibit anomalous kinetic order with respect to substrate in the presence of hydrazines when there was no preincubation of the enzyme with inhibitors prior to the addition of substrate (Figures 10-13). The $K_m$ increased with increasing inhibitor concentration, but $V_m$ remained unchanged (Figure 13). These observations imply that an interaction with substrate binding sites is involved (92).

Sigmoidal curves of velocity versus substrate concentration and parabolic Lineweaver-Burk plots result when an enzyme interacts in some way with more than one substrate molecule (7, 8, 28, 29, 30, 76, 78, 93, 105, 106, 123, 126). Since with PAO this occurs only in the presence of hydrazines, the conclusion which follows is

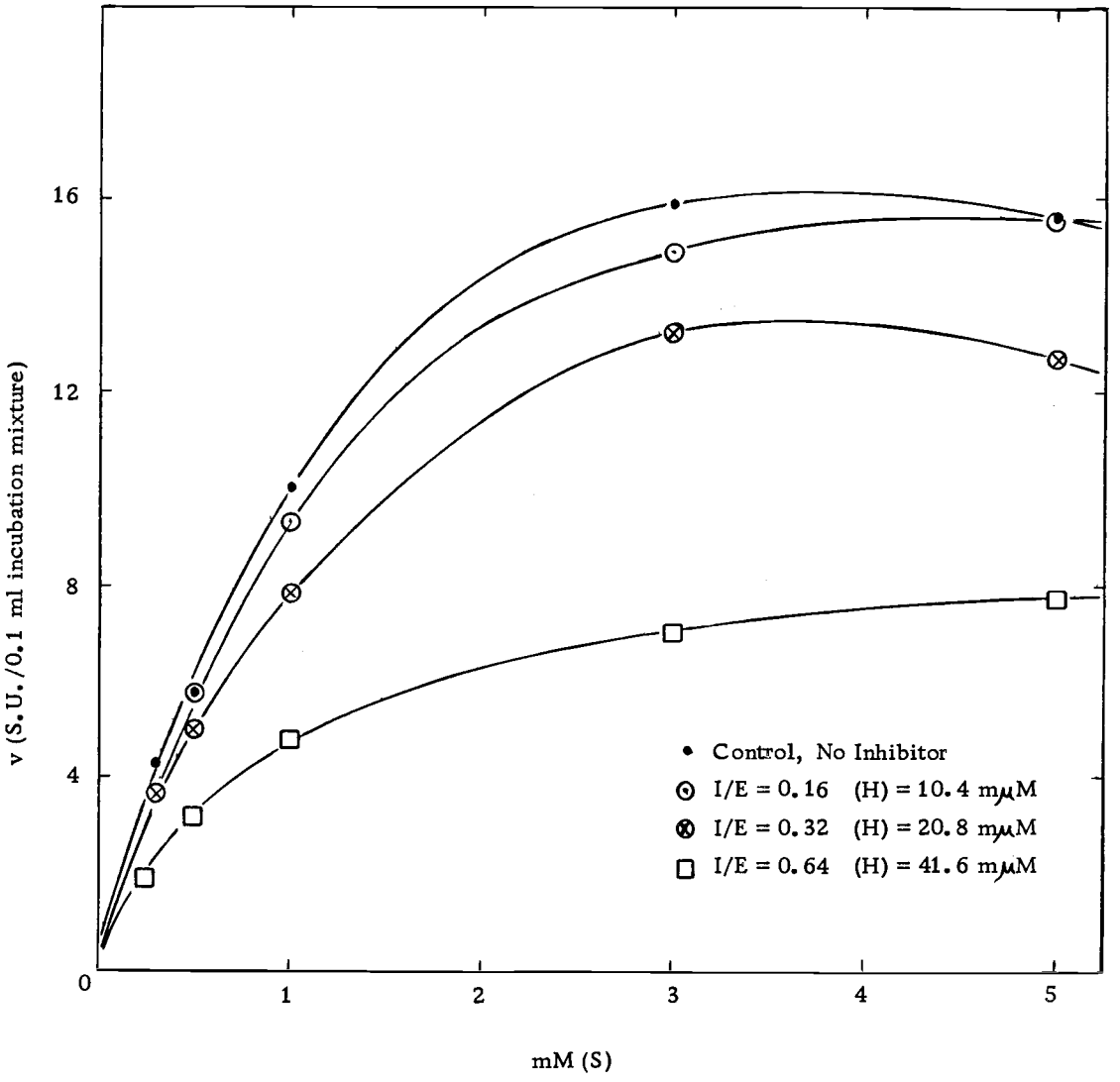


Figure 6. Effect of substrate concentration on PAO activity in the presence and absence of hydrazine. Enzyme plus inhibitor preincubated before adding substrate.

A constant amount of enzyme (0.2 ml of PAO which had a specific activity of 8.95 S.U./mg and protein concentration of 33.01 mg/ml) was incubated with varying amounts of inhibitor at room temperature in 0.06 M phosphate buffer, pH 7.0 in a total volume of 0.4 ml. At the end of a 30 minute incubation, a 0.1 ml aliquot was assayed with varying concentrations of substrate. Enzyme concentration in the 3 ml assay system was 65 mμM.

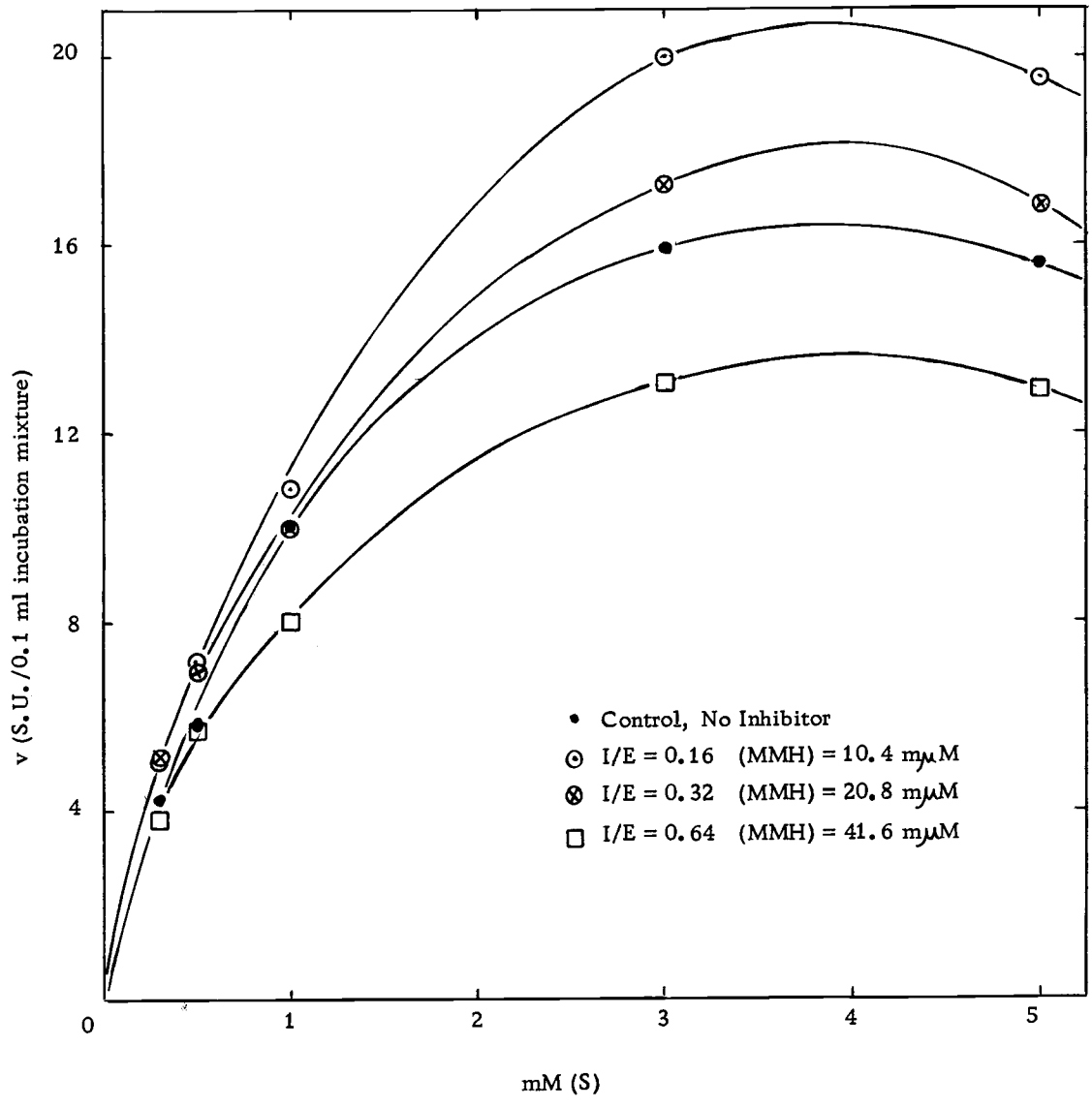


Figure 7. Effect of substrate concentration on PAO activity in the presence and absence of MMH. Enzyme plus inhibitor preincubated before adding substrate.

Experimental details are the same as Figure 6.

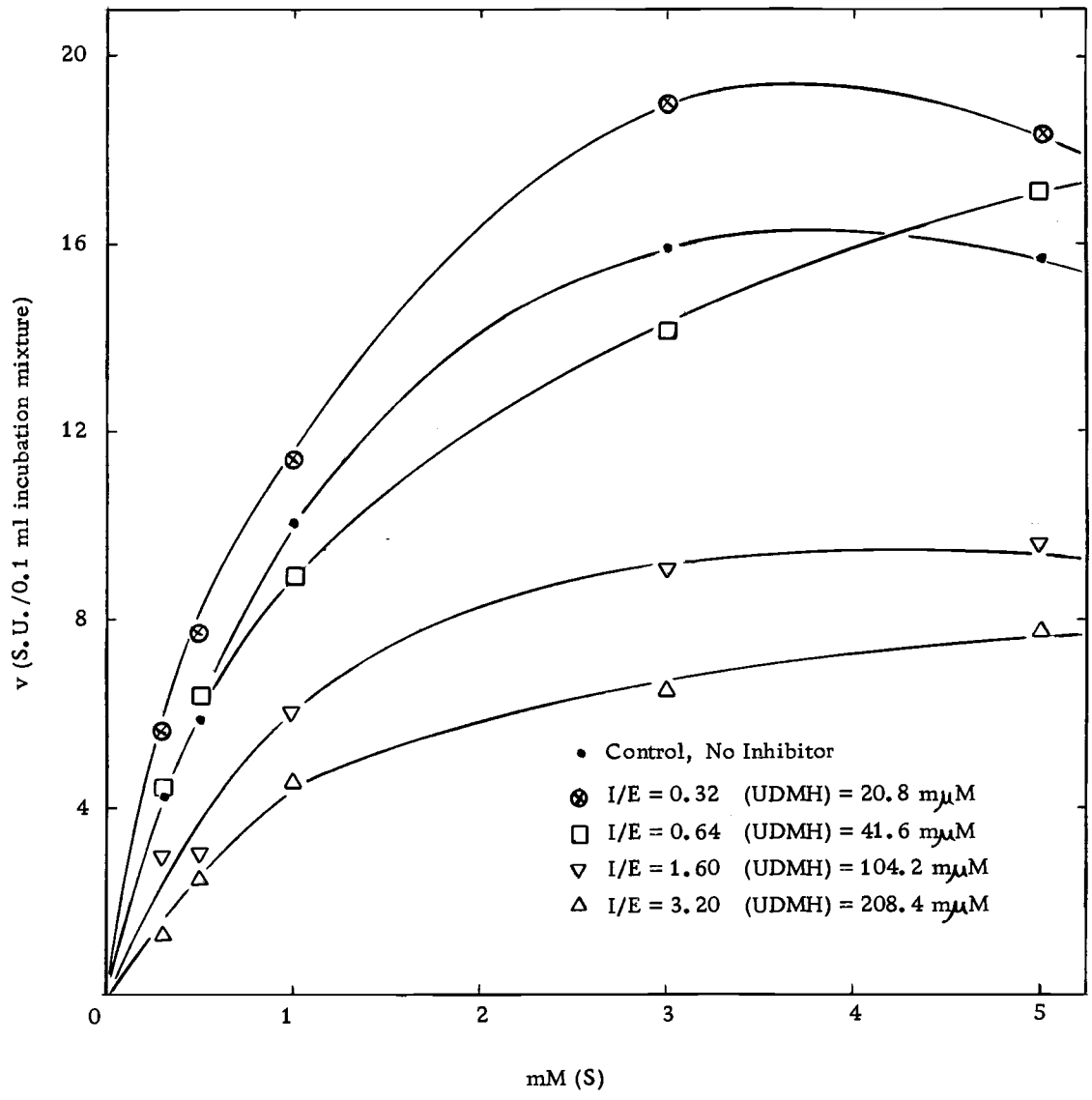


Figure 8. Effect of substrate concentration on PAO activity in the presence and absence of UDMH. Enzyme plus inhibitor preincubated before adding substrate.

Experimental details are the same as Figure 6.

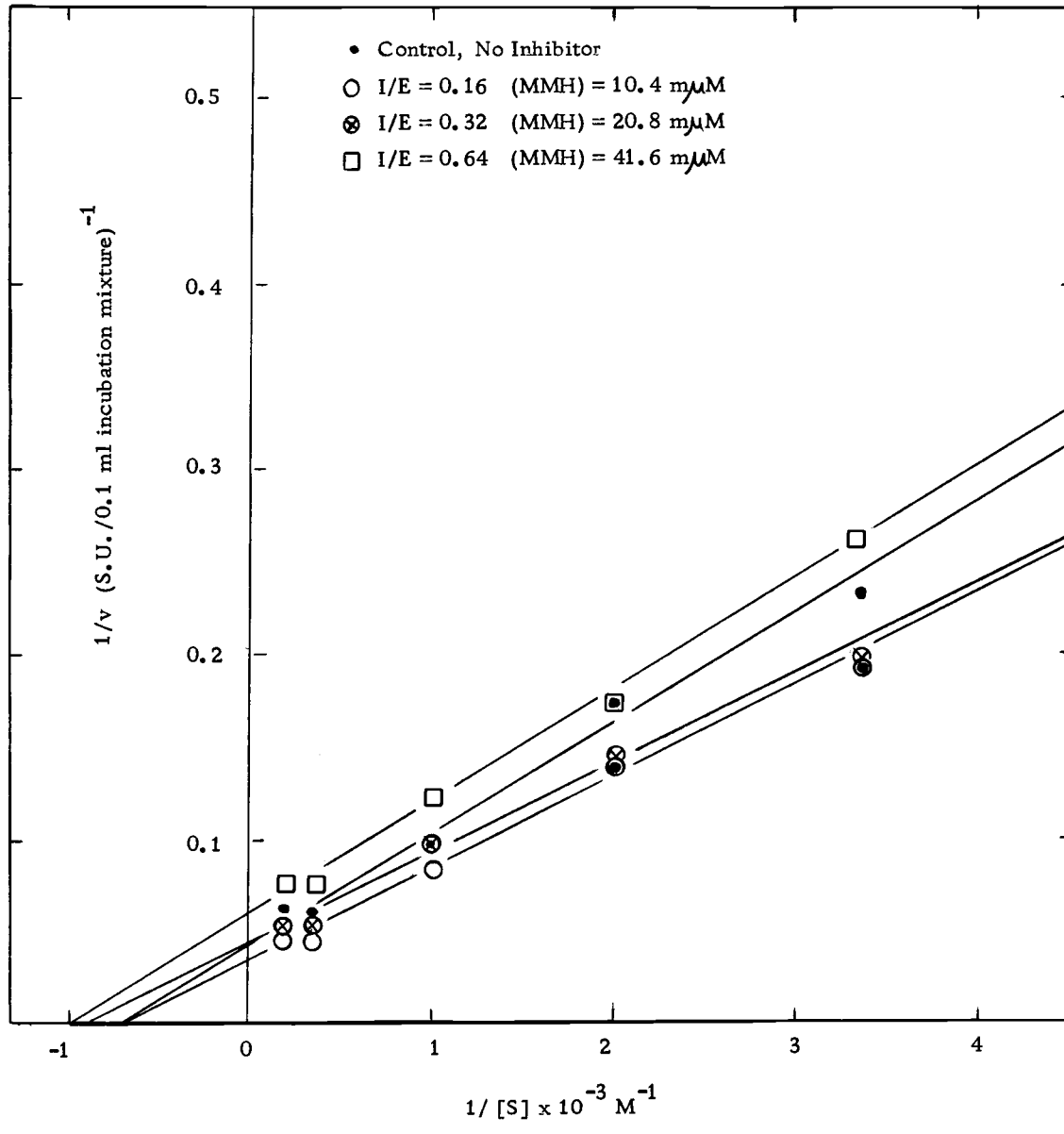


Figure 9. Lineweaver-Burk analysis of the data of Figure 7.

Experimental details are the same as Figure 6.

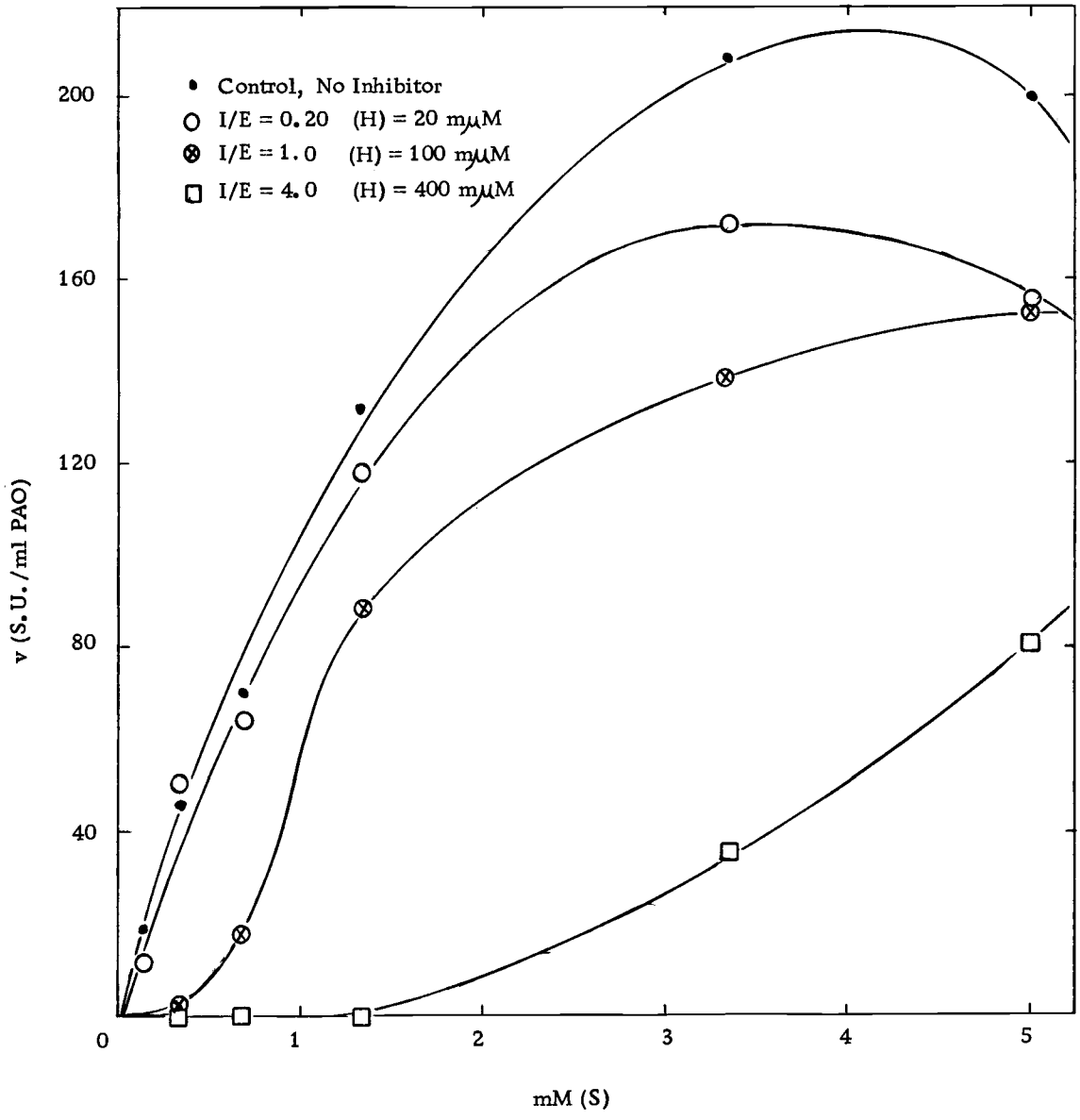


Figure 10. Effect of substrate concentration on PAO activity in the presence and absence of hydrazine. No preincubation.

A constant amount (0.1 ml) of PAO with a specific activity of 318 S.U./mg and protein concentration of 0.653 mg/ml was added to the assay cuvette containing the indicated concentrations of inhibitor and substrate. Enzyme activity was determined immediately. Benzylamine concentration varied from 0.133 to 5.0 mM and PAO concentration was constant at 100 mμM in the 3 ml assay.

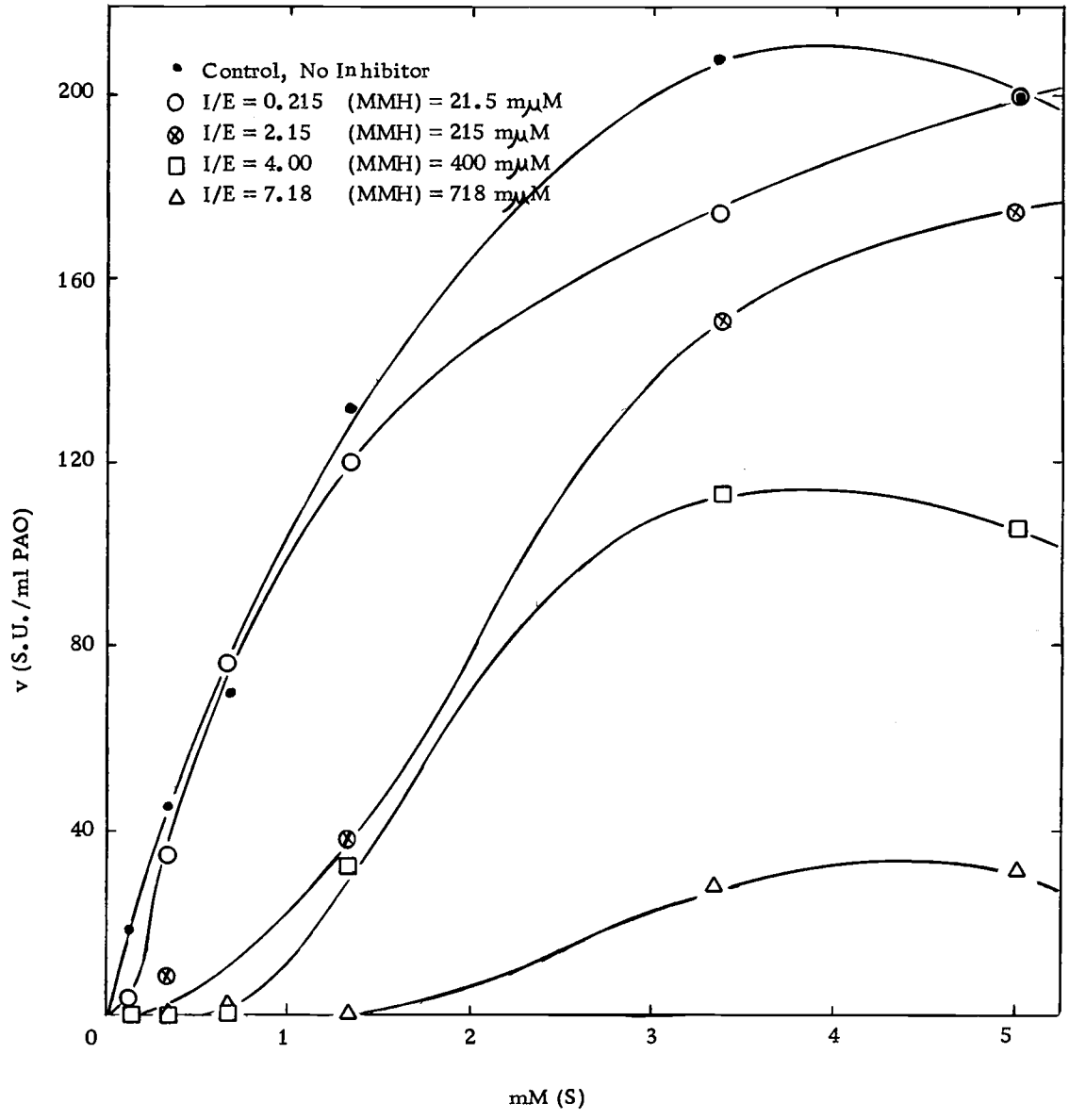


Figure 11. Effect of substrate concentration on PAO activity in the presence and absence of MMH. No preincubation.

Experimental details are the same as Figure 10.

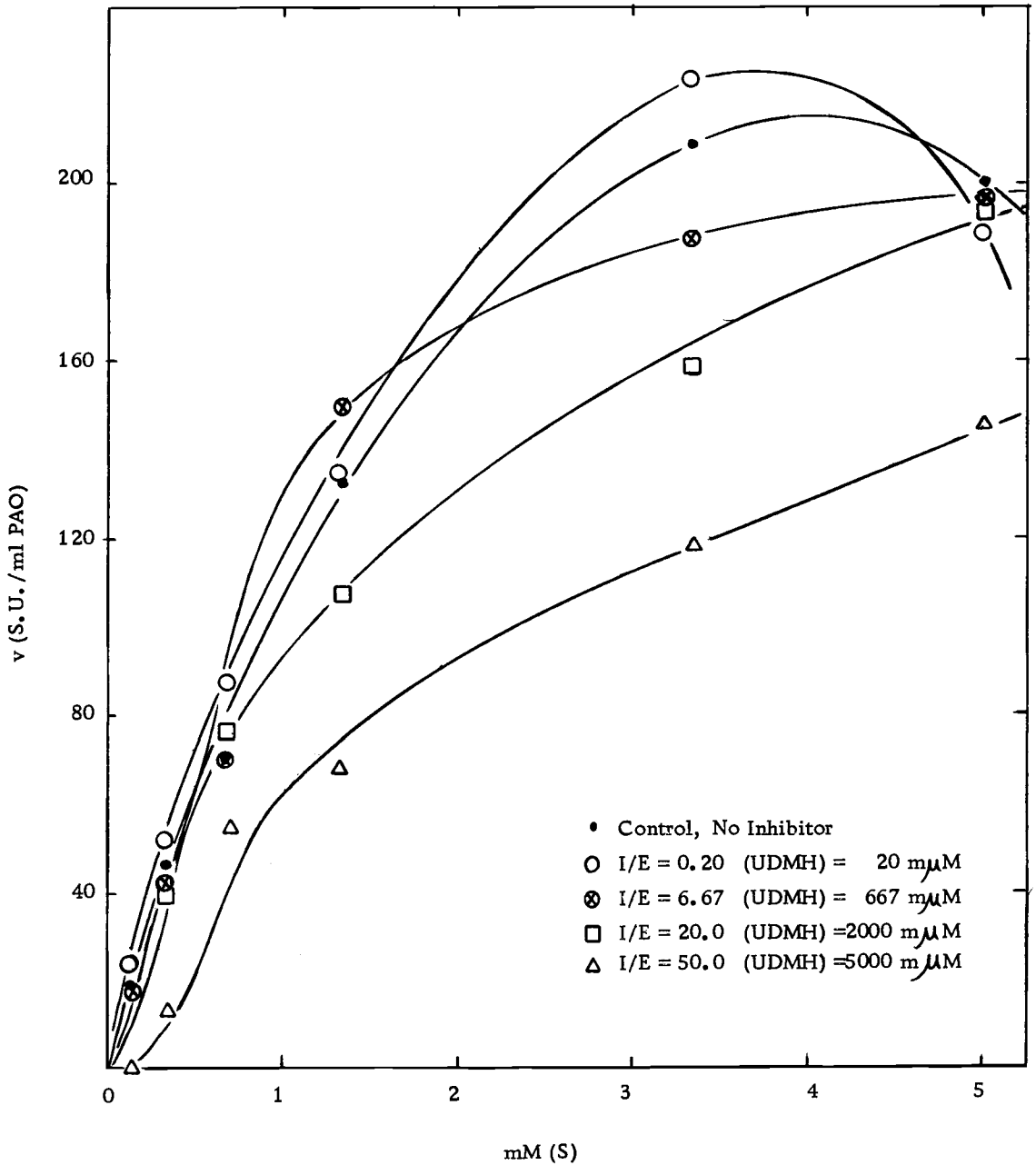


Figure 12. Effect of substrate concentration on PAO activity in the presence and absence of UDMH. No preincubation.

Experimental details are the same as Figure 10.

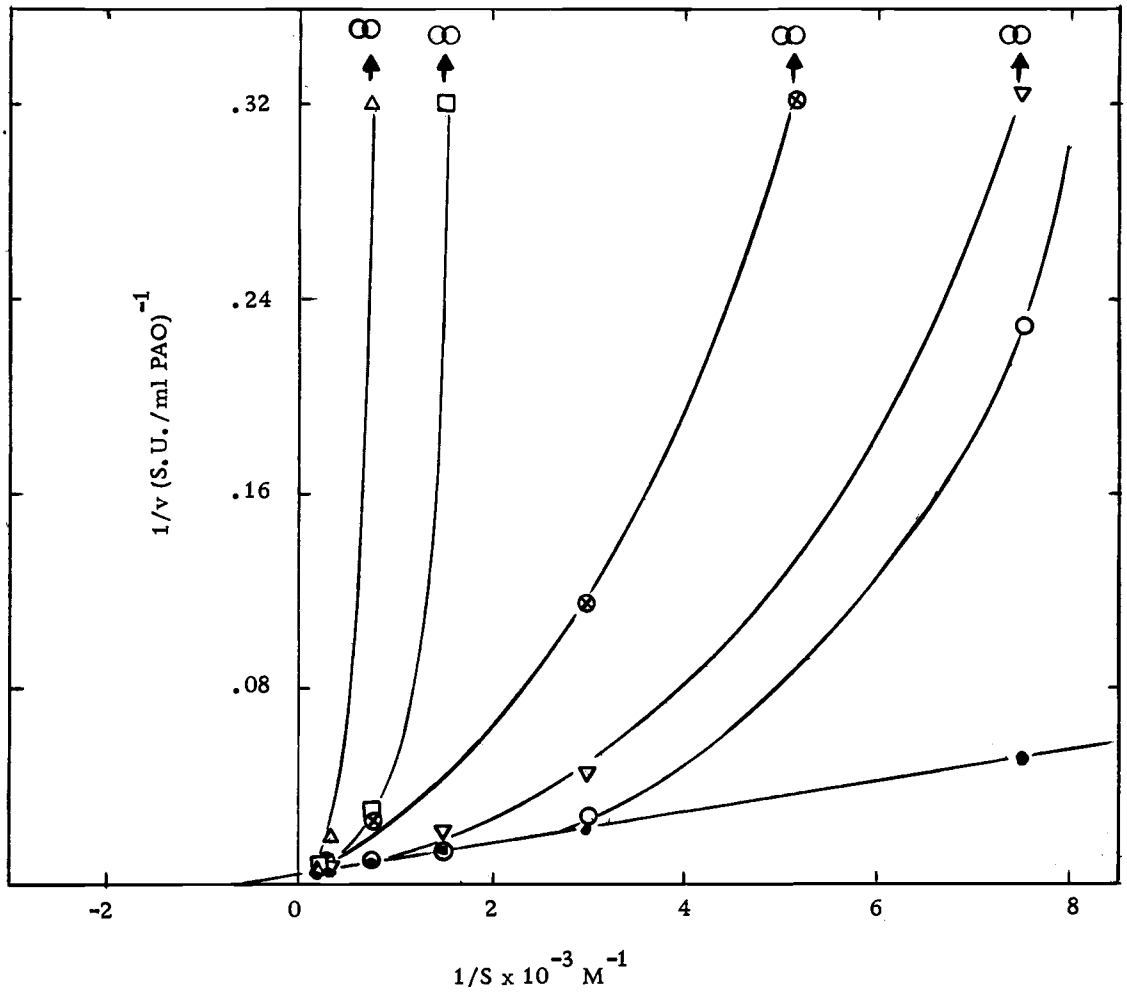


Figure 13. Lineweaver-Burk analysis of the data of Figure 11.

Experimental details are the same as Figure 10.

that these inhibitors are responsible for inducing the binding of additional substrate molecules. This behavior is characteristic of allosteric proteins for which the attachment of substances other than substrate at sites distinct from the catalytic site modifies the properties of the catalytic site (7, 28, 29, 30, 74, 76, 78, 92, 93, 103). These heterotropic interactions then would suggest that PAO might contain allosteric sites for both substrate and hydrazines.

It was mentioned previously (Section II A) that low concentrations of UDMH exhibited a substrate-dependent activation. Figure 12 shows that there are no heterotropic effects for concentrations of UDMH and benzylamine which demonstrate this stimulation. This may be related to the observation that the heterotropic cooperativity displayed by several allosteric enzyme-inhibitor systems is removed in the presence of an activator (7, 74, 80, 93, 103, 105, 106, 126, 127).

The amount of sigmoidicity of the velocity versus substrate concentration curves (or the amount of parabolicity in the double reciprocal plots) in the presence of different concentrations of a modifier may be taken as a qualitative measure of the effect of the allosteric ligand on the reaction order for substrate (45, 105, 106). For the PAO-benzylamine-hydrazine system, increasing inhibitor concentration intensifies the magnitude of the heterotropic effects, and for the same inhibitor concentration the amount of cooperativity

is H > MMH > UDMH. These observations can then be interpreted in terms of the occurrence of an increase in the reaction order for substrate.

Reaction order has been defined as a measure of the minimum number of substrate molecules that can react with an enzyme. It is a function of the number of interacting substrate-binding sites and the strength of these interactions (7, 93, 117). The increased reaction order for substrate which is produced when hydrazines are bound to PAO might then result from these inhibitors functioning to expose additional substrate binding sites or to increase the intersite interaction for those which are already accessible.

The deviations from Michaelis-Menten kinetics exhibited by PAO in the presence of hydrazines when there was no prior incubation of enzyme and inhibitor have been interpreted in terms of the existence of allosteric sites on the enzyme. The same anomalous kinetic behavior would result, however, if the inhibitor were reacting with substrate, and would be especially prominent at low substrate concentrations where the alleged allosteric effects are the greatest. This possibility can be excluded on the basis of the results presented in Section IA.

The importance of the use of initial velocities has been stressed repeatedly throughout the literature of enzymology (36, 123). Frieden (42) has pointed out that their use is especially

significant in studies of possible allosteric interactions, since measuring too large a percentage of the reaction might itself give rise to apparent allosteric effects. Initial velocities were therefore used in these studies with PAO.

Since the hydrazine inhibition of PAO appears to exhibit a kinetic competition with substrate (Section IB-5), the sigmoid relation between velocity and substrate concentration might then result from a continuing inhibition of the enzyme during assay. Unfortunately, this possibility cannot be conclusively ruled out. The validity of the entire model of PAO as an allosteric protein may rest on its proof or disproof. Although the allosteric hypothesis presented here may appear doubtful because of the time-dependent, irreversible, and kinetically competitive (with substrate) nature of the inhibition of PAO by hydrazines, a few observations on work reported for similar enzyme-inhibitor systems give it some strength.

The inhibition of rat liver mitochondrial amine oxidase by iproniazid is irreversible and time-dependent, yet a linear Lineweaver-Burk plot was obtained when inhibitor and substrate were added simultaneously to the enzyme (34). The same results were found for the irreversible inhibition of beef liver mitochondrial amine oxidase by phenylcylopropylamine (9). A Dixon plot of data obtained from the inhibition of the particulate rat liver enzyme by this same compound (no preincubation) displayed no abnormalities

(58). No deviations from Michaelis-Menten kinetics were reported in the study of the irreversible inhibition of cysteine sulphinic acid decarboxylase (a PLP enzyme) by isoniazid under similar conditions (34).

The systems described above all displayed a protective effect by substrate. All inhibitors were time-dependent, irreversible. One was a PLP-containing enzyme. Despite these similarities to the PAO-hydrazine system, none of them exhibited anomalous kinetics with respect to substrate when there was no preincubation of enzyme and inhibitor before substrate was added. These results would seem to indicate that the hydrazine inhibition of PAO is indeed unique and would lend credence to the allosteric interpretations applied to the velocity-substrate plots for PAO in the presence of hydrazines.

Further justification for this opinion comes from the fact that enzymes that are known with certainty to be allosteric exhibit the most striking cooperativity at low substrate levels. At high substrate concentrations, these effects are abolished (80, 105, 106).

The foregoing information appears to substantiate the hypothesis that PAO is an allosteric protein. However, this interpretation must be advanced with caution when based solely on the apparent heterotropic cooperative effects. The most conclusive support for this proposal comes from the biphasic nature of the hydrazine

inhibition of this enzyme discussed in the preceding section.

C. Inhibition by High Substrate Concentrations

The PAO-benzylamine system conforms to Michaelis-Menten kinetics at substrate concentrations lower than 4 mM. However, it deviates at higher substrate concentrations in that the velocity of the reaction decreases with increasing substrate concentrations (Figure 14). Of the general mechanisms proposed to explain this phenomenon of high substrate inhibition (123), three could possibly apply to PAO:

(1) The binding of more than one substrate molecule to the enzyme could produce an inactive enzyme-substrate complex. This could result if (a) the substrate in the active ES complex forms a multipoint attachment to the enzyme so that at high substrate concentrations it is possible that two or more substrate molecules can be bound simultaneously at the active center, or (b) if the substrate combines not only at the active site, but also at other distinct sites, interfering with either the binding of the substrate in an active ES complex, or with its reaction when it is so bound.

(2) Since water is one of the reactants in the reaction catalyzed by PAO, it is feasible that high levels of substrate may slow the rate because the water concentration is reduced.

(3) Amine oxidases of various sources have been shown to be

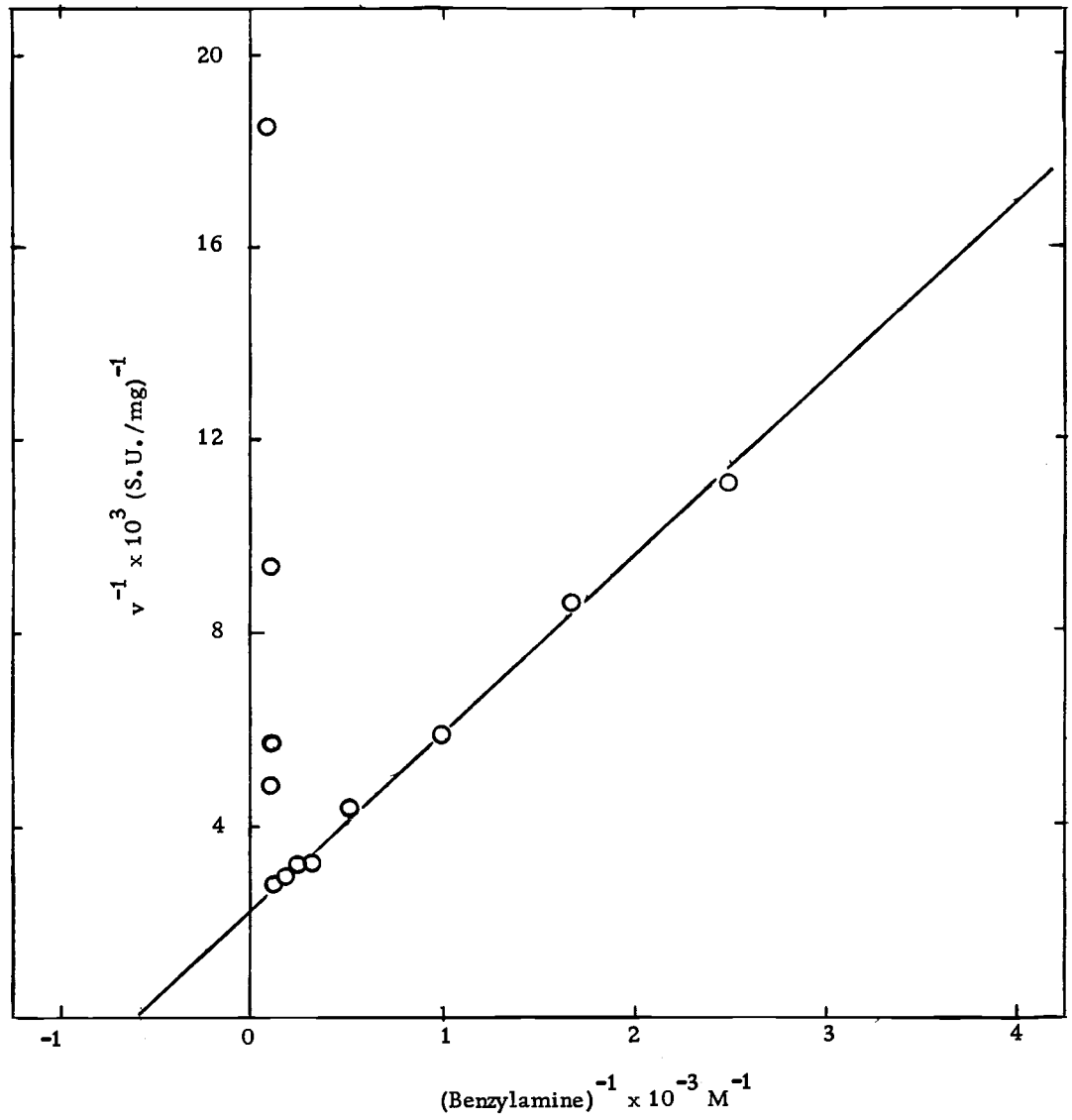


Figure 14. Lineweaver-Burk plot for the rate of benzylamine oxidation by purified PAO.

Purified PAO (Specific 496 S.U./mg) at a final concentration of 0.023 mg/ml in a 3 ml assay system was assayed with the indicated final concentrations of benzylamine. Initial velocities are recorded.

inhibited by the products of the reaction, $NH_3$ (83) and $H_2O_2$ (136). In view of the fact that increased substrate concentration would produce increased amounts of products formed, the observed inhibition of PAO activity by high substrate concentrations could potentially be caused by product inhibition.

The first possibility can be distinguished from the others by applying the theoretical treatment of Haldane (60) to the data obtained from an experiment to determine the effect of substrate concentration on the velocity of the reaction.

The general equation derived by Haldane to fit the kinetics of substrate inhibition caused by the simultaneous binding of more than one substrate molecule to the enzyme is:

$$(6) \qquad \frac{V_m}{v} = 1 + \frac{K_m}{(S)} + \frac{(S)}{K'_m}$$

It relates the observed reaction velocity (v) at substrate concentration (S) to the theoretical maximal velocity ($V_m$), the apparent dissociation constant for the substrate in the active position ($K_m$), and the apparent dissociation constant for the substrate in the inhibitory position ($K'_m$).

The experimental data for benzylamine oxidation by PAO were

shown graphically to fit this equation.[7]

Equation (6) predicts that when the velocity of the reaction is plotted against substrate concentration on a logarithmic scale a symmetrical bell-shaped curve will result having a maximum which may be expressed by the derived equation

$$(7) \quad S_o = \sqrt{K_m K_m'}$$

where $S_o$ is the optimum substrate concentration, and the dissociation constants $K_m$ and $K_m'$ are obtained from independent graphical procedures. If the Haldane mechanism holds, the theoretical value for $S_o$ calculated from Equation (7) should agree with the experimentally observed optimal substrate concentration obtained from the plot of v versus log (S).

The apparent $K_m$ for benzylamine oxidation by PAO obtained from a Lineweaver-Burk plot (Figure 14) is 1.62 mM. The apparent dissociation constant for substrate in the inhibitory position ($K_m'$) estimated from a Dixon plot of reciprocal velocity versus substrate concentration is 10.4 mM. The theoretical value of $S_o$ (4.1 mM) calculated from Equation (7) is in excellent agreement with the substrate concentration (4.2 mM) at which the curve of Figure 15 is

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[7] The mathematical analysis employed is described in detail by Haldane (60), McEwen (83), Dixon and Webb (36) and Webb (123).

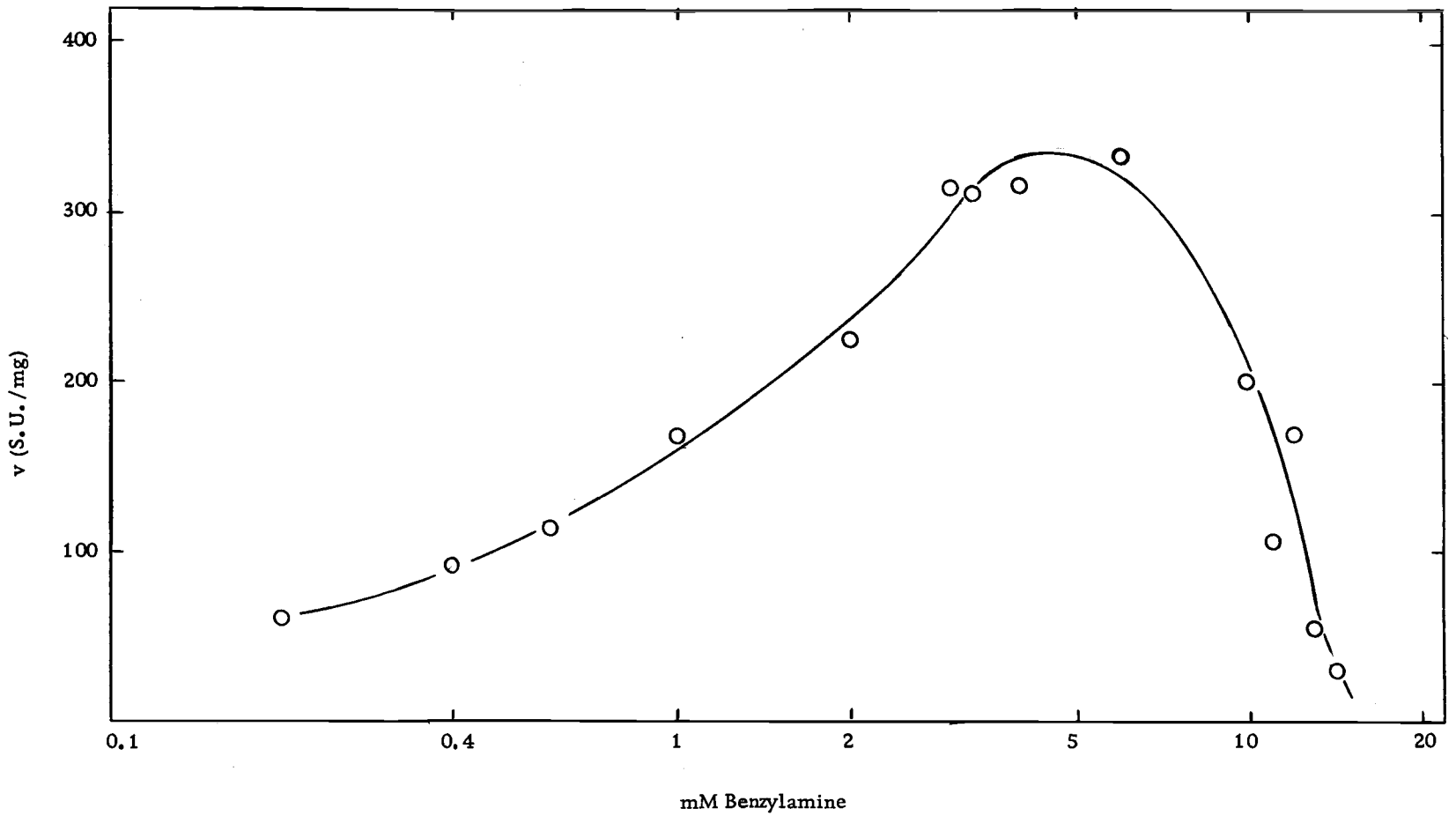


Figure 15. Semilogarithmic plot of the effect of benzylamine concentration on the velocity of the reaction catalyzed by purified PAO.

The data of Figure 14 are plotted on a semilogarithmic scale.

maximal.

The assymmetry of the curve at higher substrate concentrations can be explained if the $ES_2$ complex is partially active (94), or if the inhibition is more complex than Haldane's assumption, that two substrate molecules are bound. Distortion of symmetry will occur, for example, when more than two substrate molecules are bound (123). Thus, despite the unsymmetrical nature of the experimental curve, the close agreement of the theoretical and observed values for $S_o$ show that the experimental data are consistent with Equation (6) and it can be concluded that the high substrate inhibition of PAO follows the Haldane mechanism. Consequently, it would seem that PAO contains more than one site for the interaction with substrate, which supports the conclusions arrived at previously (Section I and Section IIB) that PAO contains more than one kinetically important substrate binding site.

## Discussion[8]

### A. Types of Inhibitor and Substrate Binding Sites on PAO

The striking heterotropic cooperativity observed in the presence of hydrazines (when there was no preincubation of enzyme and

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[8] The parenthetical symbols used throughout this discussion refer to notations in Table 9.

inhibitor before substrate was added) was interpreted to mean that these inhibitors potentiate an effect which results in the binding of more than one substrate molecule by PAO. There was no deviation from Michaelis-Menten kinetics in the absence of these inhibitors. This would indicate that the additional substrate molecules bound are associated at a site other than the catalytic site (42), thus suggesting the presence of an allosteric substrate-binding site on PAO ($A_2$).

The fact that the inhibition of PAO by high substrate concentrations was found to follow the Haldane mechanism also provided evidence that PAO could bind more than one substrate molecule. This could occur if the active site could bind more than one substrate molecule or if substrate were bound at two distinct sites. For PAO, probably both explanations hold true.

The existence of a site ($C_2$) which optimally orients inhibitor or substrate molecules for the most favorable reaction with PLP ($C_1$) was proposed in Section I. This site was thought to function through the binding of a hydrogen atom which is alpha to the attacking $-NH_2$ group of an amine or hydrazine. As a necessary consequence, this $\alpha$-H binding site ($C_2$) must be in close proximity to the site at which chemical reaction occurs ($C_1$). At high substrate concentrations, inhibition could result from the binding of two substrate molecules, one at each of these sites.

Table 9. Proposed PAO Binding Sites for Hydrazine Inhibitors (I) and Substrate (S).

| Site | Chemical Nature | Possible Function | Evidence |
|---|---|---|---|
| $C_1$ | Schiff base of PLP-Cu (II) complex | -Binds S or I through covalent forces. | -Spectral observations. |
| | | -The first step in the oxidative deamination of S. | -Stoichiometric inhibition. |
| | | -Site of irreversible binding of I responsible for the inhibition | -Competitive nature of the inhibition |
| | | | -Studies with model systems. |
| $C_2$ | unknown | -Non-covalently and reversibly binds H located alpha to $-NH_2$ of S or I. | -Structural requirements of effective S or I. |
| | | -Orients I or S for most effective reaction with $C_1$. | -Stereospecificity of I and S reactions. |
| | | -Located in close proximity to $C_1$. | -Haldane mechanism for high S inhibition. |
| | | -Binding of I at this site does not result in inhibition. | |
| $A_1$ | unknown | -Specific for the reversible binding of I through non-covalent forces. | -Heterotropic cooperative effect. |
| | | | -Homotropic cooperative effect for I. |
| | | -Binding of I at this site does not result in inhibition. | -Biphasic titration curve. |
| | | -Interacts with $C_1$. | |
| | | -Topographically removed from $C_1$. | |
| $A_2$ | unknown | -S binds here when present at high concentrations | -Haldane mechanism for high S inhibition |
| | | -S binds here in the presence of I. | -Dissymmetry of v vs. log (S) plot. |
| | | -Interacts with $C_1$. | -Heterotropic cooperative effect. |
| | | -Topographically removed from $C_1$. | -No homotropic cooperative effect for S. |

It was pointed out in Section II C that the dissymmetry of the v versus log (S) plot (Figure 15) might be attributed to the binding of more than two substrate molecules. This interpretation would be compatible with the assumption that at least three substrate binding regions are present on PAO: ($C_1$), ($C_2$), and ($A_2$). At high substrate levels substrate may be bound at all three of these sites.

Parabolic double reciprocal plots for inhibitors which react at the catalytic site but which cannot be oxidized by the enzyme (hydrazines are inhibitors of this type; see Section I) are known to occur when an enzyme reacts with more than one inhibitor molecule (28, 29, 30, 105, 106, 123, 126, 127). This situation might arise if the enzyme contains two or more inhibitor binding sites, which, if not identical with the catalytic site, interact with it, to produce deviations in the kinetic behavior of the enzyme-substrate reaction (123). Thus it appears that PAO could contain an allosteric site for hydrazines ($A_1$) as well as for substrate ($A_2$). In addition, both of these molecules react at the catalytic site ($C_1 + C_2$). A hypothesis for the function and interaction of these sites is proposed below (see also Section I) and summarized in Table 9.

## B. Nature of the Interaction between PAO Sites

Although it is not possible to determine with certainty the number of catalytic sites from kinetic data alone (8, 18, 29, 30, 42,

74, 93, 103), the conclusions put forth in Section I have pointed to only one, which is the Schiff base of a PLP-Cu (II) complex, ($C_1$). Closely associated with it is a site ($C_2$) which aids in substrate or inhibitor orientation through non-covalently binding a hydrogen alpha to the nucleophile $-NH_2$.

An obvious contradiction then arises. How can the inhibition be stoichiometric with PLP (Section IB-6 and IC) if there are other inhibitor binding sites present? This discordance can be resolved if it is assumed that the binding of hydrazines to these sites ($C_2$ and $A_1$) is through weak, reversible, non-covalent forces in contrast to their reaction at ($C_1$) which results in the formation of a stable covalent bond. The latter reaction is responsible for the irreversible nature of the inhibition, which determines the observed stoichiometry. The binding of hydrazines at $C_2$ or $A_1$ then would not result in inhibition. If this assumption holds true, the experimental results obtained would then be consistent with the binding of an additional inhibitor molecule at a site ($A_1$) which is topographically distinct but which functionally interacts with the catalytic site ($C_1$ + $C_2$).

The existence of this allosteric inhibitor binding site ($A_1$) could then account for the observed cooperative effects. The heterotropic cooperative effect might result if the binding of a hydrazine molecule at its allosteric site ($A_1$) were to induce the binding of

substrate at both its allosteric site ($A_2$) and the catalytic site ($C_1$ + $C_2$). The supposition that hydrazines but not substrates can bind at ($A_1$), would explain the paradoxical absence of homotropic cooperativity for substrate which is expected of a system where a substrate analogue produces a heterotropic cooperative effect (7, 8, 93, 103, 105, 106).

The homotropic cooperative effect reflected in the sigmoidal saturation curve of PAO by hydrazines could be explained by the conjecture that the binding of one molecule of inhibitor at its allosteric site ($A_1$) promotes the binding of others to the catalytic site (analogous to the effect with substrates). If the binding of inhibitor at low concentrations to its allosteric site ($A_1$) could produce an alteration in the enzyme molecule which would enhance the binding of substrate at the active site ($C_1$ + $C_2$), the activation component of the biphasic titration curves could likewise be explained in terms of the binding of hydrazines at ($A_1$).

Allosteric effectors are thought to act as inhibitors or activators by interacting with a second site to produce modifications in the properties of the catalytic site. According to the current major theories of allosteric transition, these alterations result from an induced conformational change or a shift in an association-dissociation equilibrium between subunits or monomers and polymers (7, 76, 78, 93).

Achee has demonstrated that PAO in solution exists in an equilibrium mixture of monomers, dimers, and trimers. The monomer was found to be more active than the dimer and trimer (1). This difference in activity might possibly result from the masking of the catalytic sites in the higher polymeric species.

In view of these considerations, the physical effect of the binding of hydrazines at their allosteric site ($A_1$) might then be due to a shift in this monomer-polymer equilibrium to expose previously masked sites, or to an induced conformational change which would alter the environment of the catalytic site. In either case, the net result would be to produce favorable conditions at the catalytic site for the binding of substrate or inhibitor.

Some other results which can be interpreted in the light of the hypothesis that PAO is an allosteric protein include the observations that:

(a) The substrate-dependency of the stimulation of PAO activity by low inhibitor concentrations was found to be contingent on whether or not a preincubation of enzyme and inhibitor preceded the addition of substrate. When there was no preincubation, this activation occurred only at low substrate levels. When a preincubation was employed, substrate concentration had no effect. This could indicate that the inhibitor-induced alterations in the PAO molecule which result in increased activity either are time-dependent (to account for

the latter), or are not observable due to the fact that in the presence of excess substrate the $E + S \rightleftarrows ES$ equilibrium is rapidly established and lies far to the right (to explain the former). The latter explanation would be compatible with the suggestion that binding of substrate at ($A_2$) might be partly responsible for the high substrate inhibition observed for PAO. If this were true, simultaneous activation (induced by the inhibitor binding at $A_1$) and inhibition might cancel each other out.

(b) For the system where enzyme was not preincubated with inhibitor, no heterotropic cooperative effect was observed for concentrations of inhibitor and substrate which exhibited an activation (Figure 12). This suggests that substrate may have a greater affinity for the catalytic site than for its allosteric site ($A_2$). At low concentrations, substrate might then bind only at the catalytic site, not at ($A_2$). Normal Michaelis-Menten kinetics would then result.

(c) The model postulated here for the nature of the interactions between PAO sites predicts than an increase in high substrate inhibition should accompany an increase in heterotropic cooperativity. However, an examination of Figures 10-12 reveals that this does not occur. This apparent discrepancy can be resolved on the basis of the physical changes described earlier. An alteration in the enzyme's conformation or a shift in the monomer-polymer equilibrium

induced by the binding of a hydrazine at ($A_1$) would favor the binding of substrate to the catalytic site rather than to ($A_2$).

Thus the existence and function of the postulated binding sites summarized in Table 9 are compatible with experimental observations on PAO-hydrazine-benzylamine interactions. The nature and number of the catalytic site components ($C_1 + C_2$) are reasonably well established (Section I). The existence of allosteric sites for both inhibitor and substrate seems the most plausible explanation of the apparent cooperative effects and is in accord with the finding that the inhibition by high substrate concentrations follows the Haldane mechanism. However, many factors are still unclear. For example, PAO may contain more than one kind of inhibitor-specific ($A_1$) site. Such an assumption would seem necessary to explain the fact that the two types of cooperative effects follow inverse relations to inhibitor structure. The chemical nature of these sites as well as the manner in which they interact with the catalytic site and with each other likewise require further deliberation.

## C. Related Properties of Other Amine Oxidases

The elegant work of McEwen on the effect of pH on substrate and inhibitor reactions with human plasma (83) and rabbit serum (85) amine oxidases has conclusively demonstrated the presence of an electrophilic binding site on these enzymes. Substrate and inhibitor

specificity studies revealed the presence of a hydrophobic region on these enzymes associated with inhibitor and substrate binding (83, 84, 85). Both of these enzymes were found to exhibit high substrate inhibition (83, 84). The human plasma enzyme conformed to the Haldane mechanism and had no anomalies in its v versus log (S) plot. Buffoni (21) has extended McEwen's interpretations to apply to the entire class of plasma amine oxidases based on the fact that these enzymes act on the non-protonated form of the substrate and a charged group in the vicinity of the amine group has a strong disturbing effect.

A model for the active site of mitochondrial amine oxidase has been proposed by Zeller and coworkers on the basis of structural studies for substrates and inhibitors. Like the plasma enzymes, it has been found to contain electrophilic and hydrophobic binding regions (10, 104, 140, 141, 142, 143, 144). In contrast, however, it does not exhibit high substrate inhibition (95).

These results demonstrate that amine oxidases from other sources also possess multiple inhibitor and substrate binding sites. However, the function of these sites can probably best be explained in terms of substrate orientation at the active site (similar to the role assigned to $C_2$ for PAO), since no deviations from simple Michaelis-Menten kinetics have been observed.

Of special interest to the possible allosteric nature of amine

oxidases is McEwen's finding that aliphatic alcohols selectively stimulate the oxidation of certain substrates by rabbit serum amine oxidase (85). The kinetic data is consistent with the hypothesis that the alcohol binding depends on hydrophobic interactions, and that alcohols increase the effectiveness of the hydrophobic areas of the enzyme for substrate binding. The two possible explanations offered by McEwen are that the aliphatic alcohols might exert their activating effect by contributing a portion of their hydrophobic residue to the active center of the enzyme or that they induce a more favorable conformation for substrate binding through an allosteric mechanism. The former was tentatively favored since no anomalous kinetic behavior was observed.

The observation that some of the properties of a given preparation of mitochondrial amine oxidase vary considerably according to the substrate used to measure its activity led to the conclusion that multiple forms of this enzyme exist. These results have been interpreted in terms of the presence of two or more distinct enzymes, one enzyme with different active sites (9, 53, 54, 110, 111, 137, 145), or the difference in the type of substrate orientation at the active site (141). The transformation of rat liver mitochondrial monoamine oxidase into a diamine oxidase-like enzyme by peroxides of higher unsaturated fatty acids (54) might even be suggestive of an allosteric mechanism. However, Gorkin's physical separation from

rat liver of two types of mitochondrial amine oxidases with different substrate specificities (50, 53) would seem to support the "two enzyme" theory. Similar results were obtained by Ragland (98) for the rabbit and beef liver mitochondrial enzymes. This interpretation would be in agreement with the fact that no high substrate inhibition has been observed for these particulate amine oxidases (95).

The sedimentation behavior of Mondovi's pig kidney amine oxidase suggests the presence of an association-dissociation phenomenon (91). This seems to be similar to that which has been observed by Achee for PAO (1). Mondovi has reported that exogenous PLP can activate purified pig kidney amine oxidase (91), which may at first appear to be in conflict with the fact that the PLP cofactor is so firmly bound to the enzyme (87). Mondovi's explanation was that PLP may exert its activating effect by promoting the dissociation into subunits (91). No kinetic data was reported, but based on the analogies with other allosteric enzymes, PLP could conceivably be behaving as an allosteric activator of pig kidney amine oxidase.

This interpretation, together with the work of McEwen on the stimulation of rabbit serum amine oxidase by aliphatic alcohols, are the only indications reported thus far that any other amine oxidases might be allosteric. Thus it seems possible that the apparent allosteric properties of PAO described in this thesis may be unique to this enzyme.

# III. ACTIVATION OF PAO IN THE PRESENCE AND ABSENCE OF HYDRAZINES

## Results

### A. Activation in the Absence of Hydrazines

PAO was found to increase in activity with time in solution at room temperature, pH 7.0. The amount and rate of activation appear to be inversely related to enzyme concentration (Figure 16) and independent of the purity of the enzyme preparation used.

Since the activation was initially detected with a crude enzyme preparation, it seemed possible that it might result from the presence of an endogenous, reversibly-bound inhibitor or activator. The following experiments were carried out with this thought in mind.

The activity of the pooled fractions eluted from the first DEAE-cellulose column (Step 3 of the PAO purification procedure described in Methods) was found to be less than the sum of the activities of the individual fractions. Following precipitation by 0.55 saturation ammonium sulfate, the activity of these combined fractions corresponded to that which was expected. Moreover, the PAO activity in the column eluate was much less stable before this ammonium sulfate step. These observations strengthened the possibility that there might be an impurity in the enzyme preparation which was removed by ammonium sulfate precipitation. However, the ammonium sulfate

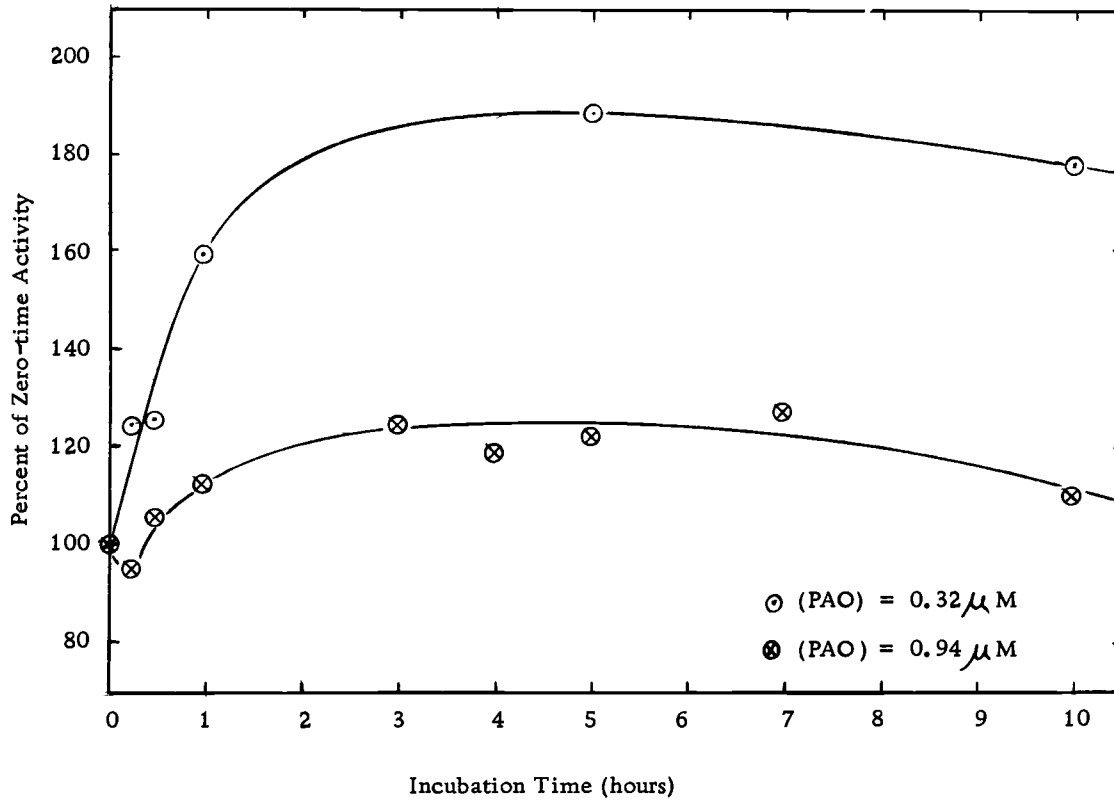


Figure 16. Time-dependent activation of PAO.

PAO of specific activity 8.95 S.U./mg and a protein concentration of 36.4 mg/ml was incubated at room temperature in 0.06 M phosphate buffer, pH 7.0 at the indicated concentrations. At chosen time intervals a 0.1 ml aliquot was assayed. The incubations were carried out in glass-stoppered tubes to prevent evaporation.

supernatant did not display any inhibitory properties when PAO was assayed in its presence, so this explanation was tentatively discarded.

The observation that velocity-concentration curves were linear over wide ranges of enzyme levels for both pure (Figure 1) and crude (Figure 17) PAO preparations is probably the most conclusive proof that an endogenous, reversibly-bound inhibitor is not responsible for the observed activation. This direct proportionality of activity to enzyme concentration likewise excludes the presence of an activator in the system.

Other possible explanations for this phenomenon are treated in the Discussion.

## B. Polymerization Studies

Sedimentation equilibrium studies on PAO by Achee and Yasunobu revealed the dependence of the sedimentation coefficient on enzyme concentration. This behavior suggested the presence of an association-dissociation phenomenon. These observations led to the proposal that the native enzyme in solution is an equilibrium mixture of monomers, dimers, and higher polymers of the monomeric unit of 170,000 molecular weight (1). These same workers also found that PAO in solution for a period of weeks to months at 0 to 5°C undergoes a time- and concentration-dependent molecular

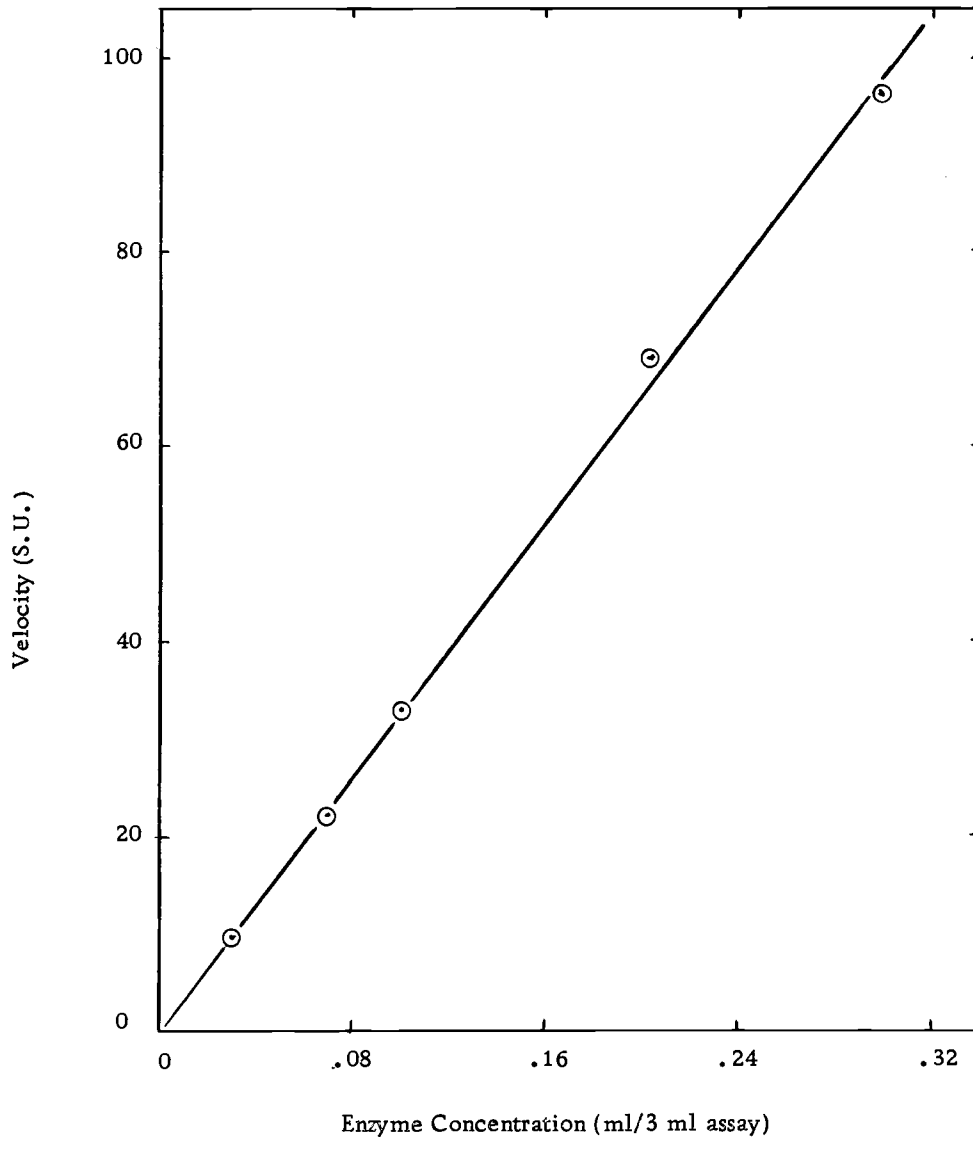


Figure 17. Effect of enzyme concentration on PAO activity.

Partially purified PAO with a specific activity of 8.86 S.U./mg and a protein concentration of 36.4 mg/ml was assayed at the indicated concentrations.

aggregation. Polymer formation was favored in more concentrated solutions, and the polymer was found to be less active than the monomer (1).

These findings gave rise to the idea that the activation of PAO at room temperature described in the preceding section might conceivably be explained in terms of such a reversibly interacting system. The observed activation might result from a shift in the monomer-polymer equilibrium to favor the formation of the more active monomer. The inverse relation to enzyme concentration displayed by the activation would be compatible with expected mass action effects on such a system.

To test this possibility, activated and unactivated PAO at different enzyme concentrations were eluted from Sephadex G-200 (see Methods).

If a deaggregation were responsible for the activation effect, active PAO species of different molecular weights should be found. In addition, the relative proportion of monomer to polymer would be expected to increase with conditions favoring activation.

Figures 18-21 show that there was only one major activity peak eluted, whether or not the enzyme was activated before it was applied to the column. Concentration[9] likewise had no effect on the elution

[9] The enzyme of concentrations used in these experiments corresponded with those for which Achee demonstrated a striking difference in the rate and position of the monomer-polymer equilibrium.

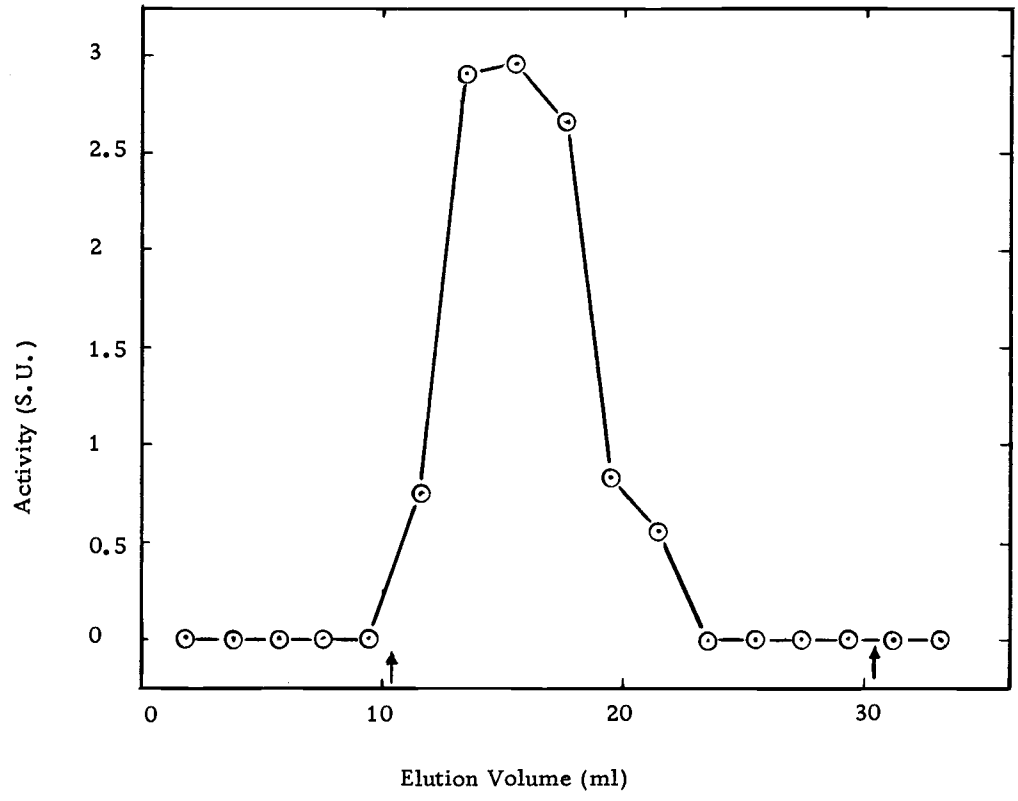


Figure 18. Elution of 0.46 μM PAO from Sephadex G-200. No Incubation.

A 0.5 ml sample of PAO with specific activity of 5.1 S.U./mg and a protein concentration of 3.84 mg/ml was applied to a Sephadex G-200 column and eluted at 5° with 0.06 M phosphate buffer, pH 7.0. Arrows indicate the void volume (10.4 ml) and total volume (30.4 ml) of the column. Further experimental details are described in Methods.

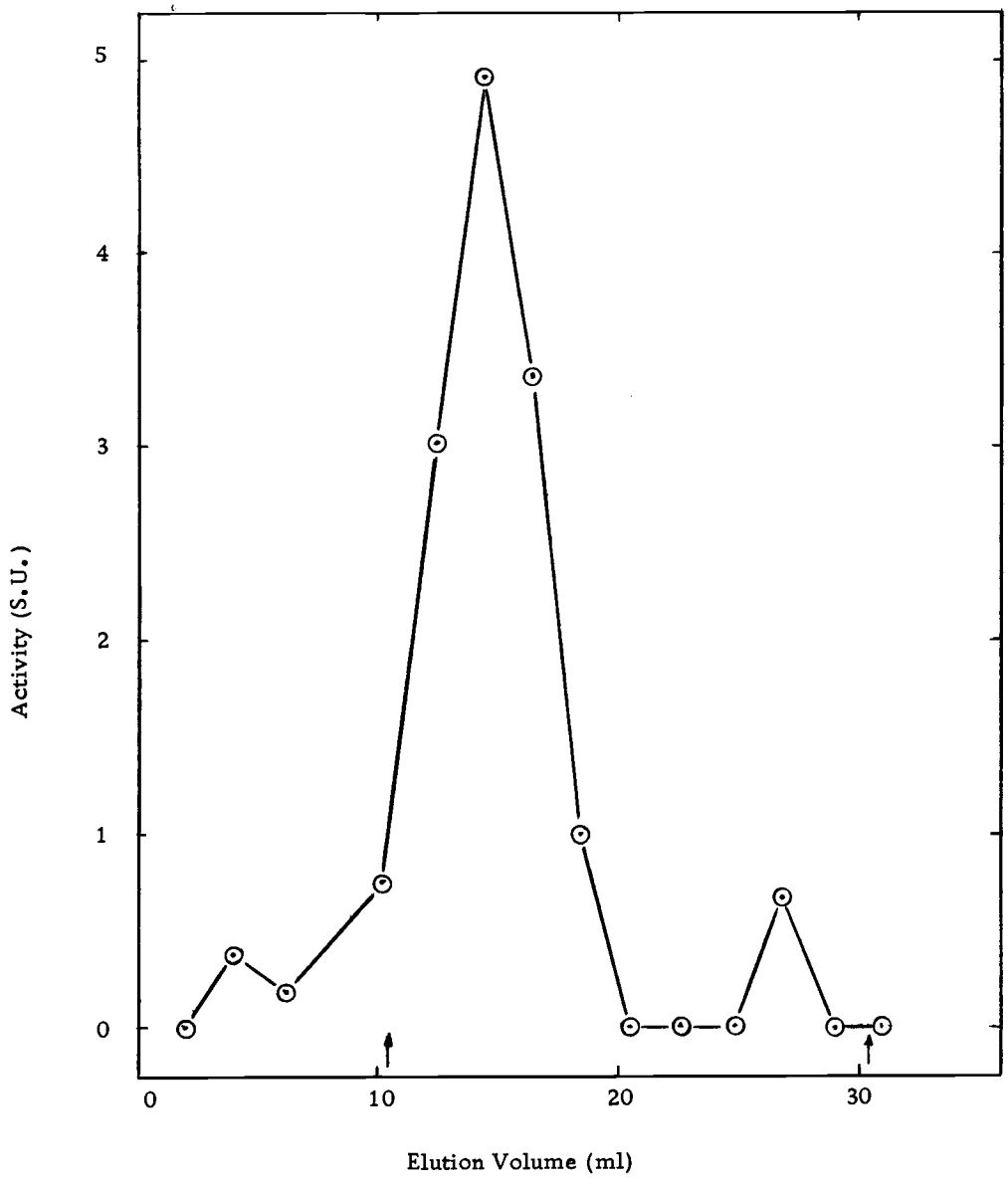


Figure 19. Elution of 0.48 μM PAO from Sephadex G-200. Prior incubation.

PAO with specific activity of 4.5 S.U./mg and a protein concentration of 4.08 mg/ml was incubated at 25° in glass-stoppered tubes. After 4-1/2 hours, the enzyme had a specific activity of 7.6 S.U./mg. A 0.5 ml sample of this activated enzyme was immediately applied to a Sephadex G-200 column and eluted at 5° with 0.06 M phosphate buffer, pH 7.0. Arrows indicate the void volume (10.4 ml) and total volume (30.4 ml) of the column. Further experimental details are described in Methods.

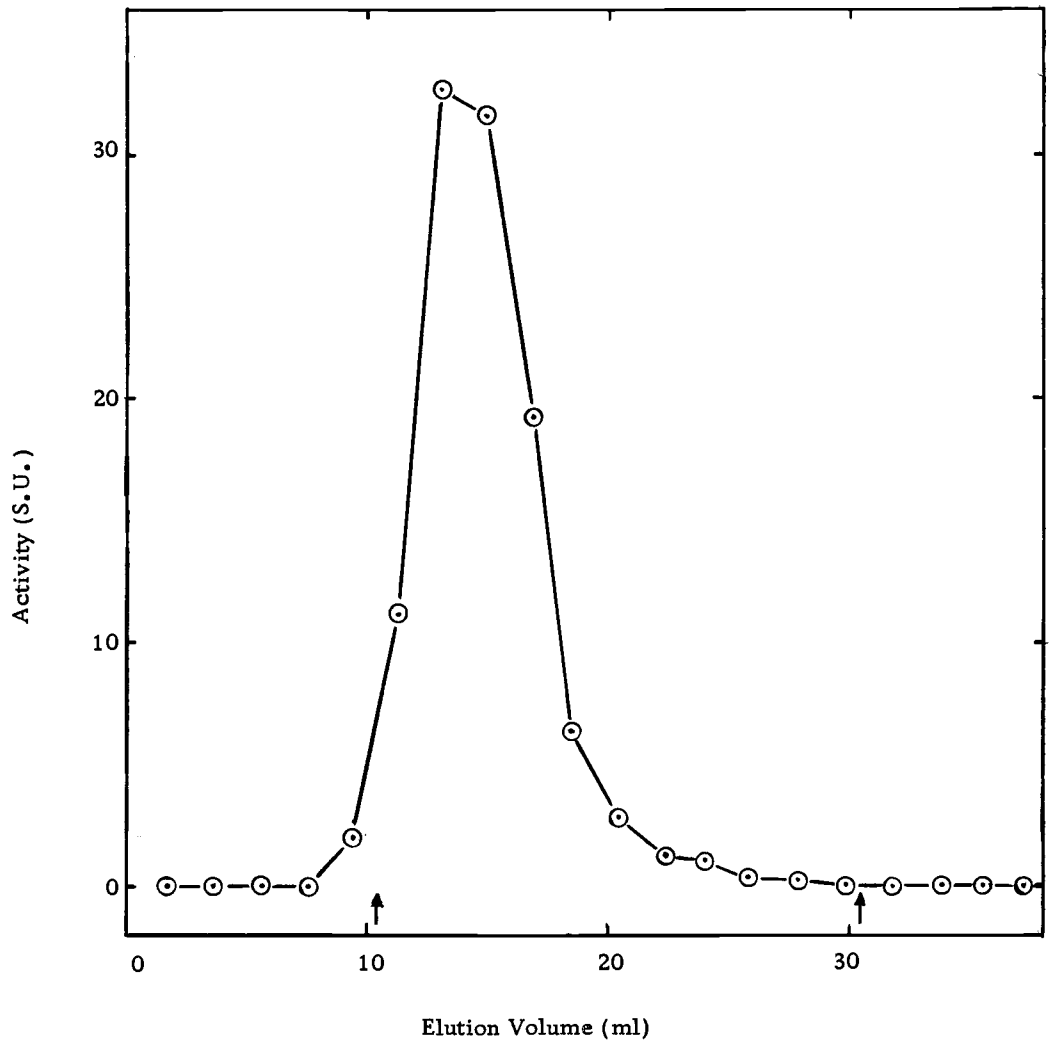


Figure 20. Elution of 4.8 μM PAO from Sephadex G-200. No incubation.

A 0.5 ml sample of PAO with specific activity of 4.9 S.U./mg and a protein concentration of 40.45 mg/ml was applied to a Sephadex G-200 column and eluted at 5° with 0.06 M phosphate buffer, pH 7.0. Arrows indicate the void volume (10.4 ml) and total volume (30.4 ml) of the column. Further experimental details are described in Methods.

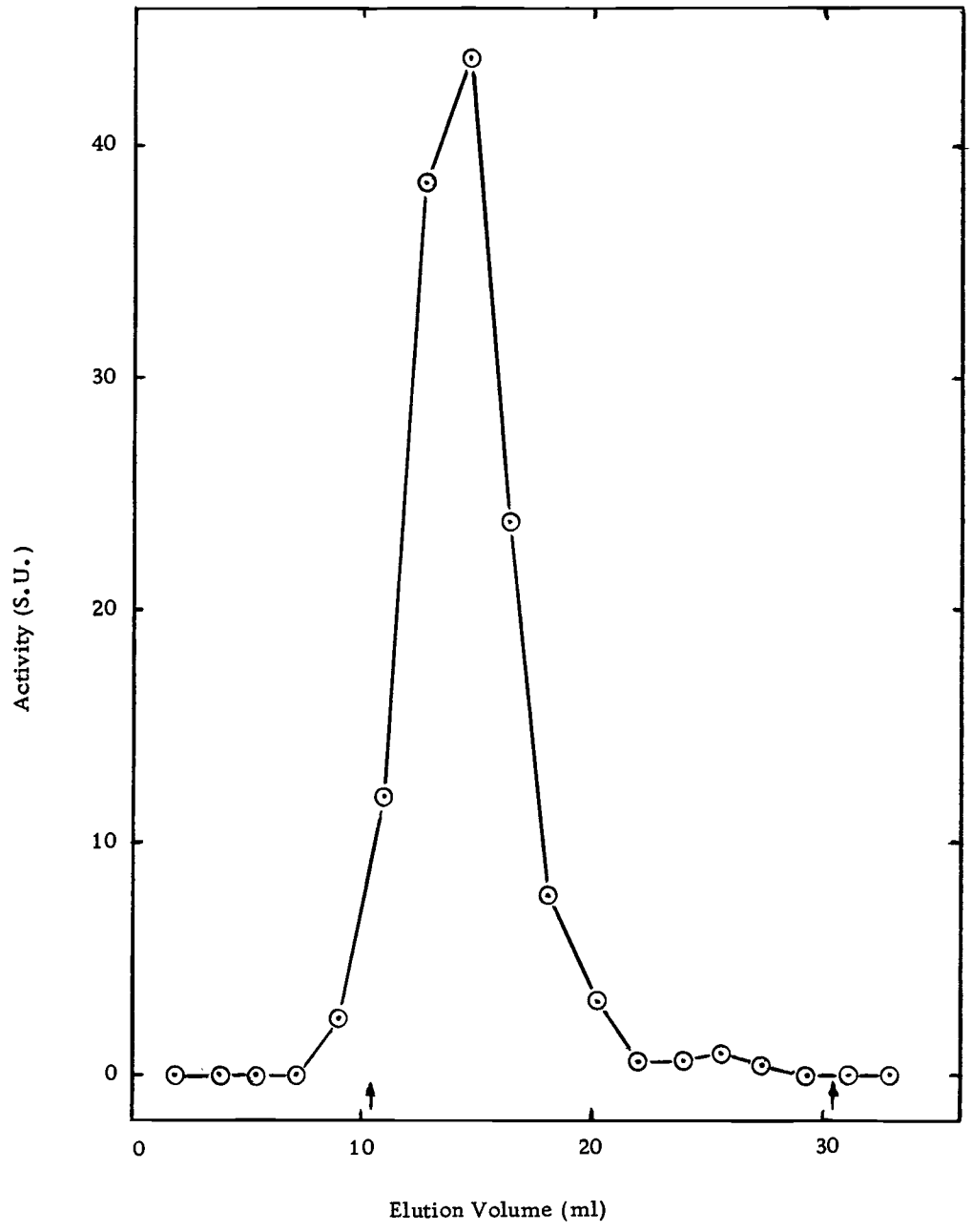


Figure 21. Elution of 4.7 $\mu$M PAO from Sephadex G-200. Prior incubation.

PAO with specific activity of 6.07 S.U./mg and a protein concentration of 39.33 mg/ml was incubated at 25° in glass-stoppered tubes. After 4-1/2 hours, the enzyme had a specific activity of 8.0 S.U./mg. A 0.5 ml sample of this activated enzyme was immediately applied to a Sephadex G-200 column and eluted at 5° with 0.06 M phosphate buffer, pH 7.0. Arrows indicate the void volume (10.4 ml) and total volume (30.4 ml) of the column. Further experimental details are described in Methods.

pattern. The molecular weight of this peak was found to be 180, 000. This value is in close agreement with the molecular weight of the monomer (170, 000) determined by Achee (1).

The elution diagrams shown in Figures 18 and 19 suggest the presence of a minor activity component which contains less than 10% of the total activity recovered. Although its occurrence cannot be explained, its molecular weight (approximately 15, 000) is too low to be any known subunit of the enzyme. Its appearance or relative proportions could not be correlated with either the concentration or incubation variables.

These results lead to the conclusion that the time- and concentration-dependent activation of PAO described here does not arise from an aggregation-deaggregation phenomenon.

## C. Activation in the Presence of Hydrazines. Apparent Reversal of Inhibition.

It was shown in Section IA that PAO in solution undergoes a time- and concentration-dependent activation. The results of Table 10 and Figures 22 and 23 demonstrate that this phenomenon is common to the hydrazine-inhibited enzyme as well. In addition, it was observed that the increase in activity with time for the inhibited enzyme was even greater than that for the control with no inhibitor present; in other words, the inhibition appeared to be reversed.

Table 10. Time Course of PAO Inhibition by BOH and UDMH. Apparent Reversal of Inhibition.

| Experiment | Mole Ratio (I/E) | μM Concentration (I) | (E) | Equilibrium: Maximum Inhibition Time (hours) | Maximum Inhibition Percent | Equilibrium: Minimum Inhibition Time (hours) | Minimum Inhibition Percent | Percent Net Reversal |
|---|---|---|---|---|---|---|---|---|
| A (BOH) | 0.29 | 0.63 | 2.18 | .25 | 38 | 4 | 1 | 37 |
| | 0.57 | 1.3 | 2.18 | .25 | 80 | 9 | 12 | 68 |
| | 2.9 | 6.3 | 2.18 | .25 | 97 | 8* | 59 | 38 |
| B (BOH) | 0.53 | 0.12 | 0.22 | 1 | 65 | 5 | 0 | 65 |
| C (UDMH) | 1.6 | 3.1 | 1.94 | .25 | 55 | 5 | -11 | 66 |
| | 9.7 | 18.8 | 1.94 | 0 | 97 | 7 | 39 | 58 |
| | 9.7 | 3.1 | 0.32 | .25 | 116 | 5 | 39 | 77 |

*Equilibrium not yet established.

Enzyme and inhibitor, at the indicated concentrations, were incubated at room temperature in 0.06 M phosphate buffer, pH 7.0. At desired time intervals, a 0.1 ml aliquot of the incubation mixture was assayed for enzyme activity. The activity of enzyme incubated under identical conditions except in the absence of inhibitor was taken as one hundred percent (zero percent inhibition). A control to correct for any change occurring in the free inhibitor was carried out by incubating the inhibitor under the same conditions in the absence of enzyme. At the appropriate time, an aliquot was added to uninhibited enzyme which had been kept at $0^{\circ}$, incubated 30 minutes to attain maximum inhibition, and then assayed. Any change with time in the percent inhibition of this system was added or subtracted to the corresponding experimental sample. Incubations were carried out in glass-stoppered tubes to prevent evaporation. PAO of specific activity 8.95 S.U./mg and a protein concentration of 36.9 mg/ml was used in Experiment A. In Experiment C, PAO of the same specific activity and a protein concentration of 36.4 mg/ml was used. PAO of specific activity 89.9 S.U./mg and a protein concentration of 0.267 mg/ml was used in Experiment B.

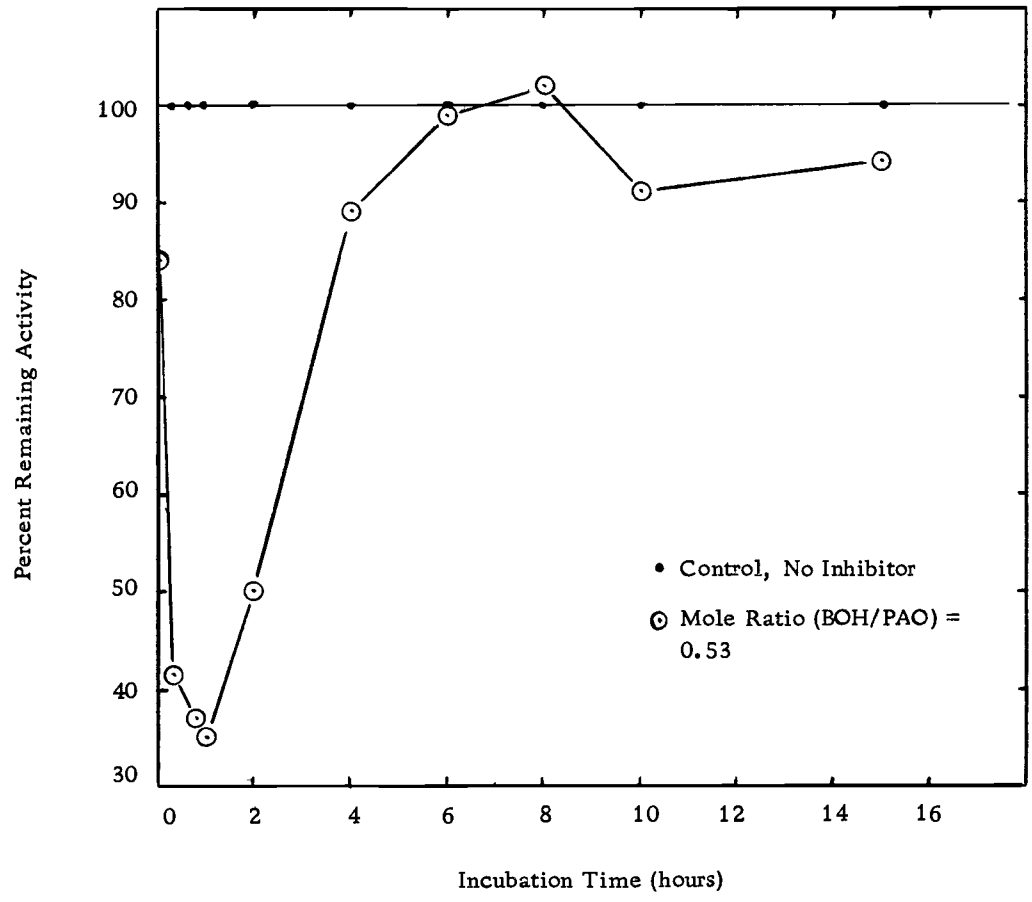


Figure 22. Effect of incubation time on PAO inhibition by BOH.

Experimental conditions are the same as those described in Table 10, Experiment B. Percent remaining activity is referred to the activity of the enzyme incubated in the absence of inhibitor under the same conditions for the same time period which was taken as 100 percent.

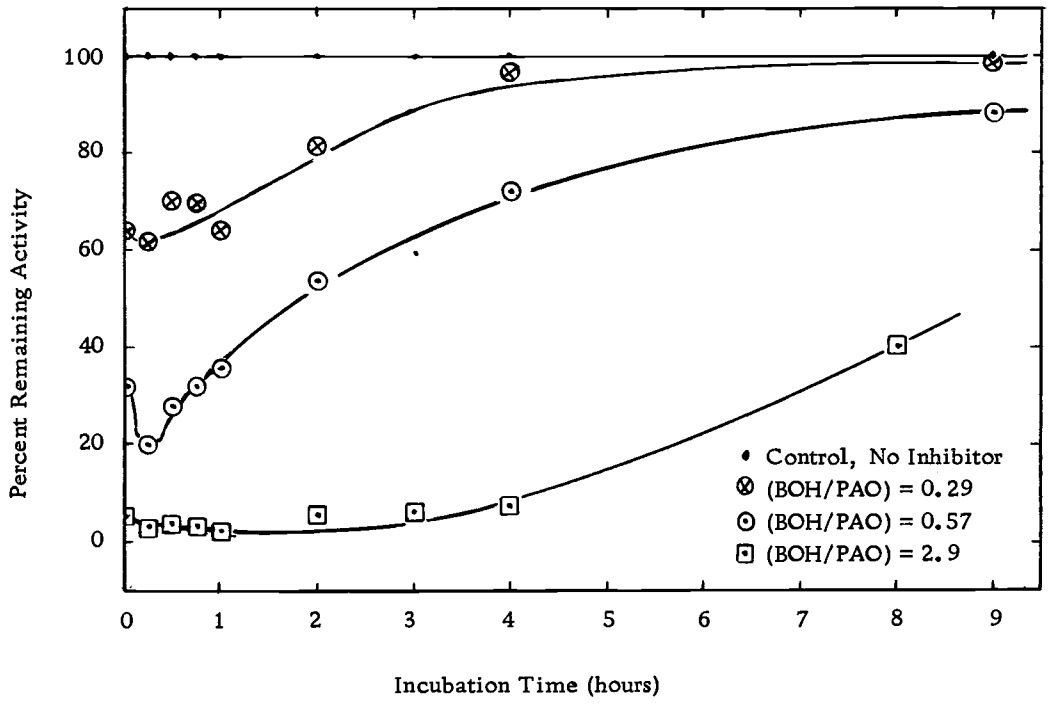


Figure 23. Effect of incubation time on PAO inhibition by BOH at different (BOH/PAO) mole ratios.

Experimental conditions are identical with those described in Table 10, Experiment A. Percent remaining activity is referred to the activity of the enzyme incubated in the absence of inhibitor for the same time period which was taken as 100 percent.

Table 10 and Figures 22 and 23 show that the maximum inhibition was reached rather quickly. Both the time and level for equilibrium inhibition depended on the concentrations of both enzyme and inhibitor, which is as expected from the results reported in Section I. Quite surprisingly, however, with longer incubation time a part of the activity was slowly regained.

An initial lag preceded the onset of reversal at high inhibitor concentrations (constant enzyme concentration). This behavior is typical of a system in which an inhibitor is decomposing, which would then suggest that decomposition of the inhibitor is responsible for the reactivation.

At the same I/E mole ratio, the initial rate of reversal was more rapid for higher concentrations of enzyme and inhibitor. This would seem to exclude the possibility that a spontaneous decomposition of the free inhibitor is occurring since increased concentration would drive the $E + I \rightleftharpoons EI$ equilibrium towards the formation of the EI complex, leaving less free inhibitor in solution available to decompose. Presumably, any spontaneous monomolecular decomposition of the inhibitor in solution would have been corrected by the "no enzyme" control (Legend of Table 10).

For the same inhibitor concentration, samples with lower enzyme levels exhibited an initial lag while those with higher enzyme concentrations did not. This would imply that the reversal is due to

a decomposition of the inhibitor which is directly dependent on enzyme concentration.

Preliminary studies have indicated that the reversal did not seem to be a function of the purity of the enzyme (compare Figures 22 and 23). The possibility that the decomposition of the inhibitor by an impurity in the enzyme preparation can then most likely be rejected.

Thus it seems that the apparent reactivation of hydrazine-inhibited PAO probably results from a chemical alteration of the inhibitor by the enzyme. Comparing systems with the same enzyme concentration, where $(I) \leq (E)$, a greater equilibrium percent net reversal was obtained for higher inhibitor levels (Table 10). Under these conditions, most inhibitor is bound, therefore the percent net reversal at equilibrium would be a measure of the decomposition of the EI complex. This may be taken as further support that the decrease in inhibition with time is caused by enzyme decomposing the inhibitor.

When $(I) > > (E)$ (at constant enzyme concentration), the amount of net reversal at a given time was inversely related to the inhibitor concentration. This too would provide evidence that the mechanism of the inhibition reversal involves the breakdown of the inhibitor by the enzyme, since for high inhibitor concentrations, equilibrium reversal will not be reached until there is no more free inhibitor to

replace that reacted by the enzyme.

A plot of log percent inhibition versus time was found to be linear for systems where $(I) \leq (E)$ at constant enzyme concentration (Figure 24). This indicates that the reversal is first order with respect to the EI complex, compatible with the scheme:

$$(8) \quad E + I \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} EI \xrightarrow{k_2} E + Q$$

which represents the case where the bound inhibitor is reacted by the enzyme. As an example, the first order rate constant at $25^{o}$ for the reversal of BOH inhibition of PAO at BOH/E = 0.57 was calculated to be $2 \times 10^{-3}$ $min^{-1}$ (Figure 24).

This explanation would seem fairly conclusive from the data obtained except for one anomaly. At constant inhibitor levels, the time required to establish equilibrium appeared to be independent of enzyme concentration (Table 10) when the same inhibitor concentration was employed. This might be explained if the time required to reach equilibrium reversal (minimum inhibition) were to depend on the magnitude of the decrease in inhibition. For the higher enzyme concentration, the activity was completely restored; no further decrease in inhibition was possible. For the lower enzyme concentration, the equilibrium level of minimum inhibition was established when the activity was not yet completely restored, thus it might

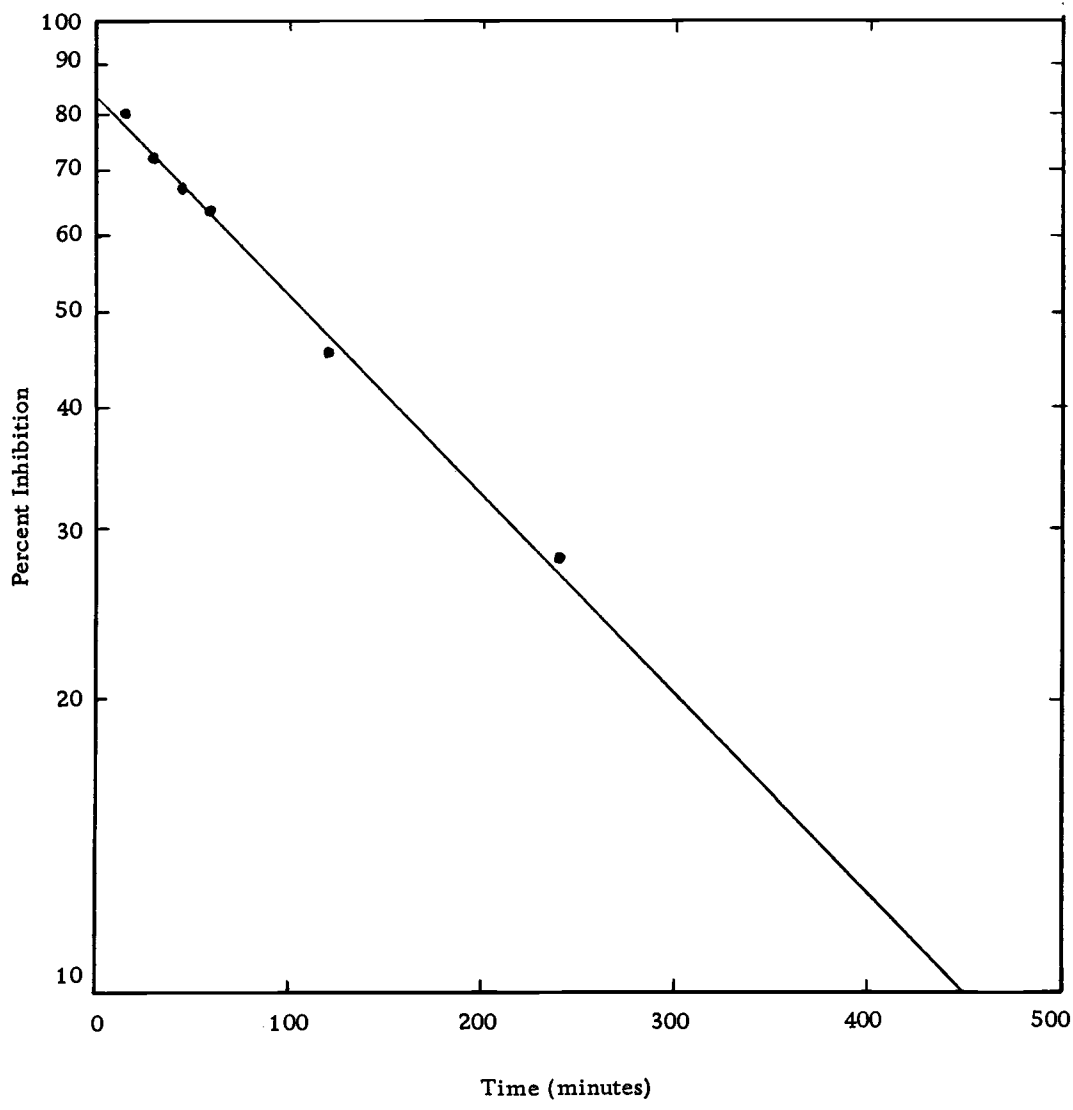


Figure 24. Reversal of BOH inhibition of PAO. First order rate plot.

The time course for the reversal of PAO inhibition by BOH is plotted on a logarithmic scale to show the first-order rate dependence. The data is from Experiment A, Table 10, where the mole ratio BOH/E = 0.57. Experimental conditions are described in the legend of Table 10.

appear to give greater net reversal than the former case (Table 10). Additional data must be obtained to elucidate the effect of enzyme concentration on the decomposition of the EI complex.

## Discussion

### A. Activation of PAO in the Absence of Hydrazines. Possible Explanations.

The results presented in Section IIIA ruled out the possibility that the activation of PAO in solution at room temperature was due to the presence of an endogenous, reversibly-bound activator or inhibitor. Velocity of benzylamine oxidation bore a linear relation to enzyme concentration, and a potentially inhibitory fraction isolated from the crude enzyme was found, in fact, not to inhibit.

The investigation of the effect of incubation and enzyme concentration on the molecular weight of PAO showed that only the monomeric form of the enzyme existed whether or not the enzyme was activated (Section IIIB). This led to the conclusion that the concentration- and time-dependent increase in enzyme activity could not be caused by a shift in the monomer-polymer equilibrium to favor the formation of the more active monomer. These results would also eliminate the possible involvement of subunit dissociation, which is compatible with the finding that separation of PAO's subunits requires rather drastic conditions (1).

Fully active soluble amine oxidases contain copper which is almost entirely in the cupric state. Reduction to the cuprous state results in a loss of enzyme activity (Section I, Discussion). If part of PAO's copper is in the Cu (I) state, the observed increase in activity could possibly arise from its oxidation by molecular $O_2$. An oxidation reaction alone would not account for the concentration dependency of the activation. It would then be necessary to assume that in dilute solutions another phenomenon is functioning to expose some copper that may not be otherwise accessible to $O_2$.

The unfolding or rearrangement of the enzyme molecule either to expose previously buried catalytic sites, or to alter the environment of the catalytic site to enhance the enzyme activity might best explain the activation phenomenon. The slow reaction indicates that it has a high energy of activation, characteristic of a conformational change. The increased degree of freedom provided by dilute solution is favorable for the occurrence of such structural alterations. This could explain the finding that the rate and level of the activation is greater for less concentrated enzyme solutions. Since PAO has such a low turnover number (Section IB-5), probably only a slight conformational change would be needed to account for the degree of activation observed.

The observation that the ESR determination of Cu (II) in soluble amine oxidases is consistently less than that obtained chemically

(24, 133, 135) could possibly be interpreted to mean that part of the enzyme's copper might be inaccessible. The enzyme is intact in the ESR procedure. The chemical method, however, involves treatments which could potentially alter the enzyme molecule to reveal previously buried, non-functional Cu (II). A conformational change thus could conceivably enhance the activity by exposing the enzyme's essential Cu (II) cofactor.

## B. Activation of PAO in the Presence of Hydrazines. Apparent Reversal of Inhibition.

The finding that the hydrazine inhibition appeared to be reversed with time initially came as somewhat of a shock after it was so laboriously demonstrated that the inhibition was stoichiometric, specific, and (pseudo-) irreversible (Section I). It now seems quite reasonable, and fits rather nicely into the overall picture of the inhibition reaction.

The data presented in Section IIIC demonstrated that the increase in activity of the inhibited enzyme is probably due to the catalytic decomposition by the enzyme.

Hill and Mann observed time-dependent changes in the spectrum of pea seedling amine oxidase in the presence of hydrazine which did not occur with the Cu (II)-free apoenzyme. The absorption maximum characteristic of the hydrazine-enzyme complex

disappeared after 90 minutes. In contrast, the peak attributed to the hydrazine derivative of the apoenzyme was stable for at least 20 hours. These results led to the conclusion that the hydrazine-enzyme complex is not stable (64). Pertinent to these observations is the fact that Cu (II) catalyzes the decomposition of hydrazines in the presence of oxygen (37).

In view of this information, it seems likely that the enzyme-dependent reactivation of hydrazine-inhibited PAO might be caused by a catalytic decomposition of the enzyme-bound inhibitor by PAO's Cu (II). Yet it appears that this can be ruled out, since the copper signal of the ESR spectrum of pig kidney amine oxidase was not diminished in the presence of hydrazines (55). The Cu (II)-catalyzed decomposition of hydrazines involves a reduction to Cu (I) (Figure 3).

Hydrazines and substrate have been shown to react at the same site and by similar mechanisms (Section I). If an inhibitor is structurally related to the substrate (as are hydrazines), it is conceivable that a reversal of inhibition could result from a reaction similar to that undergone by substrates, only much more slowly.

This mechanism for the reversal of hydrazine inhibition of PAO seems to best account for the experimental observations. It not only explains the kinetic character and the rate dependency of the time course of the reactivation, it is in accord with most all that

is known about PAO's reaction with substrates or hydrazines. The pseudo-irreversible nature of the inhibition defined in Section I arises from the fact that decomposition of the EI complex is quite slow even when a stoichiometric quantity of inhibitor is employed.

The only report in the amine oxidase literature on the spontaneous reversal of hydrazine inhibition of these enzymes was by Green (56) for the guinea pig mitochondrial enzyme. He observed that the enzyme in the presence of 20 μ M α-naphthylmethylhydrazine reached a maximum inhibition in about 30 minutes, but regained about 20% of its activity in five hours. Benzylhydrazine inhibition of this enzyme did not recover any of its activity under the same conditions.

One of the most thoroughly studied examples of an inhibited enzyme which undergoes spontaneous reactivation is the inhibition of cholinesterase by organo-phosphorus compounds. These compounds, like hydrazines, are substrate analogues which bind very strongly at the enzyme's active site. With time, however, they are reacted by the enzyme in a manner analogous to the transformation of normal substrates.

Inhibited cholinesterase, a phosphorylated enzyme, only very slowly undergoes hydrolysis. It is possible that the rate of a similar step may serve to differentiate between a substrate and an inhibitor in the PAO reaction as well.

# GENERAL DISCUSSION

The finding that the inhibition of PAO by hydrazine is both stoichiometric and specific opens a vast myriad of pharmacological applications. A control of the level of biogenic amines has been important in the treatment of both mental and physical diseased states in man and other animals, and has been linked with playing a major role in the growth of plants.

On a molecular level, the pseudo-irreversible titration of PLP by these inhibitors could serve as a useful tool to further characterize PAO's catalytic site. The covalent binding of these hydrazines might be further stabilized by a chemical reduction of the enzyme-inhibitor complex in a manner similar to that used by Buffoni (22) for amine substrates of pig plasma amine oxidase. The use of a radioactive inhibitor would label the active site. Subsequent chemical and enzymatic hydrolysis may yield a small peptide containing the bound inhibitor. The identity of the primary inhibitor binding site could be verified (is it <u>really</u> PLP?) and a determination of the sequence of amino acids in its vicinity might give insight into the nature of the site which binds the hydrogen of hydrazines and substrates which is alpha to the nucleophilic $-NH_2$.

McEwen (84) has proposed a mechanism for the oxidative deamination of amine substrates by rabbit serum amine oxidase. Its

applicability to bovine PAO can be tested in conjunction with the use of hydrazine inhibitors. In McEwen's mechanism, the substrate converts a pyridoxal Schiff base to pyridoxamine, which is then oxidized back to the aldehyde form by molecular oxygen. If this scheme is valid for PAO, hydrazines then should not inhibit in the absence of $O_2$ in the presence of excess substrate. Experiments along this line are currently being conducted in this laboratory by Dr. Reed and his students.

A quantitative study of the slow reactivation of the inhibited enzyme which may occur in a manner analogous to the transformation of normal substrates, should also provide insight into the catalytic mechanism of PAO. Much knowledge would be derived from an investigation of this reaction as a function of inhibitor structure. Energy of activation determinations for the reversal of inhibition and for the reaction of PAO with good substrates would give information about the chemical nature of the groups involved in both reactions. A spectral study of the slow reactivation could possibly serve to characterize the participating intermediates. Such studies should also determine which step in the mechanism is the rate-limiting step and makes the difference between a good substrate and a poor one.

The role of Cu (II) is probably the least understood aspect of the amine oxidase reaction. A comparison of the ESR spectra of

PAO inactivated by hydrazines of different structure as well as the difference in the Cu (II) signal observed when $^{14}$N- and $^{15}$N-hydrazines are employed (similar to Mondovi's work (90, 91) with amine substrates of hog kidney amine oxidase) could be useful in determining the function of this cofactor.

The time- and concentration-dependent activation of PAO in solution in the absence of hydrazines has been attributed to the occurrence of a conformational change which could expose previously buried, non-functional catalytic sites. A quantitative study of the effect of temperature on this process might be the simplest initial approach to verify if a conformational change is indeed responsible. If the energy of activation calculated from such experiments seems to substantiate the preliminary conclusions, the structural change in the PAO molecule might be further characterized by physical methods such as ORD, circular dichroism, or light scattering, or by experiments to determine the change in the hydrodynamic properties of PAO. The proposal that an oxidation of Cu (I) to Cu (II) might account for the increase in activity could be tested by noting the effect of various mild oxidizing agents on PAO activity.

If PAO is really an allosteric enzyme, it would be a very unique one since these properties have been thus far found only in regulatory enzymes. If the observed cooperative effects are indeed due to allosteric transitions, a whole new approach to the study of

amine oxidases would be disclosed. Two questions are imminent:

What is the chemical nature and the number of the allosteric inhibitor and substrate binding sites? A correlation of the effect of inhibitor and substrate structure as well as the effect of pH on the observed cooperativitiy should provide some preliminary information on this aspect of the investigation. In addition, a search can be made for methods which might selectively destroy one type of allosteric site, similar to the now classical example of Gerhart's and Pardee's work with aspartate transcarbamylase (45).

What is the physical nature of the allosteric transition? There are two opposing concepts of the physical changes in a protein molecule that accompany kinetically observed cooperative effects. One interprets the changes in terms of an association-dissociation phenomenon (subunit dissociation or a shift in a monomer-polymer equilibrium induced by the binding of an allosteric modifier) (93), and the other attributes them to an induced conformational change (78). The experimental validity of both of these explanations has been verified for several known allosteric proteins. These two possibilities might be most easily distinguished for the PAO-hydrazine-benzylamine system by examining the effect of the conditions which give kinetically observable cooperativity on the molecular weight of PAO. Gel-filtration techniques would provide the answer, but ultracentrifugation methods would be preferred. If the allosteric

transitions were not caused by a change in the molecular weight of PAO, then the constants obtained from the latter method would be already available to quantitate the conformational change.

In addition to these two main questions, the elucidation of the kinetic mechanism of the interaction between sites is of interest. For example, what is the sequence of reactions of substrate and hydrazines at their allosteric and at the catalytic site? Simple kinetic methods to answer questions such as these are available from an extrapolation of the studies on other allosteric enzymes.

The many similarities between PAO and cholinesterase might make a review of the properties of this thoroughly studied enzyme a profitable experience for the amine oxidase chemist. Of special interest is the reactivation of phosphorylated cholinesterase by strong nucleophiles. When the step in the amine oxidase mechanism which distinguishes between good substrates and poor ones is clearly defined, the chemical nature of compounds which can possibly split off the inhibiting group from the enzyme should also become apparent. The induced reactivation of hydrazine-inhibited amine oxidases is a possibility which opens many avenues for future research and has many important practical applications.

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